LITERATURE REVIEW

1. Cyclosporine[2,6]

Cyclosporine is a powerful immunosuppressive drug and has proved to be a potent agent in a wide variety of experimental models of tissue transplantation and in clinical organ transplantation. Cyclosporine was first isolated from two strains of imperfect fungus (*Cylindrocarpon lucidum* Booth and *Trichoderma polysporum* Rifai) from soil samples as an antifungal agent of limited activity. The latter, from which cyclosporine now is produced, is known more correctly as *Tolypocladium inflatum Gams* and was shown by Borel to have potent immunosuppressive activity in a variety of *in vitro* and *in vivo* experiments. The drug has a molecular weight of 1200 kDa and comprises of 11 amino acids, one of which is unique and most of which are hydrophobic. Cyclosporine is soluble only in lipids or organic solvents.

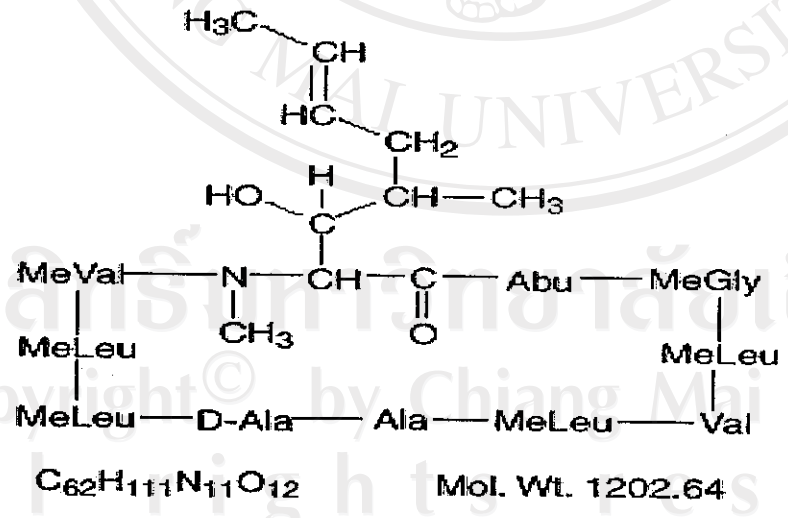

Figure 1. Structure of cyclosporine[20].

After Borel's initial description of the immunosuppressive properties of cyclosporine, it was shown to suppress rejection of vascularized organ allografts in the rat, dog and rabbit. Similar observations in various models of vascularized organ allografts in many species followed quickly. Clinical trials of the drug in renal transplantation began in Cambridge in 1978; by the early 1980s, cyclosporine was licensed for use in renal transplantation, first in Europe and then in the United States. Cyclosporine-based protocols rapidly became standard therapy in renal transplantation, unless restricted by cost, and now represent the conventional therapy against which new immunosuppressive agents are compared. Because cyclosporine was the first of the new immunosuppressive agents to enter the clinical arena, a relatively extensive review of the early experimental and clinical experience with the drug is justified; the advent of cyclosporine had an enormous impact not only on renal transplantation but also on liver and heart transplantation.

1.1 Clinical Pharmacology and Therapeutics

Cyclosporine is a potent immunomodulator that acts selectively at an early stage in the activation of T lymphocytes by inhibiting production of soluble proliferative factors, IL-2 and other cytokines. This is an important step for transplanted graft rejection or progress of autoimmune diseases. It does not suppress bone marrow function, which sets it apart from other immunosuppressive agents such as azathioprine.

Mechanism of action

Cyclosporine does not act on the initial plasma membrane events of signal reception, transduction, or calcium influx. The immunosuppressive activity of cyclosporine appears to be mediated by intracellular receptors. At therapeutic concentrations, it enters the cell passively, and binds a cytoplasmic protein termed *cyclophilin*, which is the same enzyme known as peptidyl-prolyl-cis-trans-isomerase. This enzyme catalyzes proline peptide bond isomerization, which is a rate-limiting step in protein folding. After cyclosporine forms the cyclosporine-cyclophilin complex, this complex binds to and inhibits the Ca^{2+}-dependent phosphatase calcineurin. Calcineurin is found to be the common molecular target mediating the immunosuppressive actions of cyclosporine and other immunosuppressive agents such as tacrolimus (FK506).

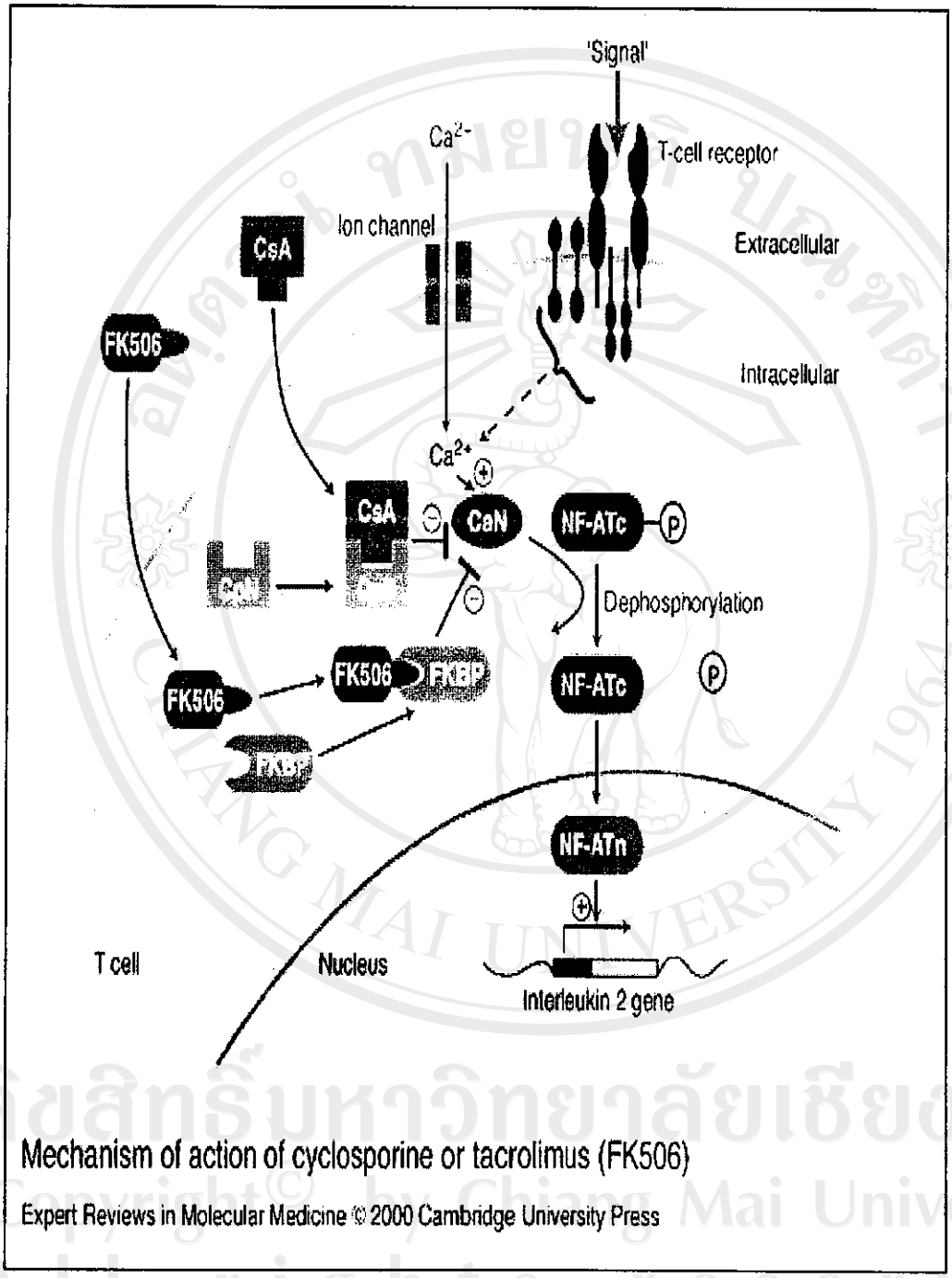

Figure 2. Mechanism of action of cyclosporine and tacrolimus[21].

Calcineurin is required for the proper assembly of a transcription factor that then binds to the IL-2 gene and initiates synthesis of IL-2 and other cytokines such as interferon-gamma. IL-2 and other cytokines are necessary for helper and cytotoxic T-cell activation, proliferation, and maturation. A lack of cytokines disrupts the activation and proliferation of the helper and cytotoxic T-cells that are essential for the rejection process. Once IL-2 gene activation has expressed on T cells, however, cyclosporine is unable to inhibit IL-2-dependent activation, because it does not block IL-2 receptor expression or binding of IL-2 to its receptor. In addition, there is some evidence that suppressor T cells are relatively spared by cyclosporine therapy, which is important for halting the progress of autoimmune diseases. Cyclosporine is used mainly for prevention of rejection; it is relatively ineffective in reversing the process once rejection develops.

