THE USE OF GLUTATHIONE IN CANINE ITRACONAZOLE-ASSOCIATED HEPATOTOXICITY

BY

NATALIE M. KIRK

THESIS

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Master's Committee:

Assistant Professor Jennifer M. Reinhart, Chair
Associate Professor Kimberly A. Selting
Clinical Assistant Professor Miranda D. Vieson

ABSTRACT

Azole antifungals are essential and effective drugs in treating a variety of superficial and systemic mycoses in veterinary species. Their activity is attributable to inhibition of fungal ergosterol by targeting cytochrome P450-dependent sterol 14α-demethylase. The most commonly used azoles in small animal veterinary medicine are itraconazole (ITZ), fluconazole (FCZ), and ketoconazole (KTZ). Owing to its greater selectivity for fungal ergosterol over mammalian cholesterol, ITZ has fewer adverse effects than KTZ. Additionally, it has a broader spectrum of activity than both KTZ and FCZ. For this reason, ITZ is used to treat a variety of fungal infections in dogs and is the treatment of choice for canine blastomycosis, a severe systemic mycosis endemic in the Midwest. Despite its efficacy, ITZ is associated with a number of adverse reactions including hepatotoxicity. The mechanism of this toxicity is unknown.

As with many drugs, oxidative damage may play an important role in ITZ-associated toxicity. In rodent studies, ITZ administration increases levels of myeloperoxidase and nitric oxide and decreases levels of hepatic glutathione (GSH) and other antioxidants. Based on this, antioxidant therapy could potentially prevent or treat ITZ-associated hepatotoxicity. Antioxidant hepatoprotectants are commonly used for a variety of liver diseases in veterinary medicine. These include GSH precursors like S-adenosylmethionine (SAMe) and N-acetylcysteine (NAC) and other antioxidants including silybin, vitamin E, and vitamin C. However, no studies have investigated the efficacy of this practice and scientific evidence is needed to provide a basis for their use. A reliable *in vitro* model to establish these principles is required prior to testing compounds in animal subjects.

The objective of this work was to develop an *in vitro* primary canine hepatocyte model of ITZ-associated hepatotoxicity and evaluate the effect of GSH using the model. We hypothesized that GSH would ameliorate ITZ-associated hepatocyte death *in vitro*. The results demonstrate dose and time-dependent cytotoxicity of cryopreserved primary canine hepatocytes exposed to ITZ. Pre-incubation with GSH caused a dose-dependent decrease in toxicity. This study establishes primary canine hepatocytes as an appropriate *in vitro* model for ITZ-associated toxicity. GSH pre-treatment partially ameliorates the toxic effect, suggesting a possible role for GSH and oxidative stress in the pathogenesis of toxicity. These findings support continued investigation into other antioxidants as prevention and/or treatment for ITZ-associated hepatotoxicity and their rational clinical use.

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CHAPTER 1: LITERATURE REVIEW

Introduction

Superficial and systemic mycoses are common infections in veterinary species, resulting in significant morbidity, mortality, and cost. A wide variety of antifungal drugs have been developed and used to treat superficial cutaneous infections such as malasseziosis and dermatophytosis, systemic mycoses such as blastomycosis and histoplasmosis, and mycoses caused by opportunistic fungi (Greene, 2012). The first effective systemic antifungals, griseofulvin and amphotericin B, were discovered in the 1960s (Smith, 1990). For a time, griseofulvin was the drug of choice for dermatophytosis in both human and veterinary medicine, but its use has gradually been replaced by the addition of oral azole antifungals to the market in the 1980s (Maertens, 2004). Similarly, the use of amphotericin B, which is associated with nephrotoxicity, decreased significantly after introduction of the azoles (Davis et al., 2018). In general, azoles are better tolerated and have a wider spectrum of action than the early antifungal drugs.

Itraconazole (ITZ), a triazole antifungal, is used to treat many types of fungal infections. It is the treatment of choice for canine blastomycosis, for which it has a reported cure rate of between 54-90% and a relapse rate of 18-20% (Legendre et al., 1996; Mazepa et al., 2011). Blastomycosis is endemic is certain areas of the United States, including the Ohio, Missouri, and Mississippi River valleys (Furcolow et al., 1970). A study in Illinois documented a mean estimated incidence of canine blastomycosis from 2001-2007 as 62.5 cases/100,000 dogs/year (Herrmann et al., 2011). In the same study, the mean estimated incidence in humans in Illinois was 7.5 cases/1 million persons/year. Trends in incidence and distribution were similar for humans and dogs, indicating that dogs may be a good sentinel species for human blastomycosis. Because blastomycosis causes significant morbidity and mortality in dogs and other mammals, including human beings, a safe and effective treatment is essential. Despite its efficacy, ITZ is associated with a number of adverse reactions, including anorexia, lethargy, vomiting, and hepatotoxicity (Legendre et al., 1996; Mazepa et al., 2011). Although drug discontinuation generally results in reversal of these changes, switching antifungal drugs can negatively impact the course of the mycosis if alternative drugs are less effective or result in their own adverse reactions.

Hepatoprotectants are commonly used to treat liver enzyme elevation due to ITZ administration in dogs. These include S-adenosyl-L-methionine (SAMe) and N-acetylcysteine, which are important in the synthesis and maintenance of cellular glutathione (GSH) levels (Bottiglieri, 2002; Lauterburg et al., 1983). GSH is an endogenous antioxidant critical for cell survival (Yuan and Kaplowitz, 2009). Other antioxidants such as silybin, vitamin E, and vitamin C are also used in dogs with clinical changes compatible with ITZ-associated hepatotoxicity. Many of these products are expensive and can lead to inappetence, so scientific evidence is needed to provide a basis for their use. The objective of the work presented in Chapter 2 is to evaluate the effect of GSH treatment on ITZ-associated hepatocyte toxicity using an *in vitro* primary canine hepatocyte model.

The Azole Antifungals

Azole antifungals are commonly utilized to treat a variety of fungal infections in dogs, as outlined above. There are two main groups of azoles, the imidazoles and the triazoles. These two groups have the same mechanism of action but differ in their structure and spectrum of activity (Table 1.1).

Mechanism of action of azole antifungals

Azoles target the fungal cell membrane by inhibiting the formation of ergosterol. Ergosterol is the primary sterol found in the fungal cell membrane, in contrast to mammalian cells that utilize cholesterol. Specifically, azoles inhibit the cytochrome P450-dependent (CYP) lanosterol 14α-demethylase (CYP51). Selective toxicity of a particular azole is dependent on its preferential binding of fungal CYP over mammalian CYP. Azoles inhibit CYP51 by binding nitrogen to the heme ferric core, preventing the demethylation of 14α-methylsterols and decreasing ergosterol synthesis (Davis et al., 2009). These perturbations lead to altered membrane fluidity and function, changes in membrane bound enzymatic activity, and disordered chitin synthesis (Vanden Bossche, 1985). At clinically achievable concentrations, azoles are fungistatic. Certain azoles, including miconazole, econazole, and clotrimazole, also alter membrane lipid organization and function, which may explain their antimicrobial activity against some Gram-positive bacteria (Vanden Bossche et al., 2003).

Imidazoles

The imidazoles have an azole ring containing two nitrogen atoms and include ketoconazole (**Figure 1.1**), clotrimazole, miconazole, and enilconazole (Davis et al., 2009). Clotrimazole, miconazole, and enilconazole are restricted to topical use for a variety of reasons. Oral clotrimazole is poorly tolerated and the pharmacokinetics are unpredictable, likely due to induction of hepatic microsomal enzymes (Burgess and Bodey, 1972). Miconazole has poor oral absorption as well as significant vehicle-related, adverse histamine-mediated effects in the parenteral form (Wade et al., 1979), which is no longer available in the United States. Miconazole and clotrimazole are mainly used for superficial mycoses, including dermatophytosis and malasseziosis. Enilconazole, on the other hand, is only approved for use in the U.S. as an environmental decontaminant in poultry facilities. However, it has been effective in topical treatment of cats infected with *Microsporum canis* (Hnilica and Medleau, 2002) and dogs with nasal aspergillosis (Sharp et al., 1993). Of the imidazoles, ketoconazole is the only drug approved for systemic use.

Ketoconazole

Ketoconazole (KTZ) was approved by the Food and Drug Administration (FDA) as a systemic antifungal drug in 1981 (Maertens, 2004). KTZ requires an acidic environment for absorption. Because feeding stimulates gastric acid secretion, absorption is increased after a meal. For nearly a decade, KTZ was the only systemic azole antifungal drug available. In human medicine, the use of KTZ has been largely replaced by the safer triazoles; in veterinary medicine, however, KTZ is still frequently used due to its efficacy and lower cost. KTZ is effective against superficial fungi like dermatophytes, *Candida*, and *Malassezia pachydermatis* as well as systemic *Candida* spp., *Blastomyces dermatitidis, Histoplasma capsulatum*, and *Coccidioides immitis*. It has limited efficacy against *Cryptococcus neoformans, Sporothrix schenkii*, and *Aspergillus* sp. (Davis et al., 2009).

In addition to inhibiting fungal CYPs, ketoconazole is a strong inhibitor of mammalian CYPs (Maurice et al., 1991; Back and Tija, 1991), including those responsible for drug metabolism and androgen and cortisol synthesis (de Coster et al., 1988). For this reason, ketoconazole administration may result in drug interactions (Hugnet et al., 2007; Mayer et al.,

2008), testosterone suppression, and basal cortisol suppression in dogs (Willard et al., 1986). KTZ's inhibition of mammalian CYPs is sometimes exploited for other uses in veterinary medicine. For example, co-administration of KTZ with cyclosporine in dogs causes CYP inhibition, which can lower the dose and cost of cyclosporine therapy (Dahlinger et al., 1998). KTZ is also effective at reducing plasma cortisol levels in dogs with hyperadrenocorticism owing to its inhibition of steroid synthesis, although alternate therapies are preferred (Feldman et al., 1990). Vomiting, anorexia, diarrhea, and lethargy are the most common side effects of ketoconazole administration in dogs, occurring in approximately 15% of patients (Mayer et al., 2008). In this retrospective study, liver enzyme elevations occurred rarely but were not associated with icterus. KTZ is also associated with hepatotoxicity in man (Lewis, et al., 1984; Duarte et al., 1984), in which the incidence of KTZ-associated hepatotoxicity is similar to that of ITZ (Lo Re et al., 2016).

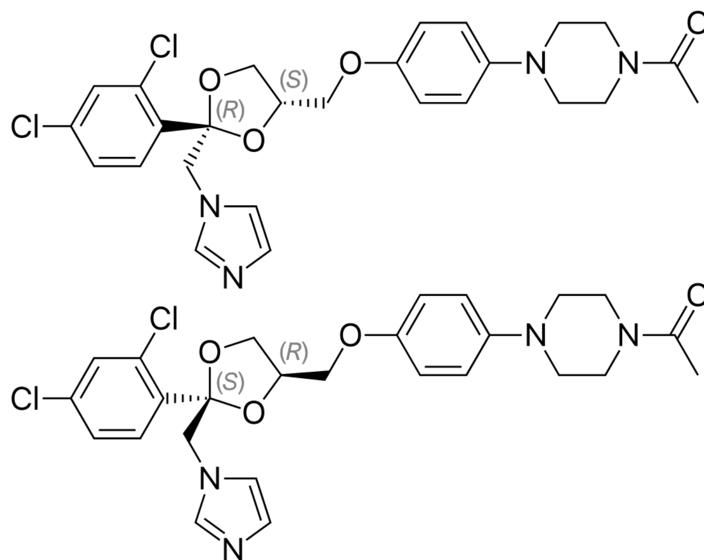


Figure 1.1. Chemical structure of KTZ (Source: https://en.wikipedia.org/wiki/Ketoconazole)

Triazoles

The triazoles have an azole ring containing three nitrogen atoms and include ITZ, fluconazole (FCZ), voriconazole, and posaconazole (**Figure 1.2**). Triazoles are more specific for fungal CYPs and have considerably less effect on mammalian steroid synthesis than the older imidazoles (Davis et al., 2009). As there are typically fewer side effects, systemic triazoles are generally preferred over KTZ.

