Hepatocyte-specific deletion of EGFR in mice reduces hepatic Abcg2 transport activity measured with $[11\text{C}]$erlotinib and positron emission tomography

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ABC, adenosine triphosphate-binding cassette

ABCG2, human ABC subfamily G member 2, also known as breast cancer resistance protein

Abcg2, rodent ABC subfamily G member 2, also known as breast cancer resistance protein

ABCB1, human ABC subfamily B member 1, also known as P-glycoprotein

Abcb1a/b, rodent ABC subfamily B member 1A and 1B, also known as P-glycoprotein

kBile, rate constant for biliary excretion of radioactivity

kuptake,kidney, rate constant for transfer of radioactivity from blood into kidney

kuptake,liver, rate constant for transfer of radioactivity from blood into liver

kurine, rate constant for urinary excretion of radioactivity

DDI, drug-drug interaction

EGFR, epidermal growth factor receptor

EGFRhep, transgenic mice lacking EGFR in hepatocytes

EGFRfl/fl, transgenic mice harboring a loxP-flanked EGFR allele

PET, positron emission tomography

TKI, tyrosine kinase inhibitor
Abstract

The epidermal growth factor receptor (EGFR) regulates cellular expression levels of breast cancer resistance protein (humans: ABCG2, rodents: Abcg2) via its downstream signaling pathways. Drugs that inhibit EGFR signaling (e.g. tyrosine kinase inhibitors, antibodies) may lead to ABCG2-mediated drug-drug interactions (DDIs) by changing disposition of concomitantly administered ABCG2 substrate drugs. In this study we used positron emission tomography (PET)/magnetic resonance (MR) imaging to compare disposition of the model Abcg2 substrate [\( ^{11}\text{C} \)]erlotinib in a mouse model of hepatocyte-specific deletion of EGFR (EGFR\(_{\text{\Delta}}\)hep mice, \( n = 5 \)) with EGFR\(_{\text{fl/fl}}\) control mice (\( n = 6 \)), which have normal EGFR expression levels in all tissues. Integration plot analysis was used to estimate the rate constants for transfer of radioactivity from liver into bile (\( k_{\text{bile}} \)) and from kidney into urine (\( k_{\text{urine}} \)). EGFR\(_{\text{\Delta}}\)hep mice showed significantly lower radioactivity concentrations in the intestine (1.6-fold) and higher radioactivity concentrations in the urinary bladder (3.2-fold) as compared to EGFR\(_{\text{fl/fl}}\) mice. \( k_{\text{bile}} \) was significantly decreased (3.0-fold) in EGFR\(_{\text{\Delta}}\)hep mice, whereas \( k_{\text{urine}} \) was by 2.2-fold increased. Western blot analysis of liver tissue confirmed deletion of EGFR and showed significant decreases in Abcg2 and increases in P-glycoprotein (Abcb1a/b) expression levels in EGFR\(_{\text{\Delta}}\)hep vs. EGFR\(_{\text{fl/fl}}\) mice. Our data show that EGFR deletion in hepatocytes leads to a reduction in Abcg2-mediated hepatobiliary clearance of a probe substrate accompanied by a shift to renal excretion of the drug, which raises the possibility that EGFR-inhibiting drugs may cause ABCG2-mediated DDIs.
Introduction

The adenosine triphosphate (ATP)-binding cassette (ABC) transporter breast cancer resistance protein (ABC subfamily G member 2, humans: ABCG2, rodents: Abcg2) influences drug disposition as it recognizes diverse drugs and drug metabolites as its substrates (e.g. rosuvastatin, sulfasalazine, mitoxantrone, topotecan, prazosin, dantrolene, ciprofloxacin, erlotinib, gefitinib) (Lee et al., 2015; Mao and Unadkat, 2015). ABCG2 is expressed in the canalicular (bile-facing) membrane of hepatocytes and in the brush border membrane of kidney proximal tubule cells, where it promotes hepatobiliary and urinary excretion of drugs or drug metabolites. ABCG2 also limits oral absorption of drugs in the small intestine and restricts brain distribution of drugs, often in concert with P-glycoprotein (ABC subfamily B member 1, humans: ABCB1, rodents: Abcb1a/b), in brain capillary endothelial cells forming the blood-brain barrier. Alterations in ABCG2 transport activity due to genetic polymorphisms or drug-drug interactions (DDIs) may lead to changes in the disposition of ABCG2 substrates, which may affect drug safety and efficacy. So far, ABCG2-mediated DDIs have been mainly studied in the intestine (Kruijtzer et al., 2002; Allred et al., 2011; Kusuhara et al., 2012; Custodio et al., 2014) and considerably less information is available with respect to DDIs leading to changes in the hepatobiliary excretion of drugs. This is most likely related to the fact that intestinal DDIs can be assessed by studying drug plasma pharmacokinetics whereas hepatic ABCG2-mediated DDIs may only lead to changes in drug concentrations in the liver and intestine without pronounced changes in drug plasma pharmacokinetics. For a better understanding of tissue DDIs non-invasive positron emission tomography (PET) imaging with radiolabeled drugs has been proposed as a useful tool (Kusuhara, 2013; Langer, 2016).

