Title
The study of FK 506 metabolism in rat lives

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Publication Date
1994

Peer reviewed|Thesis/dissertation

The Study of FK 506 Metabolism in Rat Livers

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmaceutical Chemistry

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

Date **University Librarian**

Degree Conferred: SEP 4 1994

To Ron

for your constant love, patience, encouragement and humor

Acknowledgements

During the five years I spent at UCSF, I have the good fortune to interact with several extremely talented and dedicated faculty members. My mentor Leslie Z. Benet, a staunch supporter of me to be who I am, and the prime advocate for my work, will forever be dear to me. C. Anthony Hunt, and Maria Almira Correia, committee members of this thesis, have been extremely kind to share thier knowledge and experiences with me. The frequent long discussions I had with Sven Øie, my academic advisor, have been most fruitful and enlightening. I also benefited greatly from the interactions with Emil T. Lin, Ronald A. Siegel, Martin D. Shetlar, and Davide Verotta.

The active intra- and inter-departmental interactions in UCSF also brought me together with faculty, researchers and fellows. My good friend and teacher Antony F. McDonagh has helped me tremendously in many areas, but especially in improving my presentation skills. Lester M. Bornheim has taught me a great deal about experimental design and techniques. Thomayant Prueksaritanont has been an excellent teacher and a wonderful friend to me. James De Voss and George Negrette were truly helpful in the many brainstorm sections we had together.

My sincerest thanks to K. B. Chang, Suzanne Davoll, Lolin Ip, Eric Liu, David Maltby, Mary Tse, Harrison Wong, and especially Susan Wong for their excellent technical assistance on various occassions. I also appreciate the camaraderie of Abdullah, Bill, Bob, Chao-Han, Chi-Yuan, Katy,

Kazuho, Marcelo, Martine, Narendra, Noriko, Parnian, Sara, Vince and Walter.

Special thanks to friends who are ex-LZB students: Drs. Carol Gloff, Victoria Hale, David Lau and Alice Till. Your openness and encouragement have been a constant support for me .

Last but not the least, I want to thank my dad and mom, and my sister Susannah, for their unyielding support across the miles from Montreal.

Abstract

The novel immunosuppressant FK 506, a fungal metabolite from the fermentation broth of Streptomyces Tsukubaensis was first isolated in Japan in 1984 by the Fujisawa Pharmaceutical Co. It underwent phase III clinical trials in the United States in 1989 without prior phase I and II studies. The lack of knowledge about the metabolism of this agent in humans or in animals led to this work.

This project focused on elucidating the metabolic pathways for FK 506 and its metabolites in rat livers. The lack of appropriate analytical methodology to study FK 506 metabolism required the development of an assay which would separate the parent drug from its metabolites. The successful development of a reverse phase HPLC assay allowed simultaneous monitoring of the parent drug and two interconverting metabolites. Using the tools of mass spectrometry, HPLC, as well as the enzyme linked immunoassay, the FK 506 major metabolite was identified as a 13-desmethyl FK 506.

Incubation of FK 506 in both rat hepatocytes and rat liver microsomes indicated that the formation of 13-desmethyl FK 506 is a phase I oxidative reaction mediated by cytochrome P450 enzymes. Enzyme induction and inhibition studies identified the P450 3A isozyme as the major isozyme involved in the metabolism of FK 506. FK 506 also undergoes metabolism when it is incubated with female rat liver microsomes. Metabolism of FK 506 in female rat liver microsomes provided evidence that a currently

unidentified P450 isozyme that is closely related to the P450 3A isozyme is present at a low level in female rat livers. 13-Desmethyl FK 506 also undergoes metabolism in rat liver microsomes from dexamethasone-treated rats. The metabolizing enzyme for 13-desmethyl FK 506 was shown by immunoinhibition to be P450 3A also.

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Chapter 1

Immunosuppressive Agents: What Is Known About Them?

1.a Introduction

1.a.1 Therapeutic use of immunosuppressants

Immunosuppressive agents are used primarily for the prevention of organ rejections after organ transplantation, and for the treatment of autoimmune diseases. In organ transplantation, an organ graft is exchanged between two individuals. Such a graft is called an allograft if the two individuals are of the same species, or it is called a xenograft if the two individuals are of different species. Xenografts are usually rejected, while allografts may or may not be rejected. Graft rejection is an immune response to the foreign antigens on the surface of the grafted cells. Immunosuppressants are used in these instances to deactivate the body's natural immune system in order to prevent rejection episodes from occurring, or to save the graft that is being rejected.

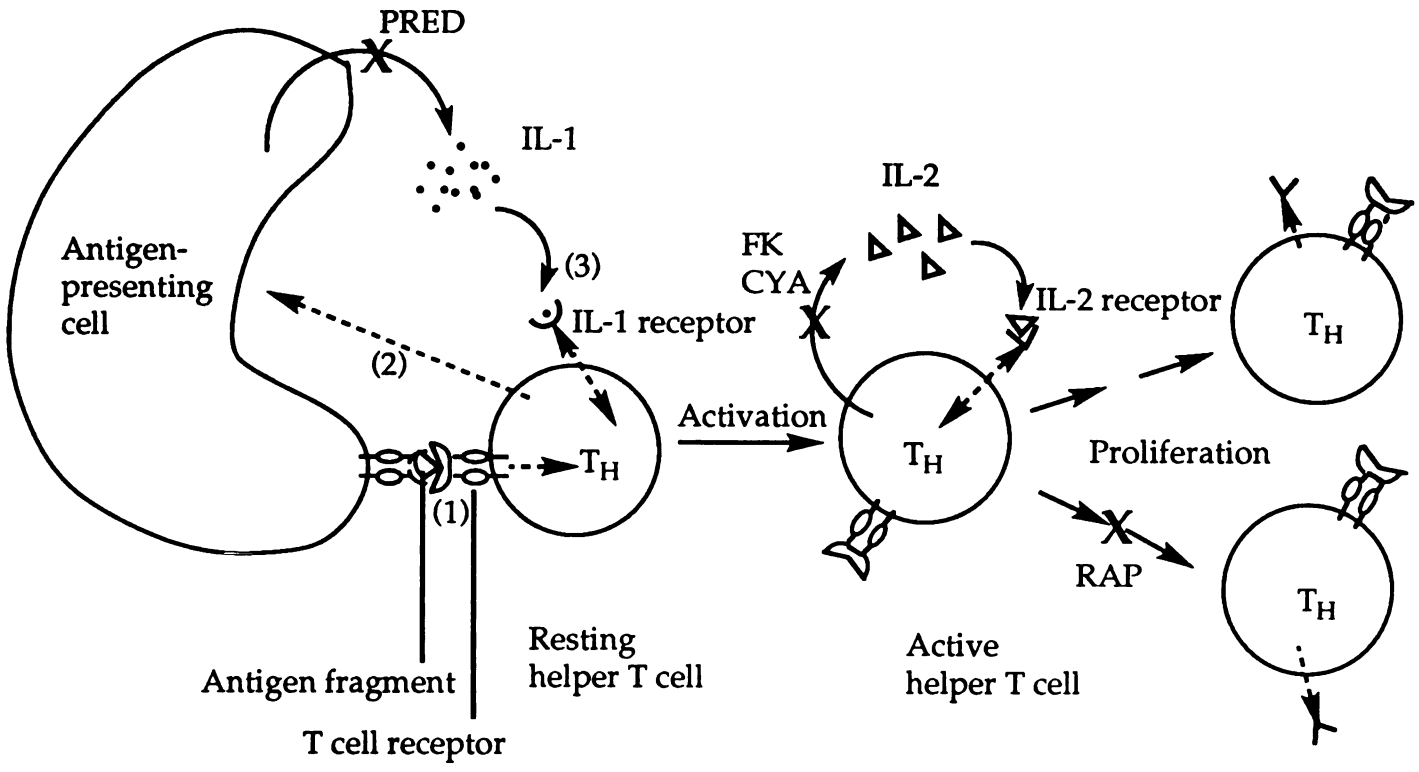
Immunosuppressants are also tested as treatments for autoimmune diseases such as systemic lupus erythematosus (Yamamoto et al., 1989), type I diabetes (Kurasawa et al., 1990), induced Heymann's nephritis (Okubo et al., 1990), and experimental autoimmune uveoretinitis (Ni et al., 1990). In addition, there have been reports of hepatotrophic properties (Francavilla et al., 1990; Mazzaferro et al., 1990) of several immunosuppressants and the ability of these drugs to interfere with T-cell apoptosis (Staruch et al., 1991).

1.a.2 Mechanism of Organ Rejection Following Transplantation

The immune reaction involved in an organ rejection is mainly mediated by T cells. The T cells are directed against genetically "foreign" versions of cell-surface glycoproteins called histocompatibility molecules on the organ graft. In humans, the histocompatibility molecules or HLA antigens (human-leukocyte-associated antigens) are a family of glycoproteins encoded by a complex of genes called the major histocompatibility complex (MHC).

T cells constitute one of the two classes of lymphocytes (white blood cells). The other class of lymphocyte is B cells. Lymphocytes are found in large numbers in the blood, the lymph, and in specialized lymphoid organs. The majority of T and B cells continuously recirculate between the blood and the secondary lymphoid organs, guided by their "homing receptors". When lymphocytes are activated by antigen, lymphocytes lose the homing receptors that mediate recirculation through lymphoid organs, and acquire new ones that guide the activated cells to sites of inflammation.

Cell-mediated immune response (figure 1.1) involves the binding of the cell surface antigen to the T cell receptor on a resting helper T cell. Antigen recognition by the T cell receptor stimulates the antigen presenting cell to secrete interleukin-1 (IL-1) which helps activate the T cell. The activated T cell makes interleukin-2 (IL-2) receptors and secretes IL-2. The binding of the IL-2 to its receptors stimulates the cell to grow and divide (figure 1.1). Active helper T cells also secrete interleukins-4, 5 and 6 (IL-4, IL-5, and IL-6, respectively) to activate, proliferate and maturate B cells to secrete



PRED: Prednisolone
CYA: Cyclosporine
RAP: Rapamycin
FK: FK 506

Figure 1.1 T-cell activation cascade: steps at which several immunosuppressants exert their immunosuppressive effect

specific antibodies to the antigens. In addition, active helper T cells secrete γ-interferon to activate macrophages. It is the coordinated effect of phagocytosing IgG-antibody coated antigenic cells by the macrophages that remove the antigenic cells.

1.a.3 Pharmacology of existing and experimental immunosuppressants

The pharmacology of the currently marketed or experimentally used immunosuppressive agents will be described in the chronological order of their introductions.

1.a.3.1 Prednisolone

Prednisone and prednisolone (figure 1.2a), patented by Schering Plough Corp. (Kenilworth, NJ), are glucocorticoids, a class of steroid hormones produced by the adrenal cortex upon stimulation by adrenocorticotrophic hormone (ACTH) from the anterior pituitary. The glucocorticoids act by altering the rate of RNA synthesis. Prednisolone poses the most proximal block in the T cell activation cascade (figure 1.1). It blocks T cell proliferation through its ability to block the activation of the interleukin 1 (IL-1) gene. Because interleukin 2 (IL-2) release is dependent upon IL-1, corticosteroids also indirectly block IL-2.

Conventional therapies for acute renal allograft rejection include high-dose pulses of glucocorticoids. Glucocorticoids have broad, nonspecific immunosuppressive and anti-inflammatory effects. In addition to their

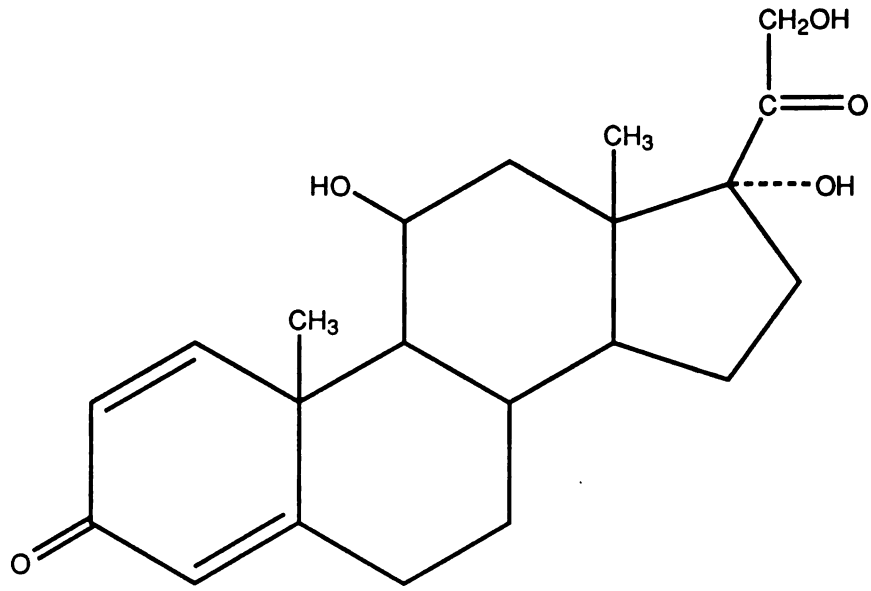


Figure 1.2a Structure of prednisolone

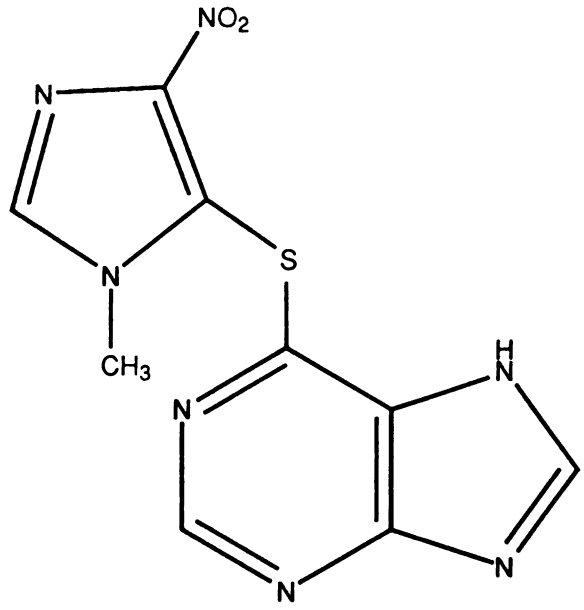


Figure 1.2b Structure of azathioprine

effects on lymphokines, glucocorticoids reduce the ability of monocytes to migrate to sites of inflammation. A major drawback to the use of prednisone in the prevention of acute rejection is that the drug inhibits the entire immune and inflammatory systems, and alters many steroid-responsive systems as well. The use of high doses of glucocorticoids can thus produce severe undesirable side effects, including decreased inflammatory and phagocytic capacity. The results of such side effects are increased susceptibility to infection, hyperglycemia, hyperkalemia, osteoporosis, increased capillary fragility, and growth suppression in children.

1.a.3.2 Azathioprine

Azathioprine (figure 1.2b) or Imuran® marketed by Burroughs Wellcome Co. (Research Triangle Park, NC) is an imidazole derivative of 6-mercaptopurine. As an anti-metabolite, it blocks DNA synthesis, and thereby inhibits gene replication and T cell activation/proliferation. Azathioprine also decreases the number of migratory mononuclear and granulocytic cells while inhibiting the proliferation of promyelocytes within bone marrow. The resulting decrease of macrophages causes severe deleterious effects such as severe leukopenia, thrombocytopenia, gastrointestinal disturbances, fever, hepatotoxicity, and an increased risk of neoplasia.

1.a.3.3 Cyclosporine

Cyclosporine or Sandimmune® patented by Sandoz AG (Basel, Switzerland) is at present the drug of choice for immunosuppression. It is a

cyclic undecapeptide (figure 1.3) derived from a fungus - the imperfect mold Tolypocladium inflatum. Cyclosporine acts by blocking the activation of the IL-2 and other lymphokine genes so that IL-2 encoding mRNA is not transcribed, and IL-2 is not secreted (figure 1.1). When IL-2 is not present, T cells do not proliferate, macrophage-activating gamma interferon is not released, and B-cell activating factors are not released. Steps proximal to the activation of IL-2 genes in the immunological cascade are not affected by cyclosporine (figure 1.1).

The major side effects of cyclosporine are nephrotoxicity, hypertension, hepatoxicity, hirsutism, tremor, increased susceptibility to infection, gingival hypertrophy and gynecomastia.

1.a.3.4 Minnesota antilymphoblast globulin

Minnesota antilymphoblast globulin (MALG) or ATGAM® (The Upjohn Co., Kalamazoo, MC) is a polyclonal immune globulin obtained by injecting animals with human lymphoid cells (e.g., B cell lymphoblasts, peripheral T cell lymphocytes or thymus lymphocytes), and then fractionating the resulting immune sera to obtain purified gamma globulins. Several possible mechanisms by which polyclonal immunoglobulins exert its immunosuppressive effect include complement mediated lysis of lymphocytes, clearance of lymphocytes due to the uptake by the reticulo-endothelium, expansion of suppressor cell populations, or the masking of T cell antigens which may result in the blocking of lymphocyte function. The elimination of circulating T cells, and the subsequent inhibition of proliferative responses result in the resolution of cell-mediated graft

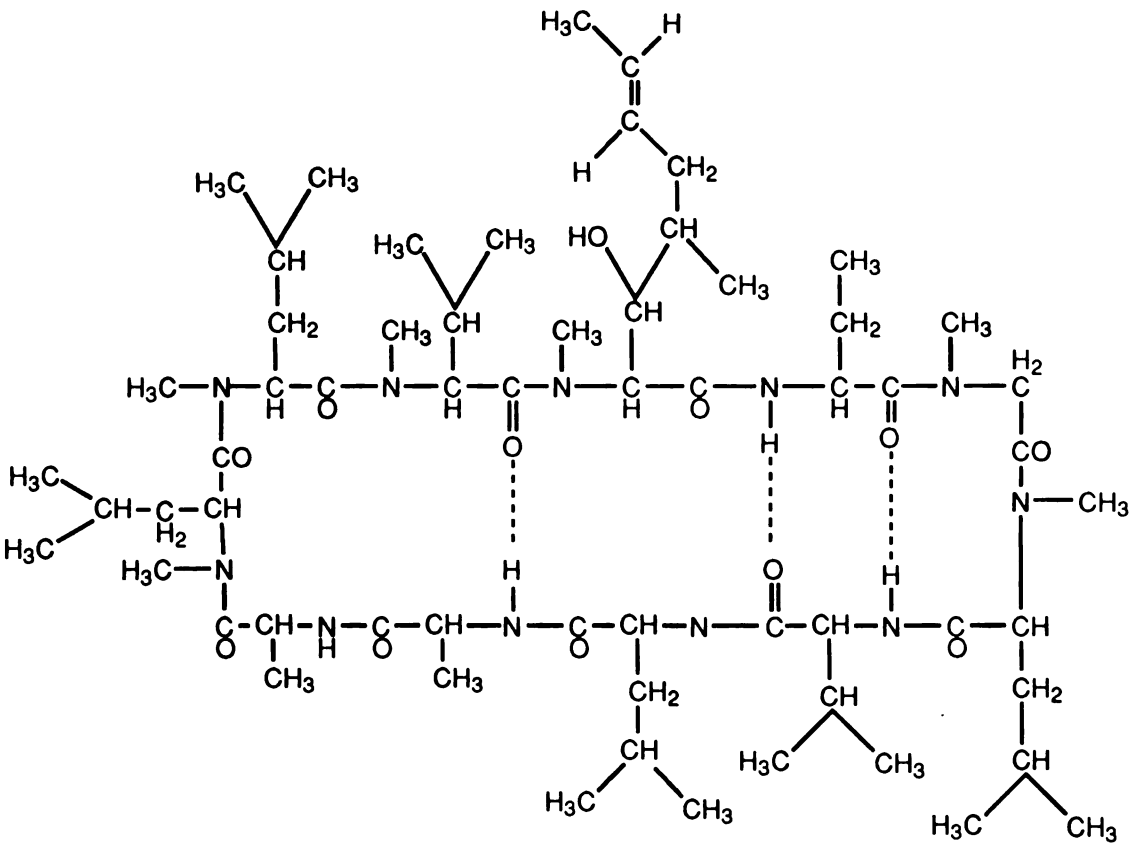


Figure 1.3 Structure of cyclosporine

rejection. The potential for over-immunosuppression is frequent, especially when multiple immunosuppressive agents are used. MALG is generally used prophylactically to reduce the number and severity of rejection episodes.

Side effects of MALG include leukopenia, thrombocytopenia, fever, chills, rigors, increased risk of infections and cancer, pulmonary edema, hematuria, hypotension, and serum sickness.

1.a.3.5 OKT3

Muromonab-CD3 or Orthoclone OKT®3 (Ortho Pharmaceutical Corp., Raritan, NJ) is a monoclonal antibody that binds to a constant component of the T-cell antigen recognition complex, and blocks cytotoxic T cell mediated cell lysis and other T lymphocyte functions. It is remarkably effective in reversing cellular rejection. The major side effects of OKT3 are hypotension, increased susceptibility to infection, severe pulmonary edema in fluid overloaded patients, dyspnea, gastrointestinal disturbances, fever, chills, tremor and headaches.

1.a.3.6 Rapamycin

Rapamycin (figure 1.4) was originally identified in 1975 as an antifungal agent (Vezina et al., 1975). Due to its structural similarity to FK 506, its potential as an immunosuppressive agent has been investigated. Currently, it is undergoing phase 1 clinical trials in the U.S. Rapamycin binds to FK binding protein (FKBP), an immunophilin that also binds to FK 506 (Koltin

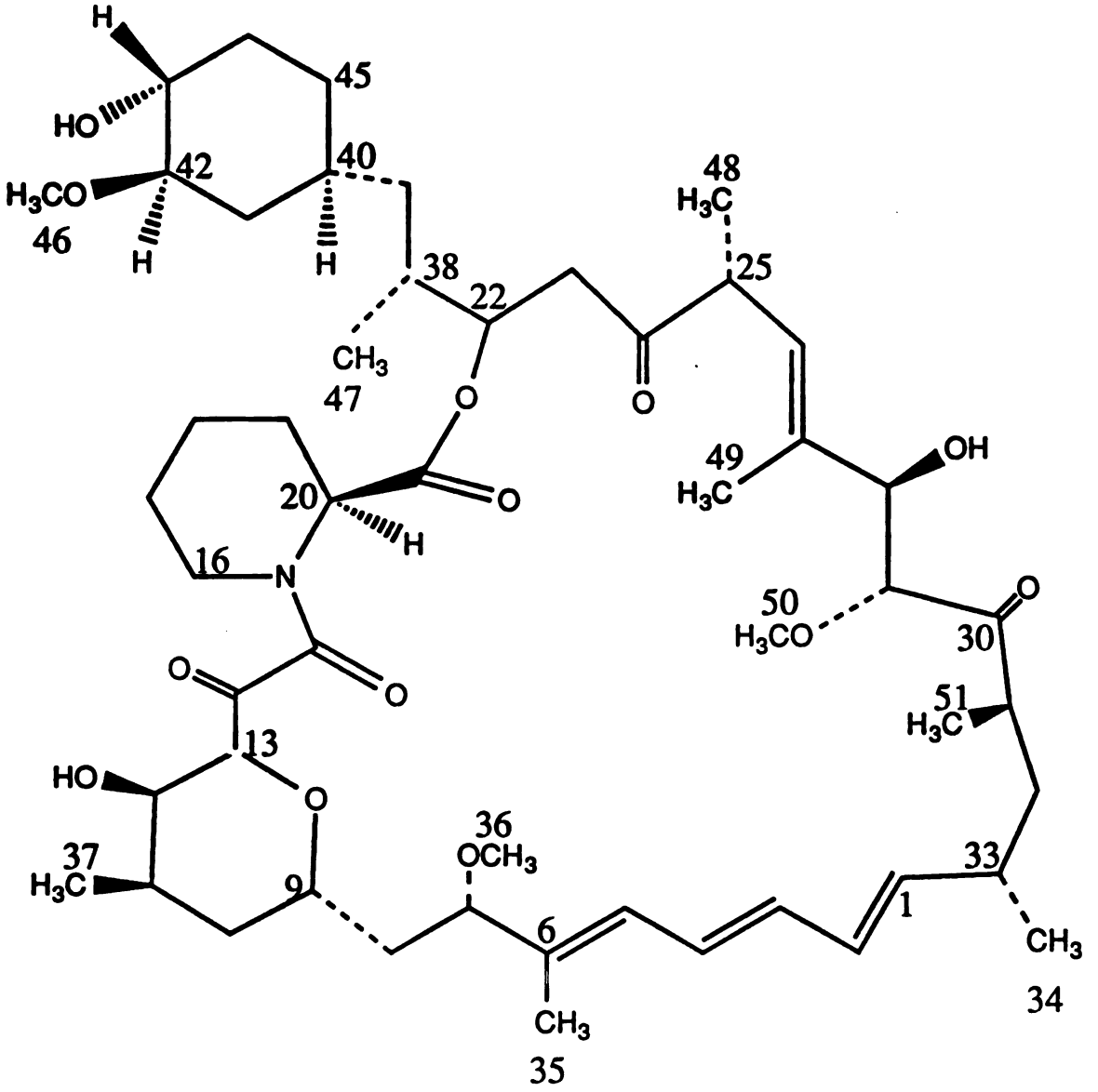


Figure 1.4 Structure of rapamycin

et al., 1991). The rapamycin-FKBP complex interacts with a third component, forming a complex which expresses its immunosuppressive activity by inhibiting the response of the T cell to activation through the IL-2 receptor (figure 1.1) (Bierer et al., 1990a; Dumont et al., 1990; Schreiber, 1991). Toxicity such as hepatic and renal functional abnormalities, including decreased creatinine clearance seen often in recipients of cyclosporine, has not been observed after rapamycin administration (Fryer et al., 1993). While myocardial necrosis was not observed in rabbits (Fryer et al., 1993), it was seen in rats (Whiting et al., 1991). In addition, weight loss, thrombocytopenia, gastrointestinal ulcerations, vasculitis and infections were some other side effects reported in man (Calne et al., 1989). Thrombocytopenia, gastrointestinal ulcerations and vasculitis were, however, not seen in pigs (Almond et al., 1993). Since only limited studies in animals have been reported, the magnitude of side effects is not clear.

1.a.3.7 Mycophenolate mofeteil

Mycophenolate mofeteil (figure 1.5a) or RS61443 (Syntex Labs, Inc., Palo Alto, CA) is an ethyl ester of mycophenolic acid. As a prodrug, it needs to be cleaved by esterases to give the active moiety - mycophenolic acid (figure 1.5b). Mycophenolic acid inhibits inosine monophosphate dehydrogenase (IMPD), thereby depleting guanosine monophosphate (GMP), guanosine triphosphate (GTP), and deoxy-guanosine triphosphate (dGTP) (figure 1.6). This depletion reduces DNA synthesis, and the consequential proliferation and functions of T and B lymphocytes (Allison and Eugui, 1993; Wang and Morris, 1991).

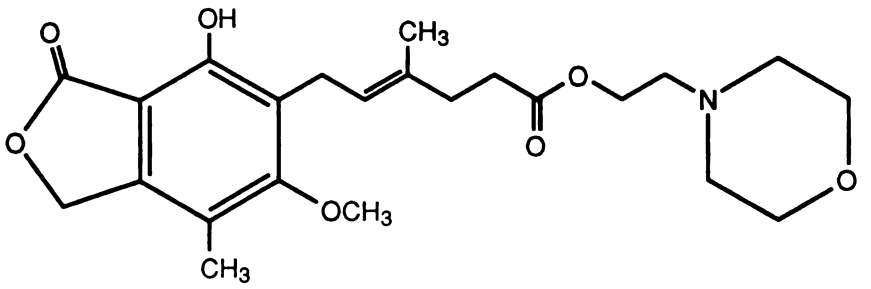


Figure 1.5a Structure of mycophenolate mofetil

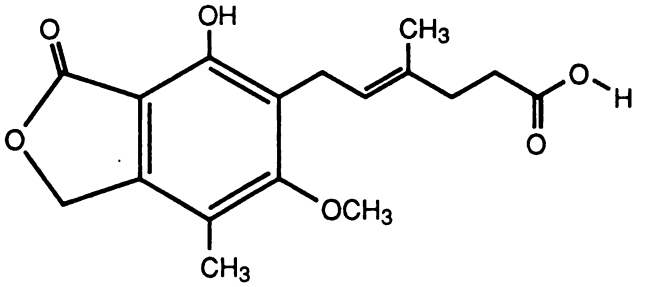
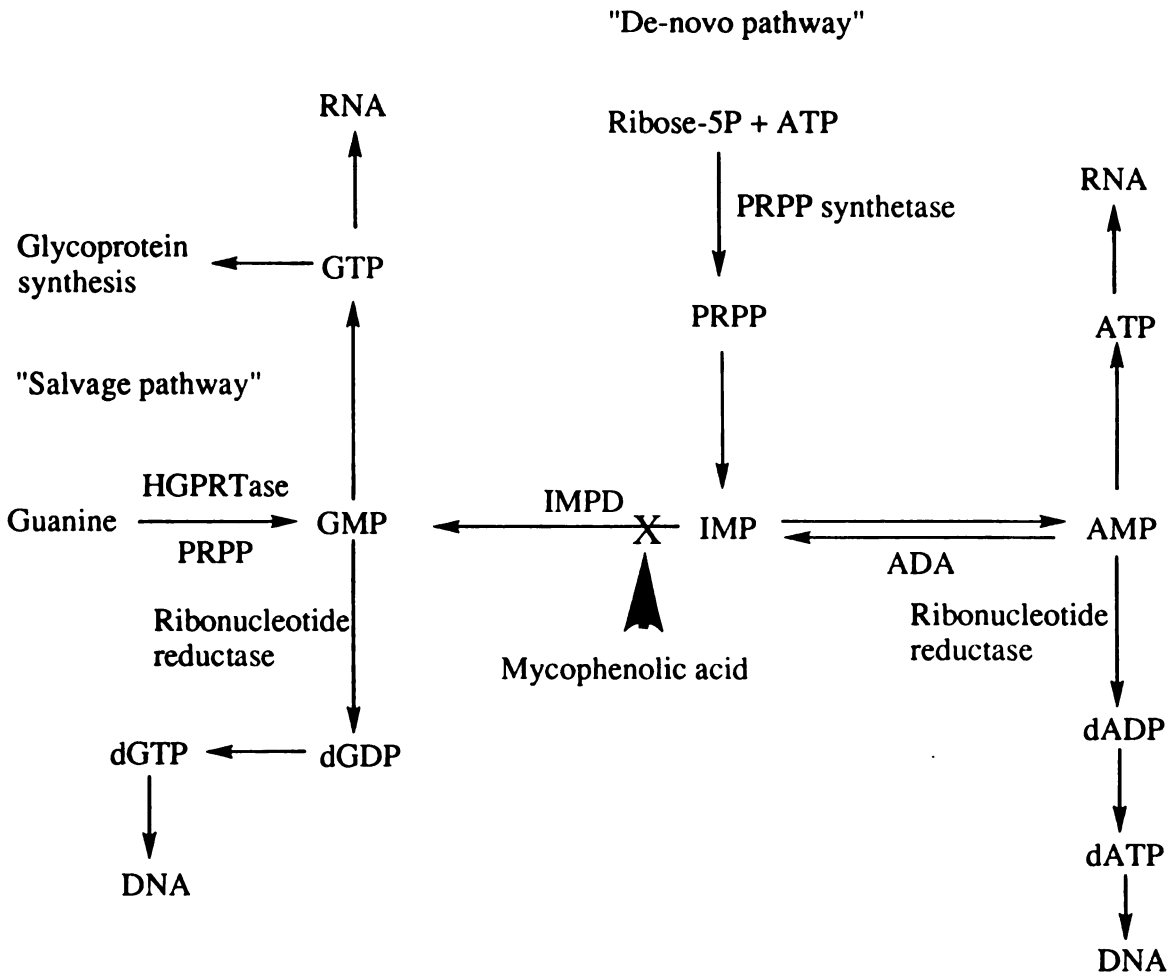


Figure 1.5b Structure of mycophenolic acid



PRPP: 5-phosphoribosyl-1-pyrophosphate; IMPD: Inosine monophosphate dehydrogenase; HGPRTase: Hypoxanthine-guanine phosphoribosyltransferase.

Figure 1.6 Mycophenolic acid blocks the de-novo pathway of purine synthesis in T cells

RS61443 is an agent in phase 2 clinical trials in the U.S. Some of the side effects noted with this agent include gastrointestinal disturbances, rash, fever, headache, muscle cramps, dizziness, weakness, joint pain, insomnia, mouth ulcers, and infections. Some of the severe side effects associated with other immunosuppressants, such as nephrotoxicity and CNS disturbances, have not been noticed with RS61443 in this early stage of clinical trials. Due to a decreased absorption of RS61443 in the presence of antacids, it is contraindicated in patients with active peptic ulcer disease.

1.a.3.8 FK 506

1.a.3.8.1 Chemical and physical properties

FK 506 (figure 1.7) or Tacrolimus® (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan) was discovered as a fungal metabolite from the fermentation broth of Streptomyces tsukubaensis by T. Goto and T. Kino in 1984 (Goto et al., 1987; Kino et al., 1987). Its chemical name according to Chemical Abstracts is [3s-[3R*[E(1S*,3S*,4S*)],4S*,5R*,8S*,9E,12R*,-14R*,15S*,16R*,18S*,19S*,26aR*]]5,6,8,11,12,13,14,15,16,17,18,19,24,-25,26,26a-hexadecahydro-5,19-dihydroxy-3-[2-(4-hydroxy-3-methoxy-cyclohexyl)-1-methylethenyl]-14,16-dimethoxy-4,10,12,18-tetramethyl-8-(2-propenyl)-15,19-epoxy-3H-pyrido[2,1-c][1,4]oxaazacyclo-tricosine-1,7,20,21(4H,23H)-tetrone hydrate. With a molecular formula of $C_{44}H_{69}NO_{12}\cdot H_2O$, it has a molecular weight of 822. FK 506 appears in the form of colorless prisms with melting point at 127-129°C. This compound is most soluble in methanol, and can also be dissolved in chloroform, acetone, ethyl acetate and ethanol. It is, however, insoluble in water or

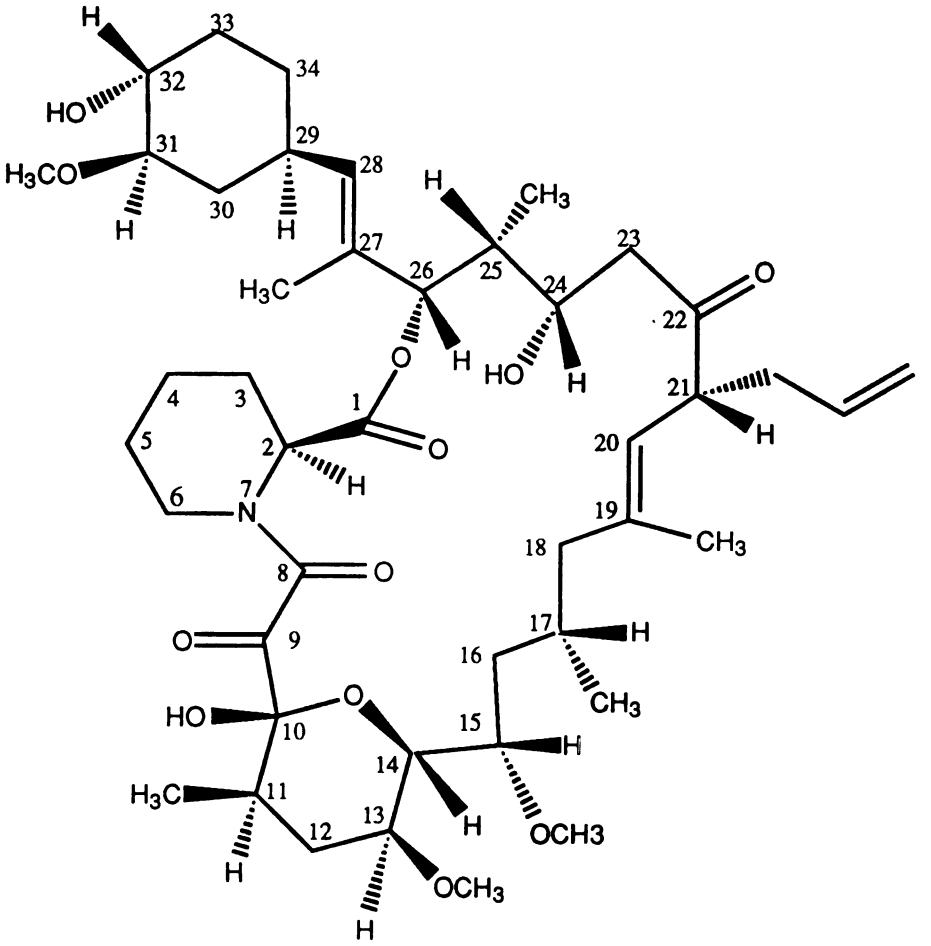


Figure 1.7 Structure of FK 506

hexane (Venkataramanan et al., 1987; Venkataramanan et al., 1991b). Results from ^{13}C-NMR spectroscopy show that FK 506 exists as an equilibrium mixture of 2 isomers in the ratio of 2 or 3 to 1 (Karuso et al., 1990; Tanaka et al., 1987) in deuterated chloroform ($CDCl_3$).