ITZ and FCZ are the most commonly used triazoles in veterinary medicine. Voriconazole and posaconazole are newer generation triazoles used for severe or drug resistant infections (Greene, 2012).

Fluconazole

FCZ was the first triazole drug approved for use in the U.S. It is small, water-soluble, has low protein binding, and is eliminated primarily through the kidneys (Davis et al., 2018). Because of these properties, FCZ readily distributes throughout the body, penetrating aqueous sites including cerebrospinal fluid (CSF), aqueous humor, and synovial fluid (Felton et al., 2014). FCZ is recommended for treatment of central nervous system (CNS) mycoses in dogs and cats due to its small size and low protein binding, which allow it to effectively cross the blood-brain-barrier (Malik et al., 1992; O'Toole et al., 2003). Accumulation of FCZ in the CSF does not equate to parenchymal penetration due to limited rates of diffusion. In comparison, ITZ is large, highly lipophilic, and has a high proportion that is bound to protein. Its lipophilicity allows it to cross the blood-brain-barrier; however, unlike FCZ, ITZ is a substrate for the P-glycoprotein efflux pump so it is efficiently removed from the CNS (Felton et al., 2014). FCZ is frequently used to treat dermatophytosis and is also effective in treating systemic mycoses, including those caused by *Candida* sp., *C. neoformans, B. dermatitiditis*, and *H. capsulatum*. It is less effective in treating *Aspergillus* sp. and *Penicillium* sp. but has been used to treat these infections in dogs (Davis et al., 2009). Successful treatment of blastomycosis may require higher doses for FCZ for a longer treatment period when compared to ITZ (Mazepa et al., 2011; Pappas et al., 1997).

Itraconazole

ITZ was approved for use by the FDA in 1992 (Maertens, 2004) and has since largely replaced the use of KTZ in human medicine. ITZ is highly lipophilic and the original encapsulated formulation requires an acidic environment, like KTZ, for absorption. Liquid formulations complexed with hydroxypropyl-β-cyclodextrin were subsequently approved for use with better absorption profiles (Boothe et al., 1997; Barone et al., 1998). Intravenous ITZ achieves high steady state plasma concentrations faster than oral ITZ (Maertens, 2004) but is no longer available in the United States.

ITZ has several benefits when compared to KTZ. First, it more selectively inhibits fungal CYPs (Vanden Bossche et al., 1987; Vanden Bossche et al., 1990). Early studies demonstrated a

lack of inhibition of mammalian CYPs in the testis, adrenal glands, and liver *in vitro* (Vanden Bossche et al., 1990) and in the hypothalamic-pituitary-adrenal axis in dogs (Phillips et al., 1987). Subsequent studies have demonstrated that ITZ is indeed a mammalian CYP inhibitor (Back and Tija 1991; Florea et al., 2003; Isoherranan et al., 2004), although less significantly than KTZ. Depression of steroid synthesis is not observed clinically and drug interactions are less common in dogs treated with ITZ. Like with KTZ, the most common side effects are anorexia, vomiting, diarrhea, lethargy, and elevated ALT with cutaneous vasculitis and ulceration occurring less frequently (Legendre et al., 1996).

ITZ is effective in treating infections with numerous fungi, including dermatophytes, *Candida* sp., *C. neoformans, Aspergillus* sp., *Malassezia* sp., *S. schenckii*, *B. dermatitiditis*, and *H. capsulatum* (Van Cutsem et al., 1987a; Davis et al., 2009). It has a broader spectrum of activity than both FCZ (Van Cutsem et al., 1990) and KTZ (Van Cutsem et al., 1987a) and is more efficacious than KTZ in treating a variety of fungal infections in animal models (Van Cutsem et al., 1987b; Van Cutsem et al., 1987c). Historically, amphotericin B was the treatment of choice for canine blastomycosis, but due to the potential for nephrotoxicity, azole antifungals are now preferred (Legendre et al., 1996). In a retrospective study comparing the efficacy of ITZ and FCZ in the treatment of canine blastomycosis, FCZ had a similar efficacy but required an average of 45 additional days of treatment (Mazepa et al., 2011). Additionally, *B. dermatitidis* is more susceptible to ITZ *in vitro* than FCZ and KTZ (Chapman et al., 1997). For these reasons, ITZ is the treatment of choice for canine blastomycosis with a reported cure rate of between 54-90% and a relapse rate of 18-20% (Legendre et al., 1996; Mazepa et al., 2011).

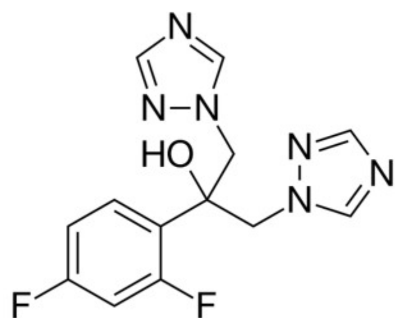
Voriconazole

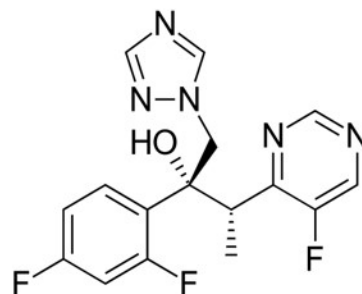
Voriconazole is a newer triazole antifungal with a similar structure to FCZ. Its spectrum of activity is similar to that of ITZ (Davis et al., 2018), although it is more potent (Greer, 2003). Voriconazole is fungicidal against *Aspergillus* sp., fungistatic against *Candida* sp., and displays *in vitro* efficacy against *C. neoformans, B. dermatitidis*, and *H. capsulatum* (Greer, 2003). In people, voriconazole is indicated for treatment of aspergillosis and other opportunistic fungi not susceptible to other antifungals (Greer, 2003). Voriconazole is small with intermediate protein binding and lipophilicity, which facilitates its movement across both the blood-CSF and blood-brain barrier (Felton et al., 2014). For these reasons, voriconazole can be used for CNS infections in veterinary patients (Bentley et al., 2011; Raghu and Reagh, 2018). It is the treatment of choice

for CNS aspergillosis in man (Felton et al., 2014). Adverse neurologic events have occurred in cats administered voriconazole orally (Quimby et al., 2010), which may be due to an extremely long half-life after short term oral administration (Vishkautsan et al., 2016). While effective, the therapeutic range is narrow in cats, so the utility of this drug is limited in feline patients (Vishkautsan et al., 2016).

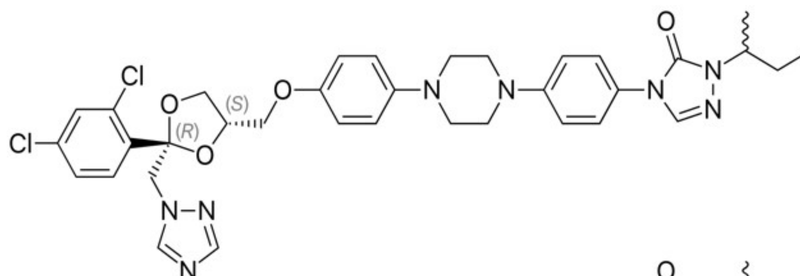
Posaconazole

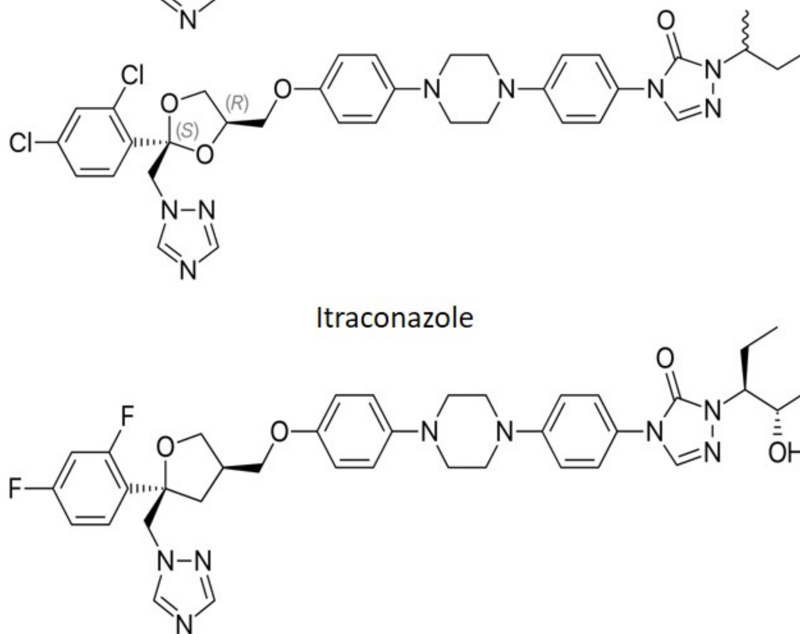
The newest triazole antifungal, posaconazole, has a similar structure to ITZ. It is used for invasive fungal infections like *Aspergillus* sp. and, unlike other azoles, has activity against Zygomycetes (Davis et al., 2018). Currently, the only FDA approved formulation in animals is the topical otic suspension Posatex®. Oral posaconazole has been used to safely treat disseminated *Aspergillus* in dogs; however, relapse was common (Corrigan et al., 2016). In a single case report, oral posaconazole successfully cured orbital aspergillosis in a Persian cat after treatment failure with ITZ and amphotericin B (McLellan et al., 2006).





Fluconazole Voriconazole





Posaconazole

Figure 1.2. Chemical structure of select imidazoles (Source: https://www.wikipedia.org/)

Table 1.1. Summary of azole antifungals

Azole	Route	Spectrum of Activity	Species	Product	Indication
Imidazoles					
Ketoconazole	PO	Dermatophytes, *Candida, Malassezia, Blastomyces, Histoplasma, Coccidioides*	D, C	Nizoral	Systemic mycoses
Clotrimazole	T	Dermatophytes, *Aspergillus, Candida, Malassezia*	D, C	Veltrim*, Otomax*, Mometamax*, Tri-Otic*, Vetro-Max*, Mometavet*	Nasal aspergillosis
Miconazole	T	Dermatophytes, *Candida Malassezia*	D, C	Conofite*, Surolan*, Easotic*, Miconosol*, Priconazole*	Topical treatment
Enilconazole	T	Dermatophytes, Aspergillosis	D, C	Clinafarm, Imaverol	Second line nasal aspergillosis
Triazoles					
Fluconazole	PO, IV	Dermatophytes, *Candida, Cryptococcus, Blastomyces, Histoplasma*,	D,C	Diflucan	CNS mycoses
Itraconazole	PO	Dermatophytes, *Candida, Cryptococcus, Blastomyces, Histoplasma, Aspergillus, Sporothrix*	D, C	Itrafungol (C)*, Sporanox	Systemic and superficial mycoses
Voriconazole	PO, IV	*Candida, Aspergillus, Cryptococcus, Blastomyces, Histoplasma*	D	Vfend	CNS mycoses, resistant aspergillosis
Posaconazole	PO, T	Zygomycetes, *Aspergillus*	D, C	Posatex (otic)*, Noxafil	Resistant aspergillosis, zygomycetosis

PO=by mouth, IV=intravenous, T=topical, D=dogs, C=cats, *=FDA approved for use in animals

<u>ITZ Metabolism</u>

ITZ is used to treat a number of systemic mycoses, but its metabolism and toxic mechanisms in animals are poorly understood. In humans and animals, ITZ is extensively metabolized by hepatic enzymes into more than 30 metabolites (Heykants et al., 1987). The main metabolite, hydroxy-ITZ (OH-ITZ) has similar antifungal activity and higher peak plasma levels than ITZ after single dose oral administration in healthy adults (Heykants et al., 1989). In man, co-administration with phenytoin, a potent CYP3A4 inducer, dramatically increases the clearance of ITZ and OH-ITZ (Ducharme et al., 1995). Similarly, serum ITZ levels are markedly decreased in patients receiving concurrent rifampicin, carbamazepine (Tucker et al., 1992), or phenobarbital (Bonay et al., 1993), other known CYP inducers. The increased clearance and decreased serum ITZ levels in the presence of CYP3A4 inducers implicates this enzyme in the *in*

vivo metabolism of ITZ. The role of CYP3A4 is further supported by the work of Isoherranen et al. (2004) who showed that ITZ is efficiently metabolized into OH-ITZ by heterologously expressed CYP3A4 Supersomes. In this study, additional unknown metabolites were identified as keto-ITZ and N-desalkyl-ITZ (ND-ITZ) by mass spectrometry. The group proposed a sequential pathway of ITZ hydroxylation into OH-ITZ followed by oxidation into keto-ITZ and subsequent oxidation into ND-ITZ (**Figure 1.3**). They also demonstrated that the metabolism of midazolam, a CYP3A4 substrate, is decreased in the presence of ITZ or OH-ITZ, showing that ITZ and its metabolites are competitive inhibitors of CYP3A4. In further support of ITZ as a CYP inhibitor, ITZ inhibits cyclosporine metabolism both *in vitro* (Back and Tija, 1991) and clinically (Florea et al., 2003). After oral administration in man, ITZ exhibits nonlinear, dose-dependent kinetics as demonstrated by disproportionately increased plasma concentrations, area under the curve (AUC), and elimination half-life after multiple doses (Van Peer et al., 1987; Hardin et al., 1988). These nonlinear, dose-dependent pharmacokinetics could be explained by competitive inhibition of ITZ metabolism by its own metabolites. A similar metabolic pathway for ITZ is suspected in dogs as OH-ITZ has been identified in canine serum after oral administration of ITZ (Yoo et al., 2002), but the specific enzyme responsible for its metabolism has not been established.

