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Tyrosine kinase inhibitors as modulators of ABC transporter-mediated drug resistance

Suneet Shukla¹, Zhe-Sheng Chen², and Suresh V. Ambudkar^{1,*}

¹Laboratory of Cell Biology, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, MD 20892, USA

²Department of Pharmaceutical Sciences, College of Pharmacy and Allied Health Professions, St. John's University, Queens, NY 11439, USA

Abstract

Tyrosine kinases (TKs) are involved in key signaling events/pathways that regulate cancer cell proliferation, apoptosis, angiogenesis and metastasis. Deregulated activity of TKs has been implicated in several types of cancers. In recent years, tyrosine kinase inhibitors (TKIs) have been developed to inhibit specific kinases whose constitutive activity results in specific cancer types. These TKIs have been found to demonstrate effective anticancer activity and some of them have been approved by the Food and Drug Administration for clinical use or are in clinical trials. However, these targeted therapeutic agents are also transported by ATP-binding cassette (ABC) transporters, resulting in altered pharmacokinetics or development of resistance to these drugs in cancer patients. This review covers the recent findings on the interactions of clinically important TKIs with ABC drug transporters. Future research efforts in the development of novel TKIs with specific targets, seeking improved activity, should consider these underlying causes of resistance to TKIs in cancer cells.

Keywords

ABC transporter; ABCG2; cancer chemotherapy; multidrug resistance; P-glycoprotein; tyrosine kinase inhibitor

1. Tyrosine kinases and their role in cancer

Tyrosine kinases (TKs) play crucial roles in physiological processes such as embryogenesis, differentiation, cell proliferation, anti-apoptotic signaling and programmed cell death (Geer et al., 1994; Levitzki and Gazit, 1995; Marshall, 1995; Pytel et al., 2009). They mediate the transduction and processing of many extra- and intra-cellular signals by catalyzing the transfer of the gamma phosphate group from ATP to target proteins (Schlessinger, 2000). These TKs can be classified as receptor TKs (RTKs) and non-receptor TKs (NRTKs). The RTKs possess an extracellular ligand binding domain and an intracellular catalytic domain with intrinsic TK activity (Krause and Van Etten, 2005). Binding of a ligand to these receptors leads to a variety of downstream effects including stimulation of other TKs,

*Author for correspondence: Suresh V. Ambudkar: Laboratory of Cell Biology, Center for Cancer Research National Cancer Institute, NIH., Bethesda, MD 20892, USA. Tel: +1-301-402-4178; ambudkar@helix.nih.gov.

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elevation of intracellular calcium levels, and activation of serine/threonine kinases, phospholipase C and phosphatidylinositol-3'-kinase, which eventually leads to changes in gene expression (Krause and Van Etten, 2005). NRTKs lack transmembrane domains and are found in the cytosol, the nucleus, and at the inner surface of the plasma membrane. NRTKs are activated by diverse intracellular signals through dissociation of inhibitors, by recruitment to transmembrane receptors (causing oligomerization and autophosphorylation), and through *trans*-phosphorylation by other kinases (Krause and Van Etten, 2005). Both RTKs and NRTKs are crucial mediators of intracellular signal transduction pathways. Three major signaling pathways that are activated by TKs have been identified as playing important roles in cell proliferation and homeostasis (Faivre et al., 2006). These include the phosphatidylinositol-3-kinase (PI3K)/AKT, protein kinase C (PKC) family, and mitogen-activated protein kinase (MAPK)/Ras signaling cascades. The PI3K pathway leads to the activation of PDK1 (3-phosphoinositide-dependent protein kinase-1) and AKT (also known as protein kinase B). The Ras/MAP pathway leads to activation of Raf1, MEK, and MAPK (Faivre et al., 2006). Any abnormality in TK structure or function leads to disruption of these signaling pathways, thereby disrupting a wide array of cellular functions which leads to increased cell proliferation and decreased apoptosis. This imbalance in cellular homeostasis that is tightly controlled by TKs in cells results in initiation of the oncogenic transformation process. This further leads to cancer development or malignancy resulting in metastasis (Aman, 2005; Travalì et al., 1990).

2. TKIs and cancer chemotherapy

One strategy to control the deregulated activity of TKs is to inhibit the catalytic activity of the kinases. A major effort in this field has been made to discover small molecule inhibitors that can block the phosphorylation mediated by these kinases. Several classes of inhibitors have been developed that interfere with the activity of unregulated kinases at different levels (Krause and Van Etten, 2005). These can be broadly divided into three categories (i) inhibitors that block the binding of ATP to the ATP binding pocket of kinases, such as the BCR-ABL kinase inhibitors imatinib, nilotinib and dasatinib, the epidermal growth factor receptor inhibitors gefitinib and erlotinib, and the cyclin-dependent kinase inhibitor roscovitine. (ii) The allosteric inhibitors block allosteric sites that are usually required for the activation of kinases, such as the p38 inhibitor BIRB 796, the Raf inhibitor BAY43-9006, and the MAP kinase inhibitor PD184352. Inhibitors from this category exhibit the highest degree of kinase selectivity, as they block the binding site and regulatory mechanism that are unique to a particular kinase. (iii) Other TKIs include drugs that may inhibit the activation of fusion of TKs by blocking their dimerization, antibodies against RTKs or their ligands, which interrupt TK signaling through neutralization of ligands, blockade of ligand binding, or receptor internalization.

A significant number of TKIs have been approved for cancer treatment or are currently being evaluated in clinical trials (Brózik et al., 2011). The most clinically advanced approaches to TK inhibition include the use of small molecule TKIs that block the ATP-binding site or allosteric site within the intracellular catalytic domain of TKs, thereby inhibiting phosphorylation and subsequent downstream signaling processes and the use of monoclonal antibodies (mAbs) directed against transmembrane RTKs, which inhibit ligand binding to the kinase and subsequently inhibit the receptor activation (Ciardiello and Tortora, 2001; Grunwald and Hidalgo, 2003; Mendelsohn and Baselga, 2003). Some recent reviews have extensively dealt with the interactions of small molecule TKIs with several target kinases in basic and clinical studies (Hantschel et al., 2008; Janne et al., 2009). This review will focus on the small molecule TKIs used currently in cancer therapy and specifically their effect on two major ABC drug transporters.

