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Carboxylesterase-Mediated Sensitization of Human Tumor Cells to CPT-11 Cannot Override ABCG2-Mediated Drug Resistance

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ABSTRACT

The recently introduced camptothecin-derived chemotherapeutic agents have demonstrated remarkable promise in cancer therapy and as such have been approved for use in humans for the treatment of ovarian, lung, and colon cancer. CPT-11 is a prodrug that is activated by esterases to yield the potent topoisomerase I inhibitor, SN-38. Considerable success has been achieved in the treatment of both naïve and drug-resistant colon cancer with CPT-11. However, mechanisms of resistance to this agent have not been explored in detail. The role of the ATP-dependent drug transporter ABCG2 in CPT-11 cytotoxicity is unclear because some ABCG2 mutants confer camptothecin resistance, whereas others do not. Because CPT-11 is activated by carboxylesterases (CEs), we assessed the relative contribution of each protein in mediating CPT-11 toxicity by both drug accumulation and cell growth-inhibition assays. Our results indicate that the expression of ABCG2 protects cells from CPT-11 toxicity, even in the presence of high levels of a rabbit liver carboxylesterase (rCE), which can efficiently activate the drug. However, this can be partially overcome by the ABCG2 inhibitor reserpine. These studies indicate that overexpression of ABCG2 in vivo would probably overcome any increased drug activation that might be achieved by gene delivery or antibody-directed enzyme prodrug therapy methods using rCE.

CPT-11 is an anticancer prodrug that is activated in vivo by esterases to yield the potent topoisomerase I poison SN-38 (Satoh et al., 1994; Tanizawa et al., 1994). SN-38 is 100- to 1000-fold more cytotoxic than CPT-11. In recent years, CPT-11 has been approved for the treatment of colon cancer, and it is currently under investigation for use in a variety of other solid malignancies, in both adults and children (Conti et al., 1996; Baker et al., 1997; Kudoh et al., 1998; Furman et al., 1999). Initial studies seem very promising, attesting to the fact that this is one of the best anticancer agents that has been introduced in the clinic in the last 30 years.

CPT-11 activation is primarily mediated by carboxylesterases (CEs) (Satoh et al., 1994; Morton et al., 1999, 2000; Khanna et al., 2000). We recently identified a rabbit liver CE (rCE) that can efficiently activate the drug (Potter et al., 1998a), and overexpression of this protein in cells in culture, or when grown as xenografts in immune-deprived mice, results in enhanced sensitivity to CPT-11 (Danks et al., 1998, 1999; Potter et al., 1998a,b). Studies using replication-deficient adenovirus harboring rCE indicate that tumor cells can be markedly sensitized to the drug after viral transduction (Wierdl et al., 2001). The potential application of this reagent to the purging of neuroblastoma cells from bone marrow has been described previously (Meck et al., 2001; Wagner et al., 2002).

Transport proteins mediate the efflux of camptothecin analogs from cells. However, the ATP-binding cassette (ABC) transporter superfamily members comprise a large group of functionally diverse proteins whose expression has been detected in prokaryotes, plants, and mammals. This gene family is defined by the presence of an approximately 250 amino acid stretch that contains Walker A and B motifs and a

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ABBREVIATIONS: CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin; SN-38, 7-ethyl-10-hydroxycamptothecin; ABC, ATP-binding cassette; CE, carboxylesterase; rCE, rabbit liver carboxylesterase; CSA, cyclosporin A_1; HPLC, high-performance liquid chromatography; m.o.i., multiplicity of infection; P-gp, P-glycoprotein; "E" gate, efflux gate; EST, expressed sequence tag; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting; AdCMVrCE, replication-deficient adenovirus expressing rCE; mutABCG2, ABCG2 containing a mutation in the Walker A domain; MK571, 3-([(3-(2-[7-chloro-2-quinolinyl]ethenyl)phenyl)-((3-dimethylamino-3- oxopropyl)-thio)-methyl]thio)propanoic acid; PSC833, 3′-oxo-4-butenyl-4-methyl-(Thr^1)-(Val^2)-cyclosporin.

conserved linker sequence termed the "ABC signature" that is essential for ATP hydrolysis (Higgins, 2001). Most ABC transporters are localized on cellular membranes and transport structurally diverse compounds. They are composed of either one or two membrane-spanning domains and one or two ATP-binding domains that function as either a single protein or a multiprotein complex (Higgins, 2001).

The so-called "half-molecule" transporters within the ABC superfamily (i.e., containing only one membrane-spanning and one ATP-binding domain) are typically localized to the membranes of intracellular organelles (Higgins, 2001). However, ABCG2 (breast cancer resistance protein, MXR, or ABCP) (Allikmets et al., 1998; Doyle et al., 1998; Bates et al., 2001) is the only drug-transporting half-molecule transporter currently localized to the plasma membrane (Rocchi et al., 2000; Maliepaard et al., 2001) and has been demonstrated to function as a homodimer (Ozvegy et al., 2001). ABCG2 is overexpressed in human tumor cell lines selected for resistance to DNA topoisomerase inhibitors such as topotecan and SN-38 (Van Hattum et al., 2002). However, drug-selected cells also have increased expression of other ABC transporters (Litman et al., 2000) that may facilitate efflux of these camptothecins, thus leading to potential confusion as to the role of ABCG2 in drug resistance.

To assess the role of ABCG2 in the resistance of cells to camptothecin analogs, we isolated a cDNA encoding the protein and expressed it in an ABCG2- and P-gp–null cell line. These cells were then characterized for transport properties and their ability to confer camptothecin resistance. More importantly, these cells were used to evaluate how ABCG2 interacts with CEs known to activate CPT-11. Because overexpression of ABCG2 would be expected to reduce intracellular concentrations of SN-38 and CPT-11, increased levels of this protein in tumors may result in drug resistance. Therefore the efficacy of enzyme/prodrug therapy approaches such as viral-directed and antibody-directed enzyme prodrug therapy with CPT-11 would depend on the expression of ABCG2. To determine the contribution of this protein in mediating resistance to the camptothecin analogs, we have performed studies on cell lines overexpressing both ABCG2 and rCE. In this series of studies, rCE was used as a tool to create high levels of SN-38 in cells and hence determine the contribution of ABCG2 toward drug resistance. Results demonstrate that reduced intracellular drug concentrations and, consequently, reduced cytotoxicity are observed in cells expressing ABCG2. Additionally and more importantly, enforced overexpression of CEs cannot overcome this drug resistance.