There are significant species differences in CYP enzymes between man and dog. Canine orthologs to human CYPs containing homologous genes may have a similar function or display variations in substrate specificity and enzyme activity (Martinez et al., 2013). Two CYP3A enzymes have been identified in dogs, CYP3A12 (Ciaccio et al., 1991) and CYP3A26 (Fraser et al., 1997). The amino acid sequences of these enzymes are 96% identical (Fraser et al., 1997). They are differentially expressed, with CYP3A26 comprising 75.2% of hepatic CYP3A and CYP3A12 comprising 99.8% of duodenal CYP3A (Mealey et al., 2008). Canine CYP3A12 also shares 79.8% of its amino acid sequence with human CYP3A4 (Ciaccio et al., 1991), one of four members of the human CYP3A family. For this reason, canine CYP3A12 is considered an ortholog of human CYP3A4. It is possible that CYP3A26 is also an ortholog for CYP3A4 or for another human CYP3A enzyme, although this has not been investigated. Despite their relation, canine CYP3A12 and human CYP3A4 enzymes may not share the same substrates. For example, midazolam, which is primarily metabolized by CYP3A4/CYP3A5 in man (Wandel et al., 1994) is metabolized primarily by CYP2B11 in dogs rather than CYP3A12 (Mills et al., 2010).

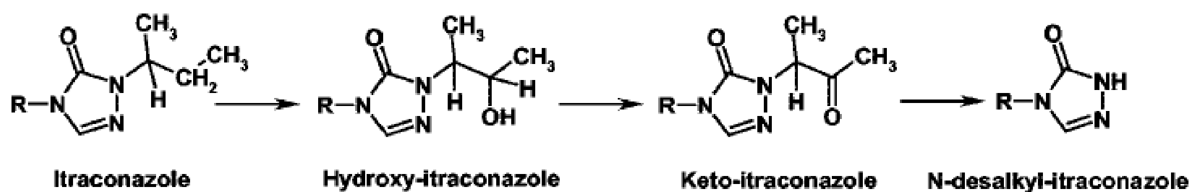


Figure 1.3. Proposed sequential metabolism of ITZ by CYP3A4 (Isoherranen et al., 2004).

ITZ-Associated Adverse Drug Reactions

Administration of ITZ is associated with multiple side effects in dogs, including nausea, vomiting, lethargy, anorexia, and ulcerative dermatitis with vasculitis (Legendre et al., 1996; Mazepa et al., 2011). These adverse drug reactions (ADR) may occur with or without concurrent hepatotoxicity, which is often asymptomatic but can lead to serious illness.

Hepatotoxicity

Hepatotoxicity, as measured by elevated serum alanine transferase (ALT) activity, is a serious and potentially life-threatening ADR that occurs in 8-42% of dogs, depending on the dose and duration of therapy (Legendre et al., 1996; Mazepa et al. 2001). The majority of these cases are asymptomatic, although the overall incidence of clinical hepatotoxicity in dogs is unknown. Based on clinical experience, clinical hepatotoxicity does occur and it is likely that intervention by veterinarians prevents the many of these cases. There is, however, considerably more data on the occurrence of hepatotoxicity in human patients receiving azole antifungal therapy.

Wang et al. (2010) conducted a systematic review and meta-analysis of hepatotoxicity caused by antifungals used to treat invasive fungal infections. In the 39 randomized controlled trials included in the study, there was a pooled risk of 1.5% in patients treated with ITZ of developing abnormal liver function tests necessitating discontinuation of treatment. The pooled risk of developing liver enzyme elevations that did not require treatment alterations in patients treated with ITZ was 17.4%. This is compatible with other smaller case studies where the majority of patients with elevated liver enzymes are asymptomatic (Restrepo et al., 1986; Perissinotti and Marini, 2018). Hepatotoxicity is generally reversible with discontinuation of therapy (Benitez and Carver, 2019), but can sometimes lead to death (Tuccori et al., 2008). The

clinical and histopathologic pattern is primarily cholestatic (Lavrijsen et al., 1992; Legras et al., 2002; Jiminez-Saenz et al., 2004; Adriaenssens et al., 2014) although panlobular necrosis may occur with long-term pulse therapy (Tuccori et al., 2008; Srebrnik et al., 2005).

The occurrence of hepatotoxicity in dogs has been reported in multiple veterinary studies, although clinical disease is poorly documented. In Legendre et al.'s (1996) prospective study of 112 dogs with blastomycosis, 8% of those receiving the lowest recommended dose of ITZ (5 mg/kg/day) had mild elevations in ALT (76 to 200 IU/L) and 4% had severe elevations in ALT (>400 IU/L). At the highest recommended dose of 10 mg/kg/day, 43% had mild, 10% had moderate (201-400 IU/L), and 8% had severe elevations in ALT. Normal serum ALT was categorized as 20 to 75 IU/L. In another, retrospective, study of 31 dogs treated with ITZ for blastomycosis, increases in ALT were identified in 6 of 23 dogs (26%). Treatment was discontinued in 1 of 23 dogs due to progressive ALT elevations (Mazepa et al., 2011). Wilson et al. (2018) recorded elevated ALT in up to 42% of dogs receiving ITZ therapy for histoplasmosis. In the majority of cases, liver enzyme elevations are not accompanied by clinical signs or dysfunction and the medication is continued; in others, progressive ALT elevations necessitate discontinuation of the drug. This is similar to what occurs in human patients experiencing hepatotoxicity.

Other Adverse Drug Reactions

Other ADRs in dogs associated with oral ITZ administration include anorexia, lethargy, vomiting, diarrhea, pollakiuria, elevated blood urea nitrogen (BUN), skin ulceration associated with vasculitis, flaky skin, and hair loss (Legendre et al., 1996). These changes can occur with or without concurrent elevations in ALT. In the study by Legendre et al. (1996), the occurrence of non-hepatic ADRs was dose dependent. Approximately 30% of dogs given the highest dose (10 mg/kg/day) experienced a non-hepatic ADR, including anorexia (14.9%), skin ulceration (7.5%), lethargy (4.5%), diarrhea (3.0%), increased BUN (3.0%), vomiting (2.8%), or flaky skin and hair loss (1.5% each). At the lowest, and currently recommended, dose (5 mg/kg/day), 7.5% of dogs experienced non-hepatic ADRs, including anorexia (2.5%), vomiting (2.5%), or pollakiuria (2.5%). As treatment efficacy was similar between the two doses, the lower dose is recommended. Similar gastrointestinal and cutaneous ADRs are reported in man (Amichai and Grunwald, 1998).

Mechanisms of Azole Hepatotoxicity

All azoles have the potential to cause hepatotoxicity. Toxic mechanisms are, however, poorly understood. Hepatotoxicity caused by KTZ is best characterized in experimental animal models. As they are a similar class of drug, details about hepatotoxic mechanisms of KTZ can be used as a guide to investigate similar mechanisms of ITZ hepatotoxicity.

Proposed Mechanisms of KTZ Hepatotoxicity

Like ITZ, metabolism of KTZ by hepatic enzymes produces several metabolites. The major metabolite that accumulates in the liver of mice is N-deacetyl ketoconazole (DAK) (Whitehouse et al., 1990). Using human liver microsomes, Fukami et al. (2016) demonstrated that KTZ is hydrolyzed into DAK by human arylacetamide deacetylase (AADAC) in the liver. In both rats (Rodriguez et al. 1999) and humans (Fukami et al., 2016), DAK is subsequently metabolized by microsomal flavin-containing monooxygenases (FMO) into N-hydroxy-DAK. N-hydroxy-DAK is susceptible to further oxidation by FMOs, which may lead to accumulation of toxic dialdehydes (Rodriguez and Acosta, 1997b; Rodriguez et al. 1999).

Rodriguez and Acosta demonstrated that KTZ (1995) and DAK (1997a) are toxic to primary rat hepatocytes in a time and concentration dependent manner. DAK was a more potent toxin than the parent compound, KTZ. Additionally, the toxicity was increased by addition of n-octylamine, a positive effector of FMO, and suppressed by methimazole, a competitive substrate for FMO. These findings suggest that DAK and subsequent toxic metabolites produced through the action of microsomal FMOs may be, in part, responsible for hepatotoxicity observed clinically.

Later studies showed a biphasic *in vivo* toxicity in rats with an early spike in serum ALT after KTZ exposure, which declined after four hours and then subsequently increased at 12 and 24 hours (Rodriguez and Buckholz, 2003). Consistent with earlier studies, this suggests initial injury caused by KTZ and later injury in response to its metabolites.

Proposed Mechanisms of ITZ Toxicity

Less is known about mechanisms of ITZ toxicity. In rats, ITZ causes dose-dependent cytotoxicity *in vitro* (Somchit et al., 2002; Somchit et al., 2009) and *in vivo* (Somchit et al., 2004; Somchit et al., 2006). As ITZ is metabolized through hepatic CYP enzymes, Somchit et al. utilized CYP inhibitors and inducers to investigate the role of CYPs in development of

hepatotoxicity. Rats were pre-treated with phenobarbital, a known CYP inducer, prior to isolation of hepatocytes for *in vitro* exposure to azoles. At the highest concentration of ITZ (1.0 mM), phenobarbital-induced hepatocytes had significantly higher viability when compared with the control (Somchit et al., 2002). In a subsequent *in vivo* study, rats were treated with either phenobarbital or SKF525A, a non-selective CYP inhibitor (Somchit et al., 2006). Similar to their 2002 *in vitro* study, they found that CYP induction with phenobarbital reduced hepatotoxicity as measured by decreased activities of ALT and γ-glutamyl transferase (GGT) and reduced inflammation and necrosis histologically. In contrast, CYP inhibition with SKF525A resulted in significantly increased hepatotoxicity as measured by elevated ALT, ALP, and GGT and increased amounts of inflammation and necrosis.

These findings shed light on possible mechanisms of toxicity. First, the protective effect of CYP induction suggests that the CYP pathway is not responsible for production of toxic metabolites. This is further supported by the deleterious effect of CYP inhibition, implicating either a possible role for the parent compound in toxicity or metabolites generated by a non-CYP metabolic pathway. For example, FMO-mediated metabolism, similar to that occurring during production of toxic metabolites from KTZ, is one possible pathway by which toxic ITZ metabolites could be generated. ITZ itself is a CYP inhibitor (Back and Tija 1991; Isoherranen et al., 2004; Florea et al., 2003; Somchit et al., 2006), so it is possible that autoinhibition of its own metabolizing CYP enzymes over time potentiates toxicity.