3. Multidrug resistance

Multidrug resistance (MDR) is a phenomenon that occurs when cancer cells develop resistance to a variety of anticancer drugs that are structurally and mechanistically unrelated (Szakacs et al., 2006). Currently, the leading cause of treatment failure in cancer is primarily due to the development of MDR to anticancer drugs. The mechanisms of MDR in cancer cells have been extensively studied and appear to be quite complex. The loss of drug carrier proteins on the cell surface and alteration/mutation of specific targets of the drugs are mechanisms of MDR in cancer cells that resist the cytotoxic effects of certain anticancer drugs (Gerrit and Rob, 1998; Gottesman, 2002). Expression of transmembrane transporters that regulate the uptake or efflux of chemotherapeutics is one such mechanism that has gained more attention recently due to increasing knowledge about the role of transporters in handling xenobiotics and other toxic compounds including natural product anticancer agents in the human body. These transporters can broadly be divided into two large families consisting of (i) efflux transporters and (ii) uptake transporters. Overexpression of efflux transporters or decreased expression of uptake transporters results in lower intracellular concentration of chemotherapeutics thereby resulting in the development of MDR. Due to the limited scope of this review, we focus here on efflux transporters. ATP-binding cassette (ABC) transporters is one family of these transporters, and is involved in ATP-dependent efflux of xenobiotics or chemotherapeutics from the cells (Higgins, 1992). In humans, the ABC protein superfamily includes 48 genes that have been assigned to a “family tree” with 7 subfamilies A-G (Dean et al., 2001). A characteristic feature of ABC proteins is the highly conserved ABC domain that is responsible for ATP binding and hydrolysis. These ABC proteins are present in all living species and can function as receptors, channels or transporters (Ueda et al., 1999). For receptors and channels, ATP binding and hydrolysis appear to have a regulatory function, whereas for active transporters, ATP hydrolysis is necessary to provide energy for the transport process. Understanding how these ABC transporters work may provide information valuable for overcoming MDR that develops during cancer chemotherapy.

3.1 The role of ABC transporters in MDR

Expression of some of the ABC transporters has been linked to development of MDR in cancer cells (Gottesman et al., 2002). Many of these ABC transporters were reported to confer resistance to anticancer drugs in cell models. They include P-gp (MDR1/ABCB1) (Juliano and Ling, 1976), Multidrug Resistance Proteins (MRPs) 1, 2, 3, 4, 5, 6, 7, and 8 ((Cole et al., 1992), reviewed in (Borst et al., 1999; Guo et al., 2003; Hopper-Borge et al., 2009; Kruh and Belinsky, 2003) and ABCG2 (BCRP/MXR/ABCP) (Allikmets et al., 1998; Doyle et al., 1998; Miyake et al., 1999). However, only three of these transporters, P-gp, MRP1 and ABCG2 appear to contribute significantly to MDR. For this reason, we will focus on these three pumps and their role in MDR.

3.1.1 P-gp—P-gp was originally isolated from the plasma membranes of Chinese hamster ovary cells displaying the MDR phenotype (Juliano and Ling, 1976). It is the most prominent and best characterized of the mammalian ABC transporters. P-gp confers resistance to and transports a wide variety of compounds that are central to most of the cytotoxic chemotherapeutic regimens such as vinca alkaloids, anthracyclines, epipodophyllotoxins and taxanes (Ambudkar et al., 1999). In addition, a wide variety of chemically dissimilar drugs including immunosuppressive agents (cyclosporine A [CysA]), calcium channel blockers (verapamil), HIV protease inhibitors (saquinavir, ritanovir), and other cytotoxic agents (paclitaxel, colchicines) are also transported by this transporter. Furthermore, in recent years, a number of studies have suggested that P-gp is also a transporter that effluxes molecularly targeted drugs such as TKIs, as we will discuss later. In

addition, P-gp is normally expressed in the apical membranes of epithelial cells of the gastrointestinal tract, liver and kidney and in endothelial cells of the blood-brain barrier (BBB) (Cordon-Cardo et al., 1990). Therefore, P-gp also plays a very important role physiologically by protecting the body from xenobiotics and other natural product toxins.

Naturally occurring genetic variants of P-gp (also known as single nucleotide polymorphisms; SNPs) also have been shown to influence the interindividual variability in pharmacokinetics and the pharmacodynamics of many drugs that are handled by P-gp (Ambudkar et al., 2003; Mickley et al., 1998). It has been proposed that the SNPs (both non-synonymous as well as synonymous) alter the protein function or expression of P-gp, thereby substantially affecting intestinal absorption, the elimination, and the penetration of drugs into the cells. For example, the C₃₄₃₅T synonymous SNP in *MDR1* was shown to be correlated with lower intestinal P-gp expression on the apical surface, which influenced the oral bioavailability of drugs that were substrates of this transporter (Hoffmeyer et al., 2000). Kimchi-Sarfaty et al. reported that a synonymous SNP in the *MDR1* gene results in functional P-gp with altered drug and inhibitor interactions (Kimchi-Sarfaty et al., 2007). A subtle alteration in the conformation of the P-gp could explain the changes in the interaction of P-gp with the substrates (Kimchi-Sarfaty et al., 2007).

