Title
Interactions of immunosuppressive drugs with P-glycoprotein (P-gp) and cytochromes P450

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INTERACTION OF IMMUNOSUPPRESSIVE DRUGS WITH P-GLYCOPROTEIN (P-GP) AND CYTOCHROMES P450

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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DEDICATION

I dedicate this thesis to my daughters, Sonal and Kuhu, for their love, patience, understanding and encouragement through my graduate studies.

PREFACE

The first successful organ transplant in modern history was a kidney transplant between identical twins (Janeway and Travers, 1994) performed at Peter Bent Brigham Hospital in Boston in 1954 (http://www.kodaorgan.com/historic.htm). In the next decade (1954-63), 30 isografts (between identical twins) were performed. In 1983, the immunosuppressive, cyclosporine was introduced. This was a major milestone and has had a dramatic impact on transplant survival and quality of life of the patient. In the decade (1983-92) following introduction of cyclosporine, the number of renal transplants rose to approximately 10,000 (Janeway and Travers, 1994). In 1996, the total number of cadaveric and living donor transplants was 20260 (http://www.gsds.org/faq/transplants.html). This staggering success is a direct consequence of improved immunosuppressive drugs in use for prevention of acute and chronic rejection.

The major specific immunosuppressant drugs currently in use for chronic therapy include cyclosporine, tacrolimus (FK 506), mycophenolate mofetil (MMF), and sirolimus (in clinical trials). These drugs are used in combination with (Katzung, 1998) non-specific immune modulators such as corticosteroids, and cytotoxic agents such as azathioprine, cyclophosphamide and/or methotrexate. Cytokines and monoclonal antibodies may also be used briefly to enhance immunosuppression. Although these immunosuppressants are widely used, the mechanism of their activity and other complex pharmacodynamic interactions are not fully elucidated. The mechanism of action

(Fruman et al., 1992) of cyclosporine was not fully understood for almost a decade after it was introduced. Despite extensive research into the mechanisms and interactions of these drugs, there are many questions.

The overall goal of this thesis is to understand some issues involving the pharmacodynamic interactions of the major immunosuppressive drugs using *in vitro* and *in vivo* systems. I have focused on two distinct problems. The first is metabolism of sirolimus and the second is mechanism of interaction of cyclosporine alone and in drug combinations with P-glycoprotein both *in vitro* and in renal transplant patients. The observations and conclusions from these studies will add to the basic understanding not only of these drugs but also drug effects and interactions.

The dissertation is organized into six chapters. The purpose of the first chapter is to discuss the current state of knowledge of the field. This detailed introduction will focus on scientific questions I have addressed. Chapters 2-5 address specific scientific hypotheses I have tested. The final chapter summarizes the findings and implications of this research.

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ABSTRACT

Interaction of Immunosuppressive Drugs With P-glycoprotein (P-gp) And Cytochromes P450

Dolly Aggarwal Parasrampuria

Immunosuppressive drugs such as cyclosporine, tacrolimus and sirolimus are specific inhibitors of T cell mediated immune responses. They are used for prevention of transplant rejection and show promise in autoimmune disorders, HIV and as modulators of multidrug resistance in anticancer therapy. The prospect of chronic usage of these drugs warrants a thorough understanding of their mechanisms of action, pharmacokinetic and pharmacodynamic interactions. In this thesis, I have addressed two different problems: the metabolism of sirolimus and the interaction of cyclosporine alone and in drug combinations with the drug efflux transporter, P-gp. The *in vitro* metabolism of sirolimus was studied in human liver microsomes. Sirolimus was extensively metabolized by CYP3A4 to first and second-generation metabolites in a time and concentration dependent manner. CYP3A5 catalyzed the formation of hydroxyl sirolimus. Sirolimus and most metabolites were detected in blood and urine samples of transplant patients. Cyclosporine and tacrolimus were potent inhibitors of P-gp mediated rhodamine 123 efflux from lymphocytes. Quinidine and verapamil were also inhibitors of rhodamine 123 efflux. Vinblastine inhibited efflux of rhodamine at low concentrations, but at high concentrations, it enhanced the efflux possibly by activating P-gp. Indomethacin, a drug that is not a P-gp inhibitor, also inhibited rhodamine 123 efflux probably by inhibiting some other anion-sensitive transporter of rhodamine. In *in vitro* proliferation assays a combination of P-gp inhibitors and cyclosporine had a synergistic inhibitory effect on T

cell proliferation and cytokine secretion. Combination with other drugs had differential effects. In transplant patients there was a clear trend towards decreased P-gp expression in CD4+ T cells from patients initiated on cyclosporine or tacrolimus. Patients who were started on tacrolimus or cyclosporine following rejection also showed an initial decline in P-gp expression in CD4+ T cells. The rhodamine efflux/retention analysis indicated that P-gp was functional in both CD4+ and CD8+ T cells. These studies indicate the possibility of numerous *in vivo* drug interactions of immunosuppressive drugs with each other and with other P-gp inhibitors. These interactions could result in modulation of immunosuppressive activity and toxicity.

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Chapter 1

IMMUNOSUPPRESSANTS

1.1 Organ Transplantation

The oldest reference to organ transplantation can be found in Hindu mythical literature (Rg Veda). The Hindu God Shiva unknowingly severed Ganesha's head but realizing his error transplanted a white elephant's head on his son. There are many other references to organ replacement/transplantation in mythological literature from various countries and cultures.

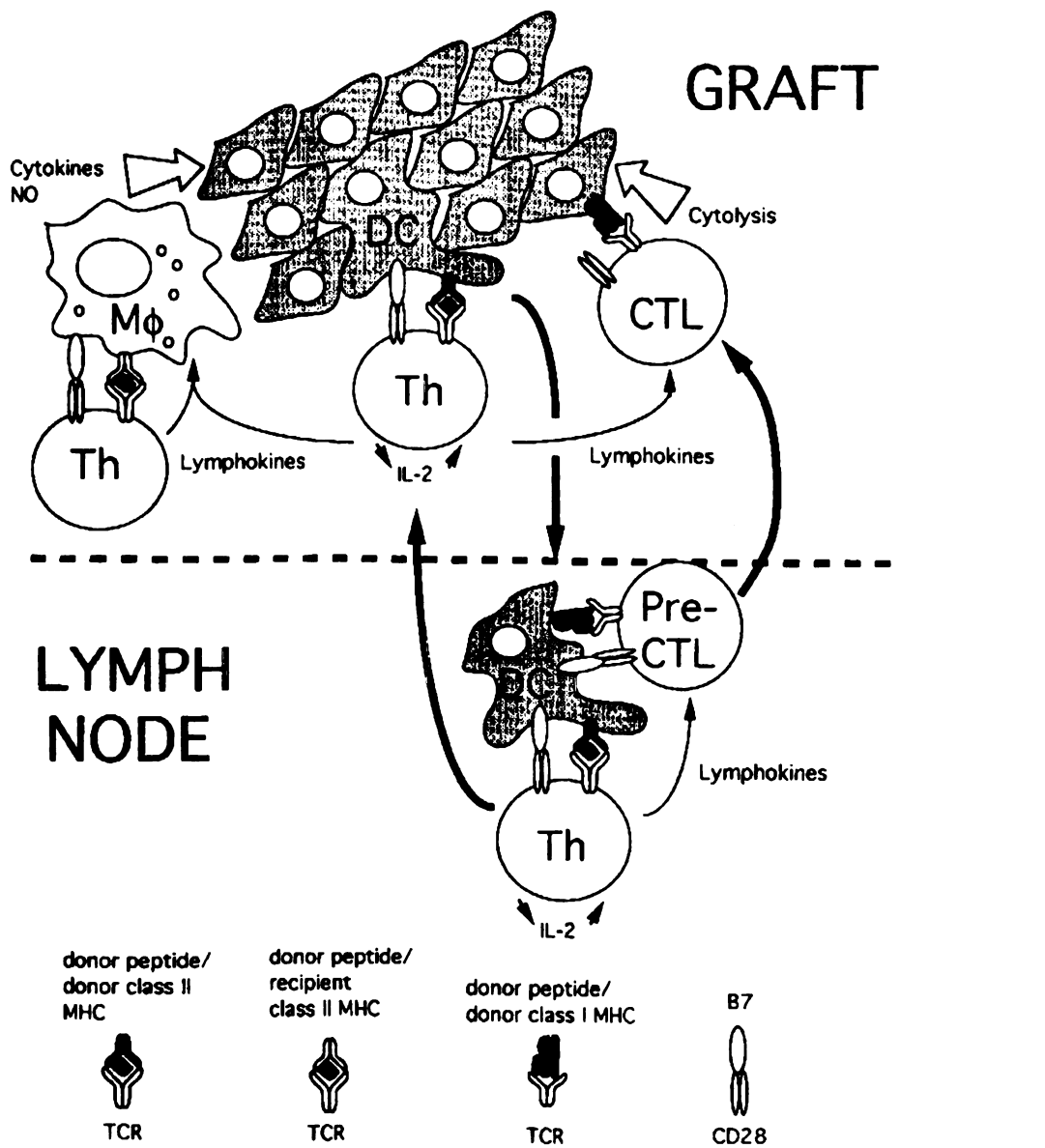
Through recorded history, there have been reports of organ transplantation to replace severed limbs and burned skin. Some of these attempts were marginally successful. The earliest accounts in modern medical literature of tissue transplantation appeared in the 1940s when Sir Peter Medawar published his seminal paper regarding skin autografts (within an individual) and homografts (between genetically identical individuals) (Medawar, 1944). Later, in treating burns with skin grafts, he demonstrated that a second graft from the same donor was rejected more rapidly than the first.

Tissues can be transplanted from one body part to another within the same individual. Such grafted tissue, known as an autograft, is not rejected since it bears the individual's own antigens. A graft from one individual to another of the same species, the

allograft, is recognized as foreign and the recipient's immune system rejects the organ in the absence of immunosuppressive therapies. Xenografts involve tissue transplants from one species to another. Each species has a single genetic locus that encodes the strongest transplantation antigens known as the major histocompatibility complex (MHC). Matching and minimizing the differences of donor and recipient for these antigens can significantly improve the graft survival.

Transplantation of nucleated cells elicits a T cell response (Fig. 1.1) to the highly polymorphic MHC. The MHC molecules are transmembrane glycoprotein heterodimers. The human MHC was originally studied in white blood cells, therefore, it is known as human leukocyte antigen (HLA). There are two classes of MHC molecules, class I (HLA -A, -B, -C) and class II (HLA -DR, -DQ, and -DP). The HLA genes in humans are located on the short arm of chromosome 6. The function of MHCs is to present a foreign antigen to the T-cells. The MHC class I molecules present viral antigens derived from cytoplasmic proteins of virus-infected cells whereas class II molecules present peptides derived from endosomal or lysosomal compartments as a result of internalization of inactivated or shed viral particles. The cytotoxic T cells ($CD8^+$) recognize antigens presented in the context of MHC class I molecules, and destroy such virus-infected cells. The majority of helper T cells ($CD4^+$) recognize antigens expressed by class II MHC. The MHCs are genetically diverse because of allelic polymorphism and isotype variation. Such highly polymorphic loci often result in heterozygosity (Lechler et al., 1995).

Fig. 1.1. Major steps involved in initiation of a cell-mediated transplant rejection response. In this figure, the graft dendritic cells (DC) present the foreign graft antigens to helper T cells (Th) of the host, thereby activating them. Once activated, Th cells synthesize and secrete cytokines (IL-2 and other lymphokines) which cause proliferation of Th cells and activation of cytotoxic T cells (CTL) and macrophages (Mϕ) that are directly responsible for cytolysis of the graft. (Figure reproduced from Jenkins, 1995)



Antigen presenting cells (APCs) such as dendritic cells, macrophages, and B cells internalize foreign peptides, and present fragments of peptide in the context of MHC II. These peptide-MHC II complexes are recognized by CD4 T cells (T-helper, Th) which undergo proliferation and release cytokines. The cytokines signal proliferation of CD8 T cells (cytotoxic T cell, Tc), activate macrophages, and antibody production. The CD8 T cells and macrophages are responsible for direct cytolysis of the graft while the B cells produce antibodies against the grafted tissue. Figure 1.2 describes the intracellular events that occur following antigen recognition. Following antigen recognition by the T cell receptor complex (TCR), there is an increase in intracellular calcium levels due to release from storage vesicles and influx. This activates an important phosphatase, calcineurin that cleaves the phosphate from the cytoplasmic component of nuclear factor of activated T-cells (NFATc) thus activating it. The activated NFATc translocates to the nucleus where it dimerizes with the nuclear component of NFAT (NFATn). This complex binds to interleukin-2 (IL-2) regulatory sites resulting in gene transcription and synthesis of IL-2.

Graft rejection can be attenuated by immunosuppressive therapy. The earliest immunosuppressive drugs (e.g. azathioprine and steroids) were proliferation inhibitors derived from cancer chemotherapy and were indiscriminate in their activity. They acted by depleting all components of the immune system such as the hematopoietic cells, polymorphonuclear cells, macrophages, activated and resting T and B cells. This non-specificity was also responsible for the toxicity and morbidity associated with the oldest drugs such as azathioprine, steroids and radiation therapy. The newer agents such as

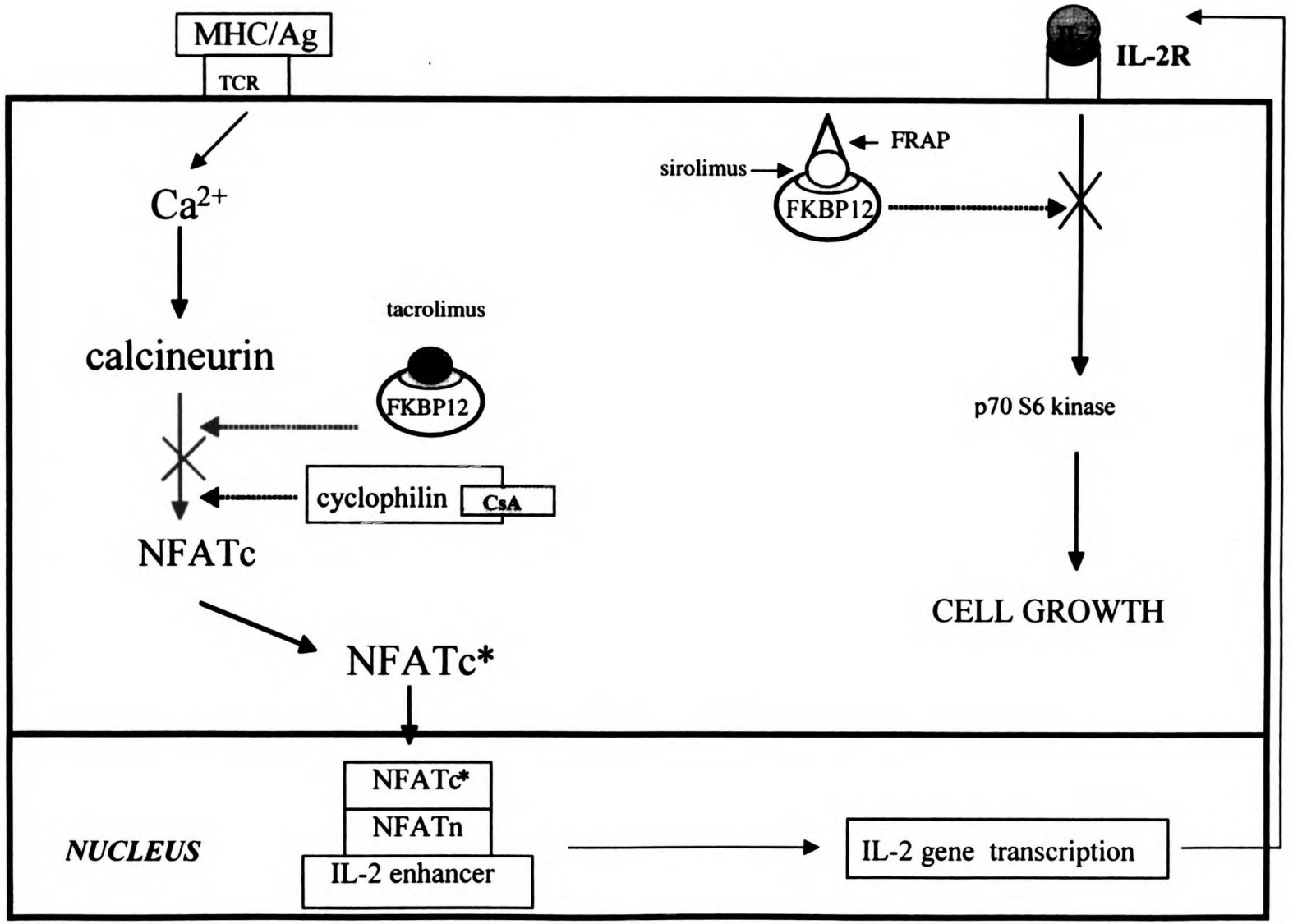


Fig. 1.2. Intracellular events in $CD4^+$ T lymphocytes and mechanism of action of cyclosporine, tacrolimus and sirolimus.(Redrawn from Roitt, 1996)

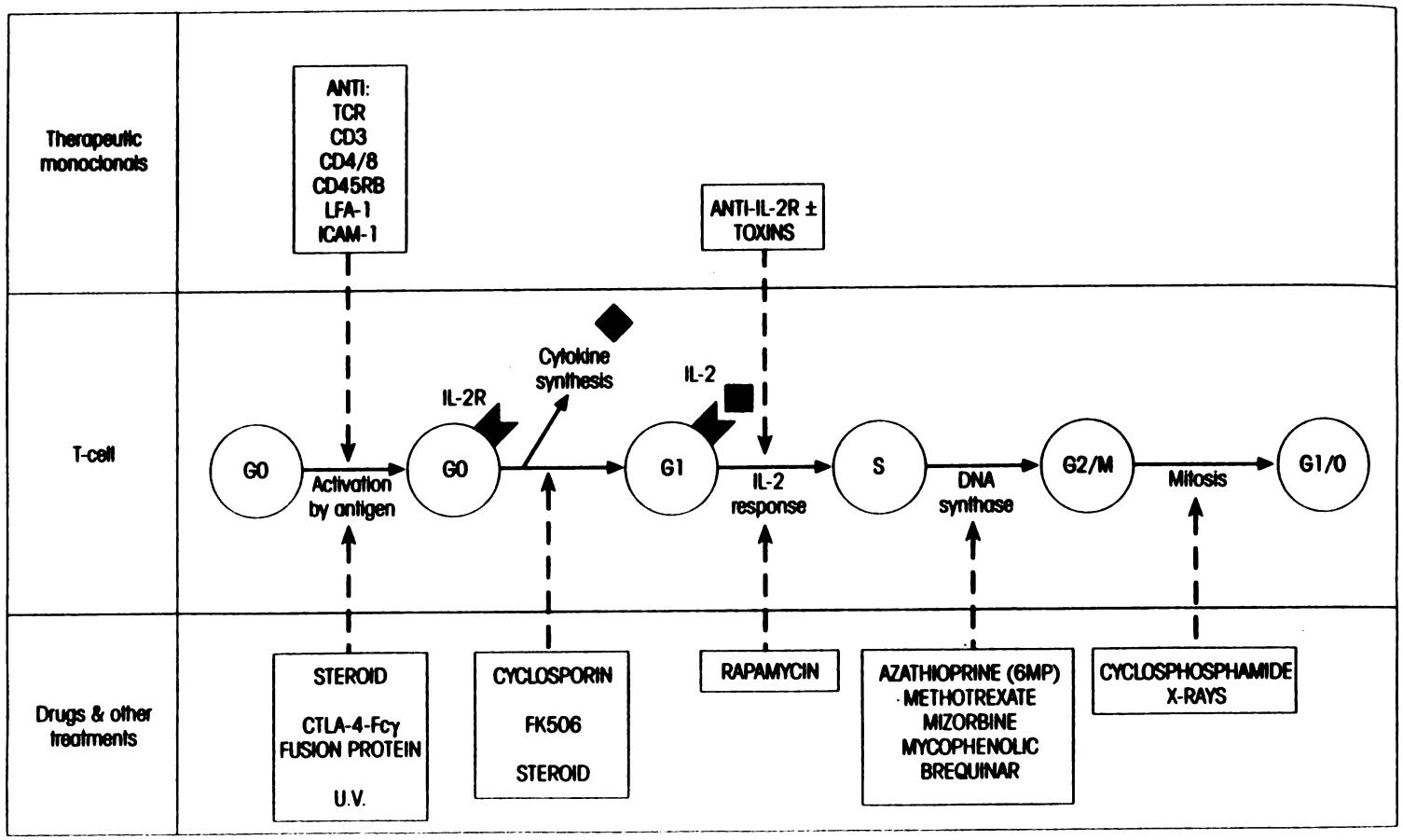
cyclosporine, tacrolimus and sirolimus aim to provide more specific immunosuppression. The proposed site of action of these drugs is the T lymphocyte. Cyclosporine and tacrolimus (FK 506) bind to cellular immunophilins (cyclophilin and FKBP12, respectively) and calcineurin thereby blocking IL-2 gene transcription by this pathway (Fig. 1.2). Following extracellular release and binding of IL-2 to its cognate receptor (CD25), p70 S6 kinase is activated which phosphorylates ribosomal S6 resulting in cell proliferation. Sirolimus binds to FKBP12 and FRAP and this complex prevents the p70 S6 kinase activation thereby preventing T cell proliferation (Fig. 1.2). Sirolimus has no action on the synthesis of IL-2.

1.2 Immunosuppressive Drugs

1.2.1 Corticosteroids

Corticosteroids are used alone or in combination with other immunosuppressive agents to prevent transplant rejection. Prednisone and prednisolone form the cornerstone of immunosuppressive steroid therapy. They produce a rapid, transient reduction in peripheral blood lymphocytes, spleen and lymph nodes. Corticosteroids act at the earliest stages of activation (Fig. 1.3) of T cells and arrest the progression of CD4 T cells from G0 to activated G0 stage, thereby inhibiting the production of inflammatory mediators. Corticosteroids can also reduce previously established antibody responses. Continuous administration of prednisone increases the catabolic rate of IgG thus lowering the

Fig. 1.3. Immunosuppressive drugs used for prevention of transplant rejection and the stages in T cell proliferation that are inhibited by these drugs (Figure reproduced from Roitt, 1997).



concentration of specific antibodies. Steroids are relatively non-toxic to proliferating myeloid or erythroid stem cells in the bone marrow and are used liberally during a rejection crisis. The usual dose for prednisone as an immunosuppressive agent is 10-100 mg orally daily. The potential side effects include adrenal suppression and serious viral, bacterial and fungal infections.

Corticosteroids may in part work by binding to the glucocorticosteroid response element present in the 5' regulatory region of some cytokine genes (Yang-Yee et al., 1990; Vacca et al., 1992; Palvogianni et al., 1993)

1.2.2 Cyclosporine

Cyclosporine was first isolated in 1973 from fungal extracts of soil samples obtained from Hardanger Vidda in Southern Norway. It is produced by the fungus species *Beauveria nivea* (PDR, 1999). Cyclosporine is a cyclic undecapeptide consisting of 11 amino acid residues. Cyclosporine is extremely insoluble in water but readily dissolves in ethanol and lipophilic solvents. The chemical structure of cyclosporine is $C_{62}H_{111}N_{11}O_{12}$ with a molecular weight of 1202.63. Cyclosporine is marketed by Novartis as Sandimmune® and the newer and more bioavailable formulation, Neoral®. Cyclosporine is approved for prophylaxis of organ rejection in kidney, liver, and heart allogeneic transplants. It is used in combination with azathioprine and corticosteroids. Cyclosporine exerts its immunosuppressive effects via inhibition of IL-2 synthesis (Figs. 1.2, 1.3).

Cyclosporine is also approved for treatment of severe active rheumatoid arthritis refractory to methotrexate, and approved for treatment of adult, nonimmunocompromised patients with severe psoriasis that has failed to respond to systemic therapy with methotrexate, PUVA, retinoids, etc. Cyclosporine has numerous side-effects but the major dose-limiting clinical toxicities associated with therapeutic doses of cyclosporine include nephrotoxicity and neurotoxicity.

A long-term complication of immunosuppressive drugs, including cyclosporine is cancer. Neoplasm progression is generally believed to occur as the result of a compromised immune system. But recently, it has been shown (Hojo et al., 1999) that cyclosporine may play a direct role via transforming growth factor-β (TGF-β) in altering cell morphology and promoting malignancy. Cyclosporine was able to induce morphological changes in an inherently non-invasive cell-line (A-549) and transform these cells to an invasive phenotype capable of: dividing readily, mobility, and growth without anchorage. In experiments using SCID-beige mice, Hojo and co-workers (1999) were able to demonstrate an increase in pulmonary metastases with dosing of cyclosporine. Cyclosporine induced the production of TGF-β by tumor cells leading to metastases, invasion and recurrence of tumors. These effects were independent of the immunosuppressive effects.

In rats, Friberg et al. (1998) have demonstrated a neuroprotective effect of cyclosporine during hypoglycemic coma. Tacrolimus was not effective. Cyclosporine

appears to act by preventing calcium-induced swelling of mitochondria in a dose dependent manner, thus preventing apoptosis and ischemia-reperfusion injury.

Cyclosporine is a potent inhibitor and substrate of the 12 transmembrane domain (TMD) drug efflux transporter known as P-glycoprotein (P-gp). This transporter is in part responsible for limiting the oral bioavailability of cyclosporine. The binding site of cyclosporine to P-glycoprotein has been characterized in the Chinese hamster ovary (CHO) cell line using a photolabeled cyclosporine analog (Demeule et al., 1998). Based on these studies cyclosporine appears to bind between the end of TM11 and the end of TM 12.

1.2.3 Tacrolimus

Tacrolimus (FK506) is a macrolide immunosuppressant produced by *Streptomyces tsukubaensis*. Commercially it is marketed as Prograf® by Fujisawa Inc. The empirical formula is $C_{44}H_{69}NO_{12}.H_2O$ and molecular weight is 822.05. It appears as white crystals or crystalline powder. It is practically insoluble in water, but freely soluble in ethanol, methanol and chloroform. Tacrolimus is approved for prophylaxis of organ rejection in patients receiving liver or renal transplants. In clinical studies (Shapiro et al., 1999a), tacrolimus has been successfully used in pediatric renal transplants. Tacrolimus was found to be effective in pediatric renal transplant recipients for both short and medium term efficacy over a period of 7 years. Tacrolimus has also been used as a long-

term primary immunosuppressant in pancreas transplantation allowing steroid withdrawal (Jordan et al., 1999). The major toxicities associated with tacrolimus include myocardial hypertrophy, insulin dependent reversible diabetes, neurotoxicity, and nephrotoxicity.

Tacrolimus exerts its immunosuppressive activity by interfering with transcription of cytokine genes in $CD4^+$ T lymphocytes (Figs. 1.2, 1.3). Tacrolimus binds to cytosolic immunophilins, the 12-kD FK506-binding protein, FKBP12 (Harding et al., 1989). Once bound to immunophilins, the FK506-FKBP12 complex binds to and inhibits calcineurin, thereby inhibiting T-cell activation (Liu et al.,1991; Fruman et al., 1992; Brazelton et al.,1996). It is believed that the cardiotoxic side-effects of tacrolimus result from inhibition of calcineurin which is essential for activation of NF-ATc and normal cardiac valve and septum morphogenesis (de la Pompa et al., 1998).

Tacrolimus demonstrates strong neuroprotective action in experimental models of stroke (Sharkey and Butcher, 1994) but the mechanisms underlying this effect are not fully understood.

1.2.4 Sirolimus

Sirolimus (rapamycin) is a macrolide immunosuppressant produced by *Streptomyces hygroscopicus* (Vezina et al., 1975). The empirical formula is $C_{51}H_{79}NO_{13}$. The molecular weight is 913.6 D. It has antifungal, antitumor, and immunosuppressive

activities. Currently sirolimus is in phase III clinical trials for adjuvant therapy with cyclosporine for prophylaxis of organ rejection following renal transplantation.

Sirolimus is structurally similar to tacrolimus (FK506) and shares the same cellular target: the 12-kD FK506-binding protein, FKBP12 (Harding et al., 1989), but its mechanism of action is distinct from that of tacrolimus. Sirolimus binds to cellular immunophilins (FKBP 12). Once bound to immunophilins, the FKBP12-sirolimus complex binds (Choi et al., 1996) to FRAP (FKBP-rapamycin-associated protein) and blocks cell proliferation by preventing the cells from progressing from G_1 to S phase (Figs 1.2, 1.3). Human FRAP is a 289 kD protein of the ATM family that regulates p70 ribosomal protein S6 kinase and is required for G_1 cell cycle progression (Brown et al., 1994; Sabatini et al., 1994; Brown et al., 1995). Thus, the binding of FKBP12-sirolimus complex to FRAP results in inactivation of p70 S6 kinase activity, resulting in selective inhibition of protein synthesis (Terada et al., 1995, Suthanthiran et al., 1996).

Sirolimus is associated with several side effects (Murgia et al., 1996; Zimmerman and Kahan, 1997) including decreased platelet count, increased total triglycerides, and elevated cholesterol. These side-effects are shown to be statistically different from those observed in patients on cyclosporine based therapy.

Sirolimus demonstrates potent antiproliferative activity (Seufferlein and Rozengurt, 1996) and has been suggested as an anticancer agent. Burton et al. (1998) report an *in vitro* inhibition in proliferation of cardiac myocytes isolated from fetal rat

hearts. This effect persisted after removal of the drug, raising concerns regarding pediatric use of this drug. Hosoi et al. (1998) found that cancer cells that overexpressed c-myc were resistant to inhibition in growth by sirolimus. West et al. (1998) have also demonstrated that mTOR (target of rapamycin, also known as FRAP, RAFT, and RAPT) was under the control of c-myc induction.

1.2.5 Mycophenolate Mofetil (MMF)

Mycophenolate mofetil is a morpholinoethyl-ester prodrug of mycophenolic acid (MPA). MMF is marketed by Roche Laboratories as CellCept®. The empirical formula of MMF is $C_{23}H_{31}NO_7$ and molecular weight is 433.5. It is a white to off-white crystalline powder. It is rapidly and completely converted to the active MPA following oral administration by plasma esterases (Lee et al., 1990; Bullingham et al. 1996). It is slightly soluble in water (43 μg/ml at pH 7.4), the solubility is higher in acidic medium (4.27 mg/ml at pH 3.6), freely soluble in methanol, and sparingly soluble in ethanol (PDR, 1999). MMF is approved for prophylaxis of organ rejection following renal transplantation concomitantly with cyclosporine and corticosteroids. The major toxicities associated with MMF include sepsis and leukopenia.

MPA is a reversible, non competitive inhibitor of inosine monophosphate dehydrogenase, an enzyme involved in de novo synthesis of guanosine triphosphate (Franklin and Cook, 1969). This results in selective inhibition of proliferation of T and B

lymphocytes (Fig. 1.3) which depend on de novo purine synthesis, but other cells are spared since they possess salvage pathways (Suthanthiran and Strom, 1997).

1.3 Cytochrome P450 Enzymes

Cytochromes P450 (CYP) constitute a superfamily of proteins responsible for the metabolism of endogenous and exogenous compounds. Both eukaryotes and prokaryotes contain numerous P450 enzymes. The amino acid sequences of CYPs are not well conserved between species but the three dimensional structures are similar. Conserved structures are usually present in the heme-binding domain and putative substrate binding region (Black and Coon, 1987; Degtyrenko, 1995). Rats have a larger number of cytochrome P450 genes, thus resulting in greater metabolism of xenobiotics compared to humans (Nebert et al., 1991). The CYP enzymes are located in the mitochondria or microsomes on the endoplasmic reticulum (ER) membrane. The microsomal enzymes are integral membrane proteins anchored via a hydrophobic N-terminal sequence such that the catalytic domain faces the cytosolic side (Sakaguchi et al., 1984; Kemper and Szczesna-Skorupa 1989; Graham-Lorence et al., 1995). The highest concentration of the CYP enzymes is found in the liver but they are present in virtually all tissues. The P450s are very specific in their catalytic activity but have a broad range of substrate specificities. Their expression is under genetic control, and is in some part regulated by hormones, diseases, drugs and environmental factors. Flavonoids and progesterone can autoactivate members of the 3A family (Domanski et al., 1998). Mutagenesis studies

have identified key substrate recognition sites for some of the CYP enzymes (He et al., 1997; Domanski et al., 1998) but a complete understanding of substrate recognition site is far from complete.

Cytochromes P450 are heme containing enzymes (Omura and Sato, 1961). They catalyze mono-oxygenation reactions by incorporating one atom of oxygen into the substrate. The other atom from molecular oxygen is reduced to water. The cytochromes P450 constitute phase I biotransforming enzymes and are responsible for detoxifying most xenobiotics by making them more hydrophilic although sometimes they create reactive toxic species. The major reactions catalyzed by CYP enzymes include oxidative and reductive dehalogenation; N-hydroxylation and oxidation; oxidative deamination; S-, N-, and O-dealkylation; and aliphatic and aromatic hydroxylation (Gonzalez, 1989). Some compounds generate toxic high energy intermediates such as epoxides producing cell transformation and mutations (Gonzalez, 1989). The major CYP enzymes include CYP1A2, 2A6, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5, 4A7, and 4A11. CYP3A4 is responsible for more than 60% of drug metabolism by the P450 system (Correia, 1998).

There is considerable variability in therapeutic drug response and toxicity of drugs that are substrates of P450s. Some of the variability arises from genetic polymorphism of CYP2D6 and CYP2C19 resulting in poor and extensive metabolizers. CYP2D6 is the characteristic of debrisoquine/sparteine metabolism initially identified by Gut et al. (1984, 1986a, b). This enzyme is absent in poor metabolizers who constitute 5-10% of the Caucasian population (Alvan et al., 1990). A genetic polymorphism in 2C19

was first described as a result of polymorphic metabolism of S-mephenytoin (Wilkinson et al., 1989). 12-23 % of Asians are poor metabolizers of CYP2C19 substrates.

1.4 P-glycoprotein

P-glycoprotein is a 170 kD transmembrane protein. It is encoded by the multidrug resistance (*MDR)* genes. The human *MDR* genes are located on chromosome 7q21.1 (Fojo et al., 1986; Chin et al., 1989), whereas the homologous mouse genes are located on chromosome 5 (Hsu et al., 1989). Humans have two genes, *MDR1* and *MDR3* (Roninson et al., 1984; Ueda et al. 1987; Chin et al., 1989; van der Bliek and Borst, 1989; Lincke et al., 1990) whereas mice have three members (*mdr1a, mdr1b, and mdr2*) (Gros et al., 1986 a, b; Devault and Gros, 1990). In humans, *MDR1* encodes the drug efflux transporter, P-gp. The entire human *MDR* locus spans ~230 kb, with *MDR1* and *MDR3* separated by 34 kb of intergenic DNA and transcribed in the same direction (Chin et al., 1989; Lincke et al., 1991). P-gp belongs to the ATP-binding cassette (ABC) family of genes and depends on ATP hydrolysis for its activity (Shapiro and Ling, 1998). P-gp is constitutively expressed in a wide variety of tissues (Table 1-1) but can also be overexpressed following exposure to xenobiotics. The acquisition of the *MDR* phenotype in mammalian cells is often associated with amplification or transcriptional activation of *MDR* genes, which results in reduced intracellular drug concentration (Pastan and Gottesman, 1987; Bradley et al., 1988; van der Bliek and Borst, 1989; Kohno et al., 1994; Kusaba et al., 1995).

Table 1-1. P-glycoprotein (P-gp) expression in normal human tissues

Tissue Type	*Organs*	*Description of Location*	*P-gp expression*	*Comments*
Epithelial Cells	Kidney	Luminal surface of proximal tubular cells	++	P-gp expression differs with type of differentiation
	Digestive Tract	Squamous epithelia of esophagus, oral cavity	-	
		Gastric, small bowel, colonic mucosa	++	P-gp expression is governed by degree of differentiation
		Pancreas – Exocrine Ductal cells Islet cells	+ ++ -	
		Liver – Biliary duct Hepatocyte	++ +	*MDR1* *MDR3, MDR1* acquired *in vitro*
	Lungs	Trachea and large airways	++	Apical surfaces
	Mammary glands		+	Focal areas in adult non-lactating mammary gland
	Prostate		+	
	Adrenal gland	Cortical epithelial cells	++	
	Skin	Epidermal keratinocytes	-	
		Sweat glands & ducts	+	Luminal expression
	CNS	Neurons, glial cells	-	
Endothelial Cells	CNS	Cerebral cortex, basal ganglia, brainstem, cerebellum, spinal cord, capillary endothelium	++	BBB
	Testes	Capillary	++	Blood-testes barrier
	Skin	Papillary dermis	++	Side effects of anticancer therapy
	Uterine cervix		+	
	Esophagus		+	
	Spleen		+	Variable
	Lymph Nodes		+	Variable
Placental Trophoblasts	Placenta	Synctial trophoblasts	++	Tissue barrier

Ref: Table summarizes the information from chapter by O'Brien and Cordon-Cardo, 1996

Wacher et al. (1995) reported a remarkable overlap in substrate specificities and tissue distribution of cytochromes P450 and P-glycoprotein but so far there is no conclusive evidence of co-regulation of these genes (Salphati and Benet, 1998; Sérée et al. 1998). Sérée and co-workers (1998) found the human hepatic CYP3A4 and *MDR1* to be coinducible by dexamethasone and the murine *mdr1b* and CYP 3a were inversely modulated in the adrenals.

P-glycoprotein constitutes an intestinal barrier to the absorption of xenobiotics thereby limiting the bioavailability of orally delivered drugs (Asperen et al., 1998). The P-gp is expressed on the apical side of the intestinal epithelium where it functions by pumping drugs out into the gastrointestinal lumen.

It has been found that changes in p53, a tumor suppressor phosphoprotein which is frequently altered in malignancies, directly affects the endogenous *MDR1* gene (Thottassery et al., 1997). An inactivation of p53 results in up-regulation of *MDR1* expression .

P-glycoprotein contains three drug-binding sites. Two of these drug-binding sites, the H site selective for Hoechst 33342 and colchicine and the R site selective for rhodamine 123 and anthracyclines, act in a cooperative manner (Shapiro and Ling, 1997). A recently identified third site has a positive allosteric effect on drug transport by the H and R sites, but doesn't appear to be directly involved in drug transport (Shapiro et al., 1999b).

The mechanism of drug transport by P-gp may involve either a "vacuum cleaner" model whereby the transporters retrieve lipophilic compounds from the lipid bilayer and pump them out (Raviv et al., 1990). They could also act as a "flippase" to bind drugs on the cytosolic side and translocate them to the outer layer (Higgins and Gottesman, 1992).

1.5 Rationale and Specific Aims

This dissertation addresses two different issues: the metabolism of sirolimus and P-gp interaction of cyclosporine alone and in combination with other drugs. The first objective is based on limited information available regarding metabolism of sirolimus in *in vitro* systems. Sirolimus is being investigated for immunosuppressive therapy in renal transplant patients. These patients receive cyclosporine as the primary immunosuppressant. While the metabolism of cyclosporine has been extensively studied (Christians and Sewing, 1993; Prueksaritanont et al., 1993), it is important to characterize the metabolite patterns of sirolimus and identify the enzymes involved. Like cyclosporine and tacrolimus, sirolimus is extensively metabolized to numerous metabolites. To be able to predict and understand the metabolism in humans, it is necessary to have a comprehensive understanding of all possible metabolites and metabolic pathways. This can be best understood using *in vitro* analysis.

The second major hypothesis of this thesis is based on various pharmacodynamic effects and clinical uses of cyclosporine reported in the literature. Cyclosporine is a

potent immunosuppressant and a good inhibitor of P-gp *in* vitro. It has been used with anticancer drugs for reversing multidrug resistance (Terwogt et al., 1998) in human clinical studies without a complete understanding of the potential effects that such a combination might have. Cyclosporine and other immunosuppressants are also capable of modulating the activity of P-gp *in vitro.* The *in vivo* effects of cyclosporine and other immunosuppressant drugs on the expression and function of P-gp at the site of action (CD4+ T lymphocytes) are not known but could be important. Using *in vitro* and *ex vivo* assays, I have studied the effect of drugs on P-gp expression and function.

The specific aims of this work include:

1. To characterize *in vitro* metabolism of sirolimus in human liver microsomes and to identify specific CYP(s) enzymes mediating the metabolism.
2. To develop an *ex-vivo* assay for quantification of P-gp expression and function using T lymphocytes.
3. To investigate the *in vitro* effect of P-gp inhibitors on T cell proliferation and secretion on IL-2, IL-4, and IFN-γ.
4. To study the effect of immunosuppressive therapy on P-gp expression and function in T lymphocytes from renal transplant patients.

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Chapter 2

SIROLIMUS METABOLISM

2.1 ***Background***

Sirolimus (rapamycin) was originally isolated from a soil sample from Rapa Nui (Easter Island). It is produced by the streptomycete, *Streptomyces hygroscopicus* (Sehgal et al., 1975). Sirolimus is a colorless solid with a melting point of 183-185 °C and has a molecular formula $C_{51}H_{79}NO_{13}$ (Fig. 2.1). Initially, sirolimus was investigated as a potential drug for anticandida activity. It has potent activity (~ 0.02 μg/ml) against a variety of strains of *Candida albicans* (Vezina et al., 1975). Sirolimus was also found to have antitumor activity and immunosuppressive activity in animal models (Martel et al., 1977; Eng et al., 1984). Sirolimus inhibits murine, porcine, and human T cell proliferation *in vitro* with IC_{50} values in the range 0.1-300 nM (Sehgal and Bansbach, 1993; Sehgal et al., 1994; Wood and Bierer, 1994). *In vitro*, sirolimus also inhibits the proliferation of natural killer cells and lymphokine-activated cytotoxic T cells at higher concentrations (Sehgal et al., 1994).

In vitro, sirolimus and cyclosporine are synergistic in suppression of human T cell proliferation (Kahan et al., 1991). In a preliminary clinical study, sirolimus did not affect the pharmacokinetics of either cyclosporine or steroids in quiescent renal transplant recipients (Kahan et al., 1995). Sirolimus is presently undergoing clinical trials for use

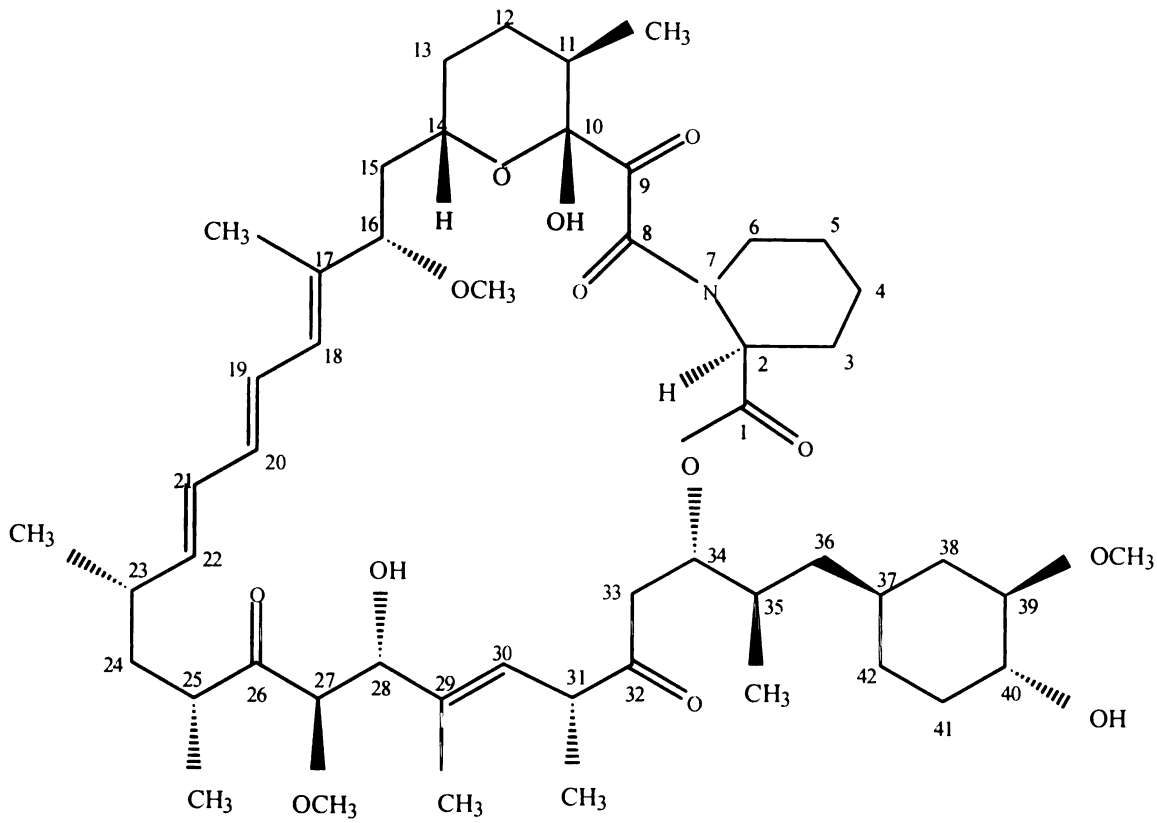


Fig. 2.1 Sirolimus

with cyclosporine in renal transplant patients. The oral dose of sirolimus in these trials ranges from 3 to 15 mg/ m^2. The population pharmacokinetics of oral sirolimus in kidney transplant patients receiving steady state oral cyclosporine are reported by Ferron et al. (1997). Sirolimus is absorbed rapidly (C_{max} between 1 and 2 hrs) followed by a bi-exponential decline. The half-life of elimination is approximately 60 hrs. Because of binding to red blood cell (RBC) proteins, it partitions extensively into RBCs with a blood/plasma ratio of 34.5. Cyclosporine pharmacokinetics are not affected by sirolimus. Systemic oral bioavailability of sirolimus is about 20%.The steady state blood volume of distribution is 1.76 L/kg and blood elimination clearance is 24 ml/hr/kg. The side effects of sirolimus observed in a phase I clinical study in stable renal transplant patients who were on oral cyclosporine have been described by Murgia et al. (1996). These include a reversible decline in platelet and white blood cell counts. The platelet toxicity was dose dependent while WBC toxicity was independent of dose. There was a dose-related increase in cholesterol levels. In another phase I clinical study, sirolimus AUC and trough levels were elevated when sirolimus and cyclosporine were dosed concomitantly as compared with dosing of the two drugs 4 hours apart (Kaplan et al., 1998).

