DMD Fast Forward. Published on March 10, 2008 as doi:10.1124/dmd.107.019687

DMD #19687

Characterization of Cytochrome P450 protein expression along the entire length of the intestine of male and female rats

Doreen Mitschke, Andreas Reichel, Gert Fricker, Ursula Moenning

DM, AR, UM: Research Pharmacokinetics, Bayer Schering Pharma AG, Berlin, Germany;

GF: Institute of Pharmacy and Molecular Biotechnology, University of Heidelberg, Germany

Running Titel: CYP P450 protein expression along the entire intestine in rat

Corresponding author:

Doreen Mitschke

Bayer Schering Pharma Ag

FPK

Muellerstr. 178

13353 Berlin

Phone: +49-30-468-16752

Fax: +49-30-468-12238

Email: Doreen.mitschke@bayerhealthcare.com

Number of text pages: 28

Number of tables: 3

Number of figures: 3

Number of references: 39

Number of words in

Abstract: 191

Introduction: 690

Discussion: 1930

Abbreviations:

CYP P450, Cytochrome P450; GI-tract, gastro-intestinal tract; room temperature, RT;

DMD Fast Forward. Published on March 10, 2008 as DOI: 10.1124/dmd.107.019687
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract:

Intestinal CYP P450 proteins play an important role in the biotransformation of drugs and may significantly limit their oral absorption and bioavailability. Therefore, we have investigated the amount of CYP P450 proteins via Western blot analysis along the entire intestine of male and female rats. Despite of the use of an inbred rat strain, controlled housing conditions for the animals and a timed sample preparation, high interindividual differences in the expression of all CYP P450 proteins was observed. **CYP3A** (135 to 243 fmol/mg protein) and **CYP2B1** (107-645fmol/mg protein) were the most abundant CYP P450 isoforms in the duodenum and jejunum of rat intestine, but were neither present in the ileum nor in the colon. Compared to CYP2B1 and CYP3A, **CYP2D1** (25-71fmol/mg protein) and **CYP2C6** (3-10fmol/mg protein) were only expressed in minor amounts. CYP2C11 could not be identified in the entire rat intestine. In conclusion, this is the first systematic evaluation and quantification of the expression of CYP P450 proteins along the entire length of the intestine in the rat both male and female. This data will provide a basis for a better understanding of the extent of intestinal metabolism along the gastrointestinal tract.

Introduction:

Absorption through the gut is a key step in the oral delivery of drugs. The main interface between gut lumen and blood stream is an epithelial cell layer consisting of polarized enterocytes controlling the passage of exogenous substances into the portal circulation. Enterocytes have a variety of structural features, including tight junctions reducing paracellular permeability, numerous drug transporters and a set of metabolic enzymes that may all affect the entry of drugs into the body. The extent to which a drug is absorbed also depends on the intrinsic properties of the compound such as solubility, permeability, efflux or uptake transport properties and susceptibility to metabolic degradation (Martinez, 2002; Benet, 2004).

Although the liver is known as the major site of first pass extraction, recent studies have indicated that the small intestine also contributes significantly to the first pass metabolism of many drugs, e.g. cylosporine (Wu, 1995), nifedipine (Iwao, 2002), midazolam (Paine, 1996) and diltiazem (Iwao, 2004). It is known that several uptake-, efflux-transporter and CYP P450 isoforms are expressed in the human and rat intestine (van de Kerkhof, EG 2007). If a drug is a substrate of efflux transporters, it may enter and exit the enterocytes several times and, with each cycle, small quantities may be metabolized by Cytochrome P450 (CYP P450) enzymes localized in the endoplasmic reticulum. The CYP P450 enzymes belong to a superfamily of hemeproteins that show a broad substrate specificity, with substrates ranging in size from ethylene (M_r 28) to cyclosporine (M_r 1201) (Isin and Guengerich, 2007). Although the liver is regarded as the main organ of drug metabolism, CYP P450 proteins are also expressed in other tissues, e.g. kidney, lung, and the gastrointestinal tract (GIT). While there is only rudimentary

information available on CYP P450 proteins in the human GIT, there is even less data on intestinal CYP P450 expression in rat. Currently, 18 CYP P450 families and 45 CYP P450 subfamilies are known in human, while 14 CYP P450 families and 29 CYP P450 subfamilies have been reported in rat (http://cpd.ibmh.msk.su/). Of those families the members of the CYP1, CYP2 and CYP3 gene family play the most important role in the phase I metabolism in liver and intestine. The analysis of CYP P450 in human GIT revealed that CYP2C, CYP1A1, CYP3A4, CYP2D6, and CYP2J2 are expressed (Obach RS, 2001; Paine MF 2006). Analysis of rat CYP P450 mRNA expression in the intestine demonstrated that CYP3A1, 2B1, 2C6, 2C11, 1A1 and 2D1-4 could be detected in significant amounts (Hiroi, 1998; Kaminsky and Zhang, 2003, Lindell, 2003). For CYP2C11 and CYP2C12 a gender-dependent protein expression has been published based on liver, with CYP2C11 being exclusively expressed in the male and CYP2C12 in the female rat (Mugford and Kedderis, 1998). However, CYP1A2, 2A1, 2B2, 2E1, 3A2 and 4A2 mRNA expression could be observed in rat liver, but not in the intestine of rats (Hiroi, 1998; Kaminsky and Zhang, 2003). While many of the CYP P450 isoforms are expressed constitutively, some are expressed as a result of induction which can occur using normal chow diet as is the case for CYP1A1 (Kaminsky and Zhang, 2003), suggesting a highly variable expression pattern of CYP P450 proteins along the GIT.

As most of the existing CYP P450 expression data in the rat GIT was obtained by mRNA expression analysis, in particular real-time PCR, there is only little information regarding the actual protein expression of CYP P450 proteins. Furthermore, most of the published data represent only selected gut segments and do not yield a comprehensive picture into the CYP P450 expression along the full

length of the intestines which in rat span a length of about 90 cm (Iatropoulos, 1986). Therefore, we have for the first time generated protein expression data of several CYP P450 proteins (CYP3A, 2B1, 2C6, 2C11, 2D1, 1A1) in full spatial resolution along the entire intestinal tract of female and male rat. As a general expression pattern, CYP3A and CYP2B1 were the predominant CYP P450 proteins in the upper intestine, while CYP2D1 and CYP2C6 dominated in the lower intestine and colon. There were high interindividual differences in the expression of all CYP P450 proteins.

