HEPATIC TRANSPORT OF PKI166, AN EPIDERMAL GROWTH FACTOR RECEPTOR KINASE INHIBITOR OF THE PYRROLO-PYRIMIDINE CLASS, AND ITS MAIN METABOLITE, ACU154

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ABSTRACT:
PKI166, a specific inhibitor of the tyrosine kinase activity of two epidermal growth factor receptors, was under development for the treatment of cancer. In preclinical studies PKI166 was mainly cleared by metabolism, and its metabolites were eliminated by biliary excretion, emphasizing the role of liver transport processes for its disposition. Here the transport properties of [14C]PKI166 and its main metabolite [14C]ACU154, an O-glucuronide, were analyzed using 1) Madin-Darby canine kidney II (MDCKII) cells stably transfected with human multidrug resistance-associated protein 2 (MRP2) and/or human organic anion-transporting peptide 2 (OATP2) and 2) liver canalicular membrane vesicles (CMVs) prepared from Wistar and mrp2-deficient TR rats. Analysis of transport through MDCKII cells revealed that [14C]ACU154 was a substrate of MRP2 and OATP2. Rat mrp2 was shown to transport [14C]ACU154 with a $K_m$ of approximately 1 $\mu$M. [14C]PKI166 efficiently crossed MDCKII cells, particularly toward the apical side, but expression of MRP2 and/or OATP2 did not increase the flux. The effect of PKI166 and ACU154 on transport of [3H]estradiol-17$\beta$-d-glucuronide (EG; via mrp2/MRP2 and OATP2) or [3H]taurocholic acid (TCA; via bile salt export pump (bsep)) was analyzed. PKI166 inhibited the transport of [3H]EG by OATP2. ACU154 did strongly inhibit [3H]TCA uptake into CMVs from Wistar but not from TR rats, demonstrating a dependence of bsep inhibition on mrp2 activity. ATP-dependent uptake of [3H]EG into CMVs from Wistar rats was inhibited by ACU154 but up to 4-fold increased by PKI166. In conclusion, OATP2 and MRP2/mrp2 were identified as transporters involved in ACU154 transport into bile. Both PKI166 and its O-glucuronide ACU154 affected mrp2/MRP2-, OATP2-, and or bsep-mediated transport processes.

The epidermal growth factor receptor family has become a widely studied pharmacological target in the therapeutic area of cancer due to its central role in the transmission of mitogenic signals, and the frequent deregulation in cancers (Fabbro et al., 2002). PKI166, a pyrrolo-pyrimidine derivative (Fig. 1) (Traxler et al., 1999), is a selective inhibitor of the tyrosine kinase activity of two epidermal growth factor receptors (HER1/HER2). It has a potent in vitro anti-proliferative activity and in vivo antitumor activity in a mouse model (Bruns et al., 2000; Solorzano et al., 2001; Baker et al., 2002; Motoyama et al., 2002). Its clinical development was hampered by liver toxicity findings (Traxler, 2003). Absorption, distribution, metabolism, elimination studies performed in rats and dogs demonstrated that PKI166 was almost exclusively excreted in feces (~90% after peroral or intravenous dosing, >50% thereof being unchanged compound), with only about 3 and 5% of the dose excreted in urine of dog and rat, respectively. Bile analysis of rat indicated extensive direct O-glucuronidation. Glucuronidates apparently were then subject to colonic bacterial deconjugation before excretion or reabsorption of PKI166, the latter leading to enterohepatic recirculation (Novartis, T. T. and H. M. W. contributed equally to the reported work. Article, publication date, and citation information can be found at http://dmd.aspetjournals.org. doi:10.1124/dmd.104.000497.

ABBREVIATIONS: MDCKII, Madin-Darby canine kidney strain II; BSEP/bsep, bile salt export pump (capital letters indicate human origin); CMV, liver canalicular membrane vesicle; EG, estradiol-17$\beta$-d-glucuronide; TCA, taurocholic acid; MRP2/mrp2, multidrug resistance-associated protein 2 (capital letters indicate human origin); OATP2, human organic anion transporting peptide 2.
Fig. 1. Structures of 14C-labeled PKI166 and its O-glucuronide ACU154.

