MOLECULAR INSIGHTS INTO XENOBIOTIC DISPOSITION IN AUSTRALIAN MARSUPIALS

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ABSTRACT
During the past two decades, studies of xenobiotic detoxification by molecular biology in diverse organisms have identified many novel environmental adaptations, providing valuable insight into habitat, dietary preferences and general physiology. While xenobiotic detoxification has been extensively studied in eutherian mammals, metabolic data concerning detoxification in Australian marsupials is limited, particularly at the molecular level of the enzymes involved. At present Australia relies heavily on overseas data to determine the possible outcomes of xenobiotic exposure in Australian native fauna. Unlike eutherian mammals, many marsupial herbivores ingest and absorb large amounts of dietary Eucalyptus terpenes. Such quantities would be toxic, even potentially fatal, to human and many other mammalian species. Specialist Eucalyptus herbivores, such as koalas and brushtail possums, have been hypothesised to utilise highly efficient enzyme systems to metabolise terpenes to non-toxic substances that can be readily excreted in the urine. Enzymes that carry out the biotransformation of Eucalyptus terpenes have been partially identified to be the cytochromes P450 (CYP). The aim of this review is to provide a summary of work being undertaken over several years in our laboratories that has provided unique insights into marsupial biology. The focuses of this study are phase I and phase II metabolisms in these unique animals, the multiplicity of metabolising enzymes/pathways involved, induction/inhibition of CYPs/other enzymes by dietary Eucalyptus terpenes and to update current knowledge of xenobiotic metabolism in Australian marsupials. The important role of marsupial genome studies in identifying evolutionary relationships and functions for mammalian genes as well as in conservation, ecology and pest management of marsupial species is also briefly highlighted.

Key words: marsupials; Eucalyptus; terpenes; metabolism; cytochrome P450 CYPs.

INTRODUCTION
During the past two decades, molecular biology has revealed the existence of a multitude of enzymes that act as the interface between an organism and its chemical environment. These enzymes, collectively termed drug- or xenobiotic-metabolising enzymes in fact participate in many critical life processes in diverse organisms including bacteria, plants and animals. Studies of these enzymes in a variety of organisms have identified many novel environmental adaptations and provided an insight into habitat, dietary preferences and general physiology (McKinnon and Nebert 1994; Dieter and Nebert 2000).

Like humans and other mammals, Australian marsupials are constantly exposed to a diverse array of exogenous compounds that are collectively termed xenobiotics. These xenobiotics come from a wide variety of sources, including natural toxins in the diet, industrial and agricultural contaminants, and other environmental exposures. Enzymatic modification of xenobiotics can potentially alter their activity and toxicity. Knowledge of these processes is vital to understanding the consequences of exposure to xenobiotics, whether these are from the xenobiotic itself or from its metabolites. To date, extensive studies of xenobiotic detoxification have been undertaken in eutherian mammals, primarily humans, mice and rats. In sharp contrast to the plethora of studies in eutherian mammals, few studies have been undertaken in marsupials.

Many of Australia’s marsupial species are experiencing population decline. One of the main reasons for this may include exposure to foreign chemicals (Bolton and Ahokas 1995), apart from loss of habitat (through urbanisation, fires or agriculture), introduced species - predation and competition for food source. Of particular interest is the exposure to foreign chemicals, as the extent of chemical exposure in Australian marsupials is unknown.

Australian marsupials are significant and unique Australian fauna. Marsupials differ greatly from eutherian (placental) mammals in many aspects of their development and physical characteristics. These differences may alter their ability to tolerate environmental chemical exposure. Relative to eutherian animals, marsupials have a significantly shorter gestation period, often within a single oestrus cycle, resulting in limited intrauterine organogenesis. This leads to a very small, extremely undeveloped neonate in whom all the organs are defined but few are functional (Tyndale-Biscoe 1973; Bolton and Ahokas 1995). With respect to most of their organs and systems, marsupial neonates appear not to reach the same stage of functional development as a neonatal eutherian until several months after birth. The majority of the marsupial neonatal growth phase happens in an air breathing environment and nutrition is obtained from the maternal milk from their mother, therefore marsupial neonates are exposed to xenobiotics at a very early stage when they have few mature organs (Bolton and Ahokas 1995). As marsupial neonates are potentially exposed to environmental chemicals earlier in

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Metabolism in Australian marsupials

El-Merhibi et al

life and are lacking the maternal detoxification system, it is postulated that the detoxifying enzyme system of marsupials may have evolved differently from that of the placental mammals. Little is known about the ability of marsupials to metabolise and detoxify these chemicals.