Materials and Methods

Cell Lines, Adenovirus, Drugs, Inhibitors, and Antibodies. Saos-2 cells were obtained from Dr. G. Zambetti (St. Jude Children's Research Hospital, Memphis, TN) and grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum under an atmosphere of 10% CO_2 at 37°C. Replication-deficient adenoviruses expressing rCE under control of the cytomegalovirus promoter were generated as described previously (Wierdl et al., 2001). CPT-11 and SN-38 were generous gifts from Dr. J. P. McGovren (Pharmacia Upjohn, Kalamazoo, MI). CPT-11 was dissolved in methanol at 10 mM and stored at −20°C. Immediately before use, the drug was diluted in serum-free media. SN-38 was dissolved in DMSO and used in a fashion identical to that of CPT-11. The fluorescent dye Hoechst 33342 was obtained from Molecular Probes (Eugene, OR). Reserpine, progesterone, probenecid, prostaglandin A1, and leukotrienes B4, C4, and D4 were purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in DMSO. Antipeptide polyclonal antibodies to ABCG2 were used as described previously (Zhou et al., 2002), and an antibody to *MRP1* was purchased from Signet Laboratories (Dedham, MA).

Isolation of ABCG2 cDNA. To determine whether an EST clone containing the ABCG2 cDNA existed, the published ABCG2 nucleotide sequence was used to search the EST database. We identified a cDNA that had significant identity (>99%) with the published sequence (dbEST clone number 52176). The clone was purchased from Incyte Systems (Palo Alto, CA), and the cDNA insert was isolated and ligated into pcDNA3 (Invitrogen, Carlsbad, CA). DNA sequence analysis confirmed the identity of the cDNA (GenBank accession number AY017168).

Generation of a Nonfunctional ABCG2 Mutant. To render the ABCG2 protein nonfunctional, the highly conserved lysine residue in the Walker A domain ($G(X)_4GKS$) was substituted with a methionine with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutation was confirmed by DNA sequence analysis. This mutant ABCG2 cDNA was transfected into Saos-2 cells as described below.

Cell Transfections. Saos-2 cells were screened by Western analysis and revealed no immunodetectable *MDR1*, P-gp, or ABCG2 protein in the membranes. The cells were transfected with the relevant plasmids (10 μg) by standard calcium phosphate coprecipitation and cultured in medium containing 1 mg/ml of G418 for at least 14 days, or until colonies were readily visible. After isolation, membrane fractions were prepared from clones, and ABCG2 expression was determined using a rabbit polyclonal antibody (Zhou et al., 2002).

Western Analysis. For analysis of MRP1 expression, 100 μg of the membrane-enriched fraction was resolved on 7.5% SDS- polyacrylamide gel electrophoresis, and after transfer to filters, proteins were immunoblotted with an anti-MRP1 antibody at a dilution of 1:300 (Signet). For ABCG2 analysis, the rabbit polyclonal antibody was used at a dilution of 1:500. For both antibodies, specific cross-reactivity was detected using a horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence.

Adsorbed ABCG2 antibody (Zhou et al., 2002) was prepared by incubation with 2 mg/ml of ABCG2 peptide overnight at 4°C and centrifugation at 10,000*g* for 5 min. The supernatant was used for Western analysis at the same dilution as the native antisera.

Carboxylesterase Assays. CE activity in whole-cell sonicates was determined spectrophotometrically using *o*-nitrophenyl acetate as a substrate (Beaufay et al., 1974; Potter et al., 1998a). Data were expressed as micromoles of *o*-nitrophenol produced per milligram of protein per minute.

Drug Accumulation and Efflux. Cells were plated at 2×10^5 cells/well in 24-well plates and were allowed to attach overnight at 37°C. After incubation with the dye (for up to 1 h, as indicated in the figure legends), cells were rapidly washed with Hanks' buffer (4°C), and after trypsinization, they were resuspended in PBS buffer (4°C). FACS analysis was performed on a Vantage flow cytometer (BD Biosciences, San Jose, CA). The following excitation and emission wavelengths were used for each dye: Hoechst 33342, 365 nm/400 nm; and mitoxantrone, 647 nm/670 nm. Only intact cells, determined from the appropriate light scatter, were analyzed, and data were collected from at least 5000 cells for each sample.

Cells lacking ABCG2 retain more dye and have greater fluorescence. Therefore, all cells with fluorescence values lower than the ABCG2-null cells were considered to efflux the dye. This was designated the efflux gate ("E" gate) and was used to quantify the proportion of cells effluxing the dye. Inhibitor calculations were made by determining the percentage of the population of cells moving from the low fluorescence "E" gate, to the high-intensity fluorescence observed in cells containing the vector plasmid.

Determination of CPT-11 and SN-38. CPT-11 and SN-38 concentrations were determined by reverse-phase HPLC as described previously (Guichard et al., 1998; Danks et al., 1999). Briefly, samples were mixed with an equal volume of ice-cold acid methanol, stored at −80°C for 1 h, and centrifuged at 30,000*g* to remove particulate matter. Drug concentrations in the supernatant were determined by separation on a NovaPak C18 column using 25% acetonitrile/75 mM ammonium acetate, pH 4.0, as a mobile phase. Detection was achieved using a Jasco 920 fluorescence detector (Jasco, Tokyo, Japan), and the sensitivity of this system was 20 pg/μl and 1.5 pg/μl for CPT-11 and SN-38, respectively.

Determination of Intracellular CPT-11 and SN-38 Concentrations. Intracellular drug concentrations were determined under the following conditions. Cells (4×10^6) were harvested by trypsinization and resuspended in 1 ml of serum-free media. CPT-11 was added to a final concentration of 1, 10, or 100 μM, and after 1 h of incubation at 37°C, cells were centrifuged, washed rapidly with cold PBS, and finally resuspended in 200 μl of 50 mM HEPES, pH 7.4. After sonication on ice, an equal volume of cold acid methanol was added, and drug concentrations were determined by HPLC as described above.

Viral Transduction of Cell Lines. Cell lines were transduced with replication-deficient adenovirus expressing rCE (AdCMVrCE), as described previously (Wierdl et al., 2001). Routinely, a multiplicity of infection (m.o.i.) of 5 was used. At this m.o.i., no viral toxicity was observed, and high yields of CE activity were produced 3 to 4 days after transduction (Wierdl et al., 2001). Cells were transduced with virus for 24 h in culture medium, at which point the reagent was removed and replaced with fresh medium. Cells were then allowed to grow for 3 to 4 days before growth-inhibition assays or drug-accumulation studies. When reserpine was used, cells were treated with 5 to 20 μM drug for 1 h at 37°C.

