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Mechanisms of the Extreme Sensitivity of Turkeys to Aflatoxin B1

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ABSTRACT

Mechanisms of the Extreme Sensitivity of Turkeys to Aflatoxin B₁

by

Sumit Rawal, Doctor of Philosophy

Utah State University, 2010

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Department: Animal, Dairy and Veterinary Sciences

The pathogenesis of hepatotoxic and hepatocarcinogenic actions of the mycotoxin aflatoxin B₁ (AFB₁) involves initial bioactivation by microsomal cytochrome P450s (P450) to a reactive and electrophilic intermediate, exo-aflatoxin B₁-8,9-epoxide (exo-AFBO). Poultry, especially turkeys, are extremely sensitive to AFB₁, a condition associated with efficient epoxidation by P450s. The purpose of this research was to 1) discover and characterize the P450s in turkey liver responsible for AFB₁ bioactivation, and 2) determine the relative importance of these P450s in turkey liver.

Initial investigations led to the discovery of CYP1A5. We then identified CYP3A37, a human CYP3A4 homologue from turkey liver, which along with CYP1A5 plays an important role in the bioactivation of AFB₁ to exo-AFBO. The E.
coli-expressed CYP3A37 possessed striking similarities to human CYP3A4, in terms of its catalytic activities and the kinetics of AFB$_1$ oxidation.

After the discovery of CYP3A37, further research evaluated its relative importance to CYP1A5, with respect to the epoxidation of AFB$_1$, to determine which of the homologues bioactivated relatively low “real world” AFB$_1$ concentrations, reflective of the potential dietary exposure. Using antibodies directed to both the enzymes as tools in immuno-inhibition experiments, we determined that CYP1A5 contributes to about 98% of the exo-AFBO formation at the low AFB$_1$ concentrations (0.1 µM), which led us to conclude that CYP1A5 is likely the dominant homologue involved in the extreme sensitivity of the turkeys to AFB$_1$. CYP3A37 also efficiently epoxidated AFB$_1$, but only at high concentrations of this mycotoxin, not likely to be achievable in turkey liver in vivo. Our research has helped shed light on the relative importance of CYP1A5 and CYP3A37 in the bioactivation of AFB$_1$ to the toxic exo-AFBO, and thus on the mechanisms of the extreme sensitivity of turkeys to AFB$_1$.

Given that AFB$_1$ is a ubiquitous component of corn-based poultry feed and contamination is practically unavoidable, we conducted further studies evaluating the chemopreventive action of probiotic bacteria, Lactobacillus, on AFB$_1$ toxicity in turkeys. Probiotic bacteria are known to bind AFB$_1$, thus reducing its bioavailability. A mix of probiotic bacteria provided protection against key endpoints of aflatoxicosis, like AFB$_1$-induced reduction in body and
liver weights. Our data demonstrate that *Lactobacillus* was protective against aflatoxicosis in turkeys, thus validating its use as a possible chemopreventive, thereby helping alleviate the significant annual losses to the poultry industry due to feed contamination by AFB$_1$. 

(161 pages)
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Sumit Rawal
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Aflatoxins

Aflatoxins (AF) are the naturally occurring mycotoxins, produced as secondary metabolites by the fungus *Aspergillus flavus*, *A. parasiticus*, and *A. nominus*. The name “aflatoxin” is derived from the first letter in *Aspergillus*, and the first three letters in *flavus* (Schoental, 1967). Structurally, AFs are difurocoumarin derivatives that fluoresce under ultraviolet light. Depending upon color of the fluorescence, AFs are divided into aflatoxin B$_1$ and B$_2$ (AFB$_1$, AFB$_2$) for blue, and G$_1$ and G$_2$ (AFG$_1$, AFG$_2$) for green (Hartley *et al.*, 1963; Dalvi, 1986) (Figure 1.1). Aflatoxin M$_1$ and M$_2$ (AFM$_1$, AFM$_2$), known as milk-AFs, are the metabolites of AFB$_1$ and AFB$_2$, respectively (Carnaghan *et al.*, 1963).

Aflatoxins are the most intensively researched group of mycotoxins, because of their demonstrated toxic and carcinogenic effects in susceptible laboratory animals and livestock and their acute toxicological and chronic hepatocarcinogenic effects in humans. Of the known AFs, AFB$_1$ is the most potent, and is a classified human carcinogen (Wogan *et al.*, 1974; Wong and Hsieh, 1976; Bondy and Pestka, 2000).
Toxicity of Aflatoxins

AFB\textsubscript{1} is toxic to a wide range of animal species. AFB\textsubscript{1} is principally an hepatotoxin and hepatocarcinogen, but it causes a myriad of other effects either directly or indirectly associated with this toxicity: immunosuppression, reduced growth rate, lowered milk and egg production, reduced reproductivity, reduced feed utilization, reduced feed efficiency, and anemia. AFB\textsubscript{1} has been shown to induce hepatocellular carcinoma in many species of animals including fish (rainbow trout, sockeye salmon, and guppy), poultry (turkeys, ducks, and geese), non-human primates (rhesus, cynomolgus, African green, and squirrel monkeys), and rodents (rats, mice, and tree shrews) (Wogan, 1992). A wide variation exists in the dose of AFs required to produce signs of liver cancer in different species. Fish and poultry, known to be extremely sensitive to AFB\textsubscript{1}, responded to doses as low as 15-30 ppb. Rats responded at concentrations of 15-1000 ppb, whereas mice showed no effects to concentrations as high as 150,000 ppb (Wogan, 1992). In rainbow trout, dietary AFB\textsubscript{1} concentrations of 20 ppb resulted in a liver tumor incidence of 62% (Bailey \textit{et al.}, 1988).

Primates showed a wide variability in AFB\textsubscript{1} susceptibility to hepatic tumors (Adamson, 1989). While squirrel monkeys developed liver cancer when fed AFB\textsubscript{1} at 2000 ppb for 13 months, much higher doses were required over a longer period of time to induce low incidence of liver carcinoma in rhesus, cynomolgus and African green monkeys (99 to 1,225 mg/animal administered
p.o. over periods of 48 to 179 months). However, AFB\textsubscript{1} induced tumors in extrahepatic tissues in the latter species.

Species susceptibility to various acute toxic manifestations, as measured by TD\textsubscript{50}, is likewise variable (Gold \textit{et al.}, 1984; Wogan, 1992). While Fisher rats appear to be extremely sensitive (TD\textsubscript{50}: 1.3 mg/kg/body weight/day), Swiss mice are highly resistant (TD\textsubscript{50}: >5300 mg/kg/body weight/day). Rhesus and cynomolgus monkeys dosed for an average of 3.3 and 14 years, respectively, yielded a TD\textsubscript{50} value of 156 and 848 mg/kg/body weight/day, respectively.

Acute aflatoxicosis in humans is manifested by vomiting, abdominal pain, pulmonary edema, coma, convulsions, and death with cerebral edema and fatty involvement of the liver, kidney, and heart (Strosnider \textit{et al.}, 2006). The occurrence of acute aflatoxicosis was evidenced by the severe outbreak in Kenya in 2004 (Probst \textit{et al.}, 2007). Epidemiological studies have consistently demonstrated that AFB\textsubscript{1} is a liver carcinogen in humans (Van Rensburg \textit{et al.}, 1985; Groopman \textit{et al.}, 1988). Studies conducted in Swaziland and Guangxi, China have linked AFB\textsubscript{1} exposure to development of liver cancer in humans (Peers \textit{et al.}, 1987; Yeh \textit{et al.}, 1989). The International Agency for Research on Cancer has concluded that there is sufficient evidence for the carcinogenicity of AFB\textsubscript{1} in humans and hence placed this mycotoxin under group I (confirmed carcinogen).
Aflatoxin B₁ is a “pro-carcinogen” in that enzymatic bioactivation is a prerequisite for carcinogenic (and toxic) activity (Garner et al., 1972). Accordingly, elucidation of the mechanisms of AFB₁ metabolism has been the focus of intense research over the years. AFB₁ is metabolized by hepatic microsomal cytochrome P450s (P450) to the reactive, electrophilic exo-AFB₁-8,9-epoxide (AFBO) which binds to DNA and other critical cellular macromolecules (Ball and Coulombe, 1991; Wogan, 1992; Coulombe, 1993; Gallagher et al., 1996; Guengerich et al., 1996a). The AFBO is highly unstable, and it reacts with DNA to form N⁷ guanine adducts by intercalation of AFBO between base pairs (Iyer et al., 1994).

In humans and most animals, the principal route of detoxification appears to be through conjugation with endogenous glutathione (GSH), a reaction catalyzed by glutathione-S-transferases (GSTs). There exists substantial amount of evidence that the conjugation of the epoxide by GSH is a major factor in determining the susceptibility of a species to AFB₁ (Hayes et al., 1991). For example, quail and rat are much more sensitive to AFB₁ toxicity and carcinogenicity than the more resistant mouse, yet all these animals exhibit high rates of P450-mediated AFBO formation. The relatively high rate of GSH conjugation by a constitutive alpha class GSTA3-3 appears to be the critical resistance factor in mice (Hayes et al., 1992). Resistance in the rat to AFB₁-induced hepatocarcinogenesis can by enhanced by induction of a similar GST in
liver which is constitutively expressed at low levels (Stresser et al., 1994). In non-human primates, evidence has been presented that GSTs in the mu-class, rather than alpha, are most efficient in conjugation of AFBO (Wang et al., 2000). The lack of a functional GST with affinity toward AFBO appears to be a major reason that poultry are extremely susceptible to AFB1 (Klein et al., 2000).

There are a number of urinary and serum biomarkers that have been validated to accurately predict AFB1 cancer risk in humans. Biomarkers for AF exposure include dose-dependent urinary excretion of aflatoxin-N7-guanine and AFM1 (Gan et al., 1988; Groopman et al., 1992; Groopman and Kensler, 1999). Urinary excretion of mercapturic acid (AFB-NAC), a product of GSH adduction of AFB1 mediated by GSTs, has also been used in field studies (Wang et al., 1999). Serum AF-albumin adducts, which are positively associated with hepatocellular carcinoma in humans (Wang et al., 1996) has found wide use in epidemiologic studies (Wild and Turner, 2001). Analysis of serum adducts indicates a positive correlation between dietary AFB1 exposure and serum AFB-albumin adducts (Gan et al., 1988; Wild et al., 1992).

Aflatoxin B1 toxicity in Poultry

Poultry, especially turkeys, are extremely sensitive to the toxic effects of AFB1 (Carnaghan et al., 1966; Arafa et al., 1981; Giambrone et al., 1985; Huff et al., 1986; Kubena et al., 1995; Klein et al., 2000). Extreme sensitivity of turkeys to AFB1 was first and graphically demonstrated by association with ‘Turkey X Disease’
which caused widespread deaths of turkeys and other poultry throughout Europe in the 1960s (Stevens et al., 1960). The disease was shown to be caused by AFB\textsubscript{1} contaminated feed (Smith, 1960). It was later reported that the contamination with AFs came from Brazilian peanut meal (Blount, 1961). Among different poultry species, turkeys were shown to be the most susceptible to AFs, quail are intermediate, while chickens are considered relatively resistant (Arafa et al., 1981; Lozano and Diaz, 2006). Although direct comparisons have not been conducted, wild turkeys appear to be less susceptible to AFB\textsubscript{1} than their commercial counterparts (Quist et al., 2000).

Dietary aflatoxins at 0.7 ppm reduced the growth rate of turkey poult, but had no effect in quails and chickens (Arafa et al., 1981). A diet containing 400 ppm AFB\textsubscript{1} severely affected body and relative liver weights in turkeys, while chickens showed no effect at this dietary concentration (Leeson et al., 1995). A study examining the effects of AFB\textsubscript{1} on the development of liver lesions in poult, indicated ducks as more susceptible than turkeys and chickens (Coker, 1979). In that study, ducks developed hepatic lesions by dietary exposure to 30 ppb, turkeys at 300 ppb, while chickens responded to 500 ppb. Another study evaluated the effects of AFB\textsubscript{1} on the development of cytopathology in the tracheal culture in day-old turkeys, Japanese quails, chicken and ducks (Colwell et al., 1973). While cultures derived from ducks developed pathology at 6 ppb of AFB\textsubscript{1}, the concentrations needed for equivalent pathology were as high as 100
ppb for those from chickens. Tracheal cultures from turkeys and quails responded to 28 and 47 ppb of AFB$_1$, respectively.

**Economic Losses to the Poultry Industry due to Aflatoxins**

Turkeys are an important international food commodity. The United States accounts for roughly one-half of the world’s turkey production at approximately 7.30 billion pounds, live weight, with an estimated value of nearly US $3 billion (National Agricultural Statistics Service, USDA). The per capita consumption of turkeys in the United States is approximately 18 pounds, and turkeys are now the 4th major food and protein source, behind chicken, beef and pork (National Turkey Federation).

Aflatoxins result in economic losses to the poultry industry from reductions in growth rate, hatchability, feed efficiency and immunity towards diseases (Richard *et al.*, 1986; Coulombe, 1993). According to a report by the Council for Agricultural Science and Technology, losses due to AFs to the US poultry industry exceeded $143 million annually (CAST, 1989). A recent study reported annual crop losses of $932 million due to mycotoxin contamination and additional losses of $466 million in efforts to prevent or reduce contamination (CAST, 2003). Although AFs are found in several feed ingredients, corn, peanut meal, cottonseed meal, and sorghum appear to be at greatest risk for introducing AFs in turkey diets (Pons and Goldblatt, 1965; Brekke *et al.*, 1977; Winn and Lane, 1978; Hill *et al.*, 1983). Crops contaminated with AFs are a worldwide problem.
and approximately 25% of world’s food supply is contaminated with mycotoxins (CAST, 1989). Conditions that favor contamination by mycotoxins include excessive moisture both in field and post harvest storage, high humidity, temperature extremes, drought stress and insect damage to crops (Coulombe, 1993). Aflatoxins are deleterious to poultry and their contamination in feed is practically unavoidable (Coulombe et al., 2005). The United States Food and Drug Administration regulates the amount of AFB$_1$ allowed in poultry feed. The current action level for corn and peanut products is 100 ppb and for cottonseed meal 300 ppb.

Consumption of AFB$_1$ contaminated feed results in poor performance, decreased body and organ weights, immunosuppression, morbidity, and mortality in turkeys (Kubena et al., 1990, 1991; Coulombe, 1993). Aflatoxins significantly affect feed consumption, total plasmatic proteins and cholesterol amounts of turkeys (Rauber et al., 2007). Furthermore, AFs lead to irreversible liver damage as indicated by decreased liver-to-body weight ratios, liver enzyme alterations, altered blood coagulation patterns, and histologic changes like hepatocellular necrosis and biliary hyperplasia in turkeys (Quist et al., 2000; Klein et al., 2002b). Dietary exposure of broiler hens to AF (10 ppm) resulted in embryonic mortality and lowered the immunity in the progeny chicks (Qureshi et al., 1998). Embryonic exposure with AFs resulted in long-term depression of the immune function in chicks (Neldon-Ortiz and Qureshi, 1992). As in other
species, the liver is the most severely affected organ in poultry, primary consequences being hepatic necrosis and neoplasias (Klein et al., 2000).

**AFB$_1$ Metabolism in Poultry: Role of Cytochrome P450s**

Cytochrome P450s are mixed-function oxidases that catalyze the biotransformation of a wide variety of xenobiotics. They are a superfamily of hemoproteins that aid in the oxidation of various substrates such as steroids, eicosanoids, pharmaceuticals, pesticides, pollutants, and carcinogens (Parikh et al., 1997). Cytochrome P450s play an important role in the formation of carcinogenic and mutagenic electrophilic intermediates from naturally occurring dietary compounds (Guengerich et al., 1996a). As mentioned previously, AFB$_1$ is not toxic *per se*, but requires metabolic conversion to the reactive and electrophilic exo-AFBO by P450s to exert its toxic effects (Ball and Coulombe, 1991; Coulombe, 1993; Gallagher et al., 1996; Guengerich et al., 1996a). This metabolite can induce mutations by alkylating DNA, principally at the N$^7$ position of guanine forming the 8,9-dihydro-8-(N$^7$-guanyl)-9-hydroxy-AFB$_1$ (Lin et al., 1977). In addition AFBO can bind to proteins and other critical cellular nucleophiles.

Turkey liver P450s are especially efficient toward AFB$_1$ bioactivation compared to other poultry species thus far examined. Turkey liver microsomes bioactivated AFs 1.8 and 3.5 times more than liver microsomes from quail and chicken, respectively (Lozano and Diaz, 2006). When comparing livers obtained
from 9, 45 and 61 day old turkeys, microsomes from younger were more active
toward AFB₁ bioactivation than that from older birds (Klein et al., 2002a).

In turkey liver, AFB₁ metabolism is mediated by homologues to human
CYP1A2 and CYP3A4 (Klein et al., 2000; Klein et al., 2003; Yip and Coulombe,
2006). Initial studies reported that concentrations of AFB₁ which are likely to be
achieved in the liver following ingestion of “real-world” concentrations of AFB₁
are bioactivated to AFBO primarily by CYP1A2, whereas much higher
concentrations are catalyzed by CYP3A4 (Gallagher et al., 1996; Kelly et al., 1997;
Van Vleet et al., 2002). CYP1A homologues also metabolize AFB₁ to produce the
detoxified metabolite AFM₁, whereas CYP3A enzymes produce another
detoxified metabolite, aflatoxin Q₁ (AFQ₁) (Campbell and Hayes, 1976;
Guengerich et al., 1996a) (Figure 1.2).

Although both CYP1A and 3A isoforms oxidize AFB₁, there are conflicting
reports on their relative importance (Shimada and Guengerich, 1989; Ramsdell
and Eaton, 1990; Gallagher et al., 1994, 1996). A recent study demonstrated a
dominant contribution of CYP3A4 homologues in AFBO production. Aflatoxin
B₁ metabolism studies in human liver microsomal preparations indicate a
predominant role for CYP3A4 and that its expression level was an important
determinant of the AFB₁ disposition in human liver (Kamdem et al., 2006).
Specific CYP3A4 inhibitors like troleandomycin have been shown to inhibit
AFBO production (Gallagher et al., 1994), while inducers of CYP3A4 activity such
as 3-methylcholanthrene and rifampicin, increase AFB\textsubscript{1} metabolism in cultured human hepatocytes (Langouet \textit{et al.}, 1995).