While most animals tend to select food low in potential toxic chemicals, several marsupials rely on Eucalyptus leaves as a major or only food source (Stupans et al. 2001). These leaves contain a high proportion of toxic plant secondary metabolites, mostly phenolics and monoterpens. Several Australian marsupials possess the capability to ingest and metabolise a range of dietary terpenes and phenols that would be toxic to many other mammalian species (Cronin 1987; McLean and Foley 1997; Foley and Moore 2005; McLean and Duncan 2006). In order to ingest and absorb such large quantities of monoterpens, Eucalyptus feeders must have evolved highly specialised detoxification mechanisms. These mechanisms presumably involve several liver enzyme systems, including the cytochromes P450 (McLean and Foley 1997).

The presence of dieldrin (organochlorine pesticide) residues was reported in an Eastern barred bandicoot (Perameles gunni) population in Western Victoria in 1995 (Bolton and Ahokas). It was also reported that organochlorine residues could be expected in kangaroo, wallaby and wombat populations which reside near farmland since farm livestock were reported to have such residues. The impact that environmental chemicals have on marsupials once ingested will ultimately depend on their ability to metabolise them. At present we rely heavily on overseas data to determine the possible outcomes of xenobiotic exposure in Australian native fauna (Bolton and Ahokas 1995; Stupans et al. 2001). This indicates that there is a clear need for metabolic studies concerning detoxification in Australian marsupials, particularly at the molecular level. The aim of this review is to provide a summary of work being undertaken over several years in our laboratories and to provide our current knowledge of xenobiotic metabolism in Australian marsupials.

XENOBIOTIC METABOLISM

The term ‘xenobiotic’ refers to chemical agents that are foreign to the body. While the enzymes that are involved in the biotransformation of xenobiotics are collectively termed xenobiotic- or drug-metabolising enzymes, many of these enzymes also participate in the metabolism of important endogenous compounds. Major enzyme families involved in xenobiotic metabolism include the cytochrome P450 superfamily (CYPs), UDP glucuronosyltransferases (UGTs) and sulphotransferases (STs). The CYPs, which were formerly referred to as Mixed Function Oxidases (MFOs), catalyse an extensive variety of reactions including hydroxylations, N-, O-, and S-dealkylations, epoxidations and N- and S-oxidations, frequently referred to as Phase I reactions. Other enzymes catalysing Phase I metabolism of xenobiotics include the flavin-containing monoxygenases (FMOs), the aldehyde and alcohol dehydrogenases and xanthine oxidase. Phase I metabolism is commonly a prerequisite for subsequent conjugative metabolism, frequently termed Phase II metabolism. Phase II metabolism is catalysed by several families of enzymes including the UDP glucuronosyltransferasexes, sulphotransferases, N-acetyl transferases and glutathione transferases. A wealth of data from studies of these enzyme families has been presented in several reviews (Nebert et al. 1987; Nebert and Gonzalez 1987; Gonzalez and Nebert 1990; Guengerich 1993; Nebert 1994; Guengerich andParikh 1997; Hirvonen 1999; Omiecinski et al. 1999; Radominska et al. 1999; Salinas and Wong 1999; Glatt et al. 2000; Oppermann and Maser 2000).

The cytochrome P450 superfamily plays the predominant role in the detoxification of most xenobiotics. The cytochromes P450 compromise a superfamily of membrane-bound haemoproteins that catalyse the oxidative metabolism of various endogenous and exogenous compounds, including steroids, fatty acids, drugs and environmental toxicants (Nebert and McKinnon 1994; Miller et al. 1996; Gonzalez and Lee 1996; Hasler et al. 1999; Roberts 1999; Ortiz De Montellano and De Voss 2005). Cytochrome P450 enzymes are of fundamental importance given their multiplicity, catalytic diversity and widespread distribution throughout the ecosystem (Hasler et al. 1999; Isin and Guengerich 2007). CYP genes have been identified in animals, plants, yeasts, fungi and bacteria with individual CYP genes regulated in a temporally controlled, xenobiotic-sensitive and tissue-specific manner.

