The HER Tyrosine Kinase Inhibitor CI1033 Enhances Cytotoxicity of 7-Ethyl-10-hydroxycamptothecin and Topotecan by Inhibiting Breast Cancer Resistance Protein-mediated Drug Efflux

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ABSTRACT

Because the activities of HER family members are elevated and/or aberrant in a variety of human neoplasms, these cell surface receptors are receiving increasing attention as potential therapeutic targets. In the present study, we examined the effect of combining the HER family tyrosine kinase inhibitor CI1033 (PD 183805) with the topoisomerase (topo) I poison 7-ethyl-10-hydroxycamptothecin (SN-38), the active metabolite of irinotecan, to inhibit HER family tyrosine kinases. Topo I poisons are used to treat a variety of malignancies. Irinotecan prolongs survival and increases quality of life in patients with metastatic colorectal cancer (1, 2). It is also active in non-small cell lung cancer (3) and is currently undergoing testing in patients with recurrent glioma (4). Topotecan is active in platinum-resistant ovarian cancer (5) as well as small cell lung cancer (6).

The activity of these agents reflects their ability to target topo I, a nuclear enzyme involved in transcription and replication (7–9). SN-38, the active metabolite of irinotecan, and other CPT derivatives stabilize covalent complexes between topo I and nuclear DNA (10–12). When moving replication complexes encounter these topo I-DNA complexes, DNA double-strand breaks result (7, 13, 14). In response to these double-strand breaks, cells initially accumulate in the S phase of the cell cycle (15–17). This arrest is followed by DNA repair or induction of apoptosis (18–21).

Sensitivity of cells to topo I poisons is modulated by a variety of factors, including topo I content, cell cycle distribution, and DNA repair capability (reviewed in Refs. 7, 14, 22, and 23). In addition, sensitivity to CPT derivatives appears to be regulated by drug accumulation. Previous studies have demonstrated that accumulation of TPT and SN-38 is decreased slightly in cells that overexpress P-glycoprotein (24–27). Higher levels of resistance to SN-38 and TPT have been observed in cell lines that were selected for mitoxantrone resistance (28–31) and subsequently shown to overexpress BCRP, a member of the ATP binding cassette family of transporters (31, 32).

Although these observations raise the possibility that BCRP might modulate accumulation and cytotoxicity of TPT and SN38, experiments that directly address this hypothesis, e.g., by analyzing drug accumulation and cytotoxicity in BCRP-transfected cell lines, have been limited. Whether BCRP modulates drug sensitivity in unsel ected human lines is also unknown. Moreover, the ability of BCRP modulators to alter sensitivity to SN-38 or TPT has not been extensively explored.

CI1033 (Fig. 1) is a quinazoline-based HER family tyrosine kinase inhibitor that is currently being evaluated as a potential anticancer agent (33). The HER family of receptors includes EGFR, HER-2, HER-3, and HER-4. Over the last decade, considerable evidence has implicated this receptor tyrosine kinase family in the development and progression of a variety of human tumors (34, 35). The founding member of this family, EGFR, is commonly amplified and mutated in high-grade glioblastomas (36). HER-2 is frequently amplified and overexpressed in breast cancer (37, 38), often in conjunction with elevated EGFR. Overexpression of these receptors is associated with antiestrogen resistance and poor prognosis of breast cancer (37, 38). Coexpression of EGFR and HER-2 has also been associated with shortened survival of patients with carcinomas of the prostate, ovary, and upper aerodigestive tract (39–41). These observations have prompted extensive efforts to target HER family members using monoclonal antibodies (37, 42) or small molecule inhibitors of receptor tyrosine kinases (43, 44).

In view of the role of HER family signaling in development of glioblastomas and colorectal cancer, there is considerable interest in evaluating CI1033 in these tumors. Because irinotecan also shown
activity against both of these tumor types, it is tempting to combine these agents. On the other hand, CI1033 would be expected to inhibit proliferation of susceptible cells, whereas topo I poisons like SN-38 are thought to be most toxic to cycling cells (45–47). These considerations raised the possibility that the two agents might antagonize each other. In the present study, we evaluated the effect of combining CI1033 and SN-38 or TPT in T98G glioblastoma cells (48) and HCT8 intestinal adenocarcinoma cells (49). Our results demonstrate that CI1033 enhances the action of these topo I poisons when the drugs are administered simultaneously. Additional experiments provide evidence that this synergism reflects enhanced drug accumulation as a consequence of diminished BCRP-mediated efflux. These results identify CI1033 as a potent and effective modulator of SN-38 and TPT accumulation.

