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Disposition of everolimus in mdr1a−/1b− mice and after a pre-treatment of lapatinib in Swiss mice

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ABSTRACT

The aim of this study was to document the *in vivo* transport of everolimus (inhibitor of mTOR) by P-glycoprotein (P-gp), and to investigate the influence of lapatinib (inhibitor of P-gp) on everolimus disposition.

Pharmacokinetics of everolimus (0.25 mg/kg) has been investigated after oral administration in mdr1a−/1b− mice compared to the wild type. Also, everolimus pharmacokinetics was characterized after oral administration on Swiss mice either alone or after 2 days of pre-treatment of lapatinib (200 mg/kg). The influence of lapatinib pre-treatment on intestinal P-gp expression was investigated by Western blot analysis. The non-compartimental analysis was performed using Winonlin® professional version 4.1 software (Pharsight, Mountain View, CA). The areas under the plasma concentration–time curve (AUC) were compared using Bailer's method.

A significant 1.3-fold increase of everolimus AUC observed in mdr1a−/1b− mice suggested that everolimus is transported *in vivo* by intestinal P-gp in mice. In addition, a 2.6-fold significant increase of everolimus AUC with lapatinib pre-treatment as compared with the everolimus alone group was noticed. The elimination half-life was comparable ($t_{1/2}$ = 5.3 h *vs.* $t_{1/2}$ = 4 h). A 38.5% significant decrease of P-gp expression was observed in duodenum segment in lapatinib pre-treated group as compared with control group.

In conclusion, lapatinib enhanced everolimus absorption by decreasing intestinal P-gp expression. An inhibition of CYP 450 could not be excluded. These results confirm the necessity of a therapeutic monitoring of everolimus combined with an inhibitor of the P-gp and CYP 450 like lapatinib in a future anti-tumor treatment.

1. Introduction

Everolimus (Certican®), 40-O-(2-hydroxyethyl)—rapamycin, is a new immunosuppressant used in cardiac and renal transplantation [1,2]. This drug belongs to the family of inhibitors of the mTOR protein (mammalian target of rapamycin) which is a regulatory protein of cells metabolism, growth and proliferation. Since mTOR protein is present both in hematopoietic cells (T cells or B) and in non-hematopoietic cells (such as smooth muscle cell), everolimus will inhibits growth factors stimulated proliferation. Several studies showed the interest of everolimus as an anti-tumor agent. An increase of survival is observed in patients in post-liver transplantation neoplasms who received everolimus as compared to patients who received only an inhibitor of calcineurine (ciclosporin or tacrolimus) with steroids at 6, 12, and 24 months (100%, 90%, 72% *vs.* 50%, 29%, 14%) [3]. Everolimus anti-tumor properties could also be explained by the presence of the protein mTOR in many tumors, an inhibition of tumor growth and angiogenesis was observed in treated human tumor cells in ovarian cancer resistant cisplatine [4].

Everolimus is highly metabolized in intestine and liver. Everolimus absolute oral bioavaibility in rats is about 16% [5]. Everolimus is a substrate of P-glycoprotein (P-gp) and also of P450 3A4, 3A5 and 2C8 cytochromes [6–8]. Cytochrome P450 3A (CYP3A) and P-glycoprotein are located in enterocytes and hepatocytes. The CYP3A/P-gp system contributes to the first-pass metabolism of many drugs, resulting in a limited bioavailability [9]. Cytochrome P450 isoenzymes are parts of the Phase I metabolizing enzymes. The CYP3A subfamily is predominant and is involved in the metabolism of more than 70% of currently