FK 506 is a novel 23-member macrolide with a hemiketal masked α,β-diketoamide (Goto et al., 1987). It is a neutral compound (Iwasaki, 1987) that is less hydrophobic than the leading immunosuppressant, cyclosporine. The hydrophobicity parameters, log *P'*, for FK 506 and cyclosporine are 0.386 and 0.545, respectively (Takada et al., 1992).

1.a.3.8.2 Pharmacology

Early reports showed that FK 506 has exciting biological properties that could be exploited for therapeutic purposes. While FK 506 does not have a strong inhibitory effect on bacteria and yeast, its antifungal activity toward Aspergillus fumigatus and Fusarium oxysporum (MIC = 0.025 and 0.05 μg/ml, respectively) is substantial (Kino et al., 1987).

In the immunological area, FK 506 has been shown to be effective in the suppression of the mixed lymphoctye reaction (MLR) in mouse (Kino et al., 1987). In addition, strong suppression of humoral and cellular immunity, as indicated by decreased plaque forming cells (PFC) and decreased delayed type hypersensitivity response (DTH) to methylated bovine serum albumin, and the suppression of alloantigen-driven proliferation [e.g., effect of graft-versus-host reaction (GVHR) in mice as reported by Kino et al, 1987]

clearly suggest that FK 506 is one of the most active immunosuppressants, both in vitro and in vivo (table 1.1).

FK 506 acts as an immunosuppressant by preventing T cell proliferation via inhibition of IL-2 gene transcription (Bierer et al., 1990b). Siekierka and coworkers showed that FK 506 must bind to a cytosolic binding protein (immunophilin) or FKBP in order to cause decreased IL-2 transcription activity (Siekierka et al., 1989a; Siekierka et al., 1989b). This 12 kDa immunophilin which has the property of a prolyl-cis-trans isomerase was initially thought to be responsible for the immunosuppressive activity of FK 506 (Harding et al., 1989; Heitman et al., 1991; Siekierka et al., 1991). Recently, other FK 506 binding proteins including two 12-kDa isoforms and several unique cytosolic proteins with molecular weights of 13, 25, 60 and 80 kDa have been reported (Fretz et al., 1991). The binding of ligand to the 12-kDa binding protein was, however, later shown to be necessary but not sufficient for immunosuppression to occur (Bierer et al., 1990a; Siekierka et al., 1991; Sigal et al., 1991a; Sigal et al., 1991b; Tocci et al., 1989). It was demonstrated by Van Duyne and coworkers (1991) that the cyclohexyl-pipecolic acid-pyranose group in FK 506 binds to a hydrophobic pocket in the FKBP, while the rest of the FK 506 macrocycle is exposed. The remainder of the FK 506 macrocycle which contains the 15-methoxy substituent and the 21-allyl substituent, has been shown to make up the "effector domain" of the molecule. The effector domain binds to the Ca^{2+}, calmodulin-dependent protein phosphatase calcineurin (Liu et al., 1991). This binding inhibits the dephosphorylating activity of calcineurin (Fruman et al., 1992), which plays a role in the activation of the IL-2 promoter (Liu et al., 1992).

	FK 506	Cyclosporine
Suppression of MLR (IC_{50})	0.32 nM	27 nM
Suppression of PFC response(ED_{50})	4.4 mg/kg	39 mg/kg
Suppression of DTH response (ED_{50})	14 mg/kg	40 mg/kg
Inhibition on GVHR at 10 mg/kg	26.1%	8.0%

MLR: Mixd lymphocyte reaction; PFC: Plaque forming cells; DTH: Delayed type hypersensitivity; GVHR: Graft-versus-host reaction.

Table 1.1 Comparison of immunosuppressive effects between FK 506 and Cyclosporine

The target for calcineurin has been proposed by Scrieber and Crabtree (1992) to be the cytosolic subunit of the nuclear factor of activated T cells (NF-AT). The dephosphorylation of the T cell specific cytosolic NF-AT allows the translocation of this transcription factor subunit to the nucleus, which then combines with the other predominantly nuclear subunit to form a functional transcription factor. This proposal was supported by McCaffrey and coworkers (1993) when they showed that the dephosphorylation of a DNA-binding protein called NF-AT_p in cell lysates is dependent on the release of calcium and activation of calcineurin. NF-AT_p specifically binds to the NF-AT sequence from the murine IL-2 promoter (McCaffrey et al., 1993; O'Keefe et al., 1992). Thus, if the transcription of IL-2 is inhibited, T cells can no longer proliferate, and the immunologic cascade is halted (figure 1.1).

1.a.3.8.3 Clinical trials

In the liver transplant program at the University of California - San Francisco, where clinical trials of FK 506 are being conducted, patients are administered the first dose of FK 506 six hours after reperfusion of the liver, and at a time when adequate urine output (>20 ml/h) is established. FK 506 is given at a dose of 0.05 mg/kg intravenously over 12 hours, repeated every 12 hours. On day 4, or on the first day that a patient is able to take oral medications, intravenous infusion of FK 506 is discontinued, and an oral dose of 0.15 mg/kg is given every 12 hours. FK 506 levels are measured at 24, 48 and 72 hours after the initiation of intravenous therapy, and three times a week thereafter.

Despite the reports of minimal toxicity associated with FK 506 in the early stage of clinical trials (table 1.2) (Thomson, 1991), patients from the University of California - San Francisco have experienced significant side effects as listed in table 1.3. The most severe deleterious effects among these include renal and CNS (central nervous system) toxicity. Nephrotoxicity that occurs upon FK 506 administration has recently been proposed to be caused by a decreased renal cortical blood flow (Ueda et al., 1993).

1.a.3.8.4 Pharmacokinetics

The determination of plasma protein binding for this drug has been a difficult task. Due to the binding of FK 506 to glass and plastic dialysis and ultrafiltration supports, the determination of plasma protein binding must be done by ultracentrifugation (Habucky et al., 1992; Piekoszewski and Jusko, 1993). Piekoszewski and Jusko have shown that FK 506 binds moderately (73% bound) to plasma proteins in human plasma over the drug concentration range of 0.1-100 ng/ml. This result is in agreement with the 78 ± 4 % found by Habucky et al (1992). Piekoszewski and Jusko (1993) also showed that FK 506 binds to α_1-acid glycoprotein at 39.1 ± 3.8%, and to albumin in a concentration-dependent manner. The binding to albumin decreases from 57.0 ± 1.7% to 44.4 ± 5.9% over a drug concentration of 0.1 to 100 ng/ml. Moreover, Habucky and coworkers (1992) demonstrated that the extent of protein binding differs between species. In rats and dogs, the unbound fraction (f_u) is 0.57 ± 0.03 and 0.23 ± 0.04, respectively.

FK 506: The Pittsburgh Experience

Primary liver transplantation

125 Patients (110 adults, 15 children)
92% Patient survival (87% graft) at 6-12 months post-transplant
Less frequent rejection episodes than cyclosporine
Reduced steroid requirement
Lower incidence of infection

Modified from Thompson, A.W. (1991). Immunol. Letters 29, 105-112.

Table 1.2 Summary of success rates of liver transplantation at the University of Pittsburgh

Toxicity Related To FK 506

Central Nervous System:

- Headache
- Tingling
- Tremors
- Insomnia

Gastrointestinal:

- Anorexia
- Diarrhea
- Nausea
- Abdominal Pain

Cardiovascular:

- Flushing
- Hypertension

Renal:

- Increased blood urea nitrogen
- Increased serum creatinine
- Increased potassium level

Table 1.3 Side effects of FK 506

FK 506 has been shown to distribute preferentially into blood cells, and redistribution of drug occurs within 20 minutes at 20° and 37°C (Beysens et al., 1991; Piekoszewski et al., 1993). The blood to plasma ratio (C_B/C_P) in humans, which ranges from 10 - 222 as reported by several authors (Beysens et al., 1991; Habucky et al., 1992; Piekoszewski and Jusko, 1993; Uchida, 1991), is dependent not only upon the temperature at which the plasma separation is performed, but also the hematocrit of the sample, and the concentration of the drug. Plasma separation at 4°C gives a smaller C_B/C_P than at 20° or 37°C. A higher hematocrit will also result in a higher C_B/C_P. Machida and coworkers (1991) indicated that the affinity of FK 506 for blood cells is at least 3 times higher at 37°C than at 20°C. Species differences in C_B/C_P were also noted. Habucky and coworkers (1992) reported C_B/C_P of 9.8 ± 1.4, 1.7 ± 0.3 and 8.9 ± 0.5 in humans, rats and dogs, respectively. The contribution of differing hematocrits among the various species to this variability in C_B/C_P was not addressed. Increasing the FK 506 concentration yields a lower C_B/C_P. FK 506 is appreciably bound to blood cells with a nonlinear C_B/C_P of 20-50 at low plasma concentrations (0-2 ng/ml) and a ratio of 11 at plasma concentrations above 5 ng/ml (Jusko and D'Ambrosio, 1991). In addition, it was demonstrated by Uchida (1991) that the drug concentration measured in plasma has a significantly higher coefficient of variation than the measurement in whole blood.

Several factors mentioned above, namely the temperature dependency of the distribution of FK 506, and the fluctuation of the drug concentration in plasma suggest that FK 506 may be more reliably monitored in whole blood in *in vivo* studies. Moreover, whole blood analysis is prudent considering

the higher accuracy that can be obtained when measuring the markedly higher blood concentrations versus the corresponding lower plasma concentrations.

Initial pharmacokinetic studies in rats by Iwasaki and coworkers (1991) using ^{14}C-labeled FK 506 have shown that the parent drug is extensively metabolized. Less than 5% of unchanged drug is recovered in the urine and feces. The great majority of the radioactivity is recovered as metabolites in the feces (>95%) resulting from biliary excretion. Nevertheless, no major metabolite but many minor metabolites were observed in the bile and feces (Iwasaki et al., 1991). Subsequent studies of portal and femoral vein infusions at 5 mg/kg in Wistar rats have been reported to yield plasma clearances of 13.7 ± 1.4 and 10.8 ±1.5 ml/min, respectively, and a hepatic extraction ratio of 0.28 (Takada et al., 1992).

In human transplant patients, Venkataramanan and coworkers (1990; 1991a; 1991b) reported plasma clearance ranges from 7 to 103 ml/min/kg, with a mean of 30 ml/min/kg; and oral bioavailability ranges from 5 to 67%, with a mean of 27%. The half-life was found to range from 3.5-40.5 hours, and the volume of distribution ranged from 5 to 65 L/kg. Jain et al. (1990) subsequently reported pharmacokinetic data from small bowel transplant patients. For a group of 5 patients, plasma clearance ranged from 12.7 to 53 ml/min/kg, oral bioavailability from 16 to 93%, with a mean of 43%, and the time to reach peak plasma concentration after an oral dose was 0.5 to 5 hours, with a mean of 2.8 hours (Jain et al., 1992).

Communications from 2 research groups have reported pharmacokinetic data from pre-kidney transplant patients. Aweeka and coworkers (1992) reported a mean blood clearance (CL_B) of 0.047 ± 0.014 L/h/kg, a mean steady state blood volume of distribution (V_{ss}) of 0.97 ± 0.18 L/kg, mean residence time (MRT) of 22 ± 7 h, and bioavailability (F) of 0.12 ± 0.06. Data from Gruber and coworkers (1993) seems to be consistent with that from Aweeka et al. (1992) . Comparison of pharmacokinetic data from pre- and post-kidney transplant recipients revealed a 2-fold higher CL_B and V_{ss} in the post-transplant patients than in the pre-transplant patients (Aweeka et al., 1992). Low bioavailabilty following oral administration has been proposed to be due primarily to limited gastrointestinal absorption and some hepatic extraction (Piekoszewski et al., 1993).

Despite the several reports of various pharmacokinetic parameters in humans, the accuracy of such parameters remains questionable since the studies summarized above utilized a non-specific analytical method, ELISA. As such, the parameters reported may give a distorted picture of the disposition of the drug and its metabolites combined. Depending on the contribution of the metabolites to the measured concentrations of the parent drug, the correlation of the currently found pharmacokinetic parameters to the observed pharmacodynamic effects may not be correct.

1.b Objectives

FK 506 is an immunosuppressant in phase 3 clinical trials in the United States. Phase 1 and 2 clinical trials of this drug were carried out primarily in the transplant unit of the University of Pittsburgh. Prior to the beginning of

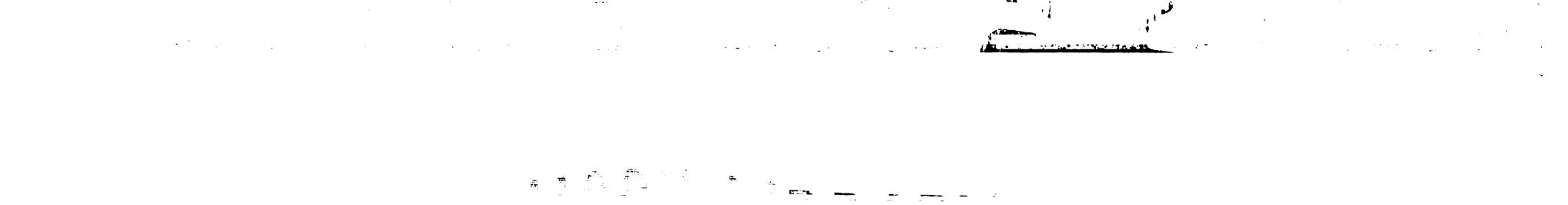
the phase 3 clinical trials in 1989, FK 506 gained a great amount of attention from the medical community because of its high potency and apparent low level of toxicity as reported in phase 1 and 2 clinical studies. While much work has been devoted to elucidate the mechanism of action of FK 506, there is, however, little metabolism data available in the literature.

Because a basic understanding of the metabolism of FK 506 is important for the understanding of the pharmacology of this drug, it is the objective of this project to find appropriate methodologies to study the metabolism of this compound, and to increase the understanding of the metabolism and the pharmacokinetics of this drug.

The overall goal of this project is to gain a better understanding of the metabolism of FK 506 in humans. However, throughout the project, rats have been used as the research subjects instead of humans due to ethical reasons, convenience, and costs. With care, results obtained from rats can often be extrapolated to humans due to existing knowledge on the similarities and the differences in the metabolic systems between rats and humans.

The specific objectives of this project are:

1. To develop an appropriate analytical methodology for the study of FK 506 metabolism.
2. To determine the structure of the major FK 506 metabolite.
3. To investigate if cytochrome P450 enzymes are involved in FK 506 metabolism. If so, identify the particular isozymes involved and determine the enzyme kinetics.



4. To investigate the possibility of sequential metabolism of the major metabolite of FK 506, the enzyme involved, and the kinetics of the reaction.

Chapter 2

Analytical Method Development

2.a Introduction

The development of a specific and sensitive assay method for FK 506 and its metabolites has been a difficult task. The low plasma concentrations observed after a clinical trial dose of 0.15 mg/kg (Jain et al., 1990; Venkataramanan et al., 1990), the lack of identification and supply of the metabolites, and the lack of any signicant spectrophotometrically useful chromophores on the drug (Tanaka et al., 1987; Venkataramanan et al., 1987) are major problems encountered in measuring FK 506 and its metabolites. Nevertheless, several analytical methods for the measurement of FK 506, and in some cases, the measurement of FK 506 metabolites, have been reported. The enzyme linked immunosorbent assay (ELISA) (Tamura et al., 1987) and the microparticle enzyme immunoassay (IM_X-MEIA) (Grenier et al., 1991), have high sensitivity but low specificity. The principle of both bioassays, ELISA and MEIA, is the recognition of the compounds of interest by an antibody. Although the monoclonal antibody that is being used is selective for a specific functional domain on FK 506, the metabolites of FK 506 may also contain the epitopes that the antibody recognizes. As such, these two bioassays detect the level of FK 506 and some of its metabolites combined. Thus far, the enzyme linked immunosorbent assay has been reported to cross react with at least 3 FK 506 metabolites (Iwasaki et al., 1993). Problems with a combined measurement of parent drug and metabolites are three fold. First, a combined measurement fails to provide specific quantification of the drug and other related compounds of interest. Second, unless the activity of the drug and its metabolites are comparable, a combined measurement would lead to misinterpretation of the correlation between measured drug concentration

and pharmacological effect. Third, the inability to follow the time course of each compound of interest will not allow the correlation of metabolite levels to possible measures of activity or manifestations of toxicity.

A radioreceptor assay (Murthy et al., 1992) which exploits the binding of FK 506 to a cytosolic protein (FK-BP) measures the competitive binding of ^{3}[H]-dihydro- FK 506 to FK-BP. This method is comparable to those for ELISA and MEIA since a similar assay sensitivity is attained, and this method also gives a combined measurement of FK 506 and its metabolites.

High performance liquid chromatographic separation in conjunction with chemiluminescence detection (Takada et al., 1990), mass spectrometric detection (Lhoest et al., 1992; Lhoest et al., 1991) or UV detection (Christians et al., 1992) have been developed. Although the HPLC separation used with the chemiluminescence detection yields high sensitivity for FK 506, it does not measure FK 506 metabolites. The method also requires complicated column switching and derivatization of FK 506. The mass spectrometric and the UV detection methods allow specific measurements of both the parent drug and the metabolites, but require gradient solvent delivery.

This chapter reports a sample preparation method for rat hepatocytes and liver microsomes, together with HPLC separation using an isocratic solvent delivery system and UV detection that allows simultaneous monitoring of FK 506 and two time-dependent metabolites. This method fills the need for a simple way to measure both the parent compound, FK 506, and its primary

metabolite in rat liver tissues using simple standard laboratory analytical equipment.

2.b Materials and methods

FK 506 was kindly supplied by Fujisawa Pharmaceutical Company (Deerfield, IL). All solvents (HPLC grade) were obtained from Fisher Chemical (Fair Lawn, NJ). All chemicals for hepatocytes isolation and metabolic incubations were obtained from Sigma Chemical Company (St. Louis, MO). FK 506 metabolite (M1) was prepared by incubation of FK 506 (10-20 μM) with rat liver microsomes, as will be described in detail in section *3.b.1* in chapter 3.

2.b.1 Instruments

For sample separation, a Beckman 110A solvent delivery module connected to a Beckman 420 controller (Beckman Instruments, Palo Alto, CA) was used. The stationary phase was a Beckman Ultrasphere octyl-decyl silane (ODS) HPLC column (particle diameter = 5 μm, column length = 250 mm, column diameter = 4.6 mm). The column was warmed to 60°C in a Waters temperature control module (Millipore Corporation, Milford, MA). The mobile phase was a degassed solvent mixture of acetonitrile/methanol/diluted o-phosphoric acid pH 3, 49/3/48 (v/v/v). The mobile phase was delivered by the solvent delivery system at an initial flow rate of 1 ml/min for the first 20 minutes, and then stepped up to 1.5 ml/min within 0.5 min and maintained for the subsequent 30 minutes. Alternatively, the mobile phase could be delivered at a constant flow rate of 1 ml/min for

60 min. Samples were injected using a Waters automatic sample injector (Millipore Corporation, Milford, MA) or a Rheodyne 7120 manual injector (Rheodyne, Cotati, CA). The compounds of interest were detected by a Beckman 160 absorbance detector at a wavelength of 214 nm. The UV light source was a zinc lamp from Beckman Instruments.

2.b.2 Sample preparation

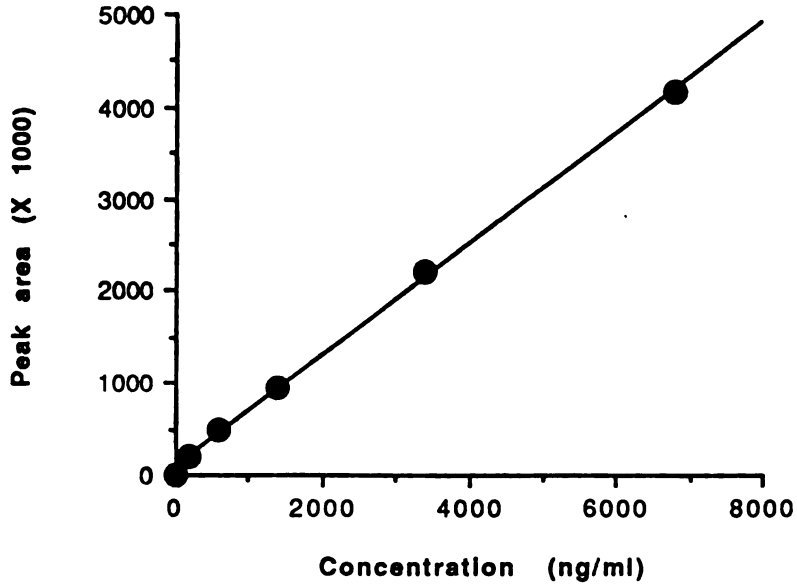
To all samples, 3 ml of protein precipitating reagent (20% v/v acetonitrile, 30 % v/v methanol and 5% w/v $ZnSO_4$ in water) was added. The mixture was vortexed and centrifuged for 10 minutes at 3500 rpm. The supernatant was passed through a BondElut C18 sample preparation column (Varian Sample Preparation Products, Harbor City, CA) that was pre-equilibrated with 2 ml of 90.5% ethyl alcohol (reagent alcohol) and 2 ml of nanopure water. This sample preparation column was then washed with 1 ml of 40% methanol in water, followed by 1 ml of hexane. Compounds of interest were eluted from the sample preparation column with 1 ml of methylene chloride. The methylene chloride was evaporated and the sample was reconstituted with the mobile phase prior to injection onto the HPLC column. A modified sample preparation procedure was also developed for samples that have a low protein concentration. All steps were similar up to the step where the supernatant was passed through a BondElut C18 sample preparation column. This sample preparation column was then washed with 1 ml of hexane, and the compound of interest was eluted from the sample preparation column with 1 ml of methanol. The methanol was evaporated and the sample was reconstituted with the mobile phase prior to injection onto the HPLC column.

2b.3 Standard curves

Standard solutions of FK 506 or FK 506 metabolite were prepared as methanolic solutions. A small quantity (100 to 1000 μg) of FK 506 powder is weighed on a small teflon disc (E & K Scientific Products, Inc., Saratoga, CA). The disc with the powder are transferred into a 7-ml borosillicate scintillation vial. Methanol is added into the vial, and the vial is capped and vortexed. The teflon disc is then removed from the solution using a clean forcep. Various amounts of the stock solution, containing 165 ng to 29 μg of the drug, were added to 250 μl polypropylene microcentrifuge tubes (E & K Scientific Products, Inc., Saratoga, CA) which are used as sample injection vials. Each aliquot was diluted to a total volume of 175 μl. A volume of 150 μl was injected onto the HPLC column for analysis. An average response factor was obtained as the ratio of the integrated peak area to the amount of standard injected. A representative standard curve of FK 506 is shown in figure 2.1.

2.c Results

An HPLC assay, with UV detection, was developed to simultaneously monitor FK 506 and its two time-dependent metabolites in rat hepatocytes and liver microsomes. Because FK 506 consists of functional groups such as ester, ketones and unsaturated aliphatic carbons which absorb in the range of 190 to 205 nm, a low wavelength was chosen for the detection of the parent compound. Light absorption at wavelengths specific to the functional groups of a compound occurs when unbonded and pi electrons are promoted from a ground state to a higher energy state. To minimize interference from



Samples were monitored at 214 nm.

Figure 2.1 Standard curve for FK 506

the mobile phase, a wavelength of 214 nm was selected. Each component of the solvent mixture used as the mobile phase was chosen for its low absorptivity at a wavelength of 214 nm, and its solvent property with respect to the compounds of interest. While acetonitrile was chosen as the primary solvent for its moderate to strong eluting power with respect to FK 506 (figure 2.2), methanol was chosen as a minor solvent for its very weak eluting power with respect to FK 506, and its ability to separate the metabolites M1 and M2 slightly farther apart. Tetrahydrofuran, which has a good resolving capacity for most hydrophobic compounds, was not selected for this assay because it tends to create a noisy baseline under the existing condition. Noisy baseline usually results in difficult detection of small amount of materials, and was therefore avoided in this method.

Previous studies from Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan) have shown that the degradation rate constant is the lowest in the pH range of 3 to 4 at the three different temperatures tested (figure 2.3). Since a pKa of phosphoric acid is 2.12, a pH 3 phosphate buffer became an obvious and convenient choice. Hence, the acidity of the aqueous phase of the solvent system was adjusted to pH 3 with o-phosphoric acid.

Joshua (1991) reported that at low column temperature, cis- and trans-conformation of the α-ketoamide moiety of FK 506 can be separated by reverse-phase HPLC. As the column temperature is increased gradually, the retention times decrease and the chromatographic peaks coalesce to a sharp symmetrical peak at 50°C. Under the current HPLC condition, a sharp peak was obtained at 60°C.

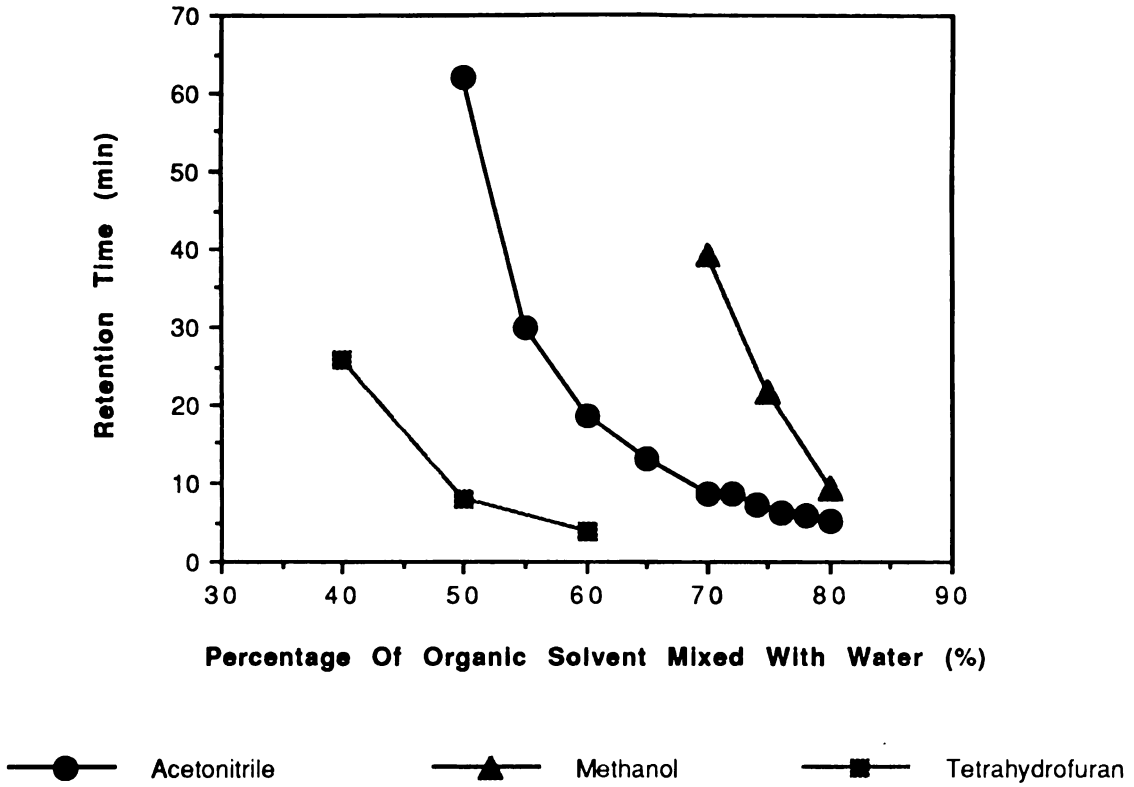
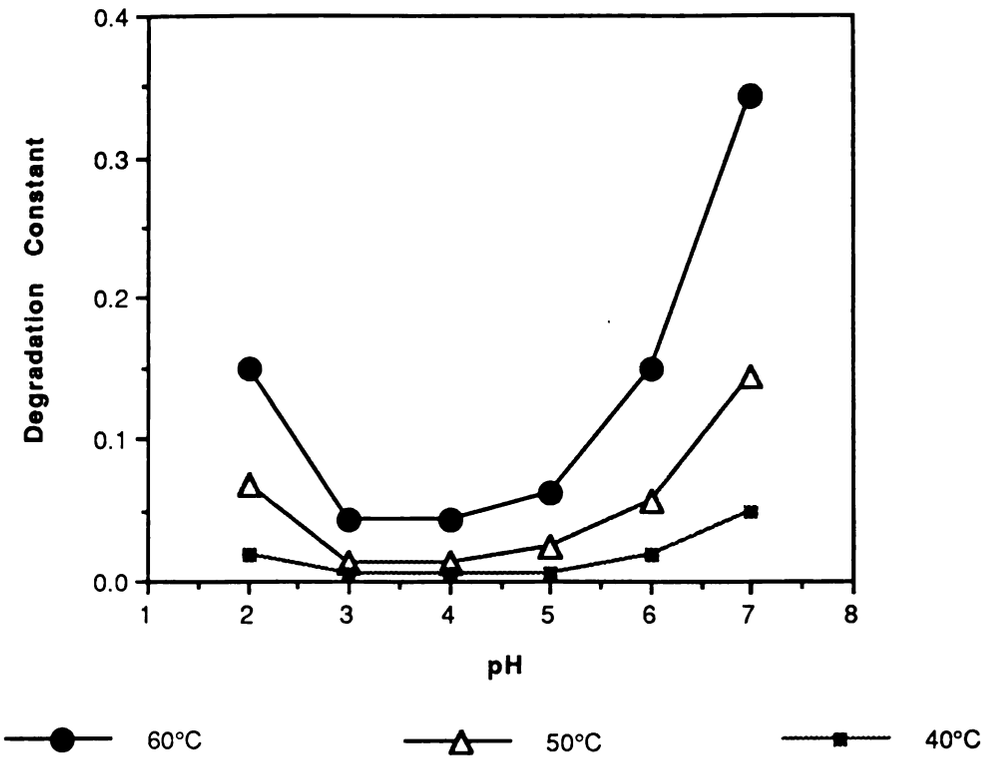


Figure 2.2 Elution power of acetonitrile, methanol and tetrahydrofuran for FK 506

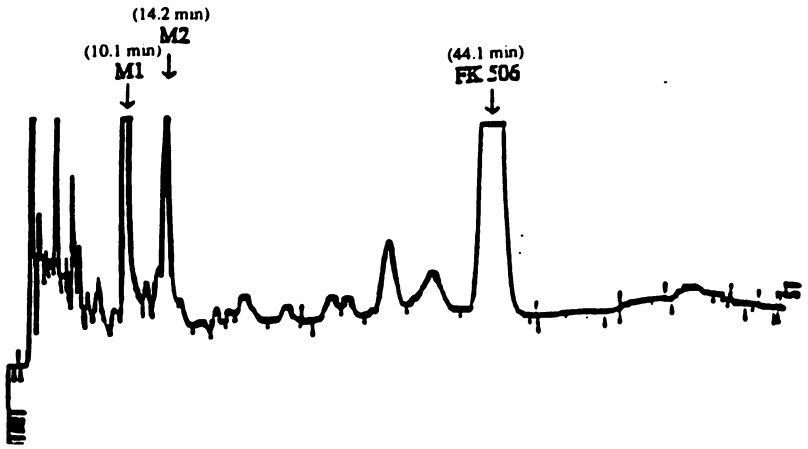


Personal communication from Dr. Masakazu Kobayashi of Fujisawa Pharmaceutical Co. Ltd.

Figure 2.3 Stability of FK 506 as a function of pH and temperature

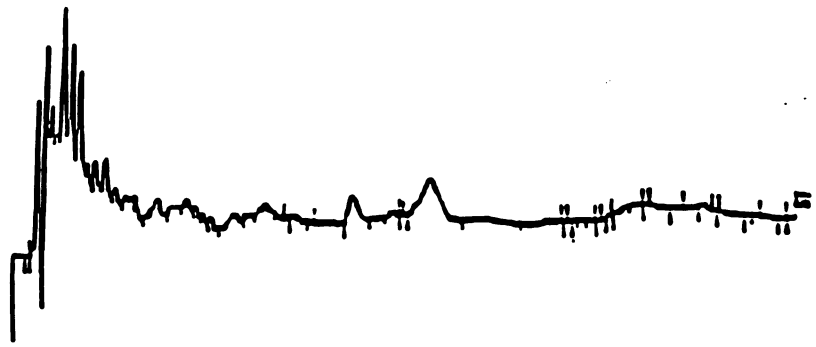
A representative chromatogram for a rat hepatocyte sample of FK 506 following a 30 min incubation, and a corresponding control sample without FK 506 are shown in figures 2.4a and 2.4b, respectively. The retention times for the two observed metabolites, M1 and M2, and the parent FK 506 are 10.1, 14.2 and 44.1 min, respectively. The retention time for the parent FK 506 is 55 min when the alternate solvent delivery schedule (a constant flow at 1 ml/min) is employed. Peak area of parent FK 506 obtained using a solvent flow of 1 ml/min is 1.5 fold of that obtained with a solvent flow of 1.5 ml/min. When comparing peak areas obtained from the two solvent delivery schedules, an adjustment is necessary.

The concentrations of FK 506 and the metabolites were quantified by the construction of external standard curves using FK 506. For assay purposes, it is assumed that the extinction coefficient is the same for FK 506 and the two metabolites. The detection limit is 0.017 nmol. The inter-day and intra-day variabilities of FK 506 are 7.3 and 7.9 %, respectively. Inter-day and intra-day coefficients of variation were estimated using FK 506 spiked microsomal samples. To estimate the absolute recovery, three different concentrations of FK 506 (1, 10, and 80 μM) and FK 506 major metabolite (M1) (0.15, 0.5, and 1.25 μM) were added to rat liver microsomal samples and extracted as described earlier. The average peak area obtained from the extracted samples was compared to the average peak area obtained from the corresponding unextracted samples. The results are summarized in table 2.1 and table 2.2 for the parent drug and metabolite, respectively.



HPLC condition: C_2H_3N/H_3PO_4, pH3/CH_4O (49/48/3), 60°C, 1-20 min @1 ml/min; 20-50 min @ 1.5 ml/min.

Figure 2.4a A representative chromatogram for a rat hepatocyte sample of FK 506 following a 30 min incubation



HPLC condition: C_2H_3N/H_3PO_4, pH3/CH_4O (49/48/3), 60°C, 1-20 min @1 ml/min; 20-50 min @ 1.5 ml/min.

Figure 2.4b A representative chromatogram for a rat hepatocyte sample without FK 506

Table 2.1 Percentages of FK 506 recovered following extraction from hepatocytes and liver microsomal samples at three different concentrations. (N=5)

	% ± S.D. Recovered in:	
Concentration (μM)	Hepatocytes	Microsomes
1	78 ± 7	76 ± 11
10	65 ± 7	65 ± 12
80	82 ± 1	85 ± 4

Table 2.2 Percentages of FK 506 metabolite recovered following extraction from hepatocytes and liver microsomal samples at three different concentrations. (N=5)

Concentration (μM)	% ± S.D. Recovered
0.15	68 ± 5
0.45	69 ± 5
1.25	70 ± 6

2.d Discussion

An HPLC assay that can simultaneously measure FK 506 and two time-dependent metabolites has been described in this chapter. This assay requires an isocratic pump and a UV detector which are standard equipment in most laboratories. While one of the previously published assays may have a slightly higher sensitivity than the present method, the simple equipment requirements, the reasonable recoveries of 75 and 69% for FK 506 and the major FK 506 metabolite, respectively, and <10% intra-day and inter-day variabilities make this assay well suited for experiments using rat liver samples. This assay will be utilized in all of the studies described in the following chapters.

Chapter 3

Structural Identification of FK 506 Metabolite

3.a Introduction

Structural identification of a metabolite is an important step in a drug metabolism study. Not only does the elucidation of the structure of the metabolite in itself provide information about the metabolic scheme of the drug of interest, knowledge of the structure of the metabolite also provides clues to the enzymes involved in the metabolism of the parent compund.

In some cases where a metabolite is thought to be biologically active or toxic, identification of the metabolite structure has been a major factor in explaining the observed activity or toxicity. For example, it has been proposed that the severe nephrotoxicity and neurotoxicity of another immunosuppressant, cyclosporine, may be related to its metabolites (Davenport et al., 1986; Kohlhaw et al., 1989; Kunzendorf et al., 1988; Leunissen et al., 1986; Rosano et al., 1988). Sewing and coworkers (1990) have reported that the di-hydroxylated and cyclized cyclosporine metabolite, AM1C9, may be one of the metabolites associated with the noted toxicities. Patients who have a high level of the cyclosporine metabolite AM1C9 seem to have a higher incidence of organ rejection and cholestasis than those who do not. Thus, the identification of metabolites is an important step in the process of understanding the metabolism and the biological effects of the drug of interest.

