Effects of antibiotic treatment on the expression of hepatic and intestinal cytochrome P450

Bruno Pereira De Carvalho
University at Albany, State University of New York, brunocarvalhoufg@gmail.com

The University at Albany community has made this article openly available. Please share how this access benefits you.

Follow this and additional works at: https://scholarsarchive.library.albany.edu/legacy-etd

Part of the Molecular Biology Commons, Pharmacology Commons, and the Toxicology Commons

Recommended Citation
https://scholarsarchive.library.albany.edu/legacy-etd/1920

This Master's Thesis is brought to you for free and open access by the The Graduate School at Scholars Archive. It has been accepted for inclusion in Legacy Theses & Dissertations (2009 - 2024) by an authorized administrator of Scholars Archive. Please see Terms of Use. For more information, please contact scholarsarchive@albany.edu.
EFFECTS OF ANTIBIOTIC TREATMENT ON THE EXPRESSION OF HEPATIC AND
INTESTINAL CYTOCHROME P450

by

Bruno Pereira de Carvalho

A Thesis
Submitted to the University at Albany, State University of New York
In Partial Fulfillment of
the Requirements for the Degree of
Master of Science

School of Public Health
Department of Environmental Health Sciences
2017
Abstract

Expression of metabolic enzymes Cytochrome P450 (CYP) is highly affected by drugs, diseases, age and gender. The Intestinal microflora has been suggested to play a role in regulating CYP expression and antibiotic treatments would lead to changes in level and composition of microbiota in mouse gut. This study aims to analyze CYP expression changes in the liver and small intestine resulted from the reduction of intestinal microflora by antibiotics. Impacts on bile acid signaling were also analyzed for a better comprehension of the mechanisms involved in this modulation. Female C57BL/6J mice (9- to 13-week old) were treated with ampicillin (AMP) or vehicle for 3 or 7 days. Analysis of mRNA expression level indicated decreased expression of CYP2B10 in liver and intestine of mice treated for 3 days. In 7 days of AMP treatment, CYP3A11 mRNA levels were decreased in liver and increased in the intestine of mice. The AMP treatment reduced the population of lithocholic acid-producing bacterial strains and led to decreased expression of fibroblast growth factor 15 (FGF15) and increased expression of apical sodium-dependent bile acid transporter (ASBT). Enlarged gallbladders and the increased levels of CYP7A1 mRNA suggested an increase of bile acid synthesis and bile secretion. This study indicates that changes in the microflora can modulate CYP expression in mouse liver and intestine. A full understanding of the role of gut microflora in xenobiotic metabolism is desirable in order to predict drug-drug interactions, drug-diet interactions, and individual pharmacokinetic differences.
Table of Contents:

Abstract..............................................................................................................................ii

Table of contents..............................................................................................................iii

List of abbreviations........................................................................................................v

1. Specific Aim..................................................................................................................1

2. Background....................................................................................................................1

   2.1 Cytochrome P450.....................................................................................................1

   2.2 Factors involved in CYP modulation.......................................................................3

   2.3 Antibiotics...............................................................................................................5

   2.4 Intestinal flora and drug metabolism.......................................................................6

   2.5 Role of bile acids and nuclear receptors in CYP modulation.................................8

3. Significance....................................................................................................................9

4. Study design..................................................................................................................10

5. Methods.........................................................................................................................11

   5.1 Animal treatment and sample collection...............................................................11

   5.2 Bacterial DNA quantification..................................................................................12

   5.3 mRNA analysis.........................................................................................................12

   5.4 Statistical analysis..................................................................................................13

6. Results...........................................................................................................................14

   6.1 Quantification of bacterial DNA in cecum..............................................................14

   6.2 Visual inspection of the gallbladder.........................................................................14

   6.3 Effects of AMP treatment on hepatic CYP expression............................................15

   6.4 Effects of AMP treatment on the expression of intestinal CYPs and several other
genes..................................................................................................................16

7. Discussion........................................................................................................19

8. Conclusion........................................................................................................23

9. References........................................................................................................24
List of abbreviations:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full name</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP or P450</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>AMP</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>CPX</td>
<td>Ciprofloxacin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PXR</td>
<td>Pregnane X receptor</td>
</tr>
<tr>
<td>FXR</td>
<td>Farnesoid X receptor</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
</tr>
<tr>
<td>CAR</td>
<td>Constitutive androstane receptor</td>
</tr>
<tr>
<td>ASBT</td>
<td>Apical sodium-dependent bile acid transporter</td>
</tr>
<tr>
<td>FGF15</td>
<td>Fibroblast growth factor 15</td>
</tr>
<tr>
<td>BA</td>
<td>Bile Acids</td>
</tr>
<tr>
<td>GF</td>
<td>Germ-free</td>
</tr>
</tbody>
</table>
1. Specific Aim

This study aims to investigate the effects of antibiotic treatment in the modulation of hepatic and intestinal drug-metabolizing enzymes. Impacts on bile acid signaling were also analyzed for a better comprehension of the mechanisms involved in this modulation.

