THE EFFECT OF RIFAMPIN ON ISONIAZID-INDUCED HEPATOTOXICITY IN RABBITS

by

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B.Sc., The University of British Columbia, 1994

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Pharmacology & Therapeutics)

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

JUNE 1997

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Date June 27, 1997

DE-6 (2/88)

Abstract

The objective of this study was to examine the effect of rifampin pretreatment on isoniazid-induced hepatotoxicity. For more than 25 years, it has been suggested that the concomitant administration of rifampin with isoniazid potentiates the hepatotoxicity induced by isoniazid. However, evidence to support this suggestion has been non-conclusive.

Isoniazid and rifampin are still the two most commonly used drugs in the prophylaxis and treatment of tuberculosis. The relatively high incidence of tuberculosis throughout the world has not diminished and with more than 30 million deaths in this decade, tuberculosis is still the major killer among all single pathogens.

Isoniazid is associated with potentially fatal liver damage in 1 - 2% of individuals exposed. However, the mechanism of isoniazid-induced hepatotoxicity remains largely unknown at the present time. Our laboratory has recently established a reliable model of isoniazid-induced hepatotoxicity in rabbits with features similar to those seen in humans.

The dosing regimen in this study consisted of seven days rifampin pretreatment (50 mg/kg/day) followed by concomitant rifampin and isoniazid (each day consisting of an initial dose of 50 mg/kg followed by 3 x 35 mg/kg doses) administration for two days. Controls for each of these drugs were also included in the study design.

The aim of the present study was to determine the effect of rifampin on the degree of isoniazid-induced hepatotoxicity, as well as to increase our understanding of the mechanism of isoniazid-induced hepatotoxicity. The following are the major findings in this thesis. Using the aforementioned protocol, rifampin significantly protected rabbits from isoniazid-induced hepatic necrosis. However, rifampin had no significant effect on plasma levels of hydrazine at 12 or 32 hours of isoniazid dosing in rabbits receiving rifampin plus isoniazid. Rifampin treatment alone significantly decreased cytochrome P-450 (CYP) 2E1 and significantly increased cytochrome P-450 reductase activities. The protective effect of rifampin in INH-induced hepatotoxicity could be due to its effect on CYP2E1 or reductase activities or another effect of rifampin not measured in this study.

In conclusion, the protective effect of rifampin on isoniazid-induced hepatic necrosis observed in this study is concordant with the results of a double-blind placebo-controlled trial in humans, adding to the support for the usefulness of this rabbit model.

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Acknowledgments

I would like to thank the following individuals for their mentorship and support throughout the last two years of my M.Sc. degree:

- Dr. James Wright, for being there when I needed to be listened to and for providing insightful criticisms and comments regarding my thesis. I would also like to thank Dr. Wright for the financial reward that he has given to me in support of my degree.

- Dr. David Godin, for being a mentor and friend, for his valuable criticisms, wisdom and understanding, for his words of encouragement, and for keeping my sense of hope and spirituality alive and strong.

- Dr. Michael Walker, for being a mentor, for his confidence in me and support, and for his valuable criticisms.

- Mr. Troy Sarich, for his valuable advice and criticisms, endless patience, wisdom, and understanding regarding my thesis, for being a mentor and friend, and for sharing his expertise of experimental techniques.

- Mr. Stephen Adams, for assisting me within the laboratory and for lending me a helping hand in learning to do various assays.

- The faculty & staff of the Department of Pharmacology & Therapeutics, UBC, for believing in me and for their support and cooperation in the last two years that I have known them.

The following M.Sc. thesis would not have been made possible without the support of my parents, Alberto & Rossana Petricca, for their patience, caring, and understanding throughout my M.Sc. degree.

Introduction

Tuberculosis, Then & Now

From a global perspective, the magnitude of the tuberculosis (TB) problem has not diminished over the last 50 years. It was estimated that in 1990, there were 7.6 million new cases in developing countries and 400,000 new cases in industrialized countries, for a world wide total of 8 million new cases (Kochi, 1991). Due to primary infection or reactivation, it has been estimated that more than 80 million new cases will occur during the nineties. Moreover, with more than 30 million deaths in this decade, tuberculosis is still the major killer amoung all infectious diseases (Bloom & Murray, 1992). TB accounts for 6.7% of all deaths in the developing world, 18.5% of all deaths in adults aged 15 to 59, and 26% of avoidable adult deaths (Murray et al., 1990 & 1992).

About 5 million new cases are estimated to occur annually in Asia, but the highest estimated case rates are in sub-Saharan Africa; the current rate is 229 per 100,000 persons per year (Snider, 1994). Overall, approximately one third of the world's population is infected with M. tuberculosis; 75% - 80% of adults in developing countries have bee infected with M. tuberculosis (Murray, 1994; Kochi, 1991).

The pandemic of HIV infection has had a profound effect on the global problem of tuberculosis. It is estimated that 9 to 11 million adults and 1 million children worldwide have HIV infection, and about 85% of this burden is in developing countries. In these countries, the combination of a high frequency of HIV infection with a high frequency of M. tuberculosis infection results in a high rate of tuberculous disease as a complication of AIDS: 20% to 40% in Africa; 18% in Haiti; and as high as 25% in Brazil, Mexico, and Argentina (Tuberculosis morbidity-United States, 1995).

Epidemiology of Tuberculosis in Europe and the United States

Most new cases of TB and the vast majority of all deaths due to TB are in developing countries in Africa and Asia (Kochi, 1991), but, in addition, the re-emergence of tuberculosis still represents a serious threat to public health in Europe and the United States (Bates & Stead, 1993). Although the epidemiological situation in Europe in 1996 seems not to be "pandemic", there is still a substantial morbidity in Europe, as reflected by an annual incidence of new cases of TB ranging between 5 per 100,000 and > 50 per 100,000 per year (Raviglione et al., 1993 & 1994).

The steadily declining incidence of TB in the United States since 1882 has been reversed since 1985 (Reider et al., 1989), with 26,283 cases reported in 1991. To the trend of increasing incidence one must add the threatening emergence of drug-resistant strains that may impede our capability of controlling the disease.

Multidrug-Resistant Tuberculosis

Multidrug-resistant tuberculosis, defined as tuberculosis (TB) that is resistant to both isoniazid and rifampin, has dreadful public health consequences. Treatment usually requires more toxic and less effective medications given for longer duration (as long as 24 months). The efficacy of currently recommended preventative regimens for multidrug-resistant TB is unknown (Nitta, 1996).

In 1994, the Centers for Disease Control and Prevention published findings of a national survey of drug resistant TB in the United States. During the first three months of 1991, a total of 3,256 cases were identified; when these were tested for sensitivity to isoniazid and rifampin, 114 (3.5%) were resistant to both drugs.

Since 1985, the Los Angeles County Health Department has used Rifamate, exclusively, as a means of preventing drug resistance to tuberculosis due to inadvertent monotherapy. Some drug companies provide fixed-dose combinations of antituberculous medications, such as INH and RIF (Rifamate), and INH, RIF, and pyrazinamide (Rifater). More recently, a group of

American physicians are recommending these fixed-dose combinations to improve compliance and decrease the chance of developing resistance (Moulding et al., 1995).

Isoniazid

History

Isoniazid or isonicotinic acid hydrazide (INH) is a drug used in the treatment of tuberculosis. INH was first introduced for the treatment of tuberculosis in 1952 and was initially considered relatively free of side effects (Dickinson et al., 1981).

During the first few years after the introduction of INH, sporadic cases suggesting possible hepatotoxicity were noted, but jaundice developing in patients on treatment was often attributed to concurrent viral hepatitis or the toxic effects of other drugs such as para-amino-salicylate (PAS) (Medical Research Council., 1962; Berte & Dewlett, 1959; Cohen et al., 1961).

Berte and associates in 1959 stated that no case of hepatotoxicity was observed in 513 patients receiving INH (Berte & Dewlett, 1959). As a result, in 1963 the American Thoracic Society recommended *all* tuberculin-positive persons should receive a year of INH chemoprophylaxis regardless of age or the duration of tuberculin positivity (American Thoracic Society., 1965).

In 1969, Scharer and Smith reported liver function abnormalities in 10.3 percent of patients receiving INH (Scharer & Smith, 1969). In spite of this, however, the problem of INH-hepatotoxicity remained generally unappreciated until 1972 when a retrospective study by Garibaldi et al. reported clinical hepatitis (equivalent potentially fatal INH-hepatotoxicity) in 19 of 2,321 patients (1%) on prophylactic INH therapy; 13 of these patients developed overt hepatitis and 2 (0.1%) died (Garibaldi et al., 1972). These findings led the United States Public Health Service (USPHS) to initiate a large, prospective, multicenter surveillance study to determine the incidence of INH-related hepatitis in 13,838 persons receiving INH for chemoprophylaxis. The overall rate of hepatitis was 10.3 per 1,000 (1%), and there were eight deaths (0.06%). The unexpectedly high mortality rate resulted in termination of the study (Kopanoff et al., 1978).

Antibacterial Activity of Isoniazid

INH was first reported to be effective against *Mycobacterium tuberculosis* and *Mycobacterium bovis* in humans in 1952 (Bernstein et al., 1952; Fox, 1952; Pansy et al., 1952). INH alone has been shown to be an effective prophylactic antituberculous drug (Robitzek & Selikoff, 1952), and the combined administration of isoniazid, rifampicin (RIF), and pyrazinamide (PZA) has proven to be the most effective chemotherapeutic regimen for the treatment of drug-sensitive M. tuberculosis infections (Combs et al., 1990). Isoniazid-resistant strains were isolated almost immediately after this antibiotic began to be used therapeutically (Middlebrook, 1952), and presently about 20% of the M. tuberculosis strains in New York City are resistant to isoniazid (Frieden et al., 1993).

Isoniazid inhibits the biosynthesis of cell fatty acids, specifically the mycolic acids, found in mycobacteria and related bacteria (Davidson & Takayama, 1979; Takayama et al., 1972).

Pharmacology of Isoniazid

INH is bacteriostatic for "resting" bacilli but is bactericidal for rapidly dividing microorganisms. Although the exact mechanism of action of INH is unknown, several hypotheses have been proposed, including inhibition of the biosynthesis of mycolic acids, cell fatty acids, nucleic acids, or inhibition of glycolysis in mycobacteria.

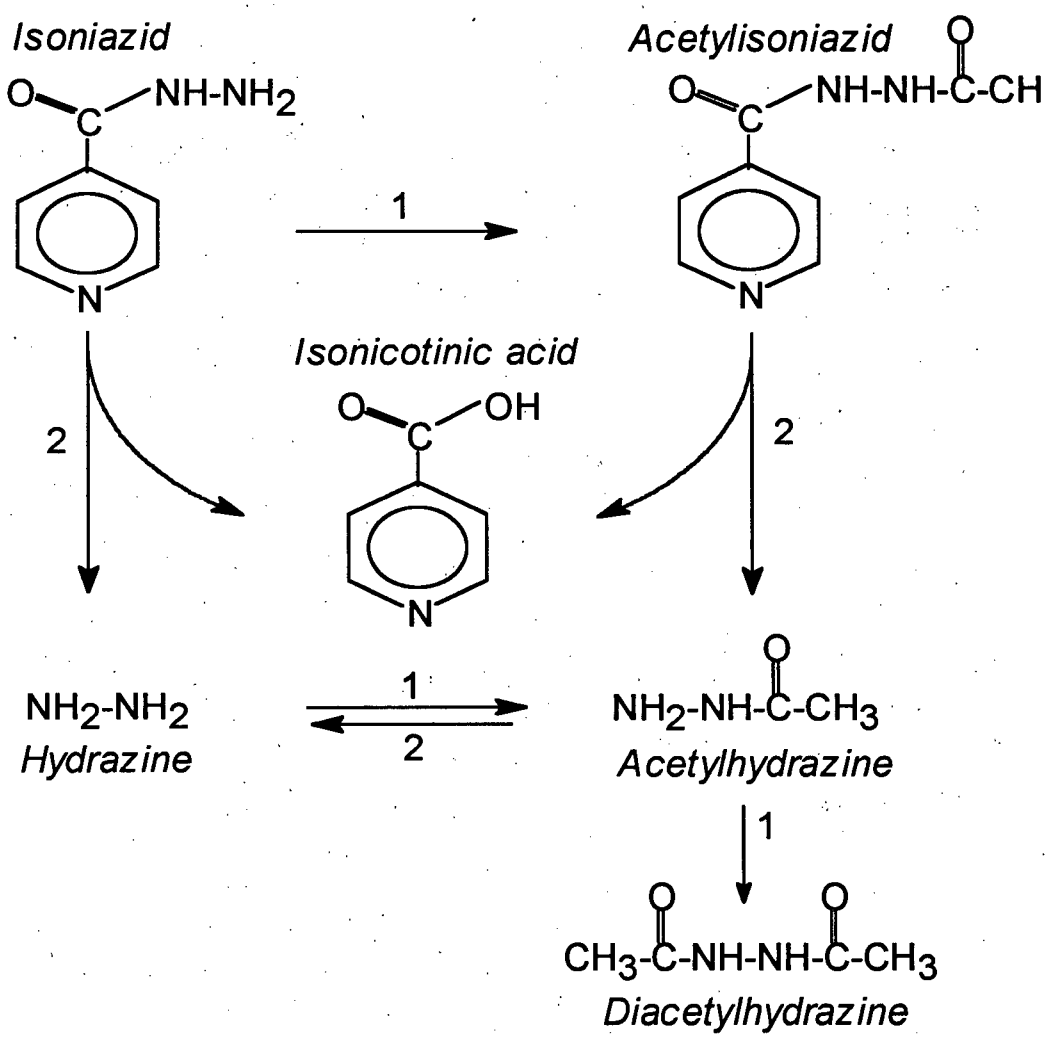
INH is readily absorbed when administered either orally or parenterally. Peak plasma concentrations occur 1 to 2 hours after oral ingestion of usual doses (Mandell & Petri, Jr., 1996). INH diffuses readily into all bodily fluids and cells. Concentrations in the CSF are similar to those in plasma (Holdiness, 1985). From 75 to 95% of a dose of INH is excreted in the urine within 24 hours, mostly as metabolites. The main excretory products in human beings are the result of enzymatic acetylation (e.g. acetylisoniazid) and enzymatic hydrolysis (e.g. isonicotinic acid) (Mandell & Petri, Jr., 1996). The major metabolic pathways of INH are shown in **Figure 1**.

Human populations show genetic heterogeneity with regard to the rate of acetylation of INH (Evans et al., 1960). The distribution of rapid and slow inactivators of the drug is bimodal

owing to differences in the activity of acetyltransferase (Mandell & Petri, Jr., 1996; Evans et al., 1960). The mean half-life in fast and slow acetylators is approximately 70 minutes and 2 to 5 hours, respectively (Mandell G L & Petri W A, Jr., 1996). The frequency of acetylator phenotype depends on race, but not age or sex. Most Orientals, Eskimos, and Amerindians are rapid acetylators; Caucasians, Blacks and Indians are predominantly slow acetylators.

Figure 1

Known Pathways of Isoniazid Metabolism



Note: This figure was taken, with permission, from Mr. Troy Sarich (Ph.D. Thesis, 1997).

Indications

INH is still considered the primary drug for the prophylaxis and treatment of tuberculosis. First-line agents combine the greatest level of efficacy with an acceptable degree of toxicity; these include INH, rifampin (RIF) (also known as rifampicin), pyrazinamide (PZA), ethambutol (EMB) and streptomycin (STM). Other agents include ethionamide (ETM) and para-aminosalicylate (PAS). Treatment requires at least two drugs (one of which is always INH) to prevent the development of drug resistance. The standard 6-month treatment regimen for drug-sensitive tuberculosis for adults and children consists of INH, RIF, and PZA for two months followed by INH and RIF for 4 additional months (Mandell & Petri, Jr., 1996). The combination of INH and RIF for 9 months is equally effective. The Center for Disease Control and Prevention (CDC) has recommended that initial therapy should be with a four-drug regimen (INH, RIF, PZA, and EMB or STM) (Centers for Disease Control., 1993) in response to an increasing frequency of drug resistance. Doses of INH used in humans for the prophylaxis and treatment of most tuberculosis infections vary from 0.036 to 0.072 mmoles/kg/day. In order to achieve therapeutic concentrations in the central nervous system, higher doses (up to 0.15 mmoles/kg) are used in children with tubercular meningitis.

Mechanism of Drug-Resistance and Proposed Treatment

Early observations suggested a link between INH resistance and the loss of mycobacterial catalase-peroxidase activity (Cohn et al., 1954; Middlebrook, 1954). Catalase-peroxidase oxidizes INH to form reactive intermediates in the mycobacterium (Johnsson & Shultz, 1994). Recent reports have demonstrated that deletion of, or point mutations in, the M. tuberculosis katG gene, which encodes a unique catalase-peroxidase, results in the acquisition of INH resistance and that transformation of INH-resistant strains of M. tuberculosis with a functional katG gene restores sensitivity to the drug (Zhang et al., 1992). Current evidence suggests that another mechanism of resistance is related to a missense mutation within the mycobacterial *inh*A gene involved in mycolic acid biosynthesis (Banerjee et al., 1994).

As a means of preventing drug resistance to tuberculosis due to unintended monotherapy, some drug companies provide fixed-dose combinations of antituberculous medications, such as INH and RIF (Rifamate), and INH, RIF, and PZA (Rifater). A group of American physicians (Moulding et al., 1995) have recently recommended these fixed-dose combinations with two or more antituberculous drugs in one capsule or tablet to improve compliance and prevent the development of resistance.

Adverse Effects of Isoniazid

The incidence of adverse reactions to INH is low, the most prominent of these reactions being hepatotoxicity, peripheral neuropathy, convulsions, hematological reactions, rash and fever. In rare instances, mental abnormalities have been noted during use of the drug; among these are euphoria, paresthesias and stupor. INH inhibits γ-amino-butyric acid (GABA) aminotransferase activity in the central nervous system and has been investigated in human neurological disorders where a GABA deficiency is postulated to contribute to the symptoms of INH administration (Perry et al., 1981; Perry et al., 1982). The prophylactic administration of pyridoxine (15-50 mg daily) prevents not only the development of peripheral neuropathy but also most other nervous system toxicities (Mandell & Petri, Jr., 1996). The hepatotoxic effect of INH is the focus of this thesis.

Hepatotoxicity of Isoniazid

Hepatotoxicity is the predominant toxic effect of INH. The characteristic pathological process is bridging, multilobular, and/or patchy necrosis. It is now recognized that INH causes a mild, evanescent focal hepatitis associated with asymptomatic aminotransferase elevation in 10 - 20% of users and clinical hepatitis (overt hepatitis resembling viral hepatitis) in approximately 0.5 to 2% of patients. INH hepatitis may manifest a full spectrum of severity, ranging from mild acute hepatitis to complete hepatic necrosis and hepatic failure or a more prolonged subacute course resulting in chronic active hepatitis (Bass & Ockner, 1990).

INH-induced liver injury is clinically, biochemically and histologically indistinguishable from viral hepatitis (Black et al., 1975).

Two reports have demonstrated both necrosis and steatosis (fat accumulation) as manifestations of INH-induced hepatotoxicity in patients taking INH and RIF (Pessayre et al., 1977; Pilheu et al., 1979). In most of the cases reported, necrosis is the major pathological feature of INH-induced hepatotoxicity.

Notably, when taken acutely at high doses, for example, in suicidal attempts, INH overdosage results in coma, seizures, metabolic acidosis, and hyperglycemia, but no hepatotoxicity. Conversely, patients receiving INH daily do not usually demonstrate signs of hepatotoxicity until day 3 - 5 of the dosing regimen, at the earliest. The reason for this delay is not fully understood. Although several hypothesis have been proposed, the mechanism of INH-induced hepatotoxicity is unknown at the present time.

Hepatotoxic Risk Factors

Age appears to be the most important factor in determining the risk of INH-induced hepatotoxicity. Hepatic damage is rare in patients less than 20 years old; the complication is observed in 0.3% of individuals 20 to 34 years old, and the incidence increases to 1.2% and 2.3% in those 35 to 49 and older than 50 years of age, respectively (American Thoracic Society., 1986; Comstock, 1983; Mandell & Petri, Jr., 1996). Greater susceptibility to INH-induced hepatotoxicity in females than males has been proposed (Moulding et al., 1989; Snider & Caras, 1992).

Ingestion of large amounts of alcohol and/or acetaminophen in patients taking INH is known to increase the risk of INH-induced hepatotoxicity (Kopanoff et al., 1978; Gronhagen-Riska et al., 1978; Cross et al., 1980).

Acetylator phenotype has also been proposed as being a major risk factor of INH-induced hepatotoxicity; however, evidence is available both in favor and against this finding. Mitchell and associates (1975b) originally suggested that isoniazid-hepatotoxicity occurs mainly in rapid acetylators, based on the observation that Orientals, 90% of whom are rapid acetylators,

showed a higher incidence of INH-induced hepatotoxicity. On the other hand, there is substantial clinical evidence to show that the risk of hepatic reactions during treatment with INH is no greater in rapid than in slow acetylators (Dickinson et al., 1981; Riska, 1976; Singapore Tuberculosis Service/British Medical Research Council., 1977; Hong Kong Chest Service/British Medical Research Council., 1977). In fact, some studies (Lal et al., 1972; Lauterburg et al., 1985; Smith et al., 1972; Musch et al., 1982) have showed that slow acetylators are at a greater risk.

In earlier clinical pharmacologic investigations, Mitchell and colleagues (1975b) suggested that acetyl isoniazid (acetyl-INH), the principal metabolite of INH, is converted into acetylhydrazine. This, in turn, they speculated, was metabolized by the influence of microsomal P-450 enzymes to a reactive intermediate which caused the hepatotoxicity. Because acetyl-INH is formed in larger amounts in rapid than in slow acetylators, they predicted a greater potential for hepatotoxicity in rapid than in slow acetylators. Studies by Ellard (Ellard, 1976) and Gurumurthy and coworkers (Gurumurthy et al., 1984) did not verify this hypothesis. While the formation of acetylhydrazine from acetyl-INH was confirmed by Ellard (1976), this group showed that the acetylhydrazine formed was rapidly acetylated to the less toxic diacetylhydrazine which was rapidly excreted. Timbrell et al. (1977) found that rapid acetylators form more acetylhydrazine *in vivo* than slow acetylators, but this is primarily excreted as diacetylhydrazine, a non-toxic metabolite of acetylhydrazine. This finding was later confirmed by Lauterburg and coworkers (Lauterburg et al., 1985). Pharmacokinetic investigation of INH therapy in humans revealed greater urinary excretion of acetylhydrazine, the suspected hepatotoxin, in slow acetylators than in rapid acetylators (Peretti et al., 1987).

Another potential risk factor increasing the risk of hepatotoxicity, which is disputed by many, is the concomitant use of RIF with INH. Some have claimed RIF to produce hepatic injury by its own toxic action; however, in most of these cases RIF was concomitantly administered with other antituberculous drugs including INH, PZA, and EMB; INH and PZA are known to be hepatotoxic (Mandell & Petri, 1996). In addition, RIF can induce the metabolism of a wide array of drugs like digitoxin, quinidine, corticosteroids, methadone, dapsone, oral anticoagulants, and verapamil (Gangadharam & Iseman, 1986). This is discussed in detail on page 24.

Inhibition and Induction of Hepatic Microsomal Enzymes by Isoniazid

INH is a potent inhibitor of several hepatic cytochrome P-450 enzymes, including cytochrome P-450 (CYP) 2E1 in humans (Epstein et al., 1991). However, Zand et al. (1993) demonstrated an induction of CYP2E1 activity in humans, two days after a one week regimen of INH, 0.03 mmoles/kg/day. Zand and associates proposed that this induction is likely due to a stabilization of the CYP2E1 enzyme during its inhibition by INH; upon termination of INH therapy, the decreased INH concentration unmasks an increased number of active enzymes (occurring with INH administration) resulting in increased enzyme activity. Concomitant ingestion of acetaminophen during this period of increased CYP2E1 activity has been shown to potentiate acetaminophen-induced hepatotoxicity (Nolan et al., 1994).

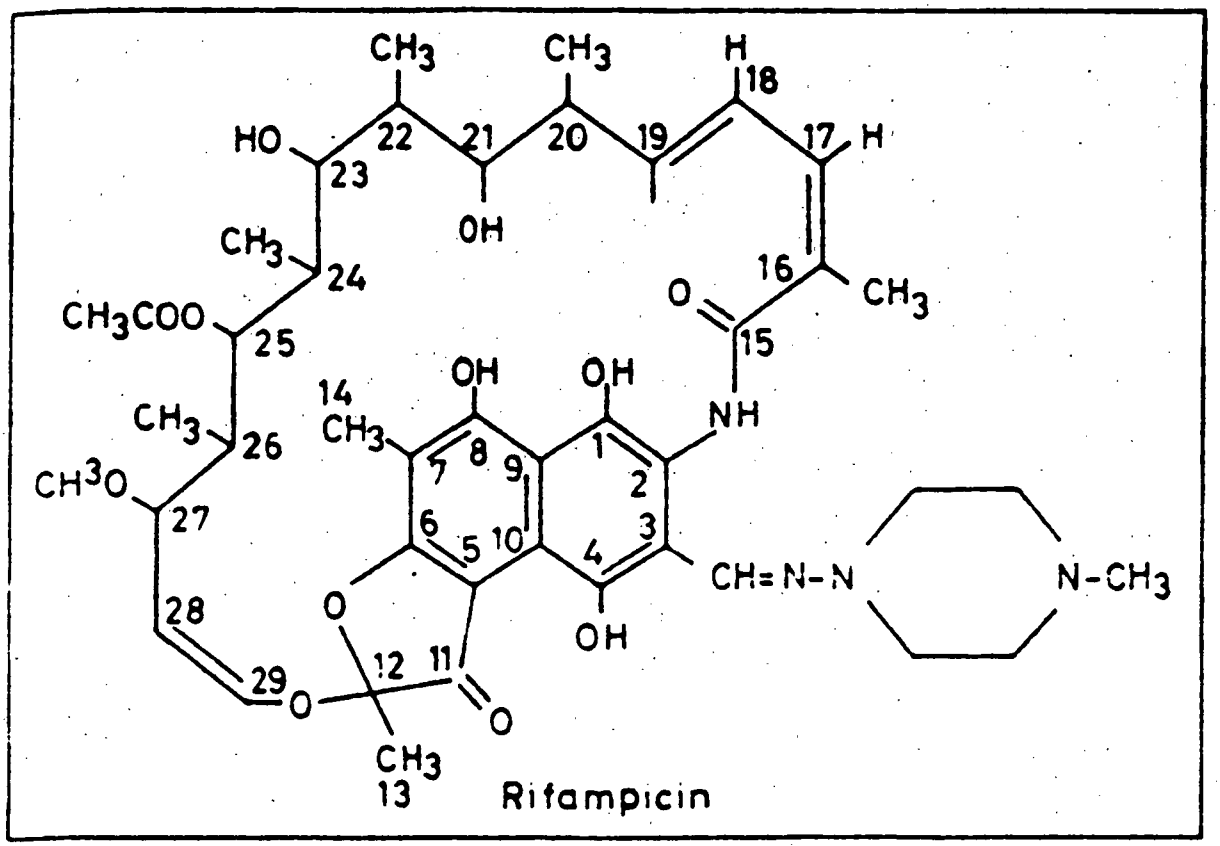
Rifampin

History

In 1957, a new strain of streptomyces (S. mediterranei) was isolated from a soil sample by Sensi in Italy (Jouhar, 1968). The culture showed high bactericidal activity against gram-positive and some gram-negative bacteria, including Mycobacterium tuberculosis and M. leprae (Virchow & Fleming, 1968). Thin layer chromatography (TLC) revealed a mixture of five antibiotic substances in the crude product (rifamycin), and only rifamycin B was obtained as a crystalline and stable substance. Further modification of rifamycin B to optimize pharmacokinetic and antibacterial characteristics finally produced, in 1963, a derivative of rifamycin which has been given the name rifampicin (rifampin)(RIF) in the U.S.A.. The structure of RIF is shown in **Figure 2** on the following page.

Figure 2

Structure of Rifampin



Antibacterial Activity of Rifampin

RIF is bactericidal for both intracellular and extracellular microorganisms including Escherichia coli, Pseudomonas, Proteus, and Klebsiella. RIF is very effective against Staphylococcus aureus in the 3 to 12 ng/ml range, as well as Neisseria meningitidis and Haemophilus influenzae in the 0.1 to 0.8 ug/ml range. At concentrations of 0.005 to 0.2 ug/ml, RIF effectively inhibits the growth of M. tuberculosis *in vitro* (Mandell & Petri, 1996).

Pharmacology of Rifampin

RIF and INH are the most effective drugs available for the treatment of tuberculosis. The dose of RIF for treatment of tuberculosis in adults is 600 mg, given once daily. In children, 10 to 20 mg/kg, with a maximum daily dose of 600 mg, is recommended. However, in patients with impaired liver function, daily doses of higher than 8 mg/kg are not recommended. Other uses of RIF include the following: treatment of brucellosis, chancroid, mycetoma, Q fever, legionellosis, chlamydial infections, and staphylococcal endocarditis; prophylaxis of epiglottis and meningitis due to H. influenzae, the prophylaxis of meningococcal meningitis, and various other staphylococcal infections for treatment or prophylactically to reduce staphylococcal carriage (Reynolds, 1996).

In most short-term standard tuberculosis regimens, RIF is given in association with INH for 6 to 9 months as recommended by the International Union Against Tuberculosis (Reynolds, 1996).

Pharmacokinetics

RIF is readily absorbed from the gastro-intestinal tract and peak plasma concentrations of about 7 to 10 ug per ml occur 2 to 4 hours after a single oral dose of 600 mg, but there is considerable interindividual variation in peak plasma levels (Acocella et., 1978; Kenny & Strates, 1981). RIF is approximately 80% bound to plasma proteins. It is widely distributed in body tissues and fluids and diffuses into the cerebrospinal fluid when the meninges are inflamed

(D'Oliveira, 1972). In pregnant women receiving the drug, RIF crosses the placenta and is distributed in breast milk. The molecule as a whole is lipid soluble and at physiological pH, only 25% of the compound is ionized (Kenny & Starts, 1981). Half-lives for RIF have been reported to range from 2 to 4 hours (Holdiness, 1984). Pharmacokinetic analysis of RIF has demonstrated that the rate of biotransformation to its desacety-lRIF derivative (Acocella et al., 1978) increases with time. Intravenous infusion of this agent in tuberculous patients has revealed similar C_{max} and T_{max} values to those obtained with oral dosage forms (Houin et al., 1983; Nitti et al., 1977). Following absorption from the G.I. tract, RIF is rapidly metabolized in the liver, the major metabolite being the desacetylated form at the C-25 position (Structure of RIF shown in **Figure 2**). Both RIF and the desacetylated metabolite are eliminated in the bile (Acocella et al., 1978). Another metabolic pathway yields 3-formyl-RIF which is excreted in the urine. DesacetylRIF retains approximately 80% of the antimicrobial activity of the parent compound (Acocella et al., 1978; Acocella & Conti, 1980). RIF, but not its desacetyl-metabolite, is then reabsorbed. After 6 hours, nearly all of the antibiotic in the bile is in the desacetylated form.