Growth-Inhibition Assays. Inhibition of cell growth by CPT-11 was assessed by cell counting using a Coulter Z2 multisizer (Beckman Coulter, Inc., Fullerton, CA). Cells were plated at a density of 5×10^4 per well in a six-well plate, and after allowing to adhere overnight, the media were removed and replaced with serum-free media containing CPT-11. After incubation for 2 h, the drug was aspirated, and fresh media were applied. After growth equivalent to three cell doublings (72 h), the cells were counted. Routinely, all data points were repeated in triplicate, and IC_{50} values were determined using the GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

Results

Isolation of the ABCG2 cDNA. The cDNA encoding human ABCG2 was isolated from brain mRNA and contained four nucleotide differences compared with ABCP (Allikmets et al., 1998). These resulted in four amino acid changes (A → V at position 24, Q → E at 166, F → S at 208, and A → P at 501) that might reflect polymorphisms in ABCG2. Nevertheless, the predicted protein sequence of our ABCG2 was 655 amino acids in length, and it was greater than 99% identical to both ABCG2 (Doyle et al., 1998) and ABCP (Allikmets et al., 1998). Importantly, this cDNA derived from brain encoded an arginine at amino acid position 482, unlike the ABCG2 isoform described by Ross and colleagues, which was isolated from a drug-resistant cell line (Doyle et al., 1998). In addition, an analysis of the human genome confirms that Arg-482 is present in the wild-type ABCG2 sequence. We are currently evaluating the role of the single amino acid substitutions on the function of ABCG2 (to be reported elsewhere), but we have evidence that the change to a noncharged amino acid at position 482 (i.e., from Arg to Thr or Gly) does not occur in the population and seems to occur only under drug selection (Zamber et al., 2003).

Characterization of Cell Lines Expressing ABCG2. To eliminate the possible confounding effects of expression of other transporters or endogenous ABCG2 in cells, we identified an ABCG2 and P-gp–null cell line by Western analysis (Saos-2). Additionally, Saos-2 cells have very low levels of endogenous CE activity (<6 μmol/min/mg) and consequently are relatively resistant to the cytotoxic effects of CPT-11 (Wierdl et al., 2003). This line was subsequently transfected with expression plasmids to yield clones that stably expressed either ABCG2 or ABCG2 containing a mutation in the Walker A domain (mutABCG2). Membrane fractions were isolated, and Western analyses were performed on these cell lines (Fig. 1). Among Saos-2–transfected clones expressing ABCG2, the rank order of protein expression was ABCG2#4 ≥ -#6 > -#7 (Fig. 1B). Two clones, ABCG2#4 and ABCG2#7, demonstrated high and low levels of ABCG2 expression, respectively, and were expanded and used for the subsequent studies described herein. Transfection with the mutant ABCG2 cDNA yielded several cell lines, with the clone mutABCG2#10 expanded for further studies. As an additional control, Saos-2 cells were transfected with pcDNA3, and after clonal selection with G418, pcDNA#2 cells were isolated. As expected, pcDNA#2 demonstrated no detectable ABCG2 expression (Fig. 1). All Saos-2 ABCG2 clones demonstrated similar growth rates and doubling times of 24 h (data not shown).

Functional Analysis of ABCG2 Cell Lines. To assess the function of ABCG2 in Saos-2 transfected cells, we performed uptake studies using the dye Hoechst 33342 and the chemotherapeutic agent mitoxantrone (Fig. 2). Cells were incubated with either drug for an hour until steady-state levels were achieved, and FACS analysis was performed to determine the levels of intracellular fluorescence. As indicated in Fig. 2A, mitoxantrone was specifically excluded from cells expressing ABCG2. In addition, these studies indicated that mitoxantrone accumulation was inversely proportional to the amount of protein expressed. With Hoechst 33342, although the drug was excluded similar to mitoxantrone, the levels of intracellular fluorescence did not directly correlate with the amounts of ABCG2 protein expressed (Fig. 2B). This discrepancy is probably caused by ABCG2 having different affinities for these drugs.

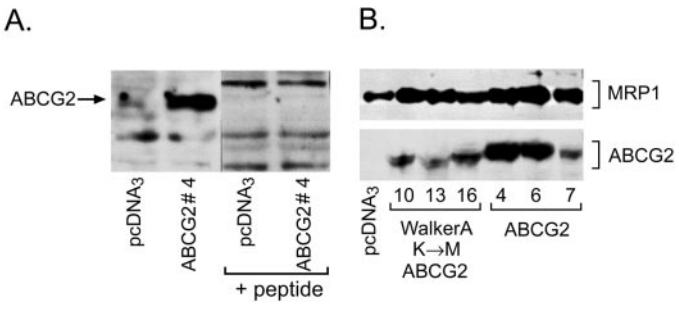

Fig. 1. Western analysis of ABCG2 expression. A, total membrane lysates of Saos-2 cells expressing human ABCG2 (ABCG2#4) and one vector control cell line (pcDNA3) cell were used for Western analysis using either an anti-ABCG2 or preadsorbed ABCG2 antisera (+peptide). The presence of ABCG2 is indicated by the arrow. B, Western analysis of expression of ABCG2 and mutABCG2 in Saos-2 cells. The filters were developed as described under *Materials and Methods* with an anti-ABCG2 or anti-MRP1 antibody.

To determine whether this was the case, we assessed the uptake of mitoxantrone and Hoechst 33342 as a function of drug concentration (Fig. 3). We observed that ABCG2#4 cells accumulated approximately 50% less mitoxantrone compared with ABCG2#7 cells at each concentration tested and that mutABCG2#10 accumulated twice as much drug as ABCG2#7 (Fig. 3A). In contrast, Hoechst 33342 showed saturation kinetics (Fig. 3B), and the concentrations required to achieve half-maximal intracellular values were estimated to be approximately 5 μM for both ABCG2#4 and ABCG2#7 cells. Nevertheless, the intracellular concentration of Hoechst 33342 in ABCG2#4 cells was approximately 50% of that observed in ABCG2#7 and was much greater than that observed in mutABCG2#10 cells. The difference in drug accumulation between ABCG2#4 and ABCG2#7 was remarkably close to the 3-fold difference in BABCG2 protein levels observed in membranes derived from these cell lines (Fig. 1B).

Analysis of Hoechst 33342 Efflux in ABCG2-Expressing Cell Lines. To further characterize cells expressing ABCG2, we measured the rate of Hoechst influx and efflux in ABCG2#4, ABCG2#7, and mutABCG2#10 cells (Fig. 3, C and D). As indicated in Fig. 3C, the steady-state intracellular dye concentrations are inversely proportional to the level of ex-

