Multidrug Transporter ABCG2 Prevents Tumor Cell Death Induced by the Epidermal Growth Factor Receptor Inhibitor Iressa (ZD1839, Gefitinib)

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Abstract
Iressa (ZD1839, Gefitinib), used in clinics to treat non–small cell lung cancer patients, is a tyrosine kinase receptor inhibitor that leads to specific decoupling of epidermal growth factor receptor (EGFR) signaling. Recent data indicate that Iressa is especially effective in tumors with certain EGFR mutations; however, a subset of these tumors does not respond to Iressa. In addition, certain populations have an elevated risk of side effects during Iressa treatment. The human ABCG2 (BCRP/MXR/ABCP) transporter causes cancer drug resistance by actively extruding a variety of cytotoxic drugs, and it functions physiologically to protect our tissues from xenobiotics. Importantly, ABCG2 modifies absorption, distribution, and toxicity of several pharmacological agents. Previously, we showed that ABCG2 displays a high-affinity interaction with several tyrosine kinase receptor inhibitors, including Iressa. Here, we show that the expression of ABCG2, but not its nonfunctional mutant, protects the EGFR signaling-dependent A431 tumor cells from death on exposure to Iressa. This protection is reversed by the ABCG2-specific inhibitor, Ko143. These data, reinforced with cell biology and biochemical experiments, strongly suggest that ABCG2 can actively pump Iressa. Therefore, variable expression and polymorphisms of ABCG2 may significantly modify the antitumor effect as well as the absorption and tissue distribution of Iressa. (Cancer Res 2005; 65(5): 1770-7)

Introduction
The epidermal growth factor factor receptor (EGFR) signaling pathway regulates diverse physiologic responses, including proliferation, differentiation, cell motility, and survival (1). However, this pathway is also used to promote tumor growth in a variety of epithelial tumors, including the most malignant form of lung carcinoma, non–small cell lung cancer (2, 3). EGFR belongs to the ErbB/HER family of tyrosine kinase receptors, which homodimerize or heterodimerize on ligand activation, resulting in transphosphorylation on tyrosine residues in the tyrosine kinase receptor cytoplasmic domain. The subsequent recruitment and activation of signaling effectors transmit the EGFR signal within the cell.

Much effort has been taken to develop specific methods of inhibiting EGFR to prevent cancerous processes. The two most promising therapeutic approaches make use of monoclonal antibodies and small molecule inhibitors that specifically target the EGFR tyrosine kinase enzymatic activity. Iressa (ZD1839, Gefitinib, AstraZeneca Pharmaceuticals, London, United Kingdom) is a small, orally active molecule that is a selective and reversible inhibitor of the EGFR tyrosine kinase activity, blocking EGFR signal transduction pathways (4, 5). As an antitumor agent, after passing phase I and II of clinical trials, Iressa failed phase III trials for the treatment of non–small cell lung cancer patients (6–8). Despite phase III failure, Iressa was the first drug of its kind to receive approval in Japan, the United States, and in Australia, and Iressa has shown promising efficacy in other solid tumors that rely on EGFR-related mechanisms for growth and survival (9).

Data explaining why Iressa works well in some cancer patients and not in others were discovered recently (10, 11). Somatic mutations clustered around the active site of the kinase domain of EGFR, present in the majority of non–small cell lung cancer tumors, correlate with hypersensitivity to Iressa. This important observation will allow enhanced success in the treatment of patients in the future. However, there is a subset of cancer patients with EGFR mutations that do not respond well to Iressa treatment. This could be explained by inadequate dosing and delivery of drug to the tumor, which may have resulted in suboptimal receptor modulation.

In addition, inappropriate dosing could also be the cause for the elevated risk of toxicity (compared with worldwide statistics) experienced by Japanese patients (12, 13). In fact, the search for factors that may cause severe side effects from Iressa treatment are now under way in Japan, where hundreds of patients have died from treatment (14). Factors affecting the absorption, distribution, metabolism, excretion, and toxicity of Iressa are of interest for study in order that a phase III clinical trial might appropriately choose patients whose tumors are likely to respond to treatment.