About 60% of a dose of RIF (including desacetylRIF) is eventually excreted in the feces. The amount excreted in the urine increases with increasing doses up to a maximum of approximately 30%.

As RIF induces its own metabolism, elimination time may decrease by up to 40% during the first two weeks resulting in shortened half-lives (2 to 3 hours) (Reynolds, 1996). Numerous studies have demonstrated increased half-life and serum concentrations in patients with liver disease. The resultant increased serum concentrations of RIF could give rise to increased serum bilirubin levels as a result of competition for common biliary excretion (Acocella & Conti, 1980; Holdiness, 1984). Administration of single therapeutic doses of RIF is also associated with a transient increase in serum bilirubin levels, also as a result of competition between RIF and bilirubin for excretion; however, with repeated administration of RIF, the bilirubin levels return to normal.

Drug Interactions of Rifampin with Isoniazid

It has been shown that there is little significant pharmacokinetic interaction between RIF and INH (Acocella et al., 1972). Although lower blood concentrations of RIF have been reported with concomitant INH administration, the effect is not considered clinically significant (Mouton et al., 1979).

Mode of Action

RIF inhibits DNA-dependent RNA synthesis in sensitive bacterial cells, but not in resistant bacteria or in mammalian cells (Staehelin et al., 1968). It does this by inhibiting DNA-dependent RNA polymerase of mycobacteria and other microbes by forming a drug-enzyme complex, leading to suppression of initiation of chain formation (but not chain elongation) in RNA synthesis. Interestingly, RIF does not significantly bind nuclear RNA polymerase of various eukaryotic cells, which likely contributes to its specificity for bacterial and some viral cells *versus* eukaryotic cells (Mandell & Petri, Jr., 1996). Additionally, RIF is required in much higher concentrations to inhibit RNA synthesis in mammalian mitochondria than bacterial cells. Higher concentrations of RIF are also effective against viral DNA-dependent RNA polymerases and reverse transcriptases.

Major Toxicities of Rifampin

In usual doses, RIF is generally well tolerated: less than 4% of patients with tuberculosis have significant adverse effects. When given at its recommended daily dose (5 - 10 mg/kg), the most common adverse reactions noted in patients are nausea and vomiting (1.5%), rash (0.8%), and fever (0.5%) (Grosset & Leventis, 1983; Mandell & Petri, Jr., 1996). The major toxicities of RIF affect the liver and the immune system. RIF-induced hepatotoxicity is dose-related and has been observed mainly in patients with underlying liver disease. The most notable problem is the development of jaundice (Scheuer et al., 1974). Sixteen deaths associated with this reaction have been recorded in 500,000 treated patients. However, hepatotoxicity from RIF rarely occurs

in patients with normal hepatic function; likewise, the combination of INH plus RIF appears generally safe in such patients (Gandadharam, 1986).

Immunoallergenic effects of RIF are usually associated with intermittent therapy. Intermittent administration of RIF (e.g. less than twice weekly) is associated with more frequent and pronounced side effects. A flu-like syndrome is seen to develop in approximately 20% of patients receiving RIF in this manner (A Hong Kong Tuberculosis Treatment Services/Brompton Hospital/British Medical Research Council., 1974a; A Hong Kong Tuberculosis Treatment Services/British Medical Research Council., 1974b; Girling & Fox, 1971; Eule et al., 1974). The syndrome often includes fever, chills, myalgias, and in rarer, more serious instances, eosinophilia, interstitial nephritis (Flynn et al., 1974; Chan et al., 1975), acute tubular necrosis, thrombocytopenia (Poole et al., 1971), hemolytic anemia (Girling, 1977), and shock (Girling & Hitze, 1979).

RIF, like many other drugs, can form immunogenic complexes with proteins which then circulate or adhere to cellular surfaces. Immunogenicity of RIF has been demonstrated in experimental animals and in humans by the development of RIF-dependent antibodies, especially IgM. The latter antibodies are directed against a side chain of RIF, but IgG, IgA, and IgE antibodies to other determinants of RIF have also been detected (Grosset & Leventis, 1983).

Hepatotoxicity of Rifampin

As mentioned earlier RIF can disturb liver function primarily due to its competition for excretion with bilirubin, but the risk of it causing serious or permanent liver damage is small, particularily among patients with no previous history of liver disease. More importantly, it is absolutely fundamental to stress that it is difficult and perhaps impossible to ascribe a hepatotoxic reaction to any one antituberculous agent because patients usually receive several drugs together. In many instances, indirect evidence has been used in the literature in attempt to assess the hepatotoxic nature of RIF. Thus, reports of the hepatic toxicity of RIF *per se* is questionable.

There are few reports of the adverse effects of RIF on liver function in humans in which patients receiving the drug had no underlying liver disease such as cirrhosis, ascites, or hepatitis associated with including alcoholism or drug-induced liver disease. Capelle and associates (1972) found that RIF competitively interfered with the elimination of bilirubin and bromsulphalein (BSP) by the liver in normal patients. In five of six subjects with normal livers, RIF caused an increase in total serum bilirubin from 0.87 ± 0.43 mg/100ml (before intake of RIF) to 1.51 ± 0.90 mg/100ml (3 hrs after a single dose of 600 mg RIF). The clearance of BSP was also significantly reduced in these patients from 0.105 ± 0.060 mg/100ml to 0.041 ± 0.009 mg/100ml on the 17th day of RIF treatment (Capelle et al., 1972). Transient increases in liver enzymes (e.g. alkaline phosphatase & serum glycolic-pyruvic & glycolic-oxalate transaminase) as well as serum bilirubin and BSP concentrations, without clinical evidence of hepatitis, have also been reported in studies in which RIF was given alone (Brickner, 1969; Cohn, 1969). In both studies, serum bilirubin elevation was usually small, and returned to normal by the conclusion of the course of treatment. Further studies determined that these abnormalities were not the result of interference by RIF or its oxidation products on the determination of bilirubin levels. When RIF and its metabolites were added to serum samples, no alterations in bilirubin levels in these samples were noted. Hemolysis was also considered as a possible mechanism. However, it was found that RIF had no effect on hepatic glutathione levels, suggesting the drug possesses no oxidizing action, as well as on the human red blood cell survival (Cohn, 1969). Similar abnormalities of liver function tests have been observed in studies in which RIF has been given in combination with other drugs (Capelle, 1972; Proust, 1971; Acocella et al., 1972; Steele et al., 1991; Hong Kong Chest Service/Tuberculosis Research Centre, Madras/British Medical Research Council., 1992). Nonetheless, both Cohn (1969) and Capelle et al. (1972) observed a transient elevation in serum bilirubin 3 hours after a single dose of RIF, returning to normal within 24 hours, demonstrating that RIF's competitive inhibition of bilirubin excretion is not due to direct hepatotoxicity.

These transient disturbances of liver function typically occur during the first few weeks of chemotherapy and suggest a mild hepatocellular dysfunction rather than cholestasis (Baron &

Bell, 1974). They are common, but are self-limiting even if RIF administration is continued, without interruption. A clinical and histological study of 11 patients during RIF administration suggests that even patients with no symptoms or signs of liver damage may have transient histological evidence of mild liver cell damage (Scheuer et al., 1974). Similar biochemical and histological changes have been reported during the administration of INH alone (Scharer & Smith, 1969).

Sherlock (Sherlock, 1968) distinguishes two types of jaundice associated with anti-tuberculosis drugs: I) due to a hepatitis-like reaction with hepatocellular injury; included under this heading are INH, ETM, PZA, EMB and cycloserine; II) due to cholestasis following disturbances of bile metabolism, transport or excretion. Under this heading is listed RIF. PAS is considered capable of causing both types of jaundice via an immune mechanism. Several investigators have concluded that jaundice that develops during combined RIF-INH therapy is not attributable to RIF (Chevrel, 1971; Decroix et al., 1971; Modai et al., 1978).

Clearly, it may be concluded that RIF administered with other drugs to patients with chronic tuberculosis is associated with toxic hepatitis in a small fraction of patients. The incidence of this hepatitis appears to be associated with certain accompanying drugs, a history of alcoholism and pre-existing liver disease.

Species Differences in the Metabolism of Rifampin

A study on the pharmacokinetics of rifapentine, a long-lasting analogue of RIF, carried out on rats, mice, and rabbits (Assandri et al., 1984) showed that, in the rat, the plasma concentration profile of the antibiotic obtained after parenteral treatment substantially reflects that previously reported in human volunteers (Birmingham et al., 1978). The rates of absorption (peak time: 8 hours), elimination ($t_{1/2}$ ~ 17 hrs), peak levels (C_{max} = 9.7 μg/ml after 10 mg/kg dose) and area under the curve (AUC)(279 μg·hour/ml after 10 mg/kg dose) values were similar to those measured in man. In mice, rifapentine showed a kinetic profile resembling that in rats, whereas in rabbits, rifapentine was eliminated ten times more rapidly than in the rat, mouse or

human. In both the rat and the mouse, AUC values of the parent drug were almost identical to those calculated for total radioactivity, indicating little presence of plasma metabolites.

Among the three animal species studied, the rabbit appears to differ greatly: the plasma half-life of the antibiotic is short ($t_{1/2}$ = 1.82 ± 0.18 hours), the volume of distribution is the lowest of all the species (V_d = 0.44 L/Kg), the AUC values for the unaltered drug are not only much lower than those calculated for the rat and the mouse (AUC = 50.54 ± 4.9 μg·hr/ml), but are also significantly less ($P<0.01$) than those measured for the total radioactivity (AUC = 69.7μg·hr/ml). The metabolite measured in this study was 25-desacetyl-rifapentine (by HPLC) and the route of administration used in each case was intravenous.

In another study in which 20 mg/kg oral doses of RIF were given to guinea pigs and rabbits, mean blood levels of RIF over 24 hours after a single dose were significantly greater in guinea pigs than rabbits (Atsunobu et al., 1972).

Acute toxicity studies of RIF in animals indicate LD_{50} values of approximately 900, 1700, and 1500 mg/kg when given orally to mice, rats, and rabbits, respectively (Furesz, 1970). Comparisons of absorption between different species is difficult because of differences in doses used. The half-life values of RIF obtained after 10 mg/kg of labelled antibiotic are 3, 4, and 7 h in the rabbit, rat, and dog, respectively (Furesz, 1970). Experiments carried out on five animals species (Binda et al., 1971) showed that biliary excretion is relatively high, except in dogs: the percentage of RIF recovered in 4hr-bile samples, after administering a dose of 25 mg/kg i.v. was 37.2% in rats, 17.4% in guinea pigs, 16.3% in rabbits, 6.8% in mice and 0.6% in dogs. In the rat, rabbit, and guinea pig peak, excretion of antibiotic occurs in the first 2 hours; in the dog, on the other hand, the rate of RIF excretion remained constant. The percentage of RIF recovered in 24-hr urine samples in mice is 18.9%; rats, 13.2%; dogs, 9.6%, rabbits, 5.4% and guinea pigs, 3.9%.

The major metabolite isolated from bile and urine in humans, guinea pig, rats, dogs, and the rabbit is 25-*o*-desacetyl-RIF (Furesz, 1970). Levels of RIF metabolites are reportedly lower in the bile of animals than in man. Only 40% of the total amount of RIF excreted is represented by products of metabolism (Furesz, 1969).

Induction and Inhibition of Hepatic Microsomal Enzymes

Among the phase I biotransforming enzymes, the cytochrome P-450 system ranks first in terms of catalytic versatility and the number of xenobiotics it detoxifies or activates to reactive intermediates (Waterman & Johnson, 1991). The highest concentration of P-450 enzymes involved in xenobiotic biotransformation is found in liver endoplasmic reticulum (microsomes), but P-450 enzymes are present in virtually all tissues.

The broad and often overlapping substrate specificity of liver microsomal P-450 enzymes precludes the possibility of naming these enzymes for the reactions they catalyze. The amino acid sequence of numerous P-450 enzymes has been determined, mainly by recombinant DNA techniques, and such sequences now form the basis for classifying and naming P-450 enzymes (Nelson et al., 1993). In general, P-450 enzymes with less than 40 percent amino acid sequence identity are assigned to different gene families (gene families 1,2,3,4, etc.). P-450 enzymes that are more than 55 percent identical are classified as members of the same subfamily (e.g. 2A1, 2A2, 2A3, etc.). The liver microsomal P-450 enzymes involved in xenobiotic biotransformation belong to three main P-450 gene families, namely CYP1, CYP2, and CYP3. Liver microsomes also contain P-450 enzymes encoded by the CYP4 gene family, substrates for which include several fatty acids and eicosanoids but relatively few xenobiotics. The liver microsomal P-450 enzymes in each of these gene families generally belong to a single subfamily (e.g. CYP1A, CYP3A and CYP4A). A notable exception is the CYP2 gene family, which contains five subfamilies (e.g. CYP2A, CYP2B, CYP2C, CYP2D, and CYP2E). The number of P-450 enzymes in each subfamily differs from one species to the next. Unfortunately, a nomenclature system based on structure does not guarantee that structurally related proteins in different species will perform the same function (Nebert et al., 1989).

The levels and activity of each P450 enzyme have been shown to vary from one individual to the next, due to environmental and/or genetic factors (Shimada et al., 1994). Decreased P-450 enzyme activity can result from: (1) a genetic mutation that either blocks the synthesis of a P-450 enzyme or leads to the synthesis of a catalytically compromised or inactive

enzyme, (2) exposure to an environmental factor (such as an infectious disease or a xenobiotic) that suppresses P-450 enzyme expression, or (3) exposure to a xenobiotic that inhibits or inactivates a pre-exisiting P-450 enzyme. Increased P-450 enzyme activity can result from: (1) gene duplication leading to overexpression of a P-450 enzyme, (2) exposure to environmental factors, such as xenobiotics, that induce the synthesis of cytochrome P-450, or (3) activation of pre-existing enzyme by a xenobiotic (Parkinson, 1996).

In Vivo and *In Vitro* Cytochrome P-450 Activities Affected by RIF in Humans

To date, human hepatic cytochrome P-450's known to be induced by RIF *in vivo* are CYP2C9, CYP2C19 and CYP3A4 (Parkinson, 1996). The most abundant P-450 enzymes in human liver microsomes belong to the CYP3A gene subfamily. All human livers appear to contain CYP3A4, although the levels vary enormously (> 10-fold) amoung individuals (Wrighton & Stevens, 1992; Shimada et al., 1994). CYP3A4 is also expressed in the small intestine.

In an *in vitro* study conducted by Morel and associates (Morel et al., 1990), hepatocytes from adult and newborn humans in primary culture were exposed to RIF. The expression of four cytochrome P-450 enzymes (or groups of enzymes, namely CYP3A, CYP2C8/9/10, CYP2E1, and CYP1A2) was investigated. These enzymes remained expressed during the study period in which the cultures were treated with RIF for three days. RIF was found to increase both CYP 2C8/9/10 mRNA transcripts and the corresponding protein. Both CYP3A mRNA and protein were also strongly induced by RIF ($P < 0.05$ in all cases). However, CYP1A2 and CYP2E1 expression was unaffected by RIF.

Induction of CYP3A by RIF in cultured hepatocytes corresponds well with *in vivo* observations (Watkins et al., 1985 & 1989). For example, pregnancies and menstrual problems have been observed in women during co-administration of RIF and the oral contraceptive 17α-ethynylestradiol, which is oxidized by CYP3A4 (Guengerich, 1988). Induction of CYP3A enzymes in human hepatocytes was later confirmed by Williams et al. (1994); an increase in

CYP3A4 activity was documented as a significant increase in testosterone 6β-hydroxylation in human hepatocytes exposed to RIF for 96 hours in culture.

Hepatic Microsomal Enzyme Activities Affected by Rifampin in Animals

The optimal dosages used to observe significant induction or inhibition of cytochrome P-450 activities in response to RIF pretreatment merits emphasis on deciding what dosing regimens are to be employed in treating the animals used in the current study. The following is a synopsis of the different dosages of RIF effective in modulating hepatic enzymes.

In male NMRI mice pretreated with 2 X 40 mg/kg/day RIF orally for 1, 3 and 10 days, Heubel and Netter (Heubel & Netter, 1979) detected significant elevations of cytochrome P-450 content, liver weight, and microsomal protein, inhibition of ethoxyresorufin de-ethylation and biphenyl-4-hydroxylation activities and induction of ethylmorphine activities. In another study in male ICR-Swiss mice (Pessayre & Mazel, 1976) in which mice were treated with 50 mg/kg RIF intraperitoneally (i.p.) for 6 days, RIF pretreatment resulted in significant increases in cytochrome P-450 total heme, liver weight, and NADPH-cytochrome c reductase (also known as cytochrome P-450 reductase) without an increase in microsomal protein. Subsequent measurement of *in vitro* microsomal drug metabolism indicated that the metabolism of ethylmorphine, zoxazolamine, benzpyrene and 17β-estradiol were all significantly increased. However, when the same dosing regimen was applied to rats no significant changes in any of the aforementioned microsomal enzyme activities or *in vitro* microsomal drug metabolism were observed.

Subsequent study (Wrighton et al., 1985) of the effect of RIF on specific hepatic cytochrome P-450 activities revealed a form of rat liver cytochrome P-450, P-450p, inducible by RIF, and sharing 73% NH_2-terminal amino acid sequence homology with rabbit cytochrome LM3c. Rats receiving intraperitoneal injections of RIF (50 mg/kg/day) for four days exhibited no significant elevation in microsomal P-450p levels as measured by the amount of triacetyloleandomycin (TAO) - P-450p complex formation (Wrighton et al., 1985). On the other hand, rabbits treated in the same manner, displayed significant increases in P-450 LM3c levels according to the degree of TAO complex formation. Subsequent measurement of the effect on

RIF pretreatment on erythromycin (EM) demethylase activity showed a lack of effect in rats and a significant increase in rabbits. EM demethylase is a catalytic activity characteristic of rat P-450p and its homologous form in rabbits, LM3c (Wrighton et al., 1985). At the same doses, gerbils, hamsters and mice were also found to have significant elevations in TAO complex formation and respective EM demethylase activity.

However, a previous report by Piriou et al. (Piriou et al., 1983) demonstrated that higher doses of RIF in female rats, 400 mg/kg/day oral RIF for 8 days, resulted in significant elevation of cytochrome b_5, cytochrome P-450, and NADPH-cytochrome c reductase levels (nmol/min/mg protein), as well as a significant increase in benzphetamine *N*-demethylase activity (nmol/min/mg protein).

Lange et al (1984) demonstrated that RIF (50 mg/kg, i.p. for 4 days) induces progesterone 6β-hydroxylation and EM N-demethylation in rabbits. They subsequently identified cytochrome P-450 3c as the principal form of cytochrome P-450 induced in the livers of these animals (Lange et al., 1985). An analysis of the sequence of P450 3c (Dalet et al., 1988a; Dalet et al., 1988b) indicates that this enzyme is associated with class 3A , and it has been designated as CYP3A6 in a uniform nomenclature based on sequence comparisons (Nebert et al., 1989). Interestingly, Lange and associates (1984) compared the effect of three different doses of RIF (25, 50, and 100 mg/kg, i.p.) for 4 days on microsomal 6β-progesterone hydroxylase activity as a measure of CYP3A6 induction in rabbits. All three doses produced significant increases in CYP3A6 activity; 50 mg/kg and 100 mg/kg doses both produced a maximal activity of approximately 5 nmol product/mg protein/minute with no significant difference of effect between these two doses.

Whitehouse and associates (Whitehouse et al., 1985) observed a significant increase in cytochrome P-450 heme content and a decrease in cytochrome b_5 and ethoxyresorufin-*o*-deethylase activities, 24 hours after the last dose (50 mg/kg/day, i.p.) of 3 days RIF pretreament. This was also confirmed earlier by Heubel and Netter (Heubel & Netter, 1979) *in vitro* with 0.05 - 0.06 M RIF.

RIF was shown previously to induce both CYP3A6 and progesterone 6β-hydroxylation in adult rabbit liver microsomes (Lange et al., 1984; Schwab et al., 1988). Potenza and colleagues (Potenza et al., 1989) found that a single injection of 100 mg/kg RIF i.p. elicited a >10-fold increase in the concentration of CYP3A6 24 hr following treatment in 1 week old animals; progesterone 6β-hydroxylase activity was also induced >10-fold. They also demonstrated that the level of expression of CYP3A6 mRNA and protein increased from infancy to adulthood in rabbits; the microsomal concentration of CYP3A6 varied over a roughly 3-fold range among untreated, adult rabbits.

In vitro investigation (Daujat et al., 1987) of the effect of RIF on hepatocytes isolated from untreated rabbits and maintained in primary monolayer cultures demonstrated a characteristic dose-response relationship with the effect of increasing concentration of RIF(0 - 50 μM) on the level of CYP2B and CYP3A6. Microsomes were isolated from primary cultures of hepatocytes which had been exposed to RIF. Concomitant with the induction of both forms, benzphetamine demethylase and 6β-progesterone hydroxylase, two monooxygenase activities specific of forms CYP2B and CYP3A6, respectively (Haugen & Coon, 1976; Lange et al., 1985), were increased according to the same dose-response pattern. Daujat et al. (1991) have recently published a protocol for the induction of CYP3A *in vivo* in rabbits and in primary cultures of animal and human hepatocytes. In the rabbit, RIF was found to significantly induce the CYP3A6 enzyme when administered intraperitoneally dissolved (50 mg/ml) in 40 mM NaOH in water at a dose of 50 mg/kg/day for 4 days.

It is important to mention that the most rigorous and accurate means of determining which particular P-45O isozyme is being induced or inhibited by a drug is to measure both the level of that particular P-450 enzyme (e.g. western immunoblot) as well as the monooxygenase activity associated with that particular isozyme. Several of the monooxygenase activities used in acertaining the induction of a cytochrome P-450 enzyme have overlapping specificities within a cytochrome P-450 family as well as between different families. As well, an increase in the level of a cytochrome P-450 enzyme does not always translate into a increase in activity of that

particular enzyme, as assayed by an increase in catalytic conversion of a substrate to a known metabolite, specific for that enzyme.

Concomitant Rifampin & Isoniazid Administration: Does Rifampin Potentiate Isoniazid-induced Hepatotoxicity ?

Several studies have shown that INH-induced hepatotoxicity occurs more frequently and severely when INH and RIF are administered in combination than when INH is given alone. However, there is evidence in support and against this premise. Recently, a meta-analysis looking at the incidence of the hepatotoxicity of INH administered without RIF and of INH administered together with RIF, based on a Medline search of English language published evidence between 1966 and December, 1989, was conducted by Steele et al. (Steele et al., 1991). When studies within the meta-analysis were pooled according to drug regimen, the incidence of drug-induced hepatotoxicity in 6,105 patients taking INH and RIF (2.55%) was higher than 2,053 patients given INH with other antituberculous drugs excluding RIF (1.6%) ($p = 0.048$). Studies in children were also pooled. 1,502 children receiving INH and RIF had a higher incidence of drug-induced hepatotoxicity (6.9%) than 477 children given multiple-drug INH regimens without RIF (1.0%) ($p<0.001$).

Historically, investigators have reported an increased incidence and severity of INH-induced hepatotoxicity in patients receiving INH and RIF as compared with INH or RIF in combination with other antituberculous drugs (Smith et al., 1972; Constans et al., 1972; Lees et al., 1972; Favez et al., 1972; Gronhagen-Riska et al., 1978; Pessayre et al., 1977). Consequently, it appears that studies looking more closely at the effect of RIF on INH-induced hepatotoxicity are based on the hypothesis that the increase in incidence and severity of hepatotoxicity with INH-RIF combination treatment is due to a potentiation of INH-induced hepatotoxicity by RIF. It is important to mention, however, that in almost all these reports, some patients had pre-existing hepatitis, alcoholism, and predisposing risk factors which could possibly potentiate the hepatotoxicities of INH and RIF. Moreover, in some of these reports (Smith et al.,

1972; Favez et al., 1972; Lees et al., 1972), investigators have claimed that *INH* potentiated RIF-induced hepatotoxicity. Surprisingly, these investigators also claimed that RIF's side effects were negligible when combined with other antituberculous drugs (e.g. ethambutol).

In contrast, a double-blind placebo-controlled clinical trial of RIF and INH conducted in patients with silicosis in Hong Kong (Hong Kong Chest Service/Tuberculosis Research Center, Madras/British Medical Research Council., 1992) demonstrated no potentiation of INH hepatotoxicity by RIF.

Numerous clinical and epidemiological studies have documented the frequent occurrence of tuberculosis amoung miners, especially those with silicosis, although the reported prevalence varies considerably from one study to another (Trasko, 1956; Monaco, 1964; Capezzuto, 1969).

Mycobacterium tuberculosis has been shown to grow more rapidly in macrophages exposed to sublethal doses of silica dust, and the bacilli are released more rapidly into the surrounding medium (Allison & D'arcy Hart, 1968). The macrophage is the major effector cell in conferring resistance to tuberculosis (Lurie, 1964).

In 1981, in a review of 358 patients with silicosis, 39% were found to have tuberculosis requiring treatment (Chen et al., 1976). Chemoprophylaxis against tuberculosis could therefore be justified in a group with such a high risk of developing tuberculosis. In the foregoing study, the population consisted of Chinese men aged < 65 yrs with a history of exposure to silica dust and a diagnosis of silicosis of any severity confirmed by an independent radiologist in London, U.K.. The patients were allocated at random to one of four regimens: (1) **RIF-only (R3):** Rifampin, 600 mg daily for 12 weeks (wk), then placebo daily for 12 wk; (2) **RIF-INH (HR3):** Isoniazid, 300 mg, and rifampin, 600 mg, daily for 12 wk, then placebo daily for 12 wk; (3) **INH-only (H6):** Isoniazid, 300 mg daily for 24 wk; (4) **Placebo (Pl):** Placebo, daily for 24 wk.

Between April 1981 and September 1987, 679 patients: 172 RIF-only/ 167 RIF-INH/ 173 INH-only/ 167 Placebo were admitted to the study. For various reasons, of the remaining 652 patients: 165 RIF only/ 161 RIF-INH/ 167 INH-only/ 159 Placebo, 520 completed their allocated regimen without known interruption. Analysis of adverse reactions demonstrated that a total of 8

patients (1%) had hepatic reactions, none in the **RIF-only** series; 7 of these including 2 in the **Placebo** series, had only an asymptomatic increase in serum alanine aminotransferase (ALT) activities, and 1 (**INH-only**) had symptomatic hepatitis but without jaundice. The regimen, including placebo, was stopped in 7 and interrupted in patient 8 (**RIF-INH**). In summary, most of the reported reactions were minor, and only 26 patients (4%) had their regimens stopped because of reactions, including 4 (2%) in the **Placebo** series. The geometric mean serum ALT activities were higher in the two INH series (**RIF-INH** and **INH-only**) than in the **RIF-only** series ($p<0.001$), and there was no evidence of any significant difference between the **RIF-only** and **Placebo** series. In the **RIF-INH** and **INH-only** series, the mean ALT activities fell once active drug administration was stopped.

Perhaps the most important observation that can be made in this study is an apparent decrease in INH-induced hepatotoxicity in **RIF-INH** treated patients *versus* **INH-only** treated patients based on a comparison of serum ALT activities. Although not discussed in the report, the geometric mean serum ALT activities (n = 272) in the **INH-only** and **RIF-INH** groups were similar during the first 8 weeks of therapy; beyond 8 weeks, ALT activities in the **RIF-INH** group fell below ALT levels in the H6 group. Similarily, the percentage of patients with raised serum ALT activities (> 28 IU/L) was significantly less in the **RIF-INH** group compared to the **INH-only** group after the first month of treatment until the end of the third month, at which point therapy in the **RIF-INH** group was stopped.

Isoniazid Hepatotoxicity - Animal Models

Pharmacological studies in mice, rats, rabbits, dogs, and guinea pigs have confirmed that the major toxicities of INH observed are convulsions, hepatic necrosis, and in guinea pigs (Heisey et al., 1980), dogs (Rubin et al., 1952), and rabbits (McKennis, Jr. et al., 1956; Whitehouse et al., 1978 & 1983) hepatic steatosis.

The difficulty with many of these studies in animals has been establishing a model for INH-induced hepatotoxicity which not only resembles the observed toxicity in humans, but which

is also reproducible. Recently, a rabbit model has reproducibly demonstrated that INH induces both histopathologically and biochemically (elevated marker liver enzymes in plasma) detectable hepatic necrosis and hepatic steatosis in greater than 50% of rabbits after repeated dosing over 33 hours (Sarich et al., 1995). This model demonstrates features of INH-induced hepatotoxicity observed in humans, including evidence of hepatic necrosis, known only to occur with repeated dosing, a delay in the onset of toxicity, as well as interindividual variability in the severity of toxicity. Interestingly, acetylation rate, in rabbits as in humans, is genetically determined, and is typically bimodally distributed into rapid and slow acetylators (acetylator phenotype) of INH (Frymoyer & Jacox, 1963).

Thus, having reviewed the aformentioned literature, the most appropriate model for the purpose of my thesis would appear to be that established by Sarich et al. (1995). Amendments to this model incorporating RIF into the dosing regimen with be discussed in detail later.

Primary Hepatotoxic Metabolite of Isonaizid

- Acetylhydrazine ?