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administered drugs. The CYP3A represent almost 30% of the cytochromes in the liver and 70% of the cytochromes in the small intestine [10]. Many studies have demonstrated that inhibitors of cytochromes 3A are leading to an increase of AUC of drug substrates with a prolongation of half time elimination [11,12]. P-gp is part of a large family of efflux transporters which is expressed in the gonads, kidneys, biliary system, intestinal epithelium, brain capillaries and lymphocytes. P-gp is coded by the multidrug resistance MDR1 genes in humans and the mdr1a and mdr1b in mice and rats. These genes encode drug-transporting protein that can cause multidrug resistance in tumor cells by decreasing intracellular drug levels. Enhanced expression of P-gp is considered to be a major mechanism of chemotherapeutics resistance [13]. Conversely, inhibitors of P-gp may increase the oral absorption of drugs transported by P-gp. For example, verapamil enhanced bioavaibility of etoposide in rats [14]. *In vitro* study using human intestinal cell line (Caco-2) has shown that everolimus is a potent substrate for P-gp-like mediated efflux and this efflux was completely inhibited by verapamil [15]. Modifications of pharmacokinetics of drugs substrates of cytochromes or P-gp by many inhibitors of these proteins are leading to therapeutic drug monitoring. As everolimus is substrate of cytochromes and P-gp, it is conceivable that pharmacokinetics parameters of this drug could be modified after a pre-treatment by P-gp inhibitor.

Lapatinib is an inhibitor of tyrosine kinase receptors (HER 1 and HER 2) which is indicated in combination with capecitabine for the treatment of patients with advanced or metastatic breast cancer whose tumors overexpress HER 2 [16,17]. Lapatinib was chosen in this pharmacological study for several reasons. First, *in vitro* and *in vivo* studies showed that lapatinib decreases the expression of P-gp and inhibits cytochromes 3A4 and BCRP [18–20]. Thus, everolimus pharmacokinetic parameters could be modified after a lapatinib pre-treatment. Second, it is relevant to study the pharmacological interaction of these two drugs acting by two different anti-tumor mechanisms (inhibition of mTOR protein and inhibition of tyrosine kinases receptor) as they could be combined for an anti-tumor treatment. As the matter of fact, a Phase I Study evaluates the combination of lapatinib and everolimus in patients with advanced solid tumors or non-Hodgkin's lymphoma [21].

The present pharmacological study was to thus undertaken with a double purpose: (a) to document the *in vivo* effect of intestinal P-gp on everolimus disposition and (b) to investigate the effect of lapatinib pre-treatment on everolimus pharmacokinetics and its influence on intestinal P-gp expression. Therefore, we have investigated the pharmacokinetics of everolimus and digoxin (substrate test of P-gp) after oral administration in mice with naturally deficient in P-gp (mdr1a−/1b−) compared to the wild type (Fvb mice). In a second time, everolimus pharmacokinetic parameters were determined after oral administration either alone or after lapatinib pre-treatment in Swiss mice.

2. Materials and methods

2.1. Drugs

Digoxin (digoxine nativelle®) was purchased from Procter Gamble Pharmaceuticals (Asnières-sur-Seine, France). 100 μL of suspension (50 μg/mL) was diluted in 900 μL of sterile water to obtain a stock solution of 5 μg/mL. Everolimus (Certican®) was purchased from Novartis Pharma SAS (Rueil-Malmaison, France). The oral preparation was obtained by dispersion of a tablet (0.25 mg) in 10 mL of sterile water (0.025 mg/mL). Lapatinib (Tyverb®) was purchased from GlaxoSmith Kline (Marly-le-Roi, France). The oral preparation was obtained by dispersion of a tablet (250 mg) in 25 mL of sterile water (10 mg/mL).

2.2. Animals

Two strains of mice were used in this study. The first strain of mice was Fvb (sensitive to the B strain of friend leukemia) mice. The Fvb mice were used in digoxin and everolimus pharmacokinetic studies to document the effect of P-gp on everolimus disposition. For each drug, the wild type was compared to mdr1a−/1b− mice (mice naturally deficient of P-gp). The second strain was Swiss mice OF1. OF1 mice were used to study the influence of lapatinib pre-treatment on everolimus disposition.

All mice used in the study were female mice (20–30 g) and provided by Charles River Laboratories (Orléans, France). They were caged under standard laboratory conditions, with tap water and regular chow provided *ad libitum* in a 12 h/12 h-light/dark cycle at a temperature of 21–23 °C. Anesthesia was induced with 5% isoflurane and maintained with 2.5% isoflurane in air. The animals were treated in accordance with the European committee standards concerning the care and use of laboratory animals.

