

Chemopotential of Temozolomide, Irinotecan, and Cisplatin Activity by CEP-6800, a Poly(ADP-Ribose) Polymerase Inhibitor

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Abstract

Poly(ADP-ribose) polymerase 1 (PARP-1) is a nuclear zinc finger DNA-binding protein that is implicated in the repair of DNA damage. Inhibition of PARP-1 through genetic knockouts causes cells to become hypersensitive to various chemotherapeutic agents. We tested the chemopotentiating ability of the PARP-1 inhibitor, CEP-6800, when used in combination with temozolomide (TMZ), irinotecan (camptothecin or SN38), and cisplatin against U251MG glioblastoma, HT29 colon carcinoma, and Calu-6 non-small cell lung carcinoma xenografts and cell lines, respectively. Exposure of tumor cells to TMZ, camptothecin (or SN38), and cisplatin before, or in the presence of, CEP-6800 significantly increased the onset and the magnitude of DNA damage, the duration for cells to effect repair, and the onset, duration, or fraction of cells arrested at the G₂/M boundary. In addition, *in vivo* biochemical efficacy studies with CEP-6800 showed that it was able to attenuate irinotecan- and TMZ-induced poly(ADP-ribose) accumulation in LoVo and HT29 xenografts, respectively. Treatment of CEP 6800 (30 mg/kg) with TMZ (17 and 34 mg/kg) resulted in 100% complete regression of U251MG tumors by day 28 versus 60% complete regression caused by TMZ alone. CEP-6800 (30 mg/kg) in combination with irinotecan (10 mg/kg) resulted in a 60% inhibition of HT29 tumor growth versus irinotecan alone by day 33. The combination therapy of cisplatin (5 mg/kg) with CEP-6800 (30 mg/kg) caused a 35% reduction in Calu-6 tumor growth versus cisplatin alone by day 28. These data suggest that CEP-6800 could be used as a chemopotentiating agent with a variety of clinically effective chemotherapeutic agents.

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Introduction

PARP-1³ is a 116-kDa nuclear zinc finger DNA-binding protein that uses NAD⁺ as a substrate to transfer ADP-ribose onto acceptor proteins such as histones, polymerases, ligases, and PARP itself (automodification; Refs. 1–3). PARP-1 belongs to a family of proteins that currently includes six members: PARP-1, PARP-2, PARP-3, PARP-4 or vault PARP, and tankyrase 1 and 2 (1, 3, 4). The most well-characterized member of this family is PARP-1. PARP-1 is activated by, and implicated in, the repair of DNA strand breaks caused directly by ionizing radiation, or indirectly after enzymatic repair of DNA lesions because of methylating agents, topoisomerase I inhibitors, and other chemotherapeutic agents such as cisplatin and bleomycin (2, 3, 5–7).

There is a substantial body of biochemical and genetic evidence demonstrating that PARP plays a role in cell survival and repair after sublethal massive DNA damage. Furthermore, the fact that PARP function in the absence of DNA damage is not critical for cell survival has made PARP a potentially viable therapeutic target for use as chemo- and/or radiopotentiators (6, 8). Early PARP inhibitors, such as 3-aminobenzamide and nicotinamide, were able to potentiate both the *in vitro* and *in vivo* cytotoxic activities of radiation, bleomycin, CPT, cisplatin, and TMZ in human and murine tumor models. The inherent limitations in the potency, selectivity, and deliverability of these compounds precluded assigning the potentiation of antitumor efficacy observed *in vitro* and *in vivo* to the inhibition of PARP (2, 9–11). These issues resulted in the development of more potent and selective structural classes of PARP inhibitors such as benzimidazole-4-carboxamides and quinazolin-4-[3H]-ones. *In vitro* and *in vivo* analyses revealed that these compounds were able to potentiate the efficacy of chemotherapeutic agents using both human and murine tumor models (6, 10, 12–15).

The objective of the studies described in this report was to evaluate the chemopotentiating activity of CEP-6800. This novel 3-aminomethyl carbazole imide is an inhibitor of both PARP-1 and PARP-2 ($K_i = 5$ nM). CEP-6800 displays an excellent selectivity profile against a variety of NAD⁺-using enzymes and protein kinases, and also demonstrates potent activity in cellular assays for inhibition of NAD⁺ depletion. The aminomethyl functionality of CEP-6800 provides a high degree of water solubility, allowing the compound to be easily evaluated in both *in vitro* and *in vivo* systems (16). On the basis of previous studies demonstrating that inhibition of

³ The abbreviations used are: PARP, poly(ADP-ribose) polymerase; CPT, camptothecin; TMZ, temozolomide; PAR, poly(ADP-ribose); ATCC, American Type Culture Collection; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2H-tetrazolium; QD, once daily.

PARP results in the potentiation of chemotherapeutic agent-induced DNA strand breaks and in the accumulation of cells at the G₂/M boundary of the cell cycle (1), we evaluated the ability of CEP-6800 to potentiate DNA strand breaks, and the fraction and accumulation of tumor cells in G₂/M as a result of *in vitro* incubation with TMZ, CPT, or SN38, and cisplatin in U251MG human glioblastoma, HT29 human colon adenocarcinoma, and Calu-6 non-small cell carcinoma cells, respectively. In addition, we evaluated the ability of CEP-6800 to attenuate *in vivo*-induced PAR accumulation caused by administration of TMZ and irinotecan using LoVo and HT29 colon carcinoma xenografts, respectively, and its ability to enhance the antitumor efficacy of TMZ, irinotecan, and cisplatin using clinically relevant s.c. xenograft models (U251MG, HT29, and Calu-6, respectively). These studies demonstrated that CEP-6800 potentiated the magnitude and/or duration of DNA damage, increased the fraction and/or the duration of time tumor cells arrested in the G₂/M phase of the cell cycle after treatment with TMZ, CPT, or SN38 or cisplatin, and potentiated the antitumor efficacy of TMZ, irinotecan, and cisplatin against s.c. human tumor xenografts in nude mice. At concentrations of and above those associated with potentiation of antitumor efficacy, CEP-6800 did not enhance the cytotoxicity of these chemotherapeutic agents on normal human intestinal epithelial or renal cells *in vitro*, and in mouse gastrointestinal and renal tissues after prolonged exposure *in vivo*. These data indicate that CEP-6800 could have therapeutic value as a chemopotentiation agent in combination with a variety of mechanistically distinct chemotherapeutic agents.

Materials and Methods

Cell Lines. The HT29 and LoVo human colon carcinoma cell lines (ATCC, Manassas, VA), the U251MG human glioblastoma cell line (ATCC), and the Calu-6 human non-small cell lung carcinoma cell line (ATCC) were cultured in McCoy's 5A (supplemented with 1.5 g/liter sodium bicarbonate; Cellgro/Mediatech, Washington, D.C.), Ham's F12K (supplemented with 2 mM L-glutamine and 1.5 g/liter sodium bicarbonate; ATCC), MEM (Cellgro/Mediatech), or MEM (supplemented with 10 mM nonessential amino acids; ATCC), respectively, containing 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA). The FHS74 INT human small intestine cell line (ATCC) was cultured in DMEM (supplemented with 10 mM nonessential amino acids, 100 mM sodium pyruvate, 200 mM glutamine, 0.1 mM oxalacetic acid, 0.2 units/ml crystalline insulin, and 30 ng/ml epidermal growth factor), and normal human renal epithelial cells (Clonetics, Walkersville, MD) were cultured in REBM Bulletkit (Clonetics). All of the cell lines were maintained at 37°C in a humidified incubator with 95% air/5% CO₂.

Reagents. For *in vivo* experiments, cisplatin (Hande Tech, Houston, TX), TMZ (Schering-Plough, Kenilworth, NJ), and irinotecan (Pharmacia & Upjohn, Kalamazoo, MI) were resuspended in 0.9% sterile saline; for *in vitro* experiments CPT (Sigma, St. Louis, MO), SN-38 (kindly provided by Dr. J. Patrick McGovern, Pharmacia & Upjohn), and TMZ were resuspended in 100% DMSO. Because of the data demonstrating that irinotecan is metabolized by carboxylesterases

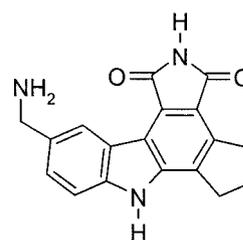


Fig. 1. Structure of CEP-6800.

and that the amount of cellular carboxylesterase is not sufficient for inducing maximal irinotecan activation, all of the *in vitro* experiments were carried out using either CPT or SN38 (17). The PARP-1 inhibitor CEP-6800 (Fig. 1) was formulated in 1× PBS (Fisher Scientific) for *in vivo* studies or 100% DMSO for *in vitro* assays. For all of the *in vitro* studies, CEP-6800 was used at a 1- μ M effective concentration based on preliminary studies with this compound. In addition, this concentration corresponds to plasma levels (C_{max}) of CEP-6800 associated with chemopotentiation of antitumor efficacy in several xenograft models (see "Chemopotentiation Studies against Human Tumor Xenografts").

Comet Assay for Evaluation of DNA Damage *in Vitro*.

Exponentially growing HT29, U251MG, or Calu-6 cells were incubated with CPT (50 μ M; 2 h), TMZ (100 μ M; 2 h) or cisplatin (100 μ M; 4 h), respectively, washed, and then incubated in medium alone with 0.1% DMSO or with CEP-6800 (1 μ M) for 0.5–30 h. The concentrations of cytotoxic agents and time points used in these studies were based on data published previously and on preliminary studies (Refs. 5, 13, 18; data not shown). Controls included incubation with 0.1% DMSO and CEP-6800 (1 μ M) alone. Cells were harvested, and counted at 0.5, 1, 4, 12, 18, and 24 h (U251), or 24, 28, 29, and 30 h (HT29 and Calu-6), then processed using the CometAssay kit as per the manufacturer's protocol (Trevigen, Inc., Gaithersburg, MD). Cells were resuspended briefly in Comet LMAgarose (Trevigen, Inc.), pipetted onto a glass microscopic slide (Trevigen, Inc.), and allowed to gel at 4°C for 30 min. The slides were then immersed in lysis solution (Trevigen, Inc.) at 4°C for 45 min, then placed in alkali solution [300 mM NaOH and 1 mM EDTA (pH >13)] for 35 min (HT29 and U251) or 60 min (Calu-6) at room temperature. Slides were then washed in 1× Tris-borate EDTA buffer (Fisher) and electrophoresed at 36 V for 10 min at room temperature in 1× Tris-borate EDTA. After electrophoreses, slides were washed in distilled water for 5 min followed by incubation in 100% ethanol for 5 min. Slides were air dried in the dark overnight and stained using the CometAssay Silver Satin kit (Trevigen, Inc.) as per the manufacturer's protocol. Comets (5 fields/slide; ~200 cells) were counted at ×100 magnification; counts were normalized to background (0.1% DMSO). Values presented are the mean \pm SE of three independent experiments.

