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High-Affinity Interaction of Tyrosine Kinase Inhibitors with the ABCG2 Multidrug Transporter

Csilla Özvegy-Laczka, Tamás Hegedűs, György Várady, Olga Ujhelly, John D. Schuetz, András Váradi, György Kéri, László Őrfi, Katalin Német, and Balázs Sarkadi

National Medical Center, Institute of Haematology and Immunology, Membrane Research Group of the Hungarian Academy of Sciences (C.Ö.-L., T.H., B.S.) and Department of Experimental Gene Therapy, Budapest, Hungary (G.V., O.U., K.N.); Department of Pharmaceutical Sciences, St. Jude Children's Research Hospital, Memphis, Tennessee (J.D.S.); Institute of Enzymology, Hungarian Academy of Sciences, Budapest, Hungary (C.Ö.-L., A.V.); and Semmelweis University, Department of Medical Chemistry, Peptide Biochemistry Research Group of the Hungarian Academy of Sciences, Budapest, Hungary (G.K., L.Ő.)

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ABSTRACT

Tyrosine kinase inhibitors (TKIs) are promising new agents for specific inhibition of malignant cell growth and metastasis formation. Because most of the TKIs have to reach an intracellular target, specific membrane transporters may significantly modulate their effectiveness. In addition, the hydrophobic TKIs may interact with so-called multidrug transporters and thus alter the cellular distribution of unrelated pharmacological agents. In the present work, we show that certain TKIs, already in the clinical phase of drug development, directly interact with the ABCG2 multidrug transporter protein with a high affinity. We found that in several in vitro assay systems, STI-571 (Gleevec; imatinib mesylate), ZD1839 (Iressa; gefitinib), and *N*-[4-[(3-bromophenyl)amino]-6-quinazolinyl]-2-butynamide (EKI-785) interacted with ABCG2 at submicromolar concentrations, whereas other multidrug transporters, human multidrug resistance protein (P-glycoprotein, ABCB1) and human multidrug resistance protein 1 (ABCC1), showed much lower reactivity toward these agents. Low concentrations of the TKIs examined selectively modulated ABCG2-ATPase activity, inhibited ABCG2-dependent active drug extrusion, and significantly affected drug resistance patterns in cells expressing ABCG2. Our results indicate that multidrug resistance protein modulation by TKIs may be an important factor in the clinical treatment of cancer patients. These data also raise the possibility that an extrusion of TKIs by multidrug transporters, e.g., ABCG2, may be involved in tumor cell TKI resistance.

In current antitumor drug research, a large variety of TKIs with increasing specificity and selectivity have been developed (Traxler, 2003). STI-571 (Gleevec; imatinib), an inhibitor of Bcr-Abl kinase, has been successfully applied in the treatment of chronic myeloid leukemia and is under clinical studies for cancers involving other deregulated kinases (Joensuu et al., 2001; van Oosterom et al., 2002). A number of epidermal growth factor receptor tyrosine kinase (EGFR-TK) inhibitors have also reached various phases of clinical or preclinical trials, including the compound ZD1839 (Iressa; gefitinib) and the irreversible TKI EKI-785 (Ranson et al., 2002; Roberts et al., 2002). The therapeutic potential of most TKIs, in addition to specific kinase-inhibitory potential, also depends on their access to intracellular targets.

An emerging question is the possible interaction of TKIs with multidrug resistance ABC transporters. These plasma membrane glycoproteins cause chemotherapy resistance by actively extruding a large variety of therapeutic compounds from the cancer cells. ABC transporters also play important protective functions against toxic compounds, e.g., in the blood-brain barrier, the gut, liver, or kidney. The three major multidrug resistance ABC proteins are MDR1 (P-glycoprotein, ABCB1), MRP1 (multidrug resistance protein 1; ABCC1) and ABCG2 (ABCP/BCRP/MXR) (Litman et al., 2001; Allen and Schinkel,

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ABBREVIATIONS: TKI, tyrosine kinase inhibitor; ABC , ATP-binding cassette; ABCP, placenta-specific ABC transporter; ADME-Tox, absorption, tissue distribution, metabolism, and toxicity; BCRP, breast cancer resistance protein; AM, acetoxymethyl ester; MOPS, 3-(*N*-morpholino)propanesulfonic acid; GS, glutathione; MDR1, human multidrug resistance protein (P-glycoprotein, ABCB1); MRP1, human multidrug resistance protein 1 (ABCC1); MXR, mitoxantrone resistance-associated protein; MX, mitoxantrone; NEM, *N*-ethylmaleimide; Sf9 cells, *Spodoptera frugiperda* ovarian cells; TK, tyrosine kinase; CI1033, 2-propenamide, *N*-[4-[(3-chloro-4-fluorophenyl)amino]-7-[3-(4-morpholinyl)propoxy]-6-quinazolinyl]-, dihydrochloride.

2002; Gottesman et al., 2002). MDR1 and MRP1 can transport a large variety of hydrophobic drugs, and MRP1 can also extrude anionic drugs or drug conjugates. The substrate specificity of ABCG2 partially overlaps with that of MDR1 and MRP1; that is, the transported compounds include mitoxantrone (MX), topotecan, flavopiridol, methotrexate, and Hoechst 33342 (Litman et al., 2001; Zhou et al., 2001; Volk et al., 2002).

The ABCG2 multidrug transporter is present in the placenta, stem cells, liver, small intestine, colon, lung, kidney, adrenal and sweat glands, and endothelia, suggesting its role in the protection/detoxification against xenobiotics (Litman et al., 2001; Maliepaard et al., 2001; Zhou et al., 2001; Cooray et al., 2002). Indeed, ABCG2 was shown to influence the absorption and secretion of topotecan (Jonker et al., 2000). ABCG2 overexpression was documented in several drug-resistant cell lines and tumors, which indicates its importance in the multidrug-resistant phenotype of cancer cells (Doyle et al., 1998; Brangi et al., 1999; Ross et al., 2000; Litman et al., 2001; Diestra et al., 2002).

Modulators of multidrug resistance ABC transporters are regarded as potential clinically applicable agents to inhibit cancer multidrug resistance, as well as to alter the absorption, tissue distribution, metabolism, and toxicity (ADME-Tox) parameters for various pharmacons (Fisher et al., 1996; Bakos et al., 2000). In our previous communication, we documented that several TKIs interacted with the human MDR1 and MRP1, and significantly inhibited their transport activities for other substrate drugs (Hegedus et al., 2002). This modulatory property may make TKIs ideal compounds for use in combination with other anticancer drugs, allowing an effective penetration of various cytotoxic agents.

In the present work, we have analyzed the interactions of the three major multidrug resistance proteins, ABCG2, MDR1, and MRP1, with three TKIs (STI-571/Gleevec, ZD1839/Iressa, and EKI-785), already in large-scale preclinical and clinical trials. In these experiments we have used several enzyme- and cell-based test systems. We measured transport-related ABC-ATPase activity, which is significantly modified by the transported substrates or inhibitors (Sarkadi et al., 1992; Bakos et al., 1998; Ozvegy et al., 2001). Another assay system was to investigate the extrusion of fluorescent dyes from mammalian cells, expressing the respective transporter. In the case of ABCG2, we measured the extrusion of the Hoechst 33342 dye (Ozvegy et al., 2002), whereas for MDR1 and MRP1 function, we analyzed the inhibition of calcein accumulation (Homolya et al., 1993; Hollo et al., 1996). These studies were complemented with direct cell toxicity assays in human cell lines selectively expressing the respective transporter proteins and, by using mitoxantrone as a cytotoxic agent, extruded by all three multidrug transporters (Litman et al., 2001).

