EFFECTS OF HUMAN MILK AND FORMULA ON THE EXPRESSION OF CYTOCHROME P450S IN CELL LINES

By

Haibo Xu

A thesis submitted in conformity with the requirements for the degree of Master of Science.
Graduate Department of Pharmacology
University of Toronto

© Copyright 2001 by Haibo Xu
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.
EFFECTS OF HUMAN MILK AND FORMULA ON THE EXPRESSION OF CYTOCHROME P450S IN CELL LINES

Haibo Xu, M. Sc., Department of Pharmacology, University of Toronto, 2001

ABSTRACT

Human milk has an intimate relation with the health of an infant. A linkage between breast-feeding and pharmacokinetics in infants has been indicated from an observation that breast-fed infants had slower caffeine metabolism compared with formula-fed infants. Since caffeine is mainly metabolized by CYP3A4 and CYP1A2, we hypothesized that human milk would inhibit the expression of these enzymes or that formula would induce their expression. We examined the effects of human milk and formula on the expression of CYP3A4, CYP1A2 and their related genes in a cell culture model of liver (HepG2) and intestine (C2BBel) cells. The mRNA of CYP3A4 and CYP1A2 was induced by formula but not by human milk in HepG2 cells. In C2BBel cells, CYP3A4 mRNA was induced by human milk but not by formula. These findings suggest that human milk may affect oral drug bioavailability and formula may influence drug biotransformation rate, which is helpful in interpreting the above clinical observation.
Acknowledgements

I would like to appreciate a variety of individuals for their advice on experimental design and technique assistance, which greatly facilitates the completion of this project. We acknowledge my advisor, Dr. Allan B. Okey, Department of Pharmacology, for his advice on hypothesis and experiment validation. Dr. Patricia A. Harper, Department of Pharmacology, for her advice on CYP1A2 regulation. Dr. Eve A. Roberts, Department of Pharmacology, for her generous gift of an adult human liver sample. Dr. Richard Rozmahel, for his advice on molecular biology techniques. Vicki Cook, for her helps in the training of cell culture and western blotting. Dr. Cindy Woodland, Department of Pharmacology, for her technical advice. Christina Tunzi, M.Sc., helped out with early technical training. Mike Woodside, Imaging Facility, Hospital for Sick Children, for his technique assistance on the software training. Yanping Wang and Suyun Yang, for their helps on PCR primer design.

On a personal level, I would like to thank the friends I have made in Department of Pharmacology for their support, advice and friendship: Bruce Kwok, Daphne Chan, Jeremy Lian, Diana Stempak, Amy Lee, Angela Wang, and Charlotte Corda. I would also like to thank my friends from other departments for their support. To my supervisor, Dr. Shinya Ito, for his kind support, advice and direction throughout the course of my research.

And, last and most importantly, my sincere thanks go to my parents, for their continuous encouragement, advice and support during the two years of abroad life.
Table of Contents

Abstract
Acknowledgements
Table of Contents
List of Tables
List of Figures
List of Abbreviations
1. Introduction
   1.1. Background
      1.1.1. Food-Drug Interaction
      1.1.2. Pharmacokinetics in Infants
      1.1.3. Human Breast Milk
         1.1.3.1. Composition
         1.1.3.2. Breast-Feeding Vs. Formula Feeding
         1.1.3.3. Drug Metabolism and Breast-Feeding
      1.1.4. Cytochrome P450 3A4
         1.1.4.1. Overview
         1.1.4.2. Regulation
         1.1.4.3. Ontogeny
      1.1.5. Cytochrome P450 1A2
         1.1.5.1. Overview
         1.1.5.2. Regulation
         1.1.5.3. Ontogeny

II
III
IV
IX
X
XII
1
1
2
3
3
4
6
6
6
7
8
8
8
11
13
IV
1.1.6. Caffeine

1.1.6.1. Clinical Application

1.1.6.2. Biotransformation

1.1.6.3. Ontogeny of Caffeine Metabolism

1.1.6.4. Delay in Caffeine Elimination in Breast-Fed Infants

1.2. Summary

1.3. Hypothesis

1.4. Project Objectives

1.5. Rationale

1.5.1. Target Genes

1.5.2. Choice of Model

1.5.3. Detection Method

1.5.4. Negative and Positive Controls

Figure 1.1-1.2,

Figure 1.3

Figure 1.4

Table 1.1

2. Material & Method

Table 2.1, 2.2

3. Results

3.1. Validation of RT-PCR

3.1.1. Calibration of PCR Cycle

3.1.2. Calibration of The Volume of The Loading cDNA
3.1.3. Detection Limit of Ethidium Bromide-Stained Agarose Gel

Figure 3.1-3.10

3.2. Basal Expressions of Cytochrome P450-Related Genes

3.2.1. CYP3A4 & CYP1A2 Expression in Human Liver

3.2.2. CYP3A7

3.2.3. CYP3A4

3.2.4. CYP3A

3.2.5. PXR

3.2.6. CYP1A2

3.2.7. AHR

Figure 3.11-3.15

3.3. Milk Studies in Hepg2 Cells

3.3.1. Effects of Formula on the Expression Of β-Actin

3.3.2. Time Course of Positive Controls

3.3.3. Effects of Human Milk and Formula on the Expression of CYP3A4 in Hepg2 Cells

3.3.4. Effects of Human Milk and Formula on the Expression of CYP3A7 in Hepg2 Cells

3.3.5. Effects of Human Milk and Formula on the Expression of CYP3A in Hepg2 Cells

3.3.6. Effects of Human Milk and Formula on the Expression of PXR in Hepg2 Cells

3.3.7. Effects of Human Milk and Formula on the Expression of
3.3.8. Effects of Human Milk and Formula on the Expression of AHR in Hepg2 Cells

Figure 3.16-3.23

3.4. Milk Studies in C2BBel Cells

3.4.1. Effects of Human Milk and Formula on the Expression of CYP3A4 in C2BBel Cells

3.4.2. Effects of Human Milk and Formula on the Expression of CYP3A7 in C2BBel Cells

3.4.3. Effects of Human Milk and Formula on the Expression of CYP3A in C2BBel Cells

3.4.4. Effects of Human Milk and Formula on the Expression of PXR in C2BBel Cells

3.4.5. Effects of Human Milk and Formula on the Expression of CYP1A2 in C2BBel Cells

3.4.6. Effects of Human Milk and Formula on the Expression of AHR in C2BBel Cells

Figure 3.24-3.28

Table 3.1

4. Discussion

4.1. Validation of RT-PCR

4.2. Basal Expression of Cytochrome P450-Related Genes

4.3. Milk Studies in Hepg2 Cells
4.3.1. Effects of Formula on Cell Viability

4.3.2. Time Course of CYP3A4 and CYP1A2 Induction by DEX or DBA

4.3.3. Effects of Human Milk and Formula on the Expression of CYP3A4 and CYP1A2 in Hepg2 Cells

4.3.4. Effects of Human Milk and Formula on the Expression of CYP3A7, CYP3A, PXR And AHR in Hepg2 Cells

4.4. Milk Studies in C2BBe1 Cells

4.4.1. Effects of Human Milk and Formula on the Expression of CYP3A4 and CYP1A2 in C2BBe1 Cells

4.4.2. Effects of Human Milk and Formula on the Expression of CYP3A7, CYP3A, PXR and AHR in C2BBe1 Cells

5. Conclusion

6. Reference
LIST OF TABLES

Table 1.1  Caffeine Disposition in Breast-fed and Formula-fed infants  18
Table 2.1  Composition of Formula  44
Table 2.2  Summary of PCR primers  45
Table 3.1  Summary of statistical analysis of the effects of human milk and formula  84
LIST OF FIGURES

Figure 1.1 Route of oral drug absorption and biotransformation 9
Figure 1.2 PXR-mediated CYP3A4 induction pathway 10
Figure 1.3 AHR-mediated CYP1A2 induction pathway 12
Figure 1.4 Metabolic pathways of caffeine 17
Figure 3.1 RNA Integrity of HepG2 & C2BBel Cells 48
Figure 3.2 Cycle Number Titration Analysis for β-Actin 49
Figure 3.3 Cycle Number Titration Analysis for CYP3A4 and CYP1A2 50
Figure 3.4 Cycle Number Titration Analysis for CYP3A7 and CYP3A 51
Figure 3.5 Cycle Number Titration Analysis for AHR and PXR 52
Figure 3.6 cDNA Titration Analysis for β-Actin 53
Figure 3.7 cDNA Titration Analysis for CYP3A4 and CYP1A2 54
Figure 3.8 cDNA Titration Analysis for CYP3A7 and CYP3A 55
Figure 3.9 cDNA Titration Analysis for PXR and AHR 56
Figure 3.10 Detection Limit of Ethidium Bromide-Stained Agarose Gel 57
Figure 3.11 CYP3A4 and CYP1A2 Expression in Human Liver 61
Figure 3.12 CYP3A4 and CYP3A7 Expression in HepG2 Cells 62
Figure 3.13 CYP3A4 and CYP3A7 Expression in C2BBel Cells 62
Figure 3.14 CYP3A and PXR Expression in HepG2 Cells and C2BBel Cells 63
Figure 3.15 CYP1A2 and AHR Expression in HepG2 Cells and C2BBel Cells 64
Figure 3.16 Effects of Formula on the Expression of β-Actin 68
Figure 3.17 Time Course of CYP3A4 and CYP1A2 induction in HepG2 Cells and C2BBel Cells 69
Figure 3.18 Effects of Human Milk and Formula on the Expression of CYP3A4 in HepG2 Cells

Figure 3.19 Effects of Human Milk and Formula on the Expression of CYP3A7 in HepG2 Cells

Figure 3.20 Effects of Human Milk and Formula on the Expression of CYP3A in HepG2 Cells

Figure 3.21 Effects of Human Milk and Formula on the Expression of PXR in HepG2 Cells

Figure 3.22 Effects of Human Milk and Formula on the Expression of CYP1A2 in HepG2 Cells

Figure 3.23 Effects of Human Milk and Formula on the Expression of AHR in HepG2 Cells

Figure 3.24 Effects of Human Milk and Formula on the Expression of CYP3A4 in C2BBel Cells

Figure 3.25 Effects of Human Milk and Formula on the Expression of CYP3A7 in C2BBel Cells

Figure 3.26 Effects of Human Milk and Formula on the Expression of CYP3A in C2BBel Cells

Figure 3.27 Effects of Human Milk and Formula on the Expression of PXR in C2BBel Cells

Figure 3.28 Effects of Human Milk and Formula on the Expression of AHR in C2BBel Cells
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3MC</td>
<td>3-Methylcholanthrene</td>
</tr>
<tr>
<td>AHR</td>
<td>Aryl Hydrocarbon Receptor</td>
</tr>
<tr>
<td>ARA9</td>
<td>Ah receptor-associated protein</td>
</tr>
<tr>
<td>ARNT</td>
<td>Aryl Hydrocarbon Nuclear Translocator</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>bHLH/PAS</td>
<td>helic-loop-helix/Per-Amt-Sim</td>
</tr>
<tr>
<td>C2BBel</td>
<td>Caco-2 Brush Border expressing cell line</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DBA</td>
<td>Dibenz[a]anthracene</td>
</tr>
<tr>
<td>ER6, -Ito, -JMP</td>
<td>Everted Repeat with a 6bp spacer, identified by Itoh S., identified Pascussi, JM.</td>
</tr>
<tr>
<td>DEX</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>HSP90</td>
<td>Heat shock protein 90</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PXR</td>
<td>Pregnane X Receptor</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse Transcription</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X Receptor</td>
</tr>
<tr>
<td>TCDD</td>
<td>2,3,7,8-tetrachlorodibenzo-p-dioxin</td>
</tr>
<tr>
<td>XREM</td>
<td>Xenobiotic Response Element Module</td>
</tr>
<tr>
<td>XREs</td>
<td>Xenobiotic Response Elements</td>
</tr>
</tbody>
</table>
INTRODUCTION

As the name “mammal” indicates, nursing is one of the central instincts for human and breast milk is a natural and adequate food for infants. Importantly, potential milk-drug interaction was suggested as the metabolism of caffeine, used therapeutically for neonatal apnoea, was compromised in breast-fed infants. However, the underlying mechanisms have yet to be elucidated. Moreover, it is unclear how breast milk influences the development of xenobiotic elimination processes. In this thesis I describe findings that provide valuable insight into this important question.

1.1. BACKGROUND

1.1.1. Food-Drug Interaction

As the major nutritional source, human milk or formula has a great potential to interact with drugs administered during neonatal period. It has been clearly demonstrated that a number of specific dietary factors can influence drug metabolism in adults by affecting absorption, regulating phase I enzymes, modulating phase II conjugating enzymes or modifying coenzyme availabilities. These include dietary protein, cruciferous vegetables, charcoal-broiled beef containing polycyclic aromatic hydrocarbons, methylxanthines and grapefruit. (Anderson, 1988; Delzenne and Verbeeck, 2001). For
example, the absorption of griseofulvin can be enhanced by high-fat meals while that of

tetracycline is inhibited by milk and by diets containing abundant calcium; The

microsomal activation of aflatoxin B1 to the exo-8,9-epoxide is reduced by a grapefruit

component flavonoid, through CYP3A4 pathway (Dresser et al., 2000; Guengerich et al.,
1994; Wang et al., 2001); ethanol induces CYP2E1, which has been shown to be

important in activating a number of potential carcinogens, such as ethyl carbamate

(urethan); polycyclic aromatic hydrocarbons and caffeine enhance the expression of

CYP1A2 (Okey, 1990); many drugs can be released from plasma albumin when a high-fat
diet is taken, due to the increased level of free fatty acid; The activity of the mixed-

function oxidase system can be enhanced by high-protein diets and thus increases the

metabolism of numerous drugs (Bidlack and Smith, 1984) (Bidlack et al.,
1986; Hathcock, 1985; Maka and Murphy, 2000; Yang and Yoo, 1988). As the sole diet for

an infant during the first half-year, breast milk or its substitute has a good potential to

interact with drugs administered during that period of time due to its consistent presence

in gastrointestinal system, complexity in composition and the presence of immune-active
molecules and cells (as discussed below). However, the potential interaction between

milk and drugs has not been clearly elucidated.


1.1.2. Pharmacokinetics in infants

Drug metabolism and its pharmacological effects during neonatal and infantile
periods often demonstrate different patterns from those of adults. Physiologically, rapid
changes occur as the transition from umbilical blood supply to portal vein system and ductus venosus shunt is closed during the first week of life, which also affects hepatic oxygenation (Gow et al., 2001). The volume of distribution during infancy is often larger than that of adulthood, partially due to the relatively increased volume of extracellular fluid and lower plasma protein binding. This factor contributes to the increased elimination half-life of some drugs (Routledge, 1994). Hepatic CYP1A2, CYP2D6, CYP2E1 and CYP3A4 develop in different maturation patterns (as discussed below) and CYP3A7 level diminishes during the first year of life (Lacroix et al., 1997; Sonnier and Cresteil, 1998; Vieira et al., 1996). Thus, pharmacokinetics of drugs, such as caffeine, is often immature or different in pattern from those of adults due to the different profile of cytochrome P450s at birth. In adults, caffeine half-life is about 6 hours, while it is prolonged in premature neonates (97.6 ± 30 hours). The hepatic elimination of caffeine seems closely related to the postconceptional age (Le Guennec et al., 1985). The fraction of unchanged caffeine in urine is about 85% in neonates while it is 2% in adults (Aldridge et al., 1979). This leads to a reduced clearance rate and a prolonged elimination half-life if biotransformation is a significant mechanism for elimination.

