Cytochrome P450 3A and ABC-transport Proteins in Horse

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Abstract
In this thesis cytochrome P450 3A (CYP3A) and the ABC-transport proteins P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), multidrug resistance protein 1 (MRP1) and multidrug resistance protein 2 (MRP2) have been studied in horse.

In the first part of the thesis the gene and protein expression, cellular localization and metabolic activity of CYP3A were investigated in the intestines, liver and upper respiratory airways. High levels of CYP3A gene and protein expression and metabolic activity were found in the small intestines. The CYP3A-related activity in the upper respiratory airways was high in spite of rather low levels of CYP3A gene and protein expression. A possible explanation in this observation is that there is an efficient electron transport from NADPH P450 reductase and cytochrome b5 to CYP3A in these tissues.

In the second part of the thesis gene and protein expression and cellular localization of P-gp, BCRP, MRP1 and MRP2 were studied in the intestines, liver and kidney. The expression and localization of the transporters in the tissues of horse showed both similarities and differences compared to other species.

Collectively the results of the present thesis indicate that CYP3A and the examined ABC-transport proteins have important roles for bioavailability and elimination of substrate compounds in horse.

Keywords: Horse, CYP3A, P-gp, BCRP, MRP1, MRP2, intestine, liver, kidney, upper respiratory airways, lymphocytes, Brunner’s gland

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Contents

1 Introduction 11

2 The Cytochrome P450 Enzymes 15
2.1 CYP3A 16

3 The ABC-transporter Family 19
3.1 P-glycoprotein 20
3.2 Breast Cancer Resistance Protein 22
3.3 The Multidrug Resistance Protein Family 23
   3.3.1 MRP1 23
   3.3.2 MRP2 24

4 Gene Regulation of CYP enzymes and ABC transporters 27

5 CYP enzymes and Transport Proteins in Horse 29
5.1 Drug interactions in horse 30

6 Morphology and Physiology of the Examined Tissues 33
6.1 The alimentary canal 33
6.2 Liver 35
6.3 Kidney 36
6.4 Tissues of the upper airways 37

7 Aims of the Thesis 39
8 **Materials and Methods** 41

8.1 Animals and sample collections 41
8.2 Microsomal preparations and determinations of enzyme activity 42
8.3 Gene expression 43
  8.3.1 RNA preparation 43
  8.3.2 Primer design 43
  8.3.3 Real-Time RT-PCR 43
8.4 Protein expression and localization 44
  8.4.1 Western blot 45
  8.4.2 Immunohistochemistry 45

9 **Results** 47

9.1 Paper I “CYP3A in horse intestines” 47
9.2 Paper II “Characterization of CYP3A-related metabolism in horse intestines and liver” 48
9.3 Paper III “Cytochrome P450 3A, NADPH cytochrome P450 reductase and cytochrome b_{5} in the upper airways in horse” 51
9.4 Paper IV “P-glycoprotein in intestines, liver, kidney and lymphocytes in horse” 52
9.5 Paper V “Expression and localization of BCRP, MRP1 and MRP2 in intestines, liver and kidney in horse” 54

10 **Discussion** 57

10.1 CYP3A 57
  10.1.1 CYP3A in the intestines and the liver 57
  10.1.2 CYP3A in the upper respiratory airways 59
10.2 ABC- transport proteins 60
  10.2.1 Protein sequence analysis 60
  10.2.2 P-gp, BCRP, MRP1 and MRP2 in the intestines 60
  10.2.3 P-gp, BCRP, MRP1 and MRP2 in the liver 62
  10.2.4 P-gp, BCRP, MRP1 and MRP2 in the kidney 63

11 **Conclusions** 65

12 **Future Research** 69

References 71

Acknowledgement 81

13 **Sammanfattning på svenska** 83
Publications included in the Thesis

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:


II  E. Tydén, H. Tjälve, P. Larsson. Characterization of CYP3A-Related Metabolism in Horse Intestines and Liver. (Submitted).


V  E. Tydén, H. Björnström, H. Tjälve, P. Larsson. Expression and localization of BCRP, MRP1 and MRP2 in intestines, liver and kidney in horse. (Manuscript).

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Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>BCRP</td>
<td>breast cancer resistance protein</td>
</tr>
<tr>
<td>DFB</td>
<td>3-(3,4-difluorobenzyloxy)-5,5-dimethyl-4-(4-methylsulphonyl)-5H-furan-2-one</td>
</tr>
<tr>
<td>CAR</td>
<td>constitutive androstane receptor</td>
</tr>
<tr>
<td>Ct</td>
<td>threshold cycle</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>DFH</td>
<td>3-hydroxy-5,5-dimethyl-4-(4-methylsulphonyl)-5H-furan-2-one</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>GADPH</td>
<td>glyceraldehyde-3-phosphatedehydrogenase</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
</tr>
<tr>
<td>MDR1</td>
<td>multidrug resistance gene 1</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MRP</td>
<td>multidrug resistance protein</td>
</tr>
<tr>
<td>MSD</td>
<td>membrane spanning domain</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotine adenine dinucleotide phosphate, reduced form</td>
</tr>
<tr>
<td>NBD</td>
<td>nucleotide binding domain</td>
</tr>
<tr>
<td>P-gp</td>
<td>permeability glycoprotein</td>
</tr>
<tr>
<td>PXR</td>
<td>pregnane X receptor</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RXR</td>
<td>retinoid X receptor</td>
</tr>
<tr>
<td>SDS-</td>
<td>sodium dodecyl sulphate-</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SYBR green</td>
<td>an asymmetrical cyanine dye used as nucleic stain</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane</td>
</tr>
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</table>
1 Introduction

Orally administrated drugs have to overcome several barriers before reaching the systemic circulation (Figure 1). Firstly the drugs are dissolved in the gastro-intestinal contents. Secondly they pass through the intestinal epithelium, where numerous efflux transporters are acting as barriers and thus prevent passage through the epithelium. Thirdly most drugs pass via the portal vein to the liver. Metabolism may take place both in the enterocytes and in the liver. Finally, the drugs or drug-metabolites are drained into the systemic circulation via the hepatic veins and the vena cava. Most drugs leave the body via the urine or bile, either non-metabolized or as polar metabolites. Several transport proteins are present in the nephrons of the kidney and may play important roles in the excretion of the drugs (Shitara et al., 2006).

Figure 1. A schematic picture of the transfer of an orally administrated drug into the systemic circulation.
The metabolic pathway of a drug/xenobiotic can be divided into three phases: phase I-III. In phase I enzymes such as cytochrome P450 (CYP) enzymes introduce a functional group, usually an OH-group, into the substrate. Sometimes reactive metabolites, which may damage DNA or other cellular constituents, are formed at the phase I metabolism. In phase II transferase enzymes use the functional group as a handle for conjugation with endogenous constituents such as glucuronic acid, sulphate or glutathione to make the substrate hydrophilic. In phase III the conjugated substrate is pumped out of the cell by efflux transporters, such as multidrug resistance protein 1 (MRP1) and multidrug resistance protein 2 (MRP2) (Xu et al., 2005). Other efflux transporters, such as P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP), may prevent that the non-metabolized drug/xenobiotic enters the cell by acting as efflux pumps at the cell membrane (Figure 2).

![Diagram](image)

Figure 2. A drug/xenobiotic that enters an enterocyte can directly be effluxed by transport proteins, such as P-gp or BCRP (1) or undergo phase I metabolism by a CYP-enzyme (2). Sometimes reactive metabolites are formed in the phase I metabolism, which may damage DNA or other cellular constituents (3). Normally, the phase I metabolite undergoes phase II metabolism (4) and is effluxed by a transport protein, such as MRP1 or MRP2 (phase III) (5).

The liver is generally accepted to be the major organ involved in drug metabolism. In recent years the small intestine has also been shown to play an important role in the metabolism of orally administrated drugs (Kolars et al., 1994). In addition to the liver and the intestines, metabolizing enzymes
are present in extrahepatic tissues such as the mucosa of the upper airways (Ding and Kaminsky, 2003).

Horses are treated with a variety of drugs, which may be substrates for the CYP-enzymes and the transport proteins. This also applies to some feed constituents as well as to xenobiotics present in the feed or in airborne particles in stables. The CYP enzymes and ABC-transporters have been extensively studied in man and laboratory animals, whereas little is known about these enzymes and efflux proteins in horse and other domestic animals. In this thesis investigations have been performed on the gene and protein expression, the cellular localization and the metabolic activity of the CYP3A in the intestines, liver and upper respiratory pathways in horse. In addition the gene and protein expression and the cellular localization of P-gp, BCRP, MRP1 and MRP2 in the intestines, liver and kidney have been examined in horse.
2 The Cytochrome P450 Enzymes

The cytochrome P450 super family (CYP) is an enzyme group critical for oxidation reactions in the phase I metabolism of xenobiotics. The name P450 was originally a description of a red pigment found in liver microsomes with an absorbance maximum at 450 nm in its reduced carbon-monoxide form (Omura and Sato, 1964). The CYP enzymes are classified into families and subfamilies based on their amino acid sequence similarity. The enzymes are denoted CYP followed by a designation of family (number), subfamily (letter) and individual enzyme (number). To be members of the same family more than 40% of the amino acid sequence should be identical (e.g. CYP3) and to be members of the same subfamily 55% of the amino acid sequence should be identical (e.g. CYP3A). Finally, each individual enzyme is represented by a number (e.g. CYP3A4). An updated list of CYP enzymes can be accessed at http://drnelson.utmcm.edu/Cytochrome_P450.htm

The CYP enzymes can functionally be divided into two groups: those involved in endogenous metabolism, such as the biosynthesis of steroids, fatty acids and bile acid and those involved in xenobiotic metabolism. There are mainly three CYP families, CYP1, CYP2 and CYP3, involved in the xenobiotic metabolism (Zhang and Benet, 2001; Zuber et al., 2002). The CYP1 family includes among others, CYP1A, which is responsible for the metabolism of several environmental toxins, such as polycyclic aromatic hydrocarbons (Ding and Kaminsky, 2003). The CYP2 family is a large family with several subfamilies among which CYP2C, CYP2D and CYP2E are involved in the metabolism of several clinically relevant drugs (Ding and Kaminsky, 2003). The third family, CYP3A, is considered the primary CYP subfamily to be involved in drug metabolism (Zhang and Benet, 2001).
In mammals the CYP enzymes are mainly anchored at the cytosolic side of
the endoplasmic reticulum, but there are also some CYP enzymes present in
the inner membranes of mitochondria (Ding and Kaminsky, 2003;
Gonzalez, 1988). The catalytic activity of the CYP enzymes is dependent on
presence of electron-donating co-factors such as NADPH P450 reductase
and cytochrome b₅. Thus, transfer of an electron from NADPH P450
reductase to CYP occurs during the metabolic CYP-cycle (Nakajima et al.,
2002; Schenkman and Jansson, 2003; Wu et al., 2005). Moreover
cytochrome b₅ has been shown to promote the metabolic activity of the
CYP-enzymes by facilitating the transfer of a second electron to the CYP-
cycle (Nakajima et al., 2002; Yamazaki et al., 1996).