3.1.2 MRP1—MRP1 was the first member of the C subfamily of ABC transporters to be identified and this subfamily now consists of ten members (ABCCs1-6 and ABCC9-12) (Cole et al., 1992; Kruh and Belinsky, 2003). Similar to P-gp, MRP1 is also able to confer resistance to vinca alkaloids, taxanes, anthracycline antibiotics and epipodophyllotoxins. However, MRP1 does not confer resistance to taxanes, which are important components of the P-gp-mediated drug resistance profile (Cole et al., 1994; Kruh and Belinsky, 2003). In addition, MRP1 confers resistance to certain heavy metal oxyanions (Chen et al., 1997; Cole et al., 1994). Only a few reports have indicated that TKIs interact with MRP1. MRP1 is expressed in a wide range of tissues, including the testis and the kidney at the basolateral side of epithelial membranes (Deeley et al., 2006; Evers et al., 1996). MRP1 transports hydrophobic drugs, drug conjugates and a variety of organic anions (Deeley et al., 2006). MRP1 transports less toxic, but more water soluble, anionic glutathione (GSH), sulfate and glucuronate drug conjugates subsequent to Phase I and II metabolism (Deeley et al., 2006). It also transports neutral/basic drugs in the presence of GSH. These drugs and GSH are presumably co-transported (Deeley et al., 2006). In addition to drug conjugate detoxification, MRP1 is essential in maintaining the redox balance of reduced GSH and oxidized glutathione (GSSG) (Deeley et al., 2006).

MRP1 may also be involved in certain aspects of the immune response, as LTC₄, a glutathione conjugate, is a substrate that has high affinity for MRP1 (Wijnholds et al., 1997).

3.1.3 BCRP—ABCG2 (BCRP) is a half ABC transporter that functions by forming a homodimer or an oligomer and confers resistance to anticancer drugs in cancer cells. ABCG2 is overexpressed in several cell lines selected by anticancer drugs such as mitoxantrone and topotecan (Doyle et al., 1998; Maliapaard et al., 1999). It is a high capacity transporter that can interact with a wide variety of substrates (Chen et al., 2003). The substrates of ABCG2 include cytotoxic anticancer drugs, toxins and carcinogens found in foods and some endogenous compounds (Doyle and Ross, 2003; Schinkel and Jonker, 2003). Although MRP1-4 are able to transport mono-glutamated methotrexate (MTX) (Chen et al., 2002), ABCG2 has been shown to extrude polyglutamated MTX (Chen et al., 2003). This result suggested that ABCG2 may confer resistance to MTX following both short- and long-term exposure (Zhao and Goldman, 2003). In addition, ABCG2 can transport some newly developed cytotoxic anticancer drugs such as CPT-11 and its active metabolite SN-38 (Kawabata et al., 2001), as well as some TKIs (Ozvegy-Laczka et al., 2005). ABCG2 is

present in many tissues, including stem cells, placental syncytiotrophoblast cells, epithelial cells of the gastrointestinal tract and liver and endothelial cells of the BBB (Cooray et al., 2002; Maliepaard et al., 2001; Zhou et al., 2001). Similar to P-gp, ABCG2 is expressed on the apical surface of epithelial cells and it can protect the body from xenobiotics, including specific compartments such as the brain and the placenta (Cooray et al., 2002; Jonker et al., 2000). Recently, ABCG2 has been shown to transport urate in the kidney and the non-synonymous polymorphism Q141→K of the gene has been found to be associated with the development of gout disease (Dehghan et al.; Woodward et al., 2009; Woodward et al., 2011). Interestingly, several reports demonstrated that ABCG2 was overexpressed only in subpopulations (also known as side population) of Acute Myelogenous Leukemia (AML) samples (Abbott et al., 2002; Suvannasankha et al., 2004). The overexpression of ABCG2 in these subpopulations of stem cells was also found in other tumors including neuroblastomas, Ewing sarcomas, breast cancer, small-cell lung cancer and glioblastomas (Hirschmann-Jax et al., 2004). These stem cells may play an important role in conferring resistance to chemotherapeutic agents, contributing to relapses. It is important to point out that the role of ABCG2 in stem cell biology is not yet well understood.

4. Role of ABC drug transporters in the development of resistance to TKIs

Resistance to TKIs has been reported, as they have been used increasingly in clinical studies and administered to patients (Janne et al., 2009; Sierra et al., 2010). Point mutations within the kinase domain that render the kinase less sensitive to TKIs are the most reported and studied mechanism of resistance to TKIs. These mutations confer resistance to TKIs by impeding inhibitor access to the ATP-binding loop and/or activation loop of the kinase. Due to the scope of this review, we will not discuss resistance to TKIs stemming from mutations in the kinases themselves. While kinase mutations do play a significant role in resistance, emerging data suggest that in addition to kinase domain mutations, other as yet not well-defined mechanisms may contribute to and may explain clinical resistance. Resistance due to the expression of ABC drug transporters is one such mechanism, which has also been reported for constitutive and acquired drug resistance to TKIs (Assef et al., 2009; Hirayama et al., 2008; Illmer et al., 2004; Mahon et al., 2003). As explained above, energy-dependent efflux of chemotherapeutics by these transporters results in the development of MDR, and efflux of TKIs mainly by P-gp and ABCG2 has also been reported as contributing factors to the development of resistance to these agents.

4.1 Interactions of TKIs with ABC drug transporters- implication in resistance or altered pharmacokinetics

Recently, we and others discovered that several TKIs are substrates or modulators of P-gp and ABCG2 (Burger et al., 2004; Dai et al., 2008; Elkind et al., 2005; Erlichman et al., 2001b; Shi et al., 2007b; Shukla et al., 2009; Shukla et al., 2008; Yanase et al., 2004). The interaction of TKIs with ABCG2 and P-gp, the role of these transporters in the development of resistance to specific TKIs, and a possible effect on the alteration of the pharmacokinetics and toxicity of clinically important TKIs are discussed below.