2. Background

2.1 Cytochrome P450

Cytochrome P450 (P450 or CYP) are heme-thiolate enzymes that have been identified across all kingdoms of life and play a fundamental role in the metabolism of many drugs and endogenous compounds. Mammalian endogenous metabolism relies on P450 for the biotransformation of vitamins, fatty acids, steroids, and eicosanoids. Exogenous compounds as pollutants, foreign natural compounds and drugs are also targets of P450 enzymes. Due to P450’s role in the metabolism of xenobiotics, this enzyme holds an outstanding importance in the fields of toxicology, pharmacology and chemical toxicity (Furge & Guengerich 2006).

P450 proteins are characterized by a ferrous heme prosthetic group (iron-protoporphyrin IX). The heme portion of these enzymes is not different from that found in heme peroxidases and hemoglobin. The number “450” stems from the reduced ferrous heme group which, due to its high affinity for CO, generates an iron-CO complex which has maximum absorption at 450nm (Hrycay & Bandiera 2015). P450s are also the major consumer of heme groups in the liver: 50% of hepatic
heme is prosthetically incorporated into P450s (Correia et al. 2011). The reactions catalyzed by these enzymes are various, including hydroxylation, dealkylation, epoxidation, heme inactivation, and group migration (Furge & Guengerich 2006).

The P450 super-family counts with over 4,500 members which 57 are present in Homo Sapiens. Members of this super-family are classified according to a systematic alphanumerical designation where the enzyme CYP3A4, for example, would belong to family 3 and subfamily 3A. The last number, 4, defines the particular enzyme (Lewis et al. 2005). Most of human P450 catalyze the metabolism of endogenous compounds. Metabolism of xenobiotics is restricted to a group of about a dozen enzymes, belonging to the families 1, 2, 3, and 4 (Zanger & Schwab 2013).

The membrane-bound characteristic of P450s is reflected in its subcellular localization and, although some exceptional families can be found in the mitochondria and cell surface, P450s are primarily found in the endoplasmic reticulum (Neve & Ingelman-Sundberg 2010). At the tissue level, they can be found in every tissue, except skeletal muscle and red blood cells (Furge & Guengerich 2006). Renaud et al. (2011) quantified 78 isoforms of P450s and concluded that 52% of the isoforms were expressed highest in the liver, 10% in the small intestine, 10% in kidney, 10% in the testes and 5% in the lung. Additional studies using human microsomes and immunoblotting concluded that the major P450 components of the liver are CYP3A, 2C and 1A2 representing respectively 40, 25, and 18% of total immunoquantified P450s. In the intestine, CYP3A and CYP2C9 are the major P450s representing 80 and 15% respectively, of total immunoquantified P450s (Paine et al. 2006).
2.2 Factors involved in CYP modulation

Various factors are able to cause changes in P450 activity and gene expression. These changes can be divided into two categories: qualitative and quantitative. The first is related to genetically pre-determined impairments (polymorphisms) and the last category is caused by diverse agents as age, gender, disease, and drugs (Toda et al., 2009a).

Interindividual differences in the metabolism of diverse drugs can be explained by polymorphic P450s. Genetic polymorphism is a stable variation, identified in a genetic locus, which can be detected in 1% or more of a specific population. Single-nucleotide polymorphisms are the most representative genetic mutation present in P450. Mutated nucleotides can result in modified amino acids and affect the metabolic activity of P450 enzymes. Polymorphisms in CYP2A6, 2C9, 2C19, and 2D6 are considered to be the ones causing the largest variations in metabolic activity (Zhou et al. 2009).

Age and gender are known to modulate P450 activity in a quantitative manner. Animal studies indicate an overall P450 decrease in rats over 20-months old (Alterman et al. 1993). The decreasing trend seems to be applicable to human monooxygenase systems despite many controversial results. Age-related diminished levels of CYP2E1, CYP3A and NADPH cytochrome c reductase in human hepatic microsomal contents was reported (Schmucker 2001). However, these results are contrasted by studies as Wynne et al. (1988), that reports absence of correlation between age and microsomal contents or activities of human liver monooxygenases. One important confounder in the investigation of age-related P450 changes is gender. An age-related feminization of male rats have been reported in diverse studies and it is known to modulate the pattern of P450 expression due to the decrease of male-specific forms of P450. Nevertheless, the role of gender in
the modulation of human P450 requires more investigation and the feminization process seems to be exclusive for rats (Kamataki et al. 1985).