1.1.3. Human milk

1.1.3.1. Composition

The complexity of the composition of human milk reflects the demands of an infant on nutrition, energy, immune defence and development. Human milk is not only composed of a broad range of minerals, vitamins, lipids and proteins, but also living cells, active enzymes, immune factors, and other unique compounds that can not be enriched in
formula. Human milk is uniquely species-specific, containing approximately 70% whey and 30% casein, which demonstrates a distinct pattern from cow milk as it has 18% whey and 82% casein. The advantage of high percentage of whey protein is that whey is much easier to absorb and more resistant to acid precipitation, usually occurring when milk is going through a gastric cavity. The amino acid composition of whey protein also differs from casein in that taurine concentration is higher while that of phenylalanine, tyrosine and methionine is lower than casein (Roberts, 2000). Moreover, it has been identified that the growth rate has a linear relationship with the milk protein density of a given species (Reeds et al., 2000). The protein concentration is approximately 1% in human milk while the concentration in cow milk-based formula is approximately 1.5% (Roberts, 2000). The profile of growth factors and hormones differs among species. The presence of growth factors is much longer in human milk than in bovine milk and the predominant form of growth factor is an Epithelium Growth Factor-like protein whereas it is not available in bovine milk (Shing and Klagsbrun, 1984). Mitogen is identified in human milk that may contribute to the development of cells in neonates and may be useful in the prevention of necrotizing enterocolitis (Klagsbrun, 1978; Tapper et al., 1979).

1.1.3. Breast-feeding vs. Formula feeding

During recent years, human milk has been gradually recognized as the best and obligatorily the first choice for neonates and infants (Roberts, 2000), as increasing intentions on the function and components of human milk have spotted the facts that: human milk supplements a complete panel of adequate quantity of species-specific nutrients to infants, including water, lipids, proteins, immunoglobulins, lysozymes,
polyamines, nucleotides, carnitine, carbohydrates, minerals, vitamins, enzymes, hormones, amino acids, cytokines and growth factors (Roberts, 2000). Breast-feeding decreases the incidence of infant morbidity and mortality from infectious disease and immune disorders, such as respiratory infection (Frank et al., 1982), otitis media (Duncan et al., 1993), necrotizing enterocolitis (Lucas and Cole, 1990). Longitudinal studies have revealed that human milk has complicated beneficial effects on the regulation of neonatal immune system as has been demonstrated by the reduction in the frequency of allergy (Halken et al., 1995; Lucas et al., 1990; Saarinen and Kajosaari, 1995), Crohn's disease (Koletzko et al., 1989; Rigas et al., 1993) and Insulin-dependent diabetes mellitus (Gerstein, 1994; Mayer et al., 1988; Virtanen et al., 1991). Furthermore, the predominant intestinal microbiota in a breast-fed, full-term infant is bifidobacteria, the amount of which overwhelms potentially harmful bacteria. Whereas in formula-fed infants, coliforms, enterococci, and bacteroides grow preferably (Dai and Walker, 1999); Breast-feeding also has been associated with potential enhancement of cognitive development (Morrow-Tlucak et al., 1988).

However, even with current wide-spread campaigns for breast-feeding, the incidence of exclusive breast-feeding is still not high for various reasons (1997). There are several medical indications for formula: 1) for an infant whose mother is unable to provide milk; 2) for an infant who cannot have breast milk due to congenital defect in metabolism; 3) for an infant whose mother has infectious disease that can be transmitted through breast milk; 4) for an infant whose mother is taking drugs, foods, or other agents that are excreted into human milk and are potentially harmful to the health of the infant (Roberts, 2000).
1.1.3.3 Drug Metabolism and Breast-Feeding

Much concern has been addressed on an infant’s exposure to drugs excreted in breast milk (Ito, 2000). However, it has not been clear whether or not human breast milk can affect the pharmacokinetics of drugs taken by infants. It has been observed that high-protein diets increase the activity of the mixed-function oxidase system and thus increase the metabolism of numerous drugs (Hathcock, 1985). Compared with formula-fed infants, the elimination of caffeine, which was used as a standard treatment for neonatal apnea, was found significantly delayed in breast-fed infants (Le Guennec and Billon, 1987). Since caffeine is primarily metabolized by a group of cytochrome P450s, it is important to know if human breast milk can exert any influence on the expression or function of these drug-metabolizing enzymes. However, there have been no reports studying the effects of human milk on the expression of cytochrome P450 systems.

1.1.4. Cytochrome P450 3A4

1.1.4.1. Overview

As the most abundantly expressed cytochrome P450 in both human liver and the small intestine, CYP3A4 composes 30-40% of the total cytochrome P450s in liver and intestine (Kolars et al., 1994b). The genetic locus of CYP3A4 was designated to chromosome 7 at band q22.1 (Inoue, 1992). The genomic structure of CYP3A4 includes 13 exons and 12 introns over a total length of 27kb (Hashimoto et al., 1993). CYP3A4
mRNA and protein are detected throughout jejunum, liver, colon and pancreas (Kolars et al., 1994b; Zhang et al., 1999b). CYP3A4, expressed along the lumen of intestine, plays a significant role in the bioavailability of oral drugs (Suzuki and Sugiyama, 2000). Human liver, where CYP3A4 is the most abundantly expressed cytochrome P450 subtype, is the major organ of the biotransformation of most drugs and plays a predominant role in the "first-pass" phenomenon. The hepatic CYP3A4 is known to metabolize a large variety of xenobiotics and endogenous biochemicals (de Wildt et al., 1999a). The catalytic function of CYP3A4 has been extensively studied, using human hepatic microsomes as well as cultured human hepatocytes. The major catalytic pathways of CYP3A4 comprise of a wide spectrum of metabolic functions, including N-oxidation, C-oxidation, N-dealkylation, O-dealkylation, nitro-reduction, dehydration and C-hydroxylation (Li et al., 1995).

1.1.4.2. Regulation

CYP3A4 inducers are composed of a broad spectrum of endogenous biochemicals, drugs, and environmental chemicals. As shown in Figure 1.2, the mechanism of the induction of CYP3A4 has been extensively studied for recent years that reveals that: 1) a variety of structurally different xenobiotics share a common signal transduction pathway of CYP3A4 induction (Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998); 2) a nuclear receptor, termed as pregnane X receptor (PXR) (Kliewer et al., 1998), transactivated by the presence of those drugs, binds to the retinoid X receptor, or RXR, which serves as a common heterodimer partner with many orphan nuclear receptors (Mangelsdorf and Evans, 1995); 3) The PXR-RXR heterodimer
binds to a response element in the promoter region that contains an ER6 motif (Everted Repeat with 6bp spacer) (TGAACT-N6-AGGTCA) in human (Barwick et al., 1996); 4) The PXR DNA-binding activity promotes the transcription of CYP3A4 (Kliewer et al., 1998). Further studies demonstrated that PXR and RXR were induced by dexamethasone with synergistic increase of CYP3A4 in human hepatocytes (Pascussi et al., 2000a). In contrast, other CYP3A4 inducers, such as RU486, rifampicin, clotrimazole, or phenobarbital failed to induce PXR expression (Pascussi et al., 2000a). Another study indicates that PXR activators are not necessarily CYP3A4 inducers (Zhang et al., 1999a), suggesting a multifactorial nature of CYP3A induction that depends on the drug, the tissue and the type of cell model employed in the study.

1.1.4.3 Ontogeny

Although the total CYP3A contents appear to be relatively stable during the transition from the fetal to neonatal life, the isozymal composition of CYP3A shifts from CYP3A7, the predominant fetal form of CYP3A, to CYP3A4. CYP3A4 mRNA level increases immediately after birth, reaches a relatively high level within the first week of life and maintains that level during the rest of the first year (Lacroix et al., 1997).

1.1.5 Cytochrome P450 1A2

1.1.5.1 Overview

Accounting for approximately 10 to 15% of the total cytochrome P450 content in human liver (Brosen, 1995; Shimada et al., 1994), cytochrome P450 1A2 (CYP1A2), is involved in a variety of biotransformation of endogenous and exogenous
Figure 1.1. Route of Oral Drug Absorption and Biotransformation. This is a schematic presentation of drug absorption and biotransformation in gastrointestinal-hepatic system. Oral drugs are absorbed through the intestinal epithelium and transported to liver through portal vein system. Liver, as the major biotransformation site of most drugs, contains a variety of cytochrome P450s that contribute to the first-pass effect, which can also occur in intestine where CYP3A4 is abundantly expressed.
Figure 1.2 PXR-mediated CYP3A4 Induction Pathway. A schematic diagram of the mechanism of PXR-mediated CYP3A4 induction, showing relative locations of nuclear receptors and their binding sites, as well as the signal transduction pathways thought to interact with them. H, Hepatocyte; N, Nucleus; L, PXR ligand; PXR, Pregnane X Receptor; RXR, Retinoid X Receptor; ER6, Everted Response element.
compounds including nitroaromatic compounds, mycotoxins and estrogens, and in metabolic activation of carcinogens, such as arylamines (Landi et al., 1999). The genetic locus of CYP1A2 is on human chromosome 15 (Corchero et al., 2001; Jaiswal et al., 1987) and the gene contains 7 exons and 6 introns with a total length of 7.8 kb (Ikeya et al., 1989). Its upstream region contains xenobiotic response elements (XREs) (Corchero et al., 2001). The extra-hepatic location of CYP1A2 is not conclusive. While it was not detected in human intestine or any other tissue by Zhang et al. (Raunio H et al., 1995; Zhang et al., 1999b), McDonnel et al. observed low expression of CYP1A2 mRNA in human duodenum (McDonnell et al., 1992).

1.1.5.2. Regulation

CYP1A2 is induced in vivo by a panel of drugs, such as phenytoin, caffeine and ritonavir (Michalets, 1998), carcinogens, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Poland and Glover, 1974), and others, such as charbroiled meat and cigarette smoke (Landi et al., 1999). As shown in Figure 1.3, one of the most important regulation pathways of CYP1A2 is through aryl hydrocarbon receptor-dependent and aryl hydrocarbon receptor-independent pathways. The protein structure of aryl hydrocarbon receptor (AHR) is demonstrated as a prototypical members of the basic helic-loop-helix/Per-Arnt-Sim (bHLH/PAS) class of transcription factors (Crews, 1998; Whitlock, Jr., 1999). AHR involves the transcriptional activation of CYP1A1, CYP1A2 and CYP1B1 (Quattrochi et al., 1994; Tang et al., 1996). Without the presence of ligands, AHR is stably located in the cytosol as one component of a protein complex that contains chaperon proteins, including HSP90, ARA9 and p23 (Gu et al., 2000). Upon
Figure 1.3 AHR-mediated CYP1A2 Induction Pathway. A schematic diagram of the mechanism of AHR-mediated CYP1A2 induction, showing relative locations of nuclear receptors and their binding sites, as well as the signal transduction pathways thought to interact with them. H, Hepatocyte; N, Nucleus; L, AHR ligand; AHR, Ah Receptor; ARNT, Ah Receptor Nuclear Translocator; ARA9, Ah receptor-associated protein; HSP90, Heat Shock Protein; XRE, Xenobiotic Response Element.
encountering its ligand, AHR is released from the complex and forms a heterodimer by binding to AHR nuclear translocator, or ARNT (Hoffman et al., 1991). The heterodimer binds to xenobiotic response elements and activates transcription (Hines et al., 1988; Reisz-Porszasz et al., 1994).

### 1.1.5.3. Ontogeny

Recent studies have clarified that the CYP1A2 is the last cytochrome P450 to develop among major hepatic CYP proteins during neonatal period. During fetal or early neonatal stage, there is no detectable CYP1A2 protein. The CYP1A2 level begins to increase gradually in the age group of 1-3 months and accounts for approximately 50% of adult contents in the group of infants aged 1 year or more. The CYP1A2 functional assays described a similar ontogenetic pattern as the ontogeny of CYP1A2 protein (Sonnier and Cresteil, 1998).

### 1.1.6. Caffeine

#### 1.1.6.1. Clinical Application

The term apnea is a description of respiration cessation longer than 20 seconds and/or associated with bradycardia, cyanosis or pallor, and occurs more often than once an hour over a 12-hour period. Many premature infants experience mild to life-threatening apneic episodes. It affects approximately 90% of premature neonates weighing under 1000 g at birth (Comer et al., 2001). Some pathological factors, which are commonly associated with lower gestational age, such as asphyxia, infection, thermal instability, hypoglycemia, seizures, or intracranial hemorrhage, have been identified that
may play a role in the pathogenesis of apnea. The duration of this disease decreases as the gestational age increases. The close relationship between age, birth weight, and the incidence and severity of apnea also underlies immaturity as a major etiological factor (Martin et al., 1986).

Caffeine has been used as a standard therapy for the treatment of apnea of prematurity (Steer and Henderson-Smart, 2000). It has been demonstrated to decrease the frequency of neonatal apneic episode. Although the therapeutic mechanism is largely unknown, there are several actions of caffeine that seem to be beneficial: caffeine 1) increases the sensitivity of the medullary respiratory centre to CO₂; 2) increases vagal activity, leading to the improvement of the contractility of respiratory muscles and their recovery from fatigue; 3) improves metabolic homeostasis; and 4) enhances the circulatory catecholamine level (Bhatia, 2000). It is rapidly and completely absorbed from gastrointestinal tract with peak serum concentration reached in 30-120 minutes. Caffeine is readily distributed into peripheral tissues including brain across the blood-brain barrier. The mean volume of distribution for caffeine in neonates (0.8-0.9 L/Kg) is larger than that reported for adults (0.4-0.6 L/Kg). It also has a smaller fluctuation range in plasma concentration than theophylline, another choice for the management of apnea (Aranda et al., 1979). However, there are still large inter-individual differences in caffeine metabolism and in its maturation process, which necessitates drug monitoring in plasma to avoid therapeutic failure or overdose.
1.1.6.2. Biotransformation

As an alkaloid, Caffeine is a xanthine derivative methylated at N-1, N-3, and N-7 positions. Caffeine is extensively biotransformed, forming numerous metabolites \textit{in vivo} (Kalow, 1985). However, there are usually only four primary metabolites in human hepatocytes derived from normal liver tissue (Figure 1.4). By the C-8 hydroxylation, 1-3-7-trimethyluric acid (137U) is formed. By the N-1, N-3, and N-7 demethylation, theobromine (37X), paraxanthine (17X), and theophylline (13X) are produced (Roberts et al., 1994). Cytochrome P450 1A2 (CYP1A2) is the high affinity N-demethylase of caffeine. Cytochrome P450 3A4 (CYP3A4) predominantly catalyzes C-8 hydroxylation. Cytochrome P450 2E1 (CYP2E1) plays a minor role in caffeine metabolism (Ha et al., 1996).

1.1.6.3. Ontogenesis of Caffeine Metabolism

In adults, the primary metabolite is paraxanthine, which represents about 50.4% of the total biotransformation; 1-3-7 trimethyluric acid represents 27.6%, theophylline and theobromine represent 11.4% and 10.6% of the total biotransformation (Cazeneuve et al., 1994). However, the primary metabolite in neonatal and infant samples was 1-3-7 trimethyluric acid (91.2%, 66.6% of the total). Furthermore, the amounts of paraxanthine, theobromine and theophylline are significantly lower in neonates and infants compared with adults. In contrast, the amount of 1-3-7 trimethyluric acid has no significant variation between those groups (Cazeneuve et al., 1994). These data, combined with others (Carrier et al., 1988; Pons et al., 1988a; Pons et al., 1988b), indicate that: 1) the maturation of the N-3 and N-7-demethylation occurs around 120-300-day
age range; 2) the N-1-demethylation is delayed compared with N-3 and N-7-demethylation; 3) the C-8-hydroxylation is not affected by the influence of maturation; 4) The delayed ontogenesis of CYP1A2 can explain why the CYP1A2 N-demethylation of caffeine is much weaker than the C8 hydroxylation mediated by CYP3A enzymes in neonates (Sonnier and Cresteil, 1998).

1.1.6.4. Delay in Caffeine Elimination in Breast-Fed Infants

Caffeine elimination is slower in infancy due to the lower activity of the cytochrome P450 monooxygenase system (Aranda et al., 1974; Pelkonen et al., 1973). Compared with formula-fed infants, breast-fed infants demonstrated a significantly longer caffeine half-life as well as significantly higher trough blood levels (approximately three- to five-fold increase) after 46 weeks’ post-conceptional age (Table 1.1) (Le Guennec and Billon, 1987).

1.2. SUMMARY

Breast-feeding is associated with nutritional, infection-preventive, and immune-regulative advantages to infants while formula has been used as a substitute under the condition that does not allow breast-feeding. The constant presence of milk and its digested products in gastrointestinal tract provides the chance to influence the microenvironment of intestine and liver, such as the enzymatic profile, which can interfere with the metabolism of drugs taken by an infant. The ontogeny of major cytochrome P450s during neonatal period shows an isozyme-specific pattern, predicting an altered scenario.
Figure 1.4 Metabolic Pathways of Caffeine. By the C-8 hydroxylation, 1-3-7-trimethyluric acid is formed. By the N-1, N-3, and N-7 demethylation, theobromine, paraxanthine, and theophylline are produced.
**Table 1.1 Caffeine Disposition in Breast-fed and Formula-fed infants** (Le Guennec et al., 1987). BF, Breast-feeding; FF, Formula-feeding.