Our data indicate that ABCG2 shows a high-affinity interaction with the three TKIs examined, whereas interaction with the MDR1 and MRP1 proteins could only be observed at much higher TKI concentrations. These in vitro data may significantly help the evaluation of the drug resistance-modulatory effects of these TKIs, as well as to predict their ADME-Tox properties.

Materials and Methods

Expression of MDR1, MRP1, and ABCG2 (R482) in Insect Cells

Recombinant baculoviruses containing the respective cDNA were prepared as described previously (Muller et al., 1996; Bakos et al., 1998; Ozvegy et al., 2002). Sf9 (*Spodoptera frugiperda*) cells were cultured and infected with a baculovirus as described by Muller et al. (1996).

Membrane Preparation and Immunoblotting

Virus-infected Sf9 cells were harvested, and their membranes were isolated and stored at −80°C. The membrane protein concentrations were determined, as described by Sarkadi et al. (1992). Immunoblot detection was performed by the specific anti-MDR1 4077, anti-MRP1 R1, or anti-ABCG2 BXP-21 antibodies, respectively. Protein-antibody interaction was determined using the enhanced chemiluminescence technique (Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK) as described earlier (Bakos et al., 1998).

Membrane ATPase Measurements

ATPase activity was measured basically as described by Sarkadi et al. (1992), by determining the liberation of inorganic phosphate from ATP with a colorimetric reaction. The incubation media contained 40 mM 4-MOPS-Tris, pH 7.0, 50 mM KCl, 2 to 5 mM dithiothreitol, 0.1 to 0.5 mM EDTA, 4 to 5 mM sodium azide, 1 mM ouabain, 10 to 20 μg of membrane, and 3.3 to 4 mM MgATP. The figures represent the mean values of at least three independent experiments with duplicates.

Multidrug Transporter Assays in Mammalian Cells

For the investigation of the function of MDR1 and MRP1 in mammalian cells, we applied well characterized HL60/PLB cell lines, all closely related derivatives of a human myelomonocytic cell line. The drug-selected HL60-MDR1 and HL60-MRP1 (adriamycin) cells singularly express MDR1 and MRP1, respectively, at constant high levels (Hollo et al., 1996). For the investigation of the function of wild-type (R482) ABCG2 in mammalian cells, we applied retrovirally transduced HL60/PLB cells (PLB-ABCG2), further selected by a low concentration of mitoxantrone, as described by Ujhelly et al. (2003). All these cells were cultured in standard RPMI media supplemented with 10% fetal calf serum and 50 U/ml penicillin and streptomycin. A constant level of multidrug resistance protein expression was periodically monitored by immunoblotting and functional flow cytometry analysis. In the latter case, selective expression of the respective multidrug resistance transporters was examined by selective transporter inhibitors (e.g., MK571 for MRP1; verapamil, as a competitive inhibitor for MDR1; and Ko143 for ABCG2).

Fluorescent Dye Uptake. The calcein assay, used for the quantitative determination of multidrug transporter activity, was performed as described earlier (Homolya et al., 1993). In brief, fluorescence intensities were determined in a fluorescence spectrophotometer (LS 50B; Applied Biosystems, Foster City, CA) after a short in vitro incubation. Cultured and well characterized MDR1- or MRP1-expressing cells were incubated with the nonfluorescent calcein AM, and with the respective concentrations of the compounds to be examined. The increase in cellular fluorescence caused by the liberation of free calcein inside the cells was determined. MDR1- and/or MRP-interactive compounds inhibit the outward transport of calcein AM, thus significantly increasing the rate of calcein accumulation. The increase in cellular fluorescence was determined in the presence of the compound (F_x), in the presence of 50 μM verapamil (or 10 μM MK571 for MRP1) completely inhibiting the transporter proteins (F_{100}), and in the absence of any inhibitor (F_0). To obtain the respective IC_{50} values for the TKIs, the inhibition values were calculated as $(F_x - F_0)/(F_{100} - F_0) \times 100$ by a computer-based evaluation protocol.

Accumulation of Hoechst 33342 dye was measured in a fluorescence spectrophotometer (LS 50B; Applied Biosystems) at 350 nm (excitation)/460 nm (emission), by using 5×10^5 cells in 2 ml of buffer A (120 mM NaCl, 5 mM KCl, 400 μM $MgCl_2$, 40 μM $CaCl_2$, 10 mM HEPES, 10 mM $NaHCO_3$, 10 mM glucose, and 5 mM Na_2HPO_4) solution. This dye becomes fluorescent only in a complex with DNA (Haugland, 1996), and the increase in cellular fluorescence reflects dye influx into the cells. In cells expressing the ABCG2 protein, dye accumulation is much slower than in the control cells (Ozvegy et al., 2002) and can be accelerated by compounds interfering with the ABCG2-dependent Hoechst dye extrusion. The intact control or ABCG2-expressing HL60/PLB cells were preincubated at 37°C in buffer A for 4 min and further incubated with 1 μM Hoechst dye for 10 min. The compounds examined subsequently were added to the cells and the altered rate of accumulation was measured for another 10 min. For maximum inhibition of the ABCG2 protein, 1 μM Ko143 was applied in each experiment, and maximum Hoechst dye binding was determined in the presence of digitonin (see Ozvegy et al., 2002 and Fig. 4). The inhibition values were calculated as in the case of calcein measurements. Figures show data compiled from at least four independent measurements.

Cytotoxicity Assays. Cytotoxicity assays were carried out by using the HL60/PLB human myelomonocytic parent and drug-resistant cell lines [HL60-MDR1, HL60-MRP1 (adriamycin), and HL60/PLB-ABCG2; see above]. The assay was performed in 24-well plates, each well containing an initial cell number of 10^5 cells, in a final volume of 1 ml. Cell culturing was performed in the presence of the agents indicated in figure legends for 120 h at 37°C in 5% CO_2, and both living and dead cells (stained by propidium iodide) were counted in a FACSCalibur cytometer (BD Biosciences, San Jose, CA). Figures represent data obtained from at least two independent experiments.

Mitoxantrone Accumulation Assay. HL60-MDR1, HL60-MRP1, HL60/PLB-ABCG2, and parental HL60 cells were suspended in buffer A (see above). Aliquots of the suspension containing 3×10^5 cells were incubated with 0.5 μM MX with or without the addition of a specific inhibitor of ABCG2, MDR1 or MRP1, or TKIs (EKI-785, ZD1839, STI-571), in concentrations indicated in the figure legends. After an incubation for 60 min at 37°C, the cells were washed and resuspended in ice-cold HPMI. Cellular MX fluorescence was determined at excitation and emission wavelengths of 635 and 661 $\pm$ 16 nm, respectively, in a FACSCalibur cytometer as above. Dead cells were excluded based on propidium iodide staining.

TK Inhibitors Investigated in the Present Study

The tyrosine kinase inhibitors used in these experiment were synthesized and characterized in the laboratory of author G. K. Figure 1 shows the structural formulas for the TKIs examined in the present experiments.

Results

ATPase Activity Measurements in Isolated Insect Cell Membranes. We have examined the effects of TKIs on the transport-related, drug-stimulated ATPase activity of the human ABCG2, as well as of MDR1 and MRP1 multidrug transporter proteins, expressed in isolated insect cell (Sf9) membranes. It has been documented that the stimulation of the multidrug transporter ATPase activity and its drug concentration dependence closely correlates with the respective transport activity of these proteins (Sarkadi et al., 1992; Bakos et al., 2000), and transport inhibitors also inhibit the ATPase activity.

The MDR1-ATPase can be stimulated by hydrophobic substrate drugs (Sarkadi et al., 1992; Muller et al., 1996), whereas the MRP1-ATPase is stimulated by various glutathione-conjugates (GS-X), glucuronate-conjugates, and anionic drugs (Bakos et al., 2000). The basal ATPase activity of ABCG2 is relatively high, with only a small drug-stimulation effect, whereas several transported compounds are inhibitory (Ozvegy et al., 2001, 2002). Therefore, the presence of endogenous (probably lipid-like) substrate of ABCG2 in the membranes has been suggested (Ozvegy et al., 2001, 2002).