MATERIALS AND METHODS

Reagents. CPT was purchased from Sigma (St. Louis, MO). SN-38 and CI1033 were provided by Pharmacia-Upjohn (Kalamazoo, MI) and Parke-Davis Pharmaceutical Research (Ann Arbor, MI), respectively. TPT was obtained from the Drug Resources Branch of the National Cancer Institute (Bethesda, MD). CPT, SN-38, TPT, and CI1033 were prepared as 1000-fold concentrated stocks in DMSO and stored at −20°C. Monoclonal antibody to topo I (50) was kindly provided by Dr. Y.-C. Cheng (Yale University School of Medicine, New Haven, CT). Reagents that recognize EGFR (SC-03) and tyrosine-phosphorylated EGFR (Y1173) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Upstate Biotechnology (Lake Placid, NY), respectively. BXP-21 murine anti-BCRP antibody was raised in balb/c mice by injection of a fusion protein containing amino acids 271–396 of BCRP (GenBank accession no. AF 09851) fused to Escherichia coli maltose binding protein using techniques reported previously for the generation of another anti-BCRP antibody (51). Fluorochrome- and peroxidase-coupled secondary antibodies were obtained from Kirkegaard and Perry Laboratories, Inc. (Rockville, MD). [Methyl-14C]Thymidine (40–60 mCi/mmol) was procured from New England Nuclear (Boston, MA). Enhanced chemiluminescence reagents were purchased from Amersham Pharmacia Biotechnology (Arlington Heights, IL). All other reagents were obtained as indicated previously (25, 52, 53).

Buffers. Medium A contained MEM, 10% heat-inactivated FBS, 1 mM sodium pyruvate, 100 units/ml penicillin G, 100 µg/ml streptomycin, and 2 mM glutamine. Buffer B consisted of serum-free RPMI 1640 containing 10 mM HEPES (pH 7.4). Cell lysis buffer (54) consisted of 6 mM guanidine hydrochloride, 250 mM Tris-HCl (pH 8.5 at 21°C), and 10 mM EDTA supplemented immediately before use with 1 mM α-phenethylmethylsulfonyl fluoride (added from a 100-fold stock in anhydrous isopropanol) and 1% (v/v) β-mercaptoethanol.

Clonogenic Assays. T98G, HCT8, MCF-7, PC-3, and Ovcar-3 cells were obtained from the American Type Culture Collection (Manassas, VA). For clonogenic assays, trypsinized T98G cells were diluted with medium A, plated in 35-mm tissue culture plates (500 cells/plate), and incubated for 14–16 h to allow cells to adhere. Drugs were added to the indicated final concentrations from 1000-fold concentrated stocks. Control plates received the corresponding volume of diluent. After a 24-h incubation, plates were washed twice with serum-free MEM, refed with medium A, and incubated for 10 days. The resulting colonies were stained with Coomassie Brilliant Blue so that visible colonies could be counted. Control plates typically contained 150–200 colonies. An identical approach was used with other cell lines, except that the number of cells plated was adjusted to 1300 (PC-3) or 2000 (Ovcar-3) to achieve 150–200 colonies in control plates, and medium A was replaced by medium consisting of RPMI 1640-5% heat-inactivated FBS (HCT8); Ham’s F-12 medium-7% heat-inactivated FBS (PC-3); MEM containing Earle’s salts, 10% heat-inactivated FBS, nonessential amino acids, 1 mM sodium pyruvate, and 10 µg/ml insulin (MCF-7); or MEM containing 20% heat-inactivated FBS (Ovcar-3), each of which was supplemented with 200 units/ml penicillin G, 100 µg/ml streptomycin, and 2 mM glutamine.

To examine the effect of sequencing, cells were plated as described above, incubated with one drug for 24 h, washed twice, incubated with the second drug for 24 h, washed, and incubated in drug-free medium for the duration of the 7–14-day incubation period. In these experiments, exposure to single agents took place at the same time as exposure to the drugs in sequence.

BCRP Transfectants. MDA-MB-231 cells were transfected with pcDNA3 encoding BCRP behind the constitutive cytomegalovirus promoter or with empty vector using techniques described previously (55, 56). Clones selected in Genetecin were maintained in 400 µg/ml Genetecin until they were subjected to colony-forming assays and drug accumulation studies as described above.

Analysis of Combined Drug Effects. Combined drug effects were analyzed by the median effect method (57). In brief, cells were treated with serial dilutions of each drug individually and with both drugs simultaneously or sequentially at a fixed ratio of doses that typically corresponded to one-half, five-eighths, three-fourths, seven-eighths, 1.0, and 1.5 times the individual IC50 values. The fractional survival (f) was calculated by dividing the number of colonies in drug-treated plates by the number of colonies in control plates. Log [1/(f − 1)] was plotted against log [drug dose]. From the resulting graphs, the x intercept (log IC50) and slope m were calculated for each drug and for the combination by the method of least squares and then used to calculate the doses of the individual drugs and the combination required to produce varying levels of cytotoxicity according to the following equation.

\[
\text{Dose} = \text{Dose} \times \left( \frac{1 - f}{f} \right)^{m}
\]

Because the two drugs were administered at a fixed ratio, the dose of the combination required to produce fractional survival f could be divided into the component doses (D1) and (D2) of drugs 1 and 2, respectively. For each level of cytotoxicity, the CI was then calculated. In this method, synergy is indicated by CI < 1, additivity is indicated by CI = 1, and antagonism is indicated by CI > 1.

Unless otherwise indicated, experiments were repeated until three replicates yielded correlation coefficients of ≥0.9 for all three median effect lines. Results of multiple experiments are summarized by indicating the mean ± SD of the CI at the indicated level of colony inhibition.