2.3. Digoxin pharmacokinetic study

Fvb and mdr1a−/1b− mice in each group orally received 0.03 mg/kg of digoxin. They were anesthetized with isoflurane and the blood samples were collected by cardiac puncture at 15, 30 min and 1, 2, 3, 4, 6, 8 and 24 h after the administration of digoxin on heparinized tubes. The samples were centrifuged at 12,000 × *g* for 8 min and the plasma was harvested into clean tubes. For digoxin quantification, an automated method was used. Plasma digoxin concentrations were measured with fluorescent polarization assay (FPIA) on TDx® using Digoxin II® reagent kit (Abbott Diagnostic, Rungis, France). This method was linear up to 5 ng/mL with a limit of quantification at 0.2 ng/mL. All plasma samples were assayed together with calibration standard and quality control.

2.4. Everolimus pharmacokinetic study

Fvb and mdr1a−/1b− mice in each group orally received 0.25 mg/kg of everolimus. The mice were anesthetized with isoflurane and the blood samples were collected by cardiac puncture at 30 min and 1, 2, 3, 5, 8 and 24 h after the administration of everolimus on EDTA tubes. For everolimus quantification, an automated method was used. Blood everolimus concentrations were measured with fluorescent polarization assay on TDx® using INNOFLUOR®CERTICAN® kit (Biomedical diagnostics, Marne la Vallée, France). This method was linear up to 40 ng/mL with a limit of quantification at 2 ng/mL. All blood samples were assayed together with calibration standard and quality control.

2.5. Everolimus pharmacokinetic after lapatinib pre-treatment

Swiss mice were divided into two groups. The first one orally received 0.25 mg/kg of everolimus, the second one orally received 100 mg/kg of lapatinib twice a day for 2 days and 0.25 mg/kg of everolimus on the third day. The collection of everolimus blood samples and the quantification of everolimus blood concentration were done in the same conditions described for everolimus pharmacokinetic studies in Fvb and mdr1a−/1b− mice.

2.6. Data analysis. Pharmacokinetic studies

As each animal provided only one sample of blood, data from animals of the same group were pooled using a naïve averaging data approach [22]. The non-compartimental analysis was performed using Winonlin® professional version 4.1 software (Pharsight, Mountain View, CA). Data were used to estimate the

mean maximal concentration (C_{max}) and the time necessary to reach maximal concentration (T_{max}). In addition, for each treatment, the elimination rate constant (k_e) and the area under the mean concentration–time curve ($AUC_{0\rightarrow Tlast}$) were estimated. The elimination rate constant (k_e) was estimated as the slope of the log-linear terminal portion of the plasma concentration *vs.* time curve, determined using unweighted linear least-squares regression analysis.

Additionally, from the estimated parameters, the terminal elimination half-life ($t_{1/2}$) for each group was derived and calculated as $t_{1/2} = \ln 2/k_e$.

2.7. Semiquantitative determination of P-gp expression by Western blot analysis

P-gp expression was measured in three segments of intestine (duodenum, jejunum, and ileum) by Western blot analysis in two groups of mice: (1) 3 female Swiss mice non-pre-treated and (2) 3 female Swiss mice with a lapatinib pre-treatment. For last group, mice orally received 100 mg/kg of lapatinib twice a day for 2 days and 100 mg/kg of lapatinib on the third day. The intestines were removed 6 h after the last administration of lapatinib on the third day.