All genes encoding CYPs are believed to have derived from a single ancestral gene existing more than 3.5 billion years ago, with many CYP genes highly conserved across species (Stoilov 2001). The presence of CYPs in organisms as diverse as bacteria and humans is a reflection of the importance of the oxygen cleavage reaction that they catalyse. Through evolution, nature has adapted this reaction to suit the requirements of numerous different synthetic and degradative pathways (Nebert and McKinnon 1994; Gonzalez and Lee 1996; Miles et al. 2000). Functionally, CYPs may be divided into three broad groups although considerable overlap exists: those that are involved primarily in xenobiotic metabolism (CYP1, CYP2, CYP3, and CYP4), those involved in steroidogenesis (CYP2, CYP11, CYP17, CYP19, CYP21) and those that participate in other important endogenous functions including calcium homeostasis (CYP4, CYP5, CYP7, CYP8, CYP24, CYP26, CYP27, CYP46, and CYP51) (Nebert and McKinnon 1994; Gonzalez and Lee 1996; McKinnon 2000). The majority of xenobiotic-metabolising enzymes are limited to families CYP1, CYP2 and CYP3. The mammalian CYP1 family has received significant attention as members of this family are readily inducible by compounds such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and polycyclic hydrocarbons such as Benzo[a]pyrene. Consequently, this has resulted in considerable interest in the use of CYP1A1 as an environmental biomarker of contamination with relevant chemical inducers. Enzymes within this subfamily are also able to activate environmental chemicals, mainly aromatic hydrocarbons and amines (Nebert and McKinnon 1994). The CYP2 family is the most complex CYP family and is involved in the metabolism of many drugs and environmental chemicals (Lewis 1998). The CYP3 family has the widest substrate specificity and is highly expressed in the liver and gastrointestinal tissues. The CYP4 family, whilst being involved to a limited degree in xenobiotic metabolism is
primarily responsible for the hydroxylation of saturated and unsaturated fatty acids (Nebert and McKinnon 1994; Hsu et al. 2007).

To date, there has been a total of 6008 named CYP sequences (Nelson 2008). This includes 2255 animal sequences, 1918 plant sequences, 205 lower eukaryote sequences, 1001 fungi sequences, and 629 bacterial sequences. Of the 2255 animal sequences, 6 belong to Australian marsupials (Nelson 2008). These include tammar wallaby (Macropus eugenii) CYP1A1, koala (Phascolarctos cinereus) CYP2C47 and CYP2C48, koala CYP4A15, and tammar wallaby CYP4B1 and CYP4B3.

FOLIVORE MARSUPIALS

Eucalyptus species are native to Australia although they have now been introduced into at least 90 other countries worldwide. They are widely distributed throughout Australia and grow in a wide range of climatic environments (Elliott and Jones 1984; reviewed in Ghisalberti 1996). The Eucalyptus forests of Australia are a food source for a number of Australian marsupial species even though Eucalyptus leaves contain low protein, high fibre and also contain high proportions of phenolics and essential oils. The composition of Eucalyptus oil depends on the species, the age of the leaf, season and locality of the trees (Cork and Foley 1997). Essential oils in Eucalyptus species are comprised of complex mixtures of monoterpene, including 1,8-cineole and significant amounts of α-pinene, d-limonene, and p-cymene (McLean et al. 1993; McLean and Foley 1997). One advantage to eating Eucalyptus foliage is the high water content present in the leaves. Mature leaves contain between 40-50% water, whilst younger leaves contain 50-60% water (Batish et al. 2006). The marsupials in Australia that feed exclusively on Eucalyptus foliage are classified as specialist feeders. Other marsupials that combine Eucalyptus foliage with other food sources are classified as generalist feeders (Hume 1982; Boyle et al. 2001).

The koala (Phascolarctos cinereus) is capable of living wholly on the leaves of certain Eucalyptus species (Lee and Martin 1988), while the brushtail possum (Trichosurus vulpecula) relies on Eucalyptus leaves as a major food source. Approximately one third of the koala’s digestible food intake has been thought to come from the digestion of phenolics, however, it is likely that the vast majority of phenolics ingested is excreted after metabolism, contributing probably little energy to the animal. Similarly, as much as 16% of the total digestible energy intake in the koala may be absorbed as essential oils (Cork et al. 1983; Cork and Sanson 1990). Other marsupial Eucalyptus feeders include the greater glider (Petauroides volans) and the ringtail possum (Pseudocheirus peregrinus) (Hume 1982; Cork et al. 1983; Foley 1987; Foley et al. 1987). It has been long postulated that specialist Eucalyptus herbivores such as the koala use highly efficient phase I and II enzyme systems to metabolise the plant secondary metabolites to non-toxic substances, which can then be readily excreted in the urine (McLean and Foley 1997). A number of studies have reported that the oils from Eucalyptus are well absorbed (Eberhard et al. 1975; Foley et al. 1987; McLean et al. 1993; McLean and Foley 1997), with the initial thought that the gut microflora may have been involved in the metabolism of Eucalyptus constituents such as terpenes (Freeland and Janzen 1974). However, studies by Foley (1987) and McLean et al. (1993) have reported that in the greater glider and the brushtail possum, Eucalyptus oils were completely absorbed before they reached the hindgut where the most abundant microflora is present. Enzymes that catalyse the biotransformation of Eucalyptus constituents of the unique diet of these herbivores animals have yet to be identified. The metabolic adaptations at the molecular level of these animals are still speculative.

