Molecularly-targeted anti-cancer drug interactions of the human ABCG2 multidrug transporter

Short PhD thesis

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Membrane Research Group of the Hungarian Academy of Sciences
Budapest, Hungary
2013

INTRODUCTION

The human ABCG2 transporter is a plasma membrane glycoprotein which belongs to the ABC (ATP Binding Cassette) protein superfamily. The ABCG2 protein is built from two structurally and functionally characteristic units. The N-terminal cytoplasmic nucleotide binding domain (NBD) is responsible for the binding and hydrolysis of ATP. The C-terminal transmembrane domain (TMD) consists of six membrane-spanning helices, and is involved in the recognition and handling of substrate molecules. ABCG2 works as a homodimer and it transports xenobiotics and intracellularly formed metabolites across membranes by harvesting the chemical energy released from the hydrolysis of ATP. ABCG2 is predominantly expressed in the plasma membrane, and is localized to the apical plasma membrane compartment in polarized cells. ABCG2 is highly expressed in placental syncytiotrophoblasts, in the epithelium of the small and large intestine and the colon, in biliary canaliculi, in the proximal tubule of the kidney, in alveolar pneumocytes of the lung, in breast tissue and in veinous and capillary endothelium. The physiological tissue distribution of ABCG2 strongly indicates that the transporter plays a crucial role in protecting the body and the fetus against xenobiotics. ABCG2 is also expressed in stem cells and is currently believed to maintain the undifferentiated state of stem cells and/or to contribute to their protection against endo- and exotoxins, various stress conditions and hypoxia. Overexpression of ABCG2 has been demonstrated in cancer cells of various origins, where, due to its protective function, it can confer cellular resistance against a wide range of chemically and target-wise unrelated drugs thus causing the emergence of the multidrug resistance (MDR) phenotype. Current studies described that the so-called side population (SP), which is defined by the function of ABCG2 and is characteristically enriched in stem cells, can be isolated not only from normal but from tumor tissues as well. The tumor-derived side population cells have been shown to recapitulate several properties of normal stem cells such as self-renewal or resistance to chemotherapeutics. Based on these findings, ABCG2 is considered to be a pivotal efflux transporter that contributes to preserving the 'cancer stem cell' sanctuary under chemotherapeutic pressure leading to the replenishment of the tumor.

Cancer cells often rely on unregulated kinase signaling, and small molecule kinase inhibitors are clinically applied to specifically target and eliminate kinase-addicted malignant cells. The constitutive tyrosine kinase activity of Bcr-Abl and Epidermal Growth Factor Receptor (EGFR) are required for the oncogenesis of chronic myeloid leukemia (CML) and various solid tumors, respectively. The multispecific ABCG2 transporter can interact with several small molecule kinase inhibitors. Being expressed at pharmacological tissue barriers and cancer cells, ABCG2 can modify both the pharmacokinetic properties and the targeted anti-tumor efficiencies of these anti-cancer agents. Here, we provided detailed biochemical characterization regarding the interaction of ABCG2 with clinically relevant small molecule inhibitors of Bcr-Abl and EGFR.

AIMS

Since at the very beginning of the *first project* presented in my thesis, data regarding the interaction of ABCG2 with the first developed and applied Bcr-Abl inhibitor imatinib were controversial, and no information was available about the interaction of ABCG2 and second generation inhibitors of Bcr-Abl, we aimed to

- investigate whether imatinib can interfere with the ATPase activity of human ABCG2,
- set up and characterize a Bcr-Abl+ K562 cellular model system showing stable overexpression of the human ABCG2 multidrug transporter,
- clarify whether the function of human ABCG2 can influence the intracellular effects of imatinib,
- by applying the Sf9 insect membrane- and Bcr-Abl+ K562 cell-based model systems which we validated by the analysis of the ABCG2-imatinib interaction, perform a comparative biochemical characterization on the interaction profile between human ABCG2 and the second generation Bcr-Abl inhibitors nilotinib, dasatinib and bosutinib.

In the *second project* presented in my thesis, using gefitinib as a well-characterized control, we set out to

- screen the potential interaction between ABCG2 and the second generation EGFR inhibitors vandetanib, pelitinib and neratinib using Sf9 insect membranes containing human ABCG2,
- explore whether ABCG2 influences the intracellular effects of vandetanib, pelitinib and neratinib in EGFR+ A431 cells showing stable overexpression of human ABCG2,
- study whether vandetanib, pelitinib and neratinib are capable of inhibiting the function of human ABCG2,
- investigate whether gefitinib exposure influences the expression of ABCG2,
- elucidate the role of the PI3K/Akt signaling axis in the rapid regulation of human ABCG2.

MATERIALS AND METHODS

Small molecule inhibitors of the Bcr-Abl and EGFR kinases were provided by VICHEM Chemie Ltd. Cell lines stably overexpressing human wild-type ABCG2 were generated by retroviral or transposon-based gene delivery in the laboratories of Drs. Katalin Német and Tamás Orbán. Total and plasma membrane expression of ABCG2 was checked by Western blot using the anti-ABCG2 BXP-21 antibody, and by flow cytometry using the anti-ABCG2 5D3 antibody which recognizes an extracellular epitope of the transporter, respectively. Specific ABCG2 function in the applied cell lines was tested by real-time fluorometric measurement of the intracellular accumulation of the Hoechst 33342 dye, which is a substrate of ABCG2. The specific ABCG2 inhibitor Fumitremorgin C (FTC) or its analogue Ko143 was used to specifically inhibit the transport function of ABCG2.

