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Disposition and Pharmacodynamics of Verapamil Enantiomers in the Presence of Inflammation.

By

Patrick Rogers Mayo

A Thesis Submitted To The Faculty Of Graduate Studies And Research In Partial Fulfillment Of The Requirements For The Degree Of Doctor Of Philosophy.

In

Pharmaceutical Sciences

Faculty of Pharmacy and Pharmaceutical Sciences

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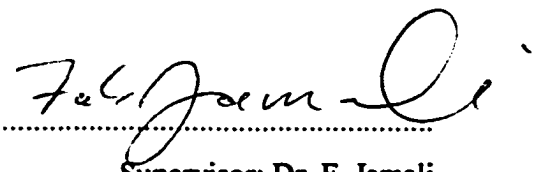

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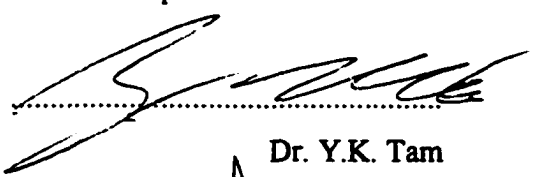

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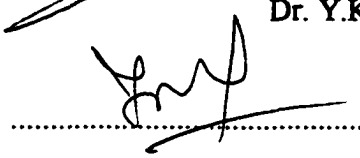

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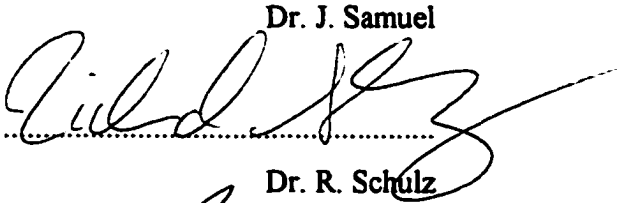

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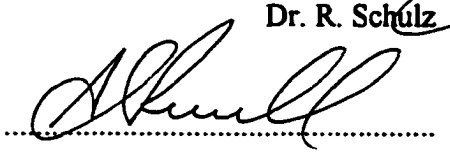

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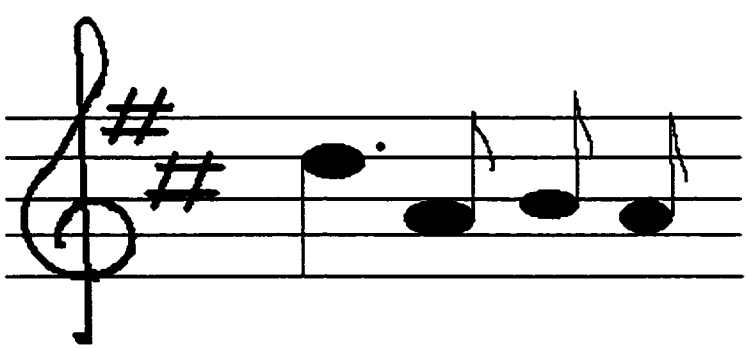
"I merely started with this one simple idea

that education should never be dissociated from life."

-Rabindranath Tagore

$S = K \, Log \, W$

- Ludwig Boltzmann



-Georg Fredrich Handel

This thesis is dedicated to my sons Tom and Max and my wife Leslie for all their patience, understanding, love and constant support.

ABSTRACT

Introduction: Animal and human studies suggest that cytochrome P450 mediated drug metabolism is diminished in the presence of inflammation or inflammatory mediators such as pro-inflammatory cytokines and nitric oxide. Thus, inflammation could increase the plasma concentration of drugs, however, the pharmacodynamic consequence of this observation is not known. Verapamil (VER) is a drug that is extensively metabolized by CYP450 isozymes and is commonly used for the treatment of hypertension and supraventricular tachyarrhythmias. The purpose of these studies was to evaluate the effect of inflammation on the pharmacokinetics and pharmacodynamics of VER enantiomers.

Methods:

The effects of inflammation were studied in a transgenic HLA-B27, human β2-microglobulin rat model of inflammation, an interferon-α2a treated rat model and in human rheumatoid arthritis patients. Serial blood samples were drawn concurrently with electrocardiograms (ECG) to determine the pharmacokinetics and pharmacodynamics of VER respectively. In all studies VER was measured using a stereospecific HPLC assay and the pharmacokinetics determined using non-compartmental analysis. The pharmacodynamic effects of VER were determined using surface ECG leads. Serum nitrite was measured as a marker for reactive nitrogen species such as nitric oxide.

Results: A decrease in the oral clearance of VER enantiomers with a corresponding rise in serum VER concentration was observed only in the presence of elevated serum nitrite. This occurred in the human rheumatoid arthritis patients and in interferon-α2a-treated rats. The HLA-B27 transgenic model of inflammation was not associated with any significant changes in VER pharmacokinetics or serum nitrite concentrations.

Surprisingly, despite elevated serum VER concentrations in the human rheumatoid arthritis patients and interferon-α2a-treated rats, less dromotropic effect was observed as measured by prolongation in PR-interval. The transgenic HLA-B27 rats also demonstrated less dromotropic effect.

Conclusions: Decrease oral clearance of VER enantiomers was observed only in the presence of elevated serum nitrite. Despite the increase in systemic drug exposure, less dromotropic activity was observed. This same decrease in dromotropic effect was observed in the transgenic HLA-B27 rats without a change in pharmacokinetics or in reactive nitrogen species. These studies suggest that inflammation-induced changes in drug disposition involve the release of reactive nitrogen species, however pharmacodynamic changes may occur in a reactive nitrogen species independent manner.

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Extra-special thanks Dr. Lise Eliot of the yellow lights and the "green-ink crew" Michael Guirguis and Dr. Ray Lovlin. These people taught me that it isn't the size of your heart, but how fast it beats. My fellow graduate students who helped me learn and put up with my strange schedules especially Ken Kulmatykci.

Finally, I would like to thank the pharmacy staff of the Cross Cancer Institute for their kind donation of cytokines, and the pharmacy and cardiology staff of the Misericordia Hospital for their continued support of my research.

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LIST OF SYMBOLS, NOMENCLATURE OR ABBREVIATIONS

AAG	α1-acid glycoprotein
$AUC_{0-\infty}$	Area under the serum concentration time curve from zero to infinity
AUC_{0-t}	Area under the serum concentration time curve from zero to time t
$AUCu_{0-\infty}$	Area under the serum concentration time curve of unbound drug from zero to infinity
Cli'	Unbound Intrinsic Clearance
CL/F	Oral Clearance
CV	Coefficient of Variation
CYP450	Cytochrome P450
C_t	Total drug concentration
C_u	Unbound drug concentration
E	Hepatic Extraction Ratio
ECG	Electrocardiogram
ESR	Erythrocyte sedimentation rate
FAD	Flavine adenine dinucleotide
fu	Fraction unbound
h	Hour
HLA	Human leukocyte antigen
IFN	Interferon
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase

Km	Michaelis-Menten Constant, Affinity, ½ Vmax
λz	Terminal Elimination rate constant
L-NAME	L-nitro-arginine-methyl-ester
L-NMMA	L-N-monomethyl-arginine
MF	Methoxyflurane
MHC	Major histocompatibility complexes
mg	Milligram
mL	Millilitre
μM	Micro-molar
min	Minute
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
ng	Nanogram
nNOS	Neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NOR	Norverapamil
NOS	Nitric Oxide Synthase
PK-PD	Pharmacokinetic-Pharmacodynamic
$ONOO^-$	Peroxynitrite
OXP	Oxprenolol
Poly I:C	Polyriboinosinic, polyribocytidylic acid
Q	Hepatic Blood Flow
r	Pearson correlation coefficient

RA	Rheumatoid arthritis
SEM	Standard error of the mean
sc	Subcutaneous
Th	T-helper cells
Tmax	Time to reach peak or maximum concentration after drug administration
t1/2	Elimination half-life
TNF-α	Tumour Necrosis Factor-α
UDPGT	Uridine-diphosphate-glucuronosyl-transferase
VER	Verapamil
Vmax	Maximum Velocity of Enzymatic Reaction

Chapter 1

INTRODUCTION

Verapamil (VER) is a calcium channel blocker that is extensively metabolized by cytochrome P450 (CYP450) isozymes. The specific isozymes involved are CYP3A4, CYP1A2, and CYP2C (Kroemer *et al.* 1993). It is widely used to treat supraventricular tachyarrythmias and has a well-defined concentration-effect relationship (Echizen *et al.* 1985). Pro-inflammatory cytokines have been shown to decrease CYP450 activity (Cawthorne, Palmer and Green 1976). Animal models of inflammation such as injection of carageen or bacterial lipopolysaccharide also results in a decrease in CYP450 activity or amount (Ferrari *et al.* 1993a, Abdel-Rassak *et al.* 1993). Decreases in CYP450 amount or activity could result in significant pharmacokinetic and pharmacodynamic changes for drugs metabolized by these enzymes. Clinically, this suggests that patients with systemic inflammatory disease such as rheumatoid arthritis (RA) may be at risk for enhanced drug effect or drug toxicity. Despite the plethora of scientific literature demonstrating decreased CYP450 amount and function little work has been done on the pharmacodynamic consequences of these observations. The scientific literature supports the hypothesis that the inflammatory process could alter both a drug's pharmacokinetics and pharmacodynamics. It is probable that the mechanism involves either direct or indirect effects of pro-inflammatory cytokines. Indirect effects could be mediated by nitric oxide (NO). Pro-inflammatory cytokines such as, interleukin-1β (IL-1β),

interleukin-2 (IL-2), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) may stimulate the production of an inducible, pathological form of nitric oxide synthase (iNOS) (Schulz and Triggle 1995). NO has been demonstrated to decrease CYP450 function through free radical destruction of the enzymatic heme site (Khatsenko *et al.* 1993). Furthermore, cytokines have been shown to directly cause the expression of iNOS resulting in significant myocardial depression in cardiac myocytes with a decrease in contractile and β-adrenergic agonist responsiveness (Balligand *et al.* 1993). Thus, cytokines could alter both drug pharmacokinetics and pharmacodynamics through the production of NO. Chronic inflammatory disease such as rheumatoid arthritis results in the release of pro-inflammatory cytokines and therefore, NO (Abbas *et al.* 1997, Harris 1990). This suggests that inflammation may change pharmacokinetics with resultant changes in pharmacodynamics, plus a direct alteration of normal pharmacodynamic response. However, some studies have suggested that NOS induction is merely a coincidental event and that cytokines have NO-independent effects on hepatic function (Sewer and Morgan 1997). Therefore, this study was undertaken to further elucidate the relationship amongst inflammation or inflammatory cytokines, reactive nitrogen species such as nitric oxide, drug metabolism and cardiac effects using the calcium channel blocker verapamil and a combined pharmacokinetic-pharmacodynamic approach.

1.0 RHEUMATOID ARTHRITIS

Rheumatoid arthritis is a chronic, systemic autoimmune disease characterized by inflammation of synovial tissues and destruction of articular cartilage (Harris 1990). It is associated with considerable morbidity and mortality.

1.1 Epidemiology

Rheumatoid arthritis demonstrates an age-related prevalence rate. The prevalence rate for adults under the age of 35 is only 0.3%, but increases to 10% in persons over the age of 65 (Mitchell *et al.* 1985, Kavanaugh 1997). This is important since the number of persons older than 65 are the most rapidly increasing segment of the North American population (U.S. Statistics). The proportion of the population over 65 was only 4% in 1900, 12.5% in 1984 and is projected to reach 21.8% by the year 2050 (US Department of Health 1985 –1986 Annual Report). Thus, the incidence of RA is expected to increase with the aging of the population. In addition, the elderly experience more disease and chronic conditions, which leads to increased drug consumption. A study by Cadieux in 1989 demonstrated that greater than 50% of persons older than 65 use between 5 to 12 drugs per day. This dramatically increases the potential of drug-drug and drug-disease interactions and emphasizes the need for of research in this area.

RA also demonstrates a gender-specific prevalence with females having a 2.5-fold greater risk of developing the disease (US Department of Health 1985 - 1986). Sex-hormones appear to be involved in this process with studies demonstrating a remission of RA during pregnancy, exacerbations post-partum, a slight decreased risk for patients on oral contraceptives and increased risk after

menopause (Dugowson *et al.*, 1988).

A genetic predisposition has also been demonstrated for RA. Studies on monozygotic twins demonstrate a 34% concordance rate whereas dizygotic twins demonstrate only a 3% concordance rate (US Department of Health 1985 –1986 Annual Report). Therefore, it appears that a genetic predisposition plus external triggers are necessary for the development of the disease. A specific genetic locus for development of RA has not been elucidated. However, 80% of RA patients are positive for the human leukocyte antigen (HLA) DR4 (Spack 1997).

1.1.1 Etiology

The cause of RA is unknown and probably involves multiple factors. A genetic predisposition plus environmental or infectious triggers is the most attractive hypothesis (Van Noort 1998). While bacteria and viruses are the most likely candidates, their involvement is inconclusive. Human T-cell lymphotropic Type-1 virus, other retroviruses, Epstein-Barr virus, herpes viruses, rubella and parvoviruses have been implicated. Bacteria such as mycoplasma, mycobacteria and enteric bacteria such as E. Coli and salmonella have also been implicated (Moreland and Koopman 1991). The infectious mechanism is further supported by the fact that certain proteins from Epstein-Barr virus and *E. Coli* bacteria (Tuckwell *et al.* 1992) mimic the beta-chain of HLA-DR4. Hence, RA could be an immune response to heat shock proteins from an infectious agent which is amplified and perpetuated due to mimicry between heat shock proteins and major histocompatibility complexes (MHC), antigens or molecules.

1.1.2 Pathology

Recently, much progress has been made in the understanding of the pathology of rheumatoid arthritis. While much work remains to be done, it is clear that rheumatoid arthritis involves the chronic activation of helper T-lymphocytes reactive against self-tissues. In this case synovial tissues and articular cartilage are damaged (Van Noort and Amor 1998, Weyand and Goronzy 1997). Initial injury of synovial microvascular endothelial cells results in leakage of leukocytes into the joints. This ultimately leads to attack of synovial tissues and destruction of articular cartilage by granulocytes such as neutrophils and monocytes. Neutrophil accumulation in synovial fluids is a common diagnostic event. Neutrophil migration is controlled by complement component C5a, leukotriene B4, platelet activating factor and interleukin-8 (IL-8). Once within the synovial joint the neutrophil will release proteinases, prostaglandins, leukotrienes and free radicals such as superoxide anion and nitric oxide (NO) that may combine to form peroxynitrite ($ONOO^-$). The endothelial cells do not have a passive role. Upon activation by pro-inflammatory cytokines such interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), endothelial cells will express "homing" molecules known as vascular adhesion molecules. These facilitate the binding of neutrophils to the endothelial wall, which is the first step of migration. Macrophages are a major component of the infiltrating mononuclear cells. They may be stimulated by IFN-γ, colony stimulating factors and IL-2. Once activated they will also produce various cytokines thus increasing the severity of the inflammation. A cycle ensues which leads to increased

inflammation, increased white cell migration, increased inflammatory mediators and increased damage to self-molecules especially articular cartilage. Since the reaction is toward self-molecules the process continues and damage is progressive.

Under normal immunological conditions the body can clearly recognize and respond to foreign antigens, but not to self-antigens. This is known as "self – nonself" discrimination. It is important to note that tolerance to self-antigens is an actively acquired process and not an inherited property (Abbas *et al.* 1997). Self-tolerance, the acquisition of non-responsiveness to ones own tissues, may be induced during lymphocyte development and activation in primary lymphyoid organs such as the thymus (central tolerance) or in secondary lymphoid tissues (peripheral tolerance). In central tolerance negative selection results in the deletion of lymphocytes specific for self-antigens. Peripheral tolerance may be due to deletion of lymphocytes that recognize self-antigen. Programmed cellular death (apoptosis) or immunosupression with anti-inflammatory cytokines may also be involved. Clearly under normal circumstances antibodies directed toward self should not be expressed. (Abbas *et al.* 1997) Paul Ehrlich, long remembered for his pioneering work on differential microbial staining, diptheria vaccine and the development of the antibiotic salvarsan (Ehrlich 606), vividly described the loss of self-tolerance and the subsequent immune reactions against self as "horror autotoxicus"(Abbas *et al.* 1997).

It is hypothesized that autoimmune diseases may occur after an environmental or infectious trigger. Antibodies are produced to deal with the

trigger, which are believed to be similar to antibodies directed towards "self" (Abbas *et al.* 1997). Therefore, a successful immunological response to a foreign trigger leaves the patient with active antibodies directed towards self-tissues. In rheumatoid arthritis, these autoantibodies are known as rheumatoid factors and are immunoglobulins, which react most commonly with the Fc portion of IgG and IgM molecules. However, it has been shown that rheumatoid factors can be formed for any immunoglobulin subclass (Harris 1990). This will lead to attack by T-cells and other immune cells of joint proteins causing an acute inflammatory response. T-cell mediated tissue injury can occur through several mechanisms. CD4+ T cells and the type 1 subset of CD8+ cells secrete proinflammatory cytokines such as IL-1β, TNF-α, IFN-γ IL-6 and IL-8 (Arend & Dayer 1990). These cytokines and others have been isolated in the inflammed synovium of RA patients where they are believed to activate resident synovial cells to produce proteolytic enzymes such as collagenase and metalloproteinases, and reactive oxygen species such as super oxide anion and hydroxyl radical. These species mediate the destruction of cartilage, ligaments and tendons of the joint. The production of growth factors by macrophages may result in fibrosis of tissues in the area further leading to dysfunction of the joint. (Abbas *et al.* 1997).

1.2 Drug Disposition in Rheumatoid Arthritis

1.2.1 Absorption

Research on inflammatory bowel disease has suggested that the absorption of drugs may be enhanced in the presence of increased intestinal permeability (Ma 1997). Intestinal epithelia exposed to cytokines such as IFN-γ, IL-4, and IL-13 *in*

vitro will demonstrate increased permeability (Sanders *et al.* 1995). While pro-inflammatory cytokines such as IL-1ß and TNF-α can increase intestinal permeability, it appears that direct exposure on the intestinal mucosa from intestinal macrophages is required (Tateishi *et al.* 1997). Therefore, patients with severe systemic inflammation could theoretically be predisposed to increased intestinal permeability and enhanced drug absorption. However, this has not been shown to occur in human patients. Patients with rheumatoid arthritis or ankylosing spondylitis without other inflammatory conditions do not demonstrate increased intestinal permeability when tested with low molecular weight polyethylene glycol (Liu *et al.* 1995). Since rheumatoid arthritis patients may also exhibit other inflammatory syndromes, increased intestinal permeability remains theoretically possible, but has not been reported. However, rheumatoid arthritis patients on non-steroidal anti-inflammatory drugs may easily develop increased intestinal permeability due to toxicity of the drug (Bjarnason and Peters 1996). Therefore, studies in this patient population should consider the possibility of altered absorption.

1.2.2 Protein Binding: Effects on Drug Clearance

It is well known that acute inflammatory conditions are associated with altered plasma protein concentrations. In particular, an acute exacerbation of rheumatoid arthritis is associated with hypoalbuminemia and an increase in α1-acid-glycoprotein and other acute phase reacting proteins such as C-reacting protein (Fey and Müller 1991). For example, an acidic drug highly bound to albumin would demonstrate an increase in free fraction with a concurrent increase

in volume of distribution. Van Den Ouweland *et al.* (1987) has observed this with naproxen. A basic drug such as propranolol is highly bound to AAG. Piquette-Miller and Jamali (1993) demonstrated increased protein binding of propranolol enantiomers to α1-acid-glycoprotein in adjuvant-induced arthritic female Wistar rats. Changes in protein binding may have other effects on a drug's pharmacokinetics. This is especially important for a drug that demonstrates restrictive clearance. That is, when the ratio of free intrinsic clearance (Cli') to the hepatic blood flow (Q) is small, then the extraction ratio is low (E). Under these conditions, the release of drug from its protein-binding site becomes the limiting factor to drug clearance (Wilkinson 1987). For a drug which is non-restrictively cleared, then Cli' is much greater than Q and clearance is much less sensitive to changes in protein binding. However, it should not be assumed that oral bioavailability is independent of protein binding since even a small change in drug extraction can have a larger effect on bioavailability (F) (Wilkinson and Shand 1975).

1.2.3 INFLAMMATION AND DRUG METABOLISM

It has become increasingly clear that autoimmune mediated inflammation has greater systemic effects than damage of articular cartilage. The mediators of this type of autoimmune inflammation have been shown to alter drug metabolism and drug response, which is the basis of this research.

1.2.3.1 Background

Serious inflammatory disease results in a plethora of biochemical responses designed for cellular protection (Harris 1990). Many of these responses

such as fever, changes in blood flow, cardiac output, renal and hepatic function and a rise in acute phase reacting proteins may also dramatically alter the normal pharmacokinetics and pharmacodynamics of drugs. This could have great clinical significance to drugs that undergo significant hepatic biotransformation, are extensively protein bound, or have a narrow therapeutic window. Historically, it has been noted that patients with rheumatoid arthritis or viral infection seem predisposed to adverse drug reactions (Poulton *et al.* 1998). Animal models of inflammatory diseases clearly demonstrate decreased cytochrome P450 (CYP450) content and activity in the liver (Cawthorne *et al.* 1976). Thus it is important to understand the inflammatory cascade not only for the treatment of inflammation, but also to understand and predict the effects of inflammatory disease on the pharmacokinetics and pharmacodynamics of drugs.

Inflammation involves an elegantly complex reaction of host cells to disease. Three cell types play a central role in host defense, immune response and homeostasis. These are polymorphonuclear leukocytes, monocyte-macrophages, and platelets (Abbas *et al.* 1997). Secretory products of these cells demonstrate the wide-ranging biochemical mediators of inflammation. These products are summarized in Table 1-1. It becomes clear that the patient with acute inflammation is a biochemically altered patient.

Table 1-1: Secretory Products of Phagocytes

Class	Examples
Polypeptide Hormones	Interleukins (IL-1 α & β) Tumor Necrosis Factor-α (Cachectin, TNF) Interferon-α ß-Endorphins IL-8, Neutrophil-activating factor
Complement Components	Classical : C1-5 Alternative: Factor B,D Inhibitors: C3b, ß-1H
Coagulation Factors	Intrinsic: IX,X,V, PT Extrinsic: VII
Bioactive Lipids	Cycloxygenase: PGE2, PGF2α Lipoxygenase: LTB4,C,D,E Platelet Activating Factors
Reactive Oxygen Species	Superoxide Anion, H_2O_2,$OH^{\bullet}$
Reactive Nitrogen Species	$NO^{\bullet}$, NO_2^-, NO_2^+, $ONOO^-$

Compiled from Arend and Dayer 1990, Harris 1990, Proulx and Du Souich 1995, Abbas *et al.* 1997. Beckman and Koppenol 1996.

Cytokines, especially the pro-inflammatory cytokines and reactive nitrogen species occupy a central role in the mediation of inflammation and possibly in the regulation of drug metabolism.

1.3 Cytokines

Antigenic stimulation results in the secretion of proteins or glycoproteins from immunocytes (Elsässer-Beile and von Kleist 1993). These proteins can communicate between different populations of leukocytes and were originally called lymphokines or simply, cytokines (Dumonde *et al.* 1969). Cytokines can be produced by virtually all nucleated cells especially lymphocytes, monocytes, immature thymocytes, astrocytes, endothelial cells, keratinocytes, fibroblasts, and

mesanglial cells (Elsässer-Beile and von Kleist 1993). Cytokine production can be induced by a wide variety of stimuli including microbial and viral antigens, plant lectins, mitogens, tumor cells, and complement factors. Additionally, cytokine production can be regulated by other cytokines (Elsässer-Beile & von Kleist, 1993). It is also important to note that cytokines are pleotropic having different effects in different tissues and/or target cells. Propagation of an *in vivo* immune response results in a characteristic lymphokine cascade. For example, the specific cascade for rheumatoid arthritis may differ from the cascade in order and amount for reactive arthritis or anklyosing spondylitis (Simon *et al.* 1994, Mohler and Butler 1990). Thus, depending on the disease-state and its specific cytokine pattern different effects on drug kinetics and dynamics could be seen. Clinically, this implies some inflammatory or infectious diseases may predispose the patient to prolonged and exaggerated therapeutic or toxic effects while other diseases may not. Thus, the specific drug effect or side effect may prove to be both drug *and* disease state-dependent.

1.4 Hepatic Drug Metabolism

Animal models using adjuvant-induced arthritis have demonstrated a marked reduction in N-demethylase, $NADPH_2$-oxidase and CYP450 activity (Morton and Chatfield 1970, Cawthorne *et al.* 1976, Whitehouse 1973). In addition to these Phase I reactions, Phase II conjugation reactions have also been reported to be diminished in the presence of inflammation (Monshouwer *et al.* 1996). For example, the excretion of acetaminophen conjugates is diminished in the presence of adjuvant-induced arthritis (Morton and Chatfield 1970). Sasaki *et*

al. 1990 and Ishikawa *et al.* 1991 found that carageenan-induced inflammation inhibited the expression of CYP450 and its enzymatic activity in male rats. Hosts of cytokines have been studied in relation to their effects on cytochrome P450 function. These studies are summarized in Table 1-2 and suggest that treatment with individual cytokines is associated with decreased expression and enzymatic activity of CYP450 isozymes (Moreno *et al.* 1987). Furthermore, decreased CYP450 expression may be due to pre-transcriptional changes resulting in decreased production of CYP450 mRNA (Morgan 1993) or post-transcriptional changes such as increased destruction of existing CYP450 (Wright and Morgan 1990). It is not clear if this is a direct or indirect effect of cytokines, but evidence suggests the possibility of multiple mechanisms. It is important to note that with the exception of IL-4, which increases CYP2E1 activity, the remaining cytokines all cause a *decrease* in CYP450 activity or amount. Furthermore, it appears that specific cytokines may affect specific CYP450 isozymes. Since different immunological or inflammatory conditions may differ in T-helper cell profile (Simon *et al.* 1994), cytokine profiles may also differ. This suggests that alteration in CYP450 isozymes may be dependent on the type and severity of the immunological insult.

Table 1-2: Cytokine Nomenclature & Known Effect on CYP450

Cytokine	Historical Name	Hepatic Effects
Interleukin - 1	Lymphocyte-activating factor Endogenous pyrogen	↓ CYP450 [1,2] ↓1A2,2C,3A[5]
Interleukin - 2	T-cell growth factor	↓ CYP450 [3]
Interleukin - 3	Multilineage growth factor (Multi-CSF)	N/A
Interleukin - 4	B-cell-stimulatory factor 1	↑ CYP2E1 [5]
Interleukin - 5	B cell growth factor T-cell-replacing factor	N/A
Interleukin - 6	B-cell-stimulatory factor 2 Interferon-β2	↓CYP3A [4] ↓1A2,2C,3A[5]
Interleukin - 7	B/T maturation factor	N/A
Interleukin - 8	Neutrophil-activating protein	N/A
Interleukin - 9		N/A
Interleukin - 10	Cytokine-synthesis-inhibitory factor	N/A
	Interferon-α	↓ CYP450 [3] ↓1A2,3A,2C,2E[7]
	Interferon-β	↓ CYP450 [3,6] ↓1A1,1A2,2E[7]
	Interferon-γ	↓ CYP450 [3] ↓1A2,1A1,2C,3A[5,6]
	Tumor Necrosis Factor-α (Cachectin)	↓ CYP1A2, ↓ CYP2C ↓ CYP3A [5]
	Tumor Necrosis Factor-β (Lymphotoxin)	N/A
	G-CSF	↓CYP450[8]
	GM-CSF	N/A
	M-CSF	N/A

[1] Ferrari *et al.* 1993a, Ferrari *et al.* 1993b[3] Ansher *et al.* 1992, [4] Chen *et al.* 1994 [5] Abdel-Rassak *et al.* 1993 [6] Delaporte and Renton 1997,[7]Cribb *et al.* 1994 [8]Proulx and Du Souich 1995 N/A: No available reports

1.4. Nitric Oxide

It has been hypothesized that cytokines inhibit CYP450 isozymes through the production of nitric oxide (NO) (Khatsenko *et al.* 1993). Pro-inflammatory cytokines such as IL-1ß and TNF-α will cause an increase in NO production through induction of the inducible form of nitric oxide synthase (iNOS) (De

Belder and Radomski 1994). The pathway for NO production involves the metabolism of L-Arginine to L-Citrulline by the enzyme, nitric oxide synthase (NOS) which exists in a constitutive, Ca^{2+}-dependant and an inducible (iNOS) Ca^{2+}-independent form (De Belder and Radomski 1994).

1.4.1 Nitrogen and Oxygen Free Radicals

Since considerable controversy exists with the field of free radical research, a brief overview of oxygen and nitrogen free radicals is warranted. A free radical is a molecule or atom that contains an unpaired electron. Unlike most stable molecules, molecular oxygen has two unpaired electrons in its outer orbitals rendering it quite reactive. During respiratory burst in neutrophils, addition of a single electron to molecular oxygen yields superoxide anion as follows;

$$2\ O_2 + NAD(P)H \xrightarrow{\text{Oxidase}} 2\ O_2^{\cdot} NAD(P) + H^+$$

Superoxide is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD)

$$2\ O_2^{\cdot} + 2\ H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

Superoxide anion and hydrogen peroxide form a more toxic and reactive radical, the hydroxyl radical ($OH^{\bullet}$) which may actually be the species most heavily involved in free radical induces tissue damage.

In addition several reactive nitrogen species can be generated from nitric oxide ($NO^{\bullet}$) and its reactions with oxygen species. NO *in vivo* rapidly combines

with oxyhemoproteins facilitating the conversion to NO_2^- and ultimately NO_3^-. In the presenceof superoxide anion, NO rapidly combines to form peroxynitrite ($ONOO^-$). It has been suggeste much of the tissue damage or enzyme inhibition attributed to NO may be due to peroxynitrite. It is important to note that both nitric oxide or peroxynitrite degrade to nitrite and ultimately nitrate. (Section summarized from Naqui *et al.* 1986, Beckman and Koppenol 1996, Debelder and Radomski 1994).

