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Advances in Brief

Imatinib Mesylate Is a Potent Inhibitor of the ABCG2 (BCRP) Transporter and Reverses Resistance to Topotecan and SN-38 *in Vitro*

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Abstract

Imatinib mesylate (Gleevec, STI571) is a kinase inhibitor selective for Bcr-Abl, activated c-Kit kinases, and platelet-derived growth factor receptor tyrosine kinase. Imatinib mesylate, similar to many other tyrosine kinase inhibitors (TKIs), such as members of the 4-anilinoquinazoline class, competes for ATP binding. Previously, 4-anilinoquinazoline TKIs have been shown to inhibit the function of the breast cancer resistance-associated drug transporter (ABCG2), reversing resistance to camptothecin derivatives topotecan and SN-38. However, the potential to inhibit ABCG2 for the 2-phenylamino-pyrimidine class of TKIs, exemplified by imatinib mesylate, has not been examined. Here, we show that imatinib mesylate potently reverses ABCG2-mediated resistance to topotecan and SN-38 and significantly increases accumulation of topotecan only in cells expressing functional ABCG2. However, overexpression of ABCG2 does not confer resistance to imatinib mesylate. Furthermore, accumulation and efflux of [^{14}C]imatinib mesylate are unaltered between ABCG2-expressing and non-ABCG2-expressing cells or by ATP depletion. These results suggest that imatinib mesylate inhibits the function of ABCG2 but is not a substrate for this transporter.

Introduction

Imatinib mesylate (STI571, Gleevec) is a tyrosine kinase inhibitor selective for Bcr-Abl expressed in Philadelphia-positive chronic myeloid leukemia (1), activated c-Kit in gastrointestinal stromal tumors, and platelet-derived growth factor receptor (2). As such, imatinib mesylate has demonstrated very significant activity in patients with chronic phase chronic myeloid leukemia as well as gastrointestinal stromal tumors. Imatinib mesylate is currently approved for these indications in the United States, Europe, and Japan and is being investigated in combination with other cytotoxic agents in clinical Phase I–III trials. Recently, Erlichman *et al.* (3) demonstrated that the ERBB1 inhibitor CI-1033, which belongs to the 4-anilinoquinazoline class of tyrosine kinase inhibitors, reverses SN-38 resistance through inhibition of the ABCG2 transporter [also termed BCRP breast cancer resistance protein (4), MXR (5), or ABCP (6)], which transports certain camptothecin analogs from cells. In addition, we reported that gefitinib (Iressa, ZD1839) also potently reversed resistance to topotecan and SN-38 in cells engineered to overexpress ABCG2, whereas it had no such effect in cells overexpressing a mutant, nonfunctional ABCG2 protein. In mice, combination of gefitinib with topotecan (9-dimethylaminomethyl-10-hydroxy camptothecin) induced significant toxicity as a consequence of decreased plasma clearance. In contrast, combination with irinotecan (CPT-11; [7-ethyl-10-(4-[1-piperidino)-1-piperidino]-carbonyloxy-camptothecin]) demonstrated greater than additive antitumor activity in xenograft models in which ERBB1 or ERBB2 expression could not be detected (7). Thus, it is probable that gefitinib was acting through inhibition of ABCG2. To determine whether other tyrosine kinase inhibitors also inhibit the function of ABCG2, we have examined the activity of imatinib mesylate in reversing ABCG2-mediated resistance to two camptothecin analogs. The results suggest that imatinib mesylate potently reverses ABCG2-mediated resistance but is not an ABCG2 substrate for efflux. These results may be of importance when considering combinations of imatinib mesylate with other anticancer cytotoxic agents that are putative ABCG2 substrates.

Materials and Methods

Cell Lines. The human osteosarcoma cell line Saos2 was obtained from American Type Culture Collection (Cat#HTB-85; Manassas, VA), and cells were maintained in DMEM (Biowhitaker, Walkersville, MD) containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 2 mM glutamine (Invitrogen, Carlsbad, CA). Saos2 derivatives were engineered to overexpress functional ABCG2 (ABCG2#4) >99% identical to both ABCG2 (4) and ABCP (6). This cDNA encodes the putative wild-type peptide (arginine at amino acid position 482), unlike the ABCG2 isoform cloned from a drug-resistant cell line (4). Saos2 cells were also engineered to express a similar level of a Walker A domain mutant [lysine residue in the Walker A domain G(X)$_4$G<u>K</u>S to methionine] that is not a functional transporter (Mut#10). Saos2pcDNA was used as a vector control cell line. These cell lines have been described in detail elsewhere (8).