Tissues were cut into small pieces and suspended in three volumes of 0.25 M sucrose, 50 mM phosphate buffer, pH 7.20, 1 mM EGTA, 2 mM $MgCl_2$, and protease inhibitors: 5 μg/mL leupeptin, 0.15 mg/mL benzamidine, 5 μg/mL aprotinin, 1 μg/mL pepstatin, and 16 mg/mL phenylmethylsulfonyl fluoride (PMSF) (Sigma–Aldrich, St. Louis, MO, USA). The suspension was then homogenized in a glass tube. The homogenate was centrifuged at 500 × *g* for 15 min. The supernatant obtained was then centrifuged at 3000 × *g* for 15 min. The pellet was washed twice with three volumes of 0.25 M sucrose, 50 mM phosphate buffer, pH 7.20, 2 mM $MgCl_2$, and protease inhibitors and centrifuged at 3000 × *g* for 15 min. Protein concentrations were determined using the colorimetric bicinchoninic assay kit (Sigma–Aldrich, St. Louis, MO, USA), with bovine serum albumin (BSA; Sigma–Aldrich) as a standard. Proteins (40 μg) were separated by SDS–polyacrylamide gel electrophoresis on an 8% polyacrylamide gel and transferred onto a nitrocellulose membrane (Amersham, Orsay, France). The nitrocellulose membranes were then incubated with a primary antibody (C219, diluted to 1:100; DakoCytomation Denmark A/S, Glostrup, Denmark), washed, and finally incubated with a peroxidase-conjugated anti-mouse IgG secondary antibody. The immunoreactive bands were visualized by the enhanced Chemi-luminescent system (PerkinElmer life science, Boston, USA). The nitrocellulose membranes were also incubated with anti-β actin (dilted to 1:5000; Sigma–Aldrich, St. Louis, USA) to normalize P-gp expression. The autoadiographs of P-gp and β actin proteins were scanned by densitometry using the image J software to obtain a quantitative evaluation of protein levels in the three segments of intestines.

2.8. Statistical analysis

Pharmacokinetic studies: The mean $AUC_{0\rightarrow Tlast}$ estimated for each study group after administration of treatment were compared using Bailer's method [23].

The test for the equality of the mean AUC between each study group after administration of treatment was performed using the standard Wald statistic. Under the null hypothesis that mean AUCs are equal, this statistic follows a normal distribution. The null hypothesis was rejected if $|Z_{obs}|$ was greater than 1.96.

Western blot studies: In the Western blot analysis of the P-gp expression, the intensity P-gp/β actin ratio was compared using Wilcoxon's test with a significant level fixed at 0.05.

Table 1
Main pharmacokinetic parameters of digoxin after oral administration (0.03 mg/kg) obtained by a non-compartimental analysis in Fvb wild type and mdr1a−/1b− mice.

	Fvb wild type	mdr1a−/1b−
Half-life (h)	5.72	5.45
$AUC_{0\rightarrow Tlast}$ (ng h/mL)	42.13	250.58
Ratio $AUC_{0\rightarrow Tlast}$[a]	5.9	
T_{max} (h)	0.25	4
C_{max} (ng/mL)	6.3	18.8
Ratio C_{max}[b]	3.0	

[a] Ratio $AUC_{0\rightarrow Tlast}$ = $AUC_{0\rightarrow Tlast}$ mdr1a−/1b− group/$AUC_{0\rightarrow Tlast}$ wild type group.
[b] Ratio C_{max} = C_{max} mdr1a−/1b− group/C_{max} wild type group.

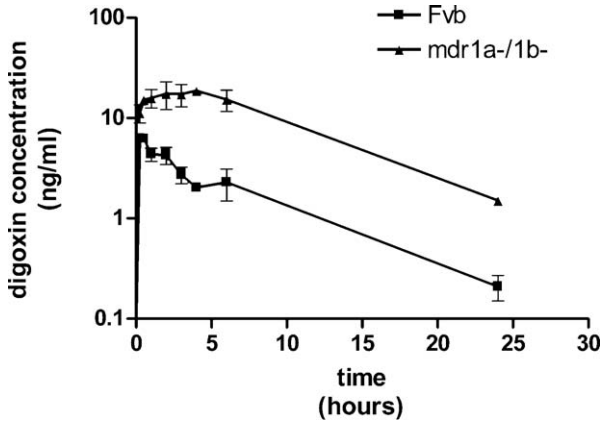

Fig. 1. Plot of time course of mean plasma digoxin concentrations (S.D.) after oral administration of digoxin at 0.03 mg/kg in Fvb and mdr1a−/1b− mice group ($n = 3$ per group).