Preliminary *in vitro* metabolism studies with human and rat liver microsomes indicate that rapamycin is metabolized by cytochromes P450 to several metabolites (Christians et al., 1992; Streit et al., 1996). Three metabolites have been identified: 39-O-demethyl sirolimus, 16-O-demethyl sirolimus, and 12-hydroxy sirolimus (Streit et al., 1996). The metabolites 16-O-demethyl sirolimus, 27-O-demethyl sirolimus, and 39-O-demethyl sirolimus demonstrate significant immunosuppressive activity *in vitro* (1/2-1/3rd

of sirolimus) (Kuhnt et al., 1997). These metabolites and 24-O-hydroxy sirolimus also bind with significant affinity to FKBP12 (0.4-4.5 times affinity with respect to sirolimus) (Kuhnt et al., 1997).

2.2 Objectives

Previous published studies represented early efforts at generation and structural identification of sirolimus metabolites, but did not emphasize enzyme-kinetics. Although, it is now known that CYP 450 enzymes mediate sirolimus metabolism (Christians et al., 1992; Sattler et al., 1992; Streit et al., 1996), the enzymes involved and kinetics of these reactions are not characterized. The initial goal of this research was to identify the major metabolites and metabolic pathways using an LC/MS based assay and to identify the enzymes mediating these reactions. This information is essential for determining the *in vivo* kinetics of sirolimus in humans. However, the actual study reported here was limited in scope in part due to difficulty in isolating metabolites for structural analysis by LC/MS and unavailability of synthesized metabolites for a comprehensive structure analysis and quantification. The identification of metabolites for this study was performed on the basis of retention times and mass to charge ratio. A complete analysis and structure identification of metabolites would require tandem mass spectrometry and NMR.

2.3 Materials and Methods

2.3.1 Chemicals and Specimens

Sirolimus was kindly provided by Wyeth-Ayerst (Radnor, PA). Acetonitrile, methanol, dichloromethane, and sulfuric acid were purchased from Fisher Scientific (Fair Lawn, NJ). All solvents were HPLC grade. cDNA overexpressed (supersomes™) human CYP3A4, CYP3A5, CYP2C19, CYP2B6, CYP2D6, and CYP2C9 were purchased from Gentest (Woburn, MA). Extraction columns (bonded phase C_{18}, 1 ml) were from Varian Sample Preparation Products (Harbor City, CA) and 250 X 4 mm 3 μm Hypersil ® C_8 analytical columns were from Keystone Scientific (Bellefonte, PA). The stock solution for protein content determination was from Bio-Rad Labs (Hercules, CA).

2.3.2 Incubation Conditions

A human liver sample was obtained after approval by the Committee on Human Research, University of California, San Francisco. Human liver microsomes were prepared from a pediatric liver by homogenization and differential centrifugation as described by Bornheim and Correia (1989). Sirolimus was metabolized by human liver microsomes using a modified published protocol (Christians et al., 1992). Briefly, sirolimus was incubated with 1 ml of microsomal suspension (3 mg/ml protein concentration) and 0.5 ml of NADPH generating system (2 mM EDTA, 10 mM $MgCl_2$,

18 mM isocitric acid, 667 U/L isocitrate dehydrogenase, 0.84 mM NADPH, 0.1 M phosphate buffer, pH=7.4) at 37°C under aerobic conditions. The reaction was stopped by adding 500 µl of acetonitrile. Samples were centrifuged at 2500g for 2 min and the supernatant loaded on 3-ml extraction columns that had been prewashed with 3 ml acetonitrile and 3 ml sulfuric acid (pH=3). Columns were dried and washed again with 3 ml sulfuric acid (0.01%) and 0.5 ml of hexane. Sirolimus and its metabolites were eluted in 2 ml methylene chloride. Methylene chloride was evaporated under nitrogen. Samples were reconstituted in 250 µl of 75:25 acetonitrile/sulfuric acid (0.01%) and washed again with 500 µl of hexane. The lower layer was withdrawn with a Pasteur pipette and analyzed using a Hewlett-Packard (Palo Alto, CA) HPLC/electrospray/MS system consisting of a series 1100 HPLC (G1322A degasser, G1312A binary pump, G1313A autosampler, and G1316A column thermostat), a 59887A electrospray interface with an Iris Hexapole Ion Guide (Analytica of Branford, Branford, CT), and a 5989B mass spectrometer (Hewlett Packard, Palo Alto, CA). The internal standard, 28-, 40- diacetyl sirolimus was synthesized as described by Streit et al. (1996).

2.3.3 Extraction of Sirolimus and Metabolites from Whole Blood

Blood samples were obtained from renal transplant patients at the University of Texas, Houston. They were shipped frozen over ice and stored at -80° C until ready for analysis. Stability studies in our laboratory indicated that there was no degradation up to 6 months at this temperature. The samples were thawed at room temperature and 1ml

blood was withdrawn for analysis. When a 1ml sample was not available, the volume was made up to 1 ml with blood from healthy volunteers. Red blood cells and proteins were precipitated with methanol/zinc sulphate (80:20). Samples were extracted and reconstituted as described for *in vitro* incubations.

2.3.4 Extraction of Sirolimus and Metabolites from Urine Samples

Urine samples were collected from a renal transplant patient with approval from the Human Research Committee, University of California, San Francisco. The urine samples represent pooled collection over two 24 hour periods within a week post-transplant. Urine samples were stored at 4-8° C until analysis. Aliquots of 200 ml of urine samples were acidified with sulfuric acid to pH=3. Sirolimus and metabolites were extracted twice by a liquid-liquid extraction process with ethyl ether. Ethyl ether was evaporated under nitrogen and samples reconstituted in 75:25 acetonitrile/sulfuric acid (0.01%). Samples were washed with hexane and 100 µl of the aqueous layer was withdrawn for analysis by LC/MS. The LC/MS assay was the same as used for *in vitro* microsomal incubations, as described in section 2.3.5.

2.3.5 Assay for Sirolimus and Metabolites

Sirolimus and its metabolites were analyzed using an assay based on solid/liquid extraction and HPLC/electrospray mass spectrometry analysis (modified from Streit et al., 1996). One hundred µl samples were injected onto the 250X4 mm analytical column filled with Hypersil MOS-1 of 3 µm particle size (Keystone Scientific, Bellefonte, PA). Methanol and 0.1 % formic acid supplemented with 1 µmol/L sodium formate were used as the mobile phase. Since sodium adducts gave the strongest signals, the mass spectrometer was set to single ion monitoring mode for detecting positive ions. The assay was linear over a range of 0.1-100 µg/L.

2.3.6 Data Analysis

The protein content of the microsomes was determined by the Bradford assay (Bradford, 1976). Cytochrome P450 content was determined using reduced carbon monoxide difference spectrum with a split beam spectrophotometer. Kinetic parameters were estimated from curves fitted using Microcalc Origin, Microsoft Corp. (Seattle, Washington). Hill plots were employed for analyzing cooperativity for the formation of metabolites. All results from human liver incubations are presented as means of 4 determinations (from the same liver sample), and cDNA expressed cytochrome P450 data are presented as the mean of 5 determinations.

2.4 ***Results***

Sirolimus metabolism was studied in human liver microsomal incubations. For all the studies sirolimus was dissolved in acetonitrile / water (75% acetonitrile / pH=3 water). The average protein content of the human liver microsome samples was 36.4 mg/ml. The cytochrome P450 content was 0.24 nmol/mg of protein. The metabolites were extracted by solid-liquid phase extraction and analyzed using electrospray LC/MS. The LC/MS analysis was based on elution times observed in single ion monitoring mode. Metabolites with the same mass to charge ratio but different retention times indicate distinct metabolites (Fig. 2.2). For this analysis, multiple metabolites with the same modification but different retention times are assigned numbers sequentially with 1 designating the metabolite with the shortest retention time followed by 2, the next shortest retention time, and so on. If only one metabolite for a particular mass to charge ratio was detected, it was not assigned any numbers.

The *in vitro* NADPH-dependent metabolism of sirolimus was studied in human liver microsomal incubations over a period of 3 hours as a stopped-assay (Fig. 2.3). The metabolism of sirolimus was time – dependent. The concentration – time profile exhibited a multi-exponential decline with a terminal rate-constant, $k = 0.14\ min^{-1}$. Several metabolites were observed; up to 12 different metabolites were detected. Both first and second generation metabolites were observed (metabolites with a single modification were considered first generation; metabolites with two or more modifications were considered second generation). A higher concentration of first

Fig. 2.2. Representative (single ion) chromatogram for *in vitro* metabolism of sirolimus. Integrated peaks represent different metabolites with similar modifications at different positions resulting in distinct retention times. The peaks represent: didemethyl sirolimus (908); demethyl sirolimus (922); demethyl hydroxy sirolimus (938); hydroxy sirolimus (952); dihydroxy sirolimus (968).

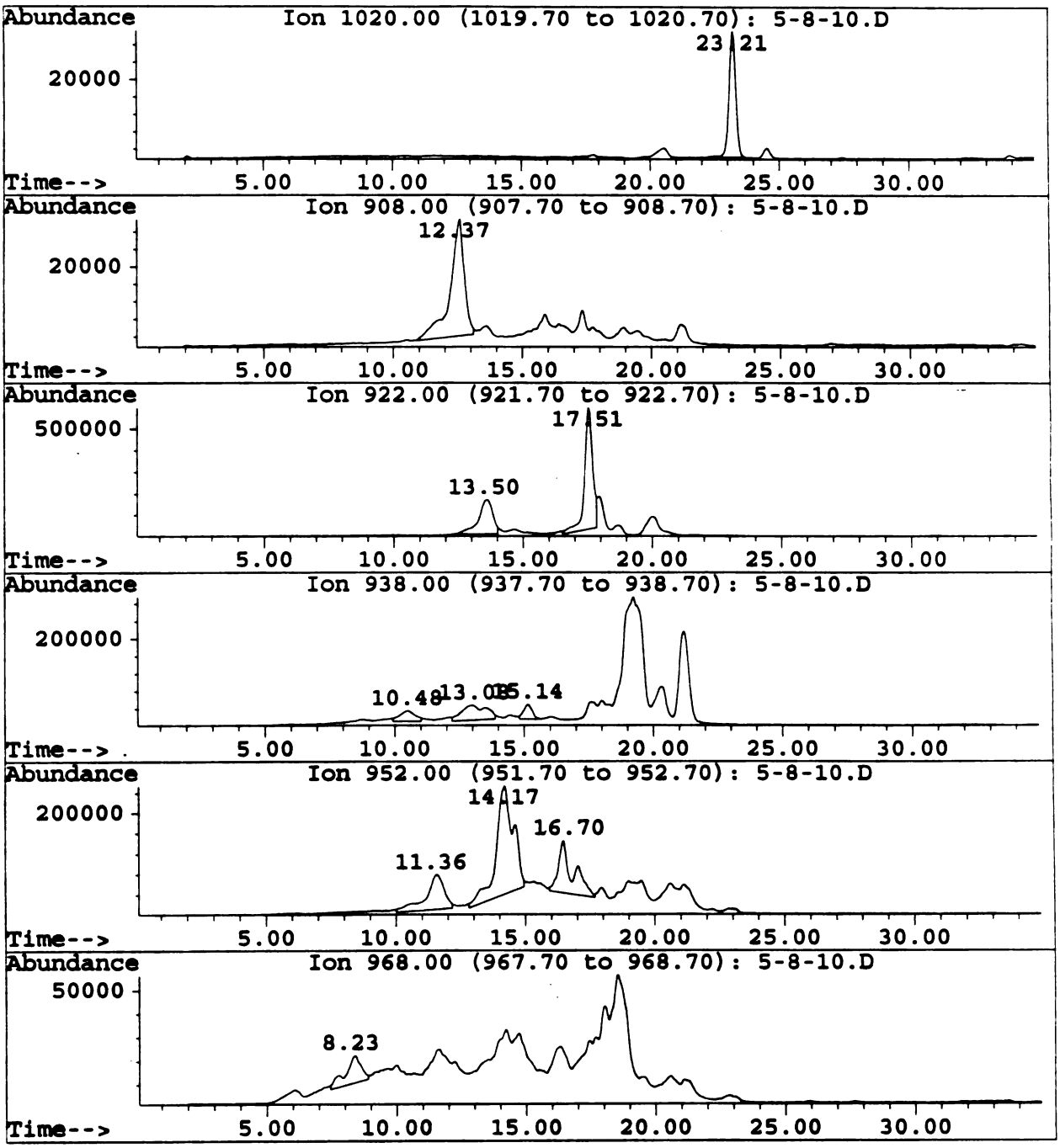
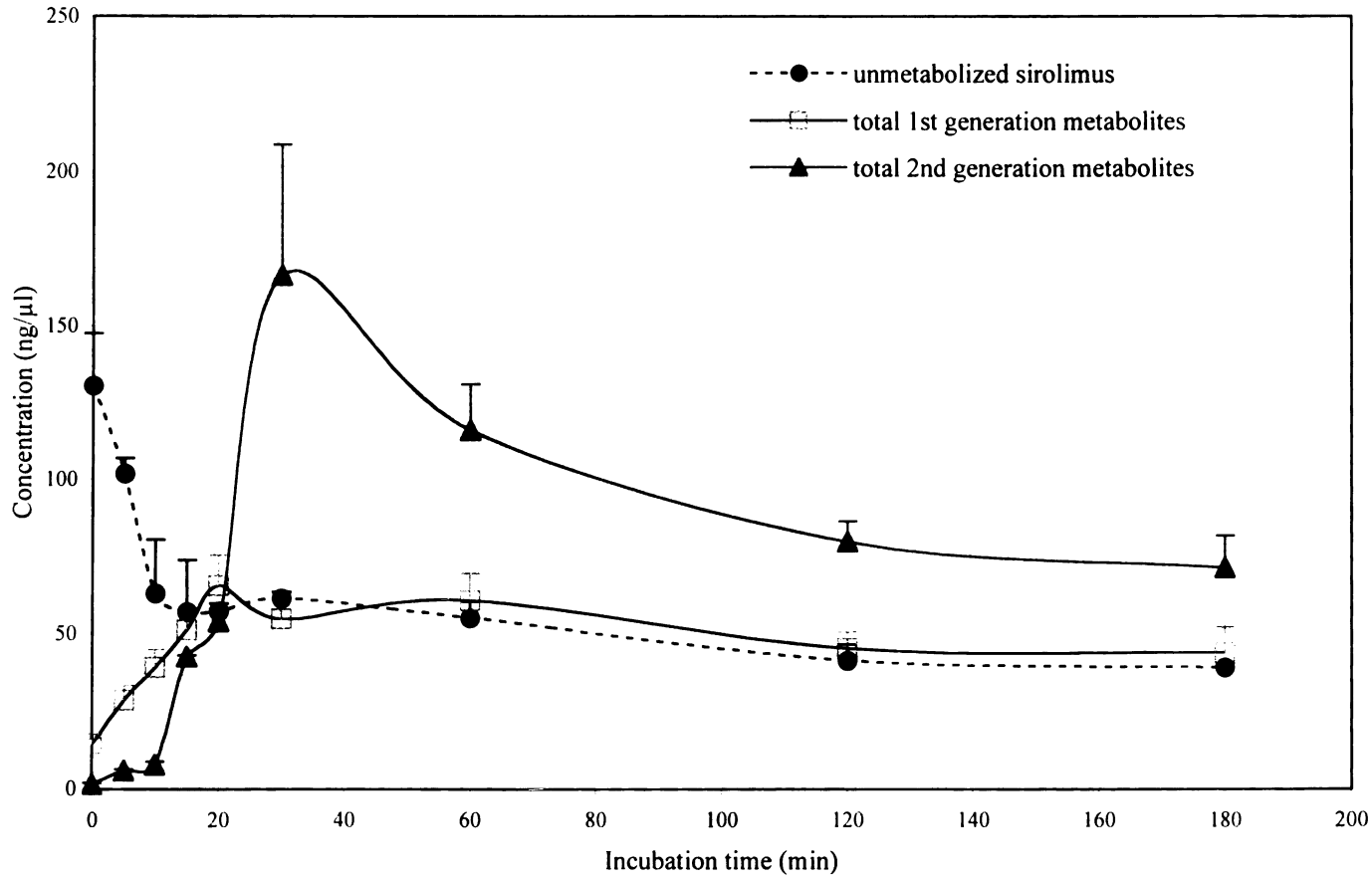


Fig. 2.3. Concentration - time profile for in vitro sirolimus metabolism.
Substrate conc. = 18.25 μM; preincubation time = 5 min; n = 4 (mean ± SD)



generation metabolites was observed at incubation times less than 20 min. Beyond 20 min incubation time, the concentration of second generation metabolites was significantly higher. The peak time for formation of first generation metabolites was 20 min; for second generation metabolites, the peak time was approximately 30 min (Fig. 2.3). The first generation metabolites (Figs. 2.4a,b) included three different demethyl sirolimus and three hydroxy sirolimus metabolites. The major second generation metabolites (Fig. 2.4c) included one demethyl hydroxy sirolimus; three different didemethyl sirolimus (only one is plotted in Fig. 2.4 c; the others were formed at very low concentrations); two different dihydroxy sirolimus metabolites; and one dihydroxy demethyl sirolimus (not plotted because of very low concentrations).

Based on the time-course data, a 15 min incubation period was selected to study the rate and apparent kinetic parameters for the formation of first generation metabolites in *in vitro* human liver microsomal incubations (Fig. 2.5). This incubation time ensured linearity for the formation of first generation metabolites. Although second generation metabolites were also formed at low substrate concentrations (Fig. 2.6), at higher substrate concentrations, the ratio of first generation metabolites was higher. The rate (v) versus substrate concentration (s) data for the formation of all first generation metabolites were best fit by sigmoidal Hill plots with Hill coefficients ranging from 1.5 – 2.25. The estimated kinetic parameters and calculated intrinsic clearances (V_{max}/K_M) are summarized in Table 2-1. Previously, Sattler and co-workers (1992) have determined the average V_{max} and K_M values for the formation of one demethyl sirolimus metabolite to be 8.1 μM (K_M) and 1.6 nmol/min/nmol P450 (V_{max}) using a Lineweaver-Burke plot. These

Fig. 2.4a. Concentration - time dependent formation of demethyl sirolimus (DS) in human liver microsomal incubations. Substrate conc. = 18.25 μM; preincubation time = 5 min; (n=4, mean ± SD)

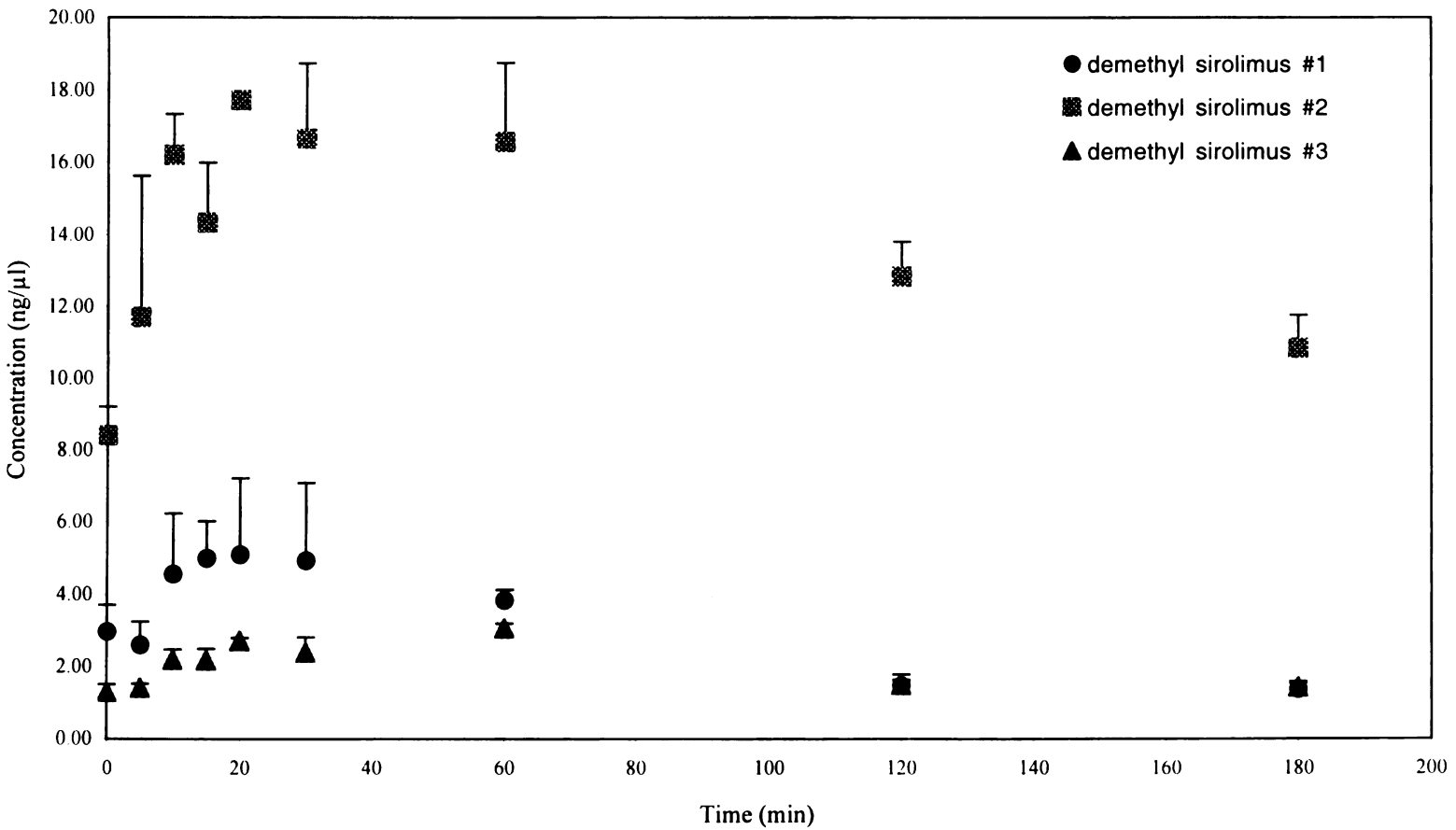


Fig. 2.4b. Concentration - time profile for the formation of 3 different hydroxy sirolimus (HS) metabolites in human liver microsomal incubations. Substrate conc. = 18.25 μM; preincubation time = 5 min; (n=4, mean ± SD)

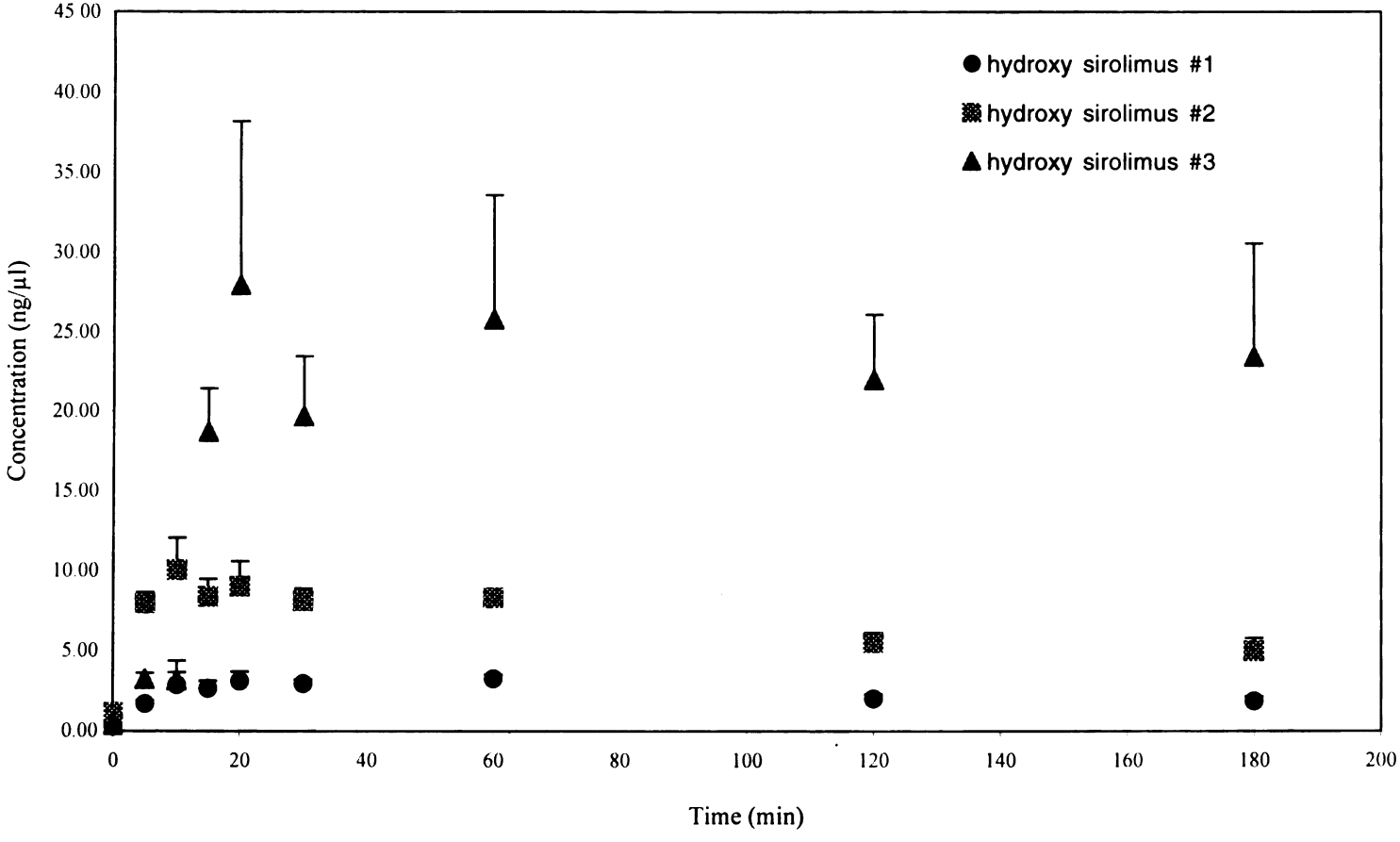


Fig. 2.4c. Concentration - time profile for the formation of major second generation metabolites in human liver microsomal incubations. Substrate conc. = 18.25 µM; preincubation time = 5 min; n=4 (mean ± SD)

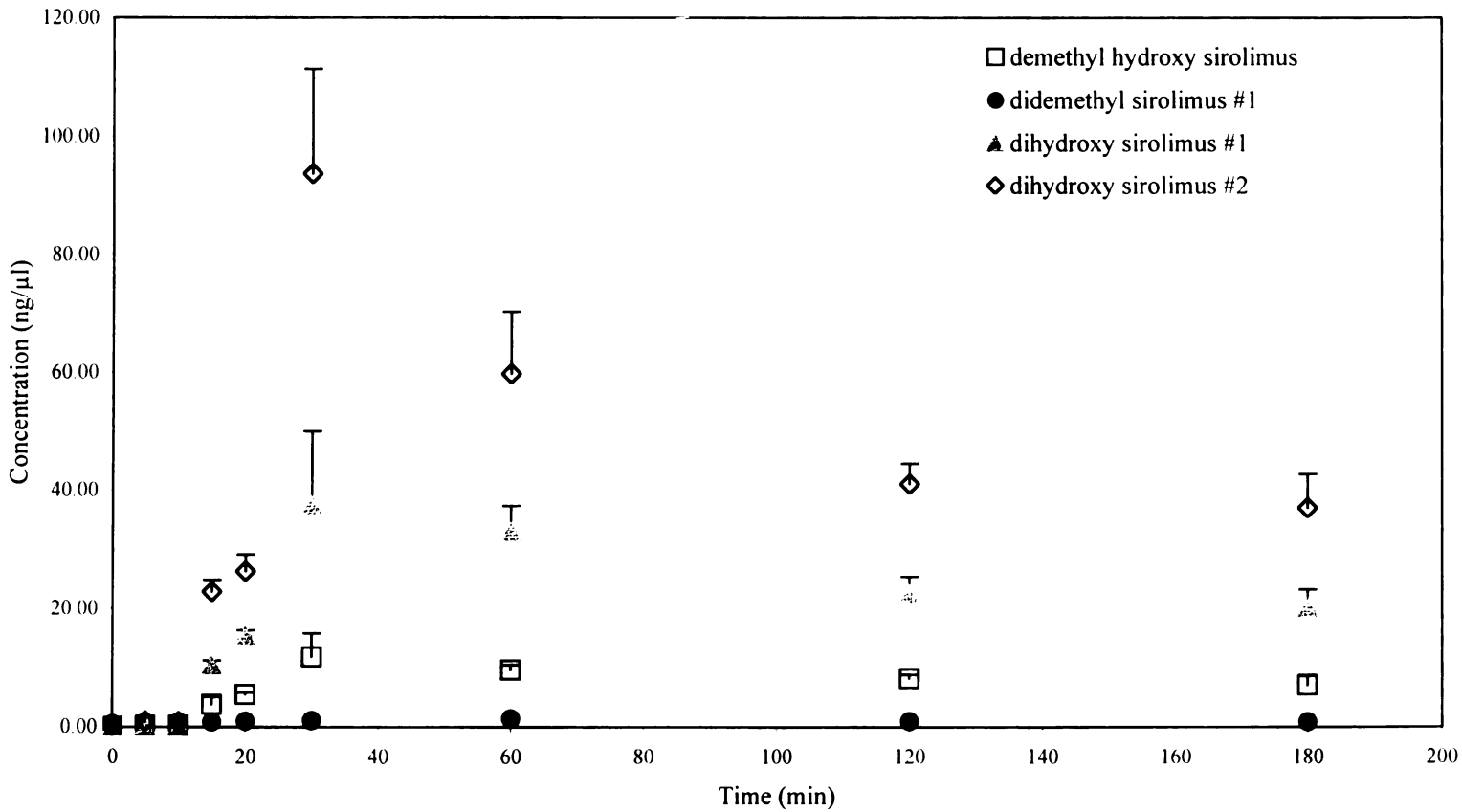


Fig. 2.5. Concentration dependent formation of first generation metabolites of sirolimus in human liver microsomal incubations. Protein conc. = 3 mg/ml; incubation time = 15 min; preincubation time = 5 min. (n=4; mean ± SD)

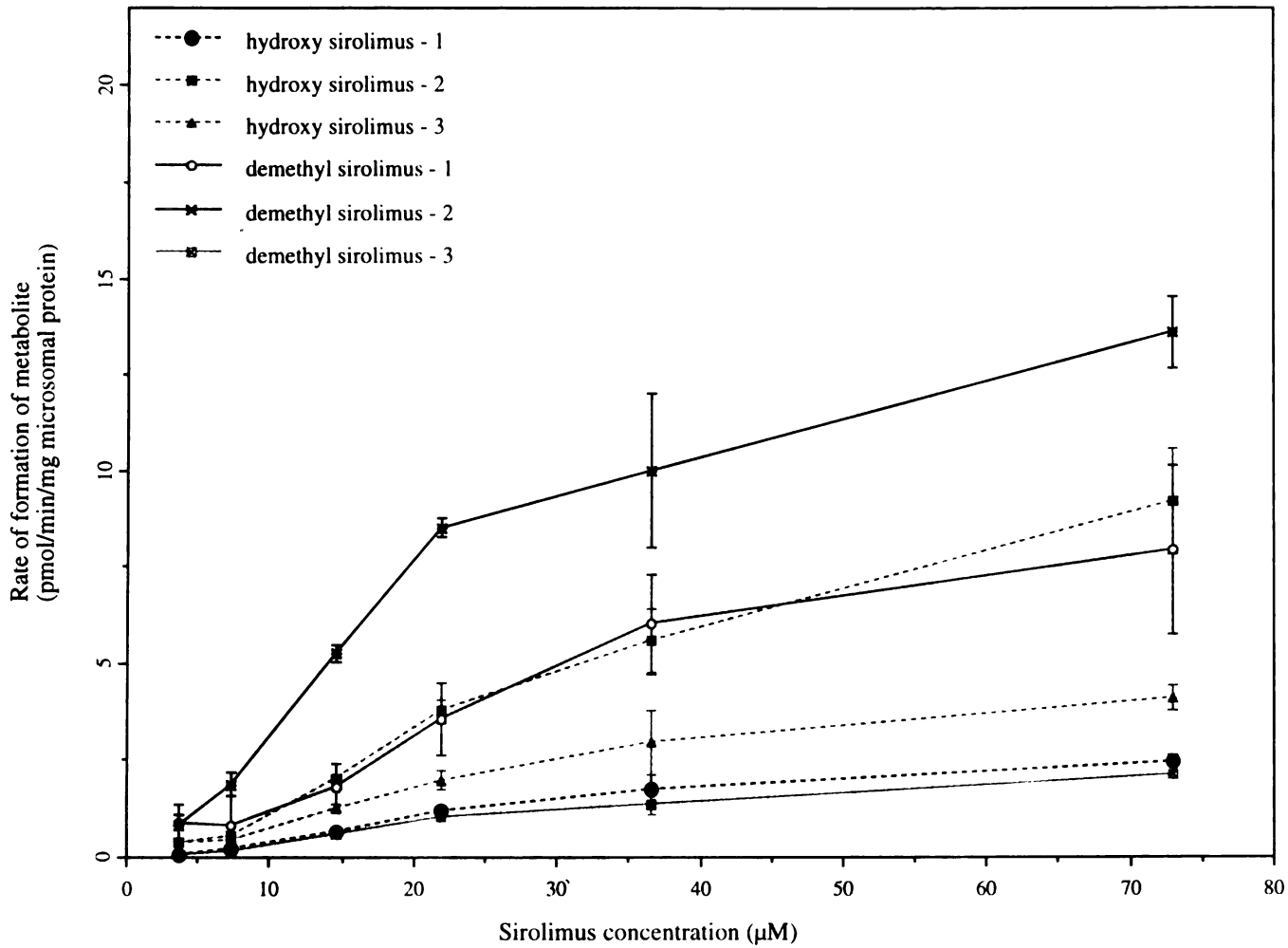


Fig. 2.6. Ratio of first and second generation metabolites to total metabolite concentration at different substrate concentrations (n=4)

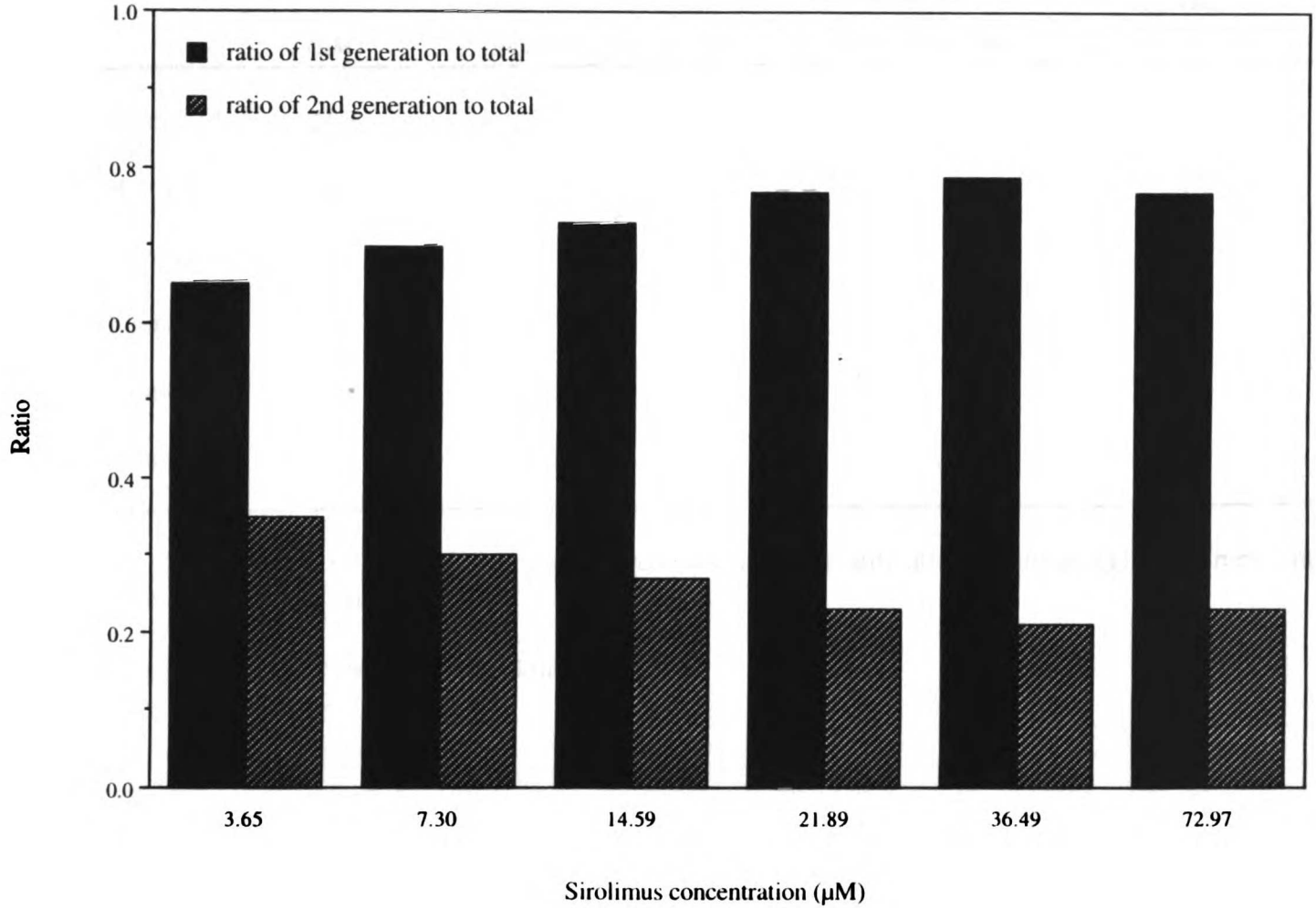


Table 2-1. Estimated kinetic parameters for the formation of first generation metabolites in human liver microsomes

Metabolite	K_M *(μM)*	V_{max} *(pmol/min/mg-protein)*	V^1_{max} *(pmol/min/nmol-pr)*	CL_{int}*(formation)* $= V_{max} / K_M$ *(μl/min/mg-protein)*	*Hill Coefficient*
demethyl sirolimus – 1	24.3	9.1	37.9	0.4	1.50
demethyl sirolimus – 2	16.1	15.5	64.6	1.0	1.64
demethyl sirolimus – 3	20.2	2.4	10.0	0.1	1.92
hydroxy sirolimus – 1	16.9	2.6	10.8	0.2	2.25
hydroxy sirolimus – 2	18.1	9.6	40.0	0.5	2.08
hydroxy sirolimus – 3	16.2	4.1	17.1	0.3	2.16

The subsets 1, 2, 3 in column 1 refer to different metabolites with similar modification but distinct elution times. The above data were obtained by sigmoidal fitting of data from Fig. 2.5.

[1] V_{max} values based on average P450 conc. = 0.24 nmol/mg

values are significantly different from the parameters determined in this study (Table 2.1).

Several different approaches can be used for identification of specific enzymes involved in metabolism. These include use of: selective chemical inhibitors, immunoinhibitors, purified enzymes, and cDNA-based individual enzymes. Each approach has its advantages and disadvantages. For studying the enzymes responsible for metabolism of sirolimus, I selected cDNA-based baculovirus expressed, commercially available enzymes. For an extensively metabolized compound such as sirolimus, this approach was considered the most sensitive and selective for detection of individual enzymes capable of metabolizing this drug. Sirolimus metabolism was evaluated at different substrate concentrations by the human cytochromes P450 -2B6, 2C9, 2C19, 2D6, 3A4, and 3A5. There was no appreciable metabolism mediated by the enzymes 2B6, 2C9, 2C19, and 2D6. The enzyme, CYP3A4 resulted in extensive metabolism of sirolimus to first and second-generation metabolites (Fig. 2.7). The formation of all metabolites was linear in the tested enzyme and substrate concentration range. However, there were differences in the detected metabolites from these incubations as compared with human liver enzymes. There were only two demethyl sirolimus (#1 and #3) metabolites formed with CYP3A4 at all tested concentrations. There were additional second-generation metabolites detected with the cDNA-based CYP3A4. The metabolism with CYP3A5 resulted in the formation of a new hydroxy sirolimus that had a distinct retention time compared to the human liver preparation mediated metabolites (Fig. 2.8). A high concentration of a distinct demethyl sirolimus was detected both in the presence

Fig. 2.7. Total first and second generation metabolites formed in in vitro incubations of sirolimus with cDNA expressed CYP3A4.
Substrate conc.=18.24 μM; incubation time = 30 min; n=5

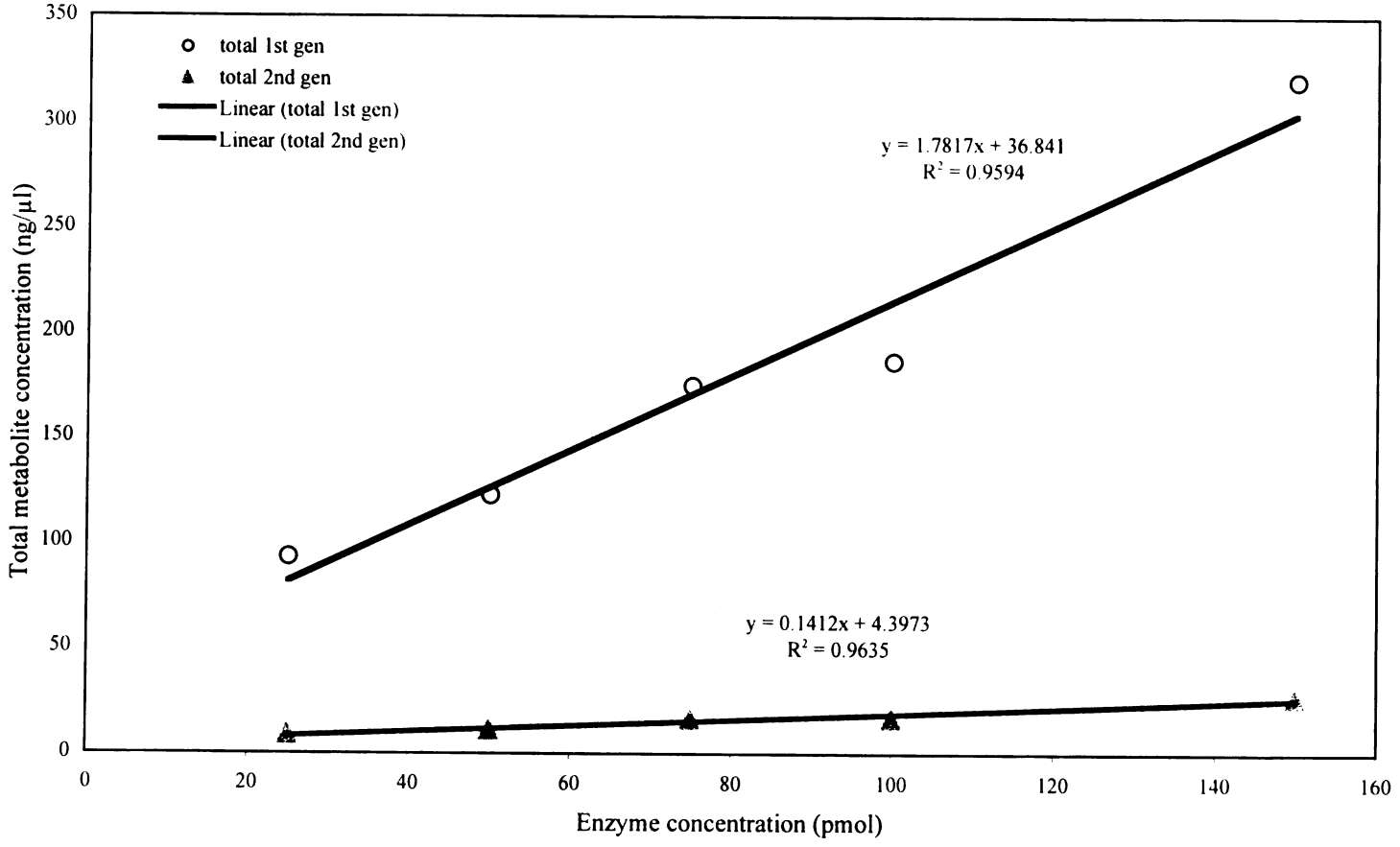
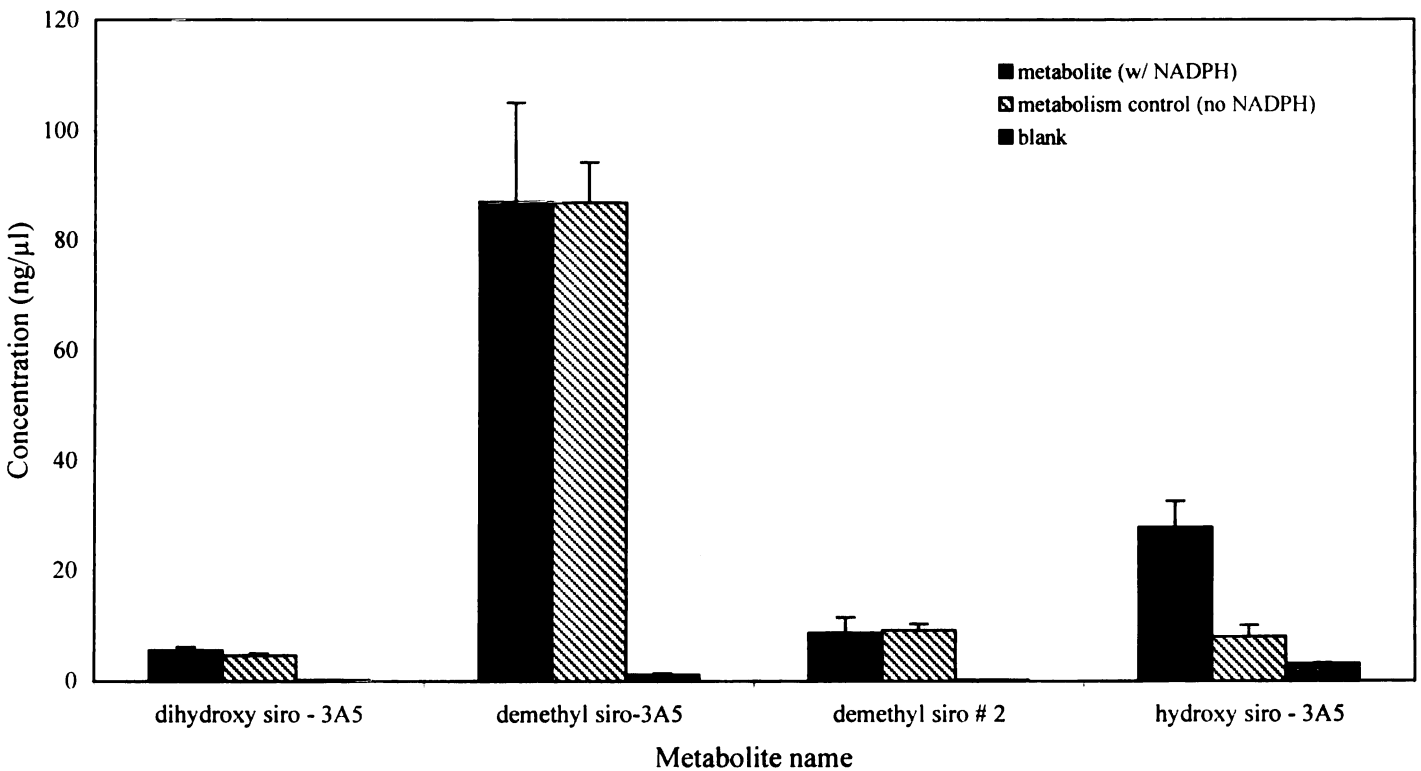


Fig. 2.8. Sirolimus reaction products from cDNA expressed CYP3A5 in vitro incubations. Substrate conc. = 37.34 μM; enzyme conc. = 50 pmol; incubation time = 30 min; n=5 (mean ± SD)

Note: Based on retention times and mass to charge ratios, dihydroxy siro-3A5; demethyl siro-3A5 and hydroxy siro-3A5 modifications of sirolimus were observed only in incubations with cDNA-based CYP3A5



and absence of NADPH. This could indicate a degradation product or a metabolite formed by an unknown pathway. Other products observed in these incubations included demethyl sirolimus #2, and a new dihydroxy sirolimus. Both these products were formed at similar amounts in the presence and absence of NADPH.

