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► **To cite this version:**

C. Chu, C. Abbara, M.S. Noël-Hudson, L. Thomas-Bourgneuf, P. Gonin, et al.. Disposition of everolimus in mdr1a-/1b- mice and after a pre-treatment of lapatinib in Swiss mice. *Biochemical Pharmacology*, 2009, 77 (10), pp.1629. <10.1016/j.bcp.2009.02.013>. <hal-00493494>

HAL Id: hal-00493494

<https://hal.science/hal-00493494v1>

Submitted on 19 Jun 2010

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

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PII: S0006-2952(09)00114-2
DOI: doi:10.1016/j.bcp.2009.02.013
Reference: BCP 10093

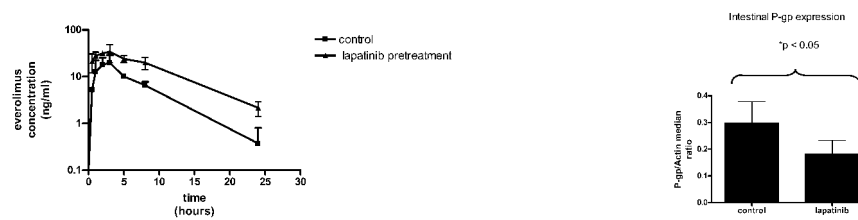
To appear in: *BCP*

Received date: 23-12-2008
Revised date: 18-2-2009
Accepted date: 18-2-2009

Please cite this article as: Chu C, Abbara C, Noël-Hudson MS, Thomas-Bourgneuf L, Gonin P, Farinotti R, Bonhomme-Faivre L, Disposition of everolimus in mdr1a-/1b- mice and after a pre-treatment of lapatinib in Swiss mice, *Biochemical Pharmacology* (2008), doi:10.1016/j.bcp.2009.02.013

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Lapatinib increases 2.5-fold everolimus AUC in mice. A 38.5% decrease of P-gp expression was observed in duodenum with a lapatinib pre-treatment. An inhibition of CYP 450 could not be excluded.



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

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ABSTRACT

1
2
3 The aim of this study was to document *in vivo* the transport of everolimus (inhibitor of
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5 mTOR) by P-glycoprotein (P-gp), and to investigate the influence of lapatinib (inhibitor of P-
6
7 gp) on everolimus disposition.
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9
10 Pharmacokinetics of everolimus (0.25 mg/kg) has been investigated after oral administration
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12 in *mdr1a-1b-* mice compared to the wild type. Also, everolimus pharmacokinetics was
13
14 characterized after oral administration on Swiss mice either alone or after 2 days of pre-
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16 treatment of lapatinib (200 mg/kg). The influence of lapatinib pre-treatment on intestinal P-gp
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18 expression was investigated by Western blot analysis. The noncompartmental analysis was
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20 performed using Winonlin[®] professional version 4.1 software (Pharsight, Mountain View,
21
22 CA). The areas under the plasma concentration-time curve (AUC) were compared using
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24 Bailer's method.
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29 A significant 1.3-fold increase of everolimus AUC observed in *mdr1a-1b-* mice suggested
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31 that everolimus is transported *in vivo* by intestinal P-gp in mice. In addition, a 2.6-fold
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33 significant increase of everolimus AUC with lapatinib pre-treatment as compared with the
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35 everolimus alone group was noticed. The elimination half-life were comparable ($t_{1/2} = 5.3$ h
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37 *vs.* $t_{1/2} = 4$ h). A 38.5% significant decrease of P-gp expression was observed in duodenum
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39 segment in lapatinib pre-treated group as compared with control group.
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43
44 In conclusion, lapatinib enhanced everolimus absorption by decreasing intestinal P-gp
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46 expression. An inhibition of CYP 450 could not be excluded. These results confirm the
47
48 necessity of a therapeutic monitoring of everolimus combined with an inhibitor of the P-gp
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50 and CYP P450 like lapatinib in a future anti-tumor treatment.
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56 Key words: lapatinib; everolimus; P-glycoprotein; pharmacokinetics; mice
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58 Category of article: Pharmacokinetics and Drug Metabolism
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1- INTRODUCTION

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2 Everolimus (Certican[®]), 40-O-(2-hydroxyethyl) - rapamycin, is a new immunosuppressant
3
4 used in cardiac and renal transplantation [1,2] This drug belongs to the family of inhibitors of
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6 the mTOR protein (mammalian target of rapamycin) which is a regulatory protein of cells
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8 metabolism, growth and proliferation. Since mTOR protein is present both in hematopoietic
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10 cells (T cells or B) and in non hematopoietic cells (such as smooth muscle cell), everolimus
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12 will inhibits growth factors stimulated proliferation. Several studies showed the interest of
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14 everolimus as an anti-tumor agent. An increase of survival is observed in patients in post liver
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16 transplantation neoplasms who received everolimus as compared to patients who received
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18 only an inhibitor of calcineurine (ciclosporin or tacrolimus) with steroids at 6, 12, and 24
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20 months (100%, 90%, 72% vs. 50%, 29%, 14%) [3]. Everolimus anti-tumor properties could
21
22 also be explained by the presence of the protein mTOR in many tumors, an inhibition of
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24 tumor growth and angiogenesis was observed in treated human tumor cells in ovarian cancer
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26 resistant cisplatin [4].
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34 Everolimus is highly metabolized in intestine and liver. Everolimus absolute oral bioavailability
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36 in rats is about 16% [5]. Everolimus is substrate of P-glycoprotein (P-gp) and also of P450
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38 3A4, 3A5 and 2C8 cytochromes [6-8]. Cytochromes P450 3A (CYP3A) and P-glycoprotein
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40 (P-gp) are located in enterocytes and hepatocytes. The CYP3A/P-gp system contributes to the
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42 first-pass metabolism of many drugs, resulting in a limited bioavailability [9]. Cytochrome
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44 P450 isoenzymes are parts of the phase I metabolizing enzymes. The CYP3A subfamily is
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46 predominant and is involved in the metabolism of more than 70% of currently administered
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48 drugs. The CYP 3A represent almost 30% of the cytochromes in the liver and 70% of the
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50 cytochromes in the small intestine [10]. Many studies have demonstrated that inhibitors of
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52 cytochromes 3A are leading to an increase of AUC of drug substrates with a prolongation of
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54 half time elimination [11,12]. P-gp is part of a large family of efflux transporters which is
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1 expressed in the gonads, kidneys, biliary system, intestinal epithelium, brain capillaries and
2 lymphocytes. P-gp is coded by the multidrug resistance MDR1 genes in humans and the
3 *mdr1a* and *mdr1b* in mice and rats. These genes encode drug-transporting protein that can
4 cause multidrug resistance in tumor cells by decreasing intracellular drug levels. Enhanced
5 expression of P-gp is considered to be a major mechanism of chemotherapeutics resistance
6 [13]. Conversely, inhibitors of P-gp may increase the oral absorption of drugs transported by
7 P-gp. For example, verapamil enhanced bioavailability of etoposide in rats [14]. *In vitro* study
8 using human intestinal cell line (Caco-2) has shown that everolimus is a potent substrate for
9 P-gp-like mediated efflux and this efflux was completely inhibited by verapamil. [15].
10 Modifications of pharmacokinetics of drugs substrates of cytochromes or P-gp by many
11 inhibitors of these proteins are leading to therapeutic drug monitoring. As everolimus is
12 substrate of cytochromes and P-gp, it is conceivable that pharmacokinetics parameters of this
13 drug could be modified after a pre-treatment by P-gp inhibitor.