<table>
<thead>
<tr>
<th>Postconceptional weeks</th>
<th>Infants</th>
<th>Half-life (hours)</th>
<th>Trough blood concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>39-42</td>
<td>BF</td>
<td>81 ± 16</td>
<td>6.8 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>FF</td>
<td>49 ± 15</td>
<td>4.8 ± 1.6</td>
</tr>
<tr>
<td>43-46</td>
<td>BF</td>
<td>68 ± 10</td>
<td>7.3 ± 3</td>
</tr>
<tr>
<td></td>
<td>FF</td>
<td>43 ± 25</td>
<td>4 ± 2.5</td>
</tr>
<tr>
<td>47-50</td>
<td>BF</td>
<td>76 ± 13</td>
<td>11.2 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>FF</td>
<td>21 ± 28</td>
<td>2.6 ± 2</td>
</tr>
<tr>
<td>51-54</td>
<td>BF</td>
<td>54 ± 9</td>
<td>10.6 ± 4</td>
</tr>
<tr>
<td></td>
<td>FF</td>
<td>16 ± 13</td>
<td>2 ± 1</td>
</tr>
</tbody>
</table>
of drug biotransformation from that of adults. One example is the metabolism of caffeine whose major catalytic pathway shifts from C8-hydroxylation during infancy to N-demethylation during adulthood. Caffeine metabolism also demonstrates an apparent difference between formula-fed infants and breast-fed infants as the caffeine half-life is about three fold longer in breast-fed infants than in formula-fed infants and this difference is not caused by caffeine consumption or oral steroid intake of those nursing mothers(Le Guennec and Billon, 1987). As caffeine is predominantly metabolized by cytochrome P450 1A2 and 3A4(Roberts et al., 1994), we suspected that the expression of these enzymes would be influenced by human milk or formula. Concerning the profile of cytochrome P450 expression during neonatal period, we considered CYP3A4 as our major focus due to a delayed ontogenesis of CYP1A2 compared with that of CYP3A4. The composition of human milk differs from bovine milk-based formula in the content of whey, casein, types of lipids and immune-active components(Roberts, 2000). It has been observed that some CYP enzymes can be down-regulated by cytokines (Abdel-Razzak et al., 1994; Muntane-Relat et al., 1995), which are present in human milk(Bottcher et al., 2000; Skansen-Saphir et al., 1993).

Since these observations suggested a potential interaction between milk components and cytochrome P450s, we suspected that human milk might affect the expression of cytochrome P450s. Interested in this aspect of milk-drug interaction, we set out to study the effects of milk, either human milk or formula, on the expression of cytochrome P450s. Furthermore, because of the nature of these enzymes, we proposed that these effects would play a developmental role in the biotransformation of drugs.
1.3. HYPOTHESES

1.3.1 Human milk inhibits the expression of cytochrome P450s in an infant’s liver and/or intestine.

1.3.2 Formula induces the expression of cytochrome P450s in an infant’s liver and/or intestine.

1.4. PROJECT OBJECTIVES

1.4.1 To ascertain the basal expression of CYP3A4, CYP3A7, CYP3A, CYP1A2, PXR and AHR in HepG2 cells and C2BBel cells.

1.4.1.1 To design specific primers for these genes.

1.4.1.2 To establish optimal RT-PCR conditions for relative quantification.

1.4.2 To ascertain the effects of human milk and formula on the expression of CYP3A4, CYP1A2 and their related genes in HepG2 and C2BBel cells.

1.4.2.1 To establish proper milk concentration for cell treatments.

1.4.2.2 To establish proper control for the expression regulation study.

1.4.2.3 To investigate the effects of formula and human milk on the gene expression.
1.5. RATIONALE

1.5.1 Target Genes

CYP1A2 is the major enzyme for the biotransformation of caffeine in adults, while the biotransformation of caffeine during neonatal period is mediated mainly by CYP3A4 due to the delayed maturation of CYP1A2 (Sonnier and Cresteil, 1998). Thus, CYP3A4 was chosen as the main focus of our study. One of the goals of this project is to examine the inductive/inhibitive effects of milk on the expression of CYP3A4 in a liver model, HepG2 cells, and an intestine model, C2BBel cells. As the fetal counterpart of CYP3A4, CYP3A7 is predominantly expressed in HepG2 cells (Sumida et al., 2000). There is a switch of CYP3A isoform from CYP3A7 to CYP3A4 during neonatal period (Lacroix et al., 1997). A comparison of the potential effects of milk on the regulation of CYP3A4 and CYP3A7 was designed to evaluate if milk could exert different effects on these two CYP3A isoforms. The examination of the expression of CYP3A7 in HepG2 cells was also used as a control for the examination of the specificity of the primers for CYP3A4 (as discussed below). The expression of CYP3A5 is not studied in this project due to the following reasons: 1) the hepatic expression of CYP3A5 is polymorphic such that it can be detected only in 10% fetal liver, 50% in infant liver and 29% in adult liver (Wrighton et al., 1990); 2) its intestinal expression also demonstrates a polymorphic pattern with considerably low activity, compared with CYP3A4 (de Wildt et al., 1999b). In order to examine the influence of the alteration in the expression of individual CYP3A subfamilies on the overall level of CYP3A, a RT-PCR analysis was developed for CYP3A. PXR is one of the most important nuclear
receptors mediating signal transduction that regulates expression of cytochrome P450s. It was observed that the PXR expression level was increased by some CYP3A4 inducers, such as dexamethasone while other CYP3A4 inducers, such as rifampicin, failed to induce PXR in human hepatocytes (Pascussi et al., 2000a). Interleukin-6, an inhibitor of CYP3A4, inhibits the expression of PXR (Pascussi et al., 2000b). Therefore, an examination of the expression level of PXR after treatment with potential CYP3A4 inducers/inhibitors would provide useful information on the type of induction/inhibition, if any. We also decided to study the mRNA expression of AHR, which is intimately associated with the signal transduction pathway of CYP1A2 regulation (Okey et al., 1994).

1.5.2 Choice of Model

The ultimate goal of this research is to examine the regulatory effects of human milk or formula on cytochrome P450 expression and function in infants. Unfortunately, it is extremely difficult to obtain these tissues. Since there was no satisfying probe for the identification of these cytochrome P450-related genes through examining easily-collectable human body fluids or other excretion samples, an in vivo human model was impractical. Another ideal model was the primary cell culture obtained from a bank of human infantile liver and intestine. However, the resource of these tissue banks was not available at current stage. An animal model was considered since they would be readily available for experimental purpose. However, the pattern of cytochrome P450 isoforms is different from species to species, from which the results obtained may not be applicable to human. Therefore, in the initiation stage of this research, in vitro cultured human cell
lines would be the best choice, given their ready availability and easy maintenance. HepG2 cells resemble fetal liver in regard to their CYP3A profile, as they predominantly express CYP3A7 (Sumida et al., 2000), the fetal form of CYP3A. This cell line has been used as an in vitro liver model for a variety of studies such as the regulation mechanism of CYP3A4 and CYP1A2 (El Sankary et al., 2000; Li et al., 1998; Pascussi et al., 1999), and gene regulating drug transportation studies (Seree et al., 1998). A transition from CYP3A7 to CYP3A4 occurs immediately within the first day of life and completes at the age around the first year (Lacroix et al., 1997). Thus, the cell line HepG2 resembles the fetal liver in the composition of CYP3A subfamily, allowing it to become a suitable model for our study. C2BBel cells, or Caco-2 Brush Border Expressing cells, is a clone from its parent cell line Caco-2, a colon carcinoma cell line. It has been documented that the differentiation of small intestinal BB (Brush Border) hydrolases resembles that of fetal colon and has been utilized for a variety of studies related to epithelial properties and enterocytic functions, including cytochrome P450 studies. This cell line is a morphologically homogeneous, BB-expressing clone from Caco-2 cells. Under the transmission electronic microscope, it predominantly resembles the structure of human colon brush borders, with a small subpopulation that is morphologically similar to human small intestine brush border. The profile of protein expression in C2BBel cells also shows resemblance to brush borders isolated from human ileum and colon (Michalets, 1998). We chose this line as an intestine model for the study of the intestinal regulation of cytochrome P450s by milk. At the time of designing the experimental setting, there were two types of cell culture vehicles of our consideration. The first type is to culture the cells on a filter that separates the apical compartment, the treatment compartment
from the basal compartment. The advantage of this system is that it resembles physiological conditions by providing nutrients from the basal side of the polarized cells and that the interference of the absorption of nutrients from the treatments is maximally reduced since the filter does not allow the passage of macromolecules such as lipids. However, the cells growing on the filter are harder to harvest and the cost is relatively expensive. Therefore, we chose the one-compartment cell culture dishes at this stage of the experiment.

1.5.3 Detection Method

We suspected that cytochrome P450s' expression or function might be affected by either human milk or formula. We decided to start the investigation from the aspect of expression because 1) the presence and relative abundance of a certain enzyme would need to be clarified first in a model; 2) The expression assay usually had the advantage of specificity due to the availability of structure-specific probes. The most ideal way to determine whether formula would affect the expression of CYP3A4 would have been to measure the protein level of CYP3A4. However, the specific antibody that could differentiate CYP3A4 from CYP3A7 was not available at the time of the initiation of this project, making this attempt impractical. Therefore, detection of the mRNA level was used to initialize this research. Since the level of the cytochrome P450s mRNA in cell lines is usually low(Carriere et al., 1994; Schmiedlin-Ren et al., 1997; Sumida et al., 2000), we decided not to use northern blotting since its cross hybridization of closely related mRNA is often a problem(Greuet et al., 1996) and its sensitivity is generally lower than that of RT-PCR. Besides, it has been shown that a relatively large amount of
total RNA per gene is required to optimize a result in the previous northern blotting protocol (>30 μg) of this laboratory and a RNase protection assay protocol (100 μg) for the quantification of CYP3A4 (Greuet et al., 1996). However, this investigation was designed to examine 7 gene expressions, demanding to harvest a large amount of RNA, which would exceed the usual yield of a cell culture if using northern blotting or RNase Protection Assay. In contrast, 10 μg of total RNA sample was found to be sufficient for RT-PCR. A RT-PCR series consisting of examining 7 genes and 5-7 treatments can usually be finished within a minimum of 5 working days. However, a northern blotting of one gene expression typically demands 4 working days. There was no satisfactory antibody that could differentiate CYP3A4 from CYP3A7 at the start of this project, which only allowed us to measure CYP3A level in these cell lines. In contrast, by designing gene-specific primers, CYP3A4 was specifically recognized and amplified by RT-PCR (Figure 3.12). Furthermore, A comparison of results obtained between northern blotting and RT-PCR has revealed that RT-PCR is a reliable method (Kim et al., 1995). Thus, we decided to employ RT-PCR, a reliable, sensitive, and time-efficient method.

A comparative Reverse Transcription-Polymerase Chain Reaction (RT-PCR) protocol was developed for use in this investigation. To make the RT-PCR quantitative, we first elucidated the mathematic formula, describing the amount of the PCR product: P, the yield of PCR reaction; R, the yield of RT reaction; R', the amount of the cDNA input for a PCR; M, the mRNA input; E<sub>RT</sub>, the RT efficiency (a value between 0 and 1); E<sub>PCR</sub>, the PCR efficiency (a value between 0 and 1); n, the PCR cycle number; The following formulas apply to RT and PCR reaction respectively (Freeman et al., 1999):

\[ R = E_{RT}(M) \]

...Equation (1)
\[ P = R'(1 + E_{\text{PCR}})^a \] ...Equation (2)

Or, by applying a log transformation, the equation (2) can be altered to:

\[ \log(P) = \log(R') + n \log(1 + E_{\text{PCR}}) \] ...Equation (3)

The yield of RT reaction R is linearly proportional to the quantity of mRNA M, as the \( E_{\text{RT}} \) can be assumed as the same with the employment of the master mix technique and using random heximer \((pd(N)_{6})\). If the PCR reaction is within linear phase, the \( E_{\text{PCR}} \), which is related to the characters of gene-specific primers and PCR parameters, will be a constant for each set of primers in one run of PCR, leaving two independent variables \( R' \) and \( n \). To illustrate the linear phase of a PCR reaction for a particular gene, we manipulated the experimental settings by holding one constant while altering the other variable:

Variable \( n \), Constant \( R' \): under these experimental settings, a typical curve is usually in a ‘S’ shape, demonstrating three phases: a bottom-left plateau phase, which is caused by insufficient amount of PCR product that is below the detection limit of ethidium bromide-stained gel; a top-right plateau phase, which is a reflection of the saturation of the PCR analysis; and a linear phase in the middle, which is the proper range suitable for quantitative detection. As determined by this method, the cycle number for \( \beta\)-Actin is 27, and 30 for CYP3A7, CYP3A, PXR and AHR, and 33 for CYP3A4 and CYP1A2.

Variable \( R' \), Constant \( n \): as the PCR cycle number is determined within the linear phase of the cycle number test, \( P \) will be linearly proportional to the \( R' \), the cDNA input. It is usually shown in a typical configuration with two phases in our experimental settings: a top-right plateau phase, demonstrating the saturation of a PCR
reaction; a linear phase, delineating that the yield of PCR analysis has a linear relationship with the initial input of cDNA. A volume of cDNA input within this linear phase was determined. For these seven genes, it was chosen as 2 μl.

As the cycle number and cDNA input volume is determined, the equation (3) could be altered to the followings by combining with equation (1):

\[ \log(P) = \log(M) + \log(E_{RT}) + n \log(1+ E_{PCR}) \]  

...Equation (4)

if sets a constant \( \log(A) = \log(E_{RT}) + n \log(1+ E_{PCR}) \), then equation (4) can be changed to:

\[ \log(P) = \log(M) + \log(A) \]  

...Equation (5)

or \( P = A \times M \)  

...Equation (6)

In equation (5), the yield of a PCR product has a linear relationship with the initial amount of mRNA, which demonstrates that the RT-PCR can practically and be conveniently used to represent and compare mRNA expression of a gene.

1.5.4 Negative and Positive Controls

PBS was chosen as the negative control for several reasons. At the time of designing the experiment, we considered several other models as candidates for negative control, such as denatured formula or whey extraction. However, it is not guaranteed that these candidates will have no positive effects on the expression of cytochrome P450s in the cultured cells, which can confuse the outcome from the treatments of human milk and formula. Therefore, they do not satisfy the criteria as a negative control at this stage of the research. Besides, due to the complexity of the components in formula, it would be improper to assume any component(s) within the
milk as inert component(s) before obtaining any concrete proof that human and/or formula can elicit a positive effect. In contrast, since the “whole formula” is our research target, PBS is a proper blank control to use because the addition of PBS does not affect the expression of the studied genes of the cell culture systems except diluting the medium to the same degree as other milk treatments does. The use of PBS provided a clear basal level of the expression of each gene.

Dexamethasone (DEX) has been widely used as a corticosteroid CYP3A4 inducer for the study of CYP3A4 regulations (Barwick et al., 1996; El Sankary et al., 2000; Pascussi et al., 2000a; Yamaguchi et al., 1999; Zhang et al., 1999a). Dibenz[a]anthracene (DBA), an aromatic hydrocarbon, is used as a positive control for the induction study of CYP1A2, through the AHR mechanism in HepG2 cells (Roberts et al., 1990). In this regards, we chose these two chemicals as positive controls for CYP3A4 or CYP1A2 respectively.
MATERIALS AND METHODS

2.1. Chemicals and Reagents

DEX, 3MC, DBA and holo-transferrin were purchased from Sigma Chemical Co, St. Louis, MO. α-Minimal Essential Medium was obtained from Department of Pathology. Dulbecco’s Modified Eagle’s Medium, Antibiotics (Penicillin-Streptomycin, liquid) and TRIzol® reagent were obtained from Gibco BRL, Burlington, ON. RNase-free DNase I, random hexamer pd(N)₆ and dNTPs were purchased from Pharmacia Biotech, Baie d’Urfe, PQ. M-MLV (Moloney Murine Leukemia Virus) Reverse Transcriptase and Low DNA Mass™ ladder were purchased from Gibco BRL, Burlington, ON. Taq DNA polymerase and GeneRuler™ 100 bp DNA ladder was purchased from MBI, Fermentas, Burlington, ON. Bio-Rad protein assay kit was purchased from Bio-Rad, Hercules, CA.