Earlier studies in rats and mice have suggested that acetylhydrazine, a metabolite of INH (following hydrolysis of acetyl-INH) is the hepatotoxin (Mitchell et al., 1976). This finding was later confirmed by Nelson et al. (1976a) who showed that ^{14}C-labeled acetylhydrazine-derived reactive intermediates bound to hepatic microsomes *in vivo*, in rats in porportion to the degree of hepatic necrosis. This was also shown, *in vitro*, using human and rat hepatocytes (Nelson et al., 1976b). These and other studies (Timbrell et al., 1977; Timbrell et al., 1980; Bahri et al., 1981) led to the proposal that INH-induced hepatotoxicity occured as a result of the conversion of acetylhydrazine by hepatic cytochrome P-450 enzymes to unknown reactive intermediates which covalently bound to intracellular proteins leading to hepatic necrosis. INH is acetylated to acetyl-INH which is then converted to acetlyhydrazine and isonicotinic acid by a microsomal amidase (Timbrell et al., 1980).

Histological studies on the proposed acetylhydrazine-induced hepatic necrosis have also suggested a hepatotoxic capacity of acetylhydrazine (Mitchell et al., 1976; Lauterburg et al.,

1979; Bahri et al., 1981; Bahri et al., 1982; Woodward & Timbrell, 1984). However, none of these studies included the measurement of hepatic aminotransferase activities in plasma, a more objective and quantitative measure of the degree of hepatic necrosis.

Other studies that followed also placed doubt on the acetylhydrazine hepatotoxicity hypothesis. For example, Wright et al. (1986) found that high doses of acetylhydrazine (4.2 mmoles/kg) given to phenobarbital-pretreated Sprague-Dawley rats do not cause significant elevations in aspartate aminotransferase and/or ALT in plasma. Phenobarbital is a potent inducer of hepatic enzymes. Additionally, concomitant administration of INH and acetylhydrazine reduced acetylhydrazine-induced hepatotoxicity (Bahri et al., 1981) and inhibited acetylhydrazine binding to protein *in vitro* (Timbrell & Wright, 1979) and gave evidence that covalent "acetyl" attachment to hepatic microsomal macromolecules does not always result in pathological damage (Woodward & Timbrell, 1984).

- Hydrazine ?

Historically, hydrazine has been known to be directly hepatotoxic. Hydrazine is a known hepatotoxin in rats (Scales & Timbrell, 1982; Timbrell et al., 1982; Jenner & Timbrell, 1994), rabbits (Yard & Mckennis, Jr., 1955; McKennis, Jr. et al, 1956; Noda et al., 1983) and monkeys (Patrick & Back, 1965).

In vitro studies by Noda and associates have identified $\bullet NHNH_2$ and diimide (NH=NH) as potentially hepatotoxic free radical intermediates of hydrazine metabolism resulting from incubation of rat microsomes with hydrazine (Noda A et al., 1985a,b & 1988). In the latter investigation, Noda et al. (1988) found that NADPH cytochrome P-450 reductase catalyzed the formation of these reactive intermediates from hydrazine.

Both acetylhydrazine and hydrazine are minor metabolites of INH. However, 24-hour excretion levels of hydrazine are reported to range from 0.4 ± 0.1 (mean ± standard error) to 1.0 ± 0.3 % of a 300 mg (2.2 mmole) dose of INH in rapid and slow acetylators, respectively (Peretti et al., 1987).

Gent et al. (1992) showed that prolonged elimination of hydrazine after administration of INH results in a slowly rising plasma baseline of hydrazine. This apparent accumulation of hydrazine is exaggerated in some patients; one patient with the highest plasma levels of hydrazine had elevated plasma bilirubin and transaminases. A progressive accumulation of hydrazine is seen with at least six weeks of treatment with INH. This possibly could explain the delayed hepatotoxicity of INH which commonly occurs within the first 8 weeks of daily INH therapy (Scharer & Smith., 1969), but which can also occur 24 weeks or longer into treatment (Byrd et al., 1972).

Recently studies in this laboratory by Sarich et al. (1996) in rabbits that were dosed subcutaneously with 0.36 mmoles/kg (50 mg/kg) INH followed by three 0.26 mmol/kg (35 mg/kg) injections at 3 hr intervals for two days demonstrated that: (1) plasma acetylhydrazine concentration at 32 hr does not correlate with plasma argininosuccinic acid lyase (ASAL) activity as AUC (r^2 = 0.02; $p<0.50$), log plasma ASAL activity at 48 hr (r^2 = 0.03; $p<0.50$) or log peak plasma ASAL activity (r^2 = 0.06; $p<0.50$); plasma acetylhydrazine at 48 hr also did not correlate with any marker of hepatic necrosis; (2) plasma hydrazine concentration at 32 hr correlated significantly with plasma ASAL activity as AUC (r^2 = 0.54; $p<0.002$), log plasma ASAL activity at 48 hr (r^2 = 0.53; $p<0.005$) and log peak plasma ASAL activity (r^2 = 0.41; $p<0.02$).

Rifampin's Role in Isoniazid-induced Hepatotoxicity

Rifampin's Effect on Hydrazine as a Hepatotoxic Metabolite of Isoniazid

Animal Studies

To further complicate the issue of elucidating the mechanism of INH-induced hepatotoxicity, Noda et al. (Noda A et al., 1983) found that rabbits pretreated with RIF (30 mg/kg, i.v.) for 6 days followed by INH (30 mg/kg, orally) exhibited no remarkable changes in plasma levels of INH metabolites, acetyl-INH, acetylhydrazine, and diacetylhydrazine, with the exception of hydrazine. After oral administration of INH or hydrazine hydrate (6.25 mg/kg, i.v.), $AUC_{0\text{-}8hr}$ values of hydrazine plasma levels in RIF-pretreated animals were significantly less than those in

the control group. The elimination rate constant (k) of hydrazine in urine was significantly greater in the RIF-pretreated group ($k = 0.55 \pm 0.03$/hr) than control ($k = 0.75 \pm 0.03$/hr)($p < 0.01$, single-compartment model). Hepatic cytochrome P-450 content in RIF-pretreated rabbits was significantly elevated relative to control with no significant effect on NADPH-cytochrome P-450 reductase activity. Interestingly, however, histological examination further showed, in RIF pretreated rabbits, more extensive hepatic necrosis than controls. Noda and associates suggested that the main metabolic course of hydrazine is oxidation and that the RIF inductive effect of cytochrome P-450 activity might prompt the oxidative degradation of hydrazine to nitrogen (N_2) via some hepatotoxic species, such as hydroxyhydrazine (H_2NNHOH), diimide (HN=NH) and/or other compounds. This was later confirmed *in vitro* by Noda and colleagues (Noda et al., 1985a,b; 1987; 1988). Noda et al. (1985a) also demonstrated, in rats, that RIF (30 mg/kg, i.p. for 6 days) and/or phenobarbital (PB) pretreatment (50 mg/kg for 3 days) followed by a single injection of INH (40 mg/kg, i.p.) resulted in a significant decrease in liver and plasma hydrazine levels, at 2 hours and 4 hours for RIF and PB pretreatment groups, respectively, as compared to control. Acetylhydrazine concentrations in both liver and plasma, however, remained unchanged for both RIF and PB pretreatment protocols. Using the same pretreament protocols outlined above, Noda et al. (1985a) also found a decrease in hydrazine plasma levels after intravenous injection of 5 mg/kg hydrazine sulfate, with phenobarbital-pretreated rats only showing a statistically significant decrease. The preceding two reports demonstrate a similarity of the effect of RIF pretreatment on the fate of INH metabolism between rats and rabbits, having used similar dosing regimens.

However, although Noda et al. (1983 & 1985a) observed that RIF pretreatment in rats and rabbits produces a decrease in plasma levels of hydrazine resulting from the metabolism of INH, neither of the studies adequately demonstrate a potentiation of INH-induced hepatotoxicity. That is, Noda et al.'s (1983) histological finding of more marked INH-induced necrosis in rabbit livers was not quantitated to any degree. For example, no grading system for assessing the degree of liver damage was utilized. Furthermore, Noda et al.'s (1985a) study in rats did not include any assessment of drug-induced hepatotoxicity by INH or RIF.

Other studies by Thomas and associates (1981) in rabbits pretreated with RIF (100 mg/kg/day, orally) for 7 days followed by a single oral dose of ^{14}C-INH 50 mg/kg showed no significant effect of RIF pretreatment on the levels of the INH urinary metabolites, hydrazine, acetylhydrazine, or diacetylhydrazine. On the other hand, a subsequent study by Thomas et al. (1987), looking at the effect of RIF pretreatment on the metabolism of acetyl-INH, showed that RIF pretreatment causes an elevation in plasma ^{14}C-acetylhydrazine and a decrease in ^{14}C-diacetylhydrazine at 6 hours following a 200 mg/kg dose of ^{14}C-acetyl-INH. A possible explanation suggested was an effect of RIF on hepatic amidase activity. However, Whitehouse et al. (1985) have shown that RIF and PB pretreatment do not affect amidase activity in the rabbit and acetyltransferase is considered non-inducible (Timbrell et al., 1980). The lack of effect of RIF pretreatment on the covalent binding of ^{14}C-acetyl-INH and its metabolites to hepatic protein (Thomas et al., 1987), as well as the lack of effect of RIF on the metabolism of INH shown by Timbrell et al. (1985), led Thomas and associates to conclude that RIF pretreatment is not likely to significantly increase the risk of hepatotoxicity, when INH is concomitantly administered to the rabbit.

Human Studies

Yet another conflicting report on the effect of RIF on INH-induced hepatotoxicity was later presented by Sarma et al. (1986), in humans. Sarma et al. (1986) measured the proportion of INH metabolized to isonicotinic acid and hydrazine in 6 slow and 8 rapid acetylators, estimated from the ratio of total isonicotinic acid to acetyl-INH in urine, in the first 6 hours after administration of INH or acetyl-INH. In slow acetylators, this proportion was approximately 3% when INH alone was administered and approximately 6% with the combination of INH and RIF ($p<0.001$). In rapid acetylators, these proportions were significantly less at <1% and 2.5%, respectively. Sarma and colleagues suggested that INH hydrolase (amidase) was induced by RIF and that the increased formation of hydrazine (based on an indirect estimate of the amount of hydrazine produced during RIF-INH therapy) could explain the increased frequency of INH-induced hepatotoxicity in slow than in rapid acetylators in tuberculous patients receiving INH plus

RIF. Furthermore, Parthasarathy et al. (1986) found that hepatotoxicity in South Indian patients during treatment of tuberculosis with short-course regimens containing INH, RIF, and PZA occured more often in slow as compared with rapid acetylators of INH, the proportions among those whose acetylator phenotype having been determined to be 11% of 317 slow acetylators and 1% of 244 rapid acetylators. Similar results were observed by other investigators who observed significantly higher levels of total and free hydrazine in plasma and whole blood respectively (by gas chromatography electron impact mass spectrometry) in slow *versus* rapid acetylators of INH (Beever et al., 1982; Blair et al., 1985).

The aforementioned human studies further add to the conundrum surrounding the mechanism of action of RIF's effect on INH-induced hepatotoxicity. Although, both animal and human studies report conflicting data regarding the effect of RIF pretreatment on the levels of INH metabolites, many claim an increase in the incidence and/or severity of INH-induced hepatotoxicity. In most cases, however, the claim to a potentiation of INH-induced hepatotoxicity is suggestive, in the sense that, there often was neither concomitant measure of plasma aminotransferase levels such as ALT or ASAL both of which are accepted as measures of the degree of necrosis in the liver, nor measurement of hepatic and/or plasma triglyceride levels which are indicators of the degree of hepatic steatosis.

It is sometimes difficult to compare human *versus* animal experimental results concerning the effect of RIF on INH-induced hepatotoxicity. However, the pharmacokinetic similarities between humans and animals for these drugs mentioned thus far should allow one to include observations in all species in attempting to explain the mechanism underlying this interaction. More importantly though, there generally does not appear to exist a study which examines all aspects of INH-induced hepatotoxicity, such as measurement of INH metabolites, phenotyping of acetylator status, and measurement of plasma aminotransferase levels. One of the aims of this thesis is to examine the effect of RIF on INH-induced hepatotoxicity by monitoring all of these variables.

Animal Models

Our laboratory has recently established a reproducible model of INH-induced hepatotoxicity in rabbits. This model has reproducibly demonstrated that INH induces both histopathologically and biochemically (elevated marker liver enzymes in plasma) detectable hepatic necrosis and hepatic steatosis in greater than 50% of rabbits after repeated dosing over 33 hours (Sarich et al., 1995). Additionally, there exists a similarity in the features of INH-induced hepatotoxicity observed in humans with those seen in this model, including evidence of hepatic necrosis, known only to occur with repeated dosing, a delay in the onset of toxicity as well as interindividual variability in the severity of toxicity. Interestingly, acetylation rate, as in humans, is genetically determined, and is typically bimodally distributed into rapid and slow acetylators (acetylator phenotype) of INH (Frymoyer & Jacox, 1963).

Thus having reviewed the aformentioned literature, the model of INH-induced hepatotoxicity in animals is that established by Sarich et al. (1995). To summarize, then, the administration schedule for INH in this model involves doses of 0.36 mmol/kg (50 mg/kg) followed by three 0.26 mmol/kg (35 mg/kg) doses at 3 hr intervals on day 1; the entire administration protocol is repeated on day 2.

Having established a dosing regimen for INH in male New Zealand white rabbits, the remaining component of the overall treatment protocol consists of a pretreatment stage with RIF, followed by concomitant use of RIF and INH, the entire protocol being representative of an acute study in rabbits.

In deciding upon what the most appropriate protocol is for the RIF component of the animal treatments, the following factors are considered: employing a dosing regimen for RIF which would: (1) significantly induce CYP3A6 levels and the corresponding 6β-progesterone hydroxylase activity as well as inhibit or induce other cytochrome P-450 activities shown to be affected by RIF, and (2) conform to the protocols of RIF pretreatment used to demonstrate significant differences in the incidence and degree of INH-induced hepatotoxicity, as determined by changes in levels of metabolites of INH and/or elevations of plasma aminotransferase levels,

in rabbits.

One of first reports of the effect of RIF pretreatment on INH metabolism in rabbits showed a reduction in acetyl-INH excreted in urine when RIF (100 mg/kg/day, p.o.) was administered for 7 days followed by a single dose of INH (50 mg/kg, p.o.) (Thomas et al., 1981). Noda et al. (1983) used a pretreatment of 30 mg/kg RIF (p.o.) for 6 days followed by a single dose of 30 mg/kg INH (orally) or hydrazine hydrate (6.25 mg/kg, i.v.) and observed no changes in plasma levels of acetyl-INH, acetylhydrazine, or diacetylhydrazine with the exception of hydrazine, the $AUC_{0\text{-}8hr}$ values in plasma being significantly less in RIF pretreated animals than control. In the same study, RIF treatment increased cytochrome P-450 levels and histological studies demonstrated more severe hepatic necrosis in RIF-pretreated rabbits compared to control.

Lange and colleagues (Lange et al., 1984 & 1985) observed significant induction of cytochrome P-450 LM3c (equivalent to CYP3A6, Nebert et al., 1989) and inhibition of LM4 (equivalent to CYP1A2: Nebert et al., 1989) after administration of 50 mg/kg/day RIF for 4 days to rabbits. LM3c induction was further confirmed by increased 6β-progesterone hydroxylase (Lange et al., 1984 &1985) and erythromycin demethylase (Lange et al., 1984 &1985; Dalet et al., 1986) activities.

RIF was also found to significantly increase cytochrome P-450 heme content, microsomal protein, and cytochrome b_5 activity with a concomitant decrease in ethoxyresorufin-*o*-deethylase activity (an activity believed to be associated with CYP1A1 gene, Nebert et al., 1989) in rabbits treated 7 days orally with 100 mg/kg RIF (Whitehouse L W et al., 1985).

Subsequent studies on the effect of RIF pretreatment on acetyl-INH metabolism in the rabbits receiving 100 mg/kg/day oral RIF for 7 days followed by a single dose of oral 200 mg/kg [^{14}C]acetyl-INH resulted in significantly less plasma diacetylhydrazine and significantly more plasma acetylhydrazine than in the control group (Thomas et al., 1987). No significant changes in covalent binding of [^{14}C]acetyl-INH to hepatic protein (a measure of INH-induced hepatotoxicity) was seen with RIF-pretreated rabbits compared to control. Still other investigators have found significant induction of CYP3A6 and corresponding EM demethylase and

6β-progesterone hydroxylase activities with 2 (Pineau et al., 1991) and 4 days (Daujat et al., 1991) of intraperitoneal treatments of rabbits with RIF (50 mg/kg/day).

Perhaps the study which best describes a dosing regimen for RIF which effectively induces CYP3A6 in rabbits is that by Lange et al. (1984). In this study, 3 different doses of RIF (25, 50, and 100 mg/kg/day, i.p.) for 4 days, were used to determine the best dose for induction of CYP3A6. Interestingly, both the 50 and 100 mg/kg doses produced maximal induction of 6β-progesterone hydroxylation as a measure of CYP3A6 activity. Most recently, a methods paper by Daujat et al. (1991) describing a protocol for the induction of CYP3A in rabbits demonstrated that RIF is a "strong inducer" when adminsitered intraperitoneally as dissolved (50 mg/ml) in 40 mM NaOH in water at a dose of 50 mg/kg/day for 4 days.

Thus, based on a review of the literature, in the present study, the RIF-INH dosing regimen chosen commences with a pretreatment stage consisting of intraperitoneal injections of 50 mg/kg/day RIF for 7 days. Then, on the 8th day of the protocol, concomitant with continued RIF dosing, administration of INH is initiated and involves subcutaneous injections of 50 mg/kg followed by 3 - 35 mg/kg doses separated by 3 hour intervals for 2 consecutive days. The experimental approach used in the animal experiments are outlined in further detail in the Methods section of this thesis.

Questions

It is quite evident that two issues remain to be resolved regarding the effect of RIF on INH-induced hepatotoxicity: (1) Does RIF potentiate INH-induced hepatotoxicity ? (2) By what mechanism does RIF modulate INH-induced hepatotoxicity, if indeed RIF does effect INH-induced hepatotoxicity ? The literature provides conflicting evidence pertaining to both issues. Thus, the following experiments aim to strengthen our understanding of RIF's effect on INH-induced hepatotoxicity, using the rabbit as a model, as well as give insight into the mechanism involved in this interaction.

Hypotheses

1. RIF pretreatment would increase the incidence and severity of INH-induced hepatotoxicity in the aforemention rabbit model.

Underlying Premises

- RIF pretreatment would result in a decrease in plasma hydrazine levels in rabbits administered RIF & INH as compared to those receiving only INH.
- RIF would cause a significant increase in hepatic cytochrome P-450 levels.
- RIF would induce cytochrome P-450 reductase activity.
- RIF would have no effect on CYP2E1 activity.
- RIF would have no effect on hepatic microsomal amidase activity.
- RIF-induced decreases in plasma hydrazine levels would be due to the conversion of hydrazine to an unknown hepatotoxic metabolite by the induction of hepatic microsomal enzymes either by:

I. an increase in cytochrome P-450 reductase activity or

II. an increase in cytochrome P-450 isozyme activities, for example CYP3A6, as seen in part by an increase in cytochrome P-450 levels or

III. a decrease in the activity of an unknown cytochrome P-450 isozyme, as seen in part by a decrease in cytochrome P-450 levels.

2. RIF alone would produce no significant signs of drug-induced hepatoxicity.

Underlying Premises

- RIF alone would not produce any significant changes in peak plasma ASAL and ALT levels (measures of hepatic necrosis) as compared to control animals.
- RIF alone would not produce any significant changes in hepatic triglyceride accumulation (a measure of hepatic steatosis) and plasma triglyceride levels as compared to control animals.

Objectives

The following is a summary of variables measured in assessing the interaction between RIF and INH using the aforementioned protocol.

- Plasma argininosuccinic acid lyase (ASAL), alanine amino transferase (ALT) and plasma and liver triglyceride (TG) levels as measures of the degree of INH-induced hepatotoxicity and the effects of RIF on this toxicity.
- Plasma hydrazine levels (12 & 32 hours after the first dose of INH) as a measure of the amount of hepatotoxic metabolite formed in response to INH and RIF-INH treatments.
- *In vitro* hydrazine production in liver microsomes (livers extracted at 48 hours of the INH component of the RIF-INH protocol) pre-incubated with INH. The purpose here is to determine the effect of INH & RIF on hepatic amidase activity.
- The effect of RIF and INH on microsomal NADPH-Cytochrome P-450 reductase activity.
- The effect of RIF and INH on total hepatic cytochrome P-450 heme content.
- The effect of RIF and INH on microsomal CYP2E1 activity.

Further Objectives

To determine:

- whether RIF alone causes significant necrosis and/or steatosis of the liver.
- whether RIF potentiates, decreases or has no effect on the degree of INH-induced hepatotoxicity.
- whether there exists any relationship (association/significant correlation) between plasma hydrazine levels and the degree of INH-induced hepatotoxicity in both Vehicle-INH and RIF-INH treated animals.
- whether there exists any relationship between the activities of the hepatic microsomal enzymes examined (CYP2E1, cytochrome P-450 reductase, and amidase) and the degree of INH-induced hepatotoxicity in both Vehicle-INH and RIF-INH treated animals.
- the mechanism by which RIF modulates INH-induced hepatotoxicity based on RIF's effect on these parameters (above).

and:

- provide further insight into the mechanism of INH-induced hepatotoxicity based on the results obtained from the parameters measured above.
- if RIF is seen to potentiate INH-induced hepatotoxicity: to attempt to elucidate whether RIF's potentiation of INH-induced hepatotoxicty is *additive* (e.g. RIF has its own mechanism of toxicity which does not affect INH-induced toxicity) or *synergistic*, for example: 1) RIF has its own mechanism of toxicity which also potentiates INH-induced hepatotoxicity or 2) RIF alone is not hepatotoxic, but has an effect which potentiates INH-induced hepatotoxicity.

Methods

Materials:

Rifampin was supplied as a gift by Ciba-Geigy Canada Ltd. Isoniazid was purchased from Sigma Chemical Co.. HPLC solvents: Acetonitrile (MeCN), Methanol (MeOH), Sodium Acetate (NaAc), and Water (H_2O) were all HPLC grade and/or prepared with HPLC grade H_2O purchased from Fisher Scientific Ltd. The alanine aminotransferase (ALT) activity assay kit was purchased from Sigma Chemical Co.. Xylene was purchased from Fisher Scientific Ltd.. Reagents for the argininosuccinic acid lyase (ASAL) activity assay include: barium argininosuccinate (converted to the sodium salt by admixture with sodium sulfate and centrifugation) and 2,4-dichloro-1-naphthol purchased from Sigma. Other reagents required for the following assays were obtained from local suppliers and were all of reagent grade.

Physical and Chemical Properties of Rifampin

In appearance, RIF is a brick-red, crystalline powder, sparingly soluble in water, but freely soluble in organic solvents such as chloroform (Lepetit., 1968). As a dry powder in a sealed container at 25 °C or less, it is stable for five years; in solution, however, it decomposes rapidly at room temperature. However, Mitchison et al. (Mitchison D A et al., 1970) reported that RIF antimicrobial activity in eight urine specimens after storage at 4°C lost only 10% of its activity in six days.

RIF was freshly prepared on a weekly basis sufficient for the treatment of a group of 10 rabbits (a total of 40 rabbits utilized in this study was divided into 4 sets of 10 rabbits each over a period of two months) at a time. The RIF solution was then aliquoted into 9 daily portions. Then, on the morning of each day of RIF dosing, an aliquot was thawed and immediately administered to the rabbits. A dose of 50 mg/kg RIF (i.p.) was achieved with a volume of 1.5 ml/kg of the RIFsolution (33.3 mg/ml in 40 mM NaOH). Previous experiments by Daujat et al.

(1991) involved dissolving RIF (50 mg/ml) in 40 mM NaOH and injecting this RIF solution at 1ml/kg volume.

Animals:

Forty New Zealand male white rabbits weighing 2 - 3 kg were obtained from the Animal Care Unit at the University of British Columbia. Throughout the entire protocol the rabbits were housed in stainless steel cages with access to food and water ad lib. The rabbits were housed and treated in groups of 10 animals. A 12 hour light/dark cycle was also included. This project was approved by the University of British Columbia Committee on Animal Care.

Phenotyping:

N-acetyltransferase-2 is the enzyme responsible for the polymorphic acetylation of both sulfamethazine and isoniazid in rabbits and humans (Blum et al., 1989; Vatsis et al., 1995). The use of sulfamethazine for acetylation phenotyping has proven to be a reliable technique based on similar acetylation characteristics of sulfmethazine in rabbits and acetylation of INH *in vivo* and *in vitro* (Gordon et al., 1973). Only rabbits in the Veh-INH and RIF-INH treatment groups were phenotyped for acetylator status 3 days prior to commencement of the dosing regimen including rifampin (RIF) and isoniazid (INH). A 3 day acclimatization period was allotted before phenotyping. The method used was adopted from a similar protocol established by Fischer and Koltz (Fischer C & Klotz U., 1978). A 0.036 mmol/kg (10 mg/kg) dose of sulfamethazine (SMZ) was injected in the lateral ear vein. Then, a 1 ml blood sample was withdrawn from the opposite lateral ear vein using a heparinized 1 ml tuberculin syringe, 20 minutes following the SMZ injection. The resulting plasma samples obtained were assayed for SMZ, and its metabolite acetylsulfamethazine (AcSMZ), using high performance liquid chromatography (HPLC). An ISCO model 2350 HPLC pump fitted with an ISCO ISIS Autoinjector/Autosampler, an ISCO V^4 ® UV-Variable Wavelength Absorbance Detector and the Chemresearch™ Chromatographic Data Management/System Controller (Version 2.4 for IBM-PC®) was employed. Prior to loading

samples onto a CSC Select ODS-2 (5 μm - 10 x 0.46 cm)(Chromatography Sciences Company Inc.) HPLC column, a work-up step in preparing the samples for injection was included.

The analytical procedure used for the quantitation of SMZ and AcSMZ in plasma samples was developed and validated in this laboratory (Sarich et al., 1997). The work-up step is as follows. 100 μL of MeCN was added to an equal volume of plasma and 10 μL of 250 μM sulfamerazine (SME). The combination was vortexed and incubated for six minutes (at room temperature) at which point 200 μL of distilled water was added and vortexed and finally centrifugated for six minutes at 12,700 x g. The resulting supernatant was injected onto the HPLC column. The solvent system used consisted of a mixture of 0.01M NaAc, 13.5% MeOH and 7.2% MeCN (pH 5.0) pumped at 1 mL/minute. This solvent is a 10% dilution of the phenotyping solvent used by Sarich et al. (1997) because of differing lengths of chromatographic columns used by Sarich and myself; the dilution was necessary to resolve the SMZ and AcSMZ peaks. All solvents were filtered prior to use (0.45 μm pore, 45 mm Nylon filters, Micron Separations Inc.) and degassed *in situ*. Room temperature ranged from 23-25 °C. The retention times of SME, SMZ, and AcSMZ in plasma were approximately 4, 6, and 7.5 minutes, respectively. The retention times of these standards did not differ significantly in propanol, blank plasma, or plasma samples.

Plasma concentrations of AcSMZ and SMZ were determined by comparison with peak areas of standard solutions of SMZ and AcSMZ both prepared as 0.01 mg/mL stock solutions (**Tables 1, 2, & 3**). Peak areas were detected at 254 nm.

Standard curves of SMZ and AcSMZ in plasma were made as follows. A standard curve of SMZ was prepared using blank rabbit plasma spiked with SMZ at concentrations of 25, 50, 75, 100, 125, 150, and 175 μM. The standard curve had a correlation coefficient (r) of 0.999, a slope of 0.216 (95% confidence interval: 0.210 to 0.221) mV·seconds/μM SMZ and a y-intercept of -0.596 (95% confidence interval: -1.173 to -0.021) mV·seconds/μM SMZ (**Table 1**; **Fig. 3**). The equation: $y = mx + b$ (where y is the peak area in mV·seconds, x is the concentration of plasma SMZ in μM, and b is the y-intercept equal to "0") was used to convert peak areas of SMZ

(mV·seconds; determined by computer-aided integration) into SMZ concentration. The slope of the standard curve obtained using this equation had a correlation coefficient (r) of 0.999 and a slope of 0.211 (95% confidence interval: 0.208 to 0.214) mV·seconds/μM SMZ.

A standard curve of AcSMZ was constructed using blank rabbit plasma spiked with AcSMZ at concentrations of 25, 50, 75, 100, 125, 150, and 175 μM. The standard curve had a correlation coefficient (r) of 0.999, a slope of 0.270 (95% confidence interval: 0.262 to 0.277) mV·seconds/μM AcSMZ, and a y-intercept of -1.551 (95% confidence interval: -2.423 to -0.678) mV·seconds (**Table 1**; **Fig. 4**). The equation $y = mx + b$ (where b, the y-intercept = 0) was used to calculate unknown concentrations of AcSMZ in plasma samples. The slope of this standard curve had a correlation coefficient (r) of 0.998 and a slope of 0.257 (95% confidence interval: 0.250 to 0.264) mV·seconds/μM AcSMZ.

A unknown peak observed in some blank plasma was subtracted from a similar interfering peak in SMZ and AcSMZ standards in plasma. This unknown peak may have accounted for the fact that some of the standard curves did not go through the origin.

Both the SMZ and AcSMZ standard curves were prepared immediately prior to running of the samples. Although the sample range for SMZ (8 - 50 μM) and AcSMZ (128 - 226 μM) was outside the standard range (25 - 175 μM) for both SMZ and AcSMZ, the slope of the AcSZM standard curve (0.257; 95% confidence interval: 0.250 to 0.264) occurs within the confidence interval for the AcSMZ standard curve constructed previously by T. C. Sarich (Ph.D. Thesis, 1997) in this laboratory. The slope of the SMZ standard curve, 0.211 (95% confidence interval: 0.208 to 0.214) mV·seconds/μM SMZ is close (< 5% difference when comparing 95% confidence intervals) to that prepared previously by T. C. Sarich (Ph.D. Thesis, 1997). Therefore, samples outside of the standard range were still included in calculating % acetylation of SMZ in determining acetylator phenotype.

The overall variance of this assay was determined by calculation of the coefficient of variance using the internal standard sulfamerazine (SME). SME was added as a 10 μL aliquot of a 250 μM solution in propanol. The coefficient of variation of the peak areas of SME over 23

sample runs was 4.5% (**Table 1**). This overall variance represents potential variance introduced into the assay by volumetric, photometric, autoinjector volume (35 μL), time and temperature variables.