The blood samples from renal transplant patients were analyzed for cyclosporine, sirolimus and their major metabolites (Figs. 2.9 a,b and 2.10 a,b). The samples were drawn from patients taking cyclosporine as their primary immunosuppressant and represent different time-points and patients. These blood samples had a high concentration of metabolites to parent ratio for both drugs, cyclosporine and sirolimus.

Two urine samples were obtained within one week post-transplant from a single transplant patient, for analysis of sirolimus and its metabolites. Sample number 1 represents early collection periods after initiating sirolimus therapy. Sample number 2 was collected over the second and third days post transplant. Both these samples indicated the presence of first and second-generation metabolites along with a small amount of sirolimus (Fig. 2.11). The metabolites were present in significantly higher amounts than sirolimus in both these samples. The first generation metabolites were demethyl and hydroxy sirolimus. The major second-generation metabolite was primarily didemethyl sirolimus, although a small amount of dihydroxy sirolimus was also detected.

Fig. 2.9a. Representative (single ion) chromatogram for cyclosporine and some of its major metabolites in blood from renal transplant patients taking cyclosporine and sirolimus.

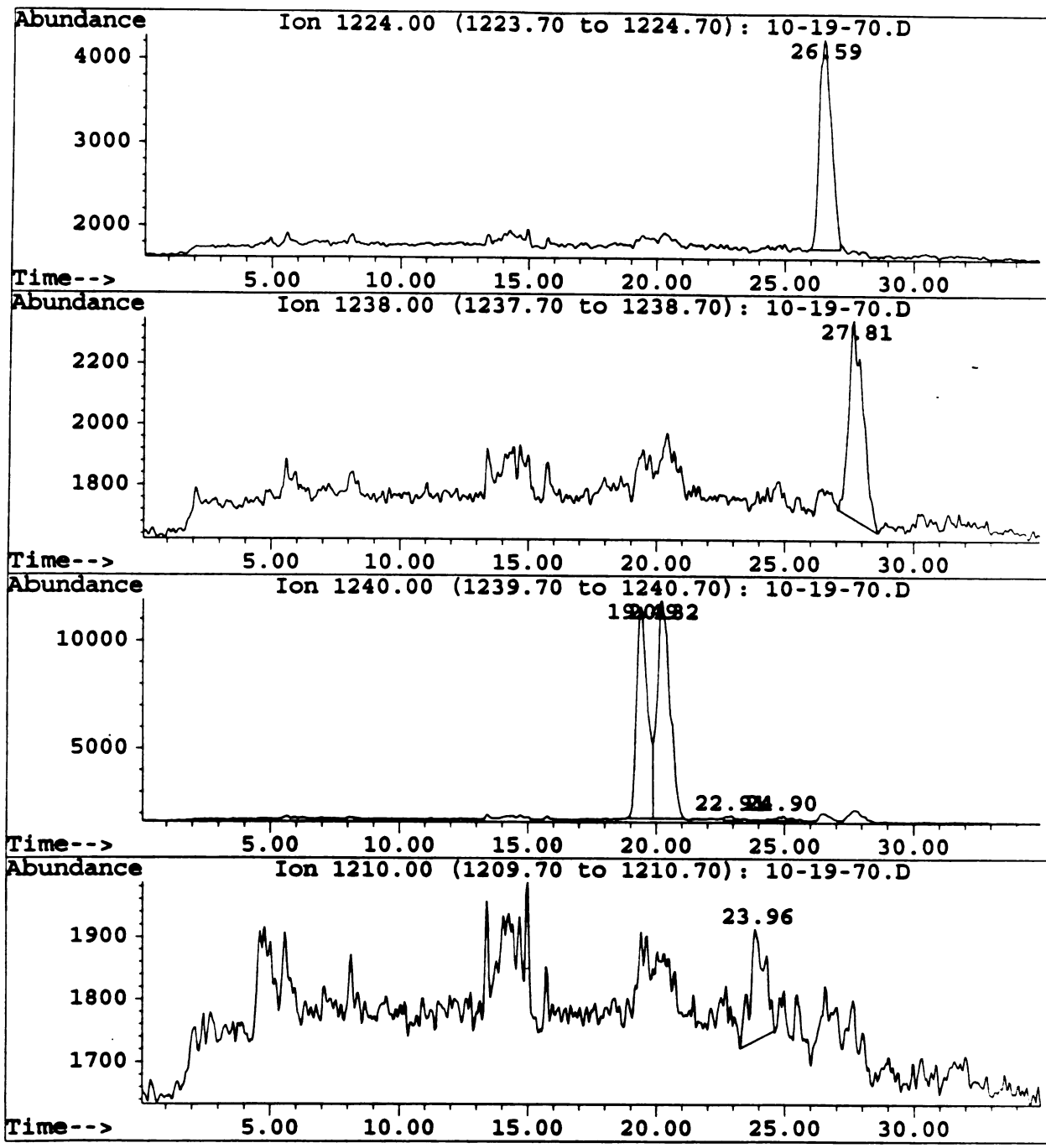


Fig. 2.9b. Cyclosporine and total metabolite concentrations in blood of kidney transplant patients in the presence of sirolimus and its metabolites in blood

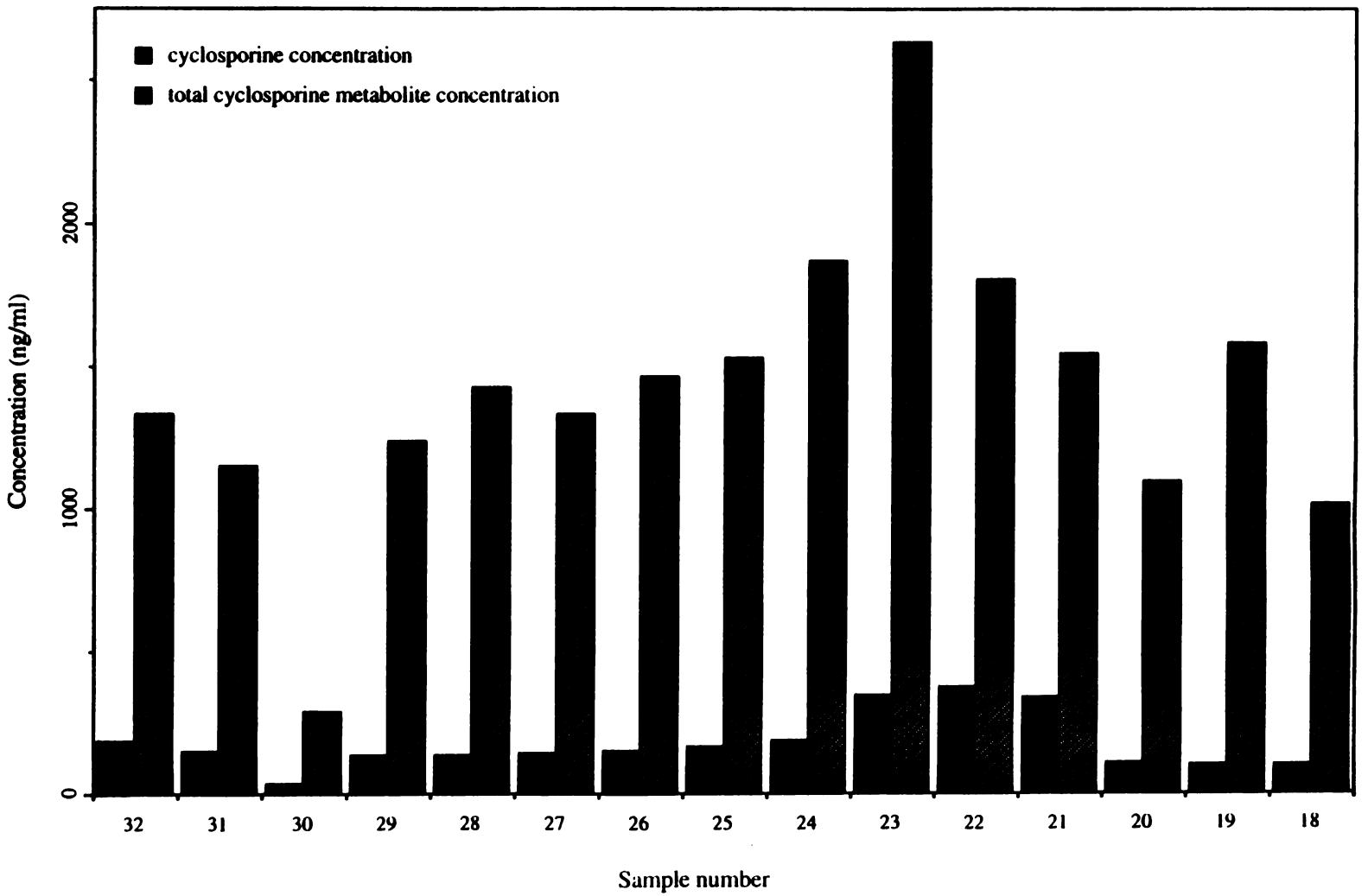


Fig. 2.10a. Representative (single ion) chromatogram for sirolimus and its metabolites in blood from renal transplant patients taking cyclosporine and sirolimus.

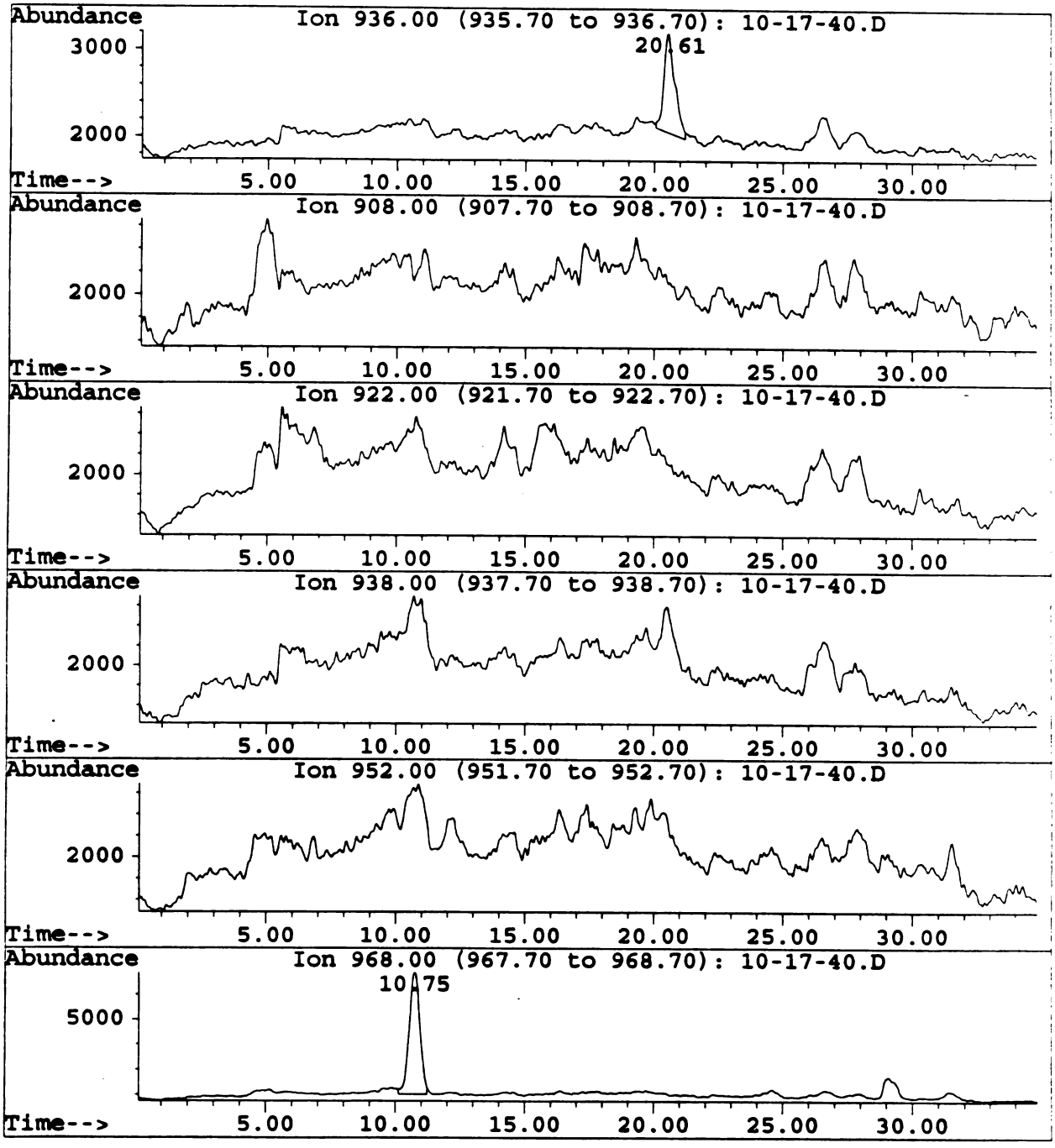


Fig. 2.10b. Sirolimus and total metabolite concentrations in blood of kidney transplant patients in the presence of cyclosporine and its metabolites in blood

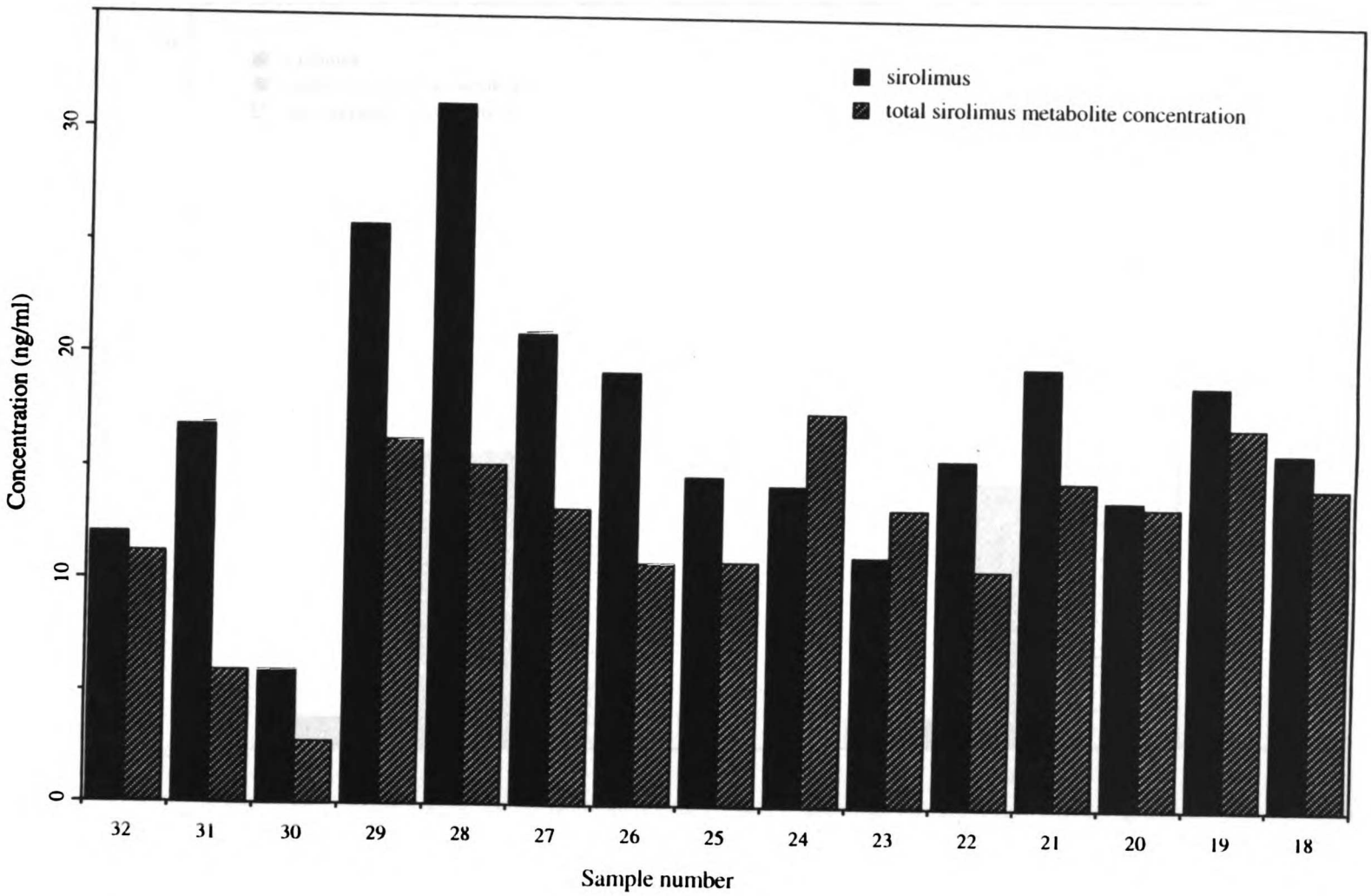
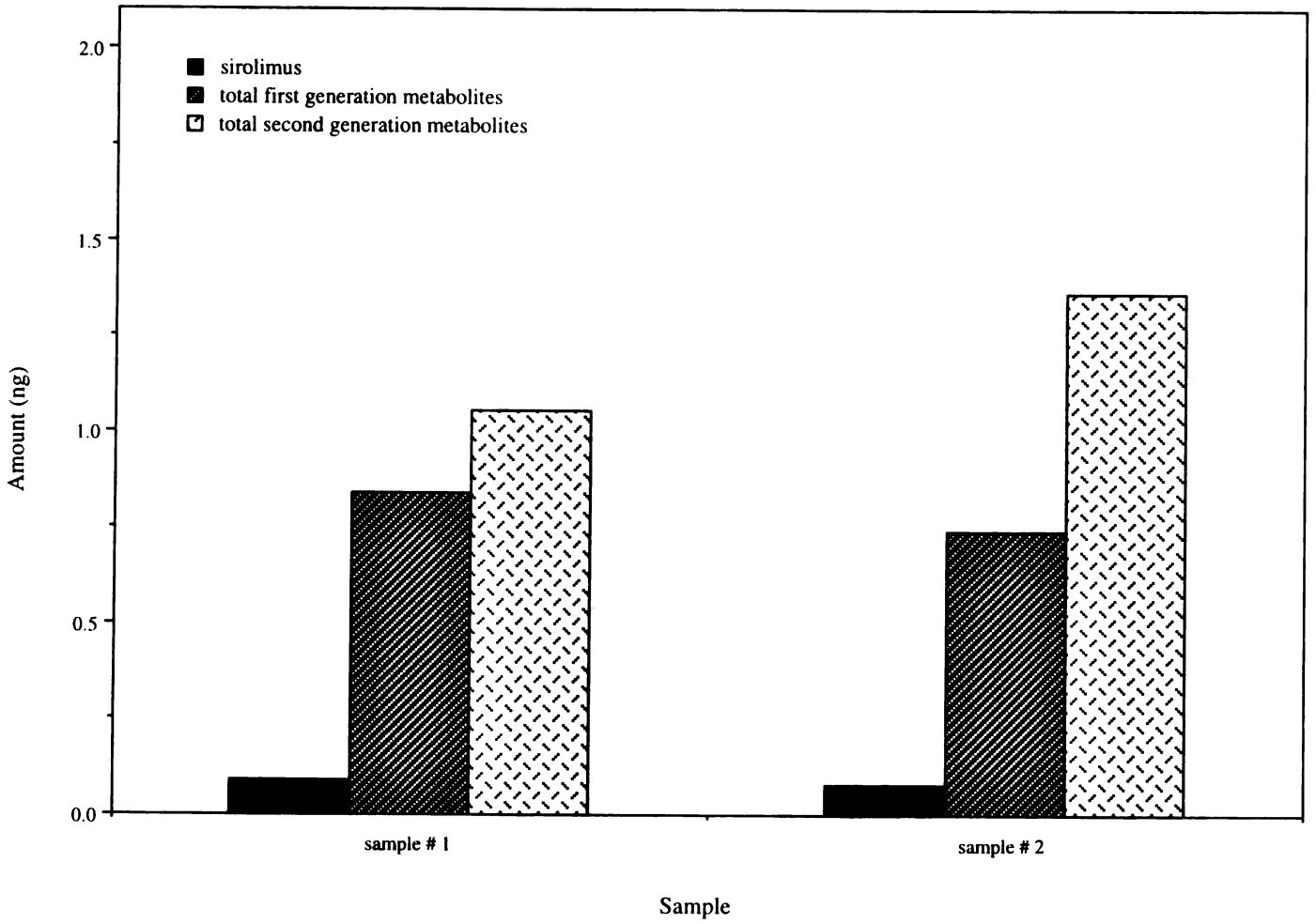


Fig. 2.11. Sirolimus and metabolites isolated from two different urine samples of a single renal transplant patient



2.5 *Discussion*

Sirolimus is a macrolide with potent immunosuppressive properties. The mechanism of action is distinct from cyclosporine, the most widely used immunosuppressant. In animal studies, it has demonstrated synergistic effects to cyclosporine (Brazelton and Morris, 1996). Sirolimus is being investigated for use with cyclosporine as an immunosuppressive agent. Previous reports suggest that sirolimus is a CYP3A substrate and undergoes gut and liver metabolism. The extent of metabolism and the kinetics for the formation of these metabolites were not determined in these previous studies. Here, I have focused on characterization of metabolism and metabolic pathways of sirolimus using human liver microsomes and individual cDNA expressed enzymes.

Sirolimus is extensively metabolized *in vitro* by human liver microsomes to form approximately 12 different phase I metabolites. The metabolism appears to be sequential. The concentration – time profile for the formation of second generation metabolites suggests that these metabolites may also be further metabolized to subsequent generation metabolites. The cDNA-based metabolism data suggest that the metabolites themselves are also substrates of CYP3A4. The first generation metabolite formation was sigmoidal (Fig. 2.5, Table 2-1) indicating a cooperative interaction between the drug and enzymes. The sigmoidal nature of metabolism reduces the concentration range over which the formation of metabolites is linear. For the first generation metabolites the linear range for product formation was between 5-20 μM. The large number of second generation metabolites observed in cDNA-based CYP3A4 metabolism studies suggests that *in vivo*,

numerous metabolites can be formed depending on access to enzymes and concentrations of drugs and metabolites. The absence of formation of demethyl sirolimus #2 in CYP3A4 incubations suggests that other enzymes, possibly CYP3A5, could be the major enzyme responsible for this metabolite formation.

The extensive metabolism of sirolimus over a narrow concentration range and the overlap of enzyme specificity between sirolimus and its metabolites raise the possibility of pharmacokinetic interactions *in vivo* between sirolimus, its metabolites and other CYP substrates/modulators in transplant patients. However, in the dose ranges evaluated in clinical studies, there is no evidence of nonlinear pharmacokinetics of sirolimus (Ferron et al., 1997).

Based on the literature (Streit et al., 1996) and the studies reported here, there appears to be a possibility of mistaking degradation products and metabolism products since both can have similar modifications. It is important to distinguish between the two by incorporating appropriate controls.

The blood samples from patients receiving sirolimus were analyzed for sirolimus and its first and second generation metabolites using LC/MS. Specifically, blood samples were analyzed for hydroxy, demethyl, didemethyl, and hydroxy demethyl sirolimus, along with sirolimus. These metabolites were selected based on *in vitro* metabolism data as most likely to be present at detectable levels in the blood samples, since the volume of blood sample available was limited (in most cases, 1ml or less). Although there was

limited information regarding the blood samples because of clinical study masking requirements, it was considered interesting and important to analyze the samples and compare the metabolite data with *in vitro* metabolism data. In human blood, there are detectable levels of first and second-generation metabolites that were previously observed in the *in vitro* metabolism studies. The considerable variation in the ratio of parent to metabolite in these samples indicates that, as observed *in vitro*, the metabolism is dependent upon the concentration-time of drug exposure to enzymes.

The apparent intrinsic clearances for formation of first generation metabolites of sirolimus are listed in Table 2-1. *In vitro* clearances have been used by various researchers for prediction of hepatic metabolic clearance in humans (Hoener, 1994; Houston, 1994; Iwatsubo et al., 1997) in order to better predict pharmacokinetic parameters. In this study, I determined the *in vivo* intrinsic clearance of sirolimus based on the methods described by Iwatsubo et al. (1997). Assumptions included the well-stirred model and negligible renal clearance of unchanged drug. Based on the *in vitro* clearance values determined in this study, the total *in vitro* clearance of sirolimus was 2.5 µl/min/mg microsomal protein. For a whole liver, this value translates to 197 ml/min. The blood to plasma ratio for sirolimus is 31 to 1 (Ferron et al., 1997), unbound drug in blood is 0.175% (Yatscoff et al., 1996). Based on these values, the calculated hepatic blood clearance of sirolimus is 0.013 ml/min. The reported total blood clearance from human data is 28 ml/min (Ferron et al., 1997). The *in vitro* clearance value of 197 ml/min (if binding is not important) or 0.013 ml/min (if partitioning into red blood cells limits access to microsomes) is significantly different from the value determined in clinical

studies. This difference could arise because of several reasons: a difference in protein binding in human liver microsomal preparations and *in vivo,* thus altering the availability of the drug to metabolizing enzymes; effect of other drugs taken by patients in whom the *in vivo* clearances were determined; transporter mediated processes; source of liver for *in vitro* studies (in this study a pediatric liver was used); or the solvent used for dissolving sirolimus for *in vitro* experiments. Other researchers (unpublished communications) have also noticed large differences in scaled up clearances based on *in vitro* metabolism data and hepatic clearance values determined *in vivo* for poorly soluble and highly protein bound drugs. More clinical information is required for a complete elucidation of the *in vivo* metabolism of sirolimus.

In conclusion, sirolimus metabolism was studied using *in vitro* human cytochromes P450 enzymes. Sirolimus is extensively metabolized by human liver microsomes to several metabolites. It undergoes sequential metabolism. In human liver microsomal incubations, several first and second generation metabolites were observed. The formation of first generation metabolites followed Hill kinetics. The data from cDNA expressed individual enzyme incubations suggests that the major enzymes responsible for metabolism of sirolimus are CYP3A4 and CYP3A5. Most of the metabolites detected in *in vitro* incubations were also detected in the blood of renal transplant patients.

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Chapter 3

AN *EX-VIVO* ASSAY FOR STUDYING MODULATION OF P-GLYCOPROTEIN (P-GP) FUNCTION

3.1 ***Background***

Organisms as diverse as yeast, bacteria, and humans possess transporters, some of which are similar in structure and function. ATP-binding cassette (ABC) transporters are one such class of energy dependent transporters (Higgins, 1992). The multidrug resistance (MDR) and multidrug resistance associated protein (MRP) groups of transporters belong to this class (Cole et al., 1992; Gottesman and Pastan, 1993). The MDR genes encode P-glycoprotein (P-gp) which has a wide tissue distribution in humans (Table 1-1). P-glycoprotein transporters are also present in human pathogens such as *Plasmodium falciparum*, *Leishmania donovani,* and *Candida albicans* (van Veen and Konigs, 1997). In humans, P-gp is predominantly involved in xenobiotic efflux, thereby protecting host tissue from toxic side effects.

Most P-gp substrates are hydrophobic molecules with a basic nitrogen atom and two planar aromatic rings, but there are exceptions (Zamora et al., 1988). The substrates include such structurally dissimilar drugs as peptides, alkaloids, steroids, immunosuppressive drugs, and calcium channel blockers (Wacher et al., 1995; Ueda et

al., 1997). The molecular weight of most substrates ranges from 300 to 2000 Da (Ueda et al., 1997).

P-glycoprotein is expressed on the plasma membranes as a 12 transmembrane domain (TMD) protein with 2 ATP binding sites present on the cytosolic side (Chen et al., 1986; Gerlach et al., 1986; Gros et al., 1986). The 12 TMDs are organized into two nucleotide-binding domains (NBDs). P-glycoprotein is glycosylated at sites on the first extracellular loop, although it is unknown whether glycosylation is essential or functional (Schinkel et al., 1993). P-glycoprotein has also been identified intracellularly in the cytosol and Golgi bodies of tumor derived cells and peripheral blood mononuclear cells (Deusing and Slate, 1994; Molinari et al., 1994; Malorni et al., 1998) where the function is unknown.

Mutational analysis has identified several residues that may be important in substrate specificity, but a comprehensive analysis is still required for complete elucidation of essential residues (Higgins et al., 1997). P-glycoprotein is structurally similar to the cystic fibrosis transmembrane conductance regulator (CFTR) gene that is a chloride channel (Riordan et al., 1989). P-glycoprotein is widely expressed in several tissues (Table 1-1) and is overexpressed in several tumors making them refractory to drug therapy. In the intestines, P-gp limits the bioavailability of orally delivered drugs such as paclitaxel, by pumping the drugs back into the gut lumen (Sparreboom et al., 1997).

P-glycoprotein is commonly believed to be a xenobiotic efflux pump with no natural substrates. But there is limited evidence suggesting it may also be involved in endogenous secretory functions. P-gp is expressed in hormone-producing cells and reproductive organs. In these cells, it may be involved in secretion of steroids or protection of cells from local high endogenous concentrations of steroids (Schinkel, 1997). In immune cells, P-gp may be involved in secretion of cytokines. *In vitro*, cytokines such as interleukin-2 (IL-2), IL-4, and interferon-γ (IFN-γ) are substrates of P-gp (Drach et al., 1996). The cytotoxic activity of natural killer (NK) cells is reduced in the presence of P-gp inhibitors (Chong et al., 1993). These observations suggest a biological role for P-gp in addition to drug transport.

A rare inherited disorder, Scott syndrome, associated with excessive bleeding episodes following exposure to phosphatidylserine, may represent a human P-gp knockout. Toti and co-workers (1997) have demonstrated a lack of expression of both MDR1 and MDR3 in B cells of Scott syndrome patients using semi-quantitative techniques such as reverse transcriptase – polymerase chain reaction (RT-PCR) and Northern blot analysis. Although P-gp is not suspected to be involved in the phenotype for this disease, it is hypothesized that a regulatory protein might be mutated resulting in the lack of expression of MDR genes.

The widespread expression of P-gp not only in malignant cells but normal human tissues has important implications in drug therapy. The drugs can be substrates of P-gp where their intracellular concentrations could be altered because of efflux; the drugs

could themselves modulate the expression and/or function of P-gp thereby altering not only their intracellular concentrations but also the function of other drugs that may be substrates of P-gp. This could result in drug-drug interactions. Defining the specific P-gp modulators and their kinetics is thus essential in order to fully characterize and understand pharmacokinetic/pharmacodynamic interactions of these drugs.

Most substrates and inhibitors of P-gp have been characterized *in vitro* using tumor derived cell lines, such as Caco-2, or transfected animal tissue derived cell-lines, such as MDCK-MDR1, which overexpress P-gp following exposure to chemotherapeutic drugs (Hollo et al., 1996; Tiberghien and Loor, 1996; Flanagan and Benet, 1999). *In vivo* experiments have been performed in normal and knockout mice (Schinkel et al., 1994). The information generated by both methods is extremely valuable but it is possible that there may be biochemical differences in the expressed P-gp in both kinds of models (Hoffman and Roepe, 1997). Thus, it is of interest to study P-gp function in normal human tissues.

3.2 Objectives

My goal was to develop and characterize an assay method using normal human tissue and to study the effect of P-gp inhibitors on a model substrate. Lymphocytes represent such a tissue since they inherently express P-gp and are relatively easy to procure. The functional activity of P-gp can be investigated by measuring rhodamine-123

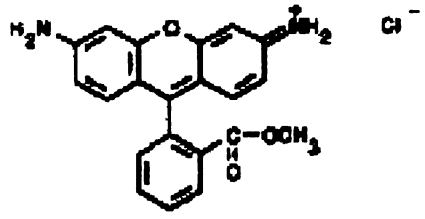
(Rh-123) retention/efflux in the presence of P-gp modulators. Rhodamine 123 is a cationic, fluorescent dye (Fig. 3.1a) that is readily taken up by cells and actively pumped out of the cells by P-gp (Shapiro and Ling, 1998). The ability of lymphocytes to efflux Rh-123 in the presence of a P-gp inhibitor is decreased (Andreana et al., 1996; Drach et al., 1996; Aggarwal et al., 1997) leading to increased intracellular accumulation of Rh-123. This increase in intracellular retention of Rh-123 is reflected in increased intensity of Rh-123. By measuring the intensity of Rh-123 in the presence and absence of inhibitors, the inhibition constants for different drugs can be determined. The drugs evaluated for these studies include known P-gp inhibitors (Wacher et al., 1995): cyclosporine, tacrolimus (FK506), quinidine, verapamil, and vinblastine (Fig. 3.1b). I also studied the effect of other drugs which have been reported not to be inhibitors of P-gp but of other drug efflux transporters (Hollo et al., 1996; Draper et al., 1997) such as multidrug resistance associated protein (MRP). These include probenecid, sulfinpyrazone, and indomethacin (Fig. 3.1b).

3.3 Materials and Methods

3.3.1 Chemicals and Specimens

Cyclosporine, quinidine, verapamil HCl, vinblastine sulfate, indomethacin, sulfinpyrazone, probenecid, sodium azide, and rhodamine 123 were purchased from Sigma Chemical Co. (St. Louis, MO). Fujisawa Pharmaceutical Company (Deerfield, IL)

Fig. 3.1a. Molecular structure of rhodamine 123 ($C_{21}H_{17}ClN_2O_3$); molecular weight 380.83



Absorption (left) and fluorescence (right) emission spectra of rhodamine 123 in methanol (Molecular probes literature http://www.probes.com)

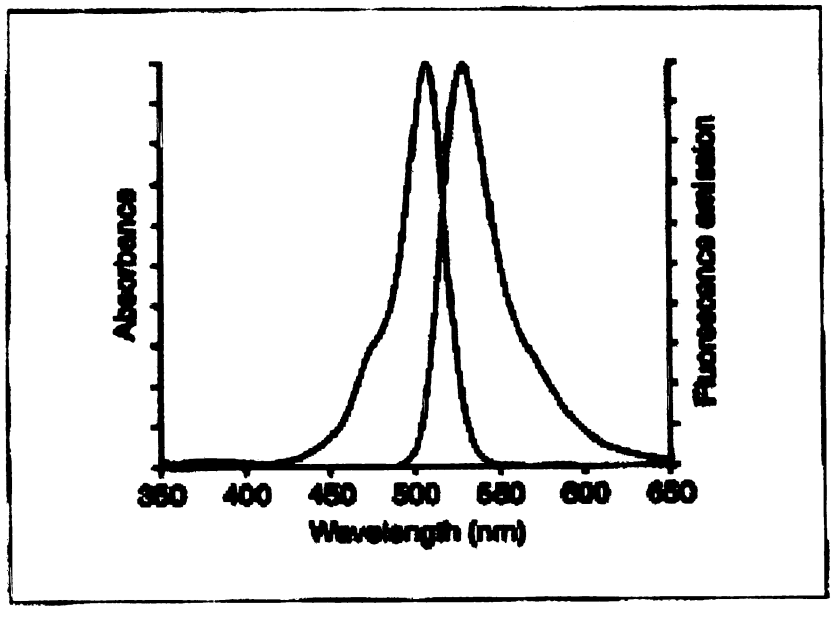
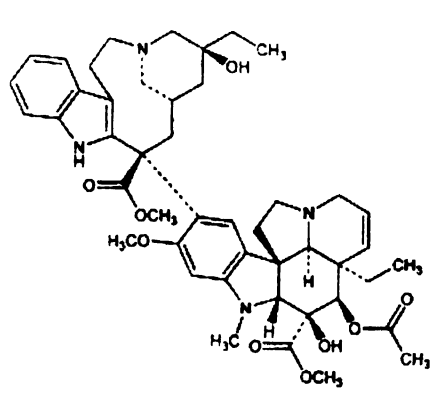
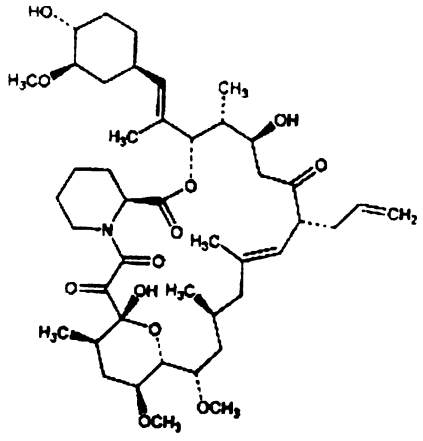


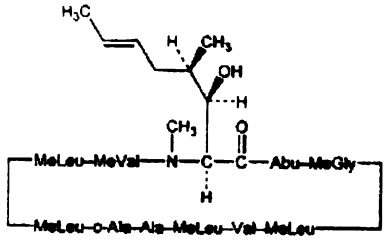
Fig. 3.1b. Molecular structures (Budavari et al., 1996)



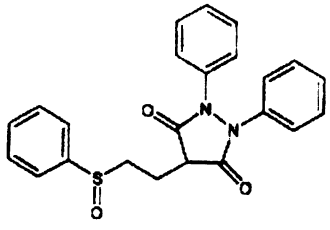
Vinblastine



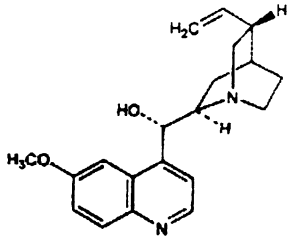
Tacrolimus



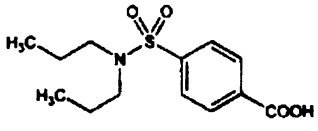
Cyclosporine



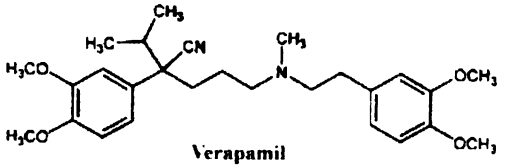
Sulfinpyrazone



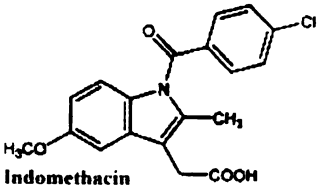
Quinidine



Probenecid



Verapamil



Indomethacin

kindly provided tacrolimus (FK 506). Mycophenolate mofetil (MMF) was a kind gift from Roche Biosciences (Palo Alto, CA). Optilyse C was purchased from Immunotech (Marseille, France). Dye-free Ca^{2+}/Mg^{2+} free Hanks BSS, RPMI 1640 with 25 mM HEPES, fetal calf serum (FCS), phosphate buffered saline (PBS), bovine serum albumin (BSA), and cell preservation medium (10% FCS, 10% DMSO in MEM EBSS, pH=7.2) were all obtained from the UCSF Cell Culture Facility (San Francisco, CA). Acridine orange, ethidium bromide, propidium iodide were procured from Molecular Probes Inc. (Eugene, OR). Ficoll-Paque® was obtained from Pharmacia Biotech AB (Uppsala, Sweden). Flow-Check™ fluorospheres were purchased from Coulter Corporation (Miami, FL). Custom ordered unconjugated P-glycoprotein antibody, 15D3, second-step goat anti-mouse Ig FITC and mouse Ig fluorescence control antibodies were purchased from Becton-Dickinson (San Jose, CA). All other solvents were obtained from Sigma Chemical Co. (St. Louis, MO).

3.3.2 Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Peripheral blood mononuclear cells were isolated by density centrifugation from buffy coats purchased from the UCSF blood bank (San Francisco, CA). Briefly, blood was diluted with dye-free Ca^{2+}/Mg^{2+} free Hanks BSS at the ratio of 1:2 (buffy coat:Hanks). The diluted blood was layered according to the manufacturer's instructions on top of Ficoll-Hypaque (Ficoll-Paque®) and centrifuged at 3000 rpm for 30 minutes. The PBMC-rich layer was withdrawn and washed twice with Hanks BSS (dye-free,

$Ca^{2+/}Mg^{2+}$ free). Cells were resuspended in cell preservation medium at 40 million cells per ml in each vial and cryo-frozen in liquid nitrogen for later use.

3.3.3 Rhodamine 123 Accumulation / Efflux

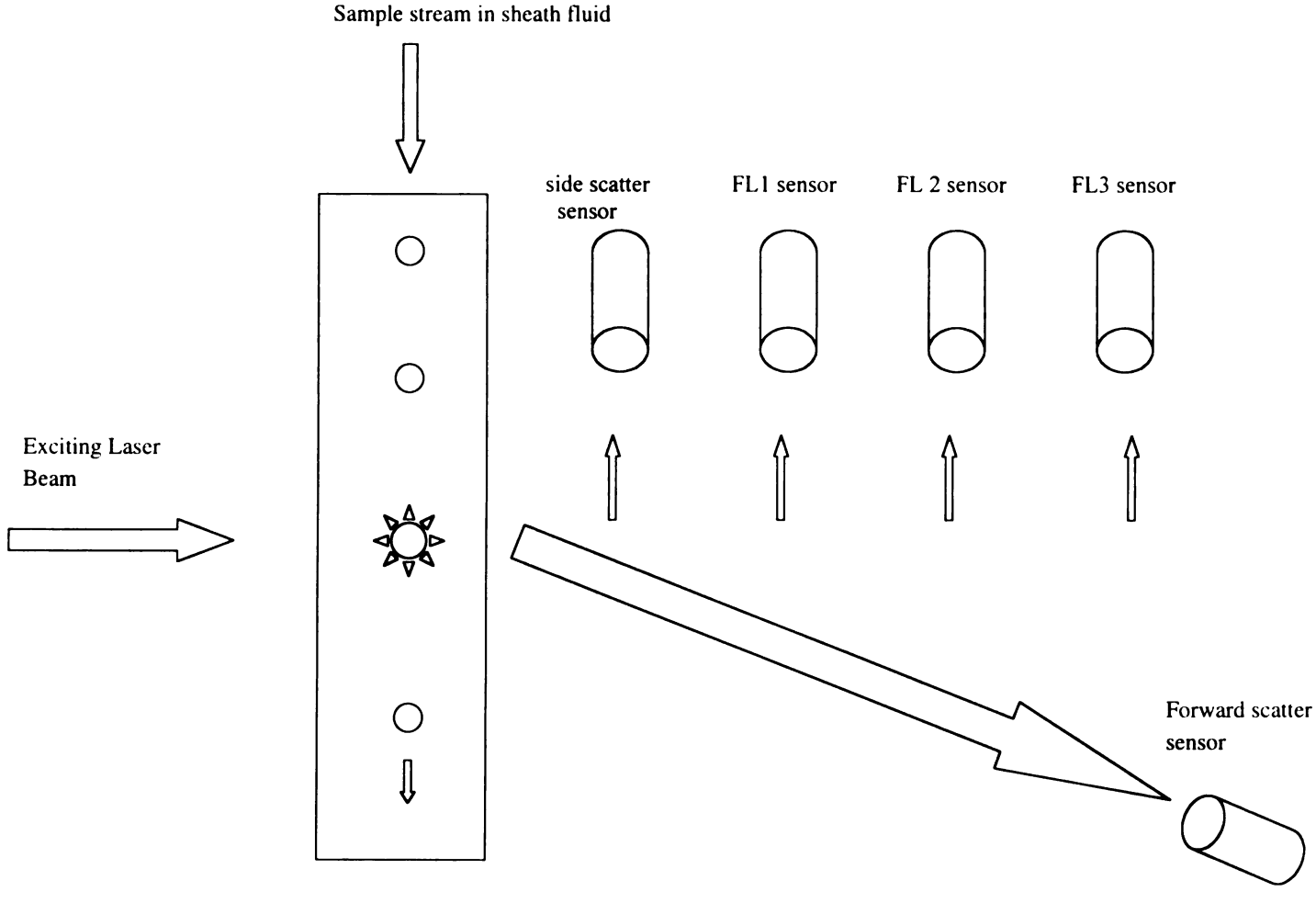
Peripheral blood mononuclear cells were thawed by rapid increase in temperature to 37°C. The cells were washed twice with RPMI 1640 (with 10%FCS). Cells were allowed to equilibrate in RPMI 1640 (with 10% FCS) for 1 hour at 37°C. After 1 hour, cell viability and cell count were assessed by ethidium bromide/acridine orange uptake. If greater than 10 % cells took up acridine orange, the dead cells were removed from the viable by Ficoll-Hypaque density centrifugation as described above. RPMI 1640 was washed off and cells were resuspended in Hanks BSS (dye-free, $Ca^{2+/}Mg^{2+}$ free) at 1 million cells/ml. Cells were incubated with or without inhibitors for 15 minutes at 37°C. After incubation with inhibitors, without washing, PBMCs were incubated with 20 ng/ml rhodamine 123 for 30 minutes at 37°C. After this incubation period, cells were placed on ice and incubated with 300 µl Optilyse C for 5 minutes to lyse contaminating erythrocytes. Following incubation, the PBMCs were washed twice with ice-cold Hanks BSS (dye-free, $Ca^{2+/}Mg^{2+}$ free) and resuspended in ice-cold PBS (with 0.5% BSA and 0.1% sodium azide). An EPICS XL flow cytometer (Coulter) with a 488-nm argon laser was used to analyze the samples. Rhodamine 123 fluorescence was collected after 525-nm bandpass filter and propidium iodide fluorescence was collected after a 575-nm bandpass filter. Data were collected for a minimum of 5,000 events per sample, and the

samples were gated on forward scatter versus side scatter to exclude non-lymphocyte populations, clumps, and debris. Propidium iodide staining was used for excluding dead cells from the analysis. The instrument was calibrated each time before analysis using Flow-Check™ fluorospheres for day-to-day and within-day stability, sensitivity, and resolution.