4.1.1 Imatinib (Gleevec, STI571)—Imatinib, the first target-based small molecule drug for treatment of BCR-ABL-positive chronic myelogenous leukemia (CML) or acute lymphoblastic leukemia (ALL) was developed in the late 1990s. It received FDA approval in May 2001 and is still being used as a first line drug for treatment of CML. Resistance to imatinib due to the overexpression of MDR1 was first reported by Mahon et al. (Mahon et al., 2000). They also found that *MDR1* gene overexpression confers resistance to imatinib in leukemia cell lines (Mahon et al., 2003). We recently reported that overexpression of P-gp is associated with imatinib resistance in K562 cells (Peng et al., 2011). Illmer et al. showed

that intracellular levels of imatinib decrease in P-gp-positive leukemic cells (Illmer et al., 2004). Decreased imatinib levels were associated with a retained phosphorylation pattern of the Bcr-Abl target Crkl and loss of effect of imatinib on cellular proliferation and apoptosis. The modulation of P-gp by CysA readily restored imatinib cytotoxicity in these cells (Illmer et al., 2004). Rumpold et al. also demonstrated that silencing the expression of P-gp in imatinib-resistant chronic myeloid leukemia (CML) cell lines resensitized the cells to both imatinib and doxorubicin (Rumpold et al., 2005). Similarly, Widmer et al. showed that the intracellular concentration of imatinib increased by 4- to 9-fold in K562 cells expressing P-gp when the expression of ABCB1 was downregulated by RNAi (Widmer et al., 2007). However, other studies showed that overexpression of P-gp in K562 cells does not confer resistance to imatinib, nor did the specific elimination of P-gp in the hematopoietic system improve the responses to imatinib in a CML animal model (Ferrao et al., 2003; Zong et al., 2005). We provided biochemical evidence for interaction of imatinib with the two major ABC drug transporters, P-gp and ABCG2, at the transport-substrate site(s) and showed that imatinib competed for [¹²⁵I]-Iodoarylazidoprazosin (IAAP) binding (a transport substrate of P-gp and ABCG2) to P-gp and ABCG2, while it did not compete for the binding of [α -³²P]-8-Azido-ATP, an ATP analog to either P-gp or ABCG2 (Shukla et al., 2008). We also used vanadate trapping and ATP hydrolysis assays to demonstrate that imatinib behaves like a transport substrate, as it stimulates ATP hydrolysis by these transporters (Shukla et al., 2008). These observations indicate that in spite of the fact that these inhibitors bind to the ATP-binding sites of the tyrosine kinase, they seem to interact at the transport-substrate site(s) instead of at the ATP or nucleotide-binding domains on the ABC transporters (Figure 1). Our data also indicated that imatinib interacts with these transporters at low micromolar concentrations, which further suggests that imatinib has a relatively high affinity for both P-gp and ABCG2. Further, Houghton et al. showed that [¹⁴C]-imatinib was not transported by ABCG2-expressing Saos2 osteosarcoma cell lines, while Burger et al. found that the accumulation of the same was significantly lower in ABCG2-expressing cell lines than in their parental counterparts (Burger et al., 2004; Houghton et al., 2004). Our work provided a possible explanation for the contradictory results reported by two different groups. We proposed that there may be a narrow concentration range in which the ABC transporters can transport the TKIs. Thus, the fact that Houghton et al. used 1 μ M of [¹⁴C]-imatinib while Burger et al used 200 nM of the labeled imatinib could explain the differences in their findings (Burger et al., 2004; Houghton et al., 2004).

One potential consequence of our findings is that the combination of imatinib and cytotoxic anticancer drugs is likely to have an additional beneficial effect by increasing the intracellular concentration of P-gp and/or ABCG2 substrates in cancer cells. Along these lines, several reports have shown that imatinib can either be used to increase the sensitivity of ABC drug transporter-expressing cells to chemotherapeutic agents or increase the bioavailability of drugs that are substrates of ABC drug transporters. Gao et al. reported that imatinib, when combined with vincristine, not only enhanced vincristine sensitivity but also significantly suppressed the tumor formation of MDR K562 cells, which overexpress P-gp, in a human nude mice xenograft model (Gao et al., 2006). Oostendorp et al. showed that while P-gp and ABCG2 have only modest effects on the ADME of imatinib in comparison to metabolic elimination in mice, co-administration of the P-gp and/or ABCG2 inhibitors, elacridar or pantoprazole, significantly increased the systemic exposure to imatinib in the presence or absence of P-gp/ABCG2 in mice (Oostendorp et al., 2009). The concentrations at which imatinib inhibits the function of Pgp and ABCG2 (low micromolar concentrations) are within the known therapeutic ranges (0.1-3.4 μ g/ml; 0.17-5.68 μ M) of plasma levels of imatinib in patients after treatment with 25-600 mg /day (Druker et al., 2001).

A list of TKIs that stimulate ATPase activity as well as block incorporation of [¹²⁵I]-IAAP into the drug substrate sites of P-gp and ABCG2 is given in Table 1. The table shows the

concentration of TKIs required for 50% stimulation of ATP hydrolysis and the concentrations required for 50% inhibition (IC_{50}) of [^{125}I]-IAAP incorporation into Pgp and ABCG2. These values indicate the affinity and nature of the interactions of TKI with these two transporters. The fact that most TKIs stimulate the basal ATPase activity of the transporters and inhibit the photolabeling of the transporters with [^{125}I]-IAAP suggests that TKIs behave as typical substrates of ABC drug transporters. It is clear from these data that TKIs that compete for ATP-catalytic sites on kinases directly interact at the drug-substrate-binding sites on P-gp, ABCG2 and possibly other ABC transporters.