1.4.2 NO and Drug Metabolism

It has been suggested that NO can directly inhibit CYP450 isozymes through binding to the heme site. Spectroscopic analysis suggests free radical binding by NO to the heme site of the CYP 450 isozyme (Khatsenko *et al.* 1993). However, studies using NO-donors and inhibitors have also demonstrated both pre- and post transcriptional changes that decrease the formation of CYP450 mRNA (Delaporte and Renton 1997). Monshouwer *et al.* in 1995 demonstrated that Phase II conjugation reactions are also affected by NO production. Using a pro-inflammatory "cocktail" of IL-1ß, TNF-α and IFN-γ, NOS activity was increased with significant inhibition of uridine-diphosphate-glucuronosyl-transferase. Since of uridine-diphosphate-glucuronosyl-transferase represents a superfamily of Phase II isozymes, this suggests even wider clinical significance as more drugs are likely to be affected. These studies do not unequivocally prove that NO is the causative agent. Since pro-inflammatory conditions favor the production of both oxygen and nitrogen free radicals, both species or an

intermediate species could be the actual causative agent. For example, doctoral work by Lovlin in 1988 demonstrated that high altitude induced the formation of oxygen free radicals with a corresponding decrease in hepatic CYP450 content. At that time little was known about nitrogen radicals thus the loss of CYP450 correlated with reactive oxygen species. Rat and human CYP2E1 is inhibited in the presence of increased NO while the formation of oxygen free radicals is diminished (Gergel *et al.* 1996). Since superoxide anion and NO combine rapidly to form peroxynitrite the observations by Gergel *et al.* (1996), could be explained by an apparent increase in NO, which rapidly reacts with superoxide anion forming $ONOO^-$ resulting in an apparent decrease in oxygen free radicals. Furthermore, it has been shown that $ONOO^-$ can impair cardiac contractile function and inhibit mitochondrial heme based enzymes (Schulz *et al.* 1997, Szaba *et al.* 1996, Xia and Zweier 1997). It is important to note that $ONOO^-$ and NO are both highly reactive and short-lived species. One method to estimate their production is to measure serum nitrite, a stable breakdown product of both reactive nitrogen species (Beckman and Koppenol 1996).

Evidence also exists for a direct cytokine effect on drug metabolism independent of NO production. Sewer and Morgan (1997) demonstrated that after blocking IL-1ß-induced NO release, NO levels returned to normal, however the observed decrease in CYP2C11 mRNA did not return to normal. Therefore, it appears that several mechanisms may be involved.

1.5 Inflammation and Drug Disposition: Animal Studies

Inflammation can be induced in animal models with a variety of

techniques. Injection of carageenan, latex or turpentine can all produce significant inflammation (Whitehouse 1988). Adjuvant-induced polyarthritis in rats can be achieved through injection of various microbes such as, *Mycobacterium butyricum*, *Bordetella pertussis*, *Corynebacterium parvum*, and attenuated *Mycobacterium tuberculosis*. All are capable of inducing inflammation and have been shown to depress CYP450 isozyme activities (Cawthorne *et al.* 1976; Descotes 1985; Moreno *et al.* 1987; Peterson & Renton 1986). Adjuvant arthritis is believed to depress drug-metabolizing enzymes through cytokines such as IL-1ß and TNF-α. Furthermore, IL-1β has been shown to stimulate the metabolism of arachadonic acid and the synthesis of acute phase proteins while decreasing total CYP450 amount in the liver (Dinarello 1992). Many studies using induced inflammation in rats report a decrease in hepatic CYP450 content and/or activity with a corresponding decrease in drug metabolism. (Abdel-Razzak *et al.* 1993, Sakai *et al.* 1992, Ferrari *et al.* 1993a, Ferrari *et al.* 1993b, Morgan 1993). Ansher *et al.* (1992) demonstrated that IL-2 and IFN-α both significantly decrease hepatic drug metabolism in mice. Furthermore, a greater depression of drug metabolism of hexobarbital was seen when IL-2 and IFN-α were administered in combination suggesting synergistic effects. This is important clinically since an inflammatory reaction would invoke an entire cytokine cascade that is likely to have greater or more complex effects. Finally, inflammation also causes an increase in acute phase reacting proteins, especially α_1-acid glycoprotein (AAG), a known binding site for many basic drugs (Belpaire *et al.* 1982). This could lead to alterations in the fraction of drug unbound (*fu*) which has the potential to alter both

pharmacokinetics and pharmacodynamics.

1.5.1 Tolbutamide

In 1992, Parent *et al.* studied the effects of turpentine induced inflammation on tolbutamide pharmacokinetics and dynamics in male New Zealand rabbits. Subcutaneous injection of turpentine resulted in a statistically significant increase in rectal temperature and rise in seromucoid levels suggesting the presence of systemic inflammatory changes. Total microsomal proteins were slightly increased. No significant differences were found in hepatic cytochrome b_5. However, animals with inflammation demonstrated significantly lower hepatic concentrations of cytochrome P450 isozymes than healthy control animals. It is important to note that alterations in cytochrome P450 isozymes appear to be specific with a decrease in CYP3C, while no change in CYP2A was observed. Activity of tolbutamide hydroxylase was also effected with a 61% decrease in V_{max}, however, there was no observed change in K_m. Compared to healthy controls, rabbits with inflammation demonstrated altered tolbutamide kinetics with significant increases in AUC and $t_{1/2}$ and a decrease in total body clearance. Pharmacodynamic measurements of hypoglycemia where not statistically different between control and inflamed groups. However, a trend towards altered glucose tolerance was observed. The study concluded that inflammation clearly decreased hepatic biotransformation of tolbutamide, but rabbits with inflammation seemed resistant to the hypoglycemic effect possibly due to the inflammatory process itself. Thus, despite an increase in drug concentration, less hypoglycemic effect was observed.

1.5.2 Propranolol

Propranolol (PRP), a β-adrenoreceptor blocker, has been extensively studied in the presence of adjuvant arthritis. Propranolol levels have been reported to be 5 to 20 times higher in humans and rats afflicted with inflammation through both increased protein binding and decreased hepatic clearance (Bishop *et al.* 1981; Schneider *et al.* 1981; Belpaire *et al.* 1989; Laethem *et al.* 1994; Piquette-Miller & Jamali 1993).

In 1986, Walker *et al.* studied propranolol pharmacokinetics in the presence adjuvant arthritis. Although the study did not address stereospecific aspects of propranolol metabolism, hepatic blood flow and response to electrically induced tachycardia were measured. They concluded that alterations in hepatic blood flow could not account for the observed increase in drug $AUC_{0\rightarrow\infty}$. They suggested that the observed elevation in propranolol drug concentration was due to increased protein binding. Interestingly, they also observed a *decrease* in pharmacological effect despite the increase in propranolol drug levels which they attributed to possible β-receptor downregulation.

In 1995, Piquette-Miller and Jamali studied the pharmacokinetics of propranolol enantiomers in the presence of inflammation and after treatment with the non-stereroidal anti-inflammatory drug, ketoprofen. Inflammation decreased the oral clearance (CL/F) of PRP with a resultant rise in $AUC_{0\rightarrow\infty}$. Treatment of the inflammation clearly increased the oral clearance of both propranolol enantiomers with a decrease in $AUC_{0\rightarrow\infty}$. Furthermore, disease severity measured by arthritic index, also correlated strongly with $AUC_{0\rightarrow\infty}$ for both enantiomers (R:

r = 0.82 and S: r = 0.81). This suggested that the severity of the inflammatory response determined the extent of pharmacokinetic alteration and that the process can be reversed through appropriate treatment. The authors suggested that the observed differences were not due solely to changes in protein binding. Since propranolol displays saturable protein binding and the observed free fraction was > 10%, then concentrations of free drug within the hepatic portal vein should be even higher. Therefore, it is not likely that protein binding alone could account for the changes in propranolol pharmacokinetics. They concluded that intrinsic clearance (Cli') may also be altered.

1.5.3 Verapamil & Oxprenolol

The effect of endotoxin-induced inflammation has also been studied on the enantioselective pharmacokinetics of oxprenolol (OXP) and verapamil (VER) (Laethem *et al.* 1994). VER and OXP were administered as racemates via gavage to inflamed and healthy control male Wistar rats. Drug binding and α1-acid-glycoprotein (AAG) levels were also measured. The results demonstrated dramatic increases in $AUC_{0\rightarrow\infty}$ for both enantiomers of OXP (R-OXP 14.6 fold increase, S-OXP 9.8 fold increase $p < 0.001$), and VER (R-VER 10 fold increase, S-VER 14 fold increase $p < 0.001$). For both drugs this coincided with a decrease in CL/F. A significant decrease in the fraction unbound (*fu*) was measured for both drugs. It was hypothesized that this was due to the rise in AAG caused by the inflammation. The authors concluded that enantioselective protein binding was likely responsible for the observed changes in pharmacokinetics. The pharmacodynamic effects of the drugs were not measured.

1.5.4 Prednisolone

Prednisolone kinetics were studied by Garg *et al.* (1993) using carrageenan-induced airpouch inflammation in male Wistar rats. The study looked at both acute and chronic effects of inflammation since biochemically the states are different. The study demonstrated a statistically significant decrease in CL/F in the chronic inflammatory group when compared to healthy control animals. A corresponding increase in prednisolone concentration was observed, however, the increase in $AUC_{0\rightarrow\infty}$ was not statistically significant. No statistically significant differences in pharmacokinetics were observed in the acute inflammatory group. Thus, both the severity and duration of inflammation may determine the magnitude of changes in pharmacokinetics. It is important to note, that the steroid effects of prednisolone may have blunted the magnitude of pharmacokinetic changes, since its glucocorticoid effects would also modify the immune response.

1.5.5 Vaccines & Viral Infections

Viral illness and vaccinations also induce pro-inflammatory cytokines. Specifically, these lead to substantial increases of both type I interferons (IFN-α and β) and type II interferon (IFN-γ). In 1978 Chang *et al.* reported prolonged theophylline elimination in influenza-A infected patients. Later, in 1980, Kraemer *et al.* reported a similar finding in children infected with influenza B. Using a rat model in 1979, Renton reported a significant decrease in phenytoin elimination in animals pre-treated with *Bordetella pertussis* or polyriboinosinic polyribocytidylic acid (Poly I:C) a known inducer of interferon's α and β. Ansher *et al.* (1990)

demonstrated a 60% decrease in CYP450 levels following treatment with diphtheria, tetanus toxoids and pertussis vaccine. This corresponded with decreased hexobarbital metabolism and increased hexobarbital induced sleep time. Specific enzyme functions were also evaluated showing decreased activity for the mixed function oxidase benzphetamine demethylase, ethylmorphine demethylase and benzo(a)pyrene monooxygenase. A similar effect was noted after the administration of Poly I:C. The suppression of hepatic drug metabolism by Poly I:C was also confirmed by Sakai *et al.* in 1992 with suppression of CYP2E1, CYP1A1, CYP1A2 and CYP2C2 isozymes. A 60% decrease in the metabolism of aminopyrine, benzphetamine, aniline, 7-ethoxy-coumarin and 7-ethoxyresorufin was also measured. Therefore, it is suggested that viral infections or vaccines, which increase interferon production (especially IFN-α) also, impair the CYP450 metabolism of xenobiotics. In addition, the pharmacodynamic effect of hexobarbital (sleep duration) was increased.

1.6 Human Studies

1.6.1 Human Hepatocyte Studies

In 1993, Abdel-Rassak *et al.* obtained human hepatic tissue from 13 adult donors. Hepatocytes were isolated and exposed to either IL1-β, IL-4, IL-6, TNF-α or IFN-γ. The effect of these cytokines on CYP450 was assessed based on expression of CYP450 mRNA and monooxygenase assay. Their results demonstrated a statistically significant reduction in mRNA expression of CYP1A2, CYP2C, CYP2E1, and CYP3A in hepatocytes treated with IL-β, IL-6, TNF-α and to a lesser extent IFN-γ. IL-1β appeared to be the most potent against

all CYP450 isozymes tested with the exception of CYP2C where TNF-α had the greatest depressant effect. It is important to note that IL-4 was shown to have a profound stimulatory effect on CYP2E1 mRNA. In addition, the study also looked at a phase II enzyme activity, namely epoxide hydroxylase. IL-1β, IL-6 and TNF-α induced decreased enzyme activity. However, IL-4 increased activity in some cultures. This study seems to support the hypothesis that acute inflammation can decrease the activity of both Phase I and II drug metabolizing pathways. This study suggests pro-inflammatory cytokines inhibit drug metabolism in humans in a manner similar to the animal models of inflammation. Clinically, it suggests that the patient with an acute inflammatory reaction such as rheumatoid arthritis, viral infection, tumor invasion, or any insult giving rise to pro-inflammatory cytokine release is at risk for increased drug toxicity due to impaired enzyme function.

1.6.2 Human Clinical Evidence

1.6.2.1 Rheumatoid Arthritis

Many studies have suggested that patients with rheumatoid arthritis (RA) appear predisposed to adverse drug reactions (Sakai, Okamoto & Kikkawa 1992). Decreased drug metabolism due to pro-inflammatory cytokines is certainly an attractive explanation. Patients with RA show a higher frequency of allergic reactions to d-penicillamine and a distinct side effect profile different from patients with crystaluria or Wilson's disease (Lyle 1979; Walshe 1974). However, a meta-analysis of 49 sulfasalazine studies by Wijnands *et al.* found no statistically significant predisposition for drug toxicity. It was suggested the other

studies had bias in their patient selection. No attempt was made in any of these studies to quantitate the severity of the inflammation or quantitate pharmacokinetics. As previously mentioned rheumatoid arthritis in humans clearly involves pro-inflammatory cytokines. Studying T-cell cytokine patterns, Simon *et al.* (1994) demonstrated that patients with RA could be distinguished from patients with reactive arthritis following infection. T helper cells (Th) can be classified into two categories, Th1 that secrete IFN-γ and IL-2 and Th2, which secrete IL-4 and IL-5. Cytokines of the Th1 spectrum are elevated after a successful response to an intracellular pathogen. Th2 cytokines are elevated in allergic and helminthic infections. (Abbas, Lichtman, and Pober 1997). Using polymerase chain reaction, Simon *et al.* (1994) demonstrated that in RA a pattern of cytokine release involved IL-10, IFN-γ and IL-2 whereas a reactive arthritis pattern shows greater amounts of IL-4 and IFN-γ, moderate amounts of IL-10 and little IL-2. They further suggested that IL-4 has an inhibitory effect on the development of RA and is involved in a protective immune response. Patients with chronic RA fail to mount this response. They proposed that IL-4 could be used as a therapeutic agent interfering with the development of chronic disease. It becomes clear that the pattern of cytokine secretion can directly influence the type and severity of inflammation. Given the plethora of animal and *in vitro* studies on inflammation and cytokines on drug metabolism, it seems likely that the patient with RA could demonstrate decreased metabolism of drugs. Intuitively, this could result in an increase in drug effect or side effect. However, to date pharmacokinetic-pharmacodynamic studies in humans are lacking.

1.6.2.2 Cyclosporine & Bone Marrow Transplant Patients

Cyclosporine represents a drug with highly variable and complex pharmacokinetics and a narrow therapeutic index. Therefore, therapeutic drug monitoring is necessary to ensure therapeutic success and minimize toxicity. In bone marrow transplant patients, acute inflammation may be encountered especially in the presence of bacterial, fungal or viral infection. Chen *et al.* (1994) monitored six bone marrow transplant patients using IL-6 as a nonspecific marker of inflammation. All patients entered the study with a normal erythrocyte sedimentation rate (ESR). Patients were treated with cyclophosphamide, total body irradiation and a continuous infusion of cyclosporine. The study monitored serum IL-6, TNF-α, C-reactive protein, α1-acid glycoprotein (AAG), and cyclosporine. The systemic clearance of cyclosporine was decreased. In addition, elevated levels of cyclosporine metabolites were also observed. Peak IL-6 and C-reactive protein levels correlated well with decreased cyclosporine clearance. The authors hypothesized that IL-6 may have inhibited CYP3A, the isozyme primarily responsible for the metabolism of cyclosporine. However, since C-reactive protein also correlated with decreasing clearance, changes in protein binding could also be involved. Therefore, human diseases that release pro-inflammatory cytokines could lead to altered drug metabolism and/or protein binding in a manner similar to animal studies. However, studies in humans are necessary to evaluate the clinical implications of inflammation on drug therapy.

1.7 Verapamil

Verapamil (VER) is a phenylalkylamine calcium channel antagonist. It is

used as a Class IIa antiarrhythmic, an antianginal, antihypertensive, and anti-migraine drug and for the potentiation of chemotherapeutic drugs (Gilman *et al.* 1990, Markham *et al.* 1996). It is believed to decrease the rate of sinus node discharge and atrioventricular (AV) conduction through antagonism of inward calcium currents through slow calcium channels (Opie 1995). Possessing a chiral center, it is commercially available as a racemate in regular and sustained release formulations.

1.7.1 Molecular Pharmacology

Pharmacologically, VER typifies the phenylalkylamines that are antagonistic at L-type Ca^{2+} channels. L-type Ca^{2+} channels are a complex of five protein subunits. (Catterall 1991). The $\alpha 1$ subunit is the central functional component of the complex of a total of six proposed α-helical transmembrane domains. These transmembrane segments are very similar to Na^{+} channel α-subunits (Tanabe 1987). The $\alpha 1$ subunit has been shown to function as a voltage-gated ion channel when expressed in appropriate cells. (Perez-Reyes *et al.* 1989, Mikami *et al.* 1989). Furthermore, studies indicate that the phenylalkylamine receptor site is located on the intracellular surface of the membrane for the following reasons:

1. Repetitive stimulation with depolarizing voltages enhances phenylalkylamine Ca^{2+} channel blockade. (Hondeghem and Katzung 1984, Lee and Tsien 1983).

2. Quaternary derivatives of phenylalkylamines are effective Ca^{2+} channel blockers when applied to the intracellular side of the channel protein. (Hescheler J, *et al.* 1982).

This implies that a typical phenylalkylamine such as verapamil must enter the transmembrane pore of the Ca^{2+} channel in order to bind to this intracellular site occluding the pore. The central transmembrane pore is lined by peptide segments found between the S5 and S6 domains of I and IV similar to a K^+ channel. It is believed that VER is an intracellular pore blocker. The structure of the phenylalkylamine-binding site may also have stereochemical implications for the activity of VER enantiomers.

1.7.2 Metabolism

VER enantiomers are extensively metabolized by the liver in both rat (McIlheny 1971) and humans (Eichelbauem *et al.* 1979). VER metabolism involves the CYP450 isozymes, CYP3A4, CYP1A2 and CYP2C (Kroemer *et al.* 1993) in man. VER metabolism in rat would be expected to involve CYP2C and CYP1A since there is no CYP3A4 expression (Fujimaki 1994, Hakkak *et al.* 1993). Flavine containing monoxygenases have also been implicated in some studies (Cashman 1989). VER possesses a chiral center as shown in Figure 1-1. This has implications for both its pharmacokinetic profile and its pharmacodynamic activity. When administered as the racemate, the majority of the drug is converted via N-dealklyation to R,S norverapamil (NOR) and R,S D-

617 shown in Figure 1-1.

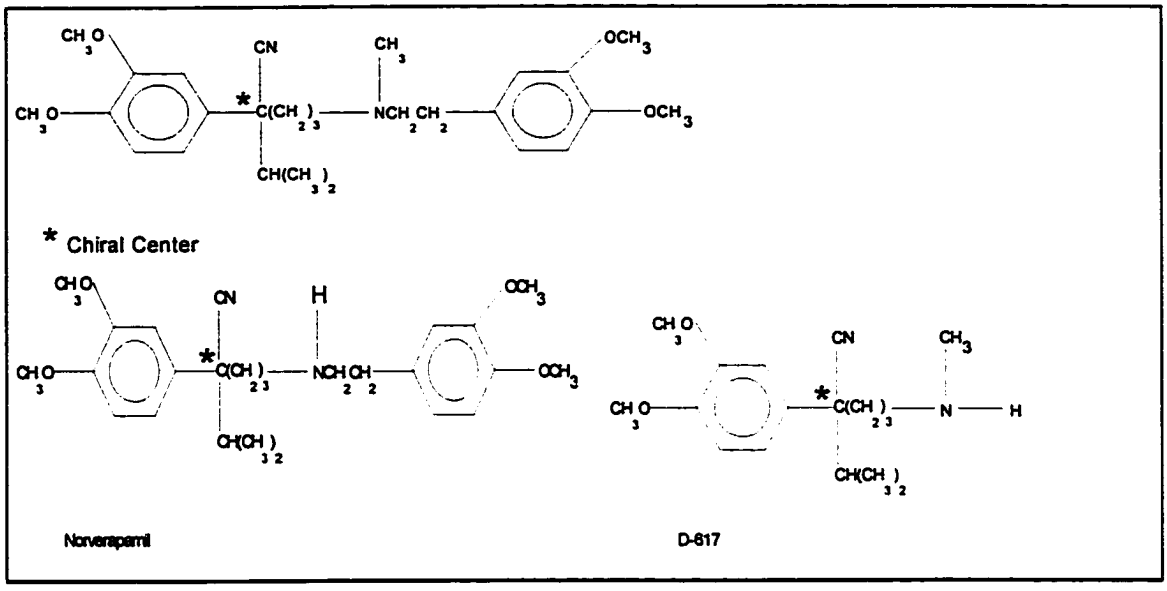

Figure 1-1: Structural Formula of Verapamil, Norverapamil and D617

To a lesser extent, O-demethylation results in the formation of two metabolites called D-702 and D-703 each demethylated on the 4 position of opposite phenyl rings. While these metabolites have been observed in other in vitro studies (Nelson *et al.* 1988, Kroemer *et al.* 1992), the clinical significance and/or activity of these metabolites are not known. To date although approximately twelve metabolites of verapamil have been identified, the greatest activity is demonstrated by the S-VER that is 10 to 20 times more potent in dromotropic and chrontropic effect than its antipode (Echizen *et al.* 1985). NOR also possesses some pharmacological activity at the AV-node, but is approximately 50 to 100 times less potent than the parent drug (Johnson *et al.* 1991).

In a study by Kroemer *et al.* in 1993 using human livers, CYP3A4 and CYP1A2 and to a lesser extent CYP2C were found to be involved in the formation of the major metabolites of VER, specifically NOR and D-617. To

varying extents these enzymes also demonstrated a stereoselective preference for S-VER over its antipode. Cashmen (1989) also demonstrated significant stereoselectivity in the oxidation of VER to D-617, but not NOR. It seems clear, based on microsomal studies and known drug interactions, that verapamil is likely metabolized primarily by CYP3A4 and to a lesser extent by CYP1A2. Known drug interactions with verapamil are largely with CYP3A4 substrates shown in Table 1-3.

Table 1-3: Verapamil- CYP3A4 Mediated Drug Interactions

Drug	CYP3A4	Effect
Cyclosporine	Substrate	Metabolism inhibited by verapamil
Quinidine	Substrate	Clearance decreased by verapamil
Rifampin	Induction	Increased verapamil clearance
Phenobarbital	Induction	Increased verapamil clearance

- Adapted from Anderson and Knoben 1998

Gross *et al.* demonstrated in 1990 that modification of VER at the 2-(3,4-dimethoxyphenyl) moiety could greatly alter stereoselective metabolism. For example, addition of a methoxy group at the meta- position forming gallopamil results in an almost complete loss of stereoselective metabolism. Therefore, it is clear that induction or inhibition of CYP450 isozymes especially CYP3A4 and CYP1A2 may have clinically significant stereopharmacokinetic and ultimately pharmacodynamic effects.

1.7.3 Stereospecific Pharmacodynamics

The need for channel entry to reach a stereospecific-binding site implies drug chirality could significantly effect pharmacodynamics (Echizen *et al.* 1985). S-VER has been show to have 10 to 20 times more dromotropic potency than its

antipode (Hescheler *et al.* 1982, Echizen *et al.* 1985). A dose of 500 to 1000 mg of R-VER has been shown to prolong PR-intervals to an extent similar to a 240 mg of racemic VER (Ahmed J *et al.* 1992). This was confirmed by Schwartz *et al.* (1993) who also demonstrated that S-VER had greater effects decreasing blood pressure and AV Wenckebach block pacing cycle length prolongation.

These differences in enantiomeric potency and pharmacology have been demonstrated to have clinical utility in cancer chemotherapy. Both R and S-VER have been shown to inhibit P-glycoprotein such that multi-drug resistance can be minimized in the presence or VER. A study by Ahmed *et al.* in 1992 demonstrated that R-VER could prevent multi-drug resistance with much less cardiac side effects.

1.7.4 Stereospecific Pharmacokinetics

The oral bioavailability of both VER enantiomers is low due to extensive stereoselective first pass metabolism (Vogelgesang *et al.* 1984). VER enantiomers are extensively metabolized by the liver (McIlheny 1971, Eichelbauem *et al.* 1979). Furthermore, hepatic metabolism of VER has been shown to be stereoselective for the more potent S-isomer as mentioned in section 7.2. Therefore, in man, lower concentrations of the more active S-VER are observed (Abernethy *et al.* 1993). However, the observed stereoselectivity in the hepatic extraction may also be explained by differences in protein binding of VER enantiomers. In man, R-VER is more highly bound than its antipode (Gross *et al.* 1990 and Schwartz *et al.* 1993). Clearance of R-VER is less than S-VER (Eichelbaum *et al.* 1985) with an oral bioavailability of 60 - 70% compared to

only 20 - 30% for S-VER (Echizen *et al.* 1985). Therefore, S-VER exhibits the characteristics of a highly extracted drug, while R-VER behaves more like a moderate to low extracted drug (Wilkinson and Shand 1975). S-VER, therefore, is expected to be more sensitive to changes in blood flow (Q) and intrinsic clearance (Cli') and less sensitive to alterations of protein binding. However, it should be emphasized that this does not mean that oral bioavailability of S-VER is independent of the extent of protein binding (Wilkinson 1987). Mehvar and Reynolds (1996) demonstrated that in the absence of protein, rat liver demonstrates the same stereoselective metabolism of S-VER that is observed in man. Thus, in the absence of protein, Cli' of S-VER was greater than that of its antipode. Addition of bovine serum albumin reversed the stereoselectivity such that R-VER was cleared faster than its antipode. This is identical to what is observed in intact Sprague-Dawley rats, namely, faster clearance of R-VER with greater protein binding of S-VER (Bhatti and Foster 1997). Mehvar and Reynolds (1996) then added human serum albumin to the liver perfusate and produced an indentical picture to that observed in man. It can be concluded that the protein binding of VER can override the stereoselectivity of the enzyme such that the elimination of VER can be said to be protein binding-dependent. Therefore, any changes in Cli' and protein binding would be expected to have significant effects on the pharmacokinetics of VER. This would be expected to have clinically significant pharmacodynamic consequences.

1.8 Interferon-α2a

Interferon-α2a (IFN-α) is a Type I IFN that is approved for a variety of

antiviral and anticancer uses. Its paracrine effects inhibit replication of viral RNA or DNA and have been used in the treatment of viral hepatitis (Perillo 1990). In higher doses, it inhibits cell proliferation and is used in the treatment of Hairy Cell leukemia and in AIDS- related Kaposi's sarcoma (Goldstein and Laszlo 1986, Krown 1987). IFN's action involves increased lytic potential of natural killer cells through increasing their O_2^- and H_2O_2 production and enhancement of cytolytic T-lymphocyte-mediated killing (Spiegel 1985). In addition, IFN-α appears to have important regulatory effects on other cytokines and their subsequent effects such as IFN-γ–induced NO production (Deguchi *et al.* 1995). Its direct effects on neutrophil function are poorly understood, but it has been shown to enhance respiratory burst with increased formation of reactive oxygen and nitrogen species (Little *et al.* 1994). NOS induction occurs with both Type I and Type II IFN's and this process is enhanced by viral infection (Kreil and Eibl 1995). IFN-α has been suggested to be both a pro-inflammatory and an anti-inflammatory cytokine (Tilg 1997). Its stimulates the production of type 1 T-helper cells leading to increases in IFN-γ, and IL-2. IL-2 in turn can increase IL-1ß and TNF-α production. Thus, IFN-α can influence pro-inflammatory activity. This is observed clinically where IFN-α therapy has resulted in the development of acute arthritis, pancreatitis, glomerulomephritis and a worsening of other autoimmune disease (Guttermann 1995). A pro-inflammatory effect in the presence of viral infection (Hepatitis C) has also been demonstrated (Pittau *et al.* 1997). Therefore, IFN-α may have complex and seemingly contradictory effects,

but can act as a pro-inflammatory cytokine.

1.8.1 IFN-α and Drug Metabolism

IFN-α has been shown to decrease the hepatic metabolism of several drugs. Monotherapy with IFN-α in cancer patients resulted in a 16% decrease in theophylline oral clearance (CL/F) which increased to 33% with an increase in the duration of therapy (Israel *et al.* 1993). Thus, chronic exposure to IFNα had a greater effect than acute exposure. In addition, no significant increases in acute phase reacting proteins were observed. Both CYP1A and CYP2E induction were down regulated by the production of IFN-α and IFN-ß in the rat (Cribb *et al.* 1994). Delaporte and Renton (1997) further suggested that CYP1A1 and CYP1A2 appear more sensitive to downregulation by IFN-α/ß production at both a pre and post-transcriptional level. Therefore, drugs metabolized by these CYP450 isozymes could be sensitive to inhibition by IFN-α.

1.9 Transgenic HLA-B27/ Human ß2-microglobulin Rat Model of Inflammation

Fischer rats can be genetically altered to provide a model of chronic inflammatory disease. Transgenic models of inflammatory disease offer another possibility to study the effects of inflammatory disease on pharmacokinetic and pharmacodynamic relationships. The Fischer 344 strain transfected with the human HLA-B27 and ß2-microglobulins spontaneously develop inflammatory abnormalities (Hammer *et al.* 1990). These abnormalities resemble the inflammatory conditions observed in humans positive for HLA-B27 such as

anklyosing spondylitis, inflammatory bowel disease (Crohn's), psoriatic skin lesions and cardiac inflammation (Lipsky and Taurog 1991). The animals develop diarrhea initially, then progressively develop joint, gut and heart lesions that are histologically similar to B27 associated disease in humans (Hammer *et al.* 1990). Since spondyloarthropathies are associated with inflammation and pro-inflammatory cytokines (Lipsky and Taurog 1991), it is possible that this disease state could have similar effects on drug pharmacokinetics and pharmacodynamics. To date, these relationships have not been extensively studied, but remain theoretically possible.

1.10 Hypothesis and Objectives

1.10.1 Rationale for Hypothesis

Inflammatory disease such as RA leads to increased production of pro-inflammatory cytokines such as IL-1ß and TNF-α. Pro-Inflammatory cytokines have been shown to decrease enzyme activity or amount of most of the cytochrome P450 isozymes either directly or through the production of nitric oxide. Potentially, this has significant clinical implications for the metabolism of many drugs. Currently, studies in humans that link pharmacokinetics to pharmacodynamic consequences are lacking. Human clinical studies on patients with rheumatoid arthritis suggest a relationship may exist between inflammatory disease and altered drug metabolism. However, no direct studies have been performed to clarify this issue. It is likely that some measure of disease severity is also required to clarify this relationship. A better understanding of this

phenomenon could lead to better treatment of the patient with inflammatory disease through the prevention of unwanted or adverse effects.

VER is extensively metabolized in a stereospecific manner by CYP450 isozymes. In man, the S-VER is cleared faster and is less extensively protein bound than its antipode. Thus, it exhibits the behavior of a highly extracted drug. In rat, the situation is reversed with more rapid clearance of R-VER and higher protein binding of its antipode. However, in both species S-VER possesses greater pharmacological activity. It has been known since the early 1970's that inflammation can decrease CYP450 enzyme activity while increasing circulation plasma proteins such as alpha-acid glycoprotein (AAG). Both decreased enzyme activity and increased protein binding could easily alter the pharmacokinetics of VER. It seems reasonable to ask if this could have a clinically significant effect on the pharmacodynamics of VER. Therefore, the purpose of this study is to determine if inflammatory disease or inflammatory cytokines cause clinically significant changes in VER pharmacokinetics and pharmacodynamics.