XENOBIOTIC METABOLISM IN AUSTRALIAN MARSUPIALS

Since Freeland and Janzen (1974) suggested that the limitations in enzyme detoxifying mechanisms would be an important factor in determining the feeding requirements of mammalian species, numerous studies have investigated the metabolism of toxic dietary compounds such as plant secondary metabolites in Australian marsupials. The focus of these studies has been on the chemical analysis of the numbers and types of metabolites excreted after ingestion of these dietary compounds as well as the implications of detoxification of plant secondary metabolites for digestive behaviour of marsupials (McLean et al. 1993; McLean and Foley 1997; Boyle et al. 1999; Dearing and Cork 1999; Boyle et al. 2000a; Boyle et al. 2000b; Stapley et al. 2000; Boyle et al. 2001; Wiggins et al. 2003; Boyle and McLean 2004; McLean et al. 2004; Boyle et al. 2005; Marsh et al. 2006; Wiggins et al. 2006a; Wiggins et al. 2006b, Wiggins et al. 2006c; McLean et al. 2007). In contrast, there have been relatively few studies investigating the enzymology of the detoxification processes, particularly at the molecular level. The few studies that have examined detoxification enzymes, such as CYPs in Australian marsupials have provided information on expression and substrate specificity of CYP families, but little work has been performed on the characterisation of individual CYP enzymes (Stupans et al. 2001).

Phase I metabolism

McManus and Ilett (1976) were the first to report the paucity of information regarding the metabolism of xenobiotics in Australian marsupials. Two primary findings were reported from experiments on the mixed function oxidase (MFO) system in the quokka (Setonix brachyurus). Firstly, the quokka generally had lower rates of oxidative metabolism compared with the rat. Secondly, induction of MFO system following ingestion of phenobarbital (barbiturate) has been found in this animal, indicating that this marsupial may be able to react to environmental contaminants by also inducing their MFO system.

These initial findings were supported in the subsequent studies by McManus and Ilett (1977) on the MFO system in the bettong (Bettongia penicillata), grey kangaroo (Macropus fuliginosus), quokka, brushtail possum and the short-nosed bandicoot (Isoodon obesulus). All of these marsupials were shown to generally have lower MFO activity when compared with rat. These initial studies highlighted the in vitro differences in xenobiotic metabolism in the liver between...
Lasiorhinus latifrons metabolism of that mediates induction of cDNAs by possums however at a slower rate when compared with humans. Midazolam was also metabolised studies revealed that the formation of the quinine metabolites to be faster in possums compared with humans. Inhibition as the primary metabolic pathway as occurs in humans, possum. The possums formed the 3-hydroxylated metabolite quinine and midazolam (CYP3A markers) in the brushtail possum. Ho et al. (1998) investigated the formation of NADPH cytochrome c reductase activity in the brushtail possum. Enzyme expression in 60-day-old possums was between 5 and 10% of the adult level. A significant increase in expression was observed between 150 and 180 days old with hepatic cytochrome P450, cytochrome b₅, glutathione transferase content, ethoxycoumarin-O-deethylase (ECOD) and ethoxyresorufin-O-deethylase (EROD) activities (CYP1A markers), and aldrin epoxidation activity all reaching adult levels by this time. However, NADPH cytochrome c reductase activity had not reached adult levels in the oldest animals studied (200 days old). These studies highlighted a significant delay in the expression of xenobiotic-metabolising enzymes in marsupials relative to eutherians. As a result of this developmental delay, marsupials may be more vulnerable to environmental chemical exposure during their comparatively extended development.

An additional study conducted by Bolton and Ahokas (1997b) compared urban and non-urban brushtail possum populations. Cytochrome P450 content, cytochrome b₅, and NADPH cytochrome c reductase as well as xenobiotic-metabolising activities for EROD and ECOD were reported to be similar to those observed in other mammalian species. The CYP content in non-urban possums was reported to be higher than that of urban possums. It was concluded that the non-urban possums may have had a higher CYP content due to the different diets that they encountered. It was assumed that the non-urban possum diet would be rich in Eucalyptus species, while the urban possum diet would comprise mainly of food scraps and fruit. A diet rich in Eucalyptus meant that the possums were exposed to toxins such as terpenes; hence the possums would have been faced with a greater detoxification challenge.

Ho et al. (1998) investigated the in vitro metabolism of quinine and midazolam (CYP3A markers) in the brushtail possum. The possums formed the 3-hydroxylated metabolite as the primary metabolic pathway as occurs in humans, however the in vitro metabolism of quinine was observed to be faster in possums compared with humans. Inhibition studies revealed that the formation of the quinine metabolites reflected CYP3A activity. Midazolam was also metabolised by possums however at a slower rate when compared with humans.

