Effect of P-Glycoprotein Expression on the Accumulation and Cytotoxicity of Topotecan (SK&F 104864), a New Camptothecin Analogue

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ABSTRACT

Topotecan (TPT, 9-dimethylaminomethyl-10-hydroxycamptothecin) is the first topoisomerase I-directed cytotoxic agent to enter clinical trials in the United States in two decades. The effect of P-glycoprotein (Pgp) overexpression on TPT cytotoxicity was examined in CHC5 (coliincible-resistant) and AuxBl (parental) Chinese hamster ovary cells. Examination of the $I_{50}$ values observed in colony-forming assays revealed that the CHC5 cells were 15-fold (SD, ±3; $n = 3$) resistant to TPT after a 1-h exposure and 3.2-fold (SD, ±1.4; $n = 4$) resistant in continuous exposure experiments. Band depletion immunoblotting revealed that 4-fold higher concentrations of extracellular TPT were required to induce the formation of topo I-DNA complexes in CHC5 cells as compared to AuxBl cells. To assess the role of Pgp in this resistance, drug accumulation and cytotoxicity assays were performed in the absence and presence of quinidine. Addition of quinidine enhanced TPT accumulation (measured by high-performance liquid chromatography) and diminished the $I_{50}$ for TPT in a greater extent in CHC5 cells than in AuxBl cells.

To examine whether similar effects could be detected in Pgp-expressing human cells, MCF-7/Adria breast cancer cells and KG-1a human acute myelogenous leukemia cells were examined. Quinidine or verapamil enhanced TPT accumulation in both of these cell lines but had no effect in parental MCF-7 cells or a variety of human leukemia cell lines that do not overexpress Pgp. Cytotoxicity measurements performed by counting the number of surviving cells (MCF-7/Adria) or employing a modified, highly stable tetrazolium dye reduction assay (leukemia cell lines) revealed that quinidine diminished the $I_{50}$ for TPT in the Pgp-overexpressing cell lines but not in the control lines.

These results suggest that Pgp overexpression diminishes TPT accumulation and TPT cytotoxicity in hamster and human cells. It should be stressed, however, that these effects were substantially smaller than the effects of Pgp overexpression on the accumulation and cytotoxicity of the anthracyclines daunorubicin and the epipodophyllotoxin etoposide in the same cell lines.

INTRODUCTION

Resistance to chemotherapeutic agents is a major problem in clinical oncology (reviewed in Refs. 1 and 2). Tumors that become resistant to one chemotherapeutic agent often exhibit resistance to structurally unrelated agents as well. One potential cause of this mdr appears to be expression of Pgp, an energy-dependent efflux pump (reviewed in Refs. 2–7). In tissue culture cells, overexpression of this pump leads to decreased intracellular accumulation of certain drugs and to diminished cytotoxicity. The compounds that are typically affected by Pgp-mediated mdr are hydrophobic natural products with complex ring structures.

CPT, a plant alkaloid isolated from Camptotheca acuminata, stabilizes covalent adducts between genomic DNA and the nuclear enzyme topo I (8–15). This agent has strong antitumor activity against a wide range of experimental tumors (16, 17). Clinical evaluation of this agent in the early 1970s revealed that leukopenia was its dose-limiting toxicity (18–21). Severe nonhematological side effects including hemorrhagic cystitis were prominent and limited the further clinical development of CPT.

Several observations have suggested that Pgp overexpression does not affect CPT activity. First, Pgp-expressing P388 murine leukemia cells remain fully sensitive to CPT in vivo (22). Likewise, the human Pgp-expressing leukemia cell line K562/ADM and its parent cell line K562 are equally sensitive to CPT in vitro (23). Finally, CPT does not inhibit [3H]vincristine binding to membrane preparations from the K562/ADM cells (23).

The realization that CPT has a novel mechanism of action and is insensitive to Pgp-mediated mdr has led to renewed interest in the development of new semisynthetic CPT analogues with superior antitumor activity and less nonhematological toxicity than CPT (15, 24–28). In contrast to CPT, several of the more hydrophilic semisynthetic CPT analogues are positively charged at physiological pH. The observation that Pgp preferentially exports positively charged hydrophobic natural products (reviewed in Refs. 4 and 6) raises the possibility that some of the new CPT analogues might be affected by Pgp overexpression. In this context, it is interesting to note that doxorubicin- and vincristine-resistant human tumor cell lines were found to demonstrate 5- to 14-fold cross-resistance to 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin, another positively charged CPT analogue (29). Conversely, a human non-small cell lung cancer cell line selected for resistance to 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin showed low-level cross-resistance (2.2-fold) to doxorubicin as well as moderate (8.6-fold) cross-resistance to CPT (30). These observations raise the possibility that Pgp might efflux certain CPT analogues.

TPT is the first of several new CPT analogues to enter clinical testing in the United States (31–34). This agent has shown activity against non-small cell lung cancer and ovarian cancer when administered on some schedules (34). Since TPT is positively charged at physiological pH, these studies were undertaken to assess TPT cytotoxicity as well as TPT accumulation in Chinese hamster ovary and human Pgp-expressing cell lines.