In the experiments presented in Fig. 2, we have examined the effects of the three TKIs on the ATPase activity in isolated membranes expressing the human ABCG2 protein. We compared the effects of the TKIs to those of a specific, high-affinity inhibitor of ABCG2, Ko143 (Allen et al., 2002), and verapamil, which is not a substrate of this protein.

As shown in Fig. 2, the vanadate-sensitive ATPase activity of ABCG2-containing isolated Sf9 cell membranes was relatively high, approximately 75 nmol/mg membrane protein/

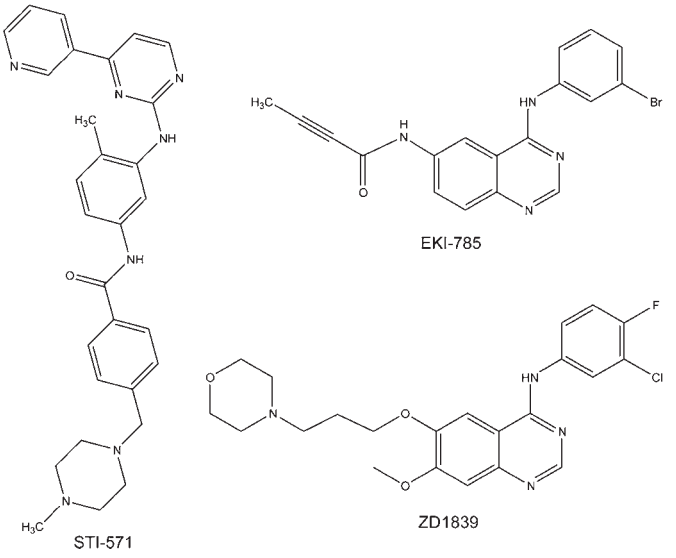

Fig. 1. Chemical formulas of the TKIs (ZD1839, STI-571, and EKI-785) examined in this article.

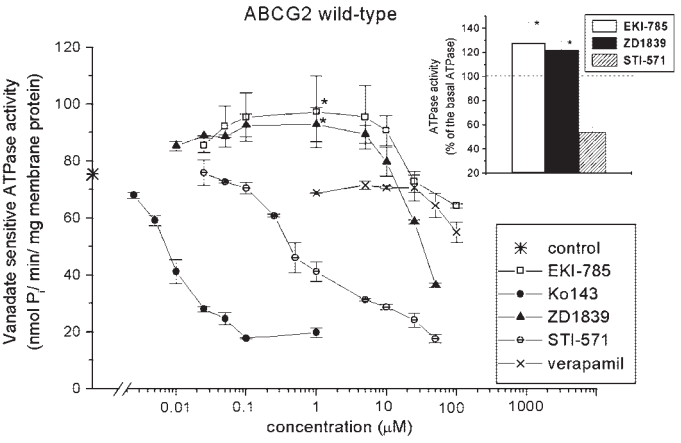

Fig. 2. Concentration dependence of the effects of the TKIs, Ko143, and verapamil, on the human ABCG2-ATPase activity in isolated membrane preparations. Inset, relative effects of the TKIs at 1 μM concentrations, compared with the basal ATPase activity. Vanadate-sensitive ATPase activity in isolated Sf9 cell membranes, containing human ABCG2, and the drug effects were measured in three independent experiments with duplicates, as described under *Materials and Methods*. Control represents the ATPase activity measured in the absence of added compounds. ⋆, Student's t test, $p < 0.05$.

min, largely exceeding that of a control, β-galactosidase-expressing Sf9 cell membrane preparation (about 6–8 nmol/mg membrane protein/min; not shown here). This ATPase was effectively inhibited by nanomolar concentrations (half-maximal inhibition observed at about 8–9 nM) of the specific inhibitor of ABCG2, Ko143 (Allen et al., 2002). In contrast, verapamil, a substrate and/or inhibitor of both MDR1 and MRP1, inhibited the ABCG2-ATPase activity only from the 50 μM concentration.

The TKIs examined in these experiments had considerably different effects on the ABCG2-ATPase. Low concentrations of EKI-785 and ZD1839 produced a small, but statistically significant ($p < 0.05$) stimulation of the ABCG2 ATPase activity (see Fig. 2 inset for 1 μM concentrations of each compound). This effect reached its maximum between 0.1 and 1 μM TKI concentrations, whereas higher EKI-785 or, even more effectively, ZD1839 concentrations were inhibitory. In contrast, STI-571 caused a strong inhibition even at low concentrations, with a half-maximal effect at about 0.5 μM. These data indicate a high-affinity interaction of ABCG2 with these TKIs, also suggesting that ZD1839 and EKI-785 may be actively transported substrates of this protein. In several cases it has been found that higher substrate concentrations inhibited the ATPase activity of both MDR1 and MRP1 (Sarkadi et al., 1992; Bakos et al., 2000).

Regarding the effects of TKIs on the MDR1 and MRP1 protein, we have already reported that STI-571 stimulated the MDR1-ATPase activity in the micromolar concentration range, whereas EKI-785 had no major effect in this assay. In contrast, both STI-571 and EKI-785 effectively inhibited the verapamil-activated MDR1-ATPase activity at concentrations between 5 and 20 μM (Hegedus et al., 2002). In the case of the MRP1-ATPase, none of these compounds stimulated this activity. The NEM-GS-stimulated MRP1-ATPase was inhibited by EKI-785 in the micromolar range (50% inhibition at about 10 μM), whereas STI-571 had a smaller effect and only at higher concentrations (50% inhibition at higher than 100 μM).

In the following experiments, we have examined the effects of ZD1839 on the ATPase activity of the MDR1 and MRP1 proteins. As documented in Fig. 3A, ZD1839 significantly stimulated the MDR1-ATPase, with a half-maximal effect at about 4 μM, although this stimulation reached only about 50% of that produced by verapamil, and a relative inhibitory effect was seen above 20 μM ZD1839 concentrations. ZD1839 had no measurable effect on the ATPase activity of MRP1 in the isolated membranes (Fig. 3A).

To study a potential inhibitory effect of ZD1839 on MDR1 and MRP1, its effect was also measured on the maximally stimulated transporters (Fig. 3B). In the case of the MDR1-ATPase, this maximum stimulation was achieved by 50 μM verapamil, whereas MRP1-ATPase activity was stimulated by a 6 mM concentration of the glutathione-conjugate, NEM-GS. In these experiments ZD1839 was effective only at concentrations between 10 and 100 μM, and its inhibitory effect was similar in the case of both MDR1 and MRP1 (Fig. 3B). All these data suggest that the examined TKIs at low micromolar concentrations show a much more pronounced interaction with the ABCG2 protein than with MDR1 or MRP1.

Fluorescent Dye Extrusion Studies. In the following experiments we applied a whole-cell screening system for studying the interactions of TKIs with the multidrug transporter proteins. In this study we used the human HL60/PLB cells, expressing large amounts of the respective multidrug transporters (see *Materials and Methods*).

As described in the literature (Zhou et al., 2001; Ozvegy et al., 2002), in cells expressing the ABCG2 protein, a decreased rate of accumulation of the Hoechst 33342 dye directly reflects the activity of the ABCG2 protein, and ABCG2 inhibition results in a rapid increase in the rate of dye accumulation.

