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Reduced expression of DNA topoisomerase I in SF295 human glioblastoma cells selected for resistance to homocamptothecin and diflomotecan

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Running title: Reduced DNA Top1 in SF295 cells for drug resistance

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Abbreviations: Top1: DNA topoisomerase I; CPT: camptothecin; hCPT: homocamptothecin; BN80915, diflomotecan: 5-ethyl 9,10-difluoro-4,5,dihydro-5hydroxy-1H-oxepino[3',4':6,7]indolizino [1,2-b]quinoline-3,15[^{13}H]-dione.

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Abstract

Homocamptothecins (hCPTs) are a novel class of topoisomerase I (Top1) inhibitors with enhanced chemical stability compared to the currently used camptothecin (CPT) analogues, irinotecan and topotecan. The hCPT derivative, diflomotecan (BN80915), is currently in clinical trials. We established two resistant human glioblastoma cell lines, SF295/hCPT50 and SF295/BN50 by stepwise exposure of the parental SF295 line to increasing concentrations of hCPT and BN80915, respectively. The two resistant cell lines were 15- to 22-fold resistant to hCPT and BN80915 as well as 7- to 27-fold cross resistant to other Top1 inhibitors, including CPT, topotecan, and the indenoisoquinolines MJ-III-65 (NSC 706744) and NSC 724998, but sensitive to the topoisomerase II inhibitors mitoxantrone and etoposide. Neither of the resistant cell lines displayed any detectable expression of the three major drug transporters Pgp, MRP1 or ABCG2 as assessed by immunoblot or flow cytometry. Reduced expression of Top1 protein occurred at the transcriptional level in both of the resistant cell lines, consistent with the reduction of Top1 enzyme level as the major contribution to the resistance phenotype in SF295/hCPT50 and SF295/BN50 cells. Treatment of the resistant cell lines with the histone deacetylase inhibitor depsipeptide or the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine alone or concomitantly did not result in re-expression of Top1. Our studies suggest that selection for resistance to hCPT or BN80915 is primarily related to reduced Top1 expression at the transcriptional level, resulting in reduced enzyme levels.

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Introduction

Homocamptothecins (hCPTs) are a new class of camptothecin (CPT) derivatives. They are different from the classical CPT by bearing a seven-membered β-hydroxylactone ring instead of the naturally occurring six-membered α-hydroxylactone observed in CPT (see Fig. 1). hCPTs are therefore more stable than CPT derivatives (Lavergne et al., 2000a; Lavergne et al., 2000b) that are readily hydrolyzed to the inactive carboxylate forms [reviewed in (Pommier, 2006)]. hCPT, like other CPT analogues, inhibits Top1 by stabilizing the covalent complex between Top1 and DNA, leading to enzyme-linked DNA strand breaks, also referred to as cleavage complexes (Pommier, 2006). Because of their increased chemical stability, hCPTs were expected to exert greater Top1 inhibition and antitumor efficacy than the current FDA-approved CPT analogues, irinotecan and topotecan [for review, see Ref (Lavergne et al., 2000b; Pommier, 2006)]. hCPTs showed very potent antitumor efficacy in many tumors including brain glioblastoma mouse models. The fluorinated hCPT, diflomotecan (BN80915) is one of the most active compounds in terms of antiproliferative activity in the hCPT series (Bailly et al., 1999; Lavergne et al., 2000b). BN80915 has been selected for further development as a highly active hCPT-based Top1 poison with an optimal balance between potency and stability. Oral and i.v. formulations of BN80915 are currently undergoing clinical evaluation in Europe (Gelderblom et al., 2003; Troconiz et al., 2006).

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Glioblastoma is the most common malignant brain tumor in adults, and is among the most lethal of all cancers (Chamberlain, 2006). Chemotherapy is one of the accepted therapeutic strategies for glioblastoma multiform (GBM) (Chamberlain, 2006; Parney and Chang, 2003; Vassal et al., 2003). The Top1 inhibitor, CPT-11 (irinotecan) is used as a second line drug for the treatment of GBM. Response rates to this drug in glioblastoma patients have been 14% to 15% of cases, and stable disease has been achieved in 14% to 55% (Chamberlain, 2002; Cloughesy et al., 2003; Friedman et al., 1999).

In order to study potential mechanisms of resistance to hCPTs that may be encountered in the clinic, we established two drug-resistant human glioblastoma sublines, SF295/hCPT50 and SF295/BN50 cell lines by stepwise exposure of parental SF295 cells to increasing concentrations of hCPT and BN80915, respectively. Here, we report that resistance to hCPTs in the SF295/hCPT50 and SF295/BN50 sublines is associated with reduced expression of Top1 protein which occurs at the transcription level.

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Materials and Methods

Chemicals Homocamptothecin and BN80915 were obtained from Dr. Paola Principe, Beaufour-Ipsen, France. NSC724998 and MJ-III-65 were from the National Cancer Institute Anticancer Drug Screen (Bethesda, MD). Topotecan was from LKT Laboratories (St. Paul, MN). Mitoxantrone, camptothecin, etoposide and bromodeoxyuridine were purchased from Sigma (St Louis, MO). Calcein-AM was from Invitrogen Corporation (Carlsbad, CA). Pheophorbide a was purchased from Frontier Scientific (Logan, UT). Fumitremorgin C was isolated by Thomas McCloud, Developmental Therapeutics Program, Natural Products Extraction Laboratory, NIH (Bethesda, MD).