A group of ATP binding cassette proteins, including P-glycoprotein, several multidrug resistance proteins, and the ABCG2 protein (also known as BCRP/MXR/ABCP), cause multidrug resistance in tumors, as they actively extrude a wide variety of anticancer drugs (for reviews, see refs. 15–17). These proteins are regarded as potential clinical targets for regulation to inhibit cancer multidrug resistance as well as to alter the...
absorption, distribution, metabolism, excretion, and toxicity variables for various chemotherapeutic drugs (18, 19). One of the key multidrug transporters, ABCG2, is a primary active transporter for mitoxantrone, topotecan, or flavopiridol, and its overexpression was documented in several drug-resistant cell lines and tumors (15, 20–23). ABCG2 is present in the plasma membrane in stem cells, placenta, liver, small intestine, colon, lung, and kidney, suggesting its role in the protection/detoxification of xenobiotics (15, 24–26). Importantly, single polymorphisms, which result in amino acid substitutions, have been identified in relatively large percentage of populations (e.g., in Japan; refs. 27, 28). These naturally occurring mutations could alter drug absorption and metabolism as well as render some cancer patients differently susceptible to chemotherapy (29).

Recent work in our laboratory showed that ABCG2 has a high-affinity (nanomolar range) interaction with several tyrosine kinase receptor inhibitors (TKRI), including Iressa. Although ABCG2 possesses a partially overlapping substrate profile with both P-glycoprotein and multidrug resistance protein-1 (15–17, 26, 30), and although all three interacted with Iressa, only ABCG2 had a high affinity to the drug (31).

In the present study, we used the A431 squamous cell carcinoma line as a model system for studying the functional interaction of ABCG2 and Iressa. This tumor cell line is dependent on EGFR signaling for survival. In fact, EGFR is amplified in these cells, allowing them to grow in serum-free medium (32–35). In contrast, drug-induced inactivation of the EGFR tyrosine kinase enzyme by Iressa or other EGFR inhibitors causes the cessation of growth and induction of mostly apoptotic cell death in A431 cells (2, 36, 37).

For our studies, we have generated retrovirally transduced A431 cells, expressing various levels of the wild-type ABCG2, or a functionally inactive mutant (R86M) ABCG2 variant. We analyzed if functional ABCG2 provided a selective growth advantage during Iressa treatment and followed the changes in the phosphorylation state of the EGFR in these cells. We also examined if the Iressa-induced cell death was modulated by ABCG2 by using Annexin V binding and confocal microscopy to detect apoptosis. Our cell biology experiments were extended by direct biochemical studies for studying the effect of Iressa on the ABCG2-ATPase activity in isolated mammalian cell membranes. The results obtained strongly suggest that the function of ABCG2 results in an active extrusion of Iressa, preventing the apoptotic effect of this molecule at the level of receptor tyrosine kinase inhibition in tumor cells.

Materials and Methods

Materials. Ko143 was a generous gift from Drs. J. Allen and G. Koomen (University of Amsterdam, Amsterdam, the Netherlands). Mitoxantrone and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO). Hoechst 33342, Alexa Fluor 488 Annexin V, and MitoTracker Deep Red 633 were purchased from Molecular Probes (Eugene, OR).

Retroviral DNA Expression Construction. The retroviral vector, SPsLdS (38), was modified previously by us to contain a neomycin resistance cassette (39). cDNA encoding wild-type (R482) ABCG2 or ABCG2K86M was inserted by blunt-end ligation into the EcoRI sites of the retroviral vector, thus removing the original gfp91 cDNA. Expression plasmids were purified from endotoxins with Endo-free Qiagen (Hilden, Germany) kits and used to transfect Phoenix-Eco packaging cells.

Cell Growth and Propagation. The human skin–derived, epidermoid carcinoma cells, A431, were maintained in α-MEM (Life Technologies, Grand Island, NY) supplemented with 10% FCS, 50 units/ml penicillin, 50 units/ml streptomycin, and 5 mmol/L glutamine at 37°C in 5% CO2.

MCF-7/MX cells (40) were a generous gift from Dr. Susan Bates (NIH, Bethesda, MD). These were maintained in DMEM supplemented with 10% FCS, 50 units/ml penicillin, 50 units/ml streptomycin, and 5 mmol/L glutamine at 37°C in 5% CO2.

Retroviral Transduction of A431 Cells with ABCG2 Constructs. The expression of ABCG2, its nonfunctional mutant variant, ABCG2K86M, and vector control in A431 cells was achieved by a method described previously by us (41). Briefly, Phoenix-Eco packaging cells were transiently transfected with retroviral DNA (see above). Viral supernatants were collected and used to transduce A431 cells. Cells expressing retroviral DNA were then selected with 10 mg/ml G418. Mitoxantrone selection of the transduced A431 cells was achieved by culturing the cells in 500 mmol/L drug.