Although mammalian P450s are well described, limited information is available on P450s in poultry, especially in the context of AFB\textsubscript{1} metabolism. Chicken P450s, CYP1A5 (Gilday \textit{et al.}, 1996) and CYP3A37 (Ourlin \textit{et al.}, 2000) have been described. This laboratory recently cloned, expressed and characterized CYP1A5 from turkey liver, the first functional protein amplified from turkeys (Yip and Coulombe, 2006). CYP1A5 is predicted to be 528 amino acids with 94.7% sequence identity to chicken CYP1A5. Like its human homologue, the \textit{E. coli}-expressed CYP1A5 efficiently bioactivated AFB\textsubscript{1} to AFBO, and in addition, produced AFM\textsubscript{1}. Recombinant P450 heterologously expressed in \textit{E. coli} proved to be very useful for the studies of turkey CYP1A5. After the discovery of a CYP1A homologue, the next task was to identify the existence of possible CYP3A4 homologue involved in AFB\textsubscript{1} oxidation and determine its relative importance compared to CYP1A5.

An important detoxification mechanism for AFB\textsubscript{1} is the GST catalyzed conjugation of AFBO with GSH (Hayes \textit{et al.}, 1991). Detoxification mediated by GSTs plays a major role in species susceptibility to AFs (Coulombe, 1993). Commercial turkeys appear to be deficient in this detoxification mechanism which is an important reason for their extreme sensitivity to AFB\textsubscript{1} (Klein \textit{et al.}, 2000). Therefore, the extreme sensitivity of turkeys to AFB\textsubscript{1}, is, in part, due to a
combination of efficient oxidation by P450s and deficient detoxification by GSTs (Klein et al., 2000; Yip and Coulombe, 2006) (Figure 1.2).

**Recombinant P450 systems to study AFB₁ Biotransformation**

The study of P450s has been greatly facilitated by their expression in heterologous systems, especially *E. coli*. The past two or three decades have seen a tremendous growth in the engineering of recombinant P450s to study their xenobiotic metabolism activity. Several expression hosts have been developed: yeast (Murakami et al., 1987), mammalian cells (Crespi et al., 1991), baculovirus/insect cells (Buters et al., 1994) and bacteria (*E. coli*). *Escherichia coli* became a popular choice for the heterologous expression of P450s. The technique started with the expression of P450 in *E. coli* through N-terminal modifications (Barnes et al., 1991). Although this technique produced significant expression levels for P450s (16 mg of spectrally detectable bovine P45017α per liter of culture) (Barnes et al., 1991), the study of drug metabolism was often limited due to the lack of endogenous electron donors NADPH-P450 reductase (NPR). While *E. coli* contains flavoproteins which could couple to some degree with P450 (Jenkins and Waterman, 1994), the rate of catalysis was often slow. To overcome this limitation P450s were co-expressed with NPR in *E. coli* (through bicistronic expression or the use of fusion proteins) producing independent functionally relevant P450 monooxygenase systems (Dong and Porter, 1996; Parikh et al.,
1997). Thus, several P450s have been co-expressed with their partner reductase successfully in *E. coli* (Parikh et al., 1997).

**N-terminal modifications of native cDNAs:** P450s were difficult to express in their native form due to their hydrophobicity. Generally P450s require alterations in the hydrophobic N-terminal nucleotide sequence (5’ end) to facilitate translation by *E. coli* (Barnes et al., 1991). This was due to the fact that N-terminal sequences serves as signal for membrane anchoring but plays no role in the catalytic activity of P450 or its interactions with the coupling enzyme. Initially, Barnes and coworkers (Barnes et al., 1991) were successful in the expression of bovine P45017α1 by modifying N-terminal sequences. Specifically, the second codon was changed to GCT (Ala) which encodes the LacZ gene, and codons 4 and 5 were changed to increase the AT content by silent mutagenesis. This reduced the free energy for base pairing and inhibited the formation of secondary structures in the mRNA which could inhibit translation (Guengerich et al., 1996a). This pioneering work led to abundant (16 mg of spectrally detectable P450 per liter of culture) bovine P45017α1 expression levels in *E. coli* (Barnes et al., 1991). Subsequently, several other P450s were expressed by modifying and/or truncating the N-terminal sequences utilizing the above mentioned strategy or using a different one (Fisher et al., 1992a, 1992b; Guo et al., 1994; Sandhu et al., 1994). The majority of the methods for heterologously expressed P450s follow a general procedure involving N-terminal sequence
alterations. Native P450s usually were expressed in insignificant amounts (Barnes et al., 1991; Dong and Porter, 1996). Another strategy of N-terminal alteration is the fusion of a bacterial ompA (outer membrane protein A) leader sequence (21 amino acids) and two additional spacer amino acids (Ala-Pro) to P450 cDNAs in-frame with P450 initiation codon. This (Pritchard et al., 1997) approach does not involve changes in the native P450 sequence and the leader sequence is removed during bacterial synthesis releasing native P450.

Another 5’ end modification is the introduction of a restriction site for cloning in a vector {usually NdeI for cloning in pCW vector (Barnes et al., 1991)}. Modifications at the 3’ end usually involve introduction of 4-6 hisitidine residues to facilitate purification of P450s by affinity Ni2+-NTA chromatography (Crowe et al., 1994) and also to include a restriction site.

N-terminal modifications have been shown to not alter the catalytic activities of the P450s, because of their prominent role in the membrane localization (Barnes et al., 1991). These peptides do not participate in formation of the active sites, which interact with the substrates. Loss of the catalytic activities of the heterologously expressed P450s were mostly associated with the lack of endogenous NPR and the necessary transfer of the electrons. Bicistronic vectors, discussed below, overcame this difficulty by producing independent functionally relevant P450s.
Expression Vector: The pCW vector (Figure 1.3) developed in the laboratory of Prof. F. W. Dahlquist has been used extensively to express modified P450 cDNAs (Gegner and Dahlquist, 1991). pCW functions by two tac promoter (inducible by isopropyl-thiogalactoside:IPTG) cassettes upstream of an NdeI (CATATG) restriction enzyme cloning site coincident with the initiation ATG codon. This vector contains a strong transcription termination mechanism and a phage M13 origin of DNA replication. Transcription prior to the addition of inducing agents is inhibited by LacI gene encoding a lac repressor molecule. pCW can also function as a bicistronic vector to co-express P450 and NADPH-P450 reductase (Parikh et al., 1997). Bicistronic operons consisting of P450 cDNA as the first cistron followed by NADPH-P450 reductase cDNA as the second cistron can be ligated into the pCW vector multicloning site (Parikh et al., 1997). The use of special restriction sites facilitates the process of subcloning in the pCW vector. This bicistronic vector proved to be extremely useful for studying the biotransformation of xenobiotics with P450s since it represented the fully functional P450 monooxygenase system in whole cells or isolated membranes. The presence of the reductase ensured that the electron transfer to P450 is not rate-limiting as it was previously for just P450 expressed vectors. Dong and Porter (1996) also produced a bicistronic vector pJL2E1/OR for expressing P4502E1 with rat NPR. They employed a different technique which involved no
modification of the N-terminal sequence. However, P450 yield was not significant which limited the catalytic studies.

Another approach of producing a fully functional P450 monooxygenase system comes with the co-expression of P450 with reductase as a fusion protein (Fisher et al., 1992b; Shet et al., 1993) like the one present in naturally occurring P450 BM3 (from Bacillus megaterium). The rationale for this approach is the mutagenesis enabled alteration of C-terminal of P450 and N-terminal of reductase to allow the fusion of the two sequences with a dipeptide linker, Ser-Thr. These fusion proteins exhibited catalytic activity only in presence of phospholipids and no apparent improvement in the activity was seen with their use.

Because of its advantages mentioned above we chose bicistronic pCW based vectors for expression in E. coli. Vectors other than pCW are also used for P450 expressions, including pBSIISK (Stratagene), pSE420 (Invitrogen), pSE380 (Invitrogen), etc.

Expression in E. coli: Several strains of E. coli are commercially available: such as JM109 (Invitrogen, Stratagene), XL 1-Blue (Stratagene), TOPP3 (Invitrogen, Stratagene), and DH5α (Invitrogen). Co-expression of P450 and reductase in a bicistronic vector appears to be most efficient in DH5α (Parikh et al., 1997; Yip and Coulombe, 2006). Expression constructs are transformed in competent E. coli cells and streaked on Luria-Bertani (LB) agar/ampicillin plates,
and allowed to grow at 37°C overnight. Starter culture consists of LB-ampicillin medium inoculated with a single colony, and grown at 37°C with shaking. Terrific broth (TB) medium has been extensively used for growing bacterial cultures containing recombinant plasmids. It is usually supplemented with thiamine, trace elements, ampicillin (50-100 μg/ml), heme precursor δ-aminolevulinic acid (δ-LA) (0.5 mM), and an inducer of transcription IPTG (1mM). Cells are grown usually for 28-48 hr at 28-32°C. While some studies indicate addition of IPTG at the start of TB culture (Guengerich et al., 1996b), others have chosen to add IPTG after the cells in main culture in TB medium have grown to an ODodor 600 of 0.3-0.5 at 37°C (Jenkins et al., 1998; Yip and Coulombe, 2006). Addition of δ-ALA seems to increase P450 expression in some cases (Gillam et al., 1995). Expressed P450s can be quantified in whole cells by CO difference spectra, which is absorbance difference at 450 nm between reduced hemoprotein (Fe2+) and the reduced hemoprotein bound to CO (Fe2+.CO) (Omura and Sato, 1964).

**Purification of P450 from membranes:** Guengerich and coworkers (Guengerich et al., 1996b), described a detailed method for the purification of recombinant P450s expressed in *E. coli*. Bacterial cells in culture are harvested and treated with lysozyme to prepare spheroplasts. Spheroplasts lysed in the presence of protease inhibitors like phenylmethylsulfonyl fluoride (PMSF) gives
rise to a lysate which is subjected to ultra centrifugation for 60 min at 4°C to separate the membrane and cytosolic fraction.

Purification of P450 from the *E. coli* membranes makes use of the oligo-His region at the C-terminal end Ni²⁺-NTA affinity chromatography (Crowe *et al.*, 1994). Membranes were first solubilized with surfactants/detergents like sodium cholate, Triton X-114, CHAPS etc., before being subjected to chromatography (via a combination of ion-exchange and affinity or just affinity) to purify enzyme expressed in *E. coli* membranes (Hosea *et al.*, 2000).

**Chemoprevention of Aflatoxicosis in Poultry**

The National Cancer Institute defines chemoprevention as the use of naturally-occurring or synthetic agents to reduce the risk of, or delay the development or recurrence of, cancer. Given that AFB₁ contamination in the feed is nearly universal, and therefore practically unavoidable (Coulombe *et al.*, 2005), chemoprevention strategies aimed at reducing AFB₁ toxicity in poultry and in other animals have been the subject of numerous studies (Klein *et al.*, 2002b, 2003; Guarisco *et al.*, 2008a, 2008b). Several chemopreventives have been evaluated in poultry for reducing symptoms of aflatoxicosis. Indeed, because of their sensitivity, poultry have been used as models in the search for novel AFB₁ chemopreventives.

Clay-based inorganic adsorbents, which prevent absorption of AFB₁ into the general circulation, hence reducing bioavailability, have been extensively
studied. In broiler chicks, adsorbents like calcium montmorillonite clay (0.5, 0.25, and 0.125% in diet), and Zeolites (1% in diet), which bind to AFB\textsubscript{1} preventing their absorption, have been shown to be protective against high AFB\textsubscript{1} exposure of 5 and 2.5 ppm, respectively (Miazzo \textit{et al.}, 2000; Pimpukdee \textit{et al.}, 2004). Hydrated sodium calcium aluminosilicate (HSCAS), fed at dietary concentrations ranging from 0.25-1%, has been shown to diminish the deleterious effects of AF (up to 5 ppm in diet) in broiler chicks (Kubena \textit{et al.}, 1993, 1998; Ledoux \textit{et al.}, 1999). Another adsorbent, clinoptilolite (15 g/kg), provided moderate amelioration in AF associated liver toxicity (hydropic degeneration and biliary hyperplasia) in broiler chicks (Ortatatli \textit{et al.}, 2005).

Detoxification of contaminated feed with microorganisms has been tested as a strategy to reduce the AF-associated toxicity. In a recent study, the detoxification potential of \textit{Nocardia corynebacteroides} (NC), as a feed additive, was evaluated in day-old Ross 308 chicks; NC significantly reduced the AF-associated lesion severity in liver, duodenum and kidneys (Tejada-Castaneda \textit{et al.}, 2008). Another report indicated the protective effect of Silymarin, a potent hepatoprotective in humans, against aflatoxicosis in broiler chicks. Although the mechanism of protection from Silymarin is uncertain, reports suggest that it acts as an antioxidant and cell membrane stabilizer and promotes the cellular synthesis of macromolecules (Tedesco \textit{et al.}, 2004). Dietary administration of a Silymarin-phospholipid complex (600 mg/kg of body weight) resulted in
significant improvement in weight gain and feed intake in broiler chicks fed AFB\(_1\) (0.8 ppm) in the diet (Tedesco et al., 2004). In addition, vitamin D and water soluble vitamins like A, E and K are shown to be protective against aflatoxicosis in quail chicks (Wilson et al., 1975, 1978).

The food antioxidant butylated hydroxytoluene (BHT) has been shown in numerous studies to reduce symptoms of aflatoxicosis in mammalian and avian models. In rodent models, BHT has been shown to reduce the development of cancer by either induction of phase II enzymes like GSTs or inhibition of phase I enzymes like P450s (Hocman, 1988; Singletary, 1990). Weight loss in chickens due to AFB\(_1\) (3000 ppb in diet) was shown to be dramatically improved by dietary BHT at a concentration of 0.39\% (Larsen et al., 1985). Likewise, dietary BHT protects against aflatoxicosis in turkeys. A diet containing 4000 ppm BHT provided significant protection against AFB\(_1\)-induced weight loss, hepatocellular necrosis, biliary hyperplasia and changes in serum enzymes indicative of hepatotoxicity, in turkeys (Klein et al., 2002b). Mechanism studies later revealed that while BHT induced GST expression and activity, none of the GSTs so induced had affinity toward AFBO (Klein et al., 2003). The protective properties of BHT appeared to be due to an inhibitory effect on the activity of P450 1A1 and 1A2, suggesting reduced bioactivation of AFB\(_1\) to the AFBO (Klein et al., 2003). Further studies revealed that dietary BHT reduced AFBO formation, as well as other CYP1A5-mediated activities (Guarisco et al., 2008b). Importantly, BHT
inhibited conversion of AFB$_1$ to AFBO, exhibiting Michaelis-Menton competitive inhibition kinetics ($K_i$=0.81 µM). Inhibition of AFBO formation was found in microsomes prepared from the birds fed 4000 ppm BHT, as well as in microsomes treated with BHT *in vitro*. Moreover, BHT (4000 ppm in the diet for 10 days) caused significant reductions in AFB$_1$ bioavailability, AFB$_1$–DNA adduct formation in the liver, and AFB$_1$ residues in tissues in turkeys (Guarisco *et al.*, 2008a).

A number of recent studies have demonstrated that probiotic bacteria offers protection against AFB$_1$ in humans and animals. The mechanism of protection is through binding of AFB$_1$ by cell wall constituents of probiotic bacteria, such as *Lactobacillus rhamnosus*. Because of its use in various dairy products including yogurt, *L. rhamnosus* is considered a safe and effective chemopreventive. *Lactobacillus rhamnosus* strain GG and LC-705 were found to be most efficient in binding a range of mycotoxins including AF (El-Nezami *et al.*, 1998, 2002a, 2002b; Haskard *et al.*, 2001; Peltonen *et al.*, 2001). In addition, *L. rhamnosus* reduced AFB$_1$ transport, metabolism, and toxicity in cultured Caco-2 cells (Gratz *et al.*, 2007). Furthermore, *L. rhamnosus* reduced AFB$_1$ bioavailability in rats, thereby decreasing its toxicity (Gratz *et al.*, 2006). Thus, there exists substantial evidence of the protective role of probiotics in preventing aflatoxicosis. Given the potential of probiotic bacteria in binding AFB$_1$, the
present study aims to evaluate their chemopreventive action on AFB₁ toxicity in turkeys, a species extremely susceptible to aflatoxicosis.

**Research Goals**

Given the importance of P450s in the metabolic activation of AFB₁, leading to its toxicity, the aim of this research was to discover and characterize the P450 homologues involved in the bioactivation of AFB₁ in turkey liver and to determine their relative importance. Given that AFB₁ is nearly ubiquitous in corn-based poultry feed and contamination is practically unavoidable, another goal was to evaluate the chemopreventive action of *Lactobacillus* on AFB₁ toxicity in turkeys.

**References**


Figure 1.1. Chemical structures of major aflatoxins
Figure 1.2. Metabolism of AFB₁: role of P450s and GSTs. The extreme sensitivity of turkeys to AFB₁ is associated with efficient AFB₁ epoxidation catalyzed by CYP1A5 and CYP3A37 coupled with deficient GST detoxification. The hydroxylated metabolites, AFM₁ and AFQ₁ are formed by CYP1A5 and CYP3A37, respectively.
Figure 1.3. A pCW based bicistronic vector for co-expression of P450 with human NADPH P450 reductase (adapted from Gillam and Guengerich, 2001). The P450 and reductase cDNAs are inserted in series in a single expression cassette under the control of tandem tac promoters. Each cDNA is optimally positioned with a ribosomal binding site. The operons are driven by IPTG inducible double tac promoters placed upstream of first cistron.
CHAPTER 2

STRUCTURE, GENETIC MAPPING, AND FUNCTION OF THE CYTOCHROME P450 3A37 GENE IN THE TURKEY (MELEAGRIS GALLOPAVO)²

Abstract

Cytochromes P450 (P450 for protein; CYP for gene) are a superfamily of membrane-bound hemoproteins that oxidize a large number of endogenous and exogenous compounds. Through oxidation reactions, these enzymes are often responsible for the toxic and carcinogenic effects of natural food-borne toxicants, such as the mycotoxin aflatoxin B₁ (AFB₁). Previous studies in our laboratory have shown that the extreme sensitivity of turkeys to AFB₁ is in part explained by efficient hepatic P450-mediated epoxidation to the toxic and reactive metabolite, exo-AFB₁-8,9-epoxide (AFBO). Using 3′-5′-rapid amplification of cDNA ends (RACE), we amplified CYP3A37 from turkey liver RNA, the E. coli-expressed protein which efficiently epoxidates AFB₁. Turkey CYP3A37 has an ORF of 1512 bp, and the protein is predicted to be 504 amino acids with 97% homology to chicken CYP3A37. The turkey gene is organized into 13 exons and 12 introns. A single nucleotide polymorphism in the 11th intron was used to assign CYP3A37 to turkey linkage group 10 (corresponding to chicken

chromosome 14, GGA14). Because of the important role of P450s in the extreme sensitivity of turkeys to the toxic effects of AFB₁, this study will contribute to the identifying allelic variants of this important gene in poultry.