Cell Lines The parental SF295 human glioblastoma cell line was obtained from the NIH Anticancer Drug Screen and was maintained in RPMI medium supplemented with 10% fetal calf serum with penicillin/streptomycin. The drug-selected SF295/BN50 and SF295/hCPT50 sublines were developed by stepwise selection and are maintained in 50 nM BN80915 or hCPT, respectively. Human breast carcinoma MCF-7 cell lines were maintained in Richter's medium with 10% fetal calf serum and penicillin/streptomycin. ABCG2-overexpresing MCF-7/FLV1000 cells (Robey et al., 2001b) and multidrug resistance-assocaited protein 1 (MRP1)-overexpressing cells (Schneider et al., 1994) were additionally maintained in 1.0 μM flavopiridol or 4.0 μM etopside, respectively. P-glycoprotein (Pgp)-overexpressing MCF-7/Tx200 cells are maintained in 200 ng/ml

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paclitaxel (Robey et al., 2004). Human embryonic kidney cells (HEK293) transfected with *MDR1* (*ABCB1*), *MRP1* (*ABCC1*) or *ABCG2* were maintained in Eagle's Minimum Essential Medium with 10% fetal calf serum and penicillin/streptomycin (Robey et al., 2006). Expression of transporter proteins was enforced by addition of 2 mg/ml G418.

Cytotoxicity Assays Four-day cytotoxicity assays were performed using the sulforhodamine B assay (Robey et al., 2006). Briefly, cells were plated in flat-bottom, 96-well plates at a density of 2500 cells per well and allowed to attach for 24 hrs at 37°C in 5% CO_2. Chemotherapeutic agents at various concentrations were added to the cells and the plates were allowed to incubate for 96 hrs at 37°C in 5% CO_2. Cells were subsequently fixed in 50% trichloroacetic acid at 4°C for 1h after which the plates were washed in water and allowed to dry. The plates were subsequently stained with sulforhodamine B solution (0.4% sulforhodamine B w/v in 1% acetic acid) and washed in 1% acetic acid in water. Sulforhodamine B was then solubilized and optical densities were read on a Bio-Rad plate reader at an absorbance of 540 nm. Each concentration was tested in quadruplicate and controls were performed in replicates of eight.

DNA-protein Crosslinks (DPC) Analysis. DPCs induced by CPT were measured by alkaline elution assays (Kohn, 1996) in SF295 and SF295/BN cells. Proliferating cells in log phase were labeled with [^{3}H]-thymidine (0.02 μCi/ml) (Perkin-Elmer Life Science Co.,

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Boston, MA) for 48 h, chased for 4 h in isotope-free medium, and exposed to CPT for 1 h at indicated concentrations. Equal number of cells were loaded onto polyvinyl chloride/acrylic copolymers (PVC) membrane filters (2 μm pore size, 25 mm diameter; Nucleopore Corporation, Livermore, CA), lysed, and subjected to alkaline elution. Cells were lysed with 5 ml SDS-lysis solution (0.1 M glycine, 2% SDS, 0.025 M Na_2EDTA, pH 10.0). After washing with 0.02 M EDTA (pH 10.0), cell lysates were eluted by 40 ml of an eluting solution containing 0.02 M H_4EDTA, 2% tetrapropyl ammoniun hydroxide (Sigma, St. Louis, MO), pH 12.1. Five fractions were collected with about 5 ml in each. After collection, filters were placed in scintillation vials to which 0.4 ml of 1 N HCl was added in each vial. The vials were sealed and heated at 60°C for 1 h to depurinate the DNA. After removing the vials from the oven, 2.5 ml of 0.4 N NaOH was added for 1 h at room temperature to release the labeled DNA from the filters. Radioactivity in the elution fractions was counted by adding 10 ml of Aquassure (Packard Instrument Company Inc., Meriden, CT) containing 0.7% glacial acetic acid into each vial. Filter retention rate was calculated and plotted *vs* elution time. The formula is:

$$DPC = [(1 - r)^{-1} – (1 - r_0)^{-1}] \times 3000$$

where "r" is the retention for drug-treated cells and "r_0" the retention for cells that have been irradiated with 3000 rads (control cells).