Therapeutic use

Cyclosporine is used for prophylaxis of allograft rejection in organ transplantation, usually in conjunction with other immunosuppressive agents such as corticosteroids and azathioprine. It has improved allograft survival significantly in most solid organ transplantation. For example, 1-year graft survival for cadaveric renal transplants has improved by approximately 10 to 20% in most cyclosporine-including protocols. Other beneficial effects include reduced incidence of acute rejection or infectious complications compared with azathioprine-prednisone protocols. Cyclosporine's beneficial effects are more significant in other solid organ transplantations, such as liver and heart transplants. An improved immunosuppression with cyclosporine-containing protocols has changed liver and heart transplantation from experimental procedures to rapidly growing therapeutic modalities. It has also been used in preventing graft-versus-host disease in bone marrow transplantation and in treating autoimmune diseases.

1.2 Clinical Pharmacokinetics

Pharmacokinetic characteristics of cyclosporine in humans are highly variable in transplant patients as well as healthy volunteers under standardized conditions. Significant factors influencing cyclosporine pharmacokinetics are illustrated in Figure 3, and pharmacokinetic parameters are summarized in Table 1. In addition, there is some confusion because the pharmacokinetic parameters are different depending on the cyclosporine analytic methods and

sample matrix. This section discusses studies using specific analytic methods with whole blood samples, unless otherwise indicated.

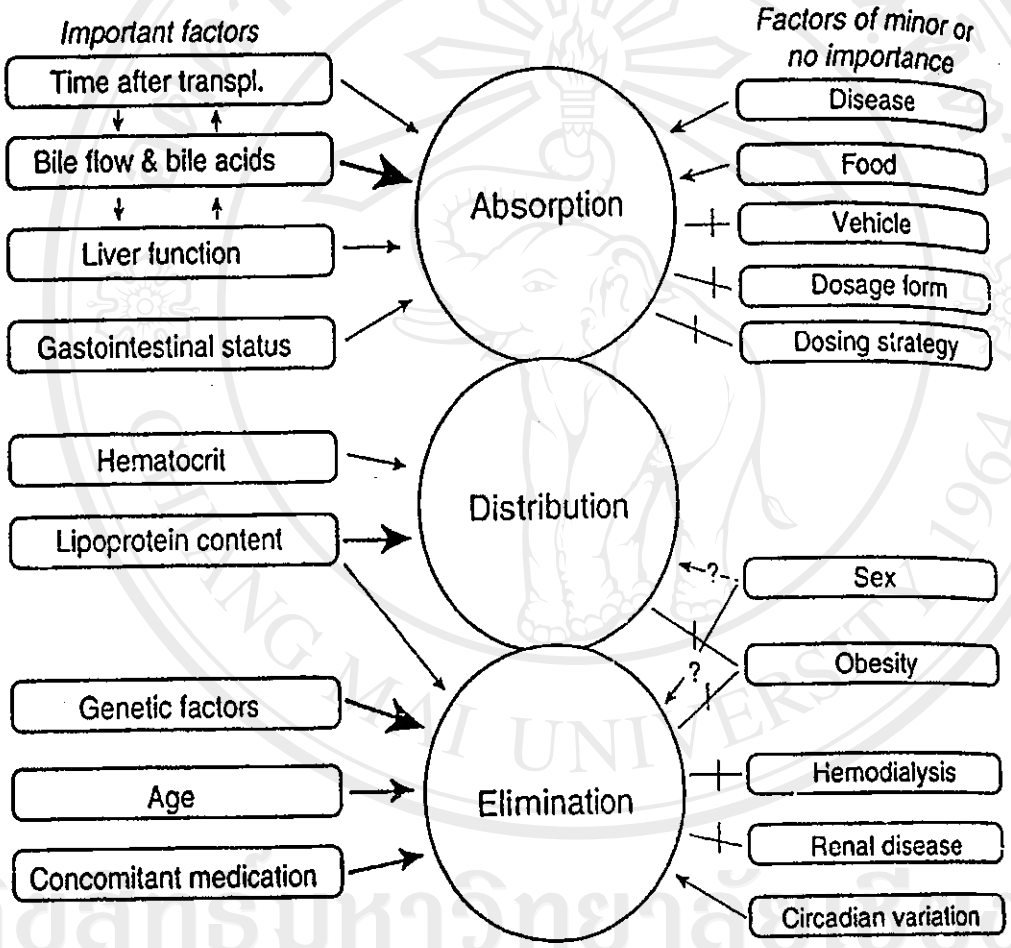

Figure 3. Factors with and without effect on the pharmacokinetics of cyclosporine[6].

Table 1. Summary of cyclosporine pharmacokinetics[a]

Parameter	Average ± SD
Bioavailability in tissue transplant patients (%)	
Renal	30 ± 13
Liver (pediatric)	<5 – 18[b]
Liver (adult)	27 ± 13
Heart	35 ± 11
Bone marrow	34[c]
First-pass metabolism (%)	10 - 27[b]
Enterohepatic circulation	Metabolites only
Protein binding (%)	>95
Clearance (ml/min/kg)	
Renal	5.7 ± 1.8
Liver	5.5[c]
Bone marrow	10.1 ± 1
T_{max} (h)	4.0 ± 1.8
C_{max} (ng/ml)[d]	1103 ± 570
V_{ss} (l/kg)	4.5 ± 1.5
Distribution $t_{1/2}\alpha$ (h)	1.1
Elimination $t_{1/2}\beta$ (h)	
Renal	11.4 ± 4.4
Uremic	15.8 ± 8.4
Liver failure	20.4 (10.8 - 48)
Hepatic metabolism (%)	>99
Biliary excretion (%)	>90
Hemodialysis	No effect
Peritoneal dialysis	No data available
Hemoperfusion	No effect

[a]All parameters based on whole blood samples and HPLC assay method except when indicated otherwise.

[b]Range.

[c]No SD reported: range = 20 - 50% (serum RIA).

[d]This C_{max} is based on dose of 10 mg/kg.

Absorption

Cyclosporine is slowly and incompletely absorbed from the gastrointestinal tract after oral or intramuscular administration. Absorption of cyclosporine in the gastrointestinal tract is highly variable, with a mean bioavailability of approximately 30% (range, <5 to 92%). The absorption process of cyclosporine is best described by a zero-order kinetic model. The zero-order absorption kinetics explain that an absorption window may exist in the upper part of the small intestine and that carrier-mediated transfer of cyclosporine across the intestinal wall may occur. It is also postulated that the decrease in bioavailability with increasing dose is due to limited solubility in the gastrointestinal tract. Cyclosporine appears in the blood after 0 to 0.9 hour, and the absorption half-life ranges from 0.5 to 2 hours. Peak concentration (C_{max}) is usually achieved 2 to 6 hours after administration of the oral solution or soft gelatin capsule. After a single dose of 14 mg/kg given once daily, peak serum concentrations reach 1103±570 ng/ml (SD) in renal transplant patients. The poor bioavailability of cyclosporine appears to be related to the outcomes of renal transplantation. Patients with cyclosporine bioavailability of less than 25% have an increased risk of renal allograft loss (63% versus 83%). The various factors influencing the bioavailability of cyclosporine are summarized in Table 2.

Table 2. Various factors affecting cyclosporine bioavailability

Factor	Comments
T-tube (bile flow)	Increase oral cyclosporine dose or use IV before T-tube clamping and reduce dose after T-tube clamping.
Time after transplant	Bioavailability increases by 39% 3 mo after transplantation.
Liver function	Bioavailability is poor (<5%) in poor liver function.
Gastric emptying	Bioavailability increases when gastric emptying is increased by metoclopramide.
High-fat diet	Bioavailability increases.
Length of small bowel	Bioavailability decreases in the patients with short bowel.

Bile acid has been shown to facilitate the bioavailability of many fat-soluble vitamins. It appears to emulsify cyclosporine, which facilitates lipophilic cyclosporine absorption.

Results on the effect of food on cyclosporine absorption are conflicting, although effect appears to be related to the content of the food. Gupta et al. showed that the fat content of food is a major determining factor. It was found that there is a significant increase in bioavailability in healthy volunteers (21-53%) when cyclosporine is given with a high-fat breakfast. It has been explained that the increased outflow of bile stimulated by fatty meals enhances the absorption of cyclosporine.

Bioavailability gradually improves over several weeks following transplantation. It is observed clinically that patients require lower doses of cyclosporine to maintain the same trough concentrations several weeks after transplantation. This phenomenon can be explained partly by prolonged ileus in the early postoperative period, which attributes to poor absorption. However, in a longitudinal pharmacokinetic study before and after renal transplantation, Odlind et al. noted that in the six patients tested 3 months posttransplantation, bioavailability has increased by almost 50% compared with the pretransplantation test. It is not clear whether the change is related to amelioration of the uremic condition or other changes in lipid absorption.

It has been observed that the absorption of cyclosporine is impaired in patients with diarrhea. Patients without vomiting or diarrhea consistently show cyclosporine serum values peaking 3 or 4 hours after an oral dose. It has been found that the area under the curve (AUC) of cyclosporine in patients with diarrhea (>500 ml/72 h) is 38% of that in patients without diarrhea.