Drug Sensitivity Determinations. To screen compounds for the ability to reverse the ABCG2 phenotype, Saos2 cells stably transfected with either functional ABCG2 (Saos2ABCG2#4), nonfunctional ABCG2 (Saos2MUT#10), or vector (Saos2pcDNA) were plated in 96-well Costar plates. Unused wells in each plate were used as blanks or medium controls. Cells (1000 cells/well) were added in 0.1 ml of 10% fetal bovine serum + DME + 500 μg/ml G418 and allowed to attach overnight. The next morning, the medium was gently aspirated, and dilutions of the compounds to be tested were added. In these experiments, each cell type was dosed with camptothecin analog (topotecan or SN-38) with or without the putative ABCG2 inhibitor. After exposure to drugs for 24 h, drug-containing medium was gently aspirated, and 0.1 ml of medium without drug was added. All of the plates were placed in a clear plastic box containing a pan filled with water for improved humidity and returned to the 37°C-5% CO_2 incubator. After an additional 5 days of incubation, 10 μl of Alamar blue (Biosource, Camarillo, CA) were added to all of the wells aseptically, and the plates were returned to the incubator for 3–6 h. The amount of the red fluorescent reduced dye produced was measured on a Cytofluor 2300 using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. The readings from blank-medium wells were subtracted from all of the other values, and the percentage of growth for treated cells, compared with untreated cells, was calculated.

Accumulation and Retention of [^{14}C]Imatinib Mesylate. Two ml of cell suspension containing either Saos2ABCG2#4 or Saos2Mut#10 (5×10^5 cells) were plated in Falcon 35 × 10-mm Multiwell 6-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ). After attachment overnight at 37°C,

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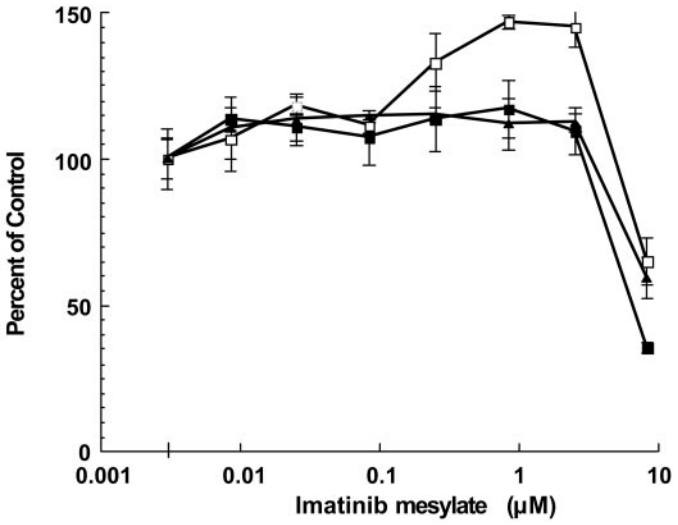

Fig. 1. Overexpression of ABCG2 does not confer resistance to imatinib mesylate. Control cells (Saos2pcDNA, ■) and Saos2Mut#10 (▲) or Saos2ABCG2#4 (□) cells overexpressing functional ABCG2 were exposed to imatinib mesylate for 24 h. Drug was removed, cells were incubated for an additional 5 days, and growth determined as described in "Materials and Methods."

medium was aspirated, and cells were washed twice with 2 ml of physiological Tris buffer [20 mM Tris containing 120 mM NaCl, 3 mM K_2HPO_4, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM glucose (pH 7.4)]. Monolayers were incubated at room temperature in physiological Tris for 10 min before aspiration of buffer and replacement with 1 ml of serum-free RPMI 1640-HEPES buffer containing 0.5 μCi/ml [^{14}C]imatinib mesylate (specific activity, 91.5 μCi/mg; final concentration, 1 μM) with or without 10 mM NaN_3 (9). After the appropriate period of incubation at room temperature (5 min), medium was rapidly aspirated to terminate drug accumulation and replaced with drug-free medium for efflux studies. To determine cellular radiolabel content, cells were washed 10 times with ice-cold PBS and drained before addition of 1 ml of trypsin-EDTA (0.05% trypsin and 0.53 mM EDTA). After 5 min, monolayers were triturated to give a uniform suspension, and 0.75 ml was used to determine radioactivity by scintillation counting. Cell number was determined with 200 μl of cell suspension.