Western Blot for Top1, Top2α, Top2β and Drug Transporter Expression. Cells were

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lysed at 4°C in buffer containing 1% SDS, 1 mM sodium vanadate, 10 mM Tris-HCl, pH 7.4, supplemented with protease inhibitors (Complete; Roche Diagnostics) and phosphatase inhibitors (Sigma, St Louis, MO). Viscosity of the samples was reduced by brief sonication, and 30 μg of protein were incubated in loading buffer (125 mM Tris-HCl, pH 6.8, 10% β-mercaptoethanol, 4.6% SDS, 20% glycerol, and 0.003% bromophenol blue), separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, MA). After blocking nonspecific binding sites for 1 h with 5% milk in PBS-T (phosphate-buffered saline, 0.5% Tween-20), the membrane was incubated for 1 h with primary antibody under the following conditions: mouse monoclonal anti-Top1 (C21 antibody from Dr. Yung-Chi Cheng at Yale university) at 1:1,000 dilution, rabbit anti-Top2α (Abcam, Cambridge, MA) at 1/10,000 dilution, mouse anti-Top2β, (BD biosciences, San Jose, CA) at 1/1,000 dilution or mouse anti-β-actin (Abcam) at 1:5,000 dilution. After three washes in PBS-T, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit (1:5,000 dilution) or anti-mouse (1:5,000 dilution) antibody (Amersham Biosciences, Buckhinhamshire, UK) for 1 h and then washed three times in PBS-T. Immunoblot was performed using an enhanced chemiluminescence detection kit (Pierce, Rockford, IL) by autoradiography.

For determination of drug transporter expression by immunoblot, microsomal membrane protein (30 μg) was obtained by nitrogen cavitation, separated by 7.5% (w/v)

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SDS-PAGE, and transferred to a PVDF membrane. The membrane was then probed with the anti-Pgp antibody C219 (Signet Laboratories, Deadham, MA) and subjected to enhanced chemiluminescence detection. After stripping with 0.2 M NaOH, the blot was subsequently probed with the anti-MRP1 antibody MRPm6 (Kamiya Biomedical, Seattle, WA) and the anti-ABCG2 antibody BXP-21 (Kamiya) in the same manner.

Flow Cytometry Determination of Drug Transporter Expression. Expression of drug transporters was determined by flow cytometry as previously described (Robey et al., 2004). Briefly, cells were incubated for 30 min in complete medium (phenol red-free Richter's medium with 10% FCS) containing 0.5 μM rhodamine with or without 3 μg/ml valspodar; 200 nM calcein-AM with or without MK-571; or 10 μM pheophorbide a with or without 10 μM fumitremorgin C (FTC) to determine P-glycoprotein, MRP1, or ABCG2 expression, respectively. Subsequently, cells were washed and incubated for 1 h in substrate-free medium continuing with or without inhibitor. Intracellular fluorescence of rhodamine, calcein or pheophorbide a was measured with a FACSort flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 488 nm argon laser and a 635 nm red diode laser. Fluorescence histograms were generated with CellQuest Software (BD Biosciences). At least 2 independent experiments were performed.

Real-time Quantitative PCR and Semi-quantitative RT-PCR. Cells were lysed and

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total RNA was extracted using RNAqueous-4PCR (Ambion, Austin, TX). Total RNA was reverse-transcribed using RETROscript kit (Ambion). Real-time quantitative PCR was done using ABsolute QPCR Mixes (Abgene, Rochester, NY) on an ABI 7900 real-time PCR instrument (AME Bioscience, Chicago, IL). Thermal cycling conditions were 50°C for 2 minutes, 95°C for 15 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Primers and probe sequences used for *Top1* were:

TGACAGCCCCGGATGAGA (sense), TGCAACAGCTCGATTGGC (antisense),

FAM- CATCCCAGCGAAGATCCTTTCTTATAACCG -TAMRA (probe), and for *18S RNA* were GATTAAGTCCCTGCCCTTTGTACA (sense), GATCCGAGGGCCTCACTAAAC (antisense), FAM-CGCCCGTCGCTACTACCGATTGG-TAMRA (probe). Gene expression was analyzed using Sequence Detection Systems software, version 1.7 (ABI PRISM). mRNA levels of *Top1* were normalized to the *18S RNA* internal standard. For semi-quantitative RT-PCR, amplification of cDNA was done using primers specific for *Top1* and *GAPDH*. Primers specific for *Top1* cDNA amplification were 5'-AGCCCAGACGGAAGC-3' (forward) and 5'-TCCAGGAAACCAGCCA-3' (reverse). The primer pair specific for *GAPDH* amplification were 5'-ACCACAGTCCATGCCATCAC (forward) and 5'-TCCACCACCCTGTTGCTGTA (reverse). Amplification of *GAPDH* cDNA served as an internal control. PCR amplification for the *Top1* and *GAPDH* mRNA was performed at an annealing temperature of 55^{o}C for 25 cycles to yield 320 and 440 bp

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products, respectively. The PCR products were resolved on 2% agarose gel, stained with ethidium bromide, and quantitated.

BrdU Incorporation and Cell Cycle Analysis. After a 30 minute pulse with 50 μM BrdU, the culture medium was removed and the cells were washed with PBS and incubated with fresh medium for the indicated times. Cells were then harvested by trypsinization and washed twice with PBS. The pellet containing 2 to 4 × 10^6 cells was suspended in 200 μl PBS and fixed with 5 ml of ice-cold 70% ethanol. After overnight storage at -20ºC, cells were centrifuged and the pellet was suspended in 3 ml 2 N HCl. After 30 minute incubation at room temperature (RT), the medium was neutralized by the addition of 6 ml 0.1 M sodium borate pH 8.5. Cells were centrifuged and the pellet washed twice in PBS containing 0.5% Tween-20 and 0.5% BSA. Cells were incubated with 15 μl anti BrdU-FITC (BD Biosciences) for 1 hour at RT in the dark. To determine DNA content, 500 μl of staining solution containing 20 μg/ml propidium iodide and 50 units of RNase A in PBS was added to the pellet. Cells were analyzed with an FACScan flow cytometer (BD Biosciences) using the CellQuest software (BD Biosciences).