Alkaline Elution. Logarithmically growing cells were labeled for 24 h with 1 µM [14C]thymidine, sedimented, and incubated for 1–2 h at 37°C in fresh medium A. To measure steady-state levels of topo I-mediated DNA single-strand breaks, aliquots were then incubated for 30 min at 37°C with the indicated concentrations of SN-38, diluted with ice-cold 75 mM NaCl-2.4 mM EDTA (pH 7.4 at 4°C), and deposited on Nucleopore phosphocellulose filters (1 µm, pore size; VWR Scientific, Minneapolis, MN) by gentle suction. All additional steps were performed as described recently (53). In brief, cells were lysed by allowing 5 ml of buffer consisting of 1% (v/v) SDS, 100 mM glycine, 25 mM EDTA (pH 10), and 0.5 mg/ml protease K to drip through the filters. After filters were washed with 20 mM EDTA (pH 10), DNA was eluted with 20 mM EDTA (adjusted to pH 12.1 with tetrapropylammonium hydroxide). The distribution of radiolabel in the eluate, the tubing of the elution apparatus, and the filter were analyzed as described by Covey et al. (58). Cells that received 150–900 cGy of γ-irradiation from a 137Cs source were included in each experiment as a standard curve.

Immunoblotting. Whole cell extracts were prepared by lysing washed cells in cell lysis buffer. After a 4–6 h incubation at 20°C, samples were sonicated to shear the viscous DNA, treated with iodooacetamide to block free
sulphydryl groups, dialyzed at 4°C into 4 mM urea and then into 0.1% (w/v) SDS (59), and lyophilized to dryness. Subsequent electrophoresis and immunoblotting were performed as described previously (60). Alternatively, detergent-soluble protein extracts for EGFR blotting were isolated by incubating cells at 4°C for 15 min in extraction buffer consisting of 150 mM NaCl, 20 mM HEPES (pH 7.5), 1.5 mM MgCl₂, 10 mM EGTA, 1 mM sodium vanadate, 10% (v/v) glycerol, and 1% (v/v) Triton X-100 supplemented with 2 µg/ml aprotinin, 5 µg/ml leupeptin, 50 µg/ml Na₂-p-tosyl-l-lysine chloromethyl ketone, 100 µg/ml Na₂-p-tosyl-l-phenylalanine chloromethyl ketone, 100 µg/ml α-phenylmethylsulfonyl fluoride, and 50 µg/ml soy bean trypsin inhibitor. After particular material was removed by centrifugation at 11,000 × g for 15 min at 4°C, protein concentrations in the supernatant were estimated (61). Aliquots containing 80 µg of protein were diluted with an equal volume of loading buffer (50 mM HEPES, 6.8% (v/v) SDS, 10% (v/v) glycerol, and 0.005% (w/v) bromophenyl blue), heated to 95°C for 5 min, and applied to SDS-polyacrylamide gels containing a 4–20% acrylamide gradient. Separated proteins were electrophoretically transferred to nitrocellulose. Membranes were incubated for 1 h at 21°C in blocking buffer consisting of 5% nonfat dry milk, 137 mM NaCl, 20 mM Tris-HCl (pH 7.6 at 21°C), and 0.1% (w/v) Tween 20. Filters were subsequently incubated overnight at 4°C with 1 µg/ml anti-EGFR or anti-phosphorylated EGFR antibody in blocking buffer, washed, and incubated for 1 h with peroxidase-conjugated secondary antibody. After additional washes, bound antibody was detected by enhanced chemiluminescence.

**Band Depletion Assay.** Replicate plates of cells were treated with 8 µM CI1033 or diluent in Medium A for 1 h. After SN-38 was added to a final concentration of 0–30 µM, samples were incubated for an additional 45 min. Proceeding one plate at a time, cells were rapidly washed twice with ice-cold buffer B and immediately solubilized by addition of 3 ml of cell lysis buffer followed by agitation. As described previously, the rapid denaturation in this guanidine hydrochloride-containing buffer traps covalent topo I-DNA complexes (60). Subsequent preparation for electrophoresis and immunoblotting was performed as described above.

**HPLC Analysis of SN-38 or CI1033 Accumulation.** Twenty-100 mM plates of cells were grown to 70% confluence for each data point. To assess the effect of CI1033 on SN-38 accumulation, T98G or BCRP-transfected MDA-MB-231 cells were treated with diluent or 8 µM CI1033 for 1 h. SN-38 was then added to a final concentration of 1 µM for 35 min. The cells were trypsinized in the continued presence of the drugs, centrifuged at 100 × g for 10 min, washed twice with ice-cold calcium- and magnesium-free Dulbecco’s PBS, and lysed in 1 ml of -20°C methanol. The precipitate was sedimented and analyzed for protein as described previously (62).