Material and Methods:

Chemicals

Dithiothreitol, sucrose, L-histidine, peroxidase conjugated goat anti-rabbit whole molecule IgG, peroxidase conjugated goat anti-mouse whole molecule IgG were purchased from Sigma Aldrich (Munich, Germany). Protease inhibitor cocktail with EDTA were purchased from Roche (Basel, Switzerland) and EDTA, Urea were purchased from VWR (Darmstadt, Germany). NuPAGE® 4x LDS sample buffer, NuPAGE® 10x sample reducing agent, NuPAGE® Novex® 4-12% Bis-Tris Gels, NuPAGE® MOPS SDS Running Buffer, Seeblue®, MagicMark™, NuPAGE® Tranfer Buffer, NuPAGE® Antioxidant, DPBS (-$MgCl_2$, -$CaCl_2$) were purchased from Invitrogen (Karlsruhe, Germany). "BCA Assay Protein Kit" was purchased from Perbio Science (Bonn, Germany). Rabbit anti-rat CYP2D1 polyclonal antibody was purchased from BIOMOL international (Hamburg, Germany). Mouse anti-rat CYP2B1/2, 2C6, 3A1 monoclonal antibodies were purchased from Abcam plc (Cambridge, UK). Mouse anti-rat CYP1A1 monoclonal antibody was purchased from Natutech GmbH (Frankfurt am Main, Germany). Mouse anti-rat CYP2C11 monoclonal antibody was purchased from Dunn Labortechnik GmbH (Asbach, Germany). Rabbit anti-rat Villin polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc (Heidelberg, Germany). CYP Supersome™ were purchased from BD Biosciences (Heidelberg, Germany)

Animal Housing

All surgical and experimental procedures were reviewed and approved by legal authorities. The study used Han-Wistar rats (Charles River, Berlin-Buch) weighing

200-340 g. Animals were kept at 22 °C with a 12 h on, 12 h off light cycle and were allowed free access to standard rat diet (ssniff Spezialitäten GmbH, Soest, Germany (V1534-000 R/M-H)) and water.

Preparation of Intestinal Homogenate

All preparations were performed starting approximately 9AM in order to avoid any diurnal changes. Rats were anesthetized with isoflurane and killed by cervical dislocation. The intestine was dissected complete from pylorus to colon, however without caecum.

Intestinal homogenate was prepared by a method described by Mohri (2001) with some modifications. The intestine was rinsed with ice cold “rinsing buffer” (pH 7.3, 1.5 mM KCl, 96 mM NaCl, 27 mM sodium citrate, 8 mM KH_2PO_4, 5.6 mM Na_2HPO_4 and protease inhibitor cocktail) and cut into pieces of three centimeter length. The first three samples after the stomach were defined as duodenum, the last sample before the caecum as ileum and all samples in-between as jejunum (Sharp and LaRegina, 1998). The samples after the caecum were set as colon. Thereafter, each piece was rinsed with “separation buffer” (pH 7.4, 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 1.5 mM EDTA, 0.5 mM dithiothreitol and protease inhibitor cocktail). Intestinal pieces were placed on an ice cold glass plate and gently squeezed out to collect the matured enterocytes. The separation procedure was repeated three times after which the separated cells were washed with “homogenizing buffer” (pH 7, 5 mM histidine, 0.25 M sucrose, 0.5 mM EDTA and protease inhibitor cocktail) and centrifuged at 800*g* for 10 min at 4 °C. The cells were disintegrated in a Potter-homogenizer at 500 rpm in 3 ml “homogenizing buffer” and centrifuged at 850 g for 5 min at 4 °C. The resulting

supernatant was carefully removed and stored in aliquots at -80 °C until use. Protein concentrations were determined with the "BCA Assay Protein Kit" according to the manufacturer's instructions.

Preparation of Liver Homogenate

Rats were anesthetized with isoflurane and killed by cervical dislocation. The liver was flushed with icecold "rinsing buffer" via the portal vein. Afterwards, the whole liver was dissected and cut to small pieces with a scalpel. The liver pieces were washed three times with icecold "rinsing buffer" and homogenized in a Potter-homogenizer at 500 rpm in 50 ml "homogenizing buffer" and centrifuged at 850 g for 5 min at 4 °C. The resulting supernatant was carefully removed and stored in aliquots at -80 °C until use. Protein concentrations were determined with the "BCA Assay Protein Kit" according to the manufacturer's instructions.

Immunoblotting

Western blot samples were obtained after precipitation of proteins from intestinal homogenates with acetone (1:5 $^{v}/_{v}$) at -80 °C overnight. Precipitated proteins were resuspended in 8 M urea (10 µl/100 µg protein) and denaturated for 30 min at 55 °C in "sample buffer" (NuPAGE® 2x LDS sample buffer, NuPAGE® 2x sample reducing agent). Samples (100 µg protein/lane) were resolved in 4-12 % Bis-Tris gradient gel (NuPAGE® Novex®); Invitrogen) by SDS gel electrophoresis.

Twenty five microgram liver homogenate were used as positive control. Proteins were electro blotted onto nitrocellulose membranes for one hour at room temperature (RT), nonspecific binding sites were blocked with 5 % (w/v) non-fat dried milk in TBS-T buffer containing 10 mM Tris-Hcl pH 7.5, 200 nM NaCl, 0.1 % (v/v) Tween 20. Blots were incubated for one hour at RT with either mouse anti-

rat CYP2C6 (1:4000), 1A1 (1:4000), 3A1 (1:2000), 2B1/2 (1:3000), 2C11 (1:4000) monoclonal antibody or rabbit anti-rat CYP2D1 (1:5000) polyclonal antibody. For reference, Villin was detected using a polyclonal antibody (1:5000). The specificity of antibodies used was tested beforehand. For the CYP2D1 and CYP3A1 antibody minor interferences were found with CYP2B1 and CYP3A2, respectively (Data not shown). Since several groups have demonstrated that CYP2B2 mRNA is not expressed in the intestine. (Kaminsky and Zhang, 2003) we assume that the Western blot signal of the CYP2B1/2 antibody reflects only the CYP2B1 expression.