14 Lapatinib is an inhibitor of tyrosine kinase receptors (HER 1 and HER 2) which is indicated
15 in combination with capecitabine for the treatment of patients with advanced or metastatic
16 breast cancer whose tumors overexpress HER2 [16,17]. Lapatinib was chosen in this
17 pharmacological study for several reasons. First, *in vitro and in vivo* studies showed that
18 lapatinib decreases the expression of P-gp and inhibits cytochrome 3A4 and BCRP [18-20].
19 Thus, everolimus pharmacokinetic parameters could be modified after a lapatinib pre-
20 treatment. Second, it is relevant to study the pharmacological interaction of these two drugs
21 acting by two different anti-tumor mechanisms (inhibition of mTor protein and inhibition of
22 tyrosine kinases receptor) as they could be combined for an anti-tumor treatment. As the
23 matter of fact, a Phase I Study evaluates the combination of lapatinib and everolimus in
24 patients with advanced solid tumors or non-Hodgkin's lymphoma [21].

25 The present pharmacological study was to thus undertaken with a double purpose: a) to

document *in vivo* the effect of intestinal P-gp on everolimus disposition, 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 (mdr 1a-/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 pretreatment in Swiss mice.

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2- MATERIALS AND METHODS

2-1. Drugs

Digoxin, (digoxine native[®]) was purchased from Procter Gamble Pharmaceuticals (Asnières-sur-Seine, France). 100 μ L of suspension (50 μ g/mL) was diluted in 900 μ L of sterile water to obtain a stock solution at 5 μ g/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 pharmacokinetics 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 pretreatment 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 hours/12 hours 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

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2 Fvb and *mdr1a*-/*b*- mice in each group orally received 0.03 mg/kg of digoxin. They were
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4 anesthetized with isoflurane and the blood samples were collected by cardiac puncture at 15,
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6 30 min and 1, 2, 3, 4, 6, 8 and 24 hr after the administration of digoxin on heparinized tubes.
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8 The samples were centrifuged at 12.000 g for 8 min and the plasma was harvested into clean
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10 tubes. For digoxin quantification, an automated method was used. Plasma digoxin
11
12 concentrations were measured with Fluorescent Polarization Assay (FPIA) on TDx[®] using
13
14 Digoxin II[®] reagent kit (Abbott Diagnostic, Rungis, France). This method was linear up to 5
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16 ng/mL with a limit of quantification at 0.2 ng/mL. All plasma samples were assayed together
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18 with calibration standard and quality control.
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2-4. Everolimus Pharmacokinetic Study

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27 Fvb and *mdr1a*-/*b*- mice in each group orally received 0.25 mg/kg of everolimus. The mice
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29 were anesthetized with isoflurane and the blood samples were collected by cardiac puncture at
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31 30 min and 1, 2, 3, 5, 8 and 24 hr after the administration of everolimus on EDTA tubes. For
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33 everolimus quantification, an automated method was used. Blood everolimus concentrations
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35 were measured with Fluorescent Polarization Assay (FPIA) on TDx[®] using
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37 INNOFLUOR[®]CERTICAN[®] kit (Biomedical diagnostics, Marne la Vallée, France). This
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39 method was linear up to 40 ng/mL with a limit of quantification at 2 ng/mL. All blood
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41 samples were assayed together with calibration standard and quality control.
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2-5. Everolimus Pharmacokinetic After Lapatinib Pre-Treatment

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52 Swiss mice were divided into two groups. The first one orally received 0.25 mg/kg of
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54 everolimus, the second one orally received 100 mg/kg of lapatinib twice a day for two days
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56 and 0.25 mg/kg of everolimus on the third day. The collection of everolimus blood samples
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1 and the quantification of everolimus blood concentration were done in the same conditions
2 described for everolimus pharmacokinetic studies in Fvb and mdr1a-/1b- mice.
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6 7 **2-6. Data Analysis. Pharmacokinetic studies**

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9 As each animal provided only one sample of blood, data from animals of the same group were
10 pooled using a naïve averaging data approach [22]. The noncompartmental analysis was
11 performed using Winonlin[®] professional version 4.1 software (Pharsight, Mountain View,
12 CA). Data were used to estimate the mean maximal concentration (C_{max}) and the time
13 necessary to reach maximal concentration (T_{max}). In addition, for each treatment, the
14 elimination rate constant (k_e) and the area under the mean concentration-time curve
15 ($AUC_{0 \rightarrow T_{last}}$) were estimated. The elimination rate constant (k_e) was estimated as the slope of
16 the log-linear terminal portion of the plasma concentration vs. time curve, determined using
17 unweighted linear least-squares regression analysis.
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20 Additionally, from the estimated parameters, the terminal elimination half-life ($t_{1/2}$) for each
21 group was derived and calculated as $t_{1/2} = \ln 2 / k_e$.
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31 32 **2-7. Semiquantitative Determination of P-gp Expression by Western Blot Analysis**