**RT-PCR.** Polyadenylated RNA (1 µg) from semiconfluent T98G, HCT8, or PC-3 cells was reverse transcribed with random hexamers and 2 units of avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) under standard conditions (64). One-twentieth of the cDNA product was used for each amplification. PCR reactions were performed in 50-µl volumes containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 20°C), 2.5 mM MgCl₂, 0.4 µM of each primer, 400 µM dNTPs, and 5 units of Taq polymerase. Reactions were amplified for a total of 33 cycles on a Perkin-Elmer DNA thermal cycler using 94°C for denaturation (1 min), 55°C for annealing (1 min), and 72°C for extension (2 min), followed by two cycles in which the extension step was lengthened to 7 min. The primer pairs used were 5’-CTTTGTTGGAAGGTC-CCGGTTA-3’ and 5’-TCCCAAGGAGTCTGGTACA-3’ for BCRP (amplify a fragment of 382 bp) or 5’-AGCTTTATGGATGATGATATCGC-5’ and 5’-CTTAAATGCTACGCACAGTTCCTC-3’ for BCRP (5’-end fragment of 90 bp) or 5’-CTTTGTTGGAAGGTC-CCGGTTA-3’ and 5’-TCCCAAGGAGTCTGGTACA-3’ for BCRP (amplify a fragment of 382 bp) or 5’-AGCTTTATGGATGATGATATCGC-5’ and 5’-CTTAAATGCTACGCACAGTTCCTC-3’ for BCRP (5’-end fragment of 90 bp). After amplification, 40% of the product was separated on a 1.2% (w/v) agarose gel in TAE buffer, stained with 0.5 µg/ml ethidium bromide, and photographed under UV light. Sequencing confirmed that the RT-PCR products corresponded to BCRP and β-actin transcripts.

**Miscellaneous Methods.** To assess the effect of CI1033 on topo I activity, log phase T98G cells in medium A were treated with 8 µM CI1033 or 0.1% DMSO for 60 min at 37°C. Nuclear extracts were then prepared and assayed for topo I activity (ability to relax supercoiled plasmid) as described previously in detail (25, 65). To evaluate the effect of CI1033 on topo I localization, cells treated for 1 h with 8 µM CI1033 were fixed and stained with C-21 anti-topo I antibody followed by rhodamine-conjugated goat antimouse IgM as described previously (66).

**RESULTS**

**Cytotoxic Effects of CI1033 and SN-38.** In an effort to evaluate the combined effects of inhibiting HER family tyrosine kinases and poisoning topo I, T98G cells were exposed to CI1033 and SN-38 for 24 h, washed, and evaluated for subsequent ability to form colonies. When T98G cells were exposed to SN-38 alone, an IC₅₀ of 8.9 ± 1.3 µM was observed (n = 8; Fig. 2A, O). Exposure to CI1033 alone resulted in an IC₅₀ of 3.7 ± 0.7 µM (n = 8; Fig. 2B, O). Treatment with both agents simultaneously for 24 h (Fig. 2, A and B, O) inhibited colony formation more than exposure to either drug alone (Fig. 2, A and B, O).

The median effect method (57) was used to compare the cytotoxicity of the combination with the cytotoxicity that would be expected if the effect of the two agents was strictly additive. In applying this method, log([1/ff] − 1) versus log([drug dose]) was plotted for each treatment (Fig. 2C). From the resulting lines, the x intercept and slope were determined and then used to calculate the CI, a parameter that indicates whether the doses of the two agents required to produce a given degree of cytotoxicity are greater than (CI > 1), equal to (CI = 1), or less than (CI < 1) the doses that would be required if the effects of the two drugs were strictly additive. When the two treatments are assumed to be mutually exclusive, results of this analysis are equivalent to the isobologram method (67). When the effects of simultaneous treatment with CI1033 + SN-38 were analyzed under this assumption, the CI was 0.6 ± 0.1 (n = 4) at the IC₅₀ of the combination and <1 over most of the concentrations examined (Fig. 2D, solid line), suggesting that the effects were synergistic on this schedule. For completeness, the CI calculated under the assumption

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that the effects of the two agents are mutually nonexclusive is also shown in Fig. 2D.

To provide an alternative assessment of the effects of combining these two agents, T98G cells were treated with varying concentrations of SN-38 in the absence or presence of 2 μM CI1033, a concentration that allowed survival of 75% of the cells when used alone. As shown in Fig. 2E, CI1033 markedly enhanced the effects of SN-38 at all drug concentrations.

In subsequent experiments, the effects of sequential treatment with CI1033 and SN-38 were examined. When CI1033 was followed by SN-38, the antiproliferative effects were slightly greater than those of either treatment alone (Fig. 3A; data not shown). The CI in this sequence was 1.4 ± 0.2 (n = 4) at the IC_{50}, and ≥1 over the entire range of concentrations examined (Fig. 3B). In the opposite sequence, the two agents again produced greater effects than either agent alone (Fig. 3C), but analysis by the median effect method revealed that the CI was 1.2 ± 0.1 (n = 4) at the IC_{50}, and close to 1 over the entire range of concentrations examined (Fig. 3D). Accordingly, the effects appeared to be synergistic only when the two agents were administered simultaneously.

To determine whether these results were unique to T98G cells, additional cell lines were treated with SN-38 and CI1033. When these two agents were administered simultaneously for 24 h, synergistic effects were observed in HCT8 colon cancer cells (CI = 0.6 ± 0.1 at the IC_{50}, n = 3; Fig. 4A) and MCF-7 breast cancer cells (CI = 0.12 ± 0.06 at the IC_{50}, n = 2), but not in PC-3 prostate cancer cells (CI = 1.1 ± 0.1 at the IC_{50}, n = 3; Fig. 4B) or Ovcar-3 ovarian cancer cells (CI = 1.2 ± 0.1 at the IC_{50}, n = 3). When the two agents were
administered in sequence, the effects in all of these cell lines were additive or less than additive (data not shown).