Introduction

Cytochromes P450 ("P450" for protein; CYP for gene) are a superfamily of hemoproteins that catalyze a large number of endogenous and exogenous compounds. Of particular relevance to toxicology, P450s are known to oxidize hundreds of environmental chemicals to form reactive intermediates that are toxic and carcinogenic. One good example is the carcinogenic mycotoxin aflatoxin B₁ that requires P450 oxidation at the 8,9 vinyl bond to produce the reactive and electrophilic exo-AFB₁-8,9-epoxide (AFBO) to exert its toxic and carcinogenic properties (Van Vleet et al., 2002). The critical lesion responsible for these properties is the 8,9-dihydro-8-(N7-guanyl)-9-hydroxy-AFB₁ adduct formed when the AFBO reacts with DNA (Iyer et al., 1994). Much of the observed interspecies variability in susceptibility to AFB₁ can be attributed to differences in AFB₁ biotransformation (Eaton and Gallagher, 1994). Aflatoxin B₁ is a recognized hepatocarcinogen and hepatotoxicant in humans and animals. In humans, AFB₁ is epoxidated principally by P4503A4 (the major P450 form in the liver), and to a lesser extent by P4501A2 (Kamdem et al., 2006).

Turkeys are extremely sensitive to the toxic effects of AFB₁ (Coulombe, 1993; Giambrone et al., 1985), a condition we have previously shown to be
associated with efficient hepatic P450-mediated epoxidation. As in humans, AFB₁ is epoxidated to AFBO by both P450 1A and 3A homologues in turkey liver (Klein et al., 2000). Aflatoxin B₁ was discovered in the early 1960s as the etiological agent of “Turkey X” disease, responsible for the widespread deaths of turkeys and other poultry throughout Europe due to contaminated Brazilian peanut meal in feed (Asao et al., 1965). We recently cloned and expressed P4501A5 from turkey liver, which like the human homologue, P4501A2, efficiently epoxidates AFB₁ to produce the exo-AFBO as well as the detoxified metabolite aflatoxin M₁ (Yip and Coulombe, 2006). Because of the importance of P4503A4 in the toxicity of AFB₁ in humans and animals, we sought to characterize the homologue of this enzyme in turkey liver. Here, we report the cloning, genetic mapping and biological activity of P4503A37 from turkey. Like P4503A4, the human homologue, the expressed protein from CYP3A37 efficiently epoxidates AFB₁ to form exo-AFBO.

Materials and Methods

**Gene amplification, sequencing, and genetic mapping.** Messenger RNAs from one-day-old male turkeys were amplified by 3’-5’ RACE as described (Yip and Coulombe, 2006). Primers used in 3’-RACE were designed based on ClustalW (www.ebi.ac.uk) sequence alignments of CYP3A4 in human and cattle, 3A4v2 in Cercopithecus aethiops (African green monkey), 3A44 in mouse, 3A66 in Macaca mulatta (rhesus monkey) and 3A45 in Onchorhynchus mykiss (rainbow
trout). The gene-specific forward primer sequence for 3′-RACE of turkey CYP3A37 was 5′-TTTGGAASTGGACCCAGRAACTGCATTGGC-3′. The gene-specific reverse primer for 5′-RACE was 5′-GCAACTTTTCAGAGTCAAGAGGCAAATCGC-3′. All reactions used a mixture of two universal primers (long primer: 5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′ and short primer: 5′-CTAATACGACTCACTATAGGGCC-3′) provided in the BD SMART RACE cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA). The full-length turkey CYP3A37 gene was cloned using forward and reverse primers designed from the sequence information obtained from 3′- and 5′-RACE (CYP3A37 full-length forward primer: 5′-CTGGGACAGATGGACAGAGAC-3′, reverse primer: 5′-GTACACCAAGAAGAAAGTCACCATCCATCTC-3′).

To identify segregating markers to genetically map CYP3A37 in the turkey genome the turkey mRNA (GenBank DQ450083) was aligned with the chicken whole genome sequence. Based on this alignment, primers were designed to amplify by PCR the intervening sequence between exons 11 and 12 of the CYP3A37 gene. Primer sequences were as follows; exon11_forward 5′-CCCAGAGGAGTTCAGACCAG-3′ and exon12_reverse 5′-CATCCCAATGCAGTTCCTG-3′. This fragment was amplified and sequenced from DNA pools representing the F1 generation of the UMN/NTBF mapping
families (Reed et al., 2003). Each DNA pool included three individuals; one sire and the two dams to which it had been crossed. Genotypes were then obtained for informative families of the UMN/NTBF mapping population by digestion of CYP3A37 intron 11 amplicons directly with restriction endonucleases followed by electrophoresis in 1% agarose (PCR/RFLP). Two-point linkage analysis of the genotypes for a SNP in intron 11 and markers already placed on the turkey linkage map (Reed et al., 2007b) was performed using Locusmap (Garbe and Da, 2003).

Analysis of biological activity. Bicistronic expression constructs consisting of the coding sequence of CYP3A37 (GenBank DQ450083) followed by that of human NADPH-P450 reductase (hNPR), the redox partner to P450, were constructed as described previously (Yip and Coulombe, 2006). Two variants of CYP3A37 were cloned: the wild-type sequence (wtCYP3A37) and an N-terminal truncated sequence (tCYP3A37). Deletion of hydrophobic N-terminal amino acids and other modifications have previously shown to increase the expression level of P450s in E. coli (Shimada et al., 1997), so we engineered the truncated sequence (tCYP3A37) by deleting 11 codons in this region. In addition, the nucleotide sequences of first eight codons were modified to enhance protein expression (Barnes et al., 1991). A 6X His tag was also added to facilitate immunodetection and purification. The expression constructs were created by digesting the original pCW1A2/hNPR vector with NdeI and XbaI to excise the
CYP1A2 coding sequence. The gel-purified vector fragment was ligated with the wild-type or truncated CYP3A37 NdeI- and XbaI-digested, PCR-amplified fragment using T4 DNA ligase. The resulting ligation mix was transformed into DH5αF’IQ cells. Individual clones were screened by colony PCR and diagnostic restriction digestion before being sequenced. Sequences in the entire reading frame of both constructs as well as the 5’- and 3’-junctions of the inserts to the vectors were verified before individual clones were used in expression. The final expression plasmids were transformed into DH5αF’IQ cells.

Heterologous expression of tCYP3A37 in E. coli (Jenkins et al., 1998) was carried out by inoculating 5 ml of Luria-Bertani ampicillin medium with a single colony of DH5αF’IQ E. coli transformants, which was then incubated for 23 hr with shaking (150 rpm) at 30 °C. Levels of P450 expression were determined by reduced CO/reduced difference spectrum (Genesys 6 UV-vis spectrophotometer, Thermo Spectronic, Rochester, NY). E. coli membranes and cytosol were then prepared for AFB1 metabolism experiments (Yip and Coulombe, 2006).

The oxidation activity toward AFB1 was determined by HPLC using conditions that we have described previously (Yip and Coulombe, 2006), which were optimized in preliminary experiments. Because of its short half-life, AFBO was indirectly measured as the glutathione (GSH)-trapped conjugate, which provides an accurate and quantitative measurement (Ramsdell and Eaton, 1990). E. coli membranes expressing tP4503A37 (0.307 μM), BHA-induced mouse
cytosol (~ 800 μg) as a source of glutathione S-transferase (GST), and 2-250 μM AFB₁ was added to the assay mixture as substrate. The reactions were incubated at 37°C for 20 min with gentle shaking and stopped by 250 μl of cold MeOH spiked with 24 μM aflatoxin G₁ (AFG₁) as an internal standard. Metabolite formation was calculated by establishing calibration curves between the peak areas in the chromatograms and the amounts of metabolites injected, using authentic AFBO-GSH HPLC standards. Kinetic data were analyzed by SigmaPlot Enzyme Kinetics Module software (Systat, San Jose, CA) and were fit using the Michaelis-Menten equation \( V = \frac{V_{\text{max}} [S]}{[S] + K_m} \) and nonlinear regression analysis to calculate the kinetic constants \( V_{\text{max}} \) and \( K_m \).

**Results**

**Sequence and structure of CYP3A37.** Sequence analysis of clones that covered the entire reading frame of CYP3A37 revealed a sequence of 1512 bp coding for 504 amino acids (Figure 2.1). Like the human homologue, the turkey CYP3A37 gene is organized into 13 exons and 12 introns. The 13 exons of the turkey gene were 124, 94, 53, 100, 114, 89, 149, 128, 73, 161, 227, 161 and 477 bases in length. Exons 1 and 13 contain 71 and 93 coding nucleotides, respectively. Our previous examination of CYP1A5 (Reed et al., 2007a) found identical gene structure between the turkey and chicken homologues. Alignment of the turkey CYP3A37 mRNA with the chicken whole genome sequence (release 2.1) indicates introns of 2561, 312, 1080, 477, 255, 718, 447, 691, 162, 429, 454, and 120 bases in
length (Figure 2.2). Alignment of the turkey mRNA sequence with that of the chicken (NM_001001751, Figure 2.3) indicated 94.2% nucleic acid sequence similarity. Most notably, the turkey CYP3A37 gene was found to be 4 amino acids shorter than the chicken homologue due to a 12 bp insertion/deletion in exon 9 (Figure 2.1). In addition to this length difference, 36 amino acid substitutions are predicted between chicken and turkey, with 92.8% similarity.

Genetic mapping of CYP3A37. Intron-containing fragments totaling 560 bp were sequenced from the turkey DNA pools (Genbank EU216548). Sequence similarity of CYP3A37 intron 11 between the turkey and chicken was 92.4%. A single G > A SNP at position 256 of the 454 bp intron was identified in the F1 individuals of the UMN/NTBF mapping population. This polymorphism occurred within the recognition sequence of the restriction endonuclease Bsl I (5'-CCNNNNN▼NGG-3') and this enzyme was used for PCR/RFLP genotyping of the mapping families. Genotypes at the CYP3A37 SNP (256G>A) were combined with the markers in the current UMN database (Reed et al., 2007b). Linkage analysis of the SNP genotypes (83 informative meioses) revealed significant linkage (two-point LOD score >3.0) between the CYP3A37 SNP and a microsatellite marker in turkey linkage group M10 (corresponding to chicken chromosome 14, GGA14). Distance between CYP3A37 and MNT323 was 9.9 cM (LOD 13.04, Figure 2.3).
**Activity of E. coli-expressed CYP3A37.** Because of their strong hydrophobicity in the N-terminal region, wild-type eukaryotic P450s without N-terminal modifications are often not expressed in *E. coli* (Guo et al., 1994), and wtCYP3A37 was no exception to this observation. Our approach of producing the truncated tCYP3A37 resulted in a relatively high level of protein expression, with 300-400 nmol tCYP3A37 per liter of culture, as estimated by the reduced CO/reduced difference spectrum of whole cells. After membrane and cytosol isolation, approximately 27% of the tCYP3A37 was recovered in the membrane fraction, and 20-40% was in the cytosol fraction. Membranes from *E. coli*-expressing tCYP3A37 also expressed active hNPR, the redox partner necessary for P450-mediated catalysis. The activity of hNPR, measured by reduction of cytochrome c, was 0.42 µmol/min/mg total protein.

The P4503A37-expressing *E. coli* membranes efficiently epoxidated AFB₁ to the exo-AFBO in our HPLC-based *in vitro* assay. Formation of this active metabolite conformed to Michaelis-Menten kinetics (Figure 2.4). The $K_m$ and $V_{max}$ values of exo-AFB₁ formation were $21.6 \pm 6.4 \, \mu M$ and $1.3 \pm 0.1 \, \text{nmol/min/nmol P450}$, respectively ($R^2 = 0.746$).

**Discussion**

Biotransformation to AFBO is the necessary first step in the development of toxic and carcinogenic activity of AFB₁ in animals, and P450s are the most important enzymes in this process (Coulombe, 1993). The adverse effects of
AFBO is due to its reactivity toward DNA and other critical cellular macromolecules (Guengerich, 2008). In turkeys, as in humans and other animals, AFB$_1$ is bioactivated mainly by P4501A2 and 3A4 homologues (Klein et al., 2000). In humans, P4503A4 is the predominant form which epoxidates AFB$_1$ at environmentally-relevant substrate concentrations (Kamdem et al., 2006). Thus, the discovery of the CYP3A4 homologue CYP3A37 in turkey liver is of substantial importance in discerning the mechanisms of the extreme sensitivity of this species to AFB$_1$.

The structure of CYP3A37 in turkey is markedly similar to that of chicken CYP3A37 and the human homologue CYP3A4 with thirteen exons and twelve introns. All 13 exons are, at least in part, translated. With the exception of exons 9 and 13, the number of amino acid residues encoded in aligned transcripts is conserved among the two poultry species and human. Turkey exon 13 was one residue longer in humans. The most notable differences occurred in exon 9 in which the turkey exon is 12 bp (4 aa) shorter than the corresponding chicken sequence. Interestingly, exon 9 in humans (NM_017460) is similar to that of turkey in that it is 18 bp shorter in the same region as the chicken protein. This region, which lies between two helix domains (human resides 271-279 and 292-323) may be less functionally constrained. Two alternate-spliced transcripts (503 and 353 residues) are reported for human CYP3A4. Splice variants were not observed in the turkey. A search of the NCBI EST database identified 19
CYP3A37 transcripts for the chicken, none of which indicated splice variants upon alignment.

Linkage of CYP3A37 to markers in turkey linkage group M10 is consistent with the position of CYP3A37 in the chicken whole genome sequence, approximately 0.9 Mbp from MNT323. In humans, CYP3A4 is found in a cluster with other P450 genes on HGA7q21.1. In the chicken (and turkey, data not shown), a second P450 gene CYP3A80 is found adjacent to CYP3A37. Investigations of this P450 gene are ongoing in our laboratory.

Membranes from *E. coli*-expressing CYP3A37 efficiently converted AFB₁ to AFBO. This confirms our previous observation of the presence of a CYP3A4 homologue in turkey liver microsomes (Klein et al., 2000). The kinetic constants for *exo*-AFB formation by tP4503A37 compared to that reported for tP4501A5 (Yip and Coulombe, 2006) indicate that the former has a higher AFB₁ epoxidation activity ($K_m = 21.6 \pm 6.4 \text{ vs. } 65 \pm 12 \mu M; V_{max} = 1.3 \pm 0.1 \text{ vs. } 0.61 \pm 0.04 \text{ nmol/min/nmol P450}$). Based simply on these kinetic constants, it is safe to assume that CYP3A37 plays an important role in AFB₁ bioactivation in turkey liver *in vivo*. However, a confirmation of the relative roles of CYP3A37 and CYP1A5 in turkey liver microsomes awaits immunoinhibition studies which are currently underway in this laboratory. By comparison, lymphoblastoid microsomes expressing human CYP3A4 cDNA exhibited slightly different kinetic constants than CYP3A37 ($K_m = 133 \mu M; V_{max} = 6.2 \text{ nmol/min/nmol P450}$).
Such interspecies rate differences are to be expected, but may also be explained by the different expression systems.

Due to extreme susceptibility of poultry, especially turkeys, to the toxic and carcinogenic effects of AFB\(_1\), development of molecular markers (SNPs and/or microsatellites) in genes coding for critical genes like CYP3A37 will be important in tracking susceptibility traits. Because of the striking similarities between the sequences of turkey and chicken, this work can be of benefit to the entire poultry industry.

**References**


Figure 2.1. Aligned coding regions of turkey (GenBank DQ450083) and chicken (GenBank NM_001001751) CYP3A37 cDNAs. Predicted amino acid sequences for each gene are given below the nucleotide sequence. Exon junctions based on alignments with genomic DNA are indicated above the chicken sequence. Position (underlined) of PCR primers used in genotyping are indicated. A 12 bp deletion in the turkey exon 9 is shown as dashes.
Figure 2.2. Idiogram of turkey CYP3A37 indicating position of exons and introns as determined by alignment of the turkey mRNA sequence (DQ450083) to the chicken whole genome sequence. Location of the 256G>A SNP used for linkage mapping is indicated by the arrow. Length of exons (above) and introns (below) are presented, coding nucleotides included in exons 1 and 13 are indicated in parentheses.
Figure 2.3. Physical map of chicken chromosome 14 (GGA14) based on positions (determined by BLASTN) of genetically mapped turkey sequences (Reed et al., 2007b) aligned with assembly 2.1 of the chicken whole-genome sequence (units = bp).
Figure 2.4. Michaelis-Menten plot for AFB$_1$ oxidation by *E. coli* membranes expressing truncated turkey CYP3A37. $K_m$ and $V_{max}$ of exo-AFBO formation are $21.6 \pm 6.4 \mu M$ and $1.3 \pm 0.1194$ nmol/min/nmol P450, respectively ($R^2 = 0.746$). Points are mean (± SE), $n = 3$. 

![Diagram of Michaelis-Menten plot for AFB$_1$ oxidation by *E. coli* membranes expressing truncated turkey CYP3A37.](image-url)

**AFB$_1$ (μM)**

**Rate (nmol/min/nmol P450)**

- **exocyclic AFB$_1$ (AFB$_1$)**
- **exo-AFBO**

ABF$_1$ (μM)
CHAPTER 3

CLONING, EXPRESSION, AND FUNCTIONAL CHARACTERIZATION OF CYTOCHROME P450 3A37 WITH HIGH AFLATOXIN B₁ EPOXIDATION ACTIVITY FROM TURKEY LIVER³

Abstract

Cytochromes P450 (P450) play an important role in the formation of carcinogenic and mutagenic electrophilic intermediates from a wide range of xenobiotics, including naturally occurring dietary compounds. The pathogenesis of hepatotoxic and hepatocarcinogenic action of the mycotoxin aflatoxin B₁ (AFB₁) involves initial bioactivation by P450s to a reactive and electrophilic intermediate exo-aflatoxin B₁-8,9-epoxide (exo-AFBO). Poultry, especially turkeys are extremely sensitive to AFB₁, a condition due, in part, to efficient epoxidation by P450 1A and 3A isoforms. We previously reported discovery of CYP1A5 from turkey liver, that like its human homologue, CYP1A2, bioactivated AFB₁ to exo-AFBO and aflatoxin M₁ (AFM₁). Here we describe CYP3A37, the CYP3A4 homologue from turkey liver. The gene has an open reading frame (ORF) of 1512 bp, and the protein is predicted to be 504 amino acids with 97% identity to chicken CYP3A37. A truncated construct of the turkey CYP3A37 with 11 amino acids deleted from the hydrophobic N-terminal region was heterologously

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expressed in *Escherichia coli*, the protein from which exhibited a CO difference spectrum typical of P450s. Like human CYP3A4, CYP3A37 biotransformed AFB₁ to *exo*-AFBO and aflatoxin Q₁ (AFQ₁) and possessed nifedipine oxidase activity, both of which were inhibited by the CYP3A4 inhibitor 17α-ethynylestradiol. Oxidation of AFB₁ to *exo*-AFBO and AFQ₁ by CYP3A37 followed sigmoidal Hill kinetics best described by Eadie-Hofstee plots, suggestive of an allosteric interaction between the enzyme and AFB₁. CYP3A37 possessed at least two binding sites (*n* = 1.9 for *exo*-AFBO and 1.6 for AFQ₁ products) for AFB₁, indicative of positive cooperativity. The calculated *Kₗ* and *Vₘₐₓ* values for the formation of *exo*-AFBO were 287 ± 21 µM and 1.45 ± 0.07 nmol/min/nmol P450, respectively, whereas those of AFQ₁ formation were 302 ± 51 µM and 7.86 ± 0.75 nmol/min/nmol P450, respectively. These data strongly suggest that CYP3A37, along with CYP1A5, plays an important role in AFB₁ epoxidation in turkey liver. And, this activity plus that of CYP1A5, previously described, likely results in the efficient bioactivation of AFB₁ that makes turkeys very susceptible to toxicosis.