4.1.2 Nilotinib (Tasigna)—Nilotinib is a recently approved BCR-ABL kinase inhibitor that has been shown to be effective in the treatment of imatinib-resistant CML. Therefore, it is considered as a second generation BCL-ABL kinase inhibitor. We were the first to report that nilotinib is a substrate of ABCG2 and it directly interacts with ABCG2 at the substrate binding sites, as it competes with the binding of [^{125}I]-IAAP and also stimulates the transporter's ATPase activity (Brendel et al., 2007; Shukla et al., 2008). In addition, we reported recently that nilotinib could reverse P-gp- and ABCG2-mediated MDR by blocking the efflux function of these transporters (Tiwari et al., 2009). These results suggest that some ABC transporters such as ABCG2 may play a role in resistance to nilotinib. Later, Mahon et al. also reported that resistance to nilotinib may be due to BCR-ABL, P-gp, or Src kinase overexpression in Philadelphia chromosome positive AR230, LAMA84, and K562 cell lines (Mahon et al., 2008). Hegedus et al. also showed that ABCG2-expressing K562 cells were 8.8-fold more resistant to nilotinib than parental K562 cells, whereas ABCB1 function only slightly modified the cytotoxic effect of this drug, resulting in a small (albeit significant) resistance of K562/ABCB1 cells. These results suggest that ABC transporters such as P-gp and ABCG2 may play a role in the resistance to nilotinib (Hegedus et al., 2009). Similar to imatinib, there also have been reports that suggest that nilotinib efflux by ABC drug transporters may not be responsible for resistance to this drug. Davies et al. demonstrated that the concentration of nilotinib in cell lines and primary CD34⁺ chronic myeloid leukemia cells is not mediated by active uptake or efflux by major drug transporters (Davies et al., 2009). More recently, Hiwase et al. showed that inhibition of Pgp activity by nilotinib can be used to increase the intracellular concentration of dasatinib in CML cells and proposed that a combination of low-dose dasatinib and nilotinib may provide an additive/synergistic anti-leukemic effect in leukemic stem cells that are refractory to TKI therapy and also express P-gp (Hiwase et al., 2010). We have very recently synthesized a Bodipy-FL derivative of nilotinib (Figure 2) and studied its interaction with P-gp and ABCG2 (Shukla et al., 2011). We showed that Bodipy-FL nilotinib (fluorescent tasigna) inhibited the BCR-ABL kinase activity in K562 cells and was also effluxed by P-gp and ABCG2 in both cultured cells and rat brain capillaries expressing P-gp and ABCG2. In addition, we demonstrated that [3H]-nilotinib was transported by P-gp-expressing polarized LLC-PK1 cells in a transepithelial transport assay. Consistent with these results, both nilotinib and Bodipy-FL nilotinib were less effective at inhibiting the phosphorylation of Crkl (a substrate of BCR-ABL kinase) in P-gp- and ABCG2-expressing K562 cells due to their reduced intracellular concentration. These studies have provided evidence that nilotinib is indeed transported by P-gp and ABCG2 (Shukla et al., 2011). In addition, availability of a Bodipy-FL derivative with similar properties as the parent drug may provide an important tool for researchers to follow the efficacy, pharmacokinetics, and toxicity of nilotinib in preclinical and clinical models. A similar approach of synthesis of a fluorescent derivative may also be used to determine whether a given TKI is transported by P-gp, ABCG2 or by other ABC transporters. The Bodipy-FL derivative of nilotinib or another TKI may be used as an *in vivo* probe for imaging P-gp- or ABCG2-expressing cancer cells in preclinical studies.

4.1.3 Dasatinib (Sprycel)—Similar to nilotinib, dasatinib is another second generation TKI that was developed for the treatment of imatinib-resistant CML. Dasatinib resembles nilotinib in that it is a substrate of both ABCB1 and ABCG2 (Giannoudis et al., 2008; Hiwase et al., 2008). In collaboration with Dr. Susan Bates and her colleagues at NCI, NIH, we demonstrated that ABCG2- and ABCB1-overexpressing K562 cells were resistant to treatment with imatinib, nilotinib, and dasatinib, indicating that all three drugs are substrates for ABCG2 and ABCB1 and that ABC transporter function in cells could cause inherent resistance to these TKIs (Dohse et al., 2010). It has also been shown very recently that penetration of dasatinib across the BBB is influenced by the presence of P-gp and ABCG2 and this uptake was significantly increased in the absence of P-gp and ABCG2 in *Abcb1 a/1b(-/-)* and *Abcg2(-/-)* mice (Chen et al., 2009; Lagas et al., 2009). The question of whether dasatinib, imatinib or nilotinib at higher concentrations still function as substrates or as inhibitory molecules is crucial for predicting the involvement of these transporters in the development of resistance to TKIs. The plasma levels of 4 μ M, 2 μ M, and 100 nM have been reported for imatinib, nilotinib, and dasatinib, respectively (Bradeen et al., 2006), the concentrations needed to inhibit ABC transporter proteins (as shown in Table 1) may thus be clinically achievable for these TKIs in patients.