**Effect of CI1033 on Action of TPT or CPT.** To determine whether CI1033 potentiated the antiproliferative effects of other topo I poisons, T98G cells were treated simultaneously with CI1033 and TPT or CPT. Results of these experiments revealed that CI1033 and TPT were synergistic (CI < 1) only when high concentrations of drugs were used (Fig. 4C). In the case of simultaneous exposure to CI1033 and CPT, the combination was less than additive throughout the entire range of concentrations examined (Fig. 4D). These results indicated that the effect of combining CI1033 with CPT derivatives varies, depending on the analogue examined.

**Effect of SN-38 on EGFR Autophosphorylation.** In an initial effort to determine the basis for the schedule-dependent synergy of CI1033 and SN-38, we evaluated potential effects of SN-38 on EGFR tyrosine phosphorylation. As indicated in Fig. 5A, treatment of T98G cells with CI1033 alone resulted in a rapid inhibition of EGFR autophosphorylation. In contrast, SN-38 did not inhibit EGFR autophosphorylation or alter EGFR polypeptide levels. These observations argue against the possibility that SN-38 is augmenting the effects of CI1033 by further inhibiting EGFR-mediated signaling.

**Effects of SN-38 and CI1033 on Cell Cycle Distribution.** Because the cytotoxicity of SN-38 is higher in S phase (45–47), we next evaluated the effects of SN-38 and CI1033 on cell cycle distribution. Flow microfluorometry revealed that 14 ± 2% and 11 ± 2% (mean, n = 3) of diluent-treated T98G cells were in S and G2 phases of the cell cycle, respectively. After a 24-h exposure to the IC50 concentration of CI1033, there was very little change in cell cycle distribution (Fig. 5B). During the 24 h after removal of CI1033 (the time when SN-38 would be present in the CI1033→SN-38 sequence), there was a transient decrease in S phase, providing a potential explanation for the antagonism observed with this sequence. A different picture emerged after treatment with SN-38. After a 24-h exposure to a concentration of SN-38 that inhibited colony formation by 90%, 74% of the cells contained >2n (S or G2-M phase) DNA content (Fig. 5B), indicating that the cells had been delayed in their progression through these phases of the cell cycle. When cells were exposed to SN-38 and CI1033 simultaneously, the cell cycle effects of SN-38 were enhanced. At the end of the 24-h exposure, the percentage of cells containing >2n DNA was increased to 86% (Fig. 5B). Moreover, the delay at G2-M phase was prolonged: Even 48 h after drug removal, 35% of cells treated with the combination were in G2-M phase, as compared with 21% of cells treated with SN-38 alone.5 These results raised the possibility that CI1033 might be enhancing the effects of SN-38.

**Effects of CI1033 on SN-38 Action.** To evaluate the possible effects of CI1033 on SN-38 action in greater detail, the effects of CI1033 on topo I polypeptide levels, topo I activity, and topo I localization were evaluated. Treatment of T98G cells with CI1033 for 1 or 19 h had no effect on topo I polypeptide levels (Fig. 6A, Lanes 1 and 7; data not shown). Likewise, treatment of cells with CI1033 for up to 24 h had no effect on the amount of topo I activity detected in nuclear extracts.5 Immunohistochemical studies failed to demonstrate any change in topo I distribution within the nucleus on CI1033 treatment.5

In additional experiments, we evaluated the effect of CI1033 on SN-38-induced stabilization of covalent topo I-DNA complexes. For these experiments, a band depletion assay (60) was initially used. The basis for this assay is the observation that topo I covalently bound to DNA migrates more slowly than free topo I on SDS-polyacrylamide gels (60). When T98G cells were incubated with increasing concentrations of SN-38, there was a dose-dependent decrease in the topo I signal at M0 ~100,000 as increasing amounts of topo I became covalently bound to DNA (Fig. 6A, Lanes 2–6). Although CI1033 alone had no effect on the topo I signal, the addition of CI1033 resulted in a decrease in topo I signal at lower concentrations of SN-38 (compare Lanes 2 and 8 or Lanes 3 and 9), raising the possibility that CI1033 was enhancing the SN-38-induced stabilization of topo I-DNA complexes.

To evaluate this possibility in greater detail, the SN-38-induced stabilization of protein-linked DNA single-strand breaks was evaluated by alkaline elution. When T98G cells were treated with SN-38 for 35 min, there was a dose-dependent increase in DNA single-strand breaks (Fig. 6B, ○). Addition of CI1033 5 min before addition of SN-38 markedly enhanced the DNA single-strand breaks induced by any particular concentration of SN-38 (Fig. 6B, ). In multiple experiments, the concentration of SN-38 required to produce a particular level of DNA damage was decreased by a factor of 13 ± 2-fold (n = 3). Additional studies (Fig. 6B, inset) indicated that CI1033 concentrations as low as 0.5 μM resulted in enhanced ability of SN-38 to stabilize topo I-DNA complexes.