2.2. Cell Cultures

HepG2 (derived from hepatocellular carcinoma) and C2BBel cells (Caco2 brush border expressing cell line, a clone of a colon adenocarcinoma Caco2 cell) were obtained from the American Type Culture Collection (ATCC). HepG2 cells were maintained in α-minimal essential medium containing 10% fetal bovine serum with 1% antibiotics in a 75 cm² flask (Becton Dickinson, Franklin Lakes, NJ). C2BBel cells were grown in Dulbecco’s modified Eagle’s medium with 4mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose and 1.0 mM sodium pyruvate, 0.01 mg/mL human
transferrin and 10% fetal bovine serum with 1% antibiotics in a 75 cm² flask (Becton Dickinson, Franklin Lakes, NJ). Both cell lines were sustained in an atmospheric mixture of 5% CO₂ and 95% O₂ at 37°C. Routinely, cells were cultured to 90-100% confluence and subcultured by trypsinization with accordance to the catalogue from ATCC.

2.3. Human Milk Sources

Human milk samples were obtained with consent from healthy nursing mothers without any medications. Milk sample one was obtained from a mother of a term neonate. Milk samples two to four were obtained from mothers of infants of 2-3 months old from women’s college hospital. All milk samples were obtained fresh and stored in –80°C until use. Milk samples were quickly thawed at 37°C and applied to cells. C2BBel cells were treated with human milk samples 1-4 for RT-PCR. HepG2 cells were treated with human milk sample 1,2, and 4 due to the limited amount of sample 3. Only sample 1 was used for protein preparation of western blotting due to the limited amount of other milk samples.

2.4. Formula

Enfalac (Bristol-Myers Squibb Canada, Ottawa, ON) and Similac (Abbott laboratories, Abbott Park, IL) were purchased from a local supermarket and stored at –80°C until use. The composition of the two brands of formula is listed in table 2.1.
2.5. Treatments of Cells

The two cell lines, HepG2 and C2BBE1, were grown on Petri dishes with corresponding medium until reaching 100% confluence and then were treated with human milk, Enfalac, Similac, DBA, 3MC, DEX or PBS at different concentrations for 72 hours or as indicated in corresponding texts or figure legends. DBA, 3MC, and DEX were dissolved in DMSO. The concentrations of human milk and formula for the study of their effects on enzyme expression were 20% (as discussed in Results and Discussion). The concentration of PBS used in cell culture was 20%. DBA and DEX were added to cells to the concentration of 10 μM in the medium.

In the time-course assay of CYP3A4 and CYP1A2 induction, 10 μM DEX or 10 μM DBA was incubated with these two cell lines and the cells were harvested at 0 hour, 8 hours, 24 hours, 48 hours and 72 hours. RT-PCR analysis was performed to examine the level of CYP3A4 and CYP1A2. Before each treatment, the cells were served with 10 ml new media added into each dish and homogenized with the media by manual swirling.

Due to the complexity of the milk compositions, we examined if the treatment with formula could influence the normal growth of the cells and their viability. Human milk or formula is the main and usually the sole food for infants for the first half-year. To investigate the effects of long-term exposure of hepatic/intestinal cells to milk products, we decided to initialize this part of the study by maximizing the milk-exposure time during which the cultured cells can be maintained properly. From our cell culture experience, since the HepG2 cells usually can be cultured no longer than 4 to 5 days after reaching confluence, we chose 72 hours as the duration of the treatment. The morphology of cultured cells during this period was examined daily. After the exposure of the cells to
the formula for 72 hours, the total RNA was harvested and RT-PCR analysis for β-Actin was developed to determine if the mRNA expression of β-Actin was affected by the presence of the different concentration of formula. As a “house-keeping” gene, β-Actin was chosen to examine if high concentration of formula could affect its expression. The purpose of using a “house-keeping” gene is 3-folds: 1) as the name indicates, the expression of a “house-keeping” gene is constant between the cells of experimental groups such that the results between these cells can be normalized on it, especially if the comparison is between two different types of tissues; 2) an apparent alteration of the expression of a “house-keeping” between cell cultures of the cell type indicates an alterations of the cell growth due to its essential function in cells; 3) the PCR amplification of a “house-keeping” gene can be used as a quality control of RT-PCR, failing of the amplification of which indicates a unsatisfying RT under some circumstances.

2.6. Isolation of Total RNA

Total RNA was used in this study as template for reverse transcription. A consistent and high quality of RNA is essential to the outcome of RT-PCR. Impure RNA, such as a result of some remaining reagents used for RNA preparation or presence of genomic DNA, could affect the reverse transcription or PCR respectively. To guarantee the quality of each RNA sample, examinations of the purity and integrity were performed as described below. Total RNA was extracted from the cells using the TRIzol® reagent
(Gibco BRL, Burlington, ON). Guanidine isothiocyanate in this reagent disrupts the integrity of cells and phenol/chloroform extracts cytosolic total RNA. All procedures were performed according to the descriptions in the instruction manual of the TRIzol® reagent. Basically, cells were lysed and homogenized by pipetting with 1.25 mL of reagent in a 100 x 15 mm tissue culture dish (Becton Dickinson, Franklin Lakes, NJ). 250 uL chloroform was added and the mixture was vortexed and homogenized for 3 min. The cell lysate was then centrifuged at 11,500 g for 15 min at 4°C. The supernatant was transferred to a new microfuge tube and the RNA was precipitated with 100% isopropyl alcohol for 25 min at room temperature and then centrifuged at 11,500 g for 10 min at 4°C. The pellet was then washed with 1 ml 75% ethanol and centrifuged at 7,500 g for 5 min at 4°C. The pellet was allowed to be partially dry and then dissolved in 100 μl of diethylpyrocarbonate-treated water. All reagents, with the exception of chloroform, used during the isolation were previously chilled on ice to lessen RNA degradation. Potential genomic DNA contamination was prevented by digesting the sample RNA with 15U/100 ul of RNase-free DNase I (Pharmacia Biotech, Baie d’Urfe, PQ) at 37°C for 15 min. The enzyme was then inactivated by incubation at 55°C for 10 min.

Spectrophotometric readings were carried out to determine the concentration and purity of the isolated RNA. The readings of the absorbance at 260 nm were calculated for the concentration of the total RNA (1 OD reading = 40 μg/ml RNA). The highest purity was indicated by a value between 1.8-2.0 of the OD_{260}/OD_{280} ratio. The integrity of the RNA was examined on a 2% agarose gel (2% agarose, 1XTAE (40 mM Tris base, 40 mM acetic acid, 1 mM EDTA, pH 8.0)) with 0.05% ethidium bromide by showing intact ribosomal 28S and 18S bands under an ultraviolet light. The RNA extract was then either
used for further experiment or stored in -80°C freezer. Since the quality of total RNA is essential to the RT-PCR quantification method, the following measures were summarized to guarantee the quality of total RNA: 1) incubation of RNA sample with DNase I for 15 min at 37°C 2) a ratio of the reading OD$_{260}$/OD$_{280}$ of 1.8-2.0 3) Visualization of RNA on agarose gel. 4) master mix of shared reagents, to reduce the variability of the addition error. The quality of total RNA extracted from HepG2 and C2BBe1 cultured with or without formula was shown in Figure 3.1.

2.7. Reverse transcription

First strand complementary DNA (cDNA) was synthesized from mRNA templates by M-MLV Reverse transcriptase (Invitrogen, Burlington, ON). A 20 µl reaction volume was used for 10 µg of total RNA. A mixture of the 10 µg total RNA, 1 µl of 140 µM random hexamers (P(dN)$_6$) (Amersham Pharmacia Biotech, Piscataway, NJ) with a total volume of 11 µl was heat-denatured at 75°C for 10 min and quickly chilled on ice for 3 minutes. Additional reagents were added for the first strand synthesis of target cDNA, containing: 1X First-Strand Buffer (250 M Tris-HCl (pH 8.3 at room temperature), 375 mM KCl, 15 mM MgCl$_2$; GibCO BRL, Burlington, ON), 1 mM dNTPs (10 mM each dATP, dCTP, dGTP, dTTP at neutral pH; Pharmacia Biotech, Baie d'Urfe, PQ), 7 µM random hexamers P(dN)$_6$ (Pharmacia Biotech, Baie d'Urfe, PQ), 1.5 µM MgCl$_2$ (25mM MgCl$_2$ stock solution; Sigma Chemical Company, St. Louis, MO), 10
mM dithiothreitol (DTT) (Pharmacia Biotech, Baie d'Urfe, PQ), 10 U/μl Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (GIBCO BRL, Burlington, ON), and DEPC-treated water. To examine if there was a genomic DNA contamination, a negative control, including all reagents excluding the RT enzyme, was employed. The reaction was incubated at room temperature for 10 min and then incubated at 42°C for 1 hr. The RT reaction products were further used as cDNA templates for polymerase chain reaction or stored at −20°C.

2.8. Primer Design

Human CYP3A family comprises CYP3A4, CYP3A5 and CYP3A7, whose homology is more than 90% between each two of them. The predominant CYP3A isoform is CYP3A7 in HepG2 cells (Sumida et al., 2000) and is CYP3A5 in Caco-2 cells (Gervot, 1996), which is the parent cell line of C2BBel. This increased the difficulty of detecting CYP3A4 due to the low expression level of CYP3A4 (Figure 3.11-3.13) in both HepG2 and C2BBel cell lines (Fisher et al., 1999; Sumida et al., 2000). This difficulty was conquered by designing specific primers that satisfied strict conditions.

All primers used in this project were designed to satisfy the special needs of this project except primers for β-Actin (Giannone et al., 1998) according to the following parameters: high specificity for each individual gene, primer length between 19-23 bp, 55-60% of (G+C)/(G+C+A+T) ratio, primer TmS (melting temperature) between 60-65°C, G or C as the nucleotide at the 3', and the annealing position of primers on different exons
such that there would be intron-exon boundary to distinguish the RT-derived cDNA from potential contaminating genomic DNA by size. All primers were commercially synthesized (DNA synthesis centre, Hospital for Sick Children, Toronto, ON). Each set of primers generated a PCR product as a single band of the expected size on a 2% agarose gel stained with 0.5mg/ml ethidium bromide and the sequence of the product was verified (DNA sequencing centre, Hospital for Sick Children, Toronto, ON)

**CYP3A**

The primers for CYP3A were designed to pick up CYP3A species, such as CYP3A4 and CYP3A7. The CYP3A primers anneal to regions within the exon 6 (forward primer) and exon 10 (reverse primer) of the genomic locus of CYP3A (AF280107, GenBank), such that they span 4 introns. The expected size of the CYP3A following PCR amplification of the first strand CYP3A cDNA with the above set of primers is 446 bp.

**CYP3A4**

The CYP3A4 primers were designed to differentiate CYP3A4 from CYP3A7, which is the predominant isoform of CYP3A family in HepG2 cells (Sumida 2000). These primers anneal to the regions within the exon 1 (forward primer) and the exon 4 (reverse primer) of the genomic locus of CYP3A4 (AF280107, GenBank), such that they span 3 introns, the expected size of the PCR product is 317 bp. This sequence is listed in Table 2.2.
CYP3A7

CYP3A7 primers were designed to differentiate the expression level of CYP3A7 from CYP3A4. These primers anneal to the regions within the exon 2 (forward primer) and the exon 4 (reverse primer) of the genomic locus of CYP3A7 (AF280107, GenBank), such that they span 2 introns, the expected size of the PCR product is 742 bp. The summary of this sequence is listed in Table 2.2.

CYP1A2

The CYP1A2 primers were designed to differentiate CYP1A2 from other CYP1A subfamily members. These primers anneal to the regions within the exon 2 (forward primer) and the exon 3 (reverse primer) of the genomic locus of CYP1A2 (AF253322, GenBank), such that they span 2 introns, the expected size of the PCR product is 262 bp. This sequence is listed in Table 2.2.

PXR

The PXR primers were designed to specifically amplify cDNA derived from the orphan nuclear receptor PXR mRNA (AF061056, GenBank). These primers anneal to the regions from 381 to 400 bp in exon 2 (forward primer) and from 741 to 760 in exon 4 (reverse primer) of the first strand cDNA derived from PXR mRNA (AF261056, GenBank), such that the expected size of the PCR product is 380 bp. This sequence is listed in Table 2.2.
The AHR primers were designed to amplify the cDNA derived from the mRNA of aromatic hydrocarbon receptor. These primers anneal to the regions from 1579 to 1598 bp in exon 2 (forward primer) and from 2454-2474 in exon 4 (reverse primer) of the first strand cDNA derived from AHR mRNA (XM_004988, GenBank), such that the expected size of the PCR product is 896 bp. This sequence is listed in Table 2.2.

β-Actin

Human β-actin was used as the internal control for all PCR amplification, using the primers published by Giannone et al. (Giannone et al., 1998). The size of the product is 450 bp. This sequence is listed in Table 2.2.

2.9. Polymerase Chain Reaction

Due to the low expression level of the cytochrome P450s in cultured cell lines, a sensitive method was required to measure and compare their levels between different treatments. To this end, RT-PCR was employed in this study. Basically, it involves ubiquitous reverse transcription of the existing mRNAs to form a pool of first strand cDNAs, which are then used as DNA amplification templates for the amplification through specific primers by Taq DNA polymerase. Although it only indirectly measures the mRNA expression level of the target genes, the combined inherent advantage of its high sensitivity and specificity outweighs other detection methods. At the initial stage of a PCR analysis, the reaction materials are in excess so that the efficiency of each cycle allows the reaction product to accumulate at a linear rate. As the amount of PCR template
increases after each cycle, the reaction components gradually become the rate-limiting factor, leading to a saturation phase. A typical curve exhibits a characteristic ‘S’-shaped profile, which has: a lower plateau on the bottom-left corner that demonstrates the lower detection limit of the ethidium bromide-stained agarose gel; a linear phase in the middle field that corresponds the linear amplification of a target gene; a higher plateau on the top-right corner that demonstrates the saturation of a PCR reaction. After the determination of a proper cycle number, which will be set as a constant, different volumes of the RT product, or first strand cDNA, will be further examined to validate a proper input volume that could guarantee the amplification is within the linear range. A typical figure will show two phases, which have: a linear phase that demonstrates a proper range that allows linear amplification of a certain target gene; a top-right plateau that demonstrates the saturation of the PCR reaction. A volume will be chosen such that it guarantees a linear relationship between the cDNA input and the amount of PCR product. A simplified formula of PCR reaction is the following one: \( P = R \times 2^n \) (\( P \): amount of the PCR product; \( R \): quantity of first strand cDNA; \( n \): cycle number)(Freeman et al., 1999). The quantity of PCR product exhibited a linear relationship with the input of the first strand cDNA. A 50 µl mixture for PCR was set up containing: 2 µl of RT reaction, 1X PCR Buffer with \((\text{NH}_4)_2\text{SO}_4\) (75 mM Tris-HCl, pH 8.8 at 25°C, 20 mM \((\text{NH}_4)_2\text{SO}_4\), 0.01% Tween 20; MBI Fermentas, Burlington, ON), 1.5 mM MgCl\(_2\) (25 mM stock solution; MBI Fermentas, Burlington, ON), 0.25 µM forward and reverse primers, 0.05 U/µl recombinant Taq DNA polymerase (MBI, Fermentas, Burlington, ON) and DEPC-treated water.

All reactions were carried out in a Perkin Elmer automated thermocycler. Due to
the high homology in the mRNA sequences between each subfamily of CYP3A, a stringent annealing temperature is required to specifically amplify the target genes. For this purpose, all PCR reactions were titrated with different annealing temperatures, ranging from 50°C-62°C (data not shown). The annealing temperature was finalized at 61°C at which all target genes were successfully amplified with a high specificity. A typical tri-phased PCR reaction was programmed with the following parameters: (1) denaturation at 94°C for 20 sec, separating the DNA double-strand templates; (2) Annealing at 61°C for 20 sec, leading to the association of the primers to the DNA templates; (3) extension at 72°C for 40 sec, causing the synthesis of new DNA. The tri-phased replication was repeated to optimize each specific signal with the following numbers of cycles: 27 (β-Actin), 30 (CYP3A, CYP3A7, AHR, PXR), 33 (CYP3A4, CYP1A2). The reaction was started from a hot start at 94°C for 5 min. Following the final cycle, the PCR reaction was ended by directly cooling down to 4°C.