Any factors influencing gastric emptying and gastrointestinal motility influence the bioavailability of cyclosporine. Drug increasing gastric emptying such as metoclopramide enhances cyclosporine absorption by 29%. Another factor found to be important in cyclosporine absorption is small bowel length. Whitington et al. concluded that the length of the small bowel is the chief determinant of the required dose of orally administered cyclosporine in children after liver transplantation and that the children require larger doses due to the limited absorptive surface of their intestines. Patients with short gut syndrome or extensive intestinal resection also have decreased cyclosporine bioavailability (3% of bioavailability).

Distribution

The distribution of cyclosporine is described by two phases: initial rapid distribution phase with a half-life of 0.1 hour ($t_{1/2\alpha}$), followed by a slower distribution phase with a half-life of 1.1 hour ($t_{1/2\beta}$). The volume of distribution is variable, ranging from 2.9 to 4.7 l/kg in renal, liver, and bone marrow transplant patients, but from 1.3 to 1.5 l/kg in healthy volunteers and heart transplant patients. Cyclosporine is widely distributed into blood cells. *In vitro* studies with radiolabeled cyclosporine show that in blood, 58% of the circulating cyclosporine is bound to red blood cells, 4% to granulocytes, 5% to lymphocytes, and 33% is in plasma. Binding of cyclosporine to red blood cells appears to be saturable and dependent on temperature and hematocrit, which may be a significant source of variability when serum or plasma samples are used for cyclosporine monitoring. Plasma cyclosporine concentrations separated at 37°C have been found to be 15% higher that those separated at 36°C. This may imply that during febrile episodes, the distribution of cyclosporine shifts from blood cells to plasma. In plasma, cyclosporine is highly bound to plasma proteins (>95%), mainly lipoproteins, which constitute 10 to 15% of all plasma proteins. Of the lipoproteins, high-density lipoprotein binds 43 to 57% of cyclosporine in plasma, low-density lipoprotein binds 25%, and very low-density lipoprotein binds 2%. Measuring the fraction of unbound cyclosporine in plasma is technically difficult because of adsorption of cyclosporine on the ultrafiltration membrane. The fraction of drug unbound in plasma ranges from 1.4 to 12% depending on the different techniques used. Lindholm and Henricsson reported a 5-fold intraindividual variability and a 2.3-fold interindividual variability in the mean free fraction of cyclosporine in plasma. They have also observed a significant correlation between the unbound fraction in plasma and the onset of acute rejection in renal transplant recipients. Cyclosporine is distributed widely throughout body tissues, concentrating in organs such as liver, pancreas, lungs, and kidneys. The highest concentrations, which are found in fat and liver tissues, are up to 10-fold higher than blood concentrations. Despite the high concentrations in fat tissue, obesity has been found to have no significant effect on cyclosporine volume of distribution based on serum or blood concentrations. This indicates that the dose of cyclosporine in obese patients should be based on lean body weight rather than actual body weight. Cyclosporine does not readily penetrate the intact blood-brain barrier and lower concentrations are found in the brain; however, liver transplant patients may

have a functional abnormality in the blood-brain barrier because of sustained liver failure. Low serum cholesterol levels as well as prolonged liver disease appear to contribute to an increased risk of serious central nervous system toxic effects, such as mental confusion and seizures. Cyclosporine passes the placenta and measurable concentrations are detected in the amniotic fluid and fetal blood, as well as in maternal breast milk.

Metabolism

Cyclosporine is extensively metabolized by the hepatic cytochrome P4503A isoenzymes. These isoenzymes are composed of at least four genes, among which P4503A4 is responsible for about 80% of cyclosporine metabolism in the liver. Cyclosporine metabolic pathways in the body involve hydroxylation, N-demethylation, cyclization, and oxidation. Cyclosporine is metabolized to the metabolites M1, M9, M4N and others. Although cytochrome P4503A is located mainly in the liver, there is some evidence that cytochrome P4503A enzymes of gastric mucosa enterocytes contribute to presystemic metabolism of cyclosporine in the gastrointestinal tract. More than 30 metabolites have been isolated and their chemical structures characterized, and all metabolites have been found to preserve the cyclic oligopeptide structure. High concentrations of the metabolites in the blood raise the question of whether these metabolites contribute to the immunosuppressive activity or toxicity of the parent drug. So far, there is no evidence that the metabolites have significant immunosuppressive activity compared with cyclosporine. Only M1, which is the predominant metabolite in the blood, has been found to have about 10% of the immunosuppresssive activity of the parent drug. None of the metabolites appears to have significant nephrotoxicity. Cyclosporine is a low- to intermediate-clearance drug, and its clearance is therefore dependent on the intrinsic enzyme activity and the unbound fraction in the blood. Cyclosporine has been reported to have average clearance of 5.7 ml/min/kg in adult kidney recipients, 5.5 ml/min/kg in adult liver recipients, and 10 to 13 ml/min/kg in bone marrow transplant patients. In healthy volunteer and heart transplant patients, clearance is slightly lower (about 4 ml/min/kg) than in other patient groups.

The mean elimination half-life ($t_{1/2\beta}$) varies from 7 to 24 hours depending on the type of transplant and concurrent disease states. In adult renal transplant recipients with near-normal renal function, the mean $t_{1/2\beta}$ of cyclosporine is about 10 hours. Because cyclosporine is

metabolized in the liver, cyclosporine clearance is significantly reduced and $t_{1/2\beta}$ is prolonged in patients with liver diseases, averaging 20 hours (range, 10-48 hours).

In one study with bone marrow recipients, a 23% reduction in clearance of cyclosporine and prolonged $t_{1/2\beta}$ (32.1 hours) have been observed in patients with hyperbilirubinemia (serum bilirubin > 20 mg/l). The clearance of cyclosporine is also decreased by 30% in renal recipients with impaired hepatic function. Cyclosporine doses should be decreased in most patients with hepatic failure. A few pharmacokinetic studies have reported a second peak, which occurs about 4 to 6 hours after the first peak. The reason for the double peak is not clear. One explanation is that undissolved cyclosporine, due to the limited solubility of cyclosporine in the gastrointestinal tract, can be resolubilized and absorbed several hours later by bile acid secretion possibly stimulated by food. The other possible explanation is that the sulfate-conjugated metabolites of cyclosporine excreted through the bile may be degraded by intestinal bacteria into parent cyclosporine, which can then be reabsorbed in the gastrointestinal tract. In usual clinical dosage ranges, cyclosporine appears to be metabolized by first-order processes, though some studies suggest nonlinear increases in plasma concentration as cyclosporine dosage is increased. A few case reports of high-dose cyclosporine intoxication, however, do not indicate any nonlinear cyclosporine elimination.

The clearance of cyclosporine is clearly dependent on the age of patients. In pediatric patients, cyclosporine clearance is generally twice as fast as in adult liver recipients. For example, Burckart et al. reported that cyclosporine clearance in pediatric liver recipients (about 1-5 years) is 9.3 ml/min/kg compared with 5.5 ml/min/kg in adult liver transplant patients. Because of this, some pediatric patients require three-times-daily dosing schedules to maintain adequate trough cyclosporine concentrations compared with twice- or once-a-day dosing schedule in adult patients.

Although circadian variations of cyclosporine clearance are observed, they appear to be clinically insignificant. Canafax et al. found that the evening dosing of cyclosporine increases the total AUC of cyclosporine and its metabolites (M1 and M9) compared with morning dosing of cyclosporine in six pancreas transplant patients. On the other hand, Venkataramanan et al. found an increased clearance of cyclosporine at nighttime in two liver transplant recipients.

Elimination

In humans, biliary excretion is the major pathway of elimination of cyclosporine. Because of extensive metabolism, most biliary excretion consists of the metabolites and less than 1% of the dose of cyclosporine is detected in the bile. Urinary excretion of cyclosporine is a minor pathway of elimination because cyclosporine is highly lipophilic and extensively metabolized by the liver. According to a study in which radiolabeling was used, only 6% of the radioactivity is excreted in the urine in 96 hours, and most of radioactivity comes from the metabolites M1, M1c, and M9, which are present in higher concentrations than the parent drug in the urine. Renal failure does not appear to impair cyclosporine excretion.

1.3 Pharmacodynamics

Drug-drug interactions

Numerous interactions between cyclosporine and other drugs can produce troublesome clinical effects. The clinically significant drug-drug interactions are summarized in Table 3.

It is not known if antacids can decrease cyclosporine absorption, but there appears to be no effect. No effects are seen when cholestyramine is given 1 hour before or 4 hours after the cyclosporine dose. Metoclopramide increases cyclosporine levels, and severe diarrhea can decrease cyclosporine levels.

As cyclosporine is extensively metabolized by the hepatic cytochrome P4503A enzyme system, various drugs that induce or inhibit these enzymes usually have significant effects on cyclosporine concentrations. Antiepileptic drugs, such as phenytoin, phenobarbital, and carbamazepine, induce these enzymes and decrease the concentration of cyclosporine. Phenytoin effects on cyclosporine metabolism begin as early as 2 days after phenytoin starts, last at least 2 weeks after its discontinuation, and usually necessitate two or three times higher cyclosporine doses. A good alternative antiepileptic drug might be valproic acid, which likely has no effect on cyclosporine concentrations. The antitubercular drugs rifampin and isoniazid are also potent inducers of the P4503A enzymes and reduce cyclosporine concentrations.