Pathogens and disease conditions are also known to modulate P450 expression. Inflammatory reactions are known to decrease hepatic P450 expression and activities in various models and clinical reports (Morgan 2009). Down-regulation of Intestinal P450s expression is also reported in mice subjected to dextran sodium sulfate-induced colitis (Kawauchi, Nakamura, Miki, et al. 2014) and rats subjected to indomethacin-induced intestinal ulcers (Kawauchi, Nakamura, Yasui, et al. 2014). Disease-induced modulation of drug metabolism is especially concerning in scenarios which eventual infections or inflammatory processes occur during a stable drug regimen.

In 1980, the influenza B outbreak caused toxicity in children under theophylline treatment. This drug has 90% of its content metabolized by P450s when ingested and the decreased P450 levels caused by influenza was responsible for adverse reactions as seizures, nausea, and vomiting (Kraemer et al. 1982).

Exogenous compounds as alcohols, environmental pollutants, and drugs are common substrates for P450 enzymes (Chang & Kam 1999). However, some of these foreign compounds can also induce or inhibit P450s, becoming key factors for human metabolism studies (Mann 2006). Glyphosate is a representative example of environmental toxicants able to influence CYP activity. This organophosphate has been reported as an inhibitor of CYP19A1 (aromatase) and CYP26A1 (Retinoic Acid-Metabolizing Cytochrome) (Samsel & Seneff 2013). The role of drugs in P450 modulation is also a topic of extreme importance in the field of drug metabolism and constitutes the basis of an important phenomenon: drug-drug interactions.
2.3 Antibiotics

Antibiotics are drugs that target bacterial organisms by diverse mechanisms as enzymatic inhibition, interference with cell membrane permeability, interference with cell wall synthesis, interference with DNA synthesis and interference with protein synthesis. Antibiotic drugs can act in a broad or narrow spectrum and exert a bacteriostatic (inhibit growth or reproduction of bacteria) and/or bactericidal (promote bacterial death) effect. Macrolides as azithromycin and erythromycin, constitute an important class of antibiotic which major mechanism relies on binding to bacterial ribosome and consequently inhibition of protein synthesis. Another representative class of antibiotics is the antitubercular, such as rifampicin. Rifampicin is capable to bind bacterial RNA polymerase and inhibit bacterial transcription. Beta-lactam antibiotics as penicillin and ampicillin (AMP) are characterized by its beta-lactam ring and are considered the most widely used group of antibiotics due to its versatility, low toxicity, and selectivity. The main mechanism of beta-lactam activity is the inhibition of bacterial cell wall synthesis (Giguere et al. 2013).

Some antibiotics can affect P450 expression and activity. This modulation is concerning, due to the fact that antibiotics are the most widely prescribed medications for treating infections and are frequently co-administered with other drugs (Cazzola et al. 2004). Some antibiotics as Rifampicin are able to induce P450 expression by activating nuclear receptors. This is an extensively studied mechanism where rifampicin, in the cytoplasm, binds to the pregnane X receptor (PXR) that function as a ligand-activated transcription factor. The activated PXR is translocated into the nucleus to form an heterodimer with retinol X receptor and bind CYP3A promoter (Chen & Raymond 2006).
The mechanisms of CYP modulation are not very clear for some antibiotics. Ishii et al. (2012) demonstrated that mice treated with Ampicillin (AMP) and ciprofloxacin (CPX), drugs that act in a wide antimicrobial spectrum, presented decreased mRNA levels of hepatic CYP3A11. CPX is also reported to decrease hepatic expression of CYP3A in rats (Xie et al. 2003). Oppositely to Rifampicin, antibiotics as AMP are not capable to induce or inhibit drug-metabolizing enzymes (Niwa et al. 2016). Therefore, the broad spectrum of this antibiotic suggests a possible role of the intestinal flora in P450. CPX and AMP are active against gram-positive bacteria and Eubacteria such as Bacteroides, Clostridium, Mycoplasma, and Mycobacterium (Toda, Ohi, et al. 2009). The role of these bacterial strains in the antibiotic-induced decrease of P450 requires more investigation.

2.4 Intestinal flora and drug metabolism

The human gut microbiota have been proved to possess fundamental roles regarding health status and lifespan. Resistance to infection and inflammation, prevention of autoimmune processes and cancer, control of metabolism, and regulation of brain-gut axis are examples of important functions being supported by the intestinal microbiota (Vaiserman et al. 2017).