33 P-gp expression was measured in three segments of intestine (duodenum, jejunum, and ileum)
34 by Western Blot analysis in two groups of mice: (1) 3 female Swiss mice non-pretreated, (2) 3
35 female Swiss mice with a lapatinib pre-treatment. For last group, mice orally received 100
36 mg/kg of lapatinib twice a day for 2 days and 100 mg/kg of lapatinib on the third day. The
37 intestines were removed 6 hours after the last administration of lapatinib on the third day.
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39 Tissues were cut into small pieces and suspended in three volumes of 0.25 M sucrose, 50 mM
40 phosphate buffer, pH 7.20, 1 mM EGTA, 2 mM $MgCl_2$, and protease inhibitors: 5 $\mu g/ml$
41 leupeptin, 0.15 mg/ml benzamidine, 5 $\mu g/ml$ aprotinin, 1 $\mu g/ml$ pepstatin, and 16 mg/ml
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1 phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, St. Louis, MO, USA). The
2 suspension was then homogenized in a glass tube. The homogenate was centrifuged at 500 g
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4 for 15 min. The supernatant obtained was then centrifuged at 3,000 g for 15 min. The pellet
5
6 was washed twice with three volumes of 0.25 M sucrose, 50 mM phosphate buffer, pH 7.20, 2
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8 mM MgCl₂, and protease inhibitors and centrifuged at 3,000 g for 15 min. Protein
9
10 concentrations were determined using the colorimetric bicinchoninic assay kit (Sigma-
11
12 Aldrich, St. Louis, MO, USA), with bovine serum albumin (BSA;Sigma-Aldrich) as a
13
14 standard. Proteins (40 µg) were separated by SDS-polyacrylamide gel electrophoresis on an
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16 8% polyacrylamide gel and transferred onto a nitrocellulose membrane (Amersham, Orsay,
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18 France). The nitrocellulose membranes were then incubated with a primary antibody (C219,
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20 diluted to 1:100; DakoCytomation Denmark A/S, Glostrup, Denmark), washed, and finally
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22 incubated with a peroxidase-conjugated anti-mouse IgG secondary antibody. The
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24 immunoreactive bands were visualized by the enhanced Chemi-luminescent system (Perkin
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26 Elmer life science, Boston, USA). The nitrocellulose membranes were also incubated with
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28 anti-β actin (dilted to 1:5000; Sigma-Aldrich, St. Louis, USA) to normalize P-gp expression.
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30 The autoadiographs of P-gp and β actin proteins were scanned by densitometry using the
31
32 image J software to obtain a quantitative evaluation of protein levels in the three segments of
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34 intestines.
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46 **2-8. Statistical Analysis**

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48 *Pharmacokinetic Studies:* The mean AUC_{0→T_{last}} estimated for each study group after
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50 administration of treatment were compared using Bailer's method [23].
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53 The test for the equality of the mean AUC between each study group after administration of
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55 treatment was performed using the standard Wald statistic. Under the null hypothesis that
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2 mean AUCs are equal, this statistic follows a normal distribution. The null hypothesis was
3 rejected if $|Z_{\text{obs}}|$ was greater than 1.96.
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5 **Western Blot Studies:** In the Western blot analysis of the P-gp expression, the
6 intensity P-gp/ β actin ratio was compared using Wilcoxon's test with a significant
7 level fixed at 0.05.
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11 3- RESULTS

12 3-1. Digoxin Pharmacokinetic Study

13 The results of the non-compartmental analysis in Fvb mice group and in mdr1a-/1b- mice
14 group after the administration of digoxin are summarized in Table 1.
15

16 As shown in Fig. 1, the oral administration of digoxin to the mdr1a-/1b- mice group resulted
17 in a mean C_{max} 3 fold higher than the mean C_{max} in the Fvb group. Also, $AUC_{0 \rightarrow T_{\text{last}}}$ of the
18 mdr1a-/1b- group is 5.9 fold greater than $AUC_{0 \rightarrow T_{\text{last}}}$ of the Fvb group ($p < 0.00001$, *Bailer's*
19 *method*). The half-life of elimination were comparable (5.7 vs. 5.5 h).
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38 3-2. Everolimus Pharmacokinetic Study

39 The results of the non-compartmental analysis in Fvb mice group and mdr1a-/1b- mice group
40 after the administration of everolimus are summarized in Table 2.
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43 As shown in Fig. 2, the oral administration of everolimus to the mdr1a-/1b- mice group
44 resulted in a mean C_{max} 1.3 fold greater than the mean C_{max} in the Fvb group. Also,
45 $AUC_{0 \rightarrow T_{\text{last}}}$ of the mdr1a-/1b- group is 1.3 fold greater than $AUC_{0 \rightarrow T_{\text{last}}}$ of the Fvb group
46 ($p < 0.001$, *Bailer's method*). The half-life of elimination were comparable (9.3 vs. 11.3 h).
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57 3-3. Everolimus Pharmacokinetic After Lapatinib Pre-Treatment

58 The results of the non-compartmental analysis after the administration of everolimus alone
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1 and after lapatinib pretreatment in Swiss mice are summarized in Table 3. As shown in Fig. 3,
2 the oral administration of everolimus following a pre-treatment of lapatinib in Swiss mice
3
4 resulted in a mean C_{\max} 1.7 fold greater than the mean C_{\max} in everolimus alone group. In
5
6 addition, the $AUC_{0 \rightarrow T_{\text{last}}}$ of the lapatinib pretreated Swiss mice group is 2.6 fold greater than
7
8 $AUC_{0 \rightarrow T_{\text{last}}}$ of everolimus alone group ($p < 0.001$, *Bailer's method*). The half-time of
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10 elimination were comparable (4.0 vs. 5.3 h).
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17 **3-4. Semiquantitative Determination of P-gp expression by Western Blot Analysis in** 18 **intestine**