Table 3. Clinically important drug interactions of cyclosporine

Drugs	Mechanism	Effects	Management
Antiepileptic drugs Phenytoin Phenobarbital Carbamazepine	Increased cyclosporine metabolism and reduced bioavailability by induction of cytochrome P-450 enzyme	Cyclosporine trough levels drop within 48 h of initiation of these drugs and remain low for at least 2 wk after discontinuation	Increase cyclosporine dose with frequent monitoring of cyclosporine levels or use valproic acid, which has no interaction.
Rifampicin or INH	Same as above	Same as above	Increase cyclosporine dose with frequent monitoring of cyclosporine levels.
Octreotide	Reduced cyclosporine absorption	Cyclosporine levels decrease within 24 to 48 h	Increase oral cyclosporine dose or use IV cyclosporine; frequent monitoring of cyclosporine levels is required.
Azole antifungal drugs Ketoconazole Fluconazole Itraconazole	Inhibition of liver cytochrome P-450 enzyme by these drugs	Cyclosporine levels significantly increase (X2-10) within 2 d, resulting in nephrotoxicity (ketoconazole>itraconazole>fluconazole)	Reduce cyclosporine dose with frequent monitoring of levels.
Macrolide antibiotics Erythromycin Josamycin	Inhibition of liver and GI cytochrome P-450 enzymes	AUC (X2) and cyclosporine trough levels (X2-3) increase	Same as above
Calcium channel blockers Verapamil Diltiazem Nicardipine	Same as above	Same as above	Reduce cyclosporine dose or use nifedipine, isradipine, or nitrendipine.
Oral contraceptives and Danazol	Same as above	Same as above	Reduce cyclosporine dose
Cholesterol-lowering drugs Lovastatin Simvastatin Pravastatin	Same as above	Incidence of rhabdomyolysis or muscle pain increases	Switch to non HMG-CoA[a] inhibitors or reduce the dose of these drugs with careful monitoring.

[a] Hydroxymethyglutaryl coenzyme A.

Drugs that inhibit the hepatic microsomal enzymes increase cyclosporine concentrations, for example, ketoconazole has caused a 10-fold increase in cyclosporine concentrations with subsequent increases in serum creatinine levels. Fluconazole can increase cyclosporine concentrations slightly after 2 weeks of therapy. Giving erythromycin to a patient on cyclosporine typically causes cyclosporine concentrations to increase up to 3-fold 2 to 14 days after the initiation of therapy.

Calcium channel blockers, such as diltiazem and nicardipine, can slightly increase cyclosporine concentrations, but nifedipine appears to have no effect. Hormones such as danazol, norethisterone, and methyltestosterone have been reported to increase cyclosporine concentrations, but the clinical significance of this interaction remains unclear. H_2 blockers such as cimetidine, ranitidine, and famotidine appear to have no effect on cyclosporine pharmacokinetics.

Amphotericin B, aminoglycosides, trimethoprim-sulfamethoxazole, melphalan, furosemide, mannitol, indomethacin, and the cephalosporins increase the incidence of adverse renal effects when given with cyclosporine therapy. Drugs that decrease cyclosporine-induced renal effects include spironolactone, enalapril, prazosin, thromboxane synthetase inhibitors, and some prostaglandins.

The combination of cyclosporine and lovastatin has been reported to cause a myalgia, rhabdomyolysis syndrome. Patients on cyclosporine are prone to gingival hyperplasia, which can be worsened by other drugs such as phenytoin and nifedipine.

1.4 Adverse reactions

Cyclosporine causes various adverse reactions, of which dose-related nephrotoxicity is the most common and serious. The major side effects of cyclosporine are summarized in Table 4.

Nephrotoxic effects

Almost all patients who receive cyclosporine have some degree of cyclosporine-related renal dysfunction at any dose. Cyclosporine-induced renal dysfunction has been well documented.

Table 4. Side effects of cyclosporine

Side effects	Incidence	Symptoms	Management
Nephrotoxicity	Most patients	Slowly rising BUN and serum creatinine, decreasing urine output	Monitor blood level; avoid nephrotoxic drugs.
Hypertension	23-100%	Increased blood pressures	Use same measures as for general hypertension.
Hepatotoxicity	Up to 50%	Hyperbilirubinemia, elevated liver enzymes	Reduce cyclosporine dose; effects are generally mild and no treatment is necessary.
Hirsutism	30%	Hair growth on the dorsum of the hand, arms, and face	
Neurotoxicity	15%	Hand tremors, paresthesis (common), seizure, mental confusion (rare)	Reduce cyclosporine dose; monitor serum magnesium and cholesterol levels.
Hyperkalemia	Uncommon	Elevated serum potassium levels, renal dysfunction is risk factor	Institute diuretic therapy with serum potassium monitoring; use Kayexalate.
Gingival hyperplasia	Uncommon	Gum overgrowth	Instruct patient on dental hygiene.

Cyclosporine-induced nephrotoxicity appears in three basic types: first, acute, reversible reduction of glomerular filtration rate; second, tubular toxicity with possible enzymuria and aminoaciduria; and third, irreversible interstitial fibrosis and arteriopathy at a later period, usually 6 months to 12 months after transplantation. The exact mechanism of cyclosporine-induced nephrotoxicity is not completely understood, but cyclosporine appears to disturb the normal activity of various vasoactive substances in the kidney. First, cyclosporine alters the balance of the vasodilator prostacycline and its antagonist thromboxane A_2 in renal cortical tissue. The

evidence to support this hypothesis is that prostacycline levels are reduced in cyclosporine-treated patients and the thromboxane A_2 systhesis inhibitor appears to improve renal function. More rare, severe forms of nephrotoxicity manifest as a form of thrombotic microangiopathy which consists of glomerular thrombosis formation and a microangiographic anemia. Cyclosporine appears to reduce prostacycline production, which causes unopposed thromboxane effects. Cyclosporine-induced thrombotic microangiopathy is a serious condition, which may cause graft loss.

Second, it has also been reported that cyclosporine may increase concentrations of the endothetial cell-derived vasoconstrictive peptide endothelin. Cyclosporine added directly to cultured human vascular cells increases the levels of endothelin in the culture medium. In the rat model, 20 mg/kg IV cyclosporine caused circulating endothelin levels to increase more than 20 times. Although endothelin appears to affect all vascular vessels, renal vessels are especially vasoconstriction, a decrease in renal blood flow, and a decrease in glomerular filtration rate, usually without morphologic changes, although chronic effects may progress to interstitial fibrosis.

There is controversy over whether cyclosporine increases the incidence of delayed graft function in cadaveric renal transplantation. It may not increase the incidence of delayed graft function per se: however, it appears to enhance ischemic injury of the kidney, require prolonged dialysis support, and delay the recovery from acute tubular necrosis once it occurs. Because of this potential additional insult of cyclosporine on the cadaveric kidney, many transplant centers use sequential initiation of cyclosporine in the immediate posttransplant peroid by avoiding cyclosporine until diuresis begins.

The possibility of long-term toxic effects of cyclosporine on the kidney and the risk of renal failure has been raised. In fact, a small number of heart and other organ transplant patients receiving cyclosporine develop chronic renal failure, but in most of patients, kidney function appears to be stabilized with a reduced dose of cyclosporine, even when cyclosporine is continued. Although some progressive loss of renal function continues for up to 7 years, the rate of decline appears to be much slower after the first year.

Other toxic effects

Some patients treated with cyclosporine develop hepatic dysfunction, which is generally subclinical and dose dependent, especially in the first 90 days posttransplant. This hepatic

toxicity is commonly characterized by mild hyperbilirubinemia and elevated serum transaminases. In the majority of patients, these alterations in liver function are rapidly reversible by reducing the dose of cyclosporine. It is recommended that liver function tests be performed regularly for patients treated with cyclosporine.

Though not common, patients may develop mild hyperkalemia during cyclosporine therapy, partly as a result of renal dysfunction. Close monitoring of serum potassium is required, particularly in early posttransplantation. If the serum potassium is less than 6 meq/l, hyperkalemia may be managed with diuretics, but if the serum potassium is greater than 6 meq/l, sodium polystyrene sulfonate (Kayexalate) with sorbitol should be initiated to reduce serum potassium. Potassium-sparing diuretics such as spironolactone should not be used in patients receiving cyclosporine.

Cyclosporine-induced neurotoxic effects are relatively common. In the early Canadian multicenter clinical study, 15% of patients receiving cyclosporine developed neurotoxic effects compared with 1% of the placebo group. Cyclosporine-induced neurotoxicity is mild in most cases, manifesting as tremors and paresthesias. Other neurotoxic effects such as seizures and mental confusion have been uncommon. Hypocholesterolemia has been identified as a significant risk factor for these patients. In that study, most patients who developed neurotoxic effects have extremely low total cholesterol levels (<120 mg/dl). It has been explained that a high-unbound fraction caused by low cholesterol, along with possible disruption of the blood-brain barrier caused by liver failure, may have contributed to these unusual neurotoxic effects.