Flow Cytometric Evaluation of Cell Cycle *in Vitro*. Exponentially growing U251MG or Calu-6 cells were incubated for 1 h with TMZ (100 μ M) or cisplatin (10 μ g/ml), then washed and incubated with medium containing 1 μ M CEP-6800 for

16, 24, 40, 48, 64, 72, 136, 144, 160, or 168 h (U251MG), or for 16, 24, 40, 48, 64, or 72 h (Calu-6). Exponentially growing HT29 cells were coincubated with SN38 (5 ng/ml) and 1 μM CEP-6800 for 16, 24, 40, 48, 64, or 72 h. Incubation times for each chemotherapeutic agent were optimized based on data published previously and on preliminary experiments (Refs. 19–21; data not shown). At each time point, cells were harvested, washed in PBS, fixed in 100% (v/v) ethanol, and stored overnight at 4°C. The next day, cells were washed in PBS followed by incubation in DNA prep stain solution (Beckman Coulter, Miami, FL) for 30 min in the dark at 37°C. Stained nuclei were then analyzed by flow cytometry (Coulter EPIC XL Detector: FL3; acquisition data 20,000 events/sample). The percentage of cells in each phase of the cell cycle was determined using MultiCycle software (Phoenix Flow Systems, San Diego, CA). Values presented are the mean \pm SE of three independent experiments.

In Vitro Analyses for Potentiation of Chemotherapeutic Induced Toxicity of Human Carcinoma Cells and Normal Human Cells. The ability of CEP-6800 to exacerbate the cytotoxic effects of the chemotherapeutic agents on human carcinoma cells (U251MG, HT29, and Calu-6), normal human intestinal cells (FHS74 NT), and normal human renal epithelial cells was evaluated using the MTS assay (Promega, Madison, WI). Cell number, reagent concentrations, and incubation times were optimized to determine that the data were in the linear range of the assay and on data published previously (Ref. 6; data not shown). The U251MG, HT29, and Calu-6 were plated at 2000 cells/well and coincubated with CEP-6800 (1.0 μM) and TMZ (50–300 μM), SN38 (10–300 ng/ml), or cisplatin (0.03–3.0 $\mu\text{g}/\text{ml}$), respectively, for 72 h. The FHS74 INT cells were plated at 3000 cells/well, and the renal epithelial cells were plated at 6000 cells/well and were analyzed in a similar manner using CEP-6800 (0.3–10 μM FHS74 INT cells or 0.1–3 μM renal epithelial cells) with the chemotherapeutic agents [FHS74 INT with TMZ (150–350 μM) or CPT (2–4 $\mu\text{g}/\text{ml}$); renal epithelial cells with cisplatin (0.625–10 $\mu\text{g}/\text{ml}$)]. A larger concentration range of CEP-6800 was used in studies with normal human cells to assess potential cytotoxic effects of this compound. After the appropriate incubation time with the compounds, the effects on cell proliferation were assayed after a 1.0-h (U251MG), 2.0-h (FHS74 INT cells), 2.5-h (HT29 and Calu-6) or a 5.0-h (renal epithelial cells) incubation in MTS solution (Promega). The absorbance was read using a microtiter plate reader at 490 nm.

Animals. Female athymic *nu/nu* mice (8–10 weeks old; Charles River, Raleigh, NC) were maintained at 5/cage in microisolator units. Animals were given a commercial diet and water *ad libitum*, housed at 48% \pm 2% humidity and 22 \pm 2°C, and light-dark cycle was set at 12-h intervals. Mice were quarantined for at least 1 week before experimental manipulation. Mice weighed between 22 and 25 g on the day of tumor cell inoculation. All of the animal experiments were performed at Cephalon, Inc., under protocol 03–023 approved by the Institutional Animal Care and Use Committee of Cephalon, Inc.

Tumor Cell Implantation and Measurement of Tumor Growth. Exponentially growing cells were cultured as described above, harvested, and injected [2×10^6 cells/mouse for HT29 and U251MG cell lines; 3×10^6 cells/mouse for the Calu-6 cell line; and 5×10^6 cells/mouse for the LoVo cell line (PAR accumulation assay only)] 1:1 with Matrigel (Fisher Scientific, Malvern, PA) into the right flank of nude mice. Tumor-bearing mice were randomized according to tumor size into the appropriate number of experimental groups with 10 mice/group (chemopotential studies) or 8 mice/group (PAR accumulation assay). For chemopotential studies, tumors were measured using a vernier caliper every 3–4 days. Tumor volumes were calculated using the following formula: $V(\text{mm}^3) = 0.5236 \times \text{length}(\text{mm}) \times \text{width}(\text{mm}) [\text{length}(\text{mm}) + \text{width}(\text{mm})/2]$.

PAR ELISA of Tumor Xenografts. Preliminary time course studies were carried out to determine the time point at which chemotherapeutic agent-induced PAR accumulation was at its peak (data not shown). Tumor-bearing nude mice were treated with one dose of irinotecan (10 mg/kg, i.p.) or TMZ (68 mg/kg, p.o.) 1 h before the administration of one dose of CEP-6800 (30 mg/kg, s.c.). Mice were sacrificed 4 h after irinotecan or TMZ dosing; tumors were frozen in liquid nitrogen and stored at -70°C until needed for analyses.

Tumors from nude mice, treated as described above, were homogenized in cold 1 N perchloric acid (Fisher) then precipitated for 1 h on ice. Samples were centrifuged for 10 min at 4°C, the supernatant removed, and the pellets were homogenized in 100% ice-cold ethanol. After the ethanol wash was repeated, the pellets were resuspended in ether (Fisher), centrifuged, and air dried for 1 h on ice. Pellets were resuspended in 1 N NaOH/50 nM EDTA and solubilized at 60°C for 1 h, then $10 \times$ radioimmunoprecipitation assay buffer (500 mM Tris-HCl, 1.5 M NaCl, 10% Triton X-100, 2.5% deoxycholic acid, and 1% SDS) and 1 N HCl were added to all of the samples. If needed, the pH of the samples was adjusted to 8.0 by using concentrated HCl. Protein concentrations were obtained using the bicinchoninic acid method (Pierce, Rockford, IL), and samples were aliquoted and frozen at -70°C until needed.

Unless otherwise stated, all of the washes were done five times with 0.05% Tween 20 in TBS. Nunc maxisorp plates (Fisher) were precoated with anti-PAR mouse monoclonal antibody (1:1000 dilution; Biomol, Plymouth Meeting, PA) in 0.1 M sodium bicarbonate for 2 h at 37°C. Plates were washed and blocked with 1% casein (Pierce) for 1 h at room temperature. After blocking, PAR polymer (Biomol) used for standard curve (0–0.08 ng/ml range) and samples (2 mg/ml) were added to the plates and incubated overnight at 4°C while rocking. The next day the plates were washed and incubated with anti-PAR rabbit polyclonal antibody (1:1000; Biomol) for 1 h at room temperature. After incubation, the plates were washed and incubated with antirabbit IgG-alkaline phosphatase conjugate (1:500; Rockland, Gilbertsville, PA) for 1 h, then washed three times in 0.05% Tween 20 in TBS, followed by washing three times in $1 \times$ TBS. Plates were incubated with 4-methylumbelliferyl phosphate (Sigma) in diethanolamine/MgCl₂ for 30 min at 37°C. Fluorescence

was read on Cytofluor with excitation filter 360 nm, emission filter 460 nm, and gain 55 (22).

Chemopotentiation Studies against Human Tumor Xenografts. The doses of the chemotherapeutic agents in the following studies were chosen to ensure the use of an active agent at a dose that would not totally suppress tumor growth, thus making it possible to evaluate additive or synergistic effects of the combination treatment (23). The dose of CEP-6800 used in these studies was based on tolerability studies and on preliminary pharmacokinetic data that would yield plasma levels 3–4-fold above the cell-based IC_{50} (data not shown).

HT29 Human Colon Carcinoma Xenografts. Nude mice were injected with tumor cells and randomized into treatment groups as described above. Upon xenografts reaching a mean tumor volume of $\sim 250 \text{ mm}^3$, mice were treated with vehicle (0.9% sterile saline, 100 $\mu\text{l}/\text{dose}$, i.p., QD, 5 days); irinotecan as a monotherapy (10 mg/kg/dose, i.p., QD, 5 days); irinotecan (10 mg/kg/dose, i.p., QD, 5 days) 1 h before CEP-6800 (30 mg/kg/dose, s.c., QD, 5 days); or CEP-6800 (30 mg/kg/dose, s.c., QD, 5 days) as a monotherapy. Dosing regimens of irinotecan were based on those described previously (17).

U251MG Human Glioblastoma Xenografts. Nude mice were injected with tumor cells and randomized into treatment groups as described above. Upon xenografts reaching a mean tumor volume of $160 \pm 10 \text{ mm}^3$, mice were treated with vehicle (0.9% sterile saline, 100 $\mu\text{l}/\text{dose}$, i.p., QD, 5 days), TMZ as a monotherapy (17 or 34 mg/kg/dose, p.o., QD, 5 days), TMZ (17 or 34 mg/kg/dose, p.o., QD, 5 days) 1 h before CEP-6800 (30 mg/kg/dose, s.c., QD, 5 days), or CEP-6800 (30 mg/kg/dose, s.c., QD, 5 days) as a monotherapy. Dosing regimens of TMZ were based on those described previously (24).

Calu-6 Human Non-Small Cell Lung Carcinoma Xenografts. Nude mice were injected with tumor cells and randomized into treatment groups as described above. Upon xenografts reaching a mean tumor volume of $145 \pm 15 \text{ mm}^3$, mice were treated with vehicle (0.9% sterile saline, 100 $\mu\text{l}/\text{dose}$, i.p., QD, days 1 and 14), cisplatin as a monotherapy (5 mg/kg/dose, i.p., QD, days 1 and 14), cisplatin (5 mg/kg/dose, i.p., QD, days 1 and 14) 1 h before CEP-6800 (30 mg/kg/dose, s.c., QD, days 1 and 14), or CEP-6800 (30 mg/kg/dose s.c., QD, days 1 and 14) as a monotherapy. Dosing regimens of irinotecan were based on those described previously and preliminary dose optimization studies done in our lab (23).

