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Interaction of the EGFR inhibitors gefitinib, vandetanib, pelitinib and neratinib with the ABCG2 multidrug transporter: Implications for the emergence and reversal of cancer drug resistance

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ABSTRACT

Human ABCG2 is a plasma membrane glycoprotein that provides physiological protection against xenobiotics. ABCG2 also significantly influences biodistribution of drugs through pharmacological tissue barriers and confers multidrug resistance to cancer cells. Moreover, ABCG2 is the molecular determinant of the side population that is characteristically enriched in normal and cancer stem cells. Numerous tumors depend on unregulated EGFR signaling, thus inhibition of this receptor by small molecular weight inhibitors such as gefitinib, and the novel second generation agents vandetanib, pelitinib and neratinib, is a promising therapeutic option. In the present study, we provide detailed biochemical characterization regarding the interaction of these EGFR inhibitors with ABCG2. We show that ABCG2 confers resistance to gefitinib and pelitinib, whereas the intracellular action of vandetanib and neratinib is unaltered by the presence of the transporter. At higher concentrations, however, all these EGFR inhibitors inhibit ABCG2 function, thereby promoting accumulation of ABCG2 substrate drugs. We also report enhanced expression of ABCG2 in gefitinib-resistant non-small cell lung cancer cells, suggesting potential clinical relevance of ABCG2 in acquired drug resistance. Since ABCG2 has important impact on both the pharmacological properties and anti-cancer efficiencies of drugs, our results regarding the novel EGFR inhibitors should provide useful information about their therapeutic applicability against ABCG2-expressing cancer cells depending on EGFR signaling. In addition, the finding that these EGFR inhibitors efficiently block ABCG2 function may help to design novel drug-combination therapeutic strategies.

1. Introduction

Human ABCG2 is a plasma membrane glycoprotein capable of eliminating xenobiotics from the cells by ATP-dependent active efflux. ABCG2 is a multispecific transporter, and its broad substrate spectrum includes several drugs currently used in anti-cancer therapy [1,2]. Being expressed at important tissue barriers, ABCG2 can influence the systemic distribution of orally administered anti-cancer agents [3]. ABCG2 was shown to be overexpressed in various cancer cells, and it can significantly reduce the intracellular efficiency of diverse structurally and target-wise unrelated anti-cancer molecules, resulting in the emergence of a multidrug resistance (MDR) phenotype [1,2]. ABCG2 is also the molecular marker of side population (SP) cells [4]. SP cells are present not

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only under physiological conditions, but could also be isolated from various hematological and solid tumors [5–8]. The SP of tumor tissues was shown to be enriched in cells recapitulating several properties of normal stem cells, such as self-renewal or resistance to chemotherapeutics [6,8–10]. According to the 'cancer stem cell' hypothesis, these drug-resistant cancer stem cells (CSC) are responsible for driving tumor re-growth, and ABCG2 is probably a pivotal efflux transporter contributing to preserving the CSC sanctuary under chemotherapeutic pressure [10].

Overly active signaling driven by the uncontrolled tyrosine kinase activity of the Epidermal Growth Factor Receptor (EGFR), a member of the EGFR/ErbB family of receptor tyrosine kinases, has been identified as a determinant factor of various cancer types, making EGFR a promising molecular cancer drug target [11–14]. Therapeutic inhibition of EGFR is achieved by ectodomain-binding monoclonal antibodies or small molecular weight inhibitors targeting the cytoplasmic kinase domain of the receptor [11,12]. The latter group includes gefitinib (Iressa/ZD1839) and the second generation inhibitors vandetanib (Zactima/ZD6474), pelitinib (EKB-569) and neratinib (HKI-272) [13,15]. Multi-kinase specificity or irreversible covalent binding to the target by these second generation inhibitors are both exploited to augment drug efficacy and target various types of solid tumors. Vandetanib is a potent inhibitor of vascular endothelial growth factor receptor 2 and 3 (VEGFR-2,3), and has additional inhibitory activity against EGFR [16]. Pelitinib, a chemical derivative of an irreversible EGFR inhibitor EKI-785 (which was previously shown to exhibit high affinity interaction with the ABCG2 transporter [17]), covalently binds to and inhibits EGFR [18]. Neratinib, another irreversible inhibitor of EGFR was synthesized on the chemical scaffold of pelitinib, and has additional inhibitory activity against another member of the EGFR/ErbB receptor tyrosine kinase family, HER-2 [19]. Gefitinib, vandetanib, pelitinib and neratinib are presently under clinical evaluation or use either as monotherapy or in combination for a histologically diverse range of tumors, including lung cancer [13], breast cancer [20–22] and colorectal cancer [23]. Expression of ABCG2 in human tumor samples [24,25], as well as presence of ABCG2-expressing cancer stem cells in cell lines or primary tumor samples of such origins [6,26,27] have also been reported.