2.1 CYP3A

CYP3A is an important CYP. In addition to the liver CYP3A is expressed
in several extrahepatic tissues such as the nasal olfactory and respiratory
mucosa, tracheal mucosa, lung and the mucosa of oesophagus and intestines
(Ding and Kaminsky, 2003). In man the CYP3A subfamily has been
reported to be responsible for approximately 60% of all oxidative drug
metabolism (Wilkinson, 2005). This subfamily has been highly conserved in
mammals. In man the most important isoenzyme within this subfamily is
CYP3A4 (Kolars et al., 1994). The horse orthologue to human CYP3A4 is
designated CYP3A89. A comparison of the amino acid sequences of the
horse CYP3A89 with the orthologous CYP3A4-enzymes in man, dog and
rat is shown in Table 1.

CYP3A4 was first identified by Watkins et al. (1985) and cloned the year
after (Molowa et al., 1986). In 2004 the crystal structure of CYP3A4 was
reported (Williams et al., 2004) and two years later it was shown that
CYP3A4 undergoes dramatic conformational changes upon ligand binding,
with an increase in the active-site volume of more than 80%. This may
explain the broad substrate specificity of CYP3A4 (Ekroos and Sjögren,
2006).
Table 1. Comparison of horse CYP3A89 with orthologous CYP3A4-enzymes in human, dog and rat.

<table>
<thead>
<tr>
<th>Species</th>
<th>Name</th>
<th>Chromosome location(^a)</th>
<th>Number of amino acids</th>
<th>Identity with horse (%)</th>
<th>Accession number(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse</td>
<td>CYP3A89</td>
<td>13</td>
<td>502</td>
<td>(100)</td>
<td>A7LGW7</td>
</tr>
<tr>
<td>Human</td>
<td>CYP3A4</td>
<td>7</td>
<td>502</td>
<td>82</td>
<td>P08684</td>
</tr>
<tr>
<td>Dog</td>
<td>CYP3A12</td>
<td>6</td>
<td>503</td>
<td>84</td>
<td>P24463</td>
</tr>
<tr>
<td>Rat</td>
<td>CYP3A23/A1</td>
<td>12</td>
<td>504</td>
<td>67</td>
<td>P04800</td>
</tr>
</tbody>
</table>

\(^a\) chromosome location www.ensembl.org. \(^b\) accession number from UniProt
3 The ABC-transporter Family

ATP-Binding Cassette (ABC) transport proteins comprise the largest superfamily of transport proteins. The ABC genes have been divided into seven subfamilies, designated A through G, based on the similarities of their amino acid sequences (Deeley et al., 2006; Klein et al., 1999). The members of the ABC family are ubiquitously present in various biological systems and are expressed in numerous organisms, ranging from bacteria to mammals. They are all involved in the trafficking of biological molecules across cell membranes (Borst et al., 2000; Higgins, 1992). The core functional unit of the ABC transporters consists of two polytrophic membrane-spanning domains (MSD) and two nucleotide-binding domains (NBD) (Figure 3). The MSD domains may vary between 5-10 transmembrane helices but usually consist of 6 transmembrane helices (Deeley et al., 2006). The two NBD utilize the energy from ATP hydrolysis to transport the substrates through pores across the cellular membrane. The ATP-binding region is highly conserved and is one the characteristic for this superfamily. In eukaryotes all ABC-transporters are efflux proteins, whereas in prokaryotes they can be either efflux or influx proteins (Higgins, 2007). Some genetic disorders in humans, such as Tagnier disease, Stargardt disease, Dubin-Johnson syndrome and cystic fibrosis are associated with dysfunctions of the ABC-transporters (Deeley et al., 2006; Keitel et al., 2003).
3.1 P-glycoprotein

P-glycoprotein (P-gp), where “P” stands for permeability, encoded by the ABCB1 gene (also referred to as the MDR1 gene), was the first eukaryotic ABC transporter discovered (Juliano and Ling, 1976). P-gp was observed to cause a multidrug-resistant phenotype in Chinese hamster ovary tumour cells (Juliano and Ling, 1976). Since then a vast number of studies have been performed on the structure, mechanism of action and physiological and pharmacological roles of P-gp. A comparison of the amino acid sequences of horse P-gp with orthologues in human, dog and rat is shown in Table 2. It can be seen that horse P-gp has about 100 amino acids more than P-gp in the other species. It is considered that P-gp plays a role in the efflux transport of various endogenous compounds, such as cholesterol, cytokines and steroids (Leslie et al., 2005). P-gp is also responsible for the transport of a variety of exogenous compounds including several drugs (Seelig, 1998).
P-gp is expressed in various tissues such as the intestine, liver, kidney, brain and placenta. The localisation of P-gp suggests a functional role in the protection against negative effects of xenobiotics by facilitating excretion of the compounds into the intestinal lumen, the bile and the urine and by preventing the uptake into the brain and the fetus. In the intestines P-gp is expressed in the apical membrane of the enterocytes in which it may limit the bioavailability of orally administrated drugs (Thiebaut et al., 1987). In the liver P-gp is present in the biliary canalicular membranes of the hepatocytes and may promote secretion of drugs into the bile (Schinkel and Jonker, 2003). In the kidney P-gp is primarily localized at the luminal surfaces of the cells of the proximal tubules and may play a role in the elimination of drugs and other xenobiotics from blood into urine (Thiebaut et al., 1987).

Table 2. Comparison of horse P-gp with orthologues in human, dog and rat

<table>
<thead>
<tr>
<th>Species</th>
<th>Names</th>
<th>Chromosome location</th>
<th>Number of amino acids</th>
<th>Identity %</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse</td>
<td>ABCB1</td>
<td>4</td>
<td>1382</td>
<td>(100)</td>
<td>XP001492073</td>
</tr>
<tr>
<td>Human</td>
<td>ABCB1</td>
<td>7</td>
<td>1280</td>
<td>90</td>
<td>P08183</td>
</tr>
<tr>
<td>Dog</td>
<td>ABCB1</td>
<td>14</td>
<td>1283</td>
<td>92</td>
<td>O46605</td>
</tr>
<tr>
<td>Rat</td>
<td>ABCB1a</td>
<td>4</td>
<td>1272</td>
<td>85</td>
<td>QPPSM0</td>
</tr>
<tr>
<td></td>
<td>ABCB1b</td>
<td>4</td>
<td>1277</td>
<td>78</td>
<td>P43245</td>
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'S chromosome location www.ensembl.org. 'UniProt.

So far, rodents are the only species known to have two P-gp. These are encoded by the genes Mdr1a (ABCB1a) and Mdr1b (ABCB1b). The tissue distribution of Mdr1a and Mdr1b differ from each other but collectively the two isoforms have the same distribution in the body as human P-gp (Leslie et al., 2005). Collies and dogs of collie-related breeds sometimes carry a single nucleotide polymorphism (SNP) within the ABCB1, which results in a defect function of the P-gp protein. When these dogs are treated with ivermectin, which is a P-gp substrate, neurotoxic effect may occur due to passage of the substance over the blood-brain barrier (Mealey, 2004).
3.2 Breast Cancer Resistance Protein

Breast cancer resistant protein (BCRP), encoded by the ABCG2 gene, was cloned from a doxorubicin-resistant breast cancer cell line in 1998 and is one of the latest discovered members of the ABC-transporter family (Doyle et al., 1998). BCRP is a “half transporter” consisting of only one set of 6 TM helices and one NBD. A homodimerisation is needed for BCRP to become a functional unit (Klein et al., 1999). BCRP is located in the plasma membrane whereas other “half transporters” often are located intracellularly (Mao and Unadkat, 2005). BCRP transports a variety of exogenous unconjugated and conjugated compounds (Mao and Unadkat, 2005; Vander Borght et al., 2006).

It has been suggested that BCRP has similar functions as P-gp and that they may act together as tissue-barrier, protecting the body against toxic xenobiotics. The substrate specificity of BCRP partly overlaps those of P-gp and the MRPs and it has become clear that BCRP is one of the major ABC transporters affecting drug disposition (Haimeur et al., 2004; Mao and Unadkat, 2005).

In humans BCRP is expressed in various organs, such as the intestines, liver, kidney, placenta, brain and mammary gland (Maliepaard et al., 2001). A comparison of the amino acid sequences of horse BCRP with orthologues in human, dog and rat is shown in Table 3. There are several known species-differences in the tissue distribution of BCRP. For example is BCRP located in the biliary canalicular membranes of hepatocytes in human liver but not in rat liver (Vander Borght et al., 2006).

Table 3. Comparison of horse BCRP with orthologues in human, dog and rat

<table>
<thead>
<tr>
<th>Species</th>
<th>Name</th>
<th>Chromosome location</th>
<th>Number of amino acids</th>
<th>Identity %</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse</td>
<td>ABCG2</td>
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<td>654</td>
<td>(100)</td>
<td>XP0011496382</td>
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<tr>
<td>Human</td>
<td>ABCG2</td>
<td>4</td>
<td>655</td>
<td>86</td>
<td>Q9UN0</td>
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<td>Dog</td>
<td>ABCG2</td>
<td>32</td>
<td>654</td>
<td>86</td>
<td>Q38JL0</td>
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<tr>
<td>Rat</td>
<td>ABCG2</td>
<td>4</td>
<td>656</td>
<td>82</td>
<td>Q800W57</td>
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</table>

*chromosome location [www.ensembl.org](http://www.ensembl.org), ^[UniProt](http://www.uniprot.org)*
3.3 The Multidrug Resistance Protein Family

The multidrug resistance protein subfamily (MRP, ABCC) comprises 13 members, where MRP1, 2, 3, 4, 5, 6 and 8 are known to be involved in transport of drugs (Schinkel and Jonker, 2003). The structure of the members of the ABCC subfamily is related to most other ABC-transporters. They contain two MSD, each followed by a nucleotide binding domain. However, MRP1 and MRP2 belong to the “long” MRPs having an additional MSD and an extracellular location of the N-terminal. The additional transmembrane domain of MRP1 and MRP2 may be required for proper function but the detailed role of this additional MSD is not yet known (Deeley et al., 2006).

3.3.1 MRP1

MRP1 (ABCC1) was the first member in the ABCC subfamily to be cloned in 1992 from a drug-selected human lung cancer cell line (Cole et al., 1992). MRP1 has been reported to be located at the plasma membrane on the basolateral side of polarized cells. In human MRP1 is expressed in several tissues (Bakos and Homolya, 2007). MRP1 orthologues show high sequence identity with one another but have some remarkable differences in the substrate specificity. A comparison of the amino acid sequence of MRP1 in horse with orthologues in human, dog and rat is shown in Table 4.