After having calculated the concentrations of SMZ and AcSMZ in the plasma samples, the ratio of AcSMZ/(SMZ + AcSMZ), a measure of the percentage of sulfamethazine acetylated in 20 minutes, was used to determine acetylator status. Rabbits were then classified as either rapid or slow acetylators if greater than 50% of SMZ was acetylated and less than 50% of SMZ was acetylated, in 20 minutes, respectively. A bimodal distribution of SMZ acetylation similar to the characteristic bimodal distribution of INH acetylation has been previously demonstrated in rabbits (Gordon et al., 1973).

Table 1

Validation Summary - Acetylator Phenotype Assay

Sulfamethazine (SMZ) & Acetylsulfamethazine (AcSMZ)		
	Sulfamethazine	**Acetylsulfamethazine**
Standard Range:	25 - 175 μM	25 - 175 μM
Sample Range:	8 - 50 μM	128 - 226 μM
Specificity:	A small peak present in blank plasma interfered to a small degree with some of the acetylsulfamethazine peaks.	
Linearity: SMZ (plasma): **AcSMZ (plasma):**	1) r = 0.999; slope = 0.216 (0.210 to 0.221); y-int = -0.596 (-1.173 to -0.017) 2) r = 0.999; slope = 0.270 (0.262 to 0.277); y-int = -1.551 (-2.423 to -0.678)	
Slope used in Calculation: Slope (m) of Standard Curve: *Equation: y = mx + b (b = 0); x = [Hz]; y = peak area*	1) r = 0.999; slope = 0.211 (0.208 to 0.214) 2) r = 0.998; slope = 0.257 (0.250 to 0.264)	
Volumetric Variance of Analytical Procedure:	4.53 % (CV% derived from sulfmerazine peak/internal standard variability over a total of 23 runs)	

•Values in Brackets ( ) represent 95% confidence interval;
•CV (Standard Deviation/mean)(SD/mean) represents the coefficient of variation.

Table 2

Sulfamethazine & Acetylsulfamethazine

Standard Peak Area Determination

Expected	Observed			
[Standard] (μM)	[SMZ] - (μM) (n = 2)	% - Accuracy	[AcSMZ] - (μM) (n = 2)	% - Accuracy
25	23.3	93.3	20.8	83.3
50	48.6	97.2	47.3	94.7
75	73.6	98.1	70.9	94.6
100	99.6	99.6	99.3	99.3
125	122.7	98.1	124.0	99.2
150	150.6	100.4	149.6	99.8
175	177.6	101.5	179.5	102.6

♦SMZ: Sulfamethazine; AcSMZ: Acetylsulfamethazine

Table 3

Sulfamethazine & Acetylsulfamethazine

Standard Peak Ratio Determination

Peak Ratio	Phenotyping Ratio	
AcSMZ/(SMZ+AcSMZ)	Expected Ratio:	Observed Ratio:
175/(25+175)	0.875	0.885
150/(50+150)	0.750	0.755
125/(75+125)	0.625	0.628
100/(100+100)	0.500	0.499
75/(125+75)	0.375	0.366
50/(150+50)	0.250	0.239
25/(175+25)	0.125	0.105

♦SMZ: Sulfamethazine; AcSMZ: Acetylsulfamethazine

Rifampin-Isoniazid Injection Protocol:

After having been acclimatized for 3 days following phenotyping, rabbits received intraperitoneal injections of 50 mg/kg/day RIF for 7 days (pretreatment period) followed by 2 days of concomitant INH and RIF administration. The RIF was dissolved using 40 mM sodium hydroxide (NaOH) (33.3 mg/ml) as previously described by Daujat et al. (1991). The INH dosing schedule was the same as that previously described by Sarich et al. (1995). Day eight involved an intraperitoneal injection of RIF (50 mg/kg/day) followed by a subcutaneous injection of 0.37 mmoles/kg (50 mg/kg) and 3 subsequent 0.26 mmol/kg (35 mg/kg) INH injections at 3 hour intervals. On day nine, the injection protocol from day eight was repeated.

Rifampin-Isoniazid Administration Design:

A total of 40 rabbits were randomized into one of 4 different treatment groups in the following manner. The **first group**, designated as **RIF-INH**, consisted of 12 rabbits given RIF (50 mg/kg/day, i.p.) for 7 days (pretreatment period) followed by 2 days of both RIF and INH. The INH dosing schedule is as described above. The **second group**, **RIF-Veh***, included 12 rabbits which received RIF (50 mg/kg/day, i.p.) for 7 days followed by 2 days of RIF and INH vehicle (saline - 0.9% NaCl) according to the same dosing regime for INH given above. The **third group**, **Veh-INH**, consisted of 12 rabbits dosed with 7 days of RIF vehicle (40 mM NaOH in distilled water) followed by 2 days of RIF vehicle and INH. The remaining 4 rabbits make up the **fourth group**, **Veh-Veh***, and were given RIF vehicle during the pretreatment period followed by RIF vehicle and INH vehicle for 2 days using the same INH dosing protocol described above. Only 4 rabbits were chosen as part of this control group since previous studies in this laboratory have shown no effect of vehicle injections on the parameters measured in this protocol (Sarich et al., 1995 & 1996).

Table 4

Treatment Groups Abbreviations and Descriptions

GROUP	n	TREATMENT
RIF-INH	11	RIF pretreatment (7 days) + Concomitant RIF plus INH
RIF-Veh*	11	RIF pretreatment (7 days) + RIF plus INH Vehicle (48 hrs)
Veh-INH	12	RIF Vehicle (7 days) + INH plus RIF Vehicle (48 hrs)
Veh-Veh*	4	RIF Vehicle (7 days) + RIF Vehicle plus INH Vehicle (48 hrs)

- **Veh* represents the vehicle for INH administration.**
- **One rabbit died in each of the RIF-INH and RIF-Veh* groups in the first 3-4 days of RIF treatment.**
- **One rabbit in the Veh-INH group was sacrificed prematurely at 40 hours of INH adminstration due to signs of severe INH toxicity.**

Table 5

Dosing Regimens, Drug Preparations, and Routes of Administration

COMPOUND	DOSING REGIMEN	PREPARATION	ROUTE
Rifampin	50 mg/kg/day	33.3 mg/ml RIF in 40 mM NaOH (1.5 ml/kg)	intraperitoneal (i.p.)
Isoniazid	Initial Dose of 50 mg/kg + 3 x 35 mg/kg doses at 3 hour intervals	Saline (0.9% NaCl)	subcutaneous (s.c.)
Rifampin Vehicle	1.5 ml/kg - 40 mM NaOH	40 mM NaOH	(i.p.)
Isoniazid Vehicle	1.0 - 2.0 ml Saline	Saline (0.9% NaCl)	(s.c.)

Blood Sampling:

1 mL blood samples were taken from the lateral ear vein using a heparinized syringe. Topical administration of xylene (Fisher Scientific Ltd.) followed by a wipe with distilled water using a cotton swab was used to dilate the vein immediately prior to blood sampling. Plasma was then isolated from blood samples by centrifugation and frozen at -60 °C until analysis was performed within two months. Blood samples were collected before initiating RIF pretreatment ("0" hr-RIF)(morning of day 1), prior to commencing INH administration ("0" hr-INH)(morning of day 8), and at 12, 24, 32, and 48 hours after the first dose of INH. The rabbits were sacrificed by cervical dislocation followed by exsanguination after the last blood sample at 48 hours (morning of day 10) was obtained.

Liver Samples:

Immediately after sacrificing of the rabbits, livers were removed, weighed and homogenized (with a glass tube and teflon pestle) with a homogenizing buffer (1.15% KCl 10mM EDTA, 10mM phosphate, pH 7.4). The resulting crude homogenate was centrifuged at 10,000xg for 20 minutes. The supernatant was subsequently centrifuged at 105,000xg for 60 minutes and the resulting pellet was washed by resuspending it in the homogenizing buffer and recentrifuging at 105,000xg for 60 minutes. The final pellet consisting of microsomes was resuspended for storage using a buffer of 20% glycerol, 1.15% KCl, 10mM EDTA, 10mM phosphate, pH 7.4. The microsomes were then frozen at -60 °C prior to analysis within 3 months. Additional samples of liver were placed in vials and flash-frozen in liquid nitrogen for determination of hepatic triglyceride levels.

Hepatotoxicity Assays:

Hepatic necrosis was quantitated by measuring peak plasma argininosuccinic acid lyase (ASAL) activity (Takahara units) and peak plasma alanine aminotransferase activity (ALT). ALT activity (units/L), a commonly used marker of liver toxicity, was determined using a kit from Sigma Diagnostics (Sigma Chemical Co., Kit#: 59-20). Plasma ASAL activity has previously been used as a marker for hepatic necrosis by Campanini et al. (1970) and Sims and Rautanen (1975). The ASAL enzyme mediates the degradation of argininosuccinic acid to arginine and fumaric acid. The reaction is reversible and is a component of the urea cycle in the liver. ASAL is largely specific to the liver and the kidney; however, in the kidney the reaction is reversed so that the enzyme tends to be a synthesizing enzyme (Campanini et al. 1970). The quantitation of plasma ASAL activity (expressed as μmoles/100 mL/hour; Takahara units) was done according to Campanini et al. (1970) with modifications as described in Sarich et al. (1995). To both the unknown and control tubes, 0.1 mL plasma was added followed by addition of 0.3 mL sodium argininosuccinic acid to the unknown tube. The unknown tube was incubated for one hour at 37 °C. To both tubes 0.2 mL trichloracetic acid solution was added. After letting stand for 5

minutes, a 0.5 mL aliquot was taken from each tube and added to separate tubes. Added to these tubes were 0.1 mL of 10% NaOH, 0.25 mL of the dichloronaphthol solution and 0.1 mL of NaOCl solution. The tubes were then placed in an ice bath for 15 minutes and the colour was subsequently read at 515 nm. ASAL activity was expressed in Takahara units. Plasma ASAL activities were logarithmically transformed to fit data to a normal distribution prior to statistical and correlational analyses.

Plasma triglyceride levels (expressed as mM triolein) were quantitated using a triglyceride kit from Sigma Diagnostics (Sigma Chemical Co.: Kit#: 336-10).

Baseline plasma ASAL and ALT activities and plasma triglyceride concentrations were determined from "0" hour-RIF plasma samples.

Hepatic triglyceride accumulation (hepatic steatosis) was quantified by analysis of crude liver homogenates using a Folch extraction (Folch et al., 1951) of triglycerides which has been modified as follows: 0.1 mL of diluted 33% (w/v) crude homogenate was added to 0.5 mL 2:1 chloroform:methanol in a test tube which was covered, placed on ice and vortexed every ten minutes for a total of 60 minutes. This mixture was then centrifuged at 1,000xg for 5 minutes yielding two distinct layers. 0.3 mL of the bottom layer was removed and evaporated to dryness using a boiling water bath. The remaining residue was then analyzed for triglyceride content using the same triglyceride assay kit used to measure plasma triglyceride levels. The hepatic triglyceride content is expressed as mg triglyceride (triolein equivalent) /g liver tissue (mg TG/ g liver).

Protein concentration of liver homogenate was determined according to the Bradford assay (Bradford et al., 1976).

Microsomal Enzymatic Activities:

Cytochrome P-450 Levels

Cytochrome P-450 levels (nmoles P-450/mg protein) were measured using carbon monoxide (CO) difference spectra according to the procedure established by Omura and Sato (1964). Reference wavelengths were 450 nm and 490 nm and the extinction coefficient used is 91 $cm^{-1}mM^{-1}$.

Cytochrome P-450 Reductase Activity

Cytochrome P-450 reductase activity (nmoles/minute/mg protein) was measured according to Phillips and Langdon (1962), using an extinction coefficient of 19.6 $cm^{-1}mM^{-1}$ and a final extraction volume of 1.575 mL.

CYP2E1 Activity

p-Nitrophenol hydroxylase activity (as a measure of CYP2E1 activity) was measured using procedures outlined in Reinke and Moyer (1985); Koop (1986); Jenner and Timbrell (1994a). A substrate concentration of 100 μM, a peak absorbance of 510 nm, and an extinction coefficient of 9.53 $cm^{-1}mM^{-1}$ were used in determining this activity.

Hepatic Amidase Activity

The analytical procedure used for the determination of hepatic amidase activity via the quantitation of hydrazine (μM) produced in microsomal samples preincubated with INH was developed and validated in this laboratory (Sarich T C, Ph.D. Thesis, 1997).

Hepatic INH-amidase has been previously identified in hepatic microsomes (Whitehouse et al., 1983; Sendo et al., 1984). It produces hydrazine via two possible routes of INH metabolism: (1) directly from INH hydrolysis to isonicotinic acid, or (2) indirectly from hydrolysis of acetyl-INH to acetylhydrazine plus hydrolysis of acetylhydrazine to hydrazine (**Figure 1, Introduction**).

Hepatic amidase activity was determined via the incubation of microsomes with INH as described by Whitehouse et al. (1983) and Sendo et al. (1984), followed by measurement of the rate of production of hydrazine (amount of hydrazine produced per mg protein per hour) using HPLC. The preparation of the sample prior to loading consists of the following steps. 150 μL hepatic microsomes (3-10 mg protein/mL) was incubated with 100 μL of INH in 67 mM KH_2PO_4 buffer (pH 7.0) (3 mM initial concentration) and 50 μL 67 mM KH_2PO_4 buffer (pH 7.0) at 37 °C for 30 minutes (300 μL total volume).

Hepatic amidase activity has been determined using incubations of INH with microsomes for up to 2 hours (Whitehouse et al., 1983). Previous work in this laboratory (Sarich T. C., Ph.D. Thesis, 1997) showed that hydrazine production was linear at 30 minutes; therefore, 30 minutes was chosen as the period for incubation of microsomes with INH. At 30 minutes the reaction was terminated with the addition of 0.3 mL MeCN, followed by vortexing, standing for 3 minutes, addition of 0.3 mL 0.6N $HClO_4$ and centrifugation at 12,700xg for 6 minutes. The supernatant was filtered using a Millipore syringe filter (Millex-LCR_4, 0.5 μm x 4 mm, Millipore Corporation, Bedford, MA 01730) mated to a 1 mL tuberculin-type syringe; 150 μL of the filtrate was combined with 150 μL HPLC grade water and 75 μL derivatizing reagent. The derivatizing reagent was made beforehand with 125 μL 3-methoxybenzaldehyde and 5 mL of 10 mM 9-fluorenone solution made up to 50 mL with propanol. The 9-fluorenone solution is included as an internal standard. This final mixture was then mixed thoroughly and incubated at room temperature for two hours. At two hours the solution was centrifuged again at 12,700xg for six minutes, filtered with a Millex- LCR_4 filter, and loaded onto an HPLC column (CSC-Select-ODS-2: 5 μm - 10 x 0.46 cm). The UV detector was set at 300 nm for detection of hydrazine. The same chromatographic system as that used for the phenotyping analysis was utilized. The solvent used consisted of 5 mM NaAc adjusted to pH 5.0 with glacial acetic acid and 65% HPLC grade MeCN in HPLC grade water. The solvent was filtered prior to use (0.45 μm pore, 45 mm Nylon filters, Micron Separations Inc.) and degassed *in situ*. The flow rate was 1 mL/minute.

Previous experiments by T. C. Sarich showed that, in control microsomes, the reaction (production of hydrazine from INH) was linear at 30 minutes when monitored over 120 minutes.

A standard curve was prepared (in triplicate) using blank rabbit microsomes spiked with hydrazine at concentrations of 2.5, 5, 12.5, 25, 50, 100, and 250 μM hydrazine. The standards were not incubated for 30 minutes as this may have affected the known concentration of hydrazine in them. Therefore, hydrazine concentrations of standards and samples (μM) are related to the concentration of hydrazine in the 300 μL incubation mixture.

The standard curve had a correlation coefficient (r) of 0.999 and a slope of 0.408 (95% confidence interval: 0.398 to 0.419) mV·seconds/μM hydrazine and a y-intercept of -0.021 (95% confidence interval: -1.121 to 1.081) mV·seconds (**Fig 5**). The equation: $y = mx + b$ (where y is the peak area in mV·seconds, x is the concentration of microsomal hydrazine in μM, and b is the y-intercept equal to "0") was used to convert peak areas of hydrazine (mV·seconds; determined by computer-aided integration) into hydrazine concentration in INH-preincubated microsomal samples. The slope of the standard curve obtained using this equation had a correlation coefficient (r) of 0.9997 and a slope of 0.408 (95% confidence interval: 0.401 to 0.416)mV·seconds/μM hydrazine. The peak area of hydrazine produced (each sample in duplicate) after incubation of actual microsomal samples was compared to the standard curve and hydrazine production was calculated as nmoles hydrazine produced/mg protein/hour. The following equation was used to calculate hydrazine produced:

mV·seconds / 0.408 mV·seconds/μM hydrazine = X μM hydrazine

X μmoles hydrazine/L x Y mg protein/mL^{-1} x 0.5 $hour^{-1}$ x 1000 mL/L^{-1} x 1000 nmoles/μmole

= nmoles hydrazine produced/mg protein/hour

All values quoted are based on a relative detector sensitivity of 0.01, the highest sensitivity used throughout the microsomal amidase activity assay. A validation summary can be found in **Table 6 & 7**.

Table 6

Validation Summary - Hepatic Amidase Activity Assay - Microsomal INH Incubation → Hydrazine Determination

Limit of Quantitation:	2.5 μM (11.0% CV); Acceptance Criteria: CV ≤ 15%
Standard Range:	2.5 - 250 μM
Sample Range:	8 - 162 μM
Specificity:	No interference of Hydrazine Peak with INH, Acetylhydrazine or other peaks.
Linearity: In Microsomes:	r = 0.999; slope = 0.408 (0.398 to 0.419); y-int = -0.021 (-1.121 to 1.081)
Slope used in Calculation: Slope (m) of Standard Curve: *Equation: y = mx + b (b = 0); x = [Hz]; y = peak area*	r = 0.9997; slope = 0.408 (0.401 to 0.416)

♦Values in Brackets ( ) represent 95% confidence interval;
♦CV (Standard Deviation/mean) (SD/mean) represents the coefficient of variation.

Table 7

Precision of Hydrazine Determination in Microsomal INH-incubated Samples

Hydrazine Standards In Microsomes*	
μM (n = 3)	CV % (SD/mean)
2.5	11
5.0	7.1
10	1.9
25	5.9
50	0.2
100	1.8
250	3.7
(mean ± SD)	4.5 ± 3.7

*** Precision in water could not be calculated because the standard curve was run in duplicate only.**

Plasma Metabolites - Hydrazine Assay

The analytical procedure used for the quantitation of hydrazine (μM) in 12 and 32 hour plasma samples was developed and validated in this laboratory. For validation of this assay, please refer to the Ph.D. thesis of T. C. Sarich (1997).

The plasma hydrazine assay is based on hydrazine largely being present in the form of azines in the plasma (R.A. Wall, personal communication) and its hydrolysis when exposed to a low pH environment. For example, pyruvate azine is generated from the reaction of hydrazine with pyruvic acid (Ellard and Gammon, 1976). In this assay, the hydrolysis of the plasma azines is done in the presence of excess derivatizing reagent, 3-methoxybenzaldehyde. Detection of conjugated hydrazine (3-methoxybenzaldehyde) is then possible at a wavelength of 300 nm.

The assay was performed in the following manner. 100 μL of MeCN (100%) was added to 100 μL of plasma, the mixture was vortexed and allowed to stand for 6 minutes. Then 100 μL of 0.6N perchloric acid and 100 μL of HPLC grade water were added and the final mixture was vortexed vigorously and centrifuged at 12,700xg for 6 minutes. The supernatant was filtered with a Millex-LCR_4 filter; a 200 μL aliquot of the supernatant was removed and combined with 50 μL of derivatizing reagent. The derivatizing reagent was prepared according to the same method used in the microsomal amidase activity assay. The resultant solution was incubated for two hours at room temperature. Then, at two hours, the solution was filtered again using a Millex-LCR_4 filter and the eluent loaded onto an HPLC column (CSC-Select-ODS-2: 5 μm - 10 x 0.46 cm). The chromatographic system used is the same as that described in the phenotyping assay. The UV detector was set at 300 nm for the detection of plasma hydrazine. The solvent used consisted of 5 mM NaAc adjusted to pH 5.0 with glacial acetic acid and 65% HPLC grade MeCN in HPLC grade water. The flow rate was 1 mL/minute. The solvent was filtered prior to use with a 0.45 μm pore, 45 mm Nylon filter (Micron Separations Inc.) and degassed *in situ*. Room temperature ranged from 23-25 °C on average.

The analytical method developed by T. C. Sarich (Ph.D. Thesis, 1997) showed that INH and acetylhydrazine elute much earlier (~ 2 minutes) than hydrazine in blank plasma and plasma

spiked with INH, acetylhydrazine, and hydrazine individually, and together. In this experiment, the hydrazine peak eluted at approximately 6 minutes as previously observed by T. C. Sarich. There were no interfering peaks observed in baseline ("0"-hour RIF) plasma samples.

A standard curve was prepared using hydrazine dissolved in helium-degassed, acidified water (in duplicate) at concentrations of 2.5, 5, 10, 25, 50, 100, and 250 μM hydrazine. The standard curve had a correlation coefficient (r) of 0.999, a slope of 1.744 (95% confidence intervals: 1.651 to 1.838) mV·seconds/μM hydrazine and a y-intercept of 1.204 (95% confidence intervals: -8.598 to 11.012) mV·seconds. Using the equation y = mx, the standard curve had a correlation coefficient (r) of 0.999 and a slope of 1.751 (95% confidence interval: 1.686 to 1.817) mV·seconds/μM hydrazine.

A standard curve was prepared using blank rabbit plasma spiked with hydrazine (in triplicate) at concentrations of 1, 2.5, 5, 10, 25, 50, 100, and 200 μM hydrazine. The standard curve had a correlation coefficient (r) of 0.999 with a slope of 1.624 (95% confidence interval: 1.581 to 1.669) mV·seconds/μM hydrazine and y-intercept of 2.063 (95% confidence interval: -8.598 to 11.012) mV·seconds (**Figure 6**). The equation: y = mx + b (where y is the peak area in mV·seconds, x is the concentration of plasma hydrazine in μM, and b is the y-intercept forced through "0") was used to convert peak areas of hydrazine (mV·seconds; determined by computer-aided integration) into hydrazine concentration in plasma samples. Using this equation, the standard curve had a correlation coefficient (r) of 0.999 and a slope of 1.642 (95% confidence interval: 1.605 to 1.674). All values quoted are based on a relative detector sensitivity of 0.01, the highest sensitivity used throughout the plasma hydrazine assay. A validation summary can be found in **Table 8 & 9**.

Table 8

Validation Summary

Determination of Hydrazine in 12 & 32 Hour Plasma Samples

Limit of Quantitation:	2.5 μM (14.4% CV); Acceptance Criteria: CV ≤ 15%
Standard Range:	2.5 - 200 μM
Sample Range:	2.5 - 160 μM
Specificity:	No interference of Hydrazine Peak with INH, Acetylhydrazine or other peaks.
Linearity: In Water:	r = 0.999; slope = 1.744 (1.651 to 1.838); y-int = 1.204 (-8.598 to 11.012)
In Plasma:	r = 0.999; slope = 1.624 (1.581 to 1.669); y-int = 2.063 (-1.823 to 5.948)
Slope used in Calculation: Slope (m) of Standard Curve: *Equation: y = mx + b (b = 0); x = [Hz]; y = peak area*	r = 0.999; slope = 1.642 (1.605 to 1.674)

•Values in Brackets ( ) represent 95% confidence interval;
•CV (Standard Deviation/mean)(SD/mean) represents the coefficient of variation.

Table 9

Precision of Hydrazine Determination in 12 & 32 Hour Plasma Samples

Hydrazine Standards In Plasma*	
μM (n = 3)	**CV % (SD/mean)**
2.5	14.4
5.0	6.8
10	11.9
25	4.2
50	7.8
100	6.7
200	3.0
(mean ± SD)	7.8 ± 4.1

*** Precision in water could not be calculated because the standard curve was run in duplicate only.**

Statistics:

For the purpose of clarity, data within the textual components of this thesis will be expressed as non-transformed, raw data, whereas graphical representations may express data which has been transformed unless otherwise indicated.

Plasma ASAL and ALT activities were logarithmically transformed so as to fit the data to a normal distribution. Then, log ASAL and log ALT activities were used for statistical analyses including the single-factor analysis of variance (ANOVA) and the Tukey test when comparing more than two treatment groups.

Regressional analyses were performed for determining the slopes of standard curves of SMZ, AcSMZ, and hydrazine.

Correlation coefficients given in the results section are Pearson's product moment correlation coefficients. In some instances, correlation coefficients (r) were converted to coefficients of determination (r^2) in order to assess the biological significance of the correlations (considered significant when $r^2 > 0.50$).

T-tests, assuming either equal or unequal variances (determined by F-tests), were used when comparing two treatment groups. ANOVA and the Tukey multiple comparison test (Zar, 1984) was done for comparison of more than two groups, unless otherwise indicated.

All data are presented as the mean ± standard error of the mean (SE).

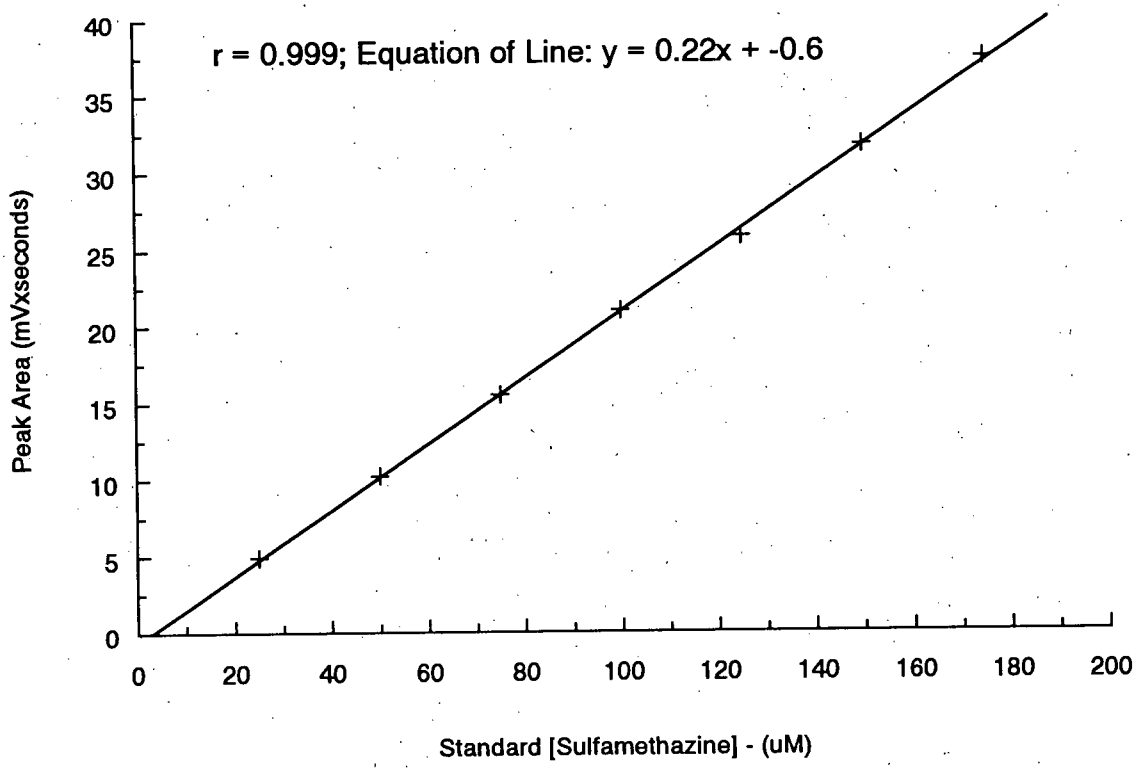


Figure 3: Standard Curve: Sulfamethazine Standards in Plasma

A standard curve was prepared (in duplicate) using blank rabbit plasma spiked with SMZ at concentrations of 25, 50, 75, 100, 125, 150, and 175 μM. The standard curve has a correlation coefficient (r) of 0.9997, a slope of 0.216 (95% confidence interval: 0.210 to 0.221) mVxseconds/μM SMZ and a y-intercept of -0.596 (95% confidence interval: -1.17 to -0.02) mVxseconds/μM SMZ. The equation: y = mx + b (where y is the peak area in mVxseconds, x is the concentration of plasma SMZ in μM, and b is the y-intercept equal to "0") was used to convert peak areas of SMZ (mVxseconds; determined by computer-aided integration) into SMZ concentration. The slope of the standard curve obtained using this equation has a correlation coefficient (r) of 0.999 and a slope of 0.211 (95% confidence interval: 0.208 to 0.214) mVxseconds/μM SMZ.

Error bars are not shown since they are too small to be visible.

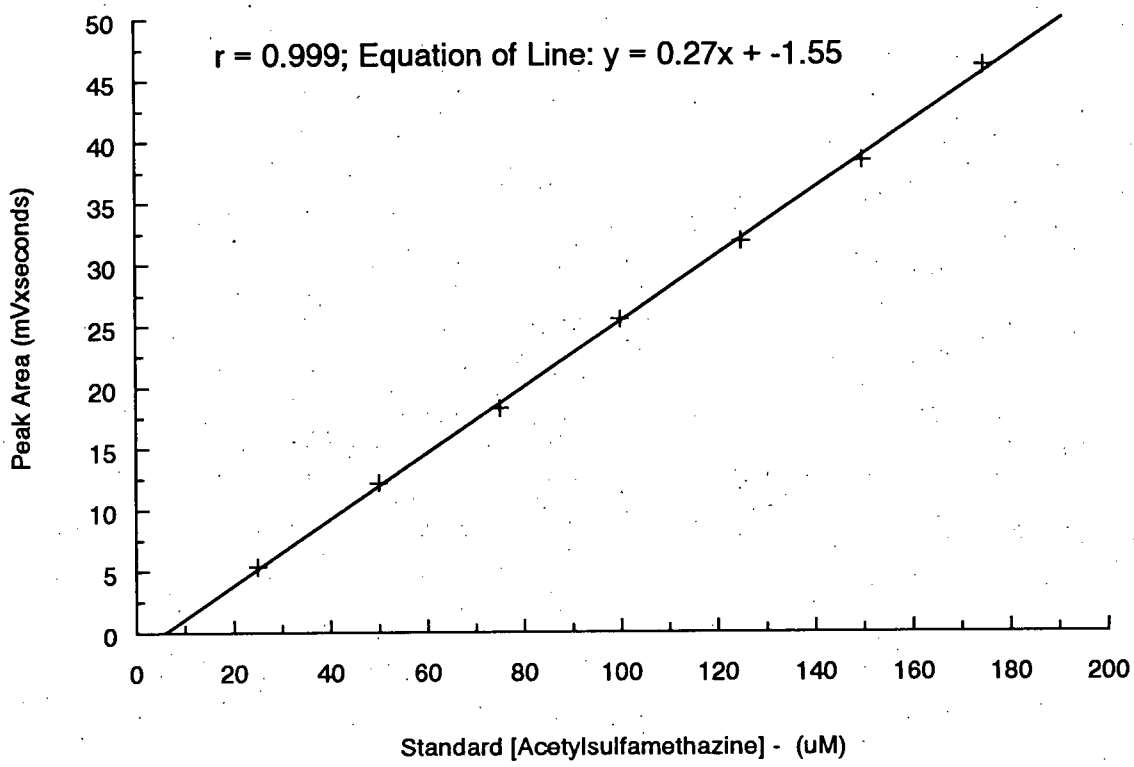


Figure 4: Standard Curve - Acetylsulfamethazine Standards in Plasma

A standard curve was constructed (in duplicate) using blank rabbit plasma spiked with AcSMZ at concentrations of 25, 50, 75, 100, 125, 150, 175 μM. The standard curve has a correlation coefficient (r) of 0.999, a slope of 0.270 (95% confidence interval: 0.262 to 0.277) mVxseconds/μM AcSMZ, and a y-intercept of -1.55 (95% confidence interval: -2.423 to -0.678) mVxseconds. The equation y = mx + b (where b, the y-intercept = 0) was used to calculate unknown concentrations of AcSMZ in plasma samples. The slope of this standard curve has a correlation coefficient (r) of 0.998 and a slope of 0.257 (95% confidence interval: 0.250 to 0.264) mVxseconds/μM AcSMZ.