Numerous analytical methodologies are available for the purpose of metabolite identification. These methods vary in their sensitivities and the kinds of information each can provide. Often times, more than one method is employed to gather structural information about the compound of interest.

The choice of the methods depends on the amount of compound available, the chemistry of the compound, and the kind of information being sought.

The structure of the observed metabolite of FK 506 was investigated by mass spectrometry and an indirect method involving the use of a 2-step enzyme immunoassay which is currently employed to clinically monitor the concentrations of FK 506 in organ transplant patients. In addition, chromatographic properties of authentic metabolites were compared with that of the observed metabolite.

3.b Materials and methods

FK 506, horseradish peroxidase-FK 506 conjugate, and FK 506 monoclonal antibody were kindly supplied by Fujisawa Pharmaceutical Company (Deerfield, IL). Antimouse IgG antibody was obtained from Atlantic Antibodies (Stillwater, MN). Bovine serum albumin (>98% (GE)) and Tween-20 were obtained from Sigma Chemical Company (St. Louis, MO). β-nicotinamide-adenine dinucleotide, reduced tetrasodium salt, 98% (NADPH) was obtained from Sigma Chemical Company or Boehringer Mannheim (Indianapolis, IN). The 96-well microtiter plate used was a Nunc immuno plate I (Applied Scientific, San Francisco, CA). A protein assay kit with albumin protein standard was obtained from Biorad (Richmond, CA). All solvents were HPLC grade and other reagents were of reagent grade. Except for phosphate buffered saline, Tween-20-phosphate buffered saline, and phosphate-citrate buffer which were prepared in advance and kept at 4°C for at least 1 week, all solutions used in the 2-step enzyme immunoassay were prepared daily. Phosphate-citrate buffer was prepared by adjusting the

pH of a 0.1 M dibasic sodium phosphate (Na_2HPO_4) aqueous solution with a 0.1M citric acid aqueous solution to pH 5.4.

FK 506 and three authentic metabolites of FK 506 (13-desmethyl FK 506, 15-desmethyl FK 506, and 31-desmethyl FK 506) were kindly supplied by Fujisawa Pharmaceutical Company (Deerfield, IL., and Osaka, Japan, respectively).

3.b.1 Metabolite isolation

FK 506 metabolites were produced by a 30 min incubation of FK 506 (10-20 μM) with rat liver microsomes (2 mg protein/ml) in 0.1 M potassium phosphate buffer at 37°C in the presence of a NADPH generating system which comprised of NADPH (1 mM), magnesium chloride (5 mM), and glucose 6-phosphate (10 mM), and glucose 6-phosphate dehydrogenase (2 U/ml). The metabolite of interest was isolated from the incubation mixture by HPLC. The HPLC system was composed of a Shimadzu LC600 liquid chromatograph (Shimadzu, Santa Clara, CA), a Shimadzu SPD-10A UV spectrophotometric detector, a Shimadzu CR601 chromatopac, and a Rheodyne manual injector (Rheodyne, Cotati, CA). The metabolite was eluted from an Econosil octyl decyl silane column (particle diameter = 10 μ, column length = 250 mm, and column diameter = 10 mm) at 3 ml/min with a solvent mixture of acetonitrile/0.01% *o*-phosphoric acid, pH 3, 55/45 (v/v). The metabolite was extracted from the collected solvent fraction into methylene chloride, which was evaporated to dryness under nitrogen. Subsequently, the metabolite sample was resuspended in methanol and injected onto a Beckman Ultrasphere octyl decyl silane column heated to

60°C in a Waters temperature control module (Millipore Corporation, Milford, MA). The metabolite was eluted at 1 ml/min with a solvent mixture of acetonitrile/methanol/0.01% o-phosphoric acid, pH 3, 49/3/48 (v/v/v). The metabolite fraction was extracted again as described, and was stored as a methanol solution at -40°C until use.

3.b.2 Mass spectrometric analysis

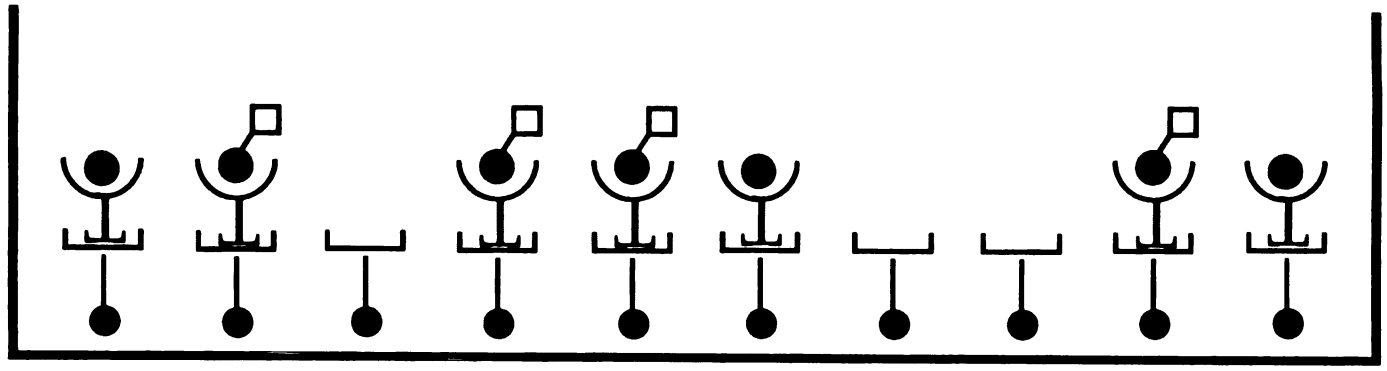
Aliquots of FK 506 or FK 506 metabolite were added to 1 µl of liquid matrix (glycerol) for molecular weight determination. Mass spectra were recorded in the negative LSIMS mode with a VG-70SE double-focusing mass spectrometer (Vacuum Generator Inc., Manchester, U.K.) equipped with a cesium ion source. The acceleration voltage was set at 6 keV, with the resolution set to 2000.

The detected $[M-H]^-$ ions were compared with the molecular weights of possible FK 506 derivatives predicted from oxidation of FK 506. Molecular weights of 790 and 776 are expected from demethylation and di-demethylation, respectively. Hydroxylation or epoxidation, will produce a molecular ion of 820. In the case that both oxidation and hydroxylation take place, a molecular ion of 806 will be observed.

3.b.3 Cross-reactivity of the 2-step enzyme immunoassay

FK 506 (0.001-10 ng/ml) and FK 506 metabolite (0.1-100 ng/ml) were used as substrates in a 2-step enzyme immunoassay currently used to monitor FK 506 levels in transplant patients (Tamura et al., 1987).

The 2-step enzyme immunoassay, as depicted in figure 3.1, was carried out as follows: First, antimouse IgG was adsorbed onto a 96-well microtiter plate by incubating the plate for 4 hours at room temperature with 200 µl per well of antimouse IgG in phosphate buffered saline (3 µg/ml). The antibody solution was then aspirated off, and the entire plate was washed 3 times with 300 µl of phosphate buffered saline in a Biorad model 1550 microplate washer (Richmond, CA). After the addition of 300 µl of 1% bovine serum albumin-phosphate buffered saline, the plate was incubated for 30 min at room temperature to cover all nonspecific binding sites. The solution was substituted with 40 µl of $2x10^5$ fold-diluted solution of horseradish peroxidase-FK 506 conjugate in 1% bovine serum albumin-Tween 20-phosphate buffered saline. After the plate was shaken for 1 min, FK 506 standard or a sample (140 µl) was added to this solution. The plate was incubated at 4°C overnight after 50 µl of monoclonal antibody solution (10 ng/ml) was added to each well. The next morning, the solution was aspirated off, and the plate was washed 3 times with 0.05% Tween 20-phosphate buffered saline, followed by 3 washes with phosphate buffered saline in the microplate washer. The enzyme substrate solution [100 mg of *O*-phenylenediamine hydrochloride and 30% hydrogen peroxide in 100 ml of phosphate-citrate buffer (pH5.4)] was added, and the plate was left in the dark (covered with foil) at room temperature for 30 to 60 min. At the end of the incubation period, 50 µl of 4N aqueous sulfuric acid was added to each well, and the plate was shaken for 30 seconds. Optical density at 492 nm was measured on a BT2000 Microkinetics Reader (Fisher-Biotech, Pittsburgh, PA) and plotted on semilogarithmic graph paper.



Anti-mouse IgG

Anti-FK 506 IgG

Free FK 506

HRP-FK 506

Figure 3.1 Illustration of a 2-step enzyme immunoassay

3.b.4 Comparison of chromatographic properties

The isolated metabolite was co-injected with three authentic metabolites (13-desmethyl FK 506, 15-desmethyl FK 506, and 31-desmethyl FK 506) on the HPLC analytical system as described in detail in section *2.b* in chapter 2. The retention times of all the injected compounds were compared.

3.b.5 Time profile of interconversion between the two metabolic peaks

The two observed metabolite peaks appeared to be interconverting peaks. In order to find out how fast the interconversion takes place, the following experiment was performed. A concentrated sample of metabolites was prepared by pooling many extracted FK 506 incubation samples immediately prior to reconstitution for HPLC injection. The sample was injected onto the Shimadzu HPLC system, and the fractions correlating to the metabolic peaks M1 and M2, as presented in section *2.c* (chapter 2), were collected into borosilicate tubes. As soon as the M1 fraction was collected, and before the M2 fraction was eluted, the M1 fraction was vortexed and aliquoted into several vials. One of these samples was immediately injected onto a separate HPLC system [the Beckman HPLC system as mentioned in section *2.b.1* (chapter 2)]. The M2 fraction collected was vortexed and aliquoted in the same manner as the M1 fraction. When the analysis of the first M1 aliquot was finished, a M2 fraction was injected for analysis. The remaining M1 and M2 fractions were injected alternately for the next 30 hours.

3.b.6 Elution profile of metabolite peaks under different pH conditions

Purified metabolite M1 reconstituted in methanol was analyzed under two different pH conditions. The first condition is identical to that described in section *2.b.1* (chapter 2). The aqueous portion of the mobile phase was prepared from diluted o-phosphoric acid adjusted to pH 3. The second condition differed from the first only in the pH of the mobile phase. The aqueous portion of the second mobile phase was prepared from a phosphate buffer adjusted to pH 7. The metabolite fraction(s) eluting from either condition was (were) collected and immediately reinjected under the alternate condition .

3.c. Results

Mass spectra of FK 506 and the major FK 506 metabolite were obtained by negative LSIMS . While the molecular ion ($[M-H]^-$) detected in the FK 506 sample has a mass of 803.5 (figure 3.2) using the ^{12}C mass scale, a molecular ion with a mass of 789.5 (figure 3.3) was detected in the major FK 506 metabolite sample. This difference of 14 mass unit suggests the metabolite to be a product from the loss of a CH_2 group from FK 506. The metabolite therefore, most probably has the structure of an O-desmethyl FK 506.

The cross reactivity of the 2-step enzyme immunoassay was tested against FK 506 and the FK 506 metabolite M1. Various concentrations of FK 506 and the major FK 506 metabolite were subjected to the

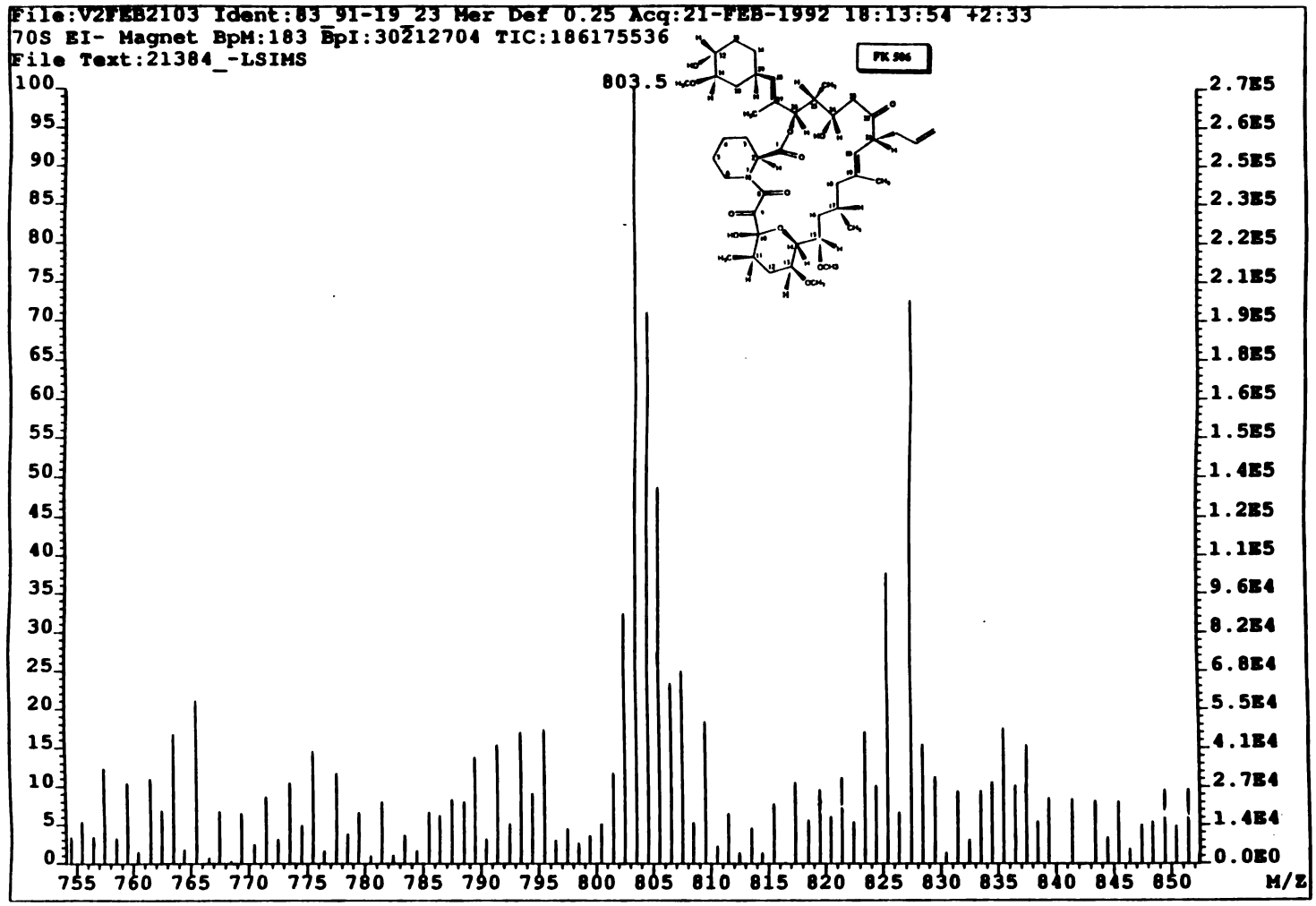


Figure 3.2 Mass spectrum of FK 506 under -LSIMS mode

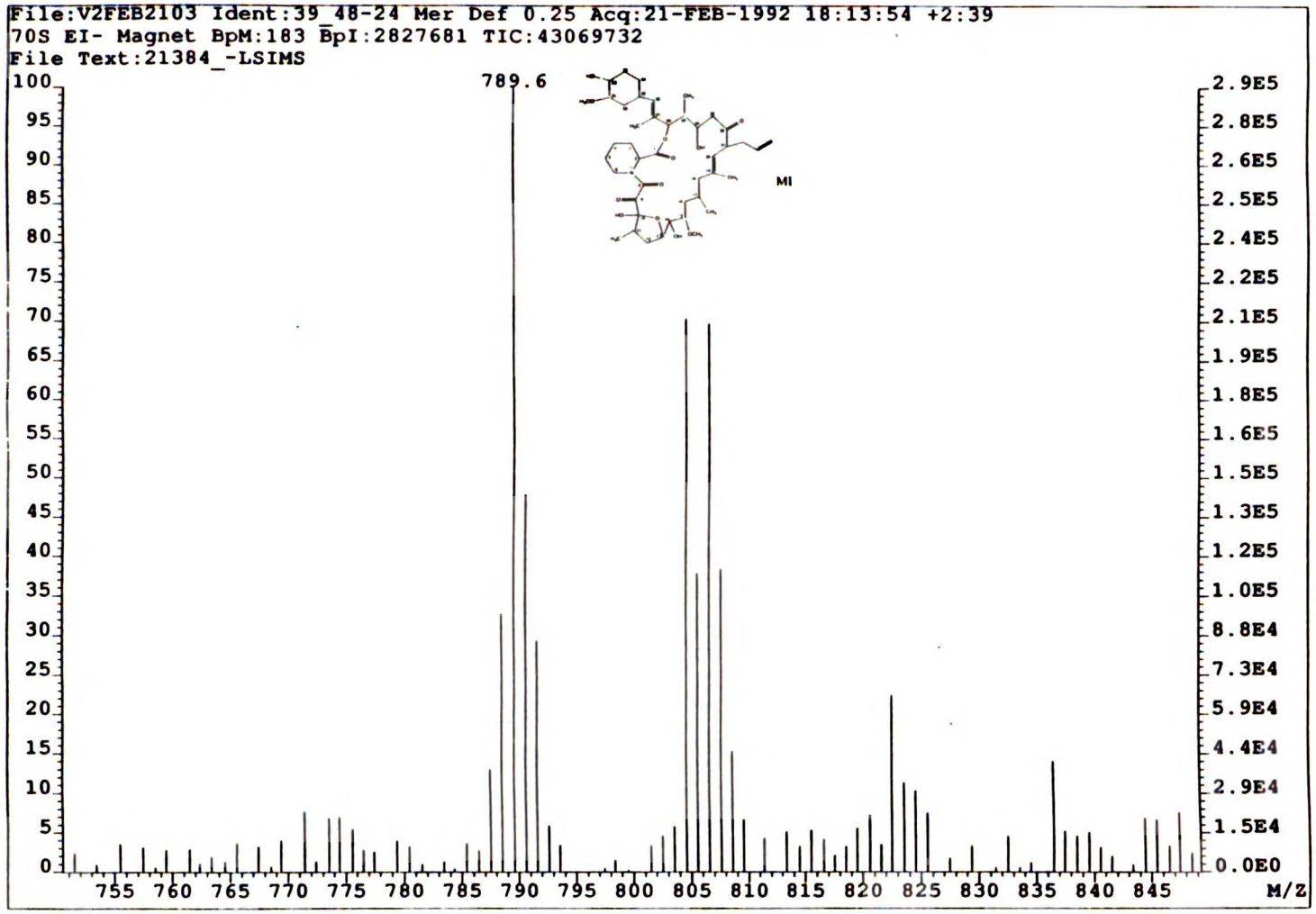


Figure 3.3 Mass spectrum of FK 506 metabolite under -LSIMS mode

immunoassay. The calibration curves of FK 506 and the FK 506 metabolites are shown in figure 3.4. At least two orders of magnitude higher concentrations of the FK 506 metabolite were needed to produce a degree of detection equivalent to that produced by parent FK 506 in the assay.

The chromatographic properties of the major metabolite were compared to those of the authentic metabolites. As shown in figure 3.5, two of the authentic metabolites, 15-desmethyl FK 506, 31-desmethyl FK 506 (figure 3.6) were eluted with retention times of 18 and 26.5 min. The third authentic metabolite, 13-desmethyl FK 506, appeared as two interconverting peaks with retention times of 9.5 and 13 min. The metabolite isolated by the method described in section *3.b.1* also eluted as two interconverting peaks with identical retention times as the authentic 13-desmethyl FK 506.

When isolated M1 was allowed to equilibrate in the mobile phase, it converted slowly with time to a mixture of M1 and M2. Likewise, isolated M2 converted to a mixture of M1 and M2 in a similar manner as M1. The time course for the interconversion is shown in figure 3.7. The total peak area of M1 and M2 at each time point remained constant throughout the study. The ratio of metabolite M1 to M2 is in an approximate range of 2 to 4 after twelve hours.

An attempt has been made to understand the cause of the interconversion between the metabolite peaks eluting with retention times of 9.5 and 13 min. Assaying the samples with an eluting solvent that contains an aqueous phase of pH 7 instead of pH 3 as described in section *2.c* (chapter 2), yields a single peak (figure 3.8). Reinjection of the fraction isolated from the single

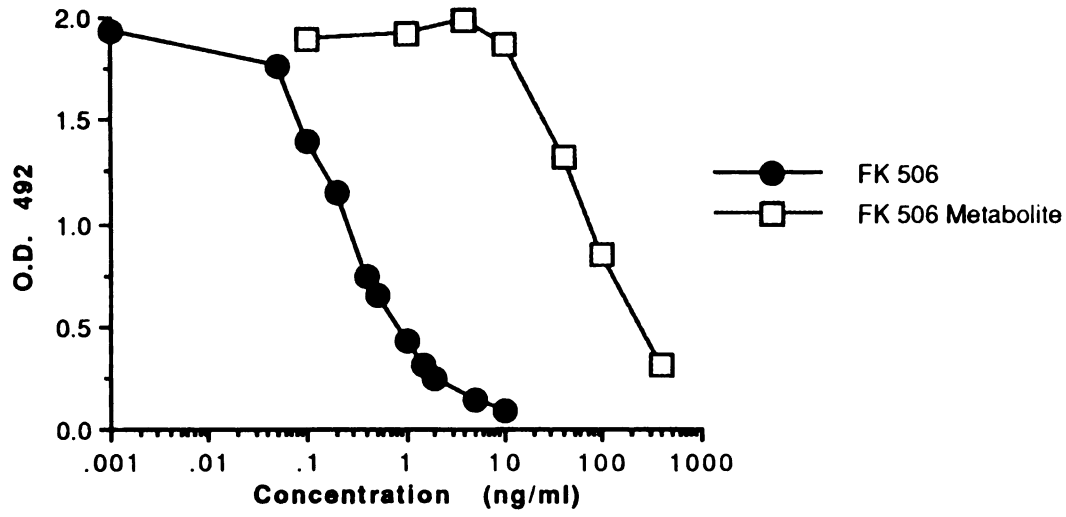


Figure 3.4 Cross reactivity of FK 506 and the isolated metabolite to the FK 506 monoclonal antibodies

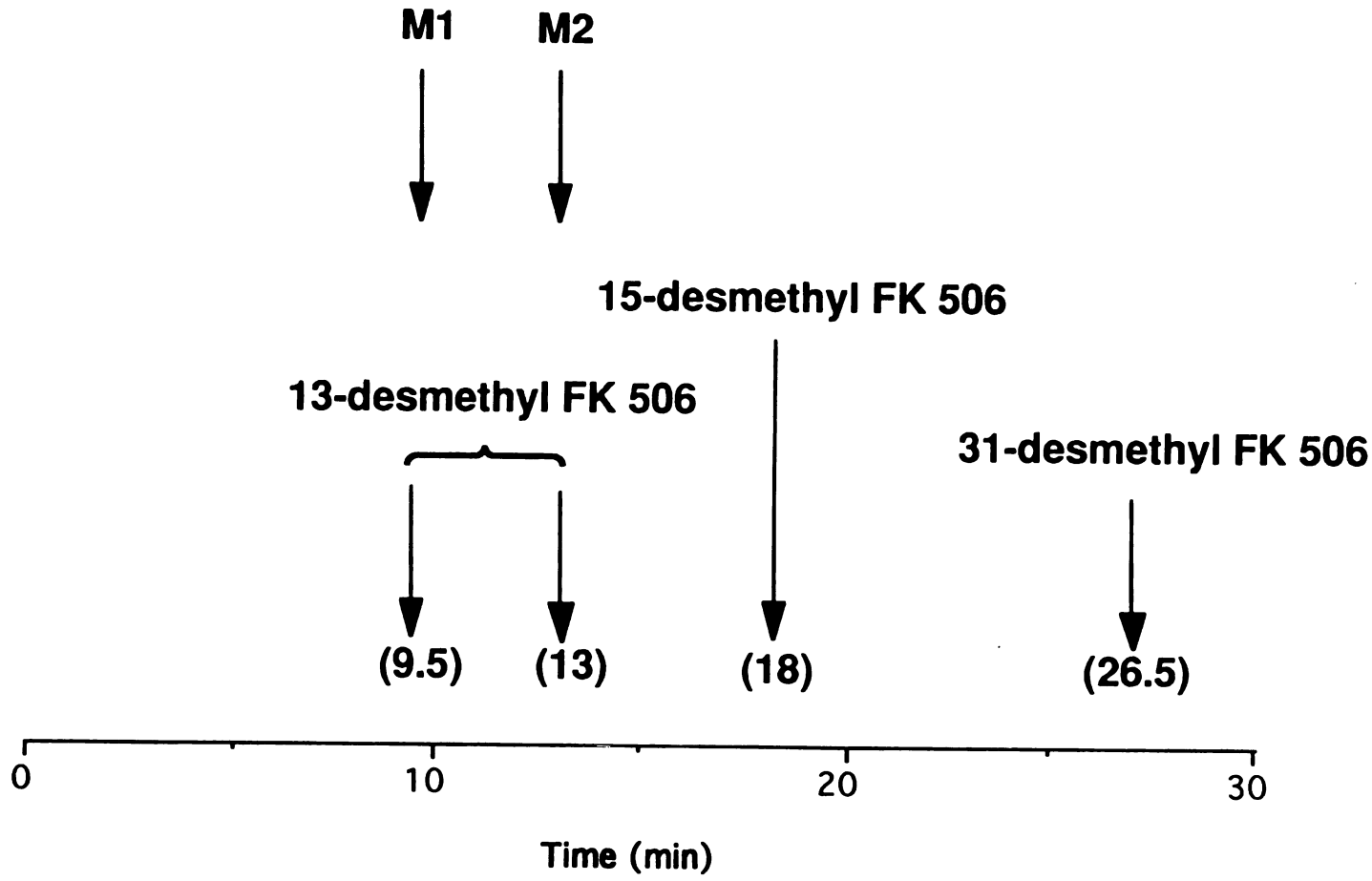
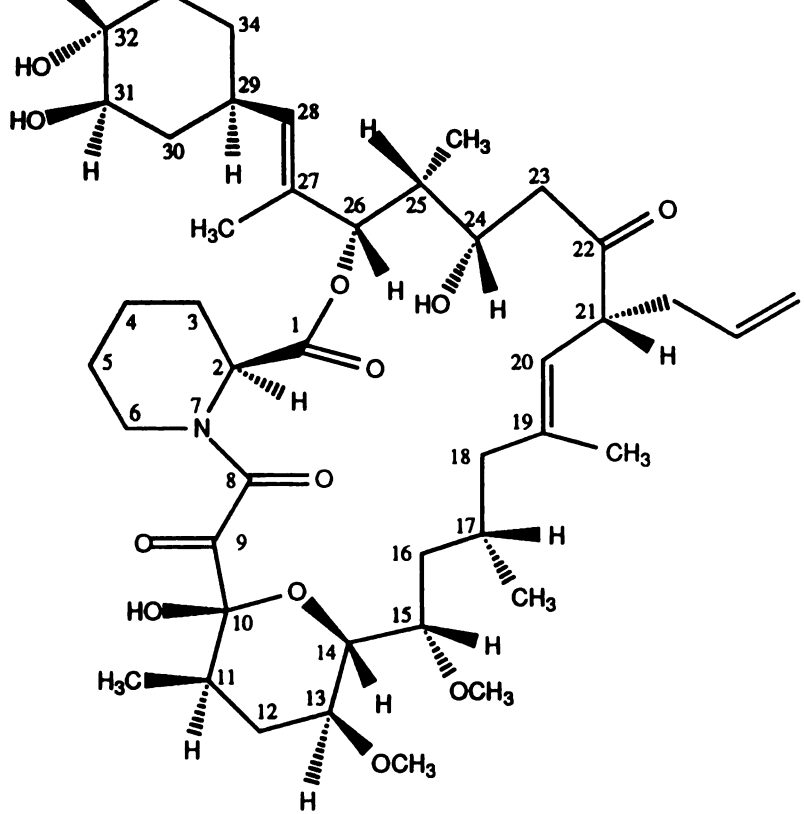
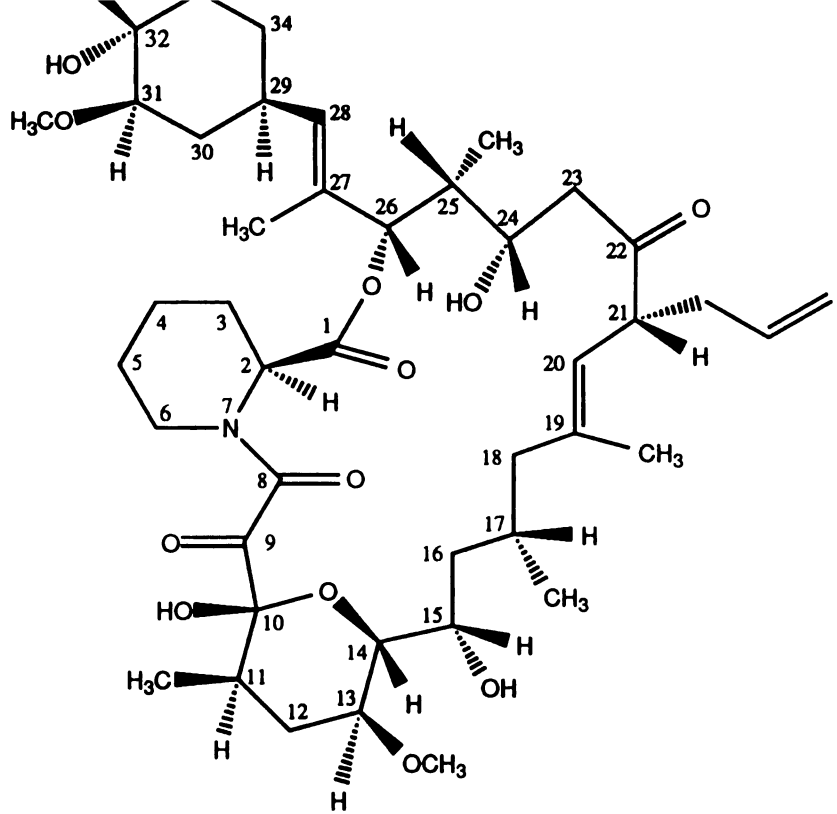


Figure 3.5 Retention times of 3 authentic FK 506 metabolites

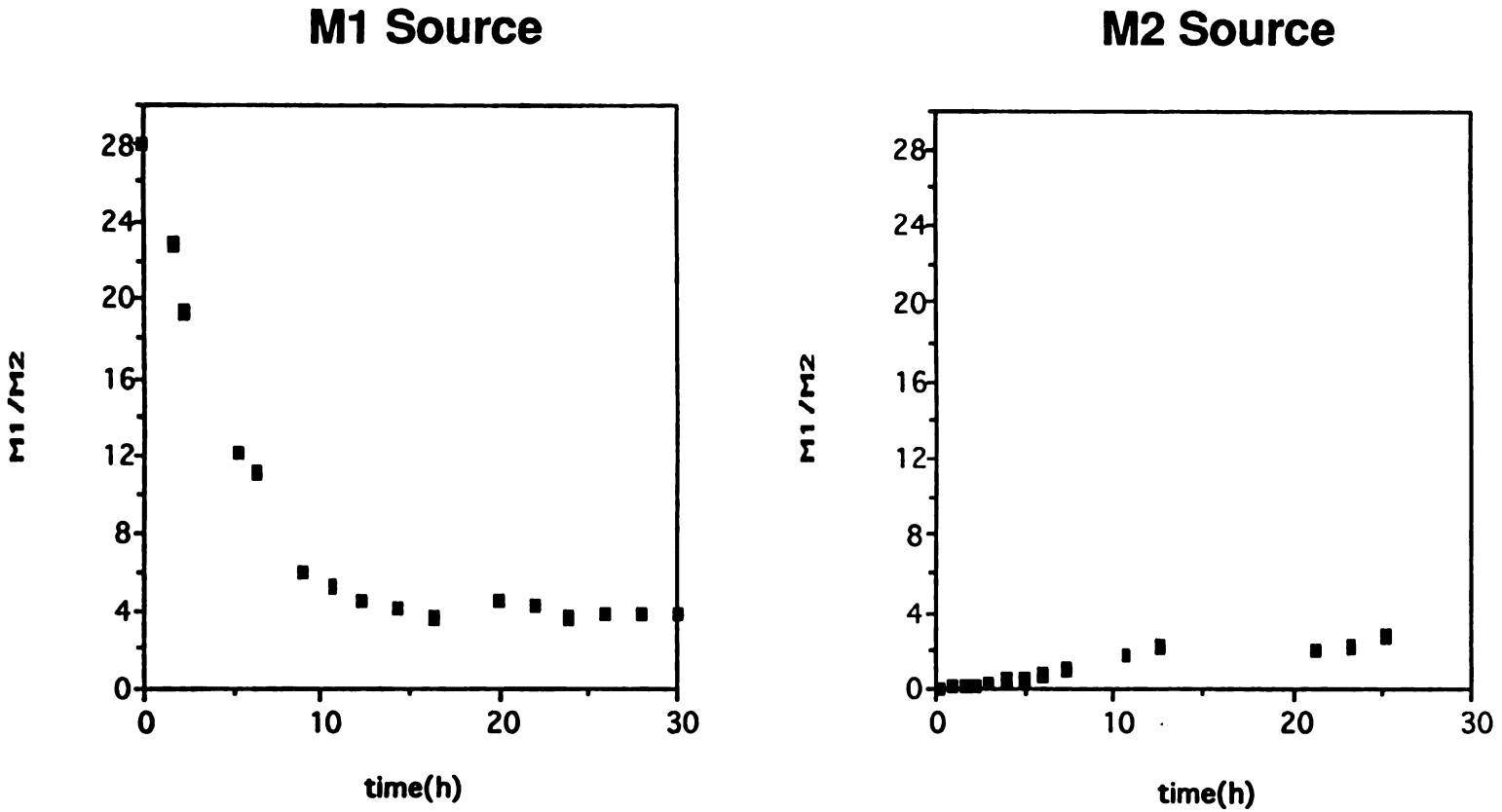


(a)



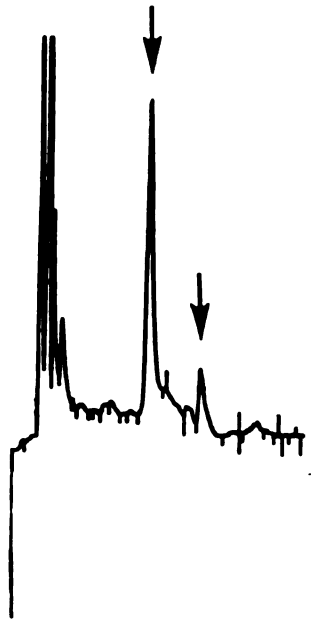
(b)

Figure 3.6 Structures of (a) 31-desmethyl and (b) 15-desmethyl FK 506

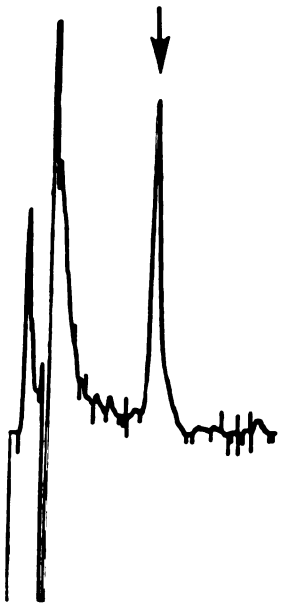


A sample of isolated metabolite M1 was injected onto the HPLC system (section *2.b.1*). From the mobile phase ($C_2H_3N/H_3PO_4/CH_4O$, 49/48/3, v/v/v) eluting off the HPLC column, a volume corresponding to M1 was collected, immediately vortexed, and aliquoted into equal volumes for serial injections. The first aliquoted sample of M1 was injected onto a second identical HPLC system within 2 min following collection. A metabolite peak corresponding to M2, which eluted from the first HPLC system, was collected and aliquoted in the same manner as M1. The first sample of M2 was injected onto the second HPLC system within 20 min following collection. The run time for each aliquoted sample of either M1 or M2 was 20 min. The peak area ratio between M1 and M2 from each aliquoted sample analyzed on HPLC system 2 was reported. No quantification of the amount of initial M1 used was made.

Figure 3.7 Time course for the interconversion between metabolites M1 and M2



(a)



(b)

Figure 3.8 Chromatogram of 13-desmethyl FK 506 eluted at (a) pH 3 and (b) pH7

peak at pH 7 yields two interconverting peaks at pH 3. Consistently, separate reinjections of the two interconverting peaks obtained at pH 3 gave a single peak at pH 7.

3.d Discussion

Several metabolites of FK 506 have been reported (Christians et al., 1991a; 1991b; 1991c; Iwasaki et al., 1991; Kobayashi et al., 1991; Lhoest et al., 1991; 1992; 1993). Among them, three products demethylated at the C13, 15, and 31 methoxy positions have been identified (Iwasaki et al., 1993). The analytical assay reported in chapter 2 can measure two interconverting metabolites at pH 3.