Somchit et al. also compared histologic changes in rats administered increasing doses of ITZ. In the 2004 study, rats were administered either a single dose of ITZ or daily dosing for 14 days. They noted a dose dependent increase in centrilobular and midzonal degeneration with focal hepatocellular necrosis, granuloma formation, and biliary hyperplasia in the subchronic dosing group. In contrast, rats in the 2006 study were administered ITZ for 4 days and had periportal to midzonal necrosis and inflammation. A wide variety of toxicants cause centrilobular injury due to the concentration of metabolizing enzymes, including CYPs, in this area. Periportal injury, however, is generally caused by direct acting toxicants that do not require biotransformation (i.e. phosphorus) (Haschek et al., 2010). Variations in oxygen tension can also impact the zonal distribution. Differences in the lobular pattern between these studies could be due to variations in experimental design, including treatment duration (14 days in 2004 study vs 4 days in 2006 study) and maximum dosage (100 mg/kg/day in 2004 study vs. 200 mg/kg/day in

the 2006 study). Centrilobular damage implicates CYP or another enzyme system concentrated in this area in producing a toxic metabolite. Given the primarily periportal damage in the 2006 study, it is possible that the parent compound may also play a role in toxicity prior to biotransformation. If conclusions can indeed be drawn about ITZ given what we know about KTZ, it is reasonable to speculate that the parent compound may cause periportal damage and toxic metabolites produced later may cause centrilobular or periportal damage depending on the enzyme system involved. A series of *in vivo* rodent studies controlling for treatment duration and dosage is required to clarify the relationship between toxicity and morphologic alterations in the liver.

In cases of clinical liver disease in man due to ITZ administration, liver damage may be massive (Tuccori et al., 2008; Srebrnik et al., 2005), cholestatic (Lavrijsen et al., 1992; Legras et al., 2002; Jiminez-Saenz et al., 2004; Adriaenssens et al., 2014), or both. Massive necrosis usually occurs as an idiosyncratic response or due to large doses of drug whereas cholestatic injury can be due to either hepatocellular damage or direct damage to the biliary epithelium (Haschek et al., 2010). Patterns of liver damage have not been documented in the canine literature. The wide variety of alterations in experimental and natural disease likely reflect differences in dose, duration, and individual susceptibility.

Proposed Mechanisms of FCZ Toxicity

In the series of studies by Somchit et al. described previously, ITZ experiments were conducted in parallel with FCZ exposure. FCZ caused dose- and time-dependent cytotoxicity in rat hepatocytes but was significantly less cytotoxic than ITZ (Somchit et al., 2002; Somchit et al., 2009). Histologically, FCZ caused only mild hepatocellular degeneration in rats at the highest dose (Somchit et al., 2004). In contrast to ITZ, CYP induction with phenobarbital was not cytoprotective against FCZ-induced cytotoxicity (Somchit et al., 2002). Because FCZ is excreted up to 80% unchanged in the urine (Grant et al., 1990) and does not undergo extensive CYP metabolism, this was an expected finding. CYP inhibition with SKF525A did, however, cause increased FCZ associated cytotoxicity (Somchit et al., 2006). Like ITZ and KTZ, the mechanism of FCZ hepatotoxicity is unknown, but could be due to toxic effects of the parent compound or an alternate, non-CYP mediated pathway.

Oxidative Damage in ITZ-Associated Hepatotoxicity

As with many drugs, oxidative damage may play an important role in ITZ-associated hepatotoxicity. Sozen et al. (2014) showed that rats administered oral ITZ had increased hepatic myeloperoxidase, an enzyme produced by neutrophils that is responsible for conversion of hydrogen peroxide to hypochlorous acid, a potent cytotoxic and oxidizing agent (Kumar et al., 2015b) and nitric oxide, a free radical that can also be converted into the highly oxidizing peroxynitrite anion (Kumar et al., 2015a). They also had decreased activities of hepatic superoxide dismutase and glutathione peroxidase, which are essential antioxidants responsible for scavenging free radicals (Kumar et al., 2015a) and increased hepatocellular degeneration, inflammation, and necrosis.

Oxidative stress and hepatocellular degeneration and necrosis have also been documented in pregnant rats and their fetuses administered ITZ at different stages of gestation (El-Shershaby et al., 2015). This study showed that both dams and fetuses had dose dependent increases in hepatic malondialdehyde, the principal product of lipid peroxidation and a common marker of oxidative stress (Del Rio et al., 2005). Dams and fetuses also had decreased levels of reduced hepatic GSH. As the major intracellular antioxidant (Yuan and Kaplowitz, 2009), decreased GSH is an indirect marker of elevated oxidative stress. Lastly, dams had decreased hepatic catalase, another enzyme important in reducing oxidative damage (Kumar et al., 2015a).

The Role of Glutathione in Azole-Associated Hepatotoxicity

GSH is an endogenous hepatic antioxidant critical to cell survival. As the most powerful reducing agent in the cell, it is essential for maintenance of redox potential and cell signaling (Yuan and Kaplowitz, 2009). GSH is an essential cofactor acting as a hydrogen donor for glutathione peroxidase, which, in turn, reduces hydrogen peroxide to water (Lubos et al., 2011).

While a definitive role for GSH in azole-associated hepatotoxicity has not been established, GSH depletion is essential to the pathogenesis of one of the best characterized hepatotoxicities, that caused by acetaminophen (APAP). In APAP toxicity, GSH is depleted by the toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI) generated by CYP metabolism, causing oxidative stress and cell damage. The GSH precursor N-acetylcysteine (NAC) is the treatment of choice for this toxicity in veterinary medicine (Webster and Cooper, 2009).

Rodriguez and Buckholz (2003) demonstrated a time dependent decrease in hepatic GSH in rats administered 90 mg/kg of KTZ. Because this decrease in GSH occurred hours after administration, the authors hypothesized that reactive KTZ metabolites (DAK and N-hydroxy-DAK) rather than the parent compound may bind to and deplete GSH. In this way, GSH may play a role in detoxification of KTZ via metabolite conjugation. If GSH is further depleted due to oxidative stress, then conjugation and removal of metabolites may be decreased, leading to subsequent liver injury.

Others, however, have proposed that GSH does not play a role in detoxification of ITZ. Rats administered ITZ and curcumin, an antioxidant that increases glutathione S-transferase (GST) activity in the liver (Piper et al., 1998), had no significant change in cytotoxicity (Somchit et al. 2009), suggesting that GSH may not be involved in detoxification of ITZ. Additional research is required to better understand what role GSH plays in ITZ-associated hepatotoxicity.

Hepatoprotectants

Hepatoprotectants are commonly used in veterinary medicine to treat a variety of liver injuries. They include the GSH precursors S-adenosyl-L-methionine (SAMe) and N-acetylcysteine (NAC) as well as the antioxidants silybin, vitamin E, and vitamin C. Their use is best described in inflammatory diseases and specific toxicities, like APAP and *Amanita* mushroom toxicity (Webster and Cooper, 2009). Hepatoprotectants are often used in cases of suspected azole-induced hepatotoxicity with anecdotal success, however, no scientific studies have been performed to date to support their use.

S-adenosyl-L-methionine

S-adenosyl-L-methionine (SAMe) (**Figure 1.4**) is formed from methionine and adenosine triphosphate (Bottiglieri, 2002). It is an endogenous molecule serving as an essential methyl donor and precursor for aminopropylation pathways and the synthesis of GSH (Bottiglieri, 2002). The product of methylation reactions involving SAMe is S-adenosylhomocysteine (SAH), which is further converted to adenosine and homocysteine by SAH hydrolase (Mato et al., 1997). Homocysteine then is either remethylated to form methionine or undergoes transsulfuration to form cystathionine, followed by hydrolysis to α-ketobutyrate and cysteine (Lieber and Packer, 2002). Cysteine, the rate-limiting factor in GSH synthesis, goes through two sequential enzymatic reactions to form GSH (Lu et al., 2000) (**Figure 1.5**).

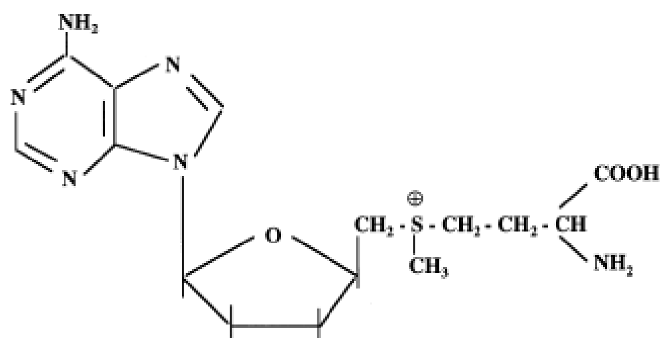


Figure 1.4. Chemical structure of SAMe (Lu et al., 2000)

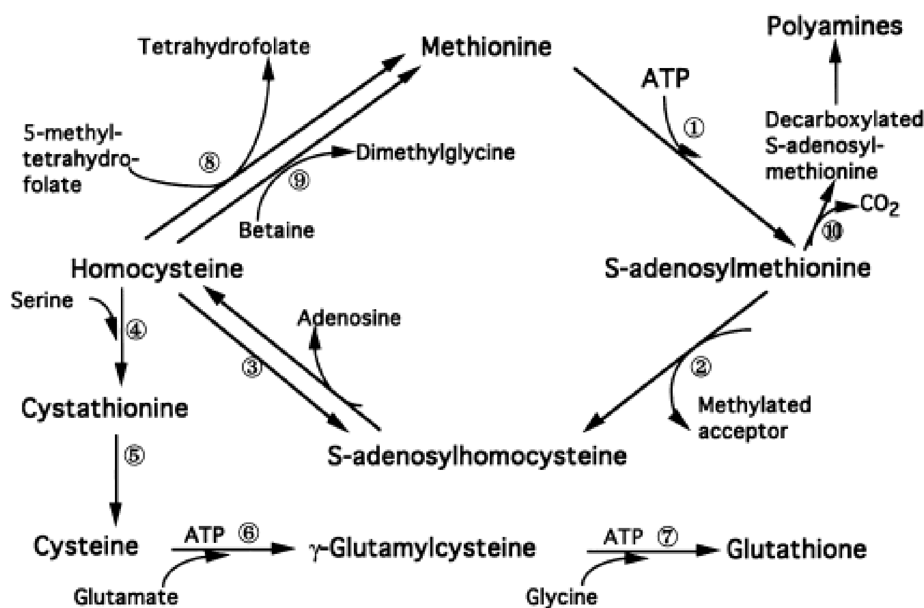


Figure 1.5. Synthesis and metabolism of SAMe (Lu et al., 2000)

The role of SAMe in non-alcoholic steatohepatitis (NASH) and alcohol induced liver injury in humans has been extensively investigated. In rodents chronically administered ethanol, SAMe administration increases liver GSH concentrations and reduces liver injury (Feo et al., 1986, Song et al., 2003). In a randomized, multicenter clinical trial, SAMe treatment in patients with alcoholic liver cirrhosis improved survival or delayed liver transplantation (Mato et al., 1999). Similarly, hepatic SAMe levels are decreased in experimental rodents models of NASH (Wortham et al., 2008). SAMe has also been used in man to treat drug-induced liver injury. For example, patients treated with bevacizumab and oxaliplatin for colorectal cancer in addition to

SAMe had significantly lowered ALT, AST, GGT, and bilirubin compared to those receiving the chemotherapeutic alone (Vincenzi et al., 2012).

The effects of SAMe administration has been evaluated in dogs and cats. In healthy cats, SAMe increased hepatic GSH and improved the redox status of both the liver and erythrocytes (Center et al., 2005a). Dogs administered oral prednisone and SAMe had increased hepatic GSH and improved hepatic antioxidant status, but no changes in clinical or histologic signs of vacuolar hepatopathy (Center et al., 2005b). SAMe also protects erythrocytes from oxidative damage in experimental acetaminophen toxicity (Webb et al., 2003) and has been used to successfully treat acetaminophen toxicity in a dog (Wallace et al., 2002). The chemotherapeutic lomustine (CCNU) causes hepatic toxicity in 6.1% of dogs (Kristal et al., 2004) and elevated ALT in up to 84% of treated dogs through unknown mechanisms (Skorupski et al., 2011). In a clinical trial by Skorupski et al. (2011), dogs administered Denamarin ®, a combination SAMe and silybin product used in dogs and cats, concurrently with CCNU had significantly decreased ALT, AST, ALP, and bilirubin compared to dogs receiving CCNU alone. These examples demonstrate the utility of SAMe with or without silybin in preventing and/or treating drug-induced hepatic injury in dogs and cats.