MATERIALS AND METHODS

Materials. Chinese hamster CHC5 cells (selected for colchicine resistance) and parental AuxBl cells (35, 36) were generously provided by Dr. Victor Ling (Ontario Cancer Institute, Toronto, Ontario, Can-

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The abbreviations used are: mdr, multidrug resistance; Pgp, P-glycoprotein; topo, topoisomerase; AuxBl, sensitive parental Chinese hamster ovary cell line; CHC5, colchicine-resistant Chinese hamster ovary cell line; MCF-7, sensitive parental human breast cancer cell line; MCF-7/Adria, doxorubicin-resistant derivative of MCF-7 cell line; CPT, camptothecin; TPT, topotecan; S9, 9-dimethylaminomethyl-10-hydroxycamptothecin; SDS, sodium dodecyl sulfate; DMSO, dimethyl sulfoxide; $I_{50}$, concentration which diminishes colony number or cell number by 50%; α-MEM, α modification of minimal essential media with ribonucleosides and deoxyribonucleosides; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PMSF, 0-phenylmethylsulfonyl fluoride; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline.

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cell lines. A and B, nuclear accumulation of daunorubicin in CHRC5 (A) and AuxBl (B) in absence (—) and presence (—) of 50 µM quindine. C, colony-forming assay in AuxBl cells (•) and CHRC5 cells (•). D, topo I titration assay in extracts containing 40 ng (Lanes 2 and 10), 20 ng (Lanes 3 and 11), 10 ng (Lanes 4 and 12), 5 ng (Lanes 5 and 13), 2.5 ng (Lanes 6 and 14), 1.25 ng (Lanes 7 and 15), and 0.62 ng (Lanes 8 and 16) of nuclear protein from the indicated cell line. Two simultaneously run minigels were juxtaposed to compose this figure. SC, location of supercoiled plasmid; R, location of relaxed plasmid. The plasmid was completely relaxed by 10 ng of nuclear protein from CHRC5 cells (Lane 12) but not by 10 ng of extract from AuxBl cells (Lane 4). Thus CHRC5 cells contain more topo I activity than AuxBl cells.

tada). MCF-7/Adria' cells and MCF-7 human breast cancer cells were provided by Drs. John Hilton and Nancy Davidson (Johns Hopkins Oncology Center). Previous studies (37) have shown that the MCF-7/Adria' cells contain an amplified human mdrl gene. KG1a human acute myelogenous leukemia cells (38) were kindly provided by Dr. Curt Civin (Johns Hopkins Oncology Center).

TPT and [3H]TPT (1.36 Ci/mmol) were kindly provided by SmithKline Beecham (King of Prussia, PA). Etoposide was a gift from Bristol-Myers (San Francisco, NY). Thioglycol was obtained from Pierce (Rockford, IL). Trasylol was purchased from Mobay Pharmaceuticals (New York, NY). Daunorubicin, verapamil, quinidine, chloroquine phosphate, polyethylene glycol (average molecular weight, 8000), and MTT were obtained from Sigma (St. Louis, MO). The sources of all other chemicals have been described previously (39).

Stock solutions of TPT, CPT, etoposide, quinidine, and verapamil were prepared in DMSO and stored frozen at —20°C. Stocks were thawed and diluted in DMSO immediately prior to use. Stocks of daunorubicin and doxorubicin were prepared immediately prior to use. Where indicated, PMSF was added to buffers from a 100 mM stock in anhydrous isopropanol.

Buffers. Medium A consisted of α-MEM, 10% (v/v) fetal bovine serum, 100 units/ml penicillin G, 100 µg/ml streptomycin, and 2 mM glutamine. Medium B contained RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, 100 µg/ml penicillin G, 100 µg/ml streptomycin, and 2 mM glutamine. Buffer C consisted of 10 mM NaCl, 10 mM Tris-HCl (pH 7.4 at 4°C), 3 mM MgSO4, 0.5 mM EDTA, 1 mM dithiothreitol, 100 units/ml Trasylol, and 1 mM freshly added PMSF. Buffer D consisted of 0.6 M KCl, 100 mM sodium phosphate (pH 7.4 at 4°C), 1 mM dithiothreitol, and 1 mM freshly added PMSF. Buffer E was prepared by combining 2 volumes of buffer D, 1 volume of 13% (w/v) polyethylene glycol, and 3 volumes of glycerol. Buffer F contained 250 mM enzyme-grade sucrose, 50 mM Tris-HCl (pH 7.4 at 4°C), 5 mM MgSO4, and 1 mM EDTA. Buffer G consist of 6 M guanidine hydrochloride, 250 mM Tris-HCl (pH 8.5 at 21°C), and 10 mM EDTA. Immediately prior to use of each aliquot of buffer G, α-phenylmethyl-sulfonyl fluoride (1 mM) and 2-mercaptoethanol (150 mM) were added. Buffer H consisted of RPMI 1640 supplemented with 10 mM N-(2-hydroxyethyl)piperazine-N'- (2-ethanesulfonic acid) (pH 7.4 at 21°C). RNA sample buffer consisted of 50% (v/v) formamide, 2.2 M formaldehyde, 20 mM 3-(N-morpholino)propanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA. Standard saline citrate buffer (1×) contained 150 mM NaCl in 15 mM sodium citrate (pH 7.0).

Extraction and Assay for Topo I. Semiconfluent AuxBl and CHRC5 cells were released from 75-cm2 tissue culture flasks by trypsinization and diluted with medium A. After sedimentation at 200 x g for 5 min, the cells were washed once with serum-free α-MEM. All further steps were performed at 4°C. Nuclei were prepared by hypotonic lysis as previously described (40). In brief, cells were resuspended at a concentration of ~107 cells/ml in buffer C, allowed to swell for 20 min at 4°C, and gently homogenized in a tight-fitting Dounce homogenizer until >98% of the cells were disrupted as assessed by phase-contrast microscopy. The nuclei were sedimented at 1400 x g for 10 min and washed twice in buffer C.