Mass spectrometric analysis of the major metabolite described showed that it is an O-desmethyl derivative, but did not distinguish between the three methoxy groups that had been demethylated (figure 3.9). The Fujisawa Pharmaceutical Company has introduced a two-step immunoassay for measuring FK 506 in clinical samples (Tamura et al., 1987). The epitope recognized by the monoclonal antibody used in the assay is thought to represent the domain bound by the C24 hydroxyl group, the C8 and C9 carbonyl groups and the C13 methoxy group (figure 3.10) (Kobayashi et al., 1991). Reportedly, the C13 O-desmethyl derivative does not cross-react with the anti-FK 506 monoclonal antibody, but the derivatives O-demethylated at C15 and C31 do (figure 3.11) (Iwasaki et al., 1993; Kobayashi et al., 1991). The desmethyl metabolite that we isolated showed no cross-reactivity in this immunoassay. This leads us to the assignment of the C13 O-desmethyl structure for our metabolite. Previous NMR studies

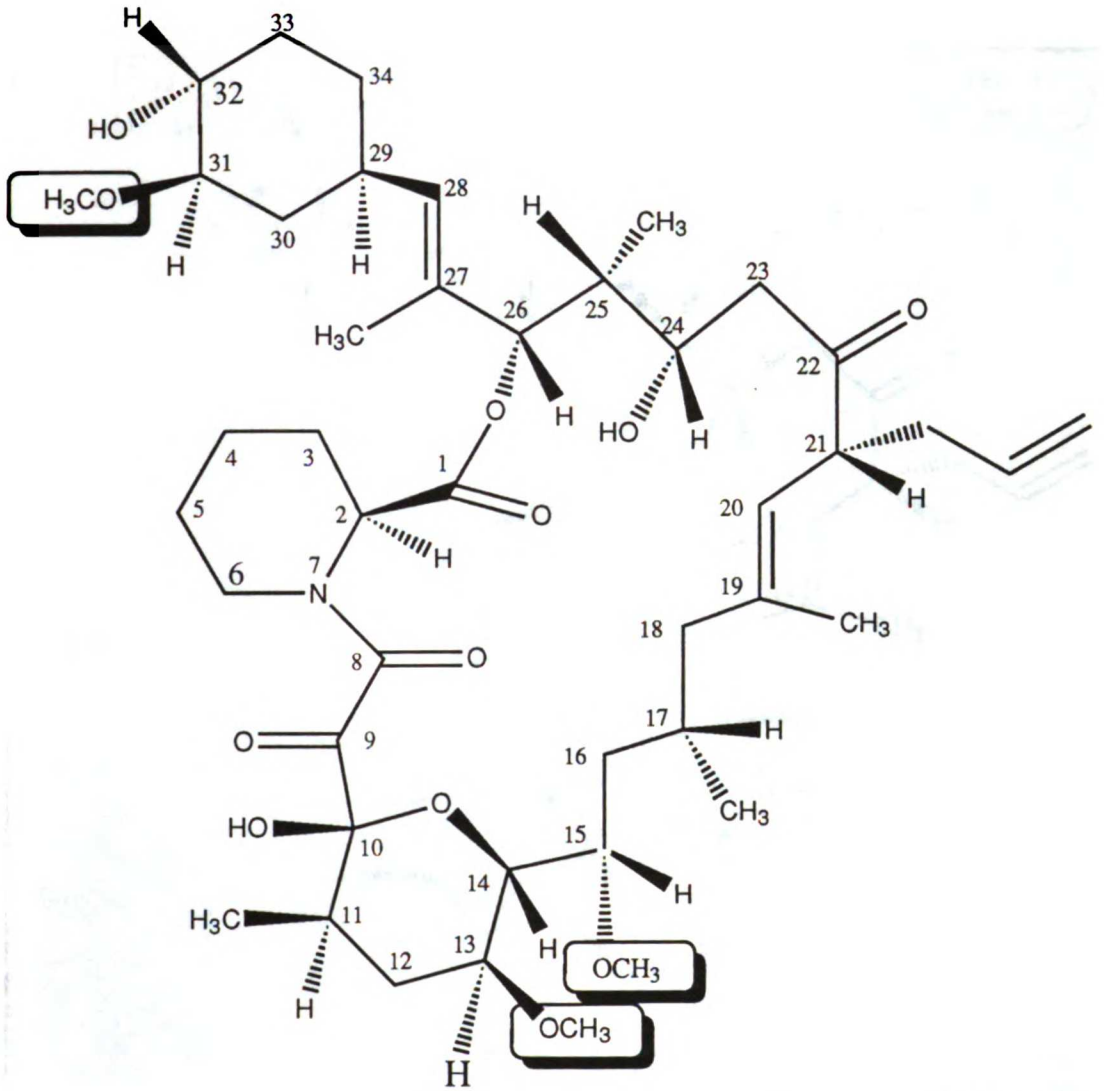


Figure 3.9 Three sites on FK 506 at which O-demethylation can take place

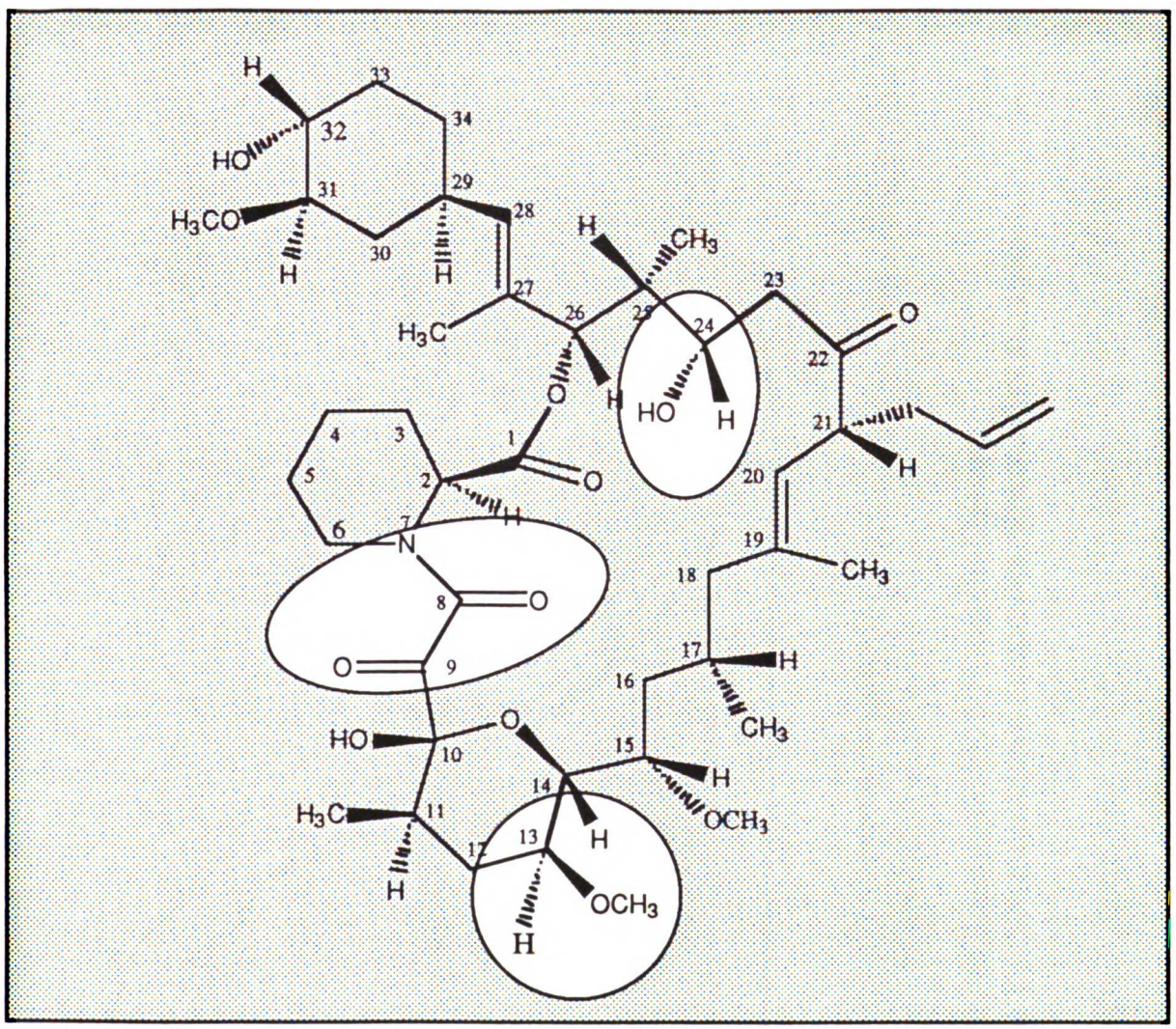


Figure 3.10 The epitopes on FK 506 that are recognized by the FK 506 monoclonal antibodies.

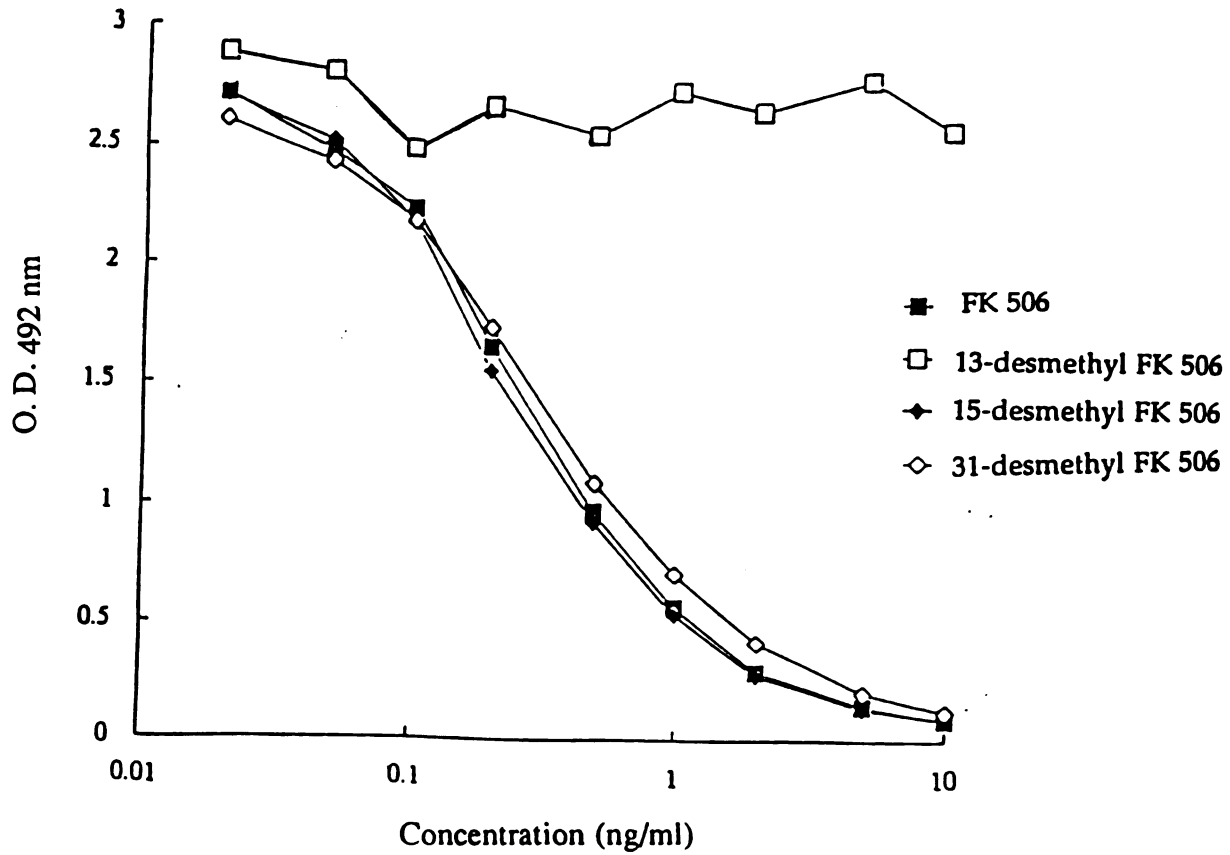


Figure 3.11 Crossreactivity of 3 authentic FK 506 metabolites and FK 506 to the FK 506 monoclonal antibodies. (Iwasaki et al., 1993)

have shown that C13 O-desmethyl FK 506 spontaneously undergoes pyran → furan rearrangement (Iwasaki et al., 1993; Vincent et al., 1992). We presume, therefore, that our metabolite, has the rearranged structure as depicted in figure 3.12, and is identical to the M1 metabolite identified by Iwasaki et al. (1993) and the C13-desmethyl FK 506 reported by Vincent et al. (1992). This assignment is further confirmed by the identical retention times and the interconversion phenomenon of the authentic 13-desmethyl FK 506 and the isolated metabolites.

The cause of the interconversion of the 2 time-dependent metabolites is presently not known. From the information obtained thus far, there are 2 possible explanations for this phenomenon. Both explanations are based on the premise that the two metabolites observed are indeed isomers. The form of isomerism between these two metabolites are most likely ring-chain tautomerism of the hemiketal. This ring-chain tautomerism is common in sugar chemistry (Solomons, 1982), as seen in the mutarotation of D-glucose (figure 3.13). Very recently, the tautomerism of FK 506 (figure 3.14) was reported by two groups (Lhoest et al., 1993; Namiki et al., 1993). Although one of the tautomeric forms reported by Namiki et al. is not truly a ring-chain tautomer of FK 506 per se, it nonetheless introduced the possibility of tautomeric FK 506. Lhoest and coworker (1993) also reported tautomeric forms of 15-desmethyl FK 506. Such observations strengthen the belief that tautomerism also exists in 13-desmethyl FK 506 which also contained a hemiketal (figure 3.15).

It is possible to detect two isomeric metabolite peaks at pH 3, but only one peak at pH 7 due to the differential resolving capability of the

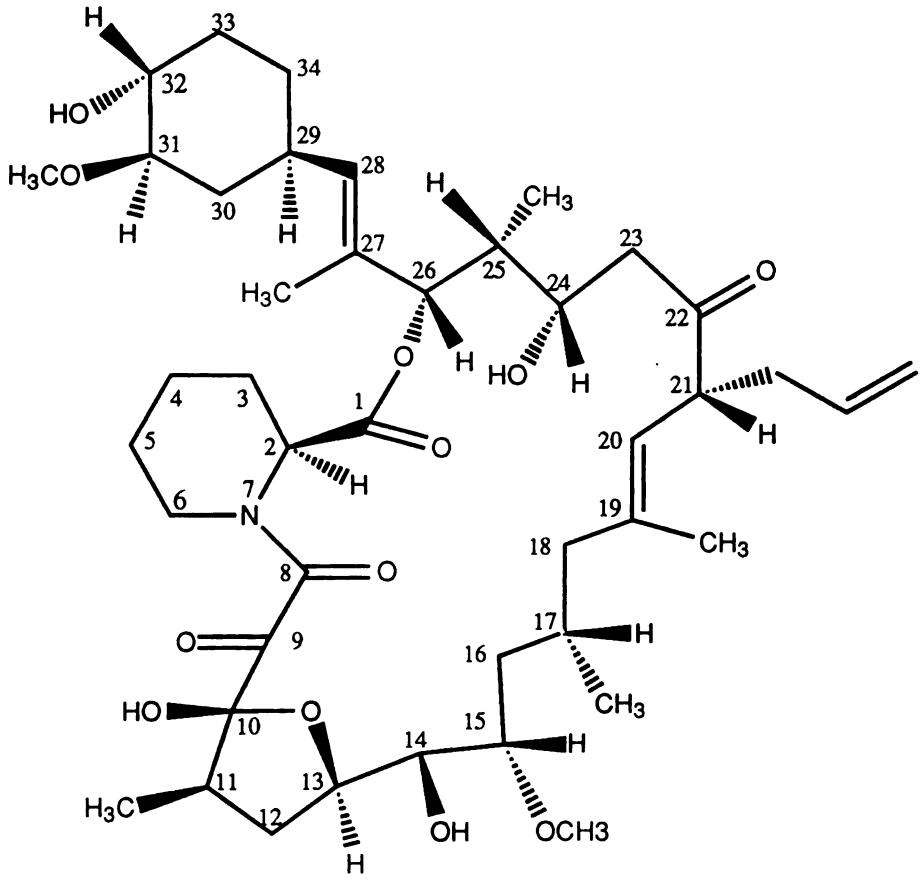


Figure 3.12 Structure of 13-desmethyl FK 506

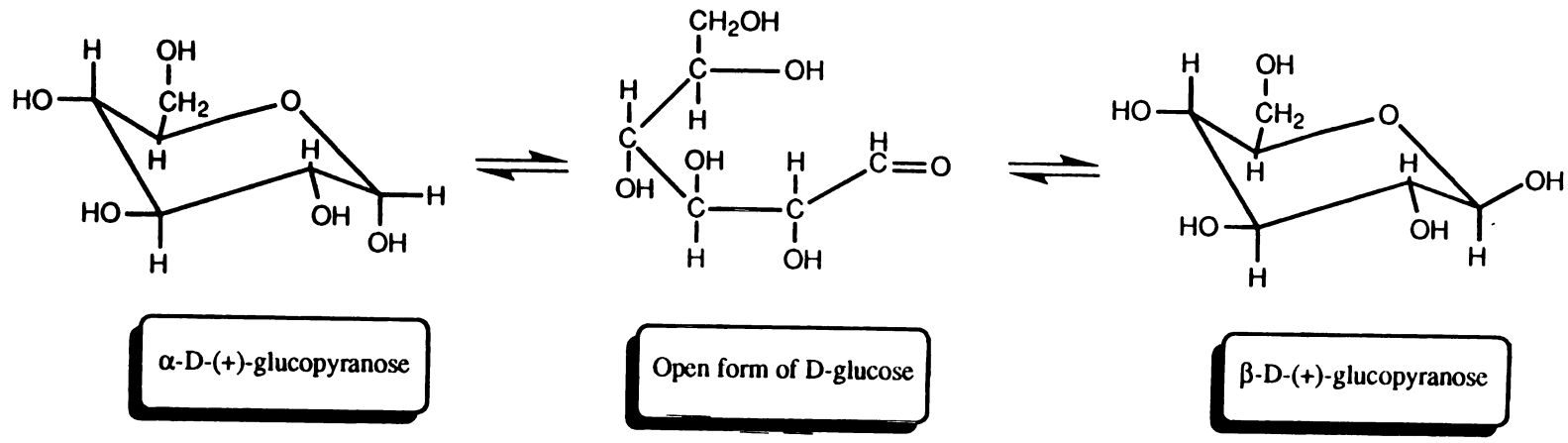


Figure 3.13 Mutarotation of D-glucose involves ring-chain tautomerism

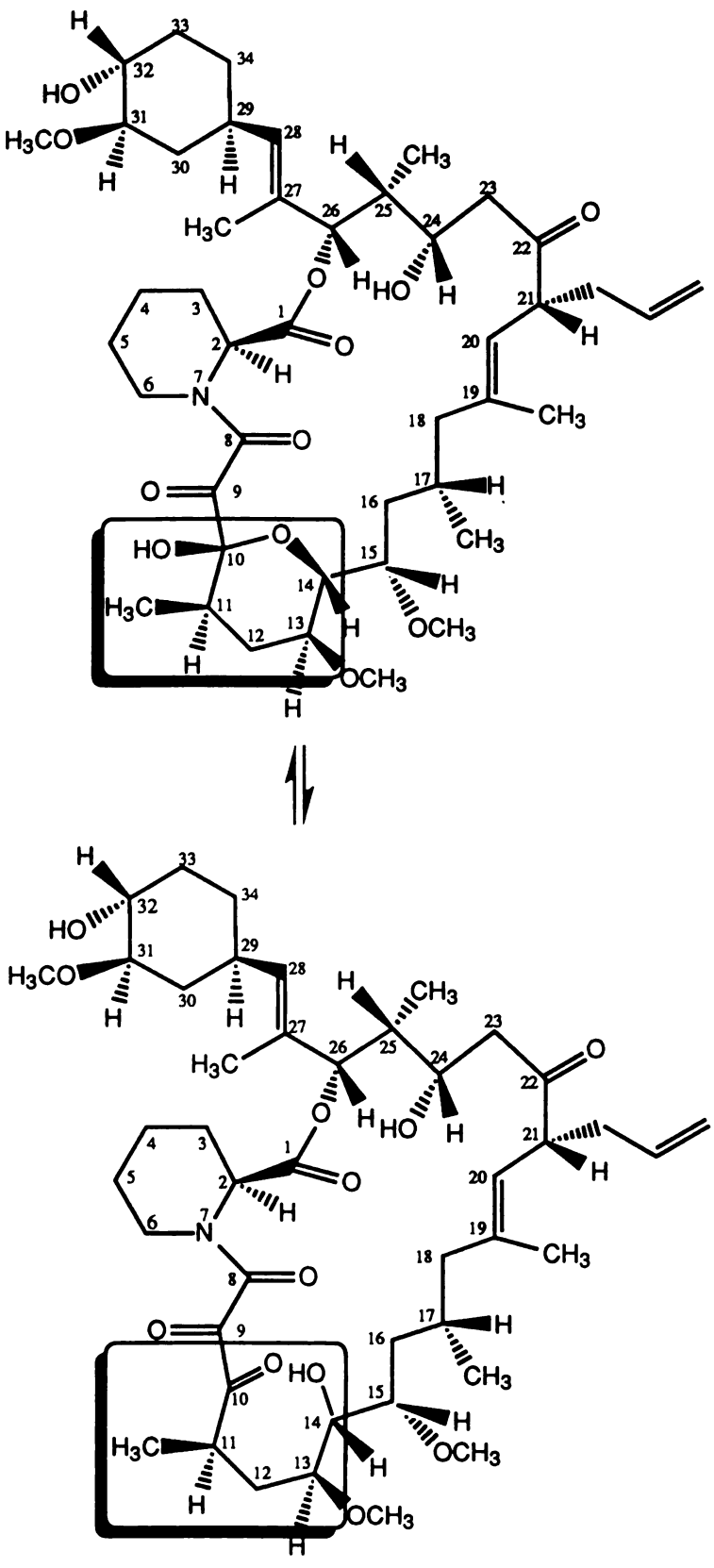


Figure 3.14 Ring -chain tautomerism of FK 506

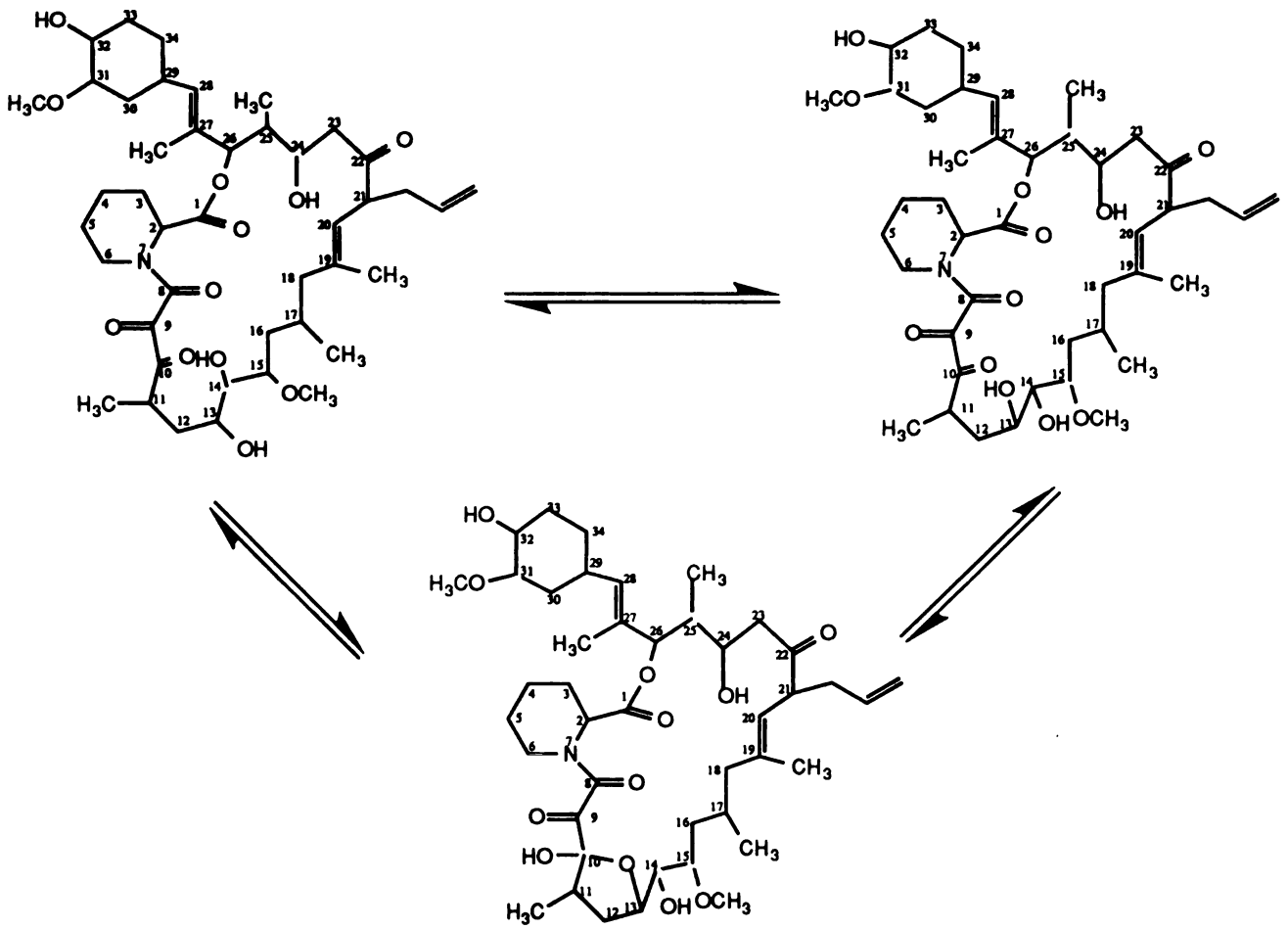


Figure 3.15 Proposed ring-chain tautomersim of 13-desmethyl FK 506

chromatographic column under different acidic conditions. For instance, the interaction between a compound and the respective stationary and mobile phases in the chromatographic system may change at different pHs due to different degrees of ionization. As seen for some peptides, it is also possible that the extent of isomerism is a function of solution pH (Henderson and Mello, 1990). At this point, it is unclear what the mechanism of interconversion might be.

Chapter 4

Metabolic Pathway and Kinetics of FK 506

4.a Introduction

Many model systems have been developed for the study of drug metabolism. As depicted in figure 4.1, an *in vivo* model is the most complex and it provides information about all phases of drug metabolism, i.e., from the absorption step to the elimination step. The *in vivo* model is generally used to establish the absorption, distribution and elimination of a drug, as well as to identify metabolites formed at various doses. *In vitro* models, on the other hand, are suitable for studying specific reactions involved in the metabolism of a drug, or the mechanism of a particular reaction.

Among the various *in vitro* models, the perfused organ model is by far the most relevant and closest to an *in vivo* model. It has the advantage of retaining an intact organ with all of its organ structure undisturbed. This model, however, is more difficult to work with than, for instance isolated cells.

Intact cells are a useful system for studying drug metabolism reactions. Because most drugs are lipophilic, they readily pass through cellular membranes and become evenly distributed in cells. Viable cells are capable of biosynthesis of cofactors such as NADPH, NADH, UDPGA and PAPS. With an adequate endogenous supply of glycogen, isolated liver cells can generate ample amounts of ATP to ensure sufficient generation of cofactors such as NADPH. Thus, isolated cell models can facilitate investigations into both phase I and phase II reactions.

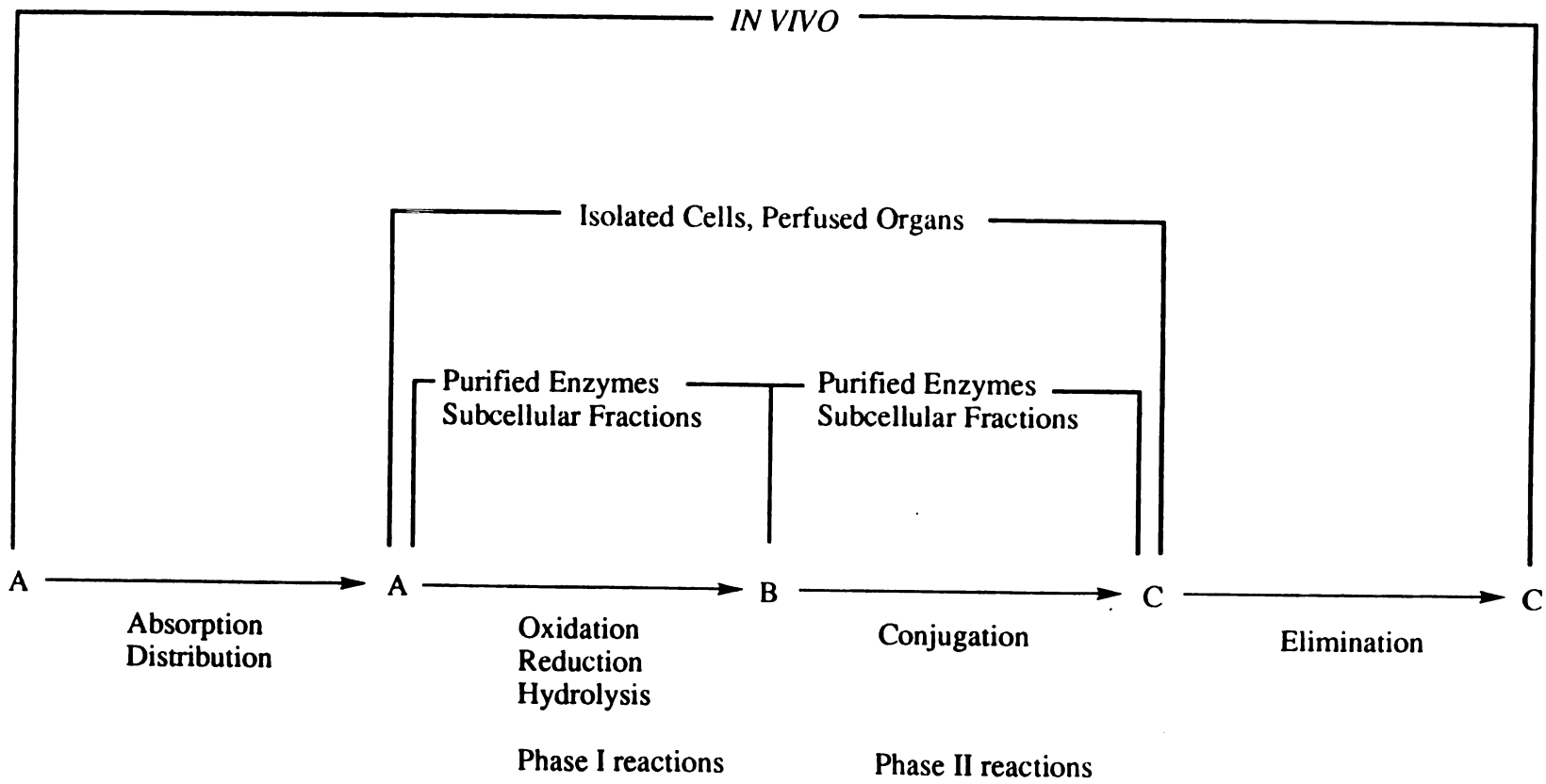


Figure 4.1 Model systems for drug metabolism

Subcellular fractions are the most frequently used and the most facile *in vitro* model to work with. Subcellular fractions can often be prepared in large quantities, and the unused portions can be stored frozen for at least one year. When stored properly, enzyme activity, such as that of cytochrome P450, in the subcellular fractions is not altered. Because each subcellular fraction has a slightly different composition of drug metabolizing enzymes (table 4.1), it is possible to narrow the number of enzymes that can possibly be responsible for a reaction that occurs in a particular subcellular fraction. The kinetics of a certain reaction can also be studied, as optimal cofactors and substrate concentrations can be varied in the incubation mixture.

Enzymes in the cytochrome P450 gene family are among the many drug metabolizing enzymes found in the liver. Cytochrome P450 was named originally on the basis of its spectral properties. It was first identified as a cellular pigment having an unusual red-shifted visible absorption maximum at about 450 nm, when present as the reduced-carbon monoxide complex (Klingenberg, 1958). Omura and Sato showed that this pigment was a b-type cytochrome containing iron-protoporphyrin IX as the prosthetic group (Omura and Sato, 1964). The P450 ferrous heme-carbonyl absorption maximum near 450 nm is quite different from those exhibited by typical hemoproteins such as myoglobin and horseradish peroxidase at 435 and 438 nm, respectively (Black and Coon, 1987). The unusual spectral properties of P450 were attributable to the presence of a thiolate (mercaptide) sulfur atom ligated to the heme iron atom at the fifth (proximal) coordination site (White and Coon, 1980).

Table 4.1 Drug metabolizing enzymes commonly found in major hepatic subcellular fractions

Microsomal Fraction	Cytosolic Fraction	Mitochondrial Fraction
	Alcohol Dehydrogenase	
Aldehyde Dehydrogenase	Aldehyde Dehydrogenase	Aldehyde Dehydrogenase
	Aldehyde/Ketone Reductase	
Carboxyl Esterase		
	Catechol-*O*-Methyl Transferase	
Epoxide Hydrolase	Epoxide Hydrolase	
Flavin Monooxygenase		
	Glutathione Peroxidase	Glutathione Peroxidase
Glutathione S-Transferase	Glutathione S-Transferase	
		Mono-Amine Oxidase
	N-Acetyl Transferase	
P450 Mixed Function Oxidases		P450 Mixed Function Oxidases
P450 Reductase		
	Sulfotransferase	
UDP-Glucuronosyl Transferase		

P450 serves as a monooxygenase which catalyzes the incorporation of one atom of molecular oxygen into a substrate and yields a molecule of water with the other atom. The catalytic cycle of P450 (figure 4.2) begins by binding the substrate to the ferric form (Fe^{3+}) of the enzyme. Electrons are donated from NADPH to NADPH-cytochrome P450 reductase which cycles between the one and the three-electron reduced forms during steady state turnover. Electrons are then transferred out from the reductase to the P450 isozymes in two 1-electron steps, first forming a ferrous (Fe^{2+}) enzyme substrate complex. Cytochrome b5 is also capable of donating the second electron in this process of electron transference. The reduced P450 substrate complex then binds O_2, which is now coordinated reversibly to the sixth heme ligand of the P450 isozymes, giving rise to a ferrous enzyme-O_2-substrate ternary complex. Upon the addition of the second electron to the ternary complex from the reductase, an iron peroxo species of unknown structure, but best represented by $Fe^{3+}O_2^{2-}$ is formed, and the molecular oxygen is cleaved. One atom of oxygen, is reduced to water, and the second oxygen atom is inserted into the substrate, giving rise to the oxidized products.

