Pharmacological Research 120 (2017) 43–50

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

Breast cancer resistance protein (BCRP/ABCG2) and P-glycoprotein (P-gp/ABCB1) transport afatinib and restrict its oral availability and brain accumulation☆

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ARTICLE INFO

Article history:
Received 12 October 2016
Received in revised form
22 December 2016
Accepted 28 January 2017
Available online 10 March 2017

Keywords:
Afatinib
Tyrosine kinase inhibitor
ABCB1
ABCG2
Brain accumulation
Oral availability

ABSTRACT

Afatinib is a highly selective, irreversible inhibitor of EGFR and HER-2. It is orally administered for the treatment of patients with EGFR mutation-positive types of metastatic NSCLC. We investigated whether afatinib is a substrate for the multidrug efflux transporters ABCB1 and ABCG2 and whether these transporters influence oral availability and brain and other tissue accumulation of afatinib.

We used *in vitro* transport assays to assess human (h)ABCB1-, hABCG2- or murine (m)Abcg2-mediated transport of afatinib. To study the single and combined roles of Abcg2 and Abcb1a/1b in oral afatinib disposition, we used appropriate knockout mouse strains.

Afatinib was transported well by hABCB1, hABCG2 and mAbcg2 *in vitro*. Upon oral administration of afatinib, Abcg2$^{-/-}$, Abcb1a/1b$^{-/-}$ and Abcb1a/1b$^{-/-}$;Abcg2$^{-/-}$ mice displayed a 4.2-, 2.4- and 7-fold increased afatinib plasma $AUC_{0\text{-}24}$ compared with wild-type mice. Abcg2-deficient strains also displayed decreased afatinib plasma clearance. At 2 h, relative brain accumulation of afatinib was not significantly altered in the single knockout strains, but 23.8-fold increased in Abcb1a/1b$^{-/-}$;Abcg2$^{-/-}$ mice compared to wild-type mice.

Abcg2 and Abcb1a/1b restrict oral availability and brain accumulation of afatinib. Inhibition of these transporters may therefore be of clinical importance for patients with brain (micro)metastases positioned behind an intact blood-brain barrier.

1. Introduction

ATP-binding cassette (ABC) transporters form a superfamily of transmembrane transport proteins. Two members, ABCB1 (MDR1, P-glycoprotein or P-gp) and ABCG2 (Breast Cancer Resistance Protein, BCRP), are especially important in pharmacokinetics [1,2]. Both affect the disposition of a wide variety of endogenous and exogenous compounds, including many anticancer drugs. They are expressed at pharmacologically important sites such as the apical membranes of enterocytes, hepatocytes and renal tubular epithelial cells, where they can limit gastrointestinal absorption or mediate direct intestinal, hepatic, or renal excretion of their substrates [1,2]. Furthermore, ABCB1 and ABCG2 are expressed on apical membranes of barriers protecting sanctuary tissues such as the blood-brain, blood-placenta and blood-testis barriers, where substrates are pumped directly out of the epithelial or endothelial cells into the blood. Consequently, several chemotherapeutic agents that are ABCB1 and/or ABCG2 substrates have restricted brain accumulation [1–4]. Improving brain accumulation of drugs is of high interest in the clinic due to the fact that current therapies are often inefficient in eradicating brain tumors or brain metastases situated in whole or in part behind an intact blood-brain barrier (BBB) [3,4].

Afatinib (Gilotrif/Giotrif, BIBW 2992) is an orally administered tyrosine kinase inhibitor (TKI) for the treatment of patients with distinct types of metastatic non-small cell lung carcinoma (NSCLC), whose tumor genes have epidermal growth factor receptor (EGFR) exon 19 deletions or exon 21 (L858R) substitution mutations [5].

☆ This work was supported by internal funds within the Netherlands Cancer Institute.

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http://dx.doi.org/10.1016/j.phrs.2017.01.035
1043-6618/

Approved by the FDA in July 2013, afatinib is a highly selective, irreversible inhibitor of the EGFR and of the human epidermal growth-factor receptor (HER)-2 [6]. It is barely metabolized, with a total recovery percentage of 89.5% of unchanged drug in the urine and feces over 72 h after dosing in humans [7]. Both EGFR and HER-2 are members of the receptor tyrosine kinase (RTK) superfamily [8] and overexpression of both receptors is often found in human cancers such as gliomas, carcinomas of the breast, ovaries, bladder, and lung, including NSCLC. The overexpression, due to gene amplification, is often associated with higher EGFR pathway signaling activity, increased proliferation of cancer cells and reduced apoptosis [9]. It has further been shown preliminarily in cell lines, that afatinib appears effective against a subset of these various cancers overexpressing EGFR and HER-2 [10–12], making it an attractive candidate for further clinical research.

Several published studies indicate that afatinib can interact with ABCB1 and ABCG2 [13–15]. It has for instance been shown that afatinib affects ABCB1 and ABCG2 in several cancer cell lines by blocking substrate transport and/or down-regulating mRNA and protein expression of the transporters [13,15]. However, these same studies reported conflicting data on the ability of afatinib to inhibit ABCB1. FDA and EMA registration information mentions that afatinib is a transported substrate and inhibitor of ABCB1 and ABCG2 [5,16,17], but the limited documentation provided does not allow an assessment of the extent of these processes. In case these efflux transporters can efficiently transport afatinib, this might potentially lead to a decreased accumulation of this drug in target tumors expressing these transporters. Moreover, brain metastases are likely to occur with a subset of cancers, the frequency being highest in lung cancer relative to other common epithelial malignancies [18]. Given the high ABCB1 and ABCG2 expression in the BBB, these transporters could potentially limit afatinib brain accumulation, which might lead to reduced therapeutic efficiency against NSCLC brain metastases. We therefore aimed in this study to investigate whether and to what extent afatinib is transported by one or both of these transporters, and how this might affect oral plasma pharmacokinetics and brain penetration of the drug in Abcb1a/1b and Abcg2 mouse models.