In our present work, we provide a biochemical characterization of the interaction between ABCG2 and the small molecular weight EGFR inhibitors gefitinib, as well as three novel inhibitors, vandetanib, pelitinib and neratinib. Susceptibility of these anti-cancer drugs to ABCG2-mediated transport and their effect on ABCG2 function were investigated in detail. Given the above outlined potential impact of ABCG2 on both pharmacological properties and intracellular efficiency of anti-cancer drugs, our *in vitro* results provide useful information about the therapeutic applicability of these agents against ABCG2-expressing cancer cells and putative cancer stem cells of the relevant EGFR-dependent tumor types.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all reagents were obtained from Sigma–Aldrich (St. Louis, MO, USA). Small molecule EGFR inhibitors were synthesized and validated by VICHEM Chemie (Budapest, Hungary). Human recombinant EGF (hEGF) was purchased from Cell Signaling Technology (Danvers, MA, USA). Alexa-Fluor-conjugated antibodies, TOPRO-3 and DAPI were obtained from Invitrogen (Eugene, OR, USA). Ko143 was obtained from Tocris Bioscience (Bristol, UK). Phycoerythrin-conjugated secondary antibodies were purchased from Beckman Coulter (Brea, CA, USA).

2.2. Cell lines, cell growth and propagation

Stable human wild-type ABCG2-expressing derivatives of A431 and PLB985 (PLB) cells were characterized previously [17,28]. Gefitinib-resistant subclones of NCI-H1650 cells were generated earlier [29]. Cells were routinely cultured. ABCG2 expression and function was checked by immunostaining with the anti-ABCG2 5D3 antibody (kind gift of Brian P. Sorrentino) and flow cytometry [30], and Hoechst 33342 transport measurements [31], respectively.

2.3. Membrane ATPase measurements

ABCG2wt was expressed in insect *Spodoptera frugiperda* (Sf9) cells [32]. Cholesterol-loading of membranes was achieved as described previously [33]. Protein yields were estimated by Western blot using the anti-ABCG2 BXP-21 antibody (kind gift of Drs. George Scheffer and Rik Scheper) [31]. Vanadate-sensitive ATPase activity of ABCG2 was measured by determining the liberation of inorganic phosphate from ATP with a colorimetric reaction [31].

2.4. Cellular viability assay

Cellular viability was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) assay. Briefly, 4000 cells were seeded in 96-well plates. The following day, drugs were added and cells were further incubated for 72 h at 37 °C in 5% CO_2. To inhibit ABCG2, 5 μM FTC or 1 μM Ko143 was used. Following staining with MTT, the formed formazan crystals were solubilized and absorbance was measured at 540 nm using a Perkin Elmer Victor X3 2030 Multilabel Plate Reader. Experiments were performed in quadruplicates. IC50 values were determined using the Prism software (GraphPad Software, Inc.) by fitting curves using nonlinear least-squares regression in a sigmoidal dose-response model with variable slope.

2.5. Detection of protein phosphorylation

Kinase phosphorylation was analyzed by Western blot. Samples were prepared as described previously [34]. Briefly, cells were seeded in serum-free medium in 6-well plates and were let to adhere. Next day, following a 15 min-hEGF-stimulation and drug-treatment for additional 15 min, cells were washed and scraped in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA-Na, 1 mM EGTA-Na, 1% Nonidet-P40) containing various inhibitors of phosphatase and protease enzyme activities [34]. 20 μg proteins were analyzed by Western blot. Blots were probed with the p-EGFR (Tyr1068) antibody (Cell Signaling Technology, Danvers, MA, USA). Beta-actin was immunostained to check sample loading.

2.6. Immunodetection of ABCG2

Total ABCG2 protein levels were measured by Western blot using the anti-ABCG2 BXP-21 antibody [31].

Cell surface ABCG2 expression was followed using the anti-ABCG2 5D3 antibody [4,30]. For flow cytometry, 5D3 labeling was performed as described previously [30], using 1 μg/mL 5D3 or mouse IgG2b (isotype control) and 3 μg/mL phycoerythrin (PE)-conjugated secondary antibodies.

Effect of drugs on 5D3 binding affinity of ABCG2 [30] was investigated using the same labeling protocol, except that following a 5-min pre-incubation, drugs were present throughout labeling with 5D3 or IgG2b. TOPRO-3 staining was also performed to allow exclusion of dead cells.

For 5D3 labeling in confocal microscopy, 4×10^4 cells/well were seeded onto Lab-Tek II chambered coverglass (Nalge Nunc International, Rochester, NY, USA) and were grown for 48 h. After