Detection of ABCG2 and Its Variant in A431 Cells by Western Analysis and Hoechst 33342 Dye Accumulation Assay. ABCG2 and its catalytically inactive variant, ABCG2K86M, were detected in A431 lysates by Western analysis as described previously (42) using the BXP-21 anti-ABCG2 monoclonal antibody. BXP-21 was a kind gift from Drs. George Scheffer and Rik Schepers (Department of Pathology, VU University Medical Center, Amsterdam, the Netherlands).

Accumulation of Hoechst 33342 dye was done by using intact A431 cells without (control) or with the overexpression of wild-type ABCG2 or its inactive K86M mutant in a fluorescence spectrophotometer (Perkin-Elmer LS-5, Boston-Elmer/Abbe Biosystems, Foster City, CA) at 350 nm (excitation)/460 nm (emission) as described (31). The increase in cellular fluorescence due to Hoechst 33342 accumulation was determined in the absence (F0) or presence of 1 mmol/L Ko143 (F100) giving 100% inhibition of ABCG2 transport activity. The value reflecting the transport activity was calculated as [(F0 – Fj)/F0] × 100.

A431/Iressa Cytotoxicity Assay. Parental A431 cells and those expressing ABCG2, ABCG2MX, and ABCG2K86M were grown to subconfluency, trypsinized, and diluted in an Eppendorf tube to 106 cells/mL serum-free medium. Cells were treated for 15 minutes at room temperature in the absence or presence of different concentrations of Ko143 and Iressa. Cells (4 × 104 per 0.4 mL) were then dispersed into a well of a 24-well plate in triplicates and incubated for 48 hours in the same Iressa- and/or Ko143-containing medium at 37°C, 5% CO2. Cells were then harvested with trypsin and collected in complete medium. Subsequently, PI (2 μg/mL) was added and the samples were counted in a FACSCalibur cytometer (Becton Dickinson, San Jose, CA) for live cells.

Iressa-Mediated EGFR Dephosphorylation. Parental A431 cells and those expressing ABCG2, ABCG2MX, and ABCG2K86M were stimulated with epidermal growth factor (20 ng/mL) for 15 minutes and subsequently incubated for an additional 15 minutes in the presence or absence of Iressa as described in A431/Iressa Cytotoxicity Assay. Cell lysates were produced as described previously (43), and proteins (30 μg) were separated by SDS-PAGE and transferred to polyvinylidine difluoride membrane using the Mini-Protean II System (Bio-Rad, Hercules, United Kingdom). Western blots were probed with mouse anti-phosphotyrosine (clone 4G-10) monoclonal antibody, which was used to detect the phosphorylation state of the EGFR. Subsequently, membranes stained with the primary antibody were incubated in TBS/0.5% Tween 20 containing 0.5% H2O2 at room temperature for 30 minutes and then washed thrice with TBS/0.5% Tween 20 to deactivate residual horseradish peroxidase activity. Then, detection by anti-EGFR rabbit polyclonal IgG (Upstate, Lake Placid, NY) was used to ensure that equal levels of EGFR were present in the lysates. Secondary antibodies were mouse and rabbit IgG horseradish peroxidase conjugate (Jackson Immunoresearch, West Grove, PA). The enhanced chemiluminescence system was used for chemiluminescence detection (Amersham, Little Chalfont, United Kingdom). For quantitative analysis of Western blots, image analysis of X-ray films was done by Bioscan version 1.0 software following digitalization with Hewlett Packard (Palo Alto, CA) 5100 C scanner.

Annexin V Binding and Confocal Microscopy. The cells (4 × 104 per well) were seeded onto eight-well Nunc Lab-Tek Chambered Coverglass
Results

Recently, we documented (31) that ABCG2 displays a high-affinity, submicromolar interaction with the tyrosine kinase inhibitor Iressa. To extend these results and show that ABCG2-mediated transport activity can protect cells from the toxic effects of Iressa in cells, we generated an appropriate cellular model system. Because Iressa efficiently acts as an anticancer agent in EGFR-dependent tumor cells, we used the human skin-derived, epidermoid carcinoma cells, A431, for studying ABCG2-Iressa interactions. This cell line, which is dependent on EGFR signaling for survival, was retrovirally transduced by ABCG2 and also by an inactive, catalytic site mutant, ABCG2K86M. We obtained several clones of these cells, expressing variable levels of ABCG2, and for comparative experiments, we further selected some of the ABCG2-transduced A431 cells in mitoxantrone, resulting in high levels of the ABCG2 protein expression (ABCG2ΔN).