2. Materials and methods

2.1. Chemicals

Afatinib (>99%) was obtained from Alsachim (Illkirch, France). Zosuquidar was purchased from Sequoia Research Products (Pangbourne, UK) and Ko143 was obtained from Tocris Bioscience (Bristol, UK). All chemicals used in the chromatographic afatinib assay were described before [19].

2.2. Transport assays

Polarized Madin-Darby Canine Kidney (MDCKII) cell lines and subclones transduced with either human (h)ABCB1, (h)ABCG2 [20], or mouse (m)Abcg2 cDNA were cultured and used in the transepithelial transport assays as described previously [21]. Transport assays were performed using 12-well Transwell® 3402 plates, 3.0 μm pore size (Corning Inc., USA) in DMEM with 10% fetal bovine serum (FBS). The parental cells and subclones were seeded at a density of 2.5×10^5 cells per well and cultured for 3 days to allow formation of an intact monolayer. Membrane tightness was assessed by measurement of transepithelial electrical resistance (TEER) using reference values that were previously established and well correlated to <1% transepithelial [^{14}C]-inulin leakage per hour [21]. The transepithelial transport experiment was started by pre-incubating the cells with or without the relevant inhibitors during 1 h, thereafter (t = 0) medium in the donor compartments was replaced with complete medium containing 2 μM afatinib alone or in combination with the appropriate inhibitors. In the inhibition experiments, 5 μM zosuquidar (ABCB1 inhibitor) and/or 5 μM Ko143 (ABCG2/Abcg2 inhibitor) were added to both apical and basolateral compartments. Plates were kept at 37 °C in 5% CO_2 during the experiment, and 50-μl aliquots were taken from the acceptor compartment at 2, 4 and 8 h for measurement of afatinib concentrations. Samples were stored at −30 °C for later LC–MS/MS measurement. Total amount of drug transported to the acceptor compartment was calculated after correction for volume loss for each sampling time point. Active transport was expressed by the transport ratio (r), which is defined as the amount of apically directed transport divided by the amount of basolaterally directed transport at a defined time point.

2.3. Animals

Female wild-type, *Abcb1a/1b*$^{-/-}$ [22], Abcg2$^{-/-}$ [23] and Abcg2$^{-/-}$;Abcb1a/1b$^{-/-}$ mice [24], all of a >99% FVB genetic background, were used. Mice between 9 and 14 weeks of age were used in groups of five mice per strain. The mice were kept in a temperature-controlled environment with a 12 h light/12 h dark cycle and received a standard diet (Transbreed, SDS Diets, Technilab – BMI) and acidified water *ad libitum*. Animals were housed and handled according to institutional guidelines in compliance with Dutch and EU legislation.

2.4. Drug solutions and pharmacokinetic experiments

Afatinib was dissolved at a concentration of 20 mg/ml in 50% (v/v) polysorbate 80, 50% (v/v) ethanol. It was then further diluted with 5% (w/v) glucose in water, to obtain a 1 mg/ml afatinib solution in water containing 2.5% (v/v) polysorbate 80, 2.5% (v/v) ethanol, and 4.75% (w/v) glucose. Afatinib was administered orally at a dose of 10 mg/kg (10 μl/g). All working solutions were prepared freshly on the day of the experiment. To minimize variation in absorption, mice were fasted for 3 h prior to oral administration of afatinib using a blunt-ended needle. For the pharmacokinetic experiment, 50-μl blood samples were drawn from the tail vein using heparin-coated capillaries (Sarstedt, Germany) at 0.5, 1, 2, 4 and 8 h or 0.25, 0.5 and 1 h. At 24 h or 2 h, mice were anesthetized using isoflurane and blood was collected via cardiac puncture. Immediately thereafter, mice where sacrificed by cervical dislocation and brain, spleen, liver and kidney were rapidly removed, weighed and subsequently frozen as whole organ at −30 °C. Prior to analysis, organs were allowed to thaw and then homogenized in appropriate volumes (1–3 ml) of 4% (w/v) bovine serum albumin (BSA) in water using a FastPrep® -24 homogenizer (MP-Biomedicals, NY, USA). Homogenates were stored at −30 °C. Blood samples were centrifuged immediately after collection at 9000*g* for 6 min at 4 °C, and plasma was collected and stored at −30 °C until analysis.

2.5. Drug analysis

Afatinib concentrations in culture medium, plasma and tissues were determined with a previously reported liquid chromatography-tandem mass spectrometric (LC–MS/MS) assay for afatinib, with a calibration curve ranging from 0.5 to 500 ng/ml for plasma [19] and tissue homogenates and from 1.5 to 1500 ng/ml for culture medium. Tissue concentrations were calculated correcting for the individual tissue weights, resulting in ng afatinib per gram tissue.