Interactions of ABCG2 (BCRP) with epidermal growth factor receptor kinase inhibitors developed for molecular imaging

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The objective of this study was to investigate in vitro the interactions between novel epidermal growth factor receptor kinase inhibitors (EGFRIs) developed for positron emission tomography (PET) imaging and the major efflux transporter breast cancer resistance protein (BCRP/ABCG2). Seven compounds were evaluated, using the ATPase activity assays and Madin-Darby canine kidney (MDCK) cells overexpressing BCRP. Five of the tested compounds activated BCRP ATPase to various extent. Overexpression of BCRP conferred resistance to ML04, ML06, methoxy-Br-ML03, and PEG6-ML05 (IC50 values for inhibition of control cell proliferation 2.1 ± 0.6, 2.2 ± 0.7, 1.8 ± 1.2, and 2.8 ± 3.1 μM, respectively, compared to >50 μM in MDCK-BCRP cells). At submicromolar concentrations, none of the EGFRIs significantly inhibited BCRP. Immunoblotting studies indicated that BCRP expression is evident in cells utilized for in vivo tumor grafting in small animal PET imaging studies. Thus, the intensity of EGFRIs radioactivity signals previously observed in tumor xenografts reflects an interplay between transporter-mediated distribution of the probe into tumor cells and target binding. Concomitant use of efflux transporter inhibitors may help distinguish between the contribution of efflux transport and EGFR binding to the tissue signal.

Keywords: epidermal growth factor receptor, epidermal growth factor receptor kinase inhibitors, breast cancer resistance protein, P-glycoprotein, imaging, positron emission tomography

INTRODUCTION

Receptor tyrosine kinases play a key role in vital cellular functions, such as cell growth, differentiation, proliferation, and survival. Therefore, it is not surprising that enhanced activity of tyrosine kinases can lead to proliferative disease, including malignant tumors (Levitzki and Gazit, 1995; Levitzki and Mishani, 2006; Yarden and Pines, 2012). Accordingly, inhibition of hyperactive tyrosine kinase signaling pathways has emerged as a promising strategy for the treatment of cancer, and several TKIs have been approved by the FDA (Levitzki and Mishani, 2006; Mishani and Hagooly, 2009; Poot et al., 2013). Nevertheless, response rate is highly variable, in particular among patients treated with small-molecule TKIs directed against the EGFR (Poot et al., 2013).

The most established cause of secondary resistance is the occurrence of mutations in the catalytic domain of the kinase, which result in the binding of currently available TKIs (Levitzki and Mishani, 2006). In addition, primary or secondary resistance may result from insufficient intracellular drug concentrations, due to poor uptake (Wang et al., 2008; Engler et al., 2011; Mandery et al., 2012) or active efflux transport (Ozvegy-Laczka et al., 2005; Eadie et al., 2014). Two prominent transporters involved in drug efflux from cells are P-gp (multidrug resistance protein 1) and the BCRP (ABCG2), members of the ABC family of membrane transporters (Szakács et al., 2006). P-gp and BCRP are expressed in tissues involved in drug absorption and elimination (intestine, liver, and kidney) and in barriers to drug distribution, such as the blood–brain barrier and the placenta. Hence, they play a key role in the pharmacokinetics of substrate drugs (Eyal et al., 2009). These transporters have also been implicated in multidrug resistance due to active removal of chemotherapeutic agents from tumor cells. P-gp and BCRP substrates include TKIs, although the relative affinities and the nature of interaction with ABC transporters vary among these compounds (Eadie et al., 2014). For example, gefitinib is a BCRP transported substrate and inhibitor at low concentrations (Ozvegy-Laczka et al., 2004), whereas nilotinib appears to be a BCRP transported substrate at submicromolar concentrations and an inhibitor at micromolar concentrations (Eadie et al., 2014).

Due to the variability in EGFRIs pharmacokinetics and patient response, PET imaging with radiolabeled EGFRIs has emerged as an important tool in the development of these compounds (Levitzki and Mishani, 2006; Mishani and Hagooy, 2009; Slobbe et al., 2012). Initial PET studies with fluorine-18-labeled reversible EGFR inhibitors in tumor-bearing animals demonstrated relatively low uptake or fast clearance of the reversible EGFRIs from the tumor area, at least in part due to active efflux from tumor cells (Mishani et al., 2008; Slobbe et al., 2012). Nevertheless, the interaction of gefitinib (Kawamura et al., 2009) and sorafenib (Asakawa et al., 2011) with P-gp and BCRP has become the basis

Abbreviations: ABC, adenosine triphosphate binding cassette; BCRP, breast cancer resistance protein; EGFR, epidermal growth factor receptor; EGFRIs, epidermal growth factor receptor kinase inhibitors; FTC, fumitremorgin C; PET, positron emission tomography; P-gp, P-glycoprotein; TKI, tyrosine kinase inhibitor.
for their evaluation as PET probes of the functional activity of these transporters at the blood-brain barrier, in which they restrict drug distribution into the brain (Eyal et al., 2009).