PCR products were quantified by electrophoresis on 2% agarose gels containing 0.05% ethidium bromide and visualized using UV light. Ethidium bromide staining allows the fluorescent detection of PCR product. To ensure the detection signal is within the linear phase of the detection limit, a calibration of the loading volume of the PCR products is necessary. The loading volume was titrated using the PCR product of Actin, due to its high level of expression in most of experimental settings. These gel saturation curves plotted the signal of PCR product (in Integrated Density Value) versus the volume loaded on the gel that ensures the each signal from the gel was within the linear phase. A similar characteristic ‘S’-shaped profile is demonstrated in these experiments, which has: a lower bottom-left plateau that demonstrates the lower detection limit of the ethidium-
bromide gel; a higher top-right plateau that demonstrates the saturation of the loading PCR products; a linear middle portion that represents the linear increase of signal to the increase of loading PCR products. A GeneRuler™ 100 bp DNA ladder (Fermentas, Burlington, ON) or a Low DNA Mass™ ladder was co-migrated to determine the size of the PCR products. To verify the amplified PCR product was not a non-specific product and to check the homology of this product, the PCR products were visualized under UV light, cut from the agarose gel and centrifuged through a Millipore PCR gel purification system. The liquid phase was then sent to the DNA sequencing centre, Hospital for Sick Children, for the analysis of the sequences. The sequencing results were confirmed with published data to ensure that the PCR products were correctly amplified from target genes. This experiment established the validity of using these sets of primers for the purpose of the amplification of individual genes.

2.10. Western Blotting

Total protein was extracted from cells by a method described by Dulic et al. Cells were lysed by RIPA buffer (50mM Tris-Cl (pH7.5), 150mM NaCl, 5mM EDTA, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS), sonicated, and centrifuged to isolate the supernatant. The concentration of total protein was measured according to the manual of the Bio-Rad protein assay kit. Western blotting was performed to quantify the protein expression levels of CYP3A as described (Muntane-Relat et al., 1995) with the following modifications: 20 µg of cytosolic protein was electrophoresed in 10% SDS polyacrylamide gel and electrotransfered to nitrocellulose (Hybond™-C, Amersham Pharmacia Biotech, Piscataway, NJ). 2.5 µg of complementary DNA (cDNA)-expressed
CYP3A4 in lymphoblastoid microsomes was loaded on the gel as a positive control for CYP3A4. The blots were blocked with 5% skim milk in 1 X TNT buffer (20 mM Tris-base, 137 mM NaCl, and 0.1% Tween-20, pH 7.6) overnight. Polyclonal antibodies against human CYP3A (Gentest Corporation, Woburn, MA) was incubated with the membrane for 3 hours to detect CYP3A. After incubation with a horse-radish-peroxidase-conjugated donkey anti-rabbit IgG (Santa Cruz Biotech, Santa Cruz, CA) for 1 hour, the membrane was immersed in Enhanced Chemiluminescence Reagent (Amersham, Burlington, ON) for 1 minute to visualize the immunopositive bands. The membrane was visualized by exposing to Kodak X-OMAT film.

2.11. Statistic Analysis

After the visualization of PCR products, the intensity of each sample was normalized to its own β-actin level and a fold induction of a gene's expression was obtained through dividing the β-actin-normalized gene expression level by that level of the PBS-treated control sample. This method was generally accepted for relative quantifications (Pascussi et al., 2000a; Sumida et al., 2000). These values were further grouped by treatments after three to four sets of experiments, depending on the availability of human milk samples (as described in Material & Methods). The results were first analyzed with ANOVA (Analysis of Variance) to test if the effect of individual treatment was equal. If the result of ANOVA demonstrated a significant difference (P<0.05), a Student's t-Test was performed between each treatment and the PBS control group to examine whether that treatment has a different effect from that of the PBS control group. As it is a multiple comparison, the significance level was adjusted with
Bonferroni correction such that the alpha level is adjusted downward to 0.0125. Thus, a significant difference is $P<0.0125$; a non-significant difference is $P>0.0125$. 
### Table 2.1 Compositions of Human Milk & Infant Formulas

<table>
<thead>
<tr>
<th></th>
<th>Unit (per 100 mL)</th>
<th>Human Milk*</th>
<th>Enfamil**</th>
<th>Similac**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy</td>
<td>kJ</td>
<td>284</td>
<td>280</td>
<td>285</td>
</tr>
<tr>
<td>Protein</td>
<td>g</td>
<td>1.0</td>
<td>1.42</td>
<td>1.4</td>
</tr>
<tr>
<td>Fat</td>
<td>g</td>
<td>3.9</td>
<td>3.58</td>
<td>3.7</td>
</tr>
<tr>
<td>Vitamin A</td>
<td>IU</td>
<td>223</td>
<td>203</td>
<td>203</td>
</tr>
<tr>
<td>Vitamin D3</td>
<td>IU</td>
<td>2.1</td>
<td>41</td>
<td>41</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>IU</td>
<td>0.3</td>
<td>1.35</td>
<td>2.0</td>
</tr>
<tr>
<td>Vitamin K1</td>
<td>mcg</td>
<td>2</td>
<td>2</td>
<td>54</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>mg</td>
<td>5</td>
<td>8</td>
<td>6.1</td>
</tr>
<tr>
<td>Vitamin B1</td>
<td>mcg</td>
<td>14</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td>Vitamin B2</td>
<td>mcg</td>
<td>40</td>
<td>90</td>
<td>-</td>
</tr>
<tr>
<td>Vitamin B6</td>
<td>mcg</td>
<td>12-15</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Folic Acid</td>
<td>mcg</td>
<td>0.14</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>µg</td>
<td>0.1</td>
<td>0.2</td>
<td>0.17</td>
</tr>
<tr>
<td>Pantothenic Acid</td>
<td>mg</td>
<td>0.24</td>
<td>0.34</td>
<td>0.304</td>
</tr>
<tr>
<td>Biotin</td>
<td>mcg</td>
<td>0.6</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Calcium</td>
<td>mg</td>
<td>28</td>
<td>53</td>
<td>53</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>mg</td>
<td>14</td>
<td>36</td>
<td>28</td>
</tr>
<tr>
<td>Magnesium</td>
<td>mg</td>
<td>3.5</td>
<td>5.4</td>
<td>4.1</td>
</tr>
<tr>
<td>Iron</td>
<td>mg</td>
<td>0.03</td>
<td>0.45</td>
<td>0.15</td>
</tr>
<tr>
<td>Zinc</td>
<td>mg</td>
<td>0.12</td>
<td>0.007</td>
<td>0.51</td>
</tr>
<tr>
<td>Copper</td>
<td>mg</td>
<td>0.025</td>
<td>0.05</td>
<td>0.061</td>
</tr>
<tr>
<td>Iodine</td>
<td>mcg</td>
<td>11</td>
<td>7</td>
<td>41</td>
</tr>
</tbody>
</table>

-, data not available.


**, according to the manufacturers.
TABLE 2.2 Gene Specific Primers

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Primer sense</th>
<th>Primer sequences 5' to 3'</th>
<th>Nucleotide Position</th>
<th>Target Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A4</td>
<td>Forward</td>
<td>GCAAAGAGCAACACAGAGCTG</td>
<td>42-62</td>
<td>317</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GTGATAGCCAGCAGCAGGCTG</td>
<td>339-358</td>
<td></td>
</tr>
<tr>
<td>CYP3A7</td>
<td>Forward</td>
<td>CCTCTGCTTTTTTGGAAATGC</td>
<td>130-152</td>
<td>742</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAGCTTTGTGGGTCTAGAGAG</td>
<td>852-871</td>
<td></td>
</tr>
<tr>
<td>CYP3A</td>
<td>Forward</td>
<td>GAAGCAGAGACAGGCAAGCC</td>
<td>556-575</td>
<td>446</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTCTGTGGTTTAGAGCCAGC</td>
<td>982-1001</td>
<td></td>
</tr>
<tr>
<td>CYP1A2</td>
<td>Forward</td>
<td>CCAACGTATTGGGTGCATG</td>
<td>598-617</td>
<td>262</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GTGATGTCCGGACACTGTTC</td>
<td>839-859</td>
<td></td>
</tr>
<tr>
<td>PXR</td>
<td>Forward</td>
<td>GCCCAGTGTCAACGCAGATG</td>
<td>381-400</td>
<td>380</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCCTGATCATCATCAGCTGC</td>
<td>741-760</td>
<td></td>
</tr>
<tr>
<td>AHR</td>
<td>Forward</td>
<td>AGCTTGAGTTAGAGCAGCCAAGG</td>
<td>1579-1598</td>
<td>896</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGCAGAGCTGTGCAGGAGAG</td>
<td>2454-2474</td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td>Forward</td>
<td>CTACAATGAGCTCCGTGTGG</td>
<td>276-295</td>
<td>450</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TAGCTTTCTCCAGGAGGAGA</td>
<td>706-725</td>
<td></td>
</tr>
</tbody>
</table>
RESULTS

3.1. Validation of RT-PCR

Before the measurement of the expression of the target genes by RT-PCR, proper validation experiments are necessary to ensure the accuracy of the quantification. To compare the levels of the mRNA expression of each target gene, a set of RT-PCR parameters was optimized to maximize the specificity, sensitivity and accuracy.

3.1.1. Calibration of PCR cycle

In order to interpret a meaningful outcome from a RT-PCR analysis, a PCR reaction must be performed within the linear range of an amplification. The proper cycle numbers chosen in these experiments guaranteed that each individual PCR signal was within the linear phase of PCR. As shown in Figure 3.2-3.5, the cycle number of β-actin was chosen as 27, that of CYP3A7, CYP3A, PXR and AHR was chosen as 30 and 33 was the cycle number for CYP3A4 and CYP1A2.

3.1.2. Calibration of the volume of the loading cDNA

To this end, a serial of volumes ranging from 0.5 µl to 12 µl of RT product were applied. As shown in Figure 3.6-3.9, 2 µl of RT product was finalized for future study.
3.1.3. Detection Limit of Ethidium Bromide-stained agarose gel

As shown in Figure 3.10, a serial of loading volumes from 2 µl to 36 µl were applied to the gel. 18 µl of PCR product was chosen as the volume for loading.

These parameters employed above allowed us to semi-quantitatively compare the yield of RT-PCR products. The expression level of each target gene was demonstrated by reverse transcribing and amplifying on the same linear scale. The saturation curves for the gene products tested in this project are presented in Figure 3.2-3.10.

3.2. Basal Expressions of Cytochrome P450-related Genes

The overall goal of this project was to determine if human milk or formula could assert any pharmacological effects on the hepatic and/or intestinal cytochrome P450s. A further question was: what could be the difference between human milk and formula in the effect(s) on those CYP enzymes? To answer these questions, we started the experiment by establishing the proper PCR conditions for specific detection of each gene expression, which also helped us to evaluate the basal expression levels of these genes.

3.2.1. CYP3A4 & CYP1A2 Expression in Human Liver

CYP3A4 is one of the most predominant cytochrome P450s in human adult liver that catalyzes more than half of the drugs commonly used in humans (Bertz and Granneman, 1997). Due to the high expression level of the CYP3A4, an adult human liver RNA was used to develop the RT-PCR assay and as a positive control. A healthy adult
Figure 3.1 Ethidium Bromide Visualization of HepG2 and C2BBel RNA samples. Total RNA was extracted from HepG2 or C2BBel cells either treated with or without Enfalac. It was loaded on a 2% agarose gel stained with 0.05% ethidium bromide, and visualized under UV light. The Integrity of RNA was determined by the visualization of intact 28S and 18S ribosomal RNA bands. Total RNA with high integrity was used for further RT-PCR analysis.
Figure 3.2 Cycle Number Titration Analysis for β-Actin. A representative RT-PCR amplification of β-actin from 2 µl of cDNA, by varying cycle number from 12 to 36. PCR products (18 µl) were loaded on a 2% agarose gel for electrophoresis and were visualized under UV light. The result shown here is from HepG2 cells. Similar cycle tests were performed for C2BBel cells (data not shown). Quantification was performed with Fluorchem™ software. A plot of IDV (Integrated Density Value) vs. cycle number is shown below the PCR figure.
Figure 3.3 Cycle Number Titration Analysis for CYP3A4 & CYP1A2. Representative RT-PCR amplifications of CYP3A4 and CYP1A2 from 2 μl of cDNA, by varying cycle number from 12 to 39. PCR products (18 μl) were loaded on a 2% agarose gel for electrophoresis and was visualized under UV light. The results shown here are from adult human liver. Quantification was performed with Fluorchem™ software. A plot of IDV (Integrated Density Value) vs. cycle number is shown below. A, electrophoresis of CYP3A4; B, plot for CYP3A4; C, electrophoresis of CYP1A2; D, plot for CYP1A2.
Figure 3.4 Cycle Number Titration Analysis for CYP3A & CYP3A7. Representative RT-PCR amplifications of CYP3A and CYP3A7 from 2 μl of cDNA, by varying cycle number from 12 to 36. PCR products (18 μl) were loaded on a 2% agarose gel for electrophoresis and were visualized under UV light. The results shown here are from HepG2 cells. Similar cycle tests were performed for C2BBel cells (data not shown). Quantification was performed with Fluorchem™ software. A plot of IDV (Integrated Density Value) vs. cycle number is shown below. A, electrophoresis of CYP3A; B, plot for CYP3A; C, electrophoresis of CYP3A7; D, plot for CYP3A7.
Figure 3.5 Cycle Number Titration Analysis for PXR & AHR. Representative RT-PCR amplifications of PXR and AHR from 2 µl of cDNA, by varying cycle number from 12 to 36. PCR products (18 µl) were loaded on a 2% agarose gel for electrophoresis and were visualized under UV light. The results shown here are from HepG2 cells. Similar cycle tests were performed for C2BBe1 cells (data not shown). Quantification was performed with Fluorchem™ software. A plot of IDV (Integrated Density Value) vs. cycle number is shown below. A, electrophoresis of PXR; B, plot for PXR; C, electrophoresis of AHR; D, plot for AHR.
Figure 3.6 cDNA Titration Analysis for β-Actin. A representative RT-PCR amplification of β-actin from 0.5µl-12µl of cDNA, with 27 cycles. PCR products (18 µl) were loaded on a 2% agarose gel for electrophoresis and were visualized under UV light. The result shown here is from HepG2 cells. Similar cycle tests were performed for C2BBel cells (data not shown). Quantification was performed with Fluorchem™ software. A plot of IDV (Integrated Density Value) vs. cDNA volume is shown below.
Figure 3.7 cDNA Volume Titration Analysis for CYP3A4 & CYP1A2. Representative RT-PCR amplifications of CYP3A4 and CYP1A2 from 0.5μl- 12μl of cDNA, with 33 cycles. PCR products (18 μl) were loaded on a 2% agarose gel for electrophoresis and were visualized under UV light. The results shown here are from adult human liver. Quantification was performed with Fluorchem™ software. A plot of IDV (Integrated Density Value) vs. cycle number is shown below. A, electrophoresis of CYP3A4; B, plot for CYP3A4; C, electrophoresis of CYP1A2; D, plot for CYP1A2.
Figure 3.8 cDNA Volume Titration Analysis for CYP3A & CYP3A7. Representative RT-PCR amplifications of CYP3A and CYP3A7 from 0.5μl-12μl of cDNA, with 30 cycles. PCR products (18 μl) were loaded on a 2% agarose gel for electrophoresis and were visualized under UV light. The results shown here are from HepG2 cells. Similar cycle tests were performed for C2BBel cells (data not shown). Quantification was performed with Fluorchem™ software. A plot of IDV (Integrated Density Value) vs. cycle number is shown below. A, electrophoresis of CYP3A; B, plot for CYP3A; C, electrophoresis of CYP3A7; D, plot for CYP3A7.
Figure 3.9: cDNA Volume Titration Analysis for PXR & AHR. Representative RT-PCR amplifications of PXR and AHR from 0.5 μl-12 μl of cDNA, with 30 cycles. PCR products (18 μl) were loaded on a 2% agarose gel for electrophoresis and were visualized under UV light. The results shown here are from HepG2 cells. Similar cycle tests were performed for C2BBe1 cells (data not shown). Quantification was performed with Fluorchem™ software. A plot of IDV (Integrated Density Value) vs. cycle number is shown below. A, electrophoresis of PXR; B, plot for PXR; C, electrophoresis of AHR; D, plot for AHR.
Figure 3.10 Detection limit of Ethidium Bromide-Stained Agarose Gel. Increasing volumes of β-actin PCR products (2μl-36μl) were loaded on a 2% agarose gel with 0.05% ethidium bromide, visualized under UV light. The result shown here is from HepG2 cells. Similar detection limit tests were performed for C2BBe1 cells (data not shown). Quantification was performed with Fluorchem™ software. A plot of IDV (Integrated Density Value) vs. PCR product loading volume is shown below.
human liver total RNA sample was kindly provided from Dr. Eve Roberts, Hospital for Sick Children. This RNA was subjected to reverse transcription and its product was used as the template for PCR, using primers specifically differentiating the CYP3A4 from the CYP3A7. This set of primers amplified a region of 317 bp for the CYP3A4 but did not amplify the CYP3A7 gene (as discussed later). The RT-PCR result is shown in Figure 3.11, which proves that CYP3A4 was expressed in this human liver sample. In a similar manner, CYP1A2 RT-PCR was developed using the same human liver RNA due to the relative abundance of CYP1A2 in human liver. This set of primers amplifies a region of 262 bp for the CYP1A2. The RT-PCR result is shown in Figure 3.11, which confirmed that CYP1A2 was expressed in this human liver sample.