In Fig. 1, we show the steady-state ABCG2 protein expression levels in the cells used in this study by Western analysis (Fig. 1A). Western blot analysis, using the specific anti-ABCG2 monoclonal antibody, BXP-21, revealed well measurable levels of ABCG2 protein in the transduced A431 cells overexpressing ABCG2 (G2) and considerably higher levels in cells further selected with the ABCG2 substrate, mitoxantrone (G2MX; ref. 42). ABCG2K86M (G2K86M) represents cells expressing the inactive, catalytic site mutant of ABCG2 at comparable levels with the cells transduced with the active, wild-type ABCG2 (G2). As a negative control, parental A431 cells were also used (control A431). As we will present below, A431 cells express low levels of endogenous ABCG2, which was not detected by Western analysis.

To detect functionality of the expressed ABCG2 protein in the transduced A431 cell lines, we used the Hoechst 33342 dye accumulation assay (Fig. 1B). Hoechst 33342 is a cell-permeable hydrophobic dye, which is a characterized substrate of ABCG2 (26, 45). Whereas drug-sensitive cells rapidly take up this dye, which becomes fluorescent on binding to DNA, cells expressing functional ABCG2 show only a low rate of increase in fluorescence. By applying the specific ABCG2 inhibitor, Ko143, the Hoechst 33342 dye eflux activity of ABCG2 can be quantified (46).

This assay is very sensitive and allowed the detection of functional endogenous ABCG2 even in control A431 cells (Fig. 1B, Ctrl A431). However, in the ABCG2 (G2)–expressing cells, a much greater Hoechst 33342 eflux activity was seen, which was further augmented after mitoxantrone selection of the transduced A431 (G2MX) cells. In contrast, the catalytic site mutant, ABCG2K86M, possessed even less Hoechst 33342 transport activity than the A431 control cells. This is likely due to the overexpressed mutant protein, which may form a dominant-negative heterodimeric complex with endogenous ABCG2. In conclusion, we have created A431 cell lines expressing differing levels of transport-competent ABCG2 as well as its inactive mutant form, ABCG2K86M.

In the following experiments, we studied if the overexpression of ABCG2 conferred a survival advantage to A431 cells exposed to Iressa. As shown in Fig. 2A, we compared the effects of Iressa on cell survival of A431 cells over a 48-hour period of exposure to various concentrations of Iressa (50–500 nmol/L). Cells were treated with Iressa and then plated for following cell growth in the surface-attached cell populations. As shown, low concentrations
ABCG2 (ABCG2 K86M) did not provide such a protection. The mediated death.

ABCG2 expression could no longer protect A431 cells from Iressa-

Under these experimental conditions, at higher Iressa concen-

parental A431 cells (control A431) survival data are the same as for the nonfunctional ABCG2-expressing cells (data not shown).

To analyze the molecular basis of these cellular effects, we did direct ABCG2-ATPase measurements in isolated membranes of ABCG2-expressing mammalian MCF-7/MX and A431 cells. We measured the vanadate-sensitive ATPase activity (44), reflecting the ABCG2 transport activity in these membranes, and studied the effects of various concentrations of Iressa as well as that of Ko143 on this ABCG2-ATPase activity.

As shown in Fig. 3, both MCF-7-ABCG2 and A431-ABCG2 membranes had a significant basal ATPase activity, which was absent in the control, MCF-7, or A431 cell membranes (data not shown) and strongly inhibited by the addition of Ko143. Low concentrations of Iressa significantly activated the ABCG2-ATPase in these membrane preparations, whereas higher Iressa concentrations (>1 μM/L) had a decreasing stimulatory effect. The same tendency was observed in ABCG2-expressing A431 cells, although

To further characterize this protective effect in terms of its specific dependence on the activity of the ABCG2 protein, we carried out a detailed study using 50 nmol/L exposure of Iressa in A431 cells expressing various levels of ABCG2 protein or its nonfunctional mutant with and without the addition of the specific, high-affinity ABCG2 inhibitor, Ko143 (47). As documented in Fig. 2A and B, ABCG2 expression protected A431 cells from Iressa-induced death, observed in the parental A431 cells or those expressing ABCG2K86M. In contrast, when the ABCG2 inhibitor Ko143 was included in the Iressa treatment (Fig. 2B, white columns), the protective effect of ABCG2 expression was removed (i.e., cell death was not significantly different from that found in the parental or ABCG2K86M-expressing A431 cells). We also observed a small increase in death rate in the parental A431 cells when Iressa was combined with Ko143, but this effect was not statistically significant. Ko143 applied alone did not cause any cell death (data not shown).