The wide substrate specificity and widespread expression of MRP1 suggests numerous possible physiological functions, which still have to be explored. MRP1 transports phase II conjugates as well as some non-conjugated compounds (Deeley and Cole, 2006). MRP1 is a transporter of cystein leukotriene C₄ (LTC₄) and thus plays a role in inflammatory response (Leslie et al., 2005). In severe liver disease MRP1 is up-regulated in the hepatocytes (Ros et al., 2003).
Table 4. Comparison of horse MRP1 with orthologues in human, dog and rat

<table>
<thead>
<tr>
<th>Species</th>
<th>Name</th>
<th>Chromosome location</th>
<th>Number of amino acids</th>
<th>Identity %</th>
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</tr>
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<td>90</td>
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<tr>
<td>Rat</td>
<td>ABCC1</td>
<td>10</td>
<td>1532</td>
<td>85</td>
<td>Q8CG09</td>
</tr>
</tbody>
</table>

a chromosome location www.ensembl.org, b UniProt,

3.3.2 MRP2

Multidrug resistance protein 2 (MRP2, ABCC2) was the second member of this subfamily to be cloned in 1996 from normal rat liver (Paulusma et al., 1996). MRP2 is also referred to as multispecific organic anion transporter (cMOAT). MRP2 is expressed in the bile canalicular membranes of hepatocytes, in the brushborder membranes of enterocytes and in the renal proximal tubules. This localization indicates a role of MRP2 in excretion of xenobiotics. Lower levels of MRP2 are found in lung, stomach and placenta and there are contradictory reports concerning the expression of MRP2 in the blood-brain barrier (Fardel et al., 2005). The substrate specificity of MRP2 is broad, and is to some extent overlapping with that of MRP1 (Keppler et al., 2000). The substrates of MRP2 include glucuronic acid and GSH conjugates of substances. MRP2 is considered to play an important role in the elimination of conjugates of various xenobiotics from intestinal epithelial cells into the intestinal lumen, from hepatocytes into the bile and from kidney proximal tubules into the urine (Keppler et al., 2000; Keppler and Köng, 1997; Nies and Keppler, 2007). Moreover, MRP2 plays an important role in the hepatic excretion of bilirubin. It has been shown that a dysfunction of MRP2 in the liver in man results in hyperbilirubinemia and may cause Dubin-Johnson syndrome (Keitel et al., 2003). A comparison of the amino acid sequence of MRP2 in horse with orthologues in human, dog and rat is shown in Table 5.
Table 5. Comparison of horse MRP2 with orthologues in human, dog and rat

<table>
<thead>
<tr>
<th>Species</th>
<th>Name</th>
<th>Chromosome location</th>
<th>Number of amino acids</th>
<th>Identity %</th>
<th>Accession number</th>
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<tr>
<td>Dog</td>
<td>ABCC2</td>
<td>28</td>
<td>1544</td>
<td>85</td>
<td>Q6PSM3</td>
</tr>
<tr>
<td>Rat</td>
<td>ABCC2</td>
<td>1</td>
<td>1541</td>
<td>77</td>
<td>Q63120</td>
</tr>
</tbody>
</table>

*chromosome location [www.ensembl.org](www.ensembl.org), ^UniProt,
4 Gene Regulation of CYP enzymes and ABC transporters

Many xenobiotics have been shown to directly or indirectly regulate the expression of the CYP enzymes as well as the expression of the ABC transporters via the nuclear receptor pathways. The orphan pregnane X receptor (PXR), is one of the key regulators for the expression of P-gp, MRP2 and CYP3A (Wilkinson, 2005). Thus, in response to activation of a ligand PXR forms a heterodimer with the retinoid X receptor (RXR). The PXR–RXR heterodimer then binds to the promoter regions of P-gp, MRP2 or CYP3A, thereby increasing transcription (Goodwin et al., 2002). The intestinal and the hepatic expression of CYP3A are highly dependent on the activity of PXR (Urquhart et al., 2007). For example the PXR ligand rifampicin has been shown to reduce the plasma concentrations of some orally co-administrated drugs by induction of intestinal P-gp. Another example is the PXR ligand hyperforin (present St. John’s wort), which induces both P-gp and CYP3A and thus may reduce the oral bioavailability of some drugs (Synold et al., 2001). An illustration of how drug interactions may occur as a result of effects on nuclear receptor pathways is shown in Figure 4.
Another nuclear receptor is the constitutive androstane receptor (CAR). CAR also forms a heterodimer with RXR, thus increasing the expression of CYP3A, P-gp and MRP2. There is an overlap in the substrate specificity of CAR and PXR (Kliewer et al., 2002; Willson and Kliewer, 2002).

The mechanism of the regulation of BCRP is not yet fully understood, but it appears that the regulation of BCRP-expression does not involve regulation by PXR or CAR (Choudhuri and Klaassen, 2006). Recently the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) has been shown to be responsible for the regulation of BCRP. PPARγ also forms a heterodimer with RXR, in this way increasing the expression of BCRP (Urquhart et al., 2007).

MRP1 is not regulated via the nuclear receptor pathway. Instead it is suggested that MRP1 is regulated by multiple regulatory mechanisms. Among others have the transcriptional factors of the antioxidant responsive element driven genes been found to be involved in the expression of MRP1 (Bakos and Homolya, 2007).
5 CYP enzymes and Transport Proteins in Horse

Little is known regarding CYP enzymes and ABC-transport proteins in horse. Concerning the CYP enzymes the few reports that are available have dealt with the metabolic capacity and protein expression of CYP in horse liver microsomes. CYP enzymes examined include CYP1A, CYP2B, CYP2E, CYP2D and CYP3A (Lakritz et al., 2000; Nebbia et al., 2004). Comparative studies on CYP dependent activities which include horses are few (Chauret et al., 1997; Nebbia et al., 2003).

Table 6. Comparisons of metabolic activities of some CYP-enzymes in liver microsomes from human, horse and dog. Data modified from Chauret et al. (1997)

<table>
<thead>
<tr>
<th>Human CYP</th>
<th>Substrates</th>
<th>Human (%)</th>
<th>Horse (% of human)</th>
<th>Dog (% of human)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A1/2</td>
<td>Phenacetin-O-deethylase</td>
<td>(100)</td>
<td>39</td>
<td>145</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>Coumarin-7-hydroxylase</td>
<td>(100)</td>
<td>24</td>
<td>17</td>
</tr>
<tr>
<td>CYP2C8/9</td>
<td>Tolbutamide hydroxylase</td>
<td>(100)</td>
<td>170</td>
<td>18</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>S-methyleno-4-hydroxylase</td>
<td>(100)</td>
<td>171</td>
<td>100</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Dextrometorphan-O-demethylase</td>
<td>(100)</td>
<td>221</td>
<td>41</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>Chlorozoxaxzone-6-hydroxylase</td>
<td>(100)</td>
<td>208</td>
<td>122</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Testosterone-6β-hydroxylase</td>
<td>(100)</td>
<td>14</td>
<td>14</td>
</tr>
</tbody>
</table>
In a study by Chauret et al. (1997) (Table 6) liver microsomes from human, horse and dog were incubated with substrates reported to be selective for particular CYP enzymes. It appears that there exist rather large differences in the metabolic CYP-related activities between these species.

Recently the first clone of a horse CYP, namely CYP2D50, was reported (DiMaio Knych and Stanley, 2008). The nucleotide sequence was highly homologous to human CYP2D6. Interestingly, there were striking differences in the dextromethorphan-O-demethylase activity between horse and human. Thus, horse CYP2D50 had a much lower capacity to metabolize dextromethorphan-O-demethylase than human CYP2D6 (DiMaio Knych and Stanley, 2008). This is in contrast to the report by Chauret et al. (1997) where horse liver microsomes had twice as high metabolic capacity of dextromethorphan-O-demethylase compared to human liver microsomes (Table 6). These data suggest that additional CYP-enzymes in horse liver microsomes can metabolize this substrate.

Regarding phase II enzymes there are also species differences between horse and other mammals. For example have the levels of glutathione- and acetyl-conjugations of various substrates shown marked differences in horse compared to other domestic animals (Gusson et al., 2006).

Concerning the ABC-transport proteins there are no reports available on species differences between horse and other species.

5.1 Drug interactions in horse

When two or more drugs that are substrates for either CYP enzymes or transport proteins are administrated together there is a potential possibility of drug interactions. Drug interactions can lead to inhibition or induction of the CYP enzymes or the transport proteins, resulting in increased or decreased tissue levels of one or both drugs. Compounds present in herbs and feed constituents or feed supplements may also inhibit or induce the CYP enzymes and the transport proteins.

As concerns the horse little is known about drug-drug interactions or drug-herb interactions. According to the review by Williams and Lamprecht (2008) there is an overall increase of the herbal supplements on the market and especially the market for horses. Various herbal supplements that are commercially available for horses may be substrates for the CYP enzymes or
transport proteins or both and could potentially interact with drugs and cause drug-herb interactions (Table 7).

Table 7. Compounds present in herb supplements that are commercially available for horses in Sweden and have been reported to inhibit or induce CYP3A and/or transport proteins

<table>
<thead>
<tr>
<th>Active plant component</th>
<th>CYP3A</th>
<th>P-gp</th>
<th>BCRP</th>
<th>MRP1</th>
<th>MRP2</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herb</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkamides</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Echinacea</td>
<td>inhibit</td>
<td>inhibit</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bergamottin</td>
<td>inhibit</td>
<td>inhibit</td>
<td></td>
<td></td>
<td></td>
<td>1, 2</td>
</tr>
<tr>
<td>Pea/clover/carrot</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gingerol</td>
<td>induce</td>
<td>induce</td>
<td></td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Ginger</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ginsenosides</td>
<td>inhibit</td>
<td>inhibit</td>
<td>inhibit</td>
<td></td>
<td></td>
<td>1, 5, 6</td>
</tr>
<tr>
<td>Ginseng</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>glabridin</td>
<td>inhibit</td>
<td>inhibit</td>
<td></td>
<td></td>
<td></td>
<td>1, 2, 7</td>
</tr>
<tr>
<td>Liquorice root</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>glyciosides</td>
<td>inhibit</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>Devils claw root</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>quercetin</td>
<td>inhibit</td>
<td>inhibit</td>
<td>inhibit</td>
<td>inhibit</td>
<td></td>
<td>1, 2, 9</td>
</tr>
<tr>
<td>Garlic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>silymarin</td>
<td>inhibit</td>
<td>inhibit</td>
<td>inhibit</td>
<td></td>
<td></td>
<td>1, 10,</td>
</tr>
<tr>
<td>Milk thistle seed/Meadow sweet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. (Zhou et al., 2004b), 2. (Zhou et al., 2004a), 3. (Hellum and Nilsen, 2008), 4. (Brandin et al., 2007), 5. (Jin et al., 2006), 6. (Hao et al., 2008), 7. (Nabekura et al., 2008), 8. (Unger and Frank, 2004), 9. (Tanaka et al., 2005), 10. (Doeher et al., 2008)

Example of drugs used in equine therapy in Sweden that are substrates for CYP3A or transport proteins are given in Table 8. It can be noted that during the time period 2005-2006 there has been an increase of 8% of the sale volumes of equine drugs in Sweden (Grunberger, 2007). The most frequently prescribed drugs to horses are antibiotics and anti-inflammatory and anti-parasitic drugs.
Table 8. Examples of drugs used in equine therapy in Sweden that are reported to be substrates, inducers or inhibitors of CYP3A and/or transport proteins.