3. Results

3.1. Digoxin pharmacokinetic study

The results of the non-compartimental analysis in Fvb mice group and in mdr1a−/1b− mice group after the administration of digoxin are summarized in Table 1.

As shown in Fig. 1, the oral administration of digoxin to the mdr1a−/1b− mice group resulted in a mean C_{max} 3-fold higher than the mean C_{max} in the Fvb group. Also, $AUC_{0\rightarrow Tlast}$ of the mdr1a−/1b− group is 5.9-fold greater than $AUC_{0\rightarrow Tlast}$ of the Fvb group ($p < 0.00001$, Bailer's method). The half-life of elimination was comparable (5.7 h *vs.* 5.5 h).

3.2. Everolimus pharmacokinetic study

The results of the non-compartimental analysis in Fvb mice group and mdr1a−/1b− mice group after the administration of everolimus are summarized in Table 2.

Table 2
Main pharmacokinetic parameters of everolimus after oral administration (0.25 mg/kg) obtained by a non-compartimental analysis in Fvb wild type and mdr1a−/1b− mice.

	Fvb wild type	mdr1a−/1b−
Half-life (h)	9.3	11.3
$AUC_{0\rightarrow Tlast}$ (ng h/mL)	515.9	709.9
Ratio $AUC_{0\rightarrow Tlast}$[a]	1.3	
T_{max} (h)	3	3
C_{max} (ng/mL)	36.5	48.1
Ratio C_{max}[b]	1.3	

[a] Ratio $AUC_{0\rightarrow Tlast}$ = $AUC_{0\rightarrow Tlast}$ mdr1a−/1b− group/$AUC_{0\rightarrow Tlast}$ wild type group.
[b] Ratio C_{max} = C_{max} mdr1a−1/1b− group/C_{max} wild type group.

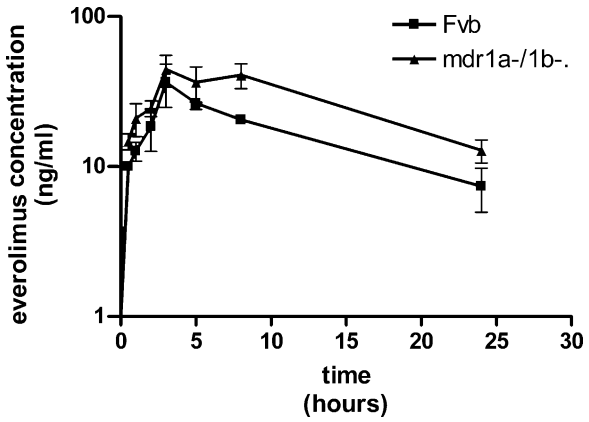

Fig. 2. Plot of time course of mean plasma everolimus concentrations (S.D.) after oral administration of everolimus at 0.25 mg/kg in Fvb and mdr1a−/1b− mice group ($n = 3$ per group).

As shown in Fig. 2, the oral administration of everolimus to the mdr1a−/1b− mice group resulted in a mean C_{max} 1.3-fold greater than the mean C_{max} in the Fvb group. Also, $AUC_{0\rightarrow Tlast}$ of the mdr1a−/1b− group is 1.3-fold greater than $AUC_{0\rightarrow Tlast}$ of the Fvb group ($p < 0.001$, Bailer's method). The half-life of elimination was comparable (9.3 h *vs.* 11.3 h).

3.3. Everolimus pharmacokinetic after lapatinib pre-treatment

The results of the non-compartimental analysis after the administration of everolimus alone and after lapatinib pre-treatment in Swiss mice are summarized in Table 3. As shown in Fig. 3, the oral administration of everolimus following a pre-treatment of lapatinib in Swiss mice resulted in a mean C_{max} 1.7-fold greater than the mean C_{max} in everolimus alone group. In addition, the $AUC_{0\rightarrow Tlast}$ of the lapatinib pre-treated Swiss mice group is 2.6-fold greater than $AUC_{0\rightarrow Tlast}$ of everolimus alone group ($p < 0.001$, Bailer's method). The half-time of elimination was comparable (4.0 h *vs.* 5.3 h).