1.10.2 Hypotheses

1. The oral clearance of VER enantiomers are decreased in the presence of IFN-α2a in rat or rheumatoid arthritis in man.

2. VER protein binding is enhanced in the presence of IFN-α2a or rheumatoid arthritis.

3. IFN-α2a and rheumatoid arthritis induced changes in drug disposition are stereoselective.

4. Elevations in serum levels of VER enantiomers will lead to increased drug effect and/or toxicity.

5. Rheumatoid arthritis and IFN-α2a will result in an increase in reactive nitrogen species as measured by serum nitrite.

6. The change in VER pharmacokinetics and pharmacodynamics involves reactive nitrogen species.

7. Serum nitrite may provide a measure of disease severity.

1.10.3 Specific Objectives

1. To determine if a reproducible electrocardiogram can be measured in the rat.
2. To determine the effect of methoxyflurane anesthesia on rat electrocardiogram.
3. To determine if any pharmacodynamic interaction exists between methoxyflurane and VER.
4. To study the effects of IFN-α2a on the pharmacokinetics of VER enantiomers in rats.
5. To study the effects of IFN-α2a plus VER on cardiac cycle, specifically PR-interval and heart rate in rats.
6. To study the effect of IFN-α2a on serum nitrite production in rats.

7. To determine if reactive nitrogen species are involved by measuring serum nitrite.
8. To study the effect of inflammation produced in transgenic HLA-B27/ Human ß2-microglobulin on the pharmacokinetics of VER enantiomers in rats.
9. To study the effects of inflammation produced in transgenic HLA-B27/ Human ß2-microglobulin plus VER on the cardiac cycle, specifically PR-interval and heart rate in rats.
10. To study the effect of inflammation produced in transgenic HLA-B27/ Human ß2-microglobulin on serum nitrite production in rats.
11. To study the effect of rheumatoid arthritis on the pharmacokinetics of VER enantiomers in humans.
12. To study the effects of RA plus VER on cardiac cycle, specifically PR-interval and heart rate and on blood pressure in humans.
13. To study the effect of RA on IL-6 and serum nitrite production in humans.

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REFERENCES

Abbas A, Lichtman A, Pober J. *Saunders Text and Review Series: Cellular and Molecular Immunology 3rd Ed.* Toronto: W.B. Saunders and Co., 1997.

Abdel-Rassak Z, Loyer P, Fautrel A, Gautier JC, Corcos L, Turlin PD, Guillouzo A. Cytokines down-regulate expression of major cytochrome P450 enzymes in adult human hepatocytes in primary culture. *Mol Pharmacol* 1993; **44**: 707-715.

Abernethy D, Wainer I, Longstreth J, Adrawis N. Stereoselective verapamil disposition and dynamics in aging during racemic verapamil administration. *J Pharmacol Exp Ther* 1993; **266**: 904-911.

Ahmed J, Godden J, Meredith P, Elliott H. R-Verapamil: pharmacokinetics and effects on PR interval, blood pressure and heart rate. *Br J Clin Pharmacol* 1993; **36**: 93-98.

Ansher S, Thompson W, Habig W. Vaccine-induced alterations in hepatic drug metabolism. *Vaccine* 1991; **9**: 277-282.

Anderson P and Knoben J. *Handbook of Clinical Drug Data 8th Ed.* Toronto: Appleton and Lange, 1998.

Ansher S, Puri R, Thompson W, Habig W. The effects of IL-2 and IFN-α administration on hepatic drug metabolism in mice. *Cancer Research* 1992; **52**: 262-266.

Arend W, Dayer J. Cytokines and cytokine inhibitors or antagonists in rheumatoid arthritis. *Arthritis Rheum* 1990; **33**: 305-315.

Balligand J, Ungureanu D, Kelly R, Kobiz L, Pimental D, Michel T, Smith T. Abnormal contractile function due to induction of nitric oxide synthesis in rat cardiac myocytes follows exposure to activated macrophage-conditioned medium. *J Clin Invest* 1993; **91**: 2314-2319.

Beckman J, Koppenol W. Nitric Oxide, Superoxide, and Peroxynitrite: The Good the Bad and the Ugly. *Am J Physiol* 1996; **271**: C1424-C1437.

Belpaire F, Bogaert M, Rosseneu M. Binding of ß-adrenoreceptor blocking drugs to human serum albumin, to α1-acid-glycoprotein and to human serum. *Eur J Clin Pharmacol* 1982; **23**: 246-253.

Belparie F, De Smet B, Chindavijal B, Fraeyman N, Bogaert MG. Effect of turpentine-induced inflammation on the disposition kinetics of propranolol, metolprolol, and antipyrine in the rat. *Fund Clin Pharmacol* 1989 **3**: 79-88.

Bhatti M, Foster R. Pharmacokinetics of the enantiomers of verapamil after intravenous and oral administration of racemic verapamil in a rat model. *Biopharm Drug Disp* 1997; **18**: 387-96.

Bishop H, Schneider RE, Welling P. Plasma propranolol concentrations in rats with adjuvant-induced arthritis. *Biopharm Drug Disp* 1981; **2**: 291- 297.

Bjarnson I, Peters T. Influence of anti-rheumatic drugs on gut permeability and on the gut-associated lymphoid tissue. *Bailliers Clin Rheum* 1996; **10**:165-176.

Cadieux R. Drug interactions in the elderly. How multiple drug use increases risk exponentially. *Post Grad Med* 1989; **86**: 179-186.

Cashman J. Enantioselective N-oxygenation of verapamil by hepatic flavin-containing monooxygenase. *Mol Pharmacol* 1989; **36**: 497-503.

Catterall WA, Striessnig J. Receptor sites for Ca2+ channel antagonists. *TiPS* 1992; **13**: 256-262.

Catterall WA. Functional subunit structure of voltage-gated calcium channels. *Science* 1991; **253**: 1499-1500.

Cawthorne M, Palmer E, Green J. Adjuvant induced arthritis and drug-metabolizing enzymes. *Biochem Pharmacol* 1976; **25**: 2683-2688.

Chang KC, Laver BA, Bell TD, Chai H. Altered theophylline pharmacokinetics during acute respiratory viral illness. *Lancet* 1978; **1**: 1132-1133.

Chen YL, Vraux VL, Leneveua A. Acute-phase reponse, IL-6 and alteration of cyclosporin pharmacokinetics. *Clin Pharmacol Ther* 1994; **55**: 649-660.

Cribb A, Delaporte E, Kim S, Novak R, Renton K. Regulation of CYP1A and CYP2E induction in rat during the production of IFNα/ß. *J Pharmacol Exp Ther* 1994; **268**: 487-494.

De Belder A, Radomski M. Nitric oxide in the clinical area. *J Hyperten* 1994; **12**: 617-624.

Deguchi M, Inaba K, Muramatsu S. Counteracting effect of IFNα and β on IFNγ-induced production of NO which is suppressive for antibody response. *Immunology Letters* 1995; **45**: 157 - 153.

Delaporte E, Renton K. CYP1A1 and CYP1A2 are downregulated at both transcriptional and post-transcriptional levels by conditions resulting in IFN-α/ß induction. *Life Sci* 1997; **60**: 787-796.

Descotes J. Immunomodulating agents and hepatic drug metabolizing enzymes. *Drug Metab Rev* 1985; **16**: 175-185.

Dinarello C, Kiskimoto T. *Biology of interleukin-1. Interleukins Molecular Biology and Immunology* Basel: Karger,1992.

Dugowson C, Koepsell T, Voigt L. Rheumatoid Arthritis in women: Incidence rate in group health cooperative, Seattle Wahington, 1987 - 1989. *Arthritis Rheum* 1989; **31**: 315-324.

Dumond D, Wolstencroft R, Panayi G, Matthew M. Morley J, Howson W. Lymphokines: Non-antibody mediators of cellular immunity generated by lymphocyte activation. *Nature* 1969; **224**: 38-42.

Eichelbaum M, Ende M, Rember G, Schomerus M, Dengler H. The metabolism of ^{14}C-D,L-verapamil in man. *Drug Metab Disp* 1979; **7**: 145-148.

Echizen H, Brecht T, Niedergesäss S, Vogelgesang B and Eichelbaum M. The effect of dextro-, levo-, and racemic verapamil on atrioventricular conduction in humans. *Am Heart J* 1985; **109**: 210-217.

Elsässer-Beile U, von Kleist S. Cytokines as therapeutic and diagnostic agents. *Tumor Bio* 1993; **14**: 69-94.

Ferrari L, Herber R, Batt A, Siest G. Differential effects of human recombinant IL-1ß and dexamethasone on hepatic drug-metabolizing enzymes in male and female rats. *Biochem Pharmacol* 1993; **45**: 2269-2277a.

Ferrari L, Jouzeau JY, Gillet P, Herber R, Fener P, Batt AM, Netter P. IL-1ß differentially repressess drug-metabolizing enzymes in arthritic female rats. *J Pharmacol Exp Ther* 1993; **264**: 1012-1020b.

Fey GF, Fuller GM. Regulation of acute phase gene expression by inflammatory mediators. *Mol Biol Med* 1987; **4**: 323-338.

Follath R, Ha HR. Pharmcokinetics of conventional and slow-release verapamil. *Br J Clin Pharmacol* 1986; **21**: 149S-153S.

Fujimaki M. Oxidation of R(+)- and S(-)-carvedilol by rat liver microsomes. Evidence for stereoselective oxidation and characterization of the cytochrome P450 isozymes involved. Drug Metab Disp 1994; **22**:700-708.

Garg V, Hon Y, Jusko W. Effects of acute and chronic inflammation on the pharmacokinetics of prednisolone in rats. *Pharm Res* 1993; **11**: 541-544.

Gergel D, Misik V, Riesz P, Cederbaum A. Inhibition of Rat and Human CYP2E1 catalytic activity and reactive oxygen free radical formation by NO. *Arch Biochem Biophys* 1997; **337**: 239-259.

Gilman A, Rall T, Nies A, Taylor P. (eds). *Goodman and Gilman's The Pharmacological Basis of Therapeutics 8th Ed.* Toronto: Pergamon Press 1990.

Goldstein D , Laszlo J. Interferon therapy in cancer: from imagination to interferon. *Cancer Res* 1986; **46**: 4315-4329.

Gross AS, Mikus G, Morkike K, Eichelbaum M. Pharmacokinetics and pharmacodynamics of the enantiomers of gallopamil. *Eur J Pharmacol* 1990; **183**: 1651-1652.

Gutterman J. Cytokine therapeutics: Lessons from interferon-α. *Proc Nat Acad Sci USA* 1995; **91**: 1198-1205.

Hakkak R, Ronis M, Badger T, Effects of enteral nutrition and ethanol on cytochrome P450 distribution in small intestine of male rats. Gastroenterology. 1993; **104**:1611-1618.

Hammer R, Maika S, Richardson J. Spontaneous inflammatory disease in transgenic rats expressing HLA-B27 and human ß2-m: an animal model of HLA-B27 associated human disorders. *Cell* 1990; **63**: 1099-1112.

Harris E. Mechanisms of Disease: Rheumatoid Arthritis - pathophysiology and implications for therapy. *New Eng J Med* 1990; **322**: 1277-1289.

Hescheler J, Pelzer D, Trube G, Trautwein W. Does the organic calcium channel blocker D600 act from inside or outside on the cardiac cell membrane. *Pflugers Arch.* 1982; **393**: 287-291.

Hondeghem LM, Katzung BG, Antiarrythmic agents: The modulated receptor mechanisms of Na^{+} and Ca^{++} channel blocking drugs. *Ann Rev Pharmacol Tox* 1984; **24**: 387-423.

Ishikawa M, Sasakiu K, Ozaki M, Watanabe K, Takayanagi Y, Sasaki K. Hepatic drug metabolizing activity in rats with carrageenan-induced inflammation. *J Pharm Dyn* 1991; **14**: 132-138.

Israel B, Blouin R, McINtyre W, Shedlofsky S. Effects of interferon-α monotherapy on hepatic drug metabolism in cancer patients. *Br J Clin Pharmacol* 1993; **36**: 229-235.

Johnson K, Balderston S, Piper J, Mann D and Reiter M. Electrophysiological effects of verapamil metabolites in the isolated heart. *J Cardiovasc Pharmacol* 1991; **17**: 830-837

Kavanaugh A. Rheumatoid arthritis in the elderly: Is it a different disease? *Am J Med* 1997; **103**: 40S-48S.

Khatsenko O, Gross S, Rifkind A, Vane J. Nitric oxide is a mediator of the decrease in CYP450-dependent metabolism caused by immunostimulants. *Pro Nat Acad Sci USA* 1993; **90**: 11147-11151.

Kraemer M, Furukawa C, Koup J, Shapiro G, Pierson W, Fierman C. Altered theophylline clearance during an influenza B outbreak. *Pediatrics* 1980; **69**: 21-36.

Kreil T, Eibl M. Viral infection of macrophages profoundly alters requirements for induction of nitric oxide synthesis. *Virology*. 1995; **212**:174 - 178.

Kroemer H, Gautier J, Beaune P, Henderson C, Wolf C, Eichelbaum M. Identification of P450 enzymes involved in metabolism of verpamil in humans. *Naunyn-Schmiedeberg's Arch Pharmacol* 1993; **348**: 332-337.

Kroemer H, Echizen H, Heidemann H, Eichelbaum M. Predictability of the in vivo metabolism of verapamil from in vitro data: contribution of individual metabolic pathways and stereoselective aspects. *J Pharmacol Exp Ther* 1992; **260**: 1052-1057.

Krown S. The role of interferon in the thereapy of epidemic Kaposi's sarcoma. *Sem Oncol* 1987; **14**: 27-33.

Laethem ME, Belpaire FM, Wijnant P, Rosseel MT, Bogaert MC. Influence of endotoxin on the steroselective pharmacokinetics of oxprenolol, propranolol, and verapamil in the rat. *Chirality* 1994; **6**: 405 - 410.

Lee B, Wong D, Benowitz N Sullam P. Altered patterns of drug metabolism in patients with acquired immunodeficiency syndrome. *Clin Pharmcol Ther* 1993; **53**: 529-535.

Lee KS, Tsien RW,Mechanism of Ca^{++} channel blockade by verapamil, D600, diltiazem and nitrendipine in single dialyzed heart cells. *Nature* 1983; **302**: 790-794.

Little R, White M, Hartshor K. IFNα enhances neutrophil respiratory burst responses to stimulation with influenza A virus and FMLP. *J Infect Diseas* 1994; **170**: 802 - 810.

Lipsky P, Taurog J. (Eds). *HLA-B27+ Spondyloarthropathies* New York: Elsevier, 1991.

Liu Y, Xu B, Cai X. The Role of intestinal permeability in the pathogenesis of ankylosing spondylitis. *Chug-Hua Nei Ko Tsa Chih Chinese J Int Med* 1995; **34**: 91-94.

Lovlin, R. The free radical effect of high altitude on rats. Thesis Dissertation: University of Alberta, 1988.

Lyle WH. Penicillamine. *Clin Rheum Dis* 1979; **5**: 569-601.

Ma T. Intestinal epithelial barrier dysfunction in Crohn's Disease. *Pro Soc Exp Bio Med* 1997; **214**: 318-327.

Markham P, Ellis T, Tambur A, Gebel H. Differential sensitivity of resting and IL-2 activated NK cells to R-verapamil. *Transplantation* 1996; **62**: 1883-1888.

McIlheny H. Metabolism of (^{14}C) verapamil. *J Med Chem* 1971; **14**:1178-1184.

Mehvar R, Reynold J. Reversal of stereoselectivity in the hepatic availability of verapamil in isolated perfused rat livers. *Drug Metab Disp* 1996; **24**: 1088-1094.

Mikami A. Primary structure and functional expression of the cardiac dihydropyridine-sensitive calcium channel. *Nature* 1989; **340**: 230-233.

Mitchell D, Utsinger PD, Zvaifler NH, Ehrlich GE. *Epidemiology, Rheumatoid Arthritis, Etiology, Diagnosis and Treatement.* Philadelphia: JB Lippincott Co., 1985.

Mohler K, Butler L. Differential production of IL-2 and IL-4 mRNA in vitro after primary sensitization. *J Immunol* 1990; **145**: 1744-1739.

Monshouwer M, Witkamp R, Nujmeijer S, Van Amsterdam J, Van Miert A. Suppression of CYP450 and UDP glucuronosyl transferase-dependent enzyme activities by pro-inflammatory cytokines and possible role of nitric oxide in primary cultures of pig hepatocytes. *Tox App Pharmacol* 1996; **137**: 237-244.

Moreland L, Koopman W. Infection as a cause of arthritis. *Curr Opin Rheum* 1991; **3**: 639-649.

Moreno J, Escofet A, Castell M, Castellote C, Queralt J. Hepatic cytochrome P-450 activities and serum biochemical changes in adjuvant arthritis. *Med Sci Res* 1987; **15**: 1469-1470.

Morgan E. Down-regulation of multiple cytochrome P450 gene products by inflammatory mediators in vivo. Independence from the hypothalamo-pituitary axis. *Biochem Pharmacol* 1993; **45**: 415-419.

Morton D, Chatfield D. The effects of adjuvant induced arthritis on the rat liver metabolism of drugs in rats. *Biochem Pharmacol* 1970; **19**: 473-481.

Nelson W, Olson L, Beitner D, Pallow J. Regiochemistry and substrate specificity of O-demethylation of verpamil in the presence of the microsomal fraction from rat and human liver. *Drug Metab Dis* 1988; **16**: 184-188.

Opie L., Frishman W, Thadani U. *Drugs for the Heart 4th Ed.* Toronto: W.B. Saunders Co., 1995.

Parent C, Belanger P, Jutras L, Du Souich P. Effect of inflammation on the rabbit hepatic cytochrome P450 isoenzymes: Alterations in the kinetics and dynamics of tolbutamide. *J Pharmacol Exp Ther* 1992; **261**: 780-787.

Perez-Reyes E. Induction of calcium currents by the expression of the α-1-subunit of the dihyropyridine receptor from skeletal muscle. *Nature* 1989; **340**: 233-236.

Perrillo R. A randomized controlled trial of interferon alfa-2b alone and after prednisone withdrawal for the treatment of chronic hepatitis B. *New Eng J Med* 1990; **323**: 295-301.

Peterson T, Renton K Kupfer cell factor mediated depression of hepatic parenchymal cell cytochrome P-450 *Biochem Pharmacol* 1986; **5**: 1491-1497.

Pittau E, Bogliolo A, Tinti A, Mela Q, Ibba G, Salis G, Perpignano G. Developement of arthritis and hypothyroidism during alpha-ineterferon therapy for chronic hepatitis C. *Clin Exp Rheum* 1997; **15**:415-419.

Piquette-Miller M, Jamali F. Selective effect of adjuvant arthritis on the disposition of propranolol enantiomers in rats detected using a stereospecific HPLC assay. *Pharm Res* 1993; **10**: 294-299.

Piquette-Miller M, Jamali F. Influence of severity of inflammation on the disposition kinetics of propranolol enantiomers in ketoprofen-treated and untreated adjuvant arthritis. *Drug Met Disp* 1995; **23**: 240-245.

Poulton K, Griffith S, Thomson W, Mattey D, Fischer J,Clarke S, Dawes P, Illier W. Adverse drug reactions in patients with rheumatoid arthritis and HLA-DR3. *Eur J Immunogen* 1998; **25**: 62-66:

Proulx M, Du Souich P. Inflammation-induced decrease in hepatic CYP450 in conscious rabbits is accompanied by an increase in hepatic oxidative stress. *Res Com Mol Path Pharmacol* 1995; **87**: 221- 236.

Renton K. The deleterious effec of Bordetella pertussis vaccine and poly (rI.rC) on the metabolism and disposition of phenytoin. *J Pharmacol Exp Ther* 1979; **208**: 267 – 270.

Sakai H, Okamoto T, Kikkawa Y. Suppression of hepatic drug metabolism by the interferon inducer, Poly I:C. *J Pharmacol Exp Ther* 1992; **263**: 381-386.

Sanders S, Madara J, McGuirk D, Gelman D, Colgan S. Assessment of inflammatory events in epithelial permeability: a rapid screening method using fluoresceine dextrans. *Epi Cell Bio* 1995; **4**:25-34.

Sasaki K, Ishikawa Y. The effect of phenobarbital on the impairment of drug metabolism by the carrageenan-induced inflammation in male rat. *Res Com Chem Path Pharmacol* 1990; **69**: 377-380.

Schneider RE, Bishop H, Kendall MJ, Quarterman CP. Effect of inflammatory disease on plasma concentrations of three ß -adrenoreceptor blocking agents. *Int J Clin Pharmacol Ther Toxicol* 1981; **19**: 158-162.

Schulz R, Dodge K, Lopaschuk G, Clanachan A. Peroxynitrite impairs cardiac contractile function by decreasing cardiac efficiency. *Am J Physio* 1997; **272**: H1212-H1219:.

Schulz R, Triggle C. Role of NO in vascular smooth muscle and cardiac muscle function. *TiPS* 1994; **15**: 255 - 259.

Schwartz J, Troconiz I, Verotte D, Liu S, Capili H. Aging effects on stereoselective pharmacokinetics and pharmacdynamics of verapamil. *J Pharmcol Exp Ther* 1993; **265**: 690 - 697.

Sewer M and Morgan E. Nitric-Oxide independent suppression of P450 2C11 expression by interleukin-1ß and endotoxin in primary rat hepatocytes. *Biochem Pharmacol* 1997; **54**: 729-737.

Shibukawa A, Wainer I. Simultaneous direct determination of the enantiomers of verapamil and norverapamil in plasma using a derivatized amylose HPLC chiral stationary phase. *J Chrom Bio App* 1992; **574**: 85-92.

Simon AK, Seipelt E, Sieper J. Divergent T-cell cytokine patterns in inflammatory arthritis *Proc Nat Acad Sci USA* 1994; **91**: 8562 - 8566.

Spack E. Treatment of autoimmune diseases through manipulation of antigen presentation. *Crit Rev Immun.* 1997; **17**: 529-536.

Spiegel R. Intron A (Interferon-alfa):Clinical Overview. *Cancer Treat Rev* 1985; **12**(SUPP B): 5 - 16.

Stitt JT: Prostaglandin E as neural mediator of the febrile response. *Yale J Bio Med* 1986; **59**: 137-149.

Szaba C, Day B, Salzman A. Evaluation of the relative contribution of nitric oxide and peroxynitrite to the suppression of mitochondrial respiration in immuno-stimulated macrophages using a manganese mesporphyrin superoxide dismutase mimetic and peroxynitrite scavenger. *FEBS Letters* 1996; **381**: 82 - 86.

Tanabe, T. Watanabe Y. Primary structure of the receptor for calcium channel blockers for skeletal muscle. *Nature* 1987; **328**: 313-318.

Tateishi H, Mitsuyama K, Toyonag A, Tomoyose M and Tanikawa K. Role of cytokines in experimental colitis: relation to intestinal permeability. *Digestion* 1997; **58**:271-81.

Tilg H. New insights into the mechanisms of interferon alfa (sic): An immuno-regulatory and anti-inflammatory cytokine. *Gastroenterology* 1997; **112**: 1017 - 1021.

Tuckwell J, Esparza L, Carson D, *et al.* The susceptibility sequence to rheumatoid arthritis is a cross-reactive B cell epitope shared by E. Coli heat shock protein dnaj and the histocompatibility leukocyte antigen DRB10401 molecule. *J Clin Invest* 1992; **89**: 327-331.

U.S. Senate Special Committee on Aging. Aging America. Trends and projections. Washington D.C., U.S. Department of Health and Human Services 1985 - 1986.

Van Noort J, Amor S. Cell biology of autoimmune fiseases. International review of cytology 1998; **178**: 127 - 206.

Van den Ouweland R, Corstens F, van de Putte L, Gribnau F. Gasterointestinal blood loss during treatment with naproxen for arthritis. *Scand J Rheum* 1987; **16**: 365-370.

Vermeulen A, Belpaire F, de Smet F, Bogaert M. Influence of human recombinant interleukin 1ß on the enantioselective dispostion of propranol in rats. *Biochem Pharmacol* 1993; **45**: 1-6.

Vogelgesang B, Echizen H, Schmidt E, Eichelbaum M. Stereoselective first-pass metabolism of highly cleared drugs: Studies of the bioavailability of L- and D-verapamil examined with a stable isotope technique. *Br J Clin Pharmacol* 1984; **18**: 733-740.

Walker K, Barber H, Hawksworth G. Mechanism responsible for altered propranolol disposition in adjuvant-induced arthritis in the rat. *Drug Met Disp* 1986; **14**: 482- 486.

Walshe JM, Chairman. Round Table Discussion-Proper use of penicillamine. *Post Grad Med J.* 1974; **14** *SUPP:* 80 - 83.

Weyand C, Goronzy J. The molecular basis of rheumatoid arthritis. *J Mol Med* 1997; **75**: 772 - 785.

Wilkinson G, Shand D. A physiological approach to hepatic drug clearance. *Clin Pharmacol Ther* 1975; **18**: 377-389.

Wilkinson G. Clearance approaches in pharmacology. *Pharmacol Rev* 1987; **39**: 1-47.

Whitehouse DM. Abnormal drug metabolism in rats after an inflammatory insult. *Agents Actions* 1976; **3**: 312-316.

Whitehouse FM. Adjuvant-induced polyarthritis in rats. In: Handbook of animal models for the rheumatic diseases. R. Greenwald and H. Diamond (eds) Vol I, pp 3 - 16. CRC Press, Boca Raton Fl, 1988.

Wijnands MJH, Van 'thof MA, Van De Putte LBA, Van Riel PL. Rheumatoid arthritis: a risk factor for sulfasalazine toxicity? A meta analysis *Brit J Rheum* 1993; **32**: 313-318.

Wright K, Morgan E. Transcriptional and post-transcriptional suppression of P450IIC11 and P450IIC12 by inflammation. *FEBS Letters* 1990; **271**: 59 - 61.

Xia Y, Zweier J. Superoxide and Peroxynitrite generation from inducible nitric oxide synthase in macrophages. *Proc Nat Acad Sci USA*. 1997; **94**: 6954-6958.

Chapter 2

METHOXYFLURANE ANESTHESIA AUGMENTS THE CHRONOTROPIC AND DROMOTROPIC EFFECTS OF VERAPAMIL.

INTRODUCTION

Inhalation anesthetics have been shown to have anti-arrhythmic or arrhythmogenic potential in both animals and man (Takada *et al.* 1993, Kroll and Knight 1984). For example, halothane and enflurane prolong refractory periods that may suppress re-entrant ventricular arrhythmia in post-myocardial infarction patients (Deutsch *et al.* 1990). However, methoxyflurane has been shown to increase the automaticity of the atrial conducting system (Warren and Morrow 1977) causing an increase in supra-ventricular tachyarrythmias. It has also been noted that a majority of patients experience some form of arrhythmia during surgical anesthesia due to multiple causes ranging from hypoventilation, electrolyte abnormalities, hypothermia and to the type of anesthesia (Feeley 1997). The addition of antiarrhythmic drugs for ventricular ectopy may actually increase the incidence of fatal ventricular arrhythmia (Miller and Mayer 1994) in the anesthetized patient. Therefore, the anesthetized patient is significantly different from the conscious patient. This results in two major problems. Firstly, the unconscious patient or the unconscious laboratory animal differs physiologically from their conscious counterparts. Most notably, autonomic tone is altered by general anesthesia (Miller and Mayer 1994). Thus, the reaction to drugs may differ from the conscious to the unconscious state. For example, it has been

shown with verapamil (VER) that the minimum effective antiarrhythmic dose is 0.5 mg/kg iv in anesthetized animals and 2 mg/kg iv in conscious animals (Curtis *et al.* 1984). The second problem involves the possibility of a drug interaction between the anesthetic agent and the test drug. A good example is the synergistic interaction between class I antiarrhythmics such as lidocaine and the inhalation anesthetic halothane (Hasimoto *et al.* 1994). An unknown drug interaction could confound the results for the basic researcher and have fatal results for the clinician. Therefore, it is important to conduct research in both anesthetized and unanesthetized animals to determine if differences and/or drug interactions exist.

Verapamil (VER) is a phenlyalklyamine L-type Ca^{2+} channel blocker. It decreases electrical conduction across the atria and slows conduction through the AV-node. Therefore, it is used for supraventricular tachyarrthymias, and has a well-described concentration effect relationship (Echizen *et al.* 1985, Harder *et al.* 1992). Methoxyflurane (MF) is a volatile inhalation anesthetic that is no longer used in humans due to renal toxicity, but is commonly used for surgical anesthesia in animals. Several structurally similar inhalation anesthetics such as halothane, isoflurane and enflurane have been shown to inhibit the Na^{+}/Ca^{2+} exhange and Ca^{2+} channels in the heart (Haworth and Goknur 1995). It is therefore possible that MF could interact with VER increasing the possibility of AV-block or supraventricular arrhythmia. In addition, pharmacokinetic-pharmacodynamic animal studies require the surgical placement of cannulae for serial blood sampling. Therefore, it is important to understand the combined effects of anesthesia and VER on pharmacodynamics. The objective of this study was to

determine the effect of MF anesthesia alone or in combination with VER on the ECG of the rat. In addition, the time to reacquire baseline values after treatment with VER, MF and MF + VER was determined.

METHODS AND MATERIALS

Verapamil hydrochloride was a gift from G.D. Searle (Skokie, Ill), manufactured by Knoll Pharmaceuticals (Stuttgart, Germany). Methoxyflurane (Metofane) was purchased from Janssen pharmaceuticals, veterinary division (North York, Canada). The braided stainless steel, Teflon coated electrodes were purchased from Cooner Wire Co (Chatsworth, California).

The study followed the guidelines established for ethical handling of live animals established by the University of Alberta. Adult, male, Sprague-Dawley rats (Charles River Colony, n = 6, 311 ± 23 g) were used in the study. The animals were acclimated to a 12-hour day-night cycle, housed in rodent cages, and fed standard rodent chow for seven days prior to the experiment.

On the morning of the experiment ECG electrodes were placed under light anesthesia using inhaled MF applied using a mask. The placement of the ECG electrodes required no more than 10 minutes in all animals. The animals were placed in a Plexiglas restraining cage and allowed to recover for a two-hour period. All animals had their ECG's recorded every 10 minutes during this 2-hour recovery period to ensure that the animal was free of any residual effects of the anesthesia. The ECG was then recorded for an additional 2-hour period to obtain baseline values. The animals were then randomized to each of three treatments:

Methoxyflurane (MF) animals had surgical anesthesia induced with inhaled MF and maintained for 2 hours while recording their ECG. The MF was then discontinued and the animal allowed to regain consciousness while their ECG was monitored until baseline values were re-established. Surgical anesthesia was defined as an absence of foot withdrawal to painful stimuli, absence of ear movement to ear prick and lack of eyelid response to brushing accompanied with deep rhythmic breathing. The VER group received VER 10 mg/kg s.c.in sterile water for injection and had their ECG's recorded over a two hour period. The VER + MF group was placed under surgical anesthesia with inhaled MF then VER 10 mg/kg s.c. was administered s.c. in sterile water for injection. The anesthesia was maintained for a 2-hour period then discontinued. ECG's were recorded until the baseline values were restored. Animals were then crossed over to the remaining treatments such that each animal served as its own control and each animal would receive only one treatment in a 24-hour period. Each treatment effect was determined by comparing the ECG measurements recorded over a two hour period and determining the maximum increase from baseline. The study is summarized in Figure 2-1.