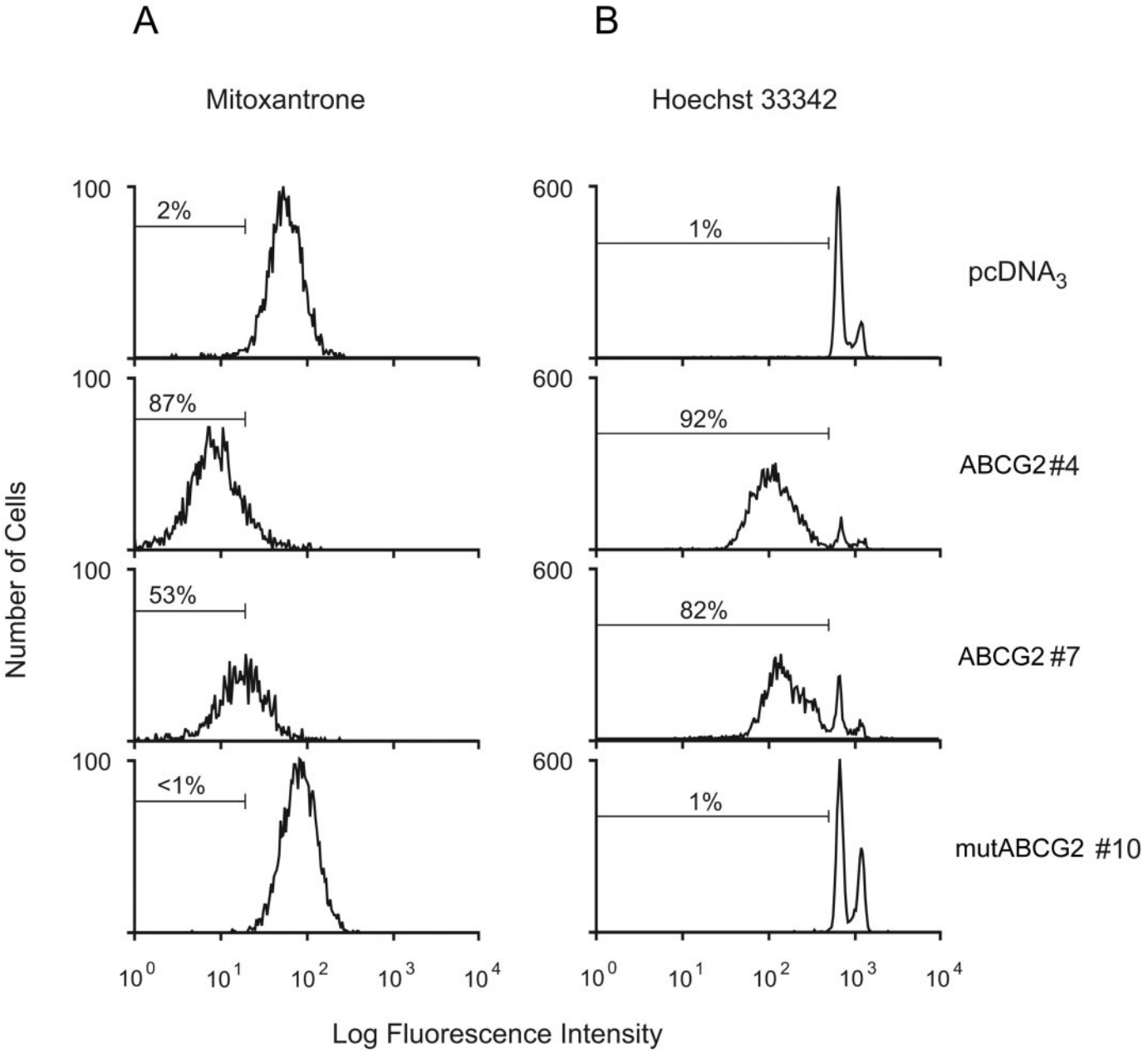

Fig. 2. Amount of functional ABCG2 expression determines the steady-state levels of drug accumulation. The Saos-2 cell lines pcDNA#2, ABCG2#4, ABCG2#7, and mutABCG2#10 were incubated for 1 h in the presence of 1 μM mitoxantrone (left) or 1 μM Hoechst 33342 (right). The proportion of cells in the population effluxing the dyes was determined by first analyzing the pcDNA3 cells. The "E" gate was defined between a fluorescent value of one and the first inflection point depicting dye retention in the vector cells. This efflux gate was individualized for each fluorescent probe (mitoxantrone or Hoechst 33342) and was used to determine the percentage of the total cell population within the "E" gate. A representative figure is shown.

pression of functional ABCG2. Hence, ABCG2#4 excludes more dye than ABCG2#7, which in turn demonstrates approximately 2-fold less Hoechst 33342 than mutABCG2#10 cells. To determine the rates of dye efflux in these cell lines, steady-state levels were achieved by incubation in media containing 10, 5, and 1 μM Hoechst 33342 for ABCG2#4, ABCG2#7, and mutABCG2#10 cells, respectively. The increased concentrations of dye were used to compensate for the activity of the ABCG2 protein in these cells. After resuspension in fresh medium, intracellular fluorescence determinations were performed at various time intervals (Fig. 3D). To estimate the initial rate of drug efflux, linear regression