Apart from direct inhibition of ABCG2, certain drugs may interfere with regulatory pathways controlling transporter expression in tissue, which could also give rise to transporter-mediated DDIs. It has for instance been shown in vitro using different cell lines that the epidermal growth factor receptor (EGFR) regulates cellular ABCG2 expression levels via its downstream signaling pathways (i.e. phosphatidyl-inositol 3-kinase (PI3K)/Akt, mitogen-activated protein kinase (MAPK)) (Takada et al., 2005; Meyer zu Schwabedissen et al., 2006; Pick and Wiese,
2012; Porcelli et al., 2014) (Fig. 1). EGFR-targeting tyrosine kinase inhibitors (TKIs) (e.g. gefitinib, erlotinib, afatinib, osimertinib), which are frequently used in the clinic for cancer treatment, inhibit EGFR-signaling pathways and may therefore cause a reduction in tissue ABCG2 expression levels, which could affect disposition of concomitantly administered ABCG2-substrate drugs. However, in vivo data on EGFR-mediated regulation of tissue ABCG2 expression levels are scarce. A possible confounding factor in the study of the influence of EGFR inhibition on ABCG2-mediated drug disposition may be the fact that most TKIs also directly inhibit ABCG2 transport activity (Hegedüs et al., 2012; D'Cunha et al., 2016). Recently, transgenic mouse models with cell type-specific hepatic deletion of EGFR have been described (Natarajan et al., 2007; Lanaya et al., 2014). Such mouse models lend themselves to studying in vivo the regulation of ABCG2 by EGFR in a “non-pharmacological” approach. A previous PET study has shown that hepatobiliary excretion of [11C]erlotinib is mediated in mice by Abcg2 (Traxl et al., 2015).

In the present study, we used [11C]erlotinib as a model Abcg2 substrate and compared its hepatic disposition, using PET and magnetic resonance (MR) imaging, in EGFR\textsuperscript{hep} mice, which specifically lack EGFR in hepatocytes, with EGFR\textsuperscript{fl/fl} mice, which have normal EGFR expression in all tissues. We hypothesized that deletion of EGFR in hepatocytes will lead to reduced hepatobiliary excretion of [11C]erlotinib due to a downregulation of hepatic Abcg2.
Materials and Methods

Chemicals and drugs

Unless otherwise stated, all chemicals were obtained from Merck (Darmstadt, Germany) or Sigma-Aldrich (St. Louis, MO, USA). All chemicals were used without further purification and were of at least analytical grade.

Animals

Healthy male EGFR\textsuperscript{\textasciitilde{hep}} (lacking EGFR in hepatocytes of the liver, but not in other tissues) and EGFR\textsuperscript{fl/fl} (harboring a loxP-flanked EGFR allele, having normal EGFR expression in all tissues) littermate control mice with a C57BL/6 background were generated as described previously (Natarajan et al., 2007) and bred in the mouse facilities of the Medical University of Vienna (Division of Decentralized Biomedical Research Facilities) according to federal guidelines and guidelines of the Medical University of Vienna (BMFW-66.009/0199-WF/II/3b/2014). At the time of experiment, animals weighed 22-33 g (EGFR\textsuperscript{fl/fl}: 31.2 ± 1.6 g, EGFR\textsuperscript{\textasciitilde{hep}}: 25.7 ± 3.3 g) and were 13-15 weeks old. Animals were housed in individual ventilated polysulfon type III cages (5-6 animals per cage) under controlled environmental conditions (24.0 ± 0.6°C, 40-70% humidity, 12 hour light/dark cycle) with free access to tap water and standard rodent diet (Ssniff Spezialdiäten, Soest, Germany). An acclimatization period of at least one week was allowed before the animals were used in the experiments. All animal experiments were approved by the national authorities (Amt der Niederösterreichischen Landesregierung) and all study procedures were performed in accordance with the European Communities Council Directive of September 22, 2010 (2010/63/EU).

Radiotracer synthesis

\[^{11}\text{C}\]Erlotinib was synthesized by \(O-[^{11}\text{C}]\)methylation of 6-O-desmethyl-erlotinib (OSI-420, Syncom BV, Groningen, The Netherlands) as described previously (Bahce et al., 2013). Specific activity at the time of injection was 74.8 ± 35.2 GBq/µmol (\(n = 11\) batches) and radiochemical purity was >98%. For intravenous (i.v.) administration to animals, \[^{11}\text{C}\]erlotinib
was formulated in 0.1 mM hydrochloric acid in physiological saline at an approximate concentration of 555 MBq/mL.