3.3.4 Flow Cytometry

Flow cytometry can be used to identify different cells by measuring the light they scatter, or the fluorescence they emit, as they flow through a laser beam; thus flow cytometry can focus analysis on cells of a particular type from a mixture. The flow cytometer can make simultaneous measurements of up to six parameters including the size and shape of a cell. Flow cytometry provides data on individual cells, each of which can be defined by a number of parameters. The limit of detection of rare cells by flow cytometry is 1 in 10^4. A dilute suspension of cells is allowed to mix with a buffer (the sheath fluid), the cells are passed single-file through a laser light beam, and the fluorescent light emitted by each cell is measured. The light scattered by each cell can be measured simultaneously; from this the size and shape of the cell can be determined (Fig. 3.2). The cells can be tagged with antibodies conjugated to fluorescent molecules. Internal features of a cell can be probed using fluorescent dyes. Active processes and kinetics of such processes can be studied using fluorescent substrates such as calcein and rhodamine 123. Rhodamine 123 is a 380.83 D molecule with excitation maxima at

Fig. 3.2. Basic principle of fluorescence flow cytometer multiparameter analysis of fluorescent labeled cells



485 nm and emission maximum at 546 nm (Fig. 3.1a). The molecular formula for Rh-123 is $C_{21}H_{17}ClN_2O_3$.

3.3.5 Data Analysis

The data were collected as mean intensity for all samples. The intensity of rhodamine fluorescence was plotted against increasing concentration of inhibitor. Data from net intensity versus inhibitor concentration were fitted to the Michaelis-Menten equation using Kaliedagraph™ 3.0.2 (Abelbeck Software) to derive inhibition constants and maximum effect.

3.4 Results

Figure 3.3 shows the flow chromatogram for rhodamine 123 accumulation by lymphocytes. In these experiments, the cells were gated (selected for analysis) on the basis of shape and size determined from initial experiments using specific antibodies for T-cells. Live cells as well as apoptosing cells took up rhodamine 123. For accurate analysis of functional P-gp, it is essential to exclude the dead and apoptosing cells from the live cell population using propidium iodide (PI) staining. The live cells did not stain with PI and appeared as a distinct population in gate G, frame 2 (Fig. 3.3). The intensity of this peak represents true accumulation of Rh 123 by viable cells and was quantified in

Fig. 3.3. Representative flow chromatogram for rhodamine 123 uptake.

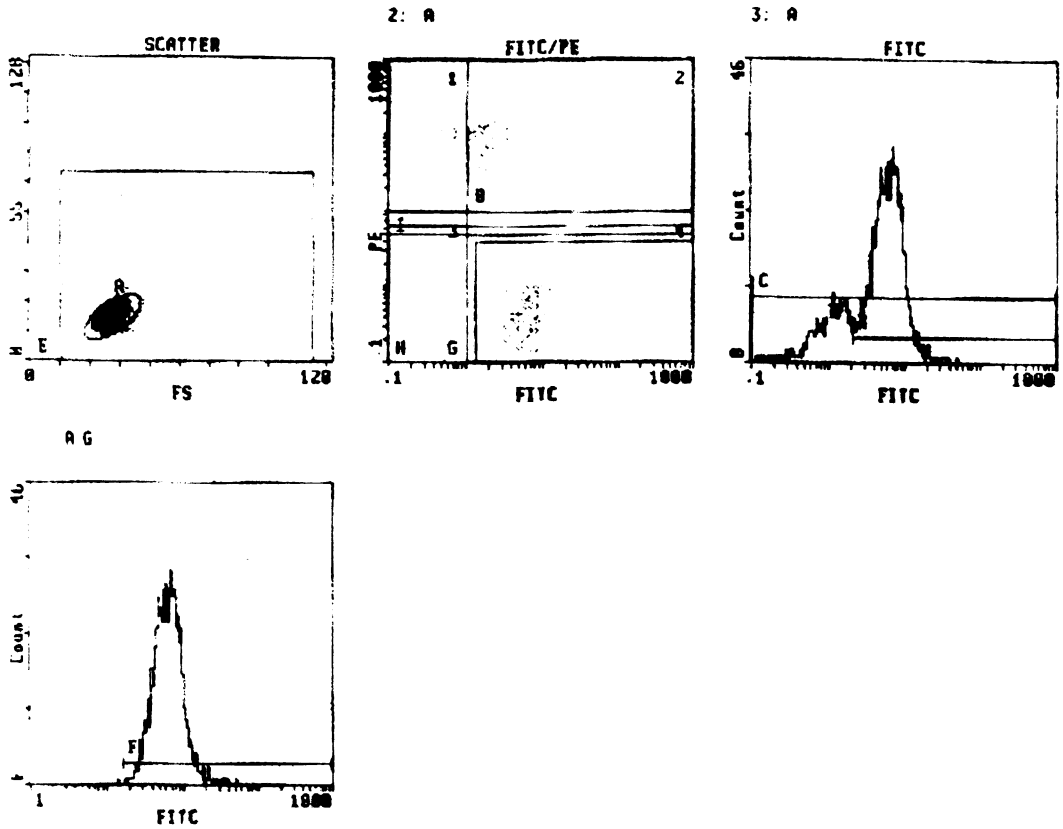
Legend

Frame 1: Gate E consists of all cells sampled from the tube
Gate A is based on size and shape and represents mostly lymphocytes

Frame 2: Cells in gate A from frame 1 are further subdivided into live (gate G) and dead (gate I) based on propidium iodide uptake by dead cells

Frame 3: Represents all cells in gate A from frame 1 which take up rhodamine 123 and segregate into two populations based on intensity at channel FL1 (505-545 nm)

Frame 4 Live cells in gate A due to rhodamine 123 uptake (FL1)



the presence and absence of an inhibitor to represent the functional characteristics of P-gp. In the presence of an inhibitor such as tacrolimus the peak intensity of Rh 123 increases (Fig. 3.4).

P-glycoprotein is an energy dependent efflux pump with maximum activity at physiological temperature (37°C). To determine if Rh 123 efflux was an active process, the accumulation/efflux was studied at two different temperatures, 4°C and 37°C, in the presence and absence of tacrolimus (Fig. 3.5). The accumulation of Rh 123 as measured by mean intensity was significantly higher at 37°C both with and without the inhibitor. The mean intensity of Rh 123 was 7-fold higher at 37°C than at 4°C without the inhibitor. In the presence of tacrolimus (P-gp inhibitor), there was a 13-fold increase in intensity of Rh 123 at 37°C compared to 4°C. At 4°C, there was no difference in the accumulation of rhodamine when an inhibitor (tacrolimus) was present.

Lymphocytes were incubated with different escalating concentrations of cyclosporine, tacrolimus, quinidine, or verapamil, resulting in a corresponding increase in Rh 123 intensity of viable cells with respect to the Rh123 intensity of the control in the presence of solvent alone (Fig. 3.4, 3.6). These analysis were performed over a wide concentration range determined by the boundary conditions of "no effect" to "extensive cell death" due to toxicity of the test compound *in vitro*. Based on these studies, the net increase in intensity of Rh 123 was determined and plotted against concentration of inhibitor to determine the inhibition constants and maximum inhibitory

Fig. 3.4. Representative chromatogram for rhodamine 123 uptake in the presence and absence of an inhibitor (tacrolimus) of P-gp

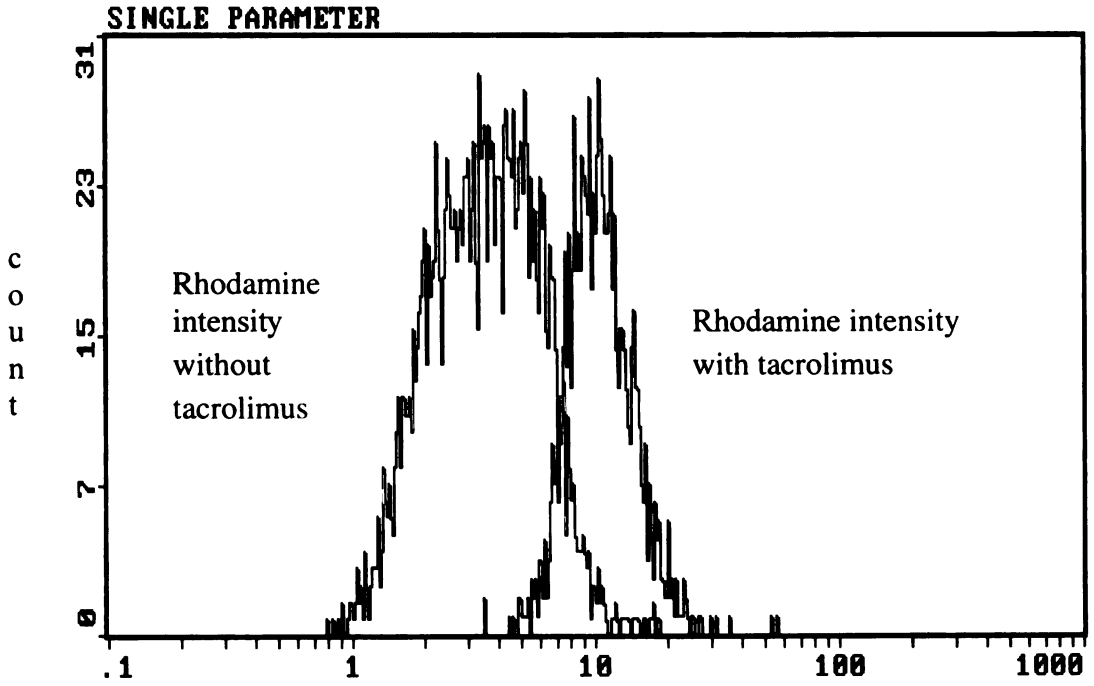


Fig. 3.5. Effect of temperature on uptake and efflux of rhodamine 123 in the presence and absence of an inhibitor (tacrolimus); n=3 (mean ± SD)

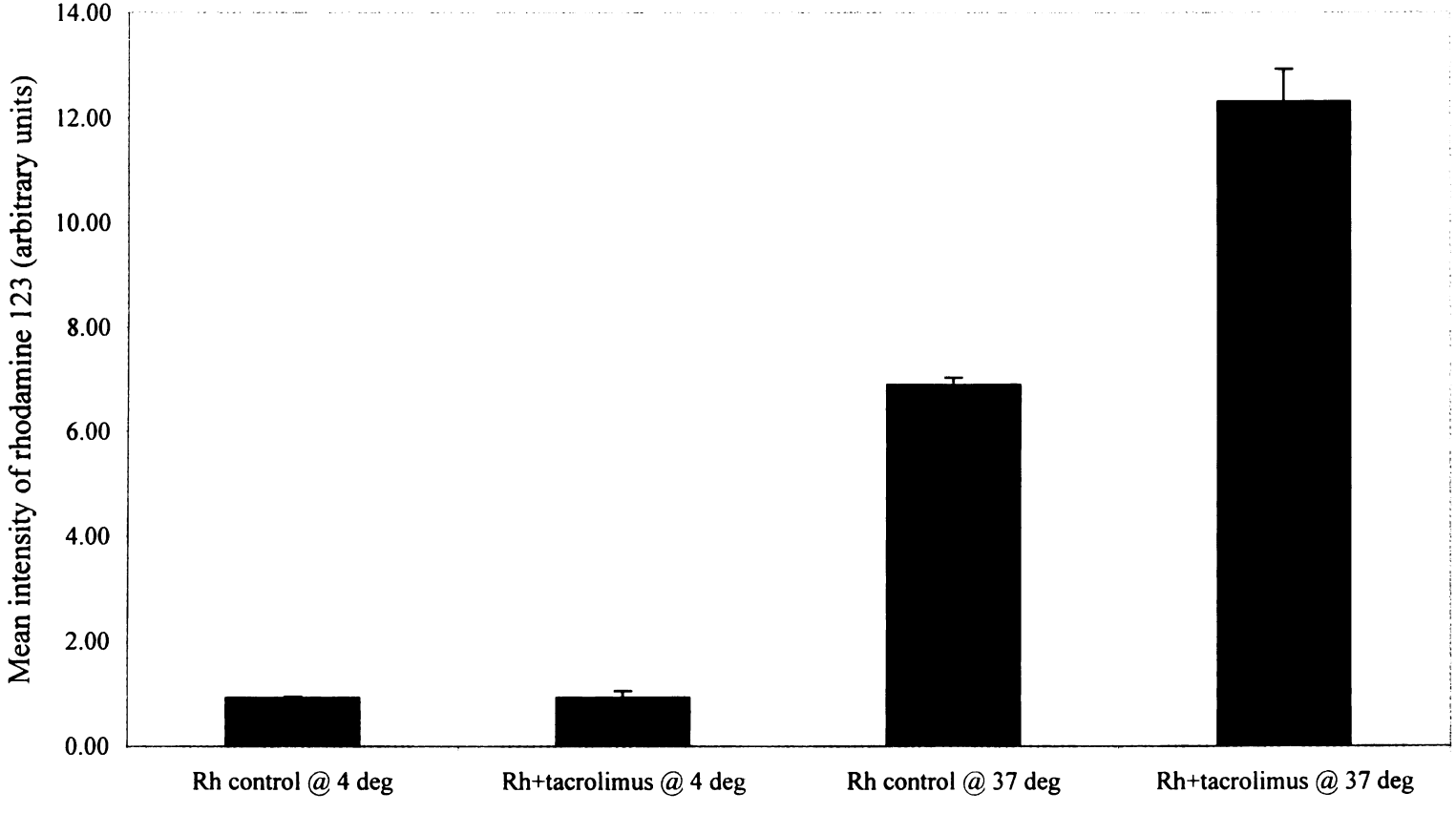
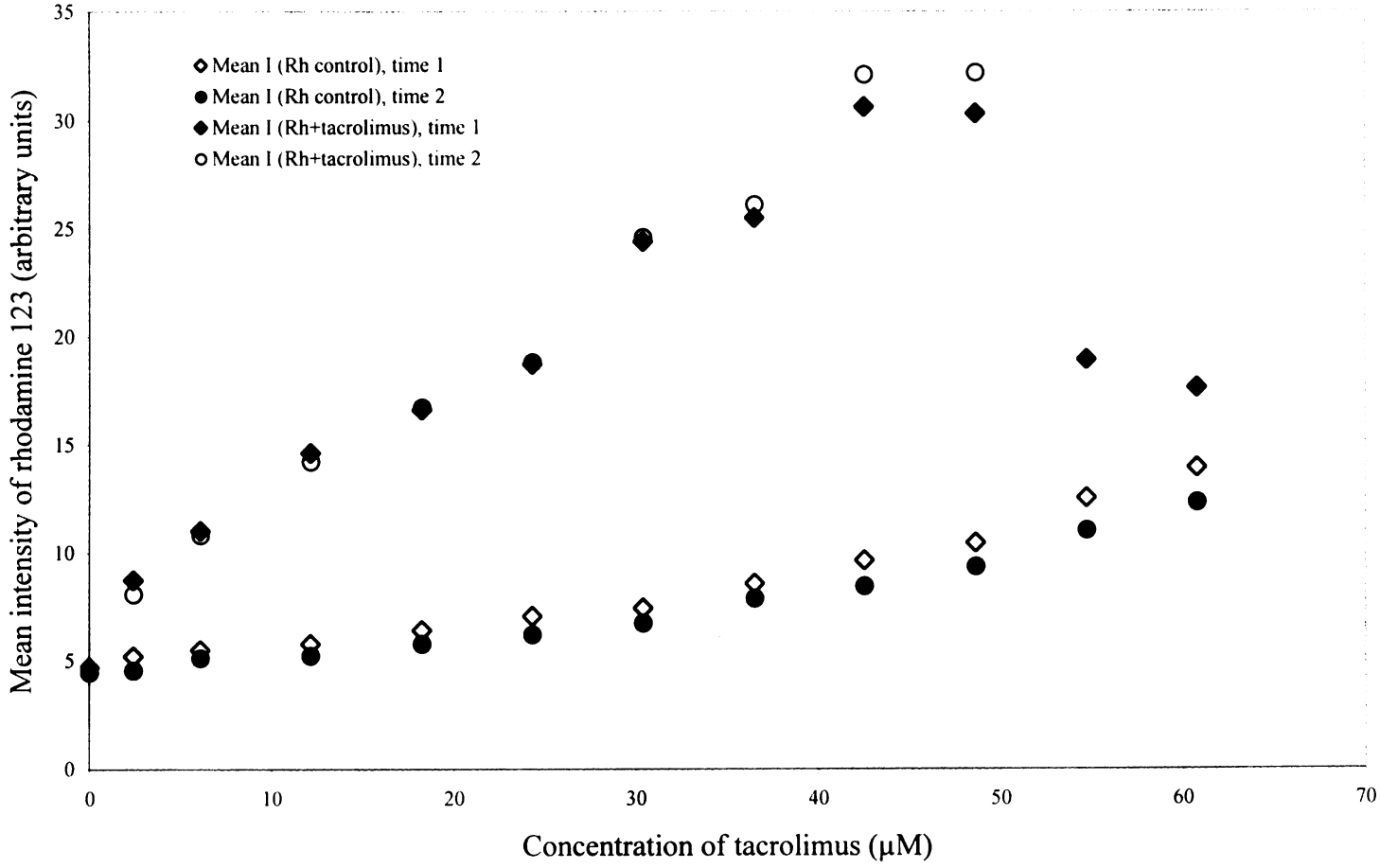


Fig. 3.6. Representative graph for P-gp inhibition by test compounds.



effect (Table 3-1). When lymphocytes were incubated with increasing concentrations of MMF, there was an apparent but not significant decrease of Rh 123 efflux (Fig. 3.7).

To investigate if the increase in Rh 123 intensity was due to inhibition of P-gp or other transporters, I investigated the effect of other compounds that are not P-gp inhibitors such as indomethacin, probenecid and sulfinpyrazone. Draper and co-workers (1997) have shown that indomethacin does not modulate P-gp mediated efflux of Rh 123 in P-gp expressing murine and human cell lines (PC-V160 and HL60/Vinc). Here, however, indomethacin (in ethanol/water) resulted in a concentration dependent, saturable increase in Rh 123 accumulation (Fig. 3.8). Over the concentration ranges tested, probenecid (in DMSO) and sulfinpyrazone (in DMSO) did not cause significant increases in accumulation of Rh 123 with respect to solvent control (Fig. 3.9).

Vinblastine is a known P-gp inhibitor. To determine the kinetics of P-gp inhibition, different concentrations of vinblastine were tested. With increasing concentrations of vinblastine, there was increased accumulation of Rh 123, but at higher concentrations of vinblastine, there was a decrease in intensity (Fig. 3.10a). This was a curious phenomenon since the lymphocytes are treated with vinblastine for less than an hour during the experiment, and no other reference has been found in the literature for such an effect. To investigate if this effect was due to increased expression of P-gp, lymphocytes were incubated with vinblastine and anti-P-gp antibody, 15D3 or the appropriate isotype control. There did not appear to be a significant change in the expression of P-gp as detected by antibody binding (Fig. 3.10b).

Table 3-1. Kinetic parameters for inhibition of rhodamine 123 efflux by lymphocytes

Inhibitor	***N (no. of blood sample sources)***	***n (data points for kinetic parameters)***	***Ki (μM)***	***Emax (arbitrary units)***
Cyclosporine	3	8	0.3 ± 0.2	3.9 ± 1.1
Tacrolimus	3	5	8.8 ± 4.2	15.1 ± 5.4
Quinidine	3	4	24.6 ± 17.4	3.8 ± 1.6
Verapamil	3	8	40.1 ± 12.7	7.0 ± 1.9
Vinblastine	1	3	82.4	21.9
Indomethacin*	1	3	199.2	30.1

*Indomethacin is not a P-gp inhibitor; it inhibits the efflux of rhodamine 123 from lymphocytes via an unknown mechanism

Fig. 3.7. Effect of MMF on rhodamine 123 uptake by lymphoyctes.

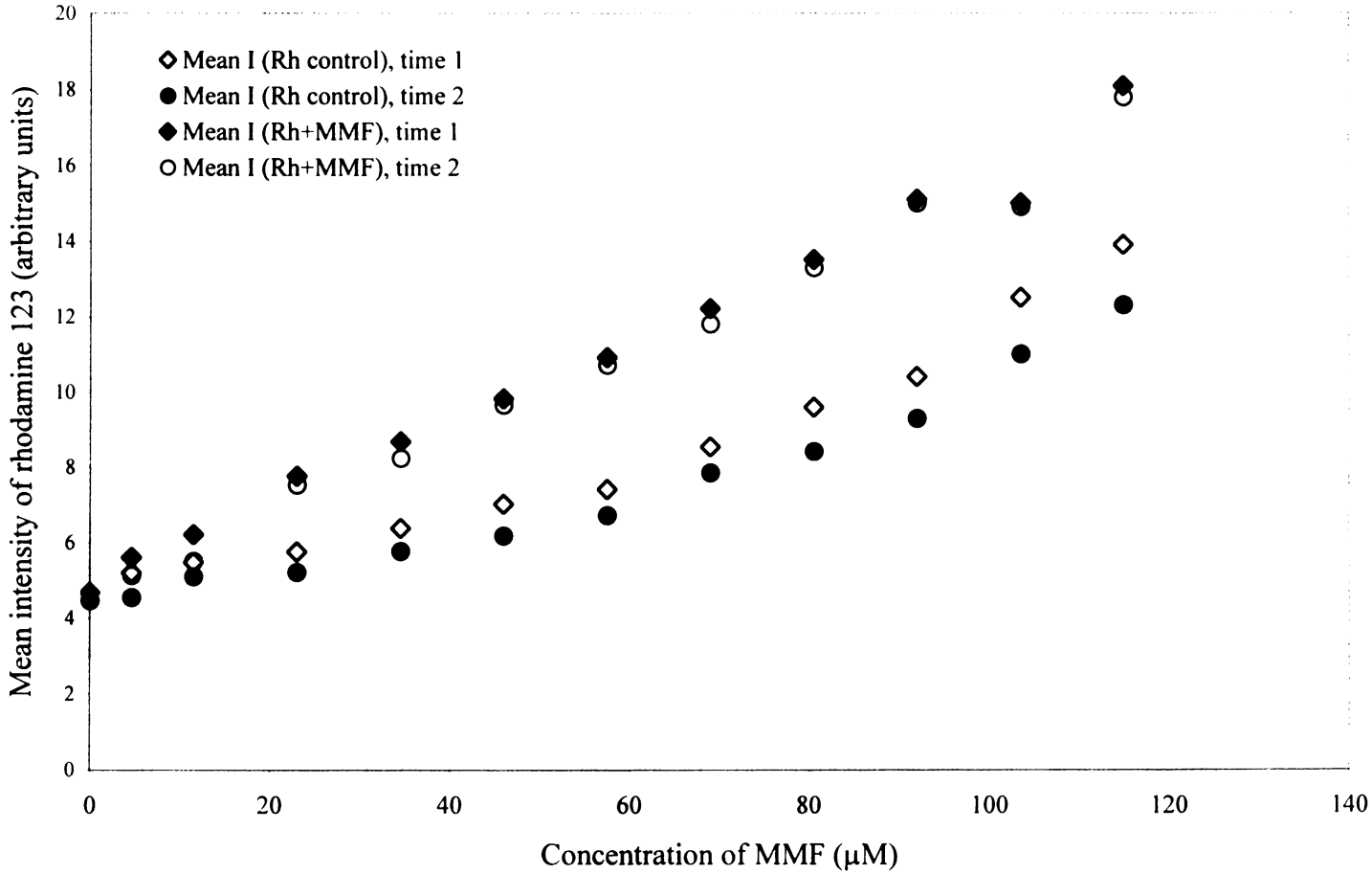


Fig. 3.8. Effect of indomethacin on rhodamine 123 uptake by lymphocytes; n=3 (mean ± SD)

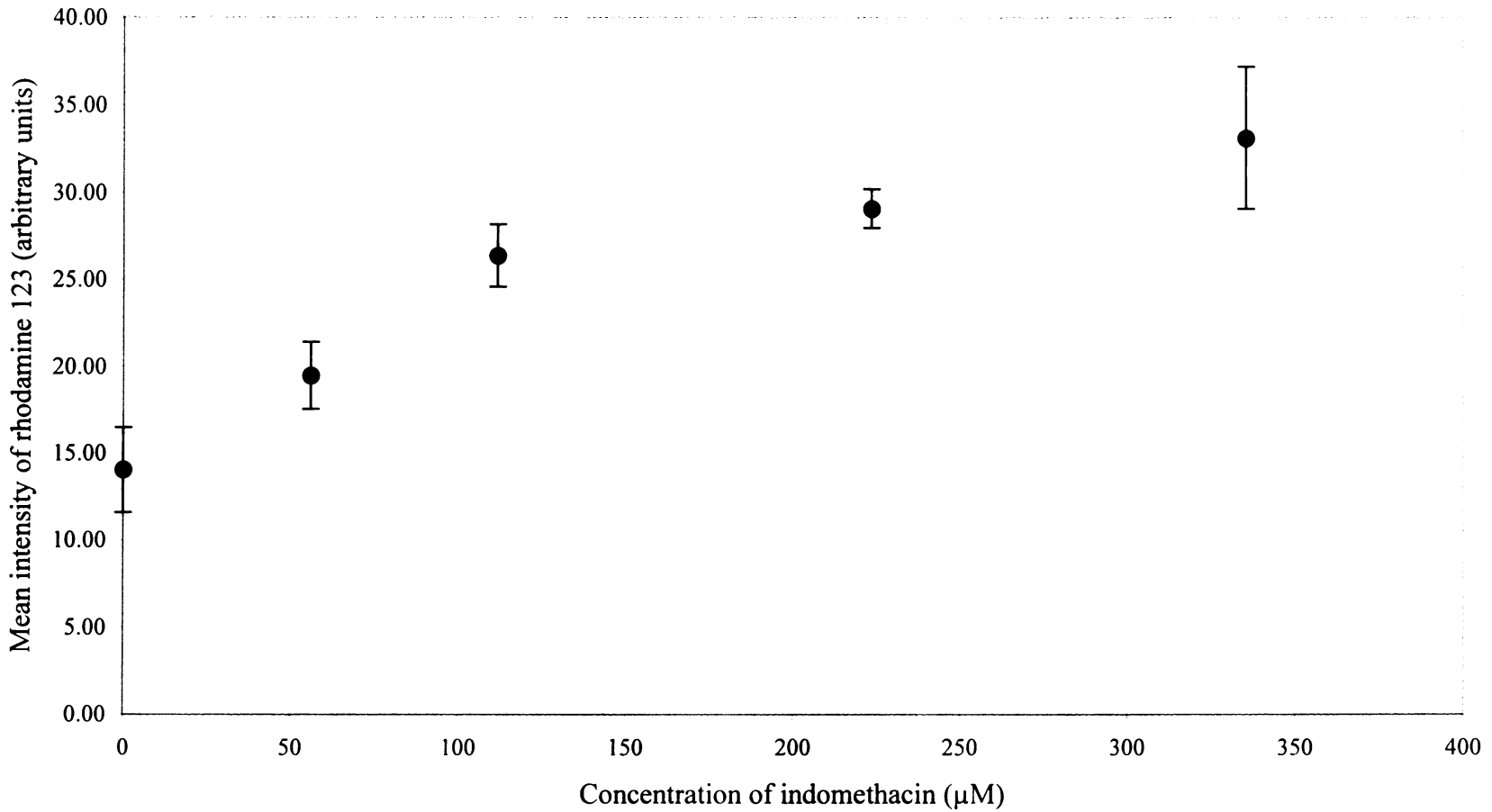


Fig. 3.9. Effect of MRP inhibitors on rhodamine 123 uptake by lymphocytes; n=3

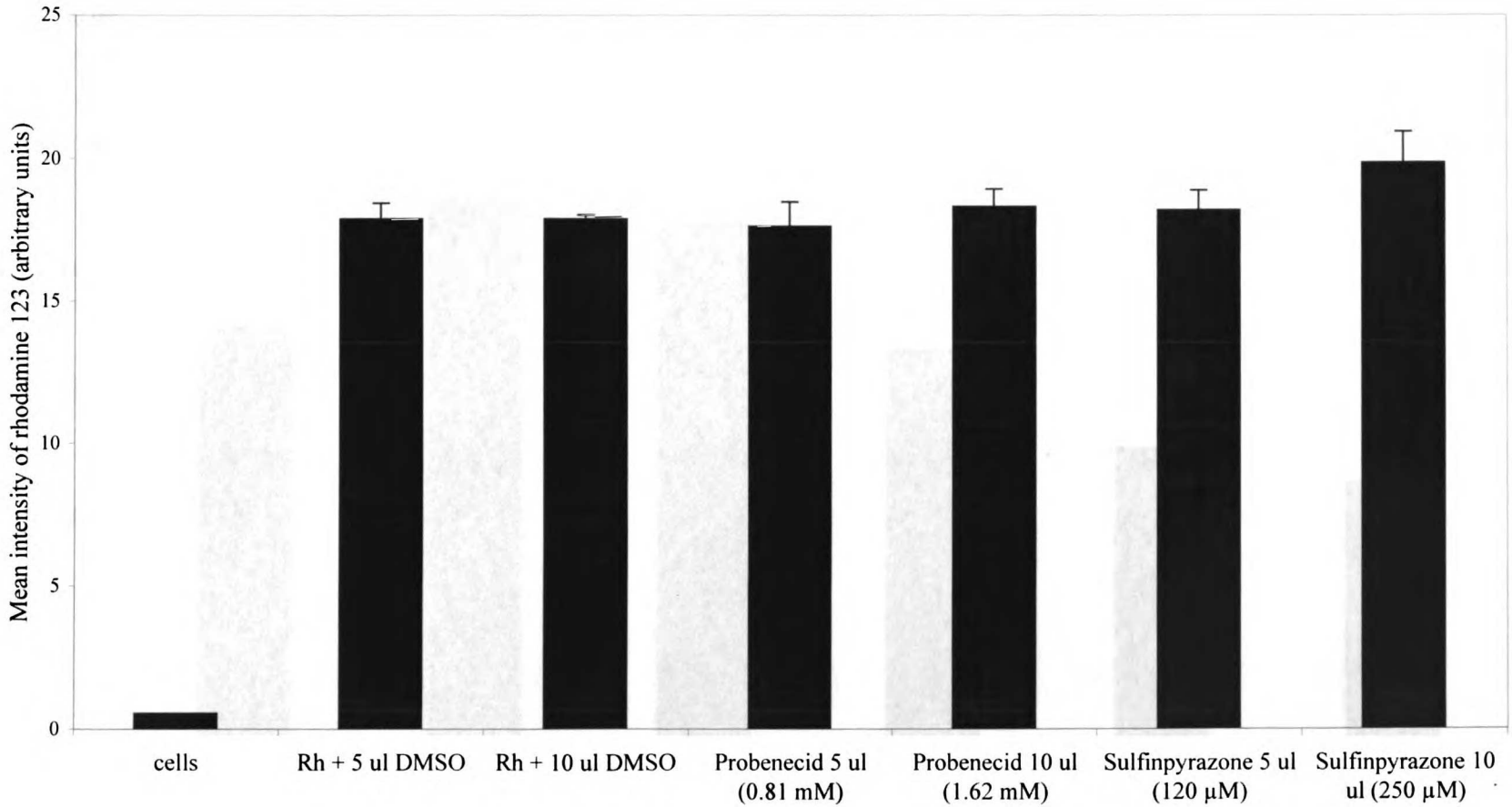


Fig. 3.10a. Effect of vinblastine on rhodamine 123 uptake by lymphocytes, n=3

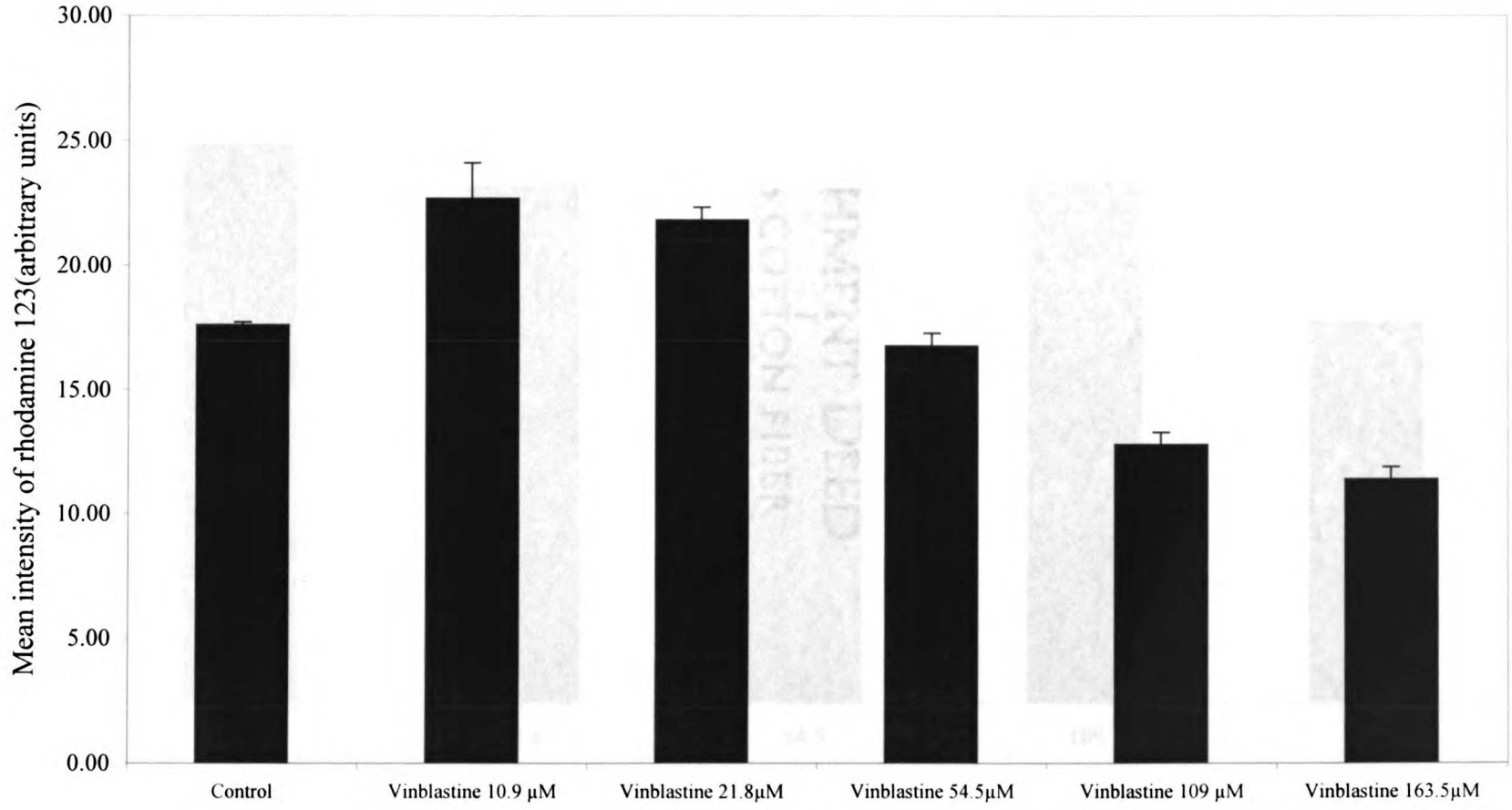
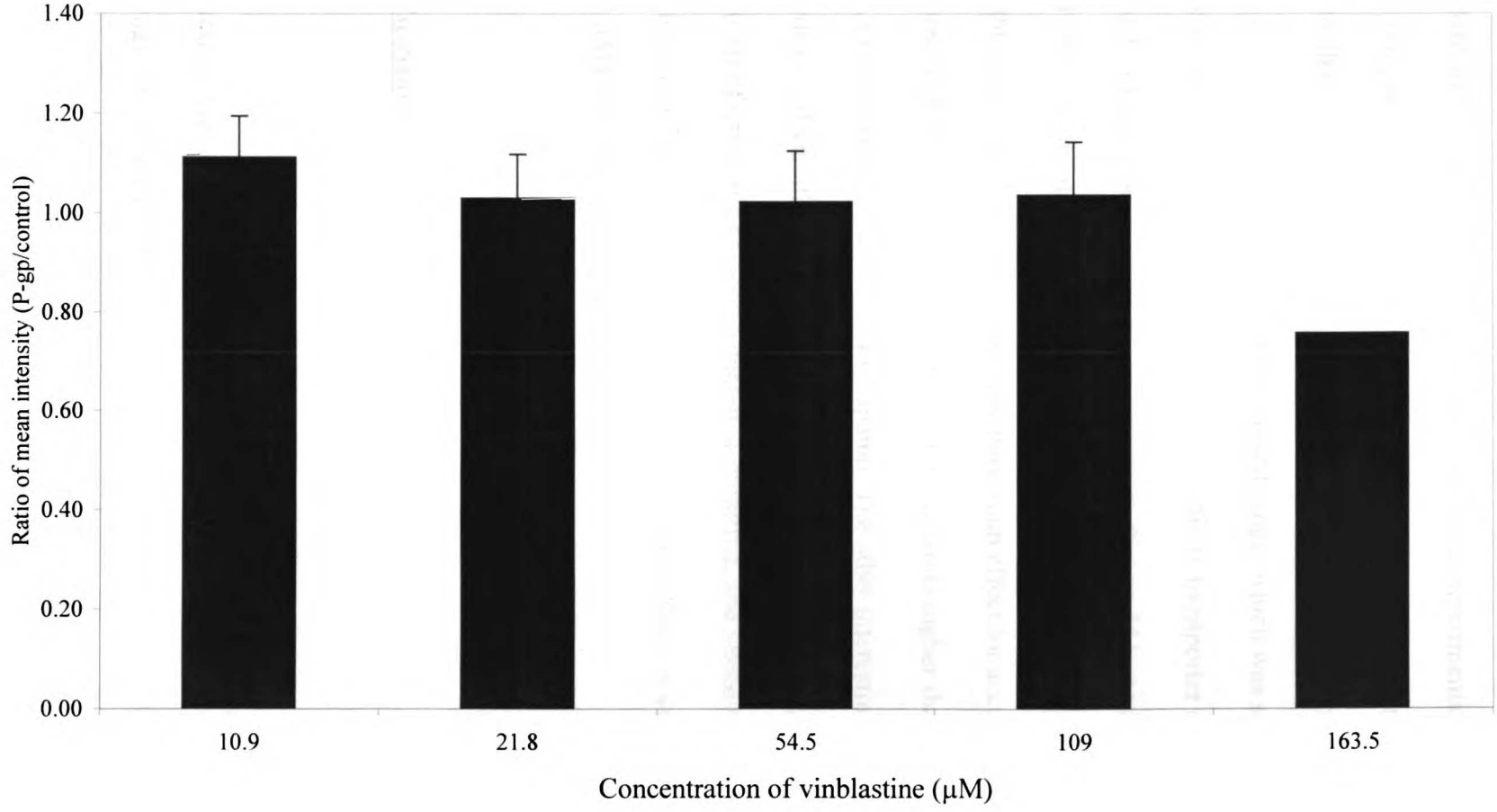


Fig. 3.10b. Effect of vinblastine on expression of P-gp on lymphocytes; n=3 (mean ± SD)

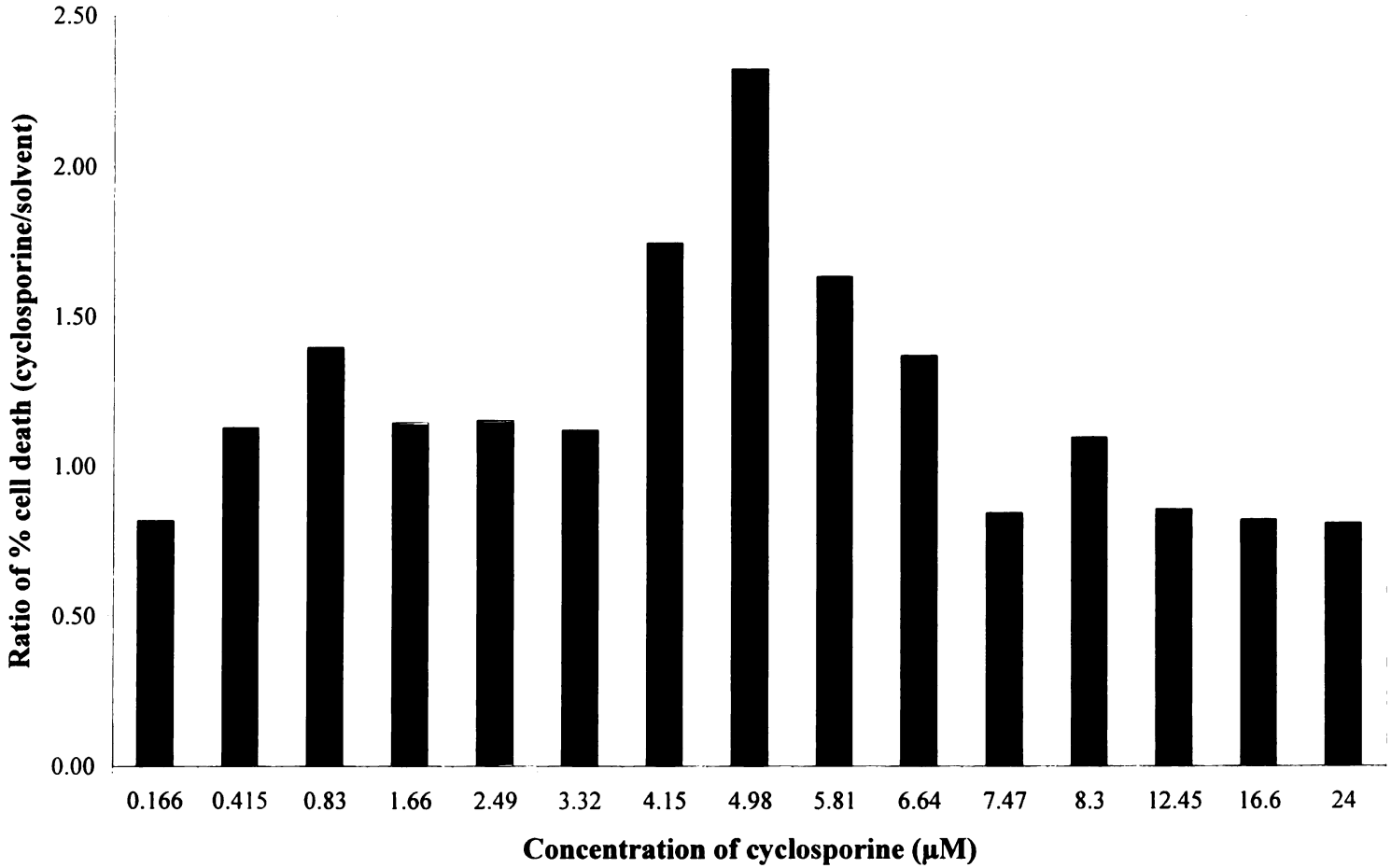


There were some interesting observations in these experiments. In experiments involving verapamil, quinidine, and cyclosporine, there appear to be at least two different transporters that are involved in Rh 123 efflux and inhibited by these compounds. One of these was a high affinity (P-gp) transporter (in μM range) which was saturated in the test inhibitor concentration range, and the other, a low affinity transporter (probably in mM range) which could not be saturated at concentrations that could be tested (because of decreased cell viability at higher concentrations). Tacrolimus does not appear to have any effect on this other unknown transporter. The maximum effect for accumulation of Rh 123 was observed with tacrolimus, which was at least 2-fold higher than the other P-gp inhibitors, cyclosporine, quinidine, and verapamil. The other interesting observation was with regards to cell viability in the presence of an inhibitor. In general, increasing concentrations of drugs and solvents (ethanol, acetonitrile, and DMSO) were toxic to cells. Cyclosporine however, appeared to have a protective effect on cells at higher (above 10 μM) concentrations (Fig. 3.11).

3.5 Discussion

These results show that lymphocytes have functional P-gp, which is inhibited by various drugs. The activity of P-gp is temperature dependent both in the presence and absence of an inhibitor. At 4°C, the P-gp was not functional and there was no effect of

Fig. 3.11. Effect of cyclosporine on cell viability in rhodamine 123 accumulation/efflux studies



adding inhibitors whereas at 37°C, Rh 123 accumulation was higher (Fig. 3.5). The uptake of Rh 123 at the lower temperature was also markedly less than at 37°C. This implies that the uptake of Rh 123 may involve active or facilitated transport processes.

Verapamil, quinidine, tacrolimus, and cyclosporine inhibit the P-gp mediated efflux of Rh 123 in the order: cyclosporine>tacrolimus>quinidine>verapamil. The maximum increase in Rh 123 accumulation did not correspond to the inhibitory potential of these drugs. The maximum effect on rhodamine accumulation was in the order: tacrolimus>verapamil>cyclosporine, quinidine. The difference in maximum activity and potency may indicate the presence of other transporters. The inhibitory effect of all these drugs was observed up to 2 1/2 hours after washing off the inhibitor. The high variabilities of the Ki and Emax values (Table 3-1) reflect in part the inherent variability in P-gp from different blood sources. Similar results are obtained when cytochrome P450 activity is compared in human liver microsome samples from different individuals. Inhibition constants (Ki) were in general higher (Table 3-2) at later time points although there was no statistically significant difference for tacrolimus, cyclosporine and verapamil. The Ki value for quinidine (Table 3-2) was significantly ($p = 0.003$) lower, 2 hours after incubation. This may reflect a slow irreversible binding of quinidine with P-gp. The maximum effect on rhodamine accumulation was the same at different times. At higher concentrations, verapamil, quinidine and cyclosporine also appear to inhibit another low affinity transporter. In Rh 123 uptake studies, it is important to measure Rh 123 intensity in live cells because including data from apoptosing/dying cells can skew the analysis.