In the present study, we measured the fluorescent dye accumulation directly in intact PLB cells by using a spectrofluorometer. Because Hoechst 33342 becomes fluorescent only in a complex with DNA, the increase in cellular fluorescence directly correlates with dye influx and DNA binding within the cells. As documented in Fig. 4, in ABCG2-expressing cells, the dye accumulation is relatively slow and is accelerated by compounds interfering with the Hoechst dye extrusion activity of ABCG2. The initial rapid dye uptake, as shown in Fig. 4, reflects a rapid Hoechst permeation and DNA-binding in damaged cells, whereas the following slow

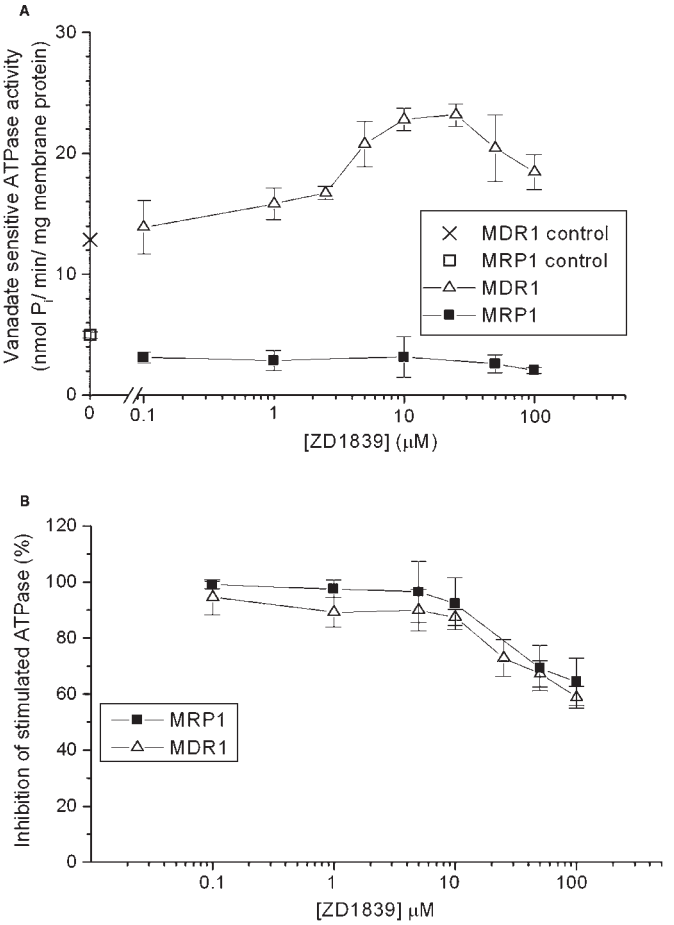

Fig. 3. Effect of ZD1839 on the human MDR1- and MRP1-ATPase activities in isolated membrane preparations. A, modulation of the vanadate-sensitive ATPase activity by ZD1839 in MDR1- or MRP1-expressing isolated Sf9 cell membranes. B, relative inhibition of the maximally stimulated MDR1- and MRP1-ATPase activities by ZD1839. The data are presented as relative values, compared with 100% activation obtained with 50 μM verapamil in the case of MDR1, and in the presence of 6 mM GS-NEM in the case of MRP1. Controls represent the ATPase activity measured in the absence of added compounds. Vanadate-sensitive ATPase activity in isolated Sf9 cell membranes, containing human MDR1 or MRP1, and the effects of ZD1839 were measured in three independent experiments with duplicates, as described under *Materials and Methods*.

increase of fluorescence reaches a linear phase. This slow, linear phase is absent in the control HL60 or PLB cells (data not shown) and is caused by the function of the overexpressed ABCG2 multidrug transporter in the cell membrane. When a compound interfering with ABCG2-dependent dye extrusion is added to the cells, the rate of Hoechst dye accumulation increases.

For maximum inhibition of the ABCG2 protein, 1 μM Ko143 is applied, whereas the maximum level of cellular Hoechst dye binding is determined by the addition of digitonin. Maximum dye loading achieved in the presence of this membrane-permeabilizing agent is much higher than that reached during the accumulation phase; thus a possible dye saturation effect can be excluded. By using this sensitive assay system, it is possible to analyze ABCG2-Hoechst dye transport inhibition, but the system does not reveal the competitive or noncompetitive nature of such an inhibition (Ozvegy et al., 2002).

As documented in Fig. 5 by the various compounds examined, inhibition of Hoechst dye extrusion could be achieved at concentrations varying by 6 orders of magnitude. The specific ABCG2 inhibitor, Ko143 (Allen et al., 2002), a derivative of the fungal toxin fumitremorgin C, inhibited dye extrusion at low nanomolar concentrations, with a half-maximal effect at about 5 nM. In contrast, verapamil had no significant inhibitory effect up to 10 μM, and half-maximal inhibition of ABCG2 function was achieved at higher than 50 μM verapamil.

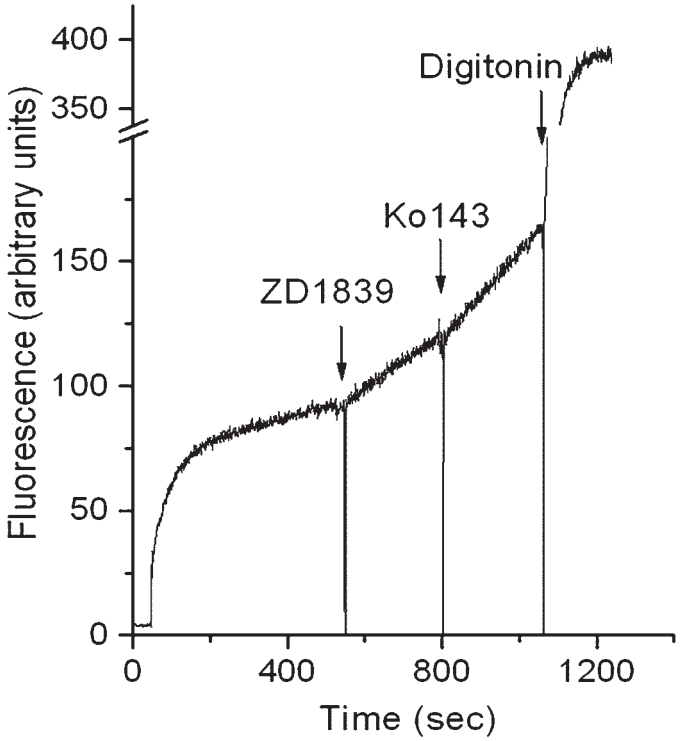

Fig. 4. Measurement of Hoechst dye uptake in a spectrofluorometry assay. Intact PLB-ABCG2 cells were preincubated for 5 min at 37°C and then further incubated at the same temperature under continuous gentle stirring. Hoechst 33342 dye (1 μM) was added at 20 s of the measurement period, and then 0.2 μM ZD1839, 1 μM Ko143, and 10 μM digitonin were added at the times indicated. Fluorescence increase is caused by the binding of Hoechst 33342 to cellular DNA. Maximum dye influx rate was estimated at the fully inhibited transporter, in the presence of Ko143. The figure shows one representative experiment.

The three TKIs examined here had a strong inhibitory effect on ABCG2-dependent dye extrusion at relatively low concentrations. Half-maximal inhibitory effects were observed at about 0.4 μM ZD1839, 0.2 μM EKI-785, and 0.9 μM STI-571. These findings indicate that the TKIs examined bind to ABCG2 and compete with Hoechst at very low concentrations, suggesting a high-affinity interaction of ABCG2 with these TKIs.

In the following experiments, the interaction of the TKIs with MDR1 and MRP1 in intact cells was analyzed by the calcein assay system (Homolya et al., 1993; Hollo et al., 1996). The nonfluorescent, hydrophobic calcein AM rapidly enters into the cells and is cleaved by nonspecific esterases to yield the fluorescent, cell-retained, free calcein. When cells expressing the multidrug transporters MDR1 or MRP1 are incubated with calcein AM, as a result of an active dye extrusion, free calcein accumulation is slow. Agents that interact with the multidrug resistance proteins inhibit dye extrusion and greatly accelerate fluorescent calcein accumulation. The concentration-dependence of this transport inhibition reflects the level of drug interaction with the drug pump proteins (see *Materials and Methods*). Again, competitive or direct inhibition of the transporters cannot be distinguished in this system.