**PET/MR imaging**

EGFR<sup>fl/fl</sup> (n = 6) and EGFR<sup>Δ</sup>hep (n = 5) mice each underwent a MR scan followed by a dynamic PET scan after i.v. injection of [11C]erlotinib. For imaging, animals were pre-anesthetized in an induction chamber using isoflurane (Abbott Laboratories, Maidenhead, UK), placed on a heated (37°C) animal bed and the lateral tail vein was cannulated. Body temperature and respiration rate were constantly monitored (SA Instruments, Stony Brook, NY, USA). Isoflurane anesthesia (1.5-2.5% in oxygen) and heating were maintained for the entire imaging period. Anatomical whole-body MR imaging was performed on a 1 Tesla benchtop ICON™ scanner (Bruker, Ettlingen, Germany) using a modified T<sub>1</sub>-weighted gradient echo sequence (T<sub>1</sub>-fast low angle shot) with the following parameters: echo time = 5 ms; repetition time = 25 ms; flip angle = 25°; field of view = 76 x 28 x 24 mm; matrix = 253 x 93; 32 slices; slice thickness = 0.75 mm; scan time = 6.25 minutes. For PET imaging, the animal bed was transferred into the gantry of a microPET Focus™ 220 system (Siemens Medical Solutions, Knoxville, TN, USA) and a 10-minute transmission scan using a <sup>57</sup>Co point source was recorded. Subsequently, [11C]erlotinib (46.9 ± 14.6 MBq, corresponding to 0.47 ± 0.54 μg (range: 0.07 μg - 2.05 μg) of unlabeled erlotinib, n = 11 batches) was administered as an i.v. bolus over 1 minute via the lateral tail vein and a 90-minute dynamic PET scan was initiated at the start of radiotracer injection with an energy window of 250-750 keV and a timing window of 6 nanoseconds.

**Postimaging procedures**

At the end of the PET scan, a terminal blood sample was withdrawn from the retro-orbital sinus vein and the animals were sacrificed by cervical dislocation while still under deep anesthesia. The liver was removed and radioactivity of blood aliquots was measured in a Wallac Wizard 1470 gamma-counter (PerkinElmer, Wellesley, MA, USA). For protein extraction, total liver tissue was homogenized using a Precellys® CK28 Lysing Kit (Bertin, Rockville, MD, USA)
containing the respective lysis buffer (for total cell extracts: RIPA buffer containing 50 mM Tris, pH 8.0, 150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS; for membrane-enriched cell extracts: 50 mM HEPES, pH 7.3, 150 mM sodium chloride, 10% glycerol, 1% Triton X-100, 1 mM EDTA and 1.5 mM magnesium chloride). The tissue was lysed using a Precellys® Evolution Homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France). Cell extracts were snap-frozen in liquid nitrogen and stored at -80°C until further processing.

**Western blotting**

For Western blotting, frozen extracts were thawed and centrifuged at 12,000 rpm at 4°C for 10 minutes. The protein concentration of the cleared lysate was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). 15 µg of lysed protein was re-suspended in denaturing protein-loading buffer and left at room temperature for 10 minutes. Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. To determine EGFR, Abcg2 and Abcb1a/b protein expression levels, the following monoclonal rabbit antibodies were used in 1:1000 dilutions: EGFR (EGF Receptor (D38B1) XP® Rabbit mAb #4267), Abcg2 (ABCG2 (D5V2K) XP® Rabbit mAb #42078) and Abcb1a/b (Anti-P Glycoprotein antibody [EPR10364-57] (ab170904)). α-Tubulin (Monoclonal Anti-α-Tubulin antibody produced in mouse) was used as loading control in a 1:500 dilution. For analysis of protein expression levels in liver tissue, all obtained bands were quantified by densitometry using ImageJ software (NIH, Bethesda, MD, USA) and normalized to α-Tubulin.

**PET data analysis**

The dynamic PET data were sorted into 25 frames, which incrementally increased in time length from 5 seconds to 20 minutes. All PET images were reconstructed using Fourier rebinning of the three-dimensional sinograms followed by a two-dimensional filtered back-projection with a ramp filter resulting in a voxel size of 0.4 x 0.4 x 0.796 mm³. The standard data correction protocol was applied to the dynamic PET data, including normalization,
injection decay correction and attenuation correction. By applying a calibration factor derived from imaging a cylindrical phantom with a known radioactivity concentration, PET units were converted into units of radioactivity. Using the medical image data examiner software AMIDE (Loening and Gambhir, 2003), left ventricle of the heart (image-derived blood curve), liver, gall bladder, duodenum, intestine, left kidney and urinary bladder were manually outlined on co-registered PET/MR images and concentration-time curves expressed in units of percent of the injected dose per milliliter or per gram (%ID/mL or %ID/g) were derived. It was assumed that the sum of radioactivity in the gallbladder, the duodenum and the intestine represented radioactivity in the bile excreted from the liver. From the concentration-time curves, the area under the curve (AUC) from 0-90 minutes was calculated using Prism 7 software (GraphPad Software, La Jolla, CA, USA).