For all of the tumor xenograft chemopotentiation studies, established tumors in each animal were individually normalized to their size at the start of the experiment, and the data calculated as the change in tumor volume relative to the day 1 volume, using the following formula: relative tumor volume = V_x/V_o where V_x is the tumor at any time point and V_o is the tumor volume at the initiation of dosing (day 1; Ref. 25). For each experimental group, the mean relative tumor volumes and SEs were calculated (SigmaStat; Jandel Scientific, San Rafael, CA). Complete regression was defined as the disappearance of a measurable tumor mass ($<14 \text{ mm}^3$) at some point after the initiation of therapy (26). Statistical anal-

yses were calculated by the Mann-Whitney rank sum test or *t* test where appropriate with $P \leq 0.05$ deemed significant.

In Vivo Evaluation of Toxicity. Renal damage was evaluated by analyzing serum samples for blood urea nitrogen, creatinine, and total protein levels (AniLytics, Gaithersburg, MD), and by histological analyses. Formalin-fixed, paraffin-embedded kidney sections were stained with H&E and evaluated by light microscopy by a board-certified pathologist (A. K-S.). Similarly, to evaluate intestinal damage, formalin-fixed, paraffin-embedded intestinal sections were stained with H&E and evaluated.

Results

Effects of PARP-1 Inhibition on DNA Damage and Repair *in Vitro* (Comet Assay).

Kinetic studies of DNA damage and repair events were conducted using a modified commercially available single cell gel electrophoresis assay (CometAssay) to evaluate the extent of DNA damage in cells exposed to genotoxic agents. The concentrations of the chemotherapeutic agents and the time points used in these *in vitro* studies were optimized based on data published previously and on preliminary studies to determine a concentration (TMZ, 100 μM ; cisplatin, 100 μM ; and CPT, 50 μM) that would result in maximal comet formation but not cause cell toxicity (Refs. 5, 13, 18; data not shown). A time-related response in DNA strand-break formation was observed in the U251MG glioblastoma cells treated with TMZ alone (Fig. 2A). The peak of strand breaks in this cell line (20–26%) was observed between 4 and 12 h, which was longer than the 2–4 h range that has been reported previously for TMZ as determined by alkaline elution using the murine leukemia cell line, L1210 (18). The combination of TMZ (100 μM) and CEP-6800 (1 μM) resulted in significant potentiation of DNA strand breaks in U251MG glioblastoma cells as compared with TMZ treatment alone (Fig. 2A). A 1.75- ($P \leq 0.05$), 2.5- ($P \leq 0.01$), 1.5- ($P \leq 0.05$), 2.7- ($P \leq 0.0001$), and a 4- ($P \leq 0.01$) fold increase in strand breaks as compared with TMZ treatment of cells alone was observed at the 30-min, 1-h, 4-h, 18-h, and 24-h time points, respectively.

In contrast to TMZ, a narrower window of comet formation was observed with cisplatin on Calu-6 non-small cell lung carcinoma cells. The peak of cisplatin (100 μM)-induced strand breaks (22%) was observed between 24 and 29 h, with strand breaks decreasing back to below initial levels by 30 h (Fig. 2B). These findings are in agreement with data published previously using different cell lines, which demonstrated the peak of strand-break formation to be at 24 h (5). The combination of cisplatin with CEP-6800 (1 μM) potentiated cisplatin-induced strand breaks at the 24-h time point (the peak of comet formation; 49%; $P \leq 0.05$) as compared with cisplatin treatment alone (Fig. 2B).

Similarly to what was observed with the Calu-6 cells in response to cisplatin, the peak of CPT (50 μM)-induced strand breaks (39%) using HT29 colon carcinoma cells was observed at 28 h (Fig. 2C) with strand breaks decreasing back to below initial levels by 30 h. These data are in agreement with data published previously using different cell lines, which demonstrated the peak of strand break formation to be at ~ 24 h (27). The combination of CPT with CEP-6800 (1

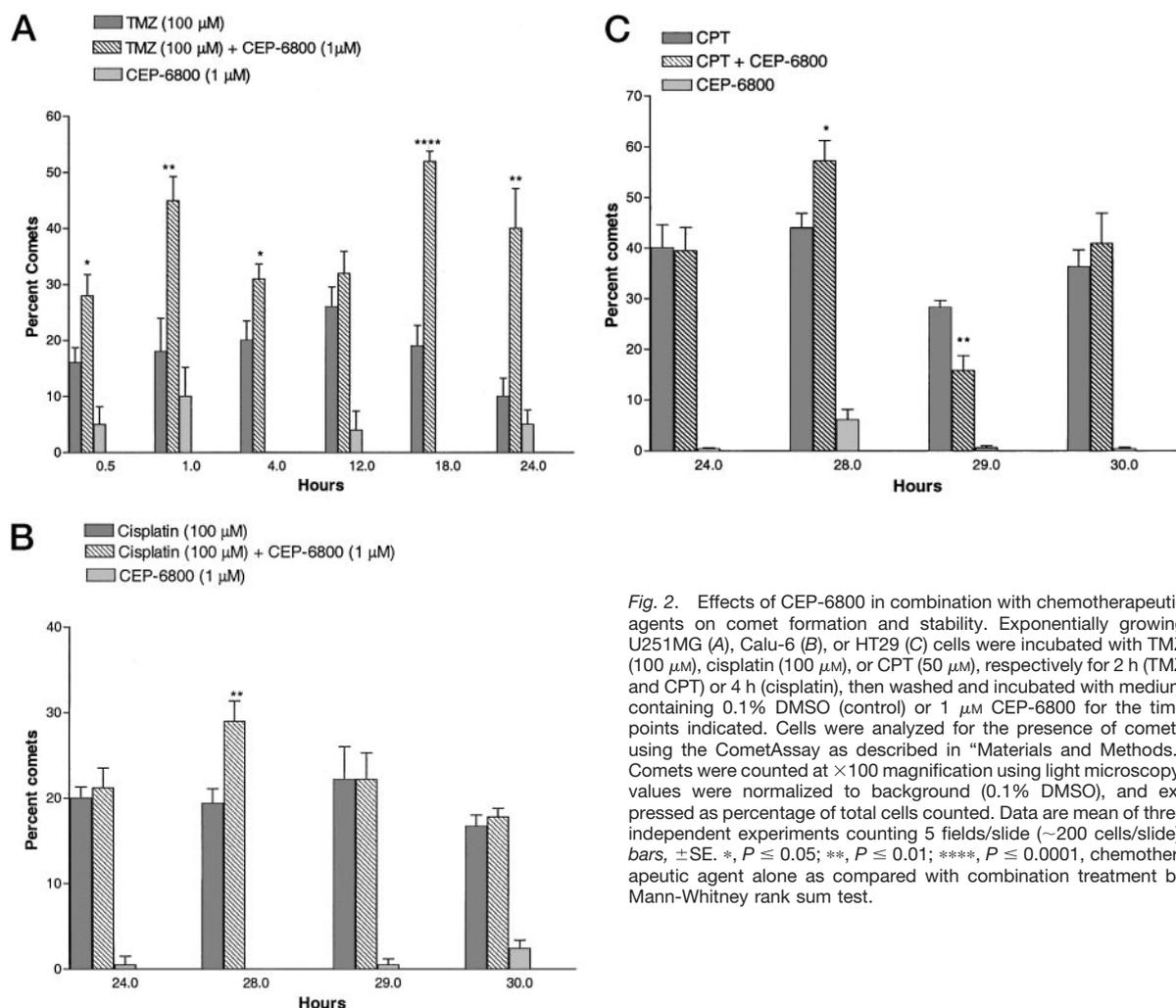


Fig. 2. Effects of CEP-6800 in combination with chemotherapeutic agents on comet formation and stability. Exponentially growing U251MG (A), Calu-6 (B), or HT29 (C) cells were incubated with TMZ (100 μ M), cisplatin (100 μ M), or CPT (50 μ M), respectively for 2 h (TMZ and CPT) or 4 h (cisplatin), then washed and incubated with medium containing 0.1% DMSO (control) or 1 μ M CEP-6800 for the time points indicated. Cells were analyzed for the presence of comets using the CometAssay as described in "Materials and Methods." Comets were counted at $\times 100$ magnification using light microscopy; values were normalized to background (0.1% DMSO), and expressed as percentage of total cells counted. Data are mean of three independent experiments counting 5 fields/slide (~ 200 cells/slide); bars, \pm SE. *, $P \leq 0.05$; **, $P \leq 0.01$; ****, $P \leq 0.0001$, chemotherapeutic agent alone as compared with combination treatment by Mann-Whitney rank sum test.

μ M) resulted in the potentiation of DNA strand breaks at the 24-h time point (44%; $P \leq 0.01$) as compared with CPT alone (Fig. 2C). In all of the cell lines used for these experiments, CEP-6800 was also tested for its ability to produce strand breaks when incubated alone. A 0.5–30 h incubation with 1 μ M CEP-6800 had minimal to no significant effect on DNA strand break formation as compared with the 0.1% DMSO control-treated cells (Fig. 2, A–C).

These data indicate that CEP-6800 exposure in combination with various chemotherapeutic agents potentiated significantly the number of DNA strand breaks at the peak period of cisplatin or CPT-induced strand breaks. In contrast, CEP-6800 exposure potentiated significantly both the number and the duration of time for effective repair of damaged DNA strand breaks in U251MG cells treated with TMZ.

The Effect of PARP-1 Inhibition on the Percentage of Tumor Cells in the G₂/M Phase of the Cell Cycle. On the basis of data implicating a role for PARP-1 in a checkpoint that monitors the genomic integrity before entering into mitosis (28, 29), we evaluated the ability of CEP-6800 to potentiate the fraction of cells arrested in G₂/M and/or the duration of time cells spend in the G₂/M phase of the cell

cycle in combination with TMZ, SN38, and cisplatin using U251MG, HT29, and Calu-6 cells, respectively. The active metabolite of irinotecan, SN38 was used for these *in vitro* studies. The concentrations of chemotherapeutic agents were optimized based on data published previously and preliminary dose finding studies (19–21).