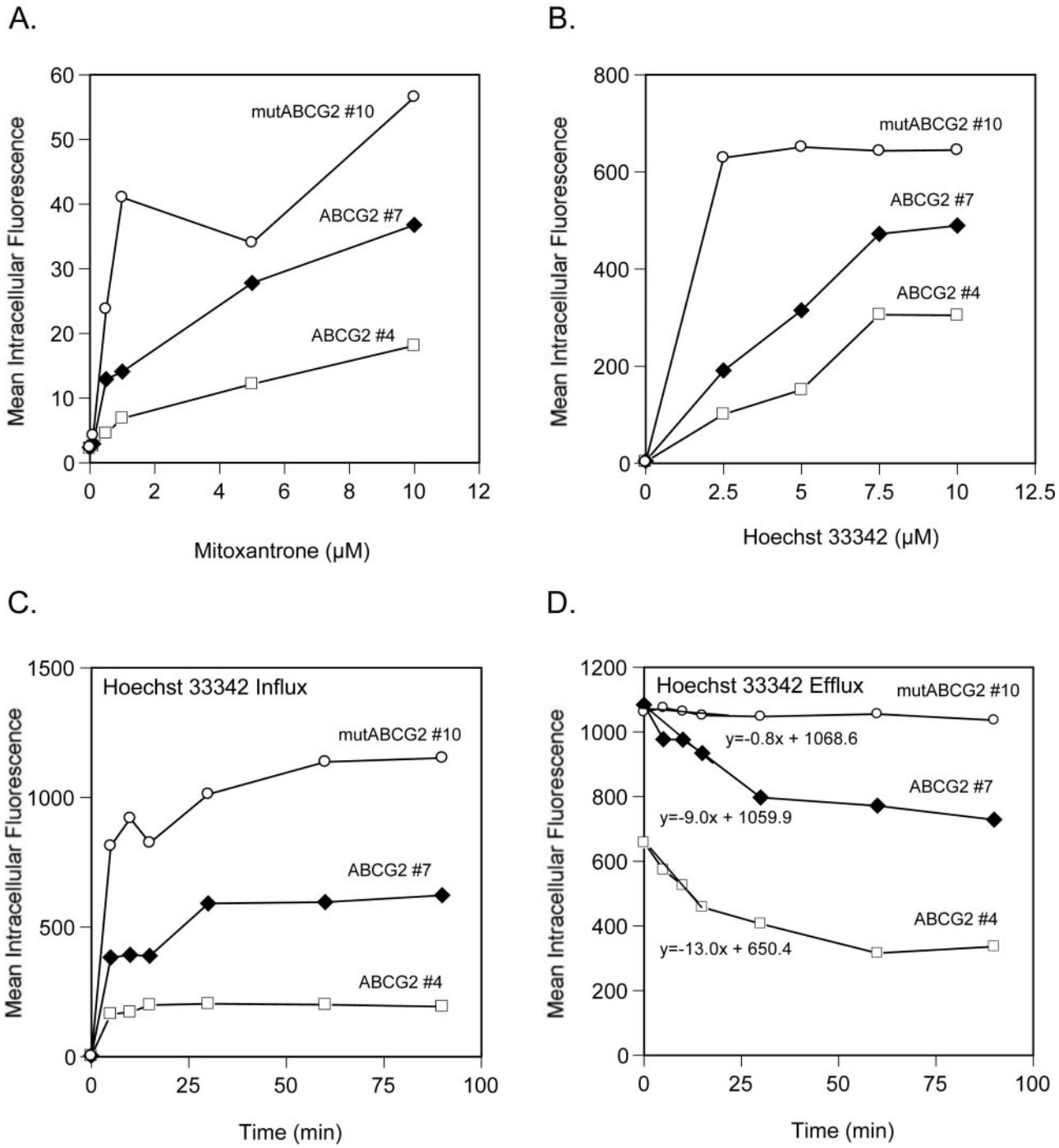

Fig. 3. Amount and function of ABCG2 affects the intracellular level of Hoechst and mitoxantrone. ABCG2#4, ABCG2#7, and mutABCG2#10 were incubated in the presence of increasing concentrations of either mitoxantrone (A) or Hoechst 33342 (B). The mean intracellular fluorescence was determined by FACS analysis. C, to determine the steady-state levels of Hoechst 33342 that can be achieved in ABCG2#4, ABCG2#7, and mutABCG2#10, cells were incubated with 5 μM dye for the indicated intervals. Mean intracellular fluorescence was determined by FACS analysis. D, to determine the rate of efflux of Hoechst 33342 from ABCG2#4, ABCG2#7, and mutABCG2#10, cells were incubated with dye for 30 min and washed with ice-cold PBS, and efflux was monitored after the addition of warmed (37°C) fresh media. To compensate for lower steady-state Hoechst 33342 levels, ABCG2#4, ABCG2#7, and mutABCG2#10 were incubated with 10, 5, and 1 μM Hoechst, respectively. Mean intracellular fluorescence was determined by FACS analysis, and initial rates of efflux were determined by linear regression analysis of the first four time points. Each point represents the mean of two values, and complete experiments have been repeated three times. A representative experiment is shown.

analysis was performed on time points up to and including 15 min. It should be noted that despite the presence of excess Hoechst 33342, the steady-state intracellular levels of drug in ABCG2#4 only reached approximately one-half that observed in ABCG2#7 and mutABCG2#10 cells (Fig. 3, C and D).Taken from the initial slopes, the rate of dye efflux was ~50% greater for ABCG2#4 than forABCG2#7. As expected, the efflux of Hoechst 33342 from mutABCG2#10 was minimal. Overall, these data indicate that the efflux of Hoechst dye is dependent on the amount of functional ABCG2 protein in cells.

Inhibition of ABCG2-Mediated Transport. To determine whether Hoechst 33342 efflux via ABCG2 could be blocked, we evaluated a variety of compounds (Table 1) for their ability to increase dye retention in ABCG2#4 cells. Of the agents tested, reserpine was found to be the most potent inhibitor, with the MRP1 and P-gp inhibitors MK571 and cyclosporine A_1 (CSA), respectively, being much less effective. The structural analogs of reserpine, yohimbine, and corynanthine were ineffective at inhibiting ABCG2. Similarly, the MDR1 inhibitor progesterone (Yang et al., 1990; Ueda et al., 1992) (Table 1) was a poor inhibitor of ABCG2. However, because of the apparent overlap between the inhibition of MDR1, MRP1, and ABCG2 by reserpine, MK571, and CSA, the relative ability of these three compounds to inhibit ABCG2 transport was assessed. In these studies, we also used both ABCG2#4 and ABCG2#7 cells to eliminate the possibility that the effectiveness of the inhibitor might be dependent on ABCG2 protein levels.

Reserpine was a very effective ABCG2 inhibitor, with the concentration required to inhibit ABCG2 being 2 μM for ABCG2#7 and approximately 5 μM for ABCG2#4 (Fig. 4A). The MRP1 inhibitor MK571 (Keppler et al., 1998) was also effective, but less so, requiring concentrations 7- to 10-fold higher than reserpine for these cells (Fig. 4B). The least effective inhibitor, CSA, required concentrations of at least 20 μM to produce 50% inhibition of dye efflux in ABCG2#7 cells (Fig. 4C) and 80 μM for ABCG2#4 (data not shown).

TABLE 1

Inhibition of Hoechst 33342 efflux by various inhibitors

All inhibitor concentrations were 10 μM, and experiments were repeated multiple times (n) as indicated. Inhibitor was able to decrease the percentage of Hoechst 33342, excluding cells in the 'E' gate, by ≥ 25% (+), by ≥ 50% (++), by ≥ 75% (+++), or by 100% (++++).

Inhibitor (n)	Effectiveness of Reversal in ABCG2 Cells
Reserpine (10)	++++
MK571 (12)	++
LY171883 (6)	+
Cyclosporine A_1 + cremophor (10)	+
Cyclosporine A_1 (8)	+
Verapamil (2)	+/−
PSC833 (3)	+/−
Structures related to reserpine	
Yohimbine (2)	−
Corynantheine (2)	−
Leukotrienes	
B_4 (4)	−
C_4 (4)	−
D_4 (4)	−
Miscellaneous compounds	
BSO (1)	−
Progesterone (1)	−
Probenecid (1)	−
Prostaglandin A_1 (1)	−

−, no change.

These studies demonstrate that ABCG2 transport inhibition is critically dependent on the amount of protein.

ABCG2 Transports SN-38. Whereas a number of reports indicate that ABCG2 transports camptothecin, some confusion exists because ABCG2 contains two different alleles [either Arg or a noncharged amino acid (e.g., Gly or Thr) at position 482] that differ in their ability to function as transporters of certain drugs (Honjo et al., 2001). Notably, in recently described studies, the expression of an ABCG2 cDNA containing arginine at position 482 was unable to confer camptothecin or mitoxantrone resistance in cells (Komatani et al., 2001). In contrast, the ABCG2 protein expressed in the Saos-2 clones described in this article, containing arginine at this position, is capable of transporting and conferring mitoxantrone resistance but not resistance to either adriamycin or daunomycin (Fig. 2 and unpublished data).