Integration plot analysis

To estimate the rate constants for transfer of radioactivity from blood into liver ($k_{uptake,liver}$, mL/min/g liver), from blood into kidney ($k_{uptake,kidney}$, mL/min/g kidney), from liver into bile ($k_{bile}$, min$^{-1}$) and from kidney into urine ($k_{urine}$, min$^{-1}$), a graphical analysis method (integration plot) was used as described previously (Shingaki et al., 2015; Traxl et al., 2015). $k_{uptake,liver}$ and $k_{uptake,kidney}$ were measured from 0.3-3.5 minutes after radiotracer injection using the integration plot method and the following equation:

$$\frac{C_{t,organ}}{C_{t,blood}} = k_{uptake,organ} \times \frac{AUC_{0-t,blood}}{C_{t,blood}} + V_e$$

where $C_{t,organ}$ is the radioactivity concentration in liver or kidney at time $t$ and $C_{t,blood}$ is the radioactivity concentration in the left ventricle of the heart at time $t$. $AUC_{0-t,blood}$ represents the area under the concentration-time curve in the left ventricle of the heart from time 0 to time $t$. $k_{uptake,organ}$ can be obtained by performing linear regression analysis of a plot of $C_{t,organ}/C_{t,blood}$.
versus $AUC_{0-t, \text{blood}}/C_{t, \text{blood}}$ and calculating the slope of the regression line. $V_E$ is the y-intercept of the integration plot.

$K_{\text{bile}}$ and $K_{\text{urine}}$ were measured from 12.5-65 minutes after radiotracer injection using the integration plot method and the following equation:

$$C_{t, \text{organ}} = k_{\text{fluid}} \times AUC_{0-t, \text{organ}} + V_E$$

where $C_{t, \text{organ}}$ is the radioactivity concentration in the intestine (including gall bladder and duodenum) or urinary bladder at time $t$. $AUC_{0-t, \text{organ}}$ represents the area under the concentration-time curve in the liver or kidney from time 0 to time $t$. $K_{\text{fluid}}$ ($K_{\text{bile}}$ or $K_{\text{urine}}$) can be obtained by performing linear regression analysis of a plot of $C_{t, \text{organ}}$ versus $AUC_{0-t, \text{organ}}$ and calculating the slope of the regression line. $V_E$ is the y-intercept of the integration plot.

**Statistical analysis**

All values are given as mean ± standard deviation (SD). Statistical differences between groups were analyzed by two-tailed unpaired t-tests using Prism 7 software. To assess correlations, the Pearson correlation coefficient ($r$) was calculated. The level of statistical significance was set to a $P$ value of less than 0.05.
Results

[¹ⁱC]Erlotinib disposition in EGFR\textsuperscript{fl/fl} and EGFR\textsuperscript{\Delta hep} mice

We acquired [¹¹C]erlotinib PET/MR scans in EGFR\textsuperscript{fl/fl} mice which have normal EGFR expression in all tissues and in EGFR\textsuperscript{\Delta hep} mice in which EGFR was deleted in hepatocytes but not in other tissues. Representative co-registered coronal whole-body PET summation and MR images are shown in Fig. 2, indicating lower radioactivity content in the duodenum and intestine and higher radioactivity content in the urinary bladder of EGFR\textsuperscript{\Delta hep} mice as compared to EGFR\textsuperscript{fl/fl} mice. In Fig. 3 and 4, concentration-time curves and AUCs of [¹¹C]erlotinib in blood, liver and intestine are shown for both mouse models. Blood radioactivity concentrations were significantly, by 1.5-fold, higher in EGFR\textsuperscript{\Delta hep} than in EGFR\textsuperscript{fl/fl} mice (blood AUC, EGFR\textsuperscript{fl/fl}: 263 ± 48 %ID/mL*min, EGFR\textsuperscript{\Delta hep}: 397 ± 93 %ID/mL*min). Also liver AUCs were significantly, by 1.4-fold, increased in EGFR\textsuperscript{\Delta hep} as compared to EGFR\textsuperscript{fl/fl} mice, but liver-to-blood AUC ratios were not significantly different between the two mouse models (Table 1). Radioactivity concentrations in the intestine (including gall bladder and duodenum) were significantly, by 1.6-fold, lower in EGFR\textsuperscript{\Delta hep} mice (intestine AUC, EGFR\textsuperscript{fl/fl}: 1,340 ± 368 %ID/g*min, EGFR\textsuperscript{\Delta hep}: 866 ± 86 %ID/g*min).

To quantitatively evaluate hepatic disposition of [¹¹C]erlotinib, we employed a graphical analysis approach (integration plot analysis) using an image-derived blood curve to estimate the rate constants for transfer of radioactivity from blood into liver ($k_{\text{uptake,liver}}$) and from liver via bile into duodenum and intestine ($k_{\text{bile}}$). Radioactivity concentrations measured in the left ventricle of the heart in the last PET frame (70-90 minutes after radiotracer injection) showed a good correlation ($r = 0.735$, $P < 0.05$) with radioactivity concentrations measured with a gamma-counter in the venous blood sample collected at the end of the PET scan (data not shown). In Fig. 5, integration plots and $k_{\text{uptake,liver}}$ and $k_{\text{bile}}$ values are shown for both mouse models. $k_{\text{uptake,liver}}$ was not significantly different, whereas $k_{\text{bile}}$ was significantly, by 3.0-fold, decreased in EGFR\textsuperscript{\Delta hep} compared to EGFR\textsuperscript{fl/fl} mice (Table 1).