Few *in vitro* studies are published examining the effects of SAMe in canine hepatocytes. Au et al. (2012) exposed canine primary hepatocytes to a combination of SAMe and silybin (see below) and interleukin 1β (IL-1β). The cells had decreased production of inflammatory mediators, including IL-8, monocyte chemotactic protein-1 (MCP-1), and prostaglandin E_2 (PGE_2) compared with hepatocytes treated with IL-1β alone. Additionally, hepatocytes treated with IL-1β had decreased intracellular levels of GSH, which was restored to control levels with addition of SAMe and silybin. This study shows the combined antioxidant and anti-inflammatory effects of SAMe and silybin in canine hepatocytes.

N-acetylcysteine

N-acetylcysteine (NAC), a variant of the amino acid L-cysteine and precursor of GSH, is an antioxidant that scavenges endogenous free radicals such as hypochlorous acid and hydroxyl radical (Aruoma et al., 1989). Animal models have demonstrated NAC's efficacy in treating hepatotoxicity, acute liver failure, ischemia-reperfusion injury, and improving microcirculation in bile duct obstruction (Zafarullah et al. 2003; Lee et al., 2009; Smyrniotis et al., 2005; Kigawa et al., 2000). Its use and efficacy are best characterized in APAP-induced toxicity (Zafarullah et

al., 2003), where it is considered the treatment of choice in small animals (Webster and Cooper, 2009; Aronson and Drobatz, 1996; Villar et al., 1998) and man (Heard, 2008). NAC restores reduced GSH levels in the cell, allowing detoxification of the toxic NAPQI metabolite (Lauterburg et al., 1983). NAC has also been effective in treating non-APAP associated acute liver failure caused by other drugs, infections, or autoimmune conditions (Lee et al., 2009).

NAC provides a protective effect against hepatotoxicity caused by cyclosporine A in rats (Kaya et al., 2008), arsenic in mice (Santra et al., 2007), and azathioprine in rat hepatocytes (Menor et al., 2004). Kaya et al. demonstrated decreased activity of endogenous antioxidants in cyclosporine A treated rats. Both Santra et al. and Menor et al. demonstrated reduced hepatic GSH after exposure to arsenic and azathioprine, respectively, and partial restoration of GSH levels with administration of NAC. As described previously, there is evidence of GSH depletion and oxidative stress in rodent models of KTZ and ITZ hepatotoxicity, so administration of NAC may ameliorate these effects. To date, there are no published studies examining the effect of NAC on azole-induced hepatotoxicity.

Silybin

Silybin is one of seven flavonolignans that makes up silymarin, a component of the milk thistle plant (Boothe, 2012). Silybin has antioxidant (Bosisio et al., 1992; Carini et al., 1992; Trouillas et al., 2008), anti-inflammatory (Bremner and Heinrich, 2002; Gharagozloo et al., 2010), and anti-fibrotic (Lieber et al., 2003) effects. However, in humans with chronic liver disease, evidence of clinical efficacy is lacking (Loguercio and Festi, 2011). In acute liver damage, silybin has been associated with increased survival in adults and children exposed to the toxic mushroom *Amanita phalloides* (Enjalbert et al., 2002) and was protective against intoxication in Beagle dogs experimentally administered *A. phalloides* (Vogel et al., 1984). It has also been used in combination with SAMe to prevent CCNU-associated hepatopathy in dogs (Skorupski et al., 2011).

Multiple experimental studies demonstrate the anti-inflammatory and antioxidant effects of silybin. Au et al. showed that canine hepatocytes exposed *in vitro* to IL-1β had increased production of the inflammatory mediators PGE_2, IL-1, and MCP-1, which was inhibited by administration of silybin (Au et al., 2011). In a study demonstrating the potential beneficial effects of silybin on ITZ-induced hepatotoxicity, rats administered a combination of ITZ and

silybin had decreased nitric oxide, myeloperoxidase, ALT, and AST levels compared to rats administered ITZ alone (Sozen et al., 2014). This study demonstrates a potential use for silybin in ITZ-associated hepatotoxicity in other species.

Vitamin E and Vitamin C

Vitamin E is a lipid soluble tocopherol existing in four isomers, the most biologically active of which is α-tocopherol (Van Metre and Callan, 2001). This tocopherol is a potent antioxidant that scavenges lipid peroxyl radicals, protecting cell membranes from oxidative damage (Jiang, 2014). Vitamin E therapy in human patients with NASH has shown modest improvement in liver enzyme tests (El Hadi et al., 2018). In a rodent study examining the effects of vitamins on APAP toxicity, pretreatment with vitamin E reduced hepatic oxidative stress (Abdulkhaleq et al., 2018).

Vitamin C has myriad functions, including improving immune function, facilitating enteric absorption of non-heme iron, synthesizing collagen, and acting as an antioxidant (Oudemans-van Straaten et al., 2014). As a hepatic antioxidant, vitamin C has beneficial effects on ischemia-reperfusion injury and endotoxin-induced oxidative damage (Seo and Lee, 2002; Cadenas et al., 1998).

Combination therapy of vitamin E and vitamin C in a prospective, randomized study of 45 adults improved fibrosis scores in patients with NASH (Harrison et al., 2003). Another randomized study of 247 adults with NASH had decreased serum ALT and AST, decreased steatosis and inflammation, but no change in fibrosis (Sanyal et al., 2010). Rodent studies of APAP toxicity have demonstrated that pretreatment with vitamins C, E, and B_{12} reduced hepatic oxidative stress. Vitamin C was more effective than vitamins E and B_{12} and *in vitro* hepatocyte survival was highest with combination therapy of all three (Abdulkhaleq et al., 2018). Treatment with vitamin E, vitamin C, or a combination of both has beneficial effects in liver diseases associated with oxidative damage in humans and animals and thus may be beneficial in animals receiving ITZ therapy.

Conclusion

Systemic azole antifungals are an essential component of a veterinary practitioner's antimicrobial arsenal. The newer triazole antifungals, including ITZ, FCZ, voriconazole, and posaconazole, are more effective and associated with fewer side effects when compared to KTZ

and non-azole antifungals. ITZ has a broad spectrum of action and is the first line treatment of choice for canine blastomycosis and other systemic mycoses. Unfortunately, all azoles are associated with hepatic and non-hepatic adverse drug reactions. Hepatic reactions can lead to interruption in therapy, significant illness, and even death.

In man, hepatic CYP3A4 is responsible for metabolism of ITZ into numerous metabolites. While a similar pathway is suspected in dogs, the responsible enzyme(s) has not been identified. Based on *in vitro* rodent studies, CYPs are not responsible for production of toxic metabolites (Somchit et al., 2002). Either the toxicity is induced by the parent drug or metabolites are produced via alternate pathways, such as the FMO-mediated pathway which generates toxic metabolites from KTZ. Regardless of the exact toxic principle, oxidative damage occurs during ITZ-associated hepatotoxicity (Sozen et al., 2014; El-Shershaby et al., 2015). This oxidative damage may be alleviated by supplementation with hepatoprotectants. These include GSH precursors like SAMe and NAC and antioxidants such as silybin, vitamin C, and vitamin E. The utility of antioxidants in canine ITZ-associated hepatotoxicity is unknown. The goal of this work is to evaluate the effect of GSH on this hepatotoxicity in an *in vitro* canine model.

CHAPTER 2: THE EFFECT OF GLUTATHIONE ON ITRACONAZOLE-ASSOCIATED HEPATOCYTE TOXICITY IN CANINE PRIMARY HEPATOCYTES

Abstract

Itraconazole (ITZ) is used to treat many types of fungal infections in dogs. However, therapy is associated with dose-dependent increases in liver enzymes in up to 42% of dogs. Glutathione (GSH) precursors are often used to treat ITZ-associated hepatotoxicity, although no studies have investigated their efficacy. The objectives of this study were (1) to develop an *in vitro* model of ITZ-associated hepatotoxicity in canine primary hepatocytes and (2) to identify the effect of GSH on cell death in hepatocytes exposed to ITZ. We hypothesized that canine primary hepatocytes would be an appropriate model for this hepatotoxicity and that GSH treatment would decrease cell death.

During model development in the first objective, three different cell viability assays were tested, including calcein AM, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and neutral red. Using a sandwich culture technique, canine primary hepatocytes were incubated with 5-fold dilutions of ITZ (0 to 50 μM) for 4 hours. Cells were plated at a concentration of 5.0 x10^5 cells/ml (calcein AM) or 2.4x10^5 cells/ml and 0.8 x10^5 (MTT and neutral red). Neutral red was the only assay that detected the expected dose-dependent cytotoxicity. The cells maintained optimum viability at a seeding concentration of 2.4x10^5 cells/ml, so this concentration was selected for future experiments. Hepatocyte phenotype was assessed by measurement of albumin production using an enzyme-linked immunosorbent assay (ELISA) for canine albumin and morphologic assessment via phase contrast microscopy. Maintenance of hepatocyte phenotype was confirmed by continued production of albumin and retention of cell morphology over time.

For the second objective, canine primary hepatocytes were incubated for 24 hours with 10-fold dilutions of GSH (0 to 500 μM) and then exposed to 5-fold dilutions of ITZ (0 to 50 μM). Cell function was determined by assessing urea production and cell viability was determined using the neutral red assay after 4 and 24 hours of ITZ exposure (n=3). A colorimetric urea assay kit did not detect a significant change in urea production with increasing ITZ concentration, time, or GSH concentration. The effect these three factors on cytotoxicity was assessed using multivariate linear regression. Hepatocyte cytotoxicity significantly increased

with ITZ concentration ($p<0.001$) and time ($p=0.004$) and significantly decreased with GSH treatment ($p<0.001$).

Using canine primary hepatocytes plated at 2.4×10^5 cells/ml in a sandwich culture, we demonstrated dose- and time-dependent ITZ-associated cytotoxicity. Pre-treating with GSH provided a protective effect against cell death. These results suggest that GSH precursors may have a role in the management or prevention ITZ-associated hepatotoxicity in dogs. Clinical trials are needed to evaluate their utility for this adverse drug reaction.

Introduction

Itraconazole (ITZ) is an azole antifungal drug used to treat a wide variety of fungal infections in dogs, including both superficial cutaneous infections such as dermatophytosis and malasseziosis, and systemic mycoses such as blastomycosis and histoplasmosis (Greene, 2012). The azoles' antifungal activity is attributable to inhibition of ergosterol by targeting the cytochrome P450-dependent sterol 14α-demethylase (Vanden Bossche, 1985). The most commonly used azoles in veterinary medicine are ITZ, fluconazole, and ketoconazole. ITZ is more selective than ketoconazole, leading to fewer side effects, and has a broader spectrum of action than both ketoconazole and fluconazole (Van Cutsem et al., 1987a; Van Cutsem et al., 1990). Additionally, ITZ is more efficacious than ketoconazole in treating a variety of fungal infections in animal models (Van Cutsem et al., 1987b; Van Cutsem et al., 1987c).

ITZ is the treatment of choice for canine blastomycosis, which is endemic in the Midwestern United States, with a reported cure rate of 54-90% and a relapse rate of 18-20% (Legendre et al., 1996; Mazepa et al., 2011). Despite its efficacy, ITZ is associated with a number of adverse reactions in dogs including nausea, vomiting, lethargy, anorexia, ulcerative dermatitis with vasculitis, and hepatotoxicity (Legendre et al., 1996; Mazepa et al., 2011). Anorexia is most common and may be associated with increases in alanine aminotransferase (ALT) (Legendre et al., 1996). Increases in ALT are reported in 12-42% of dogs depending on the dose and duration of treatment (Legendre et al., 1996; Mazepa et al., 2011; Wilson et al., 2018). Based on clinical experience, clinical hepatotoxicity does occur, although the prevalence in dogs is not reported. In a systemic review and meta-analysis of humans experiencing similar adverse reactions to ITZ, the pooled risk of developing abnormal liver function tests necessitating discontinuation of treatment was 1.5% (Wang et al., 2010). Although drug

discontinuation generally results in reversal of these changes in dogs, switching antifungal drugs can negatively affect the course of the mycosis if alternative drugs are less effective or result in their own adverse reactions.