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21 Western blot analysis of P-gp expression was performed on three parts of intestine
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23 (duodenum, jejunum, ileon) from three control mice and three mice pretreated with lapatinib
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25 (100 mg/kg of lapatinib twice a day for 2 days and 100 mg/kg of lapatinib on the third day). A
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27 38.5% significant decrease was observed in duodenum segment in lapatinib pre-treated group
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29 as compared with control group (0.298 ± 0.08 vs. 0.183 ± 0.05 ; $p < 0.05$, *Wilcoxon test*) (see
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31 Fig. 4 and Fig. 5). Nevertheless, in jejunum and ileum segment, no significant difference of P-
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33 gp expression was observed (0.58 ± 0.43 vs. 0.6 ± 0.2 and 0.47 ± 0.11 vs. 0.25 ± 0.68
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35 respectively) (data not shown).
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4- DISCUSSION

The aim of this study was to document *in vivo* the 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*-/*1-b* 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 $AUC_{0 \rightarrow T_{last}}$ 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 $AUC_{0 \rightarrow T_{last}}$ in everolimus group is lower than the ratio of $AUC_{0 \rightarrow T_{last}}$ 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 $AUC_{0 \rightarrow T_{last}}$ 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 two days (100 mg/kg, twice a day). A 2.6 fold increase of $AUC_{0 \rightarrow T_{last}}$ of everolimus was observed in mice pretreated by lapatinib as compared to everolimus alone. A 1.7 fold increase of everolimus C_{max} was also observed. The

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elimination half-lives were comparable (4.0 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 coadministered with verapamil in 16 healthy subjects, 2.3-fold increase of everolimus C_{max} (21 ± 8 vs 47 ± 18 ng/ml) was observed and accompanied by 3.5 fold increase of $AUC_{0 \rightarrow T_{last}}$ (115 ± 45 vs 392 ± 142 ng/ml.h). On the other hand, a slight prolongation of elimination half life has been noticed (32 ± 6 vs. 37 ± 6 h) [25]. When everolimus was coadministered with ketoconazole, a potent cytochrom 3A4 inhibitor, in 12 healthy volunteers, a 15-fold $AUC_{0 \rightarrow T_{last}}$ increase has been observed, as compared to everolimus group alone. Also, a 1.9-fold prolongation of elimination half-life (30 ± 4 vs 56 ± 5 h) was shown [12]. Verapamil is described like an important inhibitor of P-gp and less important inhibitor of cytochrom 3A while ketoconazole is both a potent inhibitor of P-gp and cytochrom 3A. In our results, an increase of 2.6-fold everolimus $AUC_{0 \rightarrow T_{last}}$ 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 pretreated 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

1 activity. Further investigations are needed to understand which regulators are implicated in
2 the decrease of P-gp expression. However, it has been demonstrated that a HER-2 over
3 expression leads to an induction of NF- κ B, a transcription factor controlling expression of
4 genes which promote cell growth and survival [28]. In addition, it has been showed that NF-
5 κ B induces P-gp expression [29]. These findings lead us to think that one of the mechanisms
6 of the decrease of the P-gp expression by the lapatinib could be due to an inhibition of HER-2
7 leading to a decrease of induction of NF- κ B which could result in an inhibition of P-gp
8 expression. Other mechanism of P-gp inhibition could be implicated. *In vitro* study showed
9 that lapatinib could directly inhibit the function of P-gp by binding to their ATP-binding sites
10 in order to stimulate the ATPase activity for its own transport [30]. However, the increase of
11 everolimus $AUC_{0 \rightarrow T_{last}}$ after a lapatinib pre-treatment can not be explained by a competition
12 of the two drugs transport by P-gp, as lapatinib last dose was administered 16 hours before the
13 administration of everolimus. Thus, it is conceivable that lapatinib decreases P-gp expression,
14 and so, enhances everolimus $AUC_{0 \rightarrow T_{last}}$.

15 It had been demonstrated that cyclosporine inhibits the first-pass extraction of everolimus by
16 the intestine, rather than the liver in rats. Everolimus was administered 10 minutes after
17 ciclosporin administration [31]. An inhibitory effect of lapatinib on the first-pass metabolism
18 of everolimus by cytochromes P450 in the intestine could be lead to an increase of everolimus
19 $AUC_{0 \rightarrow T_{last}}$, and C_{max} without modifying the elimination half-life as noticed in our
20 pharmacokinetic study. Nevertheless, as we discussed above, a combination of lapatinib and
21 everolimus is necessary to evaluate a competition of the two drugs on proteins. If lapatinib
22 was administered 16 hours before the administration of everolimus, a competition of the two
23 drugs on cytochromes P450 couldn't be observed. However, an inhibition of cytochromes by
24 lapatinib in mice liver could not be excluded. In order to confirm any inhibition of
25 cytochromes, metabolite quantification should be done. Also, *in vitro* studies showed that

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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 $AUC_{0 \rightarrow T_{last}}$ 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 pretreatment.

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 way. 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.

5- ACKNOWLEDGEMENT

We acknowledge Angélique Dauvin, Mirile Ganga, Aurélie Gasnier and Benoît Petit for the excellent technical assistance and Karine Ser-Leroux for the kind help in this project.