We have already documented (Hegedus et al., 2002) that the TKI inhibitors STI-571 and EKI-785 inhibited calcein AM extrusion by MDR1 at micromolar (half-maximally at about 8–30 μM) concentrations. EKI-785 also inhibited MRP1-dependent transport at micromolar concentrations (5–10 μM), whereas this transport was inhibited only at relatively high concentrations (above 20 μM) by STI-571.

Figure 6 shows the effects of ZD1839 on calcein extrusion from MDR1- and MRP1-expressing HL60 cells, respectively. As shown, calcein AM extrusion by both MDR1 and MRP1 was inhibited by micromolar concentrations of ZD1839, with an approximately half-maximal effect at ZD1839 concentrations of about 4 to 5 μM. The effective concentration of ZD1839 in the calcein assay is slightly different from that measured in the ATPase assay, when inhibition of verapamil- or NEM-GS-activated ATPase activity was measured. The two assays represent the result of the competition between

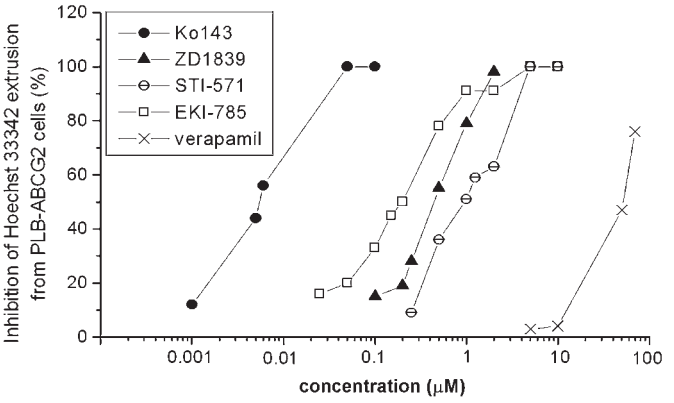

Fig. 5. Inhibition of ABCG2-dependent Hoechst 33342 dye extrusion in PLB-ABCG2 cells by various concentrations of TKIs, Ko143, and verapamil. The relative inhibition of dye extrusion was estimated as described under *Materials and Methods*. Data were compiled from at least four independent measurements for each compound. Error bars did not exceed 5 to 10% of the values.

ZD1839 and calcein AM, or ZD1839 and verapamil or NEM-GS, respectively.

Cellular Drug Toxicity Assays. To examine the direct effect of TKIs on cellular drug resistance, we applied the HL60/PLB cell lines expressing the respective multidrug transporters (see above) in a drug cytotoxicity assay. Cells were cultured in standard media on microwell plates in the presence or absence of different TKI concentrations. After 120 h of incubation, the living and dead cells were counted by flow cytometry analysis using propidium iodide staining (see *Materials and Methods*). In the presentation of these experiments we show the relative cell numbers, compared with those found in the absence of TKIs.

As shown in Fig. 7, A, the addition of up to 5 μM ZD1839 to the HL60/PLB cells, either in the case of control cells or those expressing ABCG2, MDR1, or MRP1, had no significant effect on cell growth and survival in this assay. We found a similar lack of effect of STI-571 (up to 5 μM) and EKI-785 (up to 3 μM) on the growth of these cell types. It is noteworthy that cell growth was also not significantly affected by Ko143 (up to 1 μM) and verapamil (up to 10 μM; except for a slight inhibition of cell growth in the HL60-MDR1 cells) in the cell lines examined (data not shown).

As shown in Fig. 7, B to E, the addition of 25 nM MX caused a complete growth inhibition and cell death in the parental HL60/PLB cells, whereas this MX concentration induced only a small inhibition in cell growth and survival in the ABCG2-, MDR1-, or MRP1-expressing cells (the number of live cells, compared with that in the absence of MX, was 80–90% in the ABCG2-expressing cells, whereas it was 90–100% in the MDR1 and in the MRP1 cells; not shown).

As shown in Fig. 7B, the addition of ZD1839 in the presence of 25 nM MX caused a major inhibition in cell growth in the ABCG2-expressing cells lines, and this inhibitory effect reached 50% at about 0.2 μM ZD1839. In contrast, in the MDR1 cells, a 50% inhibition could be obtained at higher than 10 μM ZD1839 (data not shown), and in the case of MRP1 cells, only a slight inhibition was caused by 10 μM ZD1839 (data not shown). Thus, in accordance with the ATP-ase and dye extrusion results, ZD1839 was a much more effective chemosensitizing agent in the case of ABCG2 than in the case of MDR1 or MRP1.

Figure 7, C and D, shows the effects of STI-571 and EKI-785 in the same cellular toxicity assay system. In the concentration range presented, neither of these TKIs had a measurable effect on the cell growth in the parental or multidrug-resistant HL60/PLB cells. However, a chemosensitizing effect was observed in the ABCG2-expressing cells, in the presence of 25 nM mitoxantrone, by both of these TKIs. As documented, STI-571 caused a half-maximal growth inhibition at 0.2 μM in the ABCG2-expressing cells, whereas at 5 μM STI-571, cell growth was inhibited by about 20% in MDR1-expressing cells and uninhibited in the MRP1-expressing cells. In the case of EKI-785 (Fig. 7D), a similar finding was obtained for the ABCG2-expressing cells (50% inhibition at 0.35 μM). In the case of MDR1-expressing cells, the combination of 0.5 to 1 μM EKI-785 and 25 nM MX actually increased cell growth by approximately 60% and was inhibitory only above 2.5 μM. There was no significant effect of MX + EKI-785 in the MRP1-expressing HL60 cells in this concentration range.

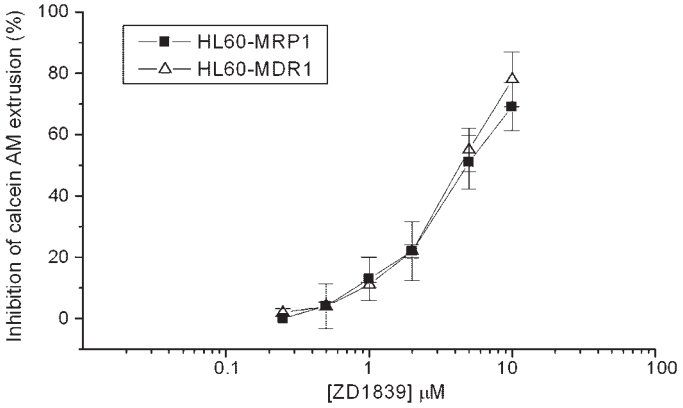

Fig. 6. Inhibition of calcein AM extrusion by the human MDR1 and MRP1 proteins by ZD1839. Calcein AM extrusion was measured in HL60-MDR1 and HL60-MRP1 cells, as described under *Materials and Methods*. Data were compiled from at least four independent measurements for each transporter.

To compare the effect of TKIs to those of Ko143, a specific, high-affinity inhibitor of ABCG2, we have performed similar cell toxicity assays with this compound as well. As documented in Fig. 7E, low concentrations of Ko143 induced a selective toxicity of 25 nM mitoxantrone in the ABCG2-expressing cells. The Ko143 concentration causing 50% growth arrest was about 6 nM, in agreement with the concentration causing 50% inhibition of Hoechst dye extrusion by ABCG2 (see Fig. 5). Again, Ko143 had no significant effect on cell growth of MDR1- or MRP1-expressing cells, even in the presence of 25 nM mitoxantrone (a slight stimulation of the growth of MDR1-expressing cells was observed at 25 nm MX + 10–100 nM Ko143).