Over the last nine years, our laboratory has been investigating xenobiotic metabolism, with particular focus on phase I metabolism. Our studies have resulted in the cloning and characterisation of marsupial members from the CYP1A, CYP2C47, CYP2C48, CYP3A, CYP4A15 and CYP4B, as well as acyl CoA oxidases (AOXs) and the peroxisome proliferator-activated receptor c that mediates induction of CYP4A and AOXs (Ngo et al. 2003a; Ngo et al. 2007). Initial studies investigated the hepatic microsomal enzyme activity in the koala and tammar wallaby (Stupans et al. 1999). The total cytochrome P450 content in koala was found to be comparable to the rat, however the tammar wallaby had significantly lower total CYP content. Microsomal activities for NADPH cytochrome P450 reductase and aminopyrine N-demethylation, generally regarded as being associated with activity of multiple CYPs (Imaoka et al. 1988), were found to be similar between the koala, tammar wallaby and rat. The tammar wallaby was found to have higher aniline hydroxylation activity (a CYP2E1 marker) compared with koala. Both tammar wallaby and rat had higher androstenedione 6β- and 16α-hydroxylation activities (CYP 2C11 markers) compared with koala.

The characterisation of tolbutamide hydroxylase activity (a CYP2C9 marker) in the brushtail possum and koala was assessed in a study conducted by Liapis et al. (2000). It was reported that tolbutamide hydroxylase activity was higher in both marsupial species compared with rat. Utilising an immunoblotting approach with anti-rat CYP2C antibodies, we demonstrated the presence of multiple CYP2C immunoreactive proteins in both the koala and tammar wallaby (Jones et al. 1998). Subsequent cloning studies have identified two full-length CYP2C cDNAs from koala, subsequently named CYP2C47 and CYP2C48. Characterisation of CYP2C47 and CYP2C48 is currently being undertaken in our laboratories.

The CYP3A family in Australian marsupials has recently been investigated in the koala, tammar wallaby, and the southern hairy-nosed wombat (Lasiorhinus latifrons) (El-Merhibi et al. 2003). CYP3A expression was detected by immunoblot analysis in all three marsupial species. Erythromycin N-demethylation activity (a CYP3A marker) was assayed in all three marsupial species. Erythromycin N-demethylation activity had significantly lower total CYP content. Microsomal activities for NADPH cytochrome P450 reductase and aminopyrine N-demethylation, generally regarded as being associated with activity of multiple CYPs (Imaoka et al. 1988), were found to be similar between the koala, tammar wallaby and rat. The tammar wallaby was found to have higher aniline hydroxylation activity (a CYP2E1 marker) compared with koala. Both tammar wallaby and rat had higher androstenedione 6β- and 16α-hydroxylation activities (CYP2C11 markers) compared with koala.

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and Ahokas (1997b) reported significant sex differences in MFO content and activity between male and female brush tail possums from a non-urban population, however these differences were not noted in the urban population.

Molecular characterisation of CYP4A family in marsupials has been investigated (Ngo et al. 2000; Ngo et al. 2006). These studies resulted in the cloning of a CYP4A member in koala, subsequently named CYP4A15. The cloned CYP4A15 cDNA of 1544 bp has approximately 70% nucleotide and deduced amino acid sequence identity to human CYP4A11. Expression of CYP4A15 in Cos-7 cells and functional analysis indicated that CYP4A15 cDNA-expressed enzyme exhibits the same capacity to metabolise lauric acid hydroxylation (a CYP4A marker) as that of rat CYP4A cDNA-expressed enzyme. Immunoblot analysis, using an anti-rat CYP4A polyclonal antibody, detected multiple CYP4A immunoreactive proteins in both the koala and tammar wallaby, with much broader immunoreactive bands detected for koala as compared to wallaby, rat and human. Multiple CYP4A isoforms have been identified in several species studied, including human, rat, rabbit and mouse (Kawashima et al. 1994, Hardwick et al. 1987, Kimura et al. 1989; Henderson et al. 1994; Okita and Okita 2001). CYP4A mRNA expression was detected by Northern blot analysis, utilising a CYP4A15 cDNA probe. A much stronger CYP4A mRNA signal was seen for koala relative to rat and human. Lauric acid activity was also assessed in koala and tammar wallaby. Lauric acid activity was significantly higher in koala compared with tammar wallaby and rat (Ngo et al. 2000). These studies suggested higher CYP4A15 protein and mRNA expression in koala, possibly reflecting dietary influences.