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Results

Establishment and Characterization of hCPT- and BN80915-resistant Cells. hCPT and BN80915-resistant cells were generated from SF295 glioblastoma cells by a stepwise increase in exposure to hCPT and BN80915, respectively. These cell lines were maintained in medium containing 50 nM hCPT or BN80915. The resistant cell lines were maintained in constant culture with selecting drug for at least 4 months before characterization by cytotoxicity assay, thus we considered them to be a homogeneous population of cells.

To determine whether the drug-selected cell lines were cross-resistant to other Top1 inhibitors, 4-day cytotoxicity assays were performed with other Top1 inhibitors as well as Top2 inhibitors. As seen in Table I, the parental SF295 cells were 35-fold more sensitive to hCPT and 8-fold more sensitive to BN80915 compared to Topotecan (TPT), although they were comparably resistant to CPT. SF295/hCPT50 cells were comparably resistant to hCPT and BN80915 (21-and 22-fold, respectively); similarly, SF295/BN50 cells were comparably resistant to both hCPT and BN80915 (15-and 21-fold, respectively). Both resistant sublines also demonstrated 9- to 20-fold cross-resistance to the indenoisoquinoline non-CPT Top1 inhibitors NSC 724998 and MJ-III-65 (NSC 706744) (Pommier, 2006) (Table I). On the other hand, both SF295/hCPT50 and SF295/BN50 cells showed 3-fold higher sensitivity to the Top2 inhibitor, mitoxantrone (MX).

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SF295/BN50 cells only showed slightly increased sensitivity to etopside (VP-16), while SF295/hCPT50 was slightly resistant to etoposide (VP-16) (Table I).

Lack of Expression of Drug Transporters in the Resistant Cells. Several groups have shown that the ATP-binding cassette (ABC) proteins P-glycoprotein (Pgp), multidrug resistance-associated protein 1 (MRP1), and ABCG2 can mediate multidrug resistance to cytotoxic drugs such as CPTs (Chen et al., 1991; Chen et al., 1999; Hoki et al., 1997) and to a lesser extent to hCPTs (Bates et al., 2004; Chauvier et al., 2002). Using MCF-7/FLV1000 (expressing ABCG2), MCF-7/VP (expressing MRP1) and MCF-7/TX200 (expressing Pgp) cells as positive controls, we observed no detectable expression of MRP1, ABCG2 or Pgp in either parental SF295 line or either of the drug-selected lines (Fig. 3A). Protein loading is shown by Ponceau staining in Figure 3B.

We next measured Pgp, MRP1 and ABCG2 expression by a flow cytometry-based functional assay, which we have concluded can detect small changes in transporter expression that may not be detected by immunoblot analysis. As shown in column 1 of Figure 3C, Pgp-overexpressing HEK293 cells readily transport rhodamine 123, as shown by the difference between the histograms depicting cells incubated without (solid line) or with (dashed line) 3 μg/ml of the Pgp inhibitor valspodar. Pgp function is not detected in the SF295 parental cells, as the solid and dashed histograms overlap. Pgp expression in the drug-selected BN50 and hCPT50 sublines was similar to that of the parental cells,

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suggesting that Pgp expression was not increased in the sublines. Similarly, in the second column of Figure 3C, MRP1-overexpressing HEK293 cells readily transport the fluorescent compound calcein AM when incubated without (solid line) or with 25 μM of the MRP1 inhibitor MK571. Parental SF295 cells and the drug-selected sublines all display similar low levels of MRP1 expression. Finally, in the last column of Figure 3C, ABCG2-overexpressing HEK293 cells readily transport the fluorescent compound pheophorbide a, shown by the difference in the histograms generated by incubating cells in pheophorbide alone (solid line) or with 10 μM FTC. Parental SF295 cells also express ABCG2, consistent with previous results (Robey et al., 2001a). In the drug-selected sublines, however, ABCG2 expression appears to be decreased compared to parental cells as evidenced by the smaller difference between the solid and dashed histgrams. Thus, the flow-cytometry results support the data obtained by immunoblot, suggesting that neither Pgp, MRP1 or ABCG2 is upregulated in the drug-selected sublines. These results suggested that the three major drug transporters associated with resistance to CPTs and hCPTs were not factors contributing to the resistance phenotype in the SF295/hCPT50 or SF295/BN50 sublines.

Reduced Formation of DNA-protein Crosslinks Induced by CPT in Resistant SF295/BN50 cells. Since drug transporters were not implicated in as the cause for drug-resistance in the hCPT-selected cells, we next examined other possible mechanisms of resistance. We measured directly the formation of DNA-protein crosslinks (DPC) in

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parent SF295 and SF295/BN50 resistant cells after different concentrations of CPT treatment with the alkaline elution assay. The amounts of DPC in SF295/BN50 cells were about one-third of those in SF295 cells after 1 h exposures to 0.1-1.0 μM of CPT (Fig. 2). This finding demonstrated that drug resistance of the SF295/hCPT50 and SF295/BN50 cells was related to reduced formation of Top1 cleavage complexes, which is generally the mechanism of resistance to CPTs (Pommier et al., 1999).