As shown in Fig. 7F, similar experiments were carried out using verapamil as a chemosensitizer for MDR1 and (although less effectively) of MRP1; in contrast, verapamil is not an effective modulator of the ABCG2 function (see Figs. 2 and 3). MK571, a specific inhibitor of MRP1, could not be applied in these cytotoxicity assays, because of the rapid loss of MK571 activity under our cell culturing conditions.

As shown, verapamil, up to 10 μM, did not cause a growth inhibition in HL60/PLB cells, including the cell lines expressing the multidrug transporter proteins (data not shown). A slight inhibition of cell growth, approaching a 30% decrease in the HL60-MDR1 cells, was observed with verapamil, which could be caused by the continuous transport of verapamil and a slight ATP-depletion in these cells (data not shown). In contrast to the TKIs or Ko143, verapamil (with 25 nM mitoxantrone) was effective in the growth inhibition of the ABCG2-expressing cells only above 5 to 10 μM concentrations. A strong growth arrest by the combination of verapamil and 25 nM MX was observed in the HL60-MDR1 cells, and a less pronounced effect in the HL60-MRP1 cells (MRP1 has been shown to be less sensitive to verapamil in several studies; see Hollo et al., 1996).

All the cytotoxicity experiments presented in Fig. 7 document the preferential modulation of the ABCG2-dependent MX resistance by Ko143 and by the TKIs, compared with verapamil, whereas the opposite effects were observed in the MDR1- and MRP1-expressing HL60 tumor cells.

Mitoxantrone Transport Experiments. In the cytotoxicity assay, we documented that the TKIs examined greatly increased the cytotoxic effect of mitoxantrone. This is most probably caused by the inhibition of the MX transport activ-

ity of ABCG2, MDR1, or MRP1. Mitoxantrone is a fluorescent compound; therefore, its accumulation in cells can be directly monitored by flow cytometry (Robey et al., 2001). We used the above described HL60/PLB cells, overexpressing ABCG2, MDR1, or MRP1, respectively, to determine mitoxantrone accumulation with or without the addition of TKIs or specific inhibitors (Ko143, verapamil, or MK571), by using a FACS-Calibur cytometer.

Figure 8 shows that after 60 min of incubation with 0.5 μM mitoxantrone, the ABCG2-expressing cells (Fig. 8B) had a much lower level of mitoxantrone accumulation than did the related control cells (Fig. 8A). This decreased accumulation is caused by an active extrusion of mitoxantrone by ABCG2, as indicated by the effect of the ABCG2 inhibitor, Ko143, which increased cellular MX fluorescence up to the level observed in the control cells. The addition of 1 μM ZD1839 (Fig. 8B; and

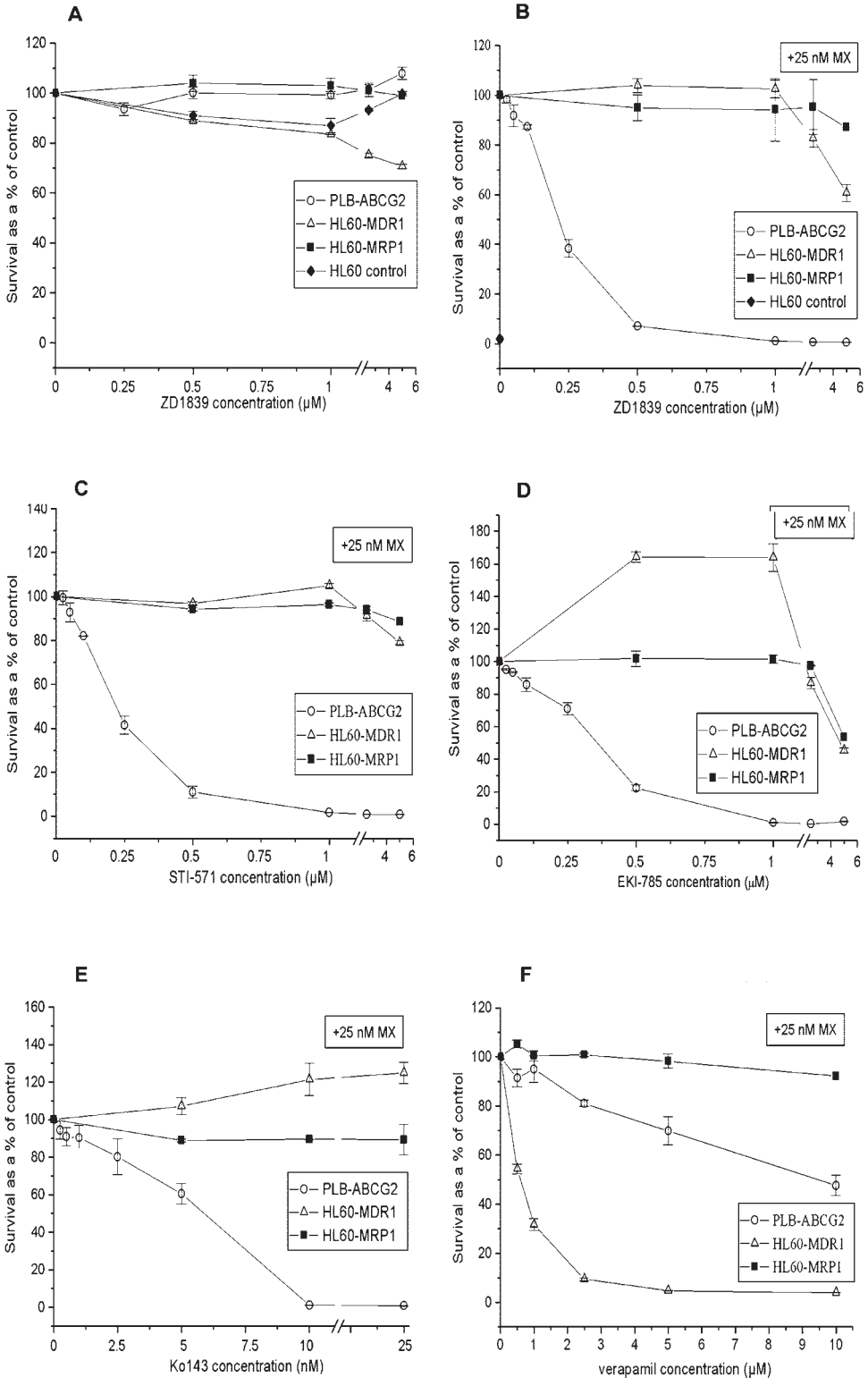

Fig. 7. Combined cellular toxicity assay performed with the TKIs in parental and multidrug-resistant HL60/PLB cell lines. A, effect of ZD1839 on the survival of PLB-ABCG2, HL60-MDR1, HL60-MRP1, and HL60/PLB (control) cells. B, combined effects of various ZD1839 concentrations and 25 nM mitoxantrone (MX) on the survival of HL60/PLB, PLB-ABCG2, HL60-MDR1, and HL60-MRP1 cells. C, combined effects of various STI-571 concentrations and 25 nM mitoxantrone (MX) on the survival of PLB-ABCG2, HL60-MDR1, and HL60-MRP1 cells. D, combined effects of various EKI-785 concentrations and 25 nM mitoxantrone (MX) on the survival of PLB-ABCG2, HL60-MDR1, and HL60-MRP1 cells. E, combined effects of various Ko143 concentrations and 25 nM mitoxantrone (MX) on the survival of PLB-ABCG2, HL60-MDR1, and HL60-MRP1 cells. F, combined effects of various verapamil concentrations and 25 nM mitoxantrone (MX) on the survival of PLB-ABCG2, HL60-MDR1, and HL60-MRP1 cells. Figures represent data obtained from at least two independent experiments.

also of 1 μM EKI-785, or 2 μM STI-571, not shown in the figure) caused a significant increase in MX accumulation in the ABCG2-expressing cells, whereas these TKIs caused no further increase of MX fluorescence in the control cells (Fig. 8A). A significant, concentration-dependent increase in MX accumulation could be observed between 0.2 and 2 μM ZD1839 in these experiments (data not shown). These data strongly suggest that an increased cytotoxicity of MX by the TKIs and Ko143 in the ABCG2-expressing PLB cells was caused by an effective modulation of mitoxantrone extrusion from these cells.