6- REFERENCES

- [1] Ruiz JC, Sanchez A, Rengel M., Beneyto I, Piazza JJ. Use of the new proliferation signal inhibitor everolimus in renal transplant patients in Spain: preliminary results of the EVERODATA registry. *Transplant Proc* 2007;39(7):2157-9 [Scopus](#).
- [2] Rothenburger M, Teerling E, Bruch C, Lehmkühl H, Suwelack B, Bara C et al. Calcineurin inhibitor-free immunosuppression using everolimus (Certican) in maintenance heart transplant recipients: 6 months' follow-up. *J Heart Lung Transplant* 2007;26(3):250-7 [Scopus](#).
- [3] Gomez-Camarero J, Salcedo M, Rincon D, Lo Iacono O, Ripoll C, Hernando A et al. Use of Everolimus as a rescue immunosuppressive therapy in liver transplant patients with neoplasms. *Transplantation* 2007;84(6):786-91 [Scopus](#).
- [4] Mabuchi S, Altomare DA, Cheung M, Zhang L, Poulikakos PI, Hensley HH et al. RAD001 inhibits human ovarian cancer cell proliferation, enhances cisplatin-induced apoptosis, and prolongs survival in an ovarian cancer model. *Clin Cancer Res* 2007;13(14):4261-70 [Scopus](#).
- [5] Crowe A, Bruelisauer A, Duerr L, Guntz P, Lemaire M. Absorption and intestinal metabolism of SDZ-RAD and rapamycin in rats. *Drug Metab Dispos* 1999;27(5):627-32 [Scopus](#).
- [6] Laplante A, Demeule M, Murphy GF, Béliveau R. Interaction of immunosuppressive agents rapamycin and its analogue SDZ-RAD with endothelial P-gp. *Transplant Proc* 2002; 34(8):3393-5 [Scopus](#).
- [7] Kovarik JM, Beyer D, Schmouder RL. Everolimus drug interactions: application of a classification system for clinical decision making. *Biopharm Drug Dispos* 2005;27(9):421-6 [Scopus](#).
- [8] Kirchner GI, Meier-Wiedenbach I, Manns MP. Clinical pharmacokinetics of everolimus. *Clin Pharmacokinet* 2004;43(2):83-95 [Scopus](#).
- [9] Wachter VJ, Silverman JA, Zhang Y, Benet LZ. Role of P-glycoprotein and cytochrome P450 3A in limiting oral absorption of peptides and peptidomimetics. *J Pharm Sci* 1998;87(11):1322-30 [Scopus](#).
- [10] Fakhoury M, Litalien C, Medard Y, Cavé H, Ezzahir N, Peuchmaur M et al. Localization and mRNA expression of CYP3A and P-glycoprotein in human duodenum as a function of age. *Drug Metab Dispos* 2005;33(11):1603-7 [Scopus](#).
- [11] Saari TI, Laine K, Bertilsson L, Neuvonen PJ, Olkkola KT. Voriconazole and fluconazole increase the exposure to oral diazepam. *Eur J Clin Pharmacol* 2007;63(10):941-9 [Scopus](#).
- [12] Kovarik JM, Beyer D, Bizot MN, Jiang Q, Shenouda M, Schmouder RL. Blood concentrations of everolimus are markedly increased by ketoconazole. *J Clin Pharmacol* 2005;45(5):514-8 [Scopus](#).

- 1 [13] Schinkel AH, Mayer U, Wagenaar E, Mol CA, van Deemter L, Smit JJ et al. Normal
2 viability and altered pharmacokinetics in mice lacking *mdr1*-type (drug – transporting) P-
3 glycoprotein. *Proc Natl Acad Sci U S A* 1997;94(8):4028-33 [Scopus](#).
- 4 [14] Piao YJ, Li X, Choi JS. Effects of verapamil on etoposide pharmacokinetics after
5 intravenous and oral administration in rats. *Eur J Drug Metab Pharmacokinet*
6 2008;33(3):159-64 [Scopus](#).
- 7
8 [15] Crowe A, Lemaire M. *In vitro* and *in situ* absorption of SDZ-RAD using a human
9 intestinal cell line (Caco-2) and a single pass perfusion model in rats: comparison with
10 rapamycin. *Pharm Res* 1998;15(11):1666-72 [Scopus](#).
- 11
12 [16] Hegde PS, Rusnak D, Bertiaux M, Alligood K, Strum J, Gagnon R et al. Delineation of
13 molecular mechanisms of sensitivity to lapatinib in breast cancer cell lines using global
14 gene expression profiles. *Mol Cancer Ther* 2007;6(5):1629-40 [Scopus](#).
- 15
16 [17] Geyer CE, Forster J, Lindquist D, Chan S, Romieu CG, Pienkowski T et al. Lapatinib
17 plus capecitabine for HER2-positive advanced breast cancer. *N Engl J Med* 2006;355(26):
18 2733-43 [Scopus](#).
- 19
20 [18] Collins D, O'Connor R, O'Donovan N, Clynes M, Crown J. Lapatinib inhibits P-
21 glycoprotein (P-GP) and synergistically enhances taxane cytotoxicity in Pgp + cell lines.
22 *Annals Onc* 2006;17(S8):57
- 23
24 [19] Polli JW, Humphreys JE, Harmon KA, Castellino S, O'Mara MJ, Olson KL et al. The
25 role of efflux and uptake transporters in N-{3-chloro-4-[(3-fluorobenzyl)oxy]phenyl}-6-
26 [5- ({[2methylsulfonyl]ethyl}amino) methyl)-2-furyl]-4-quinazolinamine (GW572016,
27 lapatinib) disposition and drug interactions. *Drug Metab Dispos* 2008;36(4):695-701
28 [Scopus](#).
- 29
30 [20] Molina JR, Kaufmann SC, Reid J, Rubin S, Galvez-Peralta M, Friedman R et al.
31 Evaluation of lapatinib and topotecan combination therapy: tissue culture, murine
32 xenograft, and phase I clinical trial data. *Clin Cancer Res* 2008;14(23):7900-8.
- 33
34 [21] <http://clinicaltrials.gov/>
- 35
36 [22] Burtin P, Mentre F, van Bree J, Steimer JL. Sparse sampling for assessment of drug
37 exposure in toxicological studies. *Eur J Drug Metab Pharmacokinet* 1996;21(2):105-11
38 [Scopus](#).
- 39
40 [23] Bailer AJ. Testing for the equality of area under the curves when using destructive
41 measurement techniques. *J Pharmacokinet Biopharm* 1998;16(3):303-9 [Scopus](#).
- 42
43 [24] Bonhomme-Faivre L, Benyamina A, Reynaud M, Farinotti R, Abbara C. Disposition of
44 Delta(9) tetrahydrocannabinol in CF1 mice deficient in *mdr1a* P-glycoprotein. *Addic Biol*
45 2008;13:295-300.
- 46
47 [25] Kovarik JM, Beyer D, Bizot MN, Jiang Q, Allison MJ, Schmouder RL. Pharmacokinetic
48 interaction between verapamil and everolimus in healthy subjects. *Br J Clin Pharmacol*
49 2005;60(4):434-7 [Scopus](#).
- 50
51 [26] Ben-Reguiga M, Bonhomme-Faivre L, Orbach-Arbouys S, Farinotti R. Modification of
52 the P-glycoprotein dependent pharmacokinetics of digoxin in rats by human recombinant
53 interferon- α . *Pharm Res* 2005;22(11):1829-36 [Scopus](#).
- 54
55 [27] Su SF, Huang JD. Inhibition of the intestinal digoxin absorption and exsorption by
56 quinidine. *Drug Metab Dispos* 1996;24(2):142-7 [Scopus](#).
- 57
58 [28] Pianetti S, Marcello A, Romieu-Mourez R, Coffey RJ, Sonenshein GE. Her-2/neu
59
60
61
62
63
64
65