Therefore, we assessed intracellular CPT-11 and SN-38 concentrations in the ABCG2#4, ABCG2#7, and pcDNA#2 Saos-2 cell lines after drug treatment. Cells were transduced with adenovirus-expressing rCE, and after 4 days, three identical aliquots were assessed for CE activity and drug accumulation studies, both with and without reserpine. After treatment with 5 μM reserpine for 1 h, concentrations of CPT-11 ranging from 1 to 100 μM were added, and intracellular drug concentrations were determined by HPLC. Table 2 demonstrates the levels of CE and drugs present in extracts of all three cell lines after treatment with 100 μM CPT-11. At all concentrations of CPT-11 tested, the intracellular levels of the parent drug were similar among all cells, even after treatment with reserpine, suggesting that CPT-11 seems to be a poor substrate for ABCG2 (data not shown). However, in ABCG2#4 cells, the levels of SN-38 were approximately 2.4-fold lower than that observed in control cells. In addition, reserpine increased the intracellular concentration of SN-38 by 79%, which is consistent with the hypothesis that this drug is a substrate for ABCG2 and that the transport activity can be inhibited by reserpine.

ABCG2#7 cells contained much higher levels of CE activity, which resulted in greater intracellular concentrations of SN-38. However, if these values are corrected for the amounts of CE protein present in the extracts, drug concentrations similar to that observed in pcDNA#2 are seen (Table 2). Because lower levels of ABCG2 are expressed in ABCG2#7, these data correlate with the results observed in the Western analyses (Fig. 1). No marked differences in drug concentrations were seen in the presence of reserpine, suggesting that the levels of ABCG2 expression in ABCG2#7 cells were not sufficient to significantly change SN-38 efflux.

ABCG2 Mediates Resistance to CPT-11 in Cultured Cells. Having determined that reduced intracellular concentrations of SN-38 were present in cells expressing ABCG2, we sought to determine what contribution this would have in CPT-11-mediated cytotoxicity. We therefore performed drug survival assays using cells expressing CE either with or without ABCG2. pcDNA#2 and ABCG2#4 cells were transduced with AdCMVrCE at an m.o.i. of 5, and after 4 days, cells were harvested and CE activities were determined. Cells were divided into two aliquots; one was treated with CPT-11, and growth inhibition was assessed. The second aliquot was incubated with 5 μM reserpine for 1 h before CPT-11 treatment. Table 3 indicates the CE activity and the

IC_{50} values for CPT-11 for these cells. As can be seen, ABCG2#4 cells are greater than 160-fold more resistant to CPT-11 compared with the control cell line (pcDNA#2), and this can partly be overcome by reserpine. Within the same cell line, reserpine made little difference in the sensitivity of pcDNA#2 cells to CPT-11. This is presumably because there is no ABCG2 present in these cells. With ABCG2#4, reserpine reduced the IC_{50} value by ~60 μM, demonstrating that cytotoxicity could be partially overcome by inhibition of ABCG2 function.

To confirm the above studies, we performed growth-inhibition studies with pcDNA#2 and ABCG2#4 cells using SN-38 as the drug. These results are indicated in Fig. 5. Because SN-38 is 100- to 1000-fold more cytotoxic than CPT-

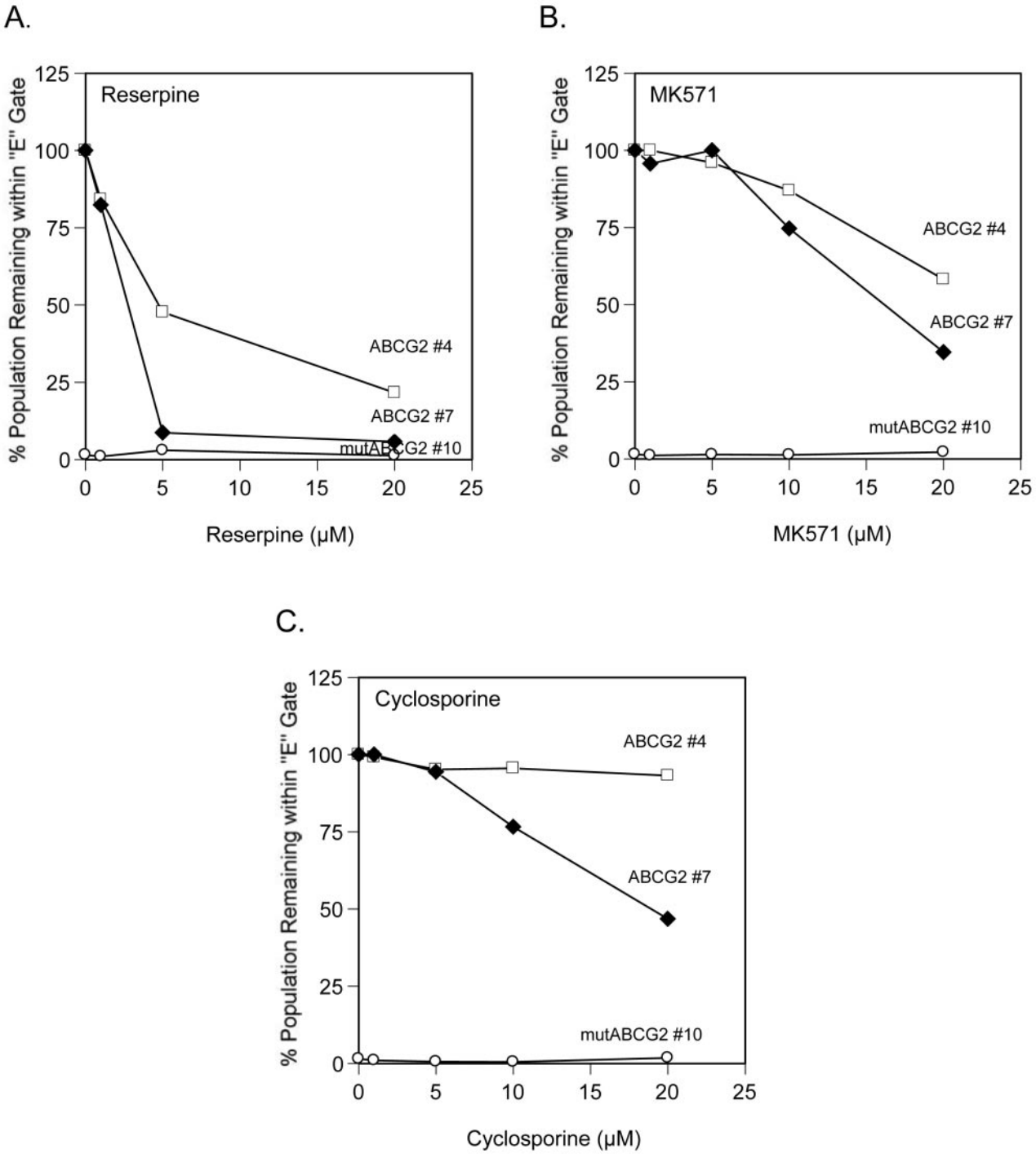

Fig. 4. Inhibitors of MDR1 and MRP1 also inhibit ABCG2 transport. A, cell lines (ABCG2#4, ABCG2#7, and mutABCG2#10) were preincubated with increasing concentrations of reserpine for 1 h, then 1 μM Hoechst 33342 was added for 30 min. Mean intracellular fluorescence was determined by FACS analysis, and values reflect the percentage of cells that shifted from the "E" population to the noneffluxing population. B, cell lines were incubated with increasing concentrations of MK571 for 1 h, then 1 μM Hoechst 33342 was added for 30 min. Fluorescence determination and calculations were performed as described in A. C, cell lines were incubated with increasing concentrations of CSA for 1 h, then 1 μM Hoechst 33342 was added for 30 min. Fluorescence was determined as described above.