Under the same experimental conditions, the HL60 cells expressing MDR1 (Fig. 8C) or MRP1 (Fig. 8D) showed somewhat greater MX accumulation than did cells with ABCG2, but significantly lower accumulation than did the HL60 control cells. When verapamil (MDR1 cells, Fig. 8C) or MK571 (MRP1 cells, Fig.8D) was also added, cellular fluorescence of MX increased up to the level observed in the HL60 control cells. Neither verapamil nor MK571 caused an increased MX accumulation in the HL60 control cells. These data indicate that both MDR1 and MRP1 can transport MX, although probably with lower effectiveness than ABCG2.

When the effects of ZD1839, EKI-785, and STI-571 were examined in the MDR1- and MRP1-expressing cells, respectively, we found a much less significant effect of these agents than in the case of ABCG2-expressing cells. In fact, TKI concentrations above 5 to 10 μM were required to obtain any increase in MX accumulation in MDR1- or MRP1-expressing cells. As shown in Fig. 8, C and D, 10 μM ZD1839 caused a detectable increase in MX accumulation both in the MDR1- and MRP1-expressing cells, but this effect was still much less than that observed in the presence of verapamil (MDR1) or MK571 (MRP1). These experimental results are in harmony with the cytotoxicity assays, indicating a lower-affinity interaction of the TKIs examined with MDR1 or MRP1, compared with that with ABCG2.

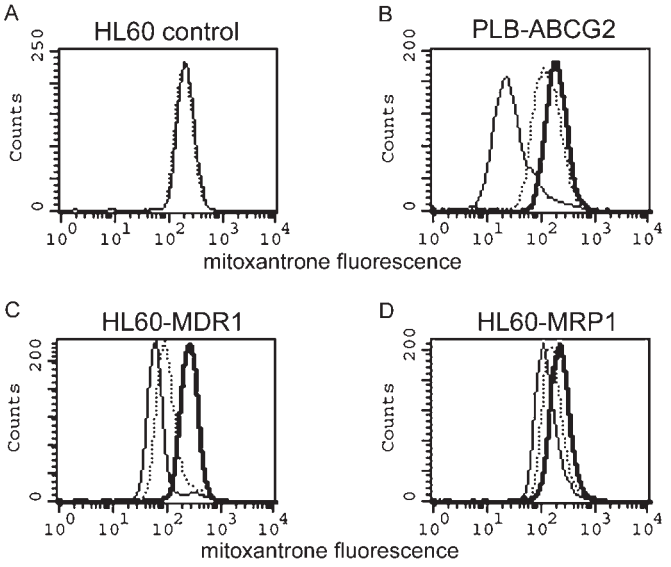

Fig. 8. Effect of TKIs on mitoxantrone accumulation in cells expressing ABCG2, MDR1, and MRP1, respectively. Parental HL60 (control), PLB-ABCG2, HL60-MDR1, and HL60-MRP1cells were incubated with 0.5 μM MX without (solid line) or with the addition of specific inhibitors (heavy solid line) or ZD1839 (dotted line). A. HL60 control cells with MX and 10 μM ZD1839; B, ABCG2 with 1 μM Ko143 or 1 μM ZD1839; C, MDR1 with 50 μM verapamil or 10 μM ZD1839; D, MRP1 with 10 μM MK571 or 10 μM ZD1839. Cellular MX fluorescence was determined as described under *Materials and Methods*. The assay was repeated three times; the histograms show data from a representative experiment.

Discussion

The application of specific TKIs is a rapidly progressing/expanding area of promising cancer therapy efforts. STI-571 (Gleevec, imatinib), which has been introduced as a selective inhibitor of the tyrosine kinase Abl and its unregulated version (Bcr-Abl), causative in the development of chronic myeloid leukemia, was found to be highly effective in these diseases and rapidly passed clinical trials to reach approved applications. Moreover, because of its inhibitory effect on signaling through platelet-derived growth factor receptor and c-Kit, this compound is also potentially effective in the treatment of cancers involving these deregulated kinases (Heinrich et al., 2000; Joensuu et al., 2001; van Oosterom et al., 2002), and clinical trials have been initiated in these directions.

A number of erythroblastic leukemia viral/EGFR/human estrogen receptor family receptor tyrosine kinase inhibitors have also reached various phases of clinical or preclinical trials, and these include the EGFR inhibitor compound ZD1839 (Iressa, gefitinib) and its relatively close structural relative, the irreversible TK inhibitor EKI-785 (Sweeney et al., 1999; Herbst, 2002; Roberts et al., 2002). The ADME-Tox properties of these TKIs are important points in their clinical application, which should be preferentially addressed during the phase of preclinical studies. All these TKIs are essentially hydrophobic compounds, which have to pass the cell membrane barrier to reach their intracellular target molecules. Therefore, their interactions with membrane transporters may be crucial in effectiveness, as well as in their absorption and tissue distribution.

Large hydrophobic molecules, such as the TKIs, have a potential to interact with the so-called multidrug resistance ABC proteins. These are ATP-dependent primary active transporters, which extrude a large variety of chemically unrelated, large, and at least partially hydrophobic compounds from the cells. When overexpressed in tumor cells, some of these proteins, especially MDR1, MRP1, and ABCG2, cause clinical multidrug resistance in cytotoxic therapy. However, these proteins also play important physiological roles, e.g., in modulating the transport properties and secretory functions of the liver and kidney, or modulating penetration of various compounds in the intestine or the blood-brain barrier. The transported substrates of the three major multidrug transporters are wide and somewhat overlapping; all of them can transport hydrophobic drugs, and MRP1 and ABCG2 may also extrude anionic drugs or drug conjugates (Bakos et al., 2000; Bates et al., 2001; Litman et al., 2001).

ABCG2 is a recently recognized drug transporter, which has been shown to extrude cytotoxic agents, e.g., mitoxantrone, topotecan, flavopiridol, and methotrexate, when overexpressed in various tumor tissues (Maliepaard et al., 1999; Litman et al., 2001; Robey et al., 2001; Volk et al., 2002). ABCG2 is abundantly expressed physiologically in the placenta, stem cells, liver, and intestine. Expression of ABCG2 has also been reported in the lung, kidney, adrenal glands, and endothelia of veins and capillaries (Litman et al., 2001; Maliepaard et al., 2001; Zhou et al., 2001; Cooray et al., 2002).

In the present experiments we have analyzed the interactions of various TKIs with the three major multidrug resistance transporters, the classical P-glycoprotein (MDR1), with MRP1 and the relatively newly recognized transporter, ABCG2 (ABCP/MXR/BCRP). These proteins are involved in cancer multidrug resistance; thus, the application of TKIs, which have to reach their intracellular targets, may be significantly modified by the presence of these transporter proteins in the cell membrane. On the other hand, the TKI interactions with the respective multidrug transporters may have a significant drug resistance-modulatory effect during combination chemotherapy. We found that in several in vitro assay systems, STI-571 (Gleevec), ZD1839 (Iressa), and EKI-785 interacted with ABCG2 at submicromolar concentrations, whereas the other multidrug transporters, MDR1 and MRP1, were much less sensitive to these agents.