4.1.4 Gefitinib (Iressa, ZD1839)—Gefitinib is the first selective inhibitor of epidermal growth factor receptors' (EGFRs) tyrosine kinase domain and was approved by the FDA in May, 2003 as mono-therapy for patients with locally advanced or metastatic NSCLC following failure of both platinum-based and docetaxel chemotherapies. The interaction of gefitinib with ABCG2 was reported by Ozvegy-Laczka et al. (Ozvegy-Laczka et al., 2004). It was then reported in independent studies that gefitinib can inhibit the function of ABCG2 and reverse resistance to 7-ethyl-10-hydroxycamptothecin (SN-38) in ABCG2-overexpressing cells. Gefitinib treatment increased the oral bioavailability of irinotecan in mice after simultaneous oral administration in both *in vitro* and *in vivo* studies (Lemos et al., 2009; Yanase et al., 2004). In addition, transduction of human epidermal carcinoma A431, human non-small cell lung cancer PC-9 and human colon cancer Caco-2 and adenocarcinoma WiDr cells with ABCG2 resulted in resistance to gefitinib (Lemos et al., 2009; Yanase et al., 2004). Another study found that the expression of functional ABCG2 protects A431 cells from the cytotoxic effects of gefitinib (Elkind et al., 2005). Yang et al. also reported that gefitinib reversed MDR mediated by both P-gp and ABCG2 (Yang et al., 2005). Recently, Agarwal et al. reported that distribution of gefitinib to the brain is limited by P-gp and ABCG2-mediated active efflux (Agarwal et al., 2010). Based on these studies, it can be extrapolated that gefitinib can potentially be used to modulate the oral bioavailability or to increase brain penetration of drugs that are substrates of ABC drug transporters and are either poorly absorbed or have poor brain penetration. A recent study demonstrated that gefitinib can be used to enhance topotecan penetration into gliomas (Carcaboso et al., 2010). Gefitinib at clinically administered doses achieves plasma concentrations of 1-2 μ M (Nakagawa et al., 2003), which is in the range for inhibiting Pgp and ABCG2 functions, therefore suggesting that expression of ABCG2 and P-gp may be an important determinant for gefitinib sensitivity in tumors and normal tissues.

4.1.5 Erlotinib (Tarceva, OSI-774)—Erlotinib is also an EGFR inhibitor that belongs to the same class as gefitinib and is used to treat non-small cell lung cancer, pancreatic cancer and several other types of cancer (Pal and Pegram, 2005). In our initial studies with this TKI, we found that it reversed vincristine and paclitaxel resistance in P-gp-overexpressing KB-C2 cells. Erlotinib also reversed mitoxantrone and SN-38 resistance in HEK293 cells that express ABCG2 (Shi et al., 2007a). In addition, it inhibited the transport of MTX and E₂17 β G in membrane vesicles prepared from cells transfected with wild-type ABCG2 (Shi et al., 2007a). Later, in *in vivo* studies done in *Abcb1 a/b(-/-)*, *Abcg2(-/-)*, *Abcb1 a/b(-/*

–) *Abcg2*(–/–), and *Abcc4*(–/–) mice, the Schellens and Stewart groups independently showed that both P-gp and *Abcg2* reduced the brain penetration of erlotinib and that the absence of P-gp and ABCG2 significantly affected the oral bioavailability of this drug in mice (Elmeliegy et al., 2011; Marchetti et al., 2008).

4.1.6 Lapatinib (Tykerb, GW572016)—Lapatinib is a reversible receptor TKI that inhibits both EGFR (erbB1/HER1) and HER2(erbB2) (Higa and Abraham, 2007). Its relatively low selective inhibition of EGFR may account for the broader spectrum of anticancer activity and improved efficacy. Lapatinib was approved by the FDA in combination with other anticancer agents for the treatment of HER2-positive breast cancers in March, 2007. We reported that lapatinib enhanced the cytotoxic effect of vincristine and paclitaxel by increasing the intracellular accumulation of these substrates in P-gp-overexpressing cell lines (Dai et al., 2008). In addition, lapatinib reversed ABCG2-mediated resistance to mitoxantrone and SN-38 (Dai et al., 2008). Lapatinib also strongly enhanced the inhibitory effect of paclitaxel on the growth of a cancer xenograft in nude mice that overexpressed P-gp (Dai et al., 2008). A synergistic interaction between lapatinib and chemotherapy agents due to its inhibition of ABCG2 activity in a panel of cell lines was then reported by Perry et al. (Perry et al., 2010). Molina et al. in *in vitro*, *in vivo* and clinical studies found that lapatinib increased the accumulation and cytotoxicity of topotecan in cells that overexpressed P-gp or ABCG2 (Molina et al., 2008). They also showed that this combination enhanced efficacy in their tumor xenografts and that the combination treatment was well tolerated in patients. Polli et al. in two reports then showed that lapatinib is a substrate of both P-gp and ABCG2, and both these transporters work in combination to modulate the CNS penetration of lapatinib in mice, resulting in a 40-fold increase in brain-to-plasma ratio in *Abcb1a/b*(–/–), *Abcg2*(–/–) mice compared with wild-type mice (Polli et al., 2008; Polli et al., 2009). The highest peak plasma levels of lapatinib and erlotinib were reported to be ~3 μM in human pharmacokinetic studies (Burris et al., 2005). This suggests that the concentrations used in *in vitro* laboratory experiments can be achieved in plasma in patients. Therefore, the concentrations used in the above studies are clinically relevant and it is possible that expression of ABC drug transporters influences the pharmacokinetic distribution of these drugs in patients and tumors.

4.1.7 Sunitinib (Sutent, SU11248)—Sunitinib malate is a small-molecule receptor TKI that inhibits the cellular signaling of multiple targets such as PDGFR and VEGFR. It is currently used in the treatment of renal cell carcinoma and imatinib-resistant GIST (Motzer et al., 2006). Similar to the TKIs mentioned above, sunitinib was found to inhibit the activity of P-gp and ABCG2 and was able to reverse P-gp-mediated and ABCG2-mediated drug resistance in cells (Dai et al., 2009; Shukla et al., 2009). Our results from *in vitro* studies were further confirmed by a recent *in vivo* study by Tang et al. in which they reported that brain accumulation of sunitinib is restricted by P-gp and ABCG2 and can be enhanced by oral elacridar and sunitinib co-administration (Tang et al., 2012). Recent studies by Hu et al. suggest that sunitinib does not appear to be a high-affinity substrate for P-gp or ABCG2 (Hu et al., 2009). Further experiments are required to determine whether sunitinib contributes to the modulation of clinical MDR mediated by P-gp and/or ABCG2.