To solubilize topo I, nuclei were resuspended in buffer D and incubated for 15 min on ice. One-half volume of 13% (w/v) polyethylene glycol was added to precipitate the DNA. After incubation at 200 x g for 5 min, the insoluble material was sedimented at 12,000 x g for 5 min. Protein was estimated in aliquots of the supernatant (41).

The ability of topo I in the extract to relax supercoiled plasmid DNA was assessed by a modification of the method of Gupta et al. (42). In brief, O67, a pBR322-based plasmid isolated by alkaline lysis and CsCl2 sedimentation (43), was found to be ~85% supercoiled. Samples (final volume, 20 µl) contained 450 ng O67, 100 mM NaCl, 50 mM Tris-HCl (pH 7.5 at room temp), 2.5 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, and 50 µg/ml bovine serum albumin. The reaction was initiated by adding nuclear extract (2 µl) containing 0.625 to 40 ng of
protein in buffer E. After a 30-min incubation at 37°C, the reaction was terminated by adding 1 µl 10% (w/v) SDS and 1 µl 10 mg/ml protease K. Samples were then incubated at 37°C for an additional 15 min and applied to 1% (w/v) agarose gels prepared in 36 mM Tris, 30 mM NaH2PO4, and 1 mM EDTA containing 2 µg/ml chloroquine (15). After electrophoresis (45 V; 5 h), gels were stained with 0.5 µg/ml ethidium bromide and photographed.

Colony-Forming Assays. Two hundred to 250 AuxBl or CH°C5 cells were seeded in triplicate or quadruplicate in 35-mm tissue culture dishes containing 1.6 ml of medium A. After a 12-h incubation at 37°C in an atmosphere containing 5% (v/v) CO2/95% air to allow the cells to adhere, DMSO lacking or containing 50 mM quinidine was added to a final DMSO concentration of 0.1% (v/v). TPT, CPT, or etoposide was then added from a 1000-fold concentrated stock prepared in DMSO. Control samples received an appropriate volume of DMSO to bring the final DMSO concentration to 0.2% (v/v). After 7–8 days, colonies were stained with Coomassie brilliant blue in 50% (v/v) methanol-7% (v/v) acetic acid and rinsed twice with water. The cloning efficiency for AuxBl and CH°C5 cells treated with DMSO or quinidine alone was typically 60–70%.

In some experiments, 10⁷ to 10⁶ AuxBl or CH°C5 cells were seeded in 5 ml of medium A on 60-mm plates. After a 12- to 24-h incubation to allow cells to adhere, cells were treated at 37°C with various concentrations of TPT for 1–24 h. At the completion of the TPT treatment, plates were washed twice with α-MEM, incubated for 7–8 days, and stained as described above. To eliminate the possibility that the TPT was an immediate determination of absorbance readings (44). This scavenger (45, 46), diminished the time-dependent increase in absorbance which is often observed with MTT assays (44). This correction obviated the need for chromatography-grade DMSO and for immediate determination of absorbance readings (44).

Topo I Depletion Assay. Band depletion immunoblotting experiments were performed as previously described (11, 39, 47) with minor modifications. The basis for this assay is the observation that TPT-induced topo I-DNA adducts exhibit diminished migration on SDS-polyacryl-
TOPOTECAN AND P-GLYCOPROTEIN

Fig. 3. Blot immunodepletion assay for the formation of topo I-DNA adducts in AuxB1 and CHRC5 cells (Lanes 1–12 and 13–24, respectively). Cells were treated with TPT at 0 (Lanes 1, 12, 13, and 24), 1.56 μM (Lanes 5 and 17), 3.12 μM (Lanes 6 and 18), 6.25 μM (Lanes 7 and 19), 12.5 μM (Lanes 8 and 20), 25 μM (Lanes 9 and 21), 50 μM (Lanes 10 and 22), and 100 μM (Lanes 11 and 23) and lysed under denaturing conditions as described in “Materials and Methods.” Aliquots containing 30 μg of protein were subjected to SDS-polyacrylamide gel electrophoresis followed by staining with Coomassie blue (A) or transfer to nitrocellulose and Western blotting with antibodies directed against topoisomerase I (B) or poly(ADP-ribose) polymerase (C). To construct panel D, the signals for topo I (arrowhead) on the blot shown in B were quantitated (absorbance x area) by image analysis (48) and compared to the signals for samples loaded with a 5–15% (w/v) acrylamide gradient. Electrophoresis, transfer of polypeptides to nitrocellulose, Western blotting, and quantitation of the autoradiographs by image analysis were performed as previously described (39, 45). Aliquots containing 30 μg of protein were applied to adjacent wells of an SDS-polyacrylamide gel prepared with a 5–15% (w/v) acrylamide gradient. Electrophoresis, transfer of polypeptides to nitrocellulose, Western blotting, and quantitation of the autoradiographs by image analysis were performed as previously described (39, 45, 48). Affinity-purified rabbit antibodies against calf thymus topo I (11) were kindly provided by Dr. Leroy Liu (Johns Hopkins University School of Medicine, Baltimore, MD). Antibodies against poly(ADP-ribose)-polymerase (Ref. 49; generously provided by Dr. Guy Poirier, Laval University, Québec, Canada) served as a control to confirm the equal loading of various lanes.