Similar studies revealed that 8 μM CI1033 also decreased the amount of TPT required to stabilize topo I-DNA complexes by a factor of 4 ± 0.4-fold (n = 2; Fig. 6C). In contrast, CI1033 had almost no effect on the ability of CPT to stabilize topo I-DNA complexes (Fig. 6D). The results provide a potential explanation for the observed synergy between CI1033 and TPT (Fig. 4C) as well as the lack of synergy between CI1033 and CPT (Fig. 4D).

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5 S. A. Boerner, C. Erlichman, and S. H. Kaufmann, unpublished observations.
CI1033 Enhances SN-38 and TPT Accumulation. The ability of CI1033 to increase SN-38-induced topo I-DNA complexes without altering topo I polypeptide levels raised the possibility that CI1033 might be affecting SN-38 accumulation. To evaluate this possibility, steady-state SN-38 levels in T98G cells were assayed by HPLC in the absence and presence of CI1033. This analysis revealed that SN-38 levels were 9.4 ± 1.9-fold higher in the presence of CI1033 (Fig. 7A). In additional studies, TPT accumulation was also examined in the absence or presence of CI1033. Because the excitation and emission spectra of TPT overlap with wavelengths commonly used for flow cytometry (25, 28, 68), we used flow microfluorometry rather than the more laborious HPLC methodology for this analysis. Treatment with CI1033 enhanced the steady-state accumulation of TPT by a factor of 1.8 ± 0.2-fold in T98G cells (n = 6; Fig. 7, B and C). Dose-response curves revealed that this effect was detectable at 0.25 μM CI1033 (Fig. 7B). Similar analysis revealed that CI1033 also enhanced drug accumulation in HCT8 cells but not in PC-3 cells (Fig. 7B, inset), providing a potential explanation for the cell line to cell line differences in whether synergy is observed (Fig. 4, A and B). When the kinetics of TPT uptake and efflux were examined in T98G cells, CI1033 had no effect on the initial rate of TPT uptake (Fig. 7C) but instead diminished the rate of TPT efflux (Fig. 7D).

CI1033 Modulates BCRP-mediated Transport. The rank order for modulation of topo I poisons by CI1033 (SN-38 > TPT > CPT) is similar to the recently published order of preference of the transporter BCRP for the same compounds (Ref. 30; see also Refs. 28, 29, and 32), raising the possibility that CI1033 might be altering SN-38 and TPT action by inhibiting BCRP. To address this possibility, drug accumulation and cytotoxicity were examined in MDA-MB-231 cells transfected with cDNA encoding BCRP behind the cytomegalovirus promoter or empty pcDNA3 vector controls (56). CI1033 enhanced steady-state SN-38 levels in MDA-MB-231/BCRP cells (Fig. 8A) by a factor of 7.8 ± 1.0 (n = 3). Likewise, TPT accumulation, which was decreased in MDA-MB-231/BCRP cells compared with parental cells, was enhanced by a factor of 2.2 ± 0.5 (n = 3) in the presence of CI1033 (Fig. 8B).

In additional experiments, CI1033 accumulation was measured by HPLC in MDA-MB-231 cells transfected with empty vector or BCRP. Results of this analysis (Fig. 8C) indicated that CI1033 accumulation was 4.9 ± 0.1-fold lower in cells transfected with BCRP. This observation suggests that CI1033 is itself a substrate for BCRP, thereby providing a potential explanation for its ability to inhibit BCRP-mediated efflux of other agents.

Consistent with the drug accumulation results presented in Fig. 8, as well as the known substrate preferences of BCRP (30, 55), CI1033 sensitized the BCRP-transfected cells to the antiproliferative effects of SN-38 (Fig. 9A), TPT (data not shown), and the positive control mitoxantrone (Fig. 9D). In contrast, CI1033 had little effect on the antiproliferative effects of SN-38 in cells transfected with empty vector (Fig. 9B). Likewise, CI1033 did not affect the action of CPT in BCRP-transfected cells (Fig. 9C).
Expression of BCRP in Previously Unselected Cell Lines. The ability of CI1033 to modulate the action SN-38 and TPT in a number of previously untreated human cancer cell lines raised the possibility that BCRP might be endogenously expressed in those lines. To assess this possibility, whole cell lysates were subjected to immunoblotting using an anti-BCRP monoclonal antibody. Results of this analysis (Fig. 10A) revealed that the BCRP polypeptide was readily detectable in HCT8 and T98G cells. In contrast, BCRP levels were much lower in PC-3 or Ovcar-3 cells, two cell lines in which CI1033 fails to modulate SN-38 sensitivity (Fig. 4B). Additional experiments (Fig. 10B) indicated that the paucity of BCRP protein in PC-3 cells reflected diminished steady-state levels of BCRP mRNA.

DISCUSSION

Results of the present study demonstrate that CI1033 enhances the cytotoxicity of SN-38 and, to a lesser extent, TPT in a number of different cell lines by enhancing steady-state drug accumulation and subsequent stabilization of topo I-DNA covalent complexes. Additional results indicate that CI1033 can also modulate drug accumulation and cytotoxicity in cells expressing the recently cloned ATP binding cassette transporter BCRP. Conversely, RT-PCR and immunoblotting (Fig. 8) demonstrated that BCRP is expressed in HCT8 and T98G cells, but not in PC-3 or Ovcar-3 cells.