**Introduction**

Cytochrome P450s (P450 for protein; CYP for gene) are the major drug metabolism enzymes involved in the metabolism of variety of endogenous and exogenous substrates (Parikh *et al.*, 1997). While converting most of the compounds into readily excreted detoxified hydrophilic form, P450s also contribute to the toxicity of some xenobiotics through bioactivation resulting in
the formation of reactive intermediates which causes toxic effects (Guengerich et al., 1996a). The carcinogenic mycotoxin aflatoxin B\textsubscript{1} (AFB\textsubscript{1}) is bioactivated by P450s to the electrophilic and highly reactive epoxide intermediate \textit{exo}-AFB\textsubscript{1}-8,9-epoxide (\textit{exo}-AFBO) (Ball and Coulombe, 1991; Coulombe, 1993; Gallagher \textit{et al.}, 1996; Guengerich \textit{et al.}, 1996a), which binds to DNA, RNA and cellular proteins and is responsible for mediating the toxic and carcinogenic effects of AFB\textsubscript{1} in humans as well as most animal species. In many species, glutathione-S-transferases (GSTs) detoxify the \textit{exo}-AFBO so produced, by conjugating it with endogenous glutathione.

Poultry, specifically turkeys, are extremely sensitive to aflatoxicosis (Carnaghan \textit{et al.}, 1966; Arafa \textit{et al.}, 1981; Giambrone \textit{et al.}, 1985; Huff \textit{et al.}, 1986; Kubena \textit{et al.}, 1995; Klein \textit{et al.}, 2000). The susceptibility of turkeys to AFB\textsubscript{1} was first demonstrated in the early 1960s as the etiological agent of “Turkey X disease” which caused widespread deaths of turkeys and other poultry in Europe (Stevens \textit{et al.}, 1960). Aflatoxins are ubiquitous in corn-based animal feeds, their presence in feed cause significant economic losses to the poultry industry every year, and contamination is practically unavoidable (CAST, 1989; Coulombe \textit{et al.}, 2005).

We have previously shown that this extreme sensitivity of turkeys to AFB\textsubscript{1}, is, in part, due to efficient oxidation mediated by homologues to human CYP1A2 and CYP3A4 (Klein \textit{et al.}, 2000, 2003; Yip and Coulombe, 2006). We
recently amplified CYP1A5 from turkey liver (Yip and Coulombe, 2006), the *E.
coli* expressed protein of which, like its human counterpart CYP1A2, oxidized
AFB1 to *exo*-AFBO and aflatoxin M1 (AFM1). Although both P450 1A and 3A
enzymes oxidize AFB1, there are conflicting reports on their relative importance
(Shimada and Guengerich, 1989; Ramsdell and Eaton, 1990; Gallagher *et al.*, 1994,
1996). In human liver, CYP3A4, which appears to account for the majority of the
formation of *exo*-AFBO and AFQ1, was an important determinant of the AFB1
disposition (Shimada and Guengerich, 1989; Kamdem *et al.*, 2006).

We recently elucidated the genetic structure of turkey CYP3A37, which
like human CYP3A4, is organized into 13 exons and 12 introns (see Chapter 2).
The exons of the turkey gene were 124, 94, 53, 100, 114, 89, 149, 128, 73, 161, 227,
163, and 475 bases in length. Exons 1 and 13 contained 71 and 93 coding
nucleotides, respectively. Because of its presumed role in AFB1 bioactivation, we
describe here the heterologous expression and functional characterization of this
CYP3A4 homologue, CYP3A37.

**Materials and Methods**

**Chemicals and Reagents.** QIAprep spin miniprep kit, QIAquick PCR
purification kit, and gel extraction kit were obtained from Qiagen (Valencia, CA).
TOPO TA cloning kit and maximum efficiency DH5αF’IQ *Escherichia coli* cells
were from Invitrogen (Carlsbad, CA). Restriction enzymes and T4 DNA ligase
were from Fermentas (Hanover, MD). *Exo-* and *endo*-AFB1-8,9-epoxide
glutathione conjugate (AFBO-GSH) standard were a generous gift from Dr. F. Peter Guengerich, Vanderbilt University School of Medicine (Nashville, TN). Anti-His tag monoclonal antibody was from Novagen (San Diego, CA). The Phototope-HRP Western Blot Detection System was from Cell Signaling (Beverley, MA). Rabbit polyclonal anti-CYP3A37 serum was raised against the peptide sequence “SQKSDSDGKNSHKA” (amino acids 278-291) by Genemed Synthesis (San Antonio, TX). This peptide sequence was selected by the manufacturer while taking into consideration that it should be antigenic, hydrophilic and readily accessible. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

**Rapid amplification of cDNA ends (RACE).** The CYP3A37 gene was amplified from 1-day-old male turkey liver by 3’-5’-RACE procedures described previously (Yip and Coulombe, 2006; Chapter 2). In brief, small segments of RNA later stored turkey liver were homogenized and RNA isolated using Poly (A) Pure mRNA purification kit (Ambion, Austin, TX). Isolated RNA was further used as a RACE template. The RACE procedure as mentioned in BD SMART RACE cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA) was followed. Primers used in 3’-RACE were designed based upon the sequence alignment of CYP3A4 in human and cattle, 3A4v2 in *Cercopithecus aethiops* (African green monkey), 3A44 in mouse, 3A66 in *Macaca mulatta* (rhesus monkey)
and 3A45 in *Onchorhynchus mykiss* (rainbow trout)\(^4\) using ClustalW (www.ebi.ac.uk). 5’-RACE primers were designed based on sequences of 3’-RACE as well as the sequence alignments. Products of the PCR were cloned into pCR 4-TOPO vector and sequenced at the CORE facility at Utah State University. Sequences were analyzed by Lasergene SeqMan II software (DNASTAR Inc., Madison, WI).

**Construction of expression plasmid and bacterial expression.** An *E. coli* based recombinant P450 system was used for CYP3A4 homologue identification (Yip and Coulombe, 2006). Expression vector construction was as described previously (Yip and Coulombe, 2006). We chose pCW vector, developed by the laboratory of Prof F. W. Dahlquist (Gegner and Dahlquist, 1991), which contains two tac promoter (inducible by isopropyl-\(\beta\)-D-thiogalactopyranoside: IPTG) cassettes upstream of an *NdeI* (CATATG) restriction enzyme cloning site coincident with the initiation ATG codon. This vector contains a strong transcription termination mechanism and a phage M13 origin of DNA replication. Transcription prior to the addition of inducing agents is inhibited by *LacI* gene encoding a lac repressor molecule. The original bicistronic pCW1A2/hNPR vector developed by Dr. F. Peter Guengerich was generously provided by Dr. Gary Yost, University of Utah (Salt Lake City, UT). Bicistronic

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expression constructs consisting of the coding sequence of CYP3A37 followed by human NADPH-P450 reductase (hNPR), the redox partner to P450, were constructed as described (Yip and Coulombe, 2006; Chapter 2). Cytochrome P450s are difficult to express in their native form due to the hydrophobic amino acid rich N-terminal region, so a truncated version of the gene was constructed to enhance its expression in E. coli (Barnes et al., 1991) (Figure 3.1). Specifically 11 codons were deleted in the N-terminus and the first 8 codons had a modified nucleotide sequence to enhance its expression. A 6X-His tag was added in the C-terminal region to facilitate immunodetection and purification (Crowe et al., 1994). The original pCW1A2/hNPR vector was digested with NdeI and XbaI to excise the CYP1A2 coding sequence for the construction of expression constructs.

Clones of CYP3A37 digested with NdeI and XbaI were ligated with the gel-purified vector fragment and PCR-amplified using T4 DNA ligase. This ligation mix was transformed into DH5αF‘IQ cells. Heterologous expression of CYP3A37 in E. coli was performed (Jenkins et al., 1998). A single colony of DH5αF‘IQ E. coli transformants was inoculated in to five milliliters of Luria-Bertani ampicillin medium. It was then allowed to grow overnight at 37 ºC with vigorous shaking. 450 mL of modified Terrific-Broth ampicillin medium with trace elements was then used for further inoculation of the starter culture at 1:100 dilutions. Cultures were grown at 37 ºC to an OD_{600} of 0.3-0.5. After an equilibration step of 30 min at 30 ºC, induction was initiated by adding 1 mM IPTG. δ-aminolevulinic acid
(0.5 mM) was also added to the cultures. Cells were incubated at 30 °C with shaking (150 rpm), harvested after 23 h and amounts of P450 expression were determined by reduced CO/reduced difference spectra (Omura and Sato, 1964) (Spectronic GENESYS 6 UV-Vis spectrophotometer, Thermo Spectronic, Rochester, NY). *E. coli* membranes and cytosol were prepared as described (Yip and Coulombe, 2006). All kinetic assays were performed with CYP3A37 *E. coli* membranes.

**Immunodetection of recombinant CYP3A37.** *E. coli* membranes expressing CYP3A37, control vector (pCWOri+ without P450 and reductase inserts) and turkey liver microsomes were run on a 4-15% Tris-HCl Ready Gel (Bio-Rad). Immunoblotting was performed according to the supplier’s instructions (Qiagen, Valencia, CA). To reduce the extraneous bands caused by the recognition of bacterial proteins, the rabbit polyclonal anti-CYP3A37 serum was first preadsorbed by pCWOri+ *E. coli* membranes (Guengerich et al., 1996b; Yip and Coulombe, 2006). Briefly, 120 µL of pCWOri+ membranes (22 mg protein) were added to 2 mL antiserum (111 mg protein) and incubated at 4 °C for 2.5 h for preadsorption. The samples were centrifuged at 13000g for 10 min at 4 °C and supernatants were used as primary antibody (1:4000). HRP-conjugated goat anti-rabbit IgG (1:5000) were used as secondary antibody.

Chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Pierce,
Rockford, IL) signal detection and images were captured using Nucleovision E20 Imaging Workstation (Nucleotech, Hayward, CA).

**Quantification of AFB<sub>1</sub> metabolites.** The *exo*-AFBO intermediate is unstable and has a short half-life, so it was determined indirectly as a stable, trapped AFBO-GSH conjugate using conditions we have described previously (Ramsdell and Eaton, 1990; Yip and Coulombe, 2006). A reaction mixture consisting of *E. coli* membranes expressing CYP3A37 (0.709 µM), butylated hydroxyanisole (BHA)-induced mouse cytosol (~800 µg total protein) as a source of GST, 2 mM NADPH, 5 mM GSH and 0.1-1000 µM AFB<sub>1</sub> in spectral grade dimethyl sulfoxide (DMSO) was made in epoxide trapping buffer (5 mM MgCl<sub>2</sub>, 25 mM KCl, 0.25 mM sucrose, and 80 mM potassium phosphate, pH 7.6) to give a final volume of 250 µL. The reactions were incubated at 37 ºC for 20 min with gentle shaking and stopped by adding 250 µL of cold methanol spiked with 24 µM aflatoxin G<sub>1</sub> (AFG<sub>1</sub>) as an internal standard. Inhibitor or anti-CYP3A37 antibodies whenever used were incubated with the membranes for 10 min in an ice-bath before adding other components of the reaction. This treatment was done to provide extra time for the inhibitor or the antibody to bind to the enzyme. The samples were kept overnight at -20 ºC to facilitate protein precipitation and then centrifuged at 13000g for 10 min. The supernatant was filtered through a 0.2 µm nylon membrane before 100 µL was injected into the HPLC. Metabolites were separated on a Shimadzu LC system (Shimadzu,
Pleasanton, CA) equipped with a model LC-20AD pump, a model SPD-20AV UV-Vis detector, and an Econosphere C$_{18}$ (150 mm X 4.6 mm) column (Alltech Associates, Deerfield, IL) which was kept at 40 ºC. The eluate was monitored by UV absorbance (λ = 254 nm). The mobile phases used in this assay were as described previously (Klein et al., 2000). Metabolites were quantified by establishing calibration curves between the peak areas in the chromatograms and the amounts of metabolites injected, using authentic AFBO-GSH and AFQ$_{1}$ standards.

**Nifedipine oxidase activity.** Nifedipine oxidase was determined as described previously (Kelly et al., 1997). Inhibitors or anti-CYP3A37 antibodies were tested on this activity in a similar way as described above.

**Data analysis.** Kinetic data were analyzed by SigmaPlot Enzyme Kinetics Module software (Systat, San Jose, CA). Data for exo-AFBO and AFQ$_{1}$ formation were analyzed by nonlinear regression using the general allosteric model, the Hill equation to calculate $V_{\text{max}}$ and $K_{m}$ [$V = V_{\text{max}} \frac{[S]^{n}}{([K_{m}]^{n} + [S]^{n})}$]. In this equation, $n$ represents the number of apparent binding sites.

**Results**

**RACE.** Sequence analysis revealed that this gene has an ORF of 1512 bp coding for 504 amino acids. In addition, it had 53 bp in the 5′-untranslated region (UTR) and 384 bp in the 3′-UTR, with a total of 1949 bp (Figure 3.2). Predicted molecular mass of this CYP3A4 homologue is 58 kDa. The heme-binding motif,
usually represented as FXXGXXXCXG, was identified in CYP3A37 as FGAGPRNCIG (Figure 3.2). Sequence identity comparison of this gene and CYP3A class genes in other species is shown in Table 3.1. These comparisons, which were made using EMBOSS-Align program (www.ebi.ac.uk), revealed that this gene has 93% and 92% nucleic acid and amino acid identity, respectively, to the chicken CYP3A37 (Ourlin et al., 2000), and was thus assigned as CYP3A37 by Dr. David Nelson of the University of Tennessee.

**Bacterial Expression and Immunodetection.** As frequently observed with unmodified P450s (Guo et al., 1994), the wild-type CYP3A37 was not expressed as detectable P450 as determined by reduced CO/reduced difference spectrum (Omura and Sato, 1964). The truncated tCYP3A37 expressed 250–400 nmol of CYP3A37 per liter of the culture, as determined by the CO difference spectra of whole cells. CYP3A37 was recovered in both membrane and cytosol, approximately 26% and 25-45%, respectively. The rest of the amounts were lost in differential centrifugation procedure used for the isolation of membrane and cytosolic fractions. *E. coli* membranes expressing recombinant CYP3A37 also expressed active hNPR (0.54 µmol/min/mg total protein) as measured by cytochrome c reduction. As compared to the activity of purified hNPR expressed in *E. coli* from the same plasmid (50 µmol/min/mg), we determined that hNPR was comprised of 2.8% (w/w) of the proteins in tCYP3A37 *E. coli* membrane (vs 8% for P450), which corresponded to a hNPR-to-P450 molar ratio of about 1:4.
Polyclonal anti-CYP3A37 antibodies detected a band with an apparent molecular mass of 58 kDa in *E. coli* expressed CYP3A37 and in turkey liver microsomes (Figure 3.3).

**AFB<sub>1</sub> Oxidation and Nifedipine Oxidase activity.** *E. coli*-expressed CYP3A37 oxidized AFB<sub>1</sub> to exo-AFBO and AFQ<sub>1</sub>, and a plot of V vs. S, exhibited a sigmoidal relationship, suggesting that the formation of both products is driven by an allosteric interaction between AFB<sub>1</sub> and CYP3A37, showing positive cooperativity (Figure 3.4A). The data were analyzed by the general allosteric model using the Hill equation, and the resultant Eadie-Hofstee plots are shown in Figures 3.4B (for exo-AFBO formation) and 3.4C (AFQ<sub>1</sub>). The n (apparent number of binding sites) values for the formation of exo-AFBO and AFQ<sub>1</sub> were 1.9 and 1.6, respectively, suggesting the presence of at least two binding sites for AFB<sub>1</sub> on CYP3A37 (Table 3.2). The mean dissociation constant (K<sub>m</sub>) for the two sites was 295 µM.

Like human CYP3A4 (Yamazaki *et al.*, 1996), CYP3A37 possessed nifedipine oxidase activity which, unlike AFB<sub>1</sub> metabolism, conformed to simple Michaelis-Menten kinetics. The K<sub>m</sub> and V<sub>max</sub> for this activity were 1.4 ± 0.5 µM and 1465 ± 289 nmol/min/nmol P450 (r<sup>2</sup> = 0.84), respectively (Figure 3.5).

**Inhibition by specific P450 inhibitors and anti-CYP3A37 antibodies.** The effects of specific inhibitors to mammalian P450s on exo-AFBO formation as well as on nifedipine oxidase activity were examined. The specific CYP3A4 inhibitor
17α-ethynylestradiol was the most effective of those tested, completely inhibiting the formation of exo-AFBO at a concentration of 200 µM (IC₅₀ = 40 µM) (Figure 3.6). Erythromycin, a CYP3A1/4 inhibitor was moderately effective. Inhibitors to other P450s were either slightly or not effective (Figure 3.6). At the same concentrations, 17α-ethynylestradiol also inhibited nifedipine oxidase activity (IC₅₀ = 74 µM) (Figure 3.7). Anti-CYP3A37 antibodies inhibited the formation of both exo-AFBO and nifedipine oxidase activity, as shown in Figure 3.8.

**Discussion**

Many P450s bioactivate xenobiotics, like AFB₁, converting them into highly reactive electrophilic intermediates, which cause mutation and carcinogenesis. Although AFB₁ is metabolized by P450s to endo- and/or exo-AFBO, it is the exo- stereoisomer which binds to DNA, RNA and other cellular macromolecules to elicit the toxic responses to this mycotoxin (Raney *et al.*, 1992). The extreme sensitivity of turkeys to AFB₁ is due, at least in part, to hepatic P450s with high epoxidation activity. In turkey liver microsomes, both CYP1A and 3A homologues are responsible for the metabolism of AFB₁ to the toxic and carcinogenic exo-AFBO, as it occurs in humans and other animals (Klein *et al.*, 2000; Yip and Coulombe, 2006; Chapter 2). Here, we report that turkey liver possesses CYP3A37, a homologue to human CYP3A4 with AFB₁ epoxidation and nifedipine oxidase activity.
The nucleic acid sequence of this gene was remarkably similar to chicken CYP3A37 (Ourlin et al., 2000), with 93% identity. While the sequence of this gene was 66% identical to human CYP3A4, the catalytic activity of the E. coli expressed CYP3A37 closely resembled that of its human counterpart. Turkey CYP3A37 possessed activity towards prototypical CYP3A4 substrate nifedipine (Yamazaki et al., 1996) and also oxidized AFB1 in a manner similar to human CYP3A4 (Gallagher et al., 1996). Inhibition of the catalytic activities of CYP3A37 by 17α-ethynylestradiol further support its designation as a CYP3A4 homologue.