1 overexpression induces NF- κ B via a PI3-kinase/ Akt pathway involving calpain-mediated
2 degradation of I κ B- α that can be inhibited by the tumor expressor PTEN. *Oncogene*
3 2001;20:1287-99 [Scopus](#).

4 [29] Bentires-Alj M, Barbu V, Fillet M, Chariot A, Relic B, Jacobs N et al. NF-kappaB
5 transcription factor induces drug resistance through MDR1 expression in cancer cells.
6 *Oncogene* 2003;22(1):90-7.
7

8 [30] Dai CL, Tiwari AK, Wu CP, Su XD, Wang SR, Liu DG et al. Lapatinib (Tykerb,
9 GW572016) reverses multidrug resistance in cancer cells by inhibiting the activity of
10 ATP-binding cassette subfamily B member 1 and G member 2. *Cancer Res* 2008;68:7905-
11 14.
12

13 [31] Yokomasu A, Yano I, Sato E, Masuda S, Katsura T, Inui K. Effect of intestinal and
14 hepatic first-pass extraction on the pharmacokinetics of everolimus in rats. *Drug Metab*
15 *Pharmacokinet* 2008;23(6):469-75.
16
17
18
19
20
21
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Table 1: Main pharmacokinetic parameters of digoxin after oral administration (0.03 mg/kg) obtained by a non-compartmental analysis in Fvb wild type and mdr1a-/1b- mice.

	Fvb wild type	mdr1a-/1b-
Half-life (hr)	5.72	5.45
AUC _{0→Tlast} (ng.hr/ml)	42.13	250.58
Ratio AUC _{0→Tlast} ^a	5.9	
T _{max} (hr)	0.25	4
C _{max} (ng/ml)	6.3	18.8
Ratio C _{max} ^b	3.0	

^a Ratio AUC_{0→Tlast} = AUC_{0→Tlast} mdr1a-1/1b- group / AUC_{0→Tlast} wild type group

^b Ratio C_{max} = C_{max} mdr1a-1/1b- group / C_{max} wild type group

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

	Fvb wild type	mdr1a-/1b-
Half-life (hr)	9.3	11.3
AUC _{0→Tlast} (ng.hr/ml)	515.9	709.9
Ratio AUC _{0→Tlast} ^a	1.3	
T _{max} (hr)	3	3
C _{max} (ng/ml)	36.5	48.1
Ratio C _{max} ^b	1.3	

^a Ratio AUC_{0→Tlast} = AUC_{0→Tlast} mdr1a-1/1b- group / AUC_{0→Tlast} wild type group

^b Ratio C_{max} = C_{max} mdr1a-1/1b- group / C_{max} wild type group

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

	everolimus	everolimus + lapatinib
Half-life (hr)	4.0	5.3
AUC _{0→Tlast} (ng.hr/ml)	128.3	332.6
Ratio AUC _{0→Tlast} ^a	2.6	
T _{max} (hr)	3	3
C _{max} (ng/ml)	19.9	34.2
Ratio C _{max} ^b	1.7	

^a Ratio AUC_{0→Tlast} = AUC_{0→Tlast} evero + lapa group / AUC_{0→Tlast} evero group

^b Ratio C_{max} = C_{max} evero+ lapa group / C_{max} evero group

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

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

Figure 3: Plot of time course of mean plasma everolimus concentrations (SD) after oral administration of everolimus at 0.25 mg / kg alone and after oral pretreatment of lapatinib (100 mg / kg twice a day) in Swiss mice ($n = 3$ per group).

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

Figure 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).