This ecological community of microorganisms is inhabited by specific bacterial species and its composition is a result of strong selective pressure. Firmicutes and Bacteroidetes are the two predominant phyla present in the gut microbiota of both human and mice (Claus et al. 2011; Miyata et al. 2011). Bardof et al. (2013) report that abundance of these phyla in murine cecum samples is 29% for Bacteroidetes and 59% for Firmicutes. The appropriate balance of different
bacterial communities is influenced by age, diet, psychogenic factors as stress, and antibiotics. Imbalance of intestinal flora can result in serious health disorders since the microbiota is shown to influence the risk of colorectal cancer, inflammatory bowel disease, and irritable bowel syndrome. Even extra-intestinal pathologies can take place in the liver and respiratory tract (Vaiserman et al. 2017; Toda, Saito, et al. 2009).

The intestinal flora is able to influence the metabolism of drugs. More investigation is required in order to completely understand the mechanisms and impacts of this influence but significant amount of information is available in the literature. The primary enterobacterial mechanism to influence the activity of drugs is the metabolism performed by its own bacterial constituents. More than 17 different chemical reactions by the gut microflora have been reported to act in the biotransformation of drugs, such as alicylosulfapridine, digoxin, L-dopa, acetaminophen, caffeic acid, phosphatidyl choline, carnitine, sorivudine, irinotecan, nonsteroidal anti-inflammatory drugs, heterocyclic amines, melamine, nitrazepam, and lovastatin (Klaassen & Cui 2015). Gut microbiota can influence drug metabolism not only by direct metabolism of xenobiotics but also indirectly through regulation of P450 metabolizing enzymes in host liver and intestine. Decreased gene expression, protein levels and metabolic activity of hepatic CYP3A in Germ-free (GF) and antibiotic-treated mice have been reported (Toda, Saito, et al. 2009; Toda, Ohi, et al. 2009). Using quantitative proteomic analysis, Kuno et al. (2016) also reported a significant decrease of hepatic CYP2B10 and CYP3A11 protein expression in both GF and antibiotic-treated mice. The mechanisms involved in P450 modulation by microbiota are not clear but one of the better studied hypothesis involves the biosynthesis of secondary bile acids in intestinal lumen. Secondary bile acids are able to activate nuclear receptors and consequently regulate P450 expression (Toda, Ohi, et al. 2009).
Previous studies demonstrated the role of enterobacteria in the modulation of intestinal bile acid transport and homeostasis. Mice submitted to antibiotic treatment presented increased mRNA levels of apical sodium-dependent bile acid transporter (ASBT), decreased mRNA levels of fibroblast growth factor 15 (FGF15) and significant differences in the expression of other ileal bile acid-related genes (Miyata et al. 2011). ASBT is a transporter that contributes to the enterohepatic circulation of bile salts by the absorption of ileal bile acids and FGF15 is a Farnesoid X receptor (FXR)-target gene involved in the suppression of intestinal ASBT and hepatic CYP7A1 expression.

2.5 Role of bile acids and nuclear receptors in CYP modulation

Bile acids (BAs) are amphipathic molecules products of cholesterol catabolism. More than its contribution to the elimination of cholesterol, BAs also play a role facilitating digestion, promoting absorption of lipid soluble vitamins and acting as signaling molecules of energy metabolism. These molecules require very controlled regulation due to its toxicity. BAs can be accumulated due to impairment of bile flow, a pathological condition called cholestasis. Apoptosis and necrosis are reported to be induced by BAs at a cellular level (Chen et al. 2014). Cholic acid and chenodeoxycholic acid are synthesized from cholesterol in the hepatocytes and are classified as primary bile acids. Lithocholic acid (LCA) and deoxycholic acid, classified as secondary bile acids, are intestinal products of bacterial metabolism of primary bile acids (Hofmann 2015).

One protective mechanism against BA-induced toxicity is PXR activation. LCA and its 3-keto metabolite are known to activate PXR. This nuclear receptor is able to regulate the expression of CYP3A, which is the main enzyme responsible to metabolize the toxic secondary BAs. Studies
have reported that LCA is able to activate also FXR and Vitamin D receptor (VDR). All these nuclear receptors are involved in the regulation of CYP3A (Toda, Ohi, et al. 2009).

Since the biosynthesis of secondary bile acids depends on intestinal bacteria, the composition of intestinal flora is suggested to play a fundamental role in the BA pool and P450 levels. Toda et al. 2009 hypothesized that down-regulation of P450 caused by antibiotics is attributed to LCA reduction and consequently reduced activation of PXR and FXR. This suggested mechanism has not yet been clarified in detail and requires more investigation.

3. SIGNIFICANCE

Antibiotics are medications widely prescribed for treating infections and are frequently administered with other drugs. Evidence indicating CYP modulation caused by changes in gut microflora require attention and further investigation due to the possibility of drug-drug interactions and adverse effects. This study investigates the effects of antibiotic-induced microflora decrease on the expression of CYP enzymes in both liver and intestine. The significance of this study is highlighted by the lack of available literature assessing the influence of microflora changes in intestinal CYPs.