Table 3-2. Time dependence of kinetic parameters for P-gp mediated inhibition of rhodamine 123 accumulation/efflux

Inhibitor	***Ki (μM)-time 1***	***Ki (μM)-time 2***	***Ki (μM)-time 3***	***Emax - time 1***	***Emax - time 2***	***Emax - time 3***
Cyclosporine	0.2 ± 0.1 (n=4)	0.2 ± 0.1 (n=3)	0.8 (n=1)	4.0 ± 1.2 (n=4)	3.8 ± 1.3 (n=3)	4.1 (n=1)
Tacrolimus	6.8 ± 4.1 (n=3)	11.9 ± 2.6 (n=2)		12.5 ± 5.6 (n=3)	19.0 ± 0.6 (n=2)	
Quinidine	39.7 ± 0.2 (n=2)	9.6 ± 2.5 (n=2)		3.4 ± 0.1 (n=2)	4.2 ± 2.6 (n=2)	
Verapamil	33.3 ± 10.5 (n=4)	44.1 ± 12.7 (n=3)	55.5 (n=1)	6.4 ± 2.3 (n=4)	7.5 ± 1.7 (n=3)	8 (n=1)

Time 1: Represents analysis within 1/2 hour after incubation (washing off inhibitor)

Time 2: Represents analysis approximately 1 1/2-2 hours after incubation

Time 3: Represents analysis after 2 1/2 hours after incubation

The activation of Rh 123 transport by P-glycoprotein at high concentrations of vinblastine was an interesting observation because it did not involve an increase in expression of P-gp. Most studies reported in the literature have used lower concentrations (usually lower than 50 μM) of vinblastine where it shows inhibitory effects on P-gp (Hollo et al., 1994; Soldner et al., 1999). The molecular mechanism underlying this phenomenon is not yet understood but could involve allosteric conformational changes in P-gp thus affecting the transport of rhodamine 123.

In lymphocytes, MRP inhibitors such as sulfinpyrazone and probenecid did not increase the accumulation of Rh 123 but indomethacin did. It is possible that indomethacin inhibits efflux of Rh 123 via its effect on some other transporter. These results are also supported by other studies from our laboratory (Flanagan et al., submitted) where indomethacin appears to inhibit an unknown transporter.

Cyclosporine at micromolar concentrations appeared to exert a protective effect on lymphocytes. This effect could be due to mitochondrial protection by inhibition of P-gp or other transporters, thereby protecting the cell from toxic effects of solvent or rhodamine. The other drugs tested did not have any such effects on cell viability. For lymphocyte based rhodamine 123 uptake studies, dye-free Ca^{2+}/Mg^{2+} free Hanks BSS buffer was the preferred medium as compared to PBS, which compromised cell viability.

The results suggest that lymphocytes can be used for studying the effect of drugs on P-gp function and expression. The advantage of using lymphocytes instead of

overexpressed cell-lines is that the lymphocytes are normal human tissue and the parameters obtained using such a tissue are likely to be a better representation of true effects of drugs. This assay method is fast and can be used in high-throughput screening of compounds. Lymphocytes can be used for studying both induction and inhibition effects of drugs on transporters. However, there are particular limitations. The level of P-gp expression in lymphocytes is low and requires the use of sensitive methods such as flow cytometer for appropriate analysis. Compared to overexpressed or transfected cell-lines, lymphocytes are fragile and difficult to use. Due to the presence of several transporters, the results can be difficult to assign to a particular transporter.

3.6 References

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Chapter 4

IN VITRO DRUG INTERACTIONS OF CYCLOSPORINE AND P-GP INHIBITORS

4.1 Background

T lymphocytes are critical in mounting immune responses against transplanted tissues and organs. Some early events in this rejection pathway include recognition of foreign antigen by CD4+ T cells resulting in synthesis and secretion of cytokines such as interleukin-2 (IL-2). Interleukin-2 causes further proliferation of CD4+ cells as well as recruitment of other components of the immune system. Cytokines are low molecular weight soluble glycoproteins that act as mediators in immune signaling. They can have local or distant effects on a variety of cells and tissues. Their effects are often pleiotropic (many different biological effects) and redundant. They play an integral role in the initiation and progression of normal immune responses. The cytokines, IL-2, IL-4, and IFN-γ are produced by activated helper T cells (CD4+); IL-2 and IFN-γ are also produced by some cytotoxic T cells (CD8+).

Cyclosporine prevents transplant rejection by inhibiting T cell proliferation and cytokine production. Transplant recipients require chronic immunosuppressive therapy for maintaining the grafted tissue. During their lifetimes, transplant patients often receive several other classes of drugs. Cyclosporine is known to interact with a wide variety of

drugs and food components (Brunner et al., 1998; Edwards et al., 1999) through poorly defined mechanisms. These interacting drugs include NSAIDS (Constantopoulos, 1999), HMG-CoA reductase inhibitors (Meuck et al., 1999), vinca alkaloids (Chan, 1998), anti-HIV drugs (Brinkman et al., 1998), antihypertensive drugs (Campana et al., 1996), antibiotics (Campana et al., 1996), calcium channel blockers (Campana et al., 1996), hypoglycemic drugs (Kaplan et al., 1998) and many more. Some of these interactions result in a change in the pharmacokinetic parameters of cyclosporine or interacting drugs and can be explained in terms of CYP3A or P-gp modulation in the gut or liver, but a large number of these interactions are not fully understood. Recently, *in vitro* (Marie et al., 1992; Grey et al., 1997; Chiodini et al., 1999) and in clinical studies (Terwogt et al., 1998), cyclosporine has been co-administered with poorly bioavailable drugs, especially anti-cancer drugs, in order to enhance their oral bioavailability. This approach relies on the capacity of cyclosporine to inhibit P-gp, thereby decreasing efflux of P-gp substrates. The clinical studies have paid little attention to potential pharmacokinetic and pharmacodynamic interactions resulting from prolonged co-administration of these drugs (Terwogt et al., 1998).

P-glycoprotein is present in lymphocytes at detectable levels (O'Brien and Cordon-Cardo, 1996). Constitutive expression of CYP3A4 enzymes is not documented in lymphocytes but 1A1 and 1B1 are expressed and inducible (Rendic and Di Carlo, 1997; Spencer et al., 1999). Drach and co-workers (1996) have shown, using *in vitro* lymphocyte assays that IL-2, IL-4, and IFN-γ are substrates of P-gp and the extracellular secretion of these cytokines is inhibited by inhibitors of P-gp. In another study, Raghu

and co-workers (1996) were able to inhibit IL-2 levels with P-gp specific antibodies in lymphocyte proliferation assays. These studies in combination with the fact that the immunosuppressive activity of cyclosporine involves inhibition of synthesis of IL-2 raise the possibility that when P-gp inhibitors are combined with cyclosporine, the combination of drugs would enhance immunosuppression. *In vitro*, these effects are best studied in the ability of drug treatments to suppress T cell proliferation and cytokine production. The P-gp inhibitors could also increase local concentrations of cyclosporine within the lymphocytes.

4.2 Objectives

The specific objective of this study was to elucidate *in vitro* pharmacodynamic interactions of cyclosporine with P-gp inhibitors and to study the effects of such interactions on the immunosuppressive activity of cyclosporine. Based on *in vitro* data (Chapter 3) for P-gp inhibition and accumulation of Rh 123, four drugs were selected: quinidine, vinblastine, indomethacin, and probenecid. Quinidine and vinblastine are known inhibitors of P-gp; indomethacin enhances Rh 123 accumulation in a concentration-dependent, P-gp independent manner and probenecid has no effect on P-gp and accumulation of Rh 123 in lymphocytes. The effect of these drugs alone and in combination with cyclosporine on T cell proliferation, expression of CD25 (receptor for binding IL-2) and extracellular secretion of soluble cytokines, interleukin-2 (IL-2), interleukin-4 (IL-4), and gamma interferon (IFN-γ) was evaluated. The concentrations of

drugs were selected based on the rhodamine uptake assays described in Chapter 3. Cyclosporine concentrations were below or equal to Ki values for P-gp inhibition. In preliminary experiments, these concentrations did not result in significant inhibition of T cell proliferation. The inhibitors were used at two concentrations above and below the Ki values for P-gp inhibition or maximum tolerated concentration without significantly compromising cell-viability. Some of these concentrations are in the therapeutic ranges. There was no attempt to characterize intracellular drug concentrations since the number of cells in the *in vitro* incubations and drug concentrations were both low.

4.3 Materials and Methods

4.3.1 Chemicals and Specimens

Ficoll-Paque® Plus was purchased from Pharmacia Biotech AB (Uppsala, Sweden). Phytohemagglutinin-P (PHA-P) was obtained from Difco Laboratories (Detroit, MI). [^{3}H] thymidine was obtained from New England Nuclear (Boston, MA). Cell media, fetal calf serum (FCS) cell preservation medium (10% FCS, 10% DMSO in MEM EBSS, pH=7.2) were obtained from UCSF Cell Culture Facility (San Francisco, CA). Propidium iodide, acridine orange and ethidium bromide were from Molecular Probes (Eugene, OR). Falcon 12-well flat bottom tissue culture plates were bought from Becton Dickinson Labware (Lincoln Park, NJ) and Costar 96-well U-bottom, sterile tissue culture plates from Corning Inc. (Corning, NY). Cytokine ELISA kits for IL-2, IL-4, and IFN-γ were

purchased from Immunotech (Marseille, France). All antibodies were from Becton – Dickinson (San Jose, CA). All other chemicals were from Sigma (St. Louis, MO).

4.3.2 T cell Proliferation Assay

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density centrifugation from buffy coats obtained from the UCSF Blood Bank (San Francisco, CA). The cells were washed and resuspended in cell preservation medium at 40 million cells per vial and cryo-frozen in liquid nitrogen at –191.2 °C. For proliferation studies, PBMCs were thawed rapidly at 37 °C. The cells were washed twice with RPMI 1640 (with 10% FCS) and allowed to equilibrate in RPMI 1640 (with 10% FCS) for 1 hour at 37 °C. The viability of cells was evaluated by ethidium bromide/acridine orange uptake. If viability was less than 95%, the dead cells were removed by Ficoll-Hypaque density centrifugation. Cells were resuspended at 10^6/ml in RPMI 1640 (0.1μm sterile filtered, with 2g/L glucose, 0.3 g/L L-glutamine, 2.0 g/L sodium bicarbonate) with 25mM HEPES, and supplemented with 10% FCS in Falcon 12-well flat bottom tissue culture plates.

Cells were stimulated with a T-cell specific mitogen (Janeway and Travers, 1994), PHA-P (at 5-10 μl PHA/ml). The concentrations and culture times were selected based on preliminary experiments for optimum proliferation, secretion of cytokines and P-gp expression. In primary T-lymphocyte culture, P-gp expression is known to decrease

to undetectable levels after 48 hours. The cyclosporine concentration was selected such that it was equal to or below the Ki value for P-gp inhibition as reported in Chapter 3, and cyclosporine when used alone, caused minimal inhibition of T cell proliferation. The order of addition of drugs was selected such that test compounds were added at the time of initiation of proliferation (along with PHA); cyclosporine was added 18 hours later; cells suspension was withdrawn for radioactive thymidine labeling 6 hours after addition of cyclosporine; and all cells were harvested 18 hours later (total incubation time = 32 hours). The time selected for addition of cyclosporine to cultured T cells (18 hours after initial activation) was unconventional and cyclosporine is not known to cause significant immune-suppression when added at this late stage during T cell activation (Ho et al., 1996).

Briefly, cells were treated with PHA and test drugs and cultured in a humidified 5% CO_2 / 95% air atmosphere at 37 °C. Cyclosporine was added to the test wells 18 hours later and cells were recultured under the same incubation conditions. Six hours later, 100 µl volume of cell suspension was withdrawn from each well and transferred into a 96-well plate. Each well was pulsed with 1 µCi/100 µl of [^{3}H] thymidine and incubated in a humidified 5% CO_2 / 95% air atmosphere at 37 °C for 18 hours before harvesting. The amount of incorporated [^{3}H] thymidine was determined using an automated Microbeta Plus liquid scintillation counter (Wallac Inc.) and Wallac software (Gaithersburg, MD).

4.3.3 Cytokine ELISA

At the conclusion of the 32 h incubation (described above), the supernatants were withdrawn, pooled, aliquoted and frozen for determination of soluble IL-2, IL-4, and IFN-γ levels by enzyme linked immunosorbent assay (ELISA) using commercially available ELISA kits. The ELISA kits were based on "sandwich" enzyme immunoassay involving two immunological steps. In this kind of assay, a standard or sample is allowed to incubate in a microtiter plate coated with an anti-cytokine antibody. This complex is further incubated with another anti-cytokine antibody linked to an enzyme capable of catalyzing a reaction with a chromogenic substrate. The intensity of the color produced is proportional to the concentration of cytokine in the standard or sample. The color change was read on a Biorad Microplate Reader (Hercules, CA) and data analyzed by Microplate Manager III software (Biorad, Hercules).

4.3.4 Flow Cytometry

Following 32-hour incubation, cells were harvested to test for expression of CD25. To measure expression of CD25, cells were labeled with phycoerythrin (PE)-conjugated anti-CD25 antibody as per the manufacturer's instructions. Appropriate Ig-isotype controls were used to account for non-specific binding. Fluorescence intensity was measured for 5000 events per sample. The viability of harvested cells was assessed

using propidium iodide (PI) uptake (dead cells take up PI). Propidium iodide fluorescence was measured using an EPICS XL Coulter flow cytometer (Miami, FL).

4.3.5 Statistical Analysis

All data are presented as means ± SD. Statistical significance of differences in mean values was assessed using the unpaired *t* test. Statistical significance was considered at a p value of 0.05.

4.4 Results

T lymphocytes can be activated to proliferate *in vitro* using appropriate mitogens. This activation mimics many developmental stages that occur during *in vivo* T cell mediated immune responses. A large number of genes are activated in preparation for protein synthesis and cell division. The T cells produce cytokines for immune regulatory function and express cell-surface molecules for signal transduction. The effect of drugs on T cells can be evaluated by measuring *in vitro* T cell proliferation, cytokine levels and expression of cell-surface immune markers such as CD25.

The incubation conditions and drug concentrations were optimized in preliminary experiments. The order of addition of test drugs and cyclosporine did not appear to cause

a significant difference in inhibition of T cell proliferation when a combination of drugs was used (Fig. 4-1). Because of high variability in numerical responses in proliferation experiments, each set of experiments was designed with all required controls for comparison. In a series of proliferation experiments, cyclosporine at 0.08μM and 0.2μM was not found to cause a significant inhibition of proliferation. Quinidine, a P-gp inhibitor, by itself caused a significant decrease in proliferation of T cells at a higher concentration (62μM; p=0.002), whereas a lower concentration of quinidine (31μM) was not immunosuppressive (Fig. 4.2). When cyclosporine and quinidine were used in combination (Fig. 4.2), there was a statistically significant decrease in T cell proliferation from the equivalent "no cyclosporine" concentration (Table 4-1). The magnitude of inhibition was greater than would be expected for non-interacting drugs. Indomethacin (56μM) caused a significant inhibition of T cell proliferation Fig. 4.3, Table 4-1). A combination of indomethacin and cyclosporine (0.08μM) demonstrated an additive effect of the two drugs, but at the higher cyclosporine concentration (0.2μM), the effect was larger than an additive effect. The viability of the T cells at the end of incubation was greater than 90%.

The effect of vinblastine on T cell proliferation, was evaluated at two concentrations, 10.9μM and 109μM. Even at the low concentration (10.9μM), vinblastine was a very strong inhibitor of T cell proliferation (Fig. 4.4, 4.5, Table 4-2). The cyclosporine and PHA data is plotted separately (Fig. 4.4) because there is a very large difference in orders of magnitude of thymidine uptake. The combined effect of vinblastine (10.9μM) and cyclosporine (0.08μM and 0.2μM) was much larger

Fig. 4.1. In vitro T cell proliferation in the presence of cyclosporine (CsA) and quinidine (Quin). Effect of order/time of addition of drugs on inhibition of proliferation; n=3 (mean ± SD)

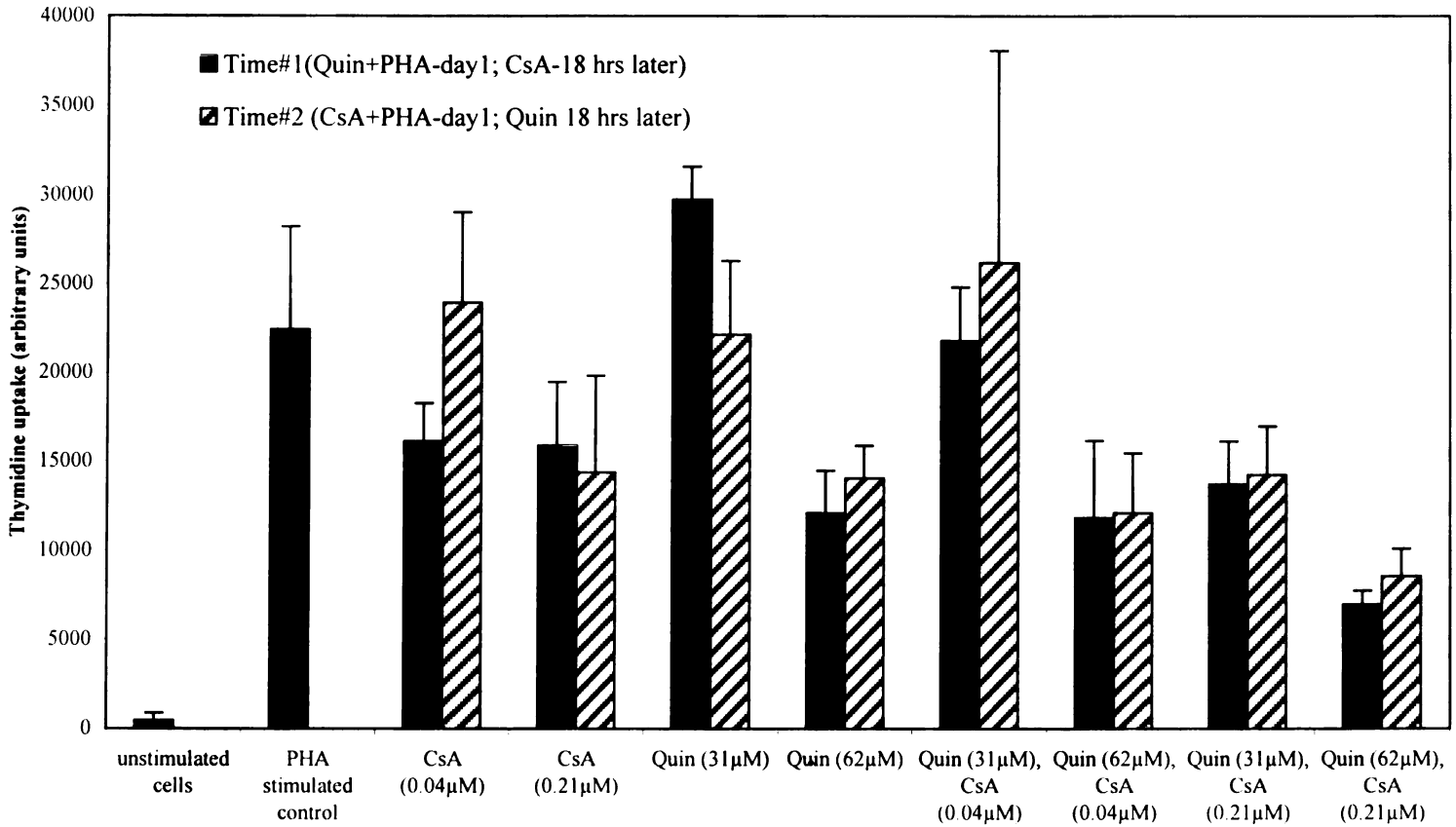


Fig. 4.2. Effect of quinidine and/or cyclosporine at higher (H) or lower (L) concentrations on in vitro T cell proliferation (mean±SD; N=3, n=9)

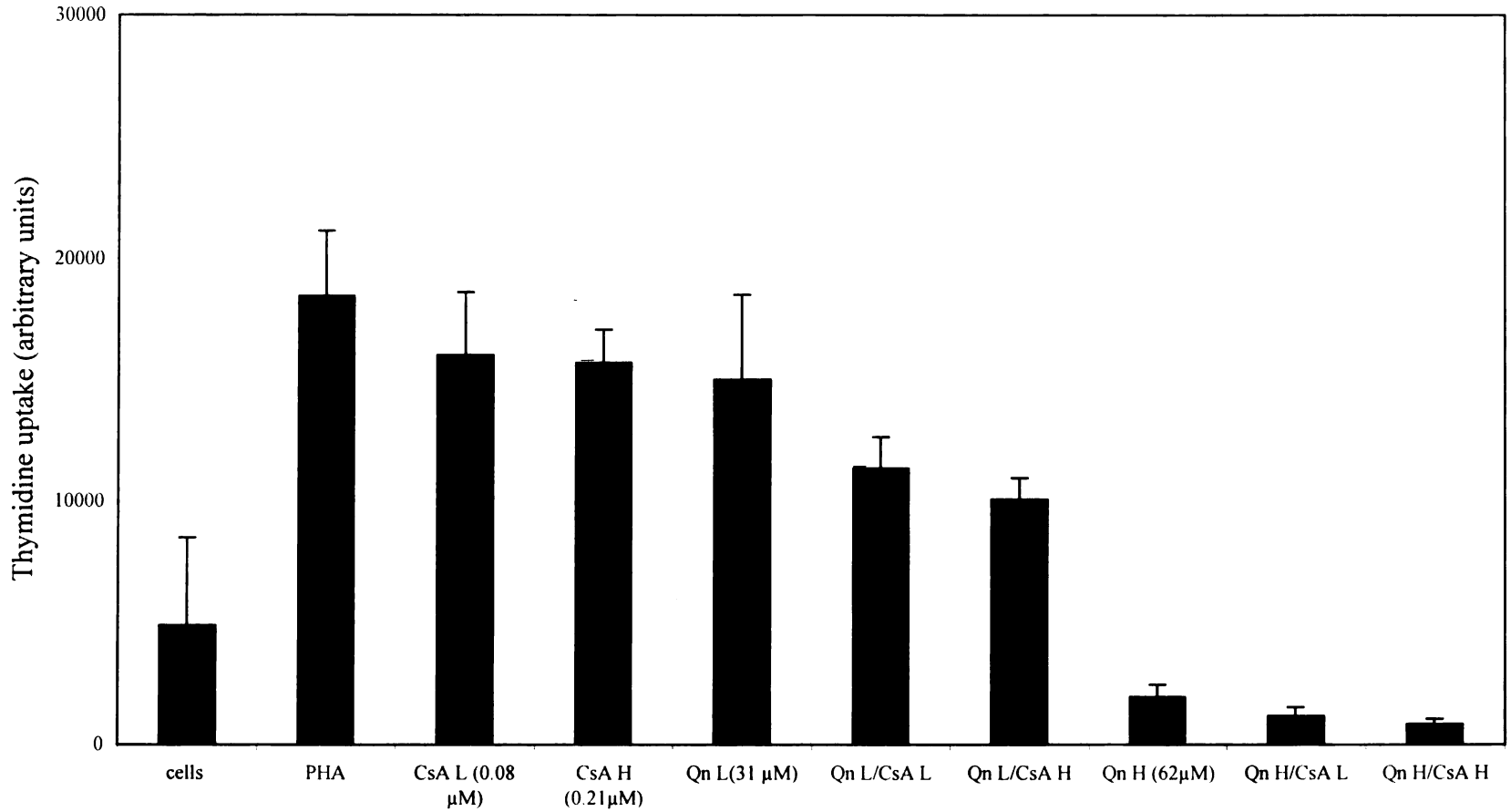


Table 4-1. Thymidine uptake by proliferating T cells in the presence of cyclosporine and quinidine or indomethacin (N=3, n=9).

Treatment	***Mean CPM (thymidine uptake)***	***SD***	***% decrease from equivalent no cyclosporine treatment****
Unstimulated cells	4877	3596	
PHA alone	18437	2693	
CsA Low (0.08 μM)	15995	2607	13.3
CsA High (0.21 μM)	15677	1386	15.0
Quin Low (31μM)	14992	3507	
Quin L / CsA L	11321	1310	24.5
Quin L / CsA H	10056	891	33.0
Quin High (62 μM)	1929	502	
Quin H / CsA L	1149	378	40.4
Quin H / CsA H	810	232	58.0
Indo (56 μM)	6960	604	
Indo / CsA L	6013	836	13.6
Indo / CsA H	5339	438	23.3

*For example, the percent decrease is calculated as follows:

$$\frac{\text{CPM (Quin alone)} - \text{CPM (Quin+CsA)}}{\text{CPM (Quin alone)}} \times 100$$

Abbreviations: CsA – cyclosporine A; Quin – quinidine; Indo – indomethacin; L – low; H - high

Fig. 4.3. Effect of indomethacin and/or cyclosporine on T cell proliferation (mean±SD; N=3, n=9)

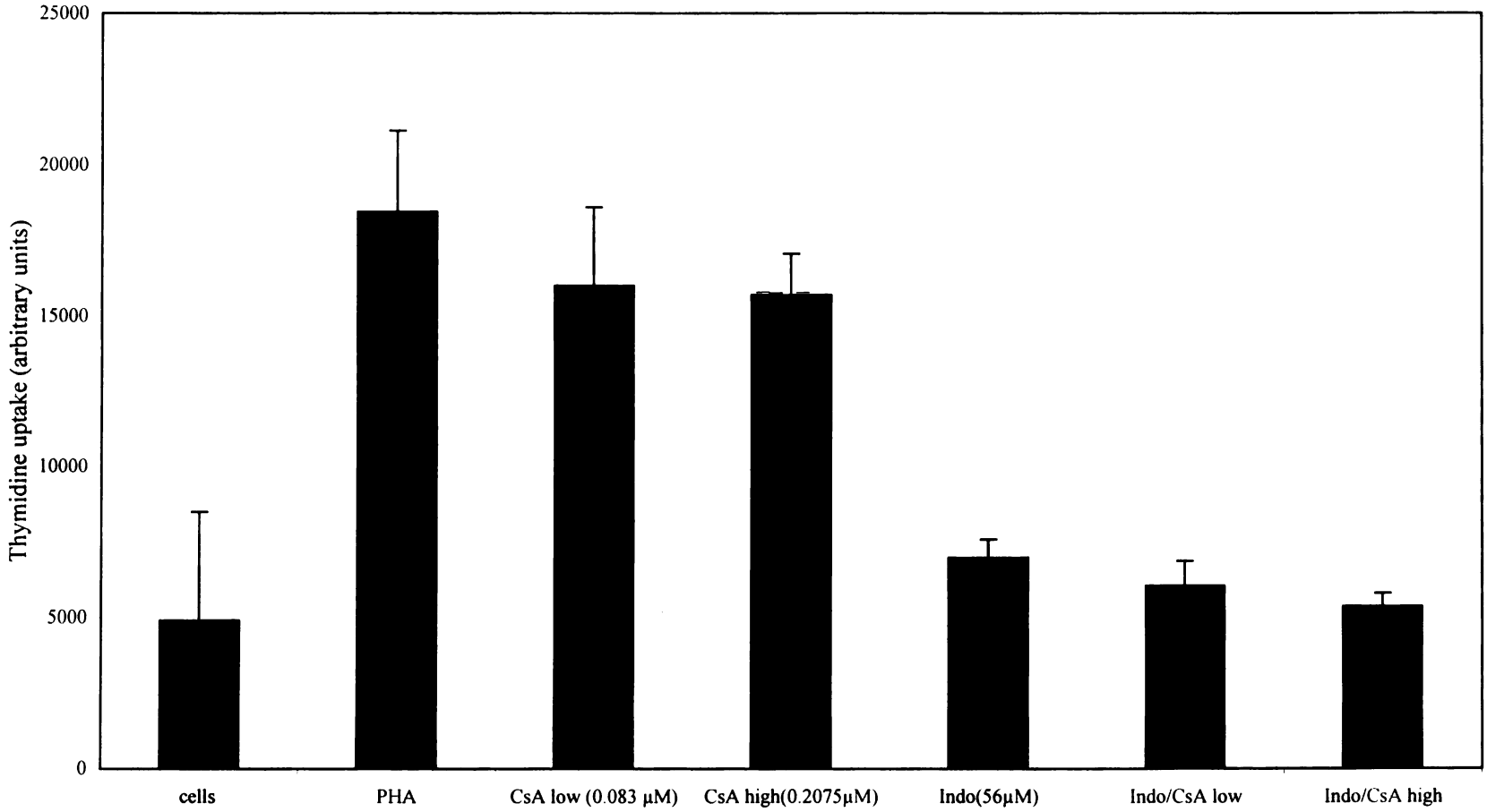


Fig. 4.4. Effect of cyclosporine on T cell proliferation (control for vinblastine/probenecid); mean ±SD (N=3, n=9)

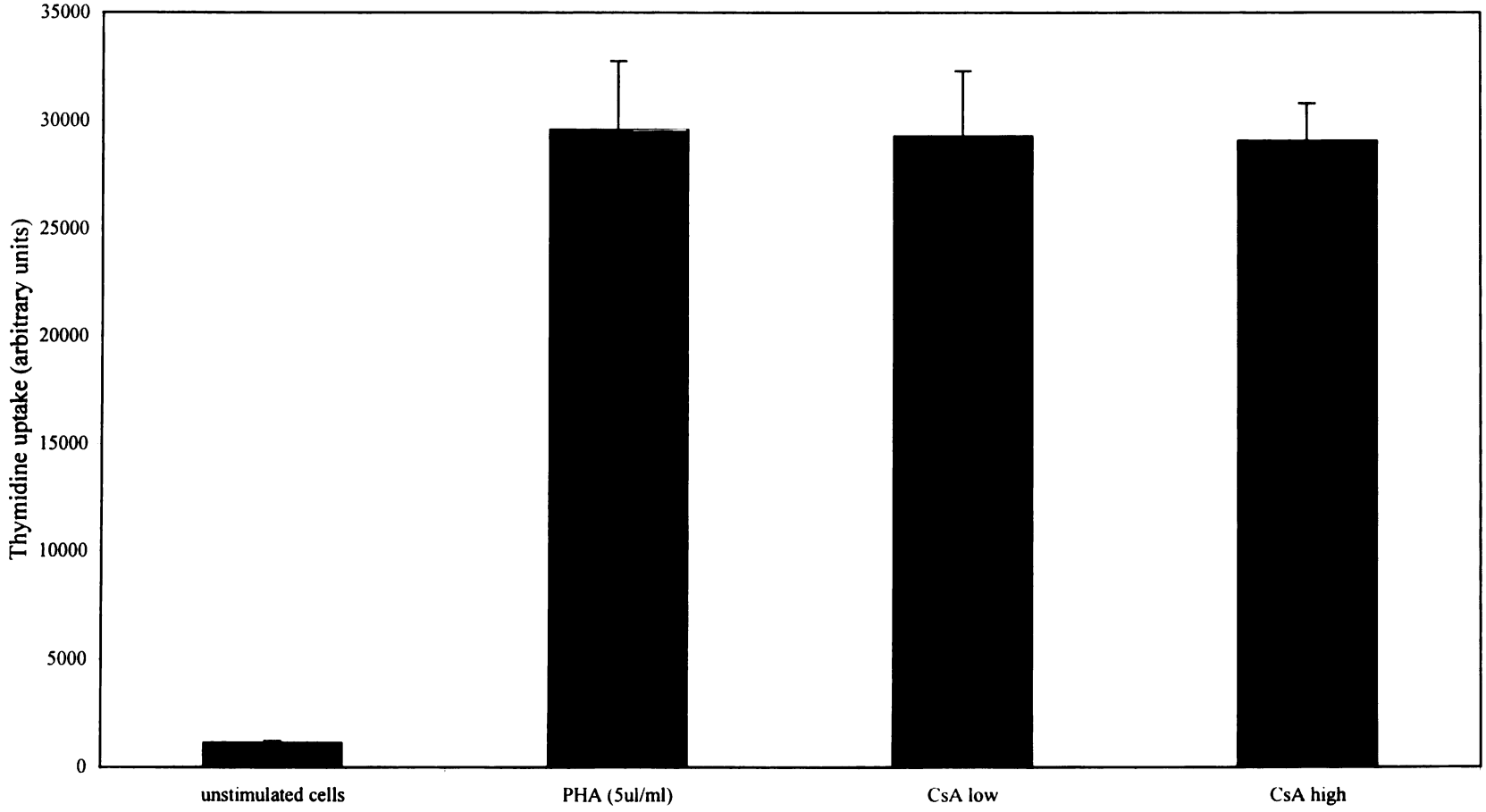


Fig. 4.5. Effect of vinblastine and cyclosporine on T cell proliferation (mean ± SD; N=3, n=9)

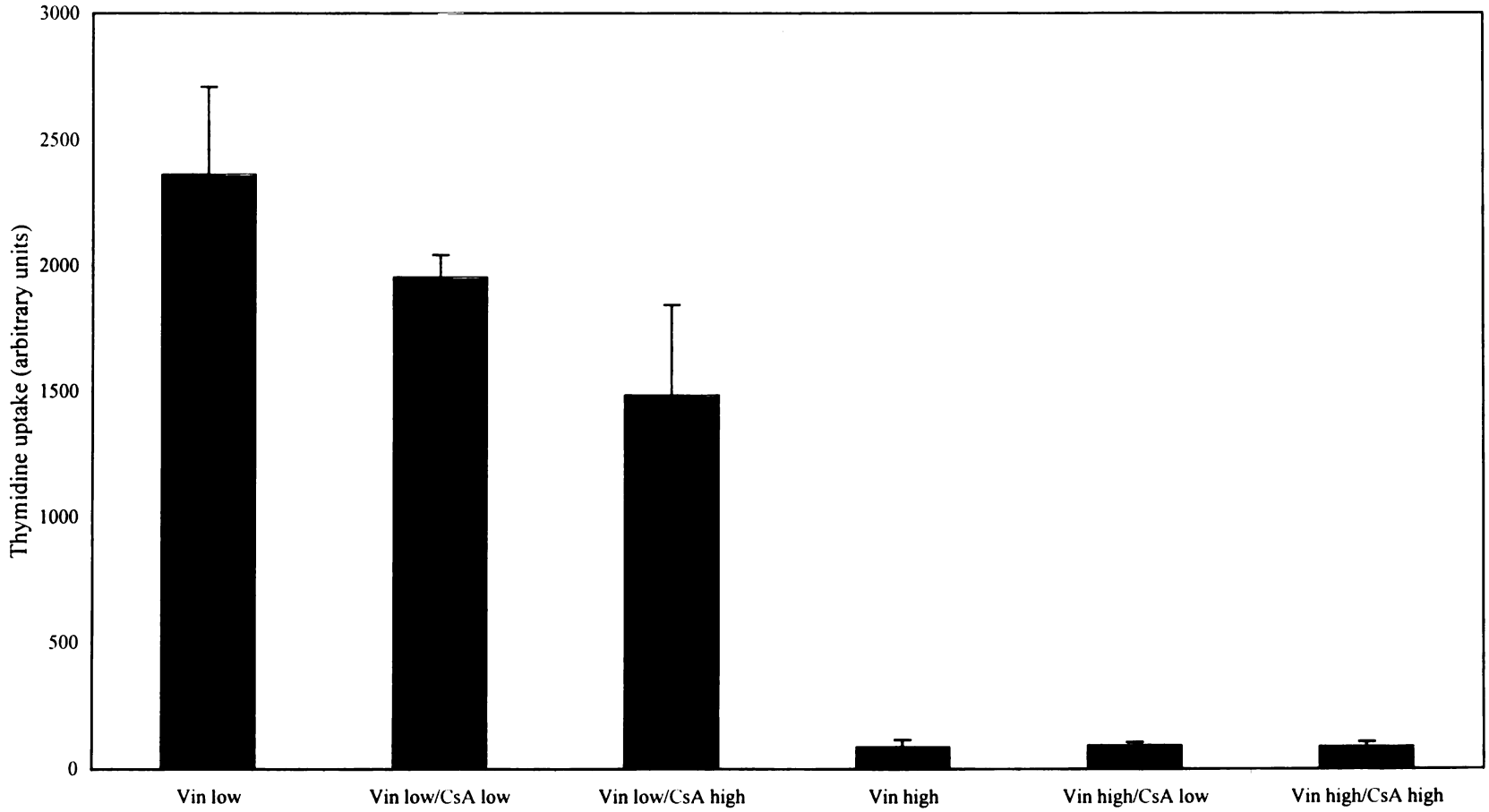


Table 4-2. Thymidine uptake by proliferating T cells in the presence of cyclosporine and vinblastine or probenecid (N=3, n=9).

Treatment	***Mean CPM (thymidine uptake)***	***SD***	***% decrease from equivalent no cyclosporine treatment****
Unstimulated cells	1097	74	
PHA alone	29572	3199	
CsA Low (0.08 μM)	29284	3026	0.97
CsA High (0.21 μM)	29083	1735	1.65
Vin Low (10.9 μM)	2362	348	
Vin L / CsA L	1952	89	17.4
Vin L / CsA H	1482	360	37.3
Vin High (109 μM)	82	31	
Vin H / CsA L	90	13	-9.6
Vin H / CsA H	85	21	-3.6
Prob L (0.81mM)	5268	904	
Prob L / CsA L	5220	813	0.9
Prob L / CsA H	4413	199	16.2
Prob H (1.62 mM)	316	65	
Prob H / CsA L	367	101	-16.2
Prob H / CsA H	433	60	-37.0

*For example, the percent decrease is calculated as follows:

$$\frac{\text{CPM (Vin alone)} - \text{CPM (Vin+CsA)}}{\text{CPM (Vin alone)}} \times 100$$

Abbreviations: CsA – cyclosporine A; Vin – vinblastine; Prob – probenecid; H-high; L-low

(Figs. 4.4, 4.5, Table 4-2) than would be expected by combining two non-interacting drugs. At higher concentrations of vinblastine (109μM), the uptake of radiolabeled thymidine was less than unstimulated cells (Fig. 4.5, Table 4-2). The addition of cyclosporine had the reverse effect and caused a proliferation of cells compared to vinblastine alone (Fig. 4.5, Table 4-2), but these effects were not statistically significant. Probenecid when used alone, caused a statistically significant decrease in proliferation of T cells ($p < 0.001$) at both tested concentrations (0.81 mM and 1.62 mM), but a combination of cyclosporine and probenecid did not result in a statistically significant inhibition of proliferation of T cells compared to probenecid alone (Figs. 4.4, 4.6, Table 4-2). The percent inhibition of the combination of low concentration of probenecid (0.81mM) and high concentration of cyclosporine (0.2μM) was additive but "low probenecid" and "high cyclosporine" was much greater than an additive effect. At high probenecid concentrations (1.62 mM), the thymidine uptake was significantly less than that of unstimulated cells, and the addition of cyclosporine enhanced the proliferation. The combination of "high probenecid" and "high cyclosporine" was statistically significant compared to probenecid alone ($p = 0.001$). The viability of cells harvested at the end of incubation was greater than 95% (Fig. 4.7).

The cells were harvested from proliferation studies to test for expression of CD25 using flow cytometry. The expression of CD25 on cells was similar in pattern to thymidine uptake studies (Figs. 4.8, 4.9). The PHA stimulated control group of cells had the highest number of cells expressing CD25 indicating a proliferating T cell population. Cyclosporine decreased the number of cells that expressed CD25 in Fig. 4.8 but there was

Fig. 4.6. Effect of probenecid and cyclosporine on T cell proliferation (mean ± SD; N=3, n=9)

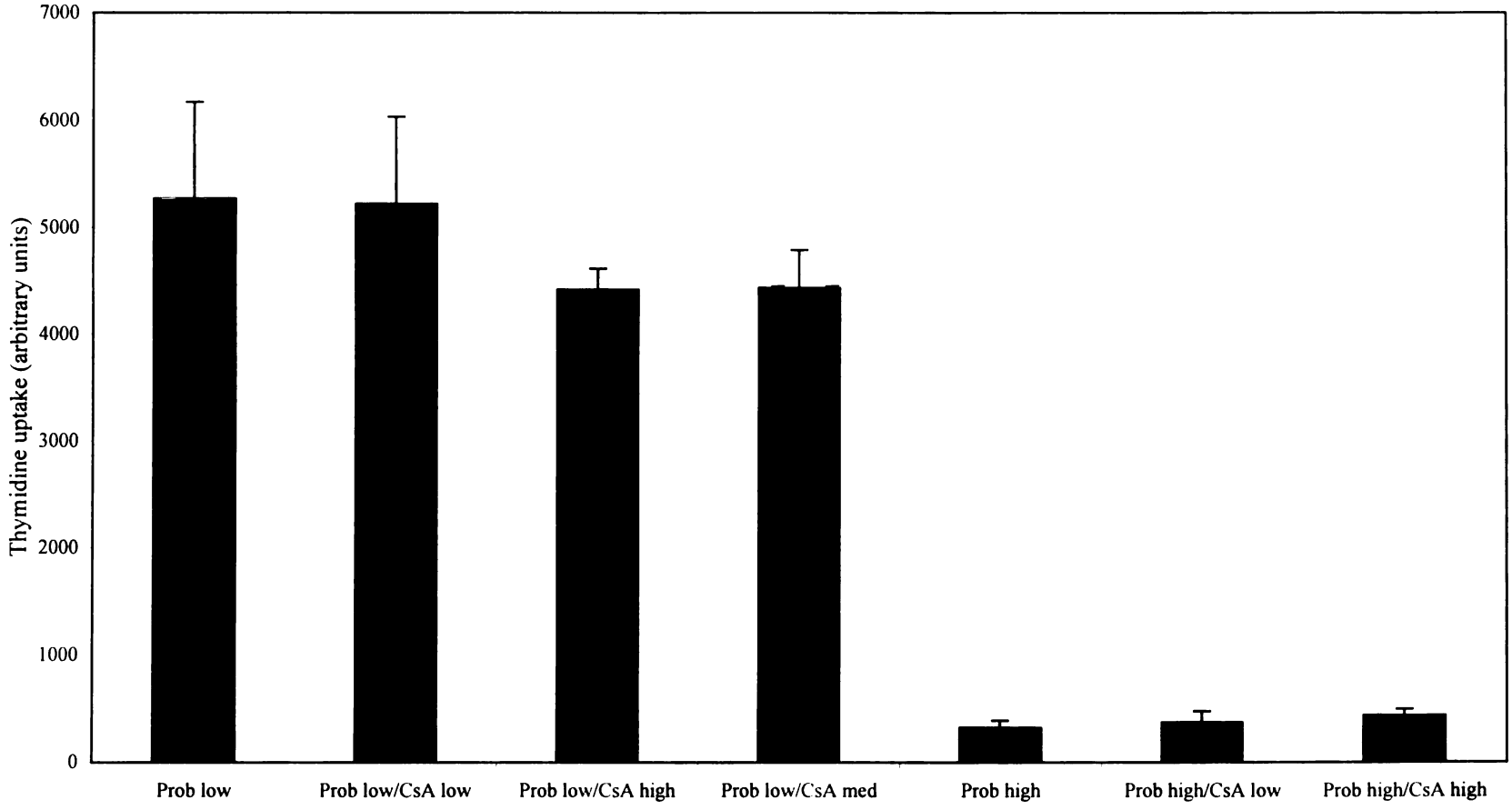


Fig. 4.7. Cell viability of proliferating T cells after 32 hour incubation with cyclosporine/vinblastine/probenecid (cells pooled from n=3)

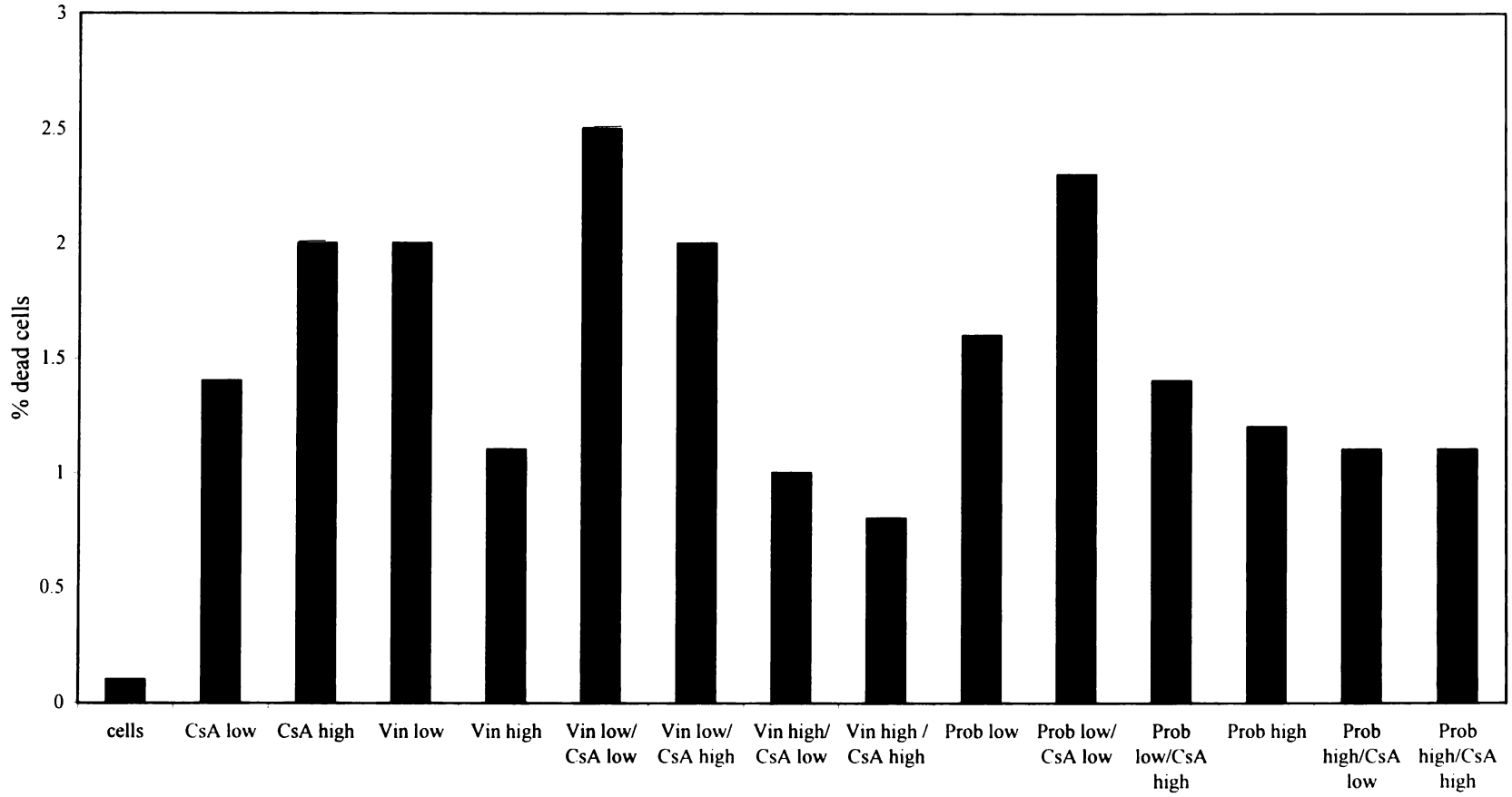


Fig. 4.8. Expression of CD25 on T cells proliferating in the presence of cyclosporine and/or quinidine, indomethacin (cells pooled from n=3)