3.2.2. CYP3A7

RT-PCR analysis was developed with primers specific for CYP3A7. These primers amplify a region of 742 bp from the cDNA template of CYP3A7. The presence of a clear signal with a length in the region of 742 bp demonstrates that HepG2 cells express CYP3A7 mRNA. The PCR results of CYP3A7 in C2BBe1 cells also showed a signal at the right region with a lower intensity (Figure 3.12).

3.2.3. CYP3A4

It is known that the expression level of CYP3A4 is low in HepG2, whereas CYP3A7, the predominant isoform of fetal CYP3A, is abundantly expressed in HepG2 cells. To denote the specificity of the CYP3A4 primers we designed in this study, we developed the RT-PCR analysis as shown in Figure 3.12. Briefly, PCR analysis was carried out for CYP3A4 and CYP3A7, with the first strand cDNA templates obtained from the same reverse transcription. The basal level of the CYP3A4 expression is too low.
to show a significant band on the ethidium bromide-stained agarose gel. However, the presence of a clear signal for CYP3A7 is shown on the same gel, suggesting that the CYP3A7 expression overwhelms that of the CYP3A4 in HepG2 cells. The basal level of C2BBel CYP3A4 was also examined by RT-PCR with human liver RNA as a positive control and it proved that CYP3A4 level was low in this cell line (Figure 3.13).

3.2.4. CYP3A

This experiment was also designed to compare with the overall protein level of the CYP3A. As shown in Figure 3.14, both HepG2 and C2BBel cells express CYP3A mRNA, as illustrated in the presence of the signal in the region of 446 bp.

3.2.5. PXR

The basal expression of PXR was evaluated both in HepG2 and C2BBel cells (Figure 3.14) and the results confirmed that both cell lines expressed PXR mRNA.

3.2.6. CYP1A2

To examine the basal mRNA level of CYP1A2 in HepG2 and C2BBel cells, a RT-PCR analysis was performed with the human liver RNA as a positive control. As shown in Figure 3.15, the basal expression of CYP1A2 mRNA in HepG2 is not detected, which resembles the condition in the human fetal period. The basal mRNA level of CYP1A2 in C2BBel cells is also low as shown in the same figure that it is not clearly detected with these experimental settings.

3.2.7. AHR

The result demonstrates that the HepG2 and C2BBel cells constitutively express
3.3. Milk Studies in HepG2 Cells

3.3.1. Effects of Formula on the Expression of beta-Actin

To elucidate the proper range of the milk concentrations that do not affect the growth of the cell lines, a range of concentrations was applied to the HepG2 cells, from 5% to 60%. The expression of the mRNA of beta-Actin, a 'house-keeping' gene, was used as a criterion for the cell viability. As shown in figure 3.16, the result reveals that the expression of beta-Actin mRNA in HepG2 cells is inhibited by a medium containing more than 40% of formula, indicating an alteration in the growth of the HepG2 cells. The beta-actin was not altered under this concentration of milk in C2BBel cells (Figure 3.16). Therefore, we chose 20%, which did not affect the expression of beta-actin, as the concentration setting of formula for the further experiments. The concentration of human breast milk that applied to both HepG2 and C2BBel cells was 20% to keep the result comparable with the ones obtained from formula.

3.3.2. Time Course of Positive Controls

As shown in Figure 3.17, CYP3A4 mRNA was firstly induced by DEX at 8 hours and the second rise reached the peak at 48 hours and maintained on that level at 72 hours in HepG2 cells. We also observed significant increase of CYP3A4 after 72 hours in C2BBel cells. CYP1A2 was induced by DBA at 8 hours and reached the peak around 24-48 hours and slightly decreased afterwards in HepG2 cells, proving that the relative
Figure 3.11 CYP3A4 & CYP1A2 Expression in Human Adult Liver. RT-PCR analysis was established for CYP3A4 and CYP1A2, using a human adult liver, a generous gift from Dr. Eve Roberts, Hospital for Sick Children. 10 μg of total RNA from this sample was reverse transcribed and PCR performed for CYP3A4 and CYP1A2 respectively. 18 μl of PCR product was loaded on a 2% agarose gel for electrophoresis and was visualized under UV light. A, electrophoresis of CYP3A4; B, electrophoresis of CYP1A2.
Figure 3.12 Expression of CYP3A4 & CYP3A7 in HepG2 Cells. RT-PCR amplifications of β-actin, CYP3A4 and CYP3A7 from 2 µl of HepG2 cDNA, with 27 cycles for β-actin, 33 cycles for CYP3A4 and 30 cycles for CYP3A7. Human adult liver RNA was used as a positive control. PCR products (18 µl) were loaded on a 2% agarose gel for electrophoresis and were visualized under UV light. This figure is representative of at least three sets of experiments. Lane 1, HepG2 β-actin; Lane 2, human liver β-actin; Lane 3, HepG2 CYP3A4; Lane 4, HepG2 CYP3A7; Lane 5, human liver CYP3A4. L: DNA ladder.

Figure 3.13 Expression of CYP3A4 & CYP3A7 in C2BBel Cells. A representative RT-PCR amplifications of β-actin, CYP3A4 and CYP3A7 from 2 µl of C2BBel cDNA, with 27 cycles for β-actin, 33 cycles for CYP3A4 and 30 cycles for CYP3A7. Human adult liver RNA was used as a positive control. PCR products (18 µl) were loaded on a 2% agarose gel for electrophoresis and were visualized under UV light. This figure is representative of at least three sets of experiments. Lane 1, C2BBel β-actin; Lane 2, human liver β-actin; Lane 3, C2BBel CYP3A4; Lane 4, C2BBel CYP3A7; Lane 5, human liver CYP3A4, Lane 6, human liver CYP3A7. L: DNA ladder.
Figure 3.14 a. Expression of PXR in HepG2 and C2BBel Cells. RT-PCR amplifications of β-actin and PXR from 2 μl of HepG2 or C2BBel cDNA, with 27 cycles for β-actin, and 30 cycles for PXR. PCR products (18 μl) were loaded on a 2% agarose gel for electrophoresis and were visualized under UV light. This figure is representative of at least three sets of experiments. Lane 1, HepG2 β-actin; Lane 2, C2BBel β-actin; Lane 3, HepG2 PXR; Lane 4, C2BBel PXR; L: DNA ladder.

Figure 3.14 b. Expression of CYP3A in HepG2 and C2BBel Cells. RT-PCR amplifications of β-actin, CYP3A from 2 μl of HepG2 and C2BBel cDNA, with 27 cycles for β-actin, and 30 cycles for CYP3A. Human adult liver RNA was used as a positive control. PCR products (18 μl) were loaded on a 2% agarose gel for electrophoresis and were visualized under UV light. This figure is representative of three sets of experiments. Lane 1, HepG2 β-actin; Lane 2, C2BBel β-actin; Lane 3, human liver β-actin; Lane 4, HepG2 CYP3A; Lane 5, C2BBel CYP3A; Lane 6, human liver CYP3A. L: DNA ladder.
Figure 3.15 a. b. Expression of CYP1A2 in HepG2 and C2BBel Cells. RT-PCR amplifications of β-actin and CYP1A2 from 2 μl of HepG2 or C2BBel cDNA, with 27 cycles for β-actin, and 33 cycles for CYP1A2. PCR products (18 μl) were loaded on a 2% agarose gel for electrophoresis and were visualized under UV light. Each figure is representative of at least three independent sets of experiments. Lane 1, HepG2/C2BBel β-actin; Lane 2, human liver β-actin; Lane 3, HepG2/C2BBel PXR; Lane 4, human liver CYP1A2; L: DNA ladder.

Figure 3.15 c. Expression of AHR in HepG2 and C2BBel Cells. RT-PCR amplifications of β-actin and AHR from 2 μl of HepG2 or C2BBel cDNA, with 27 cycles for β-actin, and 30 cycles for AHR. PCR products (18 μl) were loaded on a 2% agarose gel for electrophoresis and were visualized under UV light. Human adult liver RNA was used as a positive control. This figure is representative of at least three independent sets of experiments. Lane 1, HepG2 β-actin; Lane 2, C2BBel β-actin; Lane 3, human liver β-actin, Lane 4, HepG2 AHR; Lane 5, C2BBel AHR; Lane 6, human liver AHR; L: DNA ladder.
long-term induction was applicable in HepG2 cells. However, DBA did not induce any significant CYP1A2 mRNA during this 72-hour period in C2BBel cells.

3.3.3. Effects of Human Milk and Formula on the Expression of CYP3A4 in HepG2 cells

As shown in Figure 3.18, the results of the densitometric analysis demonstrated that: 1) in the Enfalac and Similac treated group, the expression of the HepG2 CYP3A4 mRNA was significantly increased, compared with the control group treated with PBS; 2) human milk did not induce the expression of CYP3A4 in this cell line. If expressed as a fold-induction using the level of PBS control group as the baseline, the data showed that the formula-treated HepG2 cells expressed 2.6±0.2 (Enfalac) and 1.8±0.1 (Similac) fold of CYP3A4 mRNA as the PBS-treated HepG2, and the induction of CYP3A4 in dexamethasone-treated HepG2 was 3.4±0.8 fold, compared with the PBS-treated HepG2. Due to the extremely low basal expression of CYP3A4 mRNA in HepG2 cells, it is infeasible to differentiate between a condition of inhibition and a condition of no effect on the expression. The results, shown in Figure 3.18, reveal that there is no significant induction of CYP3A4 mRNA by human breast milk.

3.3.4. Effect of Human milk and Formula on the Expression of CYP3A7 in HepG2 Cells

As shown in Figure 3.19, the result revealed that there was no significant induction of CYP3A7 mRNA after either treatment. ANOVA analysis did not reveal significant difference between treatments.
3.3.5. Effect of Human Milk and Formula on the Expression of CYP3A in HepG2 cells

To examine if the formula induction of CYP3A4 had any effect on the total expression level of CYP3A in HepG2 cells, the expression of CYP3A mRNA was examined by RT-PCR and western blotting with probes recognizing both CYP3A4 and CYP3A7. As shown in Figure 3.20, the total level of CYP3A mRNA is not significantly affected by the treatment with formula or human milk. Statistically, ANOVA analysis did not reveal any significant difference between these treatment groups. Due to the limited amount of human milk, only one set of western blotting was performed. This result revealed that there was no significant increase in the level of HepG2 CYP3A protein, which more reflected the level of CYP3A7, the predominant isoform of CYP3A in HepG2 cells.

3.3.6. Effects of Human Milk and Formula on the Expression of PXR in HepG2 Cells

The result of the effects of human milk or formula on the expression of HepG2 PXR mRNA is shown in Figure 3.21, which reveals that there is no significant alteration in PXR mRNA expression after the human milk or formula treatments in HepG2 cells.

3.3.7. Effects of Human Milk and Formula on the Expression of CYP1A2 in HepG2 Cells

As shown in Figure 3.22, while human milk treatment did not alter the expression
of CYP1A2 mRNA significantly, the expression of the CYP1A2 mRNA in HepG2 cells was found significantly increased by 72 hours treatment with Enfalac or Similac, compared with the level of the PBS-treated control group. If expressed as a fold-induction using the level of PBS control group as the baseline, the data show that the formula-treated HepG2 cells express as $2.0\pm0.1$ (Enfalac) and $2.4\pm0.5$ (Similac) folds of CYP1A2 mRNA as that of PBS-treated HepG2, and the induction of CYP1A2 in DBA-treated HepG2 is $8.4\pm2.2$ fold, compared with the PBS-treated HepG2.

3.3.8. Effects of Human Milk and Formula on the Expression of AHR in HepG2 Cells

As shown in Figure 3.23, the expression of AHR mRNA was not significantly altered by the treatment with human milk or formula after 72 hours.

3.4. Milk Studies in C2BBel Cells

In order to examine the main and alternative hypothesis in an intestine model, we studied the effects of milk treatments on expression of cytochrome P450s and their associated nuclear receptors in C2BBel cells. The treatments, including the concentrations of milk samples and CYP inducers and duration, were the same as those applied to HepG2 cells.