Generally P450s require alterations in the hydrophobic N-terminal nucleotide sequence (5’-end) to facilitate translation by E. coli (Barnes et al., 1991). The N-terminal truncated sequence of wild-type CYP3A37 was produced by deleting 11 codons and modifying the nucleotide sequence of the first 8 codons to facilitate translation by E. coli. These modifications are reported to reduce the free energy for base pairing while inhibiting the formation of secondary structures by mRNA, which could inhibit translation (Guengerich et al., 1996a). Subsequently, truncated CYP3A37 expressed in E. coli produced substantial amounts of active P450, which was confirmed by immunoblots showing a band of apparent molecular mass of about 58 kDa. The addition of hNPR, in our bicistronic constructs, ensured efficient electron transfer to P450: the hNPR-to-P450 ratio in the E. coli membranes was 1:4, similar to that we previously reported for turkey CYP1A5 (Yip and Coulombe, 2006) and greater.
than previously reported ratios of 1:5-20 in mammalian liver microsomes (Estabrook et al., 1971; Shephard et al., 1983; Parikh et al., 1997).

Striking similarities between the catalytic activities of *E. coli* expressed turkey CYP3A37 and human CYP3A4 were observed. For example, turkey CYP3A37 efficiently metabolized AFB\textsubscript{1} to *exo*-AFBO and AFQ\textsubscript{1} and also possessed nifedipine oxidase activity, as does the human enzyme. Furthermore, these catalytic activities were completely inhibited by CYP3A4 specific inhibitor 17α-ethynylestradiol. It must be emphasized that P450 prototypical substrates and inhibitors used in this study were developed for mammalian P450s, and specificities of these compounds for avian P450s have not been rigorously validated. Erythromycin, an inhibitor to CYP3A1/4, also inhibited the formation of *exo*-AFBO as well as nifedipine oxidase activity, but at concentrations higher than that observed for 17α-ethynylestradiol (≥ 800 µM). While 17α-ethynylestradiol efficiently inhibited catalytic activities of CYP3A37, erythromycin was moderately effective, which may be explained by interspecies differences among P450 enzymes.

This metabolic specificity of CYP3A37 confirms our previous observation of the involvement of a CYP3A4 homologue (along with a CYP1A2 homologue) in AFB\textsubscript{1} bioactivation in turkey liver microsomes (Klein *et al.*, 2000; Yip and Coulombe, 2006). Similar to what we previously reported for CYP1A5, turkey CYP3A37 resembled its human counterpart with respect to metabolite profile,
and to the kinetics of AFB<sub>1</sub> oxidation. Like human CYP3A4, CYP3A37 catalysis of AFB<sub>1</sub> is driven by an allosteric interaction, showing positive cooperativity.

Human CYP3A4 is reported to have a large and conformationally dynamic active site which can accommodate multiple substrates or effector molecules, the binding of which leads to allosteric kinetic behavior in that substrate binding to CYP3A4 facilitates binding of additional molecules of substrate, enhancing turnover (Roberts and Atkins, 2007). Similar to human CYP3A4 (Gallagher et al., 1996), CYP3A37 possessed at least two binding sites for AFB<sub>1</sub>. The K<sub>m</sub> values determined for CYP3A37 were almost two-fold greater than those for human CYP3A4 [302 µM vs. 139 µM for AFQ<sub>1</sub>; 287 µM for exo-AFBO in our study vs. 133 µM for AFBO reported by (Gallagher et al., 1996)], though this comparison is not accurate since those authors (Gallagher et al., 1996), did not distinguish the endo- and exo- isomers. The V<sub>max</sub> value of AFQ<sub>1</sub> formation was almost 8-fold lower for CYP3A37 than those of human CYP3A4 (7.86 vs. 61 nmol/min/nmol P450), while that of exo-AFBO formation in our study was more than 4-fold lower than that of the AFBO formation measured for human CYP3A4 (1.45 vs. 6.2 nmol/min/nmol P450) (Gallagher et al., 1996). Such kinetic differences could be explained by interspecies variations of P450s, but is more likely due to different in vitro systems used to model enzyme activity.

Although CYP3A37 and turkey CYP1A5 (Yip and Coulombe, 2006), exhibited different kinetics, a comparison of the kinetic constants of exo-AFBO
formation suggests that the former may be more efficient. The $V_{\text{max}}$ of CYP3A37 is more than two times that of CYP1A5 ($1.45 \pm 0.07 \text{ vs. } 0.61 \pm 0.04 \text{ nmol/min/nmol P450}$). However, it must be emphasized that the affinity of CYP3A37 for AFB$_1$ may be 4-fold lower than that of CYP1A5, as indicated by their $K_m$ values ($287 \pm 21 \text{ vs. } 65 \pm 12 \ \mu\text{M}$).

While there are conflicting reports as to which isoform is more important in humans in activating AFB$_1$ (Shimada and Guengerich, 1989; Ramsdell and Eaton, 1990; Gallagher et al., 1994, 1996), some reports suggest that human CYP1A2 is more active at submicromolar substrate concentrations because of its high affinity for AFB$_1$, whereas CYP3A4 activates AFB$_1$ at higher concentrations ($>50 \ \mu\text{M}$) (Crespi et al., 1991; Gallagher et al., 1996). Indeed a similar finding was observed previously in our laboratory in human bronchial cells expressing CYP 1A2 and 3A4 (Van Vleet et al., 2002).

In conclusion, CYP3A37 is important in mediating toxicity of AFB$_1$ in turkeys. This gene was heterologously expressed in E. coli, in quantities sufficient to support the catalytic activities, which were similar to the human CYP3A4. Because of the role of human CYP3A4 in AFB$_1$ oxidation to exo-AFBO (Shimada and Guengerich, 1989; Gallagher et al., 1996; Kamdem et al., 2006), we believe that the identification of this homologue from turkeys is important in discerning the mechanisms of extreme sensitivity of this species to AFB$_1$. Of particular importance was the metabolism of AFB$_1$ by the E. coli expressed protein to exo-
AFBO and AFQ<sub>1</sub>. *Exo-AFBO*, a highly reactive electrophilic intermediate, is responsible for carcinogenic and mutagenic effects of AFB<sub>1</sub>. Thus, we conclude that CYP3A37 along with CYP1A5 plays an important role in the extreme sensitivity of turkeys to AFB<sub>1</sub>.

**References**


Table 3.1. Nucleic acid and amino acid sequence identity and similarity between turkey CYP3A37 and other CYP3As (EMBOSS-Align).

<table>
<thead>
<tr>
<th>Cytochrome P450</th>
<th>Species</th>
<th>Nucleic acid</th>
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</tr>
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<tbody>
<tr>
<td></td>
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<td>% Identity</td>
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<tr>
<td>CYP3A37</td>
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<tr>
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<td>Human</td>
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<td>58</td>
</tr>
</tbody>
</table>

*Percentage similarity of amino acid sequence was determined based on EBLOSUM62 matrix.
Table 3.2. Hill Equation kinetic constants for CYP3A37 catalyzed AFB\textsubscript{1} oxidation in *E. coli* expressed CYP3A37, at AFB\textsubscript{1} concentrations of 0.1-1000 µM. “n” is number of apparent binding sites. \(V_{\text{max}}\) and \(K_m\) measured as nmol/min/nmol P450 and µM, respectively.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>(V_{\text{max}} \pm \text{SE}^*)</th>
<th>(K_m \pm \text{SE}^*)</th>
<th>(n \pm \text{SE}^*)</th>
<th>(R^2)</th>
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<tbody>
<tr>
<td>exo-AFBO</td>
<td>1.45 ± 0.07</td>
<td>287 ± 21</td>
<td>1.9 ± 0.18</td>
<td>0.98</td>
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<tr>
<td>AFQ\textsubscript{1}</td>
<td>7.86 ± 0.75</td>
<td>302 ± 51</td>
<td>1.6 ± 0.26</td>
<td>0.96</td>
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</table>

*3 replicates were used*
Figure 3.1. Expression constructs of CYP3A37 showing nucleic acid and amino acid sequences at the N- and C-terminals. Wild-type (wtCYP1A5) has 1512 bp encoding 504 amino acids; the truncated construct (tCYP1A5) has 1479 bp encoding 493 amino acids. Six histidines were added at the C-terminals to facilitate detection and purification. Nucleotides and amino acids different from the wild type sequence are bolded.
Figure 3.2. Complete cDNA and predicted amino acid sequence of turkey CYP3A37, with 5′- and 3′-UTRs. ORF: 1512bp encoding 504 aa; 5′-UTR: 53 bp; 3′-UTR: 384 bp; total cDNA length: 1949 bp. Start and stop codons are bolded, the heme-binding motif, usually represented as FXXGXXXCXG is underlined.
Figure 3.3. Immunoblot showing 60 kDa bands in both turkey liver microsomes and in CYP3A37-expressing *E. coli* membranes. A minor band which was detected at around 80 kDa was presumably a bacterial protein, present in *E. coli* membranes of CYP3A37 and in those of pCWOri+ (the empty vector control).
Figure 3.4. Kinetics of AFB₁ oxidation by *E. coli* expressed CYP3A37. A sigmoidal relationship between substrate concentration and product formation was observed, suggesting *exo*-AFBO and AFQ₁ formation are driven by an allosteric interaction between CYP3A37 and AFB₁, showing positive cooperativity. The data was analyzed by Hill’s equation. (A) V vs. S plot of *exo*-AFBO and AFQ₁ formation (B) Eadie-Hofstee plot of *exo*-AFBO formation (C) Eadie-Hofstee plot of AFQ₁ formation. Points are mean (± SE), n=3.
Figure 3.5. Nifedipine Oxidase activity of *E. coli* membranes expressing CYP3A37. Activity followed Michaelis-Menten kinetics with parameters: $V_{\text{max}} = 1465 \pm 289 \text{ nmol min}/ \text{nmol} / \text{P450}$, $K_m = 1.4 \pm 0.5 \mu \text{M}$ ($R^2 = 0.84$). Points are mean ($\pm \text{SE}$) (n=3).
Figure 3.6. Inhibition of AFB₁ oxidation to exo-AFBO by *E. coli* expressed CYP3A37, using specific inhibitors to P450s. CYP3A4 prototype inhibitor 17α-ethynylestradiol inhibited formation of exo-AFBO (IC₅₀ = 40 µM). Erythromycin was less efficient. Inhibitors to other mammalian CYPs were either slightly or not effective. Points are mean (n=3).
Figure 3.7. Inhibition of nifedipine oxidase activity of *E. coli* expressed CYP3A37 by specific inhibitors to P450s. 17α-ethynylestradiol, a specific inhibitor to CYP3A4 inhibited nifedipine oxidase activity of *E. coli* expressed CYP3A37 (IC$_{50}$ = 74 µM). Erythromycin was less effective. Points are mean (n=3).
Figure 3.8. Inhibition of CYP3A37 mediated AFB₁ epoxidation and nifedipine oxidase activity by anti-CYP3A37 antibodies (5 µg/ml/nmol P450). Points are mean (n=3).
CHAPTER 4

METABOLISM OF AFLATOXIN B₁ IN TURKEY LIVER MICROSONES: THE RELATIVE ROLES OF CYTOCHROME P450S 1A5 AND 3A37

Abstract

The extreme sensitivity of turkeys to aflatoxin B₁ (AFB₁), is associated with an efficient epoxidation by hepatic microsomal cytochrome P450s 1A5 and 3A37 to the toxic exo-aflatoxin B₁-8,9-epoxide (exo-AFBO). The combined presence of 1A5 and 3A37 in the turkey liver, both of which metabolize AFB₁ to the exo-AFBO and to detoxification products AFM₁ and AFQ₁, respectively, complicates the kinetic analysis of the oxidation of this mycotoxin in turkey liver microsomes (TLMs). Furthermore, 1A5 and 3A37 follow Michaelis-Menten and Hill’s kinetics, respectively. In the present study, we examined the reaction kinetics of AFB₁ oxidation in TLMs, using antisera directed against CYP1A5 and 3A37, for the purpose of identifying the isoform responsible for epoxidating AFB₁ at relatively low concentrations. Pretreatment with anti-CYP1A5 inhibited exo-AFBO formation, which was especially marked at low, submicromolar (~0.1 µM), while anti-CYP3A37 immune serum inhibited exo-AFBO formation, which was most evident at much higher (> 50 µM) AFB₁ concentrations. At 0.1 µM AFB₁, which is close to the range seen in the livers of animals exposed to AFB₁ concentrations typically occurring in foods and feeds, CYP1A5 contributed to about 98% of the total exo-AFBO formation. At this concentration, CYP1A5 was
responsible for a much higher ratio (50:1) of activation to detoxification product (exo-AFBO: AFM₁) compared to CYP3A37. Kinetic constants indicated that CYP1A5 acts as the higher affinity, while CYP3A37 as the lower affinity isoform for AFB₁. In total, the data presented here support the conclusion that CYP1A5 is the dominant enzyme responsible for AFB₁ bioactivation and metabolism at real-world AFB₁ concentrations, and therefore plays an important role in the extreme sensitivity of turkeys to aflatoxicosis.

Introduction

Aflatoxins, which are toxic and carcinogenic mycotoxins produced by Aspergillus fungi, are a major public health concern, especially in geographies where AFB₁ contaminated foods constitute a staple diet. Aflatoxin B₁ (AFB₁) is the most toxic and carcinogenic of all aflatoxins (Wogan et al., 1974; Wong and Hsieh, 1976; Bondy and Pestka, 2000). AFB₁ is not toxic per se, but requires bioactivation by P450s to the electrophilic and toxic intermediate exo-AFB₁-8,9-epoxide (exo-AFBO) (Ball and Coulombe, 1991; Coulombe, 1993; Gallagher et al., 1996). Poultry, especially turkeys, are extremely sensitive to the toxic effects of AFB₁ (Carnaghan et al., 1966; Arafà et al., 1981; Kubena et al., 1995; Klein et al., 2000), a condition graphically demonstrated when this mycotoxin was discovered as the etiological agent of “Turkey X Disease,” responsible for the deaths of poultry throughout Europe in the 1960s (Smith, 1960). We have previously shown that the extreme sensitivity of turkeys to AFB₁ is associated
with efficient hepatic microsomal bioactivation to \textit{exo}-AFBO by homologues to human CYP1A2 and 3A4 (Klein \textit{et al.}, 2000; Yip and Coulombe, 2006; Chapter 2; Chapter 3). We recently cloned, expressed and characterized CYP1A5 (Yip and Coulombe, 2006) and 3A37 (Chapter 2; Chapter 3) from turkey liver, which, like the human homologues, produced \textit{exo}-AFBO, and the detoxified metabolites AFM$_1$ and AFQ$_1$, respectively. Furthermore, the kinetics of \textit{exo}-AFBO formation by CYP1A5 followed Michaelis-Menton kinetics, while those for 3A37 exhibited sigmoidal Hill’s kinetics, suggestive of an allosteric interaction between AFB$_1$ and CYP3A37, showing positive cooperativity (Gallagher \textit{et al.}, 1996, Chapter 3). The relative roles of turkey CYP1A5 and 3A37 in the epoxidation of AFB$_1$ is uncertain. While the relative importance of human CYP1A2 and 3A4 in AFB$_1$ oxidation has been the subject of disagreement in the literature (Shimada and Guengerich, 1989; Ramsdell and Eaton, 1990; Ueng \textit{et al.}, 1995; Gallagher \textit{et al.}, 1996), such studies have never been performed in turkeys. In the present study, we used antisera directed against CYP1A5 and 3A37 as tools to investigate the role of these enzymes in bioactivation of AFB$_1$ in turkey liver microsomes (TLMs). The purpose of this study was to evaluate which of these isoform is most active at relatively low concentrations of AFB$_1$, more likely to be achievable in turkey liver, \textit{in vivo}. 
Material and Methods

Chemicals, reagents, and antibodies. Rabbit polyclonal anti-CYP1A5 (Yip and Coulombe, 2006) and 3A37 (Chapter 3) sera were raised against the peptide sequence “FLDFNKRFMKLLKTAVEE (amino acids 260-277)” and “SQKSDSDGKNSHKA (amino acids 278-291),” respectively (Genemed Synthesis, San Antonio, TX). These peptide sequences were selected by the manufacturer while taking into consideration that they should be antigenic, hydrophilic and readily accessible. Liver tissues were obtained from 1-day old male white turkeys, generously provided by Dr. Lynn Bagley, Moroni Feed Co. (Moroni, UT). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Microsomal metabolism and determination of AFB1 metabolites. Turkey liver microsomes were prepared as previously described (Klein et al., 2002). The exo-AFBO intermediate is unstable and has a very short half-life, so it was determined indirectly as a stable, trapped AFBO-GSH conjugate using conditions described previously (Yip and Coulombe, 2006). A reaction mixture consisting of turkey liver microsomes (~400 μg), butylated hydroxyl anisole (BHA)-induced mouse cytosol (~800 μg total protein) as a source of GST, 2mM NADPH, 5mM GSH and 0.1-1000 μM AFB1 in spectral grade dimethyl sulfoxide (DMSO) was made in epoxide trapping buffer (5 mM MgCl2, 25 mM KCl, 0.25 mM sucrose, and 80 mM potassium phosphate, pH 7.6) to give a final volume of 250 μL. The
reaction mixtures were incubated at 37°C for 20 min with gentle shaking and stopped by adding 250 μL of cold methanol spiked with 24 μM aflatoxin G₁ as an internal standard. The samples were kept overnight at -20 °C to facilitate protein precipitation and then centrifuged at 13000Xg for 10 min. The supernatants were filtered through a 0.2 μm nylon membrane before 100 μL were injected into the HPLC. Metabolites were separated on a Shimadzu LC system (Shimadzu, Pleasanton, CA), equipped with a model LC-20AD pump, a model SPD-20AV UV-vis detector, and an Econosphere C₁₈ (150 mm X 4.6 mm) column (Alltech Associates, Deerfield, IL) kept at 40°C. The elution of the peaks was monitored by UV absorbance (λ=254 nm). The mobile phases used in this assay were as described previously (Klein et al., 2000). Metabolite formation was quantified using curves between the peak areas in the chromatograms and the amounts of metabolites injected, using authentic AFBO-GSH, AFM₁ and AFQ₁ standards.