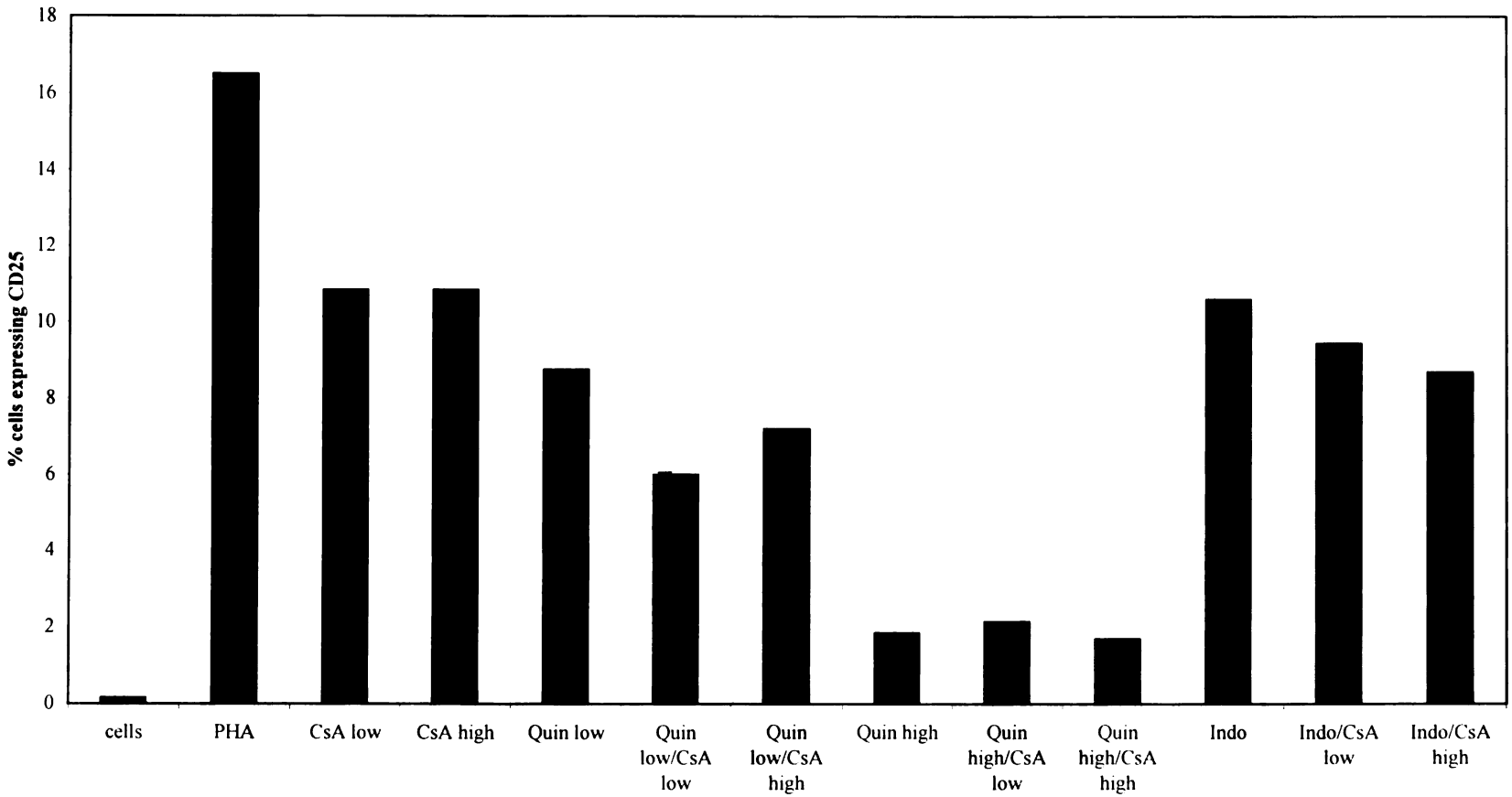
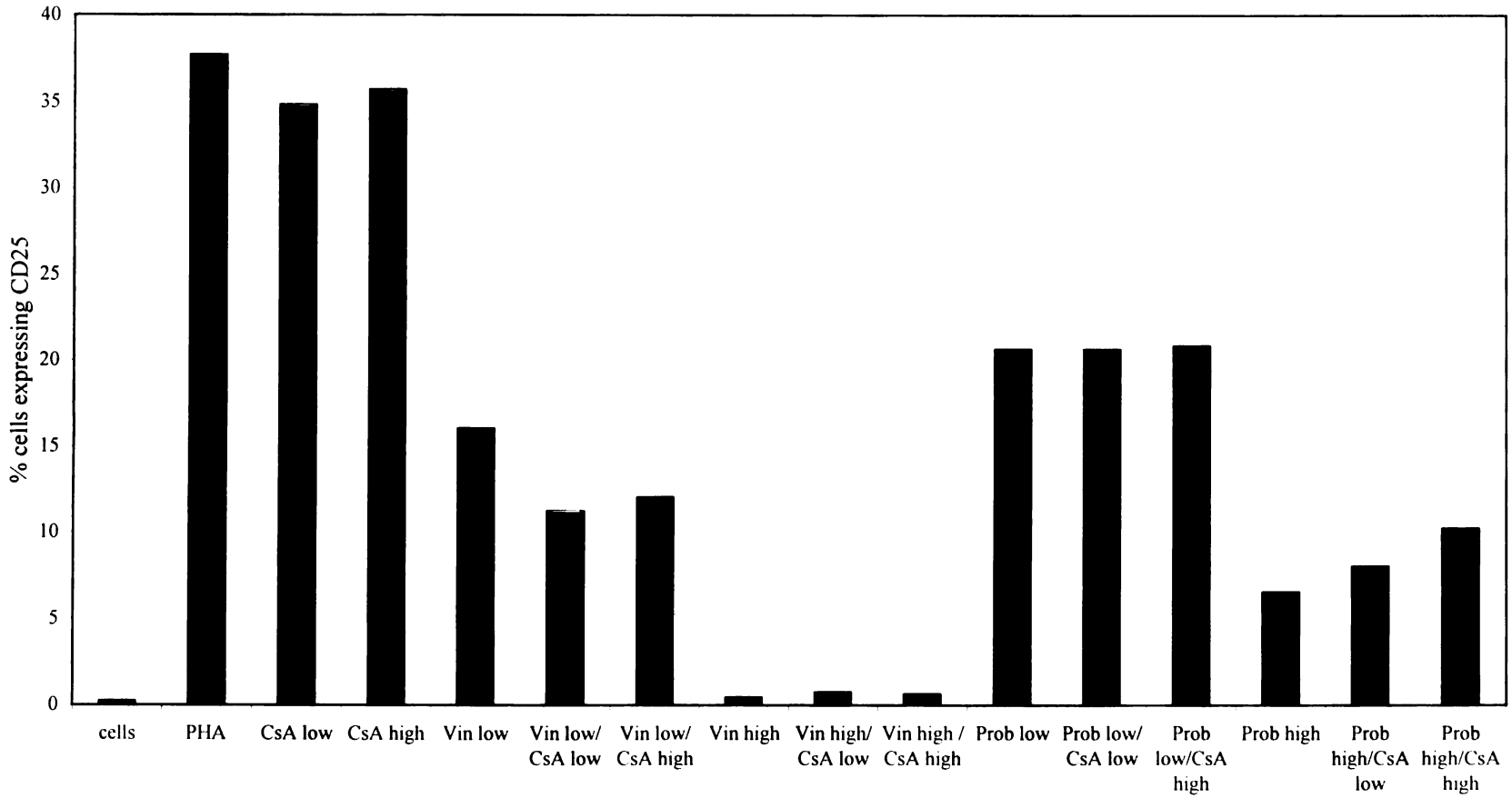


Fig. 4.9 Percentage of T cells expressing CD25 when proliferating in the presence of cyclosporine/vinblastine/probenecid (cells pooled from n=3)



no significant decrease in the number of CD25 positive cells in Fig. 4.9. These results are consistent with the known variability in response to cyclosporine in different individuals. (The blood sources used for different sets of experiments were different and PHA controls and cyclosporine controls were included with each experiment to facilitate analysis). The decrease in CD25+ cells was comparable to that observed with indomethacin. Quinidine, vinblastine and probenecid significantly decreased the number of cells expressing CD25 indicating a lack of proliferation. Combining the drugs with cyclosporine decreased the number of CD25+ T cells significantly in case of low concentration of vinblastine.

The effect of various drug treatments was evaluated on secreted extracellular cytokines. The levels of secreted IL-2 in the presence of cyclosporine (Fig. 4.10) were significantly less (at least $p < 0.05$) than PHA control cells (Tables 4-3, 4-4). This was in contrast to the results from thymidine uptake assays (Tables 4-1, 4-2) where cyclosporine did not cause a significant decrease in proliferation of cells. Quinidine at both concentrations significantly ($p < 0.001$) decreased IL-2 levels (Figs. 4.10, 4.11, Table 4-3). At the higher quinidine concentration (62μM), the concentration of IL-2 was similar to that produced by unstimulated cells, with a significant decrease in IL-2 observed for the combination of quinidine and cyclosporine ($p < 0.001$). The effect of combination of low concentration of quinidine (31μM) with either concentration of cyclosporine (0.08μM and 0.2μM) was greater than that expected from non-interacting drugs. Although indomethacin caused a significant decrease in thymidine uptake (Fig. 4.3), the concentration of IL-2 produced by cells in the presence of indomethacin was significantly

Fig. 4.10. Soluble IL-2 in pooled supernatant from 3 wells proliferating in the presence of indomethacin/cyclosporine (mean ± SD; n=2)

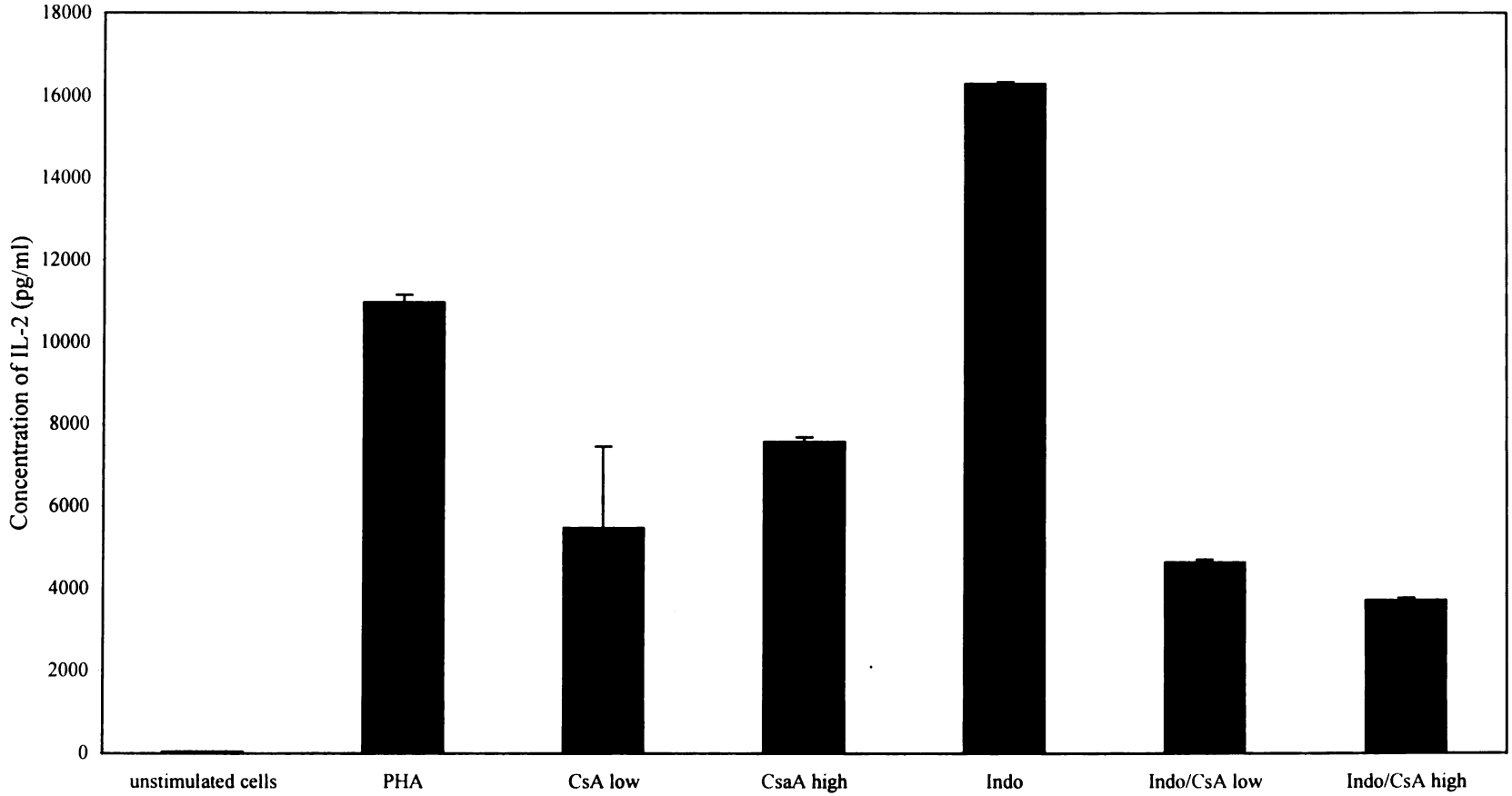


Table 4-3. Soluble IL-2 produced by cells proliferating in the presence of cyclosporine and/or quinidine, indomethacin

Treatment	***Mean conc. (pg/ml)***	***% decrease in soluble IL-2 from equivalent no cyclosporine treatment***
Unstimulated cells	35	
PHA stimulated	10958	
CsA L	5462	50.2
CsA H	7569	31.0
Quin L	573	
Quin L / CsA L	196	65.9
Quin L / CsA H	123	78.5
Quin H	50	
Quin H / CsA L	39	20.8
Quin H / CsA H	39	20.8
Indo	16294	
Indo / CsA L	4621	71.6
Indo / CsA H	3703	77.3

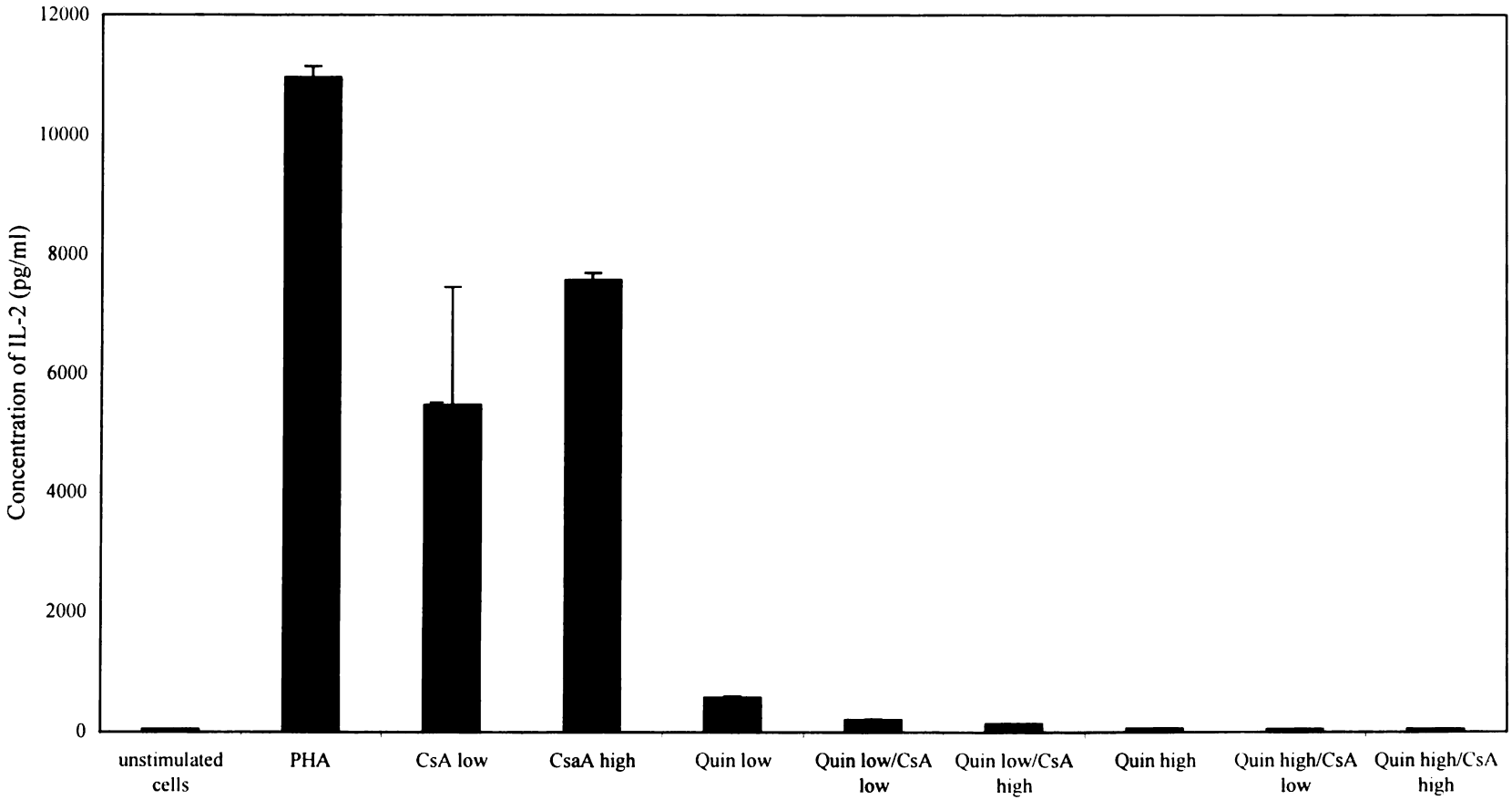
Abbreviations: CsA – cyclosporine; Quin – quinidine; Indo – indomethacin; L – low; H - high

Table 4-4. Soluble IL-2 produced by cells proliferating in the presence of cyclosporine and/or vinblastine, probenecid

Treatment	*Mean conc. (pg/ml)*	*% decrease in soluble IL-2 from equivalent no cyclosporine treatment*
Unstimulated cells	47	
PHA stimulated	12910	
CsA L	4449	65.5
CsA H	5603	56.6
Vin L	1909	
Vin L / CsA L	1010	47.1
Vin L / CsA H	1250	34.5
Vin H	51	
Vin H / CsA L	65	-28.1
Vin H / CsA H	50	2.2
Prob L	611	
Prob L / CsA L	172	71.8
Prob L / CsA H	88	85.7
Prob H	68	
Prob H / CsA L	41	40.7
Prob H / CsA H	36	47.3

Abbreviations: CsA – cyclosporine; Vin – vinblastine; Prob – probenecid; L – low; H - high

Fig. 4.11. Soluble IL-2 produced by proliferating cells in the presence of quinidine/cyclosporine (supernatant from 3 pooled samples, n=2). (Cyclosporine and PHA data in Fig. 4.10)



greater (Fig. 4.10, Table 4-3) than that produced in the PHA control group ($p < 0.001$). A combination of cyclosporine and indomethacin produced a significant decrease in IL-2 indicating a greater than additive effect (Fig. 4.10, Table 4-3). Vinblastine at both concentrations decreased IL-2 production (Figs. 4.12, 4.13, Table 4-4) as compared to the PHA control ($p < 0.001$), but there was a significant ($p < 0.01$) decrease in IL-2 when cyclosporine was combined with "low vinblastine" (10.9μM). At the high vinblastine concentration, the IL-2 levels were similar to unstimulated cell IL-2 levels and not statistically different from vinblastine alone. Probenecid also caused a significant decrease in IL-2 levels (Figs. 4.14, 4.12) at both concentrations ($p < 0.001$), but the combination of "low probenecid" (0.81 mM) and cyclosporine had a significant ($p < 0.005$) and greater than additive effect. At the high probenecid concentration (1.62 mM), the IL-2 levels were comparable to unstimulated levels but significantly ($p < 0.05$) lower for the combination than probenecid alone (Table 4-4).

All tested drugs caused a significant decrease in IFN-γ levels (Figs. 4.15 – 4.19). The effect of cyclosporine was variable (Figs. 4.15, 4.17, Tables 4-5, 4-6). The effect of combination with cyclosporine was synergistic for quinidine (31μM and 62μM), vinblastine (10.9μM and 109μM) and probenecid (significant only for "low probenecid" alone and in combination with "high cyclosporine" at $p < 0.005$). Probenecid decreased IFN-γ levels to those obtained from unstimulated levels. A combination of indomethacin and cyclosporine increased IFN-γ levels similar to cyclosporine alone (Fig. 4.15, Table 4-5).

Fig. 4.12. Soluble IL-2 produced by proliferating T cells in the presence of cyclosporine/PHA (supernatant from 3 pooled samples, n=2)

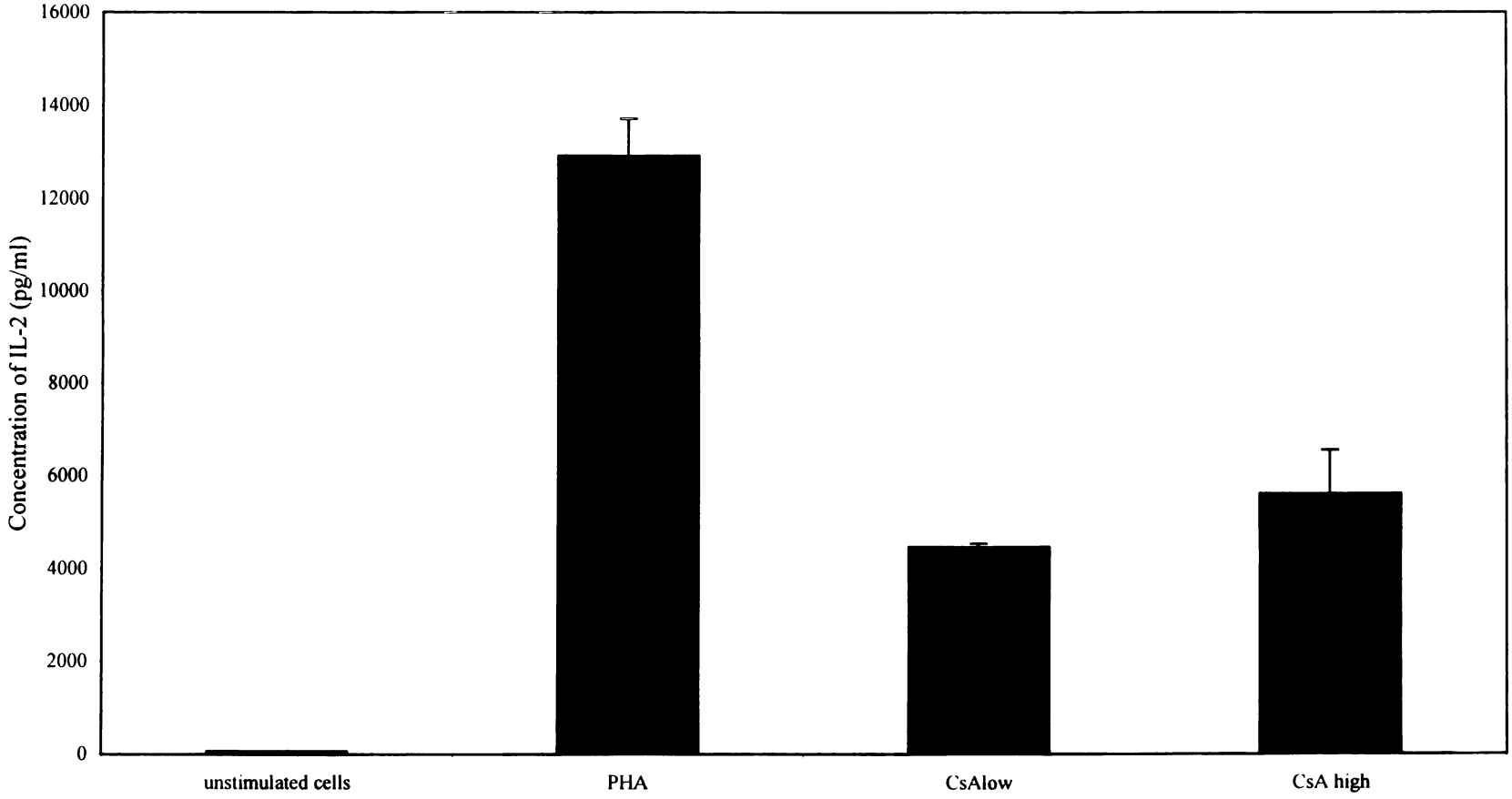


Fig. 4.13. Soluble IL-2 in pooled supernatant from 3 wells proliferating in the presence of vinblastine / cyclosporine (mean±SD; n=2).
(Corresponding cyclosporine and PHA data in Fig. 4.12)

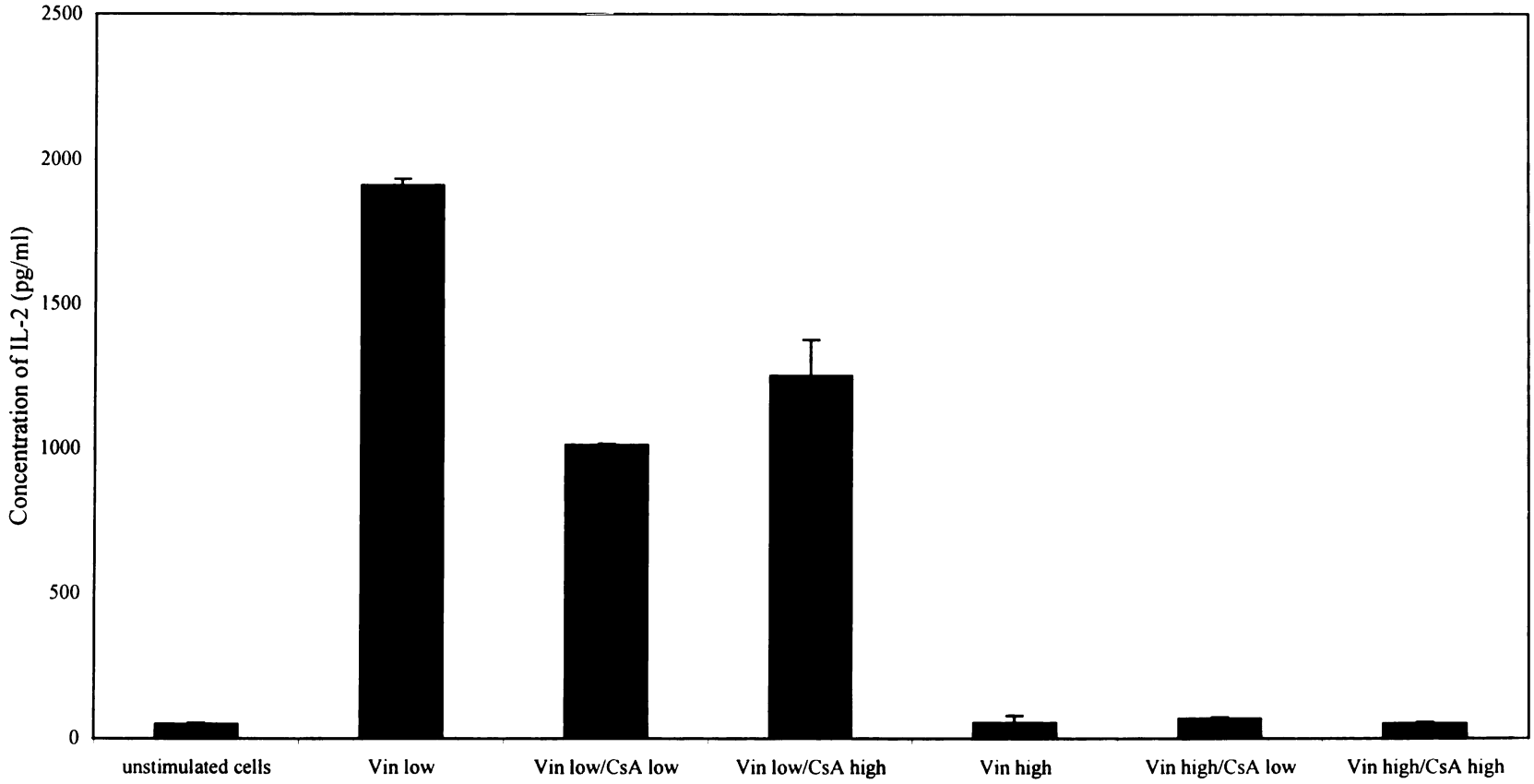


Fig. 4.14. Soluble IL-2 in pooled supernatant from 3 wells proliferating in the presence of probenecid / cyclosporine (mean±SD; n=2)
(Corresponding CsA and PHA data in Fig. 4.12)

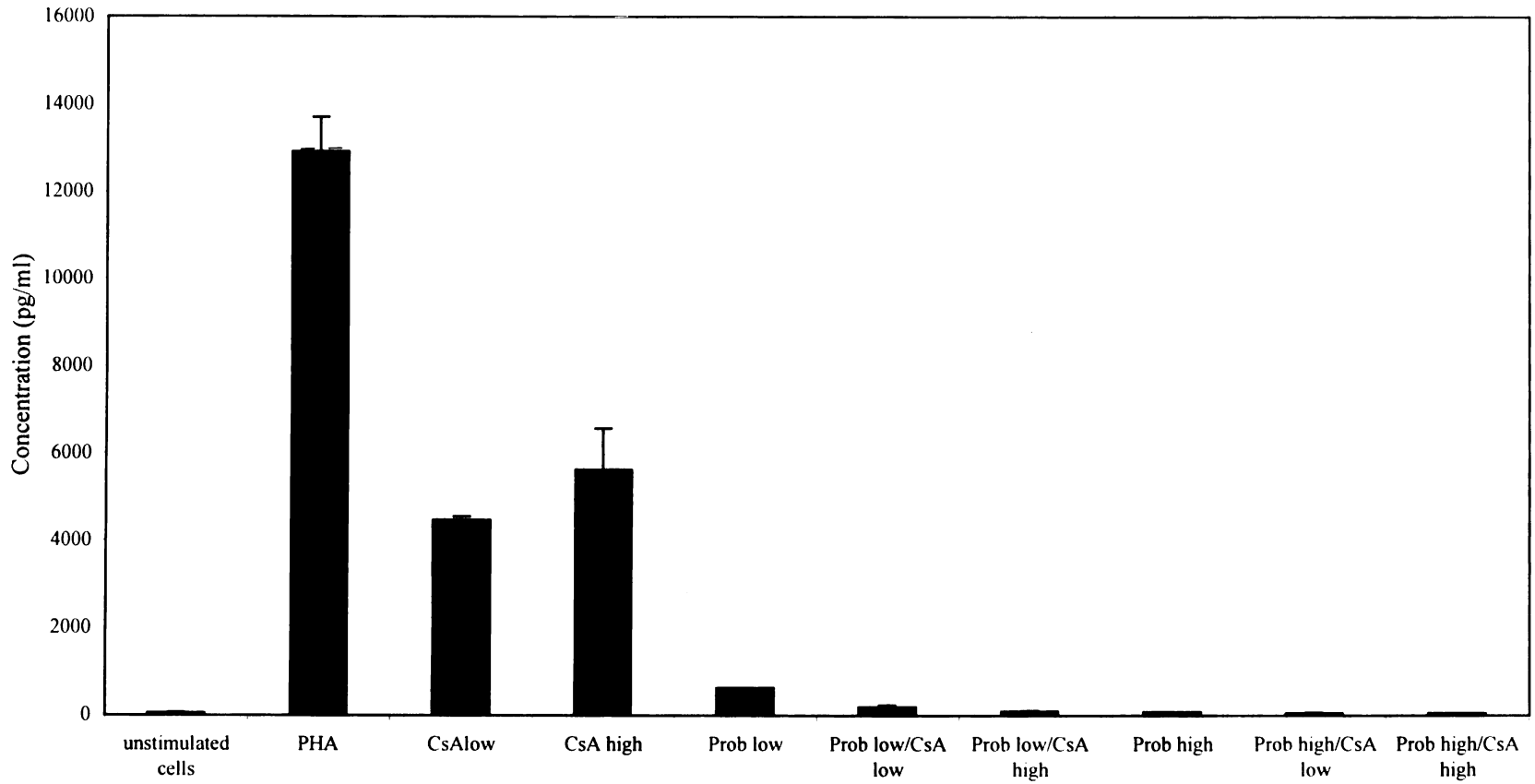


Fig. 4.15. Soluble IFN-gamma produced by proliferating T cells in the presence of indomethacin/cyclosporine (pooled supernatant from 3 samples, n=2)

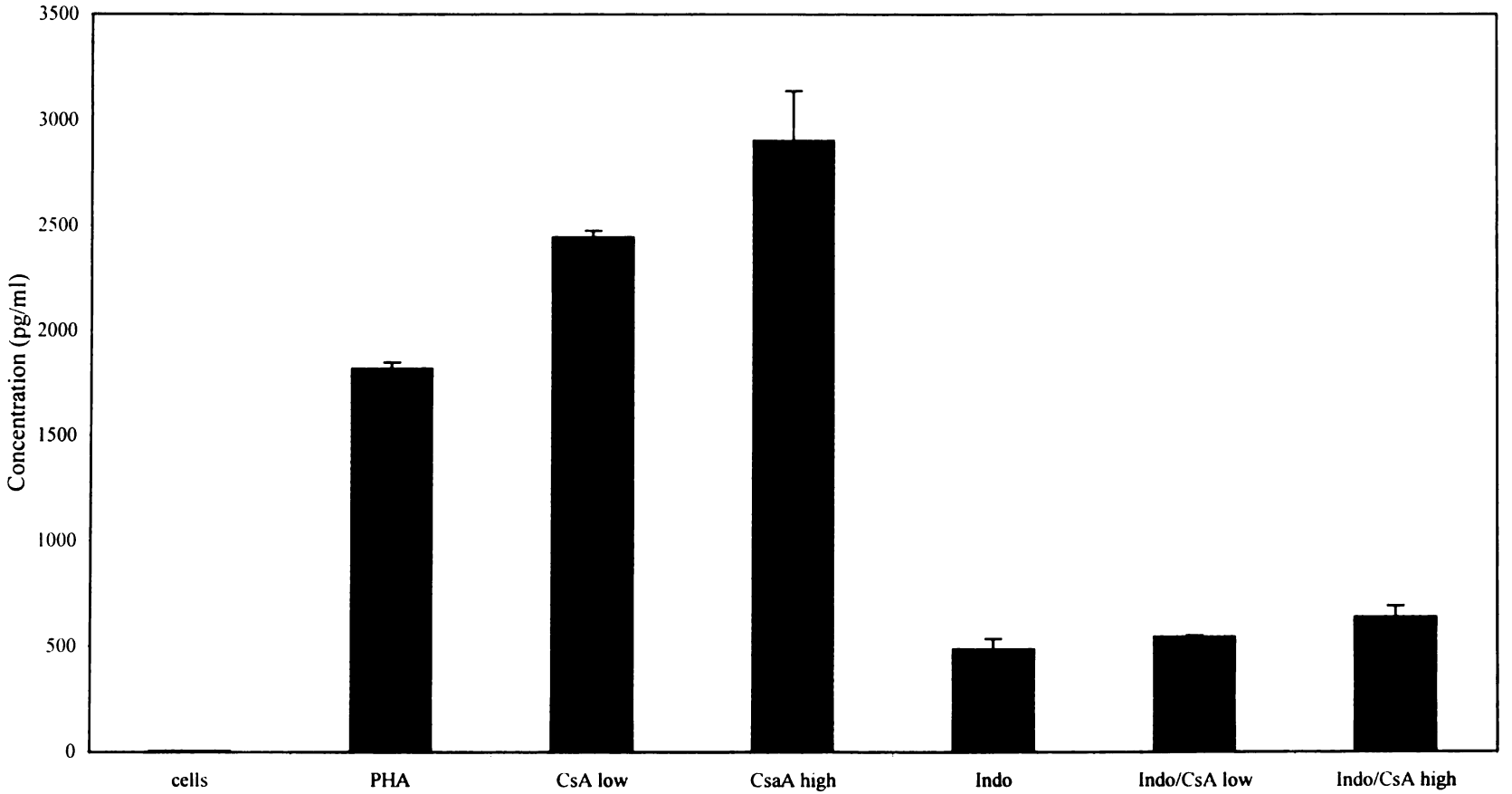


Fig. 4.16. Soluble IFN-gamma produced by proliferating cells in the presence of quinidine/CsA (supernatant from 3 pooled samples, n=2)
(Corresponding CsA and PHA data in Fig. 4.15)

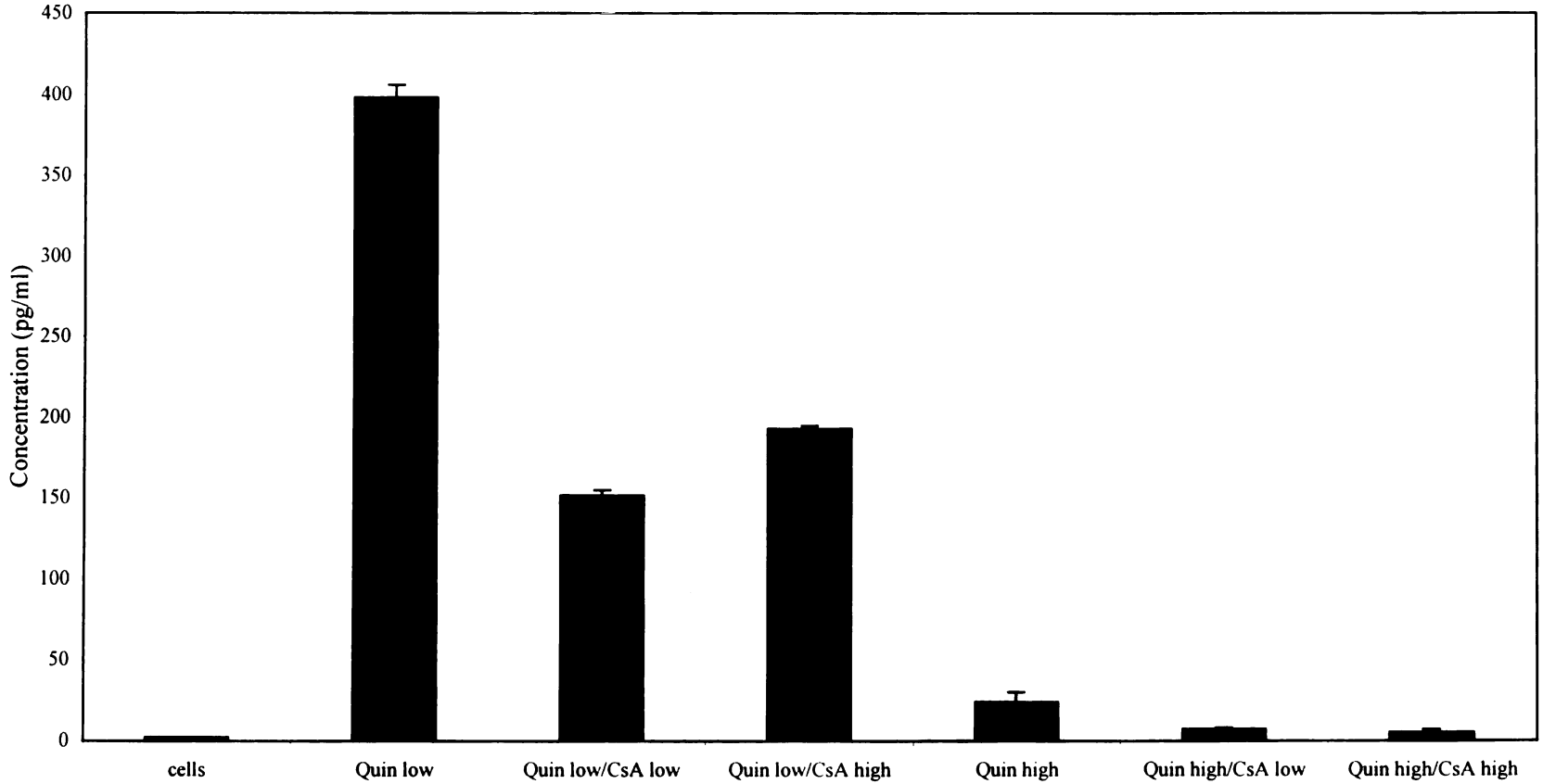


Fig. 4.17. Soluble IFN-gamma produced by proliferating T cells in the presence of cyclosporine and PHA (pooled supernatant from 3 samples, n=2)

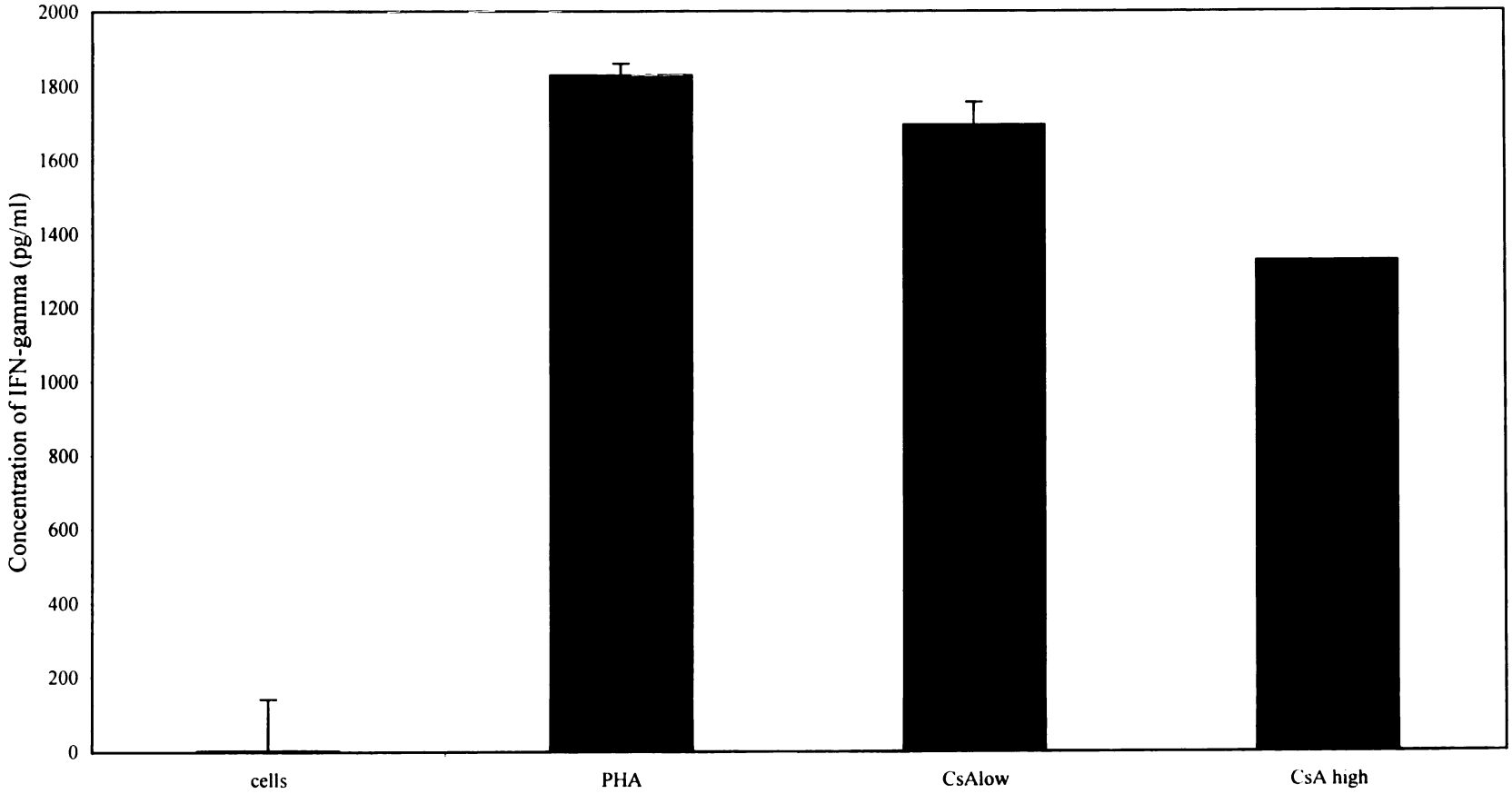


Fig. 4.18. Soluble IFN-gamma in pooled supernatant from 3 wells proliferating in the presence of vinblastine / cyclosporine (mean±SD; n=2)
(Corresponding CsA and PHA data in Fig. 4.17)