Table 3
Main pharmacokinetic parameters of everolimus (0.25 mg/kg) after oral administration (0.25 mg/kg) obtained by a non-compartimental analysis in Swiss mice.

	Everolimus	Everolimus + lapatinib
Half-life (h)	4.0	5.3
$AUC_{0\rightarrow Tlast}$ (ng h/mL)	128.3	332.6
Ratio $AUC_{0\rightarrow Tlast}$[a]	2.6	
T_{max} (h)	3	3
C_{max} (ng/ml)	19.9	34.2
Ratio C_{max}[b]	1.7	

[a] Ratio $AUC_{0\rightarrow Tlast}$ = $AUC_{0\rightarrow Tlast}$ evero + lapa group/$AUC_{0\rightarrow Tlast}$ evero group.
[b] Ratio C_{max} = C_{max} evero + lapa group/C_{max} evero group.

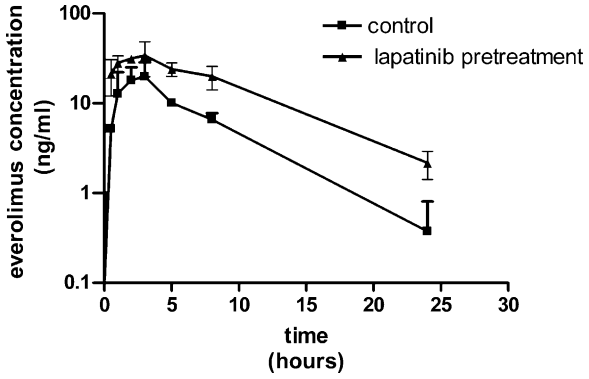

Fig. 3. Plot of time course of mean plasma everolimus concentrations (S.D.) after oral administration of everolimus at 0.25 mg/kg alone and after oral pre-treatment of lapatinib (100 mg/kg twice a day) in Swiss mice ($n = 3$ per group).

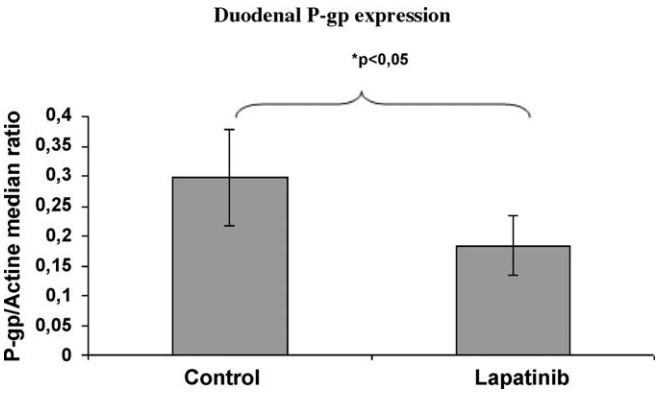

Fig. 4. Histogram of median P-gp/β actin ratio ± S.D. values from mouse duodenum ($n = 3$ per group).

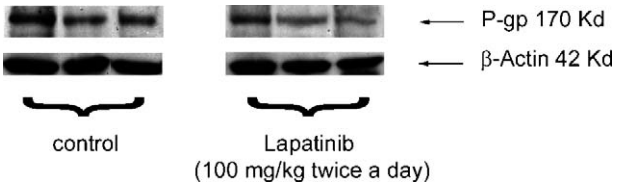

Fig. 5. P-gp immunodetection from the duodenum of mice treated with 2 days of lapatinib (100 mg/kg twice a day) or control ($n = 3$ per group).

3.4. Semiquantitative determination of P-gp expression by Western blot analysis in intestine

Western blot analysis of P-gp expression was performed on three parts of intestine (duodenum, jejunum and ileum) from three control mice and three mice pre-treated with lapatinib (100 mg/kg of lapatinib twice a day for 2 days and 100 mg/kg of lapatinib on the third day). A 38.5% significant decrease was observed in duodenum segment in lapatinib pre-treated group as compared with control group (0.298 ± 0.08 *vs.* 0.183 ± 0.05; $p < 0.05$, Wilcoxon test) (see Figs. 4 and 5). Nevertheless, in jejunum and ileum segment, no significant difference of P-gp expression was observed (0.58 ± 0.43 *vs.* 0.6 ± 0.2 and 0.47 ± 0.11 *vs.* 0.25 ± 0.68, respectively) (data not shown).