Reduced Expression of Top1 Protein and mRNA. Since we have previously observed decreased Top1 expression in CPT-selected cell lines (Fujimori et al., 1995), we next examined the hCPT and BN80915-selected lines for changes in Top1 expression. Top1 protein levels were clearly decreased in both SF295/hCPT50 and SF295/BN50 sublines (Fig. 4A). However, Top2 levels were not significantly increased (Fig. 4B & C). We then asked whether the observed reduction of the Top1 protein was due to reduced *Top1* mRNA levels. Quantitative RT-PCR analysis of total RNA extracted from exponentially growing parent SF295, resistant SF295/hCPT50 and SF295/BN50 cells revealed consistently reduced (<50%) levels of *Top1* mRNA in the resistant cells compared to SF295 cells (Fig. 4D). The mRNA results are consistent with the protein data, suggesting that the down-regulation of Top1 in the resistant cells occurs at the transcriptional level.

Low *Top1* Gene Expression in the Resistant Cell Lines cannot be Rescued by Decitabine and Depsipeptide Treatment. To determine whether the reduction of Top1

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protein and mRNA levels in both of the hCPT and BN80915-selected sublines may be caused by epigenetic changes, such as histone methylation or deacetylation, in the promoter region of *Top1* gene in these resistant cell lines (Fujimori et al., 1995), we explored the capacity of the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (decitabine) and histone deacetylase (HDAC) inhibitor depsipeptide, alone and in combination, to reactivate expression of the *Top1* gene. Parental SF295 and drug-selected cells were treated with 1.0 μM decitabine for 4 days or 2 ng/ml depsipeptide for 1 day alone or concomitantly with the two drugs and RNA was subsequently isolated. *Top1* levels were then determined by RT-PCR and normalized to GAPDH expression. Ratios of the amount of amplified DNA for the drug-treated samples as compared to the control (parental SF295 levels set to a value of 10) were calculated in order to express the relative level of gene expression after drug treatment. While decitabine treatment enhanced the *Top1* transcript level by two-thirds in parent SF295 cells, it failed to increase its level in either the resistant SF295/BN50 or SF295/hCPT50 cells (Fig. 4E). Treatment with depsipeptide alone also failed to increase expression of Top1, as did incubation with both depsipeptide and decitabine simultaneously (Fig. 4E). These results suggest that the decrease in Top1 expression is not associated with epigenetic modifications such as DNA methylation or histone deacetylation.

BrdU Incorporation and Cell Cycle Analysis. BrdU incorporation and cell cycle analyses of SF295, SF295/BN50 and SF295/hCPT50 cells were performed by FACS.

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Figure 5A shows a representative DNA histogram of BrdU-stained cells at different time points. Because BrdU is incorporated instead of thymidine into the DNA during DNA synthesis in proliferating cells, the percentage of BrdU positive cells reflects the percentage of total S-phase cells. With cell cycle analysis, we detected the percentage of S-phase cells in the three cell lines at various times after the initial BrdU pulse. BrdU positive cells in S-phase were normalized to the percent of cells that were positive 3 h after the pulse (Fig. 5B). Compared with the parent SF295 cells, both of SF295/BN50 and SF295/hCPT50 resistant cells showed slower S-phase progression. At the time of 8.5 hrs after BrdU pulse, the amount of cells that have stepped out the S phase were about 90%, 40% and 20% for SF295, SF295/BN50 and SF295/hCPT50 cells, respectively. Thereafter we measured doubling times for the three cell lines. We found that doubling times for SF295, SF295/BN50 and SF295/hCPT50 were 31.5, 99 and 69 hrs, respectively.

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Discussion

Most of the pre-clinical studies on drug resistance are carried out by using *in vitro* cellular models, including drug-resistant cell lines selected from an originally sensitive counterpart. Resistance of cancer cells to CPT is multifactorial, involving reduced drug accumulation by over-expression of drug transporters (Bates et al., 2004), reduced expression of Top1 enzyme (Fujimori et al., 1996b; Tan et al., 1989), Top1 mutation (Pommier et al., 1999; Urasaki et al., 2001; Yanase et al., 2000), elevated DNA repair (Fujimori et al., 1996a; Pommier et al., 2006) and resistance to apoptosis [for review, see Ref. (Pommier, 2006; Pommier et al., 1999)].

Besides our study, Eng et al. (Eng et al., 1990) reported a camptothecin-resistant subline of P388 murine leukemia (P388/CPT), which was developed by repeated transplantation of P388 cells in mice treated with therapeutic doses of CPT and made hyper-resistant to CPT by passage in the presence of increasing concentrations of CPT. Both *Top1* mRNA and 100 kDa Top1 enzyme levels were lower in these resistant cells. However, P388/CPT cells were not cross-resistant to other antineoplastic agents, including topoisomerase II inhibitors (Eng et al., 1990). Interestingly, the Top1-deficient leukemia P388/CPT45 cells were highly resistant to hCPT (Urasaki et al., 2000), which indicates hCPT sharing the same target Top1 with CPT. In P388/CPT45 cells, Top1 is not detectable by immunoblotting (Pourquier et al., 2000). In the present study, TPT and CPT were much less effective, comparing to hCPT and BN80915, in both SF295/hCPT and

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SF295/BN50 resistant cells. These data are consistent with previous report (Urasaki et al., 2000) that the antiproliferation activity of hCPT was greater than that of CPT in both parental and CPT-resistant cell lines. Taken together, CPT-resistant cells are cross-resistant to hCPT, and the hCPT/BN80915-resistant cells shown cross-resistance to CPTs, both cases with reduced expression of common target Top1 at mRNA and protein level.