<table>
<thead>
<tr>
<th>Active component</th>
<th>Drug</th>
<th>CYP3A</th>
<th>P-gp</th>
<th>BCRP</th>
<th>MRP1</th>
<th>MRP2</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ampicillin</td>
<td>Ampivet®</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>dexamethason</td>
<td>Opticorten®</td>
<td>induce</td>
<td>induce</td>
<td>induce/</td>
<td>induce</td>
<td>2, 3, 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vorenvet®</td>
<td></td>
<td></td>
<td>inhibit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fenbendazole</td>
<td>Axilur®</td>
<td>inhibit</td>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>ivermectin</td>
<td>Bimectin®</td>
<td>substrate</td>
<td>substrate</td>
<td>substrate</td>
<td>substrate</td>
<td>6, 7, 8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Equimax®</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Noromectin®</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ivomec®</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ivomec comp®</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ketamin</td>
<td>Ketalar®</td>
<td>substrate</td>
<td></td>
<td></td>
<td></td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ketaminol®</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lidocaine</td>
<td>Xylocain®</td>
<td>substrate</td>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>meloxicam</td>
<td>Metacam®</td>
<td>substrate</td>
<td></td>
<td></td>
<td></td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>moxidectin</td>
<td>Cydectin®</td>
<td>substrate</td>
<td>substrate</td>
<td>substrate</td>
<td>substrate</td>
<td>12, 13</td>
<td></td>
</tr>
<tr>
<td>omeprazole</td>
<td>Gastroguard®</td>
<td>induce</td>
<td>induce</td>
<td>inhibit</td>
<td></td>
<td>14, 15</td>
<td></td>
</tr>
<tr>
<td>phenylbutazone</td>
<td>Fenylbutazon®</td>
<td>induce</td>
<td></td>
<td></td>
<td></td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>praziquantel</td>
<td>Cydectin comp®</td>
<td>substrate</td>
<td>inhibit</td>
<td></td>
<td></td>
<td>17, 18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Equimax®</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ivomec comp®</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

6 Morphology and Physiology of the Examined Tissues

6.1 The alimentary canal

Being a grazing animal horse will ingest large amounts of cellulose. It can be suitable to divide the alimentary canal in horse into two parts: an anterior part (the foregut) and a posterior part (the hindgut). The foregut consists of the mouth, pharynx, oesophagus, stomach, duodenum, jejunum and ileum. The hindgut consists of the caecum, colon and rectum. The physiology of the foregut is similar to that in omnivorous species such as human and pig. To digest the cellulose in the feed horses are dependent on the large number of fermentating microorganisms, which are present in caecum and colon. The hindgut of horse has thus a similar function as the rumen and the reticulum in ruminants (Copenhaver et al., 1978; Kararli, 1995; Sisson, 1961).

The cellulose-fermenting microorganisms may also metabolize some xenobiotics, such as mycotoxins. This implies that in ruminants, some xenobiotics will be metabolized in the rumen and the reticulum, thus prior to entering the absorptive epithelium in the small intestine. This may render ruminants less sensitive to the potentially negative effects of such compounds compared to horses, in which the metabolism will not take place until in the caecum and colon, thus after the passage through the small intestine.

The structure of the small intestine provides a very large surface area (Figure 5). Firstly, the inner intestinal wall contains numerous folds. Secondly, the intestinal surface epithelium has finger-like villi. Thirdly, the epithelial enterocytes have fine extensions called microvilli that create a brush border.
and greatly increases the surface area. The epithelium of the large intestine lacks folding and villi and has a higher amount of mucous producing cells (goblet cells) than the small intestine (Copenhaver et al., 1978; Sisson, 1961).

The main cell-type in the intestinal epithelium is the single layered absorptive enterocyte, which is present at the surface epithelium and accounts for 80-90% of the total number of the epithelial cells. The enterocytes at the tip of the villi are functionally mature and non-dividing cells, whereas the crypt cells are immature and dividing. Mucous-producing goblet cells are interspersed among the absorptive enterocytes. Additional cell-types include endocrine cells and Paneth cells. The submucosa of the
duodenum and proximal jejunum contains Brunner’s glands, which in horse, in contrast to most other species, contain both mucous and serous acini (Pfeiffer and Dabareiner, 1992).

The lamina propria is the tissue layer between the surface epithelium and the muscularis mucosa. It contains smooth muscle cells, lymphatic vessels, blood vessels and a considerable number of lymphocytes (lamina propria lymphocytes; LPL), macrophages and mast cells. Lymphocytes are also interspersed among the enterocytes of the surface epithelium (intraepithelial lymphocytes; IEL) (Ferguson, 1977). The IEL and LPL consist mainly of T-cells. It has been estimated that in man there is a normal range of 10 to 30 IEL per 100 epithelial enterocytes (Ferguson, 1977). The number of IEL in relation to the number of epithelial enterocytes in horse is not known.

6.2 Liver

The liver is the largest gland in the body. The liver is involved in processing and storage of all categories of food materials (fats, carbohydrates, proteins, vitamins), removal of waste products from the blood and the synthesis of serum proteins, lipoproteins and clotting factors. It is also the major site for metabolism of xenobiotics. Horses have no gallbladder but a bile duct system with a wide calibre. The hepatic lobule, which can be considered to be the anatomical unit structure of the liver, contains a system of anastomosing blood sinusoids (Figure 6). The parenchyma is arranged in irregular cords. The duct system of the liver serves to convey the bile to duodenum. The smallest branches of the duct system are the bile canaliculi, which form a ramifying network of channels between the parenchymal cells. The canaliculi drain into bile ducts in the periphery of the lobules (Copenhaver et al., 1978; Sisson, 1961).
Figure 6. A schematic picture of the architecture in the liver. The picture also shows the membrane localization of P-gp, BCRP, MRP1 and MRP2 in the hepatocyte. The localizations of the transporters in the hepatocytes show variations between species. CYP enzymes are mainly present in the endoplasmic reticulum in hepatocytes. Modified from www.assofade.org

6.3 Kidney

Functions of the kidney include the regulation of water, electrolytes and acid-base balance, regulation of the blood pressure, metabolism of endogenous and exogenous compounds and excretion of waste products and exogenous compounds. The morphologically the equine nephrons and the basic renal processes are similar to those of most other species (Figure 7) (Toribio, 2007).
Figure 7. A schematic picture of a nephron and a tubule. The picture also indicates membrane localizations of P-gp, BCRP, MRP1 and MRP2 in proximal tubule cells (Choudhuri and Klaassen, 2006). The membrane localizations of the transporters show variations between species.

6.4 Tissues of the upper airways

The nasal olfactory epithelium is composed of three cell types: olfactory neurons, sustentacular cells and basal cells (Figure 8A). The nasal respiratory epithelium is a pseudo-stratified columnar epithelium, which contains four cell types: ciliated cells, non-ciliated (mucous) cells, goblet cells and basal cells (Figure 8B and C). The epithelium of the trachea has a similar structure as the nasal respiratory epithelium. The nasal olfactory mucosa contains Bowman’s glands localized in the lamina propria. Beneath the nasal respiratory mucosa and the tracheal mucosa serous and mucous glands are present in the submucosa (Copenhaver et al., 1978).

A potent CYP-related metabolism has been shown in the nasal olfactory and respiratory mucosa and the tracheal mucosa in several species, including the horse (Larsson et al., 1989; Larsson and Tjälve, 1996; Tydén et al., 2008). The CYP-containing cells in the nasal olfactory mucosa are the sustentacular
cells and the cells of Bowman’s glands. In the nasal respiratory and tracheal mucosa CYP-enzymes are present in the non-ciliated (mucous) cells (Grunberger, 2007; Larsson et al., 2003; Larsson et al., 1989).

Figure 8. A schematic picture of the nasal olfactory mucosa (A), nasal respiratory mucosa (B) and tracheal mucosa (C). CYP-enzymes are present in the sustentacular cells and the cells of Bowman’s glands in the nasal olfactory mucosa and in the non-ciliated (mucous) cells in the nasal respiratory mucosa and the tracheal mucosa. Legends: B, basal cell; Bg, Bowman’s gland; G, goblet cell; N, olfactory neuron; S, sustentacular; Cc, ciliated columnar cells; Mc, mucous cells.
7 Aims of the Thesis

In this thesis CYP3A and some ABC-transport proteins have been examined in horse tissues that are sites of exposure of drugs and other xenobiotics.

The specific aims of the thesis were:

- To examine the gene and protein expression, catalytic activity and cellular localization of CYP3A in the intestines and liver in horse.

- To investigate the gene and protein expression and CYP3A-related catalytic activity of the upper airways in horse.

- To explore the gene and protein expression and cellular localization of P-gp, BCRP, MRP1 and MRP2 in the intestines, liver and kidney in horse.
8 Materials and Methods

Below is a general description of the materials and methods used in this thesis. Detailed information on these matters is available in each paper.

8.1 Animals and sample collections

The tissues samples of the horses included in this thesis were collected at a slaughterhouse located in Huddunge, Sweden. All horses were standard bred trotters. Since this slaughterhouse has optimal conditions for samples preparation it was possible to collect the tissues immediately after slaughter. Tissues were taken from various parts of the intestines as shown in Table 9.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenum (D1)</td>
<td>Immediately posterior to the pyloric sphincter (Pars prima duodenii)</td>
</tr>
<tr>
<td>Duodenum (D2)</td>
<td>0.5 m posterior to the pyloric sphincter (Pars secunda duodenii)</td>
</tr>
<tr>
<td>Duodenum (D3)</td>
<td>1 m posterior to the pyloric sphincter (Pars tertia duodenii)</td>
</tr>
<tr>
<td>Jejunum (J1)</td>
<td>2.5 m posterior to the pyloric sphincter</td>
</tr>
<tr>
<td>Jejunum (J2)</td>
<td>5.5 m posterior to the pyloric sphincter</td>
</tr>
<tr>
<td>Jejunum (J3)</td>
<td>8.5 m posterior to the pyloric sphincter</td>
</tr>
<tr>
<td>Jejunum (J4)</td>
<td>13.5 m posterior to the pyloric sphincter</td>
</tr>
<tr>
<td>Ileum (I)</td>
<td>Immediately anterior to the ileo-cecal orifice</td>
</tr>
<tr>
<td>Caecum (Ca)</td>
<td>Mid-part of the parietal surface of the corpus caeci</td>
</tr>
<tr>
<td>Colon (C)</td>
<td>Origin of the small colon, posterior to the right dorsal colon</td>
</tr>
</tbody>
</table>

Tissue samples were also taken from the liver, the kidney, the nasal olfactory and respiratory mucosa and the tracheal mucosa. The horses were 3–9 years old and were either mares or geldings. All horses passed the health control required for animals, which are intended for meat production.
8.2 Microsomal preparations and determinations of enzyme activity

In the intestines about 20 cm long segments were scraped smoothly with a scalpel at the indicated sampling sites. To evaluate our scraping we have studied tissue samples of the small intestine in a microscope before and after the scraping. It was concluded that the scraping included the enterocytes from the top of the villi down to the crypts and that only few cells from the lamina propria or submucosa layer were included. Samples of the nasal olfactory mucosa, the nasal respiratory mucosa and the tracheal mucosa were also obtained by smooth scraping of the epithelial surfaces. Liver samples were taken from the middle (central) lobe and kidney samples from the cortex.