Error bars are not shown since they are too small to be visible.

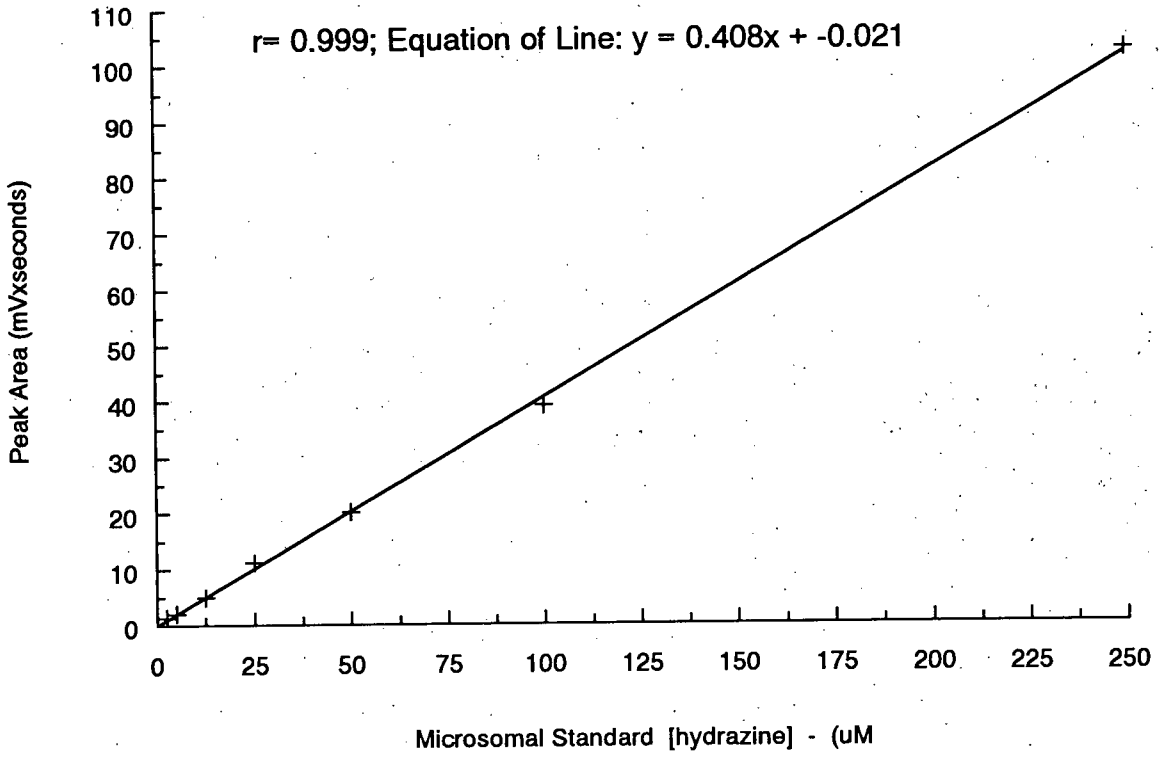


Figure 5: Standard Curve - Hydrazine Standards in Microsomes

A standard curve was prepared (in triplicate) using blank rabbit microsomes spiked with hydrazine at concentrations of 2.5, 5, 12.5, 25, 50, 100, and 250 μM hydrazine. The standard curve has a correlation coefficient (r) of 0.999 and a slope of 0.408 (95% confidence interval: 0.398 to 0.419) mVxseconds/μM hydrazine and a y-intercept of -0.02 (95% confidence interval: -1.12 to 1.08) mVxseconds . The equation: y = mx + b (where y is the peak area in mVxseconds, x is the concentration of plasma hydrazine in μM, and b is the y-intercept equal to "0") was used to convert peak areas of hydrazine (mVxseconds; determined by computer-aided integration) into hydrazine concentration in INH-preincubated microsomal samples. The slope of the standard curve obtained using this equation has a correlation coefficient (r) of 0.999 and a slope of 0.408 (95% confidence interval: 0.401 to 0.416)mVxseconds/μM hydrazine.

Error bars are not shown since they are too small to be visible.

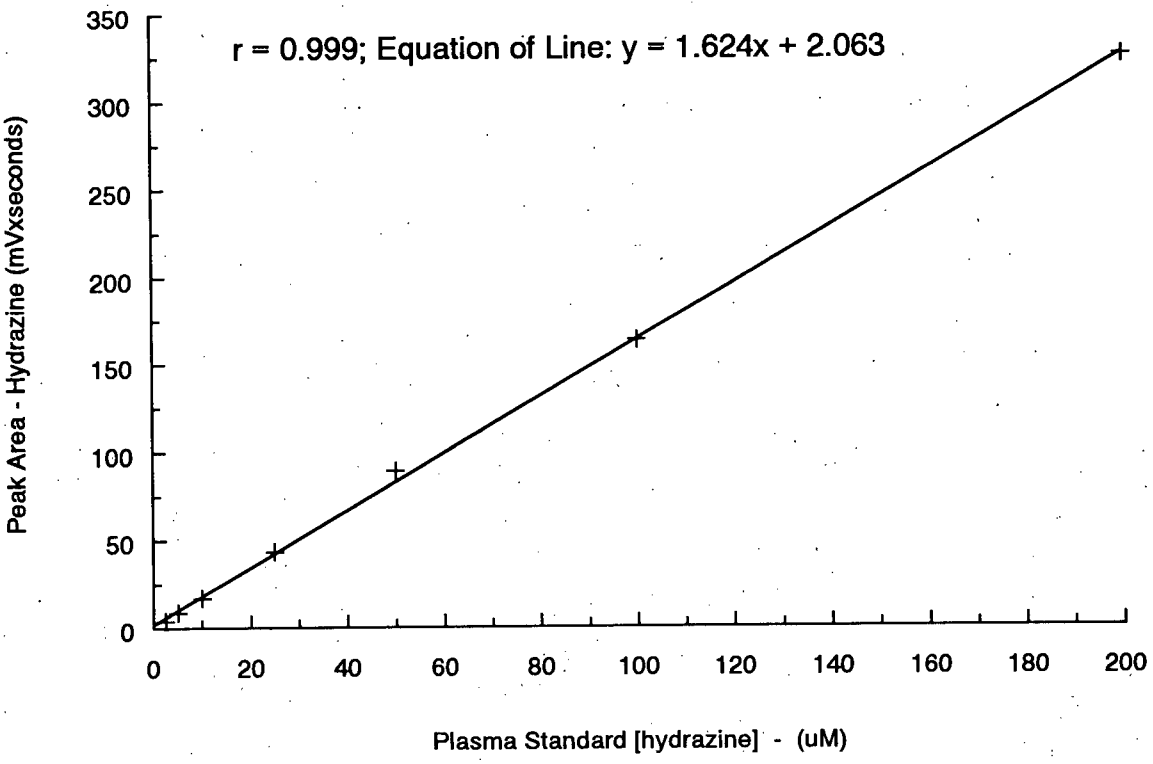


Figure 6: Standard Curve - Hydrazine Standards in Plasma

A standard curve was prepared (in triplicate) using blank rabbit plasma spiked with hydrazine at concentrations of 1, 2.5, 5, 10, 25, 50, 100, and 200 μM hydrazine. The standard curve has a correlation coefficient (r) of 0.999 with a slope of 1.624 (95% confidence interval: 1.581 to 1.669) mVxseconds/μM hydrazine and y-intercept of 2.063 (95% confidence interval: -8.598 to 11.012) mVxseconds. The equation: y = mx + b (where y is the peak area in mVxseconds, x is the concentration of plasma hydrazine in μM, and b is the y-intercept forced through "0") was used to convert peak areas of hydrazine (mVxseconds; determined by computer-aided integration) into hydrazine concentration in plasma samples. Using this equation, the standard curve has a correlation coefficient (r) of 0.999 and a slope of 1.642 (95% confidence interval: 1.605 to 1.674).

Error bars are not shown since they are too small to be visible.

Results

In the first group of ten animals studied, two (one in the RIF-INH group and one in the RIF-Veh* group) died prematurely. These rabbits died during the RIF-pretreatment period. In the final group of 10 rabbits investigated, an additional rabbit was sacrificed prior to death at approximately 40 hours of INH treatment. This rabbit had experienced severe convulsions, decreased food and water intake and weight loss, prior to death. Plasma analysis of ASAL, ALT, and hepatic and plasma triglyceride levels in this rabbit showed large elevations in all of these measures. The liver of this animal was removed and immediately frozen in liquid nitrogen at the time of sacrifice. Unless otherwise indicated, this animal was excluded in some of the statistical analyses and will from hereon be referred to as an outlier. **Unless otherwise specified, results are expressed as the mean ± standard error of the mean.**

Hepatotoxicity Assays:

Peak plasma ASAL activity was significantly increased in Veh-INH-treated animals (132.7 ± 78.7 Takahara units, n = 12) compared to Veh-Veh* controls (3.2 ± 0.1 Takahara units, n = 4)(ANOVA: $p<0.0001$; Tukey Test: $p<0.01$) (**Figure 7**). RIF-INH treated animals were not different (9.6 ± 3.0 Takahara units, n = 11) from Veh-Veh* controls and exhibited significantly less hepatotoxicity than Veh-INH treated rabbits (ANOVA: $p<0.0001$; Tukey Test: $p<0.025$).

Excluding the outlier with peak plasma ASAL activity (953.6 Takahara units) much higher than the mean (132.7 ± 78.7 Takahara units), significant but weak negative correlations could be found between ASAL activity and hepatic amidase activity ($r = -0.46$, $r^2 = 0.21$, n = 37, ANOVA: $p = 0.0043$) and CYP2E1 activity ($r = -0.45$, $r^2 = 0.2$, n = 30, ANOVA: $p = 0.013$) when all rabbits were included in the analysis. The sum of animals inclusive of all treatment groups was only n = 30 for the following reason. Measurement of CYP2E1 activity revealed eight values which were "too low" or undetectable spectrophotometrically and thus were arbitrarily assigned a value of "0" nmoles/min/mg protein. However, given the inaccuracy of this measurement, these rabbits were excluded from all correlational analyses involving CYP2E1

activity. In most correlations outlined throughout this thesis, inclusion of these values would have strengthened the correlation in question.

Peak plasma ALT activity gave a pattern which is similar to peak plasma ASAL activity. However, despite a statistically significant effect of the treatments (ANOVA: $p<0.05$), the Neuman-Keul's & Tukey tests showed no differences between the treatment groups and control (**Figure 8**).

Hepatic steatosis, measured by hepatic triglyceride content (mg TG/g liver) was not found in RIF-Veh* treated rabbits (9.1 ± 0.5 mg TG/g liver) *versus* Veh-Veh* (8.3 ± 0.4 mg TG/g liver). However, both RIF-INH and Veh-INH groups showed marked hepatic steatosis, with hepatic TG levels of 26.6 ± 2.3 mg TG/g liver (ANOVA: $p<0.0001$; Tukey test: $p<0.05$; $n = 11$) and 35.4 ± 5.4 mg TG/g liver (ANOVA: $p<0.0001$; Tukey test: $p<0.005$; $n = 12$), respectively. Although RIF-pretreatment appears to have produced less hepatic steatosis in the presence of INH, the difference between RIF-INH and Veh-INH treated animals is not significant (ANOVA: $p < 0.0001$; Tukey Test: no difference) (**Figure 9**).

A weak negative correlation was found between hepatic triglyceride (TG) content and CYP2E1 activity when all treatment groups were considered ($r = -0.66$, $r^2 = 0.44$, $n = 30$, ANOVA: $p < 0.0001$)(**Figure 15**). Hepatic TG content also negatively correlated with hepatic amidase activity ($r = -0.69$, $r^2 = 0.47$, $n = 38$, ANOVA: $p < 0.000001$) when all animals were included in the analysis (**Figure 16**).

Plasma triglyceride levels at 12, 32, and 48 hours of INH treatment did not reveal any significant differences between any of the four treatment groups (Veh-Veh*; RIF-Veh*; Veh-INH; RIF-INH) (12, 32, & 48 hours, ANOVA: $p>0.05$). However, the level of statistical significance did approach the $p<0.05$ level at 48 hours (**Figure 10**; **Table 10**, ANOVA: $p = 0.053$). The lack of significant differences between the treatment groups at 48 hours may be due to high variances found in the RIF-INH and Veh-INH treatment groups (**Refer to Table 10**). Nonetheless, there is a consistent trend of increasing levels of plasma TG's in both the RIF-INH and Veh-INH groups towards 48 hours, with the RIF-INH group showing less marked increases of plasma TG's than

the Veh-INH group. (**Table 10**).

32 hour plasma triglyceride levels correlated significantly with peak plasma ASAL activity ($r = 0.75$, $r^2 = 0.55$, $n = 11$, ANOVA: $p = 0.0086$)(**Figure 17**) for Veh-INH treated animals. One of the rabbits in this group was excluded from the analysis since it demonstrated considerably higher plasma TG levels at 32 hours (17.6 mM) and peak plasma ASAL activity (953.6 Takahara units), which were considerably higher than mean values of 3.5 ± 1.3 mM TG and 132.7 ± 78.7 Takahara units, respectively. This rabbit was sacrificed prematurely at 40 hours since it was anticipated that it would not survive beyond 40 hours of the INH dosing schedule. Statistically significant correlations were also found when all four treatment groups were pooled ($r = 0.52$, $r^2 = 0.27$, $n = 37$, ANOVA: $p = 0.001$) and INH-treated animals (RIF-INH & Veh-INH) were pooled ($r = 0.7$, $r^2 = 0.49$, $n = 22$, ANOVA: $p < 0.0005$) (**Figure 18**). The aforementioned outlier was excluded from both preceding correlations. A weak negative correlation was found between 32 hour plasma TG levels and hepatic amidase activity ($r = -0.41$, $r^2 = 0.17$, $n = 37$, ANOVA: $p = 0.012$) when all animals were included except for the aforementioned outlier.

When the outlier was excluded from the statistical analysis, a very weak correlation was observed between 48 hour plasma TG levels and peak plasma ASAL activity ($r = 0.36$, $r^2 = 0.13$, $n = 37$, ANOVA: $p = 0.029$) and hepatic TG accumulation ($r = 0.54$, $r^2 = 0.29$, $n = 37$, ANOVA: $p < 0.001$) (**Figure 19**) when all four treatment groups were combined.

Hepatic Microsomal Enzyme Activities:

Hepatic cytochrome P-450 levels were found to be significantly decreased in Veh-INH-treated animals (1.4 ± 0.1 nmoles/mg protein, n = 12) *versus* Veh-Veh* (2.9 ± 0.3 nmoles/mg protein, n = 4) (ANOVA: $p<0.0001$; Tukey test: $p<0.001$); RIF-INH (1.8 ± 0.1 nmoles/mg protein, n = 11) *versus* Veh-Veh* (ANOVA: $p<0.0001$; Tukey test: $p<0.02$) (**Figure 11**; **Table 11**). RIF alone (RIF-Veh: 2.7 ± 0.2 nmoles/mg protein, n = 11) had no significant effect on cytochrome P-450 levels.

Hepatic cytochrome P-450 reductase activity (nmoles/min/mg protein) was significantly increased in rabbits receiving RIF. The differences were as follows: RIF-INH (576 ± 31 nmoles/min/mg protein) *versus* control (Veh-Veh*: 388 ± 24 nmoles/min/mg protein) (ANOVA: $p<0.0001$; Tukey test: $p<0.001$) and RIF-Veh* (543 ± 15 nmoles/min/mg protein) *versus* control (ANOVA: $p<0.0001$; Tukey test: $p<0.005$). INH appears to have had no effect on reductase activity: Veh-INH (413 ± 16 nmoles/min/mg protein) *versus* control (ANOVA: $p<0.0001$; Tukey test: $p>0.05$) (**Figure 12**; **Table 11**).

Microsomal CYP2E1 activity was inhibited significantly by both RIF and INH, the former drug having less effect on CYP2E1 activity. The following are comparisons between the different treatments (RIF-INH; RIF-Veh*; Veh-INH) and control rabbits (Veh-Veh*): RIF-INH (0.39 ± 0.2 nmoles/min/mg protein) *versus* control (2.3 ± 0.4 nmoles/min/mg protein) (ANOVA: $p<0.0001$; Tukey Test: $p<0.001$); RIF-Veh* (1.5 ± 0.1 nmoles/min/mg protein) *versus* control (ANOVA: $p<0.0001$; Tukey Test: $p<0.05$); Veh-INH (0.47 ± 0.2 nmoles/min/mg protein) *versus* control (ANOVA: $p<0.0001$; Tukey Test: $p<0.001$). RIF-Veh* treated animals were also significantly different from both RIF-INH and Veh-INH groups (ANOVA: $p<0.00001$; Tukey test: $p < 0.005$)(**Figure 13**; **Table 11**).

Hepatic amidase activity was determined by measurement of the amount of hydrazine produced after incubation of INH with microsomes. Hydrazine production in the Veh-INH (11.5 ± 1.9 nmoles/mg protein/hour, n = 12) and RIF-INH (10.1 ± 0.1 nmoles/mg protein/hour, n = 11)

was significantly lower than in the Veh-Veh* control group (32.8 ± 4.7 nmoles/mg protein/hour, n = 4) (ANOVA: $p < 0.00001$; Tukey test: $p < 0.001$) (**Fig 14; Table 11**). RIF pretreatment had no effect on hepatic amidase activity (RIF-Veh* animals: 30.8 ± 3.1 nmoles/mg protein/hour, n = 11).

Plasma Hydrazine Levels:

Plasma hydrazine levels at 12 hours of the INH dosing regime were not significantly different between the RIF-INH (26.1 ± 2.5 μM, n = 11) and Veh-INH (20.1 ± 3.6 μM, n = 12) treatment groups (**Table 12**). Although 2 out of a total of 12 rabbits in the Veh-INH group had 12 hour hydrazine levels below the limit of quantitation (2.5 μM), they were included in the statistical analysis (F-test for variance, followed by t-Test). Similarly, no significant difference in plasma hydrazine levels at 32 hours was found between RIF-INH (26.6 ± 2.1 μM, n = 11) and Veh-INH (24.5 ± 5.1 μM, n = 12) groups (**Table 12**). Again, one of the data points within the Veh-INH group fell below the quantitation limit (2.5 μM), but was included in the statistical analyses (F-test for variance, followed by t-Test).

12 hour plasma hydrazine levels correlated significantly with hepatic triglyceride (TG) accumulation ($r = 0.79$, $r^2 = 0.62$, n = 12, ANOVA: $p = 0.0023$)(**Figure 20**) in Veh-INH treated rabbits. A significant but weak correlation was found between 12 hour plasma hydrazine and hepatic TG accumulation ($r = 0.57$, $r^2 = 0.32$, n = 23, ANOVA: $p = 0.0048$) when animals that received INH were pooled.

No significant correlation was found between 32 hour plasma hydrazine levels and hepatic TG accumulation in Veh-INH treated animals. A non-significant correlation exists between 32 hour plasma hydrazine levels and 32 hour plasma triglyceride, since a correlation would exist solely on the basis of one data point from which a linear regression is made. This data point is represented by a Veh-INH treated rabbit, which demonstrated 32 hour plasma TG (17.6 mM) and 32 hour plasma hydrazine (75.8 μM) levels which were considerably higher

than mean values of 3.5 ± 1.3 mM TG and 24.5 ± 5.1 μM, respectively. This rabbit was sacrificed prematurely at approximately 40 hours (instead of 48 hours) of INH treatment because it was believed that the rabbit would not survive. Because of this outlier, correlations could not be drawn between 32 hour plasma hydrazine levels and peak plasma ASAL activity, as well as 32 hour plasma TG levels and cytochrome P450 reductase activity. However, the fact that this animal exhibited extreme signs of INH-induced hepatotoxicity (peak plasma ASAL activity, hepatic TG accumulation, and plasma TG) does not dismiss the importance of this animal to this study. Refer to the discussion for further details.

Acetylator Phenotype:

All of the rabbits which received INH (RIF-INH & Veh-INH groups) and completed the study were rapid acetylators (range: 72.8 - 95.3% sulfamethazine acetylation in 20 minutes). Correlational analysis of the percent sulfamethazine acetylated in 20 minutes revealed non-significant correlations with peak plasma ASAL activity ($r = 0.26$, $r^2 = 0.07$, $n = 23$, ANOVA: $p > 0.10$), peak plasma ALT activity ($r = 0.16$, $r^2 = 0.03$, $n = 23$, ANOVA: $p > 0.25$), liver triglyceride accumulation ($r = 0.11$, $r^2 = 0.01$, $n = 23$, ANOVA: $p > 0.50$), and peak plasma triglyceride levels ($r = 0.19$, $r^2 = 0.04$, $n = 23$, ANOVA: $p > 0.25$).

Table 10

Comparison of Toxicological Markers:

Treatment Groups vs. Veh-Veh* Control

Marker	Veh-Veh*	Veh-INH	RIF-Veh*	RIF-INH	ANOVA
Peak Plasma ASAL Activity (Takahara Units)	3.2 ± 0.1 n = 4	132.7 ± 78.7† n = 12	2.8 ± 0.2 n = 11	9.6 ± 3 n = 11	p < 0.0001
Peak Plasma ALT Activity (Units/L)	29.6 ± 3.6 n = 4	210.6 ± 100.6 n = 12	28.5 ± 3.3 n = 11	42.4 ± 7.8 n =11	p = 0.02
Hepatic Triglycerides (mg TG/g liver)	8.3 ± 0.4 n =4	35.4 ± 5.4‡ n = 12	9.1 ± 0.5 n = 11	26.6 ± 2.3§ n = 11	p < 0.00001
12 Hour Plasma Triglycerides (mM)	0.8 ± 0.1 n =4	1.1 ± 0.2 n = 12	1.3 ± 0.2 n = 11	1.0 ± 0.1 n = 11	p = 0.46
32 Hour Plasma Triglycerides (mM)	0.7 ± 0.1 n = 4	3.5 ± 1.3 n = 12	1.6 ± 0.5 n = 11	1.6 ± 0.3 n = 11	p = 0.25
48 Hour Plasma Triglycerides (mM)	1 ± 0.1 n = 4	6.4 ± 2 n = 12	1.5 ± 0.3 n = 11	3.5 ± 1 n = 11	p = 0.053

- Values represent the mean ± standard error;
- ASAL & ALT activities were log transformed to fit a normal distribution for ANOVA & Tukey tests.

† Significantly different from the Veh-Veh* group ($p < 0.01$) using Tukey Test.
‡ Significantly different from the Veh-Veh* group ($p < 0.005$) using Tukey Test.
§ Significantly different from the Veh-Veh* group ($p < 0.05$) using Tukey Test.

Table 11

Statistical Summary - Hepatic Microsomal Enzyme Activities

ENZYME	Veh-Veh*	Veh-INH	RIF-Veh*	RIF-INH	ANOVA
Cytochrome P450 Content (nmoles/mg protein)	2.9 ± 0.3 n = 11	1.4 ± 0.1§ n = 11	2.7 ± 0.2 n = 12	1.8 ± 0.1† n = 4	p < 0.00001
CYP2E1 Activity (nmole/min/mg protein)	2.3 ± 0.4 n = 11	0.47 ± 0.2§ n = 11	1.5 ± 0.1* n = 12	0.39 ± 0.2§ n = 4	p < 0.00001
Cytochrome P450 Reductase Activity (nmole/min/mg protein)	388 ± 24 n = 11	413 ± 16 n = 11	543 ± 15‡ n = 12	576 ± 31§ n = 4	p < 0.00001
Hepatic Microsomal Amidase Activity (nmole/mg protein/hr)	32.8 ± 4.7 n = 11	11.5 ± 1.9§ n = 11	30.8 ± 3.1 n = 12	10.1 ± 1§ n = 4	p < 0.00001

◆ Values represent the mean ± standard error;
* Significantly different from the Veh-Veh* group ($p<0.05$) using Neuman-Keul's Test.
† Significantly different from the Veh-Veh* group ($p < 0.02$) using Tukey Test.
‡ Significantly different from the Veh-Veh* group ($p < 0.005$) using Tukey Test.
§ Significantly different from the Veh-Veh* group ($p < 0.001$) using Tukey Test.

Table 12

Statistical Summary - 12 & 32 Hour Plasma Hydrazine Levels

Plasma Hydrazine (μM)	Veh-INH	RIF-INH	F-test for Variance	Variance: equal - E unequal - U	t-Test
12 Hours	20.1 ± 3.6 n = 12	26.1 ± 2.5 n = 11	p = 0.11	E	p = 0.19
32 Hours	24.5 ± 5.1 n = 11	26.6 ± 2.1 n = 11	p = 0.003	U	p = 0.72

- **Values represent the mean ± standard error;**
- **Plasma hydrazine values below the limit of quantitation (2.5 μM) were included in the preceding data analysis.**

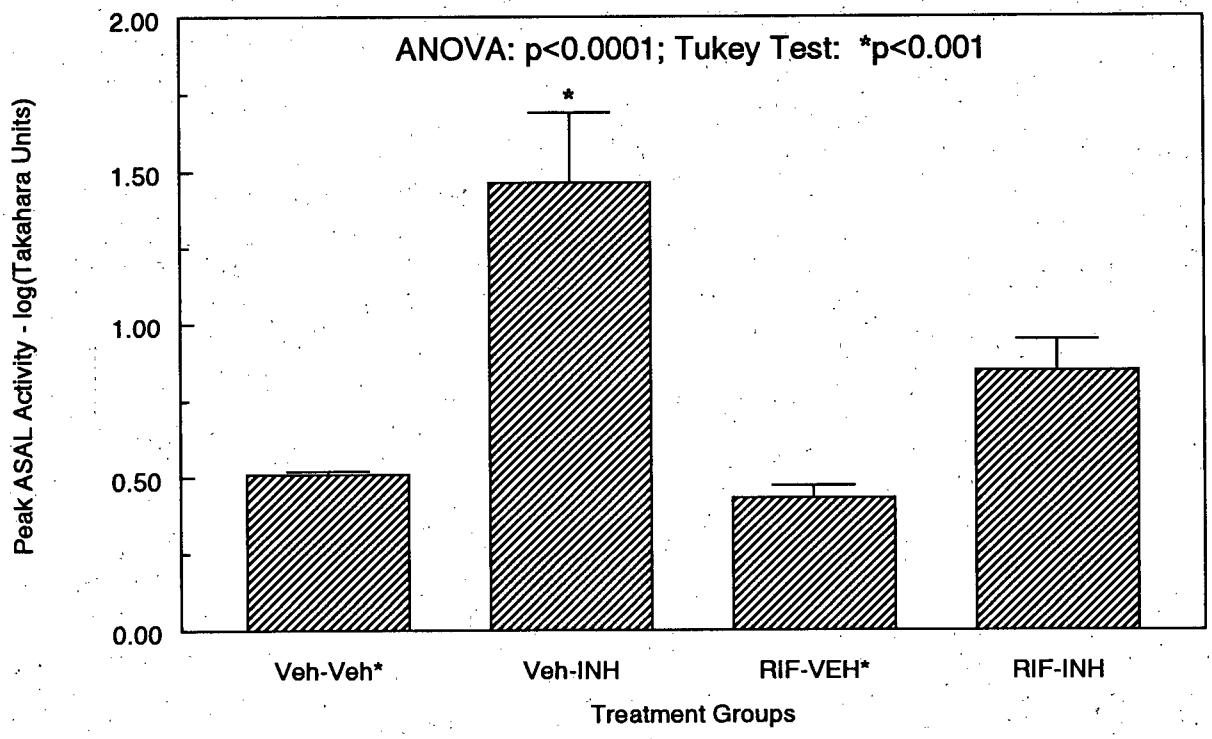


Figure 7: Differences in Peak Plasma ASAL Activities Between Treatment Groups

Peak plasma ASAL activities (Takahara units) were statistically significantly greater in Veh-INH-treated animals (132.7 ± 78.7 Takahara units, n = 12) than in controls (Veh-Veh*) (3.2 ± 0.1 Takahara units, n = 4)(ANOVA: $p<0.0001$; Tukey Test: $p<0.001$). RIF-INH-treated rabbits exhibited significantly lower elevations in peak plasma ASAL activity (9.6 ± 3 Takahara units, n = 11) than the Veh-INH group (ANOVA: $p<0.0001$; Tukey Test: $p<0.025$) and were not significantly different from controls. The RIF-Veh* (2.8 ± 0.2 Takahara units, n = 11) and RIF-INH (9.6 ± 3 Takahara units, n = 11) groups exhibited no significant hepatic necrosis.

Values represent the mean ± standard error of the mean (S.E.).