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The lower expression levels of ABCG2 in these membranes yielded lower maximum ATPase activities. The maximum stimulation obtained by Iressa was 2.6 to 3 in both membranes, and the $K_v$ for ATPase activation was also similar (~100 nmol/L in both cases). The $V_{max}$ of the vanadate-sensitive ATPase activity was 73.97 ± 6.0 nmol P/mg membrane protein/min in the MCF-7/MX membranes and 38.9 ± 2.0 nmol P/mg membrane protein/min in the A431-ABCG2 membranes. When we compared the effect of Iressa on this ATPase activity with that by other drug substrates, we found that in the A431-ABCG2 membranes prazosin gave a similar (2.5-fold) activation, whereas mitoxantrone activated the ATPase with somewhat lower effectivity (1.9-fold stimulation; data not shown).

The present data are in line with our earlier experiments carried out using ABCG2-expressing isolated S9 cell membranes (31) but, due to the lower basol ABCG2-ATPase activity in the mammalian cell membranes, the stimulatory effect of Iressa is much better appreciated here. It should be noted that because we used relatively high membrane concentrations in these ATPase experiments, most probably due to the high lipid absorption of this compound, the effective Iressa concentrations were shifted to somewhat higher levels than found in the whole cell studies.

In the following experiments, we analyzed the cellular mode of action of ABCG2 in protecting A431 tumor cells from Iressa. According to unequivocal evidence presented in the literature, Iressa exposure leads to rapid dephosphorylation of the EGFR, and this loss of specific phosphorylated tyrosine in the membrane-bound receptor is the basis of cell death induction in the EGFR-dependent A431 cells. We were interested in exploring if the presence of the functional ABCG2 transporter could protect the cells from this Iressa effect directly at the membrane receptor level.

To this end, parental A431 cells and those expressing ABCG2 (G2), ABCG2K86M (G2K86M), and ABCG2MX (G2MX) were exposed to Iressa concentrations between 25 and 100 nmol/L, and the phosphorylation status of the EGFR was directly assayed by Western blotting using anti-phosphotyrosine antibodies. As documented in Fig. 4A, EGFR in parental A431 cells (control A431) became ~80% dephosphorylated on treatment with 25 nmol/L Iressa. In ABCG2K86M (G2K86M) cells, the receptor was dephosphorylated to a greater extent (i.e., less ABCG2 protection) than the parental cells, probably owing to the dominant-negative effect of ABCG2K86M (G2K86M) on endogenous ABCG2 in A431 cells as discussed above. In contrast, ABCG2, and to a greater extent ABCG2 mitoxantrone-selected cells (ABCG2MX; G2MX), were protected significantly, and phosphorylated EGFR was still observed at 25 and 50 nmol/L Iressa treatment levels (Fig. 4A). As in the cytotoxicity experiments, ABCG2 could not protect from higher levels of Iressa treatment (Fig. 4B, 100 nmol/L Iressa). Figure 4B shows the quantitation of the Western data shown in Fig. 4A.

These data suggest that ABCG2 acts at the EGFR level by preventing the action of Iressa on tyrosine kinase activity. This action is significantly inhibited by a functional ABCG2, most likely acting through the active transport of Iressa from the plasma membrane before it reaches the plasma membrane resident EGFR.

It has been established that Iressa leads to cell death partly through apoptosis in A431 cells (2, 36, 37). To examine if ABCG2 protected against the early apoptotic steps of cell death, we used an Annexin V binding assay in conjunction with confocal microscopy. We followed the apoptotic process by staining the cells with fluorescent Annexin V, which reports the appearance of extracellular phosphatidylserine in the early apoptotic phase. Cell membrane destruction, leading to the influx and nuclear staining by PI, was also followed in the same cell samples. These experiments were carried out directly in tissue culture well–plated A431 cells, and under these conditions, cell death was prevented by ABCG2 up to Iressa concentrations of 1 μmol/L (see below). According to our experience with similar, hydrophobic drugs, due to drug absorption, higher drug concentrations are required in tissue culture plates than in cell suspensions to obtain similar cellular effects (see Materials and Methods).