When measuring multidrug transporter ATPase activity in isolated membranes, low concentrations of these TKIs selectively modulated ABCG2-ATPase activity. Moreover, in 0.1 to 1 μM concentrations, ZD1839 and EKI-785 significantly stimulated, whereas STI-571 only inhibited, this ATPase activity. The activation of ABCG2-ATPase at low concentrations of ZD1839 and EKI-785, based on earlier studies with other multidrug transporter ATPases, suggests that these agents are actually transported substrates of the ABCG2 multidrug transporter (Sarkadi et al., 1992; Bakos et al., 2000). To support such a transported substrate-like interaction, we have initiated both vesicular and whole-cell transport experiments, although no decisive data is yet available. At higher TKI concentrations, a strong inhibition of the multidrug transporters was observed in each case. Such an effect has already been observed for several substrate drugs for the various multidrug transporters and is probably caused by a less efficient dissociation of these compounds at the off-site of the transporters (Sarkadi et al., 1994).

Membrane ATPase activity of MRP1 was hardly affected by any of the TKIs examined, whereas ZD1839, similar to STI-571 (Hegedus et al., 2002), activated the MDR1 ATPase, but only at 5 to 15 μM concentrations. ZD1839 in concentrations above 10 μM inhibited both maximally stimulated MDR1 and MRP1 ATPase activities. Thus, these ATPase experiments suggested a preferential interaction of all the ZD1839, STI-571, and EKI-785 with the ABCG2 transporter, compared with their interaction with MDR1 or MRP1.

In the following experiments, we have documented that the three TKIs examined preferentially inhibited ABCG2-dependent active fluorescent dye extrusion. For studying the pump activity of MDR1 and MRP1, we used the calcein accumulation assay, whereas for examining the transport function of ABCG2, we used the Hoechst dye extrusion assay. In this latter case, we documented that, by measuring Hoechst dye fluorescence increase in cells in a fluorometry assay, the inhibitory action of various compounds could be successfully estimated. Because the changes in the rate of dye accumulation can be well measured, and the saturation of the dye uptake is only at relatively high values, this assay provides an efficient tool for such drug interaction studies.

The Hoechst 33342 dye is also efficiently extruded by the MDR1 protein, whereas it is not transported by MRP1 (Litman et al., 2001). In experiments not documented here, we have also studied the relative efficiency of ZD1839 to block Hoechst dye extrusion in MDR1- versus ABCG2-expressing HL60 cells. These experiments indicated that ZD1839 was more effective at much lower concentrations for the ABCG2-dependent Hoechst transport (50% inhibition at about 0.4 μM) than for the Hoechst extrusion caused by MDR1 expression (50% inhibition at about 5 μM ZD1839).

All these dye transport assays emphasize the high-affinity interaction of TKIs with the ABCG2 transporter. However, these assays do not reveal the competitive or noncompetitive nature of these interactions; thus, a potential TKI extrusion by ABCG2 and a direct TKI inhibition of the transporter both appear as inhibition of dye extrusion.

We have also performed detailed drug resistance assays by using cell lines selectively expressing the various multidrug transporters. In cells expressing ABCG2, all three TKIs greatly increased the cytotoxicity of low concentrations (25 nM) of mitoxantrone, which was ineffective in this cell line without these modulating compounds. A selective inhibition of ABCG2 by Ko143 in nanomolar concentrations produced a similar drug sensitivity increase, whereas higher verapamil concentrations had to be applied to modulate MX sensitivity in ABCG2-expressing cells.

Cells expressing MDR1 or MRP1 were also resistant against 25 nM mitoxantrone, and TKIs caused a decrease in this resistance. However, significantly higher concentrations of the TKIs were needed to inhibit the MX resistance in cells expressing these multidrug transporters. When the effective inhibitor verapamil (although more effective for MDR1 than for MRP1) was added, a modulation of MX toxicity that could be well measured was observed. Unfortunately, the effect of the selective MRP1 inhibitor MK571 could not be assessed, because this inhibitor rapidly lost its activity under the cytotoxicity assay conditions (data not shown). In cells expressing MDR1, EKI-785 in low concentrations caused a measurable increase in cell growth. We do not have a ready explanation for this finding, but EKI-785 competition with mitoxantrone may decrease ATP hydrolysis by MDR1, saving cellular ATP, or, alternatively, EKI-785 and mitoxantrone may be cotransported and/or may stimulate the transport of each other. In fact, in MDR1-expressing HL60 cells, the addition of low (1–5 μM) EKI-785 concentrations decreased mitoxantrone accumulation, indicating an increased MX transport activity by MDR1 (data not shown).

To directly examine MX extrusion in cell lines used in the drug resistance assays, we have also determined intracellular mitoxantrone fluorescence by flow cytometry. The findings in these assays closely correlated with those of the cytotoxicity measurements: MX accumulation in PLB-ABCG2 cells was greatly increased by low micromolar concentrations of the TKIs examined. In contrast, in cells expressing MDR1 or MRP1, which also showed a significant MX extrusion, these TKIs were much less effective in increasing MX accumulation.

The various assays in this study investigated different aspects of interactions between ABC multidrug transporters and TKIs. The stimulation or inhibition of the transporter ATPase activity represent direct interactions between TKIs and ABC transporters, whereas the inhibition of a fluorescent dye extrusion or the modulation of mitoxantrone cytotoxicity shows the net result of possible combined cellular interactions. The effective concentrations of TKIs in the three assays were found to be somewhat different, but in the same order of magnitude. Moreover, all the assays supported the

notion that the ABCG2 protein has a higher affinity toward these TKIs than does either MDR1 or MRP1.

Based on these experimental data, we suggest that multidrug resistance protein modulation by TKIs may be an important factor in the clinical treatment of cancer patients. Coadministration of TKIs with cytotoxic agents may prevent ABCG2-dependent cancer multidrug resistance and increase effectiveness for both types of intracellularly effective compounds.

The high-affinity interaction of ZD1839 with ABCG2 and its relatively low-affinity interaction with MDR1 and MRP1 may also modulate absorption and tissue distribution of this compound. In fact, the oral applicability and the intestinal absorption of ZD1839 (Ranson et al., 2002) may be caused by a strong inhibition of the ABCG2 and MDR1 multidrug transporters by high concentrations of ZD1839 at the apical surface of the intestinal epithelial cells, thus allowing a passive permeation of these hydrophobic agents into these cells. Such an effect has already been reported for ABCG2 inhibitors modulating topotecan absorption (Jonker et al., 2000).

In contrast, further cellular or tissue entry of TKIs at the relatively low plasma levels may be adversely affected by these transporters. Moreover, the removal of TKIs by multidrug transporters, e.g., ABCG2, may be involved in tumor cell TKI resistance.

The human estrogen receptor family inhibitor CI1033 has already been shown to increase the cellular accumulation and antiproliferative effectiveness of topotecan in BCRP/ABCG2-expressing tumor cells (Erlichman et al., 2001). According to a recent study with STI-571 (imatinib), the overexpression of MDR1/Pgp induced only a relatively small STI-571 resistance in intact tumor cells (Ferrao et al., 2003; Mahon et al., 2003). These findings are in line with our experiments showing a less efficient STI-571 interaction with MDR1 than with ABCG2.

The present study demonstrates the applicability of relatively simple in vitro assays for demonstrating specific, high-affinity interactions with the multidrug resistance proteins and advocates the use of such assays in further TKI drug development. Further experiments, regarding the direct extrusion of TKIs and, thus, the modulation of their cellular action by the overexpression of the ABCG2 and/or MDR1 proteins are underway in our laboratory.

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Address correspondence to: Dr. Balázs Sarkadi, National Medical Center, Institute of Hematology and Immunology, 1113 Budapest, Diószegi u. 64, Hungary. E-mail: sarkadi@biomembrane.hu