We also studied distribution of [¹¹C]erlotinib to the kidney and urinary bladder and estimated the rate constants for transfer of radioactivity from blood into kidney ($k_{\text{uptake,kidney}}$) and from
kidney into urine ($k_{\text{urine}}$) (Fig. 6 and Table 1). Radioactivity concentrations in the urinary bladder were significantly, by 3.2-fold, higher in EGFR$^{\Delta \text{hep}}$ mice (urinary bladder AUC, EGFR$^{\text{fl/fl}}$: 683 ± 441 %ID/g*min, EGFR$^{\Delta \text{hep}}$: 2,171 ± 866 %ID/g*min). $k_{\text{uptake, kidney}}$ values did not differ between the two mouse models, whereas $k_{\text{urine}}$ was significantly, by 2.2-fold, higher in EGFR$^{\Delta \text{hep}}$ mice.

**EGFR, Abcb1a/b and Abcg2 protein expression levels in liver tissue**

Western blot analysis of liver tissue collected at the end of PET imaging confirmed almost complete absence of EGFR protein expression levels in total cell extracts of EGFR$^{\Delta \text{hep}}$ mice as compared to EGFR$^{\text{fl/fl}}$ mice (on average 16.2-fold reduction, $P < 0.05$) (Fig. 7A,B). The residual, low EGFR signal in EGFR$^{\Delta \text{hep}}$ mice most likely derives from non-parenchymal cells, in which EGFR was not deleted. In EGFR$^{\Delta \text{hep}}$ mice, Abcg2 protein expression levels were significantly lower in total (on average 2.9-fold) as well as in membrane-enriched (on average 2.5-fold) cell extracts compared to EGFR$^{\text{fl/fl}}$ mice (Fig. 7A,D), suggesting that the absence of EGFR does not affect the localization of the Abcg2 protein. In contrast, Abcb1a/b protein expression levels in the liver of EGFR$^{\Delta \text{hep}}$ mice were significantly increased in total cell extracts (on average 3.1-fold), with a similar tendency in membrane-enriched cell extracts (on average 2.3-fold) (Fig. 7A,C).
Discussion