As shown in Fig. 5, in the parental control A431 cells, the addition of 1 μmol/L Iressa (Fig. 5B, D, and F) produced an early appearance of significant Annexin V staining (Fig. 5B) compared with the nontreated parental A431 cells. In apoptotic cell membranes, blebbing was observed as Annexin V–stained (green) membrane vesicles coming off the plasma membrane. Mitochondrial activity of the cells (red) was visualized by MitoTracker Deep Red 633 to demarcate cells present in the field. In apoptotic cells, mitochondria typically lose the ability to bind MitoTracker Deep Red 633 as was the case here (48).

As documented in Fig. 5, on exposure to Iressa, in the control A431 cells, both Annexin V–positive/PI-negative (apoptotic) cells...
and Annexin V–positive/PI-positive (in late phase of death) cells could be observed. Both of these phases were absent in the ABCG2-expressing A431 cells (Fig. 5D), whereas cells expressing ABCG2K86M displayed a similar cell death pattern as the control A431 cells (Fig. 5F). These microscopic data indicate that the protective effect of ABCG2 against Iressa at the EGFR level indeed removes all the following steps of the apoptotic and cell destruction process.

In conclusion, the data presented here strongly suggest that the expression of functional ABCG2 provides significant protection against low, therapeutically relevant concentrations of Iressa in causing apoptosis-related tumor cell death. This protection correlates with the active transport capacity of ABCG2 and is directly correlated with the prevention of Iressa effect at the EGFR level.

Discussion

In this study we have examined the protective effects of the human multidrug resistance transporter, ABCG2, on A431 tumor cells when exposed to the TKRI, Iressa. Iressa is a potent inhibitor of the EGFR, and when cells, such as A431, which depend on

Figure 5. Morphology of Iressa-induced cell death in control and ABCG2-expressing A431 cells. Control (A and B), ABCG2 (C and D), and ABCG2K86M (E and F) transduced A431 cells were cultured in serum-free medium for 24 hours in the absence (A, C, and E) and presence (B, D, and F) of 1 μmol/L Iressa. Confocal images show Annexin V binding (green), nuclear DNA of dead cells stained with PI (blue), and mitochondrial activity of cells (red) visualized by MitoTracker Deep Red 633.
epidermal growth factor signaling for survival, are exposed to Iressa, they die due to disruption of the essential epidermal growth factor signal. ABCG2 is an active transporter of small hydrophobic drugs and was found recently to interact with several TKRIs with high affinity (31).

Our results presented here strongly suggest that ABCG2 actively transports Iressa from A431 cells and thus protects the cells by a transport-based mechanism. ABCG2 expressed in A431 cells by retroviral transduction (Fig. 1A) was functional for Hoechst 33342 transport (Fig. 1B), which is a well-established method for determining ABCG2 transport function (31). In experiments not shown in detail, we found that 1 μmol/L Iressa interfered with Hoechst 33342 transport in A431-ABC2 cells, inhibiting this transport by >95%. Although this effect shows only an interaction between Iressa and ABCG2, the ABCG2-associated ATPase activity in isolated membranes was significantly stimulated by Iressa, indicating a direct transport activity for this compound (Fig. 3). Most importantly, abolishment of ABCG2-mediated protection of A431 cells to Iressa was shown in two ways: (a) using an ATPase- and transport-defective catalytic center mutant (ABC2kK86A) expressed at similar levels as wild-type ABCG2 (see Fig. 1) and (b) using a well-established, specific, potent inhibitor of ABCG2 activity, Ko143.