To express human wild-type ABCG2 in *Spodoptera frugiperda* (Sf9) insect cells, the Baculovirus expression vector system was used. Insect cell were incubated with the recombinant baculoviruses for 72 hours. Infected cells producing the ABCG2 protein were collected and following cholesterol-loading their membranes were isolated by ultracentrifugation. ABCG2 protein yields of the heterologous Sf9 expression system

were estimated by Western blot using the anti-ABCG2 BXP-21 antibody. The Sf9 membrane fraction containing the ABCG2 transporter was used to assess the modulatory effect of the investigated small molecule kinase inhibitors on the vanadate-sensitive ATPase activity of ABCG2. The ABCG2 ATPase was measured by determining the liberation of inorganic phosphate from ATP with a colorimetric reaction.

Cellular toxicity of the kinase inhibitors were compared in parental and ABCG2-expressing cells by staining with TOPRO-3 or MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) and subsequent flow cytometry or spectrophotometry measurements, respectively. Targeted effect of the investigated drugs was measured by following the phosphorylation status of their target or downstream kinases using phospho-specific antibodies and Western blot. Imatinib-induced erythroid differentiation was also compared in parental and ABCG2-expressing K562 cells by determining the hemoglobin-content of live cells by benzidine-staining. ABCG2-mediated intracellular accumulation of the Bcr-Abl inhibitors was determined by direct transport measurements followed by high pressure liquid chromatography-mass spectrometry (HPLC-MS) detection, in collaboration with the laboratory of Dr. Zoltán Takáts.

Effect of the applied kinase inhibitors on the conformation of the ABCG2 protein was determined by flow cytometry using the conformation sensitive anti-ABCG2 5D3 antibody that recognizes an extracellular epitope of the transporter. In each experiment, the specific ABCG2 inhibitor Ko143 was applied as a reference to achieve the ABCG2 conformation which shows maximum 5D3 binding affinity. ABCG2 inhibitory effect of the investigated drugs was measured in ABCG2-mediated Hoechst 33342 or mitoxantrone transport experiments followed by spectroflurometry or flow cytometry analysis, respectively.

Plasma membrane localization and subcellular distribution of ABCG2 was measured by flow cytometry in 5D3-labeled cells and by confocal microscopy using cells stably expressing the fluorescently tagged GFP-ABCG2 protein variant.

RESULTS

- We generated and characterized the K562 cell line stably overexpressing the human ABCG2 multidrug transporter.
- Due to their Bcr-Abl positivity, the CML-derived K562 cells are sensitive to the pharmacological inhibition of the Bcr-Abl kinase. Therefore, parental and ABCG2-expressing K562 cells could be applied to compare the intracellular toxic effect of the Bcr-Abl inhibitor imatinib. We showed that ABCG2 conferred cellular resistance to imatinib, which could be fully reversed by administration of the ABCG2 specific inhibitor Ko143. Moreover, we demonstrated that ABCG2 function prevented imatinib-induced erythroid differentiation in K562 cells. Our results strongly support the previous findings of others suggesting that imatinib is actively extruded by ABCG2.
- Applying the Bcr-Abl+ K562 model cells, we demonstrated that the cytotoxic action of the second generation Bcr-Abl inhibitors nilotinib and dasatinib, but not that of bosutinib was restricted by ABCG2.
- By following the phosphorylation status of Bcr-Abl, we showed that in K562/ABCG2 cells the Bcr-Abl inhibitors imatinib, nilotinib and dasatinib could not reach their intracellular target kinase and could not inhibit its autophosphorylation due to the specific function of ABCG2.
- Applying HPLC-MS detection based direct transport measurements, we proved that in contrast to bosutinib, ABCG2 actively transported nilotinib and dasatinib.
- We found that in EGFR+ A431 cells, ABCG2 conferred resistance to gefitinib and pelitinib, whereas intracellular action of vandetanib and neratinib was not restricted by the transporter.
- We demonstrated that the molecular basis of resistance in A431/ABCG2 cells was the highly phosphorylated EGFR even in the presence of gefitinib and pelitinib.
- We showed that at higher doses, all of the investigated Bcr-Abl and EGFR inhibitors blocked ABCG2 function and thereby promoted accumulation of the ABCG2 substrates Hoechst 33342 and mitoxantrone.

- We demonstrated that gefitinib exposure enhanced the cell surface expression of ABCG2, whereas inhibition of the EGFR/PI3K/Akt/mTOR signaling axis did not result in rapid internalization of the transporter.
- In the course of these studies, we also found that LY294002 and rapamycin, inhibitors of the PI3-kinase and mTOR kinase respectively, directly inhibit the function of ABCG2.

CONCLUSIONS

In summary, we found that the herein investigated small molecule Bcr-Abl or EGFR inhibitors all interact with ABCG2. If local drug concentrations are low, ABCG2 can confer imatinib, nilotinib, dasatinib, gefitinib or pelitinib resistance; however, anti-cancer efficiency of bosutinib, neratinib and vandetanib will most probably not be restricted by ABCG2. On the other hand, in ABCG2-expressing tissues where local drug concentrations are high, all of the investigated drugs can inhibit ABCG2 function and promote improved distribution, intracellular accumulation and cytotoxic action of simultaneously administered ABCG2 substrate chemotherapeutics, thereby reversing multidrug resistance. These phenomena might significantly influence *in vivo* Bcr-Abl or EGFR inhibitor treatment outcomes in case of kinase-addicted cancer cells or putative cancer stem cells with inherent or acquired ABCG2 expression. Our results therefore might provide useful for assessing the therapeutic applicability of the investigated drugs. Moreover, the finding that the investigated small molecule kinase inhibitors efficiently block ABCG2 function may help to design novel drug-combination therapeutic strategies.

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