After washing, membranes were incubated with peroxidase-conjugated goat anti-rabbit (1:20000) or anti-mouse (1:10000) whole molecule IgG for one hour and then washed 3 times with TBS-T. The Western Lightning® (Perkin Elmer, Waltham, Massachusetts, USA) chemiluminescent detection system in combination with the ChemiDoc™ XRS (BioRad Laboratories, Munich, Germany) were used for band-visualization. The Western blots were analyzed densitometrically using the Quantity One 1-D Software (BioRad, Laboratories, Munich, Germany). The CYP P450 signal was normalized with the Villin band density.

Intestinal CYP P450 quantification

Protein levels in intestinal and liver homogenates of CYP2B1, 3A, 2C6 and 2D1 were determined via Western blot analysis using a calibration curve with the respective recombinant rat CYP P450 enzymes (CYP Supersomes™). The amounts of CYP P450 Supersome™ for calibration were chosen according to the antibody sensitivity and in order to ensure linearity of the calibration curve. To ensure the

reproducibility of the CYP quantification, the measurement of the amount of CYP protein was repeated three times per segment per animal. As an example, the reproducibility of the quantification of the CYP3A and CYP2D1 proteins are shown in Table 1.

The CYP P450 amount was determined in intestinal homogenates from six different GIT segments (duodenum, upper jejunum, middle jejunum, lower jejunum, ileum and colon) and liver from three male and just in the intestine from three female rats.

Results:

CYP P450 expression profile along the entire intestine

The intestines of four male and female rats were prepared for Western blotting as described under Materials and Methods. Typical Western blots are shown in **FIG 1**. For CYP2C11 no signal was detected in any intestinal segment of the rat. The expression profiles of CYP3A, 2B1, 1A1, 2C6 and 2D1 were evaluated (i) along the GIT (ii) between female and male rats, and (iii) between individuals of the same sex in order to extract general expression pattern for intestinal CYP P450 proteins and to assess typical inter-subject and gender differences (**FIG 2**).

Despite of the usage of an inbred rat strain, controlled housing condition for the animals and a timed sample preparation, high interindividual differences in the expression of all CYP P450 proteins could be detected. Furthermore, the expression did often not proceed steadily along the intestine, but showed a intermittent profile with alternating peak and valley areas. This intermittent expression occurred pronounced for CYP3A and CYP2B1. Moreover, both CYP P450 proteins showed a similar expression profile insofar as they had high expression levels in the upper intestine and were hardly detectable in the lower intestine **(FIG 2a).** In some rats the highest **CYP3A** expression levels were obtained directly after the pylorus. Other animals showed an increasing expression up to the upper jejunum or even up to the middle of the jejunum. In most of the animals the CYP3A signal disappeared somewhere in the lower intestine, but the exact spot varied considerably. Apart from higher interindividual variations in the female rat, no gender dependent expression profile were detectable. In female rats **CYP2B1** protein expression

increased from the pylorus to the upper jejunum and then decreased to relatively low levels at the end of the jejunum **(FIG 2 a)**. In contrast, the expression profile of male rats undulated in the upper intestine without any concerted expression peaks among the animals. The following signal decline from the middle of the jejunum was similar in both genders. Compared to female rats the male counterparts exhibited larger interindividual variation in the CYP2B1 expression.

In contrast to CYP3A and CYP2B1 the other CYP P450 investigated (**CYP1A1, 2D1 and 2C6**) were present along the whole intestine (**FIG 2a, 2b**). The three remaining CYP P450 proteins displayed a flat expression profile with highest interindividual variations at the endings of the intestine, close to the pylorus and/or around the caecum and a rather uniform expression in the middle of the jejunum. **CYP1A1** revealed either a flat expression along the whole intestine or an expression decrease/increase in the upper/lower gut regions respectively (**FIG 2a**). Due to the high variability no gender difference for CYP1A1 was discernable, but in the female rats a distinct intermittent expression course was detected. The expression profiles of CYP2C6 and CYP2D1 were comparable (**FIG 2b**). They were generally flat. Variability occurred mostly in the lower region of the intestine and seldom in the middle part. In male rats, highest expression was found in the lower parts in particular in the colon. A tube-shape profile of CYP2D1 expression could be observed in male rats due to an additional variability in the upper part of the intestine , which cannot be seen as clearly in females.

Intestinal and Liver CYP P450 quantification

The absolute CYP P450 content was determined for four different CYP P450 enzymes in six selected intestinal 3 cm-pieces from different segments in three

female (**Table 2**) and three male rats **(Table 3).** For the male rats, also the CYP P450 content of the liver was calculated **(Table 3)**. As reference protein and calibration probe, recombinant rat CYP P450 proteins expressed in insect cells (Supersomes™/BD Bioscience) were used. An example of such a Western Blot is shown in **FIG 3**. In all intestinal segments examined no gender differences in any of the cytochrome P450 proteins were observed.

CYP3A and CYP2B1 were the most abundant CYP P450 isoforms in the duodenum and jejunum of rat intestine but were neither present in the ileum nor in the colon. They represented approximately 90% of the sum of amount of intestinal CYP P450 in duodenum and jejunum of both genders. Whereas, CYP2B1 was expressed in relative constant amounts (332-392 fmol/mg protein) in male rats, the absolute expression levels in female rats increased from the duodenum to the upper jejunum six times (107 to 645 fmol/mg protein) and dropped back to the initial expression value at the end of the jejunum (98 fmol/mg protein). In contrast to CYP2B1, CYP3A was expressed in comparable amounts in the jejunum of both genders (135 to 243 fmol/mg protein).

Compared to CYP2B1 and CYP3A, CYP2D1 and CYP2C6 were only expressed in minor amounts. Due to the absence of CYP3A and CYP2B1 in the ileum and colon, CYP2D1 is the predominantly expressed CYP P450 isoform in the lower intestinal region. Although expressed at just 44-71 fmol/mg protein it represented around 90% of the total sum of CYP P450 protein there. In all intestinal segments CYP2C6 contributed only 3-10 fmol/mg protein to total CYP P450 amount. Therefore, CYP2C6 constituted less than 1% of the total CYP P450 amount and may thus play a minor role for the metabolism in the gut.