Besides the side effects associated with cyclosporine therapy listed above, the other most common adverse reaction (21-100%) is hypertension. Cyclosporine-associated hypertension may be related to the nephrotoxic effects of the drug as well as renal vasoconstriction. Cyclosporine-induced hypertension can usually be controlled well by various antihypertensive medications. Other minor side effects include hair overgrowth and gingival hyperplasia. Some patients tend to tolerate these adverse reactions during long-term therapy, but others need special care such as antihypertensive treatment, cosmetics, and gingivectomy.

1.5 Therapeutic regimen design

Therapeutic monitoring

Table 5 summarizes the therapeutic ranges for the various analytic methods and blood matrices that are in clinical use. Therapeutic ranges of cyclosporine are at best empirical guidelines. Because cyclosporine is generally used for prevention of rejection or graft-versus-host disease, the major limitation to defining the therapeutic ranges is the lack of standards for diagnosing toxicity or degree of immunosuppression. There is no readily available marker that measures the degree of immunosuppression by cyclosporine other than acute rejection by a tissue biopsy. The only readily available test for nephrotoxicity has been serum creatinine concentration, which is insensitive to small changes in glomerular filtration rate. It is difficult to establish the therapeutic ranges of cyclosporine because the clinical studies reported in the literature have often used different analytic methods, different sample matrices (serum, plasma, or whole blood), different dosing schedules (twice daily, once daily), or different concurrent immunosuppressive agents in the various patient groups. Measuring cyclosporine concentrations, however, does help identify patients with low or high values who might benefit from dosage changes.

Table 5. Therapeutic ranges of cyclosporine

Type of transplant	Sample matrix	Analytic method[a]	Target range (ng/ml)
Kidney	Whole blood	HPLC, S-RIA, or	150-250 (<3 mo)
		S-FPIA	100-200 (>3 mo)
		NS-FPIA	400-800
	Serum/ plasma	NS-FPIA	100-250
Liver	Whole blood	HPLC or S-RIA	200-300
		NS-FPIA	400-800
Heart	Whole blood	HPLC or S-RIA	150-300
Bone marrow	Serum/ plasma	NS-FPIA or	150-300
		NS-RIA[b]	

[a]HPLC, high-performance liquid chromatography; S-RIA, monoclonal radioimmunoassay specific for cyclosporine; S-FPIA, fluorescence polarization immunoassay specific for cyclosporine; NS-FPIA, nonspecific fluorescence polarization immunoassay; NS-RIA, nonspecific polyclonal radioimmunoassay.

[b]No longer available.

Whether whole blood, plasma, or serum should be used as the sample matrix has been controversial, but whole blood is recommended for several analytic reasons. About 50% of the circulating cyclosporine in the blood is bound to erythrocytes, and concentrations depend greatly on the sample matrix chosen for analysis. Furthermore, the distribution of cyclosporine among plasma and blood cells is influenced by many factors such as temperature, hematocrit, drug concentrations, and incubation time. These factors may cause considerable variation in serum or plasma cyclosporine concentrations, when serum or plasma is separated from whole blood under the different conditions. Considerable efforts are involved in developing a consensus for these issues, but some issues remain unresolved.

High-performance liquid chromatography (HPLC), radioimmunoassay (RIA), and fluorescence polarization immunoassay (FPIA) are the principle analytic methods available for cyclosporine monitoring. HPLC is regarded as a reference standard assay; it detects cyclosporine and its metabolites separately, but requires intense labor and reliable skill for routine clinical monitoring. Specific monoclonal radioimmunoassay (S-MRIA) or specific FPIA (S-FPIA), which show good correlation to the results of HPLC, are specific for cyclosporine; however, the polyclonal radioimmunoassay (which is no longer available) and nonspecific fluorescence polarization (NS-FPIA) are nonspecific, cross-reacting with cyclosporine metabolites. There is also a nonspecific monoclonal radioimmunoassay. Results from nonspecific assay methods need to be interpreted with caution in patients with reduced hepatic function, as accumulation of metabolites occurs. Whole blood concentrations are usually much higher than serum concentrations, because cyclosporine distributes into erythrocytes. Assay accuracy is poor at concentrations of less than 50 ng/ml, which becomes important at low doses of cyclosporine.

As discussed in the previous section, the adverse reactions associated with high cyclosporine concentrations include renal dysfunction, tremor, hypertension, and hepatotoxicity. Nephrotoxicity is the most common adverse effect that is dose related; decreasing the cyclosporine dose will usually decrease the creatinine level. In renal allograft recipients, it is often difficult to differentiate between cyclosporine renal effects and acute rejection episodes. The measurement of cyclosporine concentrations may be helpful in this situation where high levels are more likely associated with cyclosporine effects and low levels with rejection.

1.6 Dosage and administration

Initial oral cyclosporine doses are usually about 8 to 14 mg/kg/d. For living donor kidney recipients, cyclosporine is given 1 to 2 days before transplantation to achieve therapeutic concentrations at the time of transplantation. This dose is divided twice daily and adjusted to maintain the desired cyclosporine concentrations. Dosage adjustment is empiric as a result of extreme intrapatient variability in absorption and clearance. A pharmacokinetic dosing program that uses each patient's pharmacokinetic parameters will soon be available. Antilymphoblast globulin or OKT3 is often used in the early posttransplant period for cadaver kidney recipients to allow cyclosporine to be held until serum creatinine concentrations are less than 3 mg/dl, in an attempt to avoid adverse renal effects of cyclosporine.

Administration of oral cyclosporine is usually preferred; however, for those patients who cannot tolerate oral therapy or whose cyclosporine absorption is poor, intravenous doses of between 3 and 6 mg/kg/d can be given. Loading doses are not used when beginning cyclosporine therapy as side effects would likely occur. Before use, intravenous cyclosporine solution should be diluted in 20 to 100 ml of normal saline or 5% dextrose for injection and given as a slow intravenous infusion over 2 to 6 hours. The intravenous cyclosporine dose can be given by a continuous 24-hour infusion which may reduce the renal effects of the drug. The intravenous dose is adjusted using trough levels. Blood samples for cyclosporine analysis should not be drawn from the intravenous lines used for administration to avoid spuriously high levels. Patients being given intravenous cyclosporine can be changed to oral therapy by giving three times the intravenous dose and measuring cyclosporine concentrations.

To make the oral cyclosporine solution more palatable, patients can dilute their dose with chocolate milk or juice. A glass container should be used for mixing to avoid adsorption of the drug to wax or plastic containers. To ensure cyclosporine absorption and assess the adequacy of the dose, a trough level should be taken within the first 2 or 3 days of starting therapy. Because absorption and clearance change during the first weeks of therapy, levels should be obtained at least two or three times weekly. After discharge from the hospital, levels should be obtained at least twice per week until the blood levels are stable usually within 1 or 2 months. Chronic

cyclosporine dosing is guided by weekly and then monthly blood concentrations and serum creatinine levels, with most patients requiring approximately 5 to 6 mg/kg cyclosporine daily.

2. Tacrolimus[2,22,23]

Tacrolimus (FK506) is a macrolide antibiotic isolated from *Streptomyces tsukubaensis*, whose immunosuppressive properties were discovered in a large-scale screening process to identify new immunosuppressive agents. In contrast to all other U.S. Food and Drug Administration (FDA)-approved immunosuppressive agents developed to date, the clinical development of tacrolimus was conducted primarily in liver rather than kidney transplant recipients. After initial FDA approval of tacrolimus in liver transplantation in 1994, pivotal phase III studies were conducted in renal transplant recipients that led to an additional indication in renal transplantation in 1996. Although indications have not been achieved in other organs, tacrolimus has shown efficacy in rejection therapy and as a primary maintenance immunosuppressive agent in heart, lung, pancreas, and small bowel transplantation. Clinical use of tacrolimus has increased markedly so that approximately three fourths of all new liver transplant recipients in the United States receive tacrolimus as their primary immunosuppressive agent. Tacrolimus is viewed widely as preferable to cyclosporine for maintenance immunosuppression in high-immunological risk renal allograft recipients (repeat renal transplant recipients, high-panel reactive antibody renal transplant recipients). More recent experiences indicated that tacrolimus may have additional properties, including steroid-sparing properties.