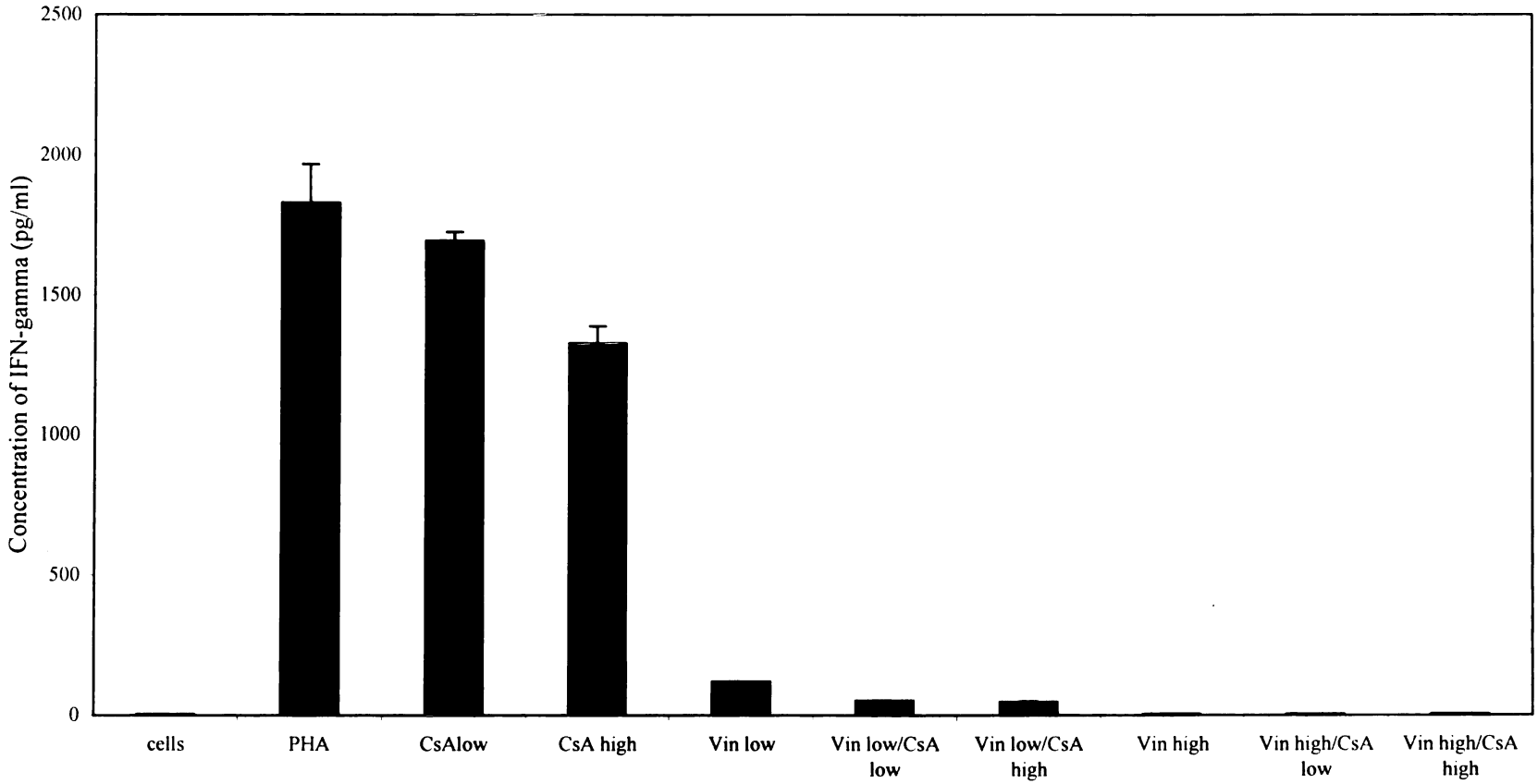


Fig. 4.19. Soluble IFN-gamma in pooled supernatant from 3 wells proliferating in the presence of probenecid / cyclosporine (mean±SD; n=2)
(Corresponding CsA and PHA data in Fig. 4.17)

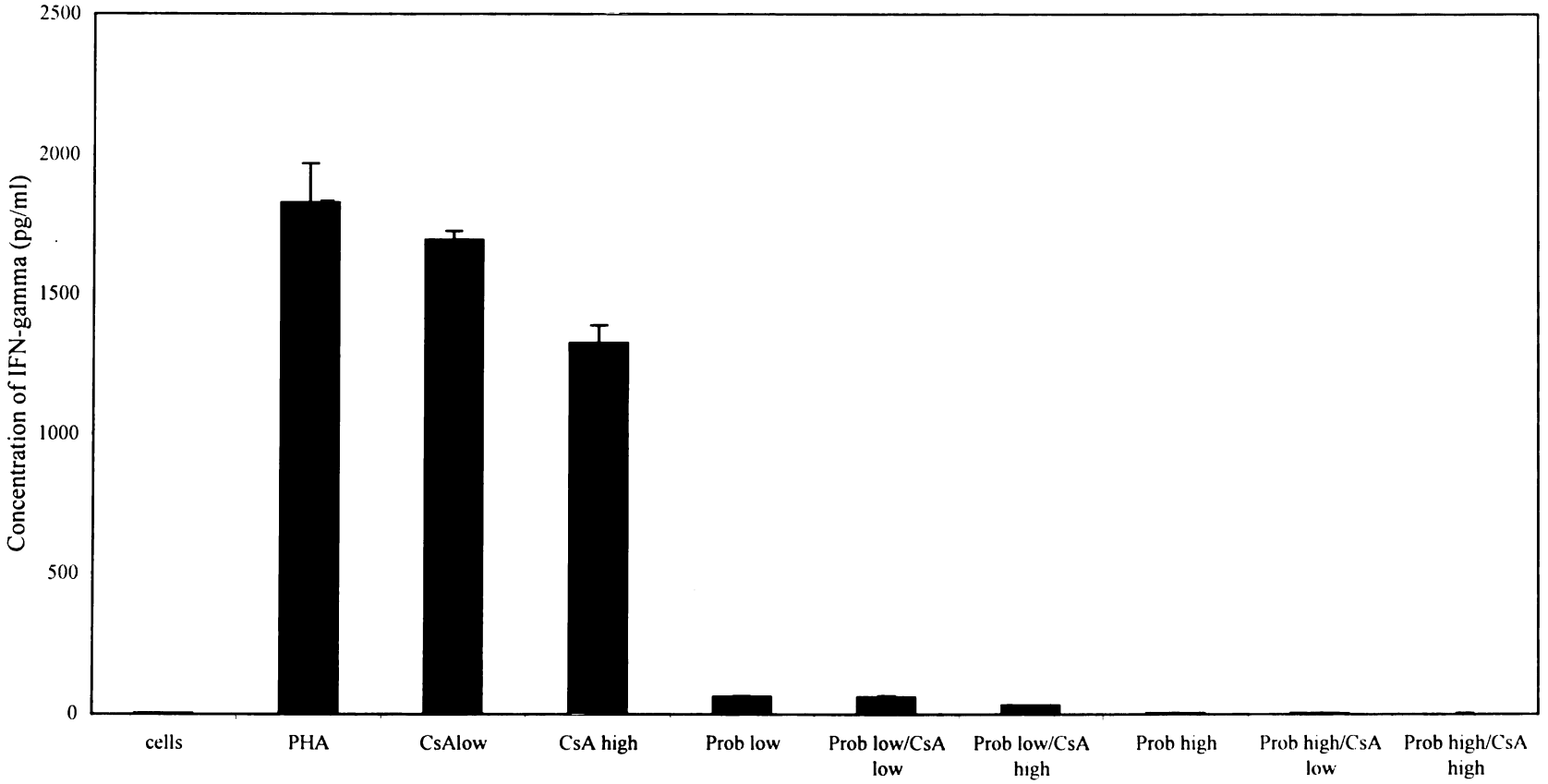


Table 4-5. Soluble IFN-γ produced by cells proliferating in the presence of cyclosporine and/or quinidine, indomethacin

Treatment	*Mean conc. (pg/ml)*	*% decrease in soluble IFN-γ from equivalent no cyclosporine treatment*
Unstimulated cells	2	
PHA stimulated	1818	
CsA L	2444	-34.4
CsA H	2903	-59.7
Quin L	398	
Quin L / CsA L	151	61.9
Quin L / CsA H	193	51.6
Quin H	24	
Quin H / CsA L	7	70.6
Quin H / CsA H	5	79.9
Indo	483	
Indo / CsA L	543	-12.5
Indo / CsA H	637	-31.8

Abbreviations: CsA – cyclosporine; Quin – quinidine; Indo – indomethacin; L – low; H - high

Table 4-6. Soluble IFN-γ produced by cells proliferating in the presence of cyclosporine and/or vinblastine, probenecid

Treatment	*Mean conc. (pg/ml)*	*% decrease in soluble IFN-γ from equivalent no cyclosporine treatment*
Unstimulated cells	2	
PHA stimulated	1828	
CsA L	1693	7.4
CsA H	1325	27.5
Vin L	117	
Vin L / CsA L	48	58.9
Vin L / CsA H	45	61.6
Vin H	2	
Vin H / CsA L	1	42.6
Vin H / CsA H	1	51.9
Prob L	60	
Prob L / CsA L	58	3.3
Prob L / CsA H	29	51.4
Prob H	2	
Prob H / CsA L	2	30.9
Prob H / CsA H	1	55.4

Abbreviations: CsA – cyclosporine; Vin – vinblastine; Prob – probenecid; L – low; H - high

The results for IL-4 levels were inconclusive because the amount of IL-4 produced in most cases was at the limit of detection and resulted in highly variable numbers.

4.5 Discussion

The immunosuppressive effects of cyclosporine are well known and the effects on different cytokines and receptors have been reported. However, the pharmacodynamic effects of combining cyclosporine with different drugs are not known. In this study, representative drugs from different classes were studied for their effects on immune responses by themselves and in combination with cyclosporine (Table 4-7). First I will briefly discuss the observed immunosuppressive activity of the test compounds.

The NSAID, indomethacin is a cyclooxygenase inhibitor and decreases prostaglandin production. In human and murine cell-lines, indomethacin acts as a MRP inhibitor but does not inhibit P-gp (Draper et al., 1997). Hatse and co-workers (1998) have demonstrated the existence of an anion-specific efflux pump that is inhibited by indomethacin in a mutant human erythroleukemia K562/PMEA-1 cell-line. In chapter 3, I have demonstrated that indomethacin blocks the efflux of rhodamine 123 from lymphocytes by inhibiting an uncharacterized transporter different from P-gp or MRP. *In vitro* experiments have shown that indomethacin increases IL-10 and IL-12 levels in isolated monocytes and mononuclear cells (Szabo et al., 1998). Yamamura and co-

Table 4-7. Summary of observed effects of drugs in combination with cyclosporine on *in vitro* T cell proliferation and secretion of cytokines in phytohemagglutinin (PHA) stimulated T cells

Drug	***Effect on T cell proliferation***	***Effect on IL-2 secretion***	***Effect on IFN-γ secretion***
Cyclosporine (CsA)	No significant decrease	Significant decrease	No significant average change
Quinidine	Significant decrease at high concentration	Significant decrease; at high concentration IL-2 level similar to that of unstimulated cells (control)	Significant decrease
Quinidine + CsA	Synergistic effect at both concentrations	Synergistic decrease at low concentration of quinidine	Synergistic decrease
Vinblastine	Significant decrease; at high concentration proliferation was less than that of control	Significant decrease; at high concentration, IL-2 level similar to that of control	Significant decrease; at high concentration, levels were similar to that of control
Vinblastine + CsA	Synergistic decrease at low concentration, slight increase at high concentration	Significant decrease but the combination was not synergistic	Synergistic decrease
Indomethacin	Significant decrease	Significant increase	Significant decrease
Indomethacin + CsA	Additive decrease	Synergistic decrease	Slight increase or similar to indomethacin alone
Probenecid	Significant decrease	Significant decrease	Significant decrease; at high concentration, levels were similar to that of control
Probenecid + CsA	Synergistic decrease only for one combination of probenecid low + CsA high	Synergistic decrease at low probenecid concentration; level similar to control at high conc.	Synergistic decrease for all combinations except probenecid low + CsA low

workers (1996) have demonstrated that in mice, high doses of NSAIDs induce an increase in granulocytes and extrathymic T cells. Serious hematological side-effects such as thrombocytopenia and aplastic anemia have been reported with indomethacin (Katzung, 1998). Tsuboi and co-workers (1995) have observed that NSAIDs including indomethacin upregulated TNF, IFN-γ and IL-2 production at protein and mRNA levels in PBMCs and T cell clones. Duffy and co-workers (1998) have observed increased cytotoxicity of some anti-tumor drugs in combination with NSAIDs such as indomethacin. This effect was independent of cyclooxygenase inhibitory activity or prostaglandin effects. They suspect that the interaction is due to inhibition of transporters such as MRP. Thus, indomethacin appears to possess immunomodulatory effects and is also reported to interact with other drugs via uncharacterized mechanisms. In my experiments, indomethacin had a significant inhibitory effect on PHA stimulated T cell proliferation. However, indomethacin had opposing effects on IL-2 and IFN-γ; it increased IL-2 production but inhibited IFN-γ. The expression of CD25 was also significantly decreased. These results suggest that the inhibitory effect of indomethacin on T cell proliferation is independent of the IL-2 synthetic pathway. Indomethacin probably interferes with CD25 expression and synthesis of other cytokines such as IFN-γ. Hence, although large amounts of IL-2 are secreted, the T cell proliferation, is inhibited probably because few receptors (CD25) are available for binding of IL-2 (an event leading to T cell proliferation).

Vinblastine is an antiproliferative, antimitotic drug, that disrupts the cytoskeleton microtubular network (North and Awwad, 1990; Dupuis et al., 1993). Vinblastine does

not affect PHA-dependent Ca^{2+} response in a T cell line (Jurkat T cells) (Dupuis et al., 1993). In a clinical study, Kellokumpu-Lehtinen and co-workers (1989) reported that co-administering vinblastine with interferon had opposite effects on the immune system. In my experiments, vinblastine was a potent inhibitor of T cell proliferation, cytokines and CD25 at two different concentrations (10.9 μM and 109 μM). Based on these experiments, it appears that vinblastine inhibits T cell proliferation and cytokines to a similar extent. Further analyses are necessary to understand the mechanisms involved in this immunosuppressive activity.

Quinidine is a class I (sodium channel-blocking) antiarrythmic drug (Katzung, 1998). It is also used intravenously in the treatment of malaria. As such, it is not known to possess any immunosuppressive activity. However, in the present work, quinidine inhibited proliferation and functions of T cells. At the concentrations tested, there was a significant decline in levels of IL-2 and IFN-γ, proliferation of T cells, and expressed CD25. Based on these data alone, a mechanism cannot be proposed, but quinidine probably interferes with early events (prior to synthesis of cytokines) in activation of T cells.

Probenecid is a uricosuric drug. Although it is also not an immune modulatory drug, in some cases it causes aplastic anemia (Katzung et al., 1998). In the data presented here, probenecid also significantly inhibited T cell proliferation, cytokine secretion or synthesis and expression of CD25 (IL2R). These results suggest a disruption in the synthesis or secretion of cytokines leading to inhibition of T cell proliferation via an

uncharacterized pathway. More detailed analysis of the various steps involved in these pathways are needed to elucidate the mechanism further.

Collectively, and surprisingly, my data suggest that several different drugs are capable of mounting immune modulatory responses based on the concentrations used. These results may also in part be able to explain some of the reported side-effects highlighted above for each drug. Of greater interest are the effects observed when some of these drugs were combined with non-immunosuppressive doses of cyclosporine in these *in vitro* experiments. At low concentrations, the P-gp inhibitors, vinblastine and quinidine demonstrated synergistic effects with cyclosporine on all T cell proliferation parameters and cytokines measured. At the higher concentrations tested, the effect of vinblastine and quinidine by themselves was equivalent to that of unstimulated cells thereby eliminating any further effects of cyclosporine.

The non-P-gp inhibiting drugs, indomethacin and probenecid had differential effects. A combination of indomethacin and cyclosporine was synergistic in terms of decreasing IL-2 levels but not for other parameters. Our hypothesis is that this decrease may be because of inhibition of a rhodamine 123 transporting inhibitor (such as MRP or another anion-specific transporter) that may also be involved in non-essential transport of IL-2. It is entirely possible however, that indomethacin inhibits P-gp in lymphocytes but these results are not consistent with cell-line data. Low concentration of probenecid and high concentration of cyclosporine was synergistic in terms of inhibition of T cell proliferation but not for other aspects.

These results in combination with previous studies (Drach et al., 1996; Raghu et al., 1996) suggest that inhibition of P-gp i*n vitro* can reduce the levels of secreted cytokines thereby inhibiting proliferation of T cells. This further implies that P-gp and possibly other transporters may in part be involved in the transport of cytokines in T cells thus suggesting an alternative biological function for P-gp. These synergistic effects also indicate that the clinical use of cyclosporine for reversing multi-drug resistance could be potentially dangerous. The results presented here suggest significant pharmacodynamic interactions of cyclosporine with different classes of drugs. The *in vitro* immunosuppressive interactions appear to be significant in the case of P-gp inhibitors. Previously, van Asperen and co-workers (1999) compared the pharmacokinetics of vinblastine in wild-type and equivalent P-gp knockout mice. They did not observe any significant differences in vinblastine concentrations in tissues other than brain, heart and intestines. Based on their studies, van Asperen and co-workers have, in fact, recommended the co-administration of vinblastine and MDR reversal agents such as cyclosporine to enhance the tumorigenic potential of these drugs. Our pharmacodynamic measures are in sharp contrast to the pharmacokinetics presented in the literature. If similar effects occur *in vivo*, such a combination could be very dangerous. It is also possible that the failure of chemotherapy is not only because of multidrug resistance but also because of the direct effects of drugs such as vinblastine on the immune system. Prior to using various combinations of drugs, it is essential to evaluate and establish their pharmacodynamic and pharmacokinetic interactions.

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Chapter 5

EFFECT OF CYCLOSPORINE THERAPY ON P-GP EXPRESSION AND FUNCTION IN LYMPHOCYTES OF RENAL TRANSPLANT PATIENTS

5.1 Background

Cyclosporine is a potent immunosuppressive drug. In animal models, it prolongs allogeneic graft survival for skin, kidney, liver, heart, pancreas, bone marrow, small intestine and lung transplants (Katzung, 1998; PDR 1999). Cyclosporine suppresses some humoral responses (antibody mediated immune responses) but the major immunosuppressive activity is related to inhibition of cell-mediated immune responses. Cyclosporine is effective against allograft rejection, delayed hypersensitivity, Freund's adjuvant arthritis, and graft versus host disease. In low doses, it is also effective against autoimmune disorders such as uveitis, rheumatoid arthritis, and type I diabetes and other diseases such as psoriasis and asthma. For immunosuppression, cyclosporine is used in conjunction with steroids, and / or monoclonal antibodies, azathioprine, and mycophenolate mofetil. In recently concluded clinical trials, cyclosporine was used with sirolimus (another potent T cell specific immunosuppressant). Cyclosporine is orally absorbed but the intersubject variability in bioavailability of cyclosporine is high (i.e., bioavailability ranges from 20-50% for the newer, more bioavailable formulation, Neoral™). Cyclosporine is extensively metabolized by cytochromes P450. Less than 6% of the drug is excreted renally, and only about 0.1% is excreted unchanged in the urine.

Cyclosporine has a biphasic disposition with an average elimination terminal half life of 8.4 hr. Cyclosporine therapy is typically initiated pre-transplant or immediately post-transplant at 2mg/kg/day and the dose is tapered gradually over a period of two months to a maintenance dose of 0.15 mg/kg/day (PDR, 1999). This represents an almost 15-fold decrease in dose over a period of two months.

Tacrolimus (FK506) is another major T cell specific immunosuppressive drug. In animal transplant models, tacrolimus prolongs liver, kidney, heart, bone marrow, small bowel and pancreas, lung and trachea, skin, cornea and limb allografts (Katzung, 1998; PDR, 1999). Tacrolimus is used in liver and kidney transplants; it is the drug of choice for liver transplants. In clinical trials , tacrolimus is being investigated for use in heart and pancreas transplantation. Tacrolimus suppresses some humoral immune responses but the main immunosuppressive action of tacrolimus is exerted via suppression of T cell mediated immune responses in allograft rejection, delayed type hypersensitivity, collagen-induced arthritis, experimental allergic encephalomyelitis and graft versus host disease. Tacrolimus is 10-100 times more potent than cyclosporine but the incidence of adverse effects with tacrolimus is high. Tacrolimus can rescue ongoing rejection and the episodes of acute rejection refractory to corticosteroids are less frequent with tacrolimus. The oral bioavailability of tacrolimus is variable ranging from 14-22% . Tacrolimus is used in combination with steroids and/or azathioprine, mycophenolate mofetil, and monoclonal antibodies. Tacrolimus is initiated not earlier than 6 hr post-transplant at an oral dose of 0.15 – 0.3 mg/kg/day with significant dose tapering over two months.

The doses of cyclosporine and tacrolimus are reduced in a way to minimize toxicity while maintaining adequate immunosuppression. However, the extent of dose reduction is based on empirical physician/hospital experience. In the literature, there is no evidence in terms of exact mechanisms that would warrant such reduction in doses. So far, there is no evidence of down-regulation of any receptors involved in mediating immune responses susceptible to these two drugs.

Several researchers have investigated the role of multidrug-resistance in transplant rejection (Kemnitz et al., 1991; Gotzl et al., 1995; Zanker et al., 1995 and 1997; Vergara et al., 1998; Melk et al., 1999). There have been conflicting reports in the literature regarding overexpression of P-gp in patients who reject their grafts. Some of the confusion arises from the use of older, less sensitive techniques that are not best suited for analyzing P-gp expression in lymphocytes since the expression is low compared to cell – lines used as positive controls. In the older studies the researchers compared P-gp expression between different groups of individuals at one time-point. In the first longitudinal study, Melk and co-workers (1999) have analyzed the P-gp expression in lymphocytes before and after kidney transplantation. They did not find any correlation of P-gp expression with rejection episodes. They observed a decline in P-gp expression in patients receiving cyclosporine and stable or increased P-gp expression in patients receiving tacrolimus.

Thus, there is a discrepancy in the literature in terms of the ability of cyclosporine and tacrolimus to exert immunosuppressive activity at lower concentrations and

overexpression of P-gp that would reduce the amount of drug available at the site of action.

5.2 Objectives

My goal was to study the effect of cyclosporine therapy on expression and function of P-gp in T lymphocytes from renal transplant patients over a period of time. The study was designed to compare such effects with those produced by other major immunosuppressive regimens commonly used at UCSF. The study design is based on a longitudinal comparison of P-gp expression and function in different groups of renal transplant patients and comparing the patterns between different groups. Based on data from the literature and preliminary results in this study, it appears that there is considerable variation in expression of P-gp among individuals and to fully understand the effect of a treatment, it is essential to compare levels within individuals at different time - points.

5.3 *Materials and Methods*

5.3.1 *Chemicals and Specimens*

All antibodies and isotype controls were obtained from Becton-Dickinson (San Jose, CA). Tacrolimus was a kind gift from Fujisawa Pharmaceutical Company (Deerfield, IL). Optilyse C was purchased from Immunotech (Marseille, France). Dye-free Ca^{2+}/Mg^{2+} free Hanks BSS, RPMI 1640 with 25 mM HEPES, fetal calf serum (FCS), phosphate buffered saline (PBS), and cell preservation medium (10% FCS, 10% DMSO in MEM EBSS, pH=7.2) were all obtained from the UCSF Cell Culture Facility (San Francisco, CA). Acridine orange, ethidium bromide, propidium iodide were procured from Molecular Probes Inc. (Eugene, OR). Ficoll-Paque® (Ficoll-Hypaque) was obtained from Pharmacia Biotech AB (Uppsala, Sweden). Flow-Check™ fluorospheres were purchased from Coulter Corporation (Miami, FL). All other chemicals and solvents were obtained from Sigma Chemical Co. (St. Louis, MO).

Blood samples (20 ml or less / blood draw) were drawn in heparinized tubes from patients after approval from the UCSF Committee on Human Research (San Francisco, CA). Patient samples were obtained from three different groups: patients receiving MMF as primary immunosuppressant; patients started on calcineurin inhibitors following rejection on MMF; patients started on cyclosporine pre-transplant. The expression and function of P-gp was also studied on lymphocytes from healthy volunteers.

5.3.2 P-glycoprotein Expression Analysis

Peripheral blood mononuclear cells were isolated from blood samples by density centrifugation using Ficoll-Hypaque as described in Chapter 3. The cells were suspended in cell preservation medium and cryo-preserved in liquid nitrogen at –191.2 °C for later use. Prior to analysis, cells were thawed at 37 °C. Cell viability was assessed using ethidium bromide/acridine orange. If more than 10% cells took up acridine orange (indicating cell death), the dead cells were removed using Ficoll-Hypaque. Cells were washed with complete medium RPMI 1640 (supplemented with 10% FCS) and allowed to equilibrate in RPMI 1640 (with FCS) at 37 °C for 1 hour prior to staining. Cells were washed with Ca^{2+}/Mg^{2+} free, dye-free Hanks' buffer and resuspended in PBS (supplemented with 0.1% sodium azide, 2% FCS). A 75 µl cell suspension was incubated with 20 µl unconjugated 15D3 anti-P-gp antibody (custom order from BD) or IgG1 - isotype control (for nonspecific binding) for 20 min at room temperature. Cells were washed once with Hanks' buffer, resuspended in PBS and stained with 7 µl FITC-conjugated goat anti-mouse IgG second antibody at room temperature for 15 min. Cells were washed once with Hanks buffer, resuspended in PBS and labeled with 20 µl PE-conjugated anti-CD4/8 antibody for 15 min at room temperature. Cells were incubated with 300 µl of Optilyse C for 5 minutes to lyse contaminating erythrocytes. After washing, cells were resuspended in PBS.

Saturating concentrations of antibodies were used for staining based on preliminary experiments. Preliminary experiments showed that Ca^{2+}/Mg^{2+} free, dye-free

Hanks' buffer was best for maintaining cell viability , PBS was next best, and supplemented PBS (with 0.1% sodium azide, 2% FCS) was the poorest. For all experiments, supplemented PBS (with sodium azide and FCS) was only used when labeling with antibody and reading on the flow cytometer. For all other purposes, Ca^{2+}/Mg^{2+} free, dye free Hanks' buffer was used.

5.3.3 Rhodamine 123 Accumulation / Efflux

Lymphocytes were thawed and equilibrated at 37 °C. Rhodamine 123 uptake studies were performed on lymphocytes with and without 12.2 μM tacrolimus as described in Chapter 3. Following rhodamine uptake, cells were washed once with ice-cold Hanks' buffer and labeled with CD4/CD8 antibody on ice as per the manufacturer's recommendation. Following incubation, cells were washed and data acquired using EPICS XL flow cytometer for 5000 events as described in Chapter 3.

5.4 Results

This study included four different groups of individuals: healthy volunteers, renal transplant patients receiving MMF and prednisone as major immunosuppressive drugs (Table 5-1), renal transplant patients who were started on MMF and prednisone but following symptoms of acute rejection presented on biopsy or clinical symptoms of

Table 5-1. Profiles of renal transplant patients receiving MMF as primary chronic immunosuppressant

Patient ID	*Age in '99 (years)*	*Race*	*Sex*	*Date of Transplantation*	*Cause leading to renal failure*	*Transplant source*
I-C	46	Hispanic	Male	8/27/97	Diabetes	Cadaver
III-C	67	Hispanic	Male	8/21/97	Hypertension	Living unrelated
V-C	61	Caucasian	Male	10/9/97	Unknown	Cadaver
XVII-C	64	Hispanic	Male	9/25/97	Diabetes	Cadaver

Table 5-2. Profiles of renal transplant patients in MMF group who were started on calcineurin inhibitors (cyclosporine or tacrolimus) after graft rejection was detected by biopsy

Patient ID	*Age in '99 (years)*	*Race*	*Sex*	*Date of Transplantation*	*Cause leading to renal failure*	*Transplant source*	*Calcineurin inhibitor*
II-C	61	Caucasian	Male	8/26/97	Unknown	Cadaver	Cyclosporine
IV-C	30	Hispanic	Female	10/2/97	IgA Nephropathy	Living unrelated	Tacrolimus
VIII-C	57	Hispanic	Male	10/31/97	Reflux Nephropathy	Cadaver	Tacrolimus
IX-C	62	Oriental	Male	12/24/97	Hypertension	Cadaver	Tacrolimus
X-C	40	Hispanic	Male	12/3/97	Glomerular Nephritis (GN)	Living unrelated	Tacrolimus
XII-C	44	Caucasian	Male	11/30/97	GN	Cadaver	Tacrolimus
XVI-C	65	Caucasian	Male	8/31/97	GN	Cadaver	Tacrolimus

rejection were started on calcineurin inhibitors (cyclosporine or tacrolimus) (Table 5-2), and renal transplant patients who were initiated on cyclosporine along with MMF and prednisone following transplant (Table 5-3). The blood samples from the last group of patients were obtained spanning pre-transplant (0-24 hours pre-transplant) to around 4 months post-transplant. The patients were initiated on cyclosporine 6 or more hours post-transplant. Other samples were drawn when the patients visited the clinic for routine checkup. The patients ranged in age from 20-65 years; included males and females; were predominantly Caucasians and Hispanics with one Oriental patient in the tacrolimus group; transplants were from cadaver, living related and living unrelated donors.

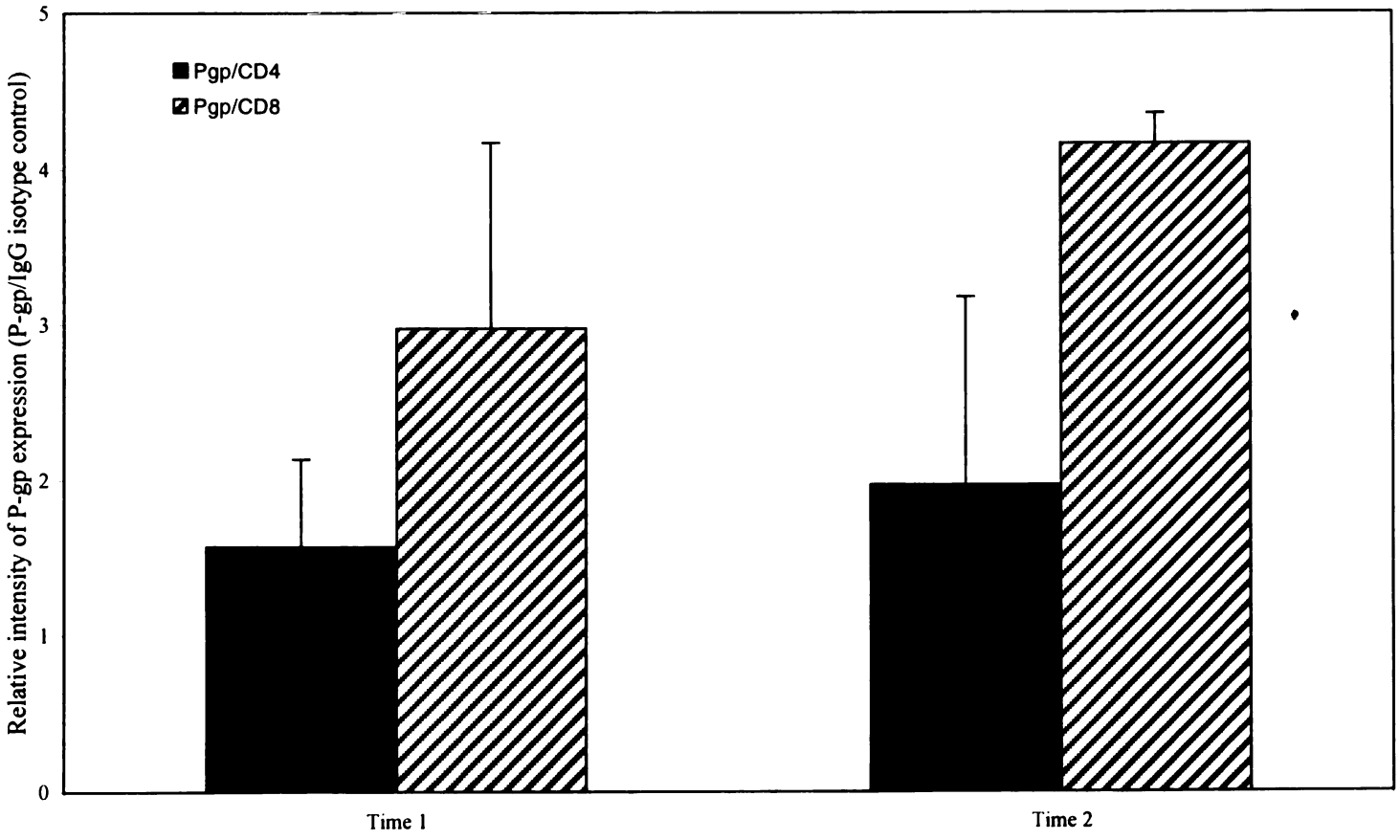
P-glycoprotein expression on T cells was measured using a two step staining process using P-gp antibody, 15D3 against an extracellular binding domain of P-gp (Shi et al., 1995). P-glycoprotein expression on lymphocytes was found to vary considerably between individuals. The data from the three healthy volunteers (all females between 20-40 years of age; one volunteer was taking birth control pills) shows higher P-gp expression on CD8+ T cells versus CD4+ T cells (Fig. 5.1). This is consistent with the literature (Aggarwal et al., 1997). The blood samples were collected 1-2 months apart but there was no significant difference in expression of P-gp on CD4+ lymphocytes. The level of P-gp expression on CD8+ T cells varied more than on CD4+ T cells but was not significantly different over time (3.0±1.2 vs. 4.1±0.2).

The P-gp expression on CD4+ lymphocytes was greater than on CD8+ T cells in transplant patients receiving MMF and prednisone as primary immunosuppressants

Table 5-3. Profiles of renal transplant patients initiated on calcineurin inhibitors (cyclosporine or tacrolimus) post-transplant

Patient ID	*Age in '99 (years)*	*Race*	*Sex*	*Date of Transplantation*	*Cause leading to renal failure*	*Transplant source*	*Calcineurin inhibitor*
I-P	50	Hispanic	Male	11/10/98	Hypertension	Living unrelated	Cyclosporine
II-P	57	Hispanic	Male	11/10/98	Diabetes	Living unrelated	Cyclosporine
III-P	38	Caucasian	Male	11/17/98	Hypertension	Living unrelated	Cyclosporine
IV-P	51	Caucasian	Female	11/18/98	Diabetes	Living related	Cyclosporine
V-P	65	Hispanic	Male	11/26/98	Diabetes	Cadaver	Cyclosporine
VI-P	50	Hispanic	Female	11/25/98	Diabetes	Cadaver	Tacrolimus
VII-P	32	Caucasian	Male	12/3/98	Glomerulo-nephritis	Cadaver	Cyclosporine
IX-P	58	Caucasian	Male	12/9/98	Diabetes	Cadaver	Cyclosporine
X-P	59	Caucasian	Male	12/10/98	Hypertension, NIDDM	Living unrelated	Cyclosporine
XI-P	46	Hispanic	Male	12/14/98	Polycystic kidney	Cadaver	Cyclosporine

Fig. 5.1. Relative P-gp expression on T cells from healthy volunteers obtained on two separate occasions (at least one month apart); n=3 (mean±SD)



(Fig. 5.2, Table 5-4). This pattern of expression is opposite to that observed in healthy volunteers. The P-gp expression on CD4+ T cells was comparable to that observed in healthy volunteers but P-gp expression on CD8+ T cells was lower in the MMF group. The samples were obtained at least one month apart, the first sample was obtained at least 6 months post-transplant on a steady dose of MMF. There was no significant difference in the expression of P-gp on either cell population.

Patients who experienced events indicative of graft rejection were initiated on tacrolimus or cyclosporine along with MMF and prednisone. In this group, one patient received cyclosporine while the others received tacrolimus. In general, the expression of P-gp on CD4+ T cells was comparable to that on CD8+ T cells. The P-gp expression was measured at two different times (Fig. 5.3, Table 5-5). The first sample was collected either before initiation of calcineurin inhibitors or soon after, the second time represents at least one month later when the patient was considered stable on calcineurin inhibitors. The level of P-gp expression on CD4+ T cells was lower after initiation of calcineurin inhibitors (2.4 ± 0.9 vs. 1.6 ± 0.4), but not significantly.

In the patient-group that was initiated on calcineurin inhibitors (one on tacrolimus, others on cyclosporine) post transplant, there was a higher expression of P-gp on CD4+ T cells compared to CD8+ T cells just prior to transplantation and initiation of calcineurin inhibitors (Fig. 5.4, Table 5-6). The expression of P-gp on CD4+ T cells decreased over a 4 month period while the expression of P-gp on CD8+ T cells increased. The pattern of P-gp expression on CD4+ and CD8+ T cells resembled that of healthy

Fig. 5.2. P-glycoprotein expression on CD4 & CD8 T lymphocytes from patients on MMF (mean±SD; n=4)

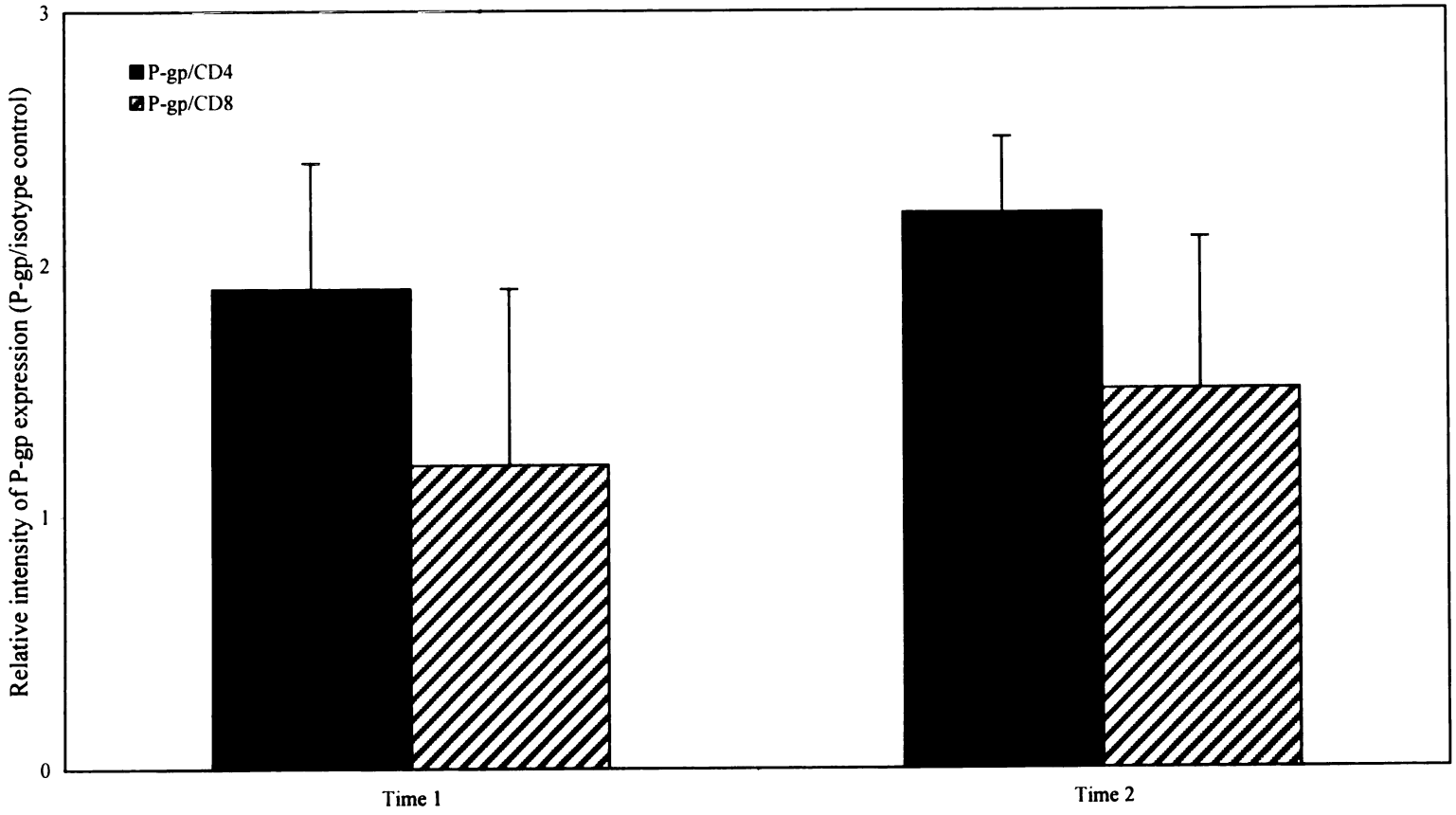


Table 5-4. P-glycoprotein expression on CD4+ and CD8+ T lymphocytes from renal transplant patients receiving MMF as their primary immunosuppressant

Patient ID	***Relative* P-gp expression on CD4+ T cells***		***Relative* P-gp expression on CD8+ T cells***	
	Time 1	***Time 2***	***Time 1***	***Time 2***
I-C	2.1	2.5	1.1	0.9
III-C	1.8	2.2	1.7	1.0
V-C	2.6	2.4	2.2	1.3
XVII-C	1.6	2.0	0.4	2.1

* Relative expression is defined as: P-gp / IgG (isotype control for non-specific binding)

Fig. 5.3. P-glycoprotein expression on CD4 & CD8 T cells from patients started on a calcineurin inhibitor (mean ± SD; n = 6)

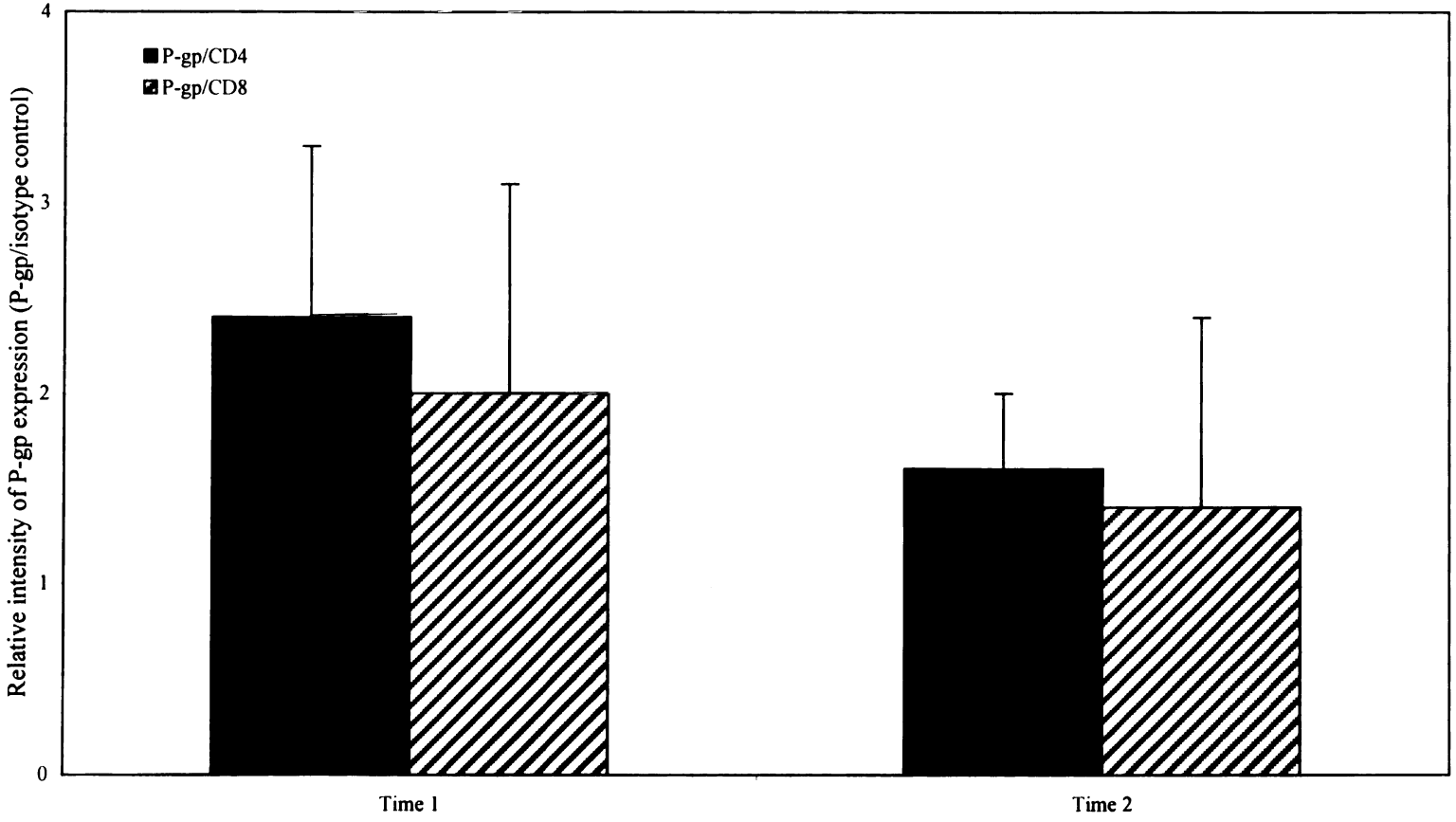


Table 5-5. P-glycoprotein expression on CD4+ and CD8+ T lymphocytes from renal transplant patients started on calcineurin inhibitors (with MMF) following rejection

Patient ID	***Relative* P-gp expression on CD4+ T cells***		***Relative* P-gp expression on CD8+ T cells***	
	Time 1[a]	***Time 2***	***Time 1[a]***	***Time 2***
II[b]–C	3.2	2.1	2.8	1.1
IV-C	3.7	1.9	3.2	3.1
IX-C	1.8	1.6	3.0	1.0
VIII-C	2.1	1.0	1.2	0.3
X-C	2.7	1.5	0.9	1.4
XII-C	1.2	1.4	0.9	1.6

* Relative expression is defined as: P-gp / IgG (isotype control for non-specific binding)

[a]These blood sample were obtained either before starting calcineurin inhibitors or soon after after starting patients on calcineurin inhibitors to control their organ rejection

[b]Patient received cyclosporine; all others received tacrolimus

Fig. 5.4. P-glycoprotein expression on CD4+/ CD8+ T cells from renal transplant patients receiving cyclosporine as the primary immunosuppressant (mean ± SD)
(indicates statistical significance in ANOVA at p=0.02)**