4. Discussion

The aim of this study was to document the *in vivo* effect of intestinal P-gp on everolimus disposition and to investigate the effect of lapatinib pre-treatment on everolimus pharmacokinetics and on intestinal P-gp expression.

In order to study the effect of P-gp on the disposition of everolimus, pharmacokinetics after oral administration of this drug was characterized in Fvb mice and mdr1a−/1b− mice. At the same time, the pharmacokinetics of digoxin, which is known to be a P-gp substrate [24], was realized.

The administration of the two molecules in mdr1a−/1b− mice resulted in higher C_{max} and $AUC_{0\rightarrow Tlast}$ as compared to those obtained in wild type mice. The application of Bailer's method has shown that the oral bioavailability of both molecules was significantly higher in the mdr1a−/1b− group than in the wild type group. In addition, half-lives of elimination in each group appear to be comparable. All these findings, added to the results obtained previously [5,6], suggest that everolimus is transported

by intestinal P-gp in mice like digoxin. However, the ratio of $AUC_{0\rightarrow Tlast}$ in everolimus group is lower than the ratio of $AUC_{0\rightarrow Tlast}$ in digoxin group. Unlike digoxin, everolimus is highly metabolized by cytochromes [7,8]. Thus, intestinal cytochromes could play a role in everolimus absorption and affect its $AUC_{0\rightarrow Tlast}$ after oral administration.

In order to document the influence of lapatinib on disposition of everolimus, pharmacokinetics study of this drug was carried in Swiss mice.

The first group of mice received everolimus alone (0.25 mg/kg). The second group of mice received everolimus after a lapatinib pre-treatment for 2 days (100 mg/kg, twice a day). A 2.6-fold increase of $AUC_{0\rightarrow Tlast}$ of everolimus was observed in mice pre-treated by lapatinib as compared to everolimus alone. A 1.7-fold increase of everolimus C_{max} was also observed. The elimination half-lives were comparable (4.0 h *vs*. 5.3 h). *In vitro* and *in vivo* studies had shown that lapatinib is a substrate and an inhibitor of the P-gp as well as cytochromes 3A and 2C8 [18–20].

Kovarick et al. have shown that when everolimus is coadministrated with verapamil in 16 healthy subjects, 2.3-fold increase of everolimus C_{max} ((21 ± 8) ng/mL *vs*. (47 ± 18) ng/mL) was observed and accompanied by 3.5-fold increase of $AUC_{0\rightarrow Tlast}$ ((115 ± 45) ng/mL h *vs*. (392 ± 142) ng/mL h). On the other hand, a slight prolongation of elimination half-life has been noticed (32 ± 6 h *vs*. 37 ± 6 h) [25]. When everolimus was coadministred with ketoconazole, a potent cytochrome 3A4 inhibitor, in 12 healthy volonteers, a 15-fold $AUC_{0\rightarrow Tlast}$ increase has been observed, as compared to everolimus group alone. Also, a 1.9-fold prolongation of elimination half-life (30 ± 4 h *vs*. 56 ± 5 h) was shown [12]. Verapamil is described like an important inhibitor of P-gp and less important inhibitor of cytochrome 3A while ketoconazole is both a potent inhibitor of P-gp and cytochrome 3A. In our results, an increase of 2.6-fold everolimus $AUC_{0\rightarrow Tlast}$ after a lapatinib pre-treatment was observed and accompanied with an slight prolongation of elimination half-life. Our results, when compared to those obtained in the previous study, suggest that lapatinib could have the same type of inhibition of P-gp and cytochromes as verapamil.