Accumulation of Topotecan. Two ml of cell suspension containing either Saos2ABCG2#4 or Saos2Mut#10 (3×10^6 cells) were plated in Falcon 35×10-mm Multiwell 6-well tissue culture plates (Becton Dickinson). After 48 h of growth at 37°C, medium was aspirated and replaced with 2 ml of medium containing 1 μM topotecan with or without 1 μM imatinib mesylate. After the appropriate period of incubation at 37°C, the medium was rapidly aspirated to terminate drug accumulation, and the monolayers were washed three times with ice-cold PBS. Ice-cold water was added (1 μl/2000 cells), and wells were scraped and transferred to microfuge tubes on ice. Samples were briefly sonicated, and 200 μl of suspension were added to 400 μl of methanol cooled on dry ice (−30°C). This mixture was vortexed vigorously and centrifuged for 2 min at $7200 \times g$. The supernatant was decanted and stored at −70°C before high-performance liquid chromatography assay. Before high-performance liquid chromatography analysis, samples were acidified with 20% H_3PO_4 (20 μl/100 μl methanol-extracted sample) to convert the carboxylate form of topotecan to the lactone form. Samples were analyzed as described previously (10). The effect of imatinib mesylate on the accumulation of total topotecan in Saos2ABCG2#4 cells was also compared with the control cell line Saos2Mut#10. Data were analyzed using three-way ANOVA, and the Holm-Sidak method was used for pairwise comparisons and comparisons *versus* the control group. Overall significance level was $P < 0.05$.

Results

ABCG2 Does Not Confer Resistance to Imatinib Mesylate. The sensitivity of Saos2 cells to imatinib mesylate was first determined in Saos2pcDNA (vector control), Saos2Mut#10 (cells with nonfunc-