**Inhibition of microsomal CYP1A5 and 3A37 activities by anti-peptide antibodies.** Anti-CYP1A5 or 3A37 immune serum (10 μg/ml/μg microsomal protein) or control preimmune serum (10 μg/ml/μg microsomal protein) were pre-incubated with the TLMs for 10 min at 4 °C before adding the other components of the epoxide trapping assay.

**Statistical analysis.** Kinetic data were analyzed by SigmaPlot Enzyme Kinetics Module software (Systat Software, Inc., San Jose, CA). Data for CYP3A37 kinetics were analyzed by nonlinear regression using the Hill equation
to calculate $V_{\text{max}}$ and $K_m \ [V = \frac{V_{\text{max}} [S]^n}{([K_m]^n + [S]^n)}].$ In this equation, $n$ represents the number of apparent binding sites. On the other hand, data for CYP1A5 kinetics were fit using the Michaelis-Menten equation $[V = \frac{V_{\text{max}} [S]}{(K_m + [S])}].$

**Results**

**Metabolism of AFB$_1$ by control TLMs.** We used microsomes from the commercial birds, as they are shown to be more susceptible than the wild varieties (Quist et al., 2000). The differences may be due to the activity of GST, as P450 mediated oxidation of AFB$_1$ from different varieties (wild, heritage and commercial) exhibited similar kinetics (data not shown). Oxidation of AFB$_1$ to different metabolites was studied over concentrations ranging from 0.1-1000 µM using TLMs. Mean product formation rates from triplicate incubations were determined simultaneously at each substrate concentration in control (preimmune serum pretreated) microsomes and in the same microsomes pretreated with anti-CYP1A5 or 3A37 immune serum, to eliminate the contribution of CYP1A5 or 3A37 to product formation, respectively. AFB$_1$ was metabolized to three products in control microsomes: *exo*-AFBO, AFM$_1$ and AFQ$_1$ in the ratio of (1: 0.53: 4.4). The V vs. S plot for the formation of these metabolites is shown in Figure 4.1. The kinetic parameters are presented in Table 4.1.
Metabolism of AFB₁ by CYP3A37 (TLMs pretreated with anti-CYP1A5 immune serum). In general, the formation of exo-AFBO and AFM₁ was inhibited by pretreatment with anti-CYP1A5 immune serum, while AFQ₁ formation was resistant to inhibition. Like the E. coli expressed CYP3A37, the rates of exo-AFBO and AFQ₁ formation (in the ratio of 1:6) followed sigmoidal Hill kinetics, indicative of positive cooperativity (Figure 4.2 and 4.3). The Eadie-Hofstee plots (Figure 4.2B for exo-AFBO, and 4.3B for AFQ₁), indicated that 3A37 metabolism of AFB₁ followed sigmoidal kinetics with a second order relationship between the substrate concentrations and product formation rates (Gallagher et al., 1996). While the Kₘ values (Table 4.1) were similar to those of E. coli expressed CYP3A37 (exo-AFBO: 281 ± 15 vs. 287 ± 21 µM; AFQ₁: 302 ± 31 vs. 302 ± 51 µM), Vₘₐₓ values (Table 4.1) were smaller (exo-AFBO: 0.41 ± 0.01 vs. 1.45 ± 0.07 nmol/min/nmol P450; AFQ₁: 2.26 ± 0.13 vs. 7.86 ± 0.75 nmol/min/nmol P450) (Chapter 3). The apparent number of binding sites (“n”) was consistent to those in the E. coli expressed CYP3A37 (exo-AFBO: 1.9 ± 0.14 vs. 1.9 ± 0.18; AFQ₁: 1.6 ± 0.15 vs. 1.6 ± 0.26) (Chapter 3). AFM₁ formation was inhibited by anti-CYP1A5 immune serum at all AFB₁ concentrations (data not shown).

Metabolism of AFB₁ by CYP1A5 (TLMs pretreated with anti-CYP3A37 immune serum). The formation of exo-AFBO and AFQ₁ were inhibited by pretreatment with anti-CYP3A37 immune serum, while AFM₁ formation was relatively resistant to inhibition. Like the E. coli expressed turkey CYP1A5 (Yip
and Coulombe, 2006), formation of \textit{exo-AFBO} and \textit{AFM\textsubscript{1}} (in the ratio of 1: 4) followed simple Michaelis-Menton kinetics (Figure 4.4 and 4.5). The kinetic parameters listed in Table 4.1 show that \(K_m\) values were similar to those of \textit{E. coli} expressed CYP1A5 (\textit{exo-AFBO}: 69 ± 26 vs. 65 ± 12 \(\mu\text{M}\); \textit{AFM\textsubscript{1}}: 37 ± 6 vs. 34 ± 9 \(\mu\text{M}\)), while \(V_{\text{max}}\) values were roughly 4-fold lower for \textit{AFM\textsubscript{1}} (0.28 ± 0.01 vs. 0.91 ± 0.07 nmol/min/nmol P450) and more than 8-fold lower for \textit{exo-AFBO} (0.07 ± 0.007 vs. 0.61 ± 0.037 nmol/min/nmol P450) (Yip and Coulombe, 2006). \textit{AFQ\textsubscript{1}} formation was inhibited by anti-CYP1A5 immune serum at all concentrations of substrate (data not shown).

**Metabolism of AFB\textsubscript{1} in TLMs pretreated with both anti-CYP1A5 and 3A37 immune serum.** After inhibition of both the enzymes, no product formation was observed.

**Relative contribution of CYP1A5 and 3A37 in the epoxidation of AFB\textsubscript{1} to \textit{exo-AFBO}**. The inhibition of \textit{exo-AFBO} formation in TLMs pretreated with anti-CYP1A5 immune serum was most marked at low concentrations of AFB\textsubscript{1}, while pretreatment with anti-CYP3A37 immune serum resulted in marked inhibition of \textit{exo-AFBO} at relatively high concentrations of AFB\textsubscript{1} (Figure 4.6). At submicromolar AFB\textsubscript{1} concentrations (0.1 \(\mu\text{M}\)), CYP1A5 contributed about 98% to the total \textit{exo-AFBO} formation (Figure 4.6). At the same concentrations, CYP1A5 produced a higher ratio (50: 1) of activation to detoxification product (\textit{exo-AFBO}: \textit{AFM\textsubscript{1}}) compared to CYP3A37 (\textit{exo-AFBO}: \textit{AFQ\textsubscript{1}} = 0.17: 1) (Table 4.2).
Discussion

Previous studies in our laboratory have established that CYP1A5 and 3A37, homologues to human CYP1A2 and 3A4, respectively, are the most important P450 isoforms responsible for the biotransformation of AFB\textsubscript{1} in turkey liver (Klein et al., 2000; Yip and Coulombe, 2006; Chapter 3). Although both CYP1A5 and 3A37 epoxidate AFB\textsubscript{1}, their relative importance is uncertain because of several complicating factors: a) both enzymes produce activation (exo-AFBO) and detoxification products (AFM\textsubscript{1} and AFQ\textsubscript{1} by 1A5 and 3A37, respectively), b) the affinities of CYP1A5 and 3A37 toward AFB\textsubscript{1} are substantially different (Yip and Coulombe, 2006; Chapter 3), and c) AFB\textsubscript{1} metabolism by these enzymes conform to different kinetic models. The relative importance of human CYP1A2 and 3A4 with respect to AFB\textsubscript{1} epoxidation have been the subject of conflicting reports mostly due to similar complications (Ramsdell and Eaton, 1990; Ueng et al., 1995; Gallagher et al., 1996; Kamdem et al., 2006). Indeed, there are striking similarities between human and turkey 1A and 3A homologues with respect to AFB\textsubscript{1} metabolism in liver microsomal and heterologously-expressed systems (Gallagher et al., 1994, 1996; Yip and Coulombe, 2006; Chapter 3).

In this study, we examined the kinetics of AFB\textsubscript{1} oxidation by CYP1A5 and 3A37 in TLMs, using anti-CYP1A5 and 3A37 immune serum as tools for sequentially isolating individual AFB\textsubscript{1}-metabolizing P450s for the purpose of identifying the predominant enzyme with particular attention to
environmentally-relevant or “real-world” concentrations seen in the livers of AFB1-exposed animals.

When the contribution of CYP3A37 was eliminated by immunoinhibition, TLMs oxidized AFB1 to just two products, exo-AFBO and AFM1, characterized by hyperbolic Michaelis-Menton kinetics. Identical characteristics have been observed in E. coli expressed CYP1A5 (Yip and Coulombe, 2006) and human CYP1A2 (Gallagher et al., 1996). Conversely, when CYP1A5 was immunoinhibited, TLMs produced exo-AFBO and AFQ1, best fitting sigmoidal Hill kinetics in agreement with the previous reports for CYPs 3A4 and 3A37 (Gallagher et al., 1996; Chapter 3). Examination of the Hill equation parameters suggested that, while the affinity of CYP3A37 for AFB1 is less than that of CYP1A5, its maximal velocity is about 6-times greater.

The sigmoidal relationship observed between the substrate concentrations and the rates of product formation, suggests that CYP3A37 interacts with AFB1 in an allosteric manner, indicative of positive cooperativity. Similar sigmoidal behavior for P450 3A in other species have been observed. Using 7-benzyloxy-4-(trifluoromethyl)-coumarin (BFC) and nonylphenol as CYP3A substrates, both homotropic and heterotropic cooperative interactions were described in fish (Kullman et al., 2004). Furthermore, human CYP3A4-catalyzed, oxidation of AFB1 to AFBO and AFQ1, and formation of temazepam from diazepam showed allostetism, with the data fitting well to sigmoidal $V_{\text{max}}$ model equivalent to the
Hill equation (Andersson et al., 1994; Gallagher et al., 1996). In addition, Gallagher et al (1996) observed sigmoidal kinetics for the formation of AFBO and AFQ₁ in human liver microsomes pretreated with furafylline, a mechanism based inhibitor of CYP1A2. Several models have been proposed for the cooperativity observed with CYP3A4 homologues, indicating the presence of a distinct allosteric site, where binding of an effector or substrate molecule enhances binding of substrate at the active site and involves a change in the conformation of the active site (Ueng et al., 1997; Davydov and Halpert, 2008).

This atypical sigmoidal second-order kinetics associated with CYP3A37 and AFB₁ implies that the rate of product formation falls off steeply at low substrate concentrations (Gallagher et al., 1996). However, such is not the case for CYP1A5 which follows first order hyperbolic Michaelis-Menten kinetics (Gallagher et al., 1996). Our results clearly show that at lower AFB₁ concentrations, such as at 0.1 µM, nearly all exo-AFBO is formed by CYP1A5 (Table 4.2). Residual AFB₁ and stable detoxified metabolites in the livers of chickens and other domesticated fowl were in the range of 0.15-7.83 and 1.54-22.34 ppb, respectively, after an 8-day exposure to a diet containing containing 3 ppm AFB₁ (Bintvihok et al., 2002). The higher range (~0.025-0.072 µM) of these in vivo concentrations of AFB₁ and of its metabolites is very close to 0.1 µM used in our assays. While in vitro metabolic systems only provide an approximation of the hepatic milieu in vivo, it seems reasonable to conclude that the formation of
*exo*-AFBO, as well as the disposition of AFB₁, at “real world” concentrations is probably dominated by CYP1A5. The probability that the *in vivo* concentration of AFB₁ at the site of the P450 enzyme would actually exceed those used in *in vitro* assay is very low; AFB₁ needs to traverse the lipid membranes in order to distribute and the total fraction of lipid at *in vivo* conditions would probably be much higher than those of *in vitro* microsomal fraction (Gallagher *et al*., 1996).

Our data shows that, at 0.1 µM AFB₁ concentrations, CYP1A5 produced a much higher ratio (50: 1) of activation (*exo*-AFBO) to detoxification product (AFM₁), relative to CYP3A37. In addition, the ratio of activation: inactivation products formed in TLMs also suggests relatively higher contribution of CYP1A5 to the AFB₁ activation as compared to CYP3A37. Previous studies examining the kinetics of AFB₁ oxidation by *E. coli* expressed CYP1A5 and 3A37 in our laboratory also indicates similar findings; CYP1A5 produced *exo*-AFBO and AFM₁ (AFB₁ concentrations: 2 - 500 µM) in a ratio of 1: 2, while CYP3A37 produced *exo*-AFBO and AFQ₁ (AFB₁ concentrations: 0.1 - 1000 µM) in a ratio of 1: 6 (Yip and Coulombe, 2006; Chapter 3). The ratios of activation: inactivation products catalyzed by CYP1A5 and 3A37 are likely to be an important factor in the biological consequences of *in vivo* AFB₁ exposure in turkeys. While CYP3A activity is higher than that of 1A in the turkey liver (Guarisco *et al*., 2008), the apparent low affinity of 3A37 at submicromolar liver AFB₁ concentrations, makes it highly unlikely that this enzyme would bioactivate AFB₁ substantially *in vivo*. 
At 0.1 µM AFB₁ concentrations, the ratio of the contributions of CYP1A5: 3A37 to the formation of exo-AFBO is 98: 2.

Based on our results, inhibition of CYP1A5 may significantly alleviate the symptoms of aflatoxicosis in poultry exposed to dietary AFB₁. In fact, butylated hydroxytoluene (BHT) added to turkey feed provided improvements in the AFB₁-associated symptoms in turkeys, in part, due to its inhibitory effect on CYP1A5, exhibiting Michaelis-Menten competitive inhibition kinetics (Kᵢ=0.81 µM) (Guarisco et al., 2008). Guarisco et al. (2008) also showed that BHT induces CYP3A, which implies that this enzyme is probably not bioactivating AFB₁ in vivo; rather it may be contributing to the protective effects of BHT by possible detoxification to AFQ₁. In conclusion, our in vitro kinetic studies of AFB₁ oxidation indicate that the dominant catalyst of AFB₁ epoxidation at in vivo dietary concentrations obtained in turkey liver is CYP1A5.

References


Table 4.1. Kinetic constants of CYP1A5 and 3A37 catalyzed AFB1 (0.1-1000 μM) metabolism in control, and immunoinhibited turkey liver microsomes.1,2,3

<table>
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<tr>
<th>Metabolite</th>
<th>Kinetic constants</th>
<th>Control</th>
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<th>+ Anti-CYP3A37</th>
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<td>exo-AFBO</td>
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<td>ND</td>
</tr>
<tr>
<td></td>
<td>$N$</td>
<td>1.7 ± 0.18</td>
<td>1.6 ± 0.15</td>
<td>ND</td>
</tr>
<tr>
<td>AFM1</td>
<td>$V_{max}$</td>
<td>0.25 ± 0.009</td>
<td>ND</td>
<td>0.28 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>$K_m$</td>
<td>41 ± 6</td>
<td>ND</td>
<td>37 ± 6</td>
</tr>
<tr>
<td></td>
<td>$N$</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1Microsomes were pretreated with anti-CYP1A5 or 3A37 immune serum (10 μg/ml/μg microsomal protein) for 10 min
2$V_{max}$ is expressed in nmol/min/nmol P450, $K_m$ in μM, $N$ is the number of apparent binding sites
3Data are mean ± SE (n=3)
4ND: Not Detected
Table 4.2. Relative contribution of CYP1A5 and 3A37 to the activation (exo-AFBO) vs. inactivation (AFM\textsubscript{1} and AFQ\textsubscript{1}) products formed in control and immunoinhibited TLMs, at 0.1 µM AFB\textsubscript{1}, which are close to those potentially encountered via real world dietary exposure.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Control (Preimmune serum)</th>
<th>CYP1A5 (anti-CYP3A37 immune serum)</th>
<th>CYP3A37 (anti-CYP1A5 immune serum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>exo-AFBO\textsuperscript{1}</td>
<td>18.8</td>
<td>18.5</td>
<td>0.19</td>
</tr>
<tr>
<td>AFM\textsubscript{1}\textsuperscript{1}</td>
<td>0.3</td>
<td>0.37</td>
<td>ND\textsuperscript{2}</td>
</tr>
<tr>
<td>AFQ\textsubscript{1}\textsuperscript{1}</td>
<td>1.06</td>
<td>ND</td>
<td>1.23</td>
</tr>
</tbody>
</table>

\textsuperscript{1}All values are pmol/min/nmol P450.
\textsuperscript{2}ND: Not detected
Figure 4.1. Kinetics of AFB$_1$ metabolism in control TLMs (pretreated with preimmune serum). Three metabolites were produced: exo-AFBO, AFM$_1$ and AFQ$_1$, a metabolic profile indicative of functioning microsomal CYP1A5 and 3A37. Points are mean ± SE (n = 3).
Figure 4.2. The rates of \textit{exo-}AFBO formation in TLMs pretreated with anti-CYP1A5 immune serum (10 μg/ml/μg microsomal protein). The resultant \textit{exo-}AFBO formation showed sigmoidal Hill’s kinetics, typical of CYP3A37. (A) \textit{V} vs. S plot of \textit{exo-}AFBO formation. (B) Eadie- Hofstee plot of \textit{exo-}AFBO formation. Points are mean ± SE (n = 3).
Figure 4.3. The rates of AFQ₁ formation in TLMs were not attenuated by pretreatment with anti-CYP1A5 immune serum. AFQ₁ formation which is exclusively by CYP3A37 followed sigmoidal Hill’s kinetics. (A) V vs. S plot of AFQ₁ formation. (B) Eadie-Hofstee plot of AFQ₁ formation. Points are mean ± SE (n = 3).
Figure 4.4. The rates of \( \text{exo-AFBO} \) formation in TLMs pretreated with anti-CYP3A37 immune serum (10 μg/ml/μg microsomal protein). After attenuation of CYP3A37, \( \text{exo-AFBO} \) formation showed Michaelis-Menton kinetics, typical of CYP1A5. The data were therefore analyzed by fitting into Michaelis-Menton equation. Points are mean ± SE (n = 3).
Figure 4.5. The rates of AFM$_1$ formation in TLMs were not attenuated by pretreatment with anti-CYP3A37 immune serum. AFM$_1$ formation which is exclusively by CYP1A5 followed simple Michaelis-Menton kinetics. Points are mean ± SE (n = 3).
Figure 4.6. The relative contribution of CYP1A5 and 3A37 on AFB₁ epoxidation can be seen by the differential effects of anti-CYP1A5 and 3A37 immune serum (10 μg/ml/μg microsomal protein) on the initial rates of *exo*-AFBO formation in TLMs. Rates of *exo*-AFBO formation in the presence of antiserum were calculated as percentage control (treatment with preimmune serum only). Each bar is mean ± SD. (n = 3).
CHAPTER 5
CHEMOPREVENTION OF AFLATOXICOSIS BY PROBIOTIC
*LACTOBACILLUS* IN TURKEYS

Abstract

Aflatoxin B1 (AFB1), a well-known human carcinogen, is a mycotoxin produced as secondary metabolite by the fungi *Aspergillus flavus*, *A. parasiticus*, and *A. nominius*. Poultry, specifically turkeys, are extremely sensitive to the toxic effects of AFB1, which makes them an excellent model to study AFB1 chemopreventives. Probiotic bacteria are able to bind AFB1 and reduce its bioavailability. Combinations of different probiotic bacteria are often used by the food industry as biopreservatives, making them a suitable candidate to study for chemopreventive actions. This study aims to investigate the protective action of probiotic *Lactobacillus*: consisting of *Lactobacillus rhamnosus* strains GG and LC-705, *Propionibacterium freundenreichii* sp shermani and *Bifidobacterium* sp (LGG), on AFB1 toxicosis in turkeys. Day-old turkeys were chosen for this study and the trial lasted for 21 days. After acclimatization for 10 days, birds were placed in one of the four groups (n=10): LGG, LGG+AFB1, Control, and AFB1. Birds were pretreated daily with 0.5 ml of either LGG (5 X 10^{10} cfu/0.5 ml PBS, prepared by directly suspending lyophilized bacteria in PBS) (groups: LGG and LGG+AFB1) or PBS (groups: Control and AFB1), by oral gavage for the first 10 days, before starting AFB1 treatment at 1 ppm in the diet. Birds received LGG, PBS and AFB1
for the rest of the period of the trial depending upon the group assignment. LGG + AFB1-treated group showed significantly higher body and liver weights than those treated with AFB1-alone. Serum profile, histopathological and AFB1-lysine adduct data supports the protective role of LGG, however the differences were not statistically significant. In conclusion, our results indicate that LGG provides protection against some of the deleterious effects of AFB1 and that this probiotic may prove to be a viable chemopreventive for aflatoxicosis in turkeys.