Fig. 8. SN-38, TPT, and CI1033 accumulation in BCRP-transfected cells. A, effect of CI1033 on SN-38 accumulation. HPLC chromatograph obtained when MDA-MB-231/BCRP cells were treated for 35 min with 1 μM SN-38 in the presence (upper tracing) or absence (lower tracing) of a 1-h pretreatment with 8 μM CI1033. Arrows, elution times of bona fide SN-38 and CI1033. Insert in A, relative SN-38 peak areas/μg protein (mean ± SD, n = 3). B, effect of CI1033 on TPT accumulation. HCT8, MDA-MB-231/BCRP, MDA-MB-231/empty vector cells were incubated for 1 h with 20 μM TPT in the presence of diluent (−) or 8 μM CI1033 (+). Mean fluorescence intensity was determined by flow microfluorometry. Results are representative of four separate experiments. C, effect of BCRP expression on CI1033 accumulation. Left panel, HPLC chromatograph obtained when extracts prepared from MDA-MB-231 cells transfected with empty vector (upper tracing) or BCRP (lower tracing) were subjected to HPLC. Extracts applied to the HPLC column were derived from equal cell numbers, based on an adjustment of extract volumes so that they corresponded to equal starting amounts of total cellular protein. Right panel, relative CI1033 accumulation/μg protein (mean ± range, n = 2) in MDA-MB-231 cells transfected with BCRP (+) or empty vector (−).

Fig. 9. Effects of CI1033 on colony formation by BCRP-transfected MDA-MB-231 cells. Cells transfected with BCRP (A, C, and D) or empty vector (B) were incubated for 24 h with the indicated concentration of SN-38 (A and B), CPT (C), or mitoxantrone (D) in the absence or presence of 2 μM CI1033, washed, and allowed to form colonies for 7 days. A and B come from a single experiment.

Fig. 10. Endogenous expression of BCRP in human cancer cell lines. A, evaluation of BCRP expression by immunoblotting. Aliquots containing 50 μg of total cellular protein from the indicated cell line were subjected to SDS-PAGE, transferred to nitrocellulose, and reacted with monoclonal anti-BCRP. The upper band detected by this antibody is BCRP, as indicated by the marked increase after transfection of BCRP into MDA-MB-231 cells. The lower band is currently unidentified. To serve as a loading control, a duplicate blot was probed with monoclonal antibody to histone H1, a polypeptide that is present in equal amounts of all diploid cells. 231, MDA-MB-231 cells transfected with cDNA encoding BCRP. B, evaluation of BCRP mRNA levels by RT-PCR. Aliquots containing 1 μg of polyadenylated RNA from the indicated cell lines were subjected to reverse transcription. One-twentieth of the total cDNA was then subjected to amplification by PCR using primers specific for BCRP or β-actin.
nblotting show that BCRP is expressed in cells that display enhanced sensitivity in the presence of CI1033. These observations have potentially important implications not only for the future clinical development of CI1033 but also for understanding resistance to topo I poisons.

Because of the role of the HER family in a variety of neoplasms, including adenocarcinomas of the breast, prostate, and ovary as well as high-grade gliomas, there has been considerable interest in combining HER family kinase inhibitors with agents that are already active in these diseases. In anticipation of these types of clinical trials, we evaluated the effect of combining CI1033, which is currently undergoing Phase I clinical testing, with cisplatin, paclitaxel, or SN-38. Clonogenic assays revealed that the combinations involving cisplatin and paclitaxel were additive at best. In contrast, combinations involving SN-38 demonstrated sequence- and cell line-dependent synergy. In particular, the antiproliferative effects of CI1033 and SN-38 were synergistic when T98G, HCT8, or MCF-7 cells were treated with both agents simultaneously (Figs. 2 and 4). When cells were treated with the two agents sequentially, however, the results were additive at best. Moreover, even with simultaneous treatment, CI1033 and the topo I poisons were additive at best in PC-3 cells. Subsequent analysis demonstrated that CI1033 concomitantly enhanced steady-state SN-38 accumulation (Fig. 7A) and SN-38-induced stabilization of topo I-DNA complexes in T98G cells (Fig. 6, A and B). Because of the relative insensitivity of the SN-38 assay, as well as the rapidity of drug influx and efflux, we were unable to assess the effect of CI1033 on SN-38 uptake and efflux. However, additional studies using flow microfluorometry also demonstrated that CI1033 enhanced the steady-state accumulation of TPT in T98G cells (Fig. 7, B and C). Subsequent analysis using this technique revealed that CI1033 did not alter the initial rate of TPT uptake (Fig. 7C) but instead inhibited drug efflux (Fig. 7D), providing an explanation for the increased steady-state accumulation of TPT in the presence of CI1033. Interestingly, drug accumulation was also enhanced in HCT8 cells (Fig. 7B, inset), which were sensitized by CI1033, but not in PC-3 cells, which were not sensitized by CI1033.