The understanding of the mechanisms which microflora modulates CYP expression can contribute towards a better understanding of how other factors known to impact the microflora – such as stress, diet, and age - can affect the metabolism of drugs.
4. STUDY DESIGN

The study design consisted of the oral dose of the antibiotic ampicillin (AMP) in female mice for 3 or 7 days. Liver tissue was collected for mRNA analysis of CYP enzymes. The small intestine was divided into two parts, proximal and distal segments. Epithelial cells of the proximal portion of small intestine were used for mRNA analysis of intestinal CYPs and nuclear receptors. Epithelial cells from the distal portion were used for determining the expression levels of two genes involved in bile acid homeostasis.

Cecum content was collected and the bacterial DNA of representative strains were quantified in order to demonstrate the efficacy of the treatment. Bile secretion was assessed by visual inspection of the gallbladders.

Figure 1. Study design scheme. The animals were treated by oral gavage with AMP at 100mg/kg body weight or the vehicle (phosphate buffered saline, PBS) for 3 or 7 days. Liver tissue, small
intestine epithelial cells, cecum content, and gallbladder were collected. Bacterial DNA quantification, mRNA analysis, and visual inspection were performed

5. Methods

5.1 Animal treatment and sample collection

Female C57BL/6J mice (9- to 13-week old) were obtained from breeding stocks maintained at the Wadsworth Center (Albany, NY) and had free access to food and water. The animals were treated once daily by oral gavage with the antibiotic ampicillin (AMP) at 100mg/kg body weight or the vehicle (phosphate buffered saline, PBS) for 3 or 7 days. Each treatment group comprised of 3 animals. Mice were euthanized by CO2 overdose prior the removal of tissues. Gallbladder was removed for visual inspection. The small intestine was evenly divided into two parts: proximal and distal. Epithelial cells of small intestine and liver tissue were collected, followed by total RNA preparation. Cecum content was collected for quantification of bacterial DNA.

Aiming the collection of intestinal epithelial cells, the two portions of small intestine were submitted to a scrapping technique previously described by Zhang et al. 2003. The small intestine was excised, divided into two parts and placed in ice-cold phosphate-buffered saline. The portions were cut longitudinally and washed with ice-cold buffered saline to remove contents. In order to loose the mucosa cells, the intestine was incubated for 20min in a solution containing phosphate-
buffered saline, pH 7.2, with 1.5 mM EDTA, 3 U/ml heparin, and 0.5 mM dithiothreitol. Epithelial cells were scrapped after the incubation and used for RNA isolation.

5.2 Bacterial DNA quantification

The collected colon content was submitted to DNA isolation using QIAamp® DNA Stool Mini Kit (QIAGEN). Quantitative real-time Polymerase Chain Reaction (qPCR) was performed using 20μg of isolated DNA and the SYBR® Select Master Mix (Applied Biosystems). The primer pairs used for qPCR are specific for the bacterial strains: *Bacteroides fragilis*, *Clostridium clostridiiforme*, and *Peptostreptococcus productus*. 16S rRNA gene-based primers were used and are listed in Table 1.

5.3 mRNA analysis

Total RNA was prepared from small intestine epithelial cells and liver tissue using TRIzol® Reagent (Invitrogen). Isolated total RNA (2μg) was used for cDNA synthesis using SuperScript® II First-Strand Synthesis System (Invitrogen) and the cDNA product was submitted to a 1:5 dilution with nuclease-free water. 2μl of the diluted cDNA product was used to perform a qPCR using the SYBR® Select Master Mix (Applied Biosystems). The expression of CYP3A11, CYP2B10 and CYP7A1 mRNA was analyzed in the liver. The expression of CYP3A11, CYP2B10, CYP1A1, PXR, FXR, VDR and Constitutive Androstane Receptor (CAR) mRNA was analyzed in the proximal portion of the small intestine and ASBT and FGF15 mRNA expression
were analyzed in the distal portion. GAPDH mRNA was used for normalization. The primer sequences used are listed in Table 1.