However, in the liver all CYP enzymes investigated were expressed in very high amounts **(Table 3)**. The sum of all CYP P450 proteins investigated was 15 times higher than in the proximal intestine, and 100 to 150 times higher than in the distal intestine.

In contrast to the intestine, CYP 2C6 was the most abundantly expressed CYP P450 isoform in the liver with 2373 fmol/mg protein. The other three CYP P450 enzymes were expressed in comparable amounts in the liver of about 1500-1900 fmol/mg protein.

Discussion:

The oral route is the most convenient drug administration route. Since intestinal metabolism may have a strong impact on the pharmacokinetics of a drug, the successful development of a drug will rely on the comprehensive knowledge on the involvement of metabolic enzymes along the intestine.

Like hepatocytes, enterocytes containe a set of different CYP P450 enzymes which are able to metabolize drugs. Indeed, intestinal drug metabolism has been proposed to play an important role in the biotransformation of drugs and may significantly limit their oral absorption and bioavailability. Although the enzymes involved, the CYP P450 proteins, have attracted a lot of attention in terms of liver metabolism, only little information exists regarding their expression in the gut wall, especially in terms of the spatial resolution along the whole length of the intestine.

A number of CYP P450 enzymes have been shown to be constitutively expressed in the GIT of several species. Most of the reported expression analysis was done based on mRNA levels (Yan and Caldwell, 2001) in rat or based on protein levels in human (Paine, 2006). These rat data, however, are not predictive for the actual protein expression and hence the functional activity. In this study protein expression data were obtained for the absolute expression of several major CYP P450 proteins along the entire intestine of the rat. The analysis was done by complete segmentation of the entire length of the intestines in both female and male rats to get the full protein expression profile.

It could be demonstrated in this study that a remarkable interindividual variability is characteristic for the expression of the major drug-metabolizing CYP P450

enzymes. This is in line with published results from other working groups (Snawder JE, 2000). The same holds true for the efflux transporters in the intestine (MacLean et al., submitted).

Nevertheless, some typical expression pattern could be discerned. **CYP3A and CYP2B1** were expressed predominately in the upper intestine and were not detectable in the ileum and colon. This is consistent with data published by Matsubara et al. (2004), Takara et al. (2003) and Takemoto et al (2003) showing a decreasing expression of CYP3A mRNA and a decreasing activity along the whole intestine. Even for the human equivalent CYP3A4 a declining mRNA expression and activity profile along the human intestine could be demonstrated (Thummel et al. 1997). The same holds true for CYP2B1 which was also found to be less expressed at lower intestinal regions (Takemoto, 2003; Rosenberg 1991).

The declining expression of the CYP enzymes at lower intestinal regions may be in part explained by the gut flora. It is known for the human intestine, that a minimum of 1000 bacteria species are present in the gut. The duodenum and jejunum have only a few bacteria, while ileum and colon contain a large and diverse microbial population (Tlasklova-Hogenova, 2004). Thus, the increase of microbial population is inversely proportional to the CYP3A and CYP2B1 protein expression along the gut. The relationship between host and gut flora is mostly harmonious and beneficial for the host. The bacteria among other things break down non digestible carbohydrates and also metabolize bile acids, bilirubin, and cholesterol (Mahida, 2003, Neish, 2002). The inverse proportionality of the gut microflora with some CYPs P450 might support the hypothesis that bacteria take over the function of some intestinal CYP P450's in the distal regions of the intestine. For example, it was shown that isoflavones such as dadzein and glycitein are extensively

metabolized by the intestinal microflora of man and rat (Rafii F, 2007; Simons AL, 2005). This hypothesis is in accordance with findings of et al (2001) who showed a decreased CYP2D2 mRNA expression in the ileum of normal mice as compared to germ-free mice.

In contrast to **CYP3A** and **CYP2B1, CYP1A1, CYP2C6** and **CYP2D1** displayed a generally flat profile with highest interindividual variations at the endings of the intestine, close to the pylorus and/or around the caecum and a rather uniform expression in the middle of the jejunum. In male rats the CYP2C6 and CYP2D1 expression seemed to increase in the lower intestine, but this could not be seen for the female animals. To our knowledge no other working group published any mRNA or protein expression profiles for CYP1A1, 2C6 and 2D1 along the rat intestine. In context with the enteric flora one might assume, that bacteria are not able to take over the substrates metabolized by CYP1A1, 2C6 and 2D1, because those enzymes are still expressed in bacteria rich areas of the gut.

In addition, we have not only examined the expression profiles, but we have also investigated the absolute amount and relative contribution of each CYP P450 in the main regions of the rat intestine (Duodenum, Jejunum, Ileum, Colon). The results showed, that CYP2B1 and CYP3A represent the major CYP P450 isoform in the upper intestine of the rat, where they account approximately 90% of the total CYP amount. CYP2D1 and CYP2C6 are expressed in lower amount in the gut and play only a minor role in the upper intestine. In the ileum/colon no CYP3A and CYP2B1 expression could be detected and therefore, CYP2D1 dominates this region with 85-91% over CYP2C6 (9-15%). CYP2C11 could not be identified in the entire rat intestine, which is consistent with other work groups, which found either a very weak signal or no signal at all (Waziers, 1990, Zhang 1996).

In the liver, however, the CYP P450 isoforms showed a completely different distribution pattern. In contrast to the intestine, CYP2C6 and CYP2D1 are expressed equally or even higher than CYP3A and CYP2B1. CYP2C6 is the major expressed isoform (32 %) in the liver of rats, followed by CYP2D1 (25 %), CYP2B1 (23 %) and CYP3A (20 %). This distribution pattern is an accordance with published data by Yan and Caldwell (2001) who demonstrated that the CYP2C family is predominant in the liver (65 %), followed by CYP3A2 (14.6 %), CYP2B1 (1.9 %) and CYP1A1/2 (1.2 %), while the expression of CYP2D1 was not examined. The low expression level of CYP2B1 is in discrepancy to our study with one possible explanation being the high inducibility of CYP2B1. Indeed, Stott et al (2004) showed that the CYP expression is dependent on the diet of the animals. They also reported differences depending on the rat strain.