**Induction/inhibition of CYPs/other enzymes by terpenes**

Numerous studies have reported that the introduction of terpenes to the diet of several species will cause an induction of CYPs. Austin et al. (1988) reported that introducing the terpenoid compounds, camphor, menthol, pinene and limonene to rats caused an induction of CYPs, specifically with members of the CYP2B subfamily. Hiroi et al. (1995) also introduced terpenoid compounds to rats and reported that although the total P450 content was not elevated, there was an induction of CYPs, specifically CYP2B1 and CYP3A2 (to a lesser extent).

Pass et al. (1999) investigated the induction of xenobiotic-metabolising enzymes by eucalyptus terpenes in the brush tail possum. The study reported that introducing a mixture of terpenes to possums that had previously been fed a diet of fruits and cereals only, caused an induction in CYP enzymes. Cytochrome P450 content in test animals was reported to be 53% higher. Aminopyrine demethylase activity (a general measure for CYPs) and androstenedione 16α-hydroxylase activity were also significantly higher in terpene fed possums compared with control possums. Aniline hydroxylase activity was also increased in the possums exposed to terpenes compared with control possums. Western blot studies utilising human CYP2E1, rat CYP2C11 and rat CYP2C6 antibodies detected the presence of CYP2E1, CYP2C11 and CYP2C6 immunoreactive proteins, with the bands being brighter in terpene-fed possums compared with control possums, thus indicating a possible increase in CYP2C and CYP2E content. The results from this study are consistent with the study conducted by Bolton and Ahokas (1997b) who also reported that diet played a role in cytochrome P450 content.

A further study by Pass et al. (2001) assessed in vitro detoxification of 1,8-cineole in koala, brush tail possum, rat and human. It was reported that detoxification through intrinsic clearance was higher in animals that included terpenes in their natural diet, supporting the hypothesis that adaptation to a *Eucalyptus* diet enables the organism to metabolise terpenes more efficiently (Pass and McLean 2002).

Ngo et al. (2003b) also investigated the induction of CYP4A by monoterpenes in the brush tail possum. Microsomal lauric acid hydroxylase activity (a measure for CYP4A) was 22% higher in possums given a diet consisting of 1,8-cineole, -pinene, d-limonene and p-cymene compared with those of possums fed a diet of fruits and cereals only. Northern blot analyses detected an insignificant increase in CYP4A mRNA level in treated possums relative to that of control possums, however acyl CoA oxidase mRNA level was significantly higher. A 202% higher acyl CoA oxidase activity was also detected in treated possums relative to that of control possums. These results suggest that *Eucalyptus* terpenes induce acyl CoA oxidase enzymes and possibly CYP4A (to a lesser extent).

Competitive inhibition of the metabolism of a substrate by another xenobiotic may indicate that the two compounds are metabolised by the same enzyme. Liapis et al. (2000) (discussed earlier) examined terpenes as potential inhibitors of CYP2C9 activity. 1,8-cineole was reported to be a competitive inhibitor for the enzyme responsible for tolbutamide hydroxylation. Known CYP inhibitors may also be used across species for which metabolism has not been characterised to elucidate potential CYP involvement. Pass et al. (2002) assessed the inhibition of microsomal metabolism of 1,8-cineole in the brush tail possum by using both specific and general inhibitors of human and rat CYPs. Ketoconazole (CYP3A inhibitor) was reported to be the most effective inhibitor, inhibiting the metabolism of 1,8-cineole in both possum and rat microsomes, suggesting a potential role of CYP3As in the metabolism of 1,8-cineole. Terpenes (p-cymene, limonene and α-pinene) were also used as inhibitors in this study. It was reported that in vitro metabolism of one terpene was inhibited by another terpene, suggesting co-metabolism of the tested terpenes by CYPs.

**Phase II metabolism**

Preliminary investigations into phase II metabolism in Australian marsupials have also been undertaken. The metabolism of phenol in the native Australian rodent and various marsupials has been reported (Baudinette et al. 1980). Phenol undergoes both oxidation (phase I) and conjugation (phase II) reactions. As phenol metabolites have been well characterised they have been used to investigate phase II metabolism in various marsupials. The study reported the metabolism of phenol was comparable between eutherians and marsupials, with the exception of the folivorous...
Marsupials and indicated that diet played a major role in the ability of a particular marsupial to metabolise phenol. Folivorous marsupials, such as the koala and the brushtail possum, showed very little sulfate conjugation of phenol compared with other marsupial species; however they were reported to form glucuronic acid conjugates. It is not clear as to whether this deficiency in sulfate conjugation observed in the koala and brushtail possum is due to the low levels of sulfate presented in their diet or their inability to use the sulfate conjugation pathway.