For preparations of microsomes the samples were homogenized in 0.05 M Tris–HCl buffer, pH 7.4 with 0.15 M KCl. As concerns preparation of intestinal microsomes complete trypsin inhibitor was also added. The homogenates were sedimented first at 10 000 g and then twice at 105 000 g. Microsomes were resuspended in the same buffer and stored at –78 ºC until used.

Total CYP and cytochrome b₅ contents were determined spectrophotometrically according to the methods of Omura and Sato (1964) and Rutten et al. (1987). (Omura and Sato, 1964) and (Rutten et al., 1987).

The NADPH P450 reductase activity was measure using cytochrome c as electron acceptor. We adapted the Cytochrome c Reductase (NADPH) Assay Kit from Sigma for usage in a 96-well microplate reader. An advantage of the 96-well plate adaptation is that less microsome are needed. This applications also implies time saving.

CYP3A activity was assayed using the CYP3A selective fluorescent probe 3-(3,4-difluorobenzyloxy)-5,5-dimethyl-4-(4-methylsulfonylphenyl)-5H-furan-2-one (DFB). We used the protocol from Chayret et al. (1999) and adopted it for the microplate reader. In this assay DFB is metabolized to the fluorescent metabolite DFH. Studies with human liver microsomes have shown that the formation of DFH from DFB shows a high selectively for CYP3A4, which is the orthologue to horse CYP3A89. Human CYP3A5 had low ability to form DFH from DFB, whereas other CYP-isoenzymes, such as CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2D6, CYP2E1, CYP2C8, CYP2C9 and CYP2C19 were found to have very low or
undetectable ability to perform this metabolism (Chauret et al., 1999). In the setup of our studies assays with human liver microsomes were run in parallel with horse liver microsomes.

8.3 Gene expression

8.3.1 RNA preparation
Tissue samples of the intestines and the liver were obtained from the horses as described under 7.2. Kidney samples were taken from the renal cortex. In paper I-IV total RNA was prepared with TRIzol. In paper V total RNA was prepared using Nucleospin RNA kit. The advantages of the latter method are that less tissue are needed, and that the method is less time consuming and easy to standardize. For both methods the RNA-purity and approximate concentration were determined spectrophotometrically by measuring the absorbance at 260 and 280 nm. Only samples with 260/280 nm ratio exceeding 1.8 were selected. To check the integrity of the RNA the samples were run on an ethidium bromide-stained 1% agarose gel containing 18% formaldehyde. The RNA samples were stored at –78°C before use.

8.3.2 Primer design
In paper I-IV primers for CYP3A and P-gp were designed based on conserved regions in human, rat, pig, dog and cattle. In 2007 the horse genome was fully sequenced and available at several non-commercial databases. In paper V primers for BCRP, MRP1 and MRP2 were designed based on the horse sequences. Primers were picked to span at least two introns. An advantage of this kind of primer design is the possibility to check for genomic contamination. An amplified product from cDNA (no introns) will be smaller than those amplified from genomic DNA (containing introns). The specificity and size of the primers were checked on an agarose gel and the PCR-products were purified and sequenced.

8.3.3 Real-Time RT-PCR
Prior to the Real-Time RT-PCR analyses samples were normalized to the exact amount of RNA using Ribogreen assay. The real-time RT-PCR reaction was carried out using the Rotor-Gene 3000 and the RT-PCR Kit One step QuantiTect™SYBR® Green from Qiagen. In paper I-IV relative quantification was used with a standard curve of known concentrations of the PCR-product. In paper V relative quantifications with the comparative
$C_{\gamma}$ method was used according to Livak and Schmittgen (2001) and data was normalized to the housekeeping gene glyceraldehyde-3-phosphatedehydrogenase (GADPH). The relative quantification with standard curve was chosen in paper I-IV since the horse genome was not sequenced at time of these studies and no known sequences of housekeeping genes were available.

### 8.4 Protein expression and localization

Antibodies with known epitope binding sequences are preferable and chosen if available. If the epitope binding sequence was not known antibodies with large species specificity were chosen (Table 10).

<table>
<thead>
<tr>
<th>Name of antibodies</th>
<th>Protein</th>
<th>Species</th>
<th>Epitope-binding sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A4</td>
<td>CYP3A4</td>
<td>rat anti-human</td>
<td>unknown</td>
</tr>
<tr>
<td>C494 P-gp</td>
<td>mouse anti-human</td>
<td>internal epitope: Human PNTLEGN, Horse PNTLEGN</td>
<td></td>
</tr>
<tr>
<td>C219 P-gp</td>
<td>mouse anti-human</td>
<td>internal epitope: Human VQUEALD, Horse VQUEALD</td>
<td></td>
</tr>
<tr>
<td>JSB-1 P-gp</td>
<td>mouse anti-human</td>
<td>internal epitope: sequence unknown</td>
<td></td>
</tr>
<tr>
<td>BXP-21 BCRP</td>
<td>mouse anti-human</td>
<td>internal epitope: sequence unknown</td>
<td></td>
</tr>
<tr>
<td>M2 III-6 MRP2</td>
<td>mouse anti-human</td>
<td>internal epitope: sequence unknown</td>
<td></td>
</tr>
<tr>
<td>MRPm6 MRP1</td>
<td>mouse anti-human</td>
<td>internal epitope: Human PSDLLQQRGL, Horse PSDLLQQKGL$^a$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ the red amino acids showing the differences between horse and human in the epitope-binding sequence of MRPm6.
8.4.1 Western blot
Protein expression was analyzed with Western Blot. CYP3A protein expression was studied using microsomal fractions. For P-gp, BCRP, MRP1 and MRP2 protein expression was studied using membrane fractions. Protein samples were diluted in Laemmli loading buffer containing β-mercapto ethanol. The proteins were separated on a 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were stained with Ponceau S to verify an even transfer of the proteins. In paper II, IV, V the proteins were visualized with the Western Blotting Detection Reagent ECL (plus or advance) and analyzed in a ChemiDoc Quantification System with Quantity-One software. In paper III proteins were visualized with the Western Blotting Detection Reagent ECL plus and detected on film.

8.4.2 Immunohistochemistry
To study the cellular localization of the proteins immunohistochemistry was preformed. Samples for immunohistochemistry (1-2 mm thick and 3-6 mm long) of the various parts of intestines and liver were fixed in 4% paraformaldehyde (4°C). To remove intestinal contents the intestinal segments were washed carefully in NaCl-solution before the fixation. In paper IV and V samples from the kidney and liver were fixed in paraformaldehyde supplemented with mercury chloride to improve epitope binding of the antibodies. In negative controls the primary antibody was omitted.
9 Results

9.1 Paper I “CYP3A in horse intestines”

The CYP3A gene expression in the intestines was high in the duodenum and jejunum and then declined rapidly in ileum, caecum and colon (Figure 9A). In the liver the CYP3A expression was about 3-5 times lower than in the anterior part of the duodenum.

The CYP3A-related metabolic activity showed a somewhat different pattern (Figure 9B). Thus, the CYP3A-related metabolic activity occurred at rather even level throughout the small intestine. Lower levels of metabolic activity were observed in the caecum and colon. In the liver the CYP3A-related metabolic activity was about equal of that in the small intestine.
The immunohistochemistry of CYP3A showed staining in the cytoplasm of enterocytes (Figure 10A and B). The immunostaining was most intense in the duodenum and the proximal parts of the jejunum. In the small intestine there was a more intense staining in the enterocytes of the tips of villi than in the enterocytes of the crypts. In the large intestine a similar pattern was seen with a more intense staining in the surface enterocytes than the enterocytes in the crypts. Within the enterocytes the strongest staining was localized in the apical parts of the cytoplasm. In the liver the immunostaining of CYP3A was highest in the centrilobular hepatocytes (Figure 10C).

![Figure 10. Immunohistochemistry of CYP3A in duodenum (A, B) and the liver (C). c, cytoplasm; e, enterocyte; cv, central vein; g, goblet cell; lp, lamina propria; n, nucleus.]

9.2 Paper II “Characterization of CYP3A-related metabolism in horse intestines and liver”

The protein expression of CYP3A was highest in the duodenum and then decreased towards in the ileum, caecum and colon (Figure 11A). In the different parts of the intestinal tract there was a strong correlation between the CYP3A protein expression and the CYP3A mRNA expression (Figure 11B). This also applied to the liver (Figure 11C).
Figure 11. (A) CYP3A protein expression in the intestines and liver. Correlations between expression of the CYP3A gene and protein in the intestines (B) and in the liver (C).

Fairly low inter-individual variations were observed among the horses included in the study both concerning the CYP3A gene expression and the CYP3A metabolic activity. The gene expression and the metabolic activity of CYP3A in the duodenum correlated with these parameters in the jejunum and ileum, but not in the caecum or colon (Table 11).

Table 11. Correlations between CYP3A mRNA expression in the duodenum and in the other intestinal segments and between CYP3A-related metabolic activity in the duodenum and in other intestinal segments

<table>
<thead>
<tr>
<th>Tissues</th>
<th>CYP3A mRNA expression</th>
<th>CYP3A-related metabolic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenum vs. Jejunum</td>
<td>r = 0.85; p &lt; 0.05</td>
<td>r = 0.90; p &lt; 0.05</td>
</tr>
<tr>
<td>Duodenum vs. Ileum</td>
<td>r = 0.94; p &lt; 0.05</td>
<td>r = 0.87; p &lt; 0.05</td>
</tr>
<tr>
<td>Duodenum vs. Caecum</td>
<td>r = 0.64; N.S</td>
<td>r = 0.1; N.S</td>
</tr>
<tr>
<td>Duodenum vs. Colon</td>
<td>r = 0.68; N.S</td>
<td>r = 0.3; N.S</td>
</tr>
</tbody>
</table>

* N.S not significant
In addition, in the liver these parameters matched with those in the small intestines, but not in the caecum and colon (Table 12).