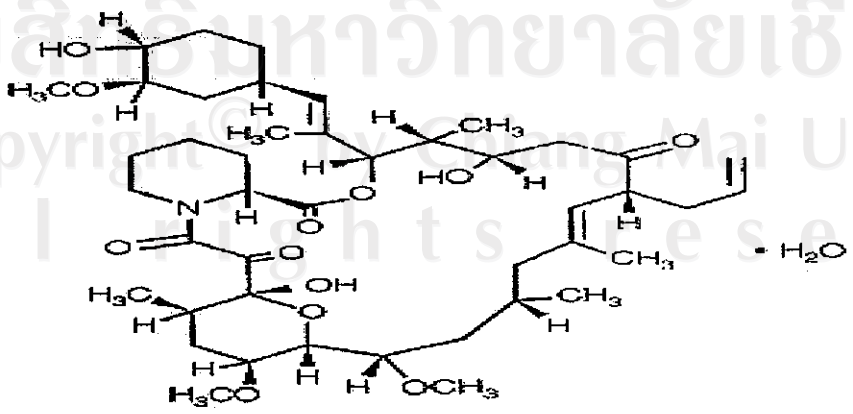

Figure 4. Structure of tacrolimus[24].

2.1 Clinical pharmacology and therapeutics

Mechanism of action

Figure 2 illustrates mechanism of action of cyclosporine and tacrolimus, which inhibit early T-cell calcium-dependent signaling events following T-cell receptor (TCR) triggering. These inhibitory effects result in inhibition of expression of several cytokine genes, including IL-2, IL-3, IL-4, interferon-gamma, and tumor necrosis factor-gamma. Although cyclosporine and tacrolimus possess similar mechanisms of action, tacrolimus is 50 to 100 times more potent than cyclosporine in inhibiting T-cell activation *in vitro*. Tacrolimus and cyclosporine bind to intracellular receptors that are members of a family of intracytoplasmic proteins termed *immunophilins*. Cyclosporine binds intracellular receptors termed *cyclophilins*, whereas tacrolimus binds intracellular receptors termed *FK506 binding proteins* (FKBPs). Binding FKBPs by tacrolimus results in formation of a drug-immunophilin complex that represents the active form of the drug.

The FK506/FKBP complex and cyclosporine/cyclophilin complexes have been shown to bind calcineurin, a serine/threonine phosphatase. These binding complexes cause a slight change in receptor structure that inactivate the phosphatase activity of calcineurin. Calcineurin is normally activated following the intracellular rise in calcium after TCR ligation. Activation of calcineurin phosphatase activity results in dephosphorylation of the cytoplasmic component of nuclear factor of activated T cells ($NF\text{-}AT_c$), allowing its subsequent translocation to the nucleus. After translocation, $NF\text{-}AT_c$ combines with the nuclear component, and the resultant complex binds to the enhancer region of the IL-2 gene, leading to up-regulation of gene transcription. When complexes inactivate the phosphatase activity of calcineurin, $NF\text{-}AT_c$ no longer binds the enhancer region of the IL-2 gene and blocks its transcription.

Therapeutic use

Tacrolimus is indicated for the prophylaxis of organ rejection in patients receiving allogeneic liver or kidney transplants. It is recommended that tacrolimus be used concomitantly with adrenal corticosteroids. Because of the risk of anaphylaxis, tacrolimus injection should be reserved for patients unable to take tacrolimus capsules orally.

2.2 Clinical pharmacokinetics

Table 6 illustrates pharmacokinetic parameters of tacrolimus. Pharmacokinetic characteristics of tacrolimus in humans are highly variable in transplant patients due to interpatient variability, and individualization of dosing regimen for optimal therapy.

Absorption

Absorption of tacrolimus from the gastrointestinal tract after oral administration is incomplete and variable. Tacrolimus C_{max} and AUC appear to increase in a dose-proportional fashion in 18 fasted healthy volunteers receiving a single oral dose of 3, 7 and 10 mg.

In 18 kidney transplant patients, tacrolimus trough concentrations from 3 to 30 ng/ml measured at 10-12 hours post-dose (C_{min}) correlate well with the AUC (correlation coefficient 0.93). In 24 liver transplant patients over a concentration range of 10 to 60 ng/ml, the correlation coefficient is 0.94.

Table 6. Summary of tacrolimus pharmacokinetics[a]

Parameter	Average ± SD
Bioavailability in tissue transplant patients (%)	
Renal	17 ± 10
Liver (adult)	22 ± 6
Protein binding (%)	99
Clearance in tissue transplant patients (l/h/kg)	
Renal	0.083
Liver	0.053
T_{max} (h)	1.5 - 3.5
V_d (l/kg)	0.85
Distribution $t_{1/2\alpha}$ (h)	5.7
Elimination $t_{1/2\beta}$ (h)	
Liver	11.7
Hepatic metabolism (%)	>99

[a]All parameters based on whole blood samples.

The rate and extent of tacrolimus absorption are greatest under fasted conditions. The presence and composition of food decrease both the rate and extent of tacrolimus absorption when administered to 15 healthy volunteers. The effect is most pronounced with a high-fat meal (848 kCal, 46% fat): mean AUC and C_{max} are decreased 37% and 77%, respectively; T_{max} is lengthened 5 fold. A high-carbohydrate meal (668 kCal, 85% carbohydrate) decreases mean AUC and mean C_{max} by 28% and 65%, respectively. In healthy volunteers (N=16), the time of the meal also affects tacrolimus bioavailability. When given immediately following the meal, mean C_{max} is reduced 71%, and mean AUC is reduced 39%, relative to the fasted condition. When administered 1.5 hours following the meal, mean C_{max} is reduced 63%, and mean AUC is reduced 39%, relative to the fasted condition. In 11 liver transplant patients, tacrolimus administered 15 minutes after a high fat (400 kCal, 34% fat) breakfast, results in decreased AUC (27 ± 18%) and C_{max} (50 ± 19%), as compared to a fasted state.

Distribution

The plasma protein binding of tacrolimus is approximately 99% and is independent of concentration over a range of 5-50 ng/ml. Tacrolimus is bound mainly to albumin and alpha-1-acid glycoprotein, and has a high level of association with erythrocytes. The distribution of tacrolimus between whole blood and plasma depends on several factors, such as hematocrit, temperature at the time of plasma separation, drug concentration, and plasma protein concentration. In a U.S. study, the ratio of whole blood concentration to plasma concentration averages 35 (range 12 to 67).

Metabolism

Tacrolimus is extensively metabolized by the mixed-function oxidase system, primarily the cytochrome P-450 system (CYP3A). A metabolic pathway leading to the formation of 8 possible metabolites has been proposed. Demethylation and hydroxylation have been identified as the primary mechanisms of biotransformation *in vitro*. The major metabolite identified in incubations with human liver microsomes is 13-demethyl tacrolimus. In *in vitro* studies, a 31-demethyl metabolite has been reported to have the same activity as tacrolimus.

Excretion

The mean clearance following IV administration of tacrolimus is 0.040, 0.083 and 0.053 ml/h/kg in healthy volunteers, adult kidney transplant patients and adult liver transplant patients, respectively. In man, less than 1% of the dose administered is excreted unchanged in urine.

2.3 Pharmacodynamics

Drug-drug interactions

Drug interaction studies with tacrolimus have not been conducted. Due to the potential for additive or synergistic impairment of renal function, care should be taken when administering tacrolimus with drugs that may be associated with renal dysfunction. These include, but are not limited to, aminoglycosides, amphotericin B, and cisplatin. Initial clinical experience with the co-administration of tacrolimus and cyclosporine results in additive/synergistic nephrotoxicity. Patients switched from cyclosporine to tacrolimus should receive the first tacrolimus dose no sooner than 24 hours after the last cyclosporine dose. Dosing may be further delayed in the presence of elevated cyclosporine levels.

Since tacrolimus is metabolized mainly by the CYP3A enzyme systems, substances known to inhibit these enzymes may decrease the metabolism of tacrolimus with resultant increases in whole blood or plasma concentrations. Drugs known to induce these enzyme systems may result in an increased metabolism of tacrolimus and decrease whole blood or plasma concentrations. Monitoring of blood concentrations and appropriate dosage adjustments are essential when such drugs are used concomitantly. Drug interactions between tacrolimus and other drugs are summarized in Table 7.

2.4 Adverse reactions

In general, the adverse reactions of tacrolimus are similar to cyclosporine (Table 8). The physiological effects including reduction in renal blood flow and glomerular filtration, are similar between tacrolimus and cyclosporine. The pathological manifestations of tacrolimus and cyclosporine toxicity are similar because they induce tubular vacuolization and arteriolar nodular hyalinosis that are indistinguishable. In general, most studies have shown that the frequency of nephrotoxicity of tacrolimus and cyclosporine are similar.

Table 7. Clinically important drug interactions of tacrolimus

Drugs	Effects	Management
Nephrotoxic agents Aminoglycosides Amphotericin B Cisplatin	The potential for additive or synergistic impairment of renal function	Take care when administering tacrolimus with drugs that may be associated with renal dysfunction.
Cyclosporine	Additive/synergistic nephrotoxicity; tacrolimus blood levels may also be increased.	Give first tacrolimus dose no sooner than 24 h after the last cyclosporine dose.
Antifungal agents Bromocriptine Calcium channel blockers Cimetidine Clarithromycin Danazol Diltiazem Erythromycin Methylprednisolone Metoclopramide	These agents may increase tacrolimus blood levels.	Reduce tacrolimus dose with frequent monitoring of levels.
Carbamazepine Phenobarbital Phenytoin Rifamycins	These agents may decrease tacrolimus blood levels.	Increase tacrolimus dose with frequent monitoring of tacrolimus levels.
Vaccines	Affected vaccination.	During treatment with tacrolimus, vaccination may be less effective; avoid the use of live vaccines.