Materials and Methods

Materials. [3H]Taurocholic acid (74 GBq/mmol) and [3H]estradiol-17β-o-glucuronide (1499 GBq/mmol) were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). [14C]PKI166 (6.6 MBq/mg) and its glucuronide [14C]ACU154 (3.2 MBq/mg) were synthesized in the Novartis Isotope Laboratories, and unlabeled PKI166 and ACU154 were synthesized in Novartis Laboratories (Basel, Switzerland).

Cell Culture and Transport Studies. Mock-transfected MDCKII cells, or MDCKII cells expressing MRPs (Sasaki et al., 2002) were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum at 37°C under 5% CO2 (Sasaki et al., 2002). Cells expressing OATPs were cultured in the presence of 200 μg/ml Zeocin; cells expressing MRP2 were cultured in the presence of 200 μg/ml G418. Transport studies were performed as described (Sasaki et al., 2002): the cells were seeded in 24-well plates (Falcon; BD Biosciences Discovery Labware, Bedford, MA) at a density of 1.4 x 10⁶ cells per well; 10 mM sodium butyrate was included in the medium 24 h before the experiment. For initiation of transport, the medium at the either the apical or basal side of the cell layer was exchanged with complete medium containing [14C]PKI166 or [14C]ACU154 (2 μM). The cells were incubated at 37°C, and aliquots of the medium were taken from each compartment at several points in time. Radioactivity in 100 μl of medium was measured in a liquid scintillation counter (TriCarb 2500 TR and 2700 TR Liquid Scintillation Systems; PerkinElmer Life and Analytical Sciences) after addition of a scintillation fluid (Irga-Safe Plus; PerkinElmer Life and Analytical Sciences). For the uptake studies, complete medium containing [14C]ACU154 (2 μM) was added at the basal side. After incubation at 37°C for 10 or 30 min, the cells were washed three times with 1.5 ml of distilled water were added before taking samples for liquid scintillation counting. The protein concentrations in cell experiments were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Preparation of CMVs and Uptake Studies. CMVs were prepared from Wistar and TR rats (Jansen et al., 1985) as described (Niinuma et al., 1997). Uptake of [14C]ACU154, [3H]PKI166, or [3H]TCA was measured in buffer (250 mM sucrose, 10 mM Tris-HCl, 10 mM MgCl2, pH 7.4) containing 10 mM creatine phosphate, 100 μM/ml creatine phosphokinase, and a 5 mM concentration of either ATP or AMP (final concentrations after addition of CMVs). After preincubation at 37°C, the reactions were started by addition of CMVs (0.15–0.5 mg/ml protein) and stopped by addition of a 50-fold excess of ice-cold stop buffer (250 mM sucrose, 10 mM Tris-HCl, 100 mM NaCl, pH 7.4). Separation of vesicle-bound and -free labeled compound was achieved by rapid filtration and washing on a sampling manifold (model 1225 with 0.45-μm filter type HA; Millipore Corporation, Bedford, MA) as described (Suzuki et al., 2003). Protein concentrations were determined according to the method of Bradford (1976) with bovine serum albumin as a standard.

Data Analysis. The kinetic parameters for transcellular transport of [14C]ACU154 were estimated from the Michaelis-Menten equation: 

\[ v = \frac{V_{\text{max}} \times S}{K_m + S} \]

where \( v \) is the initial uptake rate of substrates (pmol/min/mg of protein), \( S \) is the substrate concentration in medium (μM), \( K_m \) is the Michaelis constant (μM), and \( V_{\text{max}} \) is the maximum uptake rate (pmol/min/mg of protein); the data points determined over 2 h of incubation were used. The kinetic parameters for the inhibition of transcellular [3H]EG transport by PKI166 were estimated from the following equation: 

\[ v' = \frac{v}{1 + \frac{K_i}{I}} \]

where \( v' \) and \( v \) are the transport in the presence and absence of inhibitor, respectively; \( I \) is the inhibitor concentration (μM) and \( K_i \) the inhibition constant (μM); the data points determined over 0.5 h of incubation were used. The kinetic parameters for uptake of [14C]ACU154 into CMVs were estimated from the Michaelis-Menten equation including the nonsaturable transporter clearance \( P_{\text{diff}} \) (μM/min/mg of protein): 

\[ v = \frac{V_{\text{max}} \times S}{K_m + S} + P_{\text{diff}} \times S \]

Data were fitted to the mentioned equations by a nonlinear least-squares method with the MULTI program (Yamaoka et al., 1981) to obtain estimates of the kinetic parameters. The input data were weighted as the reciprocals of the squares of the observed values.