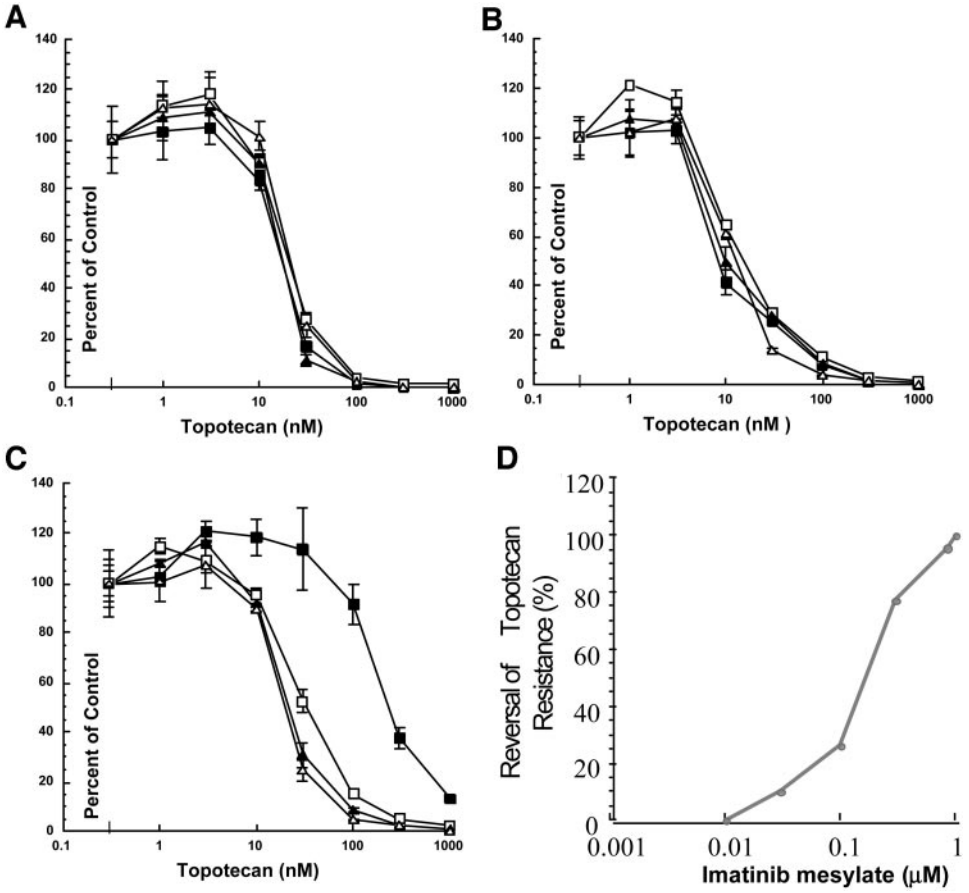

Fig. 2. Imatinib mesylate reverses ABCG2-mediated resistance to topotecan. The effect of increasing concentrations of imatinib mesylate on the sensitivity of cells to topotecan was determined for (*A*) Saos2pcDNA cells (vector control); *B*, Saos2Mut#10 cells expressing a nonfunctional ABCG2; and *C*, Saos2ABCG2#4 cells expressing functional ABCG2. Each point represents the mean ± SD for three determinations. *D* shows the percentage of reversal of ABCG2-mediated resistance as a function of imatinib mesylate concentration (■, 0 μM; □, 0.84 μM; ▲, 2.5 μM; △, 4.2 μM).

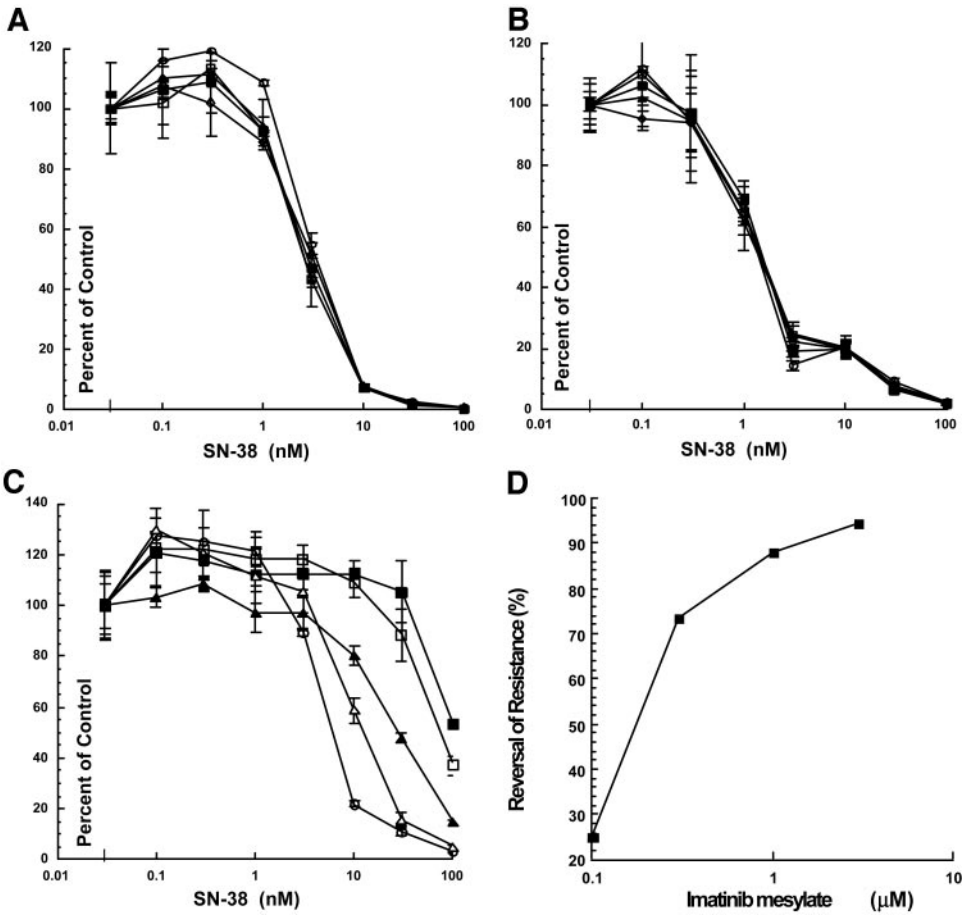

Fig. 3. Imatinib mesylate reverses ABCG2-mediated resistance to SN-38. The effect of increasing concentrations of imatinib mesylate on the sensitivity of cells to SN-38 was determined for (*A*) Saos2pcDNA cells (vector control); *B*, Saos2Mut#10 cells expressing a nonfunctional ABCG2; and *C*, Saos2ABCG2#4 cells expressing functional ABCG2. Each point represents the mean ± SD for three determinations. *D*, shows the percentage of reversal of ABCG2-mediated resistance as a function of imatinib mesylate concentration (■, 0 μM; □, 0.1 μM; △, 0.3 μM; ▲, 1.0 μM; □, 3.0 μM).