Additional experiments were performed to identify the transporter whose action was modulated by CI1033. Previous studies demonstrating that TPT and SN-38 are poor substrates for P-glycoprotein (24, 25, 27, 69, 70) made it unlikely that inhibition of P-glycoprotein is responsible for the dramatic sensitization observed in Fig. 2E. Chang et al. (50) described a CPT-selected rodent cell line that was resistant, at least in part, as a consequence of diminished drug accumulation. Although the transporter involved has not been identified, our observation that CI1033 has little or no effect on CPT-induced strand breaks (Fig. 6D) and cytotoxicity (Fig. 4D) made it unlikely that CI1033 was inhibiting this transporter. More recently, several groups have described cross-resistance between mitoxantrone and the topo I poisons TPT and SN-38 (28, 29, 31). Subsequent studies have identified BCRP/MXR (55, 71) as a transporter that might be responsible for this phenotype (30, 31, 68). In particular, the rank order of resistance to topo I poisons in cells that overexpress this transporter has been reported to be SN-38 > TPT > CPT (30, 72), which is the same rank order of sensitization observed with CI1033 (e.g., Fig. 6). These observations raised the possibility that CI1033 might be acting as a BCRP/MXR inhibitor. Consistent with this possibility, we demonstrated that CI1033 enhanced the accumulation (Fig. 8) and antiproliferative effects (Fig. 9; data not shown) of SN-38 and TPT in cells transfected with BCRP. In contrast, cells transfected with empty vector were relatively unaffected by the addition of CI1033 to SN-38 or TPT (Figs. 8 and 9).

Although BCRP has been previously implicated in resistance to SN-38 and TPT (30, 31, 68, 73), our results extend these earlier studies in several ways. First, our analysis of BCRP-transfected cell lines provides the first formal proof that BCRP affects the cytotoxicity of SN-38. Previous studies performed using cells selected in mitoxantrone or other agents demonstrated a correlation between BCRP expression and diminished action of SN-38 but could not rule out the possibility that a second transporter had been coselected during the selection procedure. The recent demonstration that methotrexate resistance in BCRP-expressing mitoxantrone-selected MCF-7 cells is mediated by another, as yet unidentified transporter (56) highlights the difficulty in determining drug resistance mechanisms using only data from drug-selected cells. The present data circumvent this problem by directly demonstrating that BCRP transfection results in SN-38 resistance (Fig. 9, A and B). Second, the present data provide the first direct evidence that BCRP is capable of altering the accumulation of topo I poisons. Previous studies have demonstrated diminished steady-state accumulation (30) or enhanced efflux (68) of TPT in drug-selected cells, but the present data clearly demonstrate that transfection of BCRP diminishes accumulation of both TPT and SN-38 (Fig. 8, A and B, respectively). Third, the present study indicates that BCRP is endogenously expressed in certain human cancer cell lines. Previous studies have demonstrated BCRP expression in cells that were extensively selected for resistance to anthracyclines, mitoxantrone, or TPT (31, 55, 71). In contrast, the present results demonstrated an effect of CI1033 on drug action in cell lines that have not previously been exposed to these agents. Further analysis confirmed the presence of BCRP mRNA and protein in these cell lines (Fig. 10), providing evidence that a transporter capable of effluxing SN-38 and TPT might be constitutively expressed in certain cancer cells.

The present studies also provide a potential explanation for the ability of CI1033 to sensitize BCRP-expressing cells. The observation that CI1033 levels are much lower in BCRP-transfected MDA-MB-231 cells than in empty vector-transfected controls (Fig. 8C) suggests that CI1033 is itself effluxed by BCRP. Accordingly, CI1033 would be expected to competitively inhibit the efflux of other agents by this transporter. This proposed mechanism of action would account for the fact that CI1033 must be present during the period of SN-38 exposure to stabilize topo I-DNA complexes and sensitize the cells (Figs. 2 and 3). We cannot, however, rule out a model in which part of the modulatory effect of CI1033 also results from the inhibition of a kinase whose activity regulates BCRP. Additional experiments are required to rule out this possibility.

Additional studies are also required to determine whether the present findings are pertinent to the modulation of clinical drug resistance. The 10-fold decrease in SN-38 accumulation and 2-fold decrease in TPT accumulation mediated by endogenous levels of BCRP (Fig. 7) are modest compared with the orders of magnitude of resistance observed in drug-selected cells (28, 29, 31, 55, 68, 71). On the other hand, it is difficult to escalate SN-38 beyond the currently recommended doses in the clinical setting because of severe nonhematological toxicities. Thus, the ability of CI1033 to enhance drug accumulation, if selective for tumor cells, might be important in modulating clinical drug effects. Only two other compounds have been reported to inhibit BCRP-mediated drug efflux to date, GF120918 (31, 72, 74) and fumitremorgin C (73). In contrast to these agents, which do not appear to have intrinsic antineoplastic activity, CI1033 is undergoing Phase I clinical testing as an antineoplastic agent in its own right. If its toxicity profile is tolerable, additional preclinical and early clinical trials of this novel sensitizing agent in combination with SN-38 appear warranted.
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The HER Tyrosine Kinase Inhibitor CI1033 Enhances Cytotoxicity of 7-Ethyl-10-hydroxycamptothecin and Topotecan by Inhibiting Breast Cancer Resistance Protein-mediated Drug Efflux

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