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward primer (5′ to 3′)</th>
<th>Reverse primer (5′ to 3′)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>AGAACATCATCCCTGCATCCA</td>
<td>CCGTTCACTCTGAGATGAC</td>
<td>Zhang et al. 2015</td>
</tr>
<tr>
<td>CYP3A11</td>
<td>GACAAACAAGGAGGATTG</td>
<td>AATGTTGGGGGCACGAGAAG</td>
<td>Zhang et al. 2003</td>
</tr>
<tr>
<td>CYP2B10</td>
<td>TGAAGCTTTCTGCCTCTCT</td>
<td>TGGAGACATGCAATTAGGAG</td>
<td>Zhang et al. 2003</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>GCCACATCCGGACATCAG</td>
<td>GCTGGACATGGCATTCGT</td>
<td>Zhang et al. 2015</td>
</tr>
<tr>
<td>CYP7A1</td>
<td>AGCAACTAAACAACCTGCAGTA</td>
<td>GTCCGGATATTCAGGATGCA</td>
<td>Miyata et al. 2011</td>
</tr>
<tr>
<td>PXR</td>
<td>CAAGGCCAAATGCTCAAC</td>
<td>CCGGTATCTCGACAGTIT</td>
<td>Zhu et al. 2014</td>
</tr>
<tr>
<td>FXR</td>
<td>CCAACCTGAGTTTTCACC</td>
<td>CACACAGCTCATCCCCCTT</td>
<td>Zhu et al. 2014</td>
</tr>
<tr>
<td>VDR</td>
<td>TCTATCATGCAATGCAATGGC</td>
<td>GTTCACCTGCCCTTCAAT</td>
<td>Zhu et al. 2014</td>
</tr>
<tr>
<td>CAR</td>
<td>GGAGCGGCTGTGGAAATTTGCT</td>
<td>TCCATCTTGTAGCAAAGAAGGCA</td>
<td>Zhu et al. 2014</td>
</tr>
<tr>
<td>ASBT</td>
<td>TGGGTTTCTCTGGCCTAGACT</td>
<td>TGTTCCTGATTCAGGTTC</td>
<td>Miyata et al. 2011</td>
</tr>
<tr>
<td>FGF15</td>
<td>GAGGACCAAACGAGCAGAAATTT</td>
<td>AGTCCTTTGATGGGCAATCG</td>
<td>Miyata et al. 2011</td>
</tr>
<tr>
<td>B. fragilis</td>
<td>CTGAACTACGCCTGTTAGCA</td>
<td>CCGCAAACTTTACACAATGTGCT</td>
<td>Liu et al. 2003</td>
</tr>
<tr>
<td>C. clostridiiforme</td>
<td>CCGATGGGAGTTTGGAAAA</td>
<td>CTGGTATAGAGGCTTACATA</td>
<td>Wang et al. 1996</td>
</tr>
<tr>
<td>P. productus</td>
<td>AACTCCGGTGGTATCGAGTG</td>
<td>GGGCTTCTGAGTCAGGTA</td>
<td>Miyata et al. 2011</td>
</tr>
</tbody>
</table>

**Table 1.** Primer sequences for mouse mRNA and bacterial DNA.

5.4 Statistical analysis

Data Analysis was performed using GraphPad Prism v.5 (GraphPad Software, San Diego, CA). Statistical significance between groups was assessed with Student’s t-test.
6. RESULTS

6.1 Quantification of bacterial DNA in cecum

Quantification of bacterial DNA was performed in cecum content of mice treated with AMP for 3 days in order to verify the efficacy of the AMP treatment in reducing bacterial content. Figure 2 indicates the significant decrease in the DNA levels of all the bacterial strains examined. Amplification of *P. productus* DNA was not detectable by the qPCR in AMP-treated mice.

6.2 Visual inspection of the gallbladder

Visual inspection of the gallbladder size presented a clear enlargement of the organ in AMP-treated mice, especially in the group treated for 7 days, as presented in Figure 3. Enlarged gallbladders are known to be indicators of increased secretion of bile acids (Akiyoshi et al. 1986).

![Graphs of bacterial DNA quantification and gallbladder size](image)

**Figure 2. Effect of Ampicillin treatment on bacterial content in Colon.** The animals were treated by oral gavage with AMP at 100mg/kg body weight or the vehicle for 3 days.
DNAs in colon content were determined by qPCR. Data are mean ± S.D. (n=3). Significant differences compared with the vehicle-treated group are indicated as: *, p < 0.05.

**Figure 3. Visual inspection of gallbladders.** Gallbladders collected from mice treated with PBS (A) and AMP (B) for 7 days presented significant enlargement.

6.3 Effects of AMP treatment on hepatic CYP expression

The mRNA levels of CYP3A11, CYP2B10, and CYP7A1 in the liver were analyzed for samples collected from 3 or 7 days of AMP treatment group. During 3 days of treatment, the antibiotics caused a significant decrease in CYP2B10 expression, and increase in CYP7A1 expression, as expected (Figure 4A). The results also suggest a decreasing trend in CYP3A11 expression but the decrease was not statistically significant. In the group treated with AMP for 7 days, the CYP3A11 expression was decreased while no changes were observed in mRNA levels of CYP2B10 and CYP7A1 (Figure 4B).
Figure 4. Effects of AMP treatment on the expression of hepatic CYPs. The mRNA levels of hepatic CYP3A11, CYP2B10 and CYP7A1 were compared between mice treated with AMP for 3 days (A) or 7 days (B) and the control group. Data are mean ± S.D. (n=3). Significant differences from the vehicle-treated group are indicated as: *, p < 0.05. ***, p < 0.001.