In this study we compared hepatic disposition of a microdose of the model Abcg2 substrate $[^{11}C]$erlotinib in EGFR$^{\Delta\text{hep}}$ and EGFR$^{\text{fl/fl}}$ mice in order to assess EGFR-mediated regulation of hepatic Abcg2 transport activity. EGFR$^{\Delta\text{hep}}$ mice have a cell type-specific deletion of EGFR in hepatocytes while EGFR$^{\text{fl/fl}}$ mice have normal EGFR expression levels in all tissues. EGFR$^{\Delta\text{hep}}$ mice are healthy and do not display any phenotypical abnormalities except for a reduction in body weight (Natarajan et al., 2007). As there is evidence for sex specific differences in EGFR pathways in the liver of mice (Wang et al., 2016) and as the assessment of these sex differences was not the subject of the present study, we focused on male mice in our experiments. Several previous studies have provided evidence for a regulatory link between EGFR signaling and ABCG2 expression (Fig. 1). It has been shown that the epidermal growth factor (EGF) can induce cell-surface expression of ABCG2 via the MAPK pathway (Meyer zu Schwabedissen et al., 2006). Moreover, the PI3K/Akt pathway, a downstream signal transduction pathway of EGFR, plays a crucial role in the posttranscriptional regulation of ABCG2 expression and its subcellular localization (Mogi et al., 2003; Takada et al., 2005; Pick and Wiese, 2012; Porcelli et al., 2014). For instance, treatment of ABCG2 overexpressing LLC-PK1 cells with a PI3K inhibitor resulted in an internalization of ABCG2 from the apical surface and a decrease in the relative expression level of ABCG2 on the cell surface (Takada et al., 2005). These regulatory pathways may either be exploited, for instance in order to overcome transporter-mediated chemoresistance of cancer cells, or they could also lead to unwanted DDIs, when a drug which interferes with EGFR signaling is combined with another drug which is transported by ABCG2. Most studies examining regulation of ABC transporters have employed cellular systems and few in vivo data are available to assess whether modulation of regulatory pathways translates into alterations of transporter-mediated disposition of probe substrates (Slosky et al., 2013; Wang et al., 2014). Recent work has shown that PET with radiolabeled transporter substrates is a powerful tool to non-invasively measure the activities of different ABC and solute carrier (SLC) transporters in the living organism (Kusuhara, 2013; Langer, 2016).
Erlotinib is a first-generation EGFR-targeting TKI, which is approved for the treatment of non-small cell lung and pancreatic cancer. Erlotinib has also been suggested as potential treatment for hepatocellular carcinoma (HCC), although a recent phase 3 study was unable to demonstrate survival improvement with erlotinib in advanced-stage HCC (Zhu et al., 2015). Erlotinib is predominantly excreted via the hepatobiliary route; in humans 83% of an i.v. dose was excreted in feces and only 8% in urine (Ling et al., 2006). Erlotinib is a substrate of ABCG2 and ABCB1 (Kodaira et al., 2010), which was shown to inhibit these transporters at higher concentrations (Shi et al., 2007). Moreover, there is evidence that erlotinib downregulates cellular ABCG2 expression levels via EGFR inhibition (Porcelli et al., 2014). We recently demonstrated that $k_{\text{bile}}$ of $[^{11}\text{C}]$erlotinib was 1.3-, 2.3- and 2.8-fold reduced in Abcb1a/b$^{-/-}$, Abcg2$^{-/-}$ and Abcb1a/b$^{-/-}$Abcg2$^{-/-}$ mice, respectively, relative to wild-type mice (Traxl et al., 2015). Moreover, we found that the majority of radioactivity in plasma, liver and bile was composed of unmetabolized $[^{11}\text{C}]$erlotinib after i.v. injection of the radiotracer into wild-type mice, whereas only radiolabeled metabolites were detected in urine. These data provided evidence that hepatobiliary excretion of $[^{11}\text{C}]$erlotinib is mediated in mice by Abcg2 and to a lesser extent by Abcb1a/b, suggesting that $[^{11}\text{C}]$erlotinib may be used as a PET probe substrate to measure Abcg2 transport activity in the liver. In the present study we used $[^{11}\text{C}]$erlotinib to investigate if deletion of EGFR in hepatocytes leads to changes in hepatic Abcg2 transport activity. To obtain quantitative pharmacokinetic parameters of hepatic disposition of $[^{11}\text{C}]$erlotinib we estimated the transfer rate constants of radioactivity from blood into liver ($k_{\text{uptake,liver}}$) and from liver into bile ($k_{\text{bile}}$) using integration plot analysis (Shingaki et al., 2015; Traxl et al., 2015) (Fig. 5). $k_{\text{bile}}$ is a parameter which has been used in other studies to assess canalicular ABC transport activities, such as that of Abcg2, in the liver of mice (Takashima et al., 2013).

$k_{\text{uptake,liver}}$ values were not significantly different between EGFR$^{\text{shep}}$ and EGFR$^{\text{fl/fl}}$ mice and ranged from 0.72-0.80 mL/min/g liver tissue corresponding to a hepatic extraction ratio of 0.72-0.80 (assuming a hepatic blood flow rate in mice of approximately 1.0 mL/min/g liver tissue) (Davies and Morris, 1993) (Fig. 5B). $k_{\text{uptake,liver}}$ values in EGFR$^{\text{fl/fl}}$ mice were comparable to
those measured in an earlier study in another mouse strain (FVB wild-type mice, $k_{\text{uptake,liver}}$: 0.734 ± 0.106 mL/min/g tissue) indicating good reproducibility of our analytical method (Traxl et al., 2015). There is evidence that hepatic uptake of [$^{11}$C]erlotinib is transporter-mediated as reflected by a decrease in $k_{\text{uptake,liver}}$ in wild-type mice co-injected with a therapeutic dose of unlabeled erlotinib as compared to mice which only received a PET microdose of [$^{11}$C]erlotinib (Traxl et al., 2015). However, as opposed to certain low permeability drugs, for which hepatic disposition is to a large extent transporter-mediated (e.g. statins), erlotinib is a highly lipophilic compound which can be expected to also penetrate cellular membranes by passive diffusion. $K_{\text{bile}}$ was 3.0-fold lower in EGFR$^{\Delta}$hep mice (Fig. 5D), suggesting a decrease in biliary excretion of [$^{11}$C]erlotinib. The decrease in total as well as in membrane-bound hepatic Abcg2 expression levels in EGFR$^{\Delta}$hep mice as revealed by Western blot analysis (Fig. 7D) suggested transcriptional regulation of Abcg2 protein levels by EGFR rather than an alteration in the membrane localization of Abcg2. Interestingly, there was an increase in hepatic Abcb1a/b expression levels (Fig. 7C). This is in line with another study which assessed changes in transporter expression levels in human conditionally immortalized proximal tubule epithelial cells following treatment with the EGFR recombinant antibody cetuximab and found significant decreases in ABCG2 mRNA and increases in ABCB1 mRNA (Caetano-Pinto et al., 2017). Our data thus support the premise that hepatobiliary excretion of [$^{11}$C]erlotinib is to a larger extent dependent on Abcg2 than on Abcb1a/b, as $k_{\text{bile}}$ was decreased in EGFR$^{\Delta}$hep mice despite an apparent upregulation of canalicular Abcb1a/b. Our data also provide evidence that [$^{11}$C]erlotinib PET is a sensitive tool to measure changes in Abcg2 transport activity in the liver. Interestingly, despite the decrease in radioactivity excreted into bile, liver profiles were parallel (Fig. 3B) and liver-to-blood AUC ratios were not significantly different between both mouse models with a tendency for lower values in EGFR$^{\Delta}$hep mice (Fig. 4D). On the other hand, blood AUC values were significantly higher in EGFR$^{\Delta}$hep mice (Fig. 4A). This could be related to an increase in the transfer of [$^{11}$C]erlotinib across the basolateral (sinusoidal) membrane from liver into blood in EGFR$^{\Delta}$hep mice, possibly involving basolateral efflux transporter(s), as potential compensatory mechanism for the decrease in biliary excretion. However, this remains
speculative as the rate constant for transfer of radioactivity from liver into blood could not be
determined with the presently employed analysis approach.