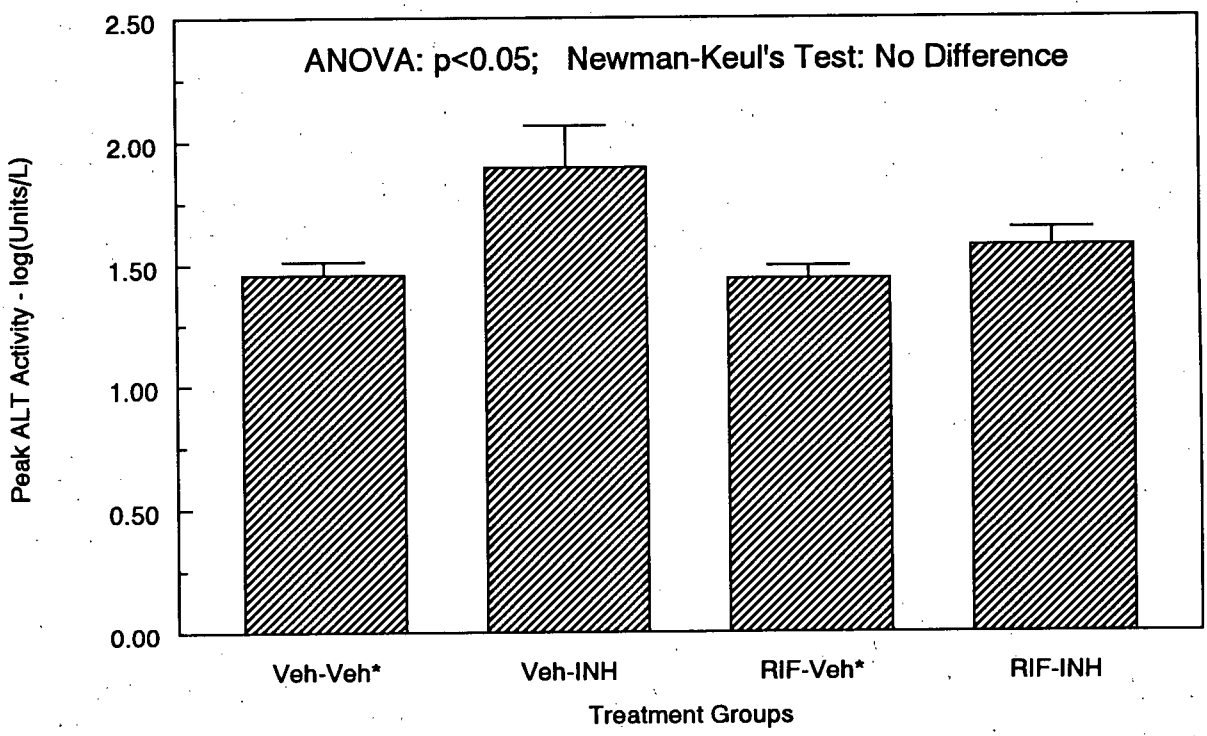


Figure 8: Differences in Peak Plasma ALT Activities Between Treatment Groups

Peak plasma ALT activity (units/L) was not significantly different between any of the treatment groups studied (ANOVA: $p<0.05$; Neuman-Keul's Test: no difference). Refer to **Table 10** for mean ALT values of different treatment groups.

Values represent the mean ± standard error of the mean (S.E.).

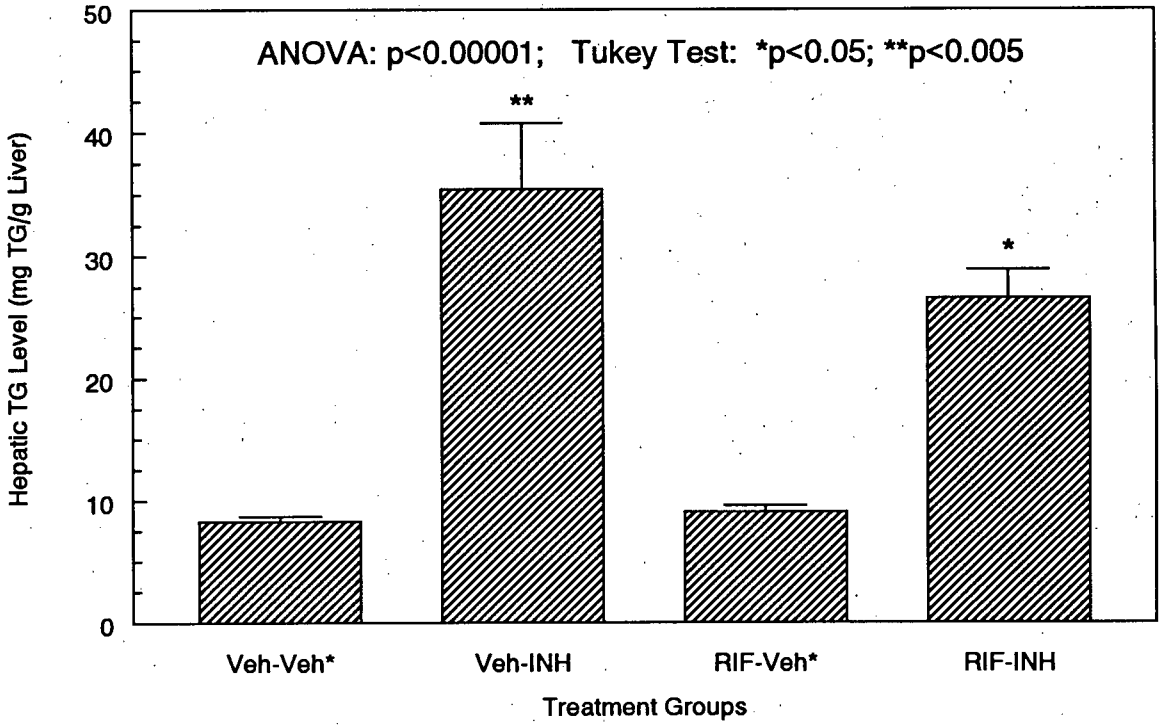


Figure 9: Differences in Hepatic Triglyceride Accumulation Between Treatment Groups

A comparison of hepatic triglyceride (TG) accumulation (mg TG/g liver) between treatment groups. Hepatic triglyceride content was significantly greater in the Veh-INH (35.4 ± 5.4 mg TG/ g liver, n = 12) group than in controls (Veh-Veh*)(8.3 ± 0.4 mg TG/g liver, n = 4). There was no significant difference between RIF-INH (26.6 ± 2.3 mg TG/g liver, n = 11) and Veh-INH treated rabbits. The RIF-Veh group (9.1 ± 0.5 mg TG/g liver, n = 11) exhibited no significant hepatic steatosis.

Values represent the mean ± standard error of the mean (S.E.).

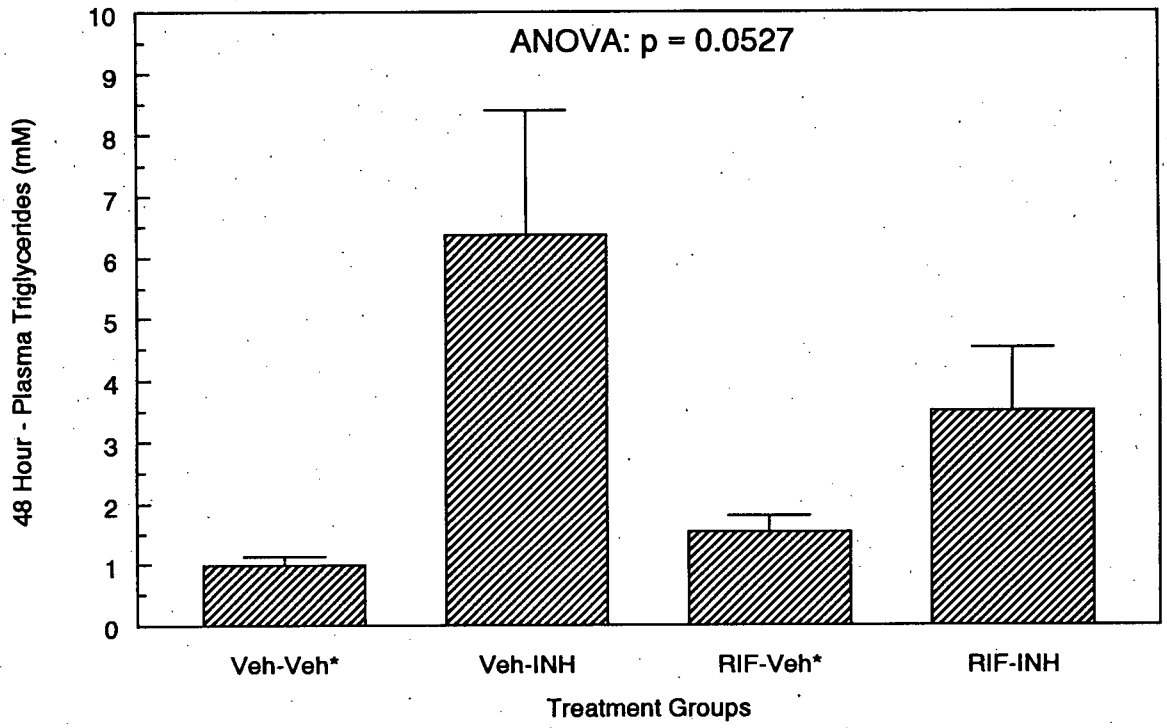


Figure 10: Differences in Plasma Triglyceride Levels Between Treatment Groups at 48 Hours of Isoniazid Treatment

A comparison of plasma triglyceride (TG) levels (mM TG) between treatment groups. Plasma triglyceride levels (mM) at 48 hours of Isoniazid treatment were not significantly different between the four treatment groups illustrated above (ANOVA: p = 0.0527). Both the Veh-INH and RIF-INH treated animals do show considerable elevations in plasma triglyceride content (6.4 ± 2 mM TG & 3.51 ± 1.01 mM TG, respectively) relative to control (Veh-Veh*: 1 ± 0.1 mM TG). However, large variances for both the Veh-INH (49.5 mM TG) and RIF-INH (11.3 mM TG) groups attributes to this statistically non-significant difference.

Values represent the mean ± standard error of the mean (S.E.).

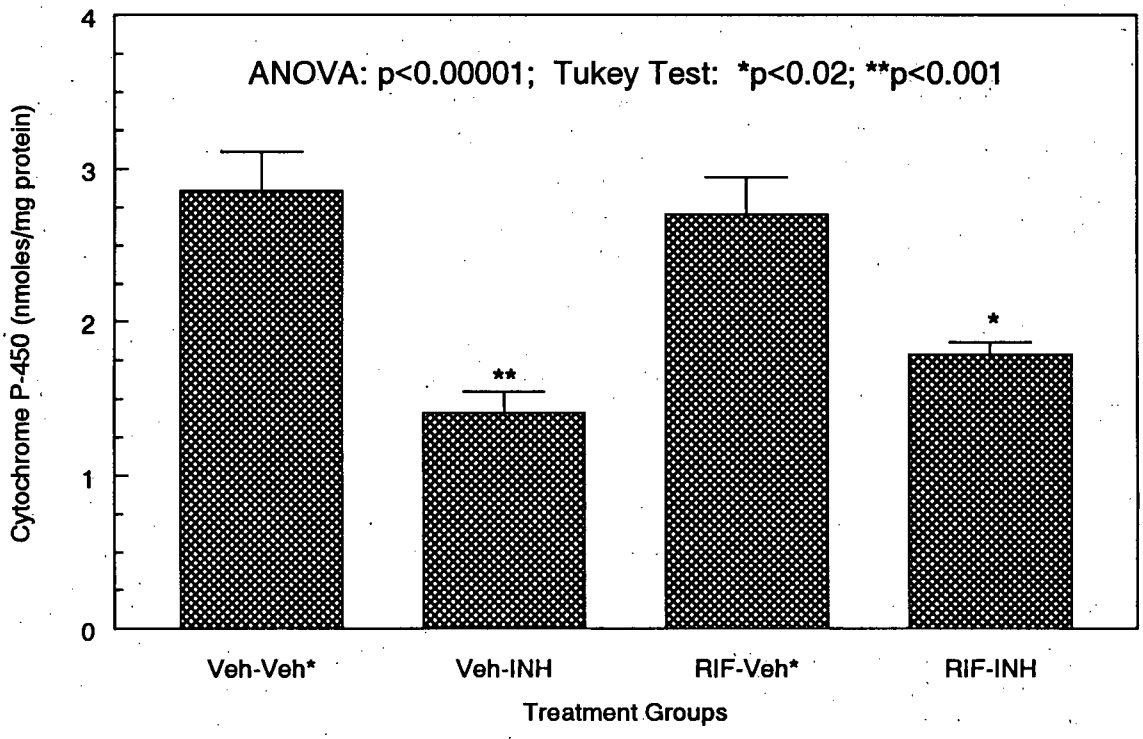


Figure 11: Differences in Cytochrome P-450 Levels Between Treatment Groups

Animals receiving isoniazid alone (Veh-INH) or in combination with RIF (RIF-INH) demonstrated significant decreases in hepatic cytochrome P-450 levels. P-450 levels in the Veh-INH group (1.4 ± 0.1 nmoles/mg protein, n = 12) and RIF/INH group (1.8 ± 0.1 nmoles/mg protein, n = 11) were significantly lower than Veh-Veh* controls (2.9 ± 0.3 nmoles/mg protein, n = 4) (ANOVA: $p<0.00001$; Tukey test: $p<0.001$ & $p<0.02$, respectively). RIF-Veh* treated rabbits showed no effect on cytochrome P-450 levels (2.7 ± 0.2 nmoles/mg protein, n = 11).

Values represent the mean ± standard error of the mean (S.E.).

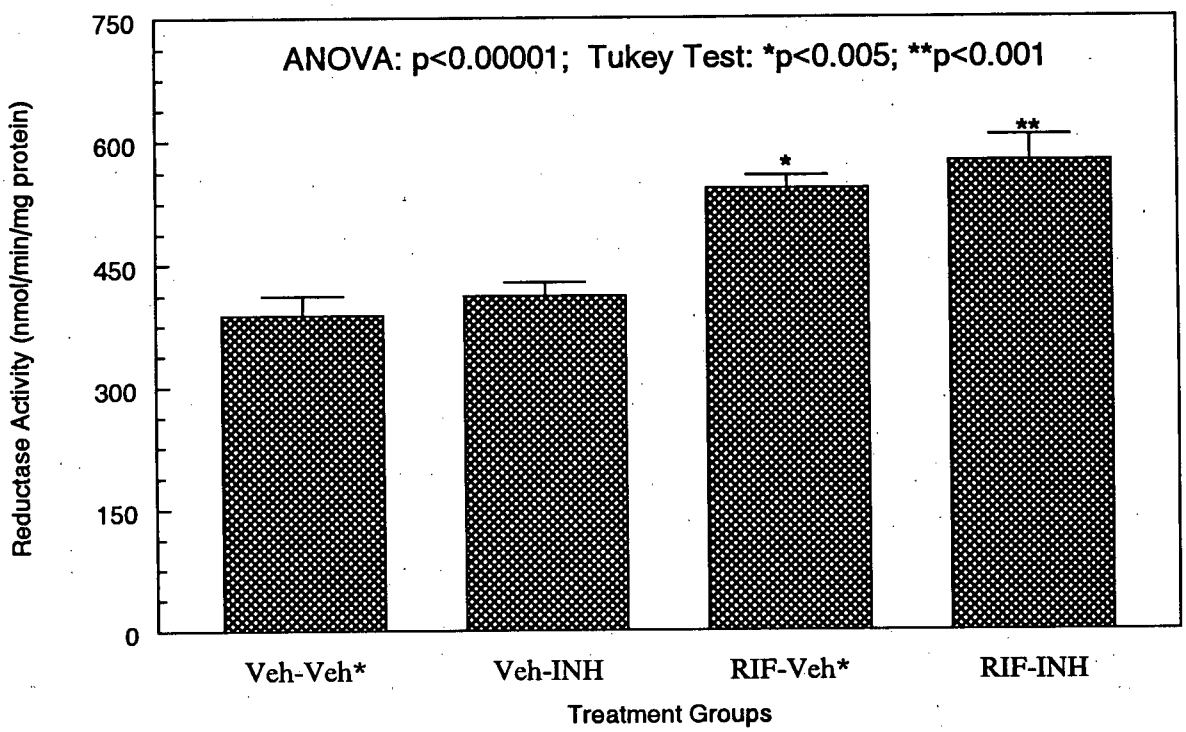


Figure 12: Differences in Hepatic Cytochrome P-450 Reductase Activities Between Treatment Groups

There was no difference in hepatic cytochrome P-450 reductase activity (nmoles/minute/mg protein) between Veh-INH (413 ± 16 nmoles/minute/mg protein, n = 12) and Veh-Veh* (388 ± 24 nmoles/minute/mg protein, n = 4) treatment groups. RIF-treated rabbits (RIF-INH & RIF-Veh* groups) showed significantly higher cytochrome P-450 reductase activities than Veh-Veh* controls (ANOVA: $p<0.00001$; Tukey test: RIF-INH vs. Veh-Veh*, $p<0.001$ & RIF-Veh* vs. Veh-Veh*, $p<0.005$). RIF-INH (n = 11) and RIF-Veh* (n = 11) groups had cytochrome P-450 reductase activities of 576 ± 31 nmoles/minute/mg protein and 543 ± 15 nmoles/minute/mg protein, respectively.

Values represent the mean ± standard error of the mean (S.E.).

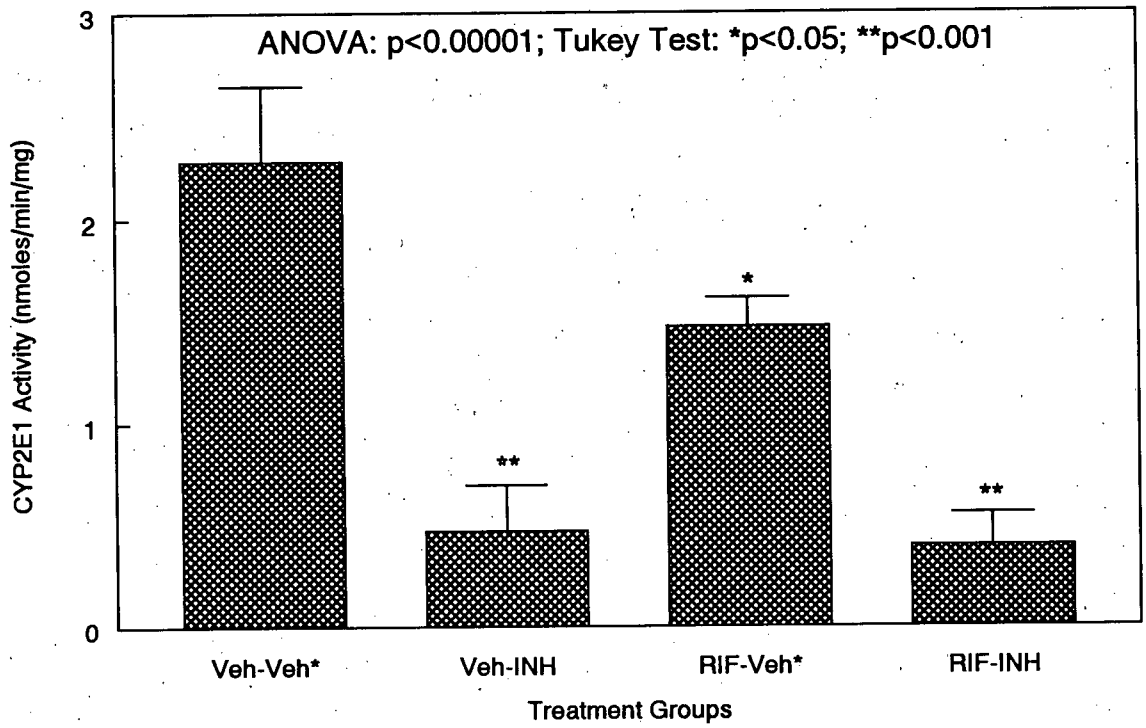


Figure 13: Differences in Hepatic CYP2E1 Activities Between Treatment Groups

CYP2E1 activity (nmoles/minute/mg protein) was significantly decreased in all treatment groups relative to Veh-Veh* controls (2.3 ± 0.4 nmoles/min/minute/mg protein, n = 4) (ANOVA: $p<0.00001$): Veh-INH (0.47 ± 0.2 nmoles/min/minute/mg protein, n = 12) *versus* control (Tukey test: $p<0.001$); RIF-INH (0.39 ± 0.2 nmoles/min/minute/mg protein, n = 11) *versus* control (Tukey test: $p<0.001$); RIF-Veh* (1.5 ± 0.1 nmoles/min/minute/mg protein, n = 11) *versus* control (Tukey test: $p<0.05$).

Values represent the mean ± standard error of the mean (S.E.).

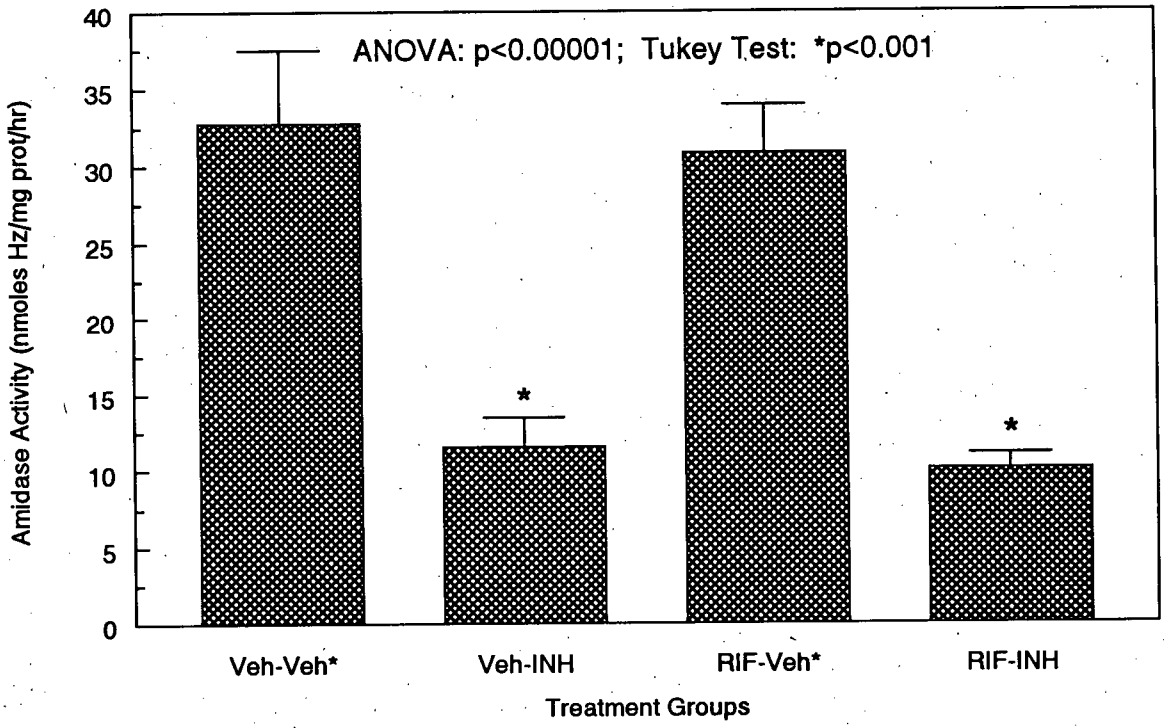


Figure 14: Differences in Hepatic Microsomal Amidase Activity Between Treatment Groups

Hepatic amidase activity (nmoles hydrazine produced/mg protein/hour) was significantly decreased in Veh-INH (11.5 ± 1.9 nmoles hydrazine/mg protein/hour, n = 12) and RIF-INH (10.1 ± 1 nmoles hydrazine/mg protein/hour, n = 11) treatment groups relative to Veh-Veh* (32.8 ± 4.7 nmoles hydrazine/mg protein/hour, n = 4) and RIF-Veh* (30.8 ± 3.1 nmoles hydrazine/mg protein/hour, n = 11) control groups (ANOVA: $p<0.00001$; Tukey test: $p<0.001$).

Values represent the mean ± standard error of the mean (S.E.).

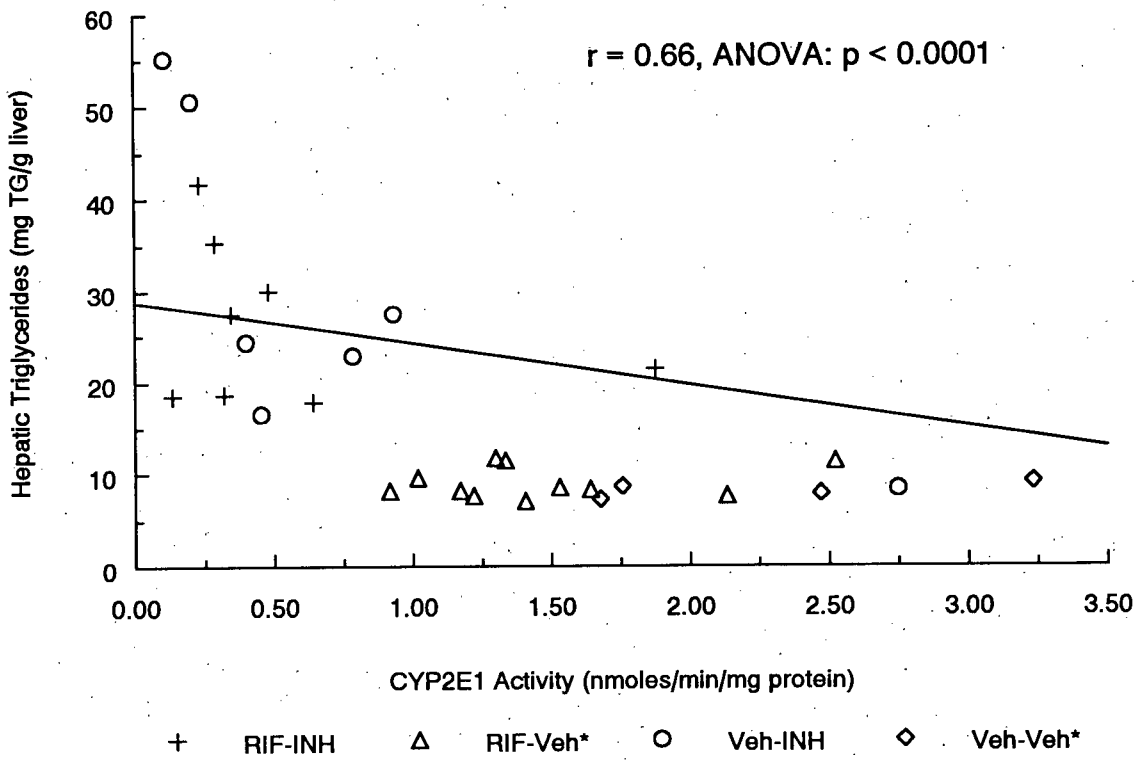


Figure 15: Scatter Plot: Hepatic Triglycerides versus CYP2E1 Activity (All Rabbits)

A weak correlation was found between hepatic triglyceride (TG) content and CYP2E1 activity when all treatment groups were considered ($r = 0.66$, $r^2 = 0.44$, $n = 30$, ANOVA: $p < 0.0001$). Values of "0" of CYP2E1 activity were excluded.

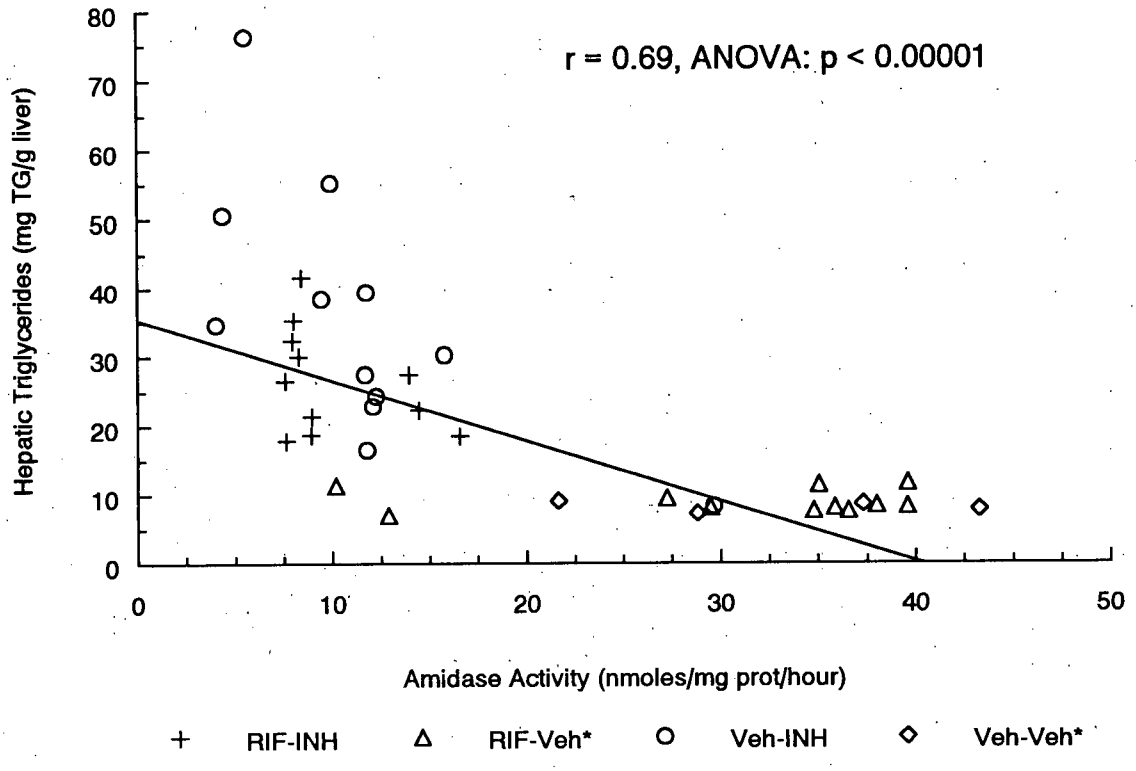


Figure 16: Scatter Plot: Hepatic Triglycerides versus Microsomal Hepatic Amidase Activity (All Rabbits)

Hepatic TG content correlates with hepatic amidase activity ($r = 0.69$, $r^2 = 0.48$, $n = 38$, ANOVA: $p < 0.00001$) when all animals are included in the analysis.