6.4 Effects of AMP treatment on the expression of intestinal CYPs and several other genes.

The mRNA levels of CYP3A11, CYP2B10 and CYP1A1 were analyzed in the epithelial cells from proximal portion of the small intestine and different effects were observed between the two treatment durations (Figure 5A and 5B). While the treatment for 3 days decreased CYP2B10 expression, similar to the changes observed in the liver, the treatment for 7 days increased
CYP3A11 expression, although in both cases, the differences are very small. There are no significant differences for CYP1A1 for both treatment groups.

Expression levels of several nuclear receptors including PXR, FXR, VDR and CAR were also analyzed in the proximal portion of the small intestine. There are no changes observed between AMP- and vehicle-treated groups (Figure 6A and 6B).

**Figure 5. Effects of AMP treatment on the expression of intestinal CYPs.** The mRNA levels of intestinal CYP3A11, CYP2B10 and CYP1A1 were compared between mice treated with AMP for 3 days (A) or 7 days (B) and the control group. Data are mean ± S.D. (n=3). Significant differences from the vehicle-treated group are indicated as: *, p < 0.05. **, p < 0.01.
Figure 6. Effects of AMP treatment on the expression of intestinal nuclear receptors. The mRNA levels of intestinal PXR, FXR, VDR and CAR were compared between mice treated with AMP for 3 days (A) or 7 days (B) and the control group. Data are mean ± S.D. (n=3).

The expression of bile acid transporter ASBT and the growth factor FGF15, two genes involved in BA homeostasis, were analyzed using the epithelial cells from the distal part of the small intestine. The treatment for 3 days decreased levels of FGF15 and increased expression of ASBT (Figure 7A and 7B).
Figure 7. Effects of AMP treatment on the expression of intestinal ASBT and FGF15. The mRNA levels of intestinal ASBT and FGF15 were compared between mice treated with AMP for 3 days (A) or 7 days (B) and the control group. Data are mean ± S.D. (n=3). Significant differences from the vehicle-treated group are indicated as: *, p < 0.05. ***, p < 0.001.

7. Discussion

The role of intestinal flora on drug-metabolizing enzymes is not well understood and few studies available in the literature investigated enterobacteria-induced changes in CYP enzymes. Previous studies reported an important decrease of hepatic CYP3A11 expression and activity in GF mice and CPX-treated mice (Toda, Saito, et al. 2009; Toda, Ohi, et al. 2009). A comprehensive RNA-seq quantification in liver of GF mice presented significant decreases of 87% and 57% in mRNA of CYP3A11 and CYP2B10, respectively (Selwyn et al. 2015). Additionally, a recent study performed quantitative proteomic analysis and reported changes in expression levels of 825
proteins in livers of GF mice and 306 proteins in livers of antibiotics-treated mice. Among the CYP proteins analyzed, the decreased levels of CYP3A11 and CYP2B10 were the most significant in both GF and antibiotics-treated models (Kuno et al. 2016). However, microbiota-induced variations in intestinal CYPs have not been reported and this study aims to better understand this influence.

The use of two different treatment times is another relevant characteristic of the present study. The BA signaling and circulation is largely known to affect CYP expression by the activation of nuclear receptors. Miyata et al. (2011) demonstrated that expression of BA transporters and other molecules responsible for BA signaling is significantly different between mice treated with AMP for 3 and 7 days and control mice. Therefore, this study administered AMP to mice for 3 or 7 days by oral gavage to investigate if the duration of treatment will affect CYP expression differently.

In mouse liver, CYP2B10 and CYP3A11 mRNA levels were significantly decreased in 3 and 7 days of treatment respectively. Decreased expression of hepatic CYP3A11 have been reported in antibiotic-treated mice (Toda, Ohi, et al. 2009) and decreased expression of hepatic CYP2B10 have been reported in GF (Selwyn et al. 2015). Changes in the expression levels of the CYP2B family of enzymes are important due to its capability to biotransform a diverse range of drug substrates such as the antidepressant bupropion, the antiretroviral efavirenz, the anaesthetic ketamine and the antineoplastic agent cyclophosphamide (Coyle & Dull 2015; Ioannides & of Chemistry (Great Britain) 2008)

Levels of CYP2B10 mRNA in the proximal portion of the small intestine presented a decrease in mice treated with AMP for 3 days, the same effect observed in the liver. No difference was observed in intestinal CYP3A11 and CYP1A1 mRNA levels. Changes in murine CYP2B10
are important due to the abundant expression of CYP2B enzymes in mouse intestine (Zhang et al. 2003) but the extrapolation of this modulation to human models may not be meaningful since CYP2B6 expression in human small intestine samples is absent or only weakly detectable (Thelen & Dressman 2009). Additionally, it is important to highlight the possibility of post-transcriptional effects influencing the observed mRNA levels. Degradation of RNA is responsible for significant underestimation of its concentration and copy number (Filion 2012).