Absorbance changes as a result of the interaction between P450 and a substrate produce spectral changes classified as type I, type II, and reverse type I. Type 1 spectral change was shown to be due to a displacement of the sixth ligand of the heme from a hydrophobic region of the apoprotein, possibly the active site, by the substrate (Schenkman and Sato, 1968). The type II spectral change was suggested to result from a primary amine binding to the ferric form of the hemoprotein at the sixth ligand (Schenkman et al., 1967). Several explanations have been put forth for the reverse type I

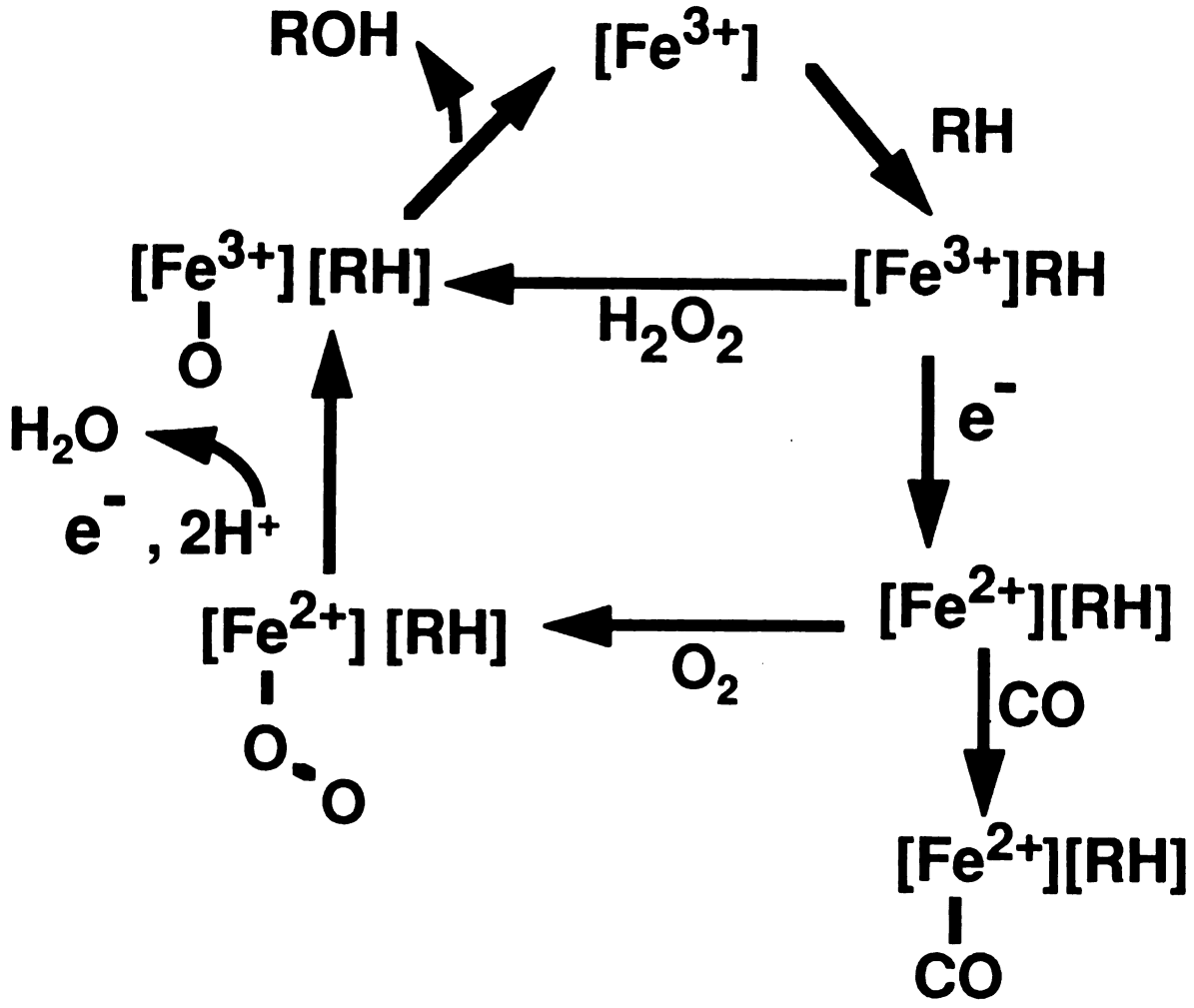


Figure 4.2 The P450 oxidation cycle

spectral change. Schenkman and coworkers (1967) proposed that this spectral change was due to the substrate binding to a site other than where endogenous substrates bind. This binding of substrate thus caused a shift in equilibrium between 2 forms of the protein (maximal absorption at 420 or 390 nm), one of which, the 390 nm form is where endogenous substrates bind. Other suggestions, such as displacement of endogenous substrates, for example, fatty acids from their binding sites (Cinti et al., 1973; Diehl et al., 1970; Powis et al., 1977; Schenkman et al., 1969), or interactions of the heme by alcohols (Yoshida and Kumaoka, 1975), have been made for the reverse type I spectra change observed.

Specific P450 isoforms are subject to exogenous and environmental regulation. The ability to inhibit (Fisher et al., 1990; Reidy et al., 1989) or induce a particular isoform (table 4.2) (Boobis et al., 1990) has facilitated the generation of much information related to preferential isozyme drug metabolism. Besides the inducers listed in table 4.2, ethinyl estradiol has been reported to induce CYP2C6, but decrease the activity of CYP3A (Prueksaritanont et al., 1993). Of importance to the biotransformation of xenobiotics in the vertebrate species are isoforms from the CYP 1, 2, and 3 families, and to a certain extent, the CYP4B subfamily. Other CYP families are believed to be selective for monooxgenation of endogenous compounds (Juchau, 1990).

While little metabolism work had been done on FK 506 when this project began, there were reports from clinical cases that a drug-drug interaction occurred between cyclosporine and FK 506 (Starzl et al., 1989). Because cyclosporine was known to be metabolized by hepatic P450 3A enzymes, the

Table 4.2 Inducibility and sex specificity of selected rat P450 enzymes

P450 Isozyme	§Selective Inducer	‡Sex Specificity (Predominance)
CYP1A1	3-Methylcholanthrene	
CYP1A2	Isosafrole	
CYP2A1		(Female)
CYP2A2		Male
CYP2B1	Phenobarbital$^{\Omega}$	
CYP2B2	Phenobarbital$^{\Omega}$	
CYP2C1	Phenobarbital$^{\Omega}$	
CYP2C2	Phenobarbital$^{\Omega}$	
CYP2C4	Phenobarbital$^{\Omega}$	
CYP2C7		(Female)
CYP2C11		Male$^{\infty}$
CYP2C12		Female
CYP2C13		Male
CYP2C16	Phenobarbital$^{\Omega}$	
CYP2E1	Acetone/Ethanol	(Female)
CYP3A1	Dexamethasone/	
CYP3A2	Pregnenolone 16α-Carbonitrile	Male$^{\Delta}$
CYP4A	Clofibrate	

§Selective inducer, except for phenobarbital, is an agent which primarily affects the protein of interest. It may also have effects on other proteins, but these other effects are small as compared to that related to the compound of interest.

$^{\Omega}$Phenobarbital is a general inducer, but appears to selectively increase the rate of transcription of the genes of several P450 isozymes.

‡Sex specificity means that the isozyme of interest is a constitutive enzyme in the indicated sex only.

$^{\infty}$Recent data (Ghosal et al., 1993) suggested that CYP2C11 is inducible by dexamethasone in female rats.

$^{\Delta}$Recent data (Ghosal et al., 1993) suggested that CYP3A2 is inducible in female rats by dexamethasone and phenytoin.

cyclosporine/FK 506 interaction led to the hypothesis that FK 506 is metabolized by a similar enzyme system. This chapter describes the metabolism studies performed in rat liver hepatocytes and rat liver microsomes to explore the possible involvement of CYP3A in FK 506 metabolism and test this hypothesis.

4.b Materials and methods

FK 506 was kindly supplied by Fujisawa Pharmaceutical Company (Deerfield, IL). Dexamethasone was a gift from The Upjohn Company (Kalamazoo, MI). Collagenase type I, bovine serum albumin (BSA), ethyleneglycol-bis-(b-aminoethyl ether)-N,N' -tetra-acetic acid (EGTA), N-2-hydroxypiperazine-N'-2-ethane sulfonic acid (HEPES), NADPH, and Scintiverse LC were obtained from Sigma Chemical Company (St. Louis, MO). Biorad protein assay kit with albumin protein standard was obtained from Biorad (Richmond, CA). Three cold testosterone metabolites, 4-androsten-6β,17β-diol-3-one, obtained from Sigma Chemical Company, and 4-androsten-16α,17β-diol-3-one, and 4-androsten-16β,17β-diol-3-one obtained from Steraloids (Wilton, NH) were used.

Male and female Sprague-Dawley rats, weighing 250-300 g, were obtained from Bantin and Kingman (San Leandro, CA).

4.b.1 Isolated rat hepatocytes

Techniques for the isolation of liver cells have been described by Labrecque and Howard (1976) and Seglen (1976). Hepatocytes used here

were prepared according to the method of Seglen, as described in detail by Irving et al. (1984).

Eight solutions were used in this hepatocyte isolation procedure. The stock solution for the perfusion medium was an Eagle's minimum essential medium without calcium (MEM-E) prepared in the laboratory with amino acids obtained from Sigma Chemical Company (St. Louis, MO) or Calbiochem-Behring (San Diego, CA). As described in table 4.3, all the amino acids were dissolved in 800 ml of distilled water with stirring. The remaining salts and glucose were added and the solution stirred until clear. After phenol red was added, the volume of the solution was brought to 875 ml with distilled water. The pH of the solution was adjusted to 6.5. The solution was then filtered, sterilized, transferred to sterile bottles labelled 10X MEM-E, and stored at 4°C.

The medium for elutriation (table 4.4) was a 1X MEM-E solution diluted approximately 10 fold from the 10X MEM-E solution. This was achieved by mixing 87.6 ml of 10X MEM-E solution with 880 ml of sterile water in an autoclaved bottle. The solution was completed by the addition of 26.6 ml of 7.5% $NaHCO_3$, 10 ml of penicillin G potassium (10,000 U/ml), and 10 ml of 1M HEPES. The perfusion medium (table 4.5) was prepared on the day of the experiment by mixing 2.5 ml 1% EGTA, 2.5 ml 1M HEPES and 250 ml of 1X MEM-E. The buffering capacity of these solutions were maintained by organic buffers such as HEPES.

A HAMS/DMEM basal medium was prepared as described in table 4.6. After all the ingredients were mixed thoroughly, the medium was filtered,

Table 4.3 Recipe for 10X Eagle's minimum essential medium without calcium (pH 6.5)

	Grams
L-arginine (base)	1.2
L-glutamine	3.0
L-histidine (base)	0.3
L-isoleucine	0.5
L-leucine	0.5
L-lysine (HCl)	0.7
L-methionine	0.2
L-phenylalanine	0.3
L-threonine	0.5
L-tryptophan	0.21
L-valine	0.5
NaCl	68.0
KCl	4.0
$MgSO_4.7H_2O$	2.0
$NaH_2PO_4.H_2O$	1.4
D-Glucose	10.0
Phenol red sodium salt	0.16
Final volume	875 ml

Table 4.4 Recipe for 1X MEM-E for elutriation

Sterile water	880.0 ml
10X MEM-E	87.6 ml
$NaHCO_3$ (7.5%)	26.6 ml
Penicillin G potassium (10,000 U/ml)	10.0 ml
HEPES (1M)	10.0 ml

Table 4.5 Recipe for 1X MEM-E perfusion medium

1X MEM-E for elutriation	250.0 ml
HEPES (1M)	10.0 ml
EGTA (1%)	2.5 ml

Table 4.6 Recipe for HAMS/DMEM basal medium

HAM'S F12 (Modified)	
with glutamine	
without sodium bicarbonate	10.64 g
Dulbecco's Modification of Eagle's Medium	
with glutamine	
without sodium bicrabonate	13.45 g
q.s. with deionized water to	2000 ml

sterilized and stored at 4°C. A HAMS/DMEM completed medium was prepared from the basal medium by the addition of penicillin and sodium bicarbonate as described in table 4.7.

A collagenase solution was prepared by the addition of 14 mg collagenase to the 150 ml HAMS/DMEM completed medium solution. Depending on the purchase date of the collagenase, an "old" batch of collagenase may have a lower enzyme activity than a new batch. Adjustments in the amount of collagenase to be added was needed at times. A DNase solution was made with 5 mg of DNase I in 25 ml of HAMS/DMEM completed medium. This solution helped prevent cell aggregation from occurring. A hepatocyte suspension buffer, which contained albumin to protect cells against mechanical stress, was prepared as described in table 4.8.

The 1X MEM-E solution and the collagenase solution used for perfusion were warmed in a water bath maintained at 37°C. Carbogen (95% O_2, 5% CO_2) was bubbled into the solutions for at least 15 minutes. Tubings that carry the medium from the reservoir to the liver were immersed in a 44.2°C water bath, and extended 2 feet from the bath to the rat liver.

Each experimental rat was placed in a large glass container covered with a lid. A large piece of cotton wool which was soaked with ether was placed into the glass container. As soon as the rat had fallen asleep (about 30 seconds), the rat was rapidly transferred onto the operating table. The abdomen of the rat was shaved, and wiped clean with 70% ethanol, followed by Betadine surgical scrub. After the skin covering the abdomen was

Table 4.7 Recipe for HAMS/DMEM completed medium

HAMS/DMEM basal medium	200 ml
Sodium bicarbonate (7.5%)	7.5 ml
Penicillin (10,000 U/cc)	2.0 ml

Table 4.8 Recipe for hepatocyte suspension buffer

	grams	w/v
Sodium chloride	4.00	(0.8%)
Potassium chloride	0.18	(0.036%)
Magnesium sulfate-7 hydrate	0.08	(0.016%)
Calcium chloride-dihydrate	0.09	(0.018%)
Glucose	0.9	(0.18%)
Bovine serum albumin -fatty-acid free	5	(1%)
HEPES	1.2	(0.24%)

q.s. with deionized water to 500 ml

Adjust pH with HCl or NaOH to 7.45.

removed, a U-shaped traverse incision was made in the abdominal muscle to open the abdomen. After the intestines were displaced to the left side of the abdominal cavity, a suture was tied loosely around the inferior vena cava and the hepatic portal vein. To prevent blood clotting, heparin was injected into the inferior vena cava that was held distally with forceps. Approximately 0.4 ml of 1000 U/ml heparin was administered by a 25 gauge needle via a 1 c.c. tuberculin syringe. The portal vein was then immediately cannulated with a 16 gauge catheter, and was tied in place with a suture. The perfusate flow was started at a rate of 20-30 (usually 28) ml per minute, and the inferior vena cava was cut below the suture to release the pressure from the perfusate. The rib cage of the rat was then cut open to expose the heart. The atrium was cannulated, and the suture which was placed around the inferior vena cava was tied. At this point, the rat organs were warmed via a surgical lamp. As soon as the liver was cleared of any visible signs of blood, the perfusion medium was switched from the 1X MEM-E to the collagenase solution. After 50 ml of the collagenase solution had passed through the liver (approximately 2-3 min), a tubing was connected to the cannula at the atrium, and the collagenase solution was recirculated for the next 16 to 18 minutes. As the liver began to swell from the collagenase perfusion, it was carefully removed from the carcass by cutting off the hepatic portal vein, both ends of the vena cava as close to the liver as possible, and by cutting some of the membranes or ligaments attached. The liver was placed on a petri dish, gently minced, and transferred to a 125 ml Erlenmyer flask filled with 50 ml of HAMS/DMEM solution. The petri dish was rinsed with 10 to 15 ml of the DNase solution, and the rinse was added to the Erlenmyer flask. The Erlenmyer flask was gently agitated at 200 rpm for 10 min at 37°C to disperse the cells. The contents of the Erlenmyer flask

were filtered twice through 2 layers of surgical gauze. A preincubation at 37°C for 10 min served to promote Kupffer cells and other debris to form aggregates. When the contents were filtered, these large aggregates or tissue fragments were removed.

The filtrate, which contained the crude collagenase-dispersion was elutriated in a Beckman J2-21 centrifuge and a JE-6B rotor (Beckman Instruments, Palo Alto, CA), equipped with a Sanderson chamber which was sterilized with 6% hydrogen peroxide, rinsed with deionized water and Levowitz-15 solution prior to each use. Elutriation was carried out with 1X-MEM-E. The cell suspension (15-20 µl) was loaded into the Sanderson chamber through the loading port . During the first pass, the rotor was set to spin at 1100 rpm, and the flow rate was initially 23 ml/min. The effluent was continuously collected into a container for later use. When the apex of the Sanderson chamber began to clear, the flow rate was increased every few minutes in increments of 2 ml/min to a final speed of 36 ml/min. At this point, all the clumped hepatocytes were left in the chamber, and the cells collected in the effluent were single cells. The clumped hepatocytes were flushed out of the chamber by stopping the rotor and pumping the 1X MEM-E at the maximum flow rate. After all the cells had been elutriated once, the pooled single cells are elutriated again. During the second pass at a flow rate of 58 ml/min, the heavier hepatocytes remained in the chamber, but the nonparenchymal cells, such as the lighter Kupffer cells, epithelial cells and the lipocytes were collected in the effluent. When the cells filled about half of the chamber, the inflow of the single cell solution was stopped, and the cells were washed with approximately 150 ml of 1X MEM-E at a flow rate of 63 ml/min. Dead cells were flushed out by instantaneously changing the

pump speed to the maximum twice, for one or two seconds each time. The hepatocytes were then recovered by flushing out the chamber. This was achieved by first stopping the centrifuge completely, and then restarting it at the maximum speed.

Hepatocytes obtained from elutriation were counted in a Coulter counter. Cell viability was checked by the trypan blue exclusion test. Only hepatocyte preparations with higher than 85% viability were used (see *section 4.b.2*). The hepatocyte suspension was centrifuged at 1000 rpm for 5 min. The supernatant was carefully removed, and the hepatocytes resuspended in the hepatocyte suspension buffer (table 4.8).

4.b.2 Determination of the structural integrity of cells

Light microscopy and retention of soluble enzymes are methods previously established to determine the structural integrity of hepatocytes. Using light microscopy, intact hepatocytes were clearly visualized as well defined spheres. Damaged cells, however, had irregular shapes. When injured cells were suspended in a 0.2% trypan blue solution, they took up the dye, and revealed a darkly stained nucleus or appeared diffusely stained. A cell with any dye uptake was considered nonviable. Cells with an intact plasma membrane excluded the trypan blue stain (LaBrecque and Howard, 1976).

Cells were suspended in the 1X MEM-E solution at 2-5 x 10^5 cells per ml. Trypan blue solution (0.5 ml of a 0.4% solution) was mixed with 0.2 ml of the cell suspension and 0.3 ml of buffer. The dilution factor in this case

was 5. The mixture was allowed to stand for 10 minutes. Since overexposure of cells to trypan blue will result in uptake of the dye by viable as well as nonviable cells, cells were not left in the dye for more than 15 minutes. Cells were counted in a hemocytometer with a cover slip placed on top of the chambers. With a Pasteur pipette, a small amount of the trypan blue-cell suspension was transferred to both chambers on the hemocytometer. By carefully touching the edge of the cover slip with the pipette tip, the cell suspension filled the chamber by capillary action. The number of viable and nonviable cells were counted separately in the 1 mm center square and four 1 mm corner squares in one of the two chambers. The counting procedure was repeated for the second chamber. The percent cell viability was calculated from the number of viable cells and the total (viable and nonviable) cell count.

The number of cells per ml =
average count per square x the dilution factor x 10^4.

4.b.3 Preparation of the cytosolic, mitochondrial, and microsomal fractions

All rats were sacrificed by decapitation. The livers were immediately removed, weighed, perfused with ice-cold 0.15 M KCl, and homogenized in 0.1 M phosphate buffer, pH 7.45 with a Dounce manual tissue grinder. The homogenate was centrifuged at 900 g for 10 minutes. The supernatant was decanted, followed by centrifugation at 10,000 g for 30 minutes. The pellet obtained from the 900 g centrifugation constituted cellular debris and nuclei. After the 10,000 g centrifugation step, the soft loose pellet was the

the mitochondria. The supernatant was centrifuged again at 100,000 g for 60 min. The resulting supernatant, which is composed mainly of the cytosolic fraction, was decanted. The pellet obtained from this step was resuspended in 0.15 M KCl and re-centrifuged at 100,000 g for 30 minutes. At the end of the 30 minute spin, the supernatant was decanted and the pellet, the microsomal fraction, was completely covered by a layer of a mixture (v/v) of 20% glycerol/80% 0.05 M Tris-HCl, pH 7.4, containing 1 mM EDTA (Gibson and Skett, 1986). The centrifugation tube containing the microsomal pellet was tightly capped and kept at -80°C until use. The mitochondrial and the cytosolic fractions were stored on ice until they were used later, but on the same day as the preparation. All steps, including centrifugation were performed on ice or at 4°C.

Microsomes were prepared from hepatocytes by the same method described above for tissue homogenates. Prior to the homogenization and ultracentrifugation steps, the hepatocyte suspension buffer was removed, and the hepatocytes were suspended in 0.1 M phosphate buffer. Microsomes prepared from the hepatocytes were only used for determining the amount of P450 and microsomal protein per million cells, and not for incubation purposes.

4.b.4 Protein concentration determination

The determination of protein concentration in all subcellular fractions was always done on the day of the experiment. The Biorad protein assay kit was used for this purpose throughout the project. A preweighed albumin standard is diluted into 20 ml of 0.1 M phosphate buffer to give a

predetermined protein stock as designated on the label of each vial. This protein standard stock was stored at 4°C in the refrigerator until use, and was stated to be stable for at least 6 months. The Biorad protein assay dye reagent concentrate, a solution containing dye, phosphoric acid, and methanol, was diluted with distilled water in a 1 to 5 ratio, and filtered. This working dye solution was stored at room temperature for up to 2 weeks. To measure the protein concentration of a sample, a standard curve was prepared. The protein standard stock was aliquoted (25, 40, 60, 90, and 120 µl) into culture tubes. To each culture tube, 145 µl of 0.1 M phosphate buffer was added. One hundred µl of the standard was aliquoted into a culture tube. To this, 5 ml of the working dye solution was added, and all mixtures were vortexed carefully. A blank was prepared by mixing 100 µl of 0.1M phosphate buffer to 5 ml of the working dye solution. Samples were appropriately diluted, and 100 µl of each sample was mixed with 5 ml of 0.1 M phosphate buffer.

The absorbance of the protein was measured in the visible range at a wavelength of 595 nm using either a Beckman Du®-64 spectrophotometer (Beckman Instruments, Palo Alto, CA) or a Perkin Elmer λ11 uv/vis spectrophotometer (Perkin Elmer Corp. Analytical Instruments, Norwalk, CT). The blank sample is first placed into the reading port, and the baseline was zeroed to the blank sample. The absorbances of the standards were measured, and a standard curve was constructed. The protein concentration of the sample was interpolated from the standard curve, with the appropriate dilution factor taken into account. A representative protein standard curve is depicted in figure 4.3.

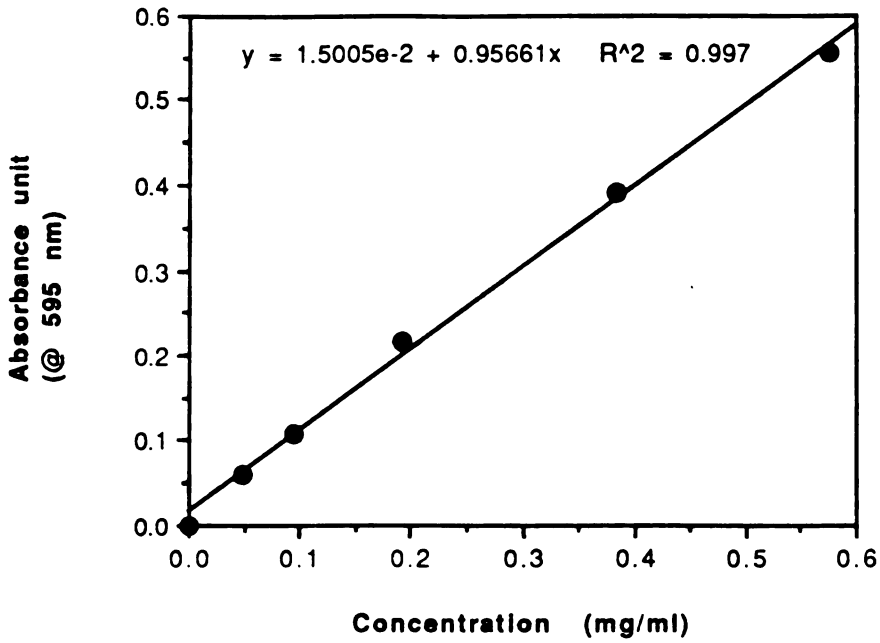


Figure 4.3 A typical protein standard curve using the Biorad protein assay kit

4.b.5 Total cytochrome P450 (P450) content measurement

Total P450 content was measured according to the method of Omura and Sato (1964), as described in detail by Schoene et al. (1970). In brief, samples diluted to approximately 0.15 mg protein/ml were bubbled with carbon monoxide for about 1 min. The carbon monoxide used was passed through an oxygen scrubber to completely remove any trace of oxygen that might have dissolved in the gas mixture. Two quartz sample cells were filled with about 1 ml of sample. Parameters for total P450 measurement on the SLM Aminco DW-2000 UV-VIS spectrophotometer were selected. After the baseline is set, the sample cell placed in front of the sample beam was removed. A small pinch (a few milligrams) of sodium dithionite was added to the contents in the sample cell. The sample cell was covered and carefully inverted several times to mix the contents. The sample cell was placed back in front of the sample beam in the reader. A difference spectrum was taken between the dithionite reduced sample and the non-reduced sample in the range of 450 and 490 nm. A representative spectrum is shown in figure 4.4.

P450 was determined from the CO difference spectrum of dithionite-reduced samples, assuming a value of 91 $cm^{-1}mM^{-1}$ for the extinction increment between 450 and 490 nm (Omura and Sato, 1964).

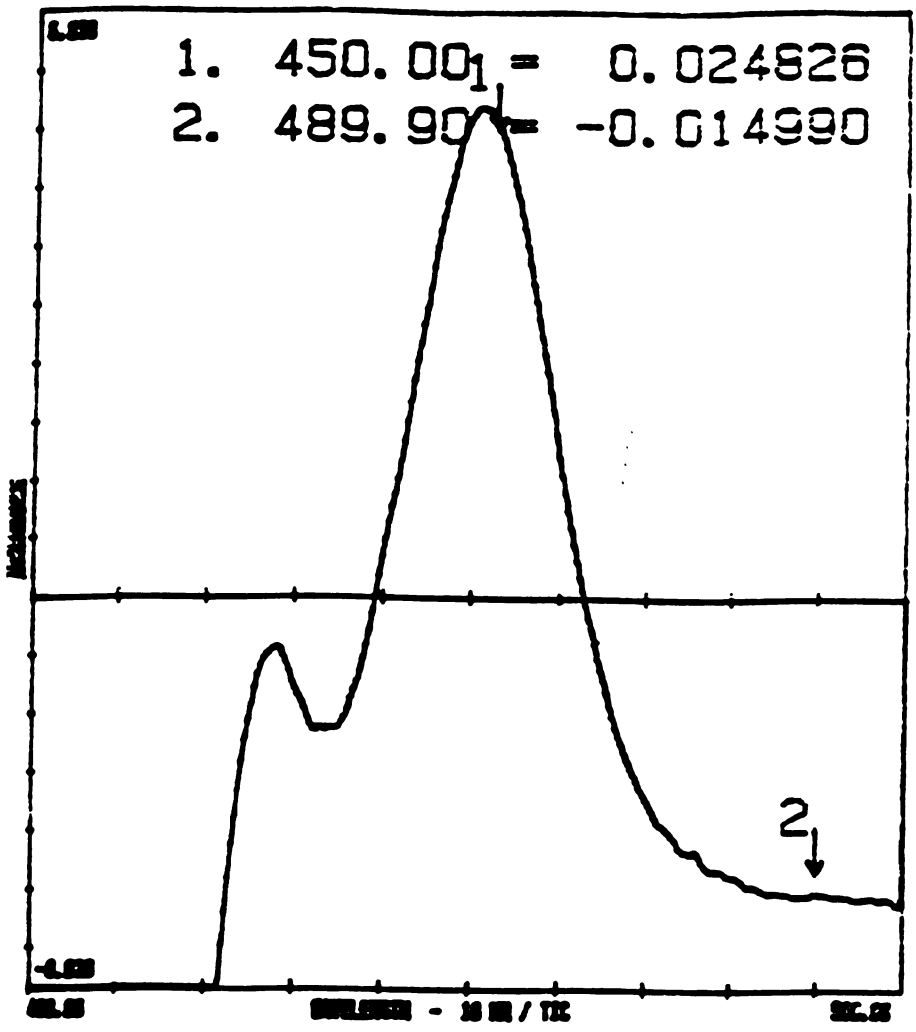


Figure 4.4 A typical carbon monoxide complexed P450 spectrum in untreated rat liver microsomes

4.b.6 Time course of FK 506 major metabolite formation in hepatocytes

Freshly harvested hepatocytes (5 million cells/ml) (Prueksaritanont et al., 1992) were pre-incubated for 5 min at 37°C. The reaction was started by the addition of FK 506 (5 μM). Duplicate or triplicate samples (0.6 ml) were taken at 0.25, 1, 3, 5, 10, 20, 30, and 60 minutes. The reaction was stopped by quick freezing the sample in an acetone-dry ice mixture. All samples were stored at -40°C until analysis.

4.b.7 Time course of 13-desmethyl FK 506 formation in liver cytosols, mitochondria and microsomes from untreated male rats

Liver mitochodria and microsomes (2 mg protein/ml) from untreated male rats and FK 506 (5 μM) were pre-incubated in the same manner as the hepatocytes. For the mitochondria and the microsomes, the reaction was started by the addition of a NADPH generating system. The NADPH generating system comprised of NADPH (1 mM), magnesium chloride (5 mM), glucose 6-phosphate (10 mM), and glucose 6-phosphate dehydrogenase (2 U/ml). In several samples, 1 mM NAD was also added to the mitochondrial incubation mixtures. Duplicate or triplicate samples (0.6 ml) were taken at 0.25, 3, 5, 10, 20, 30, and 60 minutes. The reaction was stopped by quick freezing the sample in an acetone-dry ice mixture. All samples were stored at -40°C until analysis. To test for any glutathione (GSH) dependent activity, liver cytosols (2 mg/ml) were preincubated in the presence of GSH (2 mM) for 3 minutes. The reaction was initiated by the

addition of FK 506, and was allowed to proceed as for the mitochondrial or microsomes samples.

A control study was also performed in microsomes to test if P450 enzymes were indeed involved in the metabolism of FK 506. In this study, the NADPH generating system was replaced by 0.1 M phosphate buffer.

4.b.8 Carbon monoxide inhibition study

In order for a substrate to be oxidized by a P450 isozyme, molecular oxygen coordinated to the sixth heme ligand must be present. In addition to oxygen, other ligands may bind tightly to the sixth coordination position of the ferrous form of the P450 enzymes, and thereby inhibit P450 activity. Carbon monoxide, which can reversibly bind to the sixth heme ligand in P450 (Babany et al., 1988), can bind all of the available sixth heme ligands. In the absence of oxygen, the electron transport chain cannot be completed, and the substrate cannot be oxidized. Using the carbon monoxide inhibition study, the involvement of P450 enzymes in the metabolism of FK 506 was investigated.

Hepatic microsomes (2 mg protein/ml) were placed in a vial fitted with an air-tight rubber septum. One long and one medium length catheters were inserted through the rubber septum to reach the incubation mixture. One of these catheters was connected to a supply of carbon monoxide. Carbon monoxide was bubbled into the microsomes for at least 15 min to completely saturate the incubation mixture. The second catheter serves as a pressure release outlet. This whole setup was then pre-incubated in a Dubnoff

metabolic shaker at 37°C for 3 min. When the incubation mixture was warmed, FK 506 (10 μM) was delivered from a hypodermic needle which was inserted into the septum. The reaction was started by the addition of NADPH (1 mM) via the same injection port. Duplicate or triplicate samples (0.6 ml) were taken after 10 minutes of incubation. The reaction was stopped by quick freezing the sample in an acetone-dry ice mixture. All samples were stored at -40°C until analysis.

4.b.9 Effects of differential P450 enzyme induction and inhibition on the rate of formation of 13-desmethyl FK 506

In order to determine which isoform in the P450 superfamily is responsible for the metabolism of a P450 substrate, correlation of the metabolic activity of a substrate and the induction or inhibition level of selective isoforms is often performed (Boobis et al., 1990; Kolars et al., 1992; Morrison et al., 1991; Prueksaritanont et al., 1993) . To investigate which of the major isoforms, namely isozymes in the CYP 1, 2, and 3 families, are involved in the metabolism of FK 506, male Sprague-Dawley rats were treated with dexamethasone, ethinyl estradiol, 3-methylcholanthrene, and phenobarbital.

Dexamethasone treatment involved intraperitoneal injection of dexamethasone suspended in corn oil at 100 mg/kg/day for four days (Prueksaritanont et al., 1993). Phenobarbital treatment involved intraperitoneal injection of phenobarbital dissolved in water at 80 mg/kg/day for four days. Ethinyl estradiol treatment involved subcutaneous injection of ethinyl estradiol dissolved in propylene glycol at 5 mg/kg/day for five days.

3-Methyl cholanthrene treatment involved intraperitoneal injection of 3-methyl cholanthrene suspended in corn oil at 30 mg/kg/day for five days.

All microsomes (100 μg/ml) in 0.1M phosphate buffer were pre-incubated for 3 min at 37°C with FK 506 (40 μM), diethylenetriamine penta-acetic acid (DETAPAC) (1 mM), and dithiothreitol (DTT) (1 mM). The reactions were initiated by the addition of an NADPH-generating system as described in section *4.b.7*, and were terminated after 5 min.

4.b.10 Testosterone metabolism

The activity of selective P450 isozymes can be differentiated by functional probes as listed in table 4.9. The FK 506 metabolic activity was correlated with the functional activities of various P450 isozymes, including the 6β-, 16β-, and 16α-, testosterone hydroxylase activities.

Testosterone was incubated with liver microsomes from various untreated and treated rats. Incubation mixtures (1 ml) containing liver microsomes (0.1 mg/ml) in 0.1 M phosphate buffer and testosterone (400 μM) with 0.5 μCi of ^{14}C-testosterone were pre-incubated for 5 min at 37°C. The reaction was initiated by the addition of an NADPH-generating system. The reaction was stopped after 10 min by the addition of ethyl acetate (1 ml). The mixture was vortexed and stored at 4°C until analysis.

The analysis of testosterone metabolism was adapted from the method described by Bornheim and coworkers (1987). Testosterone and its metabolites were separated using reverse phase HPLC and quantitated by

Table 4.9 Selective functional probes for P450 subfamilies and isozymes

Isozyme or subfamily	Functional probe
CYP1A1	7-Ethoxyresorufin O-deethylation
CYP 2A1	Testosterone 7α-hydroxylation
CYP2A2	Testosterone 15α-hydroxylation
CYP2B1	7-Pentoxyresorufin O-depentylation Testosterone 16β-hydroxylation Testosterone 16α-hydroxylation
CYP2C6	S-Warfarin 7-hydroxylation Progesterone 21-hydroxylation
CYP2C11	Testosterone 2α-hydroxylation Testosterone 16α-hydroxylation
CYP2C12	Steroid disulfate 15β-hydroxylation
CYP2C13	Progesterone 6β-hydroxylation Progesterone 16α-hydroxylation
CYP2D	Debrisoquine 4-hydroxylation
CYP2E1	Chlorzoxazone 6-hydroxylation
CYP3A	Testosterone 6β-hydroxylation
CYP4A	Lauric acid ω-hydroxylation

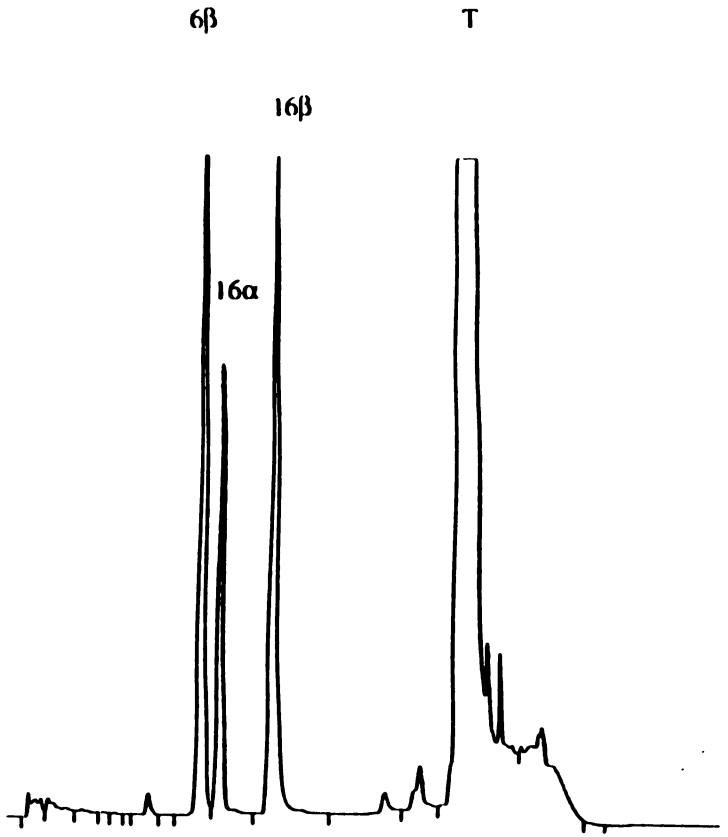
scintillation counting. Prior to extraction, 5 µl of each of the cold authentic metabolites, 6β-, 16α-, and 16β-hydroxylated testosterone, was spiked into every sample. The mixtures were vortexed and centrifuged at 1000 rpm for 5 min. The top layer was recovered for later use. To the bottom layer, 1 ml of ethyl acetate was added. The sample was capped and agitated vigorously in a VWR multitube vortexer for 5 min. After the sample was centriguged at 1000 rpm for 5 min, the top layer was carefully pipetted out and pooled with the top layer saved earlier. The procedure was repeated again for the third time, and all the ethyl acetate layers were pooled and evaporated to dryness under nitrogen.