5. Other recently reported TKIs

5.1 AG1478

The tyrphostin (tyrosine phosphorylation inhibitor) 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG1478) is a low molecular weight, reversible and selective inhibitor of EGFR (Levitzki and Gazit, 1995). It not only competes with ATP at the ATP-binding site of the kinase domain of EGFR, but also induces the formation of inactive, unphosphorylated

EGFR dimers in the presence and absence of a ligand (Arteaga et al., 1997). We have investigated the interaction of AG1478 with several ABC transporters including P-gp, ABCG2 and MRP1. AG1478 was found to modulate the sensitivity to anticancer drugs that are substrates for ABCB1 and ABCG2, as well as to increase intracellular accumulation of [³H]-paclitaxel, a substrate of P-gp, in cell lines overexpressing P-gp (Shi et al., 2009a; Shi et al., 2009b). However, AG1478 does not reverse MRP1-mediated MDR (Shi et al., 2009a; Shi et al., 2009b). Another study showed that AG1478 enhances the efficacy of radioimmunotherapy with ⁹⁰Y-CHX-A''-DTPA-hu3S193 in nude mouse xenografts comprised of A431 squamous carcinoma cells without any signs of acute toxicity (Lee et al., 2005). Whether AG1478 contributes to the reversal of clinical resistance mediated by ABC transporters remains to be determined. The determination of the modulatory effect of AG1478 in tumor xenograft models overexpressing P-gp or ABCG2 should help elucidate its importance in clinical MDR to cancer chemotherapy.

5.2 Canertinib (CI-1033)

Canertinib, a non-selective, irreversible inhibitor of pan-erbB, is active against all four HER (erbB) receptors. An *in vitro* study by Erlichman et al. showed that in cell lines overexpressing ABCG2, canertinib enhanced the cytotoxic effect of substrates for ABCG2, such as SN-38 and topotecan, by increasing the intracellular accumulation of these compounds (Erlichman et al., 2001a). In addition, they showed that canertinib was directly effluxed by the ABCG2 transporter (Erlichman et al., 2001a). These results suggest that ABCG2 may play an important role in clinical resistance to canertinib, and canertinib can modulate the effectiveness of anticancer drugs that are substrates of ABCG2. Additional studies need to be conducted to determine if canertinib is a modulator of P-gp and/or MRP1.

5.3 Cediranib (recentin, AZD2171)

Cediranib is an orally administered small-molecule TKI that targets VEGFR 1-3, PDGFR and a stem cell factor receptor, c-KIT (Wedge et al., 2005). This TKI was found to inhibit the ATPase activity of P-gp in a dose-dependent manner and it also sensitized P-gp- and MRP1-expressing cells to their respective substrate anticancer agents, suggesting that cediranib also may act as a modulator of P-gp and MRP1 (Tao et al., 2009).

5.4 Danusertib (PHA-739358)

Danusertib is a 3-aminopyrimidine derivative that potently inhibits aurora kinases as well as BCR-ABL kinase (Gontarewicz et al., 2008). Recently Balabanov et al. found that danusertib at very low concentrations was effective against BCR-ABL kinase, whereas higher concentrations were required for inhibition of aurora kinases (Balabanov et al., 2011). Interestingly, the danusertib-resistant cell lines did not show any mutations in kinases, but exhibited overexpression of ABCG2 (Balabanov et al., 2011). These authors further confirmed that overexpression of ABCG2 was indeed linked to development of resistance to this TKI. These data also suggest that danusertib is transported by ABCG2.

6. Exploiting TKIs as inhibitors of ABC transporters-a perspective

Based on our studies and other published work discussed above, TKIs are both substrates and inhibitors of ABC drug transporters, depending on the concentration of the inhibitor used and its affinity to the transporter. TKIs in general show substrate-like properties at lower concentrations but inhibit the function of the transporter at higher concentrations (Table 1). Therefore, the drug dosing schedule, systemic concentrations of the TKI, its affinity to ABC drug transporters and expression levels of these transporters in tumor cells can be important factors in clinical resistance to TKIs. These can eventually determine the potential of ABC transporters to confer resistance to TKIs in patients. Although a majority

of the research in the area of clinical drug resistance to TKIs has been focused on identifying mutations in the target regions of kinases, resistance due to the overexpression of ABC drug transporters can still be a challenge. This should be studied as an alternative mechanism by which tumor cells evade the cytotoxic effects of TKIs. Taking this into consideration, we propose that an ideal TKI would be a molecule that can inhibit the activity of the target TK but would not be a substrate or modulator for ABC drug transporters. Therefore, synthetic efforts and preclinical studies in the field should now be focused on developing such molecules. This will not only increase the probability of avoiding development of resistance to TKIs due to overexpression of ABC drug transporters, but would also lead to development of novel TKIs that kill tumor cells at much lower concentrations.

Another area of translational research that requires more *in vivo* studies is the use of TKIs as agents that can potentially modify the functional activity of ABC transporters. TKIs can possibly be exploited to improve pharmacokinetics or overcome resistance to chemotherapeutics that are substrates of ABC drug transporters in cells, tissues or tumors that express high levels of these transporters. As discussed above, a number of studies have already demonstrated that modulation of ABC transporters by inhibitors of these transporters in cancer chemotherapy may also significantly modify the pharmacokinetic and toxic profiles of anticancer drugs (Darvari and Boroujerdi, 2005; Sparreboom et al., 1999; ten Tije et al., 2003). This suggests that a combination of TKIs with cytotoxic anticancer drugs that are substrates of ABC transporters, especially P-gp and ABCG2, may offer an advantage for treating the drug-resistant cancers. Along these lines, pre-clinical studies and clinical trials are currently ongoing to evaluate the combination of anticancer drugs with EGFR TKIs to improve therapeutic outcome in cancer patients (Milano et al., 2008). It should also be mentioned that several studies have shown that the brain concentrations of TKIs such as imatinib (Breedveld et al., 2006; Breedveld et al., 2005; Kun-Eek et al., 2007), gefitinib (Heimberger et al., 2002), and lapatinib (Lagas et al., 2009; Polli et al., 2008) were relatively low. This could now be explained by the fact that these are substrates of P-gp and ABCG2, which are present abundantly at the BBB. These data imply that TKIs may have limited use for the treatment of patients with brain tumors. Based on the above strategy, the combination of known inhibitors of P-gp and/or ABCG2 with these TKIs may enhance their accumulation after repeated dosing (de Vries et al., 2006). Indeed, lapatinib and gefitinib have been shown in clinical studies to reduce CNS tumor growth (Geyer et al., 2006; Roggero et al., 2005). However, this approach may show toxic side effects as a disadvantage since TKIs, as inhibitors, will also affect the clearance of the drugs whose disposition is handled normally by ABC transporters. This may potentially result in increasing the frequency and severity of adverse effects. This is particularly important for normal tissues expressing a high level of ABCB1 and ABCG2, where the concentration and distribution of anticancer drugs might be altered.