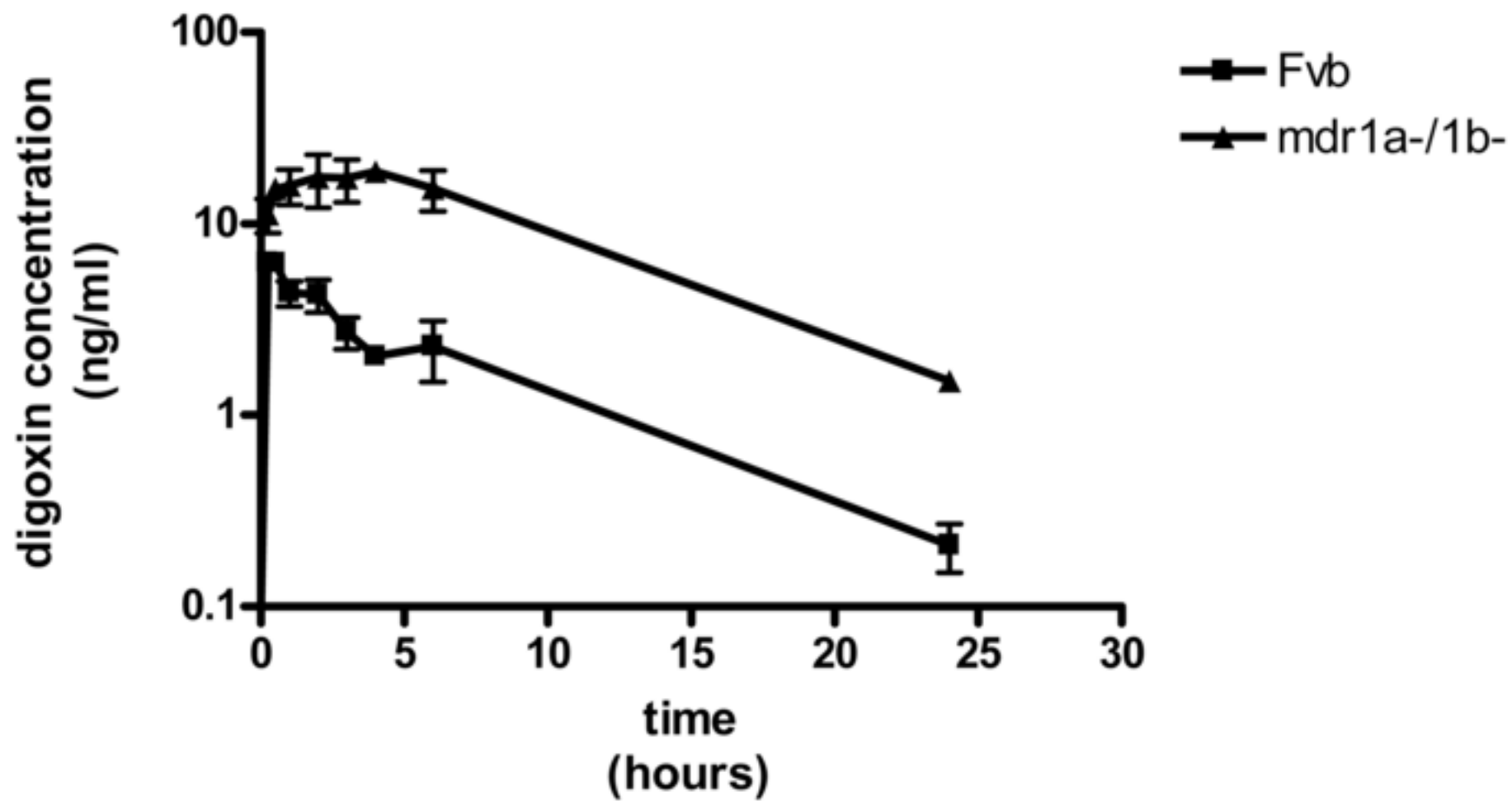


Figure 1

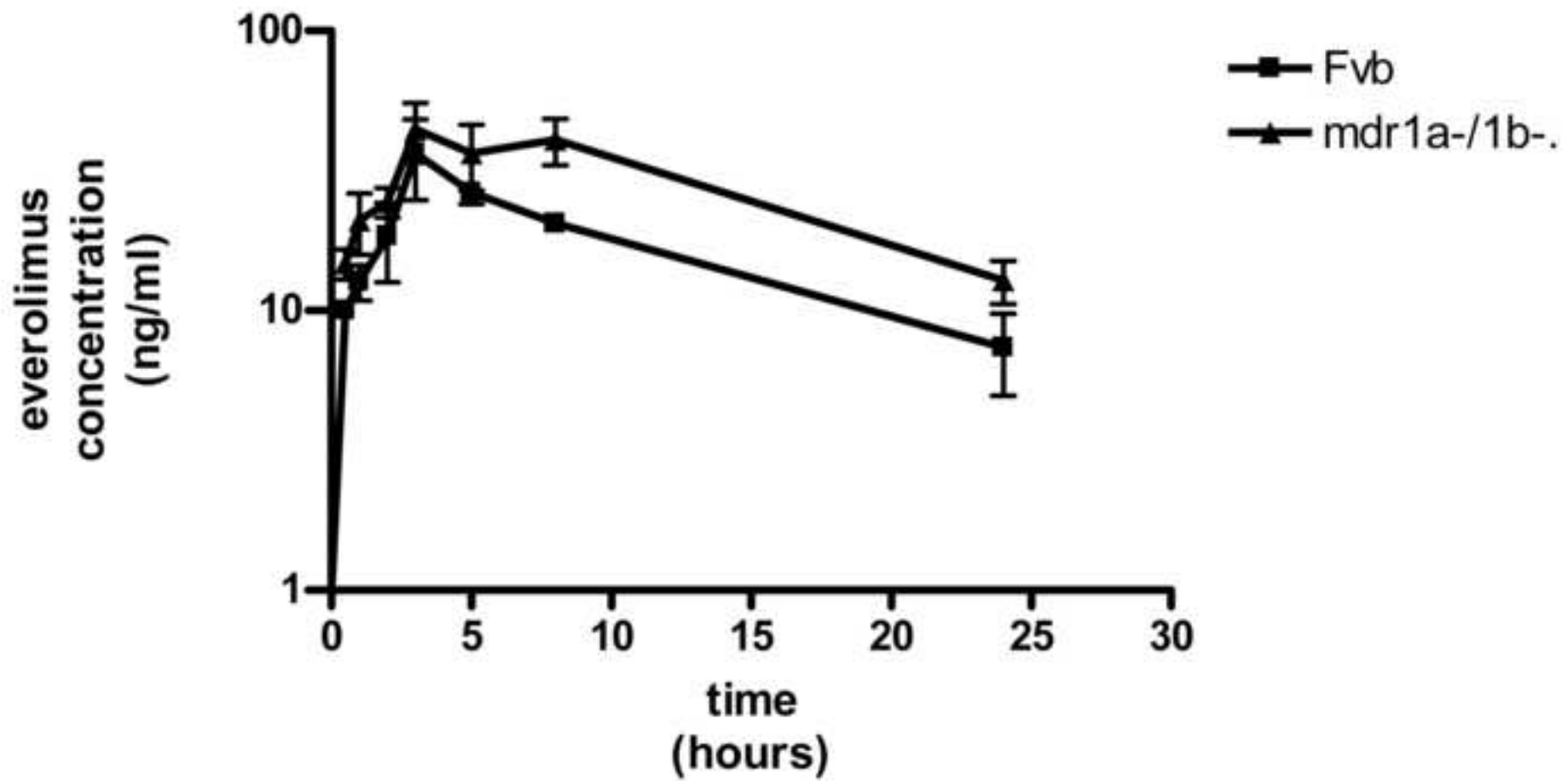


Figure 2