In our present study, both of the resistant SF295/BN50 and SF295/hCPT50 cell lines showed consistently reduced expression of Top1 protein and mRNA. The reduction of *Top1* gene expression for drug resistance may be drug structure-specific since no Top1 alteration was observed in a neuroblastoma model with *in vivo* acquired resistance to irinotecan (Calvet et al., 2004). It is reasonable that the two resistant cell lines also show cross-resistance to other Top1 inhibitors because of decreased amount of their drug target, Top1. However, the cell lines resistant to hCPTs also showed increased sensitivity to Top2 inhibitors. These data suggest that Top2 inhibitors would be beneficial in patients with acquired resistance to hCPTs as a result of Top1 reduction after hCPTs treatment.

In addition to decreased Top1 levels, we noted a slower growth rate in our resistant cell lines. This phenomenon was also observed in CPT-resistant subline of P388 leukemia with reduced Top1 content (Eng et al., 1990). In this study, slower growth rate did not seem as a major mechanism of resistance to Top1 inhibitors because the BN and hCPT resistant cells are not resistant to Top2 inhibitors mitoxatrone and etoposide. Furthermore,

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drug treatment for cytotoxicity assays were performed by continuous drug exposure for 3 days. These extended drug exposure exceeded the cellular doubling –times, which would minimize the impact of slower growth on the induction of DNA damage. Finally, DNA damage measured by alkaline elution showed reduced DNA-protein crosslinks in the resistant cells, which is most likely to contribute to drug resistance.

In mammalian cells, expression of ABC transporters such as Pgp (MDR1) and ABCG2 confers resistance to CPT and its derivatives (Brangi et al., 1999; Hoki et al., 1997; Rajendra et al., 2003). While Pgp is not implicated in resistance to hCPTs (Larsen et al., 2001; Lavergne et al., 2000a), hCPTs have been shown to be subject to transport by MRP1 (Chauvier et al., 2002) and ABCG2 (Bates et al., 2004). Additionally, selection with CPTs usually results in overexpression of ABCG2 (Kawabata et al., 2001; Maliepaard et al., 1999). In light of these previous findings, it is surprising that selection with hCPTs does not result in overexpression of ABC transporters. Thus, drug transporters may be not a major resistance challenge to hCPTs, and especially to BN80915, in clinical use.

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Footnotes:

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Legends for Figures:

Figure 1. Chemical structures of camptothecin, homocamptothecin and diflomotecan (BN80915).

Figure 2. DNA-protein crosslinks (DPCs) induced by CPT in human glioblastoma SF295 and BN80915-resistant SF295/BN50 cells. (A) Cells were prelabeled with [^{3}H]thymidine and were then treated with 0.1, 0.3 and 1.0 μM CPT for 1 hr at 37°C as described in Materials and Methods. DPCs were assayed by alkaline elution under non-deproteinizing conditions after ionizing radiation of cells with 3000 rads for reference (Diamonds); or treatment with 0.1 μM CPT (Triangles); 0.3 μM CPT (Circles) or 1.0 μM CPT (Squares) in SF295 (closed symbols) and SF295/BN50 (opened symbols) cells. (B) DPCs were measured by alkaline elution assay and are expressed as rad equivalents at 12 hrs elution time in SF295 (○) and SF295/BN50 (□) cells. The average DPCs from two independent experiments are presented; bars, SEM.

Figure 3. Analysis of drug transporter protein expression in human glioblastoma SF295 and drug resistant SF295/BN50 and SF295/hCPT50 cells. (A) For qualitative analysis of the three drug transporter proteins expression in these cell lines, 30 μg microsomal proteins were separated by SDS-PAGE and probed with anti-Pgp, -MRP1 and -ABCG2 antibodies as described in the Materials and Methods. (B) Ponceau staining of membrane shown in A. (C) SF295 parental and resistant sublines from A were incubated with 0.5

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μg/ml rhodamine 123 in the presence (dashed line) or absence (solid line) of 3 μg/ml valspodar (column 1); 200 nM calcein AM in the presence (dashed line) or absence (solid line) of 25 μM MK571 (second column); or 10 μM pheophorbide a in the presence (dashed line) or absence (solid line) of 10 μM FTC (last column) to detect Pgp, MRP1 or ABCG2, respectively. *ABCB1*-, *ABCC1*- or *ABCG2*-transfected HEK293 cells served as positive controls for Pgp, MRP1, and ABCG2, respectively (+ Control, top row of histograms). Representative results from one of two independent experiments are shown.