In U251MG cells, cell cycle arrest in the G₂/M phase of the cell cycle peaked at 60% accumulation ($P \leq 0.00001$ as compared with 15% accumulation for 0.1% DMSO control) within 40 h of exposure to TMZ (100 μ M). This accumulation was sustained over a 72-h period, with the percentage of cells arrested at G₂/M returning to initial baseline levels at the 136-h time point and continuing until the end of the study (168 h; Fig. 3A). The time to achieve peak fraction of G₂/M arrest in U251MG cells with TMZ exposure is in general agreement with previous reports using the human glioblastoma cell line, U87MG (19). Incubation with CEP-6800 after TMZ treatment resulted in an increase in the fraction of cells accumulated at G₂/M at the 16-h time point (48% accumulation, $P \leq 0.001$ as compared with 22% accumulation for TMZ alone) and continuing to the 24-h time point (40% accumulation, $P \leq 0.01$ as compared with 15% accumula-

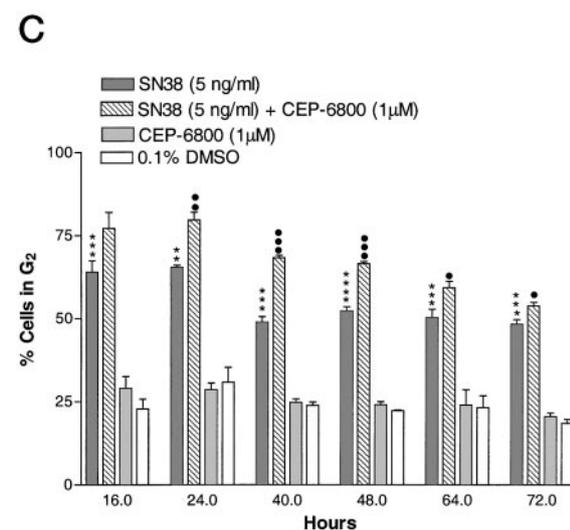
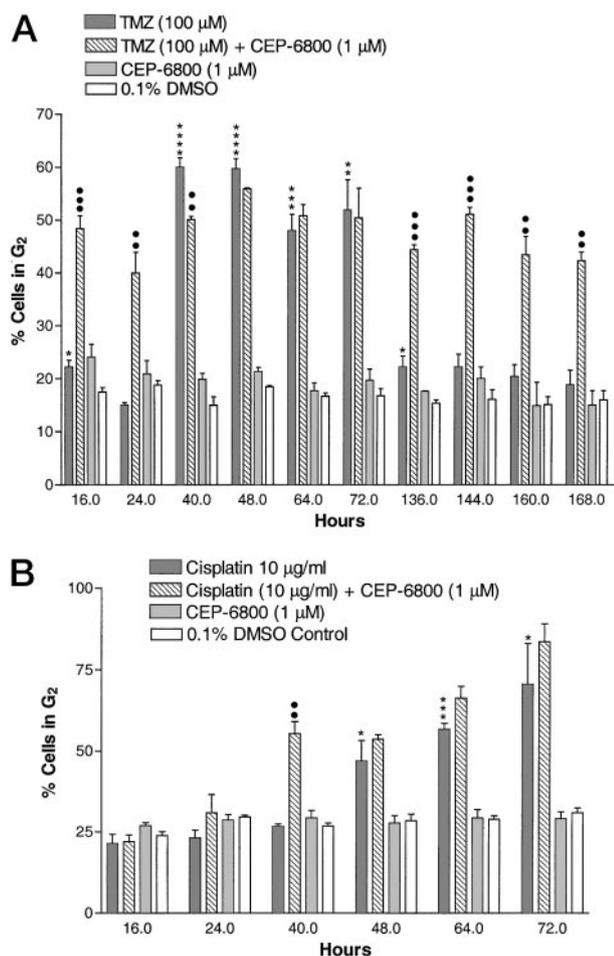


Fig. 3. Effects of CEP-6800 in combination with chemotherapeutic agents on the percentage of cells arrested in the G_2/M phase of the cell cycle. Exponentially growing U251MG (A) and Calu-6 (B) cells were incubated for 1 h with TMZ (100 μM) or cisplatin (10 $\mu\text{g}/\text{ml}$), respectively, then washed and incubated with medium containing 0.1% DMSO (control) or 1 μM CEP-6800 for the time points indicated. Exponentially growing HT29 cells (C) were incubated continuously with SN38 (5 ng/ml) and CEP-6800 1 μM for the indicated time points. All of the cells were harvested and analyzed by flow cytometry as described in "Materials and Methods" to assess the percentage of cells remaining in the G_2/M phase of the cell cycle. Values are mean of three independent experiments; bars, \pm SE. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.000$, chemotherapeutic agent as compared with 0.1% control; ●, $P \leq 0.05$; ●●, $P \leq 0.01$; ●●●, $P \leq 0.001$; ●●●●, $P \leq 0.0001$, chemotherapeutic agent alone as compared with combination treatment by Mann-Whitney rank sum test or t test where appropriate.

tion for TMZ alone). Exposure to CEP-6800 did not increase the fraction of cells arrested in G_2/M compared with the maximum levels obtained on TMZ exposure but did effect the duration of G_2/M arrest significantly. The fraction of G_2/M arrested cells treated with TMZ began to decrease by 136 h after exposure, and remained at baseline levels for the duration of the study (168 h). In contrast, cells treated with TMZ and CEP-6800 were still accumulating (42–51% accumulation as compared with 18–22% accumulation for TMZ alone; $P \leq 0.05$ to $P \leq 0.01$) at the G_2/M boundary for up to 168 h after treatment (Fig. 3A).

Incubation of Calu-6 cells with cisplatin (10 $\mu\text{g}/\text{ml}$) caused cell cycle arrest in G_2/M within 48 h (Fig. 3B; 46% accumulation, $P \leq 0.05$ as compared with 28% accumulation for 0.1% DMSO control) of exposure and peaked at 72 h (70% accumulation, $P \leq 0.05$ as compared with 29% accumulation for 0.1% DMSO alone). The time to the peak of cisplatin-induced G_2/M arrest is longer than what has been reported previously for this cell line (36–48 h; Ref. 20); however, in our experiments a longer time course was used, which could possibly account for the differences observed. CEP-6800 incubation after cisplatin treatment decreased the time it took for the cells to accumulate at G_2/M from 48 h to 40 h (Fig. 3B; 55% accumulation, $P \leq 0.01$ as compared with

cisplatin alone). Similar to what was observed with TMZ, once cisplatin treatment alone was maximally effective in inducing arrest in G_2/M , CEP-6800 did not additionally potentiate the fraction of cells at the G_2/M boundary.

Continuous exposure of HT29 cells with SN38 (5 ng/ml) resulted in peak accumulation between 16 and 24 h (Fig. 3C; 65% accumulation, $P \leq 0.01$ as compared with 22–30% accumulation for 0.1% DMSO control). After an approximate decrease of 26% at the 40-h time point, the accumulation of cells at the G_2/M boundary was sustained throughout the 72-h time course (48–53% accumulation, $P \leq 0.001$ as compared with 23–18% accumulation for 0.1% DMSO control). The magnitude of decrease in the amount of cells arrested at G_2/M is similar to what has been reported previously (21); however, the time to the peak of G_2/M arrest differs, possibly the result of continuous exposure of the cells to SN38 and the PARP-1 inhibitor in the studies described here. Coincubation of SN38 with CEP-6800 did not shift the time frame of accumulation of cells at the G_2/M phase, but resulted in a potentiation of the fraction of cells at the G_2/M boundary relative to that induced by SN38 exposure alone beginning at the 24-h time point (22% increase; $P \leq 0.01$) and continuing until the 72-h time point (11% increase; $P \leq 0.05$) with maximal potentiation of 40% ($P \leq 0.001$) at the

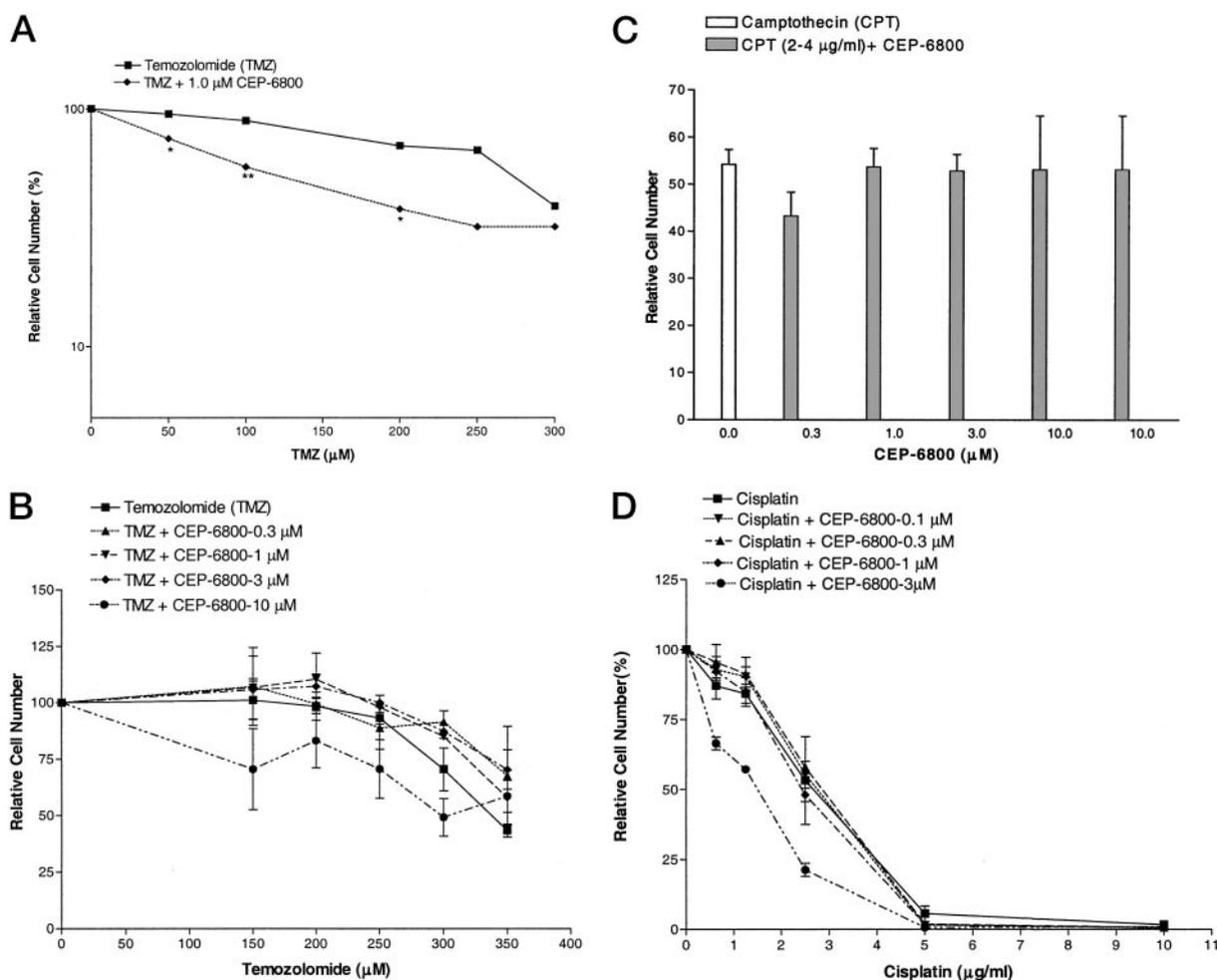


Fig. 4. Effects of CEP-6800 in combination with chemotherapeutic agents on the toxicity of normal human cells. U251MG human glioblastoma cells (A; 2000 cells/well), FHS74 cells (B and C; 3000/well), or normal human renal cells (D; 6000/well) were treated with CEP-6800 [1 μM (A); 0.3–10 μM (B and C); 0.1–3 μM (D)] in combination with TMZ (A and B; 150–350 μM), CPT (C; 2–4 μg), or cisplatin (D; 0.625–10 μg/ml) for 72 h 37°C. Effects on cell proliferation were assayed after a 1-h (U251MG), 2-h (FHS74 INT cells), or a 5-h (renal epithelial cells) incubation (in MTS solution; Promega). The absorbance was read using a microtiter plate reader at 490 nm; bars, ±SE.