Results

Transcellular Transport across MDCKII Monolayers. The transport of [14C]PKI166 across MDCKII monolayers expressing OATP2, MRP2, or both transporters was compared with the transport across control transfected MDCKII cells. In control cells the basolateral-apical flux of [14C]PKI166 was about 2-fold higher than the flux into the basolateral direction; no major changes were evident upon expression of OATP2, MRP2 or both transporters (Fig. 2). The more hydrophilic glucuronide [14C]ACU154 did permeate the monolayer of control transfected MDCKII cells much more slowly than did PKI166 itself (Figs. 2A and 3A). In the presence of both OATP2 and MRP2, the vectorial basolateral-apical flux did increase strongly and was about 5-fold higher than the apical-to-basal flux (Fig. 3D).

PKI166 inhibited the transcellular transport of [3H]EG across OATP2/MRP2 double-transfected cells in a dose-dependent way (Fig. 4). A Ki of 17 ± 2 μM was determined by nonlinear least-squares fitting. The transcellular basolateral-apical transport of [14C]ACU154 by double-transfected cells was saturable (Fig. 5), with a Km value of 42 ± 3 μM and a Vmax of 84 ± 3 pmol/min/mg protein.

Uptake into MDCKII Cells Expressing OATP2. The uptake of [14C]ACU154 into OATP2 expressing MDCKII cells was compared with the uptake into control transfected MDCKII cells. In a 30-min incubation containing 2 μM [14C]ACU154, the clearance was 8.6 ± 0.8 μM/mg protein in control cells and 11.7 ± 1.0 μM/mg protein in OATP2-expressing cells. Assays with [14C]PKI166 had a very high background due to either uptake of [14C]PKI166 or binding to the cells. No difference between control cells and OATP2-expressing cells was apparent in incubations containing 2 μM [14C]PKI166. However, the OATP2-mediated uptake of [3H]EG into MDCKII cells was reduced in the presence of 100 μM PKI166 (data not shown).

Uptake into Rat Canalicular Membrane Vesicles. To quantify the ATP-dependent transport of [14C]ACU154 and [14C]PKI166, uptake into CMVs was analyzed in the presence of either ATP or AMP. CMVs prepared from Wistar and mnp2-deficient TR rats were used, allowing us to identify the contribution of mnp2 to the active transport. For [14C]ACU154, an increased uptake in the presence of ATP as compared with AMP was evident into CMVs prepared from Wistar but not from TR rats (Fig. 6). The concentration dependence of the [14C]ACU154 uptake into CMVs prepared from Wistar rats could be best explained assuming a saturable and a nonsaturable component of uptake. A Km of approximately 1 μM and a Vmax of 95 ± 24
FIG. 2. Time profiles for the transport of [14C]PKI166 across MDCKII monolayers. Transcellular transport of [14C]PKI166 across MDCKII cells was analyzed for control transfected cells (A), OATP2-expressing cells (B), MRP2-expressing cells (C), and cells expressing both transporters (D). Open and closed circles show the transport in the basal-to-apical and the apical-to-basal direction, respectively. Data are given as means ± S.E. of triplicate determinations.

FIG. 3. Time profiles for the transport of [14C]ACU154 across MDCKII monolayers. Transcellular transport of [14C]ACU154 across MDCKII cells was analyzed for control transfected cells (A), OATP2-expressing cells (B), MRP2-expressing cells (C), and cells expressing both transporters (D). Open and closed circles show the transport in the basal-to-apical and the apical-to-basal direction, respectively. Data are given as means ± S.E. of triplicate determinations.
pmol/min/mg of protein for the saturable component and a nonsaturable transport clearance of $1.5 \pm 0.1 \, \mu l/min/mg$ of protein were determined by nonlinear least squares fitting (Fig. 7; quantification of $[14C]ACU154$ at concentrations <1 $\mu M$ so that the $K_m$ could not be defined accurately). The uptake of $[14C]PKI166$ into CMVs could not be measured. Different experimental conditions were tested (data not shown); however, since the background binding of $[14C]PKI166$ to vesicles and/or filters was high, any possibly present minor ATP-dependent uptake could not be measured.