Two solvent mixtures were used as the mobile phases in the testosterone HPLC assay. Solvents A and B were solutions of methanol:acetonitrile:deionized water in volume to volume proportions of 43:1.1:55.9, and 75:1.9:23.1, respectively. A gradient delivery was achieved according to table 4.10. Solvent was delivered by two Shimadzu LC600 liquid chromatographs (Shimadzu, Santa Clara, CA) at a constant flow rate of 1 ml/min. The stationary phase was a Rainin Microsorb C18 column (5 µ x 4.6 mm x 25 cm). The elution of metabolites and drug were monitored at 254 nm using a Shimadzu SPD-10A UV spectrophotometric detector (Shimadzu, Santa Clara, CA). Figure 4.5 shows a chromatogram of the elution pattern of the 3 cold metabolites spiked into the samples. The fractions representing 6β-, 16α-, and 16β-hydroxylated testosterone were collected in a scintillation vial, and 10 ml of a scintillation cocktail, Scintiverse LC, was added to the fraction. The vial was shaken to mix the contents, and the radioactivity of the fraction was counted using a Beckman LS7000 scintillation counter.

Table 4.10 Gradient solvent delivery program used in testosterone assay

Time[b] (min)	Flow rate (ml/min)	% B (%)	Duration[a] (min)
0	1	20	0
3	1	37.5	15
18	1	60	8
26	1	100	1.5
32	1	20	2
48	0	20	0.1

[a]Duration represents the amount of time that was taken to make a linear change in %B to the %B value specified at a certain time[b] point in the run.



6β: 6β-hydroxy-testosterone; 16α: 16α-hydroxy-testosterone; 16β: 16β-hydroxy-testosterone; T: Testosterone
Retention time for 6β-, 16α-, 16β-hydroxy-testosterones are 12.8, 14.2, and 17.1 min, respectively. Retention time for testosterone is 30.4 min.

Figure 4.5 Chromatogram of an extracted blank microsomal sample spiked with 6β-, 16α-, and 16β-hydroxy-testosterone and testosterone (T).

4.b.11 Spectral determination of substrate binding to P450

Because CYP3A was implicated as a FK 506 metabolizing enzyme, the type of binding between the substrate, FK 506, and the isozyme present in microsomes was investigated. The procedure for recording the binding spectrum differ from that for the carbon monoxide binding study, as no carbon moxide is bubbled into the liver microsomes, and no dithionite was used to reduce the samples. The baseline of the spectrum was set by placing identical samples (microsomes in 0.1 M phosphate buffer) in both the sample and reference cells of the spectrophotometer. The absorbance change was observed when substrate was added to the sample cell, and an equal volume of the solvent, which was used to dissolve the substrate, was added to the reference cell.

4.b.12 Effect of gender on the formation of 13-desmethyl FK 506

Several P450 isoforms are gender specific. Male rats, for example, have constitutive isozymes such as CYP2A2, 2C11, 2C13 and 3A2 (table 4.2). Female rats, on the other hand, have constitutive CYP2C12 (table 4.2). Because naive adult female rats do not have CYP3A2, they may serve as a negative control for CYP3A mediated reactions. Female Sprague-Dawley rats were used in this experiment. All conditions in this study were identical to those described for male rats in section *4.b.7.*

4.b.13 Kinetic study of the rate of formation of 13-desmethyl FK 506

In order to determine the optimal incubation conditions for the kinetic studies of the rate of formation of 13-desmethyl FK 506, preliminary studies were performed to find the protein concentrations and incubation periods over which the rate of formation of this metabolite was linear. The incubation mixture, composed of FK 506 (10 μM), 0.1 M phosphate buffer and liver microsomes from untreated rats (0.25 - 4mg/ml), was pre-incubated for 5 min at 37°C. The reaction was initiated by the addition of NADPH (1 mM). After incubating the untreated microsomes for 5 min, the reactions were stopped by the quick freezing method. In a separate incubation, untreated microsomes at 1 mg protein/ml was incubated in the presence of 1 mM NADPH. Samples were taken at 1, 3, 5 and 7 min to obtain a time course for the metabolite formation.

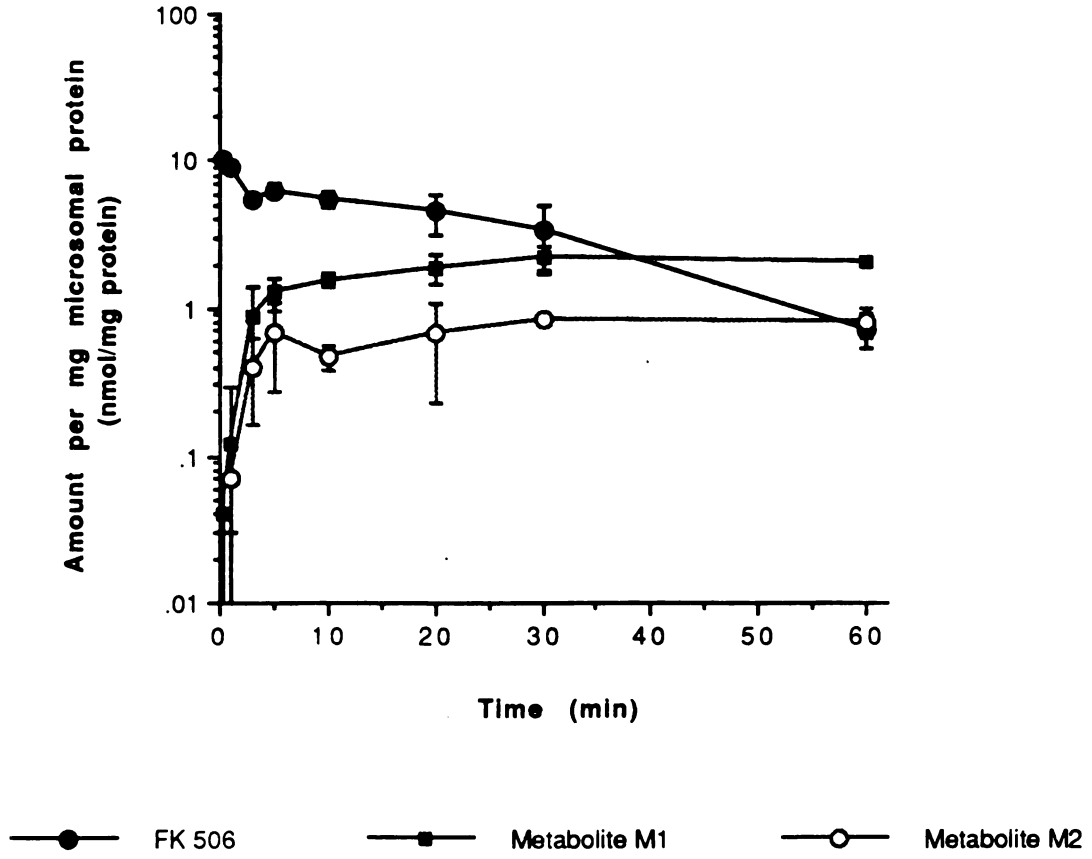
The kinetic study was carried out using incubation mixtures (0.3 ml) composed of liver microsomes from untreated male or female rats (2 mg protein/ml) in 0.1 M phosphate buffer and FK 506 (1 - 40 μM), which were preincubated for 5 min at 37°C. The reaction was initiated by the addition of NADPH (1mM), and were allowed to proceed for 5 min. The reaction was stopped by quick freezing the sample in an acetone-dry ice mixture. All samples were stored at -40°C until analysis.

4.c Results

When FK 506 (5 μM) was incubated in rat hepatocytes (5 million cells/ml) in a time course study for 60 min, two metabolites were formed. Metabolites M1 and M2 corresponded respectively to the 13-desmethyl FK 506 and the interconversion product of 13-desmethyl FK 506 which were described in chapter 3. Figure 4.6 shows the time course of FK 506 metabolism in untreated rat hepatocytes (n=3). Microsomal protein concentrations (table 4.11) were calculated from the hepatocytes used in the experiment. The amount of parent drug and metabolites were normalized to mg of microsomal protein. The parent FK 506 declined in a log linear manner, with a half-life of 14 ± 3.6 min. The two metabolites formed plateaued after 30 min.

When FK 506 was incubated in the liver cytosols from 6 fasted rats in the presence of GSH (2.5 mM) to test for GSH-dependent reactions, no metabolism was observed. No FK 506 metabolite formation was seen in the hepatic mitochondrial fraction from 6 fasted rats, in the presence of a NADPH generating system, 1 mM NAD and FK 506. In microsomes, FK 506 was metabolized in the presence of a NADPH-generating system, and formed two metabolites in the same fashion as in the hepatocytes.

Metabolism of FK 506 by liver microsomes was NADPH dependent. In control experiments wherein NADPH was not added to the incubation mixture, no metabolic activity of FK 506 was observed. In addition to being NADPH-dependent, FK 506 metabolism was also shown to be oxygen-dependent in the carbon monoxide inhibition study. When carbon monoxide



FK 506 (5 μM) was incubated in hepatocytes at 5 million cells/ml. The sampling volume was 0.6 ml.

Figure 4.6 Time course of FK 506 in isolated rat hepatocytes

Table 4.11 Liver microsomal protein and P450 content in hepatocytes prepared from Sprague-Dawley rats

rat #	nmol P450 per mg microsomal protein	pmol P450 per million cells
1	1.02	92
2	1.68	150
3	1.17	106
mean	1.29	116
sd	0.35	30

was bubbled into the incubation mixture for at least 15 min before the addition of NADPH, no metabolism of FK 506 occurred (n=2). The requirements of microsomal protein, NADPH, and oxygen in FK 506 metabolism indicated that the enzyme involved in FK 506 metabolism was a microsomal P450 enzyme.

In order to determine which P450 isozyme was involved in FK 506 metabolism, male Sprague-Dawley rats were treated with several xenobiotics to selectively inhibit or induce several P450 isozymes. FK 506 metabolic activity, as well as marker activities of isozymes were measured in the liver microsomes of the treated animals.

Rats treated with xenobiotics usually display a change in their total microsomal P450 contents, where the type and magnitude of changes depend on the xenobiotics used, and the level of induction achieved experimentally. The mean P450 concentration of liver microsomes from untreated and treated rats used in the experiments are reported in table 4.12. Dexamethasone and phenobarbital treatments in male rats significantly ($p<0.005$) induced the total P450 content of liver microsomes by 2 fold in both cases over the untreated male rats. Treatment with 3-methyl cholanthrene also induced the level of total P450 by 2.2 fold. Treatment with CYP3A inhibitor, ethinyl estradiol, did not significantly increase the level of total P450. Untreated female rats had a significantly ($p<0.05$) lower level of total P450 as compared to their male counterparts.

The relative formation of 13-desmethyl FK 506 in the FK 506 (40 μM) incubation with liver microsomes from the untreated and the variously

Table 4.12 P450 concentration in rat liver microsomes from various treatment groups

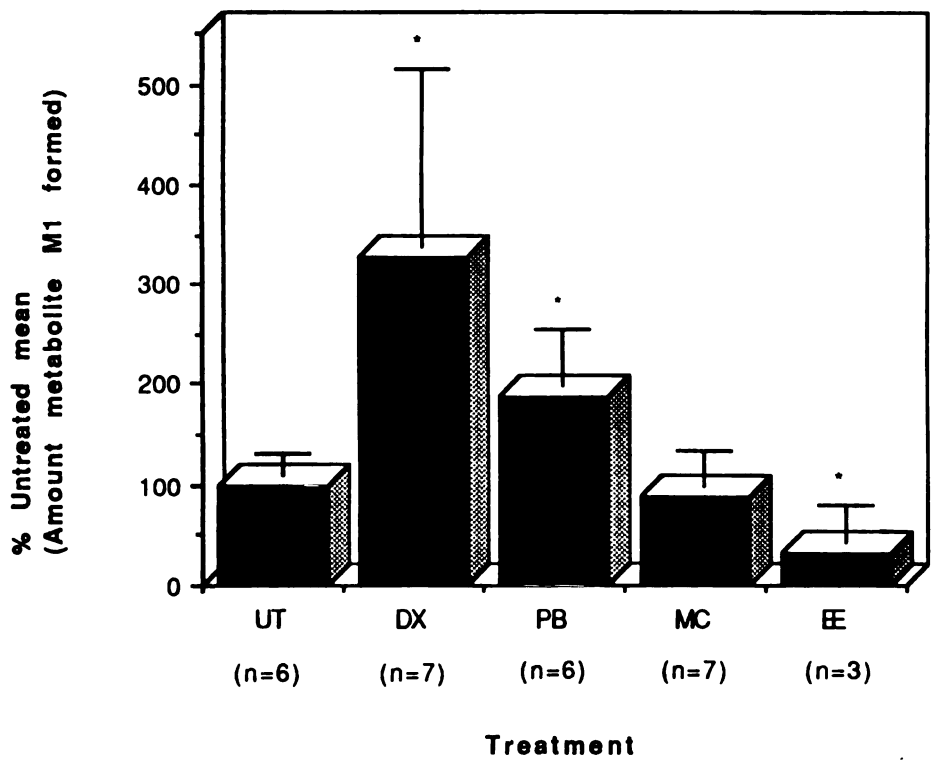
Treatment	P450 concentration (nmol/mg microsomal protein)					
	Male			Female		
	Mean	SD	N	Mean	SD	N
Untreated	1.43	0.58	30	0.87[a]	0.16	19
Dexamethasone	2.94[a]	0.71	18			
Phenobarbital	2.94[a]	0.62	14			
Ethinyl Estradiol	1.45	0.39	6			
3-Methyl Cholanthrene	3.19[a]	0.31	7			

[a] Statistically different ($P<0.005$, unpaired *t*-test) from untreated male rats

treated rats was examined. As shown in figure 4.7, dexamethasone treatment significantly ($p<0.05$) induced the appearance of the measured metabolite. Phenobarbital treatment also significantly ($p<0.05$) increased the appearance of the metabolite (figure 4.7). Treatment with ethinyl estradiol significantly inhibited the formation of the metabolite (figure 4.7). 3-Methylcholanthrene treatment did not change the amount of metabolite formed, when compared to the untreated group.

The level of enzyme induction by xenobiotics was confirmed by marker activities of selective P450 isozymes. Since 3-methylcholanthrene treatment did not have an effect on the formation of 13-desmethyl FK 506, it is assumed that CYP1A, which is normally induced by 3-methyl cholanthrene, is not involved in FK 506 metabolism. Testosterone 6β-hydroxylase activity was used as a functional marker of CYP3A, while testosterone 16β-hydroxylase activities were measured as functional markers of CYP2B. The activity of testosterone 16α-hydroxylase could be contributed by CYP2B, CYP2C6, CYP2C7, and CYP2C11.

The activities of each functional probe are summarized in table 4.13. As expected, treatment with dexamethasone which induced mainly CYP3A, but also CYP2B, significantly ($p<0.05$) increased the average testosterone 6β-, 16α-, and 16β-hydroxylase activities. Treatment with phenobarbital, an inducer primarily for CYP2B and also a general inducer, significantly ($p<0.05$) increased the average testosterone 6β-, 16α, and 16β-hydroxylase activities as well. Ethinyl estradiol, an inhibitor of CYP3A and CYP2C11 (Prueksaritanont et al., 1993), significantly ($p<0.05$) decreased the activity of testosterone 6β- and 16α-hydroxylase activities. As previously reported



UT: untreated; DX: dexamethasone-treated; PB: phenobarbital-treated; MC: 3-methylcholanthrene-treated; EE: ethinyl estradiol-treated.
*Statistically different ($p<0.05$, unpaired t-test) from the mean of the untreated group.
Microsomal protein concentration was 100 µg/ml.

Figure 4.7 Comparison of metabolite M1 formed in FK 506 incubations (40 µM) among the untreated and various treatment groups

Table 4.13 Testosterone hydroxylase activity in liver microsomes prepared from rats with various treatments

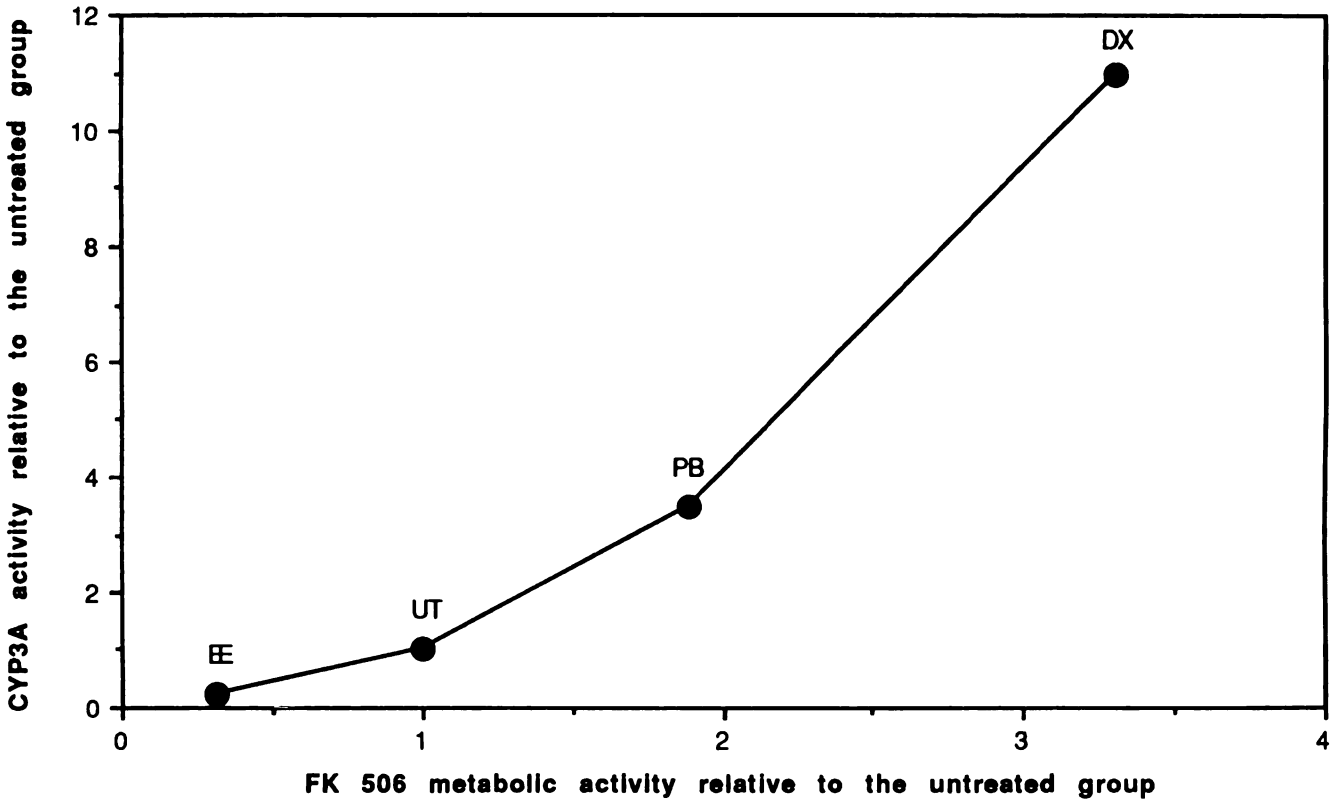
Treatment	n	Testosterone hydroxylase activity (nmol/min/mg protein) 6β	16α	16β
UT	6	2.76 **0.57**	1.73 **0.25**	0.07 **0.04**
DX	7	31.04* **6.20**	2.22* **0.40**	1.09‡* **0.54**
PB	7	9.63* **2.13**	2.54* **0.46**	1.28* **0.37**
EE	3	0.63* **0.32**	0.43* **0.10**	0.04 **0.01**

UT: untreated; DX: dexamethasone-treated; PB: phenobarbital-treated;
EE: ethinyl estradiol-treated.
Values in plain type denote the mean, and values in bold denote the standard deviation.
*Significantly ($p<0.05$, unpaired t-test) different from the respective value of the untreated group.
‡n=6

(Prueksaritanont et al., 1993), treatment with ethinyl estradiol did not significantly affect the activity of testosterone 16β-hydroxylation.

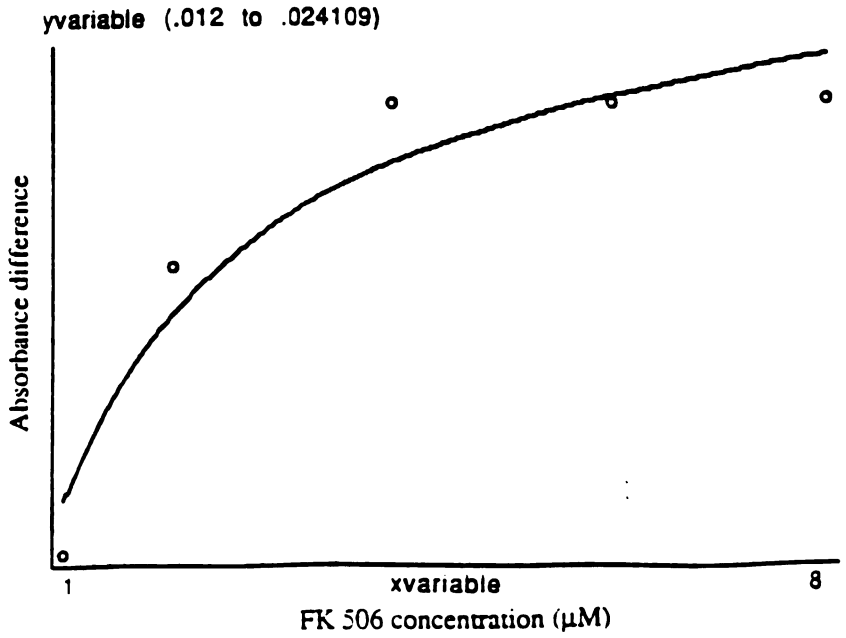
The extent of CYP3A induction by phenobarbital (350%), as measured by testosterone 6β-hydroxylase activity, is much lower than that by dexamethasone (1130%). This is consistent with the fact that dexamethasone is a more selective CYP3A inducer than phenobarbital. Treatment with ethinyl estradiol, a CYP3A inhibitor (Guengerich, 1988), significantly reduced the level of CYP3A activity to 22% of the untreated value. As depicted in figure 4.8, a positive correlation existed between the FK 506 metabolic activity and the CYP3A activity. Increases or decreases of FK 506 metabolic activities corresponded with the CYP3A activities from various CYP3A inducers and inhibitors.

Because CYP3A was indicated as the isozyme responsible for the formation of 13-desmethyl FK 506, the binding spectra of FK 506 to microsomal P450 from untreated and dexamethasone-treated rat livers were compared. As shown in figure 4.9, substrate interaction is a concentration dependent-saturable process, with half-maximal binding being independent of microsomal concentration (Schenkman et al., 1982). In liver microsomes prepared from dexamethasone treated rats (n=2), a type I spectral change (figure 4.10a), with an absorption maximum at 390 nm and an absorption minimum at 420 nm, was induced by FK 506. The absorbance changes at various substrate concentrations from one of the two experiments performed were fitted to the Michaelis-Menten equation using Minim 1.8 (figure 4.9). At 1 mg microsomal protein/ml, the half-maximal binding constant (Ks) was found to be 1.0 μM, which is similar to the 1.6 μM reported by Sattler et al.

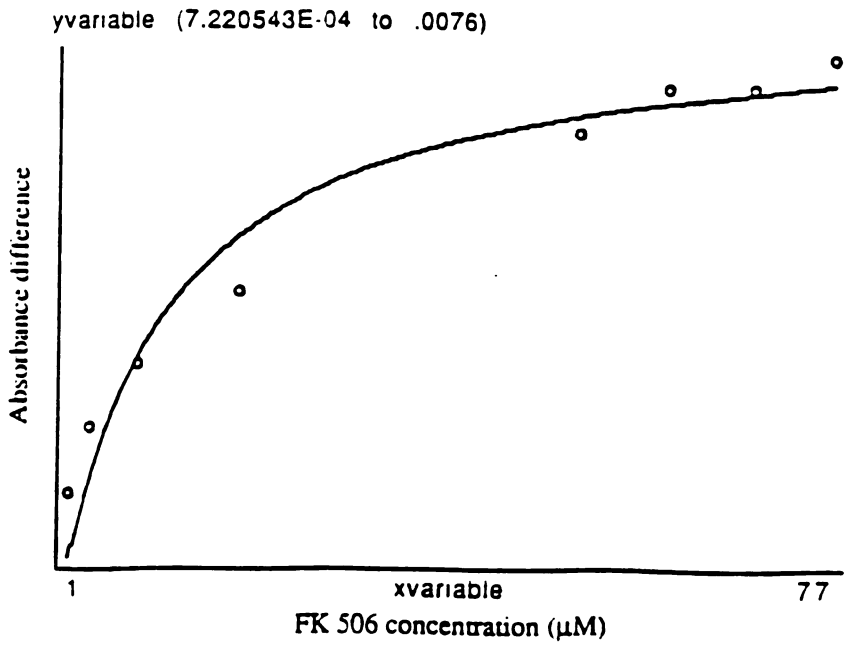


EE: ethinyl estradiol-treated; UT: untreated; PB:phenobarbital-treated; DX: dexamethasone-treated.
FK 506 metabolic activity was measured as the rate of formation of 13-desmethyl FK 506
CYP3A activity was measured as the rate of testosterone-6β hydroxylation.

Figure 4.8 Relationship between FK 506 metabolic activity and CYP3A activity

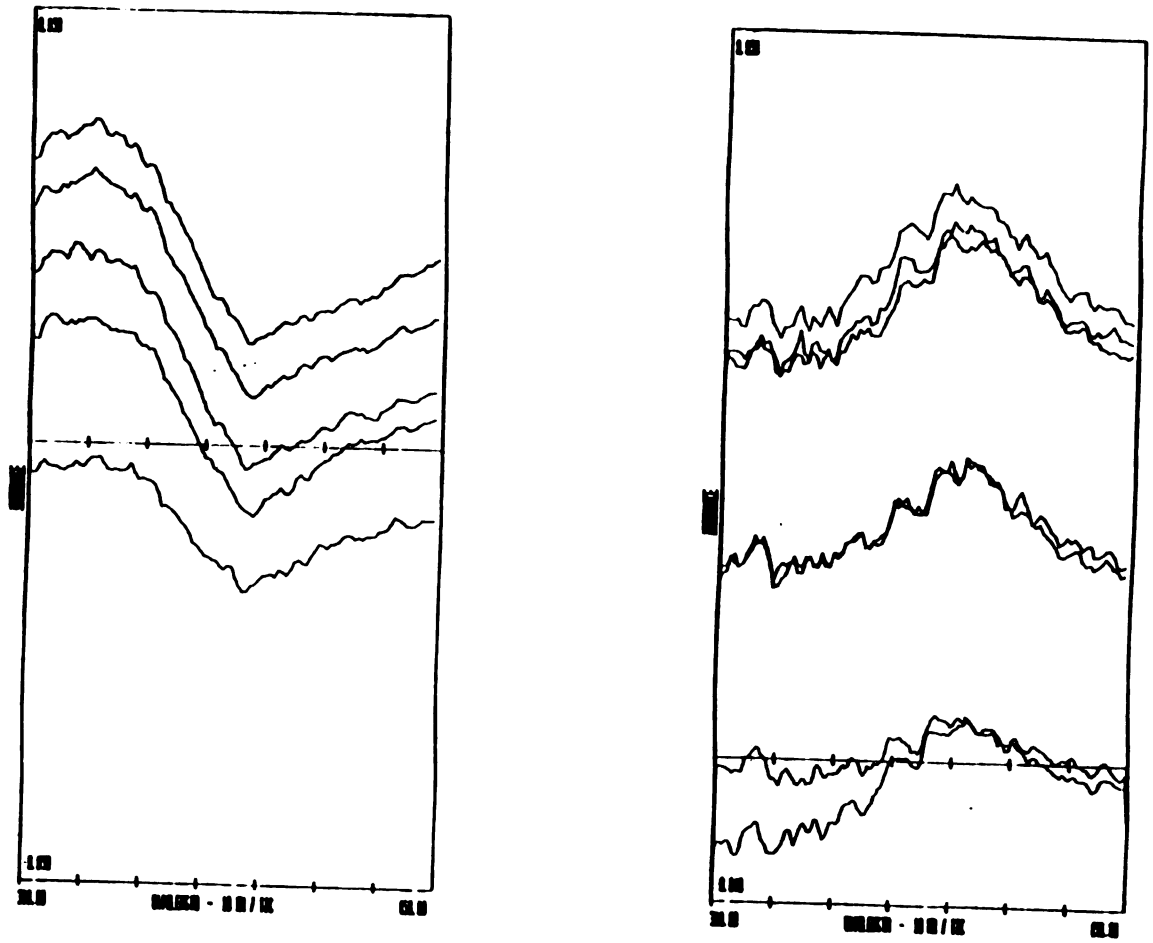


(a)



(b)

Figure 4.9 Saturable interaction between FK 506 and P450 from (a) dexamethsone-treated and (b) untreated male rat liver microsomes.



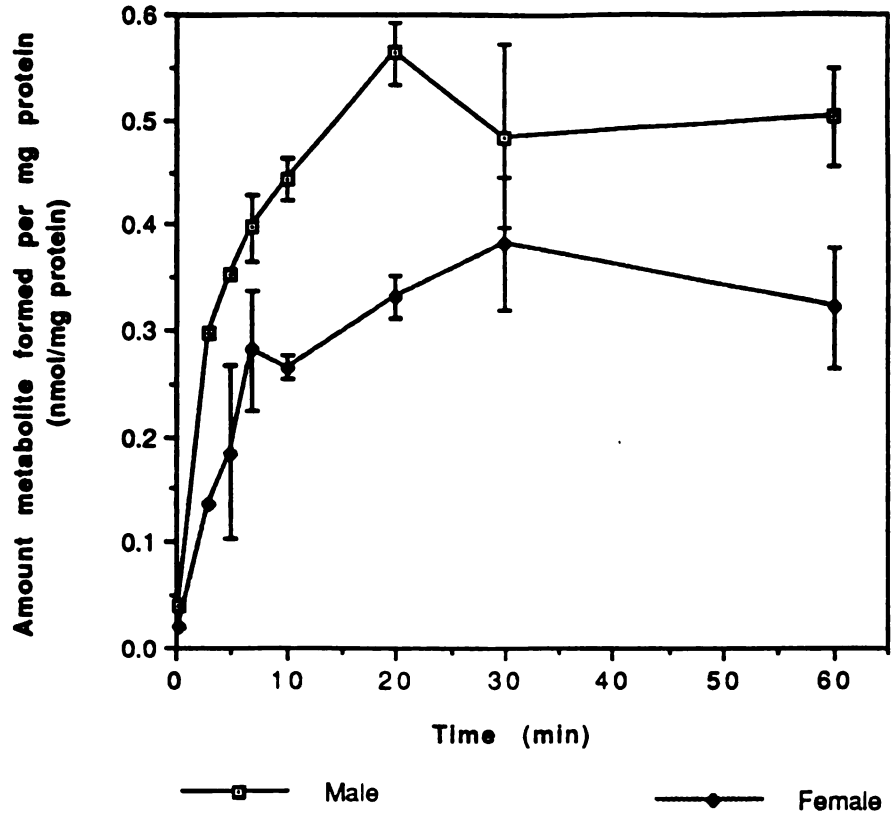
(a) (b)

Figure 4.10 Spectral changes induced by FK 506 in (a) dexamethasone-treated and (b) untreated male rat liver microsomes

(1992). In untreated rat liver microsomes (n=2), FK 506 induced a reverse type I spectral change with an absorption maximum at 420 nm and a minimum at 388-390 nm (figure 4.10b). The Ks was found to be approximately 10 μM in untreated male rats. Although no data was shown, Vincent et al. (1992) noted that no spectral change was observed in untreated rat liver microsomes, in contrast to the reverse type I spectral change reported here.

If CYP3A was the only enzyme involved in the formation of 13-desmethyl FK 506, untreated female rats that do not contain any constitutive CYP3A (Namkung et al., 1988; Waxman et al., 1985) would not be able to metabolize FK 506 into 13-desmethyl FK 506. Female rats characteristically have lower ($p<0.005$) total P450 content than the untreated male rats as previously shown in table 4.12, as well as minimal levels of CYP3A activity, as indicated by significantly lower testosterone 6β-hydroxylase activity ($p<0.005$) relative to untreated male rats (15%). These testosterone 6β-hydroxylation values are in agreement with those previously reported by others (Prueksaritanont et al., 1993; Sonderfan et al., 1987). When FK 506 (5 μM) was incubated with female rat liver microsomes that supposedly lack CYP3A activity, 13-desmethyl FK 506 was formed unexpectedly (figure 4.11). While the time course of formation of metabolite M1 appeared to be similar to that in male rats, a lower level of the metabolite was formed in the female rats.

In order to gain a better understanding of the enzyme involved in FK 506 metabolism in female rats, the kinetics of the relative formation for 13-desmethyl FK 506 was determined in male and female rats. The conditions



FK 506 (5 μM) was incubated with liver microsomes at 2 mg microsomal protein/ml. The sampling volume was 0.6 ml.

Figure 4.11 Comparison of the relative formation of metabolite M1 by male and female rat liver microsomes

used in the kinetic study were optimized with respect to incubation time and protein concentration. FK 506 over a concentration range of 1 to 40 μM was incubated with 0.3 ml of untreated rat liver microsomes (2 mg protein/ml) for 5 minutes. All samples were analyzed using the HPLC assay with the flow step-up procedure (chapter 2). Figures 4.12a and 4.12b depict the rates of formation of 13-desmethyl FK 506 with respect to time and protein concentration. Figure 4.13 depicts the initial rates of FK 506 metabolite formation in hepatic microsomes from male and female rats. The rates of formation of the metabolite M1 increased linearly at low substrate concentrations, and plateaued at higher concentrations, yielding a hyperbolic curve characteristic of saturable substrate binding. The data obtained were fitted to the Michaelis-Menten equation $v = V_{max} \cdot C_u / (K_M + C_u)$ using the fitting program Minim 1.8. The results are summarized in table 4.14. The V_{max} values, normalized to mg protein, for the formation of metabolite M1 by male and female rat liver microsomes (n = 7 each) were 0.66 ± 0.47 nmol/min/mg protein and 0.28 ± 0.15 nmol/min/mg protein, respectively. The K_M values were 24 ± 18 μM for male rat liver microsomes and 24 ± 16 μM for female rat liver microsomes. Since these samples were measured using the flow step-up procedure described in section *2.c*, the values for the kinetic parameters, V_{max}, were divided by 1.5 to adjust for the analytical discrepancy due to flow rate. The adjusted values are also summarized in bold in table 4.14.