As erlotinib partly undergoes urinary excretion (Ling et al., 2006) we also measured distribution
of [11C]erlotinib to the kidneys and urinary bladder (Fig. 6). In a previous study we found that
urinary excretion of radioactivity following i.v. injection of [11C]erlotinib was very low in wild-
type mice and markedly increased in Abcg2(-/-) and Abcb1a/b(-/-)Abcg2(-/-) mice (Traxl et al.,
2015). This suggested a shift from hepatobiliary to renal excretion (which was apparently not
mediated by renal Abcg2 and Abcb1a/b), when hepatobiliary excretion was impaired due to
knockout of Abcg2. A similar phenomenon was observed in the present study, in which km was 2.2-fold higher in EGFR\textsuperscript{hep} as compared to EGFR\textsuperscript{fl/fl} mice (Fig. 6D).

Our \textit{in vivo} data confirm previous \textit{in vitro} data that EGFR signaling can regulate ABCG2
expression levels (Mogi et al., 2003; Takada et al., 2005; Meyer zu Schwabedissen et al.,
2006; Pick and Wiese, 2012; Porcelli et al., 2014). We provide, to our knowledge, the first
evidence that EGFR deletion in hepatocytes translates \textit{in vivo} into a decrease in Abcg2
transport activity leading to changes in disposition of an Abcg2 probe substrate. Our findings
may have clinical relevance as they raise the possibility that treatment with EGFR inhibitors,
such as TKIs or antibodies, may alter hepatic ABCG2 transport activity and thereby lead to
changes in hepatobiliary clearance of concomitantly administered ABCG2 substrate drugs.
This may potentially lead to hepatotoxicity of drugs but could also prove beneficial in the
treatment of liver tumors by prolonging liver residence times of anticancer drugs, if their biliary
excretion is dependent on ABCG2 transport activity. It should be noted that our study was
conducted with a microdose of erlotinib (~ 120-fold lower than a human oral therapeutic dose
of 2 mg/kg). As erlotinib itself is a potent inhibitor of ABCG2 (half-maximum inhibitory
concentration, IC\textsubscript{50} 0.13 µM) (Noguchi et al., 2009), ABCG2 transport activity may be saturated
in the liver at therapeutic erlotinib doses, which may result in less pronounced effects of EGFR
deletion on disposition of a therapeutic erlotinib dose. Transgenic mouse models with cell type-
specific deletion of regulatory pathways or transporters may serve as valuable tools to assess
the impact of transporters on drug disposition.
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Authorship Contribution

Participated in research design: Komposch, Wanek, Langer, Sibilia

Conducted experiments: Traxl, Komposch, Glitzner, Wanek, Mairinger

Performed data analysis: Traxl, Komposch, Glitzner, Wanek, Mairinger, Langer

Wrote or contributed to the writing of the manuscript: all authors
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(Bcrp/Abcg2) in limiting the brain and testis penetration of erlotinib, flavopiridol, and mitoxantrone. *J Pharmacol Exp Ther* **333**:788-796.


Footnotes

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Alexander Traxl, Karin Komposch and Elisabeth Glitzner contributed equally to this study.

Oliver Langer and Maria Sibilia had equal responsibility for this study.
Figure Legends

Fig. 1. Schematic illustration of a simplified model showing how the epidermal growth factor receptor (EGFR) regulates cellular Abcg2 expression levels. After ligand binding, the EGFR tyrosine kinase activates several downstream signal transduction pathways, including the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. It has been shown that epidermal growth factor (EGF) increases cellular Abcg2 expression via the MAPK pathway, on both mRNA and protein levels (Meyer zu Schwabedissen et al., 2006). Furthermore, numerous studies suggested that EGF influences posttranscriptional regulation and subcellular localization of Abcg2 via the PI3K/Akt pathway (Mogi et al., 2003; Takada et al., 2005; Pick and Wiese, 2012; Porcelli et al., 2014).