Detection of Pgp by Western Blotting. AuxB1 and CHRC5 cells grown in 100-mm tissue culture plates were washed once with serum-free medium and then treated in situ with ice-cold buffer F supplemented with 1% (w/v) Nonidet P-40, 100 units/ml Trasylol, and 1 mM freshly added PMSF (45). Polypeptides in the Nonidet P-40-containing supernatants were precipitated at 4°C by the addition of trichloroacetic acid to a final concentration of 10% (w/v), washed three times with 20°C acetone, and solubilized in buffer G. Sample preparation, SDS-polyacrylamide gel electrophoresis, and transfer to nitrocellulose were performed as described above. Blots were blocked with 3% (w/v) bovine serum albumin in 150 mM NaCl containing 10 mM Tris-HCl (pH 7.4 at 21°C). Western blotting was performed with C219 (kindly provided by Dr. Victor Ling), a mouse monoclonal antibody that recognizes all identified isoforms of Pgp (50).

Detection of mdrl mRNA by Northern Blotting. After sedimentation on Ficoll-Hypaque step gradients (density = 1.119 g/cm3), human leukemia cells were washed once with serum-free RPMI 1640 and solubilized in guanidine thiocyanate (51). RNA was isolated by cesium chloride centrifugation (52). Samples containing 20 μg of total RNA were denatured by heating to 65°C for 3 min in RNA sample buffer, separated by electrophoresis in 1% (w/v) agarose gels containing 0.22 M formaldehyde, and transferred to Nytran (Schleicher and Schuell, Keene, NH). An insert containing the 683-base pair HaeIII fragment (residues −27 to 656) of the human mdrl complementary DNA (kindly provided by Dr. A. T. Fojo, National Cancer Institute) was excised with appropriate restriction endonucleases, purified by agarose gel electrophoresis, and labeled by the random primer method (53). After overnight hybridization, the blot was extensively washed with 2x standard saline citrate-0.1% (w/v) SDS at room temperature and with 0.2x standard saline citrate-0.1% (w/v) SDS for 15 min at 42°C. After autoradiography, the blot was probed for mRNA encoding phosphoglyceraldehyde dehydrogenase (54), a housekeeping gene.

Assessment of TPT Uptake by HPLC. The adherent cell lines AuxB1, CHRC5, MCF-7, and MCF-7/Adria were released from their substrata by brief treatment with trypsin-EDTA. Human leukemia cell lines were harvested by sedimentation over Ficoll-Hypaque step gradients. Cells collected from the interface were diluted with buffer H, sedimented at 200 x g for 10 min, and resuspended at a concentration of ~1 x 10⁷/ml in buffer H. TPT was added to a final concentration of 1 μM from a 1 mM stock in DMSO. Half of each sample was treated with 50 μM quinidine (added from a 50 mM stock in DMSO); the other half was treated with an equivalent amount of DMSO. After a 30-min incubation at 37°C, paired samples were sedimented at 3200 x g for 15 s, resuspended in 1 ml ice-cold PBS, immediately resedimented at 3200 x g for 15 s, and lysed by vigorous agitation for 20 s in 1 ml –20°C amide gels due to covalently bound DNA. Consequently, the signal for topo I at M, 100,000 diminishes with TPT treatment. In brief, 100-mm plates of subconfluent cells were washed once with serum-free α-MEM and treated with 10-ml aliquots of fresh α-MEM. Various dilutions of TPT were added in 100 μl DMSO to give TPT concentrations ranging from 0 to 100 μM. After a 60-min incubation at 37°C, the medium was decanted. The cells were immediately solubilized in 3 ml buffer G. Alkylation with iodoacetamide, sequential dialysis into 4 M urea followed by 0.1% (w/v) SDS, and lyophylization were performed as previously described (39, 45). Aliquots containing 30 μg of protein were applied to adjacent wells of an SDS-polyacrylamide gel prepared with a 5–15% (w/v) acrylamide gradient. Electrophoresis, transfer of polypeptides to nitrocellulose, Western blotting, and quantitation of the autoradiographs by image analysis were performed as previously described (39, 45, 48). Affinity-purified rabbit antibodies against calf thymus topo I (11) were kindly provided by Dr. Leroy Liu (Johns Hopkins University School of Medicine, Baltimore, MD). Antibodies against poly(ADP-ribose)-polymerase (Ref. 49; generously provided by Dr. Guy Poirier, Laval University, Québec, Canada) served as a control to confirm the equal loading of various lanes.

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Fig. 4. Analysis of total TPT accumulation by HPLC. A, standard curve from experiment depicted in B and C. B–D, effect of quinidine on accumulation of total TPT (lactone plus hydroxy acid) in individual cell lines. For each cell line, accumulation was measured in the absence and in the presence of 50 μM quinidine. Data are expressed as (uptake in the presence of quinidine/uptake in the absence of quinidine) × 100. A value of 100 indicates that quinidine had no effect on TPT accumulation. A value of >100 indicates that quinidine enhanced the accumulation of TPT.

Table 1 Effect of quinidine on the accumulation of TPT species in AuxBI and CHPc5 cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Quinidine</th>
<th>TPT lactone*</th>
<th>Hydroxy acid*</th>
<th>Total TPT*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AuxBI</td>
<td>–</td>
<td>18.1</td>
<td>5.7</td>
<td>23.8</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>22.2</td>
<td>10.0</td>
<td>32.2</td>
</tr>
<tr>
<td>CHPc5</td>
<td>–</td>
<td>9.3</td>
<td>3.5</td>
<td>12.8</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>35.7</td>
<td>2.9</td>
<td>38.6</td>
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*Measured by direct analysis of methanol extract.