Due to availability of suitable antibodies, we have examined four CYP P450 isoforms, but we can not exclude that other CYP P450 proteins are also expressed in remarkable amounts in the intestinal wall. For example, CYP2J3 is known to be expressed in rat intestine on the mRNA level, as well as other family members of the CYP2D family (Zeldin, 1997, Hiroi, 1998).

In human the CYP P450 intestinal “pie” chart of the proximal jejunum displays CYP3A (82 %), followed by CYP2C9 (14 %), CYP2C19 (2 %), CYP2J2 (1.4 %) and CYP2D6 (0.7 %) (Paine, 2006). Therefore, CYP3A is one of the most important CYP P450 proteins in rat and human, but in rat represent only 26-49% of the sum of CYP P450 amount in the duodenum and upper jejunum. Interestingly, the human equivalent to the most abundant CYP P450 in rat intestine, CYP2B6, is neither expressed on the mRNA nor on the protein level in the human intestine (Paine, 2006, Zhang, 1999). Furthermore, CYP2C9/19 are predominant over

CYP2D6 in human, but not in rat. CYP2D1 represented 8 to 19% in the rat upper intestine, while CYP2C6 represented less than one percent of the sum of CYP P450 amount. In conclusion, the CYP P450 distribution in the human gut is not entirely comparable with that in rat. Furthermore, the corresponding CYP P450 isoform in man often does not possess the same substrate recognition and does not catalyze the same reaction as the related isoform in rat (da Fonseca RR, 2006; Guengerich FP, 1997). For example, midazolam which is a substrate for human CYP3A4/5 and rat CYP3A1/2 (Kotegawa T, 2002) is extensively metabolized in the human intestine but not in the rat intestine. The manifestation of these differences between human and rat probably occurred to obtain the best suited CYP P450 enzymes for dietary habits and environment of each species (Lewis DF, 1998).

Taken together, the extensive amount of data of the present study both in terms of the number of intestinal segments and the number of individuals, clearly show that there is a remarkable variability in the protein expression of intestinal CYP P450 proteins along the GIT of the rat. Although there are some clear gender differences, the inter-subject variability in the CYP P450 expression within the same sex seems to be larger than between sexes making it difficult to extract simple pattern for the CYP P450 expression in the GIT. Moreover, the expression of many CYP P450s seems to resemble the shape of waves along the GIT almost as if the expression levels following the peristaltic movement of food through the gut lumen. Indeed, the animals used were not fasted and the intra-subject expression pattern of the CYP P450 proteins may relate to the individual feeding history with some segments of the GIT having had closer contact to food than others as a result of the typical intermittent motility of the gut after food intake. Thus, not every gut villus may get exposed to the food to the same extent. Considering the very short life-

span of matured enterocytes of about two days in rat, their rapid maturation during the migration to the villous tip and their exposure status at the villous tip until they are sloughed off (Iatropoulos, 1986) may well result in large local differences in the CYP P450 protein expression of intestinal villi with wave-shaped patterns along the GIT.

The present conclusions are based on very systematically collected protein expression data, however, how the in parts very strong differences in the expression translate into functional differences in local intestinal metabolism is currently subject to a subsequent study (in preparation). Furthermore, the protein expression analysis by Western blot have their limitations mainly due to availability and selectivity of antibodies used, thus that it is important to verify the data by functional data.

In summary, using a very comprehensive approach we have generated for the first time a full set of consistent data allowing the examination of the expression pattern of CYP P450 proteins more systematically than ever before. The data suggests that compounds which are substrates of CYP2B1 and CYP3A may be more subject to metabolism in the rat intestine than substrates of CYP2C6 and CYP2D1. Once the protein expression data have been confirmed by functional data and sufficient information about the expression and functional contribution of transporters along the intestines is available, this data can be used to generate quantitative scaling factors for the intestinal metabolism in the rat.

Acknowledgments:

We thank Nicole Ventz and Caroline MacLean for technical assistance.

References:

Benet LZ, Cummins CL, Wu CY (2004) Unmasking the dynamic interplay between efflux transporters and metabolic enzymes. *Int J Pharmaceut* **277**:3-9

Fonseca RR da, Antunes A, Melo A, Ramos MJ (2006) Structural divergence and adaptive evolution in mammalian cytochromes P450 2C. *Gene.* **387**(1-2):58-66

Guengerich FP (1997) Comparison of catalytic selectivity of cytochrome P450 subfamily enzymes from different species. *Chem Biol Interact* **106**(3):161-82

Hiroi T, Imaoka S, Chow T, Funae Y (1998) Tissue distribution of CYP2D1, 2D2, 2D3 and 2D4 mRNA in rats detected by RT-PCR. *Biochim Biophys Acta* **1380**(3):305-12

Hooper LV, Wong MH, Thelin A, Hansson L, Falk PG, Gordon JI (2001) Molecular analysis of commensal host-microbial relationships in the intestine. *Science* **291**(5505):881-4.

Iatropoulos MJ (1986) Morphology of the gastrointestinal tract: in Gastrointestinal Toxicology (Rozman K and Hannenen O, eds.) pp. 246-266, Elsevier, Amsterdam

Isin EM, Guengerich FP (2007) Complex reactions catalyzed by cytochrome P450 enzymes. *Biochim Biophys Acta* **1770**(3):314-29

Iwao T, Inoue K, Hayashi Y, Yuasa H, Watanabe J (2002) Metabolic extraction of nifedipine during absorption from the rat small intestine. *Drug Metab Pharmacokin* **17**(6):546-553

Iwao T, Inoue K, Hayashi Y, Yuasa H, Watanabe J (2004) Absorption and metabolic extraction of diltiazem from the perfused rat small intestine. *Drug Metab Pharmacokin* **19**(6):430-437

Kaminsky L, Zhang QY (2003) The small intestine as a xenobiotic-metabolizing organ. *Drug Meta Dispos* 31: 1520-1525

Kotegawa T, Laurijssens BE, von Moltke LL, Cotreau MM, Perloff MD, Venkatakrishnan K, Warrington JS, Granda BW, Harmatz JS, Greenblatt DJ (2002), In Vitro, Pharmacokinetic, and Pharmacodynamic Interactions of Ketoconazole and Midazolam in the Rat. *J Pharmaco Exp Ther* **302**:1228-1237