Figure 4. Top1 (A), Top2α (B), Top2β (C) protein levels by Western blotting analysis, *Top1* mRNA levels by real time quantitative-PCR analysis (D) and effect of decitabine (20 ng/ml) and depsipeptide (1 ng/ml), alone or in combination, on mRNA expression of *Top1* by semi-quantitative RT-PCR analysis (E) in SF295, SF295/BN50 and SF295/hCPT50 human glioblastoma cells. For Western blotting assay, whole-cell lysates (50 μg) isolated from each cell line were probed with monoclonal mouse Top1 and polyclonal Top2 antibodies as described in Materials and Methods. β-actin was probed to show equal loading. For real time quantitative-PCR analysis, total RNA was extracted, and real-time quantitative-PCR was done as described in Materials and Methods. mRNA levels were normalized with 18S RNA and representative of at least two independent experiments. For semi-quantitative RT-PCR analysis of *Top1* in SF295 parental cells and the respective resistant sublines SF295/BN50 and SF295/hCPT50, cells were untreated

or treated with either decitabine (1 μM) for 4 days, depsipeptide (2 ng/ml) for 1 day, or a combination of the two drugs. Representative results from three independent experiments are shown. The numbers indicates the fold difference of *Top1* relative to the untreated SF295 parental cells after normalization to *GAPDH*.

Figure 5. BrdU incorporation and cell cycle analysis in SF295, SF295/BN50 and SF295/hCPT50 cells. (A) Cells were collected at indicated times after BrdU pulse, and stained with anti-BrdU antibody coupled to FITC and Propidium Iodide/RNase A. (B) Quantification of BrdU positive cells that are still in the S-phase at indicated times after the BrdU pulse. The first time point (3 h after the BrdU pulse) is set up at 100%. The average percentages are presented from four independent experiments; bars, SEM.

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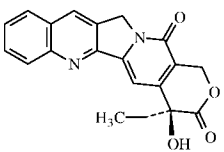
Table I. Drug sensitivity of parent (SF295) and BN80915 or hCPT-resistant (SF295/BN50 or SF295/hCPT50) human glioblastoma cancer cell lines to various anticancer drugs.

Drug	n	SF295 (IC_{50})	SF295/BN50 (IC_{50})	DR	SF295/hCPT50 (IC_{50})	DR
BN80915	3	0.3 ± 0.06	6.3 ± 4.0	21	6.5 ± 4.3	22
hCPT	3	1.3 ± 0.6	20 ± 10	15	27 ± 15	21
TPT	3	10.7 ± 3.8	70 ± 10	7	83.3 ± 15.3	8
CPT	4	1.1 ± 0.3	26.3 ± 7.5	24	30 ± 8.1	27
NSC 724998	3	0.08 ± 0.03	1.2 ± 0.7	15	0.7 ± 0.3	9
MJ-III-65	2	0.006 ± 0.005	0.13 ± 0.11	22	0.08 ± 0.03	13
MX	3	4.7 ± 1.5	1.6 ± 0.8	0.3	1.7 ± 1.0	0.3
Etoposide	3	0.7 ± 0.3	0.5 ± 0.2	0.7	1.1 ± 0.1	1.4

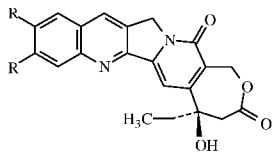
Drug sensitivity was determined by the SRB assay as outlined in the Materials and Methods section.

Degree of resistance (DR) was calculated by dividing the IC_{50} value of the resistant lines by that of the corresponding parental lines.

hCPT: homocamptothecin; TPT: topotecan; CPT, camptothecin; MJ-III-65: NSC 706744; MX: mitoxantrone



20-S-camptothecin



Homocamptothecin R = H
BN 80915 (Diflomotecan) R = F

Fig.1

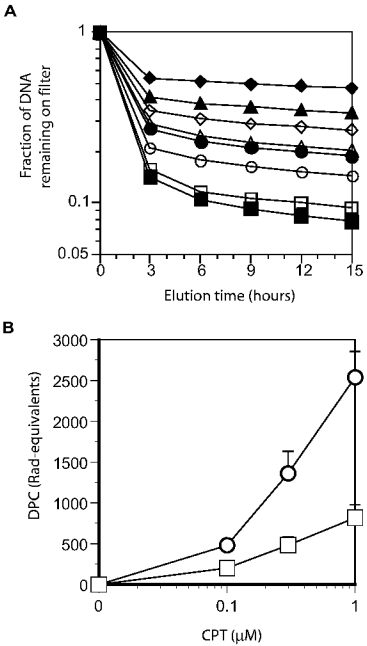

A
Fraction of DNA remaining on filter
1
0.1
0.05
0
3
6
9
12
15
Elution time (hours)
B
DPC (Rad equivalents)
3000
2500
2000
1500
1000
500
0
0
0.1
1
CPT (μM)