ISS-100 autosampler (Norwalk, CT). To prepare the mobile phase, buffer containing 1.6 mM 1-heptanesulfonic acid and 60 mM disaccharide sodium phosphate was adjusted to pH 4.0 using 20% phosphoric acid or 0.01 M NaOH and filtered through a 0.45-μm filter. This solution was mixed to a ratio of 9:1 (v/v) with MeOH and degassed with helium prior to use as the mobile phase at a flow rate of 1 ml/min. The TPT chromophore was detected using a Perkin-Elmer LC40 fluorescent detector with excitation at 382 nm and emission at 523 nm. Retention times and peak areas were calculated using the Nelson 3000 chromatographic data system (Perkin-Elmer-Nelson System, Cupertino, CA). Peak areas were fit to a linear regression of a calibration curve produced by adding 5−50 pmol of TPT in 1 ml methanol to PBS-washed cell pellets containing 1 × 10⁶ AuxBI cells.

Under the chromatographic conditions described above, TPT lactone elutes at an average retention time of 4.5 min. The open-ring hydroxy acid elutes with the solvent front. To determine the total amount of TPT (lactone plus hydroxy acid) present, 500-μl aliquots of the methanol extract were acidified with 20 μl of 20% (w/v) phosphoric acid prior to HPLC analysis or storage at −70°C. The amount of hydroxy acid was calculated by subtracting the amount of TPT lactone from total TPT. Control experiments (not shown) revealed that quinidine produced no detectable fluorescence in this assay.

Assessment of TPT Accumulation by Flow Cytometry. Adherent cell lines were released from their substrata by brief trypsinization. Non-adherent cells were harvested by sedimentation over Ficol-Hypaque step gradients. Cells collected from the interface were diluted with buffer H, sedimented at 300 × g for 10 min, and resuspended in buffer H. Various concentrations of TPT were added from 200-fold concentrated stocks in DMSO. Each sample was then divided in half. One aliquot was treated with 50 μM quinidine; the other received an equivalent amount of DMSO. After incubation at 37°C for 30 min, each sample was subjected to flow cytometry on a FACScan fluorescence-activated cell sorter (Becton Dickinson, Mountain View, CA). Excitation was at 488 nm using a 15-mW argon laser. Fluorescence in droplets containing cells was detected using a 585-nm filter with a bandwidth of 42 nm. Ten thousand events were collected per sample.

Assessment of Daunorubicin Accumulation. Nuclear accumulation of daunorubicin was measured by the method of Willingham et al. (55) as modified for flow cytometry. Subconfluent cells in 35-mm tissue culture plates were incubated for 15 min at 37°C in 1.5 ml medium B containing 10 mM N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (pH 7.4 at 37°C), 20 μM daunorubicin, and 50 μM quinidine or an equivalent volume of DMSO. At the end of the incubation, the medium was removed by aspiration and replaced with buffer H containing quinidine or DMSO. After a 10-min incubation at 37°C, the medium was replaced with trypsin-EDTA containing quinidine or DMSO. After a 20-min incubation at 37°C, cells were transferred to 1.5-ml microfuge tubes,

methanol. After insoluble macromolecules were sedimented at 12,000 × g for 2 min, the methanol extracts were immediately analyzed by HPLC or stored at −70°C until ≤30 min before injection.

HPLC was performed using a C₈ guard-pak precolumn (Waters Corp., Milford, MA) and a 4.6 mm x 25 cm analytical column of 5 μM ODS-80TM TSK-gel (TosoHaas, Philadelphia, PA). Fifty μl of the methanol extract were injected onto the column using a Perkin-Elmer

7 Preliminary experiments using H-labeled TPT indicated that steady-state levels of binding were achieved within 30 min. In addition, these preliminary experiments indicated that the half-time for efflux of TPT after removal of drug-containing medium was <2 min for the AuxBI cells at 37°C. These observations led to the decision to assess TPT accumulation in the indicated manner.
topotecan and the indicated concentration of TPT (A), doxorubicin (B), etoposide (C), or CPT (D). Bars, ± 1 SD.

For nonadherent cells, trypsinization was omitted. Total cellular accumulation of doxorubicin was measured in a similar fashion except that fixation was omitted.

Data Presentation. Cloning experiments were performed three to six times. Drug accumulation studies were performed three times for most cell lines. Individual experiments are presented, along with SD between experiments.

RESULTS

Characterization of AuxBl and CHRC5 Cell Lines. The CHRC5 Chinese hamster ovary cell line was derived from the parental AuxBl cell line by sequential mutagen treatment and colchicine selection (35). Western blotting with antibody C219 revealed that CHRC5 cells contained at least 10-fold more Pgp than the parental AuxBl cells (Ref. 56; see also Fig. 7D). Consistent with this result, the CHRC5 cells were found to accumulate 10- to 20-fold less daunorubicin (Fig. 1, A and B) when nuclear drug accumulation was assessed by the method of Willingham (55). Colony-forming assays revealed that the IC50 of CHRC5 cells for daunorubicin was 10-fold higher than that of the AuxBl cells (Fig. 1A). These results provide a baseline for comparing any effect of Pgp expression on TPT accumulation and cytotoxicity.

In addition to their differences related to Pgp expression, these two cell lines were found to manifest slight differences in characteristics which are potential determinants of topo I-mediated cytotoxicity. Growth curves revealed that the CHRC5 cells had a doubling time of 22 h compared to 18 h for the AuxBl cells (data not shown). Titration assays for topo I activity (Fig. 1D) revealed that nuclear extracts from the CHRC5 cells contained about twice the activity present in nuclear extracts from AuxBl cells (cf. Fig. 1D, Lanes 4 and 12). In agreement with previous observations that cells containing increased topo I activity are more sensitive to CPT (13, 14, 57), the CHRC5 cells were found to be an average of 1.3-fold (SD, ±0.2; n = 4) more sensitive to CPT (Fig. 2A).