**Influence of ACU154 and PKI166 on mrp2- and bsep-Mediated Transport into CMVs.** The influence of ACU154 and PKI166 on the uptake of model compounds into CMVs was assessed, using $[3H]EG$ as a substrate for mrp2 and $[3H]TCA$ as a substrate for bsep. ATP-dependent uptake of $[3H]EG$ into CMVs from Wistar rats was inhibited by ACU154 (Fig. 9A), whereas the presence of PKI166 increased the uptake (Fig. 8A). No ATP-dependent uptake of $[3H]EG$ into CMVs from TR$^{-}$ rats was evident, confirming the absence of functional mrp2 in these vesicles (Figs. 8B and 9B).

**Discussion**

In preclinical absorption, distribution, metabolism, elimination studies with $[14C]PKI166$, the O-glucuronide $[14C]ACU154$ was found in high concentrations in rat bile and was detected in mouse but not in rat and dog plasma. In patients, daily doses of 200 mg of PKI166 resulted in maximal plasma concentrations of up to 0.5 $\mu g/ml$, the plasma exposure to ACU154 was in many patients up to 5-fold higher than the exposure to PKI166 and, in some patients, even over 10-fold. Biliary excretion of ACU154 was the main route of PKI166 elimination in rat. In TR$^{-}$ rats a slower elimination of $[14C]PKI166$ from plasma and a shift toward urinary elimination was observed; in these mrp2-deficient rats, the urinary excretion amounted to about 43% compared with only 5% in Wistar rats (Novartis, unpublished results). The in vitro results of the present study help to understand 1) transport processes contributing to the hepatic elimination of PKI166 and 2) interaction of PKI166 and its main metabolite with some pivotal transport proteins in liver.

The ATP-dependent uptake of $[3H]TCA$ occurred as expected into CMVs from Wistar and TR$^{-}$ rats. This uptake was clearly inhibited by 1 $\mu M$ ACU154 in the case of CMVs prepared from Wistar rats containing functional mrp2; however, it was not inhibited in the case of CMVs prepared from TR$^{-}$ rats (Fig. 9, C and D). A similar effect was evident at 100 $\mu M$ PKI166, but much less pronounced (Fig. 8, C and D).

FIG. 4. Inhibition of transcellular transport of $[3H]EG$ by PKI166. Transcellular basal-to-apical transport of $[3H]EG$ (2 $\mu M$) across MDCKII cells was analyzed for cells expressing OATP2 and MRP2. Incubation was for 30 min in the presence of different concentrations of PKI166. C indicates the transport in control transfected cells. Data are given as means $\pm$ S.E. of triplicate determinations.

FIG. 5. Concentration dependence of transcellular transport of $[14C]ACU154$ across MDCKII monolayers. Transcellular basal-to-apical transport of $[14C]ACU154$ across MDCKII cells was analyzed for cells expressing OATP2 and MRP2 and for control transfected cells. Incubation was for 2 h. Differences between the two cell types are given as means $\pm$ S.E. of triplicate determinations. The given line results from nonlinear least squares fitting of the difference data to the Michaelis-Menten equation.

FIG. 6. Time profiles of $[14C]ACU154$ uptake by CMVs. CMVs from Wistar (A) or TR$^{-}$ rats (B) were incubated in the presence of ATP (●) or AMP (○) and 10 $\mu M [14C]ACU154$. For each point in time, total radioactivity associated with the vesicles after filtration and washing was determined in triplicate; data are given as means $\pm$ S.E.
through MDCKII cells. Thus, ACU154 depends on active transport for efficient excretion into bile. ACU154 was shown to be a substrate of human MRP2 (Fig. 3) as well as rat mrp2 (Fig. 6). This finding confirms the in vivo results obtained in rats, which indicated that mrp2 activity is critical for the hepatic elimination of PKI166. MRP2/mrp2 transports a wide range of glutathione, glucuronate, and sulfate conjugates and is overall the main transporter for excretion of conjugates from hepatocytes into bile (Oude Elferink et al., 1995). In addition ACU154 was shown to be a substrate of OATP2, by measurement of the transcellular transport as well as the uptake into OATP2-transfected MDCKII cells. ACU154 was found at high plasma concentrations in PKI166-treated patients, and its uptake into hepatocytes possibly occurs via OATP2.