In order to enhance treatment and imaging efficiency, irreversible EGFR kinase inhibitors were developed on the basis of 4-(phenylamino) quinazoline and quinoline core structures (Mishani et al., 2004, 2005, 2008; Shaul et al., 2004; Abourbeh et al., 2007; Dissoki et al., 2007; Mishani and Hagooly, 2009). However, the tumor uptake of some of these compounds was modest, and one of them, the irreversible EGFRi ML04, was found to be a P-gp substrate (Abourbeh et al., 2007).

Here, we evaluated in vitro the interactions of seven novel EGFRI developed as PET bioprobes with BCRP, in order to better understand the factors that affect their biodistribution. The evaluated compounds were ML04 (Mishani et al., 2004; Abourbeh et al., 2007); ML05 and PEG6-ML05 (Dissoki et al., 2007); ML06 (Shaul et al., 2004); methoxy-ML03 and N-[4-[(3-Bromo-phenyl)amino]-quinazoline-6-yl]-2-methoxyacetamide (“methoxy-Br-ML03”; Mishani et al., 2005); and ML10 (Mishani et al., 2008; Figure 1). The selected compounds represent those with high vs. low EGFR binding potency (e.g., IC50 = 0.05–5 nM, 10 nM, 30–45 nM, and 252 nM for ML06, ML04/ML05, PEG6-ML05, and methoxy-ML03, respectively, in A431 cell lysates; Shaul et al., 2004; Mishani et al., 2005; Dissoki et al., 2007) and various degrees of lipophilicity (e.g., Log P 3.9 for ML04, 4.12 for ML05, and 4.75 for PEG6-ML05; Dissoki et al., 2007).

MATERIALS AND METHODS

MATERIALS

The EGFRIs used in these experiments were kindly provided by Prof. Eyal Mishani (Department of Medical Biophysics and Nuclear Medicine, Hadassah-Hebrew University, Jerusalem, Israel). Gefitinib, the positive control, was purchased from Tocris Bioscience (Bristol, UK). BODIPY-prazosin was from Molecular Probes (Grand Island, NY, USA). The bicinchoninic acid (BCA) assay reagent kit was from Pierce (Rockford, IL, USA; Thermo Scientific). Skim milk was from Difco BD (Le Pont de Claix, France). Nitrocellulose membranes were from Whatman GmbH (Dassel, Germany). Cell culture reagents were from Biological Industries (Beit Haemek, Israel). All the other reagents were from Sigma–Aldrich (Rehovot, Israel).

CELL CULTURES

Madin-Darby canine kidney (MDCK) cells transfected with pcDNA empty vector (MDCK-pcDNA3; MDCK-CT) and cDNA coding for wild-type BCRP (MDCK-BCRP) cells were a generous gift from Prof. Qingcheng Mao (University of Washington, Seattle, WA, USA). The human lung carcinoma cell lines A549 and HCC827 cells were provided by Prof. Eyal Mishani.

Madin-Darby canine kidney vector (MDCK-pcDNA3) and MDCK-BCRP cells were grown in Eagle’s minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin, at 37°C and 5% CO2-humidified incubator. The BCRP positive cells were selected by supplementation of 0.05 mg/mL gentamicin to the growth medium. The adherent cells, continuously cultivated in 10 cm2 cell culture plates, were grown to nearly 80–90% confluence before they were harvested by trypsin-EDTA 0.25% solution. A549 were grown in Ham’s F12 growth medium and HCC827 were grown in RPMI-1640 growth medium. The cells were treated as described above.

ATPase ASSAY

ATPase activity was evaluated using PREDEASY kits (SOLVO Biotechnology, Szeged, Hungary), according to the manufacturer’s instructions.