11, the IC_{50} values for the former drug are markedly reduced compared with results for the latter (Table 3). Data in Fig. 5 demonstrate that cells expressing ABCG2 are 18-fold more resistant to SN-38 than are those expressing pcDNA#2. The addition of reserpine at 10 μM and 20 μM reduced the IC_{50} values from 3.6 to 1.4 and 2.7 μM, respectively, essentially confirming the inhibitory activity of this molecule on the ABCG2 transporter. It should be recalled that at 20 μM reserpine, only 80% inhibition of ABCG2 is observed in ABCG2#4 cells (Fig. 4A), which would probably only result in partial reversal of drug sensitivity to SN-38. Hence, superimposition of the survival curves for pcDNA#2 and ABCG2#4 cells would not occur at these concentrations of inhibitor.

Overall, these data indicate three points. First, overexpression of ABCG2 results in modest CPT-11 resistance but marked resistance to SN-38. Second, high-level overexpression of rCE, an enzyme highly efficient at drug activation, cannot overcome this phenotype. Third, reserpine is an inhibitor of the ABCG2 protein.

Discussion

We have demonstrated that expression of a human ABCG2 containing an arginine at position 482 results in decreased accumulation of the important chemotherapeutic agents mitoxantrone and the active metabolite of CPT-11, SN-38, in Saos-2 cells. Because overexpression and/or amplification of ABCG2 might be an important factor in the development of drug-resistant tumor cells, any approaches that circumvent this mechanism would be beneficial. We have previously proposed that viral-directed enzyme prodrug therapy with adenovirus-expressing rCE in combination with CPT-11 might be useful clinically (Danks et al., 1999; Meck et al., 2001; Wierdl et al., 2001). The studies presented here indicate that in cells which overexpress ABCG2, such an approach may be unsuccessful. However, it is currently unclear what the levels of ABCG2 are in both naïve and drug-treated human tumors, and with the development of selective inhibitors of this protein, improvements in chemotherapy may be possible.

In ABCG2, the normal allele seems to contain an arginine at amino acid 482, as determined by DNA sequence analysis of ABCG2 from different ethnic groups (Zamber et al., 2003). During drug selection, it has been hypothesized that a loss of charge at this position is critical for a change in substrate specificity (Allen et al., 2002). Loss of arginine at this position changes the substrate specificity in both human and rodent ABCG2 and further underscores the pivotal role of this amino acid in substrate selection. Mutations within ABCG2 at codon 482 have been identified in drug-selected cells during prolonged exposure to high concentrations of these agents (Bates et al., 2001; Allen et al., 2002). However, conflicting data exist in the literature concerning the true nature of the resistance properties of cells harboring ABCG2 with either noncharged amino acids (e.g., Gly and Thr) or arginine. For instance, in a recent publication, the expression of wild-type ABCG2 (arginine at position 482) was achieved in cells, but drug resistance to topotecan or mitoxantrone was not observed (Komatani et al., 2001). This discrepancy could be caused by either intrinsic properties of the recipient cells, such as the presence of other transporters known to play a role in transporting these substrates (e.g., P-gp or MRP2), or by the levels of ABCG2 protein expressed. Thus, because the mutation of amino acid 482 seems to be present only in cells exposed to selective concentrations of drug and is not found in the general population (Zamber et al., 2003), it was important to determine whether wild-type ABCG2 could transport drugs such as mitoxantrone and camptothecin analogs.

Because the ABCG2 protein used for the studies described herein contains Arg-482, our results indicate that overexpression of this protein produces resistance to SN-38 and, hence, to CPT-11. Moreover, we provide direct evidence that SN-38 is a substrate for the human ABCG2 transporter. These studies also indicate that expression of the transporter (as observed in ABCG2#4) results in more than a 160-fold increase in the IC_{50} value for CPT-11. Because drug resis-

TABLE 2

Effect of reserpine on intracellular CPT-11 and SN-38 concentrations in ABCG2-expressing Saos-2 cells

Only one CE activity is indicated per cell line because drug accumulation studies were performed with aliquots of the same batch of cells. One unit of CE activity is the amount of enzyme that converts 1 μ mol of *o*-nitrophenyl acetate to *o*-nitrophenol per minute

Cell Line	Reserpine	CE Activity	[CPT-11]	[SN-38]	SN-38 per 1000 U CE	Increase in [SN-38] by Reserpine
		μmol/min/mg	*μM*	*pM*		*%*
pcDNA#2	−	1842.7 ± 117.9	7.1	134.5	73.0	
pcDNA#2	+		4.6	142.5	77.3	5.9
ABCG2#4	−	1658.3 ± 180.6	4.8	50.6	30.5	
ABCG2#4	+		4.4	90.5	54.5	79
ABCG2#7	−	5771.3 ± 538.6	4.0	364.4	63.1	
ABCG2#7	+		4.4	370.9	64.3	1.9

TABLE 3

Carboxylesterase activity and IC_{50} values for CPT-11 with Saos-2 cells after adenoviral transduction with AdCMVrCE and treatment with reserpine

Only one CE activity is indicated per cell line because growth-inhibition studies were performed with duplicate aliquots of the same batch of cells

Cell Line	Reserpine	CE Activity	CPT-11 IC_{50}	Fold change in IC_{50}
		μmol/min/mg	*μM*	
pcDNA#2	−	1854.9 ± 41.8	0.8	
ABCG2#4	−	1766.3 ± 139.8	134.9	168
pcDNA#2	+		1.2	
ABCG2#4	+		86.4	72

tance in vivo is believed to be caused by very small changes in cell sensitivity (as low as a 2-fold change in the IC_{50} value), potentially, very marked resistance to therapeutic agents might be achieved by small increases in ABCG2 expression.

A number of different inhibitors of ABC transporters have been developed to inhibit MRP1 and MDR1, with more recent efforts directed toward identifying ABCG2 inhibitors (Bates et al., 2001). We found that ABCG2 transport of Hoechst 33342 was partially inhibited by the MRP1 inhibitor MK571. MK571 is a potent MRP1 inhibitor, and complete inhibition of transport by this protein has been observed in the presence of 10 μM drug samples (Renes et al., 2000). However, at this concentration, MK571 only inhibits 10 and 25% inhibition of Hoechst 33342 efflux in ABCG2#4 and ABCG2#7 cells, respectively (Fig. 4B). Overall, these results demonstrate that MK571 is a poor inhibitor of ABCG2.