tional ABCG2), and Saos2ABCG2#4 (ABCG2-overexpressing cells). As shown in Fig. 1, each Saos2 derivative had similar sensitivity to imatinib mesylate. Saos2pcDNA was slightly more sensitive than the other Saos2 derivatives, and imatinib mesylate inhibited Saos2pcDNA cell growth by 50% (IC_{50}) at 7.3 μM (Fig. 1). For Saos2Mut#10 and Saos2ABCG2#4, the IC_{50} was 9.5 and 9.6 μM, respectively. Thus, overexpression of ABCG2 did not confer significant resistance to this agent. To avoid toxicity in subsequent experiments, the highest concentration of imatinib mesylate was set at 4.2 μM, a concentration that caused <20% inhibition of growth in the most sensitive cell line (Saos2pcDNA).

Imatinib Mesylate Reverses ABCG2-Mediated Resistance to Camptothecin Analogs. The sensitivity of Saos2pcDNA and Saos2Mut#10 cells to topotecan was similar, with IC_{50} concentrations of 20 and 9 nM, respectively. The topotecan concentration required to inhibit Saos2ABCG2#4 cells by 50% was ~250 nM. Thus, overexpression of ABCG2 resulted in a significant increase in resistance (>12-fold) to topotecan. The sensitivity to topotecan was unchanged when Saos2pcDNA and Saos2Mut#10 cells were coincubated with imatinib mesylate at concentrations up to 4.2 μM (Fig. 2). In contrast, Saos2ABCG2#4 cells were sensitized by low concentrations of imatinib mesylate. To further characterize reversal of topotecan resistance, cells were coincubated with topotecan (0.3–1000 nM) in the presence of imatinib mesylate (0.1–3 μM), and the IC_{50} concentrations for topotecan were calculated. With an increasing concentration of imatinib mesylate, the concentration of topotecan required to inhibit cell growth by 50% decreased from 257 to 10 nM (data not shown). Reversal of topotecan resistance by 50% was achieved at a concentration of ~170 nM imatinib mesylate (Fig. 2*D*). Thus, imatinib mesylate potently reverses ABCG2-mediated resistance to topotecan. Similar results were obtained with reversal of ABCG2-mediated resistance to the active metabolite of irinotecan, SN-38 (Fig. 3). The concentration of imatinib mesylate calculated to reverse SN-38 resistance by 50% was 176 nM.

Imatinib Mesylate Increases Accumulation of Topotecan Only in Cells Expressing Functional ABCG2. Accumulation of nonradiolabeled topotecan (1 μM) was determined in Saos2ABCG2#4 and Saos2Mut#10 cells (Fig. 4*A*). In the absence of imatinib mesylate, accumulation of topotecan was low. In the presence of imatinib mesylate (1 μM), accumulation of topotecan in Saos2ABCG2#4 was significantly increased ($P < 0.001$) and was similar to that determined for Saos2Mut#10. In contrast, accumulation of topotecan in Saos2Mut#10 cells was not altered by coincubation with imatinib mesylate.

Imatinib Mesylate Is Not a Substrate for ABCG2-Mediated Efflux. Irrespective of their ABCG2 status, Saos2 cells had similar sensitivity to imatinib mesylate, suggesting that this agent is not a substrate for ABCG2-mediated efflux. However, the results shown above indicate imatinib mesylate to be a potent inhibitor of ABCG2-mediated resistance to topotecan and SN-38. To determine whether expression of ABCG2 altered the rate of accumulation, Saos2ABCG2#4 and Saos2Mut#10 cells were exposed to [^{14}C]imatinib mesylate, and accumulation was determined in the absence or presence of sodium azide. As shown in Fig. 4*B*, accumulation of radiolabeled imatinib mesylate was similar in either cell line. Addition of sodium azide reduced the rate of imatinib mesylate uptake in Saos2ABCG2#4 cells by ~30% but had a lesser effect on uptake in Saos2Mut#10 cells. Within 5 min, levels of imatinib mesylate were similar in both cell lines, irrespective of sodium azide. When cells