Table 12. Correlations between CYP3A mRNA expression in the liver and in the different intestinal segments and between the CYP3A-related metabolic activity in the liver and in different intestinal segments

<table>
<thead>
<tr>
<th>Tissues</th>
<th>CYP3A mRNA expression</th>
<th>CYP3A-related metabolic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver vs. Duodenum</td>
<td>r = 0.70; p &lt; 0.05</td>
<td>r = 0.82; p &lt; 0.05</td>
</tr>
<tr>
<td>Liver vs. Jejunum</td>
<td>r = 0.78; p &lt; 0.05</td>
<td>r = 0.91; p &lt; 0.05</td>
</tr>
<tr>
<td>Liver vs. Ileum</td>
<td>r = 0.78; p &lt; 0.05</td>
<td>r = 0.86; p &lt; 0.05</td>
</tr>
<tr>
<td>Liver vs. Caecum</td>
<td>r = 0.25; N.S</td>
<td>r = 0.26; N.S</td>
</tr>
<tr>
<td>Liver vs. Colon</td>
<td>r = 0.70; N.S</td>
<td>r = 0.66; N.S</td>
</tr>
</tbody>
</table>

N.S not significant

The NADPH P450 reductase activity was slightly higher in the small intestines than in the large intestines. The amount of cytochrome b₅ was somewhat higher in the ileum and caecum than the other intestinal segments. In the liver both the NADPH P450 reductase activity and the contents of cytochrome b₅ were much higher than in the intestines. Estimation of the quotients for the NADPH P450 reductase activity and the cytochrome b₅ content related to CYP3A gene expression showed the highest ratios in the caecum, colon and ileum (Table 13).

Table 13. Quotients of NADPH P450 reductase activity versus CYP3A mRNA expression and cytochrome b₅ contents versus CYP3A mRNA expression in the different intestinal segments and the liver

<table>
<thead>
<tr>
<th>Tissues</th>
<th>NADPH P450 reductase activity: CYP3A mRNA expression</th>
<th>cytochrome b₅ contents: CYP3A mRNA expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenum</td>
<td>1.4</td>
<td>0.03</td>
</tr>
<tr>
<td>Jejunum</td>
<td>7</td>
<td>0.1</td>
</tr>
<tr>
<td>Ileum</td>
<td>111</td>
<td>4</td>
</tr>
<tr>
<td>Caecum</td>
<td>611</td>
<td>64</td>
</tr>
<tr>
<td>Colon</td>
<td>150</td>
<td>11</td>
</tr>
<tr>
<td>Liver</td>
<td>25</td>
<td>0.6</td>
</tr>
</tbody>
</table>
9.3  Paper III “Cytochrome P450 3A, NADPH cytochrome P450 reductase and cytochrome b$_5$ in the upper airways in horse”

The gene expression of CYP3A was much higher in the liver than in the tissues of the upper respiratory airways (Figure 12A). Among the latter tissues the highest expression of the CYP3A gene was found in the nasal olfactory mucosa. Lower levels of CYP3A gene expression were observed in the nasal respiratory and tracheal mucosa (Figure 12A). An analysis of protein expression demonstrated the same pattern with highest level of the CYP3A protein in the liver, lower levels in the nasal olfactory mucosa and the weakest levels in the nasal respiratory and tracheal mucosa (Figure 12B). The CYP3A-related metabolic activity showed another pattern with considerably higher activity in the tissues of the upper respiratory airways than the liver (Figure 12C).

![Figure 12. Gene expression of CYP3A (A), Western blot of CYP3A (B) and CY3A metabolic activity (C) in the tissues of the upper respiratory airways and the liver. As a control human recombinant CYP3A4 was run in parallel in the Western blot analysis. Legends; L, liver; R, nasal respiratory mucosa; O, nasal olfactory respiratory mucosa; Tr, tracheal mucosa; HCYP, human recombinant CYP3A4.](image)

It thus appears that the pattern of the CYP3A metabolic activity in the upper respiratory tract does not correlate with the CYP3A gene and protein expression. Determinations of NADPH P450 reductase activity and cytochrome b$_5$ showed that the levels of these co-factors in the relation to the CYP3A gene and protein expression were much higher in the tissues of the upper respiratory airways than in the liver.
The highest MDR1 gene expression was found in the duodenum and the proximal parts of the jejunum. The gene expression then declined rapidly to low levels in ileum, caecum and colon (Figure 13). MDR1 gene expression was also observed in the liver and kidney with much higher levels in the liver than the kidney. Peripheral blood lymphocytes also showed MDR1 gene expression. The Western blot showed presence of P-gp protein in the intestines, liver, kidney and peripheral blood lymphocytes. There was no statistically significant correlation between the MDR1 gene expression and the P-gp protein levels in the various intestinal segments.

Immunohistochemistry showed localization of P-gp in the microvilli of the apical surface membranes of the enterocytes throughout the intestinal tract, the most intense staining being present in the proximal parts of the intestines (Figure 14A). The enterocytes of the villi in the small intestines showed a more intense staining than in the crypts. Immunostaining of P-gp was also observed in the intraepithelial and lamina propria lymphocytes as well as in lymphocytes in the blood vessels (Figure 14A). The number of intraepithelial lymphocytes was higher in the duodenum and the jejunum than in the distal parts of the intestines. In the liver P-gp was localised in the bile canalicular membranes of hepatocytes (Figure 14B). In the kidney the P-gp was localized in the luminal and basolateral surface of cells in the proximal tubules and in the luminal surfaces of the cells in the loops of Henle and the cells of the distal tubules. There was a weak staining in the mesangial cells of the glomeruli (Figure 14C).
Sequence alignment showed that P-gp in horse (XP_001592073) has a 99 amino acid long insertion about 20 amino acids from the N-terminal part of the protein, which is not present in the other examined species. Another shorter insertion (6 amino acids) is present at the amino acid positions 140-145 from the N-terminal of horse P-gp, but not in the other examined species (Figure 15).

Figure 15. Kyte-Doolittle hydropathy plots of the N-terminal regions of horse and human P-gp. The plots were drawn with a window of 19. The amino acid number is given on the horizontal axes; the hydropathy score is given on the vertical axes. Hydrophobic transmembrane regions have scores from about 1.8 (indicated by a red line). The location of the 99 amino acid insertion in horse P-gp is indicated by a dotted line. An arrow indicates the location of the 6 amino acid insertions in horse P-gp. The green arrows indicate the conserved regions of P-gp starting from amino acid 146 in horse P-gp and 41 in human P-gp.
The results showed a prominent gene expression of BCRP and MRP2 in the small intestines and liver. In contrast to BCRP the gene expression of MRP2 was low in the caecum and colon (Figure 16). In the kidney both these transporters showed a rather low expression. In all tissues the gene expression of MRP1 was lower than for BCRP and MRP2. The pattern of the intestinal MRP1 gene expression showed a slow increase from the duodenum to the caecum and colon (Figure 16).

For MRP1 and MRP2 there was a statistically significant correlation between the protein and gene expression, but no such correlation was observed for BCRP.

Immunohistochemistry showed localization of BCRP and MRP2 at the apical surfaces of the enterocytes in the small intestine with a stronger immunostaining of the cells at the tips of the intestinal villi than of the cells in the intestinal crypts (Figure 17A and B). In the large intestine there was a lack of immunostaining for MRP2, whereas BCRP showed a weak staining of the apical cellular membranes in the caecum. In addition, both in the small and large intestine BCRP immunoreactivity was observed in the cells residing in the intestinal lamina propria, which mainly consist of lymphocytes and in addition macrophages and mast cells. For MRP1 the enterocytes of the large intestine showed a marked granular immunostaining (Figure 17C).
Figure 17. Immunostaining of BCRP (A) and MRP2 (B) in the enterocytes of the small intestine and of MRP1 (C) in the enterocytes of the caecum.

In Brunner’s glands, which are present in the submucosa of the duodenum and proximal jejunum, a marked immunostaining for BCRP and MRP1 was observed in serous acini, but not in mucous acini (Figure 18A and B).

Figure 18. Immunostaining of BCRP (A) and MRP1 (B) in the cells of serous acini of Brunner’s glands.

In the liver immunostaining of MRP2 was present in the bile canalicular membranes of the hepatocytes (Figure 19A). BCRP showed immunoreactivity in the cytoplasm of hepatocytes present in the peripheral parts of the liver lobuli, in cell membranes of interlobular bile ducts and in the endothelium of interlobular veins and arterioles (Figure 19B). No specific hepatic immunostaining was observed for MRP1.

Figure 19. Immunostaining of MRP2 in the bile canalicular membranes of hepatocytes (A) and of BCRP in the peripheral parts of the lobuli in the liver (B).
In the kidney BCRP and MRP2 showed a marked immunostaining in the luminal and basolateral membranes of the cells of the loop of Henle and the cells of the distal tubules and the collecting ducts (Figure 20A and B). No specific renal immunostaining was observed for MRP1.

Figure 20. Immunostaining of BCRP (A) and MRP2 (B) in the kidney. Legends; pt, proximal tubule; dt, distal tubule; h, loop of Henle.
10 Discussion

The disposition of drugs in horses is sometimes difficult to foresee because of factors that contribute the fate of equine xenobiotics have not been well characterized. It has for example been shown that the oral absorption of some antimicrobials and antihistamines is lower in the horse compared to other animals (Davis et al., 2005; Olsén et al., 2007; Törneke et al., 2003). It is therefore a need to obtain additional information on the conditions that determine uptake, metabolism and excretion of drug is horses. The localization and expression of CYP3A and the transport proteins P-gp, BCRP, MRP1 and MRP2 have not previously been examined in tissues of horse.

10.1 CYP3A

10.1.1 CYP3A in the intestines and the liver

The results of the present thesis have shown that the gene and protein of CYP3A are highly expressed in the proximal intestines with a decreasing gradient towards the distal parts of the intestines. The immunohistochemistry showed CYP3A localization in the enterocytes, with a similar gradient from the proximal to the distal parts of the intestines. Similar patterns of CYP3A gene and protein expression and CYP3A immunostaining have been shown in man, dog and trout (Kaminsky and Zhang, 2003; Kyokawa et al., 2001; Lee et al., 2001).

The results showed relatively high levels of CYP3A-related metabolic activity in the ileum, caecum and colon in spite of low gene and protein expression in these parts of the intestines. It is known that NADPH P450 reductase is a necessary component in the CYP monooxygenase cycle and
that high levels of the reductase promote the catalytic activity of CYP enzymes by supplying electrons to the CYP cycle (Nakajima et al., 2002). In addition cytochrome b₅ is known to enhance the metabolic activity of CYP enzymes by facilitating the electron transfer to the CYP cycle (Schenkman and Jansson, 2003). Our results showed that the levels of these cofactors in the relation to the CYP3A gene and protein expression were higher in the distal than the proximal parts of the intestines. These data indicate that an efficient electron transfer from the NADPH reductase and cytochrome b₅ to CYP3A in the ileum, caecum and colon may explain the marked CYP3A-related metabolic activity in these intestinal segments.