Place Electrodes	Recovery	Measure Baseline	Treatment VER/MF/VER + MF	Monitor Return to Baseline.
10min	2 hours	2 hours	2 hours	2 hours

Figure 2-1: Study Summary

An augmented lead I electrocardiogram (ECG) was measured using braided stainless steel, Teflon coated electrodes placed subcutaneously in the left and right axilla and over the xyphoid process as shown in Figure 2-1. The ECG was recorded using a Honywell ECG amplifier (Honywell Electronics for Medicine, Edmonton, Canada) and the data recorded on a chart recorder. Cardiac intervals were measured with calipers (NewCon, Edmonton, Canada). The mean of seven cycles was taken for the measurement of PR, QRS and RR intervals. Figure 2-2 demonstrates a typical rat electrocardiogram.

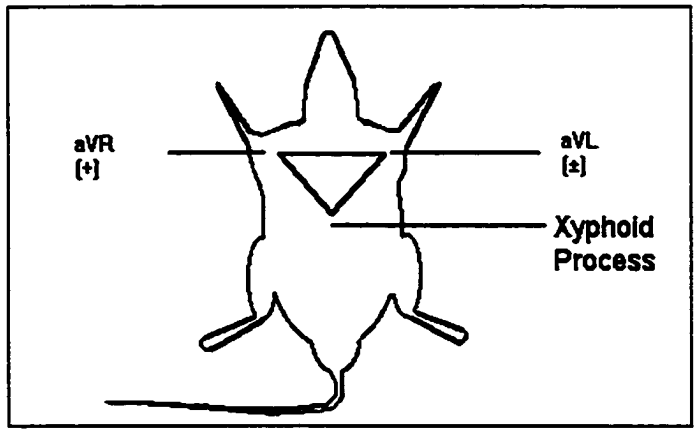


Figure 2-2: Electrode placement for the rat ECG
a = Augmented V = Voltage R = Right Axilla L = Left Axilla

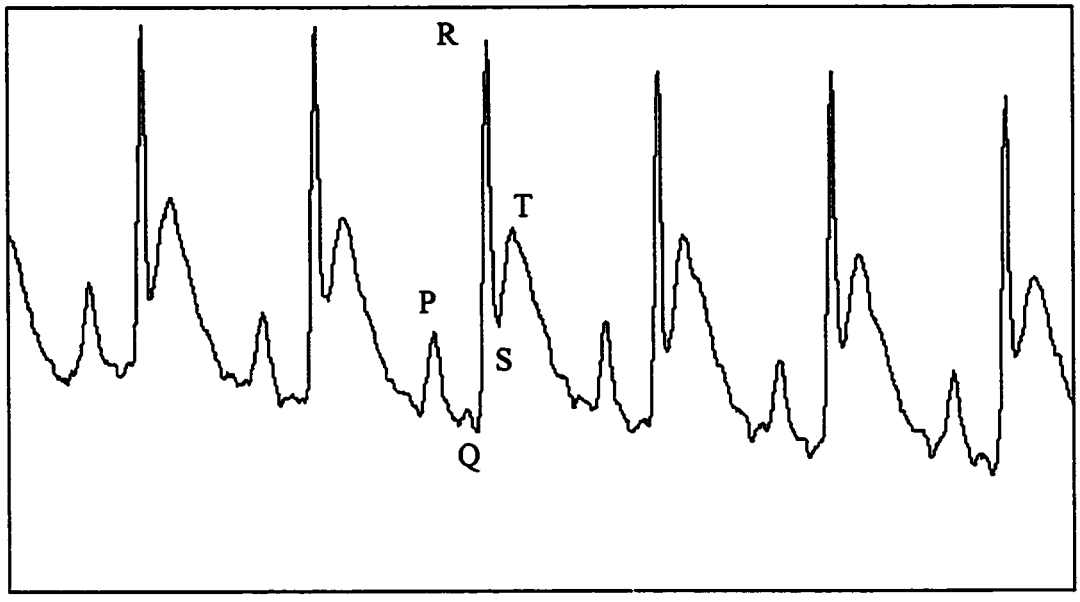


Figure 2-3: Typical rat ECG illustrating P-Wave, QRS complex and T-Wave

Statistical Analysis

Data are expressed as the mean ± SEM. A repeated partial measures ANOVA was used to test for differences amongst the treatment groups using the MANOVA univariate procedure in SPSS v6.0 software. Statistical significance was set at $p = 0.05$.

RESULTS

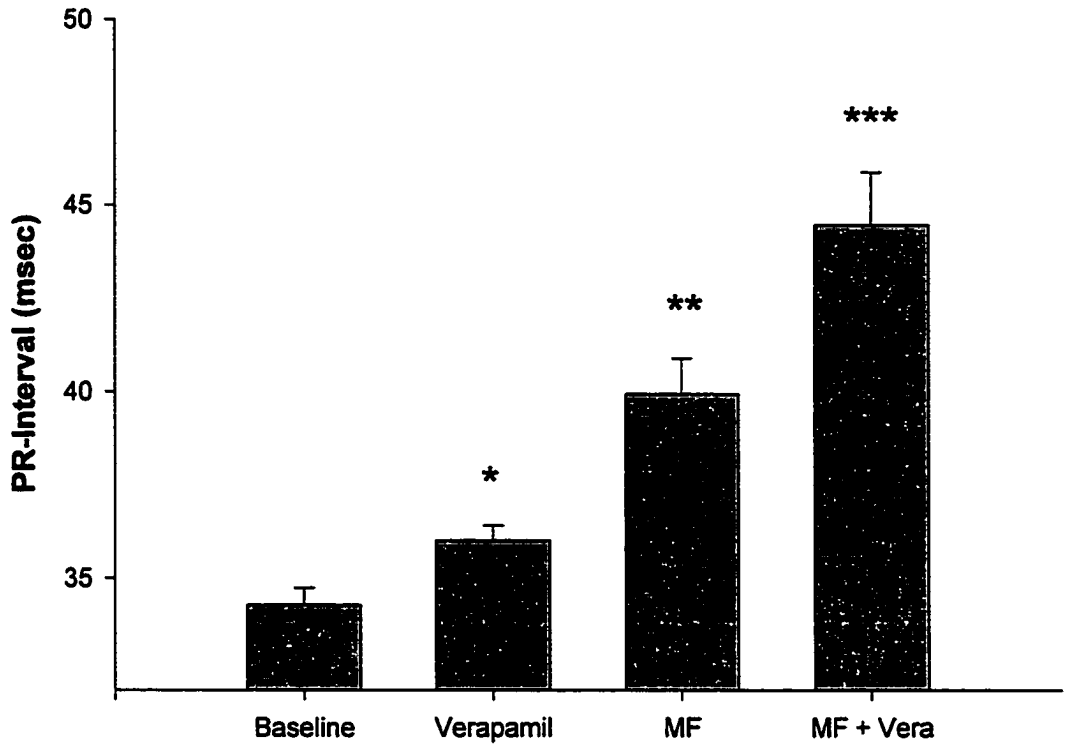
Methoxyflurane and VER both demonstrated significant dromotropic effects as measured by maximum prolongation in PR-interval (Figure 2-4). VER alone produced a significant 5% prolongation in PR-interval from baseline. The MF group demonstrated a 14.5 % maximum prolongation in PR-interval. However, VER + MF clearly demonstrated synergistic effects with a 28.7% maximum prolongation in PR-interval.

A nearly identical picture was observed for chronotropic effects as

measured by prolongation in RR-interval (Figure 2-5). VER alone resulted in a 6% prolongation in RR-interval. MF prolonged the RR-interval more than VER alone and the combined effects of VER + MF resulted in a 17.6% prolongation in RR-interval.

The effect on ventricular conduction was subtle. VER and methoxyflurane treatments caused a 5% prolongation in QRS-interval with a slight increase to 6.8% in the combined VER + MF group (Figure 2-6).

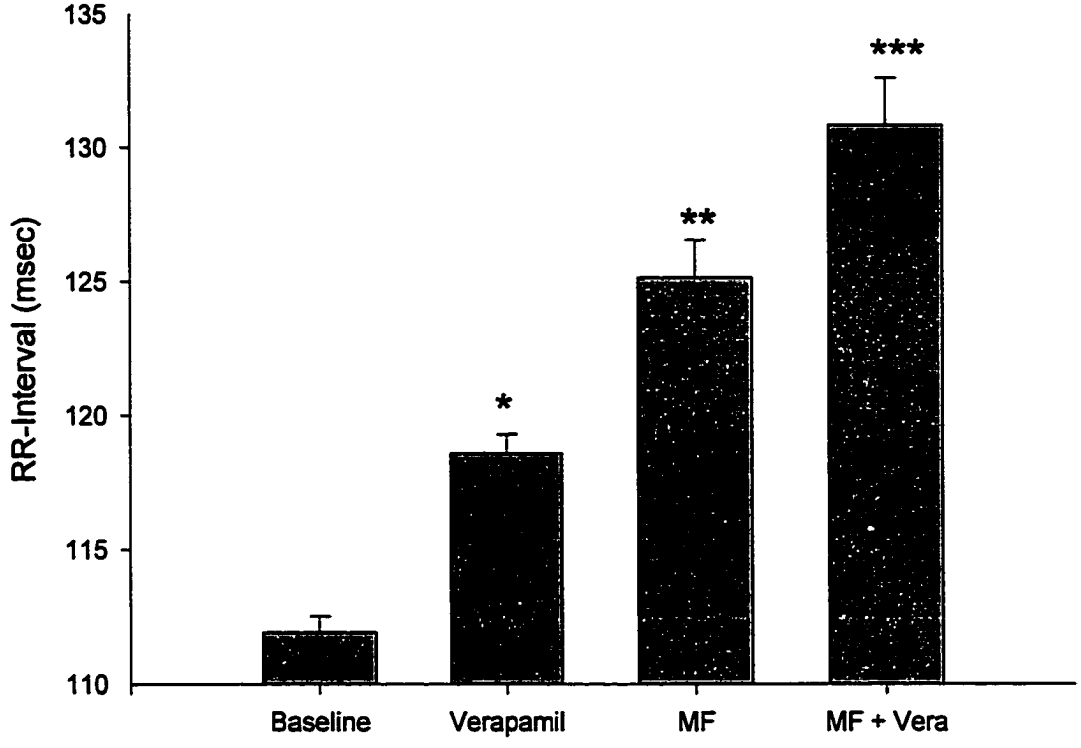
The time required to re-establish baseline, or normalize, after treatment is shown in Figure 2-7.



* Different from Baseline, $p < 0.05$
** Different from Baseline & VER, $p < 0.05$
***Different from Baseline, VER & MF, $p < 0.05$

Repeated Partial Measures ANOVA

Figure 2-4: Dromotropic effect of VER as measured by PR-interval (Mean ± SEM).



* Different from Baseline, $p < 0.05$
** Different from Baseline & VER, $p < 0.05$
***Different from Baseline, VER & MF, $p < 0.05$

Repeated Partial Measures ANOVA

Figure 2-5: Chronotropic effect of VER as measured by RR-interval (Mean ± SEM).

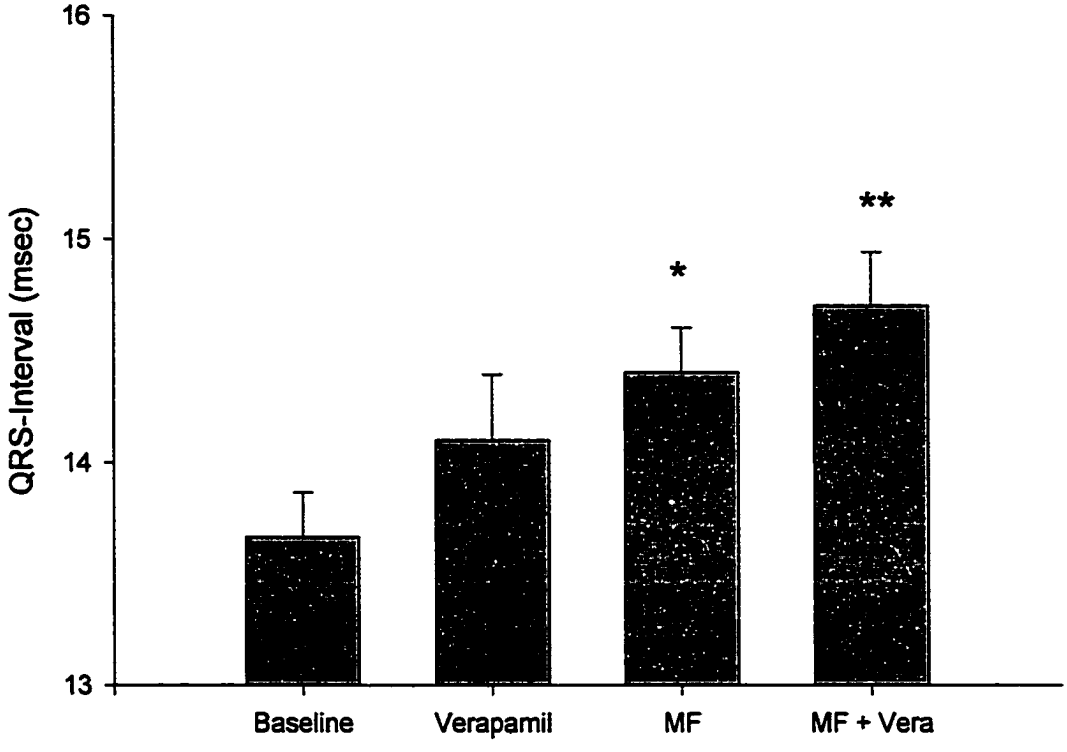


Figure 2-6: Effect of VER on ventricular conduction as measured by QRS-interval (Mean ± SEM).

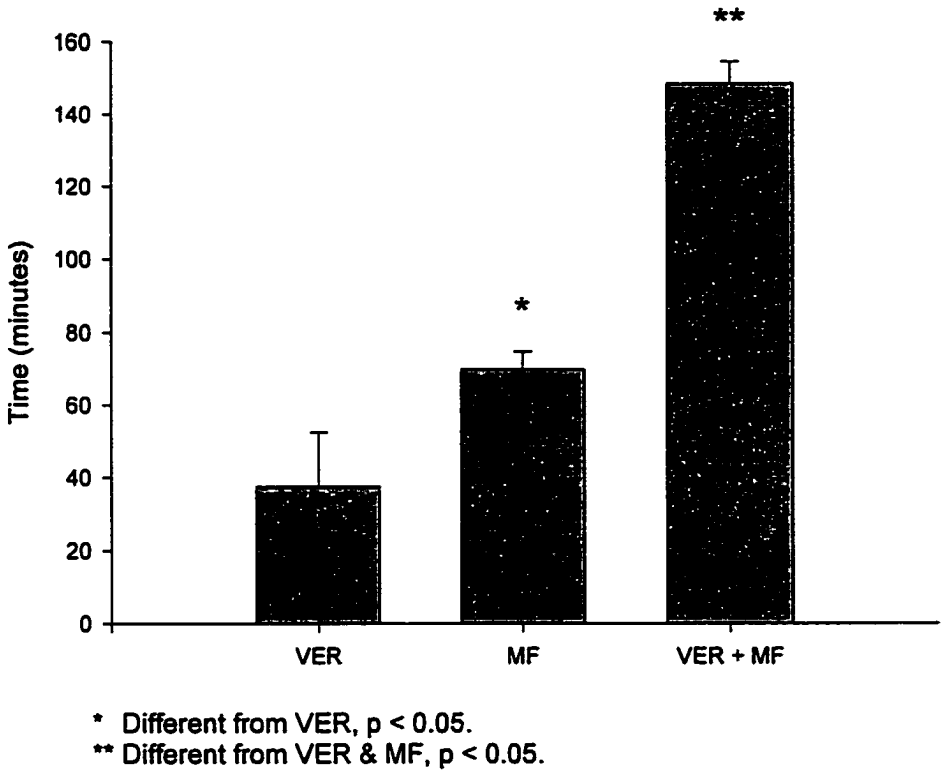


Figure 2-7: Time required to re-establish baseline (Mean ± SEM).

DISCUSSION

Methoxyflurane and verapamil appeared to have the greatest effect on dromotropism and chronotropism as measured by the PR-interval (Figure 2-4) and the RR-interval (Figure 2-5). It is generally assumed that VER has a much greater effect on the AV-node and is therefore most commonly indicated for supraventricular tachyarrythmias (Opie 1996). In our study, VER alone produced a 5% prolongation in PR-interval, which was statistically significant and reflects the relatively low dose of 10 mg/kg s.c. which was administered to the rats. A similar negative chronotropic effect was observed with a 6% prolongation in RR-

interval. This agrees well with clinical data, since VER is know to slow resting heart rate (Opie 1996).

It is clear that dose of MF required for surgical anesthesia, is sufficient to impair electrical conduction across the atria and through the AV-node as demonstrated by a 14.5 % prolongation in PR-interval (Figure 2-4). However, VER + MF clearly demonstrated synergistic effects with a 28.7% prolongation in PR-interval. MF has been shown to increase the automaticity of parts of the atrial conducting system other than the sinus either through a direct mechanism or through increased adrenergic sensitivity (Warren and Morrow 1977). VER is known to decrease the incidence of epinephrine-induced supraventricular tachyarrythmia in halothane anesthetized dogs (Kapur and Flacke 1981). However, greater prolongation in PR interval and direct depression of myocardial contractility was also observed. This appears similar to our observation of increased PR-interval prolongation with MF + VER. It is important to note that VER demonstrates "use-dependent" block of L-Type Ca^{2+} channels (Hescheler *et al.* 1982). This means that inhibition of the channel accumulates with repetitive stimuli. Furthermore, VER acts only after binding to the intracellular side of the membrane (Catterall and Streissnig 1992). Thus, VER must wait for L-type Ca^{2+} channels to open in order to reach its receptor. After the channel shuts, the drug remains trapped intracellularly and must slowly diffuse off the receptor at rest (Pelzer *et al.* 1982). This implies that VER will have a greater effect if conditions exist which increase the rate or duration of Ca^{2+} channel opening. While little has been written on methoxyflurane and Ca^{2+} channels, much work has been done on

the structurally related anesthetics halothane, enflurane and isoflurane. All three volatile anesthetics have been shown to have inhibitory effects on cardiac L-type Ca^{2+} channels (Nakao *et al.* 1989). Greater effects have been observed on low-voltage-activated calcium currents than on the high-voltage-activated L and N-types of Ca^{2+}, however halothane showed no preferential effect for low-voltage activated, L or N-type channels (McDowwell *et al.* 1996). Furthermore, halothane has been shown to increase channel open time constants in sarcoplasmic reticulum calcium channels. This suggests halothane can bind to both open and closed configurations of the channel (Bull and Marengo 1994). Thus, the negative inotropic effect of halothane and isoflurane is attributed to Ca^{2+} influx inhibition in the rat heart. This suggests that the observed synergism between methoxyflurane and VER may be due to cardiac L-type Ca^{2+} channel inhibition similar to halothane and isoflurane. Therefore, a verapamil-methoxyflurane drug interaction exists presumably at the level of the L-type Ca^{2+} channel or through indirect effects on Ca^{2+} flux through other channel types.

Methoxyflurane and VER also demonstrated a negative chronotopic effect. The R-wave is produced during contraction of the ventricles and the entire QRS complex reflects ventricular depolarization and the T-wave, ventricular repolarization (Dubin 1986). The RR-interval is used to calculate heart rate and is indicative of chronotropic effects. VER is a potent negative chronotrope (Opie 1995), and the small dose used in this study did result in a 6% prolongation in RR-interval. MF prolongs RR-interval even more than VER alone and the combined effects of both VER + MF resulted in a 17.6% prolongation in RR-

interval. This translates into a substantial slowing of heart rate suggesting that the combination can significantly depress the normal pacemaker areas of the heart.

Effects on ventricular conduction as measured by the QRS-interval were subtle. Both VER and MF caused a 5% increase in QRS-interval (Figure 2-6). The combination of VER + MF caused a slight increase to 6.8% which was different from VER alone, but not MF. These data suggest that VER and MF possess little Class IC antiarrhythmic activity. However, the rat ECG lacks a distinct S-T segment and an isoelectric line. Thus differences in QRS-intervals may be difficult to determine and it may be best to avoid a QRS measurement in the rat model (Budden 1980) unless the signal can be digitized and integrated reliably.

The time to re-establish baseline (Figure 2-7) was 37.5 ± 15.1 for VER, 69.8 ± 5.3 minutes for MF and 148.5 ± 6.6 minutes for MF + VER. This is an important measure, since any study on drug effect must include a sufficient wash-out period to ensure no residual anesthetic effects alter the pharmacodynamics of the test drug. This study only focused on pharmacodynamic interactions. No drug pharmacokinetics were measured. General anesthesia has also been shown to alter the pharmacokinetics of drugs. For example, thiopental, ketamine and propofol have been shown to increase the AUC of VER with a decrease in Vd (Orzulak-Michalak 1996). It is not known if MF has a similar effect, however the augmented effect on PR-interval by VER + MF could result from elevated concentrations of VER. However, given the study design, a rat would not be exposed to VER or anesthesia for an 18-hour period. This should have allowed

ample time for the washout of VER and anesthetic, unless severe depression of drug clearance occurred. Anesthetic use is not contraindicated in pharmacokinetic-pharmacodynamic studies, but its possible effects on the results should be understood. In addition, data collected in studies performed on an anesthetized animal should not be extrapolated to conscious animals since this could result in erroneous conclusions. If anesthesia is to be used for a procedure on the day of the study, a sufficient wash-out period should be allowed to minimize any residual effects. However the use of a control group treated in an identical manner will allow for a valid comparison.

In conclusion, a significant interaction was observed between MF and VER with an increase in dromotropic and chronotropic effects. This suggests that MF could augment the effects of VER in an unconscious animal. If the effects of VER alone are to be studied a washout period of at least 3 to 4 hours is required. This work further suggests that drug effects measured in anesthetized animals may differ significantly from studies in conscious animals. While it does not invalidate either form of experimentation, it does suggest that the two types of experiments must be interpreted cautiously. Clinically, patients may respond differently to the same drug given while under anesthesia than when awake. This suggests that in addition to changes in pharmacokinetics due to anesthesia and surgery, that pharmacodynamics may also be altered.

♦♦♦

REFERENCES

Budden R, Detweiler DK, Zbinden G, (Eds). The Rat Electrocardiogram in Pharmacology & Toxicology: Pergamon Press. Toronto (1980).

Bull R, Marengo J. Calcium-dependent halothane activation of sarcoplasmic reticulum calcium channels from frog skeletal muscle. *Am J Physiol* 1994; **266**: (2 Pt 1);C391-C396.

Catterall W, Striessnig J. Receptor sites of Ca^{2+} channel antagonists. *TiPS* 1992; **13**: 256-262.

Curtis MJ, Walker MJA. Antiarrhythmic actions of R,S-verapamil against ischaemic arrhythmia's in the rat. *Br J Pharmacol* 1984; **83**: 373-385.

Curtis MJ, Macleod BA, Walker MJA. Models for arrhythmia's resulting from myocardial ischaemia: the use of the rat. *J Mol Cell Cardiol* 1987; **19**: 399 - 419.

Dubin D. Rapid interpretation of EKG's . 3rd Ed. Cover Publishing Company, Tampa: 1986.

Deutsch N, Hantler C, Tait A, Uprichard A, Schork M,Knight P. Suppression of ventricular arrhythmia's by volatile anesthetics in a canine model of chronic myocardial infarction. *Anesthesiology* 1990; **72**: 1012 - 1021.

Echizen H, Brecht T, Niedergesäss S, Vogelgesang B,Eichelbaum M. The effect of dextro-,levo- and racemic verapamil on atrioventricular conduction in humans. *Am Heart J* 1985; **109**: 210-217.

Feeley T Management of perioperative arrhythmias. *J Cardiothor Vasc Anesth.* 1997; **11**: 10-15.

Harder S, Thürmann P, Siewart M, Blume H, Rietbrock N, vander Kleijn J, Gierend M. Concentration/effect relationship and enantioselective analysis of R,S-verapamil in hypertensive patients. *J Cardiovasc Pharmacol* 1992; **19**: 665-669.

Hashimoto H, Imamura S, Ikeda K, Nakashima M. Synergistic interaction between class I antiarrhythmic drugs and halothane. *Bio Pharm Bull* 1994; **17**: 449 - 453.

Haworth R, Goknur A. Inhibition of sodium/calcium exchange and calcium channels of heart cells by volatile anesthetics. *Anesthesiology* 1995; **82**: 1255-1265.

Heschelere J, Pelzer D, Trube G, Trautwein W. Does the organic calcium channel blocker D600 act from the inside or outside on the cardiac cell membrane? *Pflügers Arch* 1982; **393**: 287-291.

Kapur P, Flacke W. Epinephrine-induced arrhythmia's and cardiovascular function after R,S-Verapamil during halothane anesthesia in the dog. *Anesthesiology* 1981; **55**: 218-225.

Kroll D, Knight P. Antifibrillatory effects of volatile anesthetics in acute occlusion/reperfusion arrhythmias. *Anesthesiology* 1984; **61**: 657 - 661.

Miller SM. Mayer RC. Antiarrhythmic drugs should not be used to suppress ventricular ectopy in the perioperative period. *J Cardiothor Vasc Anesth* 1994; **8**: 701 - 703.

McDowwell T, Pancrazio J, Lynch C. Volatile anesthetics reduce low-voltage-activated calcium currents in a thyroid C-cell line. *Anesthesiology* 1996; **85**: 1167-1175.

Nakao S, Hirata H and Kagawa Y. Effects of volatile anesthetics on cardiac calcium channels. *Acta Anaesth Scand* 1989; **33**: 326 - 330.

Opie L, Frishman W, Thadani U. Calcium Channel Antagonists: Drugs for the Heart. 4th Ed. W.B. Saunders Company. Toronto: 1995. P 50 – 82.

Orszulak-Michalak D. The influence of selected general anesthetics on pharmacokinetic parameters of some antiarrhythmic drugs in rabbits. Part V. R,S-Verapamil. *Acta Poloniae Pharmaceutica* 1996; **53**: 57-61.

Pelzer D, Trautwein W, McDonald T. Calcium channel block and recovery from block in mammalian ventricular muscle treated with organic channel inhibitors. *Plfügers Arch* 1982; **394**: 97-105.

Takada K, Sumikawa K, Kamibayashi T, Hayashi Y, Yamatodani A, Kawabata K and Yoshiya I. Comparative efficacy of antiarrhythmic agents in preventing halothane-epinephrine arrhythmia in rats. *Anesthesiology* 1993; **79**: 563 - 570.

Warren J and Morrow D. Antiarrhythmic anesthetic action: II. The effect of methoxyflurane on ventricular automaticity. *Anesth Analg* 1977; **56**: 194-201.

Chapter 3

INTERFERON-α2a INCREASES VERAPAMIL SERUM CONCENTRATION BUT REDUCES ITS DROMOTROPIC EFFECT IN THE RAT.

INTRODUCTION

Inflammatory disease and individual proinflammatory cytokines have been shown to depress cytochrome P450 (CYP450) isozyme activities (Cawthorne *et al.* 1976; Descotes 1985; Moreno *et al.* 1987; Peterson & Renton 1986). This results in decreased drug clearance with a corresponding increase in drug concentration. Ansher *et al.* (1992) demonstrated that interferon-α2a (IFN-α) significantly decreased hexobarbital metabolism in mice with a corresponding increase in hexobarbital-induced sleep time, thus drug effect was enhanced. Furthermore, greater depression of drug metabolism of hexobarbital was observed when another pro-inflammatory cytokine interleukin-2 (IL-2) and IFN-α was added suggesting that cytokines have additive or synergistic effects. Inhibition of metabolism has also been observed in the rat with inflammation (Belpaire *et al.* 1989, Abdel-Razzak *et al.* 1993, Sakai *et al.* 1992, Ferrari *et al.* 1993a, Ferrari *et al.* 1993b). Serum levels of the ß-blocking drug propranolol, were elevated nearly 20 fold with increased protein binding in the adjuvant arthritis model of inflammation (Piquette-Miller & Jamali 1993). Furthermore, treatment of inflammation with ketoprofen, a non-steroidal anti-inflammatory drug, normalized serum levels (Piquette-Miller & Jamali 1995). Therapeutic

consequence of the inflammation-induced alteration of pharmacokinetics is not known. Nevertheless, an increase in drug concentration in the circulation is expected to result in potentiation of the effect and even toxic reactions.

The mechanism through which cytokines can alter CYP450 function is not clear. Cytokines increase the production of nitric oxide (NO) from macrophages, neutrophils, cardiac myocytes, Kupfer cells and platelets (McCall *et al.* 1989, Lapoint & Sitkins 1996,
Kubes 1995) through the inducible form of nitric oxide synthase (iNOS). It has been shown that NO can inactivate CYP450 isozymes through free radical destruction of the heme-site (Khatsenko *et al.* 1993, Parent *et al.* 1992). In addition conditions favoring iNOS induction are associated with a decrease in both pre and post-transcription of CYP450 mRNA leading to a decrease in total amount of CYP450 enzymes (Renton and Knickle 1990, Stanley *et al.* 1988, Delaporte and Renton 1996). However, NO can react quickly with superoxide anion (O_2^-) resulting in the formation of peroxynitrite ($ONOO^-$) which could also be involved in the inhibition of CYP450. Therefore, it is anticipated that immunostimulation which results in cytokine release and reactive nitrogn species generation could decrease the ability to metabolize drugs. Type I (IFN-α ,β) and Type II (IFN-γ) IFN's have been shown to be a priming signal for iNOS induction (Nathan 1992). IFN-α itself has been shown to induce iNOS and has previously been shown to decrease the clearance of several drugs metabolized by CYP450, especially CYP1A1 and CYP1A2 (Cribb *et al.* 1994).

The calcium channel blocker verapamil (VER) is extensively metabolized

in a stereospecific manner by CYP3A4, CYP1A2 and to a lesser extent CYP2C9 isozymes (Kroemer *et al.* 1993, Busse *et al.* 1995) in man. However, an absence of CYP3A4 in rat suggests VER metabolism occurs primarily through CYP2C isozymes, although CYP1A isozymes can be shown in the rat after enzyme induction (Hakkak *et al.* 1993, Fujimaki 1994). The enantiomers of VER exhibit different abilities to block the AV-node with S-VER being 10 to 20 times more potent than its antipode (Echizen *et al.* 1985). The major metabolite of VER, norverapamil (NOR), which is found in plasma of both humans and rats, also possesses pharmacological activity at the AV-node, but is approximately 50 to 100 times less potent than the parent drug (Johnson *et al.* 1991). The potential for a pharmacokinetic interaction exists between VER and IFN-α. Indeed it has been shown that endotoxin-induced inflammation results in inhibition of verapamil in the rat (Laethem *et al.*, 1994). Elevation of VER and/or NOR concentrations could, therefore, predispose the patient to drug toxicity although other pharmacodynamic effects could also occur. Therefore, the objective of this study was to examine the consequence of IFN-α-induced altered VER pharmacokinetics on the pharmacodynamics of the drug.