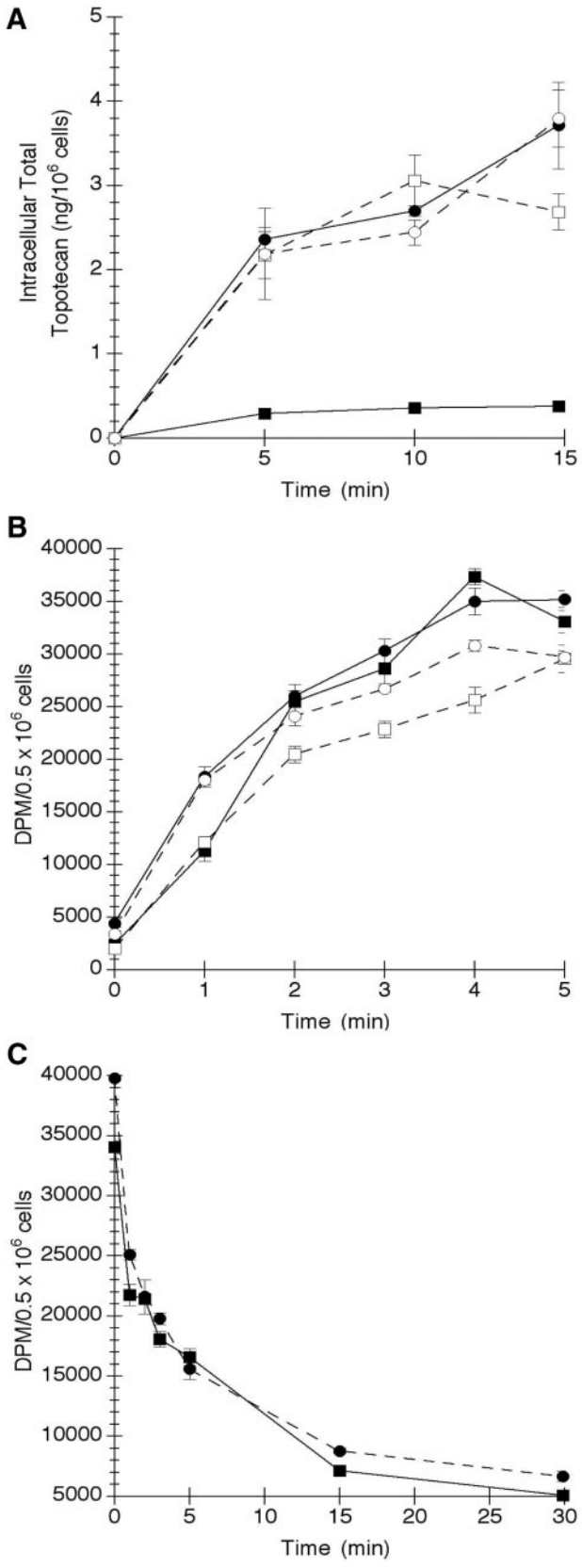

Fig. 4. Imatinib mesylate enhances topotecan accumulation in ABCG2-expressing cells but is not a substrate for ABCG2-mediated transport. *A*, accumulation of topotecan in Saos2ABCG2#4 (■ and ●) and Saos2Mut#10 (□ and ○) cells in the absence (■ and □) or presence (● and ○) of imatinib mesylate (1 μM). Results show the mean ± SD (n = 4). *B*, accumulation of [^{14}C]imatinib mesylate in Saos2ABCG2#4 (■ and □) and Saos2Mut#10 (● and ○) in the absence of NaN_3 (■ and ●) or in the presence of 10 mM NaN_3 (□ and ○). *C*, efflux of [^{14}C]imatinib mesylate from Saos2ABCG2#4 (■) or Saos2Mut#10 (●). Results show the mean ± SD (n = 3).

were preloaded with [^{14}C]imatinib mesylate, the rate of efflux was similar in Saos2ABCG2#4 and Saos2Mut#10 cells (Fig. 4*C*).