Recently, single nucleotide polymorphisms (SNPs) in ABC drug transporters, specifically P-gp and ABCG2, have also been shown to influence the interactions of drugs with these transporters (Fung and Gottesman, 2009; Morisaki et al., 2005; Noguchi et al., 2009). This suggests that the effect of the TKIs may not only be influenced by the level of expression of ABCB1 and ABCG2, but also by the polymorphic variation of these pumps. One of the most common functional SNPs of ABCG2, Q141K (421C→A), was reported to be significantly associated with a high risk of diarrhea in patients treated with oral gefitinib (Cusatis et al., 2006) and to influence plasma imatinib concentrations in patients (Takahashi et al., 2010). The above study also indicated that this functional variant of ABCG2 was associated with higher steady-state accumulation, toxicity and antitumor activity of gefitinib (Li et al., 2007). In addition, with genome-wide association studies, this SNP in ABCG2 was linked to gout disease and subsequently shown to be defective in efflux of sodium urate, which is one of the factors associated with accumulation of urate in gout patients (Dehghan et al.,

Woodward et al., 2009). Therefore, both SNPs in ABC drug transporters and the concentrations of TKIs used in patients can significantly alter the pharmacokinetics and pharmacodynamics of its substrate drugs. For these reasons, the activity of ABC transporters should be an important consideration in the development of rationally targeted TKIs. This information may be useful for the early stages of drug development, which may lead to the discovery of more selective lead compounds and broaden the clinical use of TKIs. Further research into the interplay between TKIs and other transporters including the solute carriers (SLCs) is also warranted, as this finding provides unique insight into the development of cancer treatments with TKIs as well as an increased understanding of the role of ABC transporters in the disposition of drugs.

7. Conclusions

TKIs are a class of anticancer drugs that certainly offers an advantage over conventional chemotherapeutics, as they target a specific enzyme or molecule, the function of which is altered in a specific cancer subtype. In spite of the tremendous effort that has been made to develop new TKIs, the majority of these inhibitors do not inhibit a specific target with a reasonable level of selectivity. Moreover, their interactions with ABC drug efflux transporters and uptake transporters at the physiological barriers in the body present further challenges concerning potency and selectivity, thereby resulting in resistance or altered pharmacokinetics of these drugs. For TKIs that are actively transported by ABC drug transporters, it is possible to increase their bioavailability and efficacy by co-administering a non-toxic modulator of these transporters. We suggest that further research efforts in the development of novel TKIs with specific targets, seeking improved activity, should also consider these clinical challenges as the underlying causes of development of resistance to TKIs.

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Figure 1. Schematic representation of TKI interaction with TK and ABC drug transporters
 A TKI blocks the ATP-binding pocket of either receptor (present on the cell surface) or non-receptor (present in the cytoplasm) TK and prevents the downstream phosphorylation event, thereby inhibiting the activation of the kinases. On the other hand, the TKIs discussed in this review do not interact at the ATP-binding pocket of ABC drug transporters (present on the cell surface). Instead, they interact at the substrate-binding pocket of the transporter and some are pumped out of the cells by energy derived from ATP hydrolysis by ABC drug transporters (see Table 1), resulting in reduced intracellular concentration.

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Table 1

Comparison of effect of TKIs on ATP hydrolysis and IAAP labeling of P-gp and ABCG2

TKI	ATP hydrolysis (μM) ^a		IAAP labeling (μM) ^b		Reference
	P-gp	ABCG2	P-gp	ABCG2	
Imatinib *	0.47	0.12	3.18	0.47	(Burger et al., 2004; Shukla et al., 2008; Thomas et al., 2004)
Nilotinib *	0.19	0.008	0.18	0.012	(Brendel et al., 2007; Shukla et al., 2011)
Erlotinib	4.93	0.17	No effect	No effect	(Brendel et al., 2007; Shukla et al., 2011)
Lapatinib *	1.23	0.053	2.79	3.2	(Dai et al., 2008; Polli et al., 2008; Polli et al., 2009)
AG1478	3–5	0.2–0.3	No effect	No effect	(Elkind et al., 2005; Ozvegy-Laczka et al., 2004)
Gefitinib	4.0	0.1 ^c	ND	ND	(Elkind et al., 2005; Ozvegy-Laczka et al., 2004)
Apatinib	0.95	0.25	2.9	11.0	(Mi et al., 2010)
Dasatinib *	2.54	0.23	>20	3.01	(Dohse et al., 2010; Hiwase et al., 2008)
Sunitinib *	15.1	0.18	14.2	1.33	(Hu et al., 2009; Shukla et al., 2009)

ND, Not determined.

^aThe concentration required for 50% stimulation of ATP hydrolysis.^bThe concentration required for 50% inhibition (IC₅₀) of IAAP incorporation into the indicated transporter.^cMaximum 20–30% stimulation was observed.

* These TKIs have been shown to be transported by P-gp and ABCG2.