METHODS AND MATERIALS

Materials

Verapamil hydrochloride and norverapamil were gifts from G.D. Searle (Skoki, Ill), manufactured by Knoll Pharmaceuticals (Stuttgart, Germany). The internal standard, (+) Glaucine and heptafluorobutanol were purchased from

Aldrich (Milwaukee, WI). HPLC grade hexane was purchased from Caledon Laboratories (Georgetown, Canada). HPLC grade propan-2-ol was purchased from BDH Inc. (Toronto, Canada). Triethylamine (TEA) was purchased from Sigma Chemical Co. (St. Louis, MO). Heptane was purchased from Mallinckrodt (Paris, KT) and 98% anhydrous ethanol was purchased from Stanley (Vancouver, Canada). *Aperigillus* nitrate reductase10 U/mL, 0.1 M FAD, 1 mM NADPH, 1500 U/mL LDH and 100 mM pyruvic acid were all purchased from Sigma Chemical Co. (St. Louis, MO). Human IFN-α2a 3 x 10^6 U/mL (F. Hoffmann-La Roche, Basle, Switzerland) was a generous gift from the pharmacy department at the Cross Cancer Institute, Edmonton Alberta.

Study Protocol

The study followed the guidelines established for ethical handling of live animals at the University of Alberta. Adult, male, Sprague-Dawley rats were used in the study. The animals were acclimated to a 12-hour day-night cyle, housed in rodent cages, and fed standard rodent chow. The animals were divided into a control group (331 ± 23 g, n = 8) and an IFN-α2a group (328 ± 21 g, n = 8). The animal's right jugular vein was cannulated using light ether for induction of anesthesia and methoxyflurane for maintenance. PE-50 tubing with a silastic end was advanced into the jugular vein and exteriorized to the animal's back and locked with heparin 100 U/mL. The animals were returned to their cages and allowed to recover overnight. The control group received no IFN-α on the day of surgery. The IFN-α2a group was administered two doses of 5 x 10^4 IU IFN-α2a

s.c. 12 and 2 hours prior to the verapamil dose. On day 2 racemic verapamil 20 mg/kg was given by oral gavage and the serial blood samples (approximately 175 μL) were drawn at 0, 15, 30, 45, 60, 90, 120, 180, 240, 360 and 420 minutes to determine stereospecific pharmacokinetic parameters of VER. ECG leads were placed at the time of the jugular cannulation and brought around to the animal's back. The ECG was monitored for a minimum of one hour or until a stable baseline was established. PR-interval and heart rate (HR) were recorded approximately 30 to 60 seconds prior to blood sampling. The blood sample at time zero was 500 μL to allow for measurement of erythrocyte sedimentation rate and white count. A differential white count was performed by fixing a blood smear with an equal mixture of methylene blue and eosin Y stains. The differential counts were expressed as a percentage of the raw white count. Serum nitrite was also determined just before the first blood sample to determine nitrite levels prior to the loss of blood due to sampling.

Verapamil and Norverapamil Assay.

A stereospecific verapamil assay was used as previously published by Shubikawa and Wainer (1992). Briefly, to 100 μL of plasma in a glass test tube were added

75 μL of 400 ng/mL (+) glaucine (internal standard), 100 μL 2 M NaOH, 0.4 mL sodium phosphate buffer (pH 7.0, ionic strength 0.1), and 6 mL heptane. The sample was vortexed for 1.0 minute, then centrifuged at 2000 *g* for 10 minutes. The organic layer was transferred to clean glass tubes and evaporated to dryness in a vacuum centrifuge at 60°. The resulting residue was reconstituted in 200 μL of

mobile phase (hexane-isopropanol-ethanol-TEA, 85:7.5:7.5:1.0, v/v), and 100 μL injected into the HPLC.

A Waters (Millipore-Waters, Missassuaga, Canada) HPLC apparatus was used consisting of a twin piston pump, a WISP 710B autosampler, a column oven (31^{O}) and a 470 fluorescence detector set at excitation of 272 nm and an emission of 317 nm with a bandwidth set at 18 nm. The integrator was a Hewlett-Packard (Avondale, PA) 3390A model. An achiral column (5 cm x 4.6 mm ID Supelcosil LCSi column, Supelco Inc., Bellefonte PA) was serially attached to a chiral column (250 mm x 4.6 mm I.D. 10 μm Chiralpak AD, Daicel Chemical Ind., Tokyo, Japan). Standard curves were run in duplicate and were linear over the test range (2.5 ng/mL to 200 ng/mL, $r^2 \geq 0.996$). Sensitivity for R and S-verapamil was 2.5 ng/mL (CV < 5%) and 7.5 ng/mL for R and S-norverapamil (CV < 13%).

Electrocardiogram Analysis

The electrocardiogram (ECG) was measured using braided stainless steel, Teflon coated electrodes (Cooner Wire Co, Chaterworth, CA) placed subcutaneously in the left and right axilla and over the xyphoid process. The ECG amplifier was a Honywell for Medicine ECG amplifier (Honywell Electronics for Medicine, Edmonton Canada) and the data recorded using Acknowledge software (World Precision Instruments, Miami FL) on a personal computer. The mean of five cycles was taken for the measurement of PR intervals and heart rate (HR).

Serum Nitrite Analysis

Since nitrite is a stable breakdown product of both NO and $ONOO^{-}$, it can

be used as an indirect measure of both of these reactive nitrogen species (RNS) (Beckman and Koppenol 1996). However, the ultimate metabolic fate of NO *in vivo* is nitrate. Therefore, nitrate must be reduce to nitrite for measurement. Nitrite was measured using the method of Grisham *et al.* (1995). Briefly, 100 μL of plasma was incubated with 10 U of *Asperigillus* nitrate reductase in the presence of 0.1 M flavine adenine dinucleotide (FAD), 1 mM nicotinamide adenine dinucleotide phosphate dehydrogenase (NADPH) to reduce all nitrate to nitrite. The reaction was quenched with 1500 U lactate dehydrogenase (LDH) and 100 mM pyruvic acid. This was then treated with the Griess reagent, an equal mixture of 0.2% Napthylenethylendiene and 2% sulfanilamide in 5% ortho-phosphoric acid. Absorbance measured at 540 nm using a Vmax Molecular Devices plate reader (Molecular Devices Corp, U.S.A). Calibration was performed using standard solutions of $NaNO_2$ and $NaNO_3$ to evaluate enzyme efficiency. The assay was linear from 5 μM to 200 μM ($r^2 \leq 0.996$, C.V. < 5%).

Hematological Analysis

As a measure of inflammatory response to IFN-α2a, white blood cell counts were performed after mixing the whole blood with 20 μL glacial acetic acid to lyse all red cells and staining with gentian violet. Counts were then performed manually with a Neubauer cytometer (Neubaur, Buffalo, NY) and Bausch and Lomb microscope (Bausch and Lomb, Rochester NY.) Blood smears were differentially stained with and equal mixture eosin Y and methylene blue. A total of 100 white cells were counted to determine the percentage of lymphocytes, neutrophils, segmented neutrophils, eosinophils and monocytes. Erythrocyte

sedimentation rate (ESR) was determined using the Wintrobe method.

Protein Binding

Protein binding was measured in two separate cohorts of animals, control (n = 8) and IFN-α-treated, (n = 8). The animals were prepared in a manner identical to the PK-PD study groups including R-jugular vein cannulation. The IFN-α-treated animals received 5 x 10^4 IU IFN-α2a sc x 2 days starting at the time of surgery and the following morning. Rats were anesthetized with methoxyflurane and the total blood volume removed from the R-jugular cannula. The blood was then centrifuged at 2000 g for 15 minutes. The resulting serum was adjusted to pH 7.4 with 0.1 N HCl. The serum was then spiked with 100 ng/mL of R-VER and 200 ng/mL S-VER to approximate the stereoselective drug levels and enantiomers ratio observed *in vivo*. The serum was incubated at 37 C for one hour then transferred to Amicon® micropartition chambers for ultrafiltration (Amicon Division of W.R. Grace & Co, Danvers MA). The chambers were centrifuged at 2000 g for one hour. In addition four chambers were loaded with phosphate buffer, pH 7.4, to determine the presence of any nonspecific binding or adsorption to the micropartition system. Both filtrate and nonfiltrate concentrations were measured. The fraction unbound (*fu*) was determined as the concentration unbound (Cu) divided by total concentration (Ct) where Ct equals the sum of the filtered and unfiltered concentrations. To ensure the concentrations were above the minimum quantifiable limit for the HPLC assay, four micropartition chambers were pooled allowing for a total of four measurements per group.

Pharmacokinetic Analysis

Model independent analysis of serum verapamil enantiomers was performed. Terminal elimination rate constant (λ_z) was calculated using a non-weighted nonlinear least-squares regression in WinNonLin Profession for Windows (v 2.0) (Scientific Consulting, Inc, Apex North Carolina). The $AUC_{0\rightarrow7}$ was calculated using the linear trapezoidal rule to the end of the experiment for both VER and NOR. $AUC_{0\rightarrow\infty}$ for VER was calculated from $AUC_{0\rightarrow7} + AUC_{7\rightarrow\infty}$ and $AUC_{7\rightarrow\infty} = Ct/\lambda z$, where Ct was the last experimental data point. Oral clearance (Cl/F) was calculated using $Cl / F = \frac{Dose}{AUC_{0-\infty}}$.

Statistical Analysis

The data are shown as the arithmetic mean ± S.E.M. Student's t-test for independent samples was used to compare treatment groups at $p = 0.05$.

RESULTS

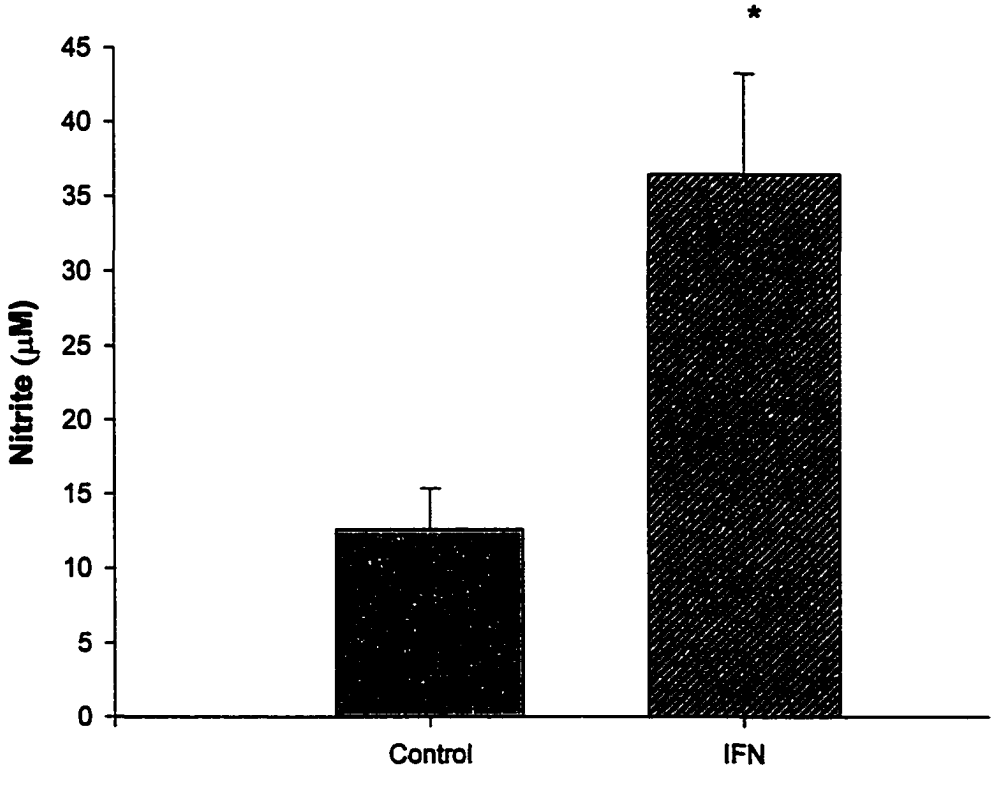
Pathophysiological Changes

Interferon treatment resulted in a six-fold increase in segmented neutrophils and a more than two-fold rise in ESR (Table 3-1). Serum nitrite was also significantly elevated by treatment with interferon (Figure 3-1).

Table 3-1: Hematologic Parameters

Parameter	Control, (Mean ± SEM)	IFN-α2a, (Mean ± SEM)
ESR (mm/h)	0.9 ± 0.1	2.3 ± 0.7*
WBC (10^9)	18.1 ± 2.4	23.9 ± 2.8
Lymphocytes (%)	81.9 ± 3.9	76.9 ± 1.9
Neutrophils (%)	15.2 ± 4.1	9.3 ± 1.3
Segmented Neutrophils (%)	1.8 ± 0.3	11.1 ± 1.9**

* Different from Control, $p < 0.05$, Student's t-test.
**Different from Control, $p < 0.001$



* Different from Control, $p < 0.01$, Student's t-test.

Figure 3-1: Serum nitrite in control and IFN-α-treated rats (Mean ± SEM).

Pharmacokinetic Changes

Pre-treatment with IFN-α clearly altered the pharmacokinetics of VER (Figure 3-2 and Table 3-2). Figure 3-2 represents the mean concentration time curves for all animals. For the IFN-α-treated animals it appears that a regression of the terminal phase would be indicate a very long t 1/2λz. However, Rat 1 and Rat 5 demonstrated very high serum levels with increasing serum levels at 7-hours. This skewed the mean concentration points at 6 and 7 hours making it appear that the serum levels have decreased very little. Therefore, a regression of the terminal phase could not be performed on these animals and are not included in the λz and $AUC_{0\rightarrow\infty}$ calculations. The effect of these two animals can be seen in the appendix at the end of this chapter (Figure 3-A-1). Additionally, a mean tail end calculation for the mean values without Rat 1 and Rat 5 are shown in Table 3-A-1. IFN-α-treatment resulted in a statistically significant 7-fold increase in R-VER $AUC_{0\rightarrow\infty}$ and a 3-fold increase in S-VER $AUC_{0\rightarrow\infty}$. A corresponding significant decrease in oral clearance (CL/F) was also observed for both enantiomers. Tmax was also altered with a 2.5-fold increase for R-VER and a 3.3-fold increase for S-VER. A 3.2-fold decrease in λ_z was observed for R-VER, but the effect on S-VER was insignificant. Protein binding as measured by *fu* was not significantly altered by the treatment with

IFN-α.

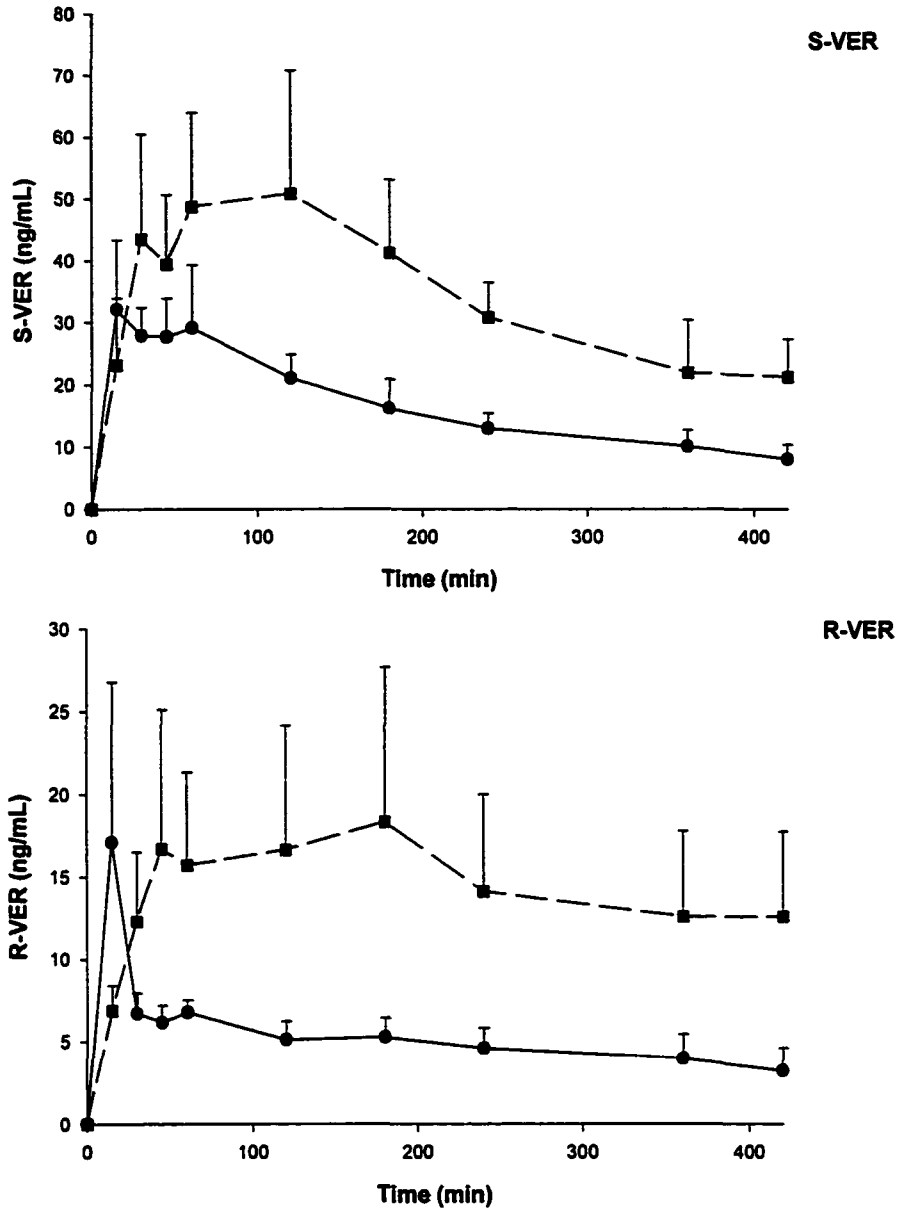


Figure 3-2: Serum VER concentration-time profile in control (●) and IFN-α2a-treated (■) rats.

Table 3-2: Verapamil Pharmacokinetic Parameters

Parameter		Control, (Mean ± SEM)	IFN-α2a, (Mean ± SEM)
Tmax (min)	S	36.4 ± 8.6	118.6 ± 25.1***
	R	28.1 ± 7.1	69.4 ± 12.3*
Cmax (ng/mL)	S	42.4 ± 9.4	68.9 ±15.4
	R	16.1 ± 8.7	19.0 ± 8.5
λz (h^{-1})	S	0.46 ± 0.19	0.37 ± 0.48
	R	0.57 ± 0.13	0.18 ± 0.04*
$AUC_{0\rightarrow 7}$ (μg.min/mL)	S	49.6 ± 4.7	163.3 ± 33.5**
	R	9.7 ± 2.4	69.7 ± 19.6**
$AUC_{0\rightarrow\infty}$ (μg.min/mL)	S	53.4 ± 6.7	178.4 ± 48.2**
	R	11.2 ± 2.8	74.4 ± 26.6*
CL/F (L/min/kg)	S	0.22 ± 0.03	0.075 ± 0.01*
	R	1.5 ± 0.5	0.27 ± 0.05*
AUC R/S	Ratio	0.19 ± 0.04	0.26 ± 0.06
fu	S	0.067 ± 0.011	0.077 ± 0.0057
	R	0.178 ± 0.017	0.184 ± 0.0096

* Different from Control, $p < 0.05$, Student's t-test.
** Different from Control, $p < 0.01$, Student's t-test.
*** Different from Control, $p < 0.001$, Student's t-test.

There was a significant correlation between serum nitrite and AUC of both VER enantiomers ($p<0.00001$) as shown in Figure 3-3 for S-VER. In addition, a negative correlation was observed between serun nitrite and PR-interval prolongation ($p < 0.02$).

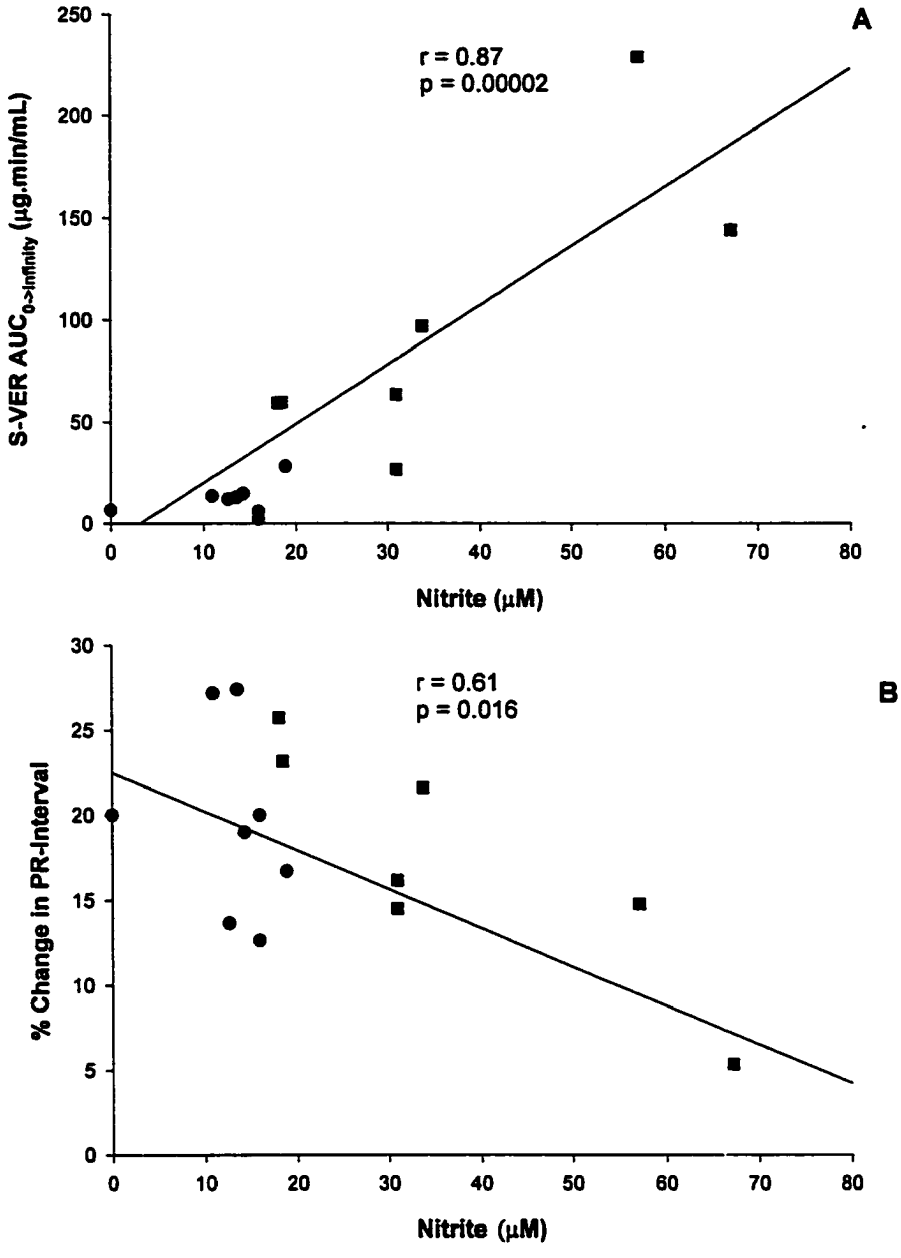


Figure 3-3: The correlation of serum nitrite with (A) S-VER $AUC_{0\rightarrow\infty}$ and (B) % change from baseline in PR-interval after 20 mg/kg VER to control (●) and IFN-α2a-treated (■) rats.

IFN-α2a causes an approximately two-fold increase in the $AUC_{0\rightarrow7}$ of both S and R-NOR (Table 3-2). In addition, a nearly two-fold increase in $AUC_{0\rightarrow7}$ was observed for S-NOR and a 2.5-fold increase was observed for R-NOR. No statistically significant changes were observed in λz. The AUC NOR/VER ratio was significantly altered for S-NOR demonstrating a 2.7-fold decrease from control values. No differences in NOR R/S ratios were observed.

Table 3-3: Norverapamil Pharmacokinetic Parameters

Parameter		Control (Mean ± SEM)	IFN-α2a (Mean ± SEM)
$AUC_{0\rightarrow7}$ (μg.min/mL)	S	62.0 ± 12.4	108.9 ± 15.7*
	R	14.2 ± 3.5	36.0 ± 8.5*
NOR/VER AUC Ratio	S	1.6 ± 0.3	0.6 ± 0.2*
	R	2.5 ± 0.9	1.3 ± 0.4
NOR AUC R/S Ratio		0.21 ± 0.05	0.33 ± 0.09
k(m) (h^{-1})	S	0.21 ± 0.06	0.18 ± 0.1
	R	0.20 ± 0.1	0.31 ± 0.1

*Different from Control, $p < 0.05$, Student's t-test.

Pharmacodynamic Changes

The IFN-α-treated group demonstrated significantly less dromotropic effect, but equal chronotropism as compared with controls (Figure 3-4).

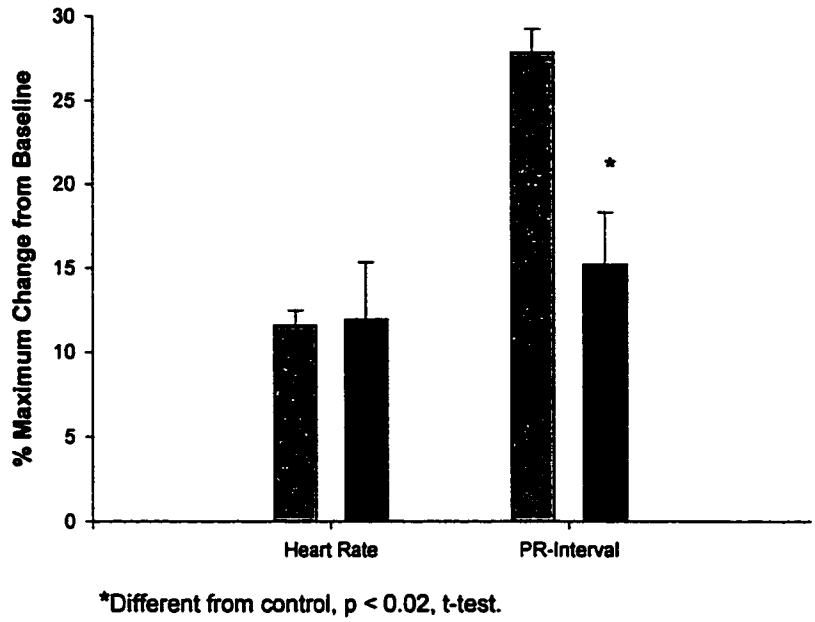


Figure 3-4: The effect of 20 mg/kg VER on % Maximum change in heart rate and PR-interval for control (▬) and IFN-α2a-treated (▬) rats.

DISCUSSION

Treatment with human IFN-α under these experimental conditions clearly caused a rise in inflammatory responses as reflected by increases in serum nitrite segmented neutrophils and ESR (Table 4-1). IFN-α has been suggested to be both a pro-inflammatory and anti-inflammatory cytokine depending on the presence of other cytokines. It stimulates the production of type 1 T-helper cells leading to increases in IFN-γ, and IL-2. IL-2 in turn can increase IL-1ß and TNF-α production (Tilg 1997). Thus, IFN-α can directly influence pro-inflammatory activity of other cytokines. This is observed clinically where IFN-α therapy has resulted in the development of acute arthritis, pancreatitis, glomerulomephritis

and a worsening of other autoimmune disease (Guttermann 1995, Pittau *et al.* 1997). The Type II IFN, IFN-γ, has been shown to be a potent inducer of iNOS such that NO has been used a bioassay for IFN-γ (Kim and Son 1996). However, considerable controversy exists on the effects of Type I IFN's (IFN-α and β). Several reports indicate that pretreatment with IFN-α can decrease NO production due to lipopolysaccaride stimulation and IFN-γ (Adler *et al.* 1995, Deguchi *et al.* 1995, Fast 1993). Thus, the environment into which the IFN-α is added becomes critically important. The experiment described here may represent not only the effects of IFN-α treatment, but the effects of IFN-α treatment after nonsterile surgery since nonsterile surgery has been shown to induce iNOS in the rat (Losonczy *et al.* 1997). However, in this experiment, the control rats were also subjected to surgical incision and demonstrated nitrite levels similar to the non-surgical rats reported by Losonczy *et al.* (1989). Furthermore, WBC, neutrophils and segmented neutrophil values in the control animals are within the reported normal value for Sprague-Dawley rats. IFN-α has been shown to directly increase neutrophil respiratory burst, a step in which the neutrophil dramatically increases oxidative metabolism prior to phagocytosis. It is believed that this step may also be antecedent to NO production especially in the presence of viral insult (Little *et al.* 1994). It is clear that IFN-α2a caused inflammatory changes within the treated animals.

Oral bioavailability of both verapamil enantiomers is low due to extensive stereoselective first pass metabolism (Vogelgesang *et al.* 1984). VER enantiomers are extensively metabolized by the liver in the rat (McIlheny 1971)

and in humans (Eichelbauem *et al.* 1979). In the rat, S-VER, the pharmacologically more active enantiomer is an intermediately cleared drug while its antipode is observed to be more efficiently cleared (extraction ratio, 0.41 and 0.74 for S- and R-VER, respectively) (Bhatti and Foster 1997). The observed stereoselectivity in the hepatic extraction ratio may be explained, at least in part, by the over one-fold difference in the plasma binding of verapamil enantiomers (Table 3-2). S-VER may be bound to plasma proteins with greater affinity than its antipode restricting its first-pass metabolism and rendering the enantiomer a drug with an intermediate hepatic extraction. Indeed, in the absence of protein, the stereoselectivity in the extraction ratios diminishes (0.96 and 0.94 for S- and R-VER, respectively) (Mehvar and Reynolds, 1996).