Fig. 2. Representative co-registered coronal whole-body [11C]erlotinib PET summation (0-90 minutes) and MR (T1-weighted gradient echo sequence) images of an EGFR^fl/fl (left) and an EGFR^Δhep (right) mouse. Anatomical structures are indicated by arrows. Radiation scale is set from 3.5 to 35 percent of the injected dose per gram tissue (%ID/g).

Fig. 3. Concentration-time curves (mean %ID/mL or %ID/g ± SD) of [11C]erlotinib in blood (A), liver (B) and intestine (C, including gall bladder and duodenum) and liver-to-blood ratios (mean ± SD) over time (D) in EGFR^fl/fl mice (filled circles, n = 6) and EGFR^Δhep mice (open triangles, n = 5).

Fig. 4. Areas under the [11C]erlotinib concentration-time curves (AUC, mean ± SD) for blood (A), liver (B) and intestine (C, including gall bladder and duodenum) and liver-to-blood (D) AUC ratios (mean ± SD) in EGFR^fl/fl mice (filled circles, n = 6) and EGFR^Δhep mice (open triangles, n = 5). ns, not significant, *P < 0.05, two-tailed unpaired t-test.

Fig. 5. Integration plots (mean ± SD) to estimate the rate constants for transfer of radioactivity from blood into liver (k_uptake,liver) (A, B) and from liver into bile (k_bile) (C, D) in EGFR^fl/fl mice (filled
circles, \( n = 6 \) and EGFR\textsuperscript{\textdagger} mice (open triangles, \( n = 5 \)) mice. See “Materials and Methods” section for the definition of variables used in the integration plots. \( K_{\text{uptake,liver}} \) and \( k_{\text{bile}} \) correspond to the slopes of the linear regression lines. \( ns \), not significant, \( ***P < 0.001 \), two-tailed unpaired t-test.

**Fig. 6.** Concentration-time curves (mean \%ID/g ± SD) of \([11C]\)erlotinib in left kidney (A) and urinary bladder (C) and rate constants for transfer of radioactivity from blood into kidney (\( K_{\text{uptake,kidney}} \)) (B) and from kidney into urine (\( k_{\text{urine}} \)) (D) in EGFR\textsuperscript{\textdagger\textdagger} mice (filled circles, \( n = 6 \)) and EGFR\textsuperscript{\textdagger} mice (open triangles, \( n = 5 \)). \( ns \), not significant, \( *P < 0.05 \), two-tailed unpaired t-test.

**Fig. 7.** Western blot analysis of EGFR, Abcb1a/b and Abcg2 protein expression levels in liver tissue collected after the PET scan from EGFR\textsuperscript{\textdagger\textdagger} mice (\( n = 3 \)) and EGFR\textsuperscript{\textdagger} mice (\( n = 3 \)) using membrane-enriched and total cell extracts. Bands were quantified by densitometry using ImageJ software and protein expression levels were normalized to \( \alpha \)-Tubulin, which was used as loading control (B-D). \( ns \), not significant, \( *P < 0.05 \), \( **P < 0.01 \), two-tailed unpaired t-test.
TABLE 1. Pharmacokinetic parameters (mean ± SD) of [11C]erlotinib in EGFR\textsuperscript{fl/fl} mice ($n = 6$) and in EGFR\textsuperscript{\Delta hep} mice ($n = 5$).

<table>
<thead>
<tr>
<th></th>
<th>AUC (min)</th>
<th>AUC ratio\textsuperscript{a}</th>
<th>$k_{\text{uptake}}$ (mL/min/g tissue)</th>
<th>$k_{\text{bile}}$ (min$^{-1}$)</th>
<th>$k_{\text{urine}}$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liver</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR\textsuperscript{fl/fl}</td>
<td>1,315 ± 270</td>
<td>5.00 ± 0.62</td>
<td>0.799 ± 0.076</td>
<td>0.018 ± 0.003</td>
<td>-</td>
</tr>
<tr>
<td>EGFR\textsuperscript{\Delta hep}</td>
<td>1,783 ± 345*</td>
<td>4.53 ± 0.29</td>
<td>0.720 ± 0.151</td>
<td>0.006 ± 0.002***</td>
<td>-</td>
</tr>
<tr>
<td><strong>Kidney</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR\textsuperscript{fl/fl}</td>
<td>677 ± 122</td>
<td>2.58 ± 0.26</td>
<td>0.458 ± 0.048</td>
<td>-</td>
<td>0.021 ± 0.015</td>
</tr>
<tr>
<td>EGFR\textsuperscript{\Delta hep}</td>
<td>1,197 ± 177***</td>
<td>3.07 ± 0.34*</td>
<td>0.434 ± 0.053</td>
<td>-</td>
<td>0.046 ± 0.013*</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Organ-to-blood AUC ratio

\* $P < 0.05$, \*** $P < 0.001$ compared to EGFR\textsuperscript{fl/fl} (two-tailed unpaired t-test)
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6

A. Kidney

B. Kidney

C. Urinary bladder

D. Urinary bladder
Figure 7