Using the above-mentioned controls, we have shown ABCG2-mediated protection at several levels. In cytotoxicity experiments (Fig. 2A), we found that parental A431 cells or those expressing catalytically inactive ABCG2 (G2K86A) die when exposed to low nanomolar concentrations, whereas cells expressing lower (G2) or higher (G2ΔAX) amounts of transporter are protected. The greatest level of protection was seen at 50 nmol/L Iressa treatment. At this concentration, we show that the ABCG2 inhibitor, Ko143, can completely reverse ABCG2-mediated protection from Iressa in these cells (Fig. 2B). An important point to note is that ABCG2 prevented the cytotoxic effects at Iressa concentrations likely to be present in tumor tissues after the oral administration of this TKRI (the pharmacologically relevant maximum plasma Iressa concentrations are between 500 nmol/L and 1 μmol/L, see ref. 8). In addition, the ABCG2 expression level in the transduced cells, especially without mitoxantrone selection, is in the range of that observed in drug-resistant tumor samples. Interestingly, at higher Iressa concentrations, a decreasing protection was seen by ABCG2. One explanation for the difficulty of ABCG2 in overcoming these excessive Iressa concentrations (>500 nmol/L) could be that the drug off-rate from the transporter is becoming slower, thus leading to a decreased transport rate. This phenomenon most probably does not affect the pharmacologic function of ABCG2 in the tumor cells with respect to Iressa because, as mentioned, there may be rather low local concentrations of Iressa during cancer therapy.

We have also explored the biochemical mechanism of the ABCG2-mediated cytotoxic protection by probing the EGFR phosphorylation state. On epidermal growth factor treatment, the EGFR was found highly phosphorylated (see Fig. 4A). Low concentrations of Iressa caused the rapid dephosphorylation of the receptor, which was reversed by functional ABCG2 (G2, G2ΔAX) but not by a nonfunctional transporter (G2ΔS65A). These data suggest that ABCG2 acts at the EGFR level, preventing the action of Iressa. ABCG2 most likely acts through the active transport of Iressa from the plasma membrane before it reaches plasma membrane resident EGFR. This finding is in line with earlier results showing that the multidrug transporters (e.g., P-glycoprotein) remove their drug substrates from the lipid phase or from the vicinity of the plasma membrane, thus preventing the cellular entry of cytotoxic agents (49).

We also did direct ABCG2-ATPase measurements in isolated membranes of ABCG2-expressing mammalian MCF-7/MX and A431 cells at various concentrations of Iressa and Ko143 concentrations (Fig. 3). We found that the ABCG2 ATPase activity was activated by low concentrations of Iressa and strongly inhibited by Ko143. These direct enzymatic studies indicate that Iressa is a transported substrate of ABCG2. In addition, higher Iressa concentrations were inhibitory for the maximum ATPase (and transport) activity of this protein. The data are also in accordance with the less effective protective effect of ABCG2 in the A431 cells at higher Iressa concentrations.

Collectively, based on all these cellular and biochemical data, we suggest that ABCG2 is actively transporting Iressa out from the cells, and this is the basis of ABCG2 protecting the tumor cells from the cytotoxic effects of this TKRI.

These results, indicating direct extrusion of Iressa by ABCG2 are somewhat unexpected based on recent studies indicating that another TKRI, STI-571 (Imatinib, Glivec), showing high-affinity interaction with ABCG2 (31), was found to be only an inhibitor and not a transported substrate of this multidrug resistance protein (50). In another study, however, the specific cytotoxic effect of STI-571 was also shown to be inhibited by ABCG2 expression (51). A possible source of this controversy is that STI-571 may have an even narrower window in the low concentration ranges, where its active transport by ABCG2 is efficient, although higher concentrations are inhibitory (31).

The findings that ABCG2 can actively protect the relevant tumor cells from Iressa have important implications regarding the clinical use of this tyrosine kinase inhibitor. First, a subset of tumors, bearing a sensitizing EGFR mutation but still unresponsive to Iressa, may be protected by an elevated level ABCG2. Second, because ABCG2 is expressed in many tissues, it could play an important role in the absorption, distribution, metabolism, excretion, and toxicity of Iressa in the patients. This would certainly affect the local concentrations of Iressa in the tumors as well as other, potentially sensitive tissues. Further, detailed clinical studies are required to examine the functional expression and the direct in vivo relevance of ABCG2 in this regard.

Importantly, inappropriate dosing could also be the cause of overall elevated risk of toxicity to Japanese patients and the hundreds of deaths that have occurred (12, 13). In fact, naturally occurring single nucleotide polymorphisms of ABCG2 in the Japanese population significantly alter the expression and/or the activity of this protein (27, 28), and these polymorphisms may be important modulators of individual therapeutic potential and toxicity (29).

In conclusion, our data strongly suggest that ABCG2 can actively extrude the clinically effective TKRI, Iressa, at low, therapeutically relevant concentrations of this compound. Our results also indicate that specific ABCG2 transporter modulation (such shown here by Ko143) may greatly increase the antitumor efficacy of Iressa.

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