The inter-individual variations in CYP3A mRNA expression and in CYP3A-related metabolic activity in the intestines and the liver were fairly low among the horses included in our study. However, in man large inter-individual variations both as concern CYP3A mRNA expression and CYP3A-related metabolic activity have been shown in the liver and the intestines (Paine et al., 1997; Rodriguez-Antona et al., 2001; Watanabe et al., 2004). The expression of CYP-enzymes is dependent on various factors, such as genetical, environmental, pathological, hormonal and dietary conditions. The horses included in our study are of the same breed and of similar age and they have been kept in a comparable environment and given similar feed. This implies that the factors affecting CYP3A expression and metabolic activity in these horses may vary to a lesser extent than in man, which in turn can result in low variation in these parameters. However, herb supplements in the horse diet may affect CYP enzymes and ABC-transport proteins activity and possibly cause herb-drug interactions.

Our results showed that the gene expression and protein expression of CYP3A correlated both in the intestines and in the liver. These data indicate that the regulation of CYP3A protein expression in these tissues mainly occurs at a pre-translational level. These results are consistent with results reported for CYP3A4 in human intestines and liver (Berggren et al., 2007; Watanabe et al., 2004). The CYP3A gene and protein expression in the liver correlated with the CYP3A-related metabolic activity in the liver. Studies in man have shown that in the liver there is a correlation between CYP3A mRNA and protein expression and CYP3A-related metabolic activity (Paine et al., 1997; Rodriguez-Antona et al., 2001; Watanabe et al., 2004).
The CYP3A mRNA expression and the CYP3A-related metabolic activity in the duodenum, jejunum and ileum were in addition found to match with these parameters in the liver. In contrast, no significant correlations were observed when the CYP3A mRNA expression or the CYP3A metabolic activity in the caecum and colon were matched with these parameters in the liver. We also found that matching of the CYP3A mRNA expression and the CYP3A-related metabolic activity in the duodenum with these parameters in the other intestinal segments gave a statistically significant correlation for the jejunum and ileum, but not for the caecum and colon. The information obtained concerning the inter-individual variations in CYP3A gene expression and metabolic activity among the horses in the study support the conclusion that CYP3A in the liver is co-regulated with CYP3A in the small intestines, but not in the large intestines. A possible explanation for these observations is that inducers or inhibitors of CYP3A, which can be present in the feed of the horses, during absorption and metabolism concomitantly, will influence CYP3A both in the liver and in the small intestines. This phenomenon will not occur in the caecum or colon, which mainly is engaged in microbial breakdown of fibrous feed components. Our results indicate that it may be possible to analyse CYP3A mRNA in duodenal pinch biopsies to estimate CYP3A-related metabolic activity in the jejunum, ileum and liver.

10.1.2 CYP3A in the upper respiratory airways

Our results showed that the CYP3A related catalytic activity in the tissues of the upper airways was considerably higher than in the liver. The CYP3A gene and protein expression, on the other hand, were found to be much higher in the liver than in the other tissues. It thus appears that the pattern of CYP3A metabolic activity does not correlate with the CYP3A gene and protein expression. A possible explanation for this is that NADPH P450 reductase and cytochrome b$_5$, which were found to be present in relatively high levels in the upper airways, may facilitate the electron transfer into the CYP-cycle in the cells in these tissues. Studies in several other species, such as cattle, sheep, swine, hamster, rat and mouse, have shown high CYP-dependent bioactivation of various xenobiotics in the upper airways (Larsson et al., 1989; Marini et al., 1998; Reed, 1993; Reed et al., 1986; Tjälve, 1991; Tydén et al., 2004b). As concern hamster and cattle both the activity of NADPH P450 reductase and the amount of cytochrome b$_5$ in relation to the CYP content have been reported to be higher in these extrahepatic tissues than in the liver (Larsson et al., 1989; Reed et al., 1986).
10.2 ABC- transport proteins

10.2.1 Protein sequence analysis

The ABC-transport proteins have been highly conserved during the evolution (Higgins, 1992). Protein sequence alignments of horse P-gp, BCRP, MRP1 and MRP2 showed high identity with corresponding orthologues in human, dog and rat.

Interestingly, two amino acid insertions, one containing 99 amino acids, the other 6 amino acids, were found to be present in the N-terminal of horse P-gp, but not in some other examined species. A Kyte-Doolittle hydrophobicity plot showed that the insertions are hydrophobic and probably located intracellularly. Further studies to explore the possible function of these insertions would be of interest.

10.2.2 P-gp, BCRP, MRP1 and MRP2 in the intestines

The studies demonstrate gene and protein expression of P-gp, BCRP, MRP1 and MRP2 along the intestinal tract in horse. It appeared that in all tissues the expression of MRP1 was lower than for P-gp, BCRP and MRP2. This observation correlates with previous reports in other species (Cherrington et al., 2002; Conrad et al., 2001; Hilgendorf et al., 2007; Langmann et al., 2003; MacLean et al., 2008; Nishimura and Naito, 2005; Peng et al., 1999).

The patterns of the distribution of the transporters along the intestinal tract, with a decreasing level of P-gp from the small intestines towards the caecum and colon, with low level of MRP2 in the caecum and colon, slowly rising levels of MRP1 from the duodenum to the caecum and colon and considerable variations of the expression of BCRP in the different intestinal segments, also correlate with reports in other species (Berggren et al., 2007; Conrad et al., 2001; MacLean et al., 2008). The gene and protein expression in the intestines showed a significant correlation for MRP1 and MRP2, but not for P-gp or BCRP. These data indicate that the expression of MRP1- and MRP2-proteins in horse intestines is regulated at a transcriptional level. The absence of such correlation for P-gp and BCRP indicates that posttranscriptional factors are involved in the expression of these proteins.

The immunohistochemistry showed localization of P-gp, BCRP and MRP2 in the apical cell membranes of enterocytes in the duodenum, jejunum and ileum. In the large intestines there was a weaker immuno-staining for P-gp
and BCRP, but no immunoreactivity was observed for MRP2. Similar patterns of intestinal localization of P-gp, BCRP and MRP2 have been observed in man and rodents (Fetsch et al., 2006; Flens et al., 1996; Peng et al., 1999). P-gp and BCRP in the enterocytes are assumed to limit the oral absorption of xenobiotics by pumping compounds back into the intestinal lumen. MRP2 is supposed to act as an out-transporter of intracellularly formed conjugates. In the large intestines the enterocytes can be presumed to be exposed to low levels of xenobiotics, which as a result would require low level of the transporters. CYP- and transferase-enzymes in the enterocytes, which are present in higher levels in the small than in the large intestines (Kaminsky and Zhang, 2003; Paine et al., 1997; Tydén et al., 2004a) may act in concert with the transporters to limit xenobiotic absorption.

MRP1 showed a cytoplasmic granular staining in enterocytes of the caecum and colon. A cytoplasmic localization of MRP1 in the enterocytes in various parts of the intestines in human has been reported by (Flens et al., 1996). Although the function of MRP1 is not fully understood, it has been assumed that it acts as an exporter of xenobiotic conjugates from cells in several tissues. It has been suggested that the role of MRP1 may be to protect the cells containing this transporter rather than the whole organism (Berggren et al., 2007).

In addition to presence of P-gp and BCRP in the apical membranes of the enterocytes our results indicated that these transporters are present in intestinal lymphocytes and macrophages. It is has been shown that P-gp and BCRP in man is expressed in lymphocytes and monocytes in the blood (Moon et al., 2007). It has been proposed that P-gp and BCRP in the blood mononuclear cells may be involved in membrane transport of leukotriene C4, platlet activating factor and some cytokines (Farrell et al., 2000; Moon et al., 2007; Tsujimura et al., 2005). In addition the transporters present in these cells may restrict drug access to these important sites of action.

BCRP and MRP1 were found in the cells of the serous acini of Brunner’s glands of the horse. This species is one of few mammals in which Brunner’s glands are comprised of both mucous and serous tubular acini (Pfeiffer and Dabareiner, 1992). It can be noted that BCRP and MRP1 have been found in serous glands at other sites, such as in salivary glands, some glands in the respiratory pathways and pancreas (Fetsch et al., 2006; Flens et al., 1996;
Köng et al., 1999). The role of these transporters in these cells is not known at present.

10.2.3 P-gp, BCRP, MRP1 and MRP2 in the liver

In the liver much higher levels of gene and protein expression were observed for P-gp, BCRP and MRP2 than for MRP1. The immunohistochemistry of P-gp and MRP2 showed localization in the bile canalicular membranes of hepatocytes. This localization has been found in other mammals (Kool et al., 1997; Li et al., 2003; Thiebaut et al., 1987). P-gp and MRP2 at these sites are considered to be involved in the extrusion of some drugs and other xenobiotics from the hepatocytes into the bile (Faber et al., 2003). MRP2 is also active in the elimination of bilirubin from hepatocyte into bile (Schinkel and Jonker, 2003).

BCRP showed a granular cytoplasmic staining in the hepatocytes in the peripheral parts of the liver lobuli and in addition a staining of the cell membranes of interlobular bile ducts and the endothelium of interlobular veins and arterioles. In contrast, studies in man and mouse have shown localization of BCRP in the bile canalicular membranes of hepatocytes (Vander Borght et al., 2006). In addition, in man immunostaining of BCRP was reported in the bile ducts and the endothelium of the blood vessels (Vander Borght et al., 2006). In normal livers of rats the hepatocytes have been reported to lack BCRP-immunostaining, whereas bile ducts and vessels were stained (Vander Borght et al., 2006). It appears that there can be large species differences in the hepatic localization of BCRP between species.

It has been shown that in human liver disease cytoplasmic expression of BCRP is present in hepatic progenitor cells, which are localized in the periphery of liver lobuli, and it was proposed that BCRP in these cells may contribute to resistance to cytotoxic agents and xenotoxins (Vander Borght et al., 2006). It is not known at present whether the BCRP immunopositive cell in horse also include the progenitor cells. However, BCRP, which is present in the peripheral parts of the liver lobuli in horse, may also act to protect the hepatocytes at these sites from potentially toxic xenobiotics.

The protein expression of MRP1 was higher in the liver than in the kidney in horse. This correlates with observations in (Flens et al., 1996). In contrast,
in dog the gene and protein expression of MRP1 is higher in the kidney than in the liver (Conrad et al., 2001).

It appears that there can be large differences in the hepatic localization and expression of the ABC-tarnsporters between species. The impact which these species differences may have on metabolism and elimination of drugs is not known as yet.

10.2.4 P-gp, BCRP, MRP1 and MRP2 in the kidney

As in the intestines and liver the levels of gene and protein expression of P-gp, BCRP and MRP2 in the kidney were much higher than for MRP1. For all the transport proteins the gene and protein expression were lower in the kidney than in the liver. As mentioned in dog the expression level of MRP1 is higher in the kidney than in the liver (Conrad et al., 2001).