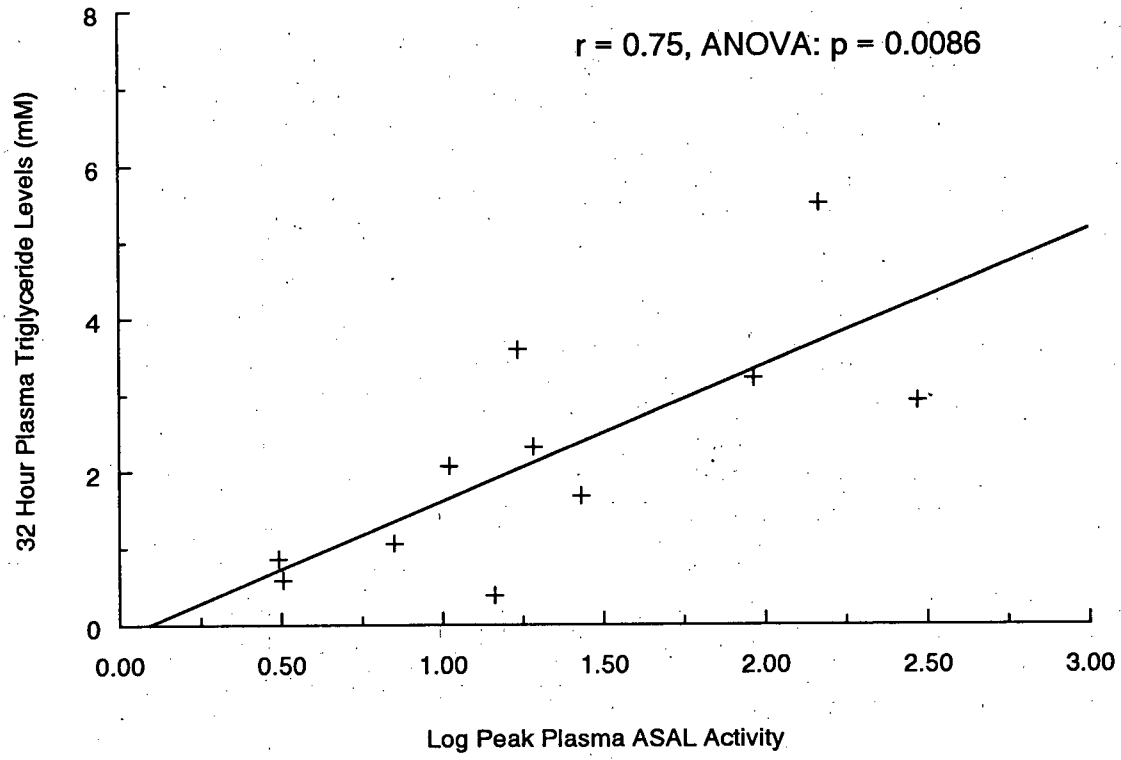


Figure 17: Correlation: 32 Hour Plasma Triglycerides versus Peak Log ASAL Activity (Veh-INH Rabbits)

32 hour plasma triglyceride levels correlate significantly with peak plasma ASAL activity ($r = 0.75$, $r^2 = 0.55$, $n = 11$, ANOVA: $p = 0.0086$) for Veh-INH treated animals. One of the rabbits in this group is excluded from the analysis since it demonstrated considerably higher plasma TG levels at 32 hours (17.6 mM) and peak plasma ASAL activity (953.6 Takahara units) which are considerably higher than mean values of 3.5 ± 1.3 mM TG and 132.7 ± 78.7 Takahara units, respectively. This rabbit was sacrificed prematurely at 40 hours since it was anticipated that it would not survive beyond 40 hours of INH administration.

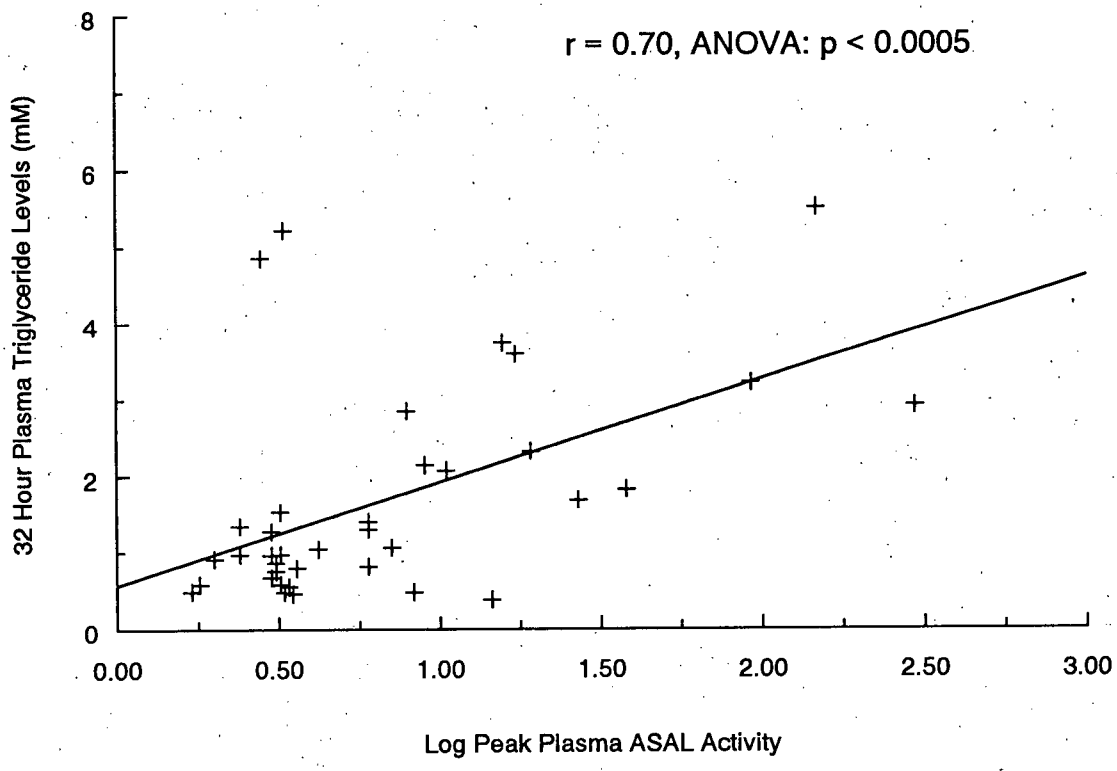


Figure 18: Correlation: 32 Hour Plasma Triglycerides versus Peak Log ASAL Activity (RIF-INH & Veh-INH Rabbits)

A statistically significant correlation was found between 32 hour plasma triglyceride levels and peak plasma ASAL activity when INH-treated animals were pooled ($r = 0.70$, $r^2 = 0.49$, $n = 22$, ANOVA: $p < 0.0005$). The outlier with extreme elevations of plasma ASAL activity and plasma TG at 32 hours was excluded.

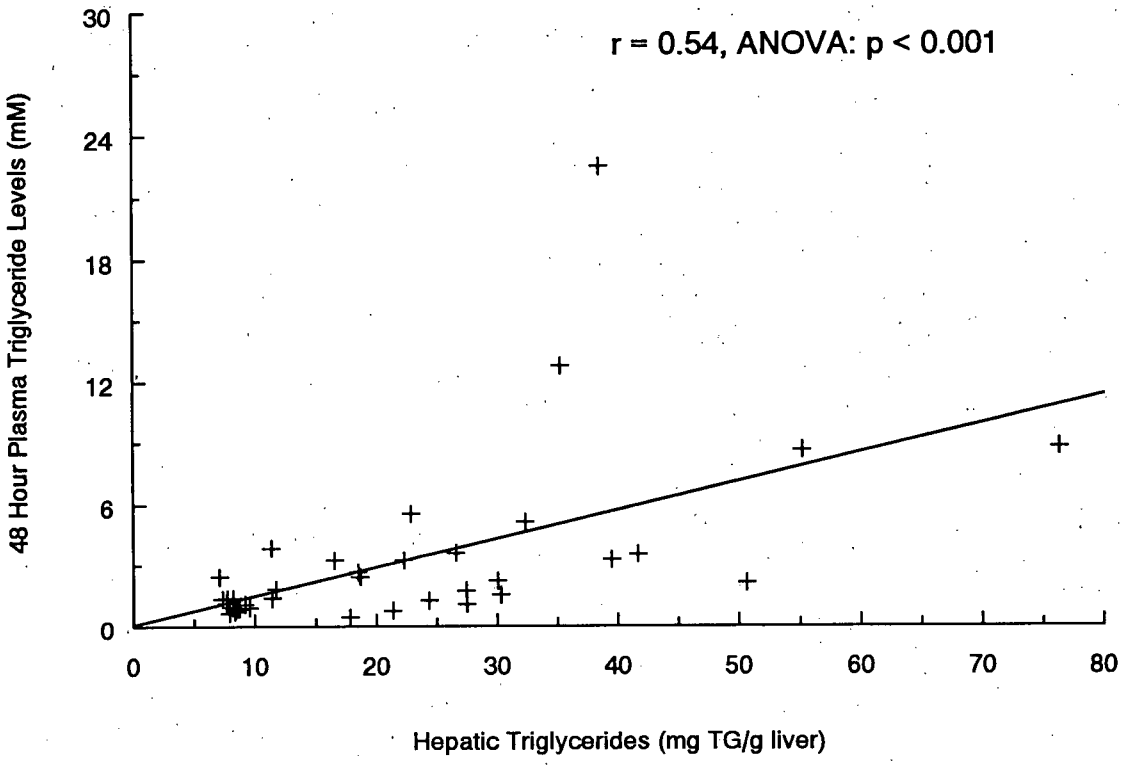


Figure 19: Scatter Plot: 48 Hour Plasma Triglycerides versus Hepatic Triglycerides (All Rabbits)

A weak correlation exists between 48 hour plasma TG levels and hepatic TG accumulation ($r = 0.54$, $r^2 = 0.29$, $n = 37$, ANOVA: $p < 0.001$) when all rabbits are included. Notably, the rabbit in the Veh-INH group with extreme signs of toxicity was excluded in this correlation.

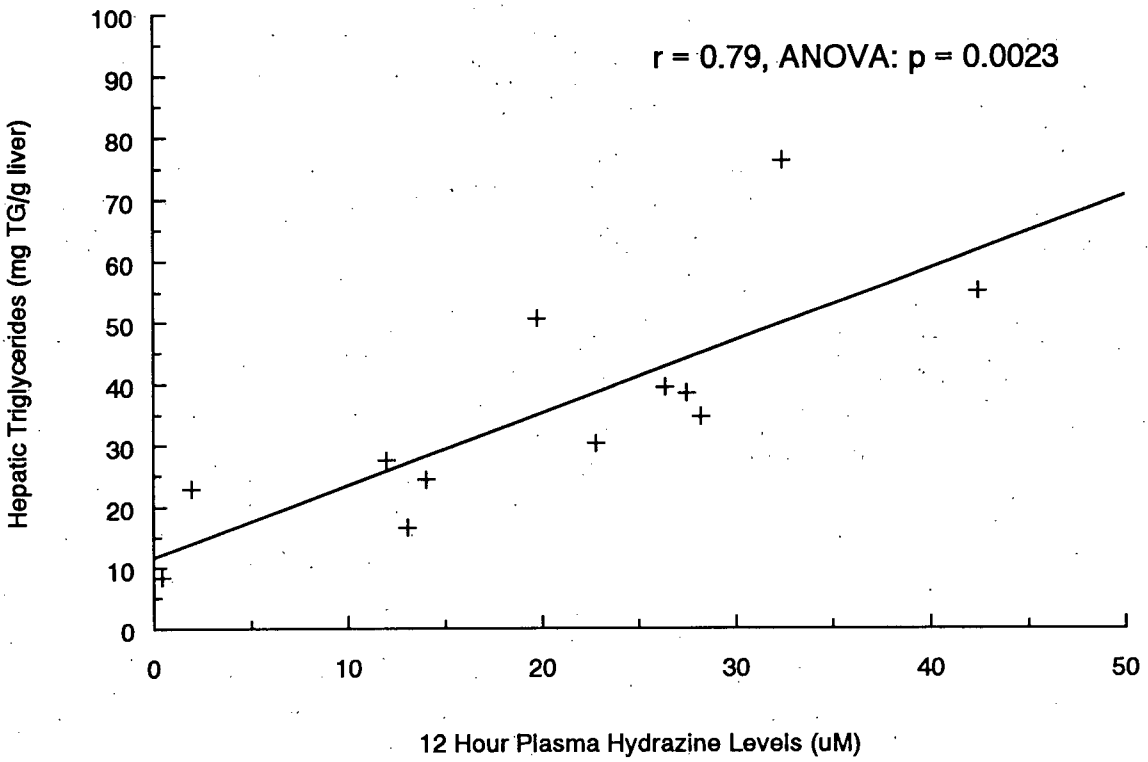


Figure 20: Correlation: 12 Hour Plasma Hydrazine versus Hepatic Triglycerides (Veh-INH Rabbits)

12 hour plasma hydrazine levels correlate significantly with hepatic triglyceride (TG) accumulation ($r = 0.79$, $r^2 = 0.62$, $n = 12$, ANOVA: $p = 0.0023$) in Veh-INH treated rabbits. Two of the rabbits in this group fell below the limit of quantitation (2.5 μM), but were still included in the statistical analysis.

Discussion

The main purpose of this study was to determine whether or not RIF pretreatment would potentiate INH-induced hepatotoxicity in the rabbit model previously developed in our laboratory. It is evident from the results obtained that RIF pretreatment significantly reduced INH-induced hepatic necrosis as seen by significantly lower peak plasma ASAL activity in RIF-INH *versus* Veh-INH-treated animals (ANOVA: $p < 0.0001$; Tukey test: $p < 0.025$). Moreover, RIF-INH-treated rabbits were not statistically different from Veh-Veh* controls (**Figure 7**).

Although analysis of the differences in peak plasma ALT levels between treatment groups did not reach statistical significance, a correlation of peak plasma ASAL with ALT levels does exist ($r = 0.86$, $r^2 = 0.74$, $n = 38$, ANOVA: $p < 0.00001$). Peak plasma ASAL activity appears to be a better discriminator of toxicity than peak plasma ALT activity for the following reasons. The coefficients of variation (CV)(standard deviation/mean) for non-transformed ASAL activity for RIF-Veh* and Veh-Veh* control groups are 27.6% and 5.3%, respectively. Even higher variability is observed for non-transformed ALT activity: CV values for RIF-Veh* and Veh-Veh* control groups are 38.2% and 24.6%, respectively. However, the opposite is true when comparing CV values of RIF-INH and Veh-INH groups between ASAL (105% & 206% respectively) and ALT (61.3% & 166% respectively). Therefore, the higher degree of variability in ALT *versus* ASAL activity within the RIF-Veh* and Veh-Veh* control groups (representing baseline ALT & ASAL activities) results in fewer animals exhibiting significant levels of ALT *versus* ASAL activity. This is further supported by the greater number of rabbits exhibiting significant levels of toxicity (criteria: 3 x mean of Veh-Veh* control group) using ASAL activity (> 10 Takahara Units) *versus* ALT activity (> 90 units/L) as markers for hepatic necrosis. 9 out of 12 rabbits in the Veh-INH group and 2 out of 11 rabbits in the RIF-INH group had significant elevations in plasma ASAL activity. Whereas only 4 out of 12 rabbits in the Veh-INH group and 1 out 11 in the RIF-INH group had significant increases in plasma ALT activity.

Finally, both peak plasma ASAL and ALT activities showed the same trends as markers of necrosis and both statistically demonstrated a significant difference between treatment groups (ASAL, ANOVA: $p < 0.0001$; ALT, ANOVA: $p < 0.05$).

Having reviewed the literature on the effects of RIF on INH-induced hepatotoxicity, my *apriori* hypothesis was that RIF pretreatment would increase INH-induced hepatotoxicity. However, the observed decrease of INH-induced hepatotoxicity by RIF is perhaps the best evidence yet, in animals. The best quality of evidence today is based on the design of the study, a double-blinded, randomized, and controlled trial. Concordant with this design is a double-blind placebo-controlled clinical trial of RIF and INH conducted in patients with silicosis in Hong Kong (Hong Kong Chest Service/Tuberculosis Research Center, Madras/British Medical Research Council, 1992). Although not discussed by the authors of this study, perhaps the most important finding in this study relevant to this thesis is a decrease of INH-induced hepatotoxicity in **RIF-INH** treated patients (HR3) versus **INH-only** treated patients (H6) based on a comparison of mean serum ALT activities. Patients in the **RIF-INH** and **RIF-only** groups received RIF-INH and RIF, respectively, for 12 weeks followed by placebo for the remaining 12 weeks of the study. On the other hand, patients in the **INH-only** group received INH for the entire study (total of 24 weeks). The geometric mean serum ALT activities ($n = 272$) were not significantly different between the **RIF-INH** and **INH-only** treatment groups from the commencement of therapy until approximately 8 weeks into treatment. During the 8 - 12 week period, ALT activities in the **RIF-INH** group fell below the H6 group. Similarily, the percentage of patients with raised serum ALT activities (> 28 IU/L) was significantly less in the **RIF-INH** group compared to the **INH-only** group after the first month of treatment up until the third month of drug administration.

An important feature of double-blind, placebo-controlled trials is that, provided the double-blind design is preserved, they enable the measurement of the adverse effects of active regimens in comparison with the placebo group. During the first 3 months of treatment, adverse reactions were resported in 22% of the patients allocated to RIF, 25% to INH and RIF, 20% to INH alone, and 17% to placebo. Thus, the majority of adverse reactions were probably not drug-

related. Only 4% of patients in the active treatment series and 2% of those in the placebo series had their regimen stopped because of adverse reactions. During the second 3 months, 5% of patients receiving INH and 3% of those receiving placebo reported reactions.

Thus, the present and previous studies provide clear evidence for the effect of RIF to decrease INH-induced hepatotoxicity.

Hepatic microsomal amidase activity was significantly inhibited by INH treatment and unaffected by RIF treatment. Amidase activities in Veh-INH (11.5 ± 1.9 nmoles hydrazine/mg protein/hour, n = 12) and RIF-INH (10.1 ± 1 nmoles hydrazine/mg protein/hour, n = 11) groups were both significantly lower than Veh-Veh* controls (32.8 ± 4.7 nmoles hydrazine/mg protein/hour, n = 11) (ANOVA: $p < 0.00001$; Tukey test: $p < 0.001$) (**Figure 14**; **Table 11**). Previous studies in this laboratory have also demonstrated significant inhibition of amidase by INH *in vivo* and *in vitro* (Sarich et al., 1997).

Only weak negative correlations could be found between amidase activity and peak log ASAL activity ($r = -0.46$, $r^2 = 0.21$, n = 37, ANOVA: $p = 0.0043$) and hepatic TG's ($r = -0.69$, $r^2 = 0.48$, n = 38, ANOVA: $p < 0.000001$). The scatter plot for the amidase *versus* hepatic TG correlation (**Figure 16**) indicates two different groups of animals, one with higher amidase activity and relatively low hepatic TG levels and the other, a range of high to low hepatic steatosis (TG's) with generally low amidase toxicity. Although amidase is likely to play a role in the mechanism of INH-induced hepatotoxicity, the enzyme itself does not explain all of the variance in the severity of the toxicity. This premise is also supported by the absence of effect of RIF treatment on amidase activity, but still a decrease in toxicity by RIF. It is also possible that the inhibition of amidase by INH is due, at least in part, to the hepatocellular damage itself caused by a metabolite of INH such as hydrazine. This damage may result in a reduction in the number of viable amidase enzymes which is seen as a decrease in the overall activity of amidase in microsomes.

Nonetheless, the mechanism of INH-induced hepatotoxicity is clearly a multifactorial one which involves several steps. RIF is likely modulating the stepwise process of INH-induced

hepatotoxicity by either influencing an existing step or adding an additional step. Therefore, a lack of correlation does not disprove any one step as an important one in the production of toxicity.

Since INH treatment significantly decreases amidase activity, baseline activity would be expected to be a determinant in INH-induced hepatotoxicity, but it can not be readily measured.

Previous studies in this laboratory (Sarich et al., 1997) have demonstrated that INH-amidase exists in rabbit plasma. However, the activity of plasma INH-amidase relative to hepatic INH-amidase is quite low, by a factor of 62 to 126 times. This finding combined with the likelihood that INH also inhibits plasma amidase makes it unlikely that plasma amidase would have a significant impact on the degree of INH-induced hepatotoxicity.

A characteristic of INH-induced hepatotoxicity in humans is that, at therapeutic doses, it only occurs after repeated daily doses of INH. This feature has also been observed in this rabbit model (Sarich et al., 1995 & 1996). This phenomenon is thought to occur, in part, as a result of the accumulation of hydrazine over time in plasma, which has been demonstrated in humans (Gent et al., 1992). Previous studies by Sarich et al. (1996 & 1997) using the same INH-dosing protocol used in this study demonstrated mean plasma levels of hydrazine at 12, 24, & 32 hours of approximately 25, 12, and 32 μM hydrazine, respectively. The drop in plasma hydrazine levels at 24 hours is due to the time interval between 12 and 24 hours in which there is no INH administration. In the present study, Veh-INH treated animals had similar levels of 20 μM and 25 μM hydrazine at *both* 12 & 32 hours, respectively, with no statistically significant differences between RIF-INH and Veh-INH-treated animals. Given the lack of difference of plasma hydrazine levels between RIF-INH and Veh-INH groups, it is likely that RIF's effect to reduce the severity of INH-induced hepatotoxicity is not due to increased formation of hydrazine or clearance of hydrazine. In fact, the slightly higher plasma hydrazine levels at 12 hours in RIF-INH (26.1 ± 2.5 μM) *versus* Veh-INH-(20.1 ± 3.6 μM) treated animals, does not support an effect on formation or clearance. Nonetheless, an effect on clearance could be better tested by measuring 24 hour plasma hydrazine levels to see if RIF enhances the degree of decline of

plasma hydrazine levels during the 12 to 24 hour period of INH dosing. If a significant difference in plasma hydrazine levels between RIF-INH and Veh-INH treated animals were to exist at 24 hours, then RIF could be acting in part by modulating the metabolism of hydrazine. Alternatively, a measurement of plasma hydrazine levels at 36 hours would also be a valuable sampling time in examining the effect of RIF on hydrazine metabolism. Recall that the last dose of INH in the present study was given at 33 hours of INH-dosing. Thus, at 36 hours we would expect to see even higher levels of plasma hydrazine than at 32 hours of INH-dosing. In his Ph.D. thesis, T.C. Sarich also predicts higher levels of plasma hydrazine at 36 *versus* 32 hours, assuming that more hydrazine would be formed and that only a small amount of hydrazine would be eliminated in this interval.

Interestingly, the rabbit referred to as an outlier (in the Veh-INH group) in the Methods and Results section of this thesis, since it demonstrated measures of toxicity (e.g. peak plasma ASAL, ALT, hepatic triglycerides, and plasma triglycerides) well beyond the mean, also exhibited the highest level of plasma hydrazine (76 μM) at 32 hours. Recall that this rabbit was sacrificed prematurely at 40 hours. The high degree of INH-induced hepatic necrosis, steatosis, and hypertriglyceridemia in association with high plasma hydrazine levels at 32 hours supports the hypothesis that hydrazine is involved in INH-induced hepatotoxicity. This is further supported by a previous study by Sarich et al. (1996) who demonstrated a significant positive correlation between plasma hydrazine levels and log ASAL activity and showed that inhibition of amidase decreased both INH-induced hepatotoxicity and the formation of hydrazine.

At present, it is still uncertain which hepatic enzyme is responsible for the metabolism of hydrazine and to what extent. In a similar study investigating the effect of RIF pretreatment on INH-induced hepatotoxicity and metabolism, Noda et al. (1983) showed significantly less area under the curve values (AUC) of plasma hydrazine between 0 - 8 hours following a single dose of oral INH 30 mg/kg in rabbits pretreated with RIF for 6 days (30 mg/kg) *versus* control (INH only). A subsequent study by Noda et al. (1988) in rats receiving 30 mg/kg RIF (i.p.) for 6 days, showed that in microsomes isolated from these rats following incubation with 4.8 mM hydrazine, cytochrome P-450 reductase (P-450 reductase) was significantly induced and involved in the

formation of a free radical derivative of hydrazine in the presence of 0_2 and NADPH *in vitro*. Assuming that this free radical analogue of hydrazine occurs *in vivo* and is also hepatotoxic *in vivo*, then Noda et al.'s (1983) finding of decreased plasma levels of hydrazine in rabbits pretreated with RIF followed by INH might support their suggested potentiation of INH-induced hepatotoxicity demonstrated by pathologically more marked INH-induced hepatic necrosis. The present study showed that RIF pretreatment decreases INH-induced hepatotoxicity and did not demonstrate significant differences in plasma hydrazine levels at 12 and 32 hours between RIF-INH and Veh-INH treated animals, calling into question the suggestion by Noda et al. (1983 & 1985a) that RIF potentiates INH-induced hepatotoxicity. Recall that, in Noda et al. (1983) did not include a grading system for the quantitative assessment of the degree of INH-induced hepatic necrosis, measured pathologically. In contrast, the current study uses ASAL and ALT activities as a quantitative measure of the degree of INH-induced hepatic necrosis.

Hydrazine is converted to acetylhydrazine and diacetylhydrazine by N-acetyltransferase. It is also possible that a metabolic pathway exists by which hydrazine is converted to a hepatotoxic intermediate of hydrazine by an unknown hepatic enzyme (e.g. a CYP-450 isozyme) and that this pathway is a minor one in relation to the clearance of hydrazine. With this assumption, one can postulate that if a hydrazine-derived reactive intermediate is highly toxic in small amounts, one would not expect to see dramatic changes in the level of clearance of hydrazine, in this case represented by plasma levels of hydrazine at 12 and 32 hours. Therefore, it is likely that RIF is not affecting the formation of hydrazine or its conversion to acetyl- and diacetyl- hydrazine. Rather, RIF is likely affecting the hepatotoxicity of hydrazine by: (1) decreasing the formation of a hepatotoxic metabolite of hydrazine, or (2) increasing the metabolism of a minor hepatotoxic metabolite of hydrazine to a non-toxic one.

The first scenario, namely decreasing the formation of a hepatotoxic metabolite of hydrazine, is possible via the decrease in CYP2E1 activity with RIF pretreatment. This hypothesis assumes, however, that CYP2E1 is involved in the conversion of hydrazine to a hepatotoxic metabolite. The second possibility, namely the conversion of a hepatotoxic metabolite of hydrazine to a non-toxic metabolite, is possible via the induction of CYP3A6

(CYP3A4 in humans). Although an induction of CYP3A6 has not been verified in the present study, one could assume that the protocol for RIF pretreatment (7 days - 50 mg/kg/day, i.p.) would have significantly induced CYP3A6. Several studies including those by Daujat et al. (1991) and Lange et al. (1984) have demonstrated significant increases in 6β-progesterone hydroxylase activity (a measure of CYP3A6 activity) with similar protocols for RIF as that used in the current study.

Both hypotheses, the inhibition of CYP2E1 and/or the induction of CYP3A6 by RIF are plausible explanations for the observed decrease in INH-induced hepatotoxicity with RIF. However, both hypotheses also assume that the "major" route of metabolism for hydrazine is its conversion to acetylhydrazine and diacetylhydrazine by N-acetyltransferase, while a minor route of metabolism is the conversion of hydrazine to a highly reactive hepatotoxic metabolite which is responsible for INH-induced hepatotoxicity.

In addition to causing necrosis, INH has also been shown, in this study, to cause fatty changes including hepatic steatosis and hypertriglyceridemia. Veh-INH-treated animals displayed significantly elevated levels of hepatic TG's (35.4 ± 5.4 mg TG/g liver, n = 12) relative to Veh-Veh* controls (8.3 ± 0.4 mg TG/g liver, n = 4) (ANOVA: $p < 0.00001$; Tukey test: $p < 0.005$) (**Figure 9**; **Table 10**). Different from its effect on INH-induced necrosis, however, RIF pretreatment did not protect INH-dosed rabbits from steatosis; RIF-INH (26.6 ± 2.3 mg TG/g liver) *versus* Veh-INH (ANOVA: $p < 0.00001$; Tukey test: No difference). Although RIF does appear to decrease INH-induced hepatic steatosis, the effect is not as great as that observed in INH-induced necrosis, which suggests that a different mechanism is involved in causing the two. Interestingly, there were no significant correlations between plasma hydrazine levels and necrosis. However, a significant correlation was found between 12 hour plasma hydrazine levels and hepatic steatosis ($r = 0.79$, $r^2 = 0.62$, $n = 12$, $p = 0.0023$) (**Figure 20**) in the Veh-INH group. A weaker correlation ($r = 0.57$, $r^2 = 0.32$, $n = 23$, $p = 0.0048$) in all INH-treated animals (RIF-INH + Veh-INH) suggests an effect of RIF on INH-induced hepatic steatosis. This is also supported by the lack of statistically significant differences in plasma TG levels at 12, 32, and 48 hours

between the different treatment groups (**Table 10**). Again, there does appear to be a decrease in the degree of hypertriglyceridemia at 32 and 48 hours with RIF-pretreatment; at 32 hours RIF-INH (1.6 ± 0.3 mM TG) *versus* Veh-INH (3.5 ± 1.3 mM TG) and at 48 hours RIF-INH (3.5 ± 1 mM TG) *versus* Veh-INH (6.4 ± 2 mM TG) (**Table 10**). The main reason for a non-statistically significant difference between the RIF-INH and Veh-INH treatment groups is probably the high variance contributing to a high standard deviation in the Veh-INH *versus* the RIF-INH and Veh-Veh groups. At 48 hours the difference between these treatment groups approaches statistical significance (ANOVA: $p < 0.053$).

Others have also reported INH-induced steatosis and/or hypertriglyceridemia in rabbits (McKennis, Jr. et al., 1956; Whitehouse et al., 1978 & 1983; Karthikeyan and Krishnamoorthy, 1991). However, unlike the preceding studies, Sarich et al. (1995 & 1996) has demonstrated concomitant INH-induced hepatic necrosis, steatosis and hypertriglyceridemia in a rabbit model similar to the present one.

One might argue that RIF itself caused hepatic steatosis which accounted for the observed lack of difference in fatty changes between RIF-INH- and Veh-INH-treated animals. However, there is only one publication which supports this hypothesis. Piriou et al. (1979) demonstrated significant increases in hepatic TG's in male rats with significant decreases in plasma TG's. However these changes occurred with a dosing regimen consisting of 400 mg/kg/day RIF for 8 days which is much higher than the present regimen. Moreover, there was no increase in hepatic TG's in this study with RIF alone.