As with many adverse drug reactions, oxidative damage may play an important role in ITZ-associated toxicity. Rodent models demonstrate increases in reactive oxygen species as well as decreases in antioxidant enzymes and glutathione (Sozen et al., 2014; El-Shershaby et al., 2015). Glutathione (GSH) is an endogenous hepatic antioxidant that is essential for maintenance of redox potential, cell signaling, and survival (Yuan and Kaplowitz, 2009). These experimental findings suggest that GSH supplementation and antioxidant therapy may be beneficial in treating or preventing ITZ-associated toxicity in animals.

Commonly used hepatoprotectants in veterinary medicine, including S-adenosyl-L-methionine (SAMe) and N-acetylcysteine, are important in the synthesis and maintenance of cellular GSH levels (Bottiglieri, 2002; Lauterburg et al., 1983). SAMe, N-acetylcysteine, and other antioxidants like silybin, α-tocopherol (vitamin E), and ascorbic acid (vitamin C) are commonly used in dogs with clinical changes consistent with ITZ-associated hepatotoxicity. However, no studies have investigated the efficacy of this practice. Because many of these products are expensive and can cause inappetence, scientific evidence is needed to provide a basis for their rational use. The first objective of this work was to develop an *in vitro* model of ITZ-associated hepatotoxicity using cryopreserved canine primary hepatocytes. The second objective was to evaluate the effect of GSH treatment on ITZ-associated hepatotoxicity using the newly established model. We hypothesized that canine primary hepatocytes would be an appropriate model of ITZ-associated hepatotoxicity and that GSH treatment would decrease cell death.

Materials and Methods

Objective 1: Assay Development

Cell Culture

Unless otherwise noted, all materials and reagents were obtained from ThermoFisher Scientific (Waltham, MA). Cryopreserved male dog (Beagle) primary hepatocytes from a single donor (Catalog number: DGCP10; Lot number: DB343) were thawed in a 37° Celsius (C) water

bath for <2 minutes and resuspended in 50 mL of thawing/plating medium (William's E medium with 5% fetal bovine serum, 1 uM dexamethasone in dimethyl sulfoxide (DMSO), 1% penicillin/streptomycin, 4 ug/ml human recombinant insulin, 2 mM GlutaMAX, and 15 mM HEPES at pH 7.4). Following resuspension, the cells were centrifuged at 60 x *g* for 4 minutes and counted using a hemocytometer and the Trypan Blue dye exclusion method. The cells were then plated into pre-collagen I coated, sterile culture plates and incubated at 37°C for 4 hours. After incubation, the supernatant was discarded and cold extracellular matrix gel from Engelbreth-Holm-Swarm murine sarcoma (Sigma-Aldrich, St. Louis) diluted to 0.35 mg/mL in incubation medium (William's E medium with 0.1 uM dexamethasone in DMSO, 0.5% penicillin/streptomycin, 6.25 ug/mL human recombinant insulin, 6.25 ug/mL human transferrin, 6.25 ng/mL selenous acid, 1.25 mg/mL bovine serum albumin, 5.35 ug/mL linoleic acid, 2 mM GlutaMAX, and 15 mM HEPES at pH 7.4,) was applied over the cells prior to an overnight incubation at 37°C.

Maintenance of Cell Phenotype

To determine maintenance of biochemical phenotype, cryopreserved canine primary hepatocytes plated as described above were incubated for 24, 48, or 72 hours. No-cell wells were included as controls. At each time point, supernatants were collected and frozen at -80°C. Frozen supernatants were later thawed on ice and albumin levels determined using a canine-specific albumin enzyme linked immunosorbent assay kit according to manufacturer's instructions (Eagle Biosciences; Amherst, NH). Absorbance was quantified using a SpectraMax iD3 multi-mode microplate reader and SoftMax Pro 7 software (Molecular Devices; San Jose, CA). To determine maintenance of cellular morphology, cells were examined for polygonal morphology and the presence of cell-to-cell junctions using Invitrogen EVOS XL Core Cell Imaging System (ThermoFisher Scientific; Waltham, MA) at 24, 48, and 72 hours of incubation. Albumin production and morphologic features were selected as they are commonly evaluated when assessing hepatic phenotype (Ware et al., 2018).

Cell Viability Assay Optimization

Three viability assays were trialed during model development: calcein AM, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and neutral ted. For these tests, cells were cultured as described above and exposed to 0, 2, 10, or 50 µm ITZ diluted in DMSO

for 4 hours. DMSO concentration was below 1% for all treatments. Cells were plated at a concentration of 5.0 x10^5 cells/ml (calcein AM) or 2.4x10^5 cells/ml and 0.8 x10^5 cells/ml (MTT and neutral red). A SpectraMax iD3 multi-mode microplate reader and SoftMax Pro 7 software were used to measure absorbance (O.D.) or fluorescence (RFUs) depending on the assay. Each condition was performed in technical triplicate and % cytotoxicity calculated as:

$$\% \text{ cytotoxicity} = \left(1 - \frac{\text{mean value}_{\text{treatment}} - \text{mean value}_{\text{blank}}}{\text{mean value}_{\text{no ITZ}} - \text{mean value}_{\text{blank}}}\right) \text{x } 100\%$$

The first assay tested was calcein AM, which is a non-fluorescent permanent dye that permeates live, intact cells. Cytosolic esterases hydrolyze calcein AM into the fluorescent molecule calcein (Weston and Parish, 1990). Following incubation with ITZ, the cells were washed with incubation medium and incubated with diluted calcein AM cell-permanent dye (ThermoFisher Scientific; Waltham, MA) at 37° C for 30 minutes. Then, the cells were washed again and fluorescence measured at an excitation wavelength of 485 nm and emission wavelength of 525 nm.

The second assay tested was 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (ThermoFisher Scientific; Waltham, MA). MTT is reduced by a metabolically active cell into insoluble formazan via mitochondrial NADPH oxidoreductase (Stockert et al., 2018). Following incubation with ITZ, the cells were washed and incubated with diluted MTT at 37° C for 4 hours. Formazan was then solubilized by the addition of 0.1% DMSO and absorbance measured at 590 nm.

Lastly, neutral red dye uptake into lysosomes was measured using a neutral red cell cytotoxicity assay kit (BioVision; Milpitas, CA) according to the manufacturer's instructions. Viable cells release the dye into the supernatant after solubilization, which is then measured at an absorbance of 540 nm.

Objective 2: The effect of GSH on ITZ-associated cytotoxicity

Exposure of cells to ITZ and GSH

Following plating and overnight incubation with the ECM gel overlay, cells were pre-treated with 0, 50, or 500 μM of GSH (Sigma-Aldrich; St. Louis, MO) diluted in incubation

medium and incubated for 24 hours. These concentrations were based on GSH concentrations previously detected in canine livers (Dedeaux et al., 2020). The cells were then exposed to 0, 2, 10, or 50 µM ITZ (Sigma-Aldrich; St. Louis, MO) diluted in DMSO for 4 or 24 hours. These concentrations were chosen based on plasma drug concentration that occur during therapeutic dosing of ITZ in dogs with significant intrahepatic accumulation expected (Mawby et al., 2013; FDA 1992). No-cell controls were included as blanks.

The neutral red cell cytotoxicity assay was used to determine cell viability at 4- and 24-hours post ITZ exposure. Following staining and dye solubilization, the absorbance was quantified and cytotoxicity calculated as described for objective 1. Each condition was performed in technical triplicate.

Urea assay

Urea is produced by hepatocytes from ammonia as the end product of protein catabolism. When hepatocytes are damaged, urea production decreases (Watson, 2014). For this reason, urea was chosen as a measure of hepatic dysfunction and possible sublethal damage in hepatocytes exposed to ITZ. Supernatants were removed from treatment wells after exposure to GSH and ITZ (see *Exposure of cells to ITZ and GSH* section) prior to assessment of cell viability and frozen at -80° C. Urea was measured in thawed supernatants using a colorimetric urea assay kit (Abnova; Taipei, Taiwan) according to the manufacturer's instructions.

Statistical Analysis

Based on both clinical evidence in dogs and experimental rodent studies (Somchit et al., 2002; Somchit et al., 2009), viability assay selection in objective 1 was based on demonstration of dose-dependent cytotoxicity in cells exposed to ITZ. The optimal cell concentration was chosen for wells in which viability and dose-dependent cytotoxicity were maintained. For maintenance of phenotype testing, an unpaired t-test was used to compare albumin production by hepatocytes and media control wells. A one-way ANOVA was used to evaluate the effect of time on albumin production. Changes in cellular morphology were assessed qualitatively. For objective 2, the effect of time, GSH concentration, and ITZ concentration on % cytotoxicity was assessed using a multivariate linear regression model. Similarly, a multivariate linear regression model was used to determine the effect of these three factors on urea production. All analyses

were performed using commercial software (Prism 8, GraphPad Software, San Diego, CA; JMP 14, SAS Institute, Cary, NC). Statistical significance was set at $p < 0.05$.

Results

Objective 1: Assay Development

Maintenance of hepatic phenotype

Albumin was detectable at all time points in significantly greater amounts than controls ($p < 0.001$). Albumin production decreased significantly over time ($p=0.0045$) (**Figure 2.1**). Hepatocytes maintained cell-to-cell adhesion and their expected polygonal morphology at all time points (**Figure 2.2**).

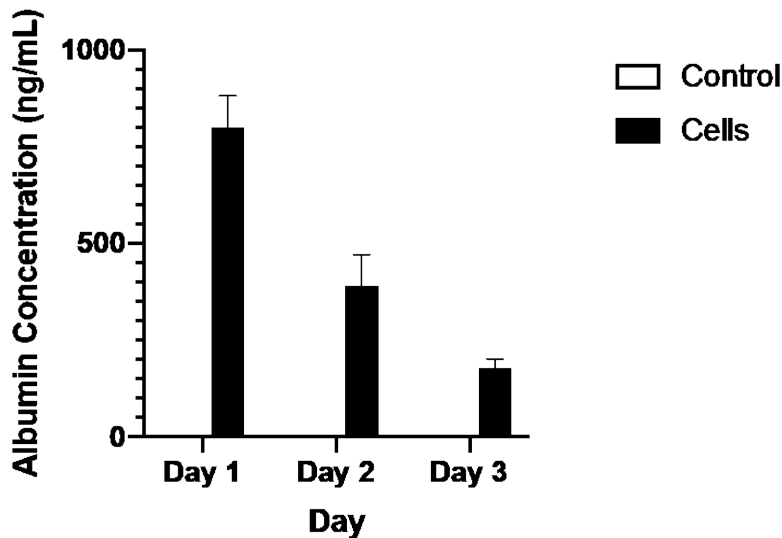


Figure 2.1. Albumin concentration (ng/ml) in supernatant of untreated hepatocytes on days 1, 2, and 3 of the experiment. Albumin production was significantly greater than controls ($p<0.001$) at all time points and decreased significantly over time ($p=0.0045$).