Table 8. Tacrolimus adverse reactions (%)[a]

Adverse reactions	Tacrolimus (N=250)	CBIR[b] (N=250)
CNS		
Headache	31 - 64	20 - 60
Tremor	44 – 56	30 - 46
Insomnia	29 – 64	21 – 68
Paresthesia	15 – 40	13 – 30
GI		
Diarrhea	32 – 72	23 - 47
Nausea	30 – 46	22 - 37
Constipation	19 – 24	20 - 27
LFT abnormal	5 – 36	2 - 30
Anorexia	6 – 34	4 - 24
Vomiting	12 – 27	9 -15
GU		
Kidney function abnormal	33 – 40	18 - 27
Creatinine increased	19 – 39	16 - 25
BUN increased	8 – 30	7 - 22
Urinary tract infection	16 – 19	18
Oliguria	16 – 18	8 - 15
Hemic/Lymphatic		
Anemia	4 – 47	1 - 38
Leukocytosis	8 – 32	7 - 26
Thrombocytopenia	10 – 24	14 - 20
Metabolic/Nutritional		
Hyperkalemia	10 – 45	7 - 26
Hypokalemia	11 – 29	14 - 34
Hyperglycemia	29 – 47	16 - 38
Hypomagnesemia	15 – 48	8 - 45
Respiratory		
Pleural effusion	30 – 32	29 - 32
Atelectasis	5 – 28	4 - 30
Dyspnea	3 – 29	2 - 23
Skin/Appendages		
Pruritus	11 – 36	5 - 20
Rash	8 – 24	3 - 19
Miscellaneous		
Abdominal pain	26 – 59	20 - 54
Hypertension	31 – 47	35 - 56
Pain	19 – 63	14 - 57
Fever	15 – 48	18 - 56
Asthenia	7 – 52	4 - 48
Back pain	13 – 30	14 - 29
Ascites	5 – 27	6 - 22
Peripheral edema	10 – 26	11 – 26

[a]Data are pooled from separate U.S. and European studies and are not necessarily comparable.
[b]CBIR = Cyclosporine-based regimen.

The metabolic effects of cyclosporine and tacrolimus and their severities are similar, including hyperkalemic metabolic acidosis and hypomagnesemia. The U.S. Multicenter Phase III trial comparing tacrolimus and cyclosporine as primary immunosuppressive agents shows a higher incidence of diabetes with tacrolimus than with cyclosporine.

Gastrointestinal disturbances (anorexia, nausea and abdominal cramping) are seen commonly with tacrolimus and are similar to those experienced with erythromycin therapy and are thought to be typical macrolide-antibiotic adverse reactions. Tacrolimus neurotoxicity is similar to cyclosporine in manifestations, severity and frequency. Typical manifestations include sleep disturbance, tremor, nightmares, seizures and coma. Seizures when observed with tacrolimus should be treated in a manner similar to those that occur under cyclosporine therapy. Peripheral neuropathy may occur with tacrolimus and when observed requires immediate conversion to cyclosporine.

2.5 Therapeutic regimen design

Therapeutic monitoring

Monitoring of tacrolimus blood concentrations in conjunction with other laboratory and clinical parameters is considered an essential aid to patient management for the evaluation of rejection, toxicity, dose adjustments and compliance. Factors influencing frequency of monitoring include but are not limited to hepatic or renal dysfunction, the addition or discontinuation of potentially interacting drugs and the posttransplant time. Blood concentration monitoring is not a replacement for renal and liver function monitoring and tissue biopsies.

Two methods have been used for the assay of tacrolimus, a microparticle enzyme immunoassay (MEIA) and an ELISA. Both methods have the same monoclonal antibody for tacrolimus. Comparison of the concentrations in published literature to patient concentrations using the current assays must be made with detailed knowledge of the assay methods and biological matrices employed. Whole blood is the matrix of choice and specimens should be collected into tubes containing ethylene diamine tetraacetic acid (EDTA) anti-coagulant. Heparin anti-coagulation is not recommended because of the tendency to form clots on storage. Samples which are not analyzed immediately should be stored at room temperature or in a refrigerator and

assayed within 7 days; if samples are to be kept longer they should be deep frozen at -20°C for up to 12 months.

2.6 Dosage and administration

Tacrolimus therapy for rejection is commonly instituted at doses of 0.1 mg/kg given twice a day. Target trough concentrations should be in the range of 10 to 15 ng/ml or up to 20 ng/ml in patients experiencing more vigorous forms of rejection. Many investigators experienced with tacrolimus therapy have observed that short-term (a few days) exposure to higher levels of tacrolimus can provide control of rejection in the most refractory cases. Primary tacrolimus therapy is usually initiated at 0.05 to 0.1 mg/kg/d given twice daily with target concentration ranges between 8 and 20 ng/ml. Long-term tacrolimus therapy can be maintained at target concentration ranges between 4 and 8 ng/ml.

Due to the potential for nephrotoxicity, patients with renal or hepatic impairment should receive doses at the lowest value of the recommended IV and oral dosing ranges. Further reductions in dose below these ranges may be required. Tacrolimus therapy usually should be delayed up to 48 hours or longer in patients with postoperative oliguria.

3. Hyaluronan (Hyaluronic acid, HA)[10,25,26,27]

In 1934, Meyer and his assistant, Palmer, described a procedure for isolating a novel glycosaminoglycan from the vitreous of bovine eyes. They showed that this substance contains a uronic acid and an aminosugar, but no sulfoesters. So they named "hyaluronic acid", which consists of hyaloid (vitreous) and uronic acid. The name "hyaluronan" (HA) should be used, when the polysaccharide is described in general term; however, commercial preparations are generally sold as the sodium salt.

In 1950s, Meyer and his colleagues determined the complete structure of hyaluronic acid. By chemical and enzymatic methods they established that HA is a linear polymer built from repeating disaccharide units with the structure..[D-glycuronic acid (1-β-3) N-acetyl-D-glucosamine (1-β-4)n]..(Figure 5).

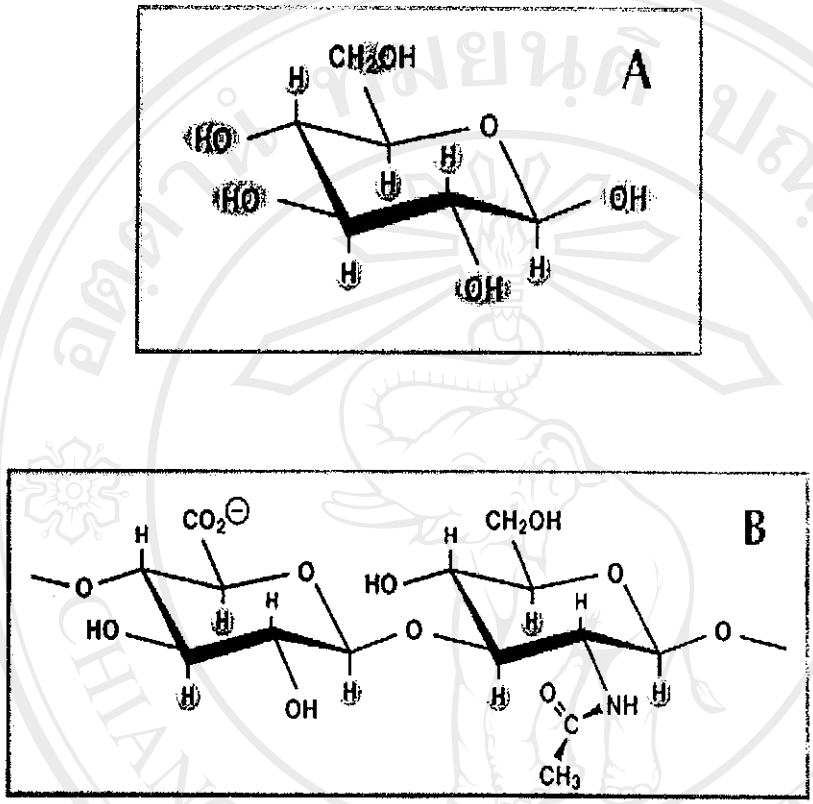

Figure 5. Relationship between beta-D-glucose (A) and the repeat disaccharide of HA, -D-glucuronic acid-beta-1, 3-N-acetylglucosamine-beta-1,4- (B)[28].

HA is present in all vertebrates, perhaps arising in animals with notochords. It is also present in the capsule of some strains of Streptococci that quite likely pirate the enzymatic machinery for its synthesis from vertebrate hosts. HA is a major constituent of the extracellular matrices in which most tissues differentiate. It is also an essential component of many extracellular matrices in mature tissues. In some cases, HA is a major constituent; as, for example, in the vitreous of the human eye, or in synovial joint fluid. In others, while representing less of the mass of the tissue, HA serves as an essential structural element in the matrix. For example, HA is present in hyaline cartilages, enough to fill the tissue volume in the absence of other constituents. However, aggrecan, the large chondroitin sulfate proteoglycan, is present at a much higher concentration and HA retains aggrecan molecules in the matrix through specific protein-HA interactions which mask the HA backbone. However, the concentration of HA varies greatly from one tissue to another (Table 9), as do the relative proportions of other glycosaminoglycan and collagens.