To analyze a possible interaction of PKI166 and ACU154 with
hepatic transport of other drugs and endogenous substances, EG and TCA were used as well characterized substrates of crucial liver transport proteins. EG is efficiently transported by OATP2 as well as MRP2/mrp2, and TCA is a substrate of bsep (Akita et al., 2001; Sasaki et al., 2002).

PKI166 inhibited the transcellular transport of [3H]EG via double-transfected MDCKII cells as well as the uptake of [3H]EG into OATP2-expressing cells in a concentration-dependent way. This indicates an inhibition of the [3H]EG transport by OATP2. However, the ATP-dependent uptake of [3H]EG into CMVs via mrp2 was enhanced by PKI166 (Fig. 8, A and B). Modulation of the MRP2/mrp2 activity by a variety of different compounds has been shown in vitro (Niinuma et al., 1997; Bodo et al., 2003; Zelcer et al., 2003). Niinuma et al. (1997) described an increased affinity of rat mrp2 for S-(2,4-dinitrophenyl)-glutathione in the presence of sulfate conjugates, whereas the corresponding glucuronides reduced the uptake and decreased the affinity. Bodo et al. (2003) reported that a number of different compounds modulate the activity of both MRP2 and MRP3, reducing transport of EG by MRP3 while stimulating its transport by MRP2. These in vitro findings indicate the potential for a considerable allosteric modulation of the MRP2/mrp2 activity. Zelcer et al. (2003) proposed that MRP2 contains two similar but nonidentical binding sites, one for the transport and one for the modulation of the transport activity (Zelcer et al., 2003). PKI166 was here identified as a stimulator of mrp2 activity toward EG, whereas its glucuronide ACU154 reduced this activity either by direct competition or an allosteric mechanism. In an earlier in vivo study in rat, increased biliary EG excretion was induced by ursodeoxycholate-3,7-disulfate (Sano et al., 1993), indicating that the reported in vitro findings might have in vivo relevance.

ACU154 inhibited the ATP-dependent uptake of [3H]EG and, even more pronouncedly, that of [3H]TCA into CMVs prepared from Wistar rats (Fig. 9, A and C). Comparison with CMVs prepared from mrp2-deficient TR/H11002 rats revealed that the inhibition of [3H]TCA uptake was dependent on the presence of functional mrp2 (Fig. 9). The latter finding suggests that TCA transport by bsep is not inhibited by cytoplasmic ACU154 but depends on the presence of mrp2, which allows ACU154 to get efficiently to the bile side of hepatocytes. Mrp2-dependent inhibition of bsep activity has been described, e.g., for EG in vitro (Stieger et al., 2000) and in vivo (Sano et al., 1993; Huang et al., 2000), and for two sulfate-conjugated bile acids that were identified as mrp2 substrates (Akita et al., 2001). Sano et al. (1993) found that the cholestatic effect of EG that is evident in Sprague-Dawley rats was absent in mrp2-deficient Eisai hyperbilirubinuria rats, in which the biliary excretion of EG was delayed; Huang et al. (2000) obtained similar results with Wistar and mrp2-deficient TR rats. The relevance of the observed inhibition of bsep activity by ACU154 for the in vivo situation remains to be determined.

In conclusion OATP2 and MRP2 were identified as transport proteins involved in ACU154 transport via hepatocytes into bile, demonstrating the usefulness of double-transfected cell lines (Sasaki et al., 2002). For PKI166, a high basal-to-apical flux was observed in MDCKII cells, indicating involvement of endogenous vescicular transport systems and an overall good permeation through the cells. Both
PKI166 and its O-glucuronide ACU154 influenced the activity of pivotal liver transport proteins, which might have an impact on the homeostasis in liver cells at a high exposure (Fig. 10).

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References


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FIG. 10. Hepatocyte trafficking of PKI166 and its main metabolite ACU154 as deduced from the reported in vitro investigations. Uptake of ACU154 (ACU) into hepatocytes can occur via OATP2. Although a transporter for PKI166 (PKI) was not identified, the results indicate a relatively easy entry into cells, possibly by active transport. Inside the hepatocyte, PKI166 is glucuronidated to ACU154, which is excreted into the bile by MRP2/mrp2. PKI166 might accelerate the excretion of its metabolite by an allosteric mechanism. Bile salt transport by BSEP/bsep might be inhibited by MRP2/mrp2 transported but not by cytoplasmic ACU154.