40-h time point (Fig. 3C). In all three of the cell lines used for these studies, CEP-6800 exposure alone had no significant effects on the cell cycle in the tumor cells evaluated.

In Vitro Analyses for Potentiation of Chemotherapeutic Induced Toxicity of Human Carcinoma Cells and Normal Human Cells. The ability of CEP-6800 to exacerbate CPT, TMZ, and cisplatin-mediated toxicities was evaluated using human carcinoma cells, normal human intestinal cells (CPT and TMZ), and normal human renal epithelial cells (cisplatin) in a modified MTS assay. Coincubation of TMZ (50–200 μM) with 1 μM CEP-6800 resulted in significant (21–45%; $P \leq 0.05$ –0.01) potentiation of U251MG tumor cell growth inhibition as compared with the corresponding concentrations of TMZ alone (Fig. 4A). Similarly, 1 μM CEP-6800 in combination with CPT was able to potentiate HT29 tumor cell growth inhibition as compared with CPT treatment alone (data not shown). CEP-6800 was not able to potentiate the cisplatin-induced inhibitory effects on Calu-6 tumor cell growth (data not shown).

To demonstrate that CEP-6800 did not exert toxic effects on normal human intestinal cells or renal cells, we incubated normal intestinal cells with TMZ (50–350 μM) or CPT (2–4 μg/ml) in combination with CEP-6800 (0.3–10 μM), and normal renal cells with cisplatin (0.625–10 μg/ml) and CEP-6800 (0.1–3 μM). Incubation of TMZ (50–350 μM) or CPT (2–4 μg/ml) in combination with CEP-6800 (0.3–10 μM) did not affect normal intestinal cell proliferation as compared with TMZ or CPT alone (Fig. 4, B and C). Similarly, incubation of cisplatin (0.625–10 μg/ml) with CEP-6800 (0.1–3 μM) had no effect on increasing toxicity in normal renal cells compared with cisplatin alone (Fig. 4D).

Incubation of CEP-6800 at low concentrations (0.1–3 μM) alone had minimal or no effect on the inhibition of U251MG and HT29 cell or normal intestinal or kidney cell growth (data not shown). However, incubation of >3 μM CEP-6800 alone on Calu-6 cells did result in tumor growth inhibition (data not shown). Similarly, 10 μM CEP-6800 did inhibit tumor cell and normal intestinal cell growth, whereas 3 μM CEP-6800 inhib-

ited the growth of normal renal cells (data not shown). These *in vitro* concentrations of CEP-6800 exceeded those observed in plasma (1–2 μM) that were associated with potentiation of the antitumor efficacy of TMZ, irinotecan, and cisplatin in the absence of apparent toxicity in tumor-bearing mice (see data below).

PAR Accumulation Assay. The ability of CEP-6800 to attenuate irinotecan and TMZ-induced PAR accumulation was evaluated using HT29 and LoVo colon carcinoma xenografts, respectively. The LoVo human colon carcinoma cell line was used to evaluate the *in vivo* biochemical efficacy based on data published previously using this cell line for *in vivo* proof-of-concept experiments (7). Preliminary experiments were performed to determine the dose of irinotecan or TMZ and the time point that gave the highest increase in PAR accumulation as an indicator of maximal activation of PARP-1 *in vivo* (data not shown). Irinotecan (10 mg/kg, i.p.) monotherapy resulted in a 26% increase in PAR accumulation in HT29 xenografts as compared with vehicle-treated controls by 4 h after irinotecan dosing (Fig. 5A). Administration of CEP-6800 1 h after irinotecan treatment resulted in a significant (48%; $P \leq 0.05$) decrease of PAR accumulation at 4 h after irinotecan dosing as compared with irinotecan monotherapy (Fig. 5A). Similarly, TMZ monotherapy resulted in a 26% increase ($P \leq 0.01$) in PAR accumulation in LoVo xenografts as compared with vehicle-treated controls by 4 h after TMZ dosing (Fig. 5B). CEP-6800 (30 mg/kg, s.c.) administered 1 h after TMZ dosing significantly inhibited (25%; $P \leq 0.001$) PAR accumulation as compared with TMZ monotherapy (Fig. 5B).

In these studies, when administered as a monotherapy to HT29 tumor-bearing animals, CEP-6800 reduced in a non-statistically significant manner the baseline levels of PAR accumulation in tumors (Fig. 5A). Similarly, in LoVo xenografts CEP-6800 monotherapy had marginal effects on baseline PAR accumulation (Fig. 5B). These combined biochemical efficacy data suggest that CEP-6800 is targeting PARP-1 activated *in vivo*, and can effectively attenuate irinotecan- and TMZ-induced PAR accumulation in HT29 and LoVo xenografts, respectively.

Chemopotentiation of Irinotecan Using HT29 Human Colon Carcinoma Xenografts.

The ability of CEP-6800 to potentiate the antitumor efficacy of the topoisomerase I inhibitor irinotecan was evaluated using the irinotecan-resistant HT29 human colon carcinoma xenografts in nude mice (30). Tumor-bearing nude mice were treated with vehicle (0.9% sterile saline, i.p., QD, 100 μl /dose), irinotecan (10 mg/kg/dose, i.p., QD, 5 days) as a monotherapy, or 1 h before treatment with CEP-6800 (30 mg/kg/dose, s.c., QD, 5 days). In this study, irinotecan or CEP-6800 monotherapy did not have any significant effects on tumor growth (Fig. 6). In contrast, the combination of irinotecan and CEP-6800 demonstrated a statistically significant reduction of tumor volume (38%; $P \leq 0.05$) compared with irinotecan alone beginning on day 19 and extending to day 33, with a maximal tumor volume reduction 60% ($P \leq 0.01$; Fig. 6).

Chemopotentiation of TMZ Using U251MG Human Glioblastoma Xenografts. The ability of CEP-6800 to potentiate the antitumor efficacy of the methylating agent TMZ

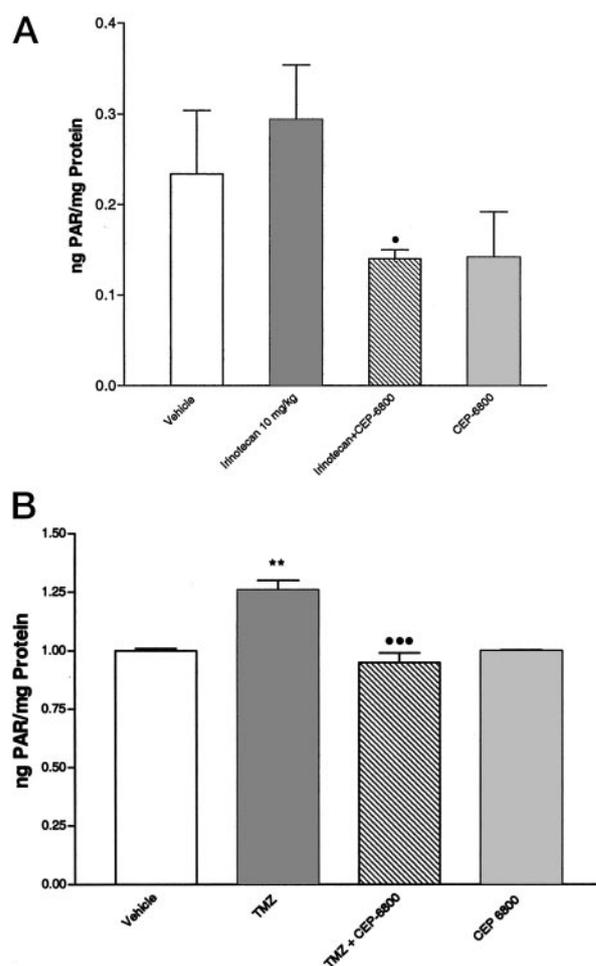


Fig. 5. Effects of CEP-6800 on the *in vivo* chemotherapeutic agent induced PAR accumulation in human colon carcinoma xenografts. Nude mice bearing HT29 (A) or LoVo (B) xenografts were administered one dose of irinotecan (10 mg/kg, i.p.) or TMZ (68 mg/kg, p.o.) alone, and 1 h before dosing with one dose of CEP-6800 (30 mg/kg, s.c.). Mice were sacrificed 4 h after dosing with the chemotherapeutic agent. Proteins were extracted from the tumor tissues and analyzed using an ELISA-based format to detect PAR accumulation as described in "Materials and Methods." **, $P \leq 0.01$, TMZ alone as compared with vehicle control; *, $P \leq 0.05$; ***, $P \leq 0.001$ chemotherapeutic agent alone as compared with combination treatment by *t* test; bars, \pm SE.

was evaluated using U251MG human glioblastoma xenografts in nude mice. Tumor-bearing nude mice were treated with vehicle (0.9% sterile saline, p.o., QD, 100 μl /dose), TMZ (17 or 34 mg/kg/dose, p.o., QD, 5 days) as a monotherapy, or 1 h before treatment with CEP-6800 (30 mg/kg/dose, s.c., QD, 5 days). TMZ monotherapy caused complete tumor regression in 60% of the mice (Fig. 7) by the termination of the study (day 28). The combination of TMZ at both doses administered plus CEP-6800 resulted in a 100% incidence of complete tumor regressions at the completion of the study as compared with 60% complete regressions as a result of TMZ treatment alone ($P \leq 0.05$; Fig. 7). CEP-6800 monotherapy did not have any significant effects on tumor growth or regressions using this dosing schedule.