The present data suggest inhibition of clearance of both enantiomers in response to IFN-α-treatment. The inhibitory effect of interferon on VER clearance, although reported here for the first time, was not unexpected. Such an effect has been reported following endotoxin-induced inflammation in the rat (Laethem *et al.* 1994), rabbits and dogs (Laethem *et al.* 1995). In the rat the reduced clearance was attributed to increased plasma protein binding and was more pronounced for S-VER (Laethem *et al.* 1995). Our data, however, suggest that the effect of interferon was more pronounced for R-VER than for S-VER as indicated by decreases in oral clearance of approximately 6 and 3 fold, respectively (Table 3-2). Inflammation is known to result in increased concentrations of α1-acid glycoprotein (AAG) (Piafsky *et al.* 1977) and decrease intrinsic hepatic clearance (Cli') of drugs (Ansher *et al.*1992, Monshouwer *et al.*

1995). In man, VER has been shown to bind to both albumin and AAG (McGowan *et al.* 1986). In general, a substantial change in Cli' may influence oral clearance of hepatically cleared drugs, and since IFN-α induced inflammation did not alter protein binding of verapamil enantiomers (Table 3-2), a diminished Cli' may explain the observed reduction in oral clearance of verapamil enantiomers (Table 3-2). In addition, increased production of NO may lead to enhanced hepatic blood flow (Q) through vasodilation (Pannen *et al.* 1997, Kakumisu *et al.* 1998). Since nitrite levels were increased in the IFN-α group (Figure 3-1), both Cli' and Q may be involved in the interaction between verapamil and IFN-α. As has been suggested for propranolol, another highly extracted drug, both decreased and increased Q may slow down clearance of the drug (Byren *et al.* 1984, Feely *et al.* 1983). A reduced Q may limit the drug supply to the site of metabolism decreasing drug extraction. However an increase in Q could increase the input rate of the drug leading to enzyme saturation and increased bioavailability. This has been observed with another High E drug, propranolol by Wagner (1985).

The influence of reduced Q is expected to be minimal on low extraction and maximal on high extraction drugs (Pang and Rowland 1977). This may explain the stereoselectivity observed in the effect of IFN-α on verapamil enantiomers. The enantiomer with higher extraction ratio in the rat (R-VER) is more affected by inflammation than its antipode with an intermediate extraction ratio.

The AUC of NOR was also significantly increased as a result of IFN

treatment (Table 3-3). This effect was more pronounced for the R enantiomer as compared to S-VER. This may be a mere reflection of increased concentration of the parent drug. The metabolite:drug ratio, however, was substantially less in the IFN-α group which reached statistical significance only for S-VER. This indicates a reduced formation and/or enhanced clearance of NOR. The present data, however, does not permit a clear interpretation of the effect of IFN-α on NOR.

The most striking observation of this study was that despite the obvious increase in the pharmacologically more active and predominant enantiomer concentrations, less dromotropism is observed (Figure 3-2 and Figure 3-3). Furthermore, this decrease in dromotropism correlated negatively with serum nitrite. Thus as serum nitrite increases dromotropic effect due to verapamil decreases. The decreased dromotropism was clearly evident in the maximum percent change in PR interval where control animals had a nearly 28% prolongation while IFN-α-treated animals exhibited only a 15%. It is important to note that the 20 mg/kg dose of VER was chosen to achieve blood levels in the rat similar to levels achieved in human after an 80 mg oral dose. Reduced response to high VER concentrations in the interferon-treated rats was unexpected since an increased drug concentration is expected to of result in increased potency and even toxicity. With VER, we expected increased prolongation of PR interval secondary to the observed elevation of the drug concentration. PR interval reflects the electrical spread of current accross the atria from the time of atrial contraction (P-wave) to the time of ventricular contraction (R-wave) and it therefore a

measure of AV-node responsiveness. AV node blockage indicates that the normal electrical spread from the atria to the ventricles is slowed. (Dubin 1986). This is a side effect of VER particularly in response to higher drug concentrations (Thomas 1996).

The observed inflammation-induced reduced response to VER may have been caused by a reduced unbound concentration of VER and/or lower sensitivity of calcium channel receptors or other effects. The former is unlikely since the extent binding of VER enantiomers to plasma proteins was not altered by inteferon treatment (Table 3-2). Therefore, the concentration of the unbound VER was still higher in the INF-α2a as compared to the control rats. Down-regulation of Ca^{2+} channels by increased expression of cytokines or NO, however, is plausible. This could also be mediated immunologically especially in the presence of cytokines which stimulate the production of NO. NO has been shown to have negative inotropic effects on the heart (Finkel *et al.* 1992). NO is powerful activator of guanylyl cyclase (GC) (Murad *et al.* 1993). Activation of GC leads to increased levels of cyclic 3'-5'-guanosine monophosphate (cGMP) which has been shown to inhibit L-Type Ca^{2+} channels (Méry *et al.* 1991, Flesch *et al.* 1997, Rivet-Bastide *et al.* 1997). Thus the extracellular Ca^{2+} current through the L-type channel ($I_{Ca\text{-}L}$) is inhibited. VER as a phenylalkylamine is believed to have negative inotropic, dromotropic and chronotropic effects through binding to the intracellular portion of the L-Type Ca^{2+} channel pore decreasing $I_{Ca\text{-}L}$ (Catterall and Striessnig 1992). Therefore, the potential exists for an IFN-induced rise in NO to alter the dromotropic effects of VER. However, this does

not preclude a direct cytokine effect on cardiac function. For example, binding of IL-2 to cardiac IL-2 receptors causes a direct increase in transmembrane Ca^{2+} ion flux. (Gross 1993). Such alterations in Ca^{2+} currents could alter the normal inotropic and dromotropic responsiveness of the mycocardium to drugs such as VER. The negative correlation of PR-interval with serum nitrite merely suggests that the down-regulation of calcium channels occurs concurrently with a rise in reactive nitrogen species. Down-regulation of β–adrenergic receptors by pro-inflammatory cytokines and inflammatory conditions has also been observed in asthma and in congestive heart failure (Meurs *et al.* 1987, Bavendiek *et al.* 1996). A decrease in receptor density is not necessary for this downregulation, but changes in receptor function due to decoupling of ß-receptor from guaninine nucleotide binding protein (G-protein) and changes in intracellular protein kinase C activity may be involved. (Strasser *et al.* 1988). Since VER also alters the intracellular function of protein kinase C (DePetrillo *et al.* 1994) this could be a common pathway for both ß-adrenergic receptor and calcium channel blocker downregulation. Finally, treatment of the inflammation with steroids has been shown to reverse the ß-receptor downregulation caused by inflammation in asthma. (Svedmyr 1990). In humans an increase in VER concentrations with a decrease in oral clearance in the elderly has been reported (Abernethy *et al.* 1993, Schwartz *et al.* 1993) with no change in plasma protein binding (Abernethy *et al.* 1993). Interestingly, similar to our interferon treated rats, despite increased in plasma concentration of VER, the dromotropic effect of VER was decreased in the elderly humans. Although Abernethy *et al.* (1993) attributed this observation

to aging, the possibility of involvement of an immunologically mediated reaction cannot be ruled out. It has been shown that cytokine production is elevated in the elderly (Fagiolo *et al.* 1993, Elsässer-Beile *et al.* 1993). Interleukin-6 (IL-6) has been found in the elderly in greater concentrations that in the young population (Roubenoff *et al.* 1998). Therefore it is possible that some changes attributed to aging may be due to the immunological status of the individual.

In an unpublished preliminary study performed in our lab, the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) was administered to the rat in an attempt to determine the role of NO. However, L-NAME proved to be a very problematic probe. Pharmacokinetics of both enantiomers were normalized in the presence of high dose L-NAME (50 mg/kg) with a signicant decrease in measured levels of nitrite. However antimuscarinic effects of L-NAME at this dose caused a significant decrease in the measured dromotropic effect of VER on the heart. Reduction in the L-NAME dose to 5 mg/kg resulted in a normalization of S-VER pharmacokinetics, but not the pharmacokinetics of R-VER which suggested a flow-dependent mechanism. Additionally, reductions in serum nitrite were in the IFN-α treated did not reach control values. It concluded, that the use of L-NAME for *in vivo* experiments of this type could lead to significant complications rendering the interpretation of the data difficult. A drug that specifically inhibits the inducible form of nitric oxide synthase (iNOS) and is devoid of other pharmacologic effects is required to unequivocally elucidate the role of NO in this interaction. Recently, compounds such as 1400W have been shown to have a high specificity for iNOS and could be used for this

type of experiment (Babu and Griffith 1998).

In conclusion, treatment with INF-α2a, or the resultant inflammation, caused inhibition of VER clearance. This, however, did not result in an increased pharmacodynamic response. In fact, a reduced response was observed which may be attributed to receptor down regulation caused by increased expression of pro-inflammatory cytokines. Implications of this observation may reach beyond a verapamil-interferon drug interaction since other inflammatory conditions (e.g., arthritis, infection, and asthma) and other receptors may be effected. For example, treatment of cardiovascular diseases in a patient with rheumatoid arthritis may require closer monitoring and the establishment of clearly defined clinical goals such as blood pressure reduction or dysrhythmia prevention to prevent a therapeutic failure on a standard therapeutic dose. Clinically, it has been observed that ventricular dysrhythmias are easier to control with drugs after the resolution of inflammation (Friedman *et al.* 1994). In addition, the increasing use of cytokines as drugs leads to the increased probability of drug-drug and drug-disease interactions.

Appendix 3 -I

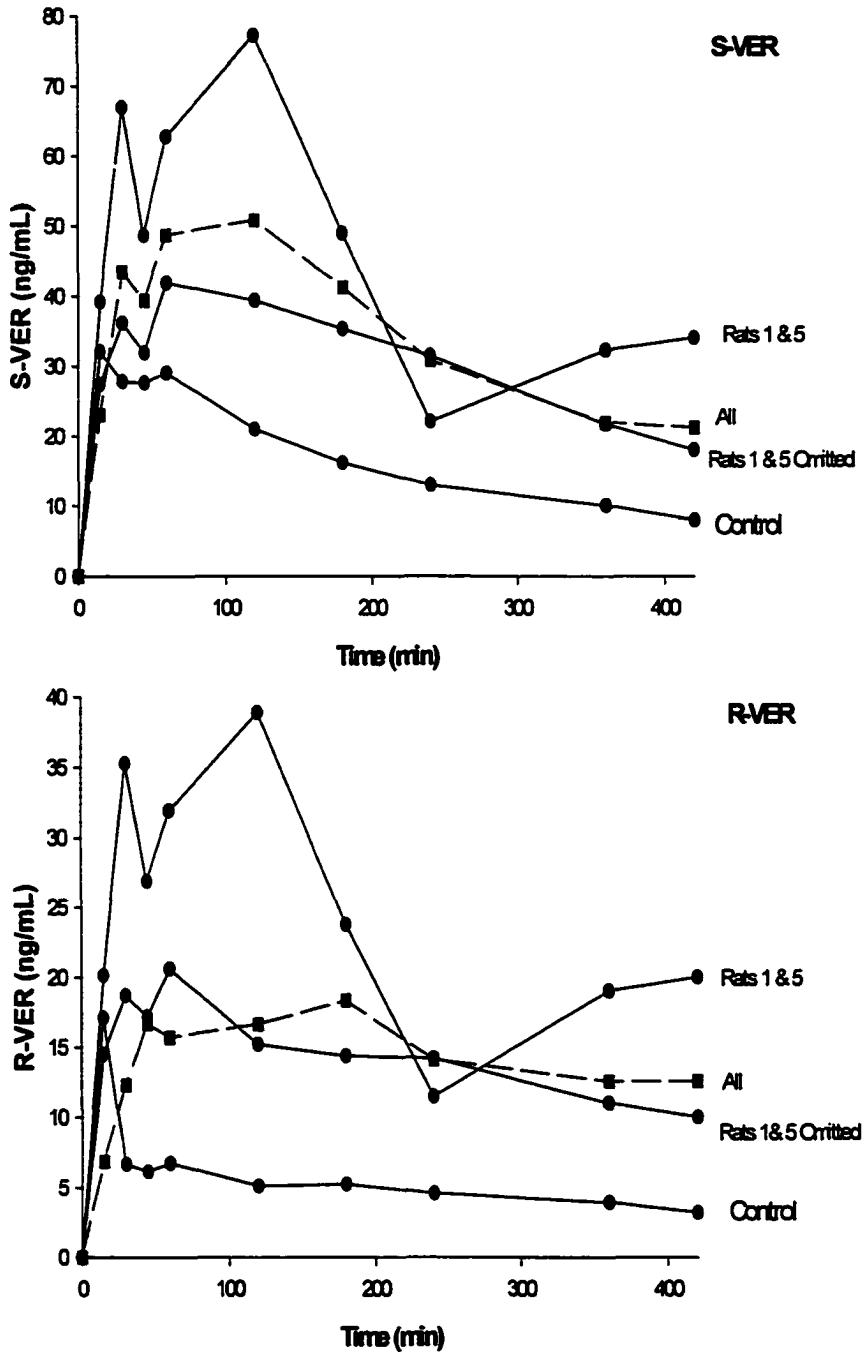


Figure 3-A-1: Serum VER concentration-time profile in control rats (●) and IFN-α2a-treated rats (■).

Table 3-A-1: Tail-End Calculations Based on Mean Values

Parameter		Mean (Rat 1 & 5 Omitted)	IFN-α2a, (Mean ± SEM)
λz (h^{-1})	S	0.35	0.37 ± 0.48
	R	0.20	0.18 ± 0.04*

REFERENCES

Abdel-Rassak Z, Loyer P, Fautrel A, Gautier JC, Corcos L, Turlin PD, Guillouzo A. Cytokines down-regulate expression of major cytochrome P450 enzymes in adult human hepatocytes in primary culture. *Mol Pharmacol* 1993; **44**: 707-715.

Abernethy D, Wainer I, Longstreth J,Adrawis N. Stereoselective verapamil disposition and dynamics in aging during racemic verapamil administration. *J Pharmacol Exp Ther* 1993; **266**: 904-911.

Adler B, Adler H, Jungi T, Peterhans E. IFNα primes macrophages for LPS-induced aptosis. *Biochem Biophys Res Comm* 1995; **215**: 921-927.

Ansher S, Puri R, Thompson W, Habig W. The effects of IL-2 and IFN-α administration on hepatic drug metabolism in mice. *Cancer Research* 1992; **52**: 262-266.

Babu R, Griffith O. Designb of isoform-selective inhibitors of nitric oxide synthase. *Cur Opin Chem Bio* 1998; **2**: 49 1 – 500.

Bavendiek U, Brixius K, Frank K, Reuter H, Pietsch M, Gross A, Muller-Ehmsen J, Edmann E, Schwinger R. Altered inotropism in the failing human myocardium. *Basic Res Cardiol* 1996; **91**: 9 - 16.

Beckman J, Koppenol W. Nitric Oxide, Superoxide, and Peroxynitrite: The Good the Bad and the Ugly. *Am J Physiol* 1996; **271**: C1424-C1437.

Belpaire FM, de Smet F, Chindavijak B, Fraeyman N, Bogaert MG. Effect of turpentine-induced inflammation on the disposition kinetics of propranolol, metoprolol, and antipyrine in the rat. *Fund Clin Pharmacol* 1989; **3**: 79 - 88.

Bhatti M, Foster R. Pharmacokinetics of the enantiomers of verapamil after intravenous and oral administration of racemic verapamil in a rat model. *Biopharm Drug Disp* 1997; **8**: 387 - 96.

Byrne A, McNeil J, Harrison P, Louis W, Tonkin A, McLean AJ. Stable oral availability of sustained release propranolol when co-administered with hydralazine or food: evidence implicating substrate delivery rate as a determinant of presystemic drug interactions. *Brit J Clin Pharmacol* 1984; **17**: :45S-50S.

Busse D, Cosme J, Beune P, Kroemer H, Eichelbaum M. Cytochromes of the P450 2C subfamily are the major enzymes involved in the O-demethylation of verapamil in Humans. *Naunyn-Schmiederberg's Arch Pharmacol* 1995; **353**: 116-121.

Catterall W and Striessnig J. Receptor sites of Ca^{2+} Channel Antagonists. *TiPS* 1992; **13**: 256 - 262.

Cawthorne MA, Palmer ED, Green J. Adjuvant induced arthritis and drug-metabolizing enzymes. *Biochem Pharmacol* 1976; **25**: 2683-2688.

Cribb A, Delaporte E, Kim S, Novak R, Renton K. Regulation of CYP1A and CYP2E induction in the rat during production of IFNα/β. *J Pharmacol Exp Ther* 1994; **268**: 487 - 494.

Delaporte E, Renton K. Cytochrome P4501A1 and P4501A2 are downregulated at both transcriptional and post-transcriptional levels by conditions resulting in interferon-α/β induction. *Life Sci* 1997; **60**: 787 - 796.

Deguchi M, Inaba K, Muramatsu S. Counteracting effect of IFNα and β on IFNγ-induced production of NO which is suppressive for antibody response. *Immunology Letters* 1995; **45**: 157 - 153.

DePitrillo P, Abernethy D, Wainer I, Andrawis N. Verapamil decreases lymphocyte protein kinase C activity in humans. *Clin Pharmacol Ther* 1994; **55**: 44-50.

Descotes J. Immunomodulating agents and hepatic drug metabolizing enzymes. *Drug Metab Rev* 1985; **16**: 175-185.

Dubin D. Rapid Interpretaion of EKG's: A programmed Course 3rd Ed. Cover Publishing Company, Tampa: 1986.

Echizen H, Brecht T, Niedergesass S, Vogelgesang B, Eichelbaum M. The effect of dextro-, levo-, and racemic verapamil on AV conduction in humans. *Am Heart Journal* 1985; **109**: 210- 217.

Eichelbaum M, Ende M, Rember G, Schomerus M and Dengler H. The metabolism of

^{14}C-D,L-verapamil in man. *Drug Metab Disp* 1979 **7**; 145-148.

Elsässer-Beile U, von Kleist S. Cytokines as therapeutic and diagnostic agents. *Tumor Biol* 1993; **14**: 69-94.

Fagiolo U, Cossarizza A, Scala E, Fanales-Belasio E, Ortolani C, Cozzie E, Monti D, Franceschi C, Paganelli R. Increased cytokine production in mononuclear cells of healthy elderly people. *Eur J Immun* 1993; **23**: 2375 - 2378.

Fast D, Lynch R, Leu R. IFNγ, but not IFNα-β synergized with TNFα and lipid A in the induction of NO production by murine L929 cells. *J Interferon Res* 1993; **13**: 271 - 277.

Feely J, Nadeau J, Wood A. Effects of feeding on the systemic clearance of indocyanine green and propranolol blood concentrations and plasma binding. *Brit J Clin Pharmacol* 1983; **15**: 383 - 5.

Ferrari L, Herber R, Batt AM, Siest G. Differential effects of human recombinant IL-1β and dexamethasone on hepatic drug-metabolizing enzymes in male and female rats. *Biochem Pharmacol* 1993; **45**: 2269 - 2277a.

Ferrari L, Jouzeau JY, Gillet P, Herber R, Fener P, Batt AM, Netter P. IL-1β differentially repressess drug-metabolizing enzymes in arthritic female rats. *J Pharmacol Exp Ther* 1993; **264**: 1012 - 1020b.

Finkel M, Oddis C, Jacob T, Watkins S, Hattler B, Simmons R. Negative inotropic effects of cytokines on the heart mediated by nitric oxide. *Science* 1992; **252**: 387-389.

Flesch M, Kilter H, Cremers B, Lenz O, Südkamp M, Kuhn-Regnier F, Böhm M.

Acute effects of nitric oxide and cGMP on human myocardial contractility. *J Pharmacol Exp Ther* 1997; **281**: 1340 - 1349.

Friedman R, Kearney D, Moak J, Fenrich A, Perry J. Persistence of ventricular arrhythmia after resolution of occult myocarditis in children and young adults. *J Am College Cardiology* 1994; **24**: 780 - 783.

Fujimaki M. Oxidation of R(+)- and S(-) carvedilol by rat liver microsomes. Evidence for stereoselective oxidation and characterization of the cytochrome P450 isozymes involved. *Drug Metab Disp* 1994;**22**:700- 708.

Grisham M, Johnson G, Gautreaux M, Berg R. Measurement of nitrate and nitrite in extracellular fluids: a window to systemic nitric oxide metabolism. *Meth Comp Meth Enzym* 1990; **7**: 84 - 90.

Gross G. Inflammatory mediators and the stunned myocardium Chapter 8 in Immunopharmacology of the Heart. Academic Press Ltd. 1993.

Gutterman J. Cytokine therapeutics: Lessons from interferon-α. *Proc Natl Acad Sci USA*1995; **91**: 1198 - 1205.

Hakkak R, Ronis MJ, Badger T. Effects of enteral nutrition and ethanol on cytochrome P450 distribution in small intesting of male rats. *Gastroenterology* 1993; **104**: 1611 –1618.

Johnson K, Balderston S, Piper J, Mann D, Reiter M. Electrophysiological effects of verpamil metabolites in the isolated heart. *J Cardiovasc Pharmacol* 1991; **17**: 830-837.

Kim Y, Son K. A nitric oxide production bioassy for IFNγ. *J Immun Meth* 1996; **7789:** 162 - 167.

Kakumisu S, Shijo H, Yokoyama M, Kim T, Akiyoshi N, Ota K, Kubara K, Okumura M, Inoue K. Effects of L-Arginine on the systemic, mesenteric and hepatic circulation in patients with cirrhosis. *Hepatology* 1998; **27**: 377 - 382.

Khatsenko O, Gross S, Rifkind A, Vane J. Nitric oxide is a mediator of the decrease in CYP450-dependent metabolism caused by immunostimulants. *Proc Nat Acad Sci USA* 1993; **90**: 11147 - 11151.

Kroemer H, Gautier J, Beaune P, Henderson C, Wolf C, Eichelbaum M. Identification of P450 enzymes involved in metabolism of verapamil in humans. *Naunyn-Schmiederberg's Arch Pharmacol* 1993; **348**: 332 - 337.

Kubes P. Nitric Oxide: A modulator of cell-cell interactions in the microcirculation. molecular biology intelligence unit. Chapter 3: Biological Significance of Nitric Oxide in Platelet Function. Radomski M ,Sales E. Springer-Verlag New York 1995.

Lapointe M, Sitkins J. Mechanisms of interleukin-1β regulation of nitric oxide synthase in cardiac myocytes. *Hypertension* 1996; **27**: 709 - 714.

Laethem ME, Belpaire FM, Wijnant P, Rosseel MT, Bogaert MC. Influence of endotoxin on the steroselective pharmacokinetics of oxprenolol, propranolol, and verapamil in the rat. *Chirality* 1994; **6**: 405 – 410.

Laethem ME, Belpaire FM, Wijnant P, Bogaert MG. Stereoselective pharmacokinetics of oxprenolol, propranolol and verapamil: species differences and influence of endotoxin. *Chirality* 1995; **7**: 616 -622.

Little R, White M, Hartshor K. IFNα enhances neutrophil respiratory burst responses to stimulation with influenza A virus and FMLP. *J Infect Diseas* 1994; **170**: 802 - 810.

Losonczy G, Bloch J, Samsell L, Schoenl M, Venuto R, Baylis C. Impact of surgery on NO in rats: Evidence for activation of iNOS. *Kidney International* 1997; **51**: 1943 - 1949.

McGowan F, Reiter M, Pritchett E, Shand D. Verapamil plasma binding: Relationship to α1-acid glycoprotein and drug efficacy. *Clin Pharmacol Ther* 1993; **33**: 485 - 490.

McIlheny H. Metabolism of (^{14}C) Verapamil. *J Med Chem* 1971; **14**: 1178-1184.

Mehvar R, Reynolds J. Reversal of stereoselectivity in the hepatic availability of verapamil in isolated perfused rat livers: the role of protein binding. *Drug Metab Disp* 1996; **24**: 1088 - 1094.

Mery P, Lohmann S, Wlater U, Fischmeister R. Ca^{2+} current is regulated by cGMP-dependent protein kinase in mammalian cardiac myocytes. *Proc Natl Acad Sci USA* 1991; **88**: 1197 - 1201.

Meurs H. Kauffman HF. Koeter GH. Timmermans A, de Vries K. Regulation of the beta-receptor-adenylate cyclase system in lymphocytes of allergic patients with asthma: possible role for protein kinase C in allergen-induced nonspecific refractoriness of adenylate cyclase. *J Allergy Clin Immunol* 1987; **80**: 326 - 39.

McCall T, Boughton-Smith N, Palmer R, *et al.*, Synthesis of nitric oxide from L-arginine by neutrophils. *Biochem J* 1989; **261**: 293 - 296.

Monshouwer M, Witkamp R, Nijmeijer S, *et al.* Suppression of cytochrome P450- and UDP glucuronosyl transferase-dependent enzyme activities by proinflammatory cytokines and possible role of nitric oxide in primary cultures of pig hepatocytes. *Toxicol Applied Pharmacol* 1996; **137**: 237 - 244.

Moreno JJ, Escofet A, Castell M, Castellote C, Queralt J. Hepatic cytochrome P-450 activities and serum biochemical changes in adjuvant arthritis. *Med Sci Res* 1987; **15**: 1469 - 1470.

Murad F, Forstermann U, Nakane M, Pollock J, Tracey R, Matsumoto T, Buechler W. The nitric oxide-cGMP signal transduction system for intracellular and intercellular communication. *Advance Second Messenger Phosphoprotein Res* 1993; **28**: 101 - 109.

Nathan C. Nitric Oxide as a secretory product of mammalian cells. *FASB J* 1992; **6**: 3051 - 3064.

Pang KS, Rowland M. Hepatic clearance of drugs. I. Theoretical considerations of a well stirred model and a parallel tube model. Influence of hepatic blood flow, plasma and blood cell binding, and the hepatocellular enzymatic activity on hepatic drug clearance. *J Pharm Biopharm* 1977; **5**: 625 - 653.

Parent C, Belanger PM, Jutras L, Du Souich P. Effect of inflammation on the rabbit hepatic cytochrome P450 isoenzymes: Alterations in the kinetics and dynamics of tolbutamide. *J Pharmacol Exp Ther* 1992; **261**: 780 – 787.

Pannen B, Bauer M, Noldge-Schomburg G, Zhang J, Robotham J, Clemens M, Geiger K. Regulation of hepatic blood flow during resusciation from hemorrhagic shock: Role of NO and endothelins. *Am J Physiol* 1997; **272**: H2736 - H2745.

Peterson TC, Renton KW. Kupfer cell factor mediated depression of hepatic parenchymal cell cytochrome P-450. *Biochem Pharmacol* 1986; **35**: 1491 - 1497.

Piafsky K. Increased Plasma Protein Binding of Propranolol and Chlorpromazine Mediated by Disease-induced Elevations of plasma 1-acid glycoprotein. *N Eng J Med* 1978; **299**: 1435-1445.

Pittau E, Bogliolo A, Tinti A, Mela Q, Ibba G, Salis G, Perpignano G. Developement of arthritis and hypothyroidism during alpha-ineterferon therapy for chronic hepatitis C. *Clin Exp Rheum* 1997; **15**:415-419.

Piquette-Miller M, Jamali F. Selective effect of adjuvant arthritis on the disposition of propranolol enantiomers in rats detected using a stereospecific HPLC assay. *Pharm. Res.* 1993; **10**: 294 - 299.

Piquette-Miller M, Jamali F. Influence of severity of inflammation on the disposition kinetics of propranolol enantiomers in ketoprofen-treated and untreated adjuvant arthritis. *Drug Met Disp* 1995; **23**: 240 - 245.

Renton K, Knickle L. Regulation of hepatic cytochrome P-450 during infectious disease. *Can J Physiol Pharmacol* 1990; **68**: 777 - 781.

Rivet-Bastide M, Vandecasteel G, Hatem S, Verde I, Benardeau A, Mercadie J, Fischmeister R. cGMP-stimulated cyclic nucleotide phosphodiesterase regulates the basal calcium current in human atrial myocytes. *J Clin Invest* 1997; **99**: 2710 - 2718.

Roubenoff R, Harris T, Abad L, Wilson P, Dallal G, Dinarello C. Monocyte cytokine production in an elderly population: Effect of age and inflammation. *J Ger Med Sci* 1998; **53A**: M20 - M26.

Sakai H, Okamoto T, Kikkawa Y. Suppression of hepatic drug metabolism by the interferon inducer, Poly I:C. *J Pharmacol Exp Ther* 1992; **263**: 381 - 386.

Shibukawa A, Wainer I. Simultaneous direct determination of the enantiomers of verapamil and norverapamil in plasma using a derivatized amylose HPLC chiral stationary phase. *J Chrom Bio App* 1992; **574**: 85 - 92.

Schulz R. Verbal communication. A specific iNOS inhibitor 1400 W October 8, 1999.

Schwartz J, Troconiz I, Verotta D, Liu S, Capili H. Age effects on stereoselective pharmacokinetics and pharmacodyanmics of verapamil *J Pharmacol Exp Ther* 1993; **265**: 690 - 697.

Stanley L, Adams D, Lindsay R. Potentiation and suppression of mouse liver CYP450 isozymes during the acute phase response induced by bacterial endotoxin. *Eur J Biochem* 1988; **174**: 31 – 36.

Strasser R, Benovic J, Lefkowitz R, Caron M. The beta-adrenergic receptor kinase: role in homologous desensitization in S49 lymphoma cells. *Adv Exp Med Bio* 1988; **232**: 503 - 517.

Svedmyr N. Action of corticosteroids on beta-adrenergic receptors: Clinical Aspects. *Am Rev Resp Dis* 1990; **141**: (2 Pt 2); S31- S38.

Thomas S, Stone C, Koury S. Cardiac dysrhythmias in severe verapamil overdose: characterization with a canine model. *Eur J Emerg Med* 1996; **3**: 9 - 13.

Tilg H. New insights into the mechanisms of interferon alfa (sic): An immuno-regulatory and anti-inflammatory cytokine. *Gastroenterology* 1997; **112**: 1017 - 1021.

Vogelgesang B, Echizen H, Schmidt E, Eichelbaum M. Stereoselective first-pass metabolism of highly cleared drugs: Studies of the bioavailability of L- and D-verapamil examined with a stable isotope technique. *Br J Clin Pharmacol* 1984; **18**: 733-740.

Wagner J. Propranolol: pooled Michaelis-Menten parameters and the effect of input rate on bioavailability. *Clin Pharm Ther* 1985;**37**:481-487.

Chapter 4

THE DROMOTROPIC EFFECT OF VERAPAMIL IS DOWNREGULATED IN A HLA B27/HUMAN ß2-MICROGLOBULIN TRANSGENIC RAT MODEL OF SPONDYLOARTHROPATHY.