WESTERN BLOT ANALYSIS

Western blot analysis was conducted as described before (Portnoy et al., 2012). Briefly, whole cell lysates were resuspended in 200 μl ice-cold lysis buffer and were shaken for 1 h at 4°C. Then, the lysate was centrifuged at 15,100 g for 15 min at 4°C. Protein concentrations were quantified by the BCA protein assay reagent kit according to the manufacturer’s instructions. Samples were run on a graded gel composed of a lower 10% separating gel layer and an upper 5% stacking gel layer. Gels were

![FIGURE 1 | Chemical structure of compounds investigated in this study.](image-url)
electrotransferred to nitrocellulose membranes and membranes were blocked in blocking buffer for 1 h at room temperature with gentle shaking, then washed three times with tris-buffered saline with tween 20 (TBST) for 5 min. Membranes were probed overnight at 4°C with BXP-21 BCRF antibody at 1:250 and anti β-actin 1:2500. The membranes were washed three times with TBST for 10 min at room temperature, then incubated with horse radish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies or goat anti-mouse IgG at 1:10000 for 1 h at room temperature. Following incubation, membranes were developed by enhanced chemiluminescence (ECL) detection and exposed to FUJI Medical Super RX X-ray films (Fujifilms, Tokyo, Japan).

RESULTS

INTERACTIONS OF THE EGFRIs WITH BCRP ATPase

Figure 2 demonstrates the effects of the tested EGFRIs on human BCRP ATPase activity in Sf9 membrane preparations. ML04, ML05, ML06, and the two methoxy derivatives of ML03 stimulated the BCRP ATPase, although activation did not exceed 40% of that produced by gefitinib (Figure 2A). PEG-6-ML05 and ML10 had no activating effect on ATPase activity. With the exception of methoxy-Br-ML03, all the compounds inhibited BCRP ATPase to various extent (Figure 2B).

EFFECTS OF BCRP OVEREXPRESS ON THE ANTI-PROLIFERATIVE ACTIVITY OF EGFRIs

Madin-Darby canine kidney II stable transfectants were used to assess the effect of human BCRP overexpression on the antiproliferative activities the EGFRIs (Figure 3). In a separate control group, 20 μM verapamil was used to block the effect of the endogenous, canine P-gp present in MDCK cells (Poller et al., 2011). Inhibition of cell proliferation by ML04, ML06, methoxy-Br-ML03, and PEG6-ML05 occurred at millimolar concentrations, and overexpression of BCRP conferred resistance to this effect (Figure 3). The other tested compounds did not affect the proliferation of MDCK-CT and MDCK-BCRP cells at concentrations up to 50 μM (data not shown).

EFFECT OF THE EGFRIs ON BODIPY-prazosin ACCUMULATION

Although some of the tested compounds partially inhibited BCRP ATPase activity, this was not reflected in the cellular assay (Figure 4). Only the positive control, FTC, significantly (p < 0.01) enhanced BODIPY-prazosin accumulation in MDCK-BCRP cells. None of the compounds significantly affected the fluorescent signal of MDCK-CT cells (data not shown).

DISCUSSION

The increasing need for personalized treatment with EGFRIs promoted the development of novel PET EGFR biomarkers. Indeed, encouraging results have been obtained through preclinical studies with both competitive and non-competitive EGFRIs. Yet, the determinants of probe distribution into tumors are not fully understood, because the specificity of tumor signal intensity may be affected by both the amount of the probe that distributes into tumor cells and its binding to its cellular targets. This study evaluated the interaction of several novel EGFR probes with BCRP, an ABC transporter that has been previously implicated in EGFRIs efflux (Ni et al., 2010). The BCRP ATPase and the BCRP inhibition assays were conducted at submicromolar concentrations, to reflect
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FIGURE 2 | Interactions of EGFRIs with BCRP ATPase. (A) Activation of BCRP-ATPase in Sf9 membrane preparations containing BCRP. Membranes were incubated with ATP and the tested compounds at 400 nM in the presence and absence of sodium orthovanadate. (B) Inhibition of sulfasalazine-stimulated BCRP-ATPase. The effect of EGFRIs was evaluated in sulfasalazine (10 μM)-stimulated membranes. Data are presented as means ± SD of the vanadate-sensitive ATPase activity from two experiments in duplicates.

FIGURE 3 | Effects of BCRP overexpression on the cytotoxicity of EGFRIs by the MTT assay. Stable MDCKII transfectants of vector alone or vectors expressing wild-type BCRP were incubated for 72 h with various concentrations of the indicated compounds. Results are shown as experimental findings (circles) and predicted model (line) in MDCK-CT cells (red), MDCK-BCRP cells (black), and MDCK-BCRP cells treated with 20 μM verapamil, to block endogenous P-gp activity (green). Data represent the means ± SD of six replicates.

clinically relevant, unbound EGFRIs plasma levels. This is particularly the case in PET imaging studies, in which ligand microdoses may be used.