Lewis DF, Watson E, Lake BG (1998) Evolution of the cytochrome P450 superfamily; sequence alignments and pharmacogenetics. *Mutat Res* **410**(3):245-70

Lindell M, Lang M, Lennernaes H (2003) Expression of genes encoding for drug metabolising cytochrome P450 enzymes and P-glycoprotein in the rat small intestine; comparison to the liver. *Eur J Drug Metab Pharmacokinet* **28**(1):41-8

Mahida YR (2004) Microbial-gut interactions in health and disease. Epithelial cell responses. *Best Pract Res Clin Gastroenterol.* **18**(2):241-53

Martinez MN, Amidon GL (2002) A mechanistic approach to understanding the factors affecting drug absorption: a review of fundamentals. *J Clin Pharmacol* **42**:620-643

Matsubara T, Kim HJ, Miyata M, Shimada K, Nagata K, Yamazoe Y (2004) Isolation and Characterization of a major intestinal CYP3A form, CYP3A62, in the rat. *J Pharmaco Exp Ther* **309**:1282-1290

Mohri K, Uesawa Y (2001) Enzymatic Activities in the Microsomes Prepared from Rat Small Intestinal Epithelial Cells by Differential Procedures. *Pharm Res* **18**(8):1232-1236

Mugford CA, Kedderis GL (1998) Sex-dependent metabolism of xenobiotics. *Drug Metab Rev* **30**:441-498

Neish AS (2002) The gut microflora and intestinal epithelial cells: a continuing dialogue. *Microbes Infect*. **4**(3):309-17

Paine MF, Shen DD, Kunze KL, Perkins JD, Marsh CL, McVicar JP, Barr DM, Gillies BS, and Thummel KE (1996) First-pass metabolism of midazolam by the human intestine. *Clin Pharmacol Ther* **60**:14-24

Obach RS, Zhang QY, Dunbar D, Kaminsky LS (2001) Metabolic Characterization Of The Major Human Small Intestinal Cytochrome P450s. *Drug Meta Dispos* **29**: 347-3521

Paine MF, Hart HL, Ludington SS, Haining RL, Rettie AE, Zeldin DC (2006) The human intestinal Cytpchrome P450 „Pie". *Drug Meta Dispos* **34**: 880-886

Rafii F, Jackson LD, Ross I, Heinze TM, Lewis SM, Aidoo A, Lyn-Cook L, Manjanatha M. (2007) Metabolism of daidzein by fecal bacteria in rats. *Comp Med*. **57**(3):282-286

Rosenberg DW (1991) Tissue-specific induction of the carcinogen inducible cytochrome P450 isoform, P450 1A1, in colonic epithelium. *Arch Biochem Biophys* **248**(1):223-226

Sharp PE, LaRegina MC (1998) *The Laboratory Rat*. CRC Press LLC, Boca Raton Florida

Simons AL, Renouf M, Hendrich S, Murphy PA. (2005) Metabolism of glycitein (7,4'-dihydroxy-6-methoxy-isoflavone) by human gut microflora. *J Agric Food Chem.* **53**(22):8519-8525

Snawder JE, Lipscomb JC. (2000) Interindividual variance of cytochrome P450 forms in human hepatic microsomes: correlation of individual forms with xenobiotic metabolism and implications in risk assessment. *Regul Toxicol Pharmacol*. **32**(2):200-209.

Stott WT, Kan HL, McFadden LG, Sparrow BR, Gollapudi BB (2004) Effect of strain and diet upon constitutive and chemically induced activities of several xenobiotic-metabolizing enzymes in rats. *Regul Toxicol Pharmacol* **39**:325-333

Takara K, Ohnishi N, Horibe S, Yokoyama T (2003) Expression profiles of drug-metabolizing emzym CYP3A and drug efflux transporter multidrug resistance 1 subfamily mRNAs in rat small intestine. *Drug Meta Dispos* **31**:1235-1239

Takemoto K, Yamazaki H, Tanaka Y, Nakajima M, Yokoi T (2003) Catalytic activities of cytochrome P450 enzymes and UDP-glucuronosyltransferases involved in drug metabolism in rat everted sacs and intestinal microsomes. *Xenobiotica* **33**:(1) 43-55

Thummel KE, Kunze KL, Shen DD (1997) Enzyme-catalyzed processes of first-pass hepatic and intestinal drug extraction. *Adv Drug Deliv Rev* **27**:99-127

Tlaskalová-Hogenová H, Stepánková R, Hudcovic T, Tucková L, Cukrowska B, Lodinová-Zádníková R, Kozáková H, Rossmann P, Bártová J, Sokol D, Funda DP, Borovská D, Reháková Z, Sinkora J, Hofman J, Drastich P, Kokesová A (2004) Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases. *Immunol Lett.* **93**(2-3):97-108

Van de Kerkhof EG, de Graaf IAM, Groothuis GMM (2007) *In Vitro* Methods to Study Intestinal Drug Metabolism. *Curr Drug Metab. ;***8**(7):658-75

Waziers I de, Cugnec PH, Yang CS, Leroux JP, Beaune PH (1990) Cytochrome P450 isoenzymes, epoxide hydrolases and glutathione transferases in rat and human hepatic and extrahepatic tissues. *J. Pharmacol. Exp. Ther.* **253**:387-394

Wu CY, Benet LZ, Hebert MF, Gupta SK, Rowland M, Gomez DY, and Wacher VJ (1995) Differentiation of absorption and first-pass gut and hepatic metabolism in humans: studies with cyclosporine. *Clin Pharmacol Ther* **58**:492-497

Yan Z, Caldwell GW (2001) Metabolism profiling, and cytochrome P450 inhibition & induction in drug discovery. *Curr Top Med Chem.* **1**(5):403-25

Zeldin DC, Foley J, Goldsworthy SM, Cook ME, Boyle JE, Ma J, Moomaw CR, Tomer KB, Steenbergen C, Wu S (1997) CYP2J subfamily cytochrome P450s in the gastrointestinal tract: expression, localization, and potential functional significance. *Mol Pharmacol* **51**:931-943