**Introduction**

Aflatoxins, produced as secondary metabolites of the fungi *Aspergillus flavus, A. parasiticus,* and *A. nomius,* are classified human carcinogens (Bondy and Pestka, 2000). Of all aflatoxins, AFB1 is the most toxic, causing growth impairment and immunosuppresion in a range of domesticated animals and livestock (Richard *et al.,* 1986; Coulombe, 1993). The toxicity of AFB1 is dependent on the formation of *exo*-AFB1-8,9-epoxide (AFBO) by cytochromes P450 (CYP for gene; P450 for protein), which is electrophilic, and binds to DNA, RNA and other critical cellular nucleophiles to cause cellular toxicosis, mutagenesis and carcinogenesis (Ball and Coulombe, 1991; Coulombe, 1993; Gallagher *et al.,* 1996; Guengerich *et al.,* 1996). Glutathione-S-Transferases (GST) are able to detoxify the AFBO, by conjugating it with endogenous glutathione. This detoxification mechanism is critical in determining the susceptibility of a species to AFB1 toxicosis (Coulombe, 1993). Poultry, specifically turkeys, are
extremely sensitive to the toxic effects of AFB\textsubscript{1} (Carnaghan \textit{et al.}, 1966; Arafa \textit{et al.}, 1981; Giambrone \textit{et al.}, 1985; Huff \textit{et al.}, 1986; Kubena \textit{et al.}, 1995; Klein \textit{et al.}, 2000). Sensitivity of turkeys to AFB\textsubscript{1} was first demonstrated by their association with the “Turkey X Disease” which caused widespread deaths of turkeys and other poultry throughout Europe in the 1960s (Stevens \textit{et al.}, 1960). We previously established that this extreme sensitivity of turkeys to AFB\textsubscript{1} is in part, due to an unfortunate combination of efficient epoxidation by P450s and deficient detoxification by GSTs (Klein \textit{et al.}, 2000; Yip and Coulombe, 2006; Chapter 2). Aflatoxin B\textsubscript{1} in turkey feed results in poor performance, decreased body and organ weights, immunosuppression, morbidity, and mortality (Kubena \textit{et al.}, 1990, 1991; Coulombe, 1993). According to a report by Council for Agricultural Science and Technology, losses due to aflatoxins to US poultry industry exceeded $143 million annually (CAST, 1989). A recent study reported annual crop losses of $932 million due to mycotoxin contamination and additional losses of $466 million in efforts to prevent or reduce contamination (CAST, 2003). Given that AFB\textsubscript{1} contamination in feed is nearly universal, and therefore practically unavoidable (Coulombe \textit{et al.}, 2005), chemoprevention strategies aimed at reducing AFB\textsubscript{1} toxicity in poultry and other animals have been the subject of numerous studies (Klein \textit{et al.}, 2002b, 2003; Guarisco \textit{et al.}, 2008a, 2008b). Indeed, because of their sensitivity, poultry have been used as models for discovery of novel AFB\textsubscript{1} chemopreventives. Our laboratory recently
studied AFB₁ chemoprevention by the antioxidant butylated hydroxytoluene (BHT). The protective properties of BHT appeared to be due to an inhibitory effect on the activity of P450 1A1 and 1A2, suggesting reduced bioactivation of AFB₁ to the AFBO (Klein et al., 2003). Further studies revealed that dietary BHT reduced AFBO formation, as well as other CYP1A5-mediated activities (Guarisco et al., 2008b). Furthermore, dietary BHT caused significant reductions in AFB₁ bioavailability, AFB₁–DNA adduct formation in the liver, and reduced AFB₁ residues in tissues (Guarisco et al., 2008a).

Probiotic bacteria, such as *Lactobacillus rhamnosus*, bind to AFB₁, and play a protective role in humans (Wollowski et al., 2001). Because of its use in various dairy products, including yogurt, *L. rhamnosus* is therefore considered a good candidate for evaluating its AFB₁ chemopreventive potential in turkeys. *Lactobacillus rhamnosus* strains GG and LC-705 were found to be the most efficient in binding a range of mycotoxins including aflatoxins (El-Nezami et al., 1998, 2002a, 2002b; Haskard et al., 2001; Peltonen et al., 2001). In addition, *L. rhamnosus* reduced AFB₁ transport, metabolism, and toxicity in Caco-2 cells (Gratz et al., 2007). Furthermore, *L. rhamnosus* reduced the AFB₁ bioavailability in rats, thereby decreasing its toxicity (Gratz et al., 2006). Thus, there exists substantial evidence of the protective role of probiotics in preventing aflatoxicosis. The present study aims to evaluate the chemopreventive action of LGG on AFB₁ toxicity in turkeys, a species extremely susceptible to aflatoxicosis.
Material and Methods

Bacterial strains. A freeze-dried mixture of probiotics, “LGG” (Valio Ltd., Helsinki, Finland), consisting of *Lactobacillus rhamnosus* GG (2.3 X 10^{10} cfu/g), *Lactobacillus rhamnosus* LC-705 (3.0 X 10^{10} cfu/g), *Propionibacterium freundenreichii* sp shermani (3.5 X 10^{10} cfu/g), and *Bifidobacterium* sp (2.9 X 10^{10} cfu/g) was generously provided by Dr. Hani El-Nezami, University of Kuopio (Kuopio, Finland). Additionally, this mixture contained 58% microcrystalline cellulose, 27% gelatin, and magnesium stearate. LGG was administered to turkeys by oral gavage in final concentrations of 5 X 10^{10} CFU/0.5 ml phosphate buffered saline (PBS), prepared by directly suspending lyophilized bacteria in PBS (Gratz et al., 2006).

Animals and Treatment. Animals were cared for under institutional approval in an AAALAC-accredited facility. The Utah State University Animal Care and Use Committee approved all procedures involving animal care, euthanasia and tissue collection. Turkeys and feed were generously provided by Moroni Feed Co. (Ephraim, UT). One day-old male Nicholas strain turkeys were used for this study because previous studies indicate that younger birds are more susceptible to aflatoxicosis than older ones (Klein et al., 2002a). Birds were maintained on a corn-based commercial starter diet for the duration of the trial, which lasted 21 days. After acclimatization for 10 days, birds were randomly placed in one of the four groups (n=10): LGG, LGG + AFB1, Control, and AFB1.
For the first 10 days, birds placed in LGG and LGG + AFB\(_1\) groups received pretreatment with 0.5 ml of LGG and those placed in Control and AFB\(_1\) groups received pretreatment with PBS (0.5 ml), once daily, by oral gavage, after which AFB\(_1\) treatment (for groups: LGG + AFB\(_1\) and AFB\(_1\)) was started at 1 ppm in the diet. Aflatoxin B\(_1\) and LGG or PBS treatment continued for the next 11 days. Thereafter, animals were euthanized by CO\(_2\) asphyxiation. Blood was collected by cardiac puncture in sterile, no-additive tubes (Becton Dickinson Vacutainer\®, Franklin Lakes, NJ) for serum and in heparinized tubes for plasma collection, and livers were rapidly removed and frozen on dry ice. The plasma and serum were removed within 1 h of blood collection. Livers were sectioned for histological analysis as described below, prior to freezing. The collected serum, plasma and tissues were stored at −80 °C until analyzed. Small sections of spleens were cut and stored in RNA later (Ambion Inc., Austin, TX) solution for cytokine gene expression analysis. Turkeys were weighed on day 1, 8, 15, 19 and before euthanasia on day 21 and livers were weighed when sampled on day 21\(^{st}\).

**Histological analysis.** Liver sections were fixed in neutral buffered 10% formalin immediately upon removal. A section of each liver was embedded in paraffin using a Model TP1050 Embedding Station (Leica Microsystems, Deerfield, IL), thin sectioned (RM 2145 Microtome, Leica Microsystems), and stained with hematoxylin and eosin (H&E) (Jung Autostainer XL, Leica Microsystems). Tissues were then fixed to the slides for histological analysis.
Each slide was evaluated for hepatic necrosis and biliary hyperplasia. A numerical score of 1 to 5 for the severity was assigned to each sample as follows (Klein et al., 2002b): hepatocellular necrosis, based on percent of viewed cells affected, 1 =< 5%, 2 = > 5 to 30%, 3 = > 30 to 60%, 4 = > 60 to 80%, or 5 = > 80%; biliary hyperplasia, 1 = normal, 2 = mild proliferation without parenchymal displacement, 3 = moderate proliferation with some mild parenchymal displacement, 4 = moderate to severe proliferation with moderate parenchymal displacement, or 5 = diffuse proliferation with severe parenchymal displacement.

**Cytokine gene expression analysis.** RNA was extracted from RNA later stored spleens, using an RNeasy Mini Kit (Qiagen). cDNA was transcribed using a high capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR was performed on an Applied Biosystems 7500 Fast machine using Fast SYBR Green (Applied Biosystems). The run conditions were as follows: 20 seconds of initial denaturation at 95°C, 40 cycles of denaturation at 95°C at 3 seconds each, and 30 seconds of annealing and extension at 60°C.

**Aflatoxin-lysine (AFB₁-lys) adducts analysis.** Samples were analyzed for AFB₁-lys by Liquid chromatography-Mass Spectrometry (LC/MS/MS) by a modified method as described previously (McCoy et al., 2005). This method involves digestion and isolation of AFB₁ adducts, but only measures AFB₁-lys. AFB₁-lys residues previously separated by LC were MS fragmented. Quantification used authentic standards.
**Statistical analysis.** Groups were compared, for differences, using appropriate ANOVA models and post-hoc tests as described under results. A level of $p < 0.05$ was chosen as statistically significant.

**Results**

Mean body weights for each treatment group over the course of the trial are summarized in Figure 5.1. LGG exerted a strong protective effect against primary endpoints of aflatoxicosis, like percent body weight gains, which were analyzed by a two-way factorial ANOVA model with main factors treatment (LGG treatment or not) and diet (AFB$_1$ or not) using proc mixed in SAS (Cary, NC). LGG-treated birds had significantly higher weight gains as compared to those which did not receive this treatment, with an estimated difference of about 83 % ($p$-value $< 0.0001$). As anticipated, birds receiving non-AFB$_1$ diets, had significantly higher weight gains than those which received AFB$_1$ in their diet, with an estimated difference of about 52 % ($p$-value $= 0.0005$). Percent body weight gain on day 21 relative to day 1 shows that, AFB$_1$-alone group experienced a significantly lower weight gain as compared to other groups (Figure 5.2) ($p$-values adjusted for multiple comparison were all 0.0002 or less), while the pairwise differences amongst groups LGG, LGG + AFB$_1$ and Control were not significant (smallest adjusted p-value for between-group comparison was 0.10, for LGG vs. Control). While AFB$_1$-alone group experienced about 350%
body weight gain, LGG, LGG + AFB\textsubscript{1} and Control groups experienced, on an average over 440\% (Figure 5.2).

Protective effects of LGG were also seen when liver weight was considered. Summary of liver and body weights and liver: body weight ratios on the day of sampling (day 21) are shown in Table 5.1. Similar to the ANOVA model for percent body weight gain, an ANOVA model was fit to the liver weight data. Model diagnostics identified the lowest liver weight in one of the birds in the LGG + AFB\textsubscript{1}-group as an outlier, so that bird was dropped from the liver weight analysis. From this model, birds receiving the non-AFB\textsubscript{1} diet had significantly higher liver weights than those with AFB\textsubscript{1} in diet (estimated difference 3.2g, p-value < 0.0001). There was no significant liver weight difference due to LGG treatment. There was no significant difference between the liver weights means amongst LGG, LGG + AFB\textsubscript{1} and Control groups (smallest adjusted p-value for between-group comparison is 0.15, for LGG + AFB\textsubscript{1} vs. Control), while even after adjusting for multiple comparisons, AFB\textsubscript{1}-alone group liver weight was significantly lower than LGG (estimated difference 4.20g, p-value=0.0003), LGG + AFB\textsubscript{1} (estimated difference 2.97g, p-value=0.02), and Control (estimated difference 5.16g, p-value < 0.0001) groups. Groups fed AFB\textsubscript{1} diet had significantly lower liver: body weight ratios than those receiving non-AFB\textsubscript{1} diets.
The principal target organ of AFB1 toxicosis is the liver. To assess the degree of hepatic damage, serum profiles were analyzed (Figure 5.3). Serum data were analyzed for within group differences using one-way ANOVA model with a post-hoc Tukey test. Serum gamma-glutamyltransferase (GGT) was significantly elevated in AFB1-treated groups as compared to the groups fed non-AFB1 diet. The mean level of GGT was slightly lower, but not significantly different; in LGG + AFB1-group as compared to those of AFB1-alone group (12.8 ± 1.6 versus 12.6 ± 5.7 U/L). Both total protein and albumin were significantly lower in AFB1-treated groups as compared to the non-AFB1 groups. Serum alkaline phosphatase, however, was significantly elevated in groups fed non-AFB1 diet than those in AFB1-treated animals. There was no statistically significant trend associated with AST, ALT, and bilirubin values (data not shown).

After the birds were sacrificed, their livers were grossly examined for hepatic changes, such as hemorrhaging, tissue firmness and color changes. Visual examination showed that livers from AFB1-alone groups were pale and firm, whereas these changes were reduced in LGG + AFB1-treated group and completely absent in groups fed non-AFB1 diet. Histology of liver sections revealed hepatic necrosis and biliary hyperplasia in AFB1- treated birds (Table 5.2), which were absent in control birds. Hepatic necrosis was mildly reduced in
turkeys treated with LGG + AFB\textsubscript{1} as compared to AFB\textsubscript{1}-alone group, but the differences were not statistically significant.

The relative gene expression of proinflammatory cytokines IL-8 and IL-1\textbeta{}, and anti-inflammatory cytokines IL-10 and IL-6 was analyzed by real-time PCR, using GAPDH as the endogenous control. AFB\textsubscript{1}-alone group showed upregulation of IL-1\textbeta{} and IL-10, and downregulation of IL-8 and IL-6, as compared to those of LGG + AFB\textsubscript{1}-group (Figure 5.4). Results were highly variable and there was no statistically significant trend associated with any of the cytokines.

Mean amounts of AFB\textsubscript{1}-lys adducts in the plasma were lower in LGG+AFB\textsubscript{1}-treated group than those in AFB\textsubscript{1}-alone group, though the difference was not statistically significant (1773 ± 215.9 versus 2169 ± 194.5 pg/mg) (Figure 5.5). These adducts were not detected in the birds fed non-AFB\textsubscript{1} diet. Visual inspection over the course of the trial indicated that birds which received LGG, were in general more active.

**Discussion**

Previous results from our laboratory has established that the extreme sensitivity of turkeys to AFB\textsubscript{1} is due, in part, to efficient P450 mediated bioactivation and deficient detoxification by GSTs (Klein et al., 2000; Yip and Coulombe, 2006; Chapter 2). Given that AFB\textsubscript{1} is an unavoidable contaminant of poultry feed (Coulombe et al., 2005), chemopreventive measures aimed to reduce
the toxicity of $\text{AFB}_1$ have been a subject of intense research. Here, we report the protective role of LGG against some end points of aflatoxicosis in turkeys. Probiotics have been previously shown to bind $\text{AFB}_1$ and subsequently reduce its toxicity in-vivo in rats and in-vitro in cultured Caco-2 cells (Gratz et al., 2006, 2007). To our knowledge, this has not been reported for turkeys thus far.

The beneficial effects of orally administered LGG were observed in key endpoints of aflatoxicosis. First, reduction in body weight gain due to $\text{AFB}_1$ returned to control values by LGG treatment, which was presumably due to the binding of $\text{AFB}_1$ by LGG leading to its excretion (Gratz et al., 2006). Turkeys are an agriculturally important production animal in the United States, thus weight gain is of critical economic importance and must be taken into consideration in the evaluation of any chemopreventive. Protective effects of LGG on $\text{AFB}_1$-induced weight loss were seen in rats (Gratz et al., 2006). Similar protective binding of $\text{AFB}_1$ by strains of *Lactobacillus* and *Propionibacterium* was seen in chickens (El-Nezami et al., 2000).

Although $\text{AFB}_1$ generally affects most systems, liver is the principal organ of effect for $\text{AFB}_1$ toxicosis. Aflatoxin B$_1$ has been shown to reduce the relative liver weights in turkeys (Klein et al., 2002b; Kubena et al., 1995). LGG provided amelioration in the $\text{AFB}_1$-associated reduction in liver weights in turkeys. Serum enzyme and histopathological data showed protection, but the differences were not significant. These findings are potentially suggestive of increased retention of
aflatoxin by LGG in the gastrointestinal tract of turkeys, which may consequently reduce the toxicity of a high AFB<sub>1</sub> exposure.