McLean et al. (1983) investigated the acetylation of sulphanilamide in four marsupial species, the Tasmanian devil (Sarcophilus harrisii), brushtail possum, pademelon (Thylogale billardierii) and the eastern barred bandicoot (Perameles gunnii). Of the marsupial species tested, only the bandicoot was unable to acetylate sulphanilamide.

Conjugation metabolism was further investigated in marsupials by Awaluddin and McLean (1985). The marsupials assessed included those with omnivorous, carnivorous, herbivorous, nectarivorous and insectivorous diets. All species were reported to metabolise benzoic acid to its glycine conjugate, hippuric acid and were able to form benzoyl glucuronide. The overall elimination of benzoic acid in marsupials was comparable to that of other mammals. No observable pattern of benzoic acid metabolism was reported between marsupial species with differing diets.

Bolton and Ahokas (1997c) purified and characterised hepatic glutathione transferases (GSTs) from the herbivorous marsupial, the brushtail possum. A single GST was reported in the brushtail possum, Possum GST 1-1, although it was noted that others may be present. Characterisation of this enzyme showed that it has a similar catalytic profile and structural homology to those belonging to the alpha class of GSTs. The most significant finding of the study was that the brushtail possum expressed a single predominant hepatic GST, with females expressing a greater amount of possum GST 1-1. This single predominant GST expression was also noted in the brown antechinus, however to a lesser extent. Five hepatic glutathione transferases were purified and characterised from the brown antechinus (Antechinus stuartii), an insectivorous marsupial (Bolton and Ahokas 1997d). Antechinus GST 1-1 which represented 71% of the total GST proteins that were purified was also reported to belong to the alpha class of GSTs. For a more detailed characterisation profile for Antechinus GST 1-1 (Ast GST A1-1), refer to Bolton et al. 1997. It has been suggested that the alpha GSTs may play a functional role in the survival of these marsupial species.

Marsupials such as koalas can excrete up to 2 to 3 grams of glucuronic acid daily. For the koala it was reported that the majority of glucuronic acid in their urine was attributable to phenols (McLean et al. 2003). Hinks and Bolliger (1957) observed an increase in the excretion of glucuronidated metabolites in the urine of the brushtail possum after being fed a diet of eucalyptus leaves. The same finding was observed in Dash (1988) who reported that glucuronic acid was excreted in the urine of the brushtail possum after being fed a diet including terpenes. However when the brushtail possums were fed a diet of bread and selected fruits and vegetables no detectable glucuronic acid was present in their urine. McLean et al. (1993) also reported that when the common ringtail possum was on a terpene free diet, little or no glucuronic acid was detected. However when terpenes were introduced into the diet glucuronic acid increased indicating that this pathway was involved in the elimination of terpenes.

Jones (2000) detected multiple UGT2 immunoreactive bands in hepatic microsomes isolated from the koala, tammar wallaby and wombat using goat anti-mouse UGT2 IgG. The immunoreactive bands were also detected in hepatic microsomes from koala pough young. Using an RT-PCR approach, UGT partial cDNAs from both the UGT1 and UGT2 family were isolated from koala, tammar wallaby and western quoll. Further work is needed to isolate the full length cDNAs from these marsupial species. Once these clones have been characterised it will add much-needed information on phase II metabolism in marsupials.

Hydroxysteroid dehydrogenases are an important group of enzymes which are not fully characterised in many species, including marsupials. Stupans et al. (1999, 2000) investigated the NADPH-mediated 17β-hydroxysteroid oxidoreductase activity in the koala and tammar wallaby. A five fold species difference in activity was reported between the koala and tammar wallaby. In these studies NADPH and NADP were reported to be the preferred cofactors for 17β-hydroxysteroid oxidoreductase activity in koala and wallaby, but for rat NAD was the preferred cofactor (Stupans et al. 1999; Stupans et al. 2000). Although 17β-hydroxysteroid oxidoreductase possibly plays a physiological role in marsupials, its actual role still remains unclear. It has been suggested that it may also play a role in enzymatic defence against plant chemicals (Stupans et al. 1999).

Type 1 11β-hydroxysteroid dehydrogenase enzymes were also investigated in the koala (Kong et al. 2002). Interestingly, 11β-hydroxysteroid dehydrogenase activity towards cortisol and cortisone was not detected in koala liver. These substrates have been previously used in other studies to determine 11β-hydroxysteroid dehydrogenase activity in mammals. The absence of this enzyme in koala was confirmed using immunoblot, northern and southern blotting and PCR amplification methods. Similar results were also obtained for the tammar wallaby. The contrast in activity or lack of between 17β-hydroxysteroid oxidoreductase and 11β-hydroxysteroid dehydrogenase is very interesting, once again highlighting the unique metabolism utilised by marsupials.