In humans, INH-induced necrosis has been accepted as the characteristic hepatotoxic effect of INH (Black et al., 1975; Mandell & Petri, Jr., 1996); however the only two studies reporting the occurrence of INH-induced hepatic necrosis and steatosis were in patients receiving both INH and RIF (Pessayre et al., 1977; Pilieu et al., 1979). As mentioned earlier, these studies did not adequately control for INH and RIF treatments. The lack of observation of hepatic steatosis in patients receiving INH does not mean that steatosis is not occurring; rather, there are no studies in humans in which hepatic steatosis with INH or plasma triglycerides were

quantitated.

The significant correlations between 12 hour plasma hydrazine levels and hepatic TG's in Veh-INH ($r = 0.79$, $r^2 = 0.62$, $n = 12$, $p = 0.0023$) (**Figure 20**) and all INH-treated animals ($r = 0.57$, $r^2 = 0.32$, $n = 23$, $p = 0.0048$) suggest that hydrazine itself is responsible for INH-induced hepatic steatosis. Although there is a weak negative correlation between hepatic TG and amidase activity ($r = -0.46$, $r^2 = 0.21$, $n = 22$, $p = 0.033$) (**Figure 16**) in all INH-treated animals, a lower amidase activity would predictably result in less hydrazine production from INH hydrolysis and hypothetically result in less INH-induced hepatic steatosis. However, the decreased amidase activity observed in this study is probably reflecting amidase which is inhibited or destroyed by the hydrazine being produced.

Hepatic steatosis and hepatic necrosis both seem to be occurring simultaneously, as there is a significant correlation between peak plasma log ASAL activity and 32 hour plasma TG's in Veh-INH ($r = 0.75$, $r^2 = 0.55$, $n = 11$, $p = 0.009$) (**Figure 17**) and all INH-treated animals ($r = 0.69$, $r^2 = 0.49$, $n = 22$, $p = 0.0003$) (**Figure 18**). Peak plasma levels of ASAL were observed to occur at 32 or 48 hours of INH-dosing. Surprisingly, a weak correlation was found between hepatic TG's and plasma TG levels, the only statistically significant one occurring at 48 hours. A correlation was found between with hepatic TG's and 48 hour plasma TG's ($r = 0.54$, $r^2 = 0.29$, $n = 37$, $p = 0.0006$) (**Figure 19**) and between hepatic TG's and peak plasma TG's ($r = 0.52$, $r^2 = 0.28$, $n = 37$, $p = 0.0009$) when all treatment groups were included.

In previous studies conducted by Sarich et al. (1996), no correlations were found between plasma hydrazine levels and INH-induced hepatic steatosis, whereas strong correlations were found between plasma hydrazine (32 hour) and hepatic necrosis (log plasma ASAL 48 hour) ($r = 0.73$, $r^2 = 0.53$, $n = 15$, $p < 0.005$) and with hepatic steatosis (mg TG/g liver) and peak plasma TG levels (mM) ($r = 0.73$, $r^2 = 0.53$, $n = 15$, $p < 0.002$).

In rats, INH appears to produce hepatic steatosis with little or no hepatic necrosis (Amenta & Johnston, 1962; Patrick & Black, 1965; Scales & Timbrell, 1982). These observations combined with the fact that, in humans, INH-induced hepatic necrosis reportedly occurs without hepatic steatosis suggests that the two toxicological processes are different mechanistically.

Although the present study suggests that INH-induced necrosis and steatosis occur by different means, there is likely to exist a common step in both. That is, it is probable that in INH-induced hepatic steatosis, hydrazine is the hepatotoxic metabolite, whereas in INH-induced necrosis, a metabolite of hydrazine is responsible. Several studies have shown that hydrazine administration to animals produces fatty changes including hepatic steatosis and hypertriglcyeridemia (Yard & McKennis, 1955; Patrick & Black, 1965; Clark et al., 1970). Pretreating rats with CYP-450 inducers such as PB or β-naphthoflavone (BNF) resulted in decreased hepatic triglyceride accumulation in response to hydrazine administration *in vivo* (Jenner & Timbrell, 1994). In contrast, inhibitors of CYP-450, such as piperonyl butoxide increased triglyceride accumulation (Jenner & Timbrell, 1994) in rats. In Jenner and Timbrell's (1994) study, hydrazine caused a dose-dependent increase in hepatic triglyceride accumulation.

Whether RIF's effect on hydrazine is via the inhibition of CYP2E1 and/or the induction of CYP3A4 or the induction of P-450 reductase is still questionable. All three enzymes are potentially involved in the metabolism of hydrazine. Nonetheless, the present study does show that hydrazine is likely to directly or indirectly cause INH-induced necrosis and steatosis.

The marked species differences between rats and rabbits, rats being less susceptible to INH-induced necrosis than rabbits while still exhibiting equivalent susceptibility to INH-induced steatosis as rabbits, might be explained by their inherent difference in amidase and acetylation activities. Rats have been shown to possess amidase activites 10 to 20 times slower and acetylation rates about 6 times slower than those found in rabbits (Whitehouse et al., 1983). The overall effect in rats, then, would be a greater proportion of hydrazine being acetylated in relation to the amount of hydrazine produced from the hydrolysis of INH. As well, the inherent difference in the RIF doses required to induce CYP3A6 in rats (200 - 400 mg/kg) versus rabbits (30 - 100 mg/kg) might also provide insight into this species difference (Benedetti & Dostert, 1994). It would be of interest to conduct a similar experiment as the present, in rats, to see which hepatic microsomal enzymes are affected (e.g. CYP2E1, CYP3A6, amidase, and P-450 reductase) and this might provide further insight into the mechanism of INH-induced steatosis *versus* necrosis.

In the present study, P-450 reductase was significantly induced by RIF (RIF-Veh group, n = 11) by approximately 40% of the mean of Veh-Veh* control rabbits (388 ± 24 nmoles/min/mg protein, n = 4) (**Figure 12**; **Table 11**). A review of the available literature reveals an absence of effect of RIF pretreatment on P-450 reductase activity in rabbits. For example, Whitehouse et al. (1985) found that RIF given for 7 days at a dose of 100 mg/kg/day (i.p.) has no significant effect on P-450 reductase activity. Noda et al. (1983) observed the same result with a dosing regimen consisting of 30 mg/kg/day RIF (parenterally) for 6 days. In contrast, others have found a significant induction of P-450 reductase in rats (Pessayre et al., 1976; Piriou et al., 1983) and mice (Dimova & Stoytchev, 1994). Thus, the present study is the first report of a significant induction of P-450 reductase in rabbits.

P-450 reductase activity did not correlate significantly with 12 or 32 hour plasma hydrazine. Interestingly, however, the rabbit in the Veh-INH group, considered an outlier because of very high signs of toxicity, also had quite a low P-450 reductase activity (263 nmoles/minute/mg protein) relative to the mean of this group (413 ± 16 nmoles/minute/mg protein). P-450 reductase activity also did not correlate significantly with any parameter of INH-induced toxicity (peak plasma ASAL & ALT, hepatic triglycerides (TG) and plasma TG). This finding may, in part, be explained by the possibility that P-450 reductase is only partly responsible for the metabolism of hydrazine or that P-450 reductase is indirectly involved in the metabolism of hydrazine. For example, P-450 reductase might be increasing the metabolism of a hepatotoxic metabolite of hydrazine to a non-toxic metabolite. One can not exclude P-450 reductase as playing a role in the metabolism of hydrazine, given the observed significant induction of P-450 reductase in the presence of both RIF and INH and the concomitant decrease in INH-induced hepatic necrosis. In rats, which appear to appear to be less susceptible to INH-induced hepatic necrosis than rabbits, P-450 reductase activities are approximately double the activity obseved in rabbits (Whitehouse et al., 1983). If there had been two or three rabbits, rather than only one, which had demonstrated extreme toxicity and low P-450 reductase activity, the aforementioned correlations might have been significant.

Previous studies in this laboratory (Sarich et al., 1997) have demonstrated that L-thyroxine (T4) mediated induction of P-450 reductase results in significantly less INH-induced hepatic necrosis and steatosis in T4-INH *versus* INH-only groups. Moreover, combining T4-INH and INH only-treated animals (n = 28) produced statistically significant negative correlations of P-450 reductase activity with peak plasma ASAL activity ($r = -0.49$, $r^2 = 0.24$, $p < 0.01$) and hepatic TG accumulation ($r = -0.63$, $r^2 = 0.40$, $n = 28$, $p < 0.001$). Kato and Takahashi (1968) and Waxman et al. (1989) have also demonstrated induction of P-450 reductase using L-thyroxine. Both the findings of T. C. Sarich (Ph.D. Thesis, 1997) and the present study strongly suggest that increased P-450 reductase activity by RIF protected against INH-induced necrosis.

An alternative explanation to the lack of correlation found between P-450 reductase activity and a marker of hepatotoxicity is that induction of P-450 reductase increased the effectiveness of an unknown P-450 enzyme in detoxifying hydrazine or a toxic intermediate of hydrazine. This would be considered an indirect effect of P-450 reductase on the hepatotoxicity of hydrazine or a metabolite of hydrazine. The possibility also exists that the induction of P-450 reductase did not confer upon the rabbits any protective mechanism against INH-induced hepatotoxicty. This hypothesis is partly supported by Noda et al.'s (1983) report that RIF pretreatment did not affect P-450 reductase activity, but still resulted in decreased plasma hydrazine levels in rabbits receiving INH. In any case, these studies in rabbits demonstrate that induction of P-450 reductase does not increase the hepatotoxicity of hydrazine as proposed in an *in vitro* study by Noda et al. (1988) in rats.

A novel finding in this study was a significant inhibition of CYP2E1 activity by RIF in rabbits. The only other finding suggesting an inhibition of CYP2E1 by RIF treatment is by Dimova and Stoytchev (1994) in mice. Dimova and Stoytchev demonstrated a significant decrease in aniline hydroxylase activity, an activity non-specific to CYP1A2 and CYP2E1 isozymes, with 50 mg/kg/day (i.p.) RIF pretreatment in mice. A review of the literature indicates that p-nitrophenol hydroxylase (used in the present study) is more selective than aniline hydroxylase for CYP2E1 activity (Koop D R et al., 1989). In the present study, CYP2E1 activity in RIF-Veh* rabbits (1.5 ± 0.1 nmole/min/mg protein, $n = 11$) was significantly lower than the

Veh-Veh* control group (2.3 ± 0.4 nmole/min/mg protein, n = 4) (ANOVA: $p < 0.00001$; Neuman-Keul's Test: $p < 0.05$) (**Figure 13**; **Table 11**). This is the first report of the inhibition of CYP2E1 by RIF in rabbits.

INH caused a greater decrease in CYP2E1 activity in Veh-INH-treated animals having a mean activity of 0.47 ± 0.2 nmole/min/mg protein (n = 12) (ANOVA: $p < 0.00001$; Tukey test: $p < 0.001$) (**Figure 13**; **Table 11**). Previous experiments by Sarich et al. (1997) demonstrated a similar degree of inhibition (Veh-INH: 0.53 ± 0.2 nmole/min/mg protein, n = 7) of CYP2E1 by INH treatment. The average dose of INH administered to the rabbits using the dosing protocol outlined in the Methods section is approximately 1.1 mmoles/kg/day for two days. Clinical studies on the effect of INH on CYP2E1 activity by Zand et al. (1993) showed that in humans, doses of INH of approximately 0.04 mmoles/kg/day for seven days causes inhibition of CYP2E1 at the time of INH administration. In contrast, at 48 hours after the last dose of INH, an induction of CYP2E1 is observed. As a possible explanation for this observation, Zand et al. (1993) proposed the CYP2E1 enzyme is likely stabilized from degradation during its induction and upon decreased exposure to INH, the now increased number of enzymes becomes unmasked, resulting in an increase in CYP2E1 activity. Previous studies in this laboratory (Sarich et al., 1997) showed an increase in CYP2E1 activity in animals pretreated with bis-*p*-nitrophenyl phosphate (BNPP), an amidohydrolase (amidase) inhibitor (Mitchell et al., 1976), followed by INH. It was proposed that INH or acetyl-INH was an inducer(s) of CYP2E1, while one or more of the INH metabolites are inhibitors of CYP2E1. Alternatively, since Jenner and Timbrell (1994b) observed increased CYP2E1 activity with low doses of hydrazine in rats, then perhaps a lower metabolite concentration of hydrazine can increase CYP2E1 activity.

It is difficult to propose a mechanism by which RIF caused an significant decrease in CYP2E1 activity since no evidence supporting the inhibitory effect of RIF on CYP2E1 activity is available. However, it has been shown that RIF administered at therapeutic doses causes significant inhibition of protein synthesis, *in vitro*, in microsomal fractions isolated from human and rat livers and this is more pronounced in humans than in rats (Buss W C et al., 1978).

Generally, a decrease in P-450 enzyme activity can result from: (1) exposure to an environmental factor (such as an infectious disease or a xenobiotic) that suppresses P-450 enzyme expression, or (2) exposure to a xenobiotic that inhibits or inactivates a pre-existing P-450 enzyme. (Parkinson, 1996). The first mechanism is most likely, as it is not uncommon for drugs which induce one CYP-450 family to decrease the expression of other CYP-450's. For example,

Interestingly, induction of CYP2E1 has also been shown to increase the toxicity of other chemicals such as carbon tetrachloride (CCl_4) in rats pretreated with ethanol (Lindros et al., 1990). In mice, RIF pretreatment at 50, 100 or 200 mg/kg for 4 days protected against acute liver injury induced by CCl_4 (Huang et al., 1995). In this study, microsomes isolated from mice pretreated with RIF 200 mg/kg orally for four days followed by a single subcutaneous injection of CCl_4 (50 μl and/or 400 μl of 5 ml/kg) exhibited a significant induction of P-450 reductase (nmol/mg protein/min). Recently, anti-(CYP2E1)-IgG experiments performed with microsomes showed that the addition of anti-(CYP2E1)-IgG to the P-450 enzyme system *in vitro* was capable of inhibiting CCl_4-mediated microsomal lipid peroxidation by 70%, suggesting that CYP2E1 is involved in the metabolic activation of CCl_4 (Johansson & Ingleman-Sundberg, 1985; Ekstrom, Von Bahr & Ingleman-Sundberg, 1989). CCl_4-mediated malondialdehyde (MDA) formation was used as a measure of microsomal lipid peroxidation (Ernster & Nordenbrand, 1967). RIF decreased MDA formation mediated by CCl_4 in a dose-dependent manner *in vitro* (RIF concentration varied from 2.2×10^{-7} to 10^{-4} M). RIF's protective effect against CCl_4-induced hepatotoxicity was also manifested as: no difference in serum ALT and aspartate transaminase (AST) activities between RIF-CCl_4 (70.23 ± 23.75 Units/L and 46.57 ± 24.49 Units/L, respectively) and untreated (40.25 ± 4.69 Units/L and 21.65 ± 4.17 Units/L, respectively) animals when 200 mg/kg doses of RIF were utilized. CCl_4-treated animals exhibited mean ALT and AST serum concentrations of 280.63 ± 58.17 Units/L and 271.36 ± 78.00 Units/L, respectively. A group of five mice were included in each treatment group analyzed. 100 mg/kg doses of RIF also significantly reduced CCl_4-induced hepatotoxicity (~50%) but to a lesser degree than 200 mg/kg (~80%).

In light of the foregoing obervations, the present study's finding of a significant decrease in CYP2E1 activity by RIF with concomitant "protection" of the rabbit from INH-induced hepatotoxicity suggests that inhibition of CYP2E1 may be partly responsible for the decrease in INH-induced hepatotoxicity by RIF in rabbits. Statistically significant negative correlations could be found with CYP2E1 activity *versus* 48 hour plasma TG ($r = -0.40$, $r^2 = 0.16$, $n = 30$, $p = 0.027$), peak log ASAL activity ($r = -0.45$, $r^2 = 0.20$, $n = 30$, $p < 0.013$), and hepatic TG levels ($r = -0.66$, $r^2 = 0.44$, $n = 30$, $p = 0.00007$) when all treatment groups were considered. Although these correlations are weak ($r^2 < 0.50$), it is important to note that: (1) those rabbits which had CYP2E1 activities that were undetectable were not included, and (2) it is likely that one of the metabolites of INH dependent on amidase activity (isonicotinic acid, acetylhydrazine, and/or hydrazine) inhibits CYP2E1. If the undetectable ("0" values) of CYP2E1 activity had been included in the analysis, the statistical and biological significance of the correlations would have been stronger. T. C. Sarich previously found a significant negative correlation between CYP2E1 activity and hepatic steatosis (liver TG; $r = -0.71$, $r^2 = 0.50$, $n = 22$, $p < 0.001$) (Ph.D. Thesis, 1997).

Induction of CYP2E1 by acetone and INH (Jenner & Timbrell, 1994a) has been shown to increase the hepatotoxicity of hydrazine *in vivo* in rats, increasing lipid accumulation compared with control animals. Jenner and Timbrell (1994a) did not see any evidence of an effect of CYP2E1 induction on INH-induced necrosis. With the following assumptions: (1) that hepatic amidase acting on INH produces hydrazine, (2) hydrazine or its metabolite is the principal metabolite of INH responsible for INH-induced hepatotoxicity, and (3) that CYP2E1 is partly responsible for the generation of a hepatotoxic metabolite of hydrazine, then one could predict that inhibition of CYP2E1 during RIF pretreatment protects those rabbits from INH-induced hepatotoxicity compared to those rabbits receiving INH without prior RIF pretreatment. That is, rabbits in the Veh-INH group will have higher baseline CYP2E1 activities and thus be prone to INH-induced hepatotoxicity before receiving INH. In contrast, rabbits in the RIF-INH group will have lower CYP2E1 activities before INH administration is initiated and thus would be "protected" to some degree from INH-induced hepatotoxicity.

Interestingly, a similar phenomenon is seen to occur with acetaminophen(APAP)-induced hepatotoxicity in mice (Snawder et al., 1994). APAP undergoes oxidative activation by the cytochrome P-450 system primarily by the 2E1, 1A2, and 3A4 isozymes (Patten et al., 1993) to the hepatotoxic reactive intermediate *N*-acetyl-*p*-benzoquinone imine (NAPQI). The activity of these P-450 isozymes is also decreased with the development of toxicity in these mice (Snawder et al., 1994).

Thus, it is possible that, in our model of INH-induced hepatotoxicity in rabbits, CYP2E1 may be involved in the production of reactive and toxic metabolites of hydrazine or other metabolites of INH and that RIF's inhibition of CYP2E1 is partly responsible for RIF's decrease of INH-induced hepatotoxicity. Alternatively, CYP2E1 may not be involved with hydrazine metabolism and the protective effect of RIF against INH-induced hepatotoxicity may be due to the induction of P-450 reductase or some other P-450 enzyme which is induced (e.g. CYP3A6 in rabbits, CYP3A4 in humans) or inhibited by RIF.

The acetylator status tested in animals receiving INH (Veh-INH and RIF-INH groups) does not correlate with any marker of toxicity. All of the rabbits phenotyped (RIF-INH & Veh-INH groups) were rapid acetylators (range: 72.8 - 95.3% sulfamethazine acetylation in 20 minutes). However, which such a small range of acetylation rates observed in the rabbits that were phenotyped, it is difficult to draw any conclusions as to whether a relationship exists between the acetylation rate of sulfamethazine and the degree of INH-induced hepatotoxicity.

The hydrazine produced from INH represents a minor metabolic pathway of INH. Recall from **Figure 1** that hydrazine can be produced from two metabolic pathways of INH. One pathway (**A**) involves the hydrolysis of INH to hydrazine by hepatic amidase. The other pathway (**B**) involves three steps, the first one requiring INH to be acetylated to acetyl-INH. In the second step, acetyl-INH is hydrolyzed to isonicotinic acid and acetlyhydrazine. Then, amidase is able once again to hydrolyze acetylhydrazine to hydrazine. In a rapid acetylator, in which more hydrazine is being produced via pathway **B**, the inhibition of amidase by INH could shunt the production of hydrazine in pathway **A** to pathway **B** resulting in even greater production of

hydrazine via this pathway. The diversity which exists in metabolic pathways leading to the production of hydrazine, combined with the potential involvement of one or more hepatic enzymes (e.g. CYP2E1, CYP3A6, and P-450 reductase) in the metabolism of hydrazine further complicates the attempt to isolate a single factor in this multi-stepped process associated with hepatotoxicity. The lack of correlation between amidase activity or acetylation rate with the degree of INH hepatotoxicity might be indicative of a multifactorial aspect to the toxicity of hydrazine and/or a metabolite of hydrazine.

Perhaps a less obvious hypothesis to explain the lack of relationships between amidase activity and toxicity is that hepatocellular damage induced by hydrazine and/or a toxic intermediate of hydrazine confers upon the liver cells a lower capacity to detoxify these metabolites. For example, the "outlier" rabbit in the Veh-INH group which was excluded in some correlational analyses exhibited extreme measures of hepatotoxicity and extremely high plasma hydrazine levels at 32 hours. This rabbit also had very low amidase activity (4 nmoles/min/mg protein) and reductase activity (263 nmole/min/mg protein) relative to the means of 11.5 ± 1.9 nmole/min/mg protein and 413 ± 16 nmole/min/mg protein, respectively. Ideally, one should measure the amidase activity prior to INH administration, since one would predict that subjects with high amidase activities could be at a higher risk of developing INH-induced hepatotoxicity. However, as mentioned earlier, it cannot readily be measured at this time.

It has been proposed that slow acetylator rabbits are more susceptible to INH-induced convulsions than rapid acetylator rabbits (Hein & Weber, 1984). In the present study, 5 out of 23 rabbits receiving INH experienced convulsions in the 24 to 48 hour period of INH dosing; only 1 out of 5 died pre-maturely (20%). These results are concordant with those of Sarich et al. (Ph. D. Thesis, 1997). Our data seem to support the hypothesis that slow acetylators are more susceptible to death from INH-induced convulsions, however, more slow acetylators need to be included in the study in order to verify this hypothesis.

Strengths and Weaknesses of the Present Rabbit Model

The rabbit model established previously in this laboratory (Sarich et al., 1995) to study the characteristics of INH-induced hepatotoxicity has been extended in the present study. Greater than 50% of the rabbits in the Veh-INH group (n = 12) demonstrated significantly elevated plasma ASAL levels (8 out of 12) and hepatic TG accumulation (10 out of 12) relative to the Veh-Veh* group (n = 4).

The present rabbit model consisting of RIF added to the previously established INH protocol has demonstrated similar characteristics as those observed in humans (Hong Kong Chest Service/Tuberculosis Research Center, Madras/British Medical Research Council, 1992): (1) RIF alone demonstrated no signs of hepatotoxicity; (2) RIF pretreatment significantly decreased INH-induced hepatic necrosis.

Although cytochrome P-450 levels were not significantly raised with RIF pretreatment, it is possible that RIF selectively induced a P-450 isozyme without affecting the overall pool of P-450 enzymes; the inhibition of CYP2E1 by RIF may have offset an increase in P-450 levels with the induction of CYP3A6, *per se*. Moreover, it is important to note that the measurement of P-450 levels according to the method described in Omura and Sato (1964) is not a measure of the level of P-450 activity, but only P-450 heme content. It would be valuable, nonetheless, to measure 6-β progesterone (or testosterone) hydroxylase activity as a measure of CYP3A6 activity to verify whether or not RIF pretreatment in this study significantly induced CYP3A6. The existing microsomal samples collected during the study can be used for this purpose.

Another aspect of the present study which might be perceived as a inherent weakness is the imbalance in numbers of animals included in each of the four respective treatment groups, Veh-Veh* (n = 4), Veh-INH (n = 12), RIF-Veh* (n = 11), and RIF-INH (n = 11). The imbalance exists due to the smaller number of rabbits in the Veh-Veh* control group (n = 4). The rationale behind using only 4 animals here is two-fold. Firstly, previous studies in this laboratory (Sarich et al., 1995) have shown that baseline characteristics for peak plasma ASAL (4.6 ± 0.8 Takahara units, n = 12) and ALT (31.3 ± 3.9 Units/L, n = 12) activities, hepatic TG's

(10.5 ± 1.6 mg TG/g liver), and plasma TG's (32 hour: 1.0 ± 0.1 mM) have little variance relative to treatment groups. Similarily, the variability in cytochrome P-450 content (2.9 ± 0.3 nmoles/mg protein), CYP2E1 activity (2.3 ± 0.4 nmole/min/mg protein), P-450 reductase activity (388 ± 24 nmole/min/mg protein), and amidase activity (32.8 ± 4.7 nmole/mg protein/hour) within the Veh-Veh* group of the present study (**Table 11**) is adequately low to justify the use of 4 animals in this control group.

Comments on the Apparent Multifactorial Nature Regarding the Mechanism of INH-induced Hepatotoxicity

It seems evident that no one particular hepatic microsomal enzyme or metabolic process involving INH is entirely responsible for INH-induced hepatotoxicity. It is likely that a number of steps are involved and that each step is present in every individual, the main difference being the degree to which a particular step is actively involved in the metabolism of INH. For example, a given species might lack a particular metabolic step and/or exhibit greater or lower activity for that step. The degree of involvement of one step over another will likely determine the severity of liver damage in response to INH. Some of these steps have been studied extensively, namely amidase and N-acetyltransferase activities. However, assuming that hydrazine is the hepatotoxic metabolite of INH, it is still difficult to predict: (1) the amount of hydrazine that will be produced from these steps, and (2) what amount of hydrazine is required for significant liver damage. For example, a high amidase activity will lead to the production of a larger amount of hydrazine, but this might be compensated by a high N-acetyltransferase activity, leading to the non-toxic metabolite of hydrazine, diacetylhydrazine. Secondly, after these two processes take place, the hydrazine remaining is likely susceptible to further metabolism to either toxic or non-toxic metabolites by P-450 reductase or CYP-450 isozymes. As was proposed earlier, it is possible that the inhibition of CYP2E1 and/or the induction of P-450 reductase may have contributed to the decrease in INH-induced hepatotoxicity with RIF pretreatment. Hepatic P-450 enzymes have been implicated in the oxidation of hydrazine in rats and rabbits (Noda et al.,

1985a; Noda et al., 1985b; Timbrell et al., 1982; Jenner & Timbrell., 1995), but the isozymes responsible are unknown. In vivo studies in rats (Noda et al., 1985a; Jenner & Timbrell, 1994) and rabbits (Noda et al., 1983) pretreated with RIF or PB showed decreased plasma hydrazine levels with concomitant increases in P-450 levels. In patients, two case reports by Wright et al. (1982 & 1984) demonstrates that the interaction between the microsomal enzyme inducer, carbamezapine, and INH can lead to significant hepatic necrosis.

Clearly, there exists greater potential to answer some of the questions or hypotheses that have been raised through the present study. The existing model used to study the effect of RIF on INH-induced hepatotoxicity can serve as a tool to resolve some of these issues. For example, various P-450 isozyme activities, including CYP3A6, can be measured to determine if any relationship exists between its activity and measures of INH-induced hepatotoxicity.

Thesis Summary:

1. The animal model of INH-induced hepatotoxicity is reproducible.

2. INH caused significant hepatic necrosis and steatosis.

3. The present study supports the hypothesis that hydrazine is involved in INH-induced hepatic steatosis, whereas a metabolite of hydrazine is likely responsible for INH-induced hepatic necrosis. There was no significant correlation between INH-induced hepatic necrosis and plasma hydrazine levels. However, a significant correlation was found between hepatic steatosis and 12 hour hydrazine plasma levels.

4. RIF itself is not directly hepatotoxic: RIF did not cause hepatic necrosis or steatosis in rabbits.

5. RIF significantly protected rabbits from INH-induced hepatic necrosis.

6. Plasma levels of hydrazine at 12 & 32 hours of INH dosing were not affected by RIF pretreatment.

7. INH treatment significantly decreased hepatic microsomal amidase activity (~65%). The mechanism for this inhibition is still unclear but might involve hydrazine or a metabolite of hydrazine.

8. RIF pretreatment did not have any effect on hepatic microsomal amidase activity. This makes an effect on amidase unlikely to be the cause of RIF's protective effect against INH toxicity.

9. RIF had no significant effect on hepatic cytochrome P-450 levels. This does not exclude the possibility that RIF significantly induced CYP3A6 as expected, given the observed inhibitory effect on CYP2E1 activity. RIF might be inhibiting and/or inducing other CYP-450 isozymes, the net effect of which is no change in overall levels of CYP-450. Moreover, measurement of CYP-450 levels is a measure of the heme portion of "potentially" active CYP-450's, not the activity of CYP-450 enzymes.

10. A novel finding is RIF's significant inhibition of CYP2E1 (~35%) in rabbits. This inhibition of CYP2E1 might play a role in RIF's decrease of INH-induced hepatotoxicity. The mechanism of this inhibition, although unknown, is likely different from INH's inhibition of CYP2E1.

11. INH treatment inhibits CYP2E1 activity.

12. A novel finding is RIF's significant increase of P-450 reductase activity by approximately 40%. P-450 reductase might be involved in hydrazine metabolism to produce a less or more toxic metabolite of hydrazine. Alternatively, the increase in P-450 reductase by RIF may have increased the effectiveness of an existing or RIF-modulated P-450 isozyme involved in the detoxification of hydrazine.

13. In this study, all rabbits receiving INH were rapid acetylators. There was no relationship between the rate of acetylation of sulfamethazine and any parameter of toxicity, suggesting that small differences in acetylator status are not critical in INH-induced hepatotoxicity.

14. The mechanism of INH-induced hepatotoxicity appears to be a multifactorial one with hydrazine being a key component of toxicity. The known factors include the enzymes leading to the production and detoxification of hydrazine, amidase and N-acetyltransferase. The unknown factors include CYP2E1, CYP3A6, P-450 reductase, and/or some other CYP-450 isozyme which leads to the production of a hepatotoxic or non-hepatotoxic metabolite of hydrazine.

15. Overall, the similarity of effect of RIF in decreasing INH-induced necrosis between humans (Hong Kong Chest Service/Tuberculosis Research Center, Madras/British Medical Research Council, 1992) and rabbits, in the present study (although using different dosing regimens), is supportive of the usefulness of the present model in examining the effect of RIF pretreatment on INH-induced hepatotoxicity.

16. This model can be used to determine which of the "unknown" factors are crucial to the mechanism of INH-induced hepatotoxicity, as well as to better define the mechanism whereby RIF decreases this hepatotoxicity.

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