Using the adjusted V_{max} and K_M values reported in table 4.14, pharmacokinetic parameters for the metabolism of FK 506 based on the formation of the measured metabolite were estimated. In the calculation for the maximum intrinsic clearance (CL_{int}):

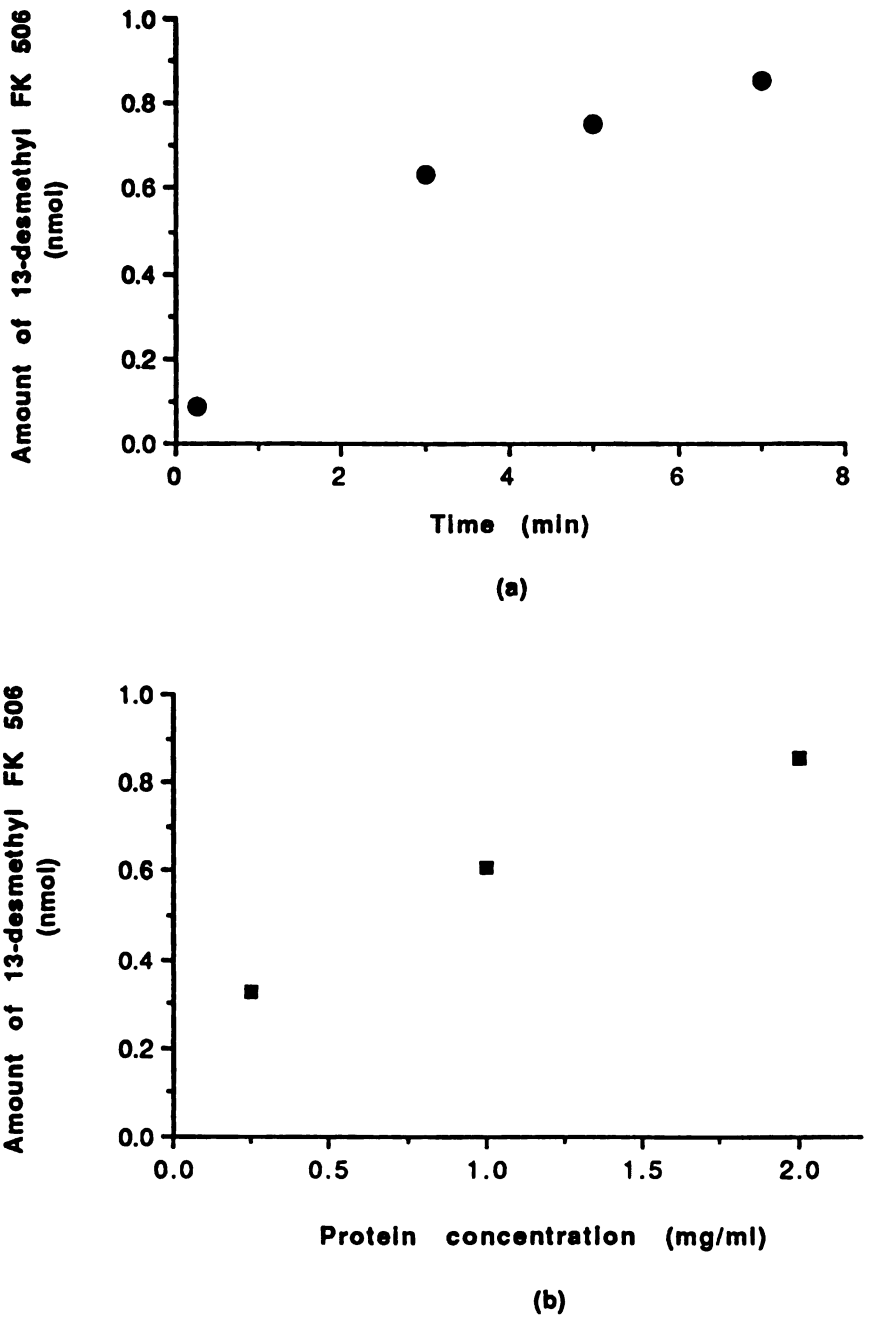


Figure 4.12 (a) Time- and (b) protein-dependent formation of 13-desmethyl FK 506 by untreated rat liver microsomes

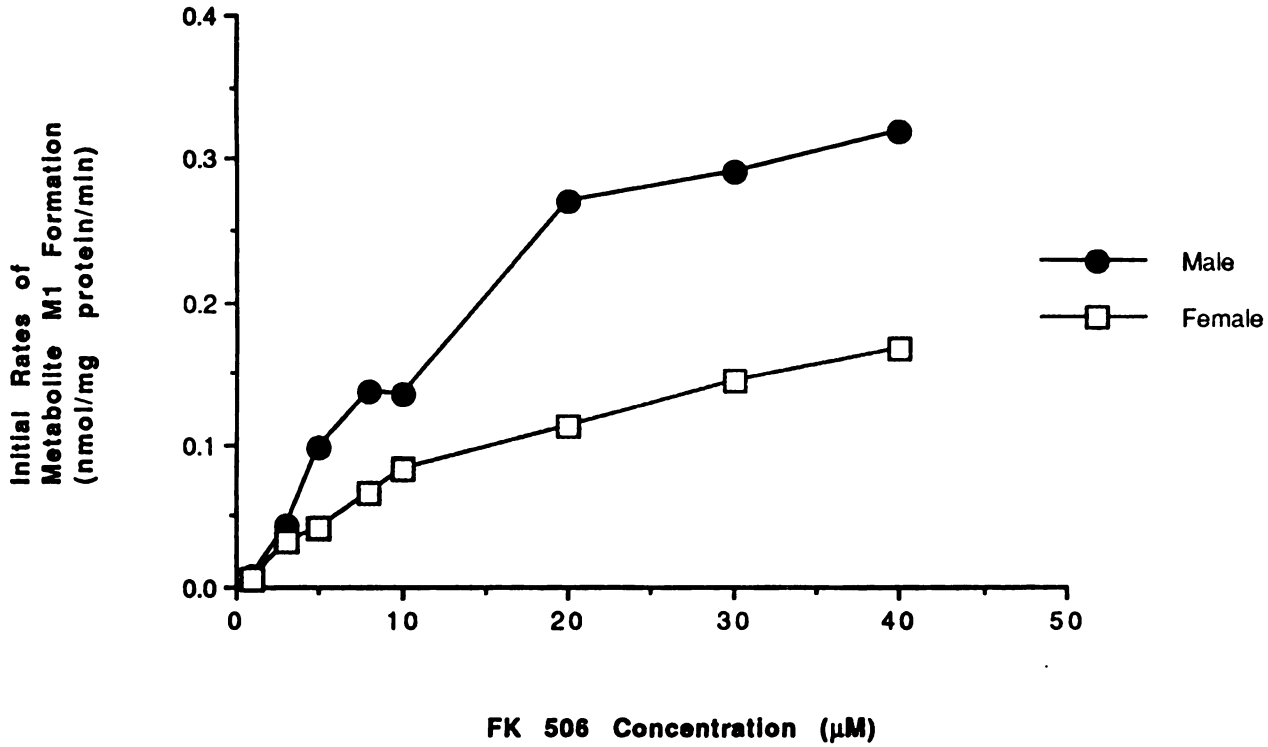


Figure 4.13 Initial (5 min) rates of 13-desmethyl FK 506 formation at various FK 506 concentrations by liver microsomes from male and female rats

Table 4.14 Kinetic values for the formation of 13-desmethyl FK 506 by liver microsomes

Group	V_{max} (nmol/mg protein/mg) mean	sd	K_M (μM) mean	sd
Untreated females n=7	0.28 **0.19**	0.15 **0.10**	24	16
Untreated males n=7	0.66 **0.44**	0.47 **0.30**	24	18

Values in bold type reflect the experimentally determined Vmax values divided by 1.5 to adjust for the analytical discrepancy due to flow rate as discussed in section *2.c.*

$$CL_{int,hep} = V_{max} / K_M$$

V_{max} was multiplied by 40 mg of hepatic microsomal protein per g rat liver (Joly et al., 1975), and the liver wet, yielding ml/min as the unit for CL_{int}. Liver wet weights averaged 12 g and 9 g in 300 g untreated male and female Sprague-Dawley rats, respectively. Hepatic blood clearance can be calculated according to the well-stirred model (Pang and Rowland, 1977) by the following equation:

$$CL_{hep} = (Q_{hep} \cdot f_{ub} \cdot CL_{int,hep}) / (Q_{hep} + f_{ub} \cdot CL_{int,hep})$$

Hepatic blood flow used in the calculation of clearance (CL_{hep}), was calculated by the allometric relationship described by Boxenbaum (1982):

$$Q_{hep} = 0.0554\ B^{0.894}$$

a hepatic blood flow value of 18.8 ml/min was assumed for a 300 gram rat. The value for fraction unbound, f_{ub} = 0.335 used in the calculation of hepatic clearance was obtained by dividing fraction unbound in plasma (f_{up}) (0.57) (Habucky et al., 1992) by the blood to plasma ratio (Cb/Cp) of 1.7 (Habucky et al., 1992). The clearance of FK 506 was estimated from the clearance associated with the formation of the metabolite:

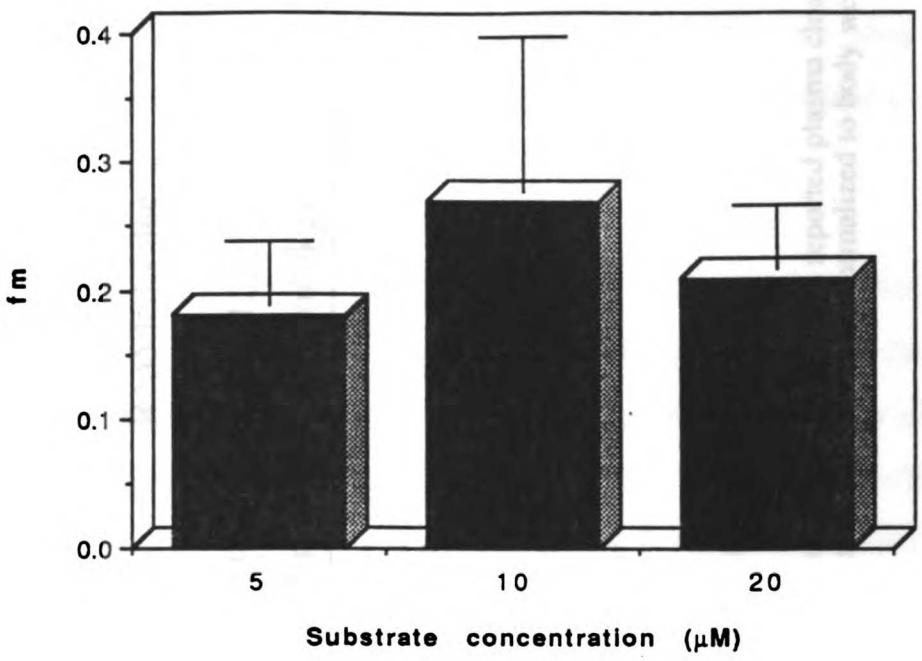
$$CL = CL_m / f_m$$

where f_m was found to be approximately 0.25 in untreated rats (figure 4.14). The report of Vincent and coworkers (1992) noted the f_m in untreated rats to be approximately 0.3, a value slightly higher than that observed here.

Table 4.15 lists the predicted values for hepatic intrinsic clearance (CL_{int}), hepatic blood clearance of FK 506 associated with the formation of metabolite M1 (CL_m), and total hepatic blood clearance (CL_{tot}). The total hepatic blood clearance (CL_{tot}) in male rats, 34 ml/min per kg body weight, predicted from the pharmacokinetic analysis appears to roughly approximate the total blood clearance (CL_{tot}), 23.2 ml/min per kg body weight, found *in vivo* by Takada et al. (1992). The *in vivo* total blood clearance was obtained by dividing the reported total plasma clearance, 13.8 ml/min (Takada et al., 1992), by the blood to plasma ratio (C_b/C_p) of 1.7 (Habucky et al., 1992) and normalized to body weight (0.35 kg) (Takada et al., 1992).

4.d Discussion

Isolated rat liver hepatocytes provide an attractive model to study the metabolic events of FK 506 in the liver *in vitro*. Maintaining the precise internal architecture and the permeability of the plasma membrane, hepatocytes allow metabolic reactions to occur in an orderly sequence inside the cell. Because intact cells retain all the membrane bound and soluble enzymes, both phase 1 and phase 2 metabolic reactions can occur in hepatocytes (Guillouzo, 1986). In using subcellular fractions such as the microsomes, the leakage of cytosolic materials, due to the disruption of the plasma membrane, makes the supplement of cofactors necessary for both phase 1 and 2 reactions. Because NADPH was the only cofactor added to



FK 506 (5, 10, and 20 μM) were incubated with untreated rat liver microsomes (1 mg/ml) for 10 min in the presence of 1mM NADPH.
f_m represents the ratio of the amount of metabolite formed to the amount of drug lost (n=7).

Figure 4.14 Fraction of drug lost converted to metabolite M1

Table 4.15 Predicted and reported values for clearance of FK 506

Groups	Predicted value			Reported value
	CL_{int} (ml/min/kg)	CL_m (ml/min/kg)	CL_{tot} (ml/min/kg)	CL_{tot} (ml/min/kg)
Untreated males	29.3	8.5	34	23[a]
Untreated females	9.7	3.0	12	NA

CL_{int} is the intrinsic clearance associated with the formation of metabolite M1.
CL_m is the clearance of FK 506 associated with the formation of metabolite M1.
CL_{tot} is the total clearance of FK 506.
[a] Plasma clearance (CL_p) of 13.8 ml/min from 350 g rats was reported by Takada et al. (1993). The reported plasma clearance was converted to blood clearance by dividing CL_p by the blood to plasma ratio in rats (1.7), and normalized to body weight (0.35 kg).
NA: No CL_{tot} has been reported in female rats.

the microsomal mixture in these experiments, the similar metabolic pattern in hepatocytes and the liver microsomes suggests that the two observed metabolites are formed via phase 1 oxidation reactions by cytochrome P450 instead of phase 2 conjugation reactions.

The NADPH- and oxygen-dependency of the FK 506 metabolic activity in the liver microsomal fraction suggests cytochrome P450 to be the metabolizing enzyme. Although flavin monooxygenase (FMO) is also a microsomal enzyme that is NADPH- and oxygen dependent, it is unlikely that FMO is the FK 506 metabolizing enzyme since the substrates for FMO are usually amines, hydrazines and organic sulfur compounds (Ziegler, 1988).

The results from the enzyme induction experiment in male rats supports the role of CYP3A in the formation of 13-desmethyl FK 506, as previously demonstrated in immunoinhibition studies by Vincent et al. (1992) in rat liver microsomes using anti-rat CYP3A IgG, and by Sattler et al. (1992) in human liver microsomes using rabbit anti-CYP3A4. The importance of CYP3A in FK 506 metabolism notwithstanding, results from the untreated female rat experiment revealed that enzyme(s) other than the currently known constitutive CYP3A2 and inducible CYP3A1 can metabolize FK 506 in female rats.

Another immunosuppressant, cyclosporine, which was thought to be primarily metabolized by CYP3A (Bertault-Peres et al., 1987; Combalbert et al., 1989), was also found to be metabolized in female rats (Prueksaritanont et al., 1993) lacking constitutive CYP3A. Earlier data from Prueksaritanont

and coworkers (1993) pointed to the possible involvement of CYP2C6/7 in both female and male rats. Preliminary findings of Jaeger[4.1] recently identified a CYP2C6-like isoform by N-terminal sequencing as the cyclosporine metabolizing isozyme purified from ethinyl estradiol treated female rats. It is, however, unlikely that the same CYP2C6-like isoform is responsible for FK 506 metabolism in female rats because less FK 506 metabolite was formed after ethinyl estradiol treatment in male rats, in contrast to the higher rate of cyclosporine metabolite formation after ethinyl estradiol treatment of male rats (Prueksaritanont et al., 1993).

The possibility of yet another CYP3A isoform metabolizing FK 506 in female rats cannot be ruled out. Recently, Gemzik et al. (1992) described the developmental changes in testosterone 6β-hydroxylation by liver microsomes from female Sprague-Dawley rats. In agreement with findings in this and other reports (Arlotto et al., 1987; Namkung et al., 1988; Sonderfan et al., 1987), Gemsik's data showed that female rats beyond 6 weeks of age exhibit low levels of testosterone 6β-hydroxylase activity. Nevertheless, by immunoblotting, Gemzik et al. (1992) also showed the presence of a small amount of a 51 kDa protein in adult female rat liver microsomes (25 μg), and a consistent level of this protein in adult male rats. While the identity of this 51 kDa protein was unknown, it was recognized by a polyclonal anti-rat CYP3A1 (Gemzik et al., 1992). During the preparation of this thesis, Strotkamp et al. (1993) also reported the possible existence of a CYP3A protein in liver microsomes from female rats. Thus, it is possible that the lower rate of FK 506 metabolism observed in adult female rats is due to a low level of expression of this 51 kDa protein.

4.1 Dr. Walter Jaeger, University of California-San Francisco, personal communication.

In contrast to the conclusion drawn in this chapter from the 3-methylcholanthrene induction studies that CYP1A is not a FK 506 metabolizing enzyme, Vincent et al. (1992) noted the possible involvement of CYP1A in FK 506 metabolism because of a 35% decrease in the total amount of metabolite formed when untreated rat liver microsomes were immunoinhibited with anti-rat CYP1A antibodies. However, Stiff et al. (1992) also demonstrated no significant changes in FK 506 metabolism after CYP1A induction by 3-methylcholanthrene, or CYP1A inhibition by α-napthoflavone. Such contrasting results could be due to possible crossreactivity of the anti-CYP1A antibody used, or possibly less than 100% specificity of enzyme inducers and inhibitors, such as 3-methylcholanthrene and α-napthoflavone, respectively. Although no definite conclusion can be drawn from such contradictory results, the fact that CYP1A is present in female rats warrants the verification of the possible involvement or non-involvement of CYP1A in FK 506 metabolism in female rats.

While untreated male rat liver microsomes consist primarily of CYP3A2 (25%), CYP2C6 and CYP2C11 (Waxman et al., 1985), glucocorticoid-treated male rat liver microsomes consist of a large percentage of CYP3A1 (46%) (Cooper et al., 1993; Waxman et al., 1985). Due to the very different P450 isozyme composition in the untreated and the dexamethasone-treated microsomes, it is perhaps not surprising that FK 506 induced different types of binding spectra in the two types of microsomes, with correspondingly different half-maximal binding constants. Moreover, in contrast to a reverse type I spectral change seen here, Vincent et al. (1992) noted no spectral change with liver microsomes from untreated rats. Their observation might have resulted from the cancellation between the type I and reverse type I

spectral changes due to the CYP2C and 3A isozymes present in their microsomal preparation.

V_{max} and K_M values derived from the kinetic studies facilitated the estimation of total clearance of FK 506 in rats within 50% of the *in vivo* value. This approach of predicting *in vivo* clearance from *in vitro* data seems to be adequate, considering the differences in the measurements between different laboratories.

Chapter 5

Sequential Metabolism of 13-Desmethyl FK 506

5.a Introduction

As discussed in section *4.c*, 13-desmethyl FK 506 is believed to be formed by CYP3A in rats. Because CYP3A can be specifically induced by dexamethasone (Wrighton et al., 1985), an increase in the amount of hepatic CYP3A enzymes in livers from rats treated with dexamethasone should give an increase in yield of 13-desmethyl FK 506, when FK 506 was incubated in hepatic microsomes.

Experiments conducted using liver microsomes from dexamethasone-treated rats, however, showed a decreased formation of 13-desmethyl FK 506 at increasing protein concentrations. In this chapter, several experiments were performed to attempt to understand the pharmacological and kinetic implications of this phenomenon.

5.b Materials and methods

FK 506 was kindly supplied by Fujisawa Pharmaceutical Company (Deerfield, IL). Dexamethasone was a gift from The Upjohn Company (Kalamazoo, MI). Testosterone and NADPH were obtained from Sigma Chemical Company (St. Louis, MO). Purified mouse cyp3A protein and anti-rat CYP3A antibody were gifts from Dr. Lester M. Bornheim from the Department of Pharmacology, University of California-San Francisco, and Dr. Steve A. Wrighton from the Lilly Research Laboratory, respectively. Biorad protein assay kit with albumin protein standard, and all chemicals used for electrophoresis and protein transfer were obtained from Biorad

(Hercules, CA). All other reagents were of reagent grade and were obtained from Fisher Scientific.

Male Sprague-Dawley rats, weighing 250-300 g, were obtained from Bantin and Kingman (San Leandro, CA). Dexamethasone-treated rats were given dexamethsaone at 100 mg/kg per day intraperitoneally for four consecutive days. All dexamethasone-treated rats were sacrificed by decapitation twenty-four hours after the last dose. Liver microsomes preparation and protein determination were performed according to the methods described previously in sections *4.b.3* and *4.b.4*, respectively.

5.b.1 Kinetic study of the rate of 13-desmethyl FK 506 formation by liver microsomes of dexamethasone-treated rats

In order to demonstrate that induction of CYP3A isozymes in microsomes from dexamethasone-treated rats increase the rate of 13-desmethyl FK506 formation, a kinetic study was performed. The kinetic parameters obtained from the dexamethasone-treated rats in this experiment were compared with those obtained from the untreated rats as described in chapter 4. To determine the optimal incubation conditions for this kinetic evaluation, studies were performed to determine the protein concentrations and incubation periods over which the rate of formation of this metabolite is linear. One group of incubation mixtures, composed of FK 506 (80 μM) in 0.1 M phosphate buffer with microsomes from dexamethasone-treated rats (10 - 200 μg protein/ml), was pre-incubated for 2 min at 37°C. The reaction was initiated by the addition of NADPH (1 mM). After an incubation period

of 5 min, the reactions were stopped by quickly freezing the samples in an acetone-dry ice mixture. A second group of incubation mixtures consisted of FK 506 (10 μM) in 0.1 M phosphate buffer with microsomes from dexamethasone-treated rats (100 - 2000 μg protein/ml). The reaction was initiated by the addition of NADPH (1mM) after a pre-incubation period of 2 min at 37°C. The reaction proceeded for 10 min. A third group of dexamethasone-treated microsomes was incubated with FK 506 (80 μM) at 100 μg protein/ml in the presence of NADPH (1 mM). Samples were taken at 0.3, 0.7, 1, 3, 5, 7, and 10 min.

The kinetic study was carried out using incubation mixtures (0.3 ml) composed of microsomes from dexamethasone-treated male rats (100 μg protein/ml) in 0.1 M phosphate buffer and FK 506 (3 - 120 μM), which were preincubated for 1 min at 37°C. The reaction was initiated by the addition of NADPH (1 mM), and was allowed to proceed for 3 min. The reaction was stopped by the quick freezing method. All samples were stored at -40°C until analysis following the analytical procedure described in section *2.b*.

5.b.2 Time course for the formation of 13-desmethyl FK 506 by liver microsomes from dexamethasone-treated rats

FK 506 (2 μM) was preincubated with liver microsomes (100 μg/ml) from dexamethasone-treated rats in 0.1 M phosphate buffer containing diethylenetriamine penta-acetic acid (DETAPAC) (1 mM) and dithiothreitol (DTT) (1 mM) for 2 min. The reaction was initiated by the addition of an NADPH generating system which consisted of NADPH (1mM), magnesium chloride (5 mM), glucose 6-phosphate (10 mM), and glucose 6-phosphate

dehydrogenase (2 U/ml). Samples (0.3 ml) were taken at 0.5, 1, 2, 3, 5, 7, 10, and 15 min. The reaction was stopped by the quick freezing method. All samples were frozen at -40°C until analysis by the method described in section *2.b.*

5.b.3 Time course of the metabolism of isolated 13-desmethyl FK 506

Incubation mixtures containing microsomal protein (100 μg/ml) prepared from dexamethasone-treated rat livers, DTT (1 mM) and DETAPAC (1 mM) in 0.1 M phosphate buffer were pre-incubated at 37°C in a Dubnoff metabolic shaking incubator for 2 min. 13-Desmethyl FK 506 (2 μM) was then added to the incubation mixtures. One minute after the addition of 13-desmethyl FK 506, the reaction was started by the addition of an NADPH generating system. Samples (0.3 ml) were taken at 0.25, 3, 5, 7, 10, and 20 minutes. The reaction was stopped by the quick freezing method. All samples were stored at -40°C until analysis as described in section *2.b.*

Two control experiments were performed to confirm that the disappearance of 13-desmethyl FK 506 was indeed a P450 mediated reaction instead of a non-enzymatic reaction. Two identical experiments as that described in the paragraph above were repeated, with either NADPH or microsomal protein replaced by 0.1 mM phosphate buffer.

5.b.4 Metabolism of 13-desmethyl FK 506 by functionally reconstituted mouse cyp3A in vitro

Mouse hepatic P450 3A (25 pmol) was mixed with rat liver NADPH-P450 reductase (1000 U), phosphatidylserine (100 μg), cytochrome b5 (50 pmol), 0.1% emulgen 911 (50 μl), and 13-desmethyl FK 506 (2 μM) in 0.1 M phosphate buffer, in a final volume of 1 ml (table 5.1). After preincubation at 37°C for 3 min, the reaction was initiated with NADPH (1 mM) and carried out for 15 min. The reaction was stopped by quick freezing, and the samples were analyzed as described in section *5.b.3*. A control expertiment was performed by replacing the 13-desmethyl FK 506 with FK 506 (5 μM) which was shown to be a CYP3A substrate.

5.b.5 Raising antibodies against mouse cyp3A isozyme

Mouse cyp3A was sent to Caltag (Healdsburg, CA), a commercial company which performs immunization treatment on animals. Sera obtained from the pre-immunized and immunized rabbit were subjected to a purification step using a Hi-Trap affinity column (Pharmacia Biotech, Inc., Alameda, CA) as described below in section *5.b.6*. The purity of these antibodies raised against mouse P450 3A was tested by using these antibodies to probe purified CYP3A, and the presence of P450 3A in dexamethasone-treated male rat liver microsomes, and untreated male and female rat liver microsomes that were separated using 9% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970), and electrophoretically transferred onto a nitrocellulose sheet (immunoblotting) (Towbin et al., 1979).

5.b.6 Purification of antibody

Anti-sera received from Caltag in a frozen state was allowed to thaw at room temperature, and then kept on ice during the purification process. Both the pre-immune sera and the anti-sera were separately passed through a Millipore 0.45 μm type HA filter which was mounted onto a small circular stainless steel filter adapter, via a syringe. A Hi-Trap affinity column was then washed and equilibrated with approximately 100 ml of 0.1M Tris/HCl, pH 8.0 (12.1 g Tris/HCl per liter). Two 5 ml volumes of pre-immune anti-sera were passed through the Hi-Trap affinity column (5 ml capacity). The effluent was collected, labelled and saved at -4°C. Aliquots (5 ml) of 0.1 M Tris/HCl (pH 8.0) were passed through the column. The absorbance of each aliquot was measured at 280 nm. The column was continuously washed with 0.1 M Tris/HCl (pH 8.0) until the absorbance returned to baseline. Suprasil quartz cuvets HS1000 (Fisher Scientific, Santa Clara, CA) were used.

Approximately 4 to 4.8 ml 1M Tris/HCl, pH 8 solution was put into each test tube to be used for IgG collection. If both pre-immune IgG and immunized IgG were to be purified on the same day, the pre-immune IgG was processed first. The IgG was eluted from the Hi-Trap affinity column, 1 ml at a time with a 0.1 M glycine/HCl, pH 2.5 solution. The mixing of the acidic IgG and the basic Tris/HCl solution helped quickly neutralize the acidic IgG. The neutralized IgG was vortexed, the absorbance measured at 280 nm, and the pH was checked. The elution step was considered complete when the absorbance returned to the baseline level. The pH of most fractions were found to be between 6.5 to 8. Prior to the processing of the

next batch of sera or storage at -4°C, the Hi-Trap affinity column was washed thoroughly with 0.1 M Tris/HCl, pH 8.0 (approximately 40 ml).

The absorbance of each fraction was plotted against the cumulative volume of the fractions. The fractions contributing to the absorbances were pooled and concentrated in an Amicon concentrator (Amicon Inc., Beverly, MA) fitted with an Amicon Diaflo^{R} Ultrafilter (YM 30 membrane).

5.b.7 Immunoblot analysis

The antibody (20 µl) prepared in section *5.b.6* was diluted into 10 ml of an antibody buffer [1% gelatin in a Tween-20 wash solution (TTBS)]. TTBS was prepared by adding 0.5 ml Tween-20 to 1 liter of TBS. The nitrocellulose paper containing electrophoretically transferred proteins was then incubated on a shaker platform in 10 ml antibody buffer for 1 to 2 hours. When the incubation was complete, the nitrocellulose paper was rinsed twice with TTBS.

A commercially available secondary antibody (goat anti-rabbit antibody) conjugated with alkaline phosphatase (Biorad, Hercules, CA) was diluted by a factor of 3000 into 10 ml antibody buffer. After the nitrocellulose paper was incubated with the secondary antibody for 1 hour, it was rinsed twice with TTBS, and once with TBS. A color developing agent (Biorad, Hercules, CA) [(100 µl each of reagent A and B diluted into 10 ml of alkaline phosphatase color developing buffer (0.1 M Tris)] was added to the blot and was agitated for about 2-10 min on a rocker platform. The color

developing reaction was stopped by quickly removing the nitrocellulose paper to a water wash.

5.b.8 Immunoinhibition study

Immunoinhibition reactions were carried out using rabbit anti-mouse cyp3A antibodies, rabbit pre-immune IgG, goat anti-rat CYP3A antibodies, and goat pre-immune IgG. Microsomal protein from rat liver was incubated with the IgGs at various IgG to protein ratios (0, 10, and 20 using anti-mouse cyp3A antibodies, and 0, 10, and 40 using anti-rat CYP3A antibodies) in the presence of DTT (1 mM) for 30 min at room temperature on a Mistral Multi-mixer (Labline Instruments, Inc., Melrose Park, IL). After DETAPAC (1 mM) in 0.1 M phosphate buffer and 13-desmethyl FK 506 (1.53 nmol) were added to the microsomal mixtures, the mixtures were preincubated at 37°C for 1 min. The metabolic reaction of 13-desmethyl FK 506, conducted in a total volume of 300 µl, was initiated by the addition of a NADPH generating system. A total of 2.5 µg of dexamethasone-treated microsomal protein was used. After a reaction period of 120 min, the reactions were stopped and the samples were analyzed.

5.c Results

When FK 506 (10 µM) was incubated with untreated rat liver microsomes for 10 min, the amount of 13-desmethyl FK 506 obtained increased almost linearly over the protein concentration range of 100 to 500

μg/ml (figure 5.1). Between 500 and 2000 μg/ml, the rate of 13-desmethyl FK 506 formed began to slow as predicted by saturable kinetics. Within this same range of protein concentrations (100-2000 μg/ml) of microsomes from dexamethasone-treated rats, a decreasing amount of 13-desmethyl FK 506 was obtained.

When FK 506 (80 μM) was incubated with liver microsomes prepared from dexamethasone-treated rats for 5 min, the amount of 13-desmethyl FK 506 formed appeared linear with respect to protein concentrations over the low range of 10 - 200 μg/ml (figure 5.2). The rate of formation of 13-desmethyl FK 506 also appeared linear with respect to the incubation times studied (figure 5.3). Thus, optimal incubation conditions for the kinetic study of 13-desmethyl FK 506 formation (100 μg protein/ml and 3 min incubation) were established in the dexamethasone-treated rat liver microsomes.

The initial rates of 13-desmethyl FK 506 formation in the dexamethasone-treated group increased linearly at low substrate concentration, but plateaued at higher concentrations (figure 5.4). The data obtained were fitted to the Michaelis Menten equation $v = V_{max} \cdot C_u / (K_M + C_u)$ using the program Minim 1.8. The V_{max} and K_M were 2.81 ± 1.07 nmol/min/mg protein, and 29 ± 11 μM, respectively (n=4). To adjust for the analytical discrepancy due to flow rate as discussed in section *2.c*, the experimentally determined V_{max} was divided by 1.5, yielding a value of 1.87 ± 0.71 nmol/min/mg protein for V_{max}.

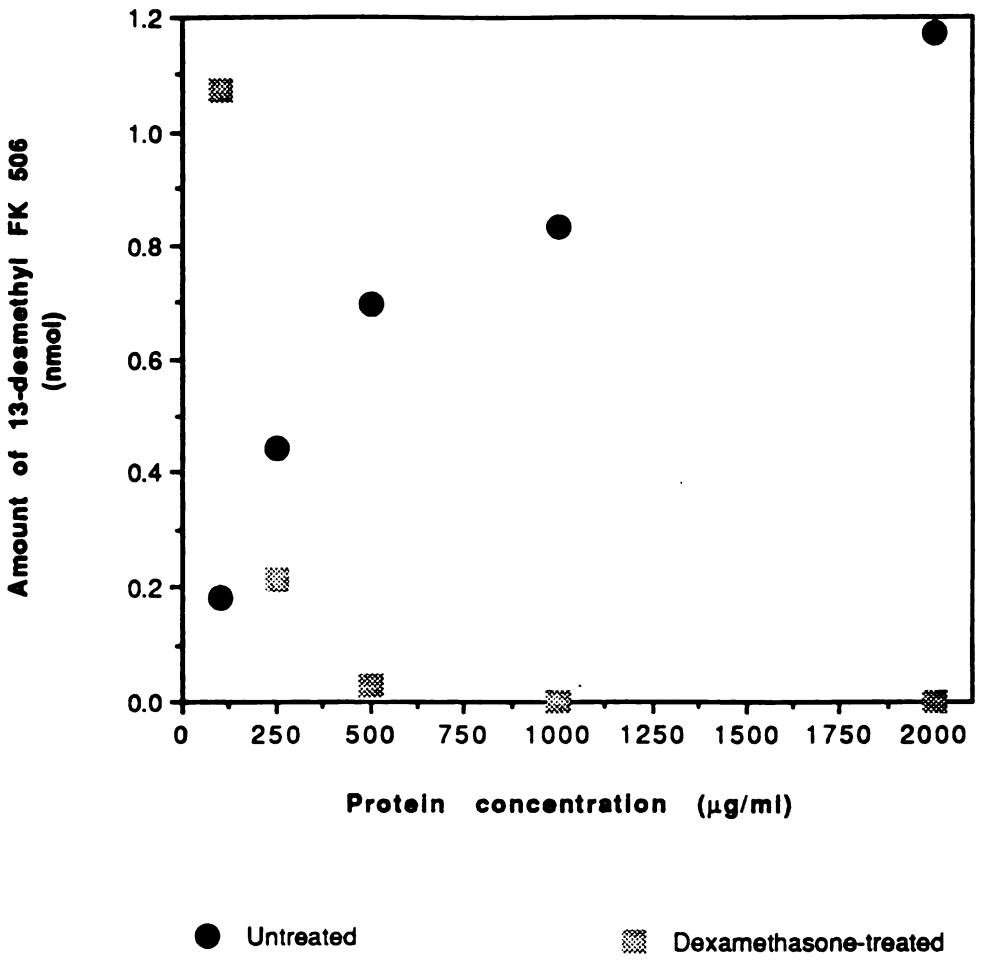


Figure 5.1 Formation of 13-desmethyl FK 506 over the protein concentration range of 100 to 2000 µg/ml of untreated and dexamethasone-treated rat liver microsomes in FK 506 incubations (10 µM) for 10 min

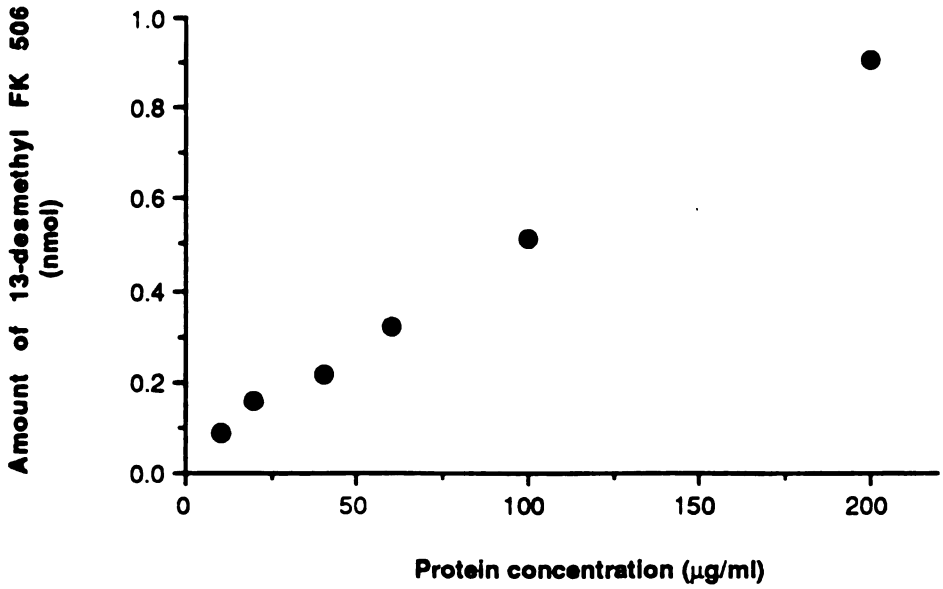


Figure 5.2 Formation of 13-desmethyl FK 506 over the protein concentration range of 10 to 200 µg/ml in dexamethasone-treated rat liver microsomes in FK 506 incubations (10 µM) for 5 min

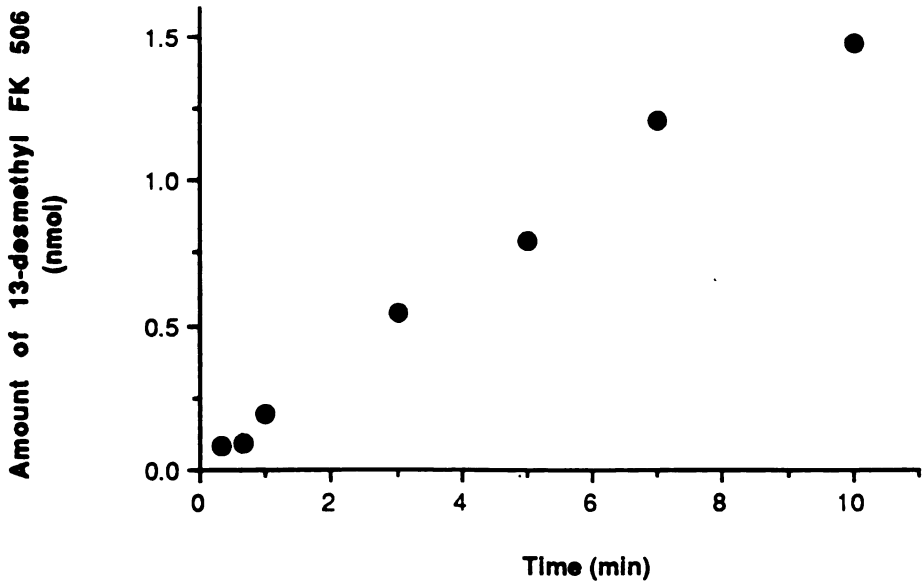


Figure 5.3 Formation of 13-desmethyl FK 506 in FK 506 incubations (10 μM) over an incubation period of 0.5 to 10 min in dexamethasone-treated rat liver microsomes (100 μg/ml)

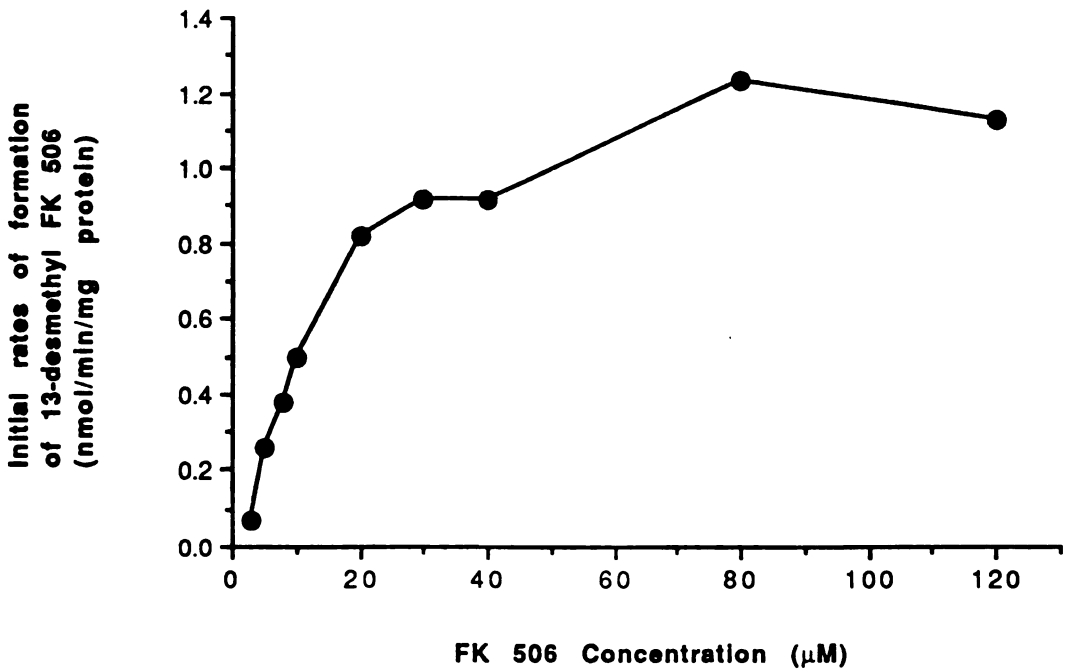


Figure 5.4 Initial (3 min) rates of formation of 13-desmethyl FK 506 at various FK 506 concentrations by liver microsomes (100 μg/ml) from dexamethasone-treated male rats

Figure 5.5 shows that FK 506 (2 μM) incubated with liver microsomes from dexamethasone-treated rats (100 μg/ml) exhibited a log linear decline with a half-life of 1.4 ± 0.4 min (n=8). The level of 13-desmethyl FK 506, metabolite M1, increased with time up to approximately 4 min. Subsequently, it decreased with a terminal half-life of 9.7 ± 2.5 min (n=6). When the metabolite time profile was analyzed by the method of residuals, the residual plot yielded a half-life of 1.4 ± 0.43 min (n=6), corresponding to the half-life of the parent FK 506.