INTRODUCTION

Transgenic models of inflammatory disease offer the possibility to study the effects of inflammatory disease on pharmacokinetic and pharmacodynamic of drugs. The Fischer 344 rat strain transfected with the human HLA-B27 and human ß2-microglobulins (HB2M) spontaneously develop inflammatory abnormalities (Hammer *et al.* 1990). These abnormalities resemble the inflammatory conditions observed in humans positive for HLA-B27 such as anklyosing spondylitis, inflammatory bowel disease (Crohn's), psoriatic skin lesions, and cardiac inflammation (Lipsky and Taurog 1991). The animals demonstrate diarrhea initially, then progressively develop joint, gut and heart lesions that are histologically similar to B27 associated disease in humans (Hammer *et al.* 1990). It has previously been shown that systemic inflammation resulting from arthritic conditions or following administration of individual pro-inflammatory cytokines can decrease the amount or function of cytochrome P450 (CYP450) isozymes (Cawthorne *et al.* 1976, Ferrari *et al.* 1993). A decrease in CYP450 function can result in decreased drug metabolism. For example, the oral clearance of propranolol is decreased by adjuvant arthritis, presumably due to decreased intrinsic clearance (Piquette-Miller and Jamali 1995). Verapamil

(VER) is a Ca^{2+} channel blocker that is extensively metabolized by cytochrome P450 isozymes (Kroemer *et al.* 1993). It is widely used to treat supraventricular tachyarrythmias and has a well-defined concentration-effect relationship (Echizen *et al.* 1985). Thus, a decrease in CYP450 amount or function would be expected to alter the pharmacokinetics of VER with pharmacodynamic consequences. As observed in the previous chapter, treatment with IFN-α2a caused a rise in VER serum concentrations with a concurrent decrease in dromotropic effect. While the precise mechanism of CYP450 inhibition remains unclear, many researchers have suggested that cytokine-induced nitric oxide (NO) or other free radical generation may be responsible (Proulx and du Souich 1995, Khatsenko *et al.* 1985). Reactive nitrogen species such as NO and peroxynitrite ($ONOO^-$) have been shown to inhibit heme-based enzymes (Parent *et al.* 1992, Szaba *et al.* 1996). However, decreases in the amount of CYP450 mRNA have been shown to occur in an NO-independent fashion (Sewer and Morgan 1997). Therefore, the objectives of this study were to determine if the HLA-B27/HB2M model of spondyloarthropathies alters the pharmacokinetics and/or pharmacodynamics of VER. In addition, the role of reactive nitrogen species such as NO or peroxynitrite ($ONOO^-$) was evaluated by measuring serum nitrite which is a stable breakdown product of both NO and $ONOO^-$. Because nitrite is ultimately converted to nitrate, all nitrate was reduced to nitrite.

METHODS

Materials

Verapamil hydrochloride and norverapamil (NOR) were gifts from G.D.

Searle (Skokie, Ill), manufactured by Knoll Pharmaceuticals (Stuttgart, Germany). The internal standard (+)Glaucine and heptafluorobutanol were purchased from Aldrich (Milwaukee, WI, USA). HPLC grade hexane was purchased from Caledon Laboratories (Georgetown Ontario, Canada). HPLC grade propan-2-ol was purchased from BDH Inc. (Toronto, Ontario, Canada). Triethylamine (TEA) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Heptane was purchased from Mallinckrodt (Paris, Kentucky, USA) and 98% anhydrous ethanol was acquired from Stanley (Vancouver, BC). *Aperigillus* nitrate reductase10 U/mL, 0.1 M FAD, 1 mM NADPH, 1500 U/mL LDH and 100 mM pyruvic acid were all purchased from Sigma Chemical Co. (St. Louis, MO, USA).

The study was carried out according to the guidelines established for ethical handling of live animals. Adult, male (281 ± 5.4 g, n=4) Fischer rats transgenic for HLA-B27 and HB2M and healthy adult male Fischer rats (289 ± 4.3 g, n = 4, Charles River Colony). All transgenic animals tested positive for the expression of the HLA-B27 and HB2M genes based on a DNA analysis of a tail tip tissue sample. The animals were acclimated to a 12 hour day-night cycle, housed in rodent cages, and fed standard rodent chow for a 7 day period prior to the experiment. The right jugular vein was cannulated using light ether for induction of anesthesia and methoxyflurane for maintenance. PE-50 tubing with a silastic end was advanced into the jugular vein and exteriorized to the animal's back and locked with heparin 100 U/mL. The animals were returned to their cages and allowed to recover overnight.

On day 2 racemic verapamil 25 mg/kg was administered orally by gavage

and serial blood samples (175 μL) were drawn at 0, 15, 30, 45, 60, 90, 120, 180, 240, and 360 minutes to determine pharmacokinetic parameters. ECG leads were placed at the time of the jugular cannulation and brought around to the animal's back. PR-interval and heart rate (HR) were recorded approximately 30 to 60 seconds prior to blood sampling. Serum nitrite was measured in the time zero blood sample to determine nitrite levels prior to the loss of blood due to sampling.

Verapamil and Norverapamil Assay.

A stereospecific verapamil assay was used as previously published by Shubikawa and Wainer (1992). Briefly, to 100 μL of plasma in a glass test tube were added

75 μL of a 400 ng/mL (+) glaucine (internal standard), 100 μL 2 M NaOH, 0.4 mL sodium phosphate buffer (pH 7.0, ionic strength 0.1), and 6 mL heptane. The sample was vortexed for 1.0 minute, then centrifuged at 2000 *g* for 10 minutes. The organic layer was transferred to clean glass tubes and evaporated to dryness in a vacuum centrifuge at 60°. The resulting residue was reconstituted in 200 μL of mobile phase (hexane-isopropanol-ethanol-TEA, 85:7.5:7.5:1.0, v/v), and 100 μL injected into the HPLC.

A Waters (Millipore-Waters, Missassuaga, Canada) HPLC apparatus was used consisting of a twin piston pump, a WISP 710B autosampler, a column oven (31°) and a 470 fluorescence detector set at excitation of 272 nm and an emission of 317 nm with a bandwidth set at 18 nm. The integrator was a Hewlett-Packard (Avondale, PA) 3390A model. An achiral column (5 cm x 4.6 mm ID Supelcosil LCSi column, Supelco Inc., Bellefonte PA) was serially attached to a chiral

column (250 mm x 4.6 mm I.D. 10 μm Chiralpak AD, Daicel Chemical Ind., Tokyo, Japan). Standard curves were run in duplicate and were linear over the test range (2.5 ng/mL to 200 ng/mL, $r^2 \geq 0.996$). Sensitivity for R and S-VER was 2.5 ng/mL (CV < 5%) and 7.5 ng/mL for R and S-NOR (CV < 13%).

Electrocardiogram Analysis

The electrocardiogram (ECG) was measured using braided stainless steel, Teflon coated electrodes (Cooner Wire Co, Chatsworth, CA) placed subcutaneously in the left and right axilla and over the xyphoid process. The ECG amplifier was a Honeywell for Medicine ECG amplifier (Honeywell Electronics for Medicine, Edmonton, Alberta) and the data recorded using Acknowledge software (World Precision Instruments, Miami Florida) on a personal computer. The mean of five cycles was taken for the measurement of PR intervals and heart rate (HR).

Serum Nitrite Analysis

Serum nitrite was measured using the method of Grisham *et al.* (1995). However, the ultimate metabolic fate of NO *in vivo* is nitrate. Therefore, nitrate must be reduce to nitrite for measurement. Briefly, 100 μL of plasma was incubated with 10 Units of *Asperigillus* nitrate reductase in the presence of 0.1 M flavine adenine dinucleotide (FAD), 1 mM nicotinamide adenine dinucleotide phosphate dehydrogenase (NADPH) to reduce all nitrate to nitrite. The reaction was quenched with 1500 U lactate dehydrogenase (LDH) and 100 mM pyruvic acid. This was then treated with the Griess reagent (an equal mixture of 0.2% Napthylenethylendiene and 2% sulfanilamide in 5% ortho-phosphoric acid).

Absorbance measured at 540 nm using Vmax plate reader (Molecular Devices Corp, Fisher Scientific, Edmonton, Canada). Calibration was performed using standard solutions of $NaNO_2$ and $NaNO_3$ to evaluate reductase enzyme efficiency. The assay was linear from 5 μM to 200 μM with a coefficient of variation less than 5%, $r^2 \geq 0.997$.

Pharmacokinetic Analysis

Model independent analysis of serum verapamil enantiomers was performed. Terminal elimination rate constant (λ_z) was calculated using a non-weighted nonlinear least-squares regression in WinNonLin Professional for Windows (v 2.0) (Scientific Consulting, Inc, Apex North Carolina). The $AUC_{0\to 6}$ was calculated using the linear trapezoidal rule and estimating $AUC_{6\to\infty}$ using Ct/λz where Ct is the concentration at the end of the experiment. Oral clearance (Cl/F) was calculated using $Cl / F = \frac{Dose}{AUC_{0-\infty}}$.

Statistical Analysis

The data are shown as the mean ± S.E.M. The Student's t-test for two independent groups at p = 0.05 was used to compare control with transgenic animals.

RESULTS

No statistically significant differences were observed in any pharmacokinetic parameters for VER (Table 4-1, Fig 4-1). $AUC_{0\to 6}$ accounted for

approximately 50% of AUC $_{0\rightarrow\infty}$, for S-VER and 70% for R-VER $AUC_{0\rightarrow6}$. R-VER demonstrated higher oral clearance and less variability than its antipode. Significantly less NOR was observed in the TG animals as measured by $AUC_{(0\text{-}6)}$ (Table 4-1). The transgenic rat $AUC_{0\rightarrow6}$ was more than half of control values for S-NOR and R-NOR. The NOR/VER $AUC_{0\rightarrow6}$ ratios demonstrated a 2-fold decrease for the S-enantiomers, and a nearly 3-fold decrease for R-enantiomers.

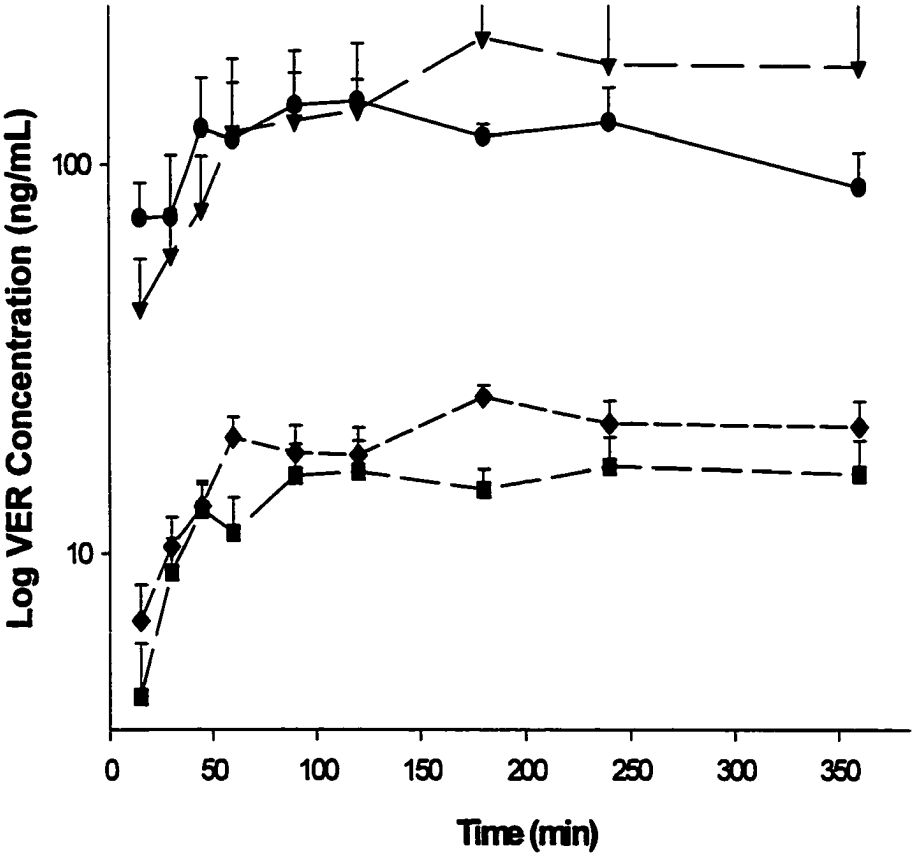


Figure 4-1: Verapamil concentration vs. time profiles.
S-VER: Control Rats ●—● Transgenic Rats ▼--▼.
R-VER: Control Rats ■—■ Transgenic Rats ◆--◆.

Table 4-1: Verapamil Pharmacokinetic Parameters

Verapamil		Control (Mean ± SEM)	Transgenic (Mean ± SEM)
tmax (min)	S	116.7 ± 9.6	178.4 ± 26.4
	R	180.1 ± 8.4	179.6 ± 24.5
Cmax (ng/mL)	S	144.5 ± 19.3	209.7 ± 64.8
	R	16.7 ± 3.5	25.1± 17.7
$AUC_{0\rightarrow 6}$ μg.min/mL	S	41.1 ± 9.1	53.2 ± 23.5
	R	5.2 ± 0.8	8.3 ± 2.9
$AUC_{0\rightarrow\infty}$ μg.min/mL	S	88.3 ± 32.1	90.8 ± 52.5
	R	6.8 ± 1.1	11.8 ± 4.6
CL/F (mL/min/kg)	S	0.057 ± 0.2	0.137 ± 0.112
	R	0.21 ± 0.165	0.83 ± 0.6
λ_z (h^{-1})	S	0.16 ± 0.06	0.20 ± 0.06
	R	0.12 ± 0.06	0.18 ± 0.001
$AUC_{0\rightarrow 6}$ R/S Ratio		0.13 ± 0.0003	0.17 ± 0.003
Norverapamil		**Control**	**Transgenic**
$AUC_{0\rightarrow 6}$ μg.min/mL	S	34.6 ± 6.8	16.9 ± 3.8*
	R	17.7 ± 3.4	7.6 ± 2.0*
NOR/VER AUC Ratio	S	0.77 ± 0.02	0.31 ± 0.01*
	R	3.28 ± 0.05	0.91 ± 0.2*

* Different from Control, $p < 0.05$

Transgenic modification had no significant effect on percent change in heart rate (44.6 ± 12.6 % for control vs. 38.4 ± 24.3% for transgenic), however PR-interval prolongation was nearly five fold less than control values ($p < 0.05$) (Figure 4-2). It is important to note that two of the four control animals developed 2nd degree AV-block that lasted for approximately one hour. No transgenic

animals developed AV-block.

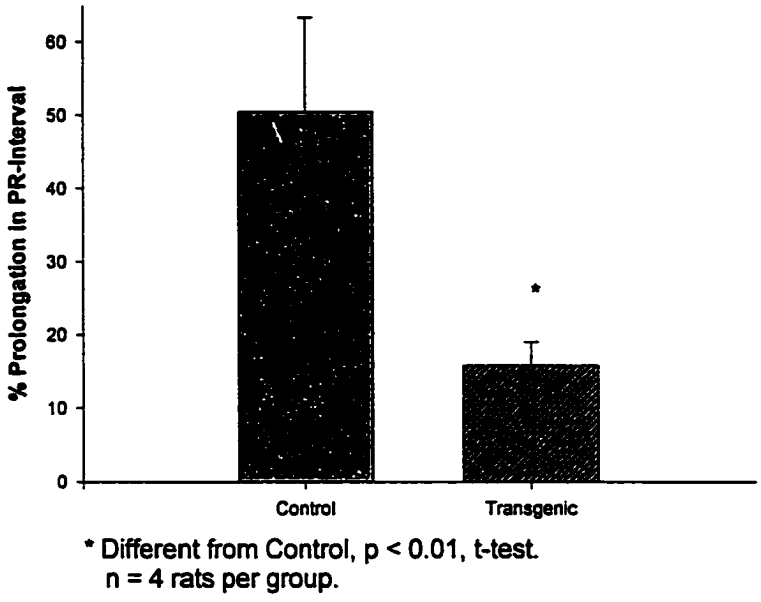


Figure 4-2: The effect of 25 mg/kg VER on PR-interval prolongation.

No significant differences were detected in serum nitrite levels (Figure 4-3). Furthermore, no significant correlation was observed between serum nitrite and VER AUC's.

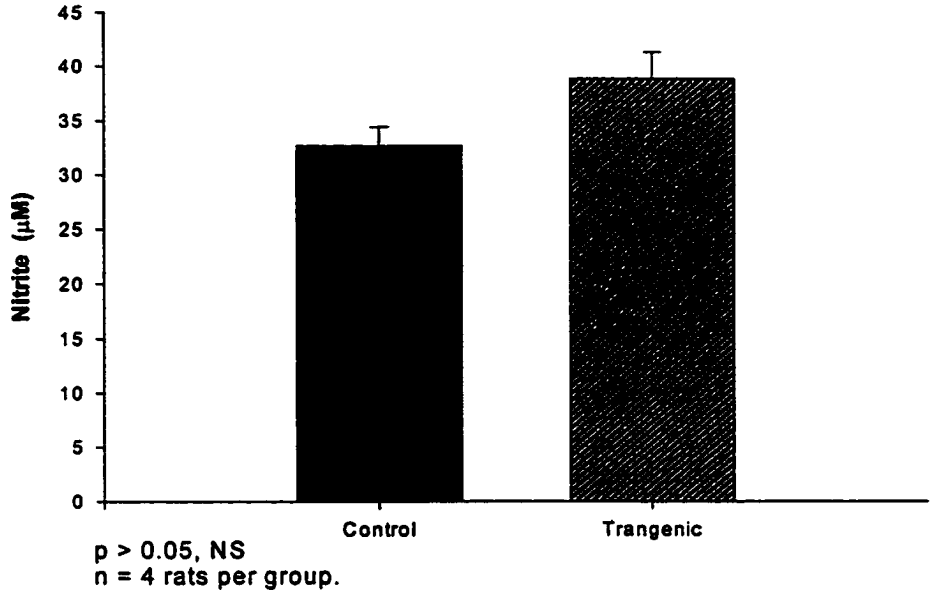


Figure 4-3: Serum nitrite for Fischer control and transgenic rats (Mean ± SEM).

DISCUSSION

No significant differences were observed in VER pharmacokinetics due to transgenic modification. This is in contrast with our observation for interferon-α2a-treated animals, where a decrease in oral clearance was observed with a statistically significant rise in the serum concentrations of VER enantiomers. In addition, patients with rheumatoid arthritis demonstrated an increase in VER AUC with a decrease in fraction unbound. Therefore, pro-inflammatory changes in both an animal model and in human RA patients clearly demonstrated a change in VER pharmacokinetics. This was not observed in transgenic animals. However, metabolite pharmacokinetics were altered in the transgenic animals. The transgenic $AUC_{0\rightarrow6}$ was approximately half of control values for both R- and

S-NOR. Metabolite/parent ratios for both R and S-enantiomers indicate that the transgenic group had significantly less metabolite in comparison to parent drug. This could be due to decreased metabolite formation, which could suggest inhibition of at least one metabolic pathway leading to the formation of R and S-NOR. It is possible the metabolism of NOR is more sensitive to the effects of transgenic-induced inflammation. It has been shown that different inflammatory diseases such as rheumatoid arthritis and ankylosing spondylitis exhibit differences in the expression of cytokines (Simon *et al.* 1994, Mohler & Butler 1990). Therefore, a differential effect by specific cytokines is possible.

VER has been shown to be extensively metabolized by CYP3A4, CYP1A2 and CYP2C (Kroemer *et al.* 1993) in humans. However, Sprague-Dawley rats lack CYP3A4, therefore VER metabolism would be expected to proceed via CYP2C and CYP1A isozymes (Fujimaki 1994, Hakkak *et al.* 1993. Norverapamil undergoes further dealkylation that appears to be heavily dependent on CYP2C (Nelson *et al.* 1988, Kroemer *et al.* 1993). Thus it is possible that differences in cytokine expression could differentially alter CYP450 function. For example, interleukin-1ß (IL-1ß), and IL-6 have been shown to decrease activity of CYP3A4, CYP1A2 and CYP2C (Chen *et al.* 1994, Abdel-Rassak *et al.* 1993) whereas interleukin 4 (IL-4) has been shown to increase CYP450 activity (Abdel-Rassak *et al.* 1993). This raises the possibility that some cytokines may stimulate CYP450 activity. It has been shown that interleukin-8 has a significant role in inflammatory bowel disease of a chronic nature (McLaughlan *et al.* 1997). Thus it is possible that a different pattern of cytokine expression could also alter

CYP450 function in a different manner. In this study, only NOR pharmacokinetics were shown to be altered. This suggests a greater effect on CYP2C since CYP3A and CYP1A are more involved in the metabolism of the parent drug, VER and rats do not produce a CYP3A4 isozyme (Kroemer *et al.* 1993). It is therefore possible that this model of inflammation is different from rheumatoid arthritis or human ankylosing spondylitis.

The transgenic animals did not demonstrate a significant elevation in serum nitrite over control animals. This suggests that the production of reactive nitrogen species may not be elevated by transgenic modification. It may also indicate that HLA B27/HB2M model of inflammation is a less severe form of inflammation or that the animals were not severely afflicted. Aiko and Grisham (1995) demonstrated that elevations in serum nitrite in HLA-B27 transgenic rats occurred only in the presence of significant bowel inflammation. It has also been shown in humans that less difference is observed in serum nitrite levels due to inflammatory bowel disease than with infectious gastroenteritis (Dykhuizen *et al.* 1996). Furthermore, in a study of children with chronic inflammatory bowel disease, elevations of stool nitrites and nitrates was much greater than elevations in the serum and urine (Levine *et al.* 1998). Therefore, the lack of a significant rise in serum nitrite in our study may suggest that despite the positive genotype for HLA-B27/HB2M, the animals did not have severe inflammation. This is in contrast with our research on humans with rheumatoid arthritis and interferon-α2a-treated rats in which serum nitrite was significantly elevated. Furthermore, serum nitrite was highly correlated with the rise in VER AUC ($r = 0.8$, $p < 0.05$)

for both animals and humans.

Despite the observed unchanged VER pharmacokinetics, the downregulation in dromotropic effect was the most striking observation of this study. The control animals responded to the 25 mg/kg dose of VER with a significant 50% increase in PR-interval with two animals developing 2nd degree AV-block that lasted until serum levels began to fall. The same dose in the transgenic animals resulted in no toxicity and only an 11% prolongation in PR-intervals. However, a significant decrease in NOR was observed. NOR also possesses pharmacological activity at the AV-node, but is approximately 50 to 100 times less potent than the parent drug (Johnson *et al.* 1991). Therefore, a decrease in NOR levels is not likely to account for the observed decrease in dromotropic effect. This same decrease in VER dromotropism was observed in interferon-α2a (IFN-α2a) treated rats (Chapter 3). In addition the IFN-α2a - treated animals demonstrated elevations of both VER and NOR levels. Thus the decreased dromotropism occurred in the presence of elevated drug concentration. Nitric oxide has been shown to downregulate Ca^{2+} channels through a cGMP-protein kinase C interaction (Méry *et al.* 1991). However in this study no significant differences in serum nitrite, a stable degradation product of NO, were observed. Therefore, it appears that the downregulation incurred by the transgenic modification occurs independently of NO production. This suggests that an inflammatory mediator such as a pro-inflammatory cytokine could have a modulating effect on cardiac function, which is independent of nitric oxide production. For example, it has been demonstrated that interleukin-2 binds to IL-

2 cardiac receptors altering normal Ca^{2+} currents (Gross 1993). This could certainly alter the pharmacodynamic effects of VER. Further work with this model of inflammation would be useful to further explore the changes in VER pharmacodynamics and to further understand the regulation of Ca^{2+} channels in the presence of systemic inflammation.

This work suggests that even subtle inflammation may alter cardiac function and pharmacodynamic response to cardiac drugs. Furthermore, this change may occur in an NO-independent manner suggesting a direct effect of inflammation on cardiac function. If extrapolated to humans, inflammatory conditions may render a patient less sensitive to the cardiac effects of calcium channel blockers leading to therapeutic failures at normal dosing levels. Therefore, the patient with inflammatory disease requiring calcium channel blocker therapy should be monitored closely and titrated directly to therapeutic effect.

♦♦♦

REFERENCES

Abbas A, Lichtman A, and Pober J. Saunders Text and Review Series: Cellular and Molecular Immunology 3rd Ed. W.B. Saunders and Co. Toronto Chapters 1, 5 & 19: 1997.

Abdel-Rassak Z, Loyer P, Fautrel A, Gautier JC, Corcos L, Turlin PD, Guillouzo A. Cytokines down-regulate expression of major cytochrome P450 enzymes in adult human hepatocytes in primary culture. *Mol Pharmacol* 1993; **44**: 707-715.

Aiko S, Grisham M. Spontaneous intestinal inflammation and nitric oxide metabolism in HLA-B27 transgenic rats. *Gastroenterology* 1995; **109**: 142 – 50.

Cawthorne MA, Palmer ED, Green J. Adjuvant induced arthritis and drug-metabolizing enzymes. *Biochem Pharmacol* 1976; **25**: 2683 - 2688.

Chen YL, Vraux VL, Leneveua A, *et al.* Acute-phase reponse, IL-6 and alteration of cyclosporine pharmacokinetics. *Clin Pharmacol Ther* 1994; **55**: 649 – 660.

Dykhuizen R, Masson J, McKnight G, Mowat A, Smith C, Smith L, Benjamin N. Plasma nitrate concentration in infective gastroenteritis and inflammatory bowel disease. *Gut* 1996; **39**: 393 - 395.

Echizen H, Brecht T, Niedergesäss S, Vogelgesang B, Eichelbaum M. The effect of dextro-, levo-, and racemic verapamil on atrioventricular conduction in humans. *Am Heart J* 1985; **109**: 210 - 217.

Ferrari L, Jouzeau JY, Gillet P, Herber R, Fener P, Batt AM, Netter P. IL-1β differentially repressess drug-metabolizing enzymes in arthritic female rats. J Pharmacol Exp Ther 1993; **264**: 1012 - 1020.

Fujimaki M. Oxidation of R(+)- and S(-)-carvedilol by rat liver microsomes. Evidence for stereoselective oxidation and characterization of the cytochrome P450 isozymes involved. Drug Metab Disp 1994; **22**:700-708.

Gross G. Inflammatory mediators and the stunned myocardium Chapter 8 in Immunopharmacology of the Heart. Academic Press Ltd. 1993

Hakkak R, Ronis M, Badger T, Effects of enteral nutrition and ethanol on cytochrome P450 distribution in small intestine of male rats. Gastroenterology. 1993; **104**:1611-1618.

Hammer R, Maika S, Richardson J. Spontaneous inflammatory disease in transgenic rats expressing HLA-B27 and human ß2-microglobulin: an animal model of HLA-B27 associated human disorders. *Cell* 1990; **63**: 1099 - 1112.

Johnson K, Balderston S, Piper J, Mann D, Reiter M. Electrophysiological effects of verapamil metabolites in the isolated heart. *J Cardiovasc Pharmacol* 1991; 17: 830 – 837.

Kroemer H, Gautier J, Beaune P, Henderson C, Wolf C, Eichelbaum M. Identification of P450 enzymes involved in metabolism of verpamil in humans. *Naunyn-Schmiedeberg's Arch Pharmacol* 1993; **348**: 332 - 337.

Levine J, Pettel M, Valderrama E, Gold D, Kessler B, Trachtman H. Nitric oxide and inflammatory bowel disease: Evidence for local intestinal production in children with active colonic disease. *J Ped Gastro Nutrition* 1998; 26: 34 - 38.

Lipsky P and Taurog J. (Eds). HLA-B27+ Spondyloarthropathies New York Elsevier, 1991.Chapter 3.

McLaughlan J, Seth R, Vautier G, Robins R, Scott B, Hawkey C, Jenkins D. Interleukin-8 and inducible nitric oxide synthase mRNA levels in inflammatory bowel disease at first presentation. *J Pathology* 1997; **181**: 87 - 92.

Mery PF, Lohmann S, Walter U, Fischmeister R. Ca^{2+} current is regulated by cGMP-dependent protein kinase in mammalian cardiac myocytes. *Proc Nat Acad Sci USA* 1991; **88**: 1197 - 1201.

Mohler K, Butler L. Differential production of IL-2 and IL-4 mRNA in vitro after primary sensitization. *J Immunol* 1990; **145**: 1744 - 1739.

Nelson W, Olson L, Beitner D, Pallow J. Regiochemistry and substrate specificity of O-demethylation of verpamil in the presence of the microsomal fraction from rat and human liver. *Drug Metab Dis* 1988; **16**: 184 – 188.

Piquette-Miller M, Jamali F. Influence of severity of inflammation on the disposition kinetics of propranolol enantiomers in ketoprofen-treated and untreated adjuvant arthritis. *Drug Met Disp* 1995; **23**: 240 - 245.

Proulx M, du Souich P. Inflammation-induced decrease in hepatic CYP450 in conscious rabbits is accompanied by an increase in hepatic oxidative stress. *Res Com Mol Path Pharmacol* 1995; **87**: 221 - 236.

Sewer M, Morgan E. Nitric-Oxide independent suppression of P450 2C11 expression by interleukin-1ß and endotoxin in primary rat hepatocytes. *Biochem Pharmacol* 1997; **54**: 729 - 737.

Simon AK, Seipelt E, Sieper J. Divergent T-cell cytokine patterns in inflammatory arthritis. *Proc Nat Acad Sci USA* 1994; **91**: 8562 - 8566.

Szaba C, Day B, Salzman A. Evaluation of the relative contribution of nitric oxide and peroxynitrite to the suppression of mitochondrial respiration in immunostimulated macrophages using a manganese mesoporphyrin superoxide dismutase mimetic and peroxynitrite scavenger. *FEBS Letters* 1996; **381**: 82 - 86.

Chapter 5

RHEUMATOID ARTHRITIS DECREASES THE ORAL CLEARANCE OF VERAPAMIL ENANTIOMERS AND DOWNREGULATES DROMOTROPIC EFFECTS IN HUMANS.

INTRODUCTION

Inflammatory diseases such as rheumatoid arthritis (RA) and Crohn's disease have been shown to alter the pharmacokinetics of several drugs. The therapeutic consequence of the effect is not known. It has been suggested that patients with rheumatoid arthritis are more predisposed to adverse drug reactions than the general population (Wolfe 1997, Poulton *et al.* 1998). For example, patients with RA demonstrate a higher frequency of allergic reactions to d-penicillamine and a side effect profile which is distinctively different from patients with crystaluria or Wilson's disease (Lyle 1979; Walshe 1974). For another drug, sulfasalazine, however, significant differences were not found between RA patients and the general population (Wijnands *et al.* 1993). It is important to note that no attempt was made in these studies to quantitate the severity of the inflammation. This is critical since it is highly probable that disease severity determines the extent of biochemical alterations and therefore may directly control pharmacokinetic-pharmacodynamic relationships. Another important point is that all these studies have concentrated only on the drugs used to treat inflammation. The possibility of multiple diseases exists particularly with aging population. It is imperative, therefore, to consider the potential effect of

inflammatory diseases on drugs used in the treatment of other conditions. For example, inhibition of clearance of cardiovascular drugs has been frequently reported (Schneider *et al.* 1981).

Inflammation has been shown to influence both metabolism and plasma protein binding of several drugs in humans and animals. In the rat afflicted with adjuvant induced arthritis, for example, marked reduction in N-demethylase, $NADPH_2$-oxidase, CYP450 and Phase I and II conjugation activity have been observed (Cawthorne *et al.* 1976, Whitehouse 1973, Morton and Chatfield 1970). Sasaki *et al.* 1990 and Ishikawa *et al.* 1991 found that carageenan-induced inflammation inhibited drug-metabolizing activity by decreasing CYP450 content in the rat liver. In human microsome studies, pro-inflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) have been shown to decrease the amount and/or activity of several CYP450 isozymes such as CYP1A2, CYP2C, CYP3A and CYP2E (Ferrari *et al.* 1993a, Ferrari *et al.* 1993b, Abdel-Rassak *et al.* 1993). The precise mechanism of action remains unclear, but may involve the increased production of nitric oxide (NO) due to. pro-inflammatory cytokines (de Belder and Radomski 1994).