P-gp, BCRP and MRP2 were localized both in the apical and basolateral membranes of cells in Henles loop and in the cells of the distal tubules and collecting ducts P-gp was also present in the luminal membranes of the cells of the proximal tubules. The role of P-gp and BCRP in the kidney in horse may be to participate in active tubular excretion of xenobiotics reaching the kidneys via the blood, pumping of xenobiotics absorbed from the urine back into the tubular lumen as well as transport of compounds from tubular cells into the blood. Similar functions may be assumed for MRP2, although the substrates for this transporter can be presumed to mainly involve conjugates of drug and other xenobiotic. P-gp, BCRP and MRP2 have been reported to be present solely in the apical membranes of the cells in the proximal tubules in man and rodents (Huls et al., 2008; Schaub et al., 1997). It is important to recognize that the differential renal patterns of the tissue expression of the transporters also imply that variations in the functions of these transporters in the kidneys can vary between species.

Paper IV and V highlights that marked species differences may exist between horse and other species as concern gene and protein expression and cellular localization of the transporters in the tissues. This indicates that the effects of the transporters on the kinetics and dynamics of drugs may vary between horse and other species. Further studies aiming at exploring these issues would be of great interest.
11 Conclusions

Some salient results, which have emerged in this thesis, are:

➢ The CYP3A gene and protein expression are high in the small intestines of horse. The gene expression of CYP3A in the first part of duodenum was even higher than in the liver.

➢ There are correlations between CYP3A gene and protein expression both in the intestines and the liver, indicating transcriptional regulation of CYP3A protein expression in these tissues in horse.

➢ The CYP3A gene expression and the CYP3A-related metabolic activity in the duodenum, jejunum and ileum were found to match with these parameters in the liver. This observation may have clinical relevance, since it may be possible to analyze the CYP3A expression in duodenal pinch biopsies and predict CYP3A-related metabolic activity in the liver in horse.

➢ In contrast to the correlation described above between CYP3A gene expression and the CYP3A-related metabolic activity in the small intestines and liver, no such correlation was observed when these parameters were matched with those in the caecum and colon. It is possible that inducers or inhibitors of CYP3A, which can be present in the feed of horse, concomitantly will influence CYP3A both in the small intestines and in the liver. This may not occur in the caecum and colon, which mainly are engaged in microbial breakdown of fibrous feed components.
The results showed high levels of CYP3A-related metabolic activity in the upper airways in spite of low levels of gene and protein expression of CYP3A in these tissues. A possible explanation for this observation is that the CYP3A-related metabolic activity in the upper airways will be facilitated by an efficient electron transfer into the CYP-cycle due to presence of high levels of NADPH P450 reductase and cytochrome b$_5$ in these tissues.

CYP3A gene expression and CYP3A-related metabolic activity in the intestines and the liver showed rather low inter-individual variations among the horses included in the study. This implies that environmental and other factors, which may affect CYP3A expression and metabolic activity, may vary to a lesser extent than in man, in whom higher variations in these parameters have been observed.

The amino acid sequences of the proteins of the ABC-transporter family are generally highly conserved between species. However, protein sequence alignment showed that in horse P-gp has two amino acid sequences, respectively 99 and 20 amino acids long, inserted at the N-terminal region of the protein, which were absent in the other species examined. The possible function of these insertions is not known at present. Protein sequence alignment of BCRP, MRP1 and MRP2 showed that these proteins are highly conserved between horses and other species.

In horse gene and protein expression of P-gp, BCRP, MRP1 and MRP2 were found in the intestines as well as in the liver and kidney. The expression and the localization of the transporters in these issues of horse showed both similarities and differences compared to other species.

Immunohistochemistry showed localization of P-gp, BCRP and MRP2 in the apical cell membranes of the enterocytes in the small intestines, in which these transport proteins may play a role in the regulation of oral bioavailability of some xenobiotics by pumping compounds from the inside of the enterocytes back into lumen of the intestines. Additional observations were that P-gp and BCRP are present in intraepithelial and lamina propria intestinal lymphocytes and that BCRP and MRP1 are present in the cells of serous acini of Brunner’s glands.
In the liver P-gp and MP2 were localized in the bile canalicular membranes, in which they may act to excrete drugs and other xenobiotics from the hepatocytes into the bile. BCRP was localized in the cytoplasm of cells in the peripheral parts of the liver lobuli. The role of this transporter in these cells is not known at present.

In the kidney the transporters were found in different parts of the nephrons, in which they may function to promote tubular secretion of xenobiotics into urine or transport of xenobiotics from the tubular cells into the blood.

Collectively the results of the present thesis indicate that CYP3A and the examined ABC-transport proteins have important roles for bioavailability and elimination of substrate compounds in horse.
12 Future Research

The results of this thesis indicate that CYP-enzymes and transport proteins are of great importance for the disposition of drugs and other xenobiotics in horse. Further studies aiming at exploring the roles of CYP-enzymes and transport proteins for the fate of xenobiotics in horse as well as in other domestic animals would be of interest. A few examples are given below.

- The CYP-enzymes involved in the metabolism of xenobiotics belong to the CYP-families 1, 2 and 3. It would be of interest to explore the occurrence of some CYP-enzymes in these families (e.g. CYP1A, CYP2C, CYP2D and CYP2E) in the intestines and liver in horse. Studies on the occurrence of these enzymes in the upper respiratory airways of horse would also be of interest.

- It would be of interest to examine the occurrence and metabolic activities of CYP-enzymes of the 1, 2 and 3 families in the intestines, liver and upper airways in other domestic animals.

- It would be of interest to explore the occurrence of additional transport proteins in the tissues of horse, as well as in other domestic animals.

- It would be of interest to map in detail the spectrum of drugs used in horse and other domestic animals that are substrate for different CYP-enzymes and transport proteins. “Model drugs” may then be used in in vivo studies to explore the roles of the CYP-enzymes and the transporters for the disposition of drugs.
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I avhandlingen har cytokrom P450 3A (CYP3A) och ABC-transportproteiner P-glykoprotein (P-gp), breast cancer resistance protein (BCRP), multidrug resistance protein 1 (MRP1) och multidrug resistance protein 2 (MRP2) studerats hos häst. CYP3A och transportproteiner har stor betydelse för upptag, metabolism och utsöndring av läkemedel och andra främmande ämnen. Det finns många undersökningar där CYP3A och transportproteiner har studerats hos gnagare och människa, medan däremot undersökningar hos häst saknas.

I den första delen av avhandlingen (arbete I-III) studerades gen- och proteinuttryck och den metaboliska aktiviteten hos CYP3A i tarmarna, levern och de övre luftvägarna hos häst. I tarmen fanns det högsta gen- och proteinuttrycket i duodenum och jejunum. En intressant observation var att genuttrycket i den första delen av duodenum var ca. 4,5 gånger högre än i levern. Det fanns en korrelation mellan genuttrycket och proteinuttrycket för CYP3A både i tarmarna och i levern, vilket indikerar att det är en pre-translationell reglering av proteinuttrycket för CYP3A i dessa vävnader hos häst. Genuttryck och metabolisk aktivitet för CYP3A i duodenum, jejunum och ileum korrelerade med dessa parametrar i levern. Denna observation kan ha klinisk relevans, eftersom det kan vara möjligt att analysera genuttrycket för CYP3A i biopsier från duodenum och på basis av detta avgöra den CYP3A-relaterade metabolismen i levern hos häst.

Man vet att CYP-relaterad metabolisk aktivitet är beroende av närvaro av elektrondonerande enzymer såsom NADPH P450-reduktas och cytokrom b₅. Våra resultat visade relativt höga nivåer av CYP3A-relaterad metabolism i de distala delarna av tarmkanalen och i de övre luftvägarna hos häst, trots låga nivåer av gen- och proteinuttryck i dessa vävnader. En möjlig förklaring
till dessa observationer är att den CYP3A-relaterade metaboliska aktiviteten i dessa delar av tarmen och i de övre luftvägarna stimuleras av en effektiv elektronöverföring till CYP3A från NADPH P450-reduktas och cytochrom b₅, som återfanns i relativt höga halter i dessa vävnader.

Den CYP3A-relaterade metaboliska aktiviteten i tarmarna och i levern visade relativt låga interindividuella variationer hos hästarna som ingick i undersökningen. Det kan vara så att genetiska faktorer, liksom miljöfaktorer, som kan påverka uttryck och metabolisk aktivitet hos CYP3A, varierar mindre hos de undersökta hästarna än hos människa, där större variationer har observerats för dessa parametrar.

I den andra delen av avhandlingen (arbete IV-V) studerades gen- och proteinuttrycket och den cellulära lokalisationen av transportproteinerna P-gp, BCRP, MRP1 och MRP2 i tarmar, lever och njurar hos häst. Aminosyrasekvenserna för dessa proteiner är i allmänhet högt konserverade mellan olika djurslag. Emellertid visade proteinsekvensjämförelser att det för P-gp hos häst finns två aminosyrasekvenser, som består av respektive 99 och 6 aminosyror, i den N-terminala delen av protein som inte finns hos ett par andra undersökta djurslag eller hos människa. Den funktionella betydelsen av detta är ännu okänd. Proteinsekvensjämförelser för BCRP, MRP1 och MRP2 visade att dessa proteiner hos häst är mycket lika de hos ett par andra undersökta djurslag och hos människa.

Höga nivåer av gen- och proteinuttryck för P-gp och MRP2 återfanns i duodenum, jejunum och ileum, medan däremot nivåerna i caecum och kolon var låga. Gen- och proteinuttrycket för BCRP varierade i olika tarmavsnitt. För MRP1 sågs ett långsamt ökande gen- och proteinuttryck från duodenum till kolon. För alla transportproteinerna var gen- och proteinuttrycket högre i levern än i njurarerna. En annan observation var att gen- och proteinuttrycket för MRP1 var lägre i alla vävnader jämfört med de andra transportörerna.

Immunohistokemi visade förekomst av P-gp, BCRP och MRP2 i de apikala cellmembranen i tarmens enterocyter. Dessa transportprotein kan ha stor betydelse för regleringen av det orala upptaget av läkemedel och andra främmande ämnen genom pumpa substanser som tagits upp i cellerna tillbaka till tarmlumen. Ytterligare observationer var att P-gp och BCRP återfinns i de lymfocyter som finns i tarmslemhinnan och att BCRP och
MRP1 återfinns i serösa acini i Brunners körtlar i duodenum och början av jejunum.

I levern återfanns P-gp och MRP2 i levercellernas gallkanalikuli, i vilka de kan verka vid utsöndringen av främmande ämnen i gallan. BCRP återfanns i cytoplasman i cellerna i de perifera delarna av leverlobuli. Det är möjligt att BCRP i dess celler kan ge skydd mot negativa effekter av hepatotoxiska substanser.

I njurarna återfanns transporproteinerna i olika delar av nefronen. Funktioner av transportproteinerna i njuren kan vara att medverka i utsöndringen av främmande ämnen från blodet till urinen, pumpa tillbaka substanser som tagits upp i cellerna i nefronen till urinen, samt transportera ämnen från cellerna i nefronen ut i blodet.

Sammantaget indikerar resultaten i avhandlingen att CYP3A och de undersöpta ABC-transportproteinerna har stor betydelse för upptag, metabolism, vävnadsfördelning och utsöndring av läkemedel och andra främmande ämnen hos häst.