Aflatoxins are immunosuppressive in poultry, in part, through their effects on cytokines (Qureshi et al., 1998; Yarru et al., 2009). Our results indicate downregulation of anti-inflammatory cytokine, IL-6, in AFB<sub>1</sub>-alone group, which was consistent with earlier reports (Yarru et al., 2009). AFB<sub>1</sub>-induced alteration of cytokine expression among species appears to be highly variable (Rossano et al., 1999; Liu et al., 2002). Although the gene expression of cytokines was not significantly altered due to LGG treatment, our results provide an additional evidence for a species-dependent immune response to AFB<sub>1</sub>.

Treatment with LGG lowered, but did not alter the AFB<sub>1</sub>-lys adduct amounts significantly. Similar findings were reported by Gratz et al. (2006), suggesting a saturation of the hepatic metabolizing capacity within the dosing regimen used.

Thus, LGG provided some evidence of its protective effects in AFB<sub>1</sub>-associated toxicity in turkeys. Several strains of probiotic bacteria are known to bind a range of mycotoxins including aflatoxins. Of these strains, Lactobacillus rhamnosus strain GG and LC-705 appears to be most efficient in binding AFB<sub>1</sub> to the bacterial surface (El-Nezami et al., 1998, 2002a, 2002b; Haskard et al., 2001). In conclusion, LGG provides protection against AFB<sub>1</sub>-induced reduction in body
and liver weights in turkeys, however chronic studies are required to evaluate its potential role as a possible chemopreventive against aflatoxicosis in turkeys.

References


Table 5.1. The effect of orally administered LGG on liver, body, and liver to body weight ratio in turkeys on the day (21st) of sampling.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Liver Weight(^2)</th>
<th>Body Weight(^2)</th>
<th>Liver/body weight ratio(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGG</td>
<td>19.23 (1.91)(^a)</td>
<td>854.11 (83.05)(^a,c)</td>
<td>0.03 (0.002)(^a)</td>
</tr>
<tr>
<td>LGG+AFB(_1)</td>
<td>18.00 (2.18)(^a)</td>
<td>901.15 (94.97)(^a,c)</td>
<td>0.02 (0.001)(^b)</td>
</tr>
<tr>
<td>Control</td>
<td>20.19 (2.98)(^a)</td>
<td>825.79 (129.44)(^b,c)</td>
<td>0.03 (0.002)(^a)</td>
</tr>
<tr>
<td>AFB(_1)</td>
<td>15.03 (0.98)(^b)</td>
<td>738.71 (60.55)(^b)</td>
<td>0.02 (0.001)(^b)</td>
</tr>
</tbody>
</table>

\(^1\)Male turkeys were placed in one of the four groups: LGG, LGG+AFB\(_1\), Control, and AFB\(_1\). After 10 days of pretreatment with either LGG (groups: LGG and LGG+AFB\(_1\)) or PBS (groups: Control and AFB\(_1\)), 1 ppm AFB\(_1\) was added to the diets of two AFB\(_1\)-treated groups. Birds received LGG, PBS and AFB\(_1\) for the next 11 days, depending upon the group assignment.

\(^2\)Values are mean (+ SD) \(n = \text{at least 9}.\) Different superscripts indicate significant differences among groups \((p < 0.05)\).
Table 5.2. Histopathologic lesion severity scoring of turkeys in different groups.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Biliary Hyperplasia</th>
<th>Hepatic Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGG</td>
<td>1.00 (0.00)(^a)</td>
<td>1.10 (0.31)(^a)</td>
</tr>
<tr>
<td>LGG+AFB(_1)</td>
<td>3.33 (1.12)(^b)</td>
<td>2.00 (0.50)(^b)</td>
</tr>
<tr>
<td>Control</td>
<td>1.00 (0.00)(^a)</td>
<td>1.00 (0.00)(^a)</td>
</tr>
<tr>
<td>AFB(_1)</td>
<td>3.09 (0.94)(^b)</td>
<td>2.18 (0.40)(^b)</td>
</tr>
</tbody>
</table>

\(^1\)Male turkeys were placed in one of the four groups: LGG, LGG+AFB\(_1\), Control, and AFB\(_1\). After 10 days of pretreatment with either LGG (groups: LGG and LGG+AFB\(_1\)) or PBS (groups: Control and AFB\(_1\)), 1 ppm AFB\(_1\) was added to the diets of two AFB\(_1\)-treated groups. Birds received LGG, PBS and AFB\(_1\) for the next 11 days, depending upon the group assignment.

\(^2\)Values are mean (± SD) \(n = \text{atleast 9}\). Different superscripts indicate a significant difference among groups \((p < 0.05)\).
Figure 5.1. The protective effect of orally administered LGG on AFB1-induced declines in mean body weights in turkeys over the course of the study. On day 1, LGG, LGG + AFB1 and Control groups had 10 birds each, while AFB1-alone group had 11. One bird in LGG + AFB1 died on day 3rd after the start and no other mortalities were seen during the course of the trial.
Figure 5.2. The chemopreventive effect of orally administered LGG on AFB$_1$-induced reduction in percent body weight gain (on day 21 relative to day 1) in turkeys. AFB$_1$-alone group had significantly lower weight gain than rest of the groups. Male turkeys were placed in one of the four groups: LGG, LGG+AFB$_1$, Control, and AFB$_1$. After 10 days of pretreatment with either LGG (groups: LGG and LGG+AFB$_1$) or PBS (groups: Control and AFB$_1$), 1 ppm AFB$_1$ was added to the diets of two AFB$_1$-treated groups. Birds received LGG, PBS and AFB$_1$ for the next 11 days, depending upon the group assignment. Each box represents the mean ± SD (n = at least 9). Different superscripts indicate significant difference among groups (p<0.05) (n = at least 9).
Figure 5.3. The effect of orally administered LGG on serum chemistries in turkeys. Male turkeys were placed in one of the four groups: LGG, LGG+AFB₁, Control, and AFB₁. After 10 days of pretreatment with either LGG (groups: LGG and LGG+AFB₁) or PBS (groups: Control and AFB₁), 1 ppm AFB₁ was added to the diets of two AFB₁-treated groups. Birds received LGG, PBS and AFB₁ for the next 11 days, depending upon the group assignment. (A) ALP, (B) GGT, (C) Total protein, (D) Total albumin. Each bar represents the mean ± SD (n = at least 9). Different superscripts indicate a significant difference among groups (p < 0.05).
Figure 5.4. The effect of orally administered LGG on Relative Quantification of cytokines as assessed by RT-PCR in turkeys. Male turkeys were placed in one of the four groups: LGG, LGG+AFB₁, Control, and AFB₁. After 10 days of pretreatment with either LGG (groups: LGG and LGG+AFB₁) or PBS (groups: Control and AFB₁), 1 ppm AFB₁ was added to the diets of two AFB₁-treated groups. Birds received LGG, PBS and AFB₁ for the next 11 days, depending upon the group assignment.
Figure 5.5. The effect of orally administered LGG on AFB1-lys adducts in turkeys. AFB1-lys adducts were detected in plasma of groups receiving AFB1 in diet and were absent in those receiving AFB1-free diet. Male turkeys were placed in one of the four groups: LGG, LGG+AFB1, Control, and AFB1. After 10 days of pretreatment with either LGG (groups: LGG and LGG+AFB1) or PBS (groups: Control and AFB1), 1 ppm AFB1 was added to the diets of two AFB1-treated groups. Birds received LGG, PBS and AFB1 for the next 11 days, depending upon the group assignment. Each bar represents the mean ± SD (n = at least 9).
CHAPTER 6
SUMMARY AND FUTURE WORK

Extreme sensitivity of turkeys to AFB\textsubscript{1} is explained in part, by the presence of efficient epoxidating P450 1A and 3A homologues. This research project aimed to identify and characterize these P450s in turkeys. Further research focused on determining the relative importance of P450 1A5 and 3A37 in AFB\textsubscript{1} epoxidation and to elucidate the detailed metabolism of AFB\textsubscript{1} in turkey liver microsomes. In another task, the chemopreventive action of probiotic bacteria, \textit{Lactobacillus}, was studied on AFB\textsubscript{1} toxicity in turkeys. Because of their extreme sensitivity, turkeys make an excellent model to study AFB\textsubscript{1} chemopreventives.

The results of this study identified a CYP3A4 homologue, CYP3A37, from the turkey liver. The gene for this P450 was found to have an ORF of 1512 bp, and the protein was predicted to be 504 amino acids with 97% identity to chicken CYP3A37. Our results demonstrates the utility of deletion of hydrophobic N-terminal nucleotide sequences (5’ – end) to enhance the expression of P450s in \textit{E. coli}. The N-terminal truncated CYP3A37 expressed in \textit{E. coli} produced substantial amounts of active P450 to support the catalytic activities. The sequence of CYP3A37 was 66% identical to human CYP3A4 and the catalytic activities of the \textit{E. coli} expressed protein closely resembled that of its human counterpart. For example, CYP3A37 biotransformed AFB\textsubscript{1} to \textit{exo}-AFBO and AFQ\textsubscript{1} and possessed
nifedipine oxidase activity, both of which were inhibited by CYP3A4 inhibitor 17α-ethynylestradiol. Like the human CYP3A4, the kinetics of the oxidation of AFB₁ to exo-AFBO and AFQ₁ by CYP3A37 conformed to sigmoidal Hill kinetics, suggesting that there be an allosteric interaction between AFB₁ and CYP3A37, indicative of positive cooperativity. Of particular importance was the metabolism of AFB₁ by the *E. coli* expressed protein of CYP3A37 to exo-AFBO. Exo-AFBO, a highly reactive electrophilic intermediate, is responsible for the carcinogenic and mutagenic effects of AFB₁. Thus, we concluded that CYP3A37 along with CYP1A5 plays an important role in the extreme sensitivity of turkeys to AFB₁.

After the characterization of both CYP1A and 3A homologues from the turkey liver, we attempted to determine their relative importance in AFB₁ epoxidation, in turkey liver microsomes. The combined presence of CYP1A5 and 3A37 in turkey liver, both of which metabolized AFB₁ to the toxic exo-AFBO and to detoxification products AFM₁ and AFQ₁, respectively, complicated the kinetic analysis of the oxidation of this mycotoxin in turkey liver microsomes. Although both the enzymes are efficient epoxidators of AFB₁, the observed kinetics of the AFB₁ oxidation for CYP1A5 differed from those of CYP3A37. While CYP1A5 showed hyperbolic Michaelis-Menton, CYP3A37 exhibited sigmoidal Hill’s kinetics, indicative of an allosteric interaction, for the formation of AFB₁ oxidation products.
Immunoinhibition studies, utilizing anti-peptide sera directed against CYP1A5 and 3A37, revealed that CYP1A5 very likely is the dominant homologue in the formation of *exo*-AFBO at real world *in vivo* concentrations of AFB₁, in the turkey liver. Microsomal metabolism studies, in the presence or absence of antiserum, using AFB₁ concentrations ranging over 0.1-1000 µM, showed that CYP1A5 is the major contributor to the formation of *exo*-AFBO at relatively low concentrations of AFB₁, reflective of those potentially encountered through dietary exposure. CYP3A37, however, efficiently activated higher AFB₁ concentrations, not likely to be achievable in turkey liver *in vivo*. We also concluded that CYP1A5 acts as the higher affinity and CYP3A37 as the lower affinity enzyme for AFB₁, as indicated by their $K_m$ values. In these experiments, the kinetics of the formation of different products of AFB₁ metabolism, in turkey liver microsomes, pretreated with either anti-CYP1A5 or 3A37 immune serum was consistent with those observed with the individual P450s expressed in *E. coli*. These studied helped us in not only determining the relative importance of CYP1A5 and 3A37, but also to confirm their metabolic specificity, in the formation of the metabolites of AFB₁.

Since AFB₁ is ubiquitous in corn-based poultry feed, we evaluated the chemopreventive potential of probiotic, LGG, on AFB₁ toxicity in turkeys. LGG binds to AFB₁, thus reducing its bioavailability and toxicity. Turkeys fed LGG in their diet showed improvements in some key endpoints of aflatoxicosis. Birds
which received LGG + AFB₁ had significantly higher body and liver weights than those of AFB₁-alone. Serum profile, histopathological analysis and AFB₁-adduct analysis showed trends toward protective effects of LGG, but the differences were not statistically significant. In conclusion, LGG offers some protection against aflatoxicosis, thus validating its use as a possible chemopreventive; however, chronic studies are required to confirm its use as a dietary additive.

The results of this dissertation have provided insight into the role of P450s in the extreme sensitivity of turkeys to AFB₁. Commercial turkeys possess P450s with high AFB₁ epoxidation potential, which is associated with their sensitivity to this mycotoxin. Our studies have helped unravel the relative importance of CYP1A5 and 3A37, which will contribute toward the development of strategies to protect poultry against the deleterious effects of AFB₁. Because CYP1A5 is likely the predominating isoform bioactivating AFB₁ at \textit{in vivo} dietary concentrations, further research investigating the chemopreventives which could inhibit CYP1A5, therefore merits study. Another direction may include development of CYP1A5 gene knock-out models for poultry and studying their susceptibility to AFB₁. Based on the results of our studies, inhibition of CYP1A5 may provide significant improvements in AFB₁-associated toxicity in turkeys. In fact, the dietary antioxidant BHT was found to have protective effects on AFB₁ toxicity, in part, because of its inhibitory effect on CYP1A5.
Several evidences point towards the presence of P450s capable of metabolizing xenobiotics in the intestines in other species; although in very small quantities as compared to liver. Therefore, future work may include examination of those P450 isoforms in the intestines from turkeys, which may play a role in AFB\textsubscript{1} epoxidation, before it reaches the liver.

Our laboratory has established that the extreme sensitivity of turkeys to AFB\textsubscript{1} is due, in part, to an unfortunate combination of efficient P450 mediated epoxidation and deficient detoxification by GSTs. Commercial turkeys lack isoforms of GSTs capable of conjugating the exo-AFBO. Future work, which is currently on going in our laboratory, includes examination of the GSTs in wild turkeys. Although direct comparisons have not been made, wild turkeys appear to be relatively resistant to AFB\textsubscript{1} as compared to their commercial counterparts. The protective traits might have been lost in commercial turkeys, due to the intense pressure of selective breeding. By identifying the relevant genetic markers for the AF resistance, the protective traits in the commercial varieties, may well be restored.

Additional work may be done to study other metabolic detoxification enzymes, for example, epoxide hydrolase (EH). This enzyme acts on exo-AFBO and converts it into a dihydrodiol derivative, which is hydrophilic and thus could be excreted from the body. Single nucleotide polymorphisms in the microsomal EH have been associated with the risk of development of
hepatocellular carcinomas in humans; especially in regions where AFB1 contaminated foods constitute a staple diet.
January 28, 2010

Sumit Rawal
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Dear Dr. Kim,

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Utah State University
Logan UT 84322

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I am glad to hear you will be defending your thesis this Spring. I have received your request to use the published manuscript "Rawal S, Reed KM, Mendoza KM and Coulombe RA Jr. (2009). Structure, genetic mapping, and function of the Cytochrome P450 3A37 gene in the turkey (Meleagris gallopavo). Cytogenetics and Genome Research; 125:67-73." as a chapter in your thesis. I see no reason why this should not included as part of your thesis and give you my permission to do so.

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February 16, 2010
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Shirley Yip <shirleyyipsm@gmail.com>  
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Hi, Sumit,
I approved your request to include the following paper in your dissertation. Congratulations to your successful thesis defense. Good luck in your next endeavor!

Shirley.

On Mon, Mar 15, 2010 at 12:49 PM, Sumit Rawal <sumit.rawal@aggiemail.usu.edu> wrote:
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> Thanks.
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> Best Regards,
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1998-2002: Bachelor of Pharmacy: Delhi Institute of Pharmaceutical Sciences and Research, University of Delhi, New Delhi, India

Research Experience:
Research areas: Molecular and biochemical toxicology; Xenobiotic metabolism; Enzyme kinetics and inhibition; Toxicokinetics/Pharmacokinetics; chemoprevention

Skills:
• Molecular biology: RNA and DNA isolation, 3’ and 5’ RACE, PCR, primer construction, molecular cloning, expression/plasmid vector construction, site directed mutagenesis, heterologous gene expression, protein purification, western blotting
• Instrumentation: HPLC, UV-visible spectrophotometry, fluorescence spectrophotometry, ELISA
• Laboratory animal handling: rats, mice, rabbits, poultry etc; dosing (various routes) and dissection, sample collection, tissue preparation for assays, histology, protein quantification
• In-vivo animal studies, In-vitro drug metabolism assays and Enzyme kinetics
• BLAST analysis, ClustalW multiple sequence alignment and ExPASy protein sequence analysis
• Software proficiency: Microsoft office, SigmaPlot, GraphPad Prism, SAS programming, EndNote and DNASTAR Lasergene
Work Experience:

- **Graduate Teaching and Research assistant**: Utah State University, Logan, Utah, 2005-Present
- **Clinical Research Associate**: CliniRx Research Pvt Ltd, Gurgaon, India, 2004-2005
  - Skills: ICH/GCP & FDA guidelines for conduct of clinical trials, knowledge of clinical drug development process, phase II & III trials and pharmacovigilance
  - Assisted project manager in preparation of clinical protocol
  - Responsible for site management and monitoring
  - Verified study documents to ensure data accuracy
  - Provided continuous training of site personnel on GCPs
  - Giving presentations in investigator meetings
  - Ensured adherence of clinical study to established protocols, SOPs, IRBs and regulatory requirements

Publications:

- **Rawal S** and Coulombe RA Jr. Metabolism of Aflatoxin B1 in turkey liver microsomes: the relative roles of Cytochrome P450 1A5 and 3A37. *In Preparation* Toxicological Sciences

Abstracts and Presentations:

- **Rawal S** and Coulombe RA Jr. Cloning, Expression and Partial Characterization of Cytochrome P450 3A37 from Turkey liver that oxidizes

• Strategies in the new drug discovery. Toxicology Seminar Series, Utah State University, 2006.

• Informed Consent in clinical trials. Investigator’s Meeting for a global cardiology trial. Imperial Hotel, New Delhi, India 2005.

Awards and Honors:

• Utah State University, E.L. and Inez Waldron Biotechnology Endowment Fund travel award (2008).

• Food Safety Specialty section best poster award, presented at Society of Toxicology annual meeting in Seattle (2008).

• Utah State University, Awarded competitive Graduate Departmental Assistantship (2005).

• GATE-2002 (Graduate Aptitude Test in Engineering): 99.69 percentile (All India Rank: 57 out of approximately 12000 applicants), conducted by Indian Institute of Science, Bangalore.

• University Grants Commission, University of Delhi, Awarded two years scholarship for pursuing Master of Pharmacy (2002). An eligibility criterion is excellent performance in GATE.

Professional Affiliations:

• Society of Toxicology, Reston, VA USA, 2006-present

• Delhi Pharmacy Council, New Delhi, India (Registered Pharmacist), 2002-present

• Indian Pharmacological Society