Zhang QY, Wikoff J, Dunbar D, Kaminsky L (1996) Characterization of rat small intestinal cytochrome P450 composition and inducibility. *Drug Meta Dispos* **24**(3):322-328

Zhang QY, Dunbar D, Ostrowska A, Zeisloft S, Yang J, Kaminsky LS (1999) Characterization of human small intestinal Cytochromes P450. Drug Meta Dispos **27**(7):804-809

Footnotes:

Corresponding author:

Doreen Mitschke, Research Pharmacokinetics, Bayer Schering Pharma AG, 13343 Berlin, Germany

Addresses author:

Doreen Mitschke, Andreas Reichel, Gert Fricker, Ursula Moenning

DM, AR, UM: Research Pharmacokinetics, Bayer Schering Pharma AG, Berlin, Germany;

GF: Institute of Pharmacy and Molecular Biotechnology, University of Heidelberg, Germany

Legends for figures:

FIG 1: Representative Western blots of all analyzed CYP P450 enzymes and villin of a male rat with 33 intestinal segments is depicted in this picture. The villin band was taken as a marker protein for enterocytes. CYP3A: Two immunoreactive bands were detected after incubation with the CYP3A1 antibody used. The double band was detected in all samples as well as in liver (data not shown) suggesting a cross reactivity with other isoforms of the CYP3A family which all share a high amino acid sequence identity (70%-97%). For data evaluation, the density of both bands was taken and named CYP3A.

FIG 2: The figure displays the expression profiles of CYP3A, 2B1 and CYP1A1 **(2A)** and CYP2C6 and CYP2D1 **(2B)** along the entire intestine. The relative abundance of each intestinal segment (a 3 cm) are depicted and assigned to the main intestinal sections (duodenum, jejunum, ileum, colon) on the x axis. The data are given for all individual animals tested to give an idea of the CYP P450 expression variability. The density of each Western blot signal was measured and the ratio of the CYP P450 signal with the responding villin signal was calculated. In order to show the dynamics of the expression the ratio of the lowest detectable band was put to one and all other signals were compared to it. Samples with no band at all were put to zero.

FIG 3: Quantification of the absolute CYP P450 amount in intestinal homogenates of one male rat by Western blot analysis. In **3A** an example of such a Western blot

is shown. The quantification was done based on a CYP Supersome™ calibration curve. In **3B** an evaluation example is given for CYP2B1 in a female rat. Seventy five µg homogenate of selected intestinal regions were loaded on each gel: D stands for duodenum, J1-3 for upper, middle and lower jejunum, I and C for Ileum and Colon, respectively. S1-S4 are Supersomes™ standard CYP P450 proteins in the four different amounts loaded (CYP2B1 S1: 62.5, S2:125, S3: 250, S4:500fmol; CYP3A1 S1: 3.1, S2: 6.3, S3: 12.5, S4: 25fmol; CYP2D1 S1: 0.3, S2: 0.5, S3: 1, S4: 2fmol; CYP2C6 S1:0.6, S2: 1.3, S3: 2.5, S4: 5fmol).

Table 1:

Tab 1: Reproducibility of CYP quantification by Western blot

The data of three independent assays are given. The repeated quantification was done exemplary for 6 segments and two different CYP P450 isoforms (CYP3A and CYP2D1) in one animal.

	CYP3A (*fmol / mg protein)*					**CYP2D1** *(fmol / mg protein)*				
	replication			**AV**	**SD**	**replication**			**AV**	**SD**
	1	**2**	**3**			**1**	**2**	**3**		
Duodenum	165	173	163	**167**	**5**	46	59	63	**56**	**9**
upper Jejunum	188	235	257	**227**	**35**	63	62	62	**63**	**1**
middle Jejunum	171	191	163	**175**	**14**	41	52	37	**43**	**8**
lower Jejunum	175	186	160	**174**	**13**	23	27	20	**23**	**3**
Ileum	-	-	-	**-**	**-**	35	39	27	**33**	**6**
Colon	-	-	-	**-**	**-**	62	53	40	**51**	**11**

AV: average, SD: standard deviation

DMD Fast Forward. Published on March 10, 2008 as DOI: 10.1124/dmd.107.019687
This article has not been copyedited and formatted. The final version may differ from this version.

Table 2:

Tab 2: The CYP P450 amount of the intestinal main section of female rat. Results from three female in each case are expressed as means ± SD. The percentage shown in brackets refers to the relative contribution of the given CYP P450 to the sum of CYP P450 amount in the respective segment.

	Female rat					
	Duodenum	**upper Jejunum**	**middle Jejunum**	**lower Jejunum**	**Ileum**	**Colon**
	fmol / mg protein					
CYP2B1	107 ± 62 (30%)	645 ± 252 (67%)	333 ± 80 (59%)	98 ± 75 (31%)	-	-
CYP3A	176 ± 56 (49%)	243 ± 44 (49%)	187 ± 39 (33%)	160 ± 23 (51%)	-	-
CYP2D1	70 ± 25 (20%)	71 ± 17 (8%)	43 ± 5 (8%)	55 ± 29 (17%)	44 ± 9 (85%)	58 ± 6 (91%)
CYP2C6	3 ± 1 (1%)	3 ± 1 (<1%)	3 ± 1 (1%)	4 ± 2 (1%)	8 ± 5 (15%)	6 ± 5 (9%)
sum of CYP	356 ± 144	962 ± 314	566 ± 125	317 ± 129	52 ± 14	64 ± 11

DMD Fast Forward. Published on March 10, 2008 as DOI: 10.1124/dmd.107.019687
This article has not been copyedited and formatted. The final version may differ from this version.

Table 3:

Tab 3: The CYP P450 amount of the intestinal main section of male rat. Results from three male in each case are expressed as means ± SD. The percentage shown in brackets refers to the relative contribution of the given CYP P450 to the sum of CYP P450 amount in the respective segment.