When 13-desmethyl FK 506 (2 μM) was incubated in the presence of NADPH with liver microsomes (100 μg/ml) from dexamethasone-treated rats, it disappeared in a log linear manner (figure 5.6) with a half-life of 8.9 ± 1.3 min (n=8). When the cofactor NADPH was omitted from the system, no metabolism of 13-desmethyl FK 506 occurred.

An attempt was made to reconstitute the metabolism of 13-desmethyl FK 506 and FK 506 using purified mouse cyp3A isozyme. Neither of these two compounds was metabolized in the reconstituted mouse cyp3A system.

When microsomal protein and purified CYP3A were probed with the antibody purified as described in section *5.b.6*, a single band near 50-52 kDa appeared in the purified rat CYP3A sample (lane 8), and the dexamethasone-treated samples (lanes 9, 10 and 11) (figure 5.7). The density of the protein bands increased with the increasing amount of microsomal protein added (lanes 9, 10, and 11). Similar bands expected in the untreated male group were not detectable, possibly due to insufficient protein loading. No protein band was detected in the female groups. All the proteins that were present in

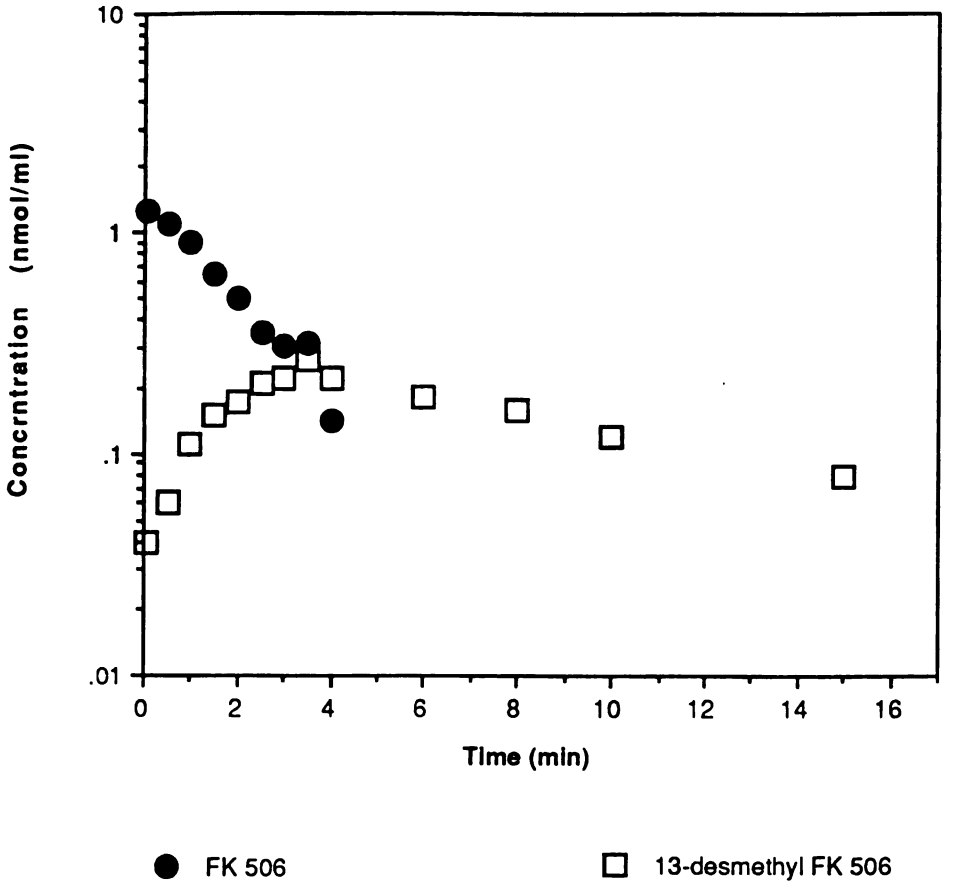


Figure 5.5 Time course for the disappearance of FK 506 and the formation of 13-desmethyl FK 506 in incubations with liver microsomes (100 μg/ml) from dexamethasone-treated rats

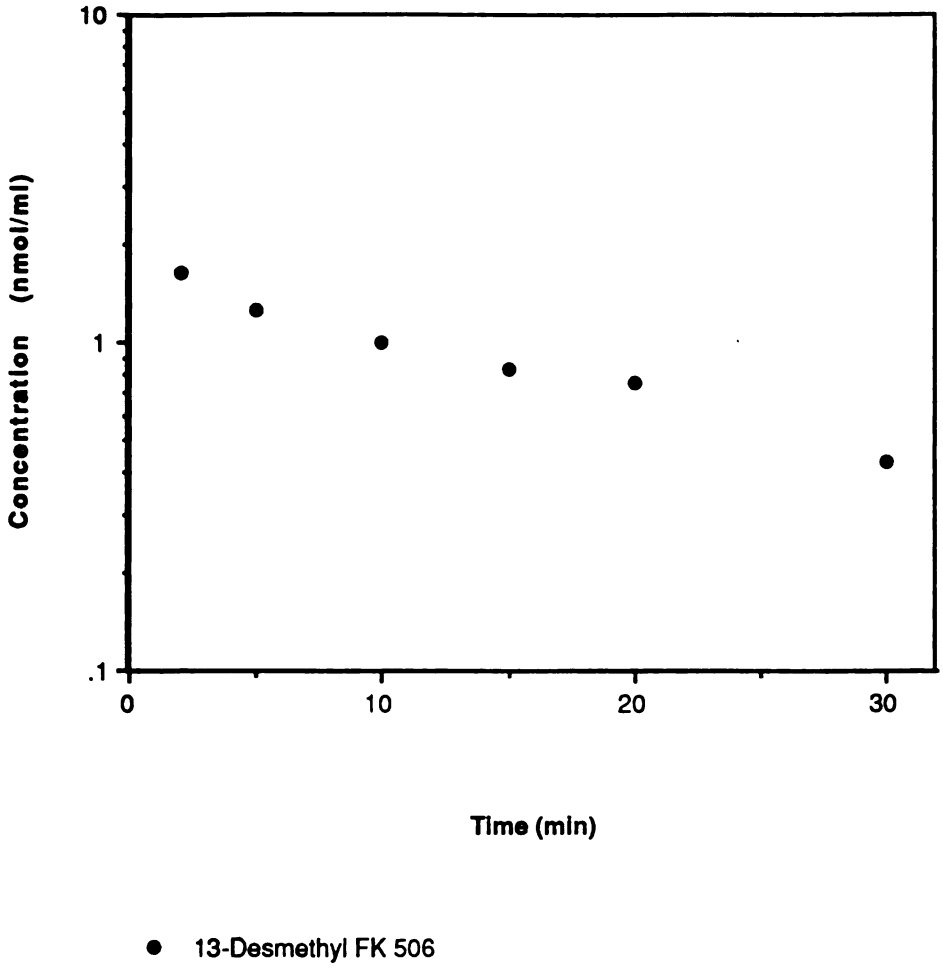
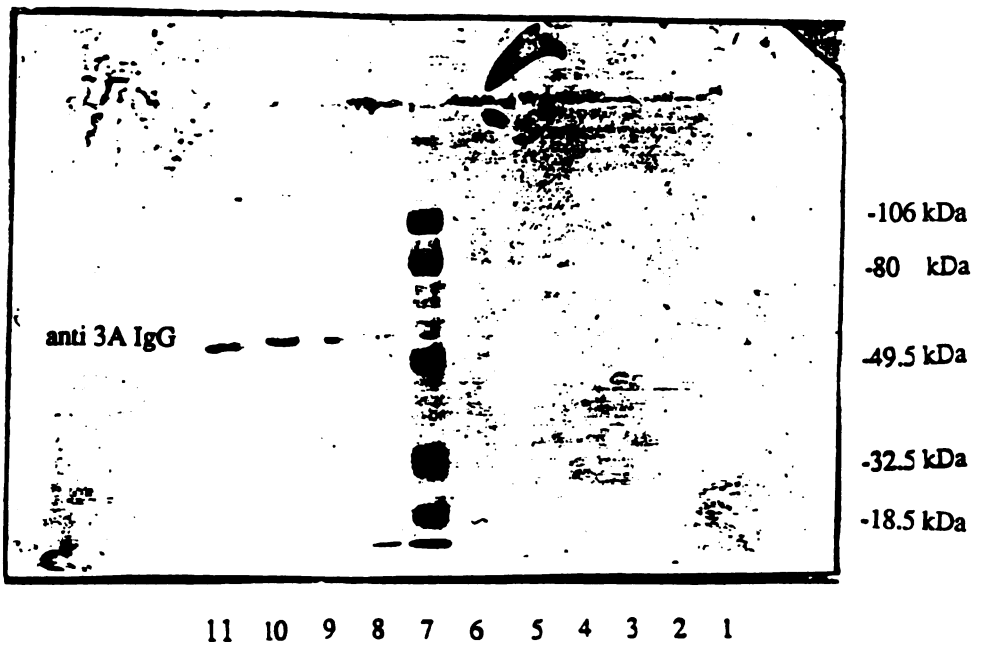


Figure 5.6 Time course of the disappearance of 13-desmethyl FK 506 (2 μM) in incubations of liver microsomes from dexamethasone-treated rats



Lanes 1, 2, and 3 contained untreated female rat liver microsomes (1, 2, and 4 μg, respectively). Lanes 4, 5, and 6 contained untreated male rat liver microsomes (1, 2, and 4 μg, respectively). Lanes 7 and 8 contained standards-low range and purified rat CYP3A (0.8 pmol). Lanes 9, 10, and 11 were dexamethasone-treated male rat liver microsomes (1, 2, and 4 μg, respectively). Microsomes from untreated male and female rats did not react with the rabbit anti-mouse cyp3A antibody, probably due to insufficient loading.

Figure 5.7 Immunoblot of purified rat CYP3A and liver microsomes from dexamethasone-treated male rats, as well as untreated male and female rats, using antibodies raised against purified mouse cyp3A

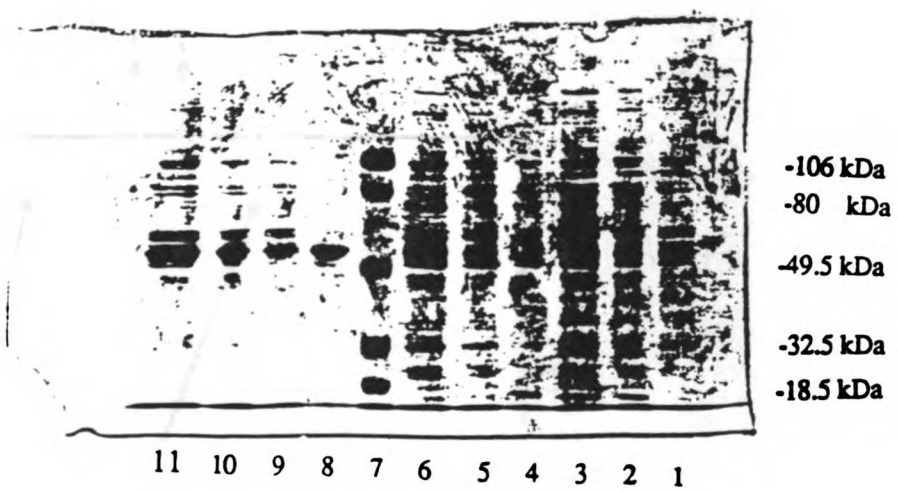
the microsomal samples were separated by SDS-PAGE, and stained separately by Coomasie brilliant blue as shown in figure 5.8.

Immunoinhibition studies using anti-mouse P450 3A antibodies and goat anti-rat CYP3A antibodies showed dose-dependent inhibition of 13-desmethyl FK 506 metabolizing activity. The metabolism of 13-desmethyl FK 506 by dexamethasone-treated microsomes, which were pre-incubated with anti-mouse P450 3A antibodies at mg-IgG to mg-protein ratios of 10 and 20, was inhibited to 63 and 42% of the original activity (figure 5.9). Pre-immune IgG inhibited the metabolism by 22% at 20 mg-IgG/mg-protein (figure 5.9).

When goat anti-rat CYP3A antibody was pre-incubated with dexamethasone-treated microsomes at 10 and 40 mg-IgG/mg-protein , the metabolism of 13-desmethyl FK 506 was inhibited to 55 and 0 % of the original activity as depicted in figure 5.10. Pre-incubation with pre-immune IgG reduced the metabolic activity to 78 and 55 % of the original activity (figure 5.10).

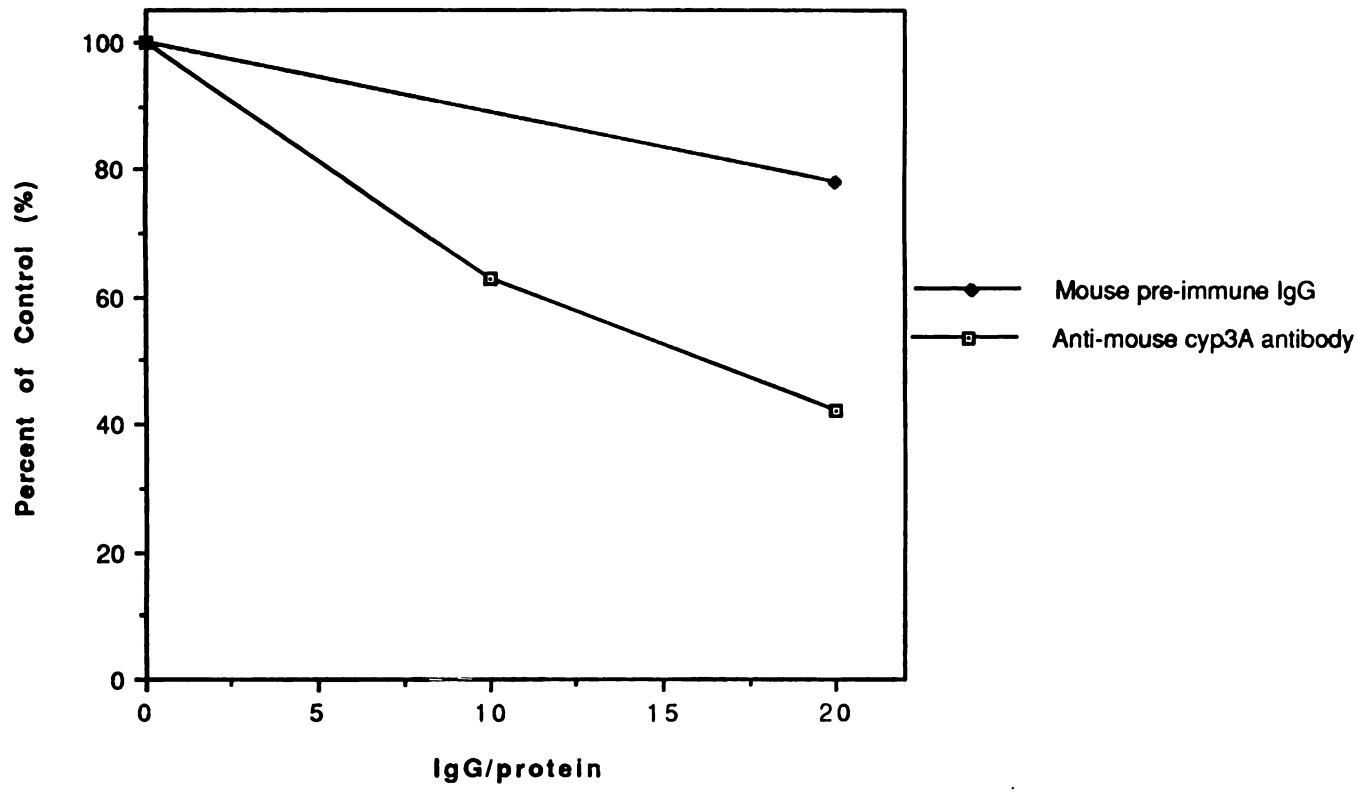
5.d Discussion

The higher ($p \leq 0.001$) rate of 13-desmethyl FK 506 formation by dexamethasone-treated microsomes than untreated microsomes as shown in the kinetic studies presented in this chapter and chapter 4 (table 5.1), was consistent with hypothesis that 13-desmethyl FK 506 formation is a CYP3A catalyzed reaction in male rats.



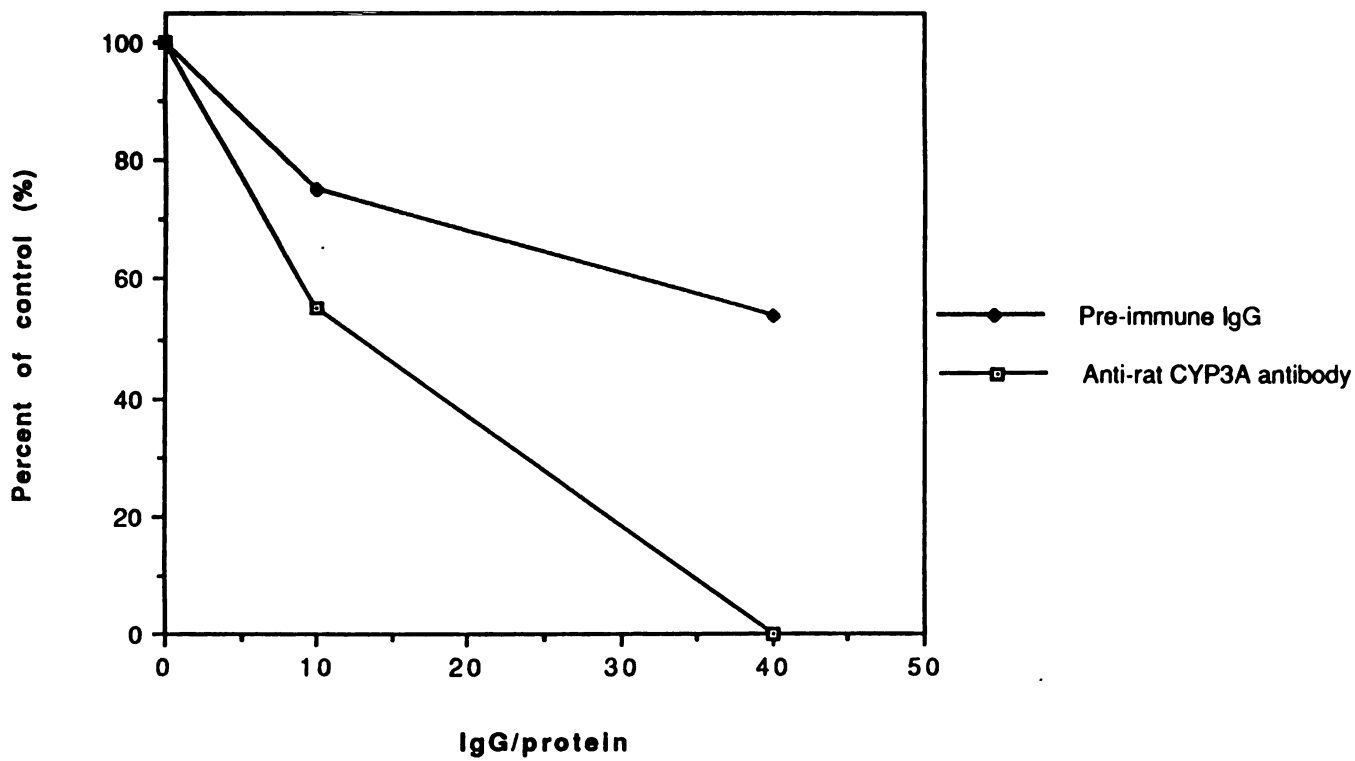
Lanes 1, 2, and 3 contained untreated female rat liver microsomes (1, 2, and 4 μg, respectively). Lanes 4, 5, and 6 contained untreated male rat liver microsomes (1, 2, and 4 μg, respectively). Lanes 7 and 8 contained standards-low range and purified rat CYP3A (1.6 pmol). Lanes 9, 10, and 11 were dexamethasone treated male rat liver microsomes (1, 2, and 4 μg, respectively).

Figure 5.8 Proteins (purified rat CYP3A and liver microsomes from dexamethasone-treated male rats and untreated male and female rats) separated on a 9% SDS-PAGE were stained by Coomassie brilliant blue



Activities were expressed as a relative percentage of the control value with no IgG used. The100 % value was 1.23 nmol/min/mg protein.

Figure 5.9 Inhibition of 13-desmethyl FK 506 metabolism in liver microsomes from dexamethasone-treated male rats using anti-mouse cyp3A antibody



Activities were expressed as a relative percentage of the control value with no IgG used. The100 % value was 1.23 nmol/min/mg protein.

Figure 5.10 Inhibition of 13-desmethyl FK 506 metabolism in liver microsomes from dexamethasone-treated male rats using anti-rat CYP3A antibody

Table 5.1 Comparison of kinetic values for the formation of 13-desmethyl FK 506 obtained from untreated and dexamethasone-treated male rats

Treatment Group	V_{MAX} (nmol/min/mg protein)	K_M (μM)
Untreated (n=7)	0.44 ± 0.30	24 ± 18
Dexamethasone-treated (n=4)	1.89 ± 0.71‡	29 ± 11

‡Significantly ($p \leq 0.001$) different than the untreated group.

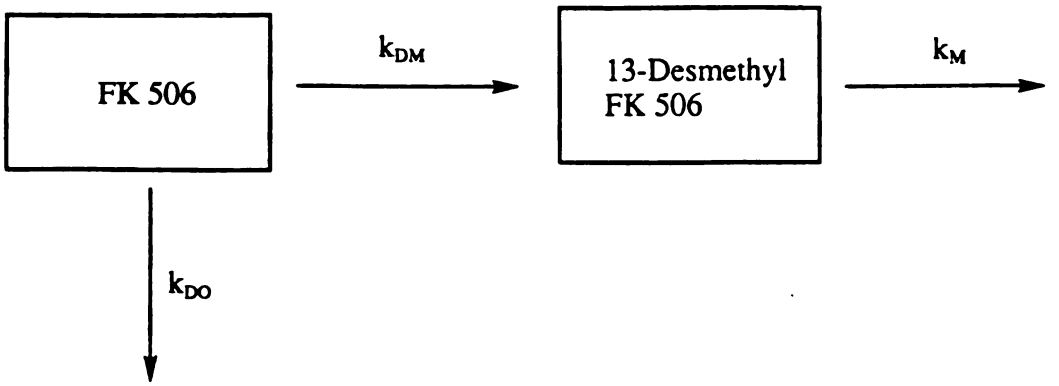
To represent the disposition of parent drug and 13-desmethyl FK 506 in liver microsomes, a kinetic model such as the one depicted in figure 5.11 was set up. An assumption was made that the parent drug (D) was metabolized to 13-desmethyl FK 506 (M), which was monitored, and other metabolites that were not monitored. The rate constant for the transformation of parent drug to M was denoted as k_{DM}, and the rate constant for the disappearance of metabolite M was denoted as k_M. The rate constant for the transformation of parent drug into all other metabolites was represented by k_{DO}. It was also assumed that $k_D = k_{DM} + k_{DO}$, where k_D is the total rate constant for the elimination of the parent drug in the system.

Therefore the disposition of the 13-desmethyl FK 506 (M) can be described by a biexponential equation:

$$M = [k_{DM}D / (k_D - k_M)] \bullet [e^{-k_M t} - e^{-k_D t}]$$

Thus, the metabolite time profile would be characterized by the rate constants k_D and k_M, and the parent drug time profile would be characterized by the rate constant k_D. Since k = 0.693 / half-life, k could be found if the half-life of a process was known.

Inspection of the half-life for the elimination of 13-desmethyl FK 506 (8.9 ± 1.3 min) revealed that it corresponded to the terminal half-life for the time profile of 13-desmethyl FK 506 upon formation from FK 506 incubation (9.7 ± 2.5 min). The mean rate constant k_M at 100 μg protein/ml was 0.071 min^{-1}. Because the half-life for the elimination of FK 506 (1.4 ± 0.4 min) coincided with the half-life of the residual plot of the metabolite



k_{DM}: Rate constant for drug (FK 506) going to metabolite (13-desmethyl-FK 506)
k_{DO} : Rate constant for drug (FK 506) going to metabolites other than 13-desmethyl-FK 506
k_M : Rate constant for metabolite (13-desmethyl FK 506) going to other unidentified metabolites

Figure 5.11 Kinetic model for the disposition of FK 506 and 13-desmethyl FK 506 in rat liver microsomes

time profile, this half-life was associated with the rate constant k_D, being 0.50 min^{-1} at 100 μg protein/ml. As the rate constant for the elimination of the parent drug was higher than the rate constant for the elimination of the metabolite, the elimination of 13-desmethyl FK 506 was therefore not rate limited by its formation from the parent drug.

The inhibition of the metabolic activity of 13-desmethyl FK 506, using both rabbit anti-mouse cyp3A antibodies and goat anti-rat CYP3A antibodies, indicated that the sequential metabolism of the FK 506 metabolite, 13-desmethyl FK 506, was a P450 3A-mediated reaction.

Because P450 3A appeared to be responsible for both the formation and the sequential metabolism of the FK 506 metabolite, 13-desmethyl FK 506, the apparent instantaneous rate of metabolite formed is the difference between the rate of metabolite formed and the subsequent rate of metabolism. The decreasing amount of metabolite formed at increasing protein concentrations (above 200 μg/ml) was probably due to its sequential metabolism.

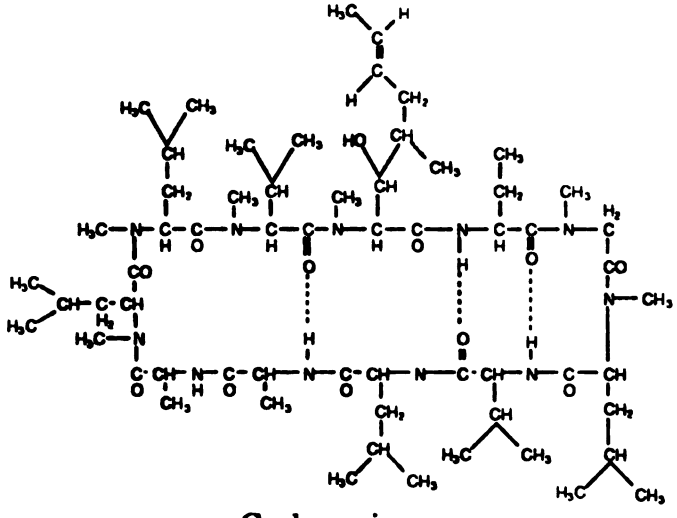
Chapter 6

Summary of Findings

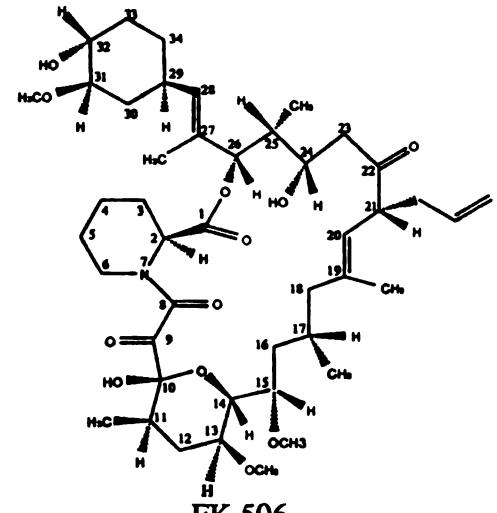
As depicted in figure 6.1, the hepatic metabolism of the current immunosuppressant of choice, cyclosporine, has been extensively studied. On the other hand, as recently as 1990, little information on the metabolism of the novel immunosuppressant FK 506, which had been going through phase III clinical trials since 1989, was available. Thus, the lack of information on the metabolism of FK 506 led to the initiation of this project.

Because all the experiments performed in this project were carried out with intact cells and subcellular fractions of rat livers, the first task in this project was to develop appropriate methodology for the measurement of FK 506 and its metabolites in rat liver samples. The analytical method used for the measurement of FK 506 clinically was inadequate for metabolism studies. The enzyme linked immunosorbent assay (ELISA) used clinically to monitor patients' blood and plasma samples measures a combined level of FK 506 and its metabolites, and is not capable of quantifying the parent drug or individual metabolites separately.

An assay was developed for the simultaneous measurement of FK 506 and two of its metabolites. The sample preparation was a quick and simple solid-liquid extraction procedure, which yielded satisfactory recoveries of 75 and 69% for FK 506 and the major FK 506 metabolite, respectively. Samples were analyzed utilizing reverse phase high performance liquid chromatography and ultraviolet detection. The compounds of interest were



Cyclosporine



FK 506

CYP3A

AM4N — N-demethylation

AM1, AM9 — monohydroxylation

AM14N, AM1A, AM1C, AM1C9
AM19, AM69,AM49, AM4N69

enzyme ?

Metabolites ?

enzyme ?

Secondary metabolites ?

Figure 6.1 The lack of metabolic information for FK 506 as compared to Cyclosporine in 1990

eluted with a mobile phase composed of acetonitrile, methanol and dilute *o*-phosphoric acid pH 3 solution (49/3/48, v/v/v), which was delivered at 60°C through a stationary phase C18 column. Both the interday and intraday variabilities were less than 10%. The detection limit for FK 506 was 0.017 nmol in this assay.

Because the assay utilized in this project can detect 2 metabolite peaks, the second task was to elucidate the structure of these 2 metabolites. The three methods utilized for structural identification in this project were mass spectrometry, retention time comparison and the ELISA. The major metabolite was identified to be a 13-desmethyl FK 506 which undergoes ring-chain tautomerization as does the parent drug. The second metabolite was the ring-chain tautomer of the first metabolite.

In addition to metabolic incubations of FK 506 with rat hepatocytes and rat liver subcellular fractions, carbon monoxide inhibition studies showed that the metabolic enzyme of FK 506 was related to the cytochrome P450 supergene family. By correlating the metabolic activities of FK 506 and testosterone in liver microsomes from untreated, dexamethasone-treated, phenobarbital-treated and ethinyl estradiol-treated rats, the cytochrome P450 isozyme responsible for the formation of the major metabolite, 13-desmethyl FK 506, was identified as CYP3A. Kinetic studies of the formation of 13-desmethyl FK 506 in male rat liver microsomes also facilitated the prediction of the total clearance of FK 506 *in vivo* from *in vitro* data.

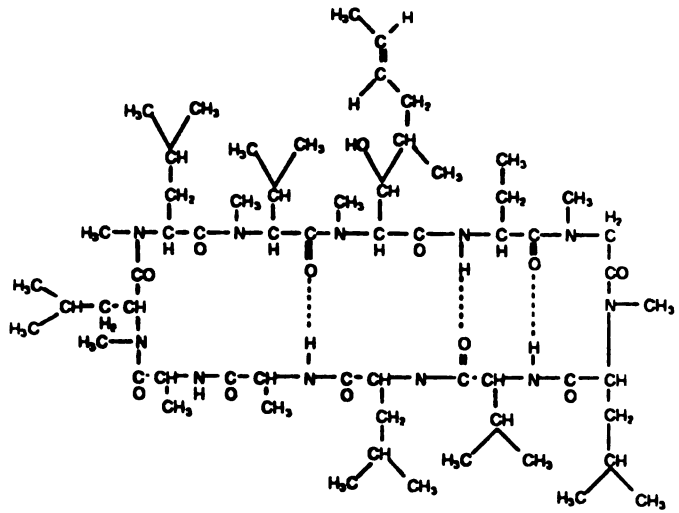
The formation of 13-desmethyl FK 506 upon FK 506 incubation in liver microsomes from female rats which are generally believed to lack CYP3A,

provided evidence that either FK 506 can be metabolized by non-CYP3A isozymes, or that female rats have constitutive CYP3A isozymes that were previously not-known or non-detectable.

Incubation of 13-desmethyl FK 506 with dexamethasone-treated rat liver microsomes, as well as immunoinhibition reactions with anti-CYP3A antibodies, also showed that this metabolite could be sequentially metabolized by CYP3A. Comparison of the elimination rate constants for the metabolism of FK 506 and 13-desmethyl FK 506 in dexamethasone-treated rat liver microsomes revealed that the elimination of 13-desmethyl FK 506 was not rate limited by the metabolism of the parent drug FK 506. The metabolic pathway of FK 506 known to date is depicted in figure 6.2.

Of all the substrates that are metabolized by the P450 enzymes, at least 50% of them involve CYP3A[5.1]. Because FK 506 is currently known as a CYP3A substrate, concomitant use of FK 506 with other therapeutic agents that are CYP3A substrates may result in drug-drug interactions. As such, the dosing regimens of this potent immunosuppressant should be carefully designed to avoid toxicity due to elevated levels of the drug in the presence of CYP3A inhibitors, or impairment of pharmacological response due to reduced levels of the drug in the presence of CYP3A inducers.

5.1 Dr. Dennis Smith, Pfizer Central Research, U.K., personal communication.



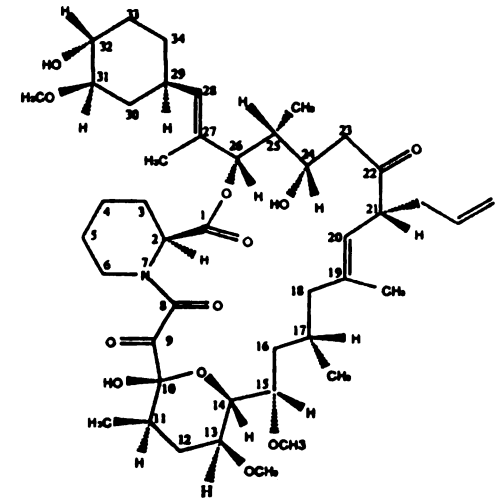
Cyclosporine

CYP3A

AM4N — N-demethylation

AM1, AM9 — monohydroxylation

AM14N, AM1A, AM1C, AM1C9
AM19, AM69,AM49, AM4N69



FK 506

CYP3A

15-desmethyl FK 506
31-desmethyl FK 506
12-hydroxy FK 506

13-desmethyl FK 506

CYP3A

Secondary metabolites - yes
(structures not yet known)

Figure 6.2 Simplified metabolic schemes for Cyclosporine and FK 506 known to date

'Tis sad to see the sons of learning
In everlasting Hell fire burning
While he that never read a line
Doth in eternal glory shine

Robin Flower; from the Irish
9th Century

學無止境

Live and Learn

Ancient Chinese Idiom

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