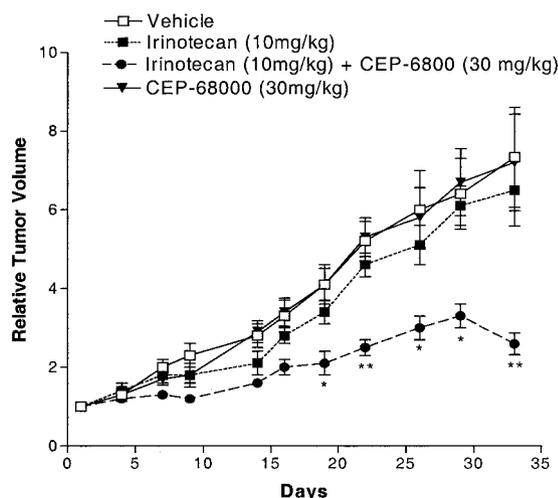


Fig. 6. Chemopotentiation of the antitumor efficacy of irinotecan on HT29 human colon carcinoma xenografts. Athymic nude mice were injected s.c. with 2×10^6 HT29 tumor cells. Upon xenografts reaching a mean tumor volume of ~ 250 mm³, mice were treated with vehicle (0.9% sterile saline), irinotecan alone (10 mg/kg/dose, i.p., QD, 5 days), irinotecan (10 mg/kg/dose, i.p., QD, 5 days) plus CEP-6800 (30 mg/kg/dose, s.c., QD, 5 days), or CEP-6800 alone (30 mg/kg/dose, s.c., QD, 5 days). In the combination group, CEP-6800 was administered 1 h after irinotecan dosing. Tumor volumes were determined every 3–4 days. Tumor volumes were normalized to those at the initiation of dosing. Values are mean relative tumor volume; *, $P \leq 0.05$; **, $P \leq 0.01$, irinotecan alone as compared with combination treatment by Mann-Whitney rank sum test or *t* test where appropriate; bars, \pm SE.

Chemopotentiation of Cisplatin Using Calu-6 Human Non-Small Cell Lung Carcinoma Xenografts. The ability of CEP-6800 to potentiate the antitumor efficacy of the DNA cross-linking agent cisplatin was evaluated using Calu-6 human non-small cell lung carcinoma xenografts in nude mice. Tumor-bearing nude mice were treated with vehicle (0.9% sterile saline, i.p., QD, 100 μ l/dose), cisplatin (5 mg/kg/dose, i.p., QD, days 1 and 14) as a monotherapy, or 1 h before treatment with CEP-6800 (30 mg/kg/dose, s.c., QD, days 1 and 14). Cisplatin therapy alone on this dosing regime caused a significant and sustained inhibition of tumor xenograft growth as compared with vehicle-treated tumors beginning on day 7 (55%; $P \leq 0.001$) and extending to day 28 (76%; $P \leq 0.001$), the termination of the study. The combination treatment of cisplatin plus CEP-6800 resulted in a statistically significant reduction of tumor volume as compared with cisplatin monotherapy (32%; $P \leq 0.05$) beginning on day 11 and extending to day 28 (35%; $P \leq 0.05$; Fig. 8). CEP-6800 monotherapy did not have any significant effects on tumor growth using this dosing schedule.

In Vivo Toxicity. On the basis of previous data indicating that the most common nonhematological side effect of TMZ treatment was gastrointestinal toxicity (31) and that the major reported toxicity from cisplatin treatment is nephrotoxicity (32), we evaluated intestinal tissues from all of the mice receiving TMZ and/or CEP-6800, and kidney tissue from all of the mice receiving cisplatin and/or CEP-6800 to determine whether the combination treatment exacerbated the effects of the single agent alone. In addition, we obtained serum samples from mice receiving cisplatin and/or CEP-6800 to

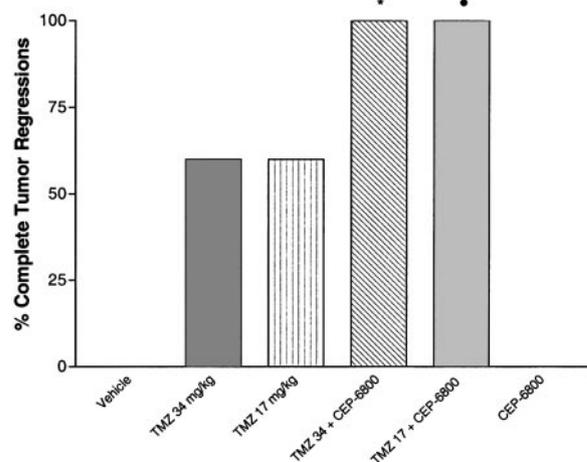


Fig. 7. Chemopotentiation of the antitumor efficacy of TMZ on U251MG human glioblastoma xenografts. Athymic nude mice were injected s.c. with 5×10^6 U251MG tumor cells. Upon xenografts reaching a mean tumor volume of ~ 160 mm³, mice were treated with vehicle (0.9% sterile saline), TMZ alone (17 or 34 mg/kg/dose, p.o., QD, 5 days), TMZ (17 or 34 mg/kg/dose, p.o., QD, 5 days) plus CEP-6800 (30 mg/kg/dose, s.c., QD, 5 days), or CEP-6800 alone (30 mg/kg/dose, s.c., QD, 5 days). In the combination group, CEP-6800 was administered 1 h after TMZ dosing. Tumor volumes were determined every 3–4 days. Tumor volumes were normalized to those at the initiation of dosing. Values are percentage of complete tumor regression by day 28. *, $P \leq 0.05$ -TMZ (17 mg/kg/dose) as compared with TMZ (17 mg/kg/dose) plus CEP-6800; ●, $P \leq 0.05$ TMZ (34 mg/kg/dose) as compared with TMZ (34 mg/kg/dose) plus CEP-6800 by *t* test.

evaluate for serum levels of indicators of kidney function. Histological evaluation of intestines from mice treated with TMZ alone, or of kidneys from mice treated with cisplatin alone or in combination with CEP-6800 revealed no indications of chemotherapeutic-induced toxicity in these tissues (data not shown). In addition, all of the serum levels for kidney function were within normal limits (data not shown).

In general the combination treatment of TMZ (5 days), irinotecan (5 days), or cisplatin (days 1 and 14) and CEP-6800 was well tolerated. Dosing with TMZ or cisplatin in combination with CEP-6800 resulted in significant body weight loss (10–16%; $P \leq 0.05$ –0.001); however, the body weight loss observed was reversible over a 5–7-day period after dosing (*i.e.*, returning to body weight levels of vehicle-treated controls; data not shown). In addition, no mortality was observed in any of the treatment groups. These combined data indicate that the dosing schedules are well tolerated, and the administration of CEP-6800 exhibits sustained and significant potentiation of the antitumor efficacy of TMZ, irinotecan, and cisplatin in U251MG human glioblastoma, HT29 human colon carcinoma, and Calu-6 human non-small cell carcinoma xenografts, respectively.

Discussion

The evaluation of PARP inhibitors as radio- and chemopotentiation has been ongoing for >20 years based on an extensive body of biochemical and genetic evidence linking PARP-1 (10, 12, 13) and more recently PARP-2 (33) in DNA damage and repair mechanisms in response to genotoxic

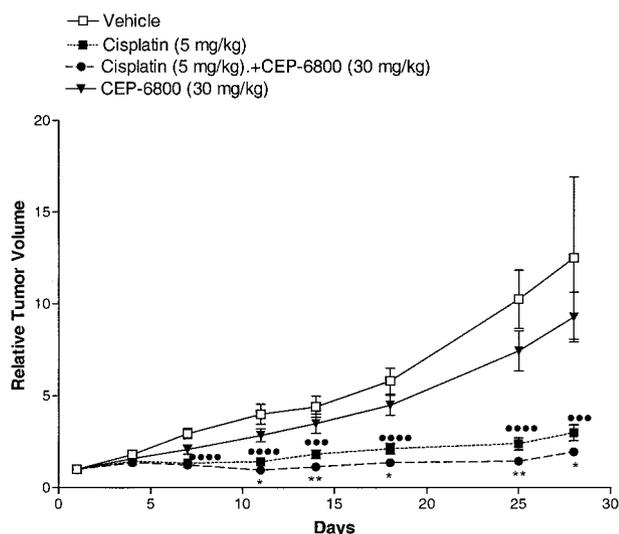


Fig. 8. Chemopotentiation of the antitumor efficacy of cisplatin on Calu-6 human non-small cell lung carcinoma xenografts. Athymic nude mice were injected s.c. with 3×10^6 Calu-6 tumor cells. Upon xenografts reaching a mean tumor volume of $\sim 145 \text{ mm}^3$, mice were treated with vehicle (0.9% sterile saline), cisplatin alone (5 mg/kg/dose, i.p., QD, days 1 and 14), cisplatin (5 mg/kg/dose, i.p., QD, days 1 and 14) plus CEP-6800 (30 mg/kg/dose, s.c., QD, days 1 and 14), or CEP-6800 alone (30 mg/kg/dose, s.c., QD, days 1 and 14). In the combination group, CEP-6800 was administered 1 h after cisplatin dosing. Tumor volumes were determined every 3–4 days. Tumor volumes were normalized to those at the initiation of dosing. Values are mean relative tumor volume. ●●●, $P \leq 0.001$, ●●●●, $P \leq 0.0001$, cisplatin alone as compared with vehicle; *, $P \leq 0.05$; **, $P \leq 0.01$, cisplatin alone as compared with combination treatment by Mann-Whitney rank sum test or t test where appropriate; bars, \pm SE.

insults (reviewed in Refs. 1, 3). The potential application of PARP inhibitors therapeutically as chemo- and radiopotentiators has, until relatively recently, been limited by the potency, selectivity, and pharmaceutical properties of these agents (2, 6, 9–14, 32). More potent and selective PARP inhibitors (benzimidazole-4-carboxamides and quinazolin-4-[3H]-ones) have been developed recently that have demonstrated the ability to potentiate the effects of radiation and of chemotherapeutic agents such as CPT, topotecan, irinotecan, cisplatin, etoposide, bleomycin, 1,3-bis(2-chloroethyl)-1-nitrosourea, and TMZ *in vitro* and *in vivo* using both human and murine tumor models of leukemia, lymphoma metastases to the central nervous system, and colon, lung, and breast carcinomas (6, 10, 12–15).