In the group treated with AMP for 7 days, intestinal CYP3A11 expression was unusually increased. Induction of intestinal CYP3A11 by antibiotics has not been reported in the literature and can have important effects on the metabolism of drugs such as the anticancer agent docetaxel, which the low oral bioavailability is main caused by CYP-mediated first pass metabolism (Thummel 2007). More studies are required to understand the mechanism responsible for the intestinal CYP3A11 increase in response to 7 days of AMP treatment.

A possible mechanism to explain CYP decreased levels caused by antibiotic treatments relies on the ability of nuclear receptors, such as PXR, FXR, VDR, and CAR, to induce expression of CYPs. Enterobacteria are responsible for the synthesis of secondary bile acids, potent activators of some nuclear receptors. Therefore, the decrease of gut microbiota caused by antibiotic exposure could reduce the synthesis of secondary bile acids, attenuate the activation of nuclear receptors and consequently decrease CYP expression (Figure 8). This study analyzed the mRNA levels of the nuclear receptors PXR, FXR, VDR and CAR in the small intestine of mice exposed to AMP treatment for 3 and 7 days but the treated group presented no statistically significant differences from control group. It is possible that changes in microflora only affect the hepatic expression of nuclear receptors, as demonstrated by Toda, Saito, et al. (2009), which results presented decreased mRNA levels of PXR, FXR, and CAR in the liver of germ-free mice.
Figure 8. Proposed mechanism for enterobacteria-mediated modulation of CYPs. The antibiotic treatment reduces bacterial content, including LCA-producing strains as *bacteroides* and *clostridium*. Decreased levels of LCA and other secondary bile acids result in reduced activation of nuclear receptors and consequently decrease of CYP expression.

The antibiotic treatment successfully reduced the bacterial content in the colon of AMP-treated mice. DNA quantification of the strains *b. fragilis*, *c. clostridiiforme* and *p. productus* demonstrated a significant decrease in the DNA levels of these representative strains. More than a representative group of intestinal bacteria, *Clostridium* and *Bacteroides* are strains active in the synthesis of lithocholic acid (LCA), a potent ligand for PXR and FXR. (Toda, Saito, et al. 2009) Thus, the reduction of LCA-producing bacteria may explain the role of bile acids in the CYP modulations observed.

Mice treated with AMP presented an enlarged gallbladder, indicating changes in bile flow. Enlargement of gallbladders are associated with increased secretion of bile acids and this effect is
consistent with the higher expression levels of CYP7A1 observed in the liver of AMP-treated mice (Akiyoshi et al. 1986). CYP7A1 is responsible for the synthesis of primary bile acids in the liver.

In the distal portion of small intestine, ASBT and FGF15 mRNA levels were increased and decreased, respectively. Decreased enterobacteria may reduce activation of nuclear receptors causing decreased expression of FGF15 and increasing expression of ASBT (Miyata et al. 2011).

8. Conclusion

This study concludes that AMP treatment for 3 or 7 days is able to modulate expression of drug-metabolizing enzymes in the liver and intestine of mice. In the liver, CYP2B10 and CYP3A4 mRNA levels were reduced after 3- and 7-days treatment, respectively. In the intestine of mice treated with AMP for 3 days, CYP2B10 expression was decreased and the group treated for 7 days surprisingly presented increased expression of intestinal CYP3A11. These influences need to be carefully accounted upon the necessity of co-treatment of antibiotics with other drugs.

Although the increased mRNA levels of intestinal CYP3A11 need further explanation, the decreased expression levels of CYPs may be explained by reduced production of secondary BAs after antibiotic treatment. The AMP treatment reduced the population of LCA-producing bacterial strains. Further investigation of CYP protein levels and enzymatic activity are desirable for a better understanding of the effect of antibiotics on drug-metabolizing enzymes.
9. References


Ioannides, C. & of Chemistry (Great Britain), R.S., 2008. Cytochromes P450: Role in the Metabolism and Toxicity of Drugs and Other Xenobiotics, RSC Pub.


Zhu, Y. et al., 2014. Regulation of intestinal cytochrome P450 expression by hepatic cytochrome