3.4.1. Effects of Human Milk and Formula on the Expression of CYP3A4 in C2BBel Cells

The primary goal of this part of study is to examine whether human milk down-regulates
Figure 3.16 Effects of Formula on the Expression of β-actin in HepG2 and C2BBel cells. HepG2 and C2BBel cells were treated with a serial of concentrations of Enfalac for 72 hours. PCR amplification of β-actin was performed from 2 μl of HepG2 or C2BBel cDNA with 27 cycles. PCR products (18 μl) were loaded on a 2% agarose gel for electrophoresis and were visualized under UV light. This figure is representative of three repeats. Lane 1, HepG2 treated with 60% Enfalac; Lane 2, HepG2 treated with 40% Enfalac; Lane 3, HepG2 treated with 20% Enfalac; Lane 4, HepG2 without any treatment; Lane 5: C2BBel treated with 60% Enfalac; Lane 6: C2BBel treated with 40% Enfalac; Lane 7: C2BBel treated with 20% Enfalac; Lane 8: C2BBel without any treatment; L: DNA ladder.
Figure 3.17 Time Course of CYP3A4 and CYP1A2 Induction in C2BBe1 Cells and HepG2 Cells. C2BBe1 cells and HepG2 cells were treated with DBA (for CYP1A2) or DEX (for CYP3A4) in a time-course assay. After exposure to 10 μM DBA or 10 μM DEX for five different time periods, cells were harvested, and total RNA was extracted. RT-PCR was performed as described in Materials & Methods. PCR product (18 μl) was loaded on a 2% agarose gel for electrophoresis and was visualized under UV light. h, hour.
Figure 3.18. Effects of Human Milk and Infant Formulas on the Expression of CYP3A4 Level in HepG2 Cells. After the treatment with 20% human milk, 20% Enfalac, 20% Similac, 10 μM DEX or 20% PBS for 72 hr, cells were harvested and 10 μg total RNA was reverse transcribed and PCR performed for CYP3A4. Results of one replicate are shown in the gel above. PCR products were loaded on a 2% agarose gel with 0.05% ethidium bromide and quantified using AlphaEaseTM analysis software. The results are shown in the graphic representation below. All CYP3A4 signals were normalized to β-actin and the results are expressed as a fold-induction above PBS-treated levels. The data represent mean ± standard deviation (n=3). "*" indicates P<0.0125. Statistical analysis of the relative levels of CYP3A4 mRNA is shown in Table 3.1.
Figure 3.19. Effects of Human Milk and Infant Formulas on the Expression of CYP3A7 Level in HepG2 Cells. After the treatment with 20% human milk, 20% Enfalac, 20% Similac, 10 μM DEX or 20% PBS for 72 hr, cells were harvested and 10 μg total RNA was reverse transcribed and PCR performed for CYP3A7. Results of one replicate are shown in the gel above. PCR products were loaded on a 2% agarose gel with 0.05% ethidium bromide and quantified using AlphaEase™ analysis software. The results are shown in the graphic representation below. All CYP3A7 signals were normalized to β-actin and the results are expressed as a fold-induction above PBS-treated levels. The data represent mean ± standard deviation (n=3). ** indicates P<0.0125. Statistical analysis of the relative levels of CYP3A7 mRNA is shown in Table 3.1.
Figure 3.20. Effects of Human Milk and Infant Formulas on the Expression of CYP3A Level in HepG2 Cells. A, after the treatment with 20% human milk, 20% Enfalac, 20% Similac, 10 μM DEX or 20% PBS for 72 hr, cells were harvested and 10 μg total RNA was reverse transcribed and PCR performed for CYP3A. Results of one replicate are shown in the gel above. PCR products were loaded on a 2% agarose gel with 0.05% ethidium bromide and quantified using AlphaEaseTM analysis software. The results are shown in the graphic representation below. All CYP3A signals were normalized to β-actin and the results are expressed as a fold-induction above PBS-treated levels. The data represent mean ± standard deviation (n=3). "*" indicates P<0.0125. Statistical analysis of the relative levels of CYP3A mRNA are shown in Table 3.1. B, after the same treatments mentioned above, total protein was harvested and western blotting with 20 μg of total protein sample or 2.5 μg of positive control was performed to examine the CYP3A protein level. Due to the limited resource of human milk, only sample number 1 was used for this experiment.
Figure 3.21. Effects of Human Milk and Infant Formulas on the Expression of PXR Level in HepG2 Cells. After the treatment with 20% human milk, 20% Enfamil, 20% Similac, 10 μM DEX or 20% PBS for 72 hr, cells were harvested and 10 μg total RNA was reverse transcribed and PCR performed for PXR. Results of one replicate are shown in the gel above. PCR products were loaded on a 2% agarose gel with 0.05% ethidium bromide and quantified using Alpha Ease™ analysis software. The results are shown in the graphic representation below. All PXR signals were normalized to β-actin and the results are expressed as a fold-induction above PBS-treated levels. The data represent mean ± standard deviation (n=3). “*” indicates P<0.0125. Statistical analysis of the relative levels of PXR mRNA is shown in Table 3.1.
Figure 3.22. Effects of Human Milk and Infant Formulas on the Expression of CYP1A2 Level in HepG2 Cells. After the treatment with 20% human milk, 20% Enfalac, 20% Similac, 10 μM DBA or 20% PBS for 72 hr, cells were harvested and 10 μg total RNA was reverse transcribed and PCR performed for CYP1A2. Results of one replicate are shown in the gel above. PCR products were loaded on a 2% agarose gel with 0.05% ethidium bromide and quantified using AlphaEaseTM analysis software. The results are shown in the graphic representation below. All CYP1A2 signals were normalized to β-actin and the results are expressed as a fold-induction above PBS-treated levels. The data represent mean ± standard deviation (n=3). "*" indicates P<0.0125. Statistical analysis of the relative levels of CYP1A2 mRNA is shown in Table 3.1.
Figure 3.23. Effects of Human Milk and Infant Formulas on the Expression of AHR Level in HepG2 Cells. After the treatment with 20% human milk, 20% Enfamil, 20% Similac, 10 μM DBA or 20% PBS for 72 hr, cells were harvested and 10 μg total RNA was reverse transcribed and PCR performed for AHR. Results of one replicate are shown in the gel above. PCR products were loaded on a 2% agarose gel with 0.05% ethidium bromide and quantified using AlphaEaseTM analysis software. The results are shown in the graphic representation below. All AHR signals were normalized to β-Actin and the results are expressed as a fold-induction above PBS-treated levels. The data represent mean ± standard deviation (n=3). * indicates P<0.0125. Statistical analysis of the relative levels of AHR mRNA is shown in Table 3.1
CYP3A4 expression and/or formula induces its expression. Four human milk samples were collected as described in Material and Method section. RT-PCR analysis was performed. As shown in Figure 3.24, the results of the densitometric analysis demonstrated that: 1) in the Enfalac and Similac treated group, the expression of the C2BBel CYP3A4 mRNA was not significantly increased, compared with the control group only treated with PBS; 2) human milk significantly induced the mRNA expression of CYP3A4 in this cell line. If expressed as a fold-induction using the level of PBS control group as the baseline, the data show that there is a 1.4±0.1-folds induction of CYP3A4 in cells treated with the human milk samples 2-4, compared with the PBS-treated C2BBel. Interestingly, the PCR results obtained from sample 1, which was obtained from a mother of a term neonate, demonstrated a 6.0-fold induction of CYP3A4. The induction of CYP3A4 in DEX-treated HepG2 was 3.7±0.8 fold, compared with the PBS-treated C2BBel cells. The results revealed that CYP3A4 mRNA was significantly induced by human milk after 72 hours of treatment. The protein, harvested from the treatment of human milk sample 1, was used for western blotting and the result indicated a similar pattern of increase of total CYP3A. (Figure 3.24 B)

3.4.2. Effects of Human Milk and Formula on the Expression of CYP3A7 in C2BBel Cells

The results of the densitometric analysis demonstrated that: 1) in the Enfalac and Similac treated group, the expression of the C2BBel CYP3A7 mRNA was significantly increased, compared with the control group treated with PBS; 2) human milk also significantly induced mRNA expression of CYP3A7 in this cell line; If expressed as a fold-induction using the level of PBS control group as the baseline, the data showed that
the milk-treated C2BBel cells expressed 2.6±0.6 fold (human milk), 2.7±0.4 fold (Enfalac), 2.8±0.5 fold (Similac) of CYP3A7 mRNA, compared with the PBS-treated C2BBel cells, and the induction of CYP3A7 in DEX-treated C2BBel cells was 3.3±0.8 fold. These results revealed that CYP3A7 mRNA is significantly induced by human milk and formula at 72 hours of treatment.

3.4.3. Effects of Human Milk and Formula on the Expression of CYP3A in C2BBel Cells

To examine the overall level of CYP3A after the treatments with human milk or formula, RT-PCR analysis was performed. As shown in Figure 3.26, the overall mRNA level of CYP3A was not significantly increased after the treatment with human milk or formula.

3.4.4. Effects of Human Milk and Formula on the Expression of PXR in C2BBel Cells

There was no statistically significant increase in PXR mRNA expression in the human milk-treated group compared with PBS control group. However, PXR mRNA was significantly induced in the formula-treated groups (Figure 3.27). The data demonstrated that the formula-treated C2BBel cells expressed 2.2±0.4 fold (Enfalac), or 1.7±0.1 fold (Similac) of PXR mRNA, compared with the PBS-treated C2BBel cells. However, as shown in Figure 3.27, the mRNA expression of PXR was not significantly altered by DEX treatment.
3.4.5. Effects of Human Milk and Formula on the Expression of CYP1A2 in C2BBel Cells

Although DBA failed to induce CYP1A2 in this cell line (Figure 3.17), we still examined if human milk treatment would affect the expression of CYP1A2 by RT-PCR, since the chance that human milk or formula might exert their regulative effects different from these inducers cannot be ruled out. However, we did not observe any induction of CYP1A2 mRNA in C2BBel cells after 72 hours of milk treatment (data not shown).

3.4.6. Effects of Human Milk and Formula on the Expression of AHR in C2BBel Cells

The result revealed that there was no significant alteration in the mRNA expression level of AHR (Figure 3.28).

The statistical significance of this project is summarized in Table 3.1.
Figure 3.24. Effects of Human Milk and Infant Formulas on the Expression of CYP3A4 Level in C2BBEl Cells. After the treatment with 20% human milk, 20% Enfalac, 20% Similac, 10 µM DEX or 20% PBS for 72 hr, cells were harvested and 10 µg total RNA was reverse transcribed and PCR performed for CYP3A4. Results of one replicate are shown in the gel above. PCR products were loaded on a 2% agarose gel with 0.05% ethidium bromide and quantified using AlphaEaseTM analysis software. A, represent human milk sample 2-4 B, represent results from human milk sample 1. Row 1, RT-PCR Row 2, Western blotting was carried out for this sample with 20 µg of total protein or 2.5 µg of cDNA-expressed CYP3A4 protein as positive control. C, a graphic representation of the mRNA quantification results. All CYP3A4 signals were normalized to β-Actin and the results are expressed as a fold-induction above PBS-treated levels. “*” indicates P<0.0125. Statistical analysis of the relative levels of CYP3A4 mRNA is shown in Table 3.1. The data represent mean ± standard deviation (n=3) (except human milk sample 1).
Figure 3.25. Effects of Human Milk and Infant Formulas on the Expression of CYP3A7 Level in C2BBel Cells. After the treatment with 20% human milk, 20% Enfalac, 20% Similac, 10 μM DEX or 20% PBS for 72 hr, cells were harvested and 10 μg total RNA was reverse transcribed and PCR performed for CYP3A7. Results of one replicate are shown in the gel above. PCR products were loaded on a 2% agarose gel with 0.05% ethidium bromide and quantified using AlphaEaseTM analysis software. The results are shown in the graphic representation below. All CYP3A7 signals were normalized to β-actin and the results are expressed as a fold-induction above PBS-treated levels. The data represent mean ± standard deviation (n=4). “*” indicates P<0.0125. Statistical analysis of the relative levels of CYP3A7 mRNA is shown in Table 3.1.
Figure 3.26. Effects of Human Milk and Infant Formulas on the Expression of CYP3A Level in C2BBel Cells. After the treatment with 20% human milk, 20% Enfalac, 20% Similac, 10 μM DEX or 20% PBS for 72 hr, cells were harvested and 10 μg total RNA was reverse transcribed and PCR performed for CYP3A. Results of one replicate are shown in the gel above. PCR products were loaded on a 2% agarose gel with 0.05% ethidium bromide and quantified using AlphaEaseTM analysis software. The results are shown in the graphic representation below. All CYP3A signals were normalized to β-Actin and the results are expressed as a fold-induction above PBS-treated levels. The data represent mean ± standard deviation (n=4). "*" indicates P<0.0125. Statistical analysis of the relative levels of CYP3A mRNA is shown in Table 3.1.
Figure 3.27. Effects of Human Milk and Infant Formulas on the Expression of PXR Level in C2BBe1 Cells. After the treatment with 20% human milk, 20% Enfalac, 20% Similac, 10 μM DEX or 20% PBS for 72 hr, cells were harvested and 10 μg total RNA was reverse transcribed and PCR performed for PXR. Results of one replicate are shown in the gel above. PCR products were loaded on a 2% agarose gel with 0.05% ethidium bromide and quantified using AlphaEaseTM analysis software. The results are shown in the graphic representation below. All PXR signals were normalized to β-actin and the results are expressed as a fold-induction above PBS-treated levels. The data represent mean ± standard deviation (n=4). "*" indicates P<0.0125. Statistical analysis of the relative levels of PXR mRNA is shown in Table 3.1
Figure 3.28. Effects of Human Milk and Infant Formulas on the Expression of AHR Level in C2BBel Cells. After the treatment with 20% human milk, 20% Enfamil, 20% Similac, 10 μM DBA or 20% PBS for 72 hr, cells were harvested and 10 μg total RNA was reverse transcribed and PCR performed for CYP3A4. Results of one replicate are shown in the gel above. PCR products were loaded on a 2% agarose gel with 0.05% ethidium bromide and quantified using AlphaEaseTM analysis software. The results are shown in the graphic representation below. All AHR signals were normalized to β-actin and the results are expressed as a fold-induction above PBS-treated levels. The data represent mean ± standard deviation (n=4). "*" indicates P<0.0125. Statistical analysis of the relative levels of AHR mRNA is shown in Table 3.1.
<table>
<thead>
<tr>
<th></th>
<th>CYP3A4</th>
<th>CYP3A7</th>
<th>CYP3A</th>
<th>PXR</th>
<th>CYP1A2</th>
<th>AHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>20% Human Milk</td>
<td>H</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>Significant</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>20% Enfalac</td>
<td>H</td>
<td>Significant</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>NS</td>
<td>Significant</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>20% Similac</td>
<td>H</td>
<td>Significant</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>NS</td>
<td>Significant</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>10 μM DEX</td>
<td>H</td>
<td>Significant</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>Significant</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>-</td>
</tr>
<tr>
<td>10 μM DBA</td>
<td>H</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Significant</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 3.1. Summary of Statistical Analyses of the Effects of Human Milk and Formula. There were five treatments for each set of experiment: 20% human milk, 20% Enfalac, 20% Similac, 10 μM DEX/DBA and 20% PBS. Analysis of variance (ANOVA) was first performed. If the result shows a significant difference (P<0.05), a Student’s t-test was performed between each treatment and the PBS control group to examine whether that treatment has a different effect from the PBS control group. The significant level was adjusted with Bonferroni correction so that the alpha level is adjusted downward to 0.0125. Thus, a significant difference is P<0.0125; a non-significant difference is P>0.0125. H, HepG2 cells; C, C2BBel cells; NS, non-significant; -, not evaluated;
DISCUSSION

4.1. Validation of RT-PCR

As shown in Figure 3.2 to Figure 3.9, the generation of standard curves for the determination of the cDNA input and the cycle number as those discussed above established the optimal experimental parameters for the relative quantifications. Specifically, as the inter-sample variability of $E_{RT}$ and $E_{PCR}$ is greatly reduced with optimal technique measures, the yield of a PCR analysis can be assumed proportional to the amount of cDNA template available in a RT product. As the setting of a RT is strictly defined and performed, each available mRNA in a RT preparation can be assumed to be amplified equally. Thus, a strictly controlled RT-PCR is valid to be used to compare the level of mRNA expression of a gene between samples. These measures maximally reduce the chance of false positive results, an important error associated with the RT-PCR method. Also, as the detection of the limitation of ethidium bromide staining was performed, the chance of false negative results from the maximum detection limit of the gel was also reduced (Figure 3.10). Thus, with the parameters determined by a standard curve, we safely assumed that the yield of PCR reaction resulting from this study was dependent on the initial concentration of mRNA template in a linear manner.

Due to the special profile of CYP3A subfamilies in HepG2 cells, we started the RT-PCR by designing CYP3A4-specific primers. Since there is a high homology between CYP3A4 and CYP3A7, an initial set of CYP3A4 primers was proved to be
able to amplify both CYP3A4 and CYP3A7 (data not shown) that was confirmed by sequencing (data not shown). A modification was made that the forward primer was designed to anneal to a region close to the 5' end of the CYP3A4 cDNA, the sequence of which was only available to CYP3A4. As shown in Figure 3.12, this modification successfully differentiated CYP3A4 from CYP3A7 in HepG2 cells, which eliminated the interference of CYP3A7 in the quantification procedure of CYP3A4 mRNA expression. The low expression of cytochrome P450s in cultured cell lines not only made the gene-specific amplification difficult, but also under astringent conditions, opened the window for unspecific amplification, which interfered with the amplification efficiency. In this regard, we decided to use high stringency parameters to design primers for each gene, which reduced the chance of unspecific amplification under the circumstance that the expression of a certain gene was low. We also used unified settings for primer designs, which allowed us to use a unitary PCR parameter for all target genes except the cycle number, which was discussed above.