Fig. 2

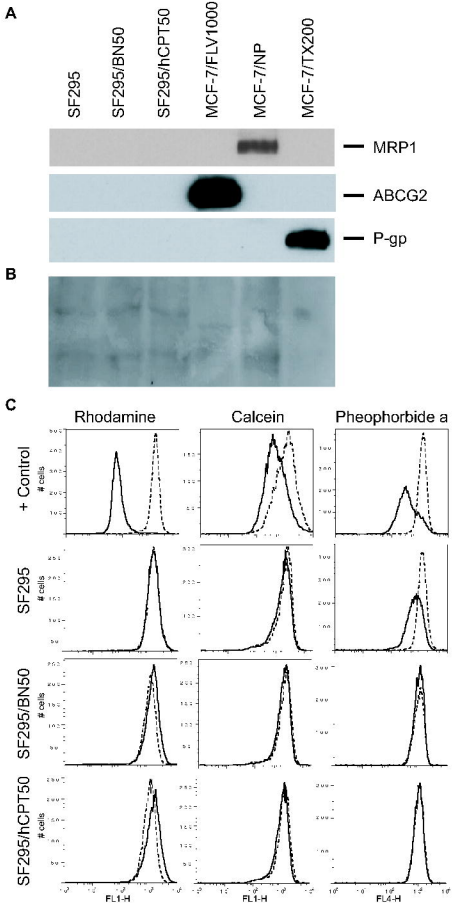

A
SF295
SF295/BN50
SF295/hCPT50
MCF-7/FLV1000
MCF-7/NP
MCF-7/TX200
MRP1
ABCG2
P-gp
B
C
Rhodamine
Calcein
Pheophorbide a
+ Control
SF295
SF295/BN50
SF295/hCPT50
cells
FL1-H
FL4-H

Fig. 3

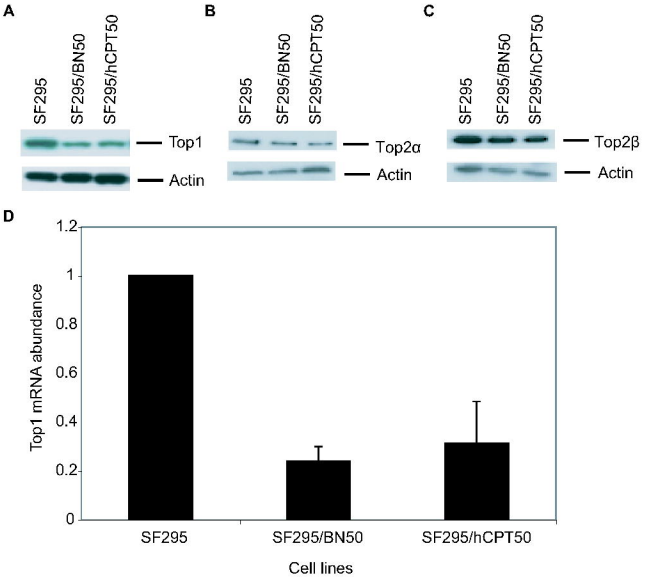
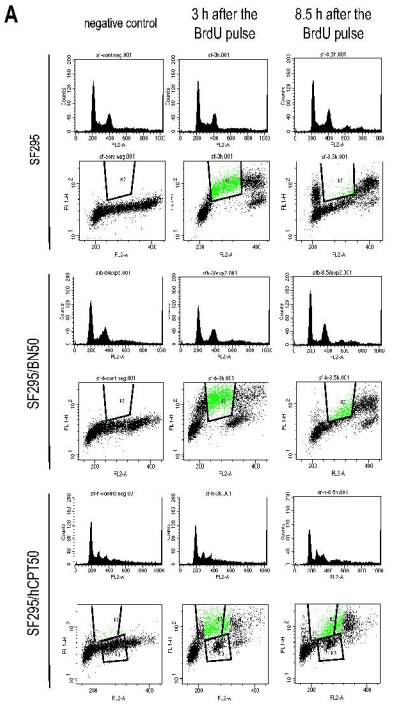

A
SF295
SF295/BN50
SF295/hCPT50
Top1
Actin
B
SF295
SF295/BN50
SF295/hCPT50
Top2α
Actin
C
SF295
SF295/BN50
SF295/hCPT50
Top2β
Actin
D
Top1 mRNA abundance
1.2
1
0.8
0.6
0.4
0.2
0
SF295
SF295/BN50
SF295/hCPT50
Cell lines
E
SF295
SF295/BN50
SF295/hCPT50
Decitabine - + - + - + - + - + - +
Depsipeptide - - + + - - + + - - + +
10 16.7 5.7 6.5
7.4 5.2 2.0 2.2
7.3 6.7 3.7 3.9
Topo I
GAPDH

Fig. 4


A
negative control
3 h after the BrdU pulse
8.5 h after the BrdU pulse
SF295
SF295/BN50
SF295/hCPT50

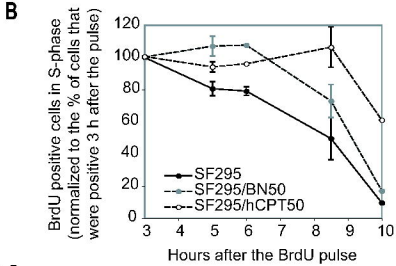

B
BrdU positive cells in S-phase (normalized to the % of cells that were positive 3 h after the pulse)
120
100
80
60
40
20
0
3
4
5
6
7
8
9
10
Hours after the BrdU pulse
SF295
SF295/BN50
SF295/hCPT50

Fig. 5