Table 9. Concentration of HA in some tissue and fluid

Source	Concentration (mg/l)
Human umbilical cord	4,100
Human synovial fluid	1,420-3,600
Bovine nasal cartilage	1,200
Human vitreous body	140-338
Human dermis	200
Rabbit brain	64
Rabbit muscle	27
Human thoracic lymph	8.5-18
Human urine	0.1-0.5
Human serum	0.01-0.1

3.1 Biosynthesis

Several reports have shown that HA is synthesized in the plasma membrane, in contrast to other glycosaminoglycans made in the golgi. The latter also differ in that they are covalently linked to proteins, forming proteoglycans, and usually carry sulfate groups. In eukaryotes, the HA synthase is located in the plasma membrane where HA is synthesized directly into the extracellular matrix. Prehm et al. demonstrated that HA synthesis differs from that of other glycosaminoglycans; its chains are elongated at the reducing end by alternate transfer to the substrates UDP-glucuronic acid (UDP-GlcA) and UDP-N-acetylglucosamine (UDP-GlcNAc). This occurs inside the plasma membrane, and the chain (with the nonreducing end ahead) is translocated to the pericellular space. The other glycosaminoglycans grow by addition of sugars to the nonreducing end. The eukaryotic synthase is identified as a 50 Da protein and shows immunological cross-reaction with the streptococcal enzyme and affinity labelling. The activity of the HA synthase correlates with a variety of cellular parameters, such as cell growth, transformation, metastasis and mitosis.

3.2 Physiological and cell biological functions

The main biological function of HA is still unknown but several roles have been assigned to it. The cartilage proteoglycan, aggrecan, is bound specifically to HA chains, the bond being stabilized by so-called link proteins. The aggregated forms have masses on the order of 10^8 Da and are deposited within the collagen framework. Without this interaction, the proteoglycans would not be retained in cartilage. HA has been assigned various roles in the homeostasis of the extracellular space. HA and other polysaccharides regulate the distribution and transportation of plasma proteins in the tissues. The synthesis of HA seems to be maximal during mitosis and whenever rapid tissue proliferation and regeneration occur. A possible function would be that HA, growing out from the cell surface, detachment of the cell from its supporting matrix so that it can divide more easily. In 1989, it has been reported that the lymphocyte homing receptor CD44 shows a striking homology, with cartilage proteins that bind HA. It was soon verified that CD44 can bind HA and that CD44 apparently is identical with the HA receptor characterized by Underhill and Toole. In 1993, Thomas suggested that CD44 may play a role in stimulating *in vivo* aggressiveness of tumors through HA-rich stroma. HA has been connected with various

processes during morphogenesis and differentiation. HA concentration often increases in compartment where cellular migration is going to take place- one example is the neural crest cell migration- and it has been suggested by Toole et al. that the polysaccharide opens up migration paths. Tumors are often enriched in HA and in some tumors elevated levels are correlated with invasiveness. Tumor cells do not always make HA themselves but can stimulate other cells to produce the polysaccharide.

3.3 Turnover and catabolism

The major route of transport of HA from the tissues to the circulation is via the lymphatics. The turnover of HA in the bloodstream is normally in the range of 0.3-10 mg/min/body weight. It has been calculated that 10-100 mg of HA enters the bloodstream everyday in the adult human. However, a considerable part of HA is already metabolized in the lymph node. Lymph nodes may extract as much as 80-90% of HA from peripheral lymph before it can reach the bloodstream, more than 90% of ^{3}H-HA injected into afferent lymph vessel of peripheral sheep lymph node is cleared in the node, indicating that the normal total daily turnover of HA from the tissue may be very high. By the use of a ^{3}H-labeled HA, it has been shown that the normal half-life of HA in the circulation in man and rabbit is 2-6 minutes, indicating that about 25% of plasma HA contents is eliminated each minute.

The elimination of circulation HA takes place mainly in the liver via receptor-mediated endocytosis in the endothelial cells. HA is subsequently degraded in the lysosomes that contain hyaluronidase, β-glucuronidase, and β-N-acetyl-glucosaminidase, the end products are lactate and acetate. HA receptors on liver endothelial cells have greater affinity to higher than lower molecular weight HA, the higher molecular weight HA is more rapidly cleared from the bloodstream than low molecular weight material. The average molecular weight of HA in serum is approximately 140-160 kDa.

Recently has also been observed that there is a diurnal variation in the serum concentration of HA in man, and that this increase is seen during the first hour after rising. Early morning physical activity may cause an increase lymphatic outflow of HA that has accumulated in the tissues during the night, it was shown that exercise can increase the serum HA concentration.

A small amount of HA is found in the urine, approximately 1% of the total daily turnover in the circulation is excreted via the kidney. The HA that is eliminated in urine has a considerably lower molecular weight than that in plasma.

3.4 Measurement of HA

Earlier methods of serum HA assay often used multistep procedures. The first step usually precipitated total glycosaminoglycan with cetyl pyridinium chloride. The precipitate is separated by electrophoresis and the individual components quantified by densitometry. An alternative procedure measures the cetyl pyridinium complexes by nephelometry, before and after digestion with streptomyces hyaluronidase. HPLC techniques have also been described for the measurement of HA.

The development of radioimmunometric assays lead to a great increase of information about HA in health and diseases. These assays are based on the isolation of binding proteins for HA from bovine cartilage and human brain, called hyaluronectin (HN). In the assay developed by Laurent and Tengblad, bovine cartilage HA binding protein is incubated with varying amounts of free HA and a fixed amount of HA bound to Sepharose gel. The binding protein is allowed to partition between free and bound polysaccharides. The amount of radioactivity pelleted with the gel is a function of the amount of free HA in the sample. This assay has been developed into a commercial assay by Pharmacia Diagnostics AB, Sweden, with a working range of 4-50 mg/l and a coefficient of variation of about 10%.

Delpech has described an enzyme-linked immunosorbent assay (ELISA) using microtitre plates coated with HA, exploiting the inhibition of hyaluronectin binding by soluble HA. The HN bound to the plate is revealed by alkaline phosphatase conjugated anti-HN antibodies. This assay and that of Pharmacia detect high molecular weight fragments. A recent ELISA method takes advantage of an antikeratan sulfate antibody to differentiate between coated aggregating rat chrondrosarcoma proteoglycan, which captures the HA, and keratan sulfate-bearing aggregating proteoglycan subsequently added. The range of this assay is linear to the logarithmic concentration of HA in the range of 15-10,000 mg/l, and measures HA > 10,000 mg/l molecular weight. Another ELISA has been described which depends on the specific binding of HA to the hyaluronic acid binding region of proteoglycan. The remaining uncomplexed proteoglycan

monomers are determined by incubation with specific monoclonal antibodies to hyaluronic acid binding region followed by the addition of polyclonal antibodies against proteoglycan monomers and enzyme conjugated antibodies. Sample HA are quantified by their inhibitory capacity in the assay. The inhibition test using biotinylation technique for determination of HA in serum has been developed based on the specific interaction of hyaluronic acid binding protein with HA in the range of 10-10,000 ng/ml.

3.5 Clinical application of HA

The introduction of immunologically based assay has enabled serum and urinary HA levels to be studies in a wide spectrum of diseases. Several areas have been investigated so far, the main clinical application at present are related to liver disease, chronic renal failure, rheumatology, and some forms of neoplastic diseases. The other disorders, such as respiratory diseases are still at an early stage of investigation.

Markedly high serum levels are noted in certain liver diseases, especially in patients with cirrhosis, when the clearance is impaired. In these cases serum HA can be used to follow the development of the disease. Serum HA is also a sensitive marker for impending rejection of liver transplants. Patients with rheumatoid arthritis constitute another major group with increased serum HA, but in this case the level varies markedly during the day corresponding to physical activity. There are good indications that in these subjects the excess HA comes from the joints. Under stringent sampling conditions of serum, it should be possible to extract interesting information on the inflammatory joint process. Increased HA levels are also seen in other inflammatory diseases and it is of special interest that high HA levels in patients with septic conditions is a sign of poor prognosis. Certain tumors, notably Wilms' tumor and mesothelioma, produce factors that activate synthesis of HA and increase its serum level. Rare hereditary diseases with disturbances of HA metabolism and elevated blood levels have also been discovered, e.g., Werner's syndrome and cutaneous hyaluronanosis. Information accumulated during the last decade regarding the metabolism of HA has made this polysaccharide an interesting clinical marker for a number of pathological conditions.

OBJECTIVES

1. To investigate the correlation of hyaluronan level and immunosuppressive drug levels in renal transplant patients.

2. To study the level of hyaluronan in renal transplant patients as a prognostic marker for monitoring rejection.