Collectively, these studies highlight that marsupials possess diverse, fully functional xenobiotic metabolising systems, which warrant further exploration. Studies concerning the metabolism of xenobiatics in marsupials may be increasing but the number of studies still falls well behind their counterparts, the eutherians. The studies have highlighted important interspecies differences in xenobiotic metabolism among marsupials and in identifying the multiplicity of metabolising enzymes/pathways involved.
MARSUPIAL GENOME STUDIES

The complete genome sequence of the gray shorttailed opossum (Monodelphis domestica), a member of the South American marsupial family Didelphidae, has recently been reported (Mikkelsen et al. 2007). Since the generation of this first marsupial genome, significant progress has been made in the area of marsupial genomics, including the discoveries concerning detoxification and genes associated with xenobiotic detoxification. Utilising the data from the opossum genome, Holmes and coworkers subsequently reported the characterisation and cloning of opossum carboxylesterase, a phase I enzyme that has diverse metabolic roles in the detoxification of a wide range of drugs and xenobiotics (2008). An array of data describing many recent advances in marsupial genetic research has been presented in several studies (Baker et al. 2007; Belov et al. 2007; Davidow et al. 2007; Duke et al. 2007; Duncan et al. 2007; Gentles et al. 2007; Goodstadt et al. 2007; Gu et al. 2007; Lemos 2007; Mahony et al. 2007; Devor and Samollow 2008). It has also been suggested that it is important to sequence another genome from an Australian marsupial as this will extend the data from the opossum genome and allow further detection of marsupial specific coding and noncoding elements. In addition, sampling of both the American and Australasian lineages would allow the reconstruction of the genome of their common ancestor (Mikkelsen et al. 2007).

Previously, the kangaroo genome project has been proposed by Wakefield and Graves (2003). This proposed project aimed at sequencing the entire genome of the tammar wallaby, a member of the Australian marsupial family Macropodidae. The opossum and the tammar wallaby were reported last sharing a common ancestor about 60 to 70 million years ago (Kirsch et al. 1997; Nilsson et al. 2004). According to the emergence of traits along the mammalian lineage, mammals evolved from a branch of reptiles that left no other descendants, therefore they are all equally related to birds and reptiles, with a divergence date of 315 million years (Myr) ago. Marsupials make up a single taxonomic lineages would allow the reconstruction of the genome of their common ancestor (Mikkelsen et al. 2007).

FUTURE DIRECTIONS

The current study provides an overview of research being undertaken over the last several years that has provided unique insights into xenobiotic metabolism in Australian marsupials, including studies from our laboratories. The focuses here are phase I and phase II metabolisms, the multiplicity of detoxifying enzymes/pathways involved, and induction/inhibition of CYPs by dietary Eucalyptus terpenes. The results of preliminary studies on marsupial metabolisms demonstrated that, relative to eutherian species such as humans and rats, marsupials display several significant differences in xenobiotic metabolising enzymes at both the biochemical and molecular level. Further investigations into enzymes concerning dietary Eucalyptus terpene detoxification in marsupials are critically important in identifying the significances of these preliminary observations and in understanding the adaptation of marsupial herbivores to their unique Eucalyptus diet. The cloning of marsupial CYP1A (Wooly 2004), CYP2C47 (Jones et al. 2008), CYP2C48 (Jones et al. 2008), CYP3A (El-Merhibi 2007), CYP4A15 (Ngo et al. 2006), CYP4B (Mile 2007), and AOXs (Ngo et al. 2003a) has provided useful tools for further biochemical and molecular studies. Future studies of interest include: study to examine which CYP enzymes catalyse the biotransformation of dietary Eucalyptus constituents, study to investigate the regulation of CYPs of interest at protein and mRNA levels in relevant tissues, functional analyses concerning the metabolic capacity of marsupial CYPs, and further investigation into whether dietary Eucalyptus terpenes are also inducers of other enzymes associated with terpene detoxification, apart from the already identified CYP enzymes (Pass and McLean 2002). These studies can be performed using CYP cDNA-expressed proteins, animal cell lines, enzyme assays with appropriate substrates, including dietary Eucalyptus terpenes, and cotransfection and reporter gene assay or transgenic construct containing CYP gene of interest and corresponding receptor or promoter region. Further findings that dietary Eucalyptus terpenes induce CYP enzymes would be of interest to a wider research area, which might lead to the initiation of more research work related to human and other animal species, as induction of CYP enzymes has significant toxicological implications. The availability of the opossum genome and future advances in marsupial genetic studies will also enable further molecular cloning and characterisation of marsupial genes associated with xenobiotics to be carried out, providing further invaluable insights into the metabolic capabilities of these unique animals.
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Metabolism in Australian marsupials


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