Resistance of CHRC5 Cells to TPT. If TPT sensitivity were unaffacted by Pgp overexpression, the CHRC5 cells would be expected to be 1.3-fold more sensitive to TPT as well. Contrary to this prediction, the CHRC5 cells were consistently less sensitive to the cytotoxic effects of TPT than the AuxBl cells (Fig. 2, B and C). In one group of experiments, the two cell lines were treated with various concentrations of TPT for 1 h and washed extensively (Fig. 2B). Under these conditions, the IC50 for TPT in AuxBl cells was 40 ± 10 (SD) nM (n = 3), and the IC50 in CHRC5 cells was 15-fold higher (SD, ±3; n = 3).

The camptothecins are known to be particularly toxic to cells in the S phase of the cell cycle (58-62). To assess the possibility that differences in growth and cell cycle characteristics of the two cell lines might account in part for the apparent resistance of CHRC5 cells to TPT during a 1-h incubation with drug, the exposure time was lengthened to 24 h (not shown) or to 7 days (Fig. 2C). These prolonged exposure times markedly increased the cytotoxicity of TPT in both cell lines. Nonetheless, the IC50 for the Pgp-overexpressing CHRC5 cells remained an average of 10-fold higher than that of the AuxBl cells after a 24-h exposure to TPT (not shown) and 3.2-fold higher (SD, ±1.4; n = 4) after a 7-day exposure (Fig. 2C). Although differences in cell cycle kinetics might account in part for the difference in sensitivity observed between the two cell lines during brief exposures, the Pgp-overexpressing CHRC5 cells continued to display modest resistance to TPT even with prolonged exposure.

Band Depletion Immunoblotting. If Pgp overexpression were altering the uptake of TPT, one would expect that higher concentrations of TPT would be required to stabilize topo I-DNA complexes in the CHRC5 cells. To assess this possibility, band depletion immunoblotting was performed. Cells were incubated with increasing concentrations of TPT for 60 min and immediately lysed under denaturing conditions. In the absence of TPT, topo I migrated with an apparent molecular weight of 100,000 (Fig. 3B, Lanes 1 and 13). When TPT was progressively added to the cells, more topo I became covalently bound to DNA, and less topo I migrated with a molecular weight of 100,000 in each cell line (Fig. 3B, Lanes 5-11 and 17-23). Quantitation of the signal for topo I, however, revealed that 4-fold higher concentrations of TPT were required to deplete the topo I signal in CHRC5 cells by 50% as compared to AuxBl cells (Fig. 3D).

Effect of Quinidine on TPT Accumulation and Cytotoxicity. The preceding results are consistent with the possibility that TPT accumulation might be diminished by Pgp overexpression.

Interestingly, the proportion of AuxBl cells killed by TPT increased from 1 log to >3 logs as the exposure time was increased from 1 h to 24 h to continuous exposure, but the IC50 for TPT (~40 nM) did not change appreciably (cf. Fig. 2, B and C). This is consistent with the suggested S-phase selectivity of this class of agents (58-62).
To further examine this possibility, drug accumulation studies and cytotoxicity experiments were performed in the absence and presence of 50 μM quinidine. Quinidine is one of many compounds that have been shown to modulate drug resistance in some mdrl lines by diminishing the ability of Pgp to efflux cytotoxic agents (reviewed in 2–7, 55, 63, 64).

Cell-associated TPT was detected fluorimetrically after methanol extraction and HPLC separation. A calibration curve obtained with this assay is shown in Fig. 4A. Because the opening hydroxy acid is not detected unless the methanolic cell extract is acidified, this method readily distinguishes between the lactone, which is thought to be the active form of the drug, and the hydroxy acid, which is thought to be inactive (65). Results obtained in one experiment utilizing this assay are shown in Table 1. Quinidine treatment enhanced the accumulation of TPT lactone in the AuxB1 cells by a factor of 1.2 and the accumulation of total TPT (lactone plus hydroxy acid) by a factor of 1.4. In contrast, quinidine treatment enhanced the accumulation of TPT lactone in the CHC5 cells by a factor of 3.8 and accumulation of total TPT by a factor of 3.0. These results are consistent with the view that Pgp overexpression can affect TPT accumulation. Furthermore, in these experiments, there was no evidence for excess conversion of TPT lactone to the (inactive) hydroxy acid in the CHC5 cells in the absence of quinidine. No additional fluorescent peaks were detected in the CHC5 cells. These observations appear to rule out differences in drug metabolism between the two cell lines as a cause for the differences in drug sensitivity.

When the colony-forming assays were repeated, addition of 50 μM quinidine diminished the IC50 for TPT in CHC5 cells (Fig. 2D) by a factor of 4.2 ± 0.5 (n = 3). Similar results were obtained with 10 μM verapamil (data not shown). As a consequence, the CHC5 cells became about as sensitive to TPT as AuxB1 cells grown under the same conditions (Fig. 2D).

For purposes of comparison, the effect of quinidine on the cytotoxicity of etoposide, another drug effluxed by Pgp, was also examined (Fig. 2E). In contrast to the 4-fold change in IC50 observed for TPT, quinidine diminished the IC50 for etoposide in CHC5 cells by a factor of 27 ± 8 (n = 3).

Effect of Pgp Expression on TPT Accumulation and Cytotoxicity in Human MCF-7/Adriar Breast Cancer Cells. To determine whether Pgp expression also affected TPT accumulation and cytotoxicity in human cells, Pgp-expressing MCF-7/Adriar breast cancer cells (37) and parental MCF-7 cells were examined. Control experiments revealed that the MCF-7/Adriar cells were 20- to 30-fold resistant to the selecting agent doxorubicin (Fig. 5A). The accumulation of doxorubicin in MCF-7/Adriar cells was enhanced 5- to 8-fold by treatment with verapamil (Fig. 5B) or quinidine (not shown). In contrast, these agents had no effect on the accumulation of doxorubicin in MCF-7 cells (Fig. 5C).