Discussion

Small molecule inhibitors of tyrosine kinases are currently a major focus in the development of novel cancer-directed therapeutic strategies. Most, but not all, of these agents are designed to compete with ATP, thus preventing activation of kinase activity. However, there are many ATP-dependent cellular processes, including ABC transporters responsible for modulating cellular levels of xenobiotics. Erlichman *et al.* (3) demonstrated that CI-1033, an irreversible inhibitor of ERBB1, reversed ABCG2-mediated resistance to the camptothecin SN-38. We also demonstrated reversal of topotecan and SN-38 resistance by another 4-anilinoquinazoline, gefitinib (7). In this study, we examined the ability of imatinib mesylate, a 2-phenylamino-pyrimidine derivative, to reverse topotecan resistance. Previously, it has been shown (11) that imatinb mesylate inhibited MDR1- and MRP1-mediated extrusion of calcein AM dye (K_i, ~8 μM; K_i, ~20–30 μM), respectively. However, this study did not determine whether imatinib mesylate directly inhibited the transporter or was a competitive substrate. Here, we examined the ability of imatinib mesylate to reverse resistance to topotecan and SN-38 in a series of Saos2 human osteosarcoma cell lines engineered to overexpress functional ABCG2 or a nonfunctional mutant ABCG2. Neither ABCG2 nor P-glycoprotein can be detected in parental Saos2 cells (8). Our initial experiments demonstrated that each Saos2 derivative had similar sensitivity to imatinib mesylate; thus, overexpression of functional ABCG2 did not confer significant resistance. In contrast, overexpression of functional ABCG2 conferred 12- to 20-fold resistance to topotecan and ~50-fold resistance to SN-38. Coincubation with nontoxic concentrations of imatinib mesylate reversed resistance to topotecan and SN-38 in cells expressing functional ABCG2 but not in Saos2Mut#10 or vector control cells. In the absence of imatinib mesylate, the IC_{50} concentration for topotecan and SN-38 in Saos2ABCG2#4 cells was 257 and 109 nM, respectively. Increasing the concentration of imatinib mesylate from 3 to 4200 nM progressively decreased the IC_{50} concentration for topotecan and SN-38, with 50% reversal of resistance being achieved at ~170 nM imatinib mesylate.

Resistance to topotecan was associated with decreased accumulation of drug in ABCG2-expressing cells, and accumulation was increased by imatinib mesylate to the same level as that determined in Saos2Mut#10 cells. Imatinib mesylate did not modulate the accumulation of topotecan in cells expressing a nonfunctional ABCG2.

These results suggest that ABCG2 does not confer resistance to imatinib mesylate, but this agent may inhibit ABCG2-mediated transport. The inference is that imatinib mesylate is an inhibitor without being a competitive substrate. To investigate this, we examined the rate of accumulation of radiolabeled imatinib mesylate in cells expressing functional or nonfunctional ABCG2. Accumulation of [^{14}C]imatinib mesylate was similar in both cell lines. Furthermore, depletion of ATP by preincubation of cells with sodium azide slightly decreased the rate of drug accumulation. In contrast, depletion of ATP leads to enhanced accumulation of substrates that are actively extruded by ATP-dependent transporters (12). Results from the efflux studies further support the conjecture that imatinib mesylate is not a substrate for the ABCG2 transporter because the rate of efflux was similar in cells expressing functional or nonfunctional transporter.

As a single agent, imatinib mesylate has demonstrated very significant activity against several human cancer types. Currently, there are ongoing and proposed clinical studies combining imatinib mesylate with conventional cytotoxic agents. Our study suggests that imatinib mesylate is a potent inhibitor of the ABCG2 transporter that mediates

transport of certain camptothecin derivatives and mitoxantrone. Inhibition of ABCG2-mediated resistance is achieved at pharmacologically relevant concentrations of imatinib mesylate that are achieved in patient plasma, hence there is the potential for imatinib mesylate to modulate the pharmacology of ABCG2 substrates such as topotecan, SN-38, and mitoxantrone. Furthermore, it raises the concern that enhanced antitumor activity of imatinib mesylate combined with such ABCG2 substrates may not be indicative of activity of the kinase inhibitor against its primary (putative) molecular target. Thus, understanding the clinical results from studies in which a tyrosine kinase inhibitor such as imatinib mesylate, gefitinib, or CI-1033 is combined with cytotoxic agents associated with ABCG2 transport may require careful analysis.

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