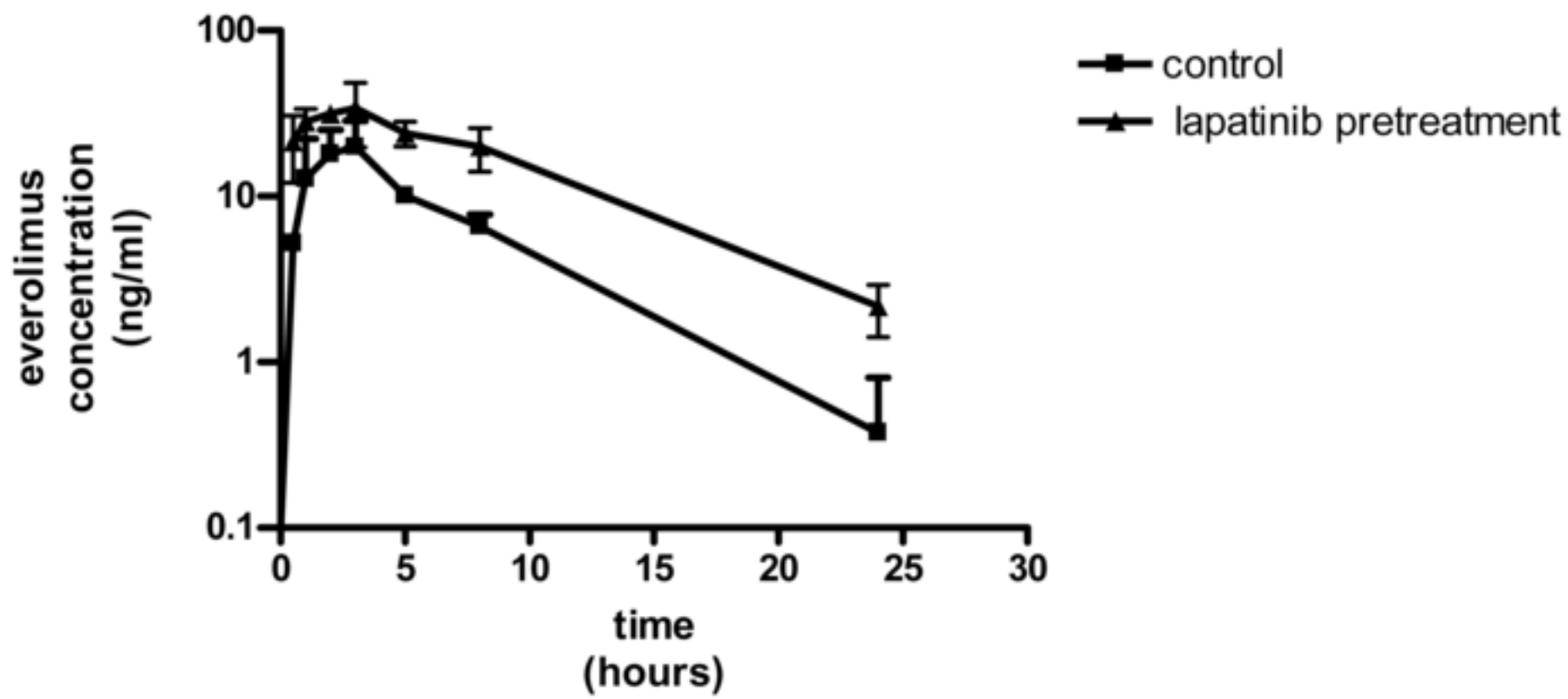


Figure 3

Figure 4

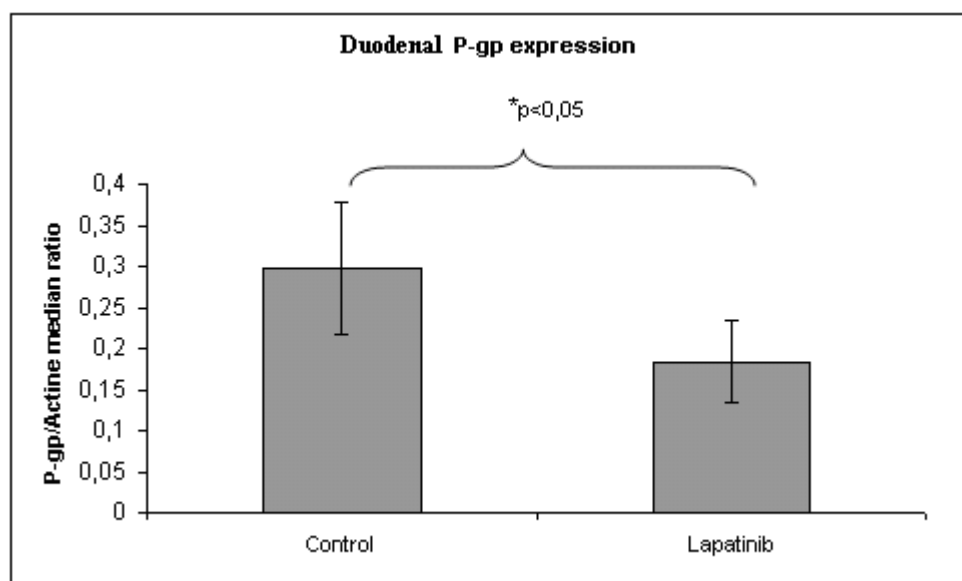


figure 5