TPT accumulation in MCF-7 and MCF-7/Adriar cells was examined using the HPLC assay described above. Quinidine had no effect on the accumulation of TPT in MCF-7 cells (Fig. 4C). In the Pgp-expressing MCF-7/Adriar cells, on the other hand, quinidine increased TPT accumulation by a factor of 3 (Fig. 4C). Consistent with the preceding drug accumulation studies, cytotoxicity assays (Fig. 6A) revealed that verapamil decreased the IC50 of TPT in MCF-7/Adriar cells by a factor of 3.6 ± 1.4 (n = 4). For comparison, the same concentration of

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Fig. 7. Characterization of KG1a cells. A and B, Northern blot showing expression of Pgp message in KG1a cells. Samples containing 20 μg of total RNA were separated by agarose gel electrophoresis and probed with an mdrl partial complementary DNA (A). The same blot was reprobed with a complementary DNA that hybridizes with phosphoglyceraldehyde dehydrogenase message (B). KG1a cells expressed more mdrl message than the other cell lines. C and D, detection of Pgp in KG1a cells by Western blotting. Nonidet P-40 extracts from CHC5 cells (Lanes 1–4 and A), AuxBl cells (Lane 5), KG1a cells (Lane 6), and HL-60 cells (Lane 7) were subjected to SDS-polyacrylamide gel electrophoresis staining with Coomassie blue (C) or blotting with antibody C219 (D). Arrowhead, M, 170,000 Pgp signal. Samples contained protein from 5 × 10^6 cells (Lanes 1 and 5–8), 2.5 × 10^6 cells (Lane 2), 1.25 × 10^6 cells (Lane 3), and 0.5 × 10^6 cells (Lane 4). Nonadjacent lanes from a single gel and corresponding autoradiograph were juxtaposed to compose this figure.

*The effect of quinidine on TPT accumulation and cytotoxicity in AuxBl cells (Table 1 and Fig. 2, C versus D) presumably arises from the inhibition of the small but detectable amount of Pgp expressed in these cells (Ref. 74; also observable in Fig. 7D on prolonged exposure).
verapamil decreased the IC$_{50}$ of doxorubicin (Fig. 6B) by a factor of 17.6 ± 3.6 ($n = 3$) and the IC$_{50}$ of etoposide (Fig. 6C) by a factor of 6.5 ± 1.3 ($n = 4$). In contrast, verapamil had no effect on the IC$_{50}$ of CPT in these cells (Fig. 6D). Likewise, verapamil had no effect on the cytotoxicity of TPT in the MCF-7 cells (not shown). These results are consistent with the view that Pgp overexpression can affect TPT accumulation (Fig. 4C) and cytotoxicity (Fig. 6) in human cancer cells.

Effect of Pgp Expression on TPT Accumulation and Cytotoxicity in a Human Acute Leukemia Cell Line. To determine whether more modest levels of Pgp expression also affected TPT accumulation and cytotoxicity, a panel of human acute leukemia cell lines was examined. Northern blotting (Fig. 7A) and Western blotting (Fig. 7D) revealed that unselected KG1a cells expressed detectable amounts of mdr1 mRNA and protein. In contrast, a number of other cell lines did not (Fig. 7).

The accumulation of TPT in these cell lines was examined in the absence and presence of quinidine. Quinidine had no effect on the accumulation of TPT in HL-60, Molt 3, K562, or CEM cells (Fig. 4D). In contrast, quinidine increased the accumulation of TPT in Pgp-expressing KG1a cells by a factor of 1.8 ± 0.2 ($n = 3$) (see Fig. 4D).

The HPLC assay described above is not routinely available in many clinical laboratories. For other fluorescent drugs such as daunorubicin, flow cytometry is a widely available method of analyzing cellular drug accumulation (63, 64, 66 and references therein). To assess whether flow cytometry could be utilized to assess TPT accumulation, KG1a and HL-60 cells were incubated with TPT in the absence or presence of quinidine and then subjected to flow cytometry. The intensity of the cellular fluorescence was directly related to the extracellular TPT accumulation over a wide range of TPT concentrations (Fig. 8A, inset). In KG1a cells, fluorescence attributable to TPT accumulation was 1.6-fold higher (SD, ±0.1; $n = 6$) in the presence of quinidine than in its absence (Fig. 8A). In contrast, quinidine had no effect on the fluorescence of HL-60 cells (Fig. 8B). Thus the effect of Pgp overexpression on TPT accumulation can be detected by flow cytometry as well as HPLC.

To confirm that the changes in drug accumulation resulted in changes in cytotoxicity, cell proliferation assays were performed using a modified MTT dye reduction assay (Fig. 9). In contrast to the originally published MTT assay (44), this procedure yields a relatively stable product (Fig. 9A). Absorbance was a linear function of viable cell number over a 10-fold range of cell concentrations (Fig. 9A). When this assay was utilized to assess TPT cytotoxicity in KG1a cells (Fig. 9B), quinidine was found to decrease the IC$_{50}$ of TPT in Pgp-expressing KG1a cells (Fig. 9B) by a factor of 1.8 ± 0.5 ($n = 3$). The same concentration of quinidine was found to decrease the IC$_{50}$ of etoposide in KG1a cells by a factor of 3 (Fig. 9C). In contrast, quinidine had no effect on the IC$_{50}$ of CPT in these cells (Fig. 9D). Moreover, quinidine had no effect on the cytotoxicity of TPT in the cell lines that did not express mdr1 (not shown).