In this study, we evaluated the effects of CEP-6800 (C_3 -aminomethyl carbozole imide), a potent and selective PARP inhibitor (enzyme-based IC_{50} of 17 nM and a cell-based IC_{50} of 300 nM), on the extent and duration of DNA damage, repair, G_2/M cell cycle arrest, and *in vivo* biochemical and antitumor efficacy induced by three mechanistically distinct chemotherapeutic agents in clinically relevant tumor xenograft models. The chemotherapeutic agents used in these studies, the alkylating agent TMZ, the topoisomerase I inhibitor irinotecan, and the DNA intercalating agent cisplatin, were chosen based on their ability to activate PARP-1 *in vitro* in tumor cells, induce DNA lesions *in vitro*, and/or enhance the antitumor efficacy of known PARP inhibitors (benzamide and nicotinamide analogues and quinazolines; Refs. 2, 3, 6,

7, 13, 14). As stated previously, PARP inhibitors have been demonstrated to potentiate a wide variety of mechanistically distinct chemotherapeutic agents. The tumor models (U251MG, HT29, and Calu-6) used to evaluate these agents in combination with CEP-6800 were chosen based on clinical relevance and previous data demonstrating an *in vivo* response to the specific chemotherapeutic agent (21, 23, 34).

In these studies, we provide evidence that the PARP-1 inhibitor CEP-6800 attenuated PARP-1 activation (as measured by PAR accumulation) induced in colon tumor xenografts by exposure to TMZ and irinotecan. Evidence exists demonstrating that disruption of PARP-1-mediated base excision repair mechanisms induced by TMZ and PARP-1-mediated ADP-ribosylation of topoisomerase I in response to CPT exposure, may be responsible for the potentiation of the cytotoxic effect of these chemotherapeutic agents on otherwise resistant tumor cell types (6, 14, 15). The direct observations of *in vivo* biochemical efficacy of CEP-6800 on modulating PARP-1 activation in tumors in response to TMZ (and irinotecan) in a time and dose-related manner, and the observation that CEP-6800 can attenuate baseline PAR accumulation, are consistent with the proposed PARP-1-mediated mechanism of action of CEP-6800 as a chemopotentiating agent.

Potentiation of DNA strand breaks and/or disruption of DNA repair in tumor cells have been observed on exposure to earlier generations of less selective and less potent PARP-1 inhibitors (2, 9, 10). In the *in vitro* studies described here, U251MG glioblastoma, HT29 colon carcinoma, and Calu-6 non-small cell lung carcinoma cells were exposed to CEP-6800 and chemotherapeutic agents in the comet single cell electrophoresis assay, to assess time- and dose-dependent effects of DNA strand breaks and the kinetic of DNA repair. Exposure of tumor cells to TMZ, CPT, and cisplatin before incubation with CEP-6800 had profound effects on the onset of DNA damage, the magnitude of damage, and the duration for cells to effect repair of DNA damage, depending on the chemotherapeutic agent and tumor cell type. In no instance did exposure to CEP-6800 alone induce DNA strand breaks in cells. In general, the kinetics and magnitude of DNA strand breaks observed with the concentrations of TMZ, CPT, and cisplatin evaluated *in vitro* were consistent with that observed previously with these agents in other tumor cell types including TMZ in combination with L1210 murine leukemia cells, CPT in combination with L5178Y murine lymphoma cells, and cisplatin in combination with O-342 rat ovarian tumor cells (5, 18, 27). In the case of CPT and cisplatin exposure on DNA damage, in HT29 and Calu-6 cells, respectively, CEP-6800 potentiated DNA strand breaks significantly during a relatively narrow time frame of exposure. In contrast, exposure of U251MG cells to TMZ and CEP-6800 potentiated significantly the magnitude and the onset of appearance of DNA strand breaks, and the duration of time in which significant DNA damage was observed relative to that observed on exposure to TMZ alone. In U251MG cells treated with TMZ and CEP-6800, and HT29 cells treated with CPT and CEP-6800, decreases in the amount of DNA strand breaks were observed between 4 and 12 h. This observation may be an attempt of the cells to repair their damaged DNA; however, because of the continuous exposure to CEP-6800, the cells were unable to effectively repair the damage, which may ultimately lead to the

death of the cells. These data with TMZ and CEP-6800 in particular, are suggestive that PARP-1 inhibition delays the repair of DNA strand breaks on exposure of cells to alkylating agents, findings supported by similar observations in PARP-1 and PARP-2 knockout mice exposed to alkylating agents, and consistent with the role of PARP-1 and -2 in base excision repair (29, 33).

A number of laboratories have demonstrated that inhibition of PARP-1 using PARP-/- immortalized mouse embryonic fibroblasts in combination with the alkylating agent methylnitrosourea or radiation results in accumulation of tumor cells with DNA damage at the G₂/M boundary of the cell cycle (1, 29). These data are suggestive for a role of PARP-1 inhibition in promoting genomic instability in cancer cells exposed to DNA damaging agents. The present studies expanded on these observations, demonstrating that the effects of PARP-1 inhibition with CEP-6800 exposure *in vitro* on the onset, magnitude, and duration of tumor cell accumulation at the G₂/M boundary is both cell type- and chemotherapeutic agent-dependent, as observed by other laboratories using glioblastoma cell lines (35). In this instance, the most profound effects were again observed with TMZ and CEP-6800 in glioblastoma cells. Exposure to TMZ and CEP-6800 resulted in a pronounced shift in time for the significant accumulation of tumor cells at G₂/M, from 40 h with TMZ alone, to 16 h with TMZ and CEP-6800. Although exposure to CEP-6800 and TMZ did not potentiate the fraction of cells accumulating in G₂/M relative to that maximally achieved with TMZ exposure alone, the duration of time cells remained at the G₂/M boundary was prolonged by coexposure to TMZ and CEP-6800 relative to cells exposed to TMZ alone. In contrast, CEP-6800 potentiated the effects of SN38 on HT29 colon carcinoma cells arrested in G₂/M relative to that observed with SN38 alone, but did not affect the kinetics of accumulation in or the exit from the G₂/M boundary. The current studies demonstrated that the effects of CEP-6800 in cisplatin-mediated G₂/M arrest in Calu-6 cells were subtle, affecting the onset of accumulation of cisplatin-treated cells in G₂/M, but neither potentiating nor prolonging the accumulation of tumor cells at G₂/M. In all instances, CEP-6800 exposure in the absence of genotoxic insult had no significant cell cycle effects on any of the tumor cell types examined.

The observed biochemical efficacy of CEP-6800 on attenuating PARP-1 in tumors by TMZ and irinotecan *in vivo*, and the cell type-dependent and chemotherapeutic agent-dependent effects of PARP-1 inhibition by CEP-6800 *in vitro* on DNA damage and G₂/M arrest in tumor cells would be predicted to result in significant chemopotentialization of antitumor efficacy relative to that observed with each of the distinct chemotherapeutic agents evaluated in relevant preclinical tumor models. This was the case in the studies described here, where CEP-6800 was administered to mice at a dose predicted to achieve plasma concentrations (1–1.5 μM) 3–4-fold above the cell-based IC₅₀ for PARP-1 inhibition by this compound (300 nM). This dose was administered with active, albeit suboptimal doses, of each chemotherapeutic agent in proof-of-concept experiments to discern the degree and extent of chemopotentialization possible without causing toxicity to the mice.

The combination of CEP-6800 with all of the chemotherapeutics generated a statistically significant response in all of the

tumor models; however, the magnitude of the response varied between each model. Treatment with TMZ at both doses administered (17 and 34 mg/kg/dose) in combination with CEP-6800 resulted in 100% complete regression by the termination of the study as compared with 60% complete regression caused by TMZ (17 and 34 mg/kg/dose) monotherapy. Also, U251MG tumor xenografts in the mice receiving the combination of TMZ (34 mg/kg/dose) with CEP-6800 began to regress ~5 days earlier than with TMZ monotherapy (data not shown). In contrast, CEP-6800 in combination with irinotecan (10 mg/kg/dose ×5 days) did not result in HT29 xenograft regression; however, the combination treatment did result in a significant 60% inhibition of tumor growth by the end of the experiment as compared with irinotecan monotherapy. The dosing schedule used for the irinotecan monotherapy did not cause tumor growth inhibition in this xenograft model. The combination therapy of cisplatin (5 mg/kg/dose days 1 and 14) plus CEP-6800 caused a significant and sustained (35%) reduction in tumor growth as compared with cisplatin monotherapy. Interestingly, in this model the response to cisplatin monotherapy was quite robust; a 76% inhibition of tumor growth as compared with vehicle was observed using this dosing schedule. These combined data indicate that CEP-6800 can potentiate significantly the actions of three mechanistically distinct chemotherapeutics in proof-of-concept studies. Additional dose- and schedule-dependent optimization of CEP-6800 and chemotherapeutic agents may be required in some models to observe a maximal chemopotentialization response.

An important characteristic of a chemopotentializer is that it should not cause any toxicity on its own or exacerbate any toxicity associated with the chemotherapeutic agent. The toxicities associated with the chemotherapeutic agents used in these studies are myelosuppression, gastrointestinal (TMZ, irinotecan, and CPT), and nephrotoxicity (cisplatin; Refs. 31, 36, 37). As demonstrated through both *in vitro* and/or *in vivo* analyses, CEP-6800 did not exacerbate TMZ- or CPT- (*in vitro* analyses only) induced intestinal toxicity or cisplatin-induced nephrotoxicity. The data from the preliminary toxicity studies in combination with the antitumor efficacy data suggest that the dosing schedules used are well tolerated, and that the administration of CEP-6800 exhibits sustained and significant potentiation of the antitumor efficacy in three clinically relevant tumor models.

In conclusion, our data provide a comprehensive preclinical *in vitro* and *in vivo* evaluation of CEP-6800, a prototype in the carbazole imide family of PARP inhibitors. The effects of CEP-6800 in combination with three mechanistically distinct chemotherapeutic agents on DNA damage/repair, cell cycle arrest at the G₂/M boundary, and *in vivo* antitumor efficacy, along with its acceptable pharmacological profile, make it a potential candidate for use as a chemopotentializing agent in a wide variety of cancers where these agents are used as a standard of care.

References

1. D'Amours, D., Desnoyers, S., D'Silva, I., and Poirier, G. G. Poly(ADP-ribose)ylation reactions in the regulation of nuclear functions. *Biochem. J.*, 342: 249–268, 1999.
2. Griffin, R. J., Curtin, N. J., Newell, D. R., Golding, B. T., Durkacz, B. W., and Calvert, A. H. The role of inhibitors of poly(ADP-ribose) polymerase as resistance-modifying agents in cancer therapy. *Biochimie*, 77: 408–422, 1995.