EGFRIs INTERACTIONS WITH BCRP

Five of the tested compounds, namely ML04, ML05, ML06, methoxy-ML03, and methoxy-Br-ML03, activated BCRP ATPase at submicromolar concentrations, suggesting that these compounds are transported by BCRP. The values obtained with ML10 and PEG6-ML05 could result from inhibition of basal Pi release (manufacturer’s brochure). A partial overlap observed between ATPase stimulation and inhibition indicated that the evaluated compounds may function as competitive inhibitors which are also transported substrates (Eadie et al., 2014).

Compared to the relatively low concentrations which stimulated the BCRP ATPase, greater EGFRIs concentrations were required to affect the proliferation of MDCK-CT cells. This reflects both the distributional barrier into cells and the degree sensitivity to the toxic effects...
Our study could have been strengthened by using the efflux ratio assay to distinguish between inhibitors and substrates and to gain a better understanding of the mechanisms that control the distribution of the compounds of interest into cells. We used the ATPase assay due to its simplicity, and subsequently evaluated the cellular effects of the TKIs as a marker of their BCRP-mediated transport. However, future characterization of these compounds will involve the use of bidirectional transport assays.

Due to the relatively small number of molecules evaluated in our study, it is difficult to assess the impact of structural modifications on the molecule interactions with BCRP. Furthermore, BCRP is predicted to have multiple binding sites with overlapping specificities (Ni et al., 2010). Thus, the interactions of substrates with the transporter cannot be precisely identified. It has been suggested that substrate recognition is based on global physiochemical properties, such as carbon size length and lipophilicity, although the importance of log P has been debated (Ni et al., 2010; Szafraniec et al., 2014). A more sophisticated structure activity relationship study with SN-38 and its analogs suggested that one amine bound to one carbon of a heterocyclic ring, fused heterocyclic rings, and two substituents on a carbocyclic ring of the fused heterocyclic are important factors in the molecule’s interaction with BCRP (Saito et al., 2006). That study also predicted a strong interaction of gefitinib with BCRP based on these features. Chemical modifications such as those aimed to achieve irreversible EGFR binding may reduce the potency of the EGFRi in binding BCRP. This is supported by the results obtained in the ATPase activation assay (Figure 2A). For example, PEGylation of ML04 and ML05, aimed at reducing their log P with resultant lower non-specific binding (Dissoki et al., 2007; Mishani et al., 2008; Mishani and Hagooly, 2009), could have affected the recognition of these compounds by both BCRP (Figure 2) and P-gp. Nevertheless, no trend was observed with regard to the relationships between EGFR inhibition potency or lipophilicity of the compound and its interactions with BCRP.
TUMOR CELLS EXPRESSION OF P-gp AND BCRP
Preclinical PET and biodistribution studies in animal bearing tumor xenografts demonstrated high and sustained uptake of 11C-erlotinib in HCC827, which harbor an activating mutation in exon 19, compared to A549 and NC1358 tumors (Memon et al., 2009). In order to better understand the impact of BCRP on the distribution of the studied compounds in experimental tumor models, we also examined BCRP expression in HCC827 and A549 cells. In both cell lines, BCRP expression was comparable to that observed in the positive control, MDCK-BCRP. Therefore, it appears that the irreversibly binding of the EGFRIs to its intracellular target could potentially contribute to overcoming the distributional restriction, at least in part, due to relatively longer residence within cells.

CONCLUSION
Our data suggest that some of the EGFRIs evaluated in this study interact with BCRP. Whether the compound functions as a transported substrate or an inhibitor may depend on its concentrations, the cell type and the experimental or clinical setting. Accordingly, low radioligand concentrations, in contrast to concentrations aimed to clinically inhibit EGFRIs, could have resulted in a more pronounced BCRP/P-gp efflux and may be one explanation for the failure of some successful targeted EGFRIs as tracer imaging agents (Mishani et al., 2008). Therefore, using a mixture of radiolabeled tracer and a "cold" compound to reflect clinically relevant concentrations might improve the outcome of the preclinical imaging studies.

The relative role of transporter-mediated efflux vs. target binding in probe accumulation within tumors is currently unknown. To address this issue, the activity of novel compounds developed as EGFR probes can be tested in vitro in the presence and the absence of ABC transporters inhibitors. Likewise, given the expression of BCRP and P-gp in cell lines commonly used for tumor grafting in small animals, in vivo studies conducted in the presence and the absence of transporter inhibitors may help distinguish between pharmacokinetic and pharmacodynamic contribution to the radioactivity signal within tumors. Those radioligands which are found to be good P-gp/BCRP substrates and do not avidly bind EGFRIs may be utilized for in vivo imaging studies of P-gp/BCRP functional activity at the blood brain barrier. Importantly, further interactions with other efflux transporters (multidrug resistance-associated proteins; MRPs) and relevant uptake transporters should be investigated in order to better understand the biodistribution of these probes.

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