DISCUSSION

The camptothecins are a class of cytotoxic agents which poison the nuclear enzyme topo I (reviewed in Ref. 67). These agents have a unique mechanism of cytotoxicity (reviewed in Refs. 61, 62, 68). In addition, the parent compound CPT is unaffected by Pgp (Refs. 22 and 23 and this study). In light of these observations, the camptothecins are undergoing renewed investigation as potential antineoplastic agents.

TPT is the first member of this class of antineoplastic agents to undergo clinical testing in the United States in 20 years. Phase I trials of this agent have been completed (31–34). As an adjunct to these studies, we have examined the effect of Pgp expression on TPT accumulation and cytotoxicity. Chinese hamster CH$_{9}$C5 cells which overexpress Pgp were observed to be resistant to TPT relative to the parental AuxB1 cells (Fig. 2, B and C). Differences in cell cycle kinetics might account for part of this resistance (cf. Fig. 2, B and C). Several observations, however, indicate that an effect of Pgp on the cellular accumulation of TPT also contributes to this resistance. First, low-level resistance of CH$_{9}$C5 cells persisted even with continuous exposure to TPT (Fig. 2C). Second, when the cells were coincubated with quinidine, a well-characterized modulator of Pgp-mediated mdr (2–7, 55), both the accumulation (Fig. 4B) and the cytotoxicity (Fig. 2D) of TPT increased. Finally, quinidine and verapamil were observed to have similar effects on TPT accumulation and cytotoxicity in two additional Pgp-expressing cell lines (Figs. 4, 6, and 9) but not in six control human cell lines that do not overexpress Pgp. Collectively, these observations suggest that Pgp overexpression can result in the diminished accumulation and the reduced cytotoxicity of TPT.

This Pgp-mediated resistance, although modest in magnitude, might have clinical implications. Pgp overexpression has been commonly observed in colorectal carcinoma but not in non-small cell lung cancer or platinum-resistant ovarian cancer (69, 70). In a phase I clinical trial of TPT which investigated a clinically active schedule, no responses were observed in patients with colorectal carcinoma who received TPT at the maximal tolerated dose (34). In contrast, the same schedule of administration produced responses in patients with non-small cell lung cancer and platinum-resistant ovarian cancer (34). This preliminary observation regarding Pgp overexpression and clinical resistance to TPT requires further investigation during phase II trials of this agent.

During the course of the studies described above, a number of new assays were developed. The HPLC assay for TPT (Fig. 4) was modified from the original procedure of Beijnan et al. (71). The modified assay is quantitatively reliable and highly sensitive. Under conditions where the HPLC assay is not available, TPT accumulation can be followed by flow cytometry (Fig. 8). This assay will detect the effect of Pgp modulators on the accumulation of TPT in CH$_{9}$C5 and MCF-7/Adriamycin 'cell lines (not shown) as well as the KG1a cells (Fig. 8A). Flow cytometry does not, of course, distinguish between TPT lactone and the open-ring hydroxy acid. After the 30-min incubation with TPT utilized in this assay, however, most of the intracellular drug remains in the lactone form (Table 1). Finally, a modified...
version of the MTT dye reduction assay was developed (Fig. 9). Addition of the free radical scavenger thiodiglycol improved the stability of the reduced formazan (Fig. 9A) and obviated the need for expensive HPLC-grade DMSO in this assay. These assays should prove useful for investigating the accumulation and cytotoxicity of TPT in a variety of clinical and research settings.

The present studies suggest that some analogues of CPT might be affected by Pgp overexpression and others might not. While this manuscript was being revised, a report by Chen et al. (72) was published comparing the activity of several CPT analogues in Pgp-expressing human KB V1 cells and the parental KB 3-1 cell line. Consistent with earlier observations (22, 23), the cytotoxicities of CPT and several analogues were shown to be unaffected by Pgp overexpression. In contrast, the cytotoxicity of TPT was diminished in the Pgp-expressing cell line (72). The results of the present study complement and extend these recent findings by showing that the diminished cytotoxicity of TPT is observed in a variety of Pgp-expressing cell lines rather than a single cell line. In addition, the present study demonstrates that the diminished cytotoxicity is accompanied by diminished accumulation of TPT lactone rather than a change in the conversion of the lactone to inactive hydroxy acid or any other detectable metabolites.

Finally, we have compared the effect of Pgp overexpression on the accumulation and cytotoxicity of TPT to the effect on other drugs that are classically considered to be substrates for the multidrug transporter. The effect of Pgp overexpression on TPT accumulation and cytotoxicity is substantially smaller than the effect of Pgp overexpression on daunorubicin, doxorubicin, or etoposide action in the same cell lines (Figs. 1, 2, 5, 6, and 9). It is important to note that TPT kills several logs of Pgp-resistant KB 3-1 cells. Consistent with earlier observations (22, 23), the cytotoxicities of CPT and several analogues were shown to be unaffected by Pgp overexpression. In contrast, the cytotoxicity of TPT was diminished in the Pgp-expressing cell line (72). The results of the present study complement and extend these recent findings by showing that the diminished cytotoxicity of TPT is observed in a variety of Pgp-expressing cell lines rather than a single cell line. In addition, the present study demonstrates that the diminished cytotoxicity is accompanied by diminished accumulation of TPT lactone rather than a change in the conversion of the lactone to inactive hydroxy acid or any other detectable metabolites.

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