Cyclosporine A and its potent derivative PSC833 inhibit P-glycoprotein but have marginal effects on ABCG2. Even at 20 μM, CSA was ineffective in inhibiting Hoechst 33342 dye export in ABCG2#4 cells (Fig. 4C). In contrast, 50% inhibition was seen in ABCG2#7 cells. This strongly supports the concept that ABCG2 inhibition is dependent on the amount of protein expressed.

Of all of the compounds tested, reserpine was the most efficacious at inhibiting ABCG2-mediated Hoechst dye transport. However, the analogs yohimbine and corynanthine were ineffective. Because the most prominent difference between the latter compounds and reserpine is the absence of a benzoyl ring, structure-activity relationships can be developed that will assist in the design of potentially novel ABCG2 inhibitors. Thus, exploiting reserpine as a basis for a lead compound in the development of ABCG2 inhibitors seems possible.

Growth-inhibition studies with cells expressing ABCG2 using SN-38 as the drug indicated that significant decreases in cytotoxicity were observed compared with control cells. This drug resistance could be partially overcome by reserpine in a dose-dependent fashion (Fig. 5). However, even at con-

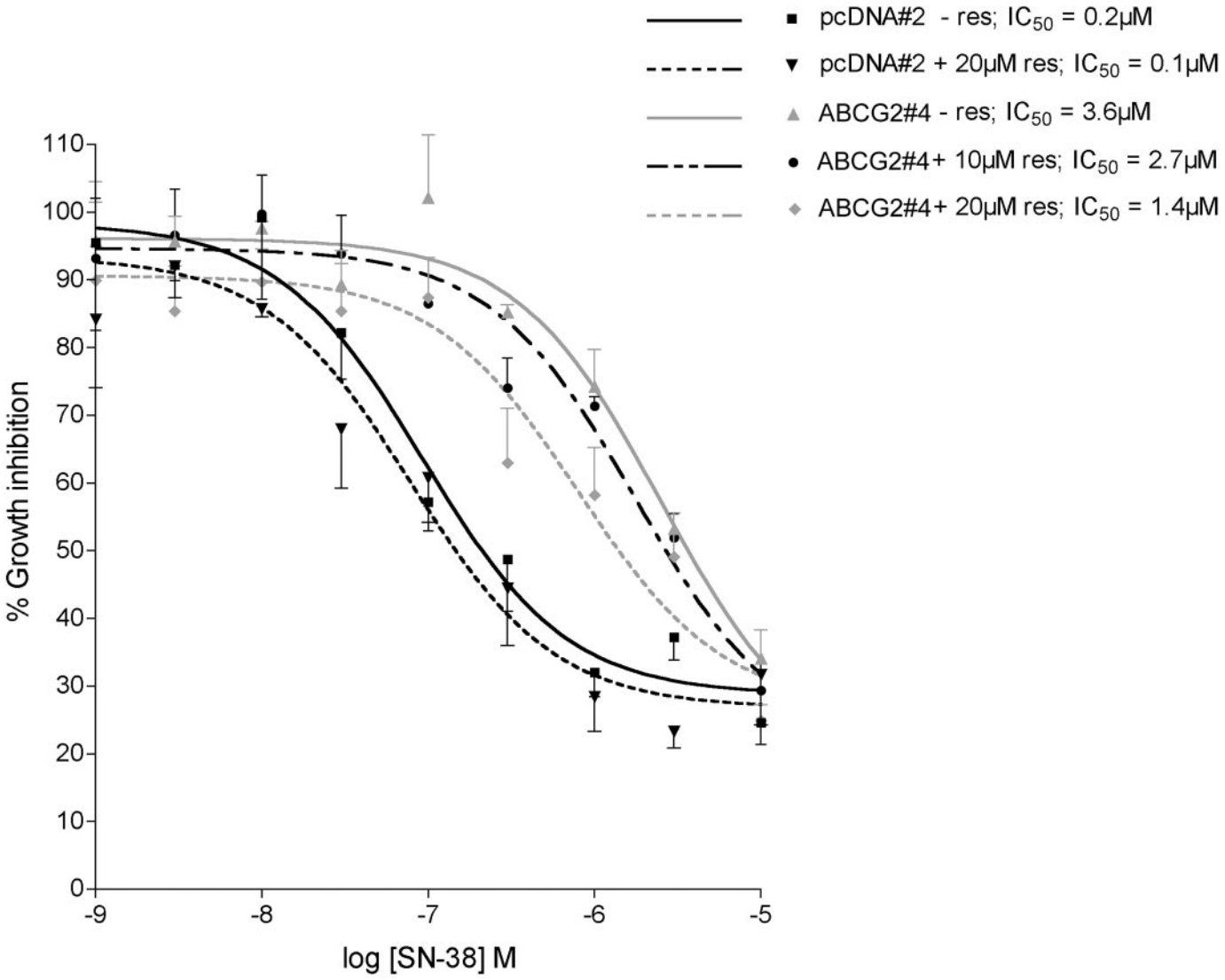

Fig. 5. Growth inhibition of cells expressing ABCG2 to SN-38. Cells expressing ABCG2 (ABCG2#4), or control cells (pcDNA#2) were exposed to different concentrations of SN-38 for 2 h. For cells treated with reserpine (res), the inhibitor was added 1 h before SN-38 addition and remained on the cells throughout drug exposure. Three days later, growth inhibition was calculated as the percentage of untreated cells. All data points were repeated in triplicate.

centrations of 20 μM reserpine, ABCG2-expressing cells were still 7-fold more resistant than cells lacking expression of the transporter. This is in part because at this concentration of reserpine, only ~80% inhibition of ABCG2 is observed in ABCG2#4 cells. However, a 7-fold increase in drug resistance would almost certainly result in the development of insensitive tumor cells in vivo, and it is apparent, therefore, that more potent and potentially more specific inhibitors of ABCG2 might be necessary. Ultimately, the contribution of ABCG2 to drug resistance in patients treated with camptothecin analogs will have to be evaluated to assess whether modulation of this efflux function could be a viable approach to improving cancer therapy.

Overall, our studies demonstrate the role of ABCG2 in the resistance to CPT-11, probably via the specific efflux of SN-38, and indicate the potential reversal of this phenotype by reserpine. However, enforced overexpression of rCE, which can efficiently activate CPT-11, could not overcome this drug resistance. Hence, an analysis of expression of ABCG2 in tumors of patients undergoing or about to undergo therapy with topotecan or CPT-11, might indicate the potential for resistance to these agents. Additionally, the development of specific inhibitors of ABCG2 may prove useful in overcoming any observed resistance to topotecan or CPT-11 in vivo.

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