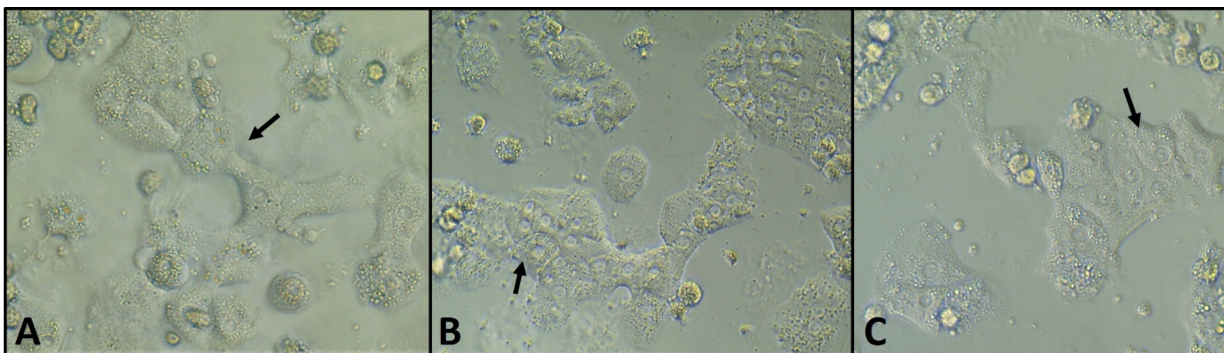


Figure 2.2. Phase contrast microscopy of untreated hepatocytes at 24 hours (A), 48 hours (B), and 72 hours (C) of incubation. The cells maintained cell-to-cell adhesion (arrows) and the polygonal morphology characteristic of hepatocytes.

Cell viability assay optimization

The results of cell viability optimization are presented graphically in **Figure 2.3.** A dose-dependent increase in hepatocyte cytotoxicity was not detected after 4 hours of ITZ exposure using calcein AM or MTT assays. In contrast, dose-dependent cytotoxicity was detected after 4 hours of ITZ exposure using the neutral red assay. For both the MTT and neutral red assays, overall viability was lower in cells plated at 0.8 x10^5 cells/ml compared to 2.4x10^5 cells/ml.

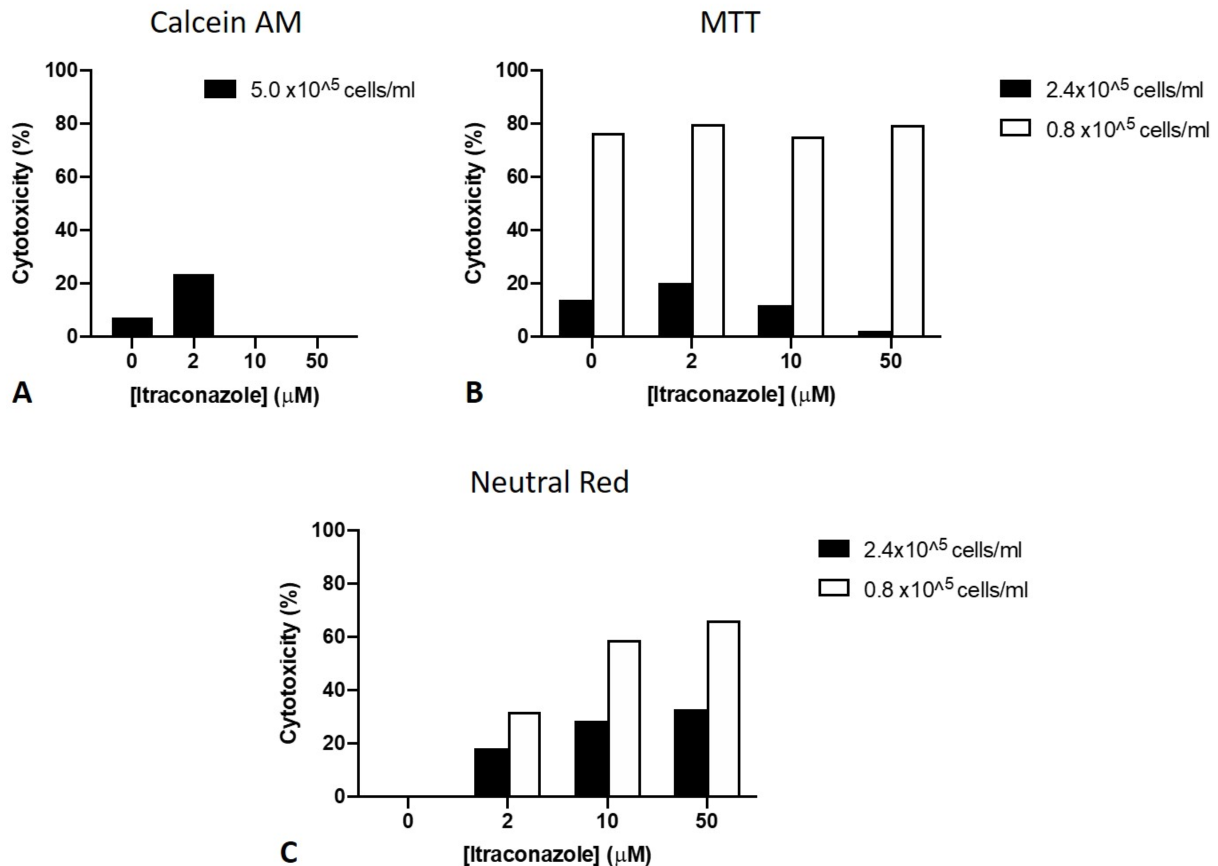


Figure 2.3. Percent cytotoxicity of hepatocytes treated with ITZ for 4 hours and viability detected using (A) calcein AM, (B) MTT, or (C) neutral red dyes.

Objective 2: The effect of GSH on ITZ-associated cytotoxicity

Exposure of cells to ITZ and GSH

Cytotoxicity results are graphically presented in **Figure 2.4**. Hepatocyte cytotoxicity significantly increased with time ($p = 0.004$) and ITZ concentration ($p < 0.001$). Cytotoxicity significantly decreased with increasing GSH concentration ($p < 0.001$). A significant interaction was identified between time and ITZ concentration ($p = 0.014$). No other significant interactions were observed ($p = 0.306 – 0.999$) (**Table 2.1**).

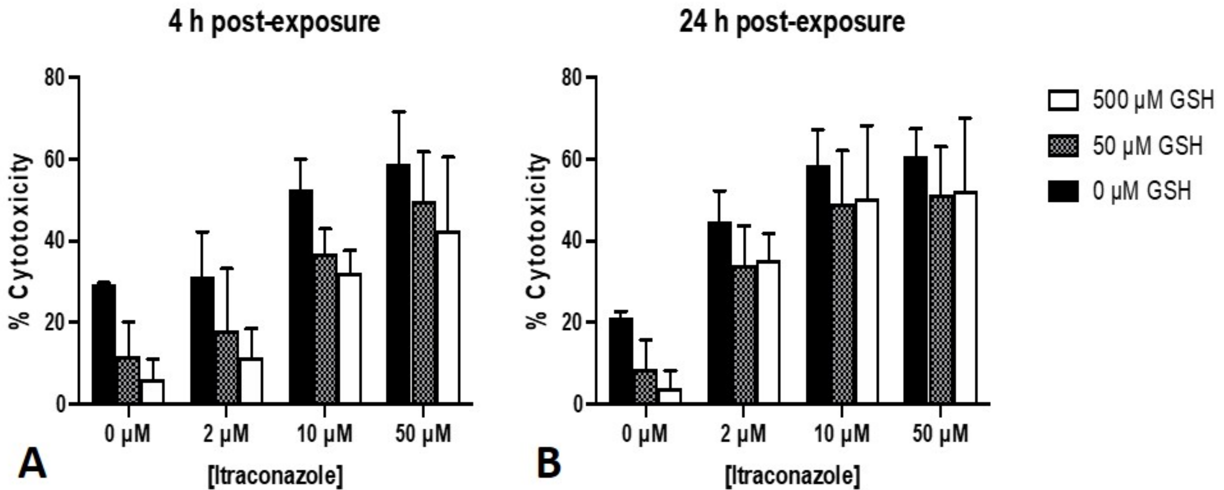


Figure 2.4. Percent cytotoxicity of hepatocytes treated with GSH and ITZ (A) 4 hours and (B) 24 hours after ITZ exposure.

Factor/Interaction	P-Value
ITZ	< 0.001
Time	0.004
GSH	< 0.001
ITZ x Time	0.014
ITZ x GSH	0.980
Time x GSH	0.306
ITZ x Time x GSH	0.999

Table 2.1. Results of multivariate linear regression analysis for % cytotoxicity with the factors ITZ concentration, time, and GSH concentrations. The effect of all three individual factors on % cytotoxicity was significant. A significant interaction was observed between ITZ and time, but not between GSH and ITZ, GSH and time, or all three factors together.

Urea assay

The amount of urea produced by hepatocytes is represented graphically in **Figure 2.5.** Urea production did not significantly decrease with increasing ITZ concentration (p=0.9880) nor

did it significantly increase with increasing GSH concentration (p=0.9300). Urea production significantly increased over time (p<0.001) (**Table 2.2**).

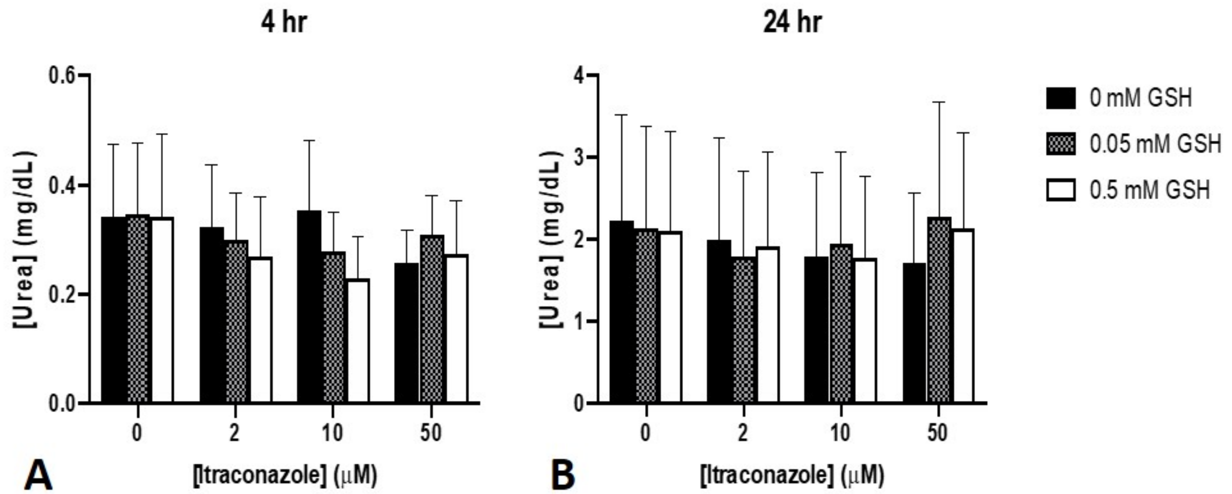


Figure 2.5. Urea (mg/dL) production in hepatocytes treated with GSH and ITZ for 4 hours (A) and 24 hours (B).

Factor	P-Value
ITZ	0.9980
Time	<0.001
GSH	0.9300

Table 2.2. Results of multivariate linear regression analysis for urea production with the factors ITZ concentration, time, and GSH concentrations. The only significant factor was time.

Discussion

In the first objective of this study, we showed that cryopreserved canine primary hepatocytes in a sandwich culture are an appropriate *in vitro* model for canine ITZ-associated hepatotoxicity. Over the course of 72 hours, cells maintained lineage-specific albumin production and morphologic features, thus confirming maintenance of hepatocyte phenotype over the course of the experiment. Albumin production did decrease over time, which is likely due to decreasing viability rather than dedifferentiation as the cells maintained hepatic

morphology. Detection of cell death was best accomplished by measuring the uptake of neutral red dye by viable cells. Using this detection method, ITZ caused significant dose- and time-dependent cytotoxicity *in vitro,* which mimics the dose-dependent hepatotoxicity seen *in vivo* (Legendre et al., 1996). Similar results have also been demonstrated in rodent models (Somchit et al., 2002; Somchit et al., 2009). The second objective of this study investigated the potential cytoprotective role of GSH in canine ITZ-associated hepatotoxicity. Pre-treatment of the cells with GSH for 24 hours prior to ITZ exposure had a significant dose-dependent protective effect against ITZ-associated cytotoxicity. These results suggest that GSH or its precursors may play a role in preventing or treating this toxicity *in vivo*.