The determination of P-gp expression in intestines segments by Western blot analysis showed that P-gp expression decreased only in duodenum in the lapatinib pre-treated group as compared with the control group. No significant difference of P-gp expression was observed in jejunum and ileum. The concept of anatomical region-dependant modification of P-gp was already proposed to account for the inhibiting effect of some drugs on intestinal P-gp. For instance, IFN-α substantially inhibits P-glycoprotein expression in rats jejunum and ileum [26]. Quinidine increases ileal and jejunal digoxin absorption by inhibiting P-gp in rats [27]. An inhibition of duodenal P-gp expression by lapatinib could explain a decrease of the protein activity. Further investigations are needed to understand which regulators are implicated in the decrease of P-gp expression. However, it has been demonstrated that a HER 2 over expression leads to an induction of NF-κB, a transcription factor controlling expression of genes which promote cell growth and survival [28]. In addition, it has been showed that NF-κB induces P-gp expression [29]. These findings lead us to think that one of the mechanisms of the decrease of the P-gp expression by the lapatinib could be due to an inhibition of HER 2 leading to a decrease of induction of NF-κB which could result in an inhibition of P-gp expression. Other mechanism of P-gp inhibition could be implicated. *In vitro* study showed that lapatinib could directly inhibit the function of P-gp by binding to their ATP-binding sites in order to stimulate the ATPase activity for its own transport [30]. However, the increase of everolimus $AUC_{0\rightarrow Tlast}$ after a lapatinib pre-treatment can not be explained by a competition of the two drugs transport by P-gp, as lapatinib last dose was administered 16 h before the administration of everolimus. Thus, it is conceivable that lapatinib decreases P-gp expression, and so, enhances everolimus $AUC_{0\rightarrow Tlast}$.

It had been demonstrated that cyclosporine inhibits the first-pass extraction of everolimus by the intestine, rather than the liver in rats. Everolimus was administered 10 min after ciclosporin administration [31]. An inhibitory effect of lapatinib on the first-pass metabolism of everolimus by cytochrome P450 in the intestine could be lead to an increase of everolimus $AUC_{0\rightarrow Tlast}$, and C_{max} without modifying the elimination half-life as noticed in our pharmacokinetic study. Nevertheless, as we discussed above, a combination of lapatinib and everolimus is necessary to evaluate a competition of the two drugs on proteins. If lapatinib was administered 16 h before the administration of everolimus, a competition of the two drugs on cytochromes P450 could not be observed. However, an inhibition of cytochromes by lapatinib in mice liver could not be excluded. In order to confirm any inhibition of cytochromes, metabolite quantification should be done. Also, *in vitro* studies showed that lapatinib inhibits BCRP transporter [19]. However, no study has documented the influence of BCRP on everolimus disposition. Thus, the results obtained from our pharmacokinetic study and from the Western blot analysis concerning everolimus and lapatinib interaction, suggest that lapatinib could enhance everolimus disposition by decreasing P-gp expression.

Administration of everolimus in Fvb mice resulted in an increase of 4.1-fold of $AUC_{0\rightarrow Tlast}$ and 1.8-fold of C_{max} higher than in Swiss mice. Obviously, absorption is enhanced, but also the elimination process appears to be different as half times of elimination are not comparable in the two strains. Many hypotheses could explain these results: physiological changes, differences in enzymatic arsenal, and protein expression according to the strains of mice.

In conclusion, this study showed first that everolimus is transported by P-gp in mice. In addition, a 2.6-fold significant increase of everolimus AUC was noticed after a lapatinib pre-treatment.

These results show the necessity of a therapeutic monitoring of everolimus when it is administered with an inhibitor of P-gp and/or cytochromes like lapatinib in an anti-tumor treatment. Moreover, it could be relevant to document the pharmacological interaction of these two drugs in combination in order to observe an eventually competition on P-gp and cytochromes and therefore, an increase of a toxicity effect. Finally, everolimus and lapatinib are two drugs with anti-tumor properties, acting on two different pathogenesis ways. Thus, lapatinib may increase the risk of toxicity associated with a higher concentration of everolimus in blood but equally the efficiency. Further investigations are necessary to document the pharmacodynamic effect of a pre-treatment of lapatinib on the anti-tumor activity of everolimus.

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