	Male rat						
	Duodenum	**upper Jejunum**	**middle Jejunum**	**lower Jejunum**	**Ileum**	**Colon**	**Liver**
	fmol / mg protein						
CYP2B1	338 ± 113 (62%)	392 ± 102 (66%)	346 ± 106 (67%)	332 ± 46 (64%)	-	-	1718 ± 992 (23%)
CYP3A	150 ± 20 (28%)	153 ± 52 (26%)	141 ± 35 (27%)	135 ± 41 (26%)	-	-	1458 ± 540 (20%)
CYP2D1	51 ± 21 (9%)	46 ± 8 (8%)	25 ± 9 (5%)	46 ± 30 (9%)	44 ± 37 (86%)	71 ± 17 (88%)	1897 ± 307 (25%)
CYP2C6	4 ± 1 (1%)	4 ± 1 (<1%)	4 ± 1 (1%)	5 ± 3 (1%)	7 ± 4 (14%)	10 ± 6 (12%)	2373 ± 288 (32%)
sum of CYP	543 ± 155	595 ± 163	516 ± 151	518 ± 120	51 ± 41	81 ± 23	7446 ± 2127

FIG 1:

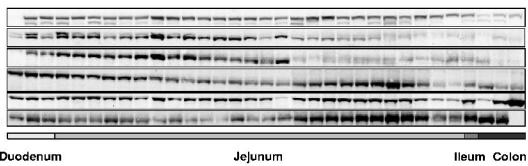

Villin
CYP3A
CYP2B1
CYP1A1
CYP2D1
CYP2C6
Duodenum
Jejunum
Ileum
Colon

FIG 2A:

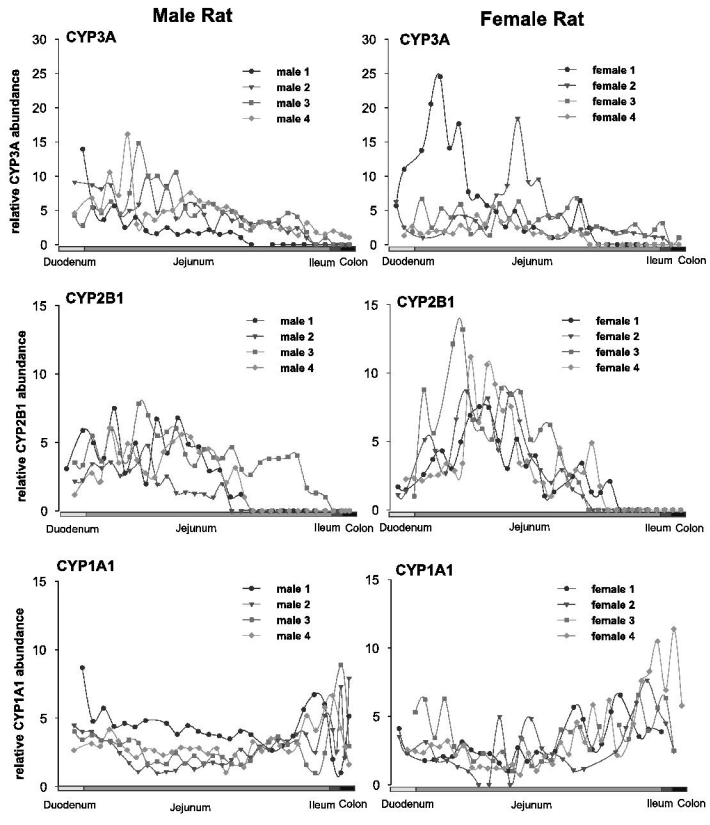

Male Rat
Female Rat
CYP3A
CYP2B1
CYP1A1
relative CYP3A abundance
relative CYP2B1 abundance
relative CYP1A1 abundance
male 1
male 2
male 3
male 4
female 1
female 2
female 3
female 4
Duodenum
Jejunum
Ileum
Colon

FIG 2B:

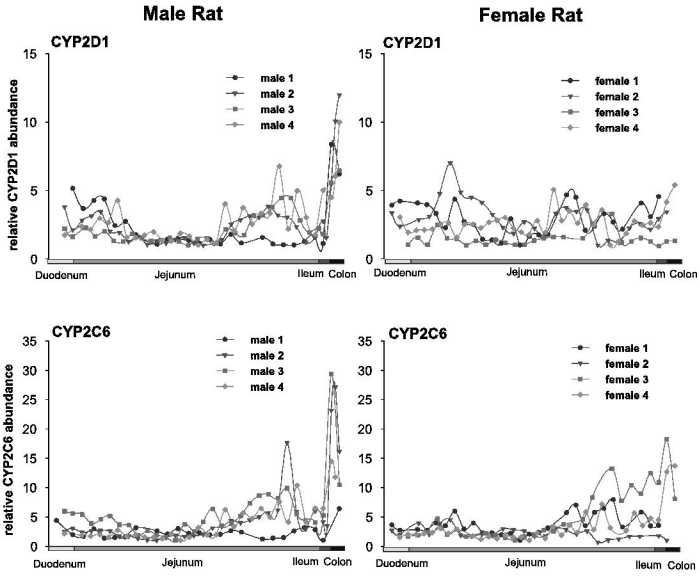
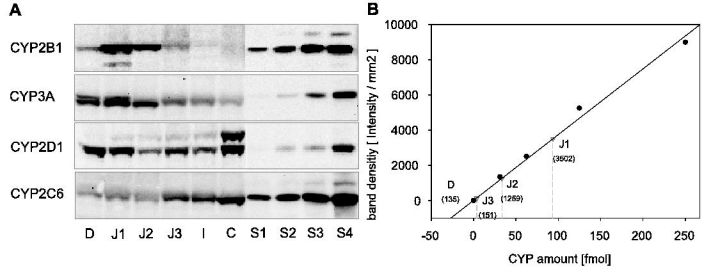

Male Rat
Female Rat
CYP2D1
relative CYP2D1 abundance
male 1
male 2
male 3
male 4
female 1
female 2
female 3
female 4
CYP2C6
relative CYP2C6 abundance
Duodenum
Jejunum
Ileum
Colon

FIG 3:


A
CYP2B1
CYP3A
CYP2D1
CYP2C6
D J1 J2 J3 I C S1 S2 S3 S4
B
band density [Intensity / mm2]
10000
8000
6000
4000
2000
0
-50 0 50 100 150 200 250
CYP amount [fmol]
D
(135)
J3
(151)
J2
(1269)
J1
(3502)