4.2. Basal Expression of Cytochrome P450-related Genes

To study the regulation of the cytochrome P450-related expressions, we first determined the relative basal expression levels of each gene. As the most predominantly expressed cytochrome P450 in human liver and small intestine, CYP3A4 accounts for approximately 40% of the total CYP content in both liver and intestine(Kolars et al., 1994a; Watkins et al., 1987). Liver is also the site where CYP1A2 is most abundantly expressed(Landi et al., 1999). For this reason, a normal
human liver RNA sample was utilized to set up the RT-PCR parameters for CYP3A4 and CYP1A2 (Figure 3.11). After sequencing, this result proved the accuracy of the primers to amplify CYP3A4 and CYP1A2 and confirmed their abundance in human adult liver. We further examined the expression levels of CYP3A4 in HepG2 cells and C2BBel cells along with CYP3A7. The results indicated that the expression pattern of CYP3A4 of these two cell lines was similarly low, especially in HepG2 cells, of which CYP3A4 mRNA expression was too low to be detected. However, the patterns of CYP3A7 expression between these two cell lines are distinct. RT-PCR analysis showed that CYP3A7 was abundantly expressed in HepG2 cells, while the level of CYP3A7 in C2BBel cells was similar to that of adult liver. As it is known that the predominant form of CYP3A in Caco-2 cells is CYP3A5(Schmiedlin-Ren et al., 1997), these two experiments also indicated the specificity of the primers for CYP3A4 and CYP3A7 respectively. Although the total level of CYP3A cannot be used as an index of CYP3A4 in these cell lines, the information on the total CYP3A expression can be used as a reference to indicate the influence of the variation of a subfamily on the total level of CYP3A. Besides, since there were no specific antibodies that could differentiate CYP3A4 from CYP3A7 at the time of the investigation, only total CYP3A protein level was measured. In this regard, the total CYP3A mRNA level was also used to compare with the level of CYP3A protein. As shown in figure 3.14, the overall level of CYP3A mRNA is similar between HepG2 and C2BBel cells.

As one of the most important component in CYP3A4 signal transduction pathway, PXR(Kliwer et al., 1998) mediates the induction of CYP3A4(Bertilsson et al., 1998;Blumberg et al., 1998). It binds to a variety of structurally diverse,
exogenous and endogenous ligands and forms a heterodimer with RXR to activate the ER6 (Everted Repeat with a 6 bp spacer), which is located in the promoter region of CYP3A4 (Waxman, 1999). To study the regulation of CYP3A4, the level of PXR is of particular interest since some inducers, like DEX, induce its expression while other inducers do not, such as rifampicin. In this regard, we examined the basal level of the PXR expression pattern in this two cell lines. As shown in figure 3.14, the basal level of PXR is higher in HepG2 cells than in C2BBel cells.

As mentioned above, the ontogeny of CYP1A2 in human displays a delayed pattern compared with other cytochrome P450s, such as CYP3A4, CYP2C, CYP2D6 and CYP2E1 (Sonnier and Cresteil, 1998). This leads to the altered biotransformation pattern of drug metabolized by CYP1A2, such as imipramine and caffeine, from CYP1A2 to other cytochrome P450s, such as CYP3A4. However, CYP1A2 is highly inducible (Michalets, 1998). In this regard, we examined the mRNA expression of CYP1A2 in these two cell lines. As shown in Figure 3.15, the basal expression of CYP1A2 was not detected with RT-PCR method. Human liver was used as a positive control, which illustrated a distinct difference between the expression of CYP1A2 in these in vitro cell line HepG2 and the in vivo liver tissue.

CYP1A2 is inducible by aromatic hydrocarbons such as DBA and 3MC through the AHR mechanism in HepG2 cells (Roberts et al., 1990). The induction is via a translocation of AHR protein from cytosol to nucleus, which shows an apparent reduction of total cellular AHR (Giannone et al., 1998). It was also found that the greatest reduction in the level of AHR protein occurred at the concentrations of aromatic hydrocarbons that caused the maximum induction of CYP1A2 in LS180 and
mouse Hep-1 cells while the AHR mRNA levels were not affected (Li et al., 1998). We suspected that AHR mRNA expression would not be altered even if CYP1A2 was induced; As shown in Figure 3.15, AHR was constitutively expressed in both cell lines and in human liver.

4.3. **Milk Studies in HepG2 Cells**

To our knowledge, this is the first investigation that focuses on the potential that human milk and formula affect the expression of cytochrome P450s, which can further influence the biotransformation of drugs metabolized by these enzymes.

4.3.1 *Effects of Formula on Cell Viability*

As shown in Figure 3.16, there was no significant reduction in the expression of β-actin mRNA in HepG2 cells until the concentration of formula rose to 40% and above while C2BBe1 cells apparently were more tenable under high concentrations of milk which reflected its nature as an intestinal cell line. The morphology of cultured cells treated with 20% of formula was not altered and no dead cells or detached cells were found under this concentration. Therefore, we decided to treat the cells with 20% formula, which was also used in several cell lines in a previous study (Steimer et al., 1981). Further experiments also proved that the β-actin expression was not altered by the treatment with human milk at this concentration, which further proved that this concentration of milk does not affect the cellular growth.
4.3.2 Time Course of CYP3A4 and CYP1A2 Induction by DEX or DBA

Maximal CYP3A4 induction can be maintained up to 120 hours in HepG2 cells by rifampicin (Sumida et al., 2000). We conducted a similar experiment in our cell culture system and found CYP3A4 mRNA level was induced with a biphasic pattern such that it was induced as early as 8 hours and second increase was maintained up to 72 hours in HepG2 cells. The induction of CYP3A4 was also strong at 72 hours in C2BBel cells (Figure 3.17). The initial increase can be explained by the activation of pre-existing factors, such as PXR. The second increase probably requires de novo protein biosynthesis which has a very similar pattern observed in RFP-mediated induction of MDR (Multi-Drug Resistance) (Geick et al., 2001). This result further proved that the 72 hours treatment with DEX was proper to be used in this project. The 3MC-mediated induction of CYP1A2 mRNA was reported to peak around 20 hours in cultured cell lines, such as LS180 or HepG2 (Li et al., 1998). We conducted a similar time-course study with DBA in HepG2 and C2BBel cells. DBA-mediated CYP1A2 mRNA induction was observed as early as 8 hours and reached a peak at around 24-48 hours and declined slightly afterwards. Similar phenomenon were also observed for other CYP1A2 inducers such as 3MC (Li et al., 1998). The decline of mRNA level appeared to be relatively rapid inactivation by biotransformation in the liver (Riddick et al., 1994). To maximize the experimental efficiency, treatment for 72 hours was chosen. These experiments demonstrated that HepG2 cells were long-term inducible. In C2BBel cells, we did not observe appreciable induction by DBA during 72 hours period, which indicated that the AHR-mediated mechanism of CYP1A2 induction was not intact. As shown in Figure 3.18
and Figure 3.24, DEX significantly induced CYP3A4 with a 3.4 and 3.7-fold induction in HepG2 and C2BBel cells respectively. DBA significantly induced CYP1A2 with a 8.4-fold induction as mean value in Figure 3.22.

4.3.3 Effects of Human milk and Formula on the Expression of CYP3A4 and CYP1A2

Both brands of formula, Enfalac and Similac, exerted a similar effect that significantly increased CYP3A4 mRNA expression with 1.8-2.6-fold inductions and 2.0-2.4-fold of CYP1A2 mRNA in HepG2 after 72 hours treatments. It is not clear which component is responsible for this inductive effect at this stage of the project which needs further investigation in the future. As the two major enzymes responsible for the metabolism of caffeine in neonates and in adults respectively (Sonnier and Cresteil, 1998), the induction of both enzymes by 72 hour treatment with formula in this in vitro liver model indicates that caffeine metabolism may be enhanced through this induction mechanism. Thus, these results can be used to explain the phenomenon that caffeine half-life is shortened in formula-fed infants. Also, the biotransformation of drugs that are substrates of CYP3A4 and CYP1A2, such as carbamazepine and cyclosporine, may be affected if formula is the major diet for an infant at the time of drug administration.

4.3.4 Effects of Human milk and Formula on the Expression of CYP3A7, CYP3A, PXR and AHR

As it is known that CYP3A7 is the major isoform of CYP3A in HepG2 cells, we examined whether milk treatments would affect its expression since we observed an
induction of CYP3A4. We did not observe any statistically significant induction of CYP3A7 either by human milk, formula, or by DEX-treated group. CYP3A7 shares 90% of mRNA sequence with CYP3A4. Also, the promoter region of CYP3A7 exhibit 90% sequence identity with CYP3A4 and functional PXR response element was found in CYP3A7 promoter region(Bertilsson et al., 2001;Pascussi et al., 1999) as well as a distal functional xenobiotic responsive enhancer module (XREM), which is conserved with the XREM of CYP3A4(Bertilsson et al., 2001), indicating a close evolutionary distance. CYP3A7 was also observed to be inducible in cultured human adult hepatocytes(Greuet et al., 1996). These results indicate that CYP3A7 may have a similar induction mechanism as that of CYP3A4. However, a previous study reported a similar CYP3A7 ER6(Itoh et al., 1992) with one different nucleotide at the 3'-half site from that reported by Pascussi(Pascussi et al., 1999). The CYP3A7 ER6-Ito element could not bind with PXR:RXR complex(Blumberg et al., 1998;Pascussi et al., 1999) while the CYP3A7 ER6-JMP element was observed to bind with PXR:RXR efficiently. Besides, there was one out of total three human adult liver samples in which CYP3A7 was not induced(Greuet et al., 1996). Considering all the observations above, we suspect that there might be a polymorphism in CYP3A7 promoter region, which may lead to different outcomes on the inducibility of CYP3A7. Furthermore, the affinity of the PXR:RXR heterodimer was found greater for CYP3A4 ER6 compared with its affinity for CYP3A7 ER6-JMP(Pascussi et al., 1999), which indicated that the PXR-mediated induction of CYP3A could preferentially favour that of CYP3A4. Concerning the relative abundance of CYP3A7 and the lower affinity of its ER6 to PXR:RXR heterodimer, we conclude that CYP3A7 is not significantly induced in HepG2 cells. It has been observed that a switch
from CYP3A7 to CYP3A4 occurs in the neonatal period with unknown reasons. With the observation we found here, we suspected that postnatal initiation of oral feeding might play a role in this enzyme switching.

The total expression level of CYP3A was observed not significantly increased by formula treatments or DEX in HepG2 cells (Figure 3.20). It is not surprising since the major isoform of CYP3A is CYP3A7 in HepG2 cells, which is a reflection of a disturbed regulation of CYP3A subfamily in this carcinoma cell line.

PXR mRNA was not induced by human milk, formula or DEX in HepG2 cells. It has been reported that DEX induces CYP3A4 through activating glucocorticoid receptor (GR) and PXR (El Sankary et al., 2000) and it also induces PXR and RXR mRNA expression in a human primary cell culture environment (Pascussi et al., 2000a), the heterodimer of which binds to ER6 in the promoter region of CYP3A4, up-regulating the transcription of CYP3A4 mRNA. These studies had some contradictory findings. The study of cotransfection of GR and PXR revealed that functionally, there was no additive or synergistic effect between the GR- or PXR-mediated CYP3A induction pathway in HepG2 cells (El Sankary et al., 2000). However, in a primary hepatocyte culture system, DEX induced PXR expression and thus was claimed to account for a synergistic effect of DEX and PXR activators on CYP3A induction (Pascussi et al., 2000a). A possible interpretation of this result is that this inductive effect is an unspecific effect that reflects the natural homeostatic function of corticosteroid in maintaining the differentiated phenotype rather than a pharmacological “induction” especially when the authors used a concentration (100 nM) close to the physiological concentration (200 nM) (Williams D
and Marks V, 1994) while its ED$_{50}$ is 10 $\mu$M as an activator of PXR(Lehmann et al., 1998).

We did not observe any significant alteration of AHR by the treatments of human milk, formula or DBA, which is in agreement with previous studies(Giannone et al., 1998; Li et al., 1998).

Although formula induced CYP3A4 and CYP1A2, the total level of PXR and AHR was not altered by such treatments, indicating the changes of CYP3A4 and CYP1A2 was specific.

4.4. Milk Studies in C2BBel Cells

Intestine is the location where the absorption of milk occurs and it is also an enzymatic barrier where the types of the presence of cytochrome P450s influences bioavailability of oral drugs. For this purpose, we chose C2BBel cells as an in vitro model for intestine.

4.4.1 Effects of Human milk and Formula on the Expression of CYP3A4 and CYP1A2

The human milk studies revealed two different patterns of induction of CYP3A4 in C2BBel cells: 1) one human milk sample obtained from a mother of a term neonate demonstrated a 6-fold induction of CYP3A4 mRNA. Western blotting from this result was also demonstrated an increase in CYP3A level; 2) other three milk samples obtained from a breastfeeding clinic demonstrated a 1.4±0.1-fold induction. However, formula did
not have any significant inductive effect on CYP3A4 in C2BBel cells (Figure 3.24). These results, combined with results in HepG2 cells, suggest that: 1) there might be an inter-individual variation in the composition of human milk; 2) human milk might alter the bioavailability of oral drugs by inducing CYP3A4 in intestine; 3) there might be a tissue-specific response to diets. Although the mechanism of these effects is not clear at this stage, this differential influence of milk diets on the cytochrome P450 expressions in cells from two major digestive tissues provides valuable information on the milk-drug interaction.

As we demonstrated above, DBA failed to induce CYP1A2 in C2BBel cells after 72 hours. Thus, adding the observations that CYP1A2 was not expressed in normal human intestine(Zhang et al., 1999b), we considered that C2BBel resembled normal human intestine tissue in term of its CYP1A2 profile. However, to rule out the possibility that milk exert effects by pathways different from DBA, we examined the level of CYP1A2 expression after the treatment of human milk or formula for 72 hours. The result indicated that there was no induction of CYP1A2 mRNA.

4.4.2 Effects of Human milk and Formula on the Expression of CYP3A7, CYP3A, PXR and AHR

Interestingly, we observed that all three milk treatments significantly induced CYP3A7 mRNA. DEX was also observed to have an inductive effect on CYP3A7 mRNA with 3.4-fold induction. The different patterns of CYP3A7 induction between C2BBel and HepG2 cells might be due to the difference in basal expression of CYP3A7 in two cell lines. CYP3A7 is the predominant isoform of CYP3A in HepG2 cells (Figure
3.12) while its level in C2BBel cells is relatively low and comparable to that in adult human liver (Figure 3.13). As described above, we observed some increase in CYP3A7 level in HepG2 cells without any statistical significance. Thus, a small amount of increase of CYP3A7 from these two cell lines can demonstrate a significant difference since the fold induction must be based on its level of constitutive expression. However, by far, we cannot quantify the absolute quantity of a gene expression with the available experimental facilities. The other possibility is that there might be a difference in the CYP3A7 promoter region between this two cell lines, which can determine the affinity to PXR:RXR complex and the inducibility.

We further examined the level of PXR after these treatments. As shown in Figure 3.14, the level of PXR expression in C2BBel cells is lower than that in HepG2 cells. PXR mRNA expression was significantly increased only in the formula groups with 2.2±0.4-fold (Similac) and 1.7±0.1-fold (Enfalac) but not in the rest of groups. The mechanism of this induction is unknown since we did not find any induction of CYP3A4 by these formulas in C2BBel cells. Interestingly, it was found that inducers of PXR was not necessarily CYP3A inducers, such as two non-CYP3A inducers, isoniazide and perfluorodecanoic acid(Zhang et al., 1999a).

The total CYP3A level was not altered (Figure 3.26), which indicated that the induction of CYP3A4 by human milk did not significantly alter the total level of CYP3A, which might be attributed to the disturbed regulation of CYP3A subfamily in this carcinoma cell line, as shown by the low level of CYP3A4, the adult isoform of CYP3A. We did not observe any significant alteration of AHR mRNA by the treatments of human milk, formula or DBA after 72 hours.
CONCLUSION

In summary, results of this project revealed complex effects of human milk and formula. First of all, the mRNA of CYP3A4 and CYP1A2, two major enzymes responsible for the biotransformation of caffeine, were significantly induced in HepG2 cells by formula but not by human milk. In contrast, CYP3A4 mRNA was observed to be significantly induced in C2BBel cells by human milk but not by formula. These results indicate that human breast milk might be able to influence bioavailability of oral drugs by increasing the "first-pass" effect in intestine. In contrast, formula might be able to affect biotransformation rates of drugs as suggested by its effects on CYP3A4 and CYP1A2 in the hepatic model, HepG2 cells. These results provide insight into a developmental role of human milk and formula in the ontogeny of drug-metabolizing enzymes and are helpful in interpreting the clinical observation of increased half-life of caffeine in breast-fed premature infants, compared with formula-fed infants. Further in vivo experiments and functional assays are necessary to thoroughly explain this interesting phenomenon.
Reference List


35. Gervot, L. CYP3A5 is the major cytochrome P450 3A expressed in human colon and colonic cell lines. Environmental Toxicology and Pharmacology 2, 381-388. 1996.