3. Tentori, L., Portarena, I., and Graziani, G. Potential clinical applications of poly(ADP-ribose) polymerase (PARP) inhibitors. *Pharmacol. Res.*, 45: 73–85, 2002.
4. Smith, S. The world according to PARP. *Trends Biochem. Sci.*, 26: 174–179, 2001.
5. Bürkle, A., Chen, G., Küpper, J.-H., Grube, K., and Zeller, W. J. Increased poly(ADP-ribosylation) in intact cells by cisplatin treatment. *Carcinogenesis (Lond.)*, 14: 559–561, 1993.
6. Delaney, C. A., Wang, L.-Z., Kyle, S., White, A. W., Calvert, A. H., Curtin, N. J., Durkacz, B. W., Hostomsky, Z., and Newell, D. R. Potentiation of temozolomide and topotecan growth inhibition and cytotoxicity by novel poly(adenosine diphosphoribose)polymerase inhibitors in a panel of human tumor lines. *Clin. Cancer Res.*, 6: 2860–2867, 2000.
7. Canan-Koch, S., Thoresen, L., Tikhe, J., Maegley, K., Li, J., Yu, X.-H., Almassy, R., Zhang, K., Zook, S., Kumpf, R., Zhang, R., Curtin, N. J., Delaney, C. A., Kyle, S., Thomas, H., Wang, L.-Z., Newell, D. R., Hostomsky, Z., and Webber, S. Structure activity relationship, crystal structure, and biological evaluation of potent poly(ADP-ribose) polymerase-1 inhibitors. *Am. Chem. Soc. 220th National Meeting, MEDI-007*: 2000.
8. Masutani, M., Nozaki, T., Nakamoto, K., Nakagama, H., Suzuki, H., Kusuoka, O., Tsutsumi, M., and Sugimura, T. The response of Parp knockout mice against DNA damaging agents. *Mutat. Res.*, 462: 159–166, 2000.
9. Kato, T., Suzumura, Y., and Fukushima, M. Enhancement of bleomycin activity by 3-aminobenzamide, a poly(ADP-ribose) synthesis inhibitor, *in vitro* and *in vivo*. *Anticancer Res.*, 8: 239–244, 1988.
10. Griffin, R. J., Srinivasan, S., Bowman, K. J., Calvert, A. H., Curtin, N. J., Newell, D. R., Pemberton, L. C., and Golding, B. T. Resistance-modifying agents. 5. Synthesis and biological properties of quinazolinone inhibitors of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP). *J. Med. Chem.*, 5247–5256, 1998.
11. Mattern, M. R., Mong, S.-M., Bartus, H. F., Mirabelli, C. K., Crooke, S. T., and Johnson, R. K. Relationship between the intracellular effects of camptothecin and the inhibition of DNA topoisomerase I in cultured L1210 cells. *Cancer Res.*, 47: 1793–1798, 1987.
12. Bowman, K. J., White, A., Golding, B. T., Griffin, R. J., and Curtin, N. J. Potentiation of anti-cancer agent cytotoxicity by the potent poly(ADP-ribose)polymerase inhibitors NU 1025 and NU 1064. *Br. J. Cancer*, 78: 1269–1277, 1998.
13. Bowman, K. J., Newell, D. R., and Curtin, N. J. Differential effects of the poly(ADP-ribose) polymerase (PARP) inhibitor NU 1025 on topoisomerase I and II inhibitor cytotoxicity in L1210 cells *in vitro*. *Br. J. Cancer*, 84: 106–112, 2001.
14. Liu, L., Taverna, P., Whitacre, C. M., Chatterjee, S., and Gerson, S. L. Pharmacologic disruption of base excision repair sensitizes mismatch repair-deficient and -proficient colon cancer cells to methylating agents. *Clin. Cancer Res.*, 5: 2908–2917, 1999.
15. Tentori, L., Leonetti, C., Scarsella, M., d'Amati, G., Portarena, I., Zupi, G., Bonmassar, E., and Graziani, G. Combined treatment with temozolomide and poly(ADP-ribose) polymerase inhibitor enhances survival of mice bearing hematologic malignancy at the central nervous system site. *Blood*, 15: 2241–2244, 2002.
16. Ator, M., Bihovsky, R., Chatterjee, S., Dunn, D., and Hudkins, R. Novel multicyclic compounds and their use thereof. Patent WO 01/85686 A2, 2001.
17. Jansen, J. M., Zwart, B., Hulscher, S. T. M., Giaccone, G., Pinedo, H. M., and Boven, E. CPT-11 in human colon-cancer cell lines and xenografts: characterization of cellular sensitivity determinants. *Int. J. Cancer*, 70: 335–340, 1997.
18. Boulton, S., Pemberton, L. C., Curtin, N. J., Griffin, R. J., Golding, B. T., and Durkacz, B. W. Potentiation of temozolomide-induced cytotoxicity: a comparative study of the biological effects of poly(ADP) polymerase inhibitors. *Br. J. Cancer*, 72: 849–856, 1995.
19. Hirose, H., Berger, M. S., and Pieper, R. O. p53 effects both the duration of G₂/M arrest and the fate of temozolomide-treated human glioblastoma cells. *Cancer Res.*, 61: 1975–1963, 2001.
20. Links, M., Ribeiro, J., Friedlander, M., and Russell, P. J. Regulation and deregulation of G2 checkpoint proteins with cisplatin. *Anticancer Res.*, 18: 4057–4066, 1998.
21. te Poele, R. H., and Joel, S. P. Schedule-dependent cytotoxicity of SN-38 in p53 wild-type and mutant colon adenocarcinoma cell lines. *Br. J. Cancer*, 81: 1285–1293, 1999.
22. Birchler, M., Fiocchi, E. M., and Jones, B. E. A quantitative method for determining *in vivo* activation of PARP and biological activity of inhibitors of the enzyme. *Society of Neurosci. Abs.*, 26: 17, 2000.
23. Schiller, J. H., and Bittner, G. Potentiation of platinum antitumor effects in human lung tumor xenografts by the angiogenesis inhibitor squalamine: Effects on tumor neovascularization. *Clin. Cancer Res.*, 5: 4287–4294, 1999.
24. Houghton, P. J., Stewart, C. F., Cheshire, P. J., Richmond, L. B., Kristein, M. N., Poquette, C. A., Tan, M., Friedman, S., and Brent, T. P. Antitumor activity of temozolomide combined with irinotecan is partly independent of O⁶-Methylguanine-DNA methyltransferase and mismatch repair phenotypes in xenograft models. *Clin. Cancer Res.*, 6: 4110–4118, 2000.
25. Dionne, C. A., Camoratto, A. M., Emerson, E., Neff, N., Vaught, J., Murakata, C., Djakiew, D., Lamb, J., George, D., and Isaacs, J. T. Cell cycle-independent death of prostate adenocarcinoma is induced by the trk tyrosine kinase inhibitor CEP-751 (KT6587). *Clin. Cancer Res.*, 4: 1887–1898, 1998.
26. Thompson, J., Zamboni, W. C., Cheshire, P. J., Lutz, L., Luo, X., Li, Y., Houghton, J., Stewart, C. F., and Houghton, P. J. Efficacy of systemic administration of irinotecan against neuroblastoma xenografts. *Clin. Cancer Res.*, 3: 423–431, 1997.
27. Gradzka, I., Skierski, J., and Szumiel, I. DNA repair, cell cycle progression and cell death following camptothecin treatment in two murine lymphoma L5178Y sublines. *Cell Biochem. Func.*, 16: 239–252, 1998.
28. Menissier-de Murcia, J., Niedergang, C., Trucco, C., Ricoul, M., Dutrillaux, B., Marks, M., Oliver, J., Masson, M., Dierich, A., LeMeur, M., Walztinger, C., Chambon, P., and de Murcia, G. Requirement of poly(ADP-ribose) polymerase in recovery from DNA damage in mice and cells. *Proc. Natl. Acad. Sci. USA*, 94: 7303–7307, 1997.
29. Trucco, C., Oliver, J., de Murcia, G., and Menissier-de Murcia, J. DNA repair defect in poly(ADP-ribose) polymerase-deficient cell lines. *Nucleic Acid Res.*, 26: 2644–2649, 1998.
30. Goldwasser, F., Bae, I., Valenti, M., Torres, K., and Pommier, Y. Topoisomerase I-related parameters and camptothecin activity in the colon carcinoma cell lines from the National Cancer Institute anticancer screen. *Cancer Res.*, 55: 2116–2121, 1995.
31. Brada, M., Judson, I., Beale, P., Reidenberg, P., Statkevich, P., Dugan, M., Batra, V., and Cutler, D. Phase I dose-escalation and pharmacokinetic study of temozolomide (SCH 52365) for refractory or relapsing malignancies. *Br. J. Cancer*, 81: 1022–1030, 1999.
32. Chen, G., and Pan, Q. Potentiation of the antitumor activity of cisplatin in mice by 3-aminobenzamide and nicotinamide. *Cancer Chemother. Pharmacol.*, 22: 303–307, 1988.
33. Schreiber, V., Ame, J.-C., Dolle, P., Schultz, I., Rinaldi, B., Fraulob, V., Menissier-de Murcia, J., and de Murcia, G. Poly(ADP-ribose) polymerase-2 (PARP-2) is required for efficient base excision DNA repair in association with PARP-1 and XRCC. *J. Biol. Chem.*, 277: 23028–23036, 2002.
34. Plowman, J., Waud, W. R., Koutsoukos, A. D., Rubinstein, L. V., Moore, T. D., and Grever, M. R. Preclinical antitumor activity of temozolomide in mice: efficacy against human brain tumor xenografts in synergism with 1, 3-Bis(2-chloroethyl)-1-nitrosourea. *Cancer Res.*, 54: 3793–3799, 1994.
35. Tentori, L., Portarena, I., Torino, F., Scerrati, M., Navarra, P., and Graziani, G. Poly(ADP-ribose) polymerase inhibitor increases growth inhibition and reduces G₂/M cell accumulation induced by Temozolomide in malignant glioma cells. *Glia*, 40: 44–54, 2002.
36. Furman, W. L., Stewart, C. F., Poquette, C. A., Pratt, C. B., Santana, V. M., Zamboni, W. C., Bowman, L. C., Ma, M. K., Hoffer, F. A., Meyer, W. H., Pappo, A. S., Walter, A. W., and Houghton, P. J. Direct translation of a protracted irinotecan schedule from a xenograft model to a phase I trial in children. *J. Clin. Oncol.*, 17: 1815–1824, 1999.
37. Racz, I., Tory, K., Gallyas F., Jr., Berente, Z., Osz, E., Laszlo, J., Bernath, S., Sumegi, B., Rablóczy, G., and Nagy-Literati, P. BGP-15- a novel poly(ADP-ribose) polymerase inhibitor-protects against nephrotoxicity of cisplatin without compromising its antitumor activity. *Biochem. Pharmacol.*, 63: 1099–1111, 2002.

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