During the model development stage, we investigated three different markers of cell viability. These included calcein AM, a dye that is converted into a fluorescent molecule in live cells, and two colorimetric assays, MTT and neutral red. MTT is a salt that is converted into formazan in metabolically active cells and neutral red is a dye that is actively incorporated into lysosomes. Cells were plated at 5.0 x10^5 cells/ml for the calcein AM assay per the manufacturer's instructions and at 2.4 x10^5 cells/ml and 0.8 x10^5 cells/ml for the MTT and neutral red assays. Viability was markedly decreased at the lowest concentration, likely as a result of low signal detection. Based on this, a plating concentration of 2.4x10^5 cells/ml was selected for subsequent assays as a balance between viability and conservation of resources. Of the three viability assays, only neutral red yielded reproducible results depicting a dose-dependent increase in hepatocyte toxicity after 4 hours of ITZ exposure. Therefore, we elected to use neutral red dye uptake as a measure of cell viability to investigate the role of GSH in mitigating cell death.

The dose-dependent protective effect provided by GSH was most pronounced at the 4-hour time point; at 24 hours, there was minimal difference between the 50 μM and 500 μM GSH groups. The reason for this is unknown, but it is possible that by the end of the experiment, the cells had reached their maximum capacity to uptake or respond to GSH regardless of the amount provided. The protective effect was also seen in vehicle control cells, which likely experienced some cytotoxicity attributable to the toxic effects of the DMSO vehicle (Sumida et al., 2011). Reactive oxygen and nitrogen species can accumulate due to a number of cellular insults, so it is expected that GSH, the most abundant hepatic antioxidant, would have a protective effect against cellular stress of any cause, whether by ITZ or the vehicle control.

The protective effect of GSH suggests that oxidative damage may play a role in the mechanism of ITZ-associated hepatotoxicity. GSH precursors such as SAMe and N-acetylcysteine are frequently used as hepatoprotectants in veterinary medicine. Other antioxidants, including silybin, α-tocopherol, and ascorbic acid, are also used treat canine liver disease. SAMe and silybin in particular have been shown to increase hepatic GSH and decrease the production of pro-inflammatory mediators *in vitro (*Au et al., 2010, Au et al., 2012). The beneficial effect of GSH demonstrated here provides evidence for the rational use of hepatoprotectants in dogs with suspected ITZ-induced hepatotoxicity and may also provide insight into mechanisms of toxicity, which are poorly understood. Hepatic oxidative damage has been demonstrated in rodent models of ITZ-induced hepatotoxicity, indicating a potential role for depletion of GSH and other antioxidants (Sozen et al., 2014; El-Shershaby et al., 2015). GSH could be directly involved in detoxification by binding toxic metabolites, which has been proposed in ketoconazole-mediated hepatotoxicity (Rodriguez and Buckholz 2003). Alternatively, it is possible that depletion of antioxidants occurs by another mechanism such that GSH supplementation improves cellular antioxidant status and, thus, viability.

This study sets the groundwork for additional investigation into the use of antioxidants in ITZ-associated hepatotoxicity in dogs. There are, however, some limitations to these *in vitro* experiments. Hepatocytes were pre-incubated with GSH to achieve maximum effect despite pre-treatment not being a practical expectation in dogs requiring immediate antifungal therapy. However, daily co-administration of GSH precursors with ITZ *in vivo* is expected to provide a long-term effect over time. Additionally, canine primary hepatocytes were obtained from a single donor, which does not take into account inter-individual variability. To address this, future studies will assess pharmacogenetic variation *in vivo*.

Urea production was measured in an attempt to account for sublethal damage, which is another possible outcome of ITZ therapy. Urea is a marker of hepatocyte function, so we hypothesized that cells exposed to ITZ would have a dose-dependent decrease in urea production, which would be mitigated by the addition of GSH. However, contrary to the results from cytotoxicity experiments, a dose dependent decrease in urea production in hepatocytes exposed to ITZ was not detected and GSH supplementation did not mitigate this effect. There are several possible explanations for this discrepancy. First, the timing of supernatant collection allowed urea to accumulate from when the cells were plated until the end of the experiment at

either 4- or 24-hours post-exposure to ITZ. This explains why urea production significantly increased over time. The accumulation of urea over time may have masked subtle changes in production. This could be addressed in the future by washing the cells 4 hours prior to supernatant collection to allow a consistent time frame for urea to accumulate. If this protocol alteration does not produce the expected results, then using an alternative urea kit through a different manufacturer may be required. It is also possible that endogenous urea production is not a useful functional marker in this particular model, so other tests like albumin production could be assessed. Indeed, we demonstrated albumin production by the cells when assessing hepatic phenotype, so this assay could be used as a measure of function in future experiments.

Another limitation inherent to all *in vitro* experiments is that our results may not reflect *in vivo* biologic behavior in dogs experiencing clinical ITZ-associated hepatotoxicity. To address this concern, several features of our assay were specifically designed to best mimic the *in vivo* environment. Primary hepatocytes were chosen for this study over immortalized cells because they maintain liver-specific functions and enzyme activity that more accurately represent *in vivo* function (Hewitt et al., 2007; Guo et al., 2011). We also chose a sandwich configuration where cells are cultured between two layers of collagen. This is the preferred method for *in vitro* drug disposition and hepatotoxicity studies (Keemink et al., 2015). Compared to cells cultured on a single layer of collagen, sandwich-cultured hepatocytes produce albumin for longer, maintain their polygonal morphology (Dunn et al., 1989), and repolarize to form bile canaliculi (LeCluyse et al., 1994; Bi et al., 2006), thus more closely reflecting their *in vivo* functionality. These findings have been consistently demonstrated in fresh, sandwich-cultured rat (LeCluyse et al., 1994; Dunn et al., 1989) and dog (Rose et al., 2006) hepatocytes as well as cryopreserved human hepatocytes (Bi et al., 2006).

Future *in vitro* studies will assess additional antioxidants (ascorbic acid and α-tocopherol) to select the most effective compound prior to clinical trials in canine patients. Results of this and future studies are critical to developing guidelines for the use of antioxidants in ITZ-associated hepatotoxicity in dogs. Additional studies are underway in our laboratory to determine how ITZ is metabolized in dogs and the possible contribution of genetics in predicting this drug reaction. Between breed and within breed polymorphisms are responsible for a number of differences in drug metabolism. A classic example is ivermectin sensitivity in collie dogs where a deletion in the multidrug resistance gene encoding for the efflux pump P-glycoprotein results in

neurotoxicity (Mealey et al., 2001). Others involve differences in CYP enzymes. In Greyhound dogs, CYP2B11 deficiency has been implicated in poor clearance of the anesthetic propofol. Recently, three CYP2B11 haplotypes were identified across 63 dog breeds; a specific haplotype in Greyhounds was associated with reduced CYP2B11 expression (Martinez et al., 2020). These examples highlight the importance of first identifying the enzyme responsible for ITZ metabolism and then screening dogs for genetic polymorphisms that may predict hepatotoxicity.

In this study, we showed that ITZ causes dose- and time-dependent hepatocyte toxicity *in vitro*. These findings demonstrate that appropriately cultured canine primary hepatocytes are a viable model in which to study ITZ-associated hepatotoxicity in dogs. Most importantly, ITZ-induced cytotoxicity is mitigated by pre-incubation with GSH in a dose-dependent manner, suggesting a possible role for GSH or its precursors in the management of ITZ-associated hepatotoxicity in dogs.

CHAPTER 3: CONCLUSIONS AND FUTURE DIRECTIONS

Hepatotoxicity associated with the use of azole antifungals is a serious potential treatment complication in both veterinary species and man. While ITZ and other triazole antifungals are associated with fewer adverse reactions when compared with KTZ, these reactions do occur and can lead to a delay in therapy or significant morbidity and mortality. The mechanism of hepatotoxicity in dogs is unknown. In Chapter 2, we demonstrated that GSH exerts a dose-dependent protective effect against ITZ-associated cytotoxicity in canine primary hepatocytes. GSH is the most abundant hepatocellular antioxidant and is responsible for maintaining redox potential and cell signaling. Rodent studies have shown increased hepatic oxidative stress and decreased levels of GSH and other antioxidants in rats administered ITZ (Sozen et al., 2014; El-Shershaby et al., 2015). Similar to the study presented in Chapter 2, rats also experience dose- and time-dependent ITZ-associated hepatotoxicity *in vivo* (Somchit et al., 2002; Somchit et al., 2009). The protective effect provided by GSH *in vitro* suggests that the clinical use of antioxidants in dogs may prevent or alleviate this toxicity. Future *in vitro* studies in our lab will characterize the effects of other antioxidants (ascorbic acid and α-tocopherol) using this model. Once the optimum antioxidant has been identified, we will begin clinical trials in canine patients receiving ITZ.

There are many other parameters that could be evaluated in this model. First, the presence of sublethal damage could be assessed by measuring ALT in the supernatant. GSH was co-administered with ITZ in the study presented in Chapter 2; future studies could investigate the effect of GSH administered before, with, and after ITZ. This would help direct the timing of treatment in clinical situations. Lastly, it is not known if endogenous GSH decreases in canine hepatocytes exposed to ITZ. Rodriguez and Buckholz (2003) hypothesized that GSH may play a role in KTZ detoxification, so it is possible that progressive GSH depletion may be a mechanism of toxicity. If GSH is indeed depleted *in vitro*, this would establish a plausible mechanistic explanation for why supplementation is protective.

A vital part of understanding ITZ-associated hepatotoxicity is identifying the major pathways of its metabolism in the liver. ITZ is metabolized by CYP3A4 in man. The major metabolite, hydroxy-ITZ, has been identified in canine serum (Yoo et al., 2002). Based on this information, our laboratory hypothesizes that canine CYP3A12 and/or CYP3A26 are responsible

for metabolism in dogs. Studies are underway using canine liver microsomes incubated with ITZ to identify what metabolites are produced and what specific CYP(s) is involved using commercially available recombinant enzymes. ITZ and its metabolites inhibit CYP3A4 (Back and Tija, 1991; Isoherranan et al., 2004), so CYP inhibition in dogs will be assessed using a probe known to be metabolized by the enzyme (i.e. diazepam for CYP3A12). Once the metabolizing CYP has been established, that enzyme can be depleted in canine hepatocytes *in vitro* followed by exposure to ITZ and viability assessment as outlined in Chapter 2. Pharmacogenetic studies in dogs receiving ITZ therapy may identify genetic polymorphisms in genes encoding for CYPs that predispose to development of hepatotoxicity.

Rodent studies may aid in better understanding ITZ-associated hepatotoxicity. Studies by Somchit et al. (2004, 2006) identified varying patterns of hepatic degeneration and necrosis caused by ITZ in rats. A study controlling for dose and duration of ITZ treatment will help to clarify these divergent morphologic findings. Drug metabolizing enzymes are stratified in the liver, causing specific patterns of injury depending on the location of the enzyme. For example, if CYP enzymes produce a toxic metabolite, hepatocyte damage is expected to occur where CYP enzymes are concentrated (centrilobular regions). Alternatively, if progressive autoinhibition of CYP leads to accumulation of the parent compound and subsequent toxicity, damage is expected to occur where cells first contact ITZ (periportal regions). Both KTZ and its metabolites have been shown to be toxic to rat hepatocytes (Rodriguez and Acosta, 1995; Rodriguez and Acosta, 1997a), so it is reasonable to hypothesize that mixed periportal and centrilobular damage may occur with ITZ toxicity. Correlating the findings of *in vitro* enzyme and viability assays with morphologic changes in the liver will provide evidence that *in vitro* results are consistent with what occurs *in vivo*.

The study discussed in Chapter 2 provides a basis for future *in vitro* studies to elucidate mechanisms of ITZ-associated hepatotoxicity and the effect of antioxidant therapy in dogs. The protective effect of GSH identified here establishes that GSH or its precursors may be an effective prevention or therapeutic strategy for this toxicity. Future studies in our laboratory will explore the effect other antioxidants both *in vitro* and in clinical trials, investigate mechanisms of metabolism and toxicity, and identify potential genetic risk factors in dogs. We hope this work will provide veterinary practitioners with rational prevention and treatment strategies of this potentially devastating toxic disease.

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