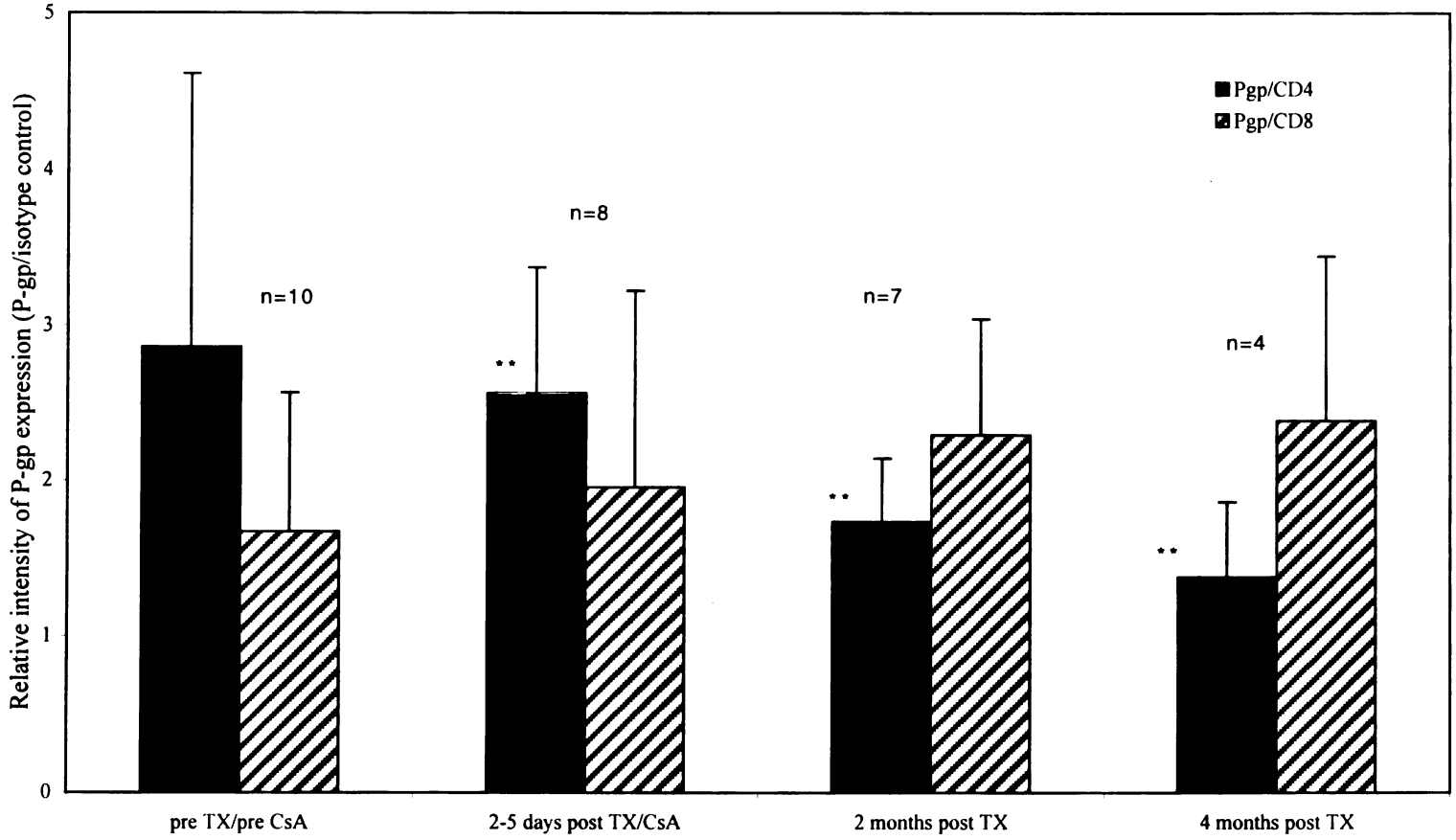


Table 5-6. P-glycoprotein expression on CD4+ and CD8+ T lymphocytes in renal transplant patients receiving calcineurin inhibitors starting immediately after transplant (along with other treatments such as monoclonal antibodies, steroids, and MMF)

Patient ID	*Relative* P-gp expression on CD4+ T cells*				*Relative* P-gp expression on CD8+ T cells*			
	Pre TX	*Post TX*	*2 Months post TX*	*4 months post TX*	*Pre TX*	*Post TX*	*2 Months post TX*	*4 months post TX*
I-P	5.5	3.2	2.2	0.9	1.9	2.4	3.6	3.2
II-P	1.5	2.8			1.4	2.2		
III-P	1.4	2.3	2.4	1.2	1.1	1.5	2.7	1.3
IV-P	2.4	1.6			1.0	1.0		
V-P	2.1	2.6	1.7		2.1	2.3	1.7	
VI-P	1.5			1.4	1.4			3.4
VII-P	6.5	3.9	1.5		3.9	4.6	2.2	
IX-P	2.4	2.7	1.7		0.9	0.9	2.4	
X-P	3.1	1.4	1.5	2.0	1.0	0.7	2.1	1.6
XI-P	2.2		1.3		2.0		1.3	

*Relative expression is defined as: P-gp / IgG (isotype control for non-specific binding)

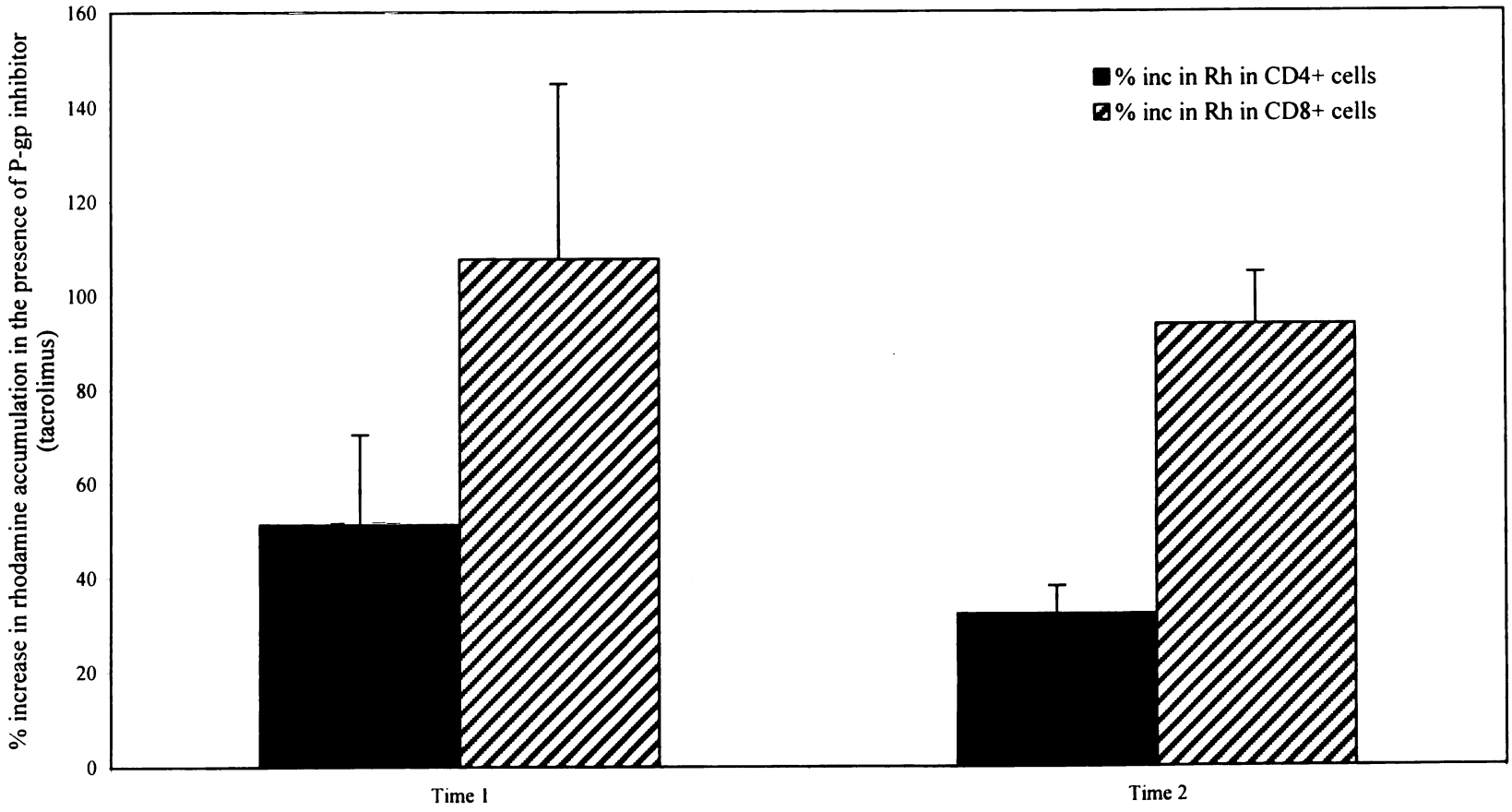
Abbreviations: TX – transplantation

volunteers at 4 months. The variability of P-gp expression was very high pre-transplant but decreased post-transplant.

Fluorescent dyes such as rhodamine 123 can be used to characterize the functional capability of P-gp (Chapter 3). Cells take up rhodamine 123 by passive diffusion but it is actively pumped out of the cells by P-gp. Using inhibitors such as tacrolimus, the efflux of rhodamine 123 from cells can be blocked. Such a blockage results in increased fluorescent intensity due to accumulation of rhodamine 123 within cells. In the absence of functional transporters, there would be no increase in accumulation of rhodamine 123 in cells. Using different concentrations of an inhibitor, it is possible to study the kinetics of inhibition of P-gp and develop a semi-quantitative understanding of functional P-gp. However, for such analysis, a large volume of lymphocytes are needed. The patient samples available were limited in volume (a maximum of 20 ml/blood draw). The number of lymphocytes present in this volume of patient blood is very low. Hence, a quantitative analysis of P-gp function could not be performed. However, by analyzing rhodamine 123 accumulation in the samples with or without a single high inhibitor concentration, it was possible to detect if functional P-gp is expressed on T cell populations of patients and healthy volunteers.

The lymphocytes from healthy volunteers exhibited marked increase in accumulation of rhodamine 123 in CD8+ T cells compared to CD4+ T cells (Fig. 5.5). At different times, the accumulation of rhodamine 123 was similar in both cell populations. In the MMF group, the rhodamine 123 retention in CD4+ T cells was similar at different

Fig. 5.5. Ex-vivo functional activity of P-gp in T cells from healthy volunteers, measured by rhodamine 123 accumulation in the presence and absence of 12.2 μM tacrolimus (mean ± SD)



times (Fig. 5.6, Table 5-7) and similar to that of the healthy volunteers, but the accumulation of rhodamine 123 in CD8+ T cells was considerably higher in CD8+ T cells at the later time-point. Patients started on calcineurin inhibitors (Fig. 5.7, Table 5-8) exhibited similar levels of rhodamine accumulation as healthy volunteers in both CD4+ and CD8+ T cells. The retention of rhodamine 123 in CD8+ T cells was greater than in the CD4+ T cells. The retention of rhodamine 123 in both CD4+ and CD8+ T cell populations from patients in the cyclosporine group was highly variable at all the time-points measured (Fig. 5.8, Table 5-9). In the first few days post-transplant, the level of accumulation of rhodamine 123 was similar in both CD4+ and CD8+ T cells, but at 4 months post-transplant, the percent increase in rhodamine accumulation was greater in CD8+ T cells.

As a control, I also studied the expression of CD25 on T cells. This is indicative of T cell responses. Actively proliferating cells have higher CD25 expression. An increase in CD25 expression indicates proliferating T cells that can mount immune responses against the transplanted tissue. Figures 5.9 and 5.10 demonstrate the expression of CD5 (IL2R) on lymphocytes from the different patient groups. The expression of CD25 was essentially constant and similar in both MMF and tacrolimus groups indicating good immunosuppressive control over cell-mediated immune responses (Fig. 5.9). Before transplantation, the patients exhibited high levels of CD25 expression (Fig. 5.10), and the levels declined with time post-transplant indicating effective immunosuppression.

Fig. 5.6. Ex-vivo functional activity of P-gp in T cells from patients receiving MMF, measured by rhodamine 123 accumulation in the presence and absence of 12.2μM tacrolimus (mean ± SD; n=4)

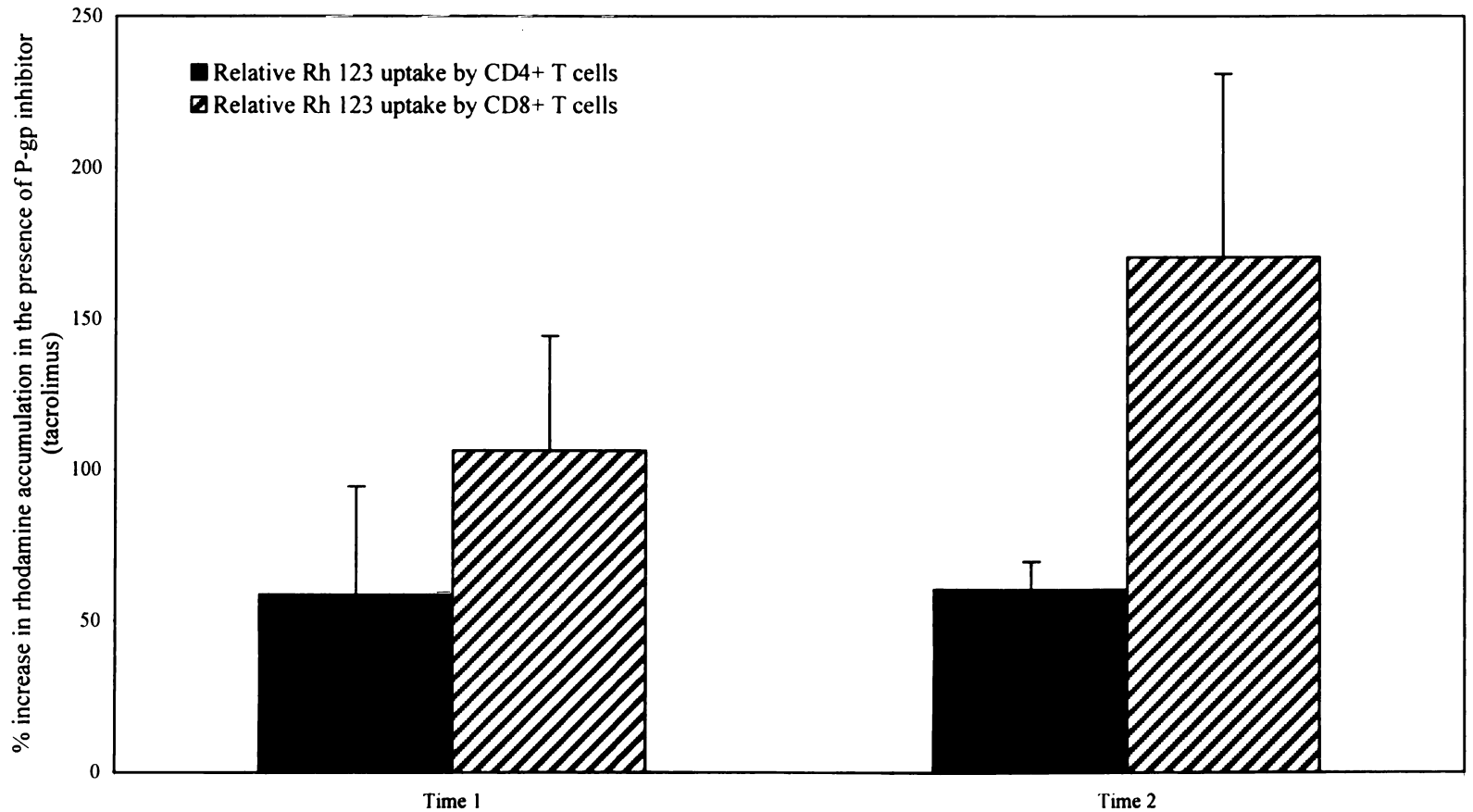


Table 5-7. Rhodamine accumulation in the presence and absence of a P-gp inhibitor by CD4+ and CD8+ T lymphocytes from renal transplant patients receiving MMF as their primary immunosuppressant

Patient ID	***% increase* in rhodamine retention in the presence of a P-gp inhibitor by CD4+ T cells***		***% increase* in rhodamine retention in the presence of a P-gp inhibitor by CD8+ T cells***	
	Time 1	***Time 2***	***Time 1***	***Time 2***
I-C	10.6	54.3	49.4	221.7
V-C	87.3	60.5	130.1	221.3
VI-C	51.2	51.9	126.1	101.8
XVII-C	84.6	72.8	118.7	136.3

* % increase is defined as: 100 X [(Rh + inh) – (Rh control)] / (Rh control)

Fig. 5.7. Ex-vivo functional activity of P-gp in T cells from patients receiving calcineurin inhibitors, measured by rhodamine 123 accumulation in the presence and absence of 12.2 μM tacrolimus (mean ± SD; n=6)

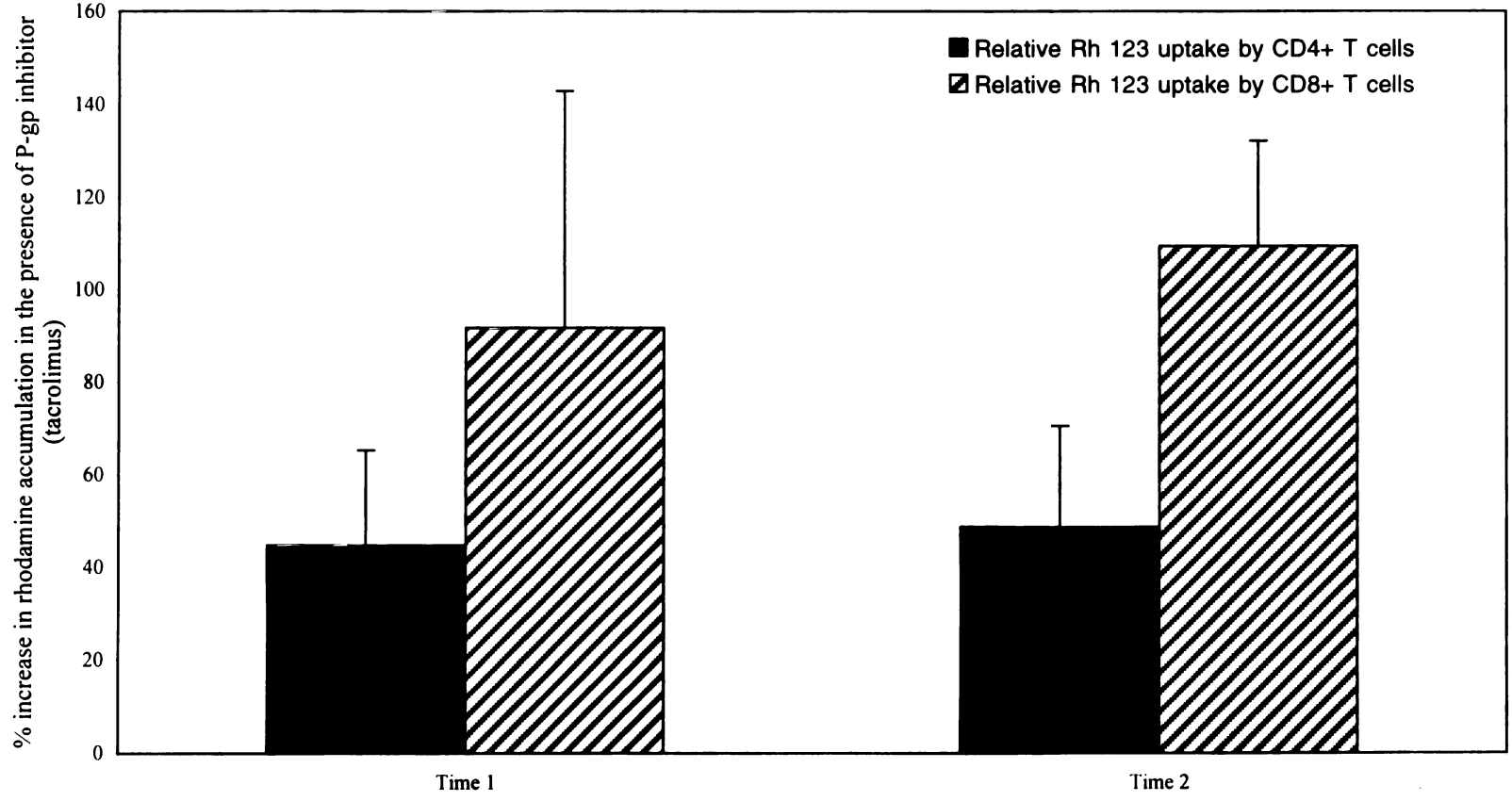


Table 5-8. Rhodamine accumulation in the presence and absence of a P-gp inhibitor by CD4+ and CD8+ T lymphocytes from renal transplant patients started on calcineurin inhibitors (with MMF) following rejection

Patient ID	***% increase* in rhodamine retention in the presence of a P-gp inhibitor by CD4+ T cells***		***% increase* in rhodamine retention in the presence of a P-gp inhibitor by CD8+ T cells***	
	Time 1[a]	***Time 2***	***Time 1[a]***	***Time 2***
II[b]–C	19.7	10.6	29.8	115.6
IV-C	48.1	77.1	165.9	137.2
VIII-C	39.8	57.7	85.3	69.2
IX-C	82.0	50.7	133.1	111.8
X-C	37.8	43.9	47.1	120.0
XII-C	40.5	51.4	89.3	101.9

* % increase is defined as: 100 X [(Rh + inh) – (Rh control)] / (Rh control)

[a]These blood sample were obtained either before starting calcineurin inhibitors or soon after after starting patients on calcineurin inhibitors to control their organ rejection

[b]Patient received cyclosporine; all others received tacrolimus

Fig. 5.8. Ex-vivo functional activity of P-gp in T cells from renal transplant patients receiving calcineurin inhibitors as primary immunosuppressant, measured by rhodamine 123 accumulation in the presence and absence of 12.2 μM tacrolimus (mean ± SD)

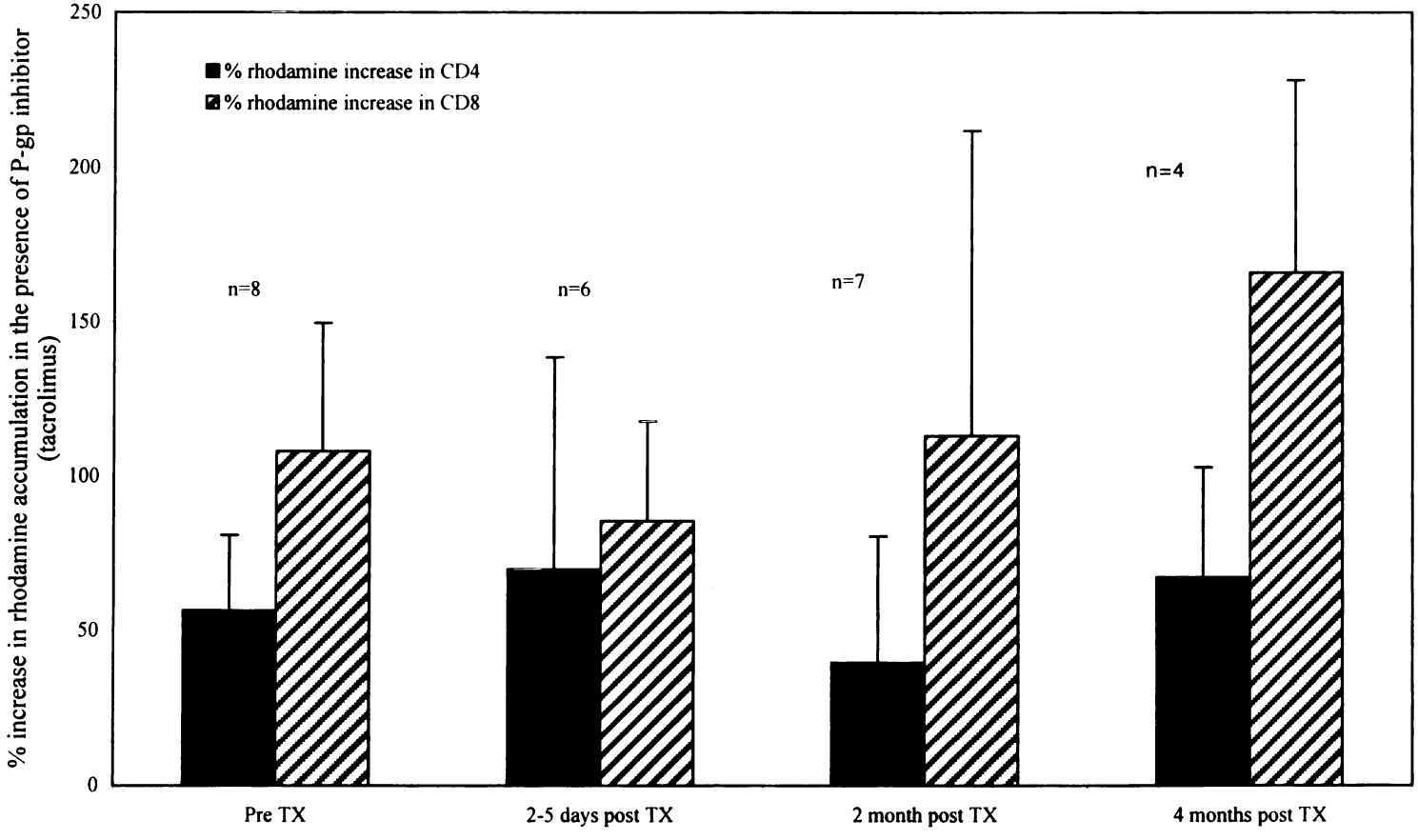


Table 5-9. Rhodamine accumulation in the presence and absence of a P-gp inhibitor by CD4+ and CD8+ T lymphocytes in renal transplant patients receiving calcineurin inhibitors starting immediately after transplant (along with other treatments such as monoclonal antibodies, steroids, and MMF)

Patient ID	*% increase* in rhodamine retention in the presence of a P-gp inhibitor by CD4+ T cells*				*% increase* in rhodamine retention in the presence of a P-gp inhibitor by CD8+ T cells*			
	Pre TX	*Post TX*	*2 Months post TX*	*4 months post TX*	*Pre TX*	*Post TX*	*2 Months post TX*	*4 months post TX*
I-P	76.0	76.1	36.9	37.4	154.8	97.7	161.5	140.2
III-P	46.4	30.1	5.3	71.2	54.4	49.6	124.7	132.2
V-P	59.7	203.0	118.2		110.8	141.0	275.8	
VI-P	44.4			115.9	82.0			115.9
VII-P	50.0	60.2	-4.0		80.1	80.1	-54.5	
IX-P	25.7	15.9	19.8		181.1	60.2	75.1	
X-P	43.0	32.4	42.5	44.0	106.1	83.6	96.1	44.0
XI-P	105.5		58.0		94.8		111.3	

*% increase is defined as: 100 X [(Rh + inh) – (Rh control)] / (Rh control)

Abbreviations: TX – transplantation

Fig. 5.9. Expression of CD25 on T lymphocytes from renal transplant patients (mean ± SD)

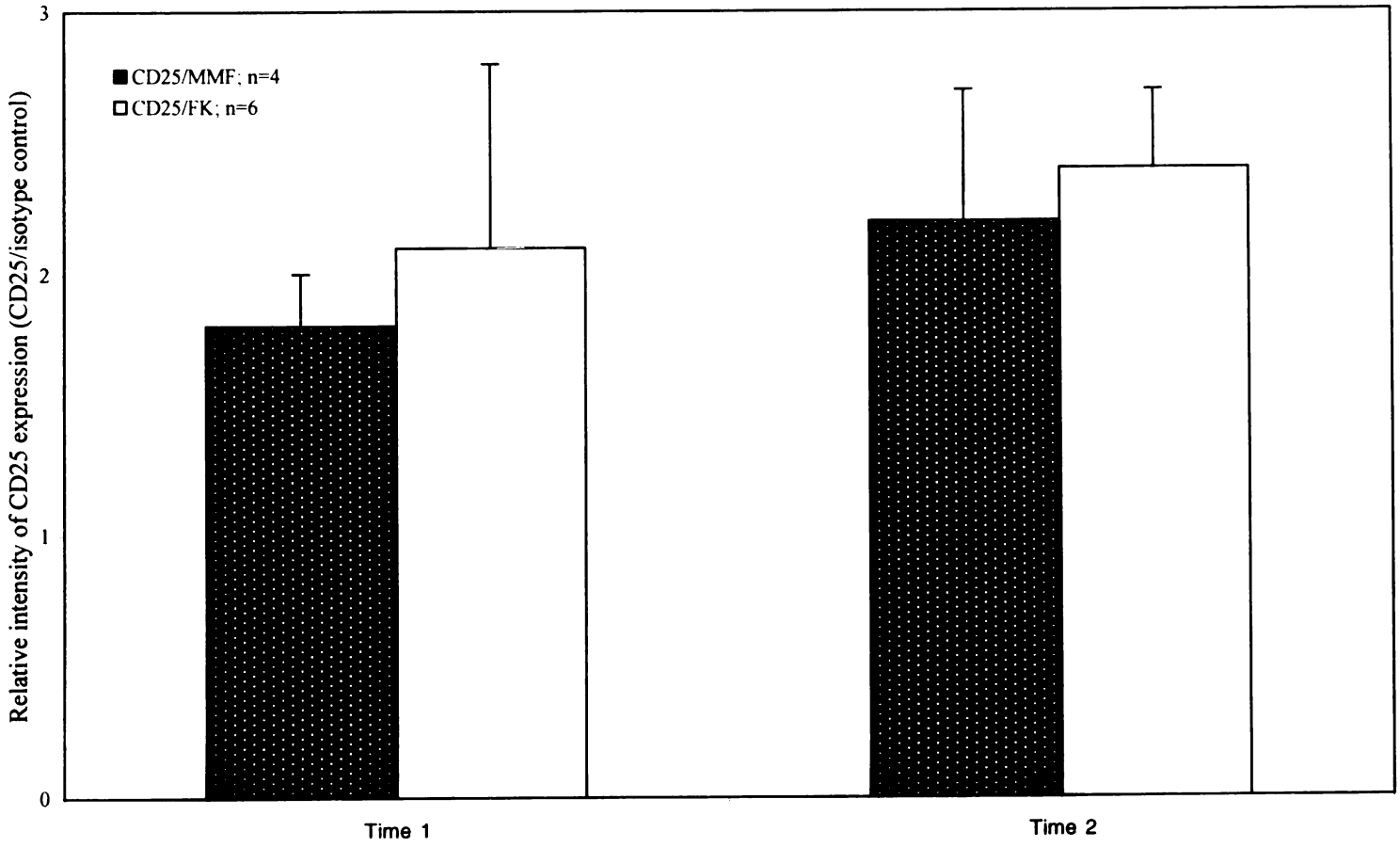
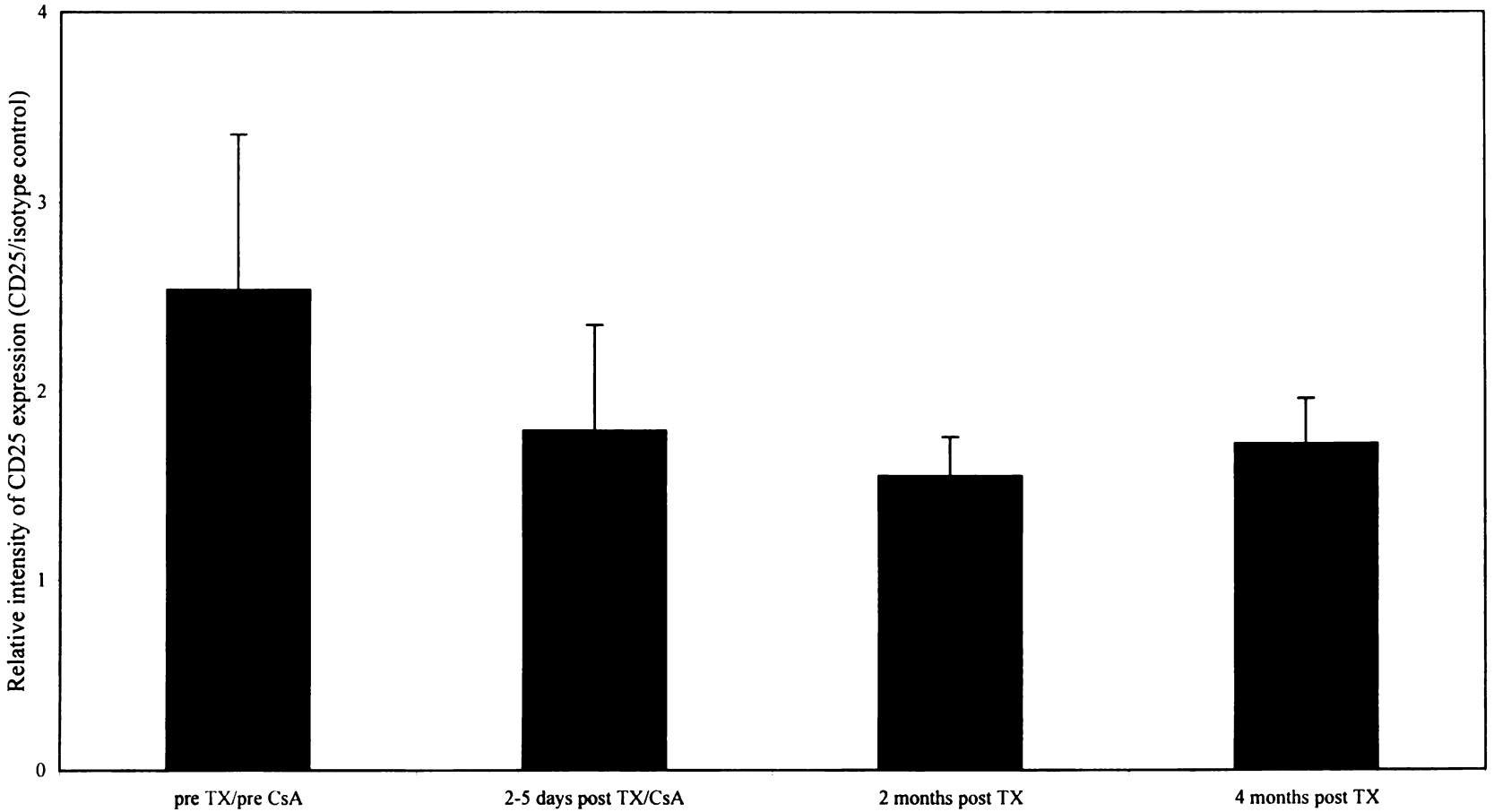


Fig. 5.10. Expression of CD25 on T lymphocytes from renal transplant patients receiving cyclosporine (mean ± SD)



5.5 *Discussion*

Transplant patients receive a combination of drugs including calcineurin inhibitors such as cyclosporine or tacrolimus, steroids such as prednisone, monoclonal antibodies such as OKT3 or HAT and MMF or azathioprine. These drugs are initiated at high doses and within days, the doses of calcineurin inhibitors are tapered down with or without a dose adjustment for other drugs. This dose adjustment is made to limit toxicity while maintaining therapeutic efficacy. The patient blood levels are monitored and doses adjusted in part to stay within the therapeutic range. Further dose-adjustments are made empirically by the physicians. Within the first six months, most patients can be stably maintained on low immunosuppressive calcineurin inhibitor dosage regimens. There is no clear explanation for this downward dose adjustment. In this study, I have investigated the role that P-gp might play in limiting/enhancing the availability of calcineurin inhibitors in T cells, especially CD4+ T cells. The calcineurin inhibitors act by blocking Ca^{2+} dependent calcineurin activation of CD4+ T cells.

These experiments demonstrate the functional expression of P-gp in CD4+ and CD8+ T cell populations of renal transplant patients. Due to the limited number of samples and high variability of expression of P-gp, these studies cannot demonstrate statistical significance. However, some preliminary trends can be detected. In general, when individual patient trends were considered, it appears that initiation of calcineurin inhibitors decreases the expression of P-gp on CD4+ T cells. This decrease could result in increased intracellular concentrations of cyclosporine and tacrolimus. Due to high local

concentrations of these drugs at the site of action (CD4+ T cells), a lower dose would be effective in maintaining adequate immunosuppression. Alternatively, as discussed in Chapter 4, lower levels of P-gp could also limit the secretion of cytokines such as IL-2 if P-gp is involved *in vivo* in secreting cytokines. By decreasing the amount of IL-2 secreted, T cell proliferation will be inhibited.

The data from the rhodamine efflux/retention assays demonstrate that P-gp in both CD4+ and CD8+ T cells is functional. It however, does not provide a quantitative measure of expression of P-gp in these cells. Although the expression of P-gp in CD4+ T cells was higher than the CD8+ T cells in patients, the functional studies indicate significantly higher functionality of P-gp in CD8+ T cells. Similar disparities in expression and function have been observed by others as a function of disease or other unknown parameters (Andreana et al., 1996; Demur et al., 1998).

The exact mechanisms responsible for altering P-gp expression are not known. P-glycoprotein expression is known to be developmentally regulated and also altered in diseases such as AIDS (Andreana et al., 1996; Aggarwal et al., 1997). Further analysis of mRNA levels would be helpful in identifying the stage at which P-gp expression is altered. It is possible that cyclosporine and tacrolimus inhibit transcription thereby decreasing the level of expressed protein on the surface. Alternatively, the inhibitors could bind to and inhibit the translocation of P-gp to the membrane surface. This study was a preliminary analysis of specific effects of P-gp inhibitors on the expression and function of expressed P-gp on the surface of lymphocytes. Further detailed analysis with

larger groups of patients and healthy volunteers and better and more controlled sample availability is necessary in further understanding the complex mechanisms involved in the P-gp expression and function and their effects on immunosuppression. Since P-gp expression appeared to be different in different individuals, a more comprehensive analysis of different ethnic groups and genders will also be helpful in understanding if P-gp expression is polymorphic or follows definite patterns.

5.6 References

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Chapter 6

SUMMARY AND PERSPECTIVES

Immunosuppressive drugs form the cornerstone of transplant immunology. These drugs have made a tremendous difference in extending the life of transplant tissue and the patient. Cyclosporine, tacrolimus and sirolimus belong to a new class of immunosuppressive drugs that specifically target cell-mediated immune responses. Due to their potency and specific activity, these drugs offer a clear advantage over the older non-specific anti-rejection therapy that inhibited all components of the immune system such as bone marrow, platelet, polymorphonuclear cells, macrophages, memory and resting T and B lymphoyctes, activated T and B lymphocytes, and T and B cell clones. Cyclosporine, tacrolimus and sirolimus only affect activated and clonal T and B cells. These drugs spare the other components of the immune system thereby limiting toxicity and mortality.

Cyclosporine and tacrolimus are approved by FDA for use in transplant patients; sirolimus is in clinical trials, recently receiving a positive review by a FDA advisory committee. Although these drugs have been in use for sometime, their mechanisms of activity and toxicity, pharmacodynamic and pharmacokinetic interactions are not fully understood. Although these drugs belong to different classes and have structural differences, they share several common features. Cyclosporine and tacrolimus are different in structure and whereas cyclosporine is a peptide, tacrolimus is a macrolide.

Despite the difference in structure and binding proteins, both inhibit the same calcium-dependent calcineurin pathway leading to synthesis of cytokines such as IL-2. Tacrolimus and sirolimus on the other hand share extensive structural similarities, but bind to completely different cytosolic proteins and exert immunosuppressive activity via different mechanisms. These drugs share other similarities in terms of their interactions with cytochromes P450 and transporters such as P-glycoprotein which act in limiting the bioavailability of the immunosuppressive drugs. In part these enzymes and transporters are also responsible for the variability in response to these drugs.

In my thesis, I have tested hypotheses related to interaction of the immunosuppressive drugs with transporters *in vitro* and in renal transplant patients. I also investigated the phase I metabolism of sirolimus. Based on these studies a few conclusions can be drawn. Sirolimus, a T cell specific immunosuppressant drug is extensively metabolized to greater than 10 first and second-generation metabolites *in vitro*. The major enzymes responsible for this metabolism include CYP3A4 and CYP3A5. Although *in vitro*, CYP3A5 was responsible for an insignificant fraction of total sirolimus metabolism, it could play an important role *in vivo* where drug concentrations are below saturating conditions. Since sirolimus is indicated for use in conjunction with cyclosporine, another CYP3A4 substrate, there is a real possibility of drug interactions of these two drugs especially in cases when the availability of enzymes is limited either due to inhibition or presence of several substrates (both parent and metabolite). The *in vitro* analysis using human liver microsomes and cDNA based individual enzymes provides evidence of both first and second generation phase I

metabolites and also a suggestion that there may be subsequent generation metabolites observed *in vivo*. Sirolimus and some of its metabolites appear to be substrates of the same CYP enzymes. *In vivo*, this would result in competition for gut and liver metabolism between parent and metabolites leading to variable metabolism patterns.

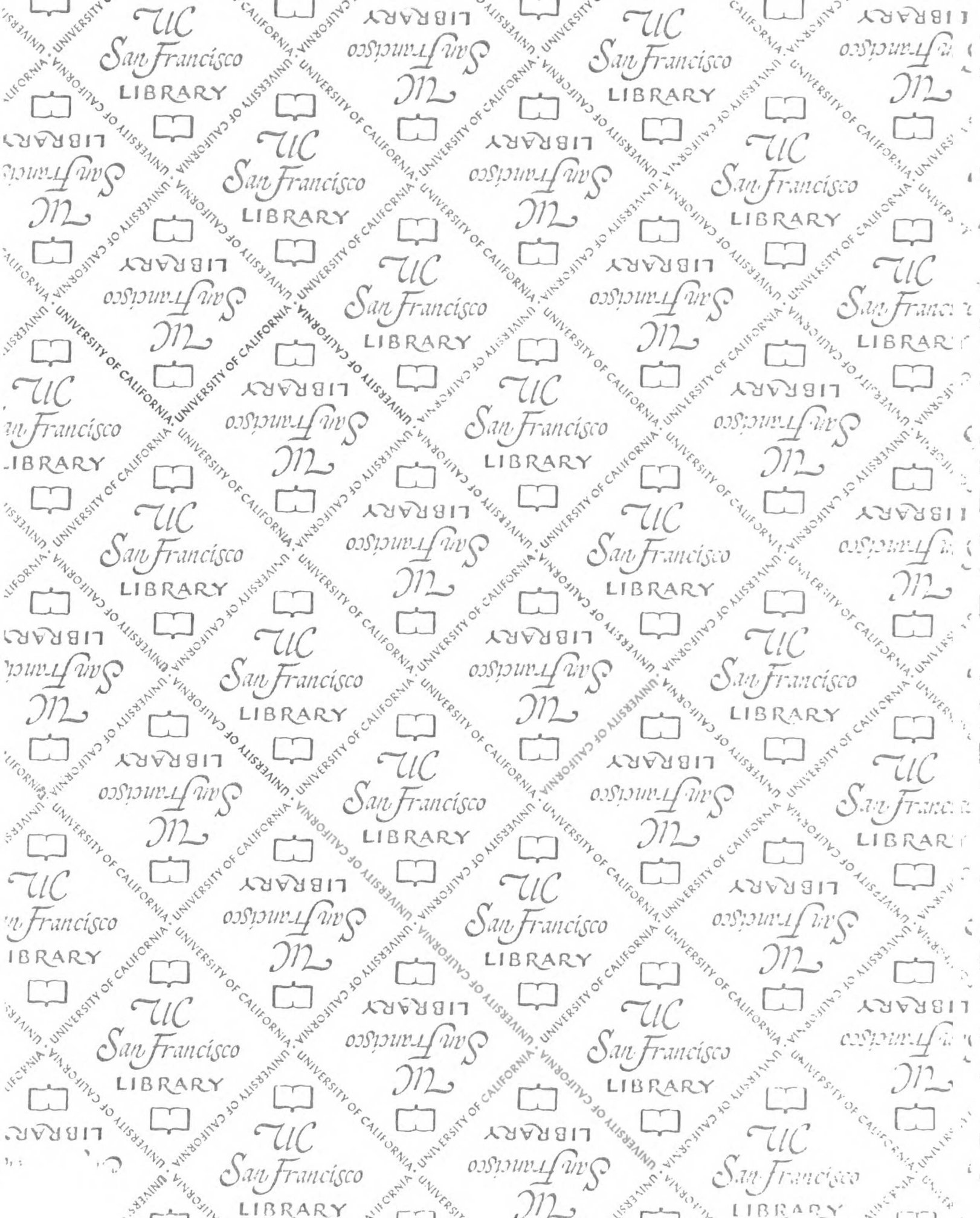
The immunosuppressive drugs, cyclosporine, tacrolimus and sirolimus prevent transplant rejection by inhibiting T cell mediated immune responses. These drugs block specific pathways involved in signal transduction leading to cytokine synthesis and T cell proliferation. In cell-line based assays, these drugs are potent inhibitors of P-gp, a drug-efflux transporter with a wide tissue distribution. Lymphocytes were investigated as an *ex-vivo* model for studying the P-gp modulatory effects of immunosuppressants and other classes of drugs. Based on a rhodamine 123 efflux/retention assay, data for P-gp inhibition constants for different drugs were determined. Since lymphocytes represent an easily accessible normal human tissue, data obtained in this model might be a better representation of actual physiological processes as compared to data generated using modified animal or human cell-lines. In the data presented in Chapter 3, different drugs inhibited P-gp to a different extent. There was evidence of an indomethacin-sensitive rhodamine efflux pump that appears to be distinct from P-gp or MRP. Vinblastine caused an enhanced efflux of rhodamine 123 from lymphocytes suggesting an activation of P-gp. The most potent inhibitor tested, cyclosporine, did not result in the largest rhodamine 123 accumulation in these cells, indicating the presence of different cyclosporine sensitive transporters. It would be interesting to compare the results obtained in the *ex-vivo* assay with other normal tissues. If there exists a good correlation between these results,

lymphocytes can be used for screening of compounds for P-gp modulatory activity. Early use of such ex-vivo techniques can provide a good understanding of mechanisms involved in pharmacokinetic interactions and poor bioavailability of drugs.

The utility of lymphocyte proliferation assays to assess effects of drugs on immune responses is well established. In the studies reported in Chapter 4, pharmacodynamic effects of drugs alone or in combination with cyclosporine were studied. Based on these studies, there is clear evidence of significant *in vitro* drug interactions between P-gp inhibitors and cyclosporine. Combinations of drugs are often used in transplant patients, some of these combinations could result in enhanced immune-suppression putting the patient at risk for other diseases. Since cyclosporine is a potent P-gp inhibitor, it has been used clinically in cancer patients to reverse multidrug resistance or enhance oral bioavailability of drugs. Such combinations of drugs may result in an immune-compromised cancer patient unable to mount an effective response against the tumor. It is possible that some of the cancers that are refractory to such combination therapy may not be resistant to treatment only on the basis of low drug concentrations, but also because of an ineffective immune system. An interesting and unexpected result from these studies was the concentration dependent immunosuppressive effects of various drugs. In these studies, the mechanisms involved in this immunosuppression were not fully understood. Further detailed analysis of individual steps in T cell activation / proliferation and the analysis of messenger RNA for cytokines would help elucidate these mechanisms further. The significant interactions observed with relatively low levels of cyclosporine were surprising but interesting and could have implications in the use of

immunosuppressants in autoimmune diseases where lower concentrations of cyclosporine may be combined with NSAIDs.

The expression of P-gp in lymphocytes from renal transplant patients and healthy volunteers was highly variable. Patients vary considerably in their dose-response to cyclosporine and tacrolimus. The variability in expression of P-gp may in part be responsible for such effects. The findings suggest that immunosuppressive drugs such as cyclosporine and tacrolimus may modulate the expression of P-gp and also its functional capability soon after initiation of such therapy. The patients receiving MMF and healthy volunteers had stable expression as compared to patients on cyclosporine or tacrolimus. The decrease in expression of P-gp from pre-transplant to post-transplant clearly indicates that cyclosporine may be directly responsible for such effects. Although these studies were not statistically significant, they provide a rationale for undertaking a larger study with greater control over the patient groups. It would be interesting to determine if the expression of P-gp follows any particular pattern between genders and races. An understanding of the interplay between drugs or disease conditions with expression and function of transporters will be important as we move towards development of safer and more selective therapies.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