In addition to the effects on drug metabolism, inflammation may also effect protein binding. Fey and Müller (1991) reported in an increase in α1-acid glycoprotein (AAG) and other acute phase proteins in the presence of inflammation. A rise in AAG could lead to alterations in the fraction unbound (*fu*) which could alter both pharmacokinetics and pharmacodynamics. Piquette-Miller and Jamali (1993) demonstrated increased protein binding of propranolol

to AAG in adjuvant arthritic female rats.

The consequence of inflammation-induced increased protein binding and/or reduction in metabolism on the pharmacokinetics of drugs is expected to be altered drug concentration thereby changing therapeutic outcomes.

Verapamil (VER), a phenylalkylamine calcium channel blocker is marketed as the racemate. The S-enantiomer possesses 10 to 20 times greater dromotropic, inotropic and chronotropic activity than its antipode (Echizen *et al.* 1985). VER is extensively metabolized in a stereoselective manner by CYP3A4, CYP1A2 and CYP2C9 (Kroemer *et al.* 1993, Nelson *et al.* 1988) in man. Oral clearance (CL/F) of S- and R-VER are approximately 7.9 and 2.5 L/min, respectively, indicating substantial but stereoselective first-pass metabolism (Kroemer *et al.* 1992). VER is extensively protein bound primarily to AAG and, to a lesser extent, albumin. Protein binding is also stereoselective with *fu* for S-VER being 0.23 and R-VER only 0.13 (Gross A *et al.* 1990). Pharmacokinetics of VER, therefore, are expected to be affected by inflammatory diseases. The purpose of this study was to determine if rheumatoid arthritis in humans influences pharmacokinetics of VER, and whether this brings about changes in pharmacodynamics of the drug.

METHODS

Materials

Verapamil hydrochloride and norverapamil were gifts from G.D. Searle (Skokie, Ill). (+) Glaucine (internal standard) and heptafluorobutanol were purchased from Aldrich (Milwaukee, WI). HPLC grade hexane was purchased

from Caledon Laboratories (Georgetown, Canada). HPLC grade propan-2-ol was purchased from BDH Inc. (Toronto, Canada). Triethylamine (TEA) was purchased from Sigma Chemical Co. (St. Louis, MO). Heptane was purchased from Mallinckrodt (Paris, KT) and 98% anhydrous ethanol was purchased from Stanley (Vancouver, Canada). *Aperigillus* nitrate reductase 10 U/mL, 0.1 M FAD, 1 mM NADPH, 1500 U/mL LDH and 100 mM pyruvic acid were all purchased from Sigma Chemical Co (St. Louis, MO).

Subjects and Study Protocol

Eight RA patients and 8 corresponding age- and sex-matched healthy volunteers completed the study (Table 5-1). A small but statistically significant difference in weight was observed between the two groups.

Table 5-1: Patient Characteristics

Parameter	Control	RA
Age (y)	43.0 ± 5.1	43.0 ± 5.1
Height (cm)	173.6 ± 1.4	174.1 ± 1.7
Weight (kg)	76.7 ± 2.9	63.5 ± 3.4 *
Gender	5 Male 3 Female	5 Male 3 Female
# Joints Involved	0	16.3 ± 8.0*
Arthritic Index	0	3.9 ± 2.0*
Other medications	1-Acetaminophen PRN	2 - Hydroxychloroquine
	1- Loratidine PRN	4 - Methotrexate
		1 - Sulfasalazine
		1 – Cyclosporine

*Significantly different from Controls

The study was performed in accordance with the Declaration of Helsinki.

The protocol was approved by the University of Alberta Hospital Research and Ethics Committee. All participants provided written, informed consent and were included in the study only after physical and laboratory examination. No patients had any signs of hepatic or renal disease. The subjects fasted on the evening prior to the study and reported to the clinical investigation unit at 0730 h. They were weighed and temperature recorded. An i.v. line was established in the antecubital vein for serial blood samples. Surface electrodes were placed to record a standard lead II, and aV5 ECG. The patients were then monitored for a minimum of one hour or until a stable baseline was established for heart rate, PR-Interval and blood pressure. At time zero 80 mg of racemic verapamil (Isoptin, Lot: 6C384, Expiry 03/01, Searle Oakville, Canada) was administered with 200 mL water. Serial blood samples (7 mL) were drawn at 0, 20, 40, 60, 90, 180, 240, 320, 480 and 720 minutes. Blood pressure and ECG measurements were recorded one min prior to each blood sample. Subjects had a standard breakfast after the 90-minute sample and lunch after the 320-minute sample. Serum nitrite and IL-6 levels were also measured in the time zero blood samples.

Disease severity was clinically assessed according to the American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis (Schumacher *et al.* 1993). An arthritic index was calculated using the number of joints involved and the severity of the involvement as follows; 0: No joints involved, 1: 1 - 4 joints involved, 2: 5 - 9 joints and 3: > 10 joints involved. Swelling was assessed as 0: None, 1: Mild, 2: Moderate, 3:Severe. Thus a maximum score of 6 would indicate severe disease

(Table 5-1).

Verapamil and Norverapamil Assay.

A stereospecific verapamil assay was used as previously published by Shubikawa and Wainer (1992). Briefly, to 1.0 mL of plasma in a glass test tube were added

75 μL of 400 ng/mL (+) glaucine (internal standard), 100 μL 2 M NaOH, 0.4 mL sodium phosphate buffer (pH 7.0, ionic strength 0.1), and 6 mL heptane. The sample was vortexed for 1.0 minute, then centrifuged at 2000 *g* for 10 minutes. The organic layer was transferred to clean glass tubes and evaporated to dryness in a vacuum centrifuge at 60 C. The resulting residue was reconstituted in 200 μL of mobile phase (hexane-isopropanol-ethanol-TEA, 85:7.5:7.5:1.0, v/v), and 100 μL injected into the HPLC.

A Waters (Millipore-Waters, Missassuaga, Canada) HPLC apparatus was used consisting of a twin piston pump, a WISP 710B autosampler, a column oven (31^{O}) and a 470 fluorescence detector set at excitation of 272 nm and an emission of 317 nm with a bandwidth set at 18 nm. The integrator was a Hewlett-Packard (Avondale, PA) 3390A model. An achiral column (5 cm x 4.6 mm ID Supelcosil LCSi column, Supelco Inc., Bellefonte PA) was serially attached to a chiral column (250 mm x 4.6 mm I.D. 10 μm Chiralpak AD, Daicel Chemical Ind., Tokyo, Japan). Standard curves were run in duplicate and were linear over the test range (2.5 ng/mL to 200 ng/mL, $r^2 \geq 0.9996$). Sensitivity for R and S-verapamil was 2.5 ng/mL (CV < 5%) and 7.5 ng/mL for R and S-norverapamil (CV < 13%).

Electrocardiogram and Hemodynamic Analysis

A lead II and aV5 electrocardiogram was recorded using a Hewlett-Packard Digital Holter with full disclosure (Hewlett-Packard, Avondale, PA). The mean of five cardiac cycles was taken for the measurement of PR intervals and heart rate (HR). Blood pressure was determined using a Dynamap (IVAC Instruments, Toronto, Canada) automated cuff syphgmomanometer. Mean arterial pressure (MAP) was calculated from systolic blood pressure (SBP) and diastolic blood pressure (DBP) using $MAP = \frac{(2 * DBP) + (SBP)}{3}$. Subjects were in the supine state for 10 minutes prior to measuring ECG and blood pressure.

Serum Nitrite Analysis

Serum nitrite was measured using the method of Grisham *et al.* (1995). However, the ultimate metabolic fate of NO *in vivo* is nitrate. Therefore, nitrate must be reduce to nitrite for measurement. Briefly, 100 μL of plasma was incubated with 10 Units of *Asperigillus* nitrate reductase in the presence of 0.1 M flavine adenine dinucleotide (FAD), 1 mM nicotinamide adenine dinucleotide phosphate dehydrogenase (NADPH) to reduce all nitrate to nitrite. The reaction was quenched with 1500 U lactate dehydrogenase (LDH) and 100 mM pyruvic acid. This was then treated with the Griess reagent, an equal mixture of 0.2% Napthylenethylendiene and 2% sulfanilamide in 5% ortho-phosphoric acid. Absorbance was measured at 540 nm using a Vmax microtiter plate reader (Molecular Devices Corp., Fisher Scientific, Edmonton, Canada). Calibration was

performed using standard solutions of $NaNO_2$ and $NaNO_3$ to evaluate the dehydrogenase efficiency. The assay was linear within the examined range of 5 to 200 μM (CV< 5%, $r^2 \geq 0.995$).

Interleukin 6 Assay

Interleukin 6 levels were measured using an enzyme linked immunoassay available from Medgenix Diagnostics (Medgenix Diagnostics, Fleurus, Belgium). This allowed for the quantitation of IL-6 to 2.0 pg/mL (CV < 8%). Due to an accidental destruction of samples, only four controls and four RA patients had IL-6 measurements performed.

Protein Binding

Serum for the protein binding study was pooled from the time zero blood sample of the two groups (n = 8/group). The resulting serum was adjusted to pH 7.4 with 0.1N HCl. The serum was then spiked with 100 ng/mL of S-VER and 200 ng/mL R-VER to approximate *in vivo* drug concentrations. The serum was incubated at 37° for one hour then transferred to micropartition chambers (Amicon Division of W.R. Grace & Co, Danvers MA) for ultrafiltration. The chambers were centrifuged at 2000 g for one hour. In addition four chambers were loaded with phosphate buffer, pH 7.4, to determine the presence of any nonspecific binding or adsorption to the micropartition system. Both filtrate and nonfiltrate concentrations were measured and the fraction unbound (*fu*) determined as the concentration unbound (Cu) divided by total concentration (Ct). To ensure the samples were above the minimum quantifiable limit for the HPLC assay, three micropartition chambers were pooled allowing for a total of four measurements

per group.

Pharmacokinetic Analysis

Model independent analysis of serum verapamil enantiomers was performed. The terminal elimination rate constant (λ_z) was calculated using a nonweighted nonlinear least-squares regression. $AUC_{0\rightarrow\infty}$ was calculated using the trapezoidal rule until the last experimental data point (C_{last}) plus $C_{last}/\lambda z$. AUC of the unbound fraction was calculated from

$AUCu_{o\rightarrow\infty} = AUC_{o\rightarrow\infty} * fu$ assuming concentration independent protein binding (Keefe *et al.* 1981). Oral clearance (Cl/F) was from $Cl / F = \frac{Dose}{AUC_{0-\infty}}$.

Serum S-VER and PR interval and data were fitted to the direct link sigmoid Emax Model using $E = \frac{E\max * C'}{C' + EC_{50}'}$ where E and Emax are effects at t and the maximum effect, C is concentration at t and EC50 is the concentration at 50% maximal effect. An indirect link pharmacokinetic-pharmacodynamic (PK-PD) model was also used consisting of a single PK and an effect compartment (Whiting, Holford and Sheiner 1980). Goodness of fit was evaluated using r-correlation, visual inspection of the residuals, standardized residuals, the Akaike information criteria (AIC) and the Schwarz criteria (SC) (Yamaoka *et al.* 1978).

Statistical Analyses

The data are expressed as the mean ± S.E.M. Student's t-test for two independent groups was used to compare the two groups. A least-squares linear regression was performed on the serum nitrite vs S-VER AUC and arthritic index

using a Pearson's r to determine goodness of fit. In the event of non-linearity a binomial function was also used. A multivariate discriminate analysis was used to assess serum IL-6 and nitrite as indicators of disease severity. A discriminate analysis is a technique in which a discriminant function is calculated from linear combinations of the independent variables based on their sums-of-squares to create canonical functions. It is used to predict group differences and group membership. The relative importance of each independent variable within the discriminant function can be evaluated from the correlation between the independent variable and the canonical functions (Tatsuoka 1970). Statistical significance was set at $p<0.05$.

RESULTS

Pharmacokinetics

RA caused a significant and substantial rise in the serum concentratios of both enantiomers (Figure 5-1) as reflected in an approximately four and three-fold increase in $AUC_{0-\infty}$ of S- and R-VER, respectively (Table 5-2). A similar increase was observed for Cmax that reached significance for R-VER but not for S-VER due to substantial variation. Terminal elimination t1/2, was not significantly altered for either enantiomer. Protein binding was significantly altered by rheumatoid arthritis with a 6-fold decrease in *fu* for R-VER and a 5-fold decrease in S-VER. However, calculation of $AUCu_{0\rightarrow\infty}$ resulted in a significant alteration for R-VER (2.7 ± 0.3 vs 1.2 ± 0.6, $p < 0.05$) and not for S-VER (1.2 ± 0.1 vs 1.1 ± 0.6).

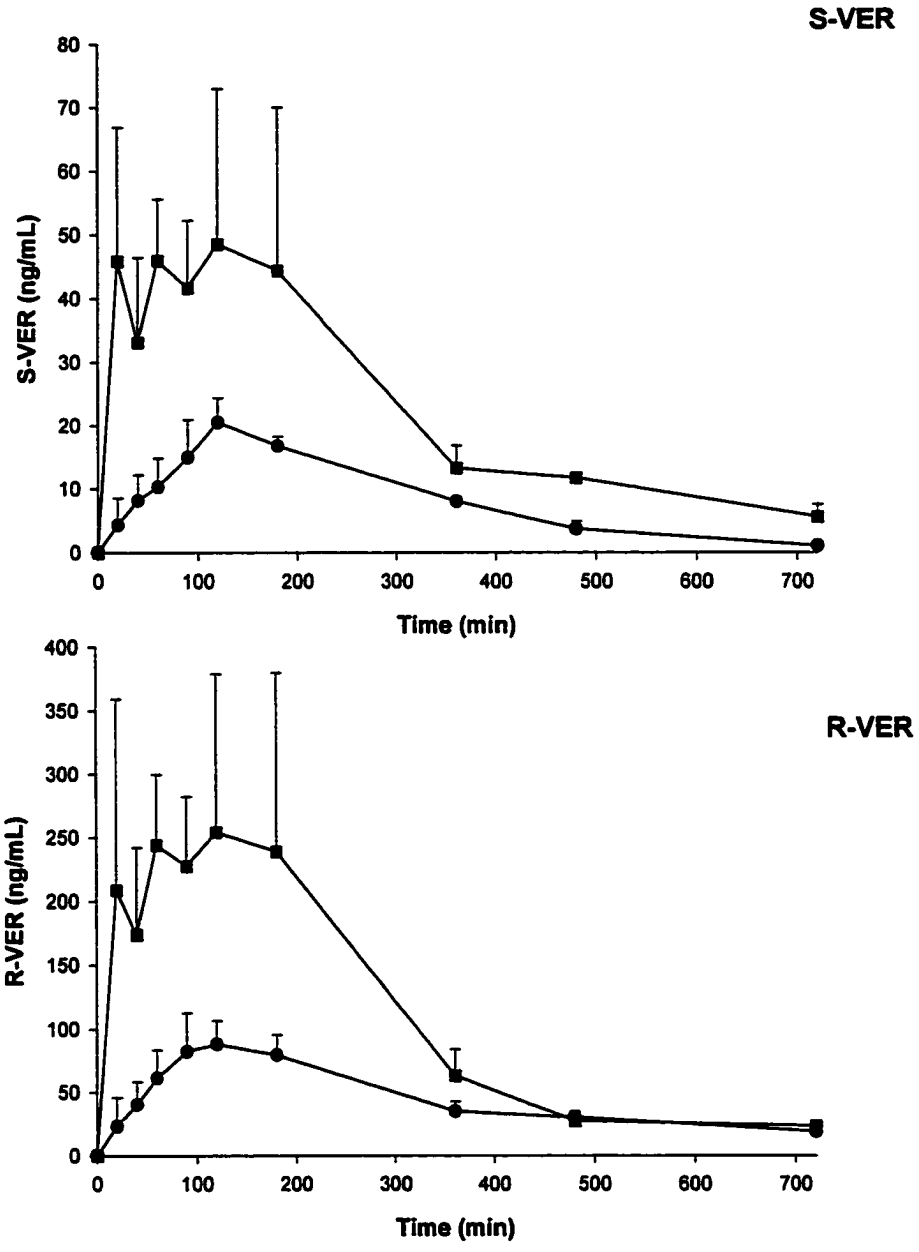


Figure 5-1: Serum VER concentration-time profile in healthy control patients (●) and rheumatoid arthritis patients (■) after receiving 80 mg VER orally.

Norverapamil pharmacokinetics were also altered by rheumatoid arthritis. The terminal $t_{1/2}$ of NOR was not estimated due to serum fluctuations. NOR/VER AUC ratios were not altered by the disease.

Table 5-2: Verapamil Pharmacokinetic Parameters

Parameter		Control (Mean ± SEM)	Rheumatoid (Mean ± SEM)
tmax (h)	S	1.6 ± 0.3	1.6 ± 0.6
	R	1.5 ± 0.3	1.7 ± 0.6
Cmax (ng/mL)	S	29.2 ± 4.1	95.3 ± 41.1
	R	143.9 ± 27.2	528.2 ± 27.2*
$t_{½\lambda z}$ (h^{-1})	S	4.1 ± 1.2	8.5 ± 0.7
	R	4.9 ± 0.7	3.6 ± 0.7
$AUC_{0\rightarrow12}$	S	6.9 ± 0.7	31.8 ± 7.4*
(μg.min/mL)	R	32.7 ± 3.4	112.4 ± 32.2*
$AUC_{0\rightarrow\infty}$	S	8.1 ± 1.2	33.6 ± 6.8*
(μg.min/mL)	R	39.1 ± 4.6	124.6 ± 34.1*
fu	S	0.138 ± 0.007	0.028 ± 0.005*
	R	0.073 ± 0.001	0.011 ± 0.001*
VER $AUC_{0\rightarrow\infty}$ R/S Ratio		5.6 ± 0.8	5.1 ± 0.9
CL/F (L/h)	S	360.7 ± 68.6	62.2 ± 18.1*
	R	67.0 ± 6.9	43.1 ± 8.7*
NOR $AUC_{0\rightarrow12}$	S	30.8 ± 12.5	68.7 ± 9.8*
(μg.min/mL)	R	668.6 ± 120.7	1892.8 ± 254.1*
NOR/VER $AUC_{0\rightarrow12}$ Ratio			
	S	4.5 ± 1.9	4.4 ± 1.3
	R	2.3 ± 0.3	2.2 ± 0.6

* Significantly different from Control.

Pharmacodynamics

Despite the elevation in serum VER and NOR levels, less dromotropic

effect as measured by PR-interval prolongation was observed in RA (Figure 5-2). AV block was observed only in the control and not in RA patients. No other cardiac effects were significantly different (Table 5-3).

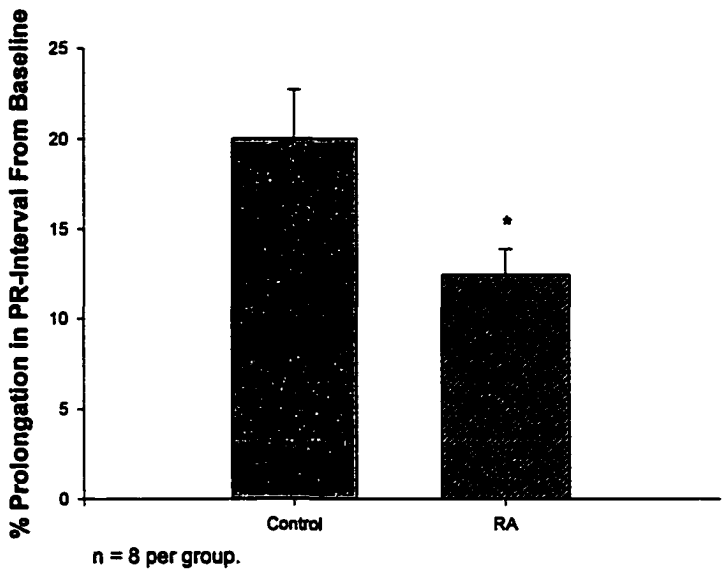


Figure 5-2: The effect of 80 mg oral VER on PR-interval in control and rheumatoid patients. * Significantly different from control $p < 0.05$.

Table 5-3: Cardiac Data: Percent Reduction from Baseline Values

Parameter		Control	Rheumatoid
% Reduction Heart Rate		22.6 ± 3.6	24.3 ± 7.7
% Reduction Blood Pressure	SBP	10.3 ± 1.9	8.7 ± 2.3
	DBP	8.1 ± 1.2	12.4 ± 1.8
% Reduction MAP		10.2 ± 1.6	9.6 ± 1.8
First Degree AV node Block		2 out of 8	Nil

No statistically significant differences were observed in the baseline data between the two groups.

PK-PD Data

As expected PR interval-concentration plots exhibited a great deal of variability (Appendix I, Figure 5-A.1 and 5-A.2). While in general significant and positive linear effect-concentration correlation ($r = 0.98$ to 0.71; $p<0.05$ to 0.001) was observed, three patients in the RA group demonstrated pronounced counter-clockwise hystereses. In all three cases the hysteresis collapsed to concave curves when the data were fitted to a one-compartment model with oral input linked to a theoretical effect compartment (Appendix I, Figure 5-A.3). When the control and the remaining patients data were fitted to an Emax model (Wagner 1968), sigmoidal curves were observed (Appendix I, Figure 5-A.4 and 5-A.5).

Serum Nitrite and Interleukin-6

Serum IL-6 and nitrite were significantly elevated in the RA group as compared with the control subjects (Figure 5-3).

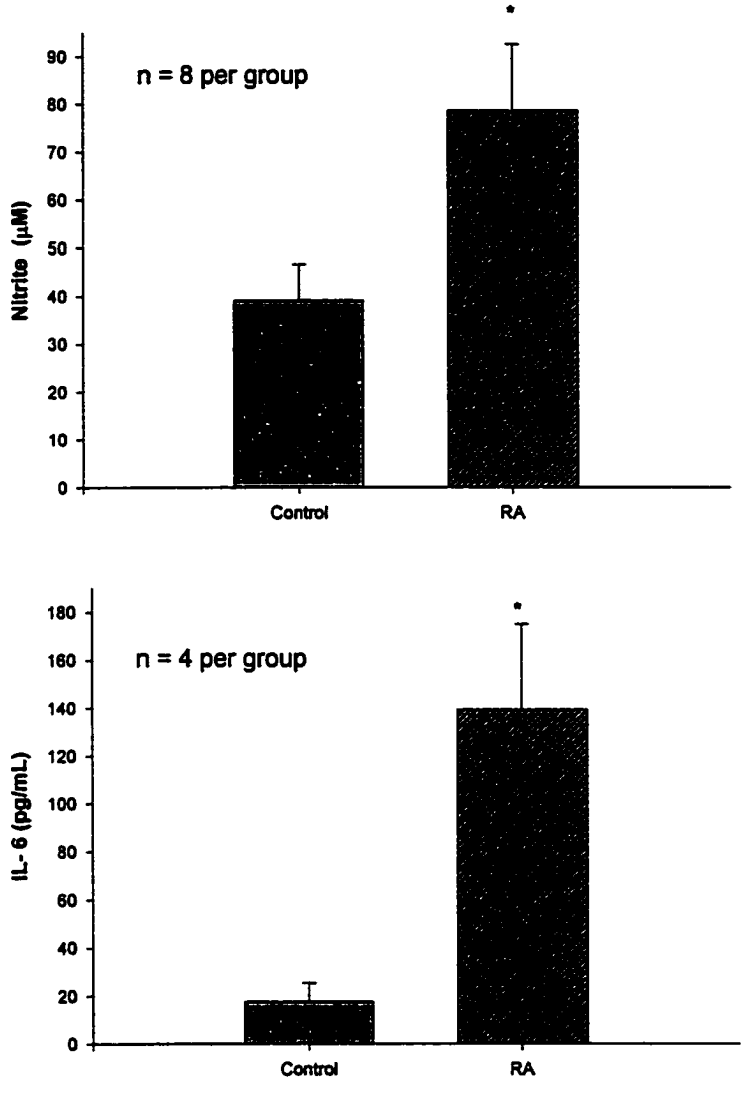


Figure 5-3: Serum nitrite and interleukin-6 (IL-6) levels for control and RA patients. *Significantly Different from Control $p < 0.05$.

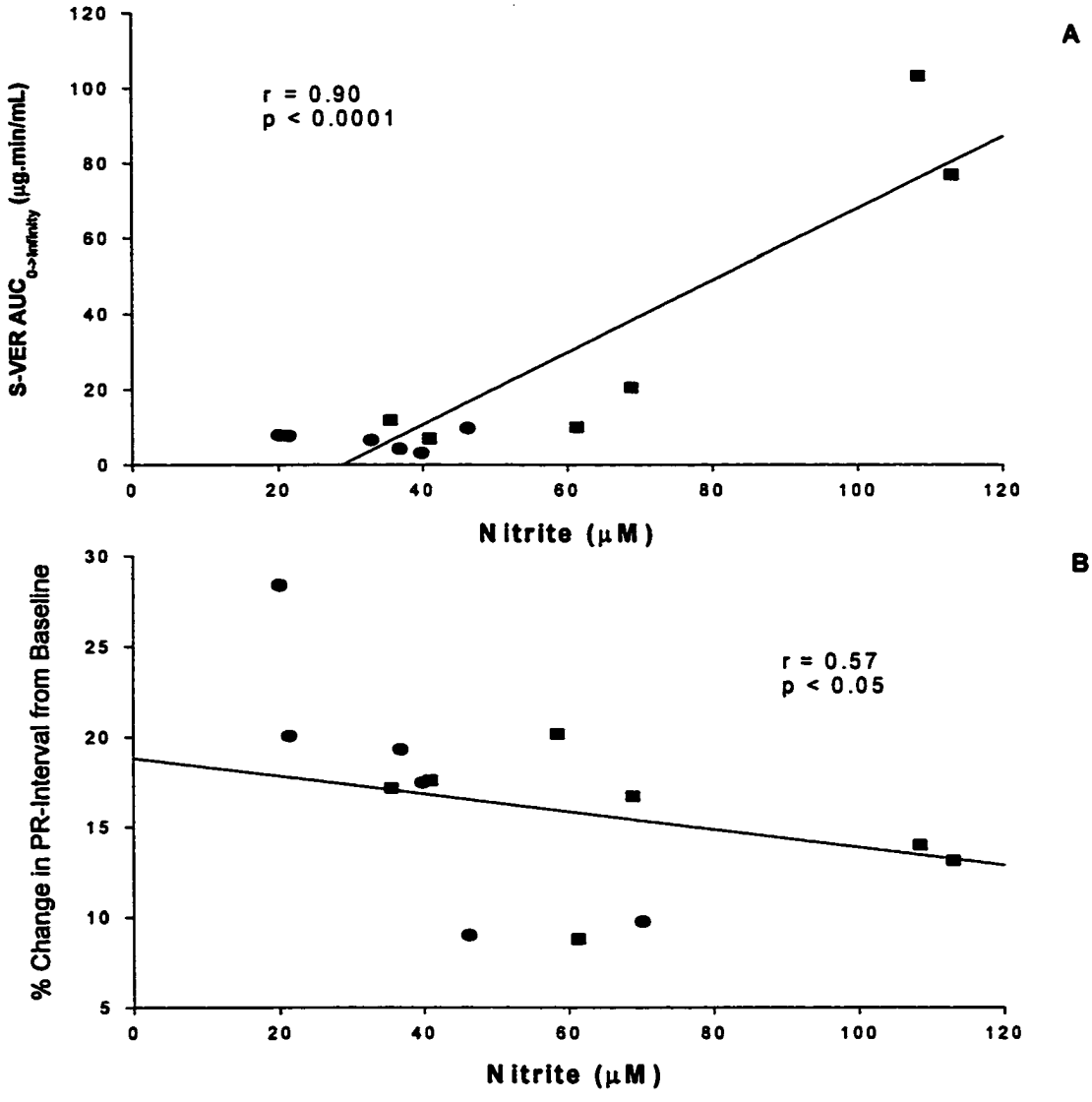


Figure 5-4: The Correlation of Serum nitrite with (A) S-VER $AUC_{0\rightarrow\infty}$ and (B)%Change in PR-Interval after 80 mg orally of VER for Healthy Subjects (•) and Rheumatoid Patients (■).

Positive and negative linear correlations were observed between serum nitrite and the pharmacologically more active enantiomer S-VER $AUC_{0\rightarrow\infty}$ ($r= 0.90$, $p<0.0001$) and % change in PR-Interval from baseline ($r= 0.57$, $p<0.05$), respectively. In addition, serum nitrite strongly correlated with disease severity. Attempts to correlate IL-6 with disease severity were unsuccessful largely due to a paucity of data.

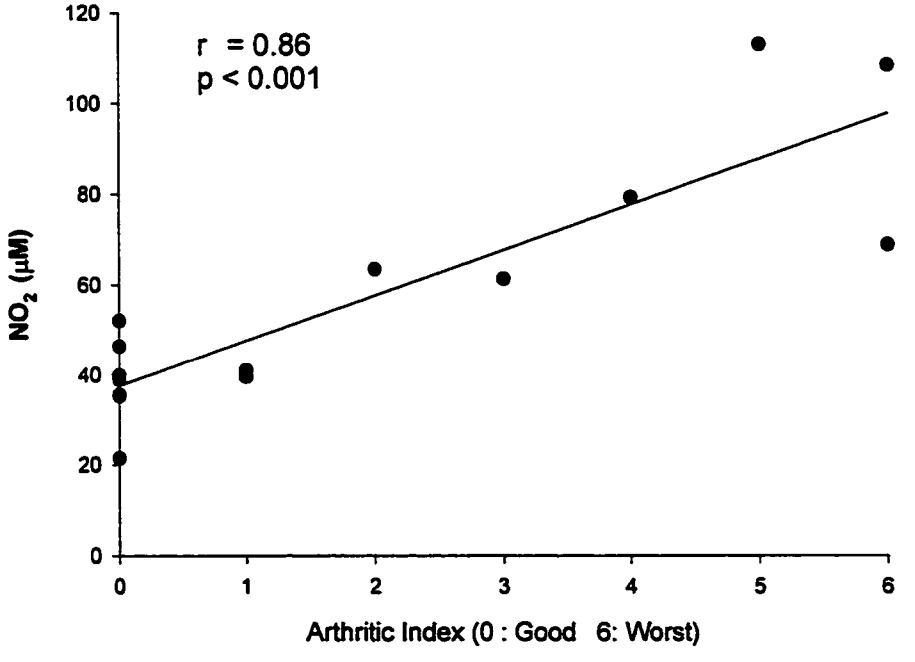


Figure 5-5: Arthritic index vs Serum nitrite (μM).
Healthy control patients all had an Arthritic Index of 0.

Using a discriminant analysis, a subject could be correctly identified as

control or rheumatoid 100% of the time ($p < 0.04$). The correlation's between the discriminating variables and canonical functions were nitrite (r=.99), IL-6 (r= 0.56), and S-VER AUC

(r=-0.46).

DISCUSSION

Pharmacokinetics

This study clearly demonstrates that RA causes an increase in drug AUC with a concurrent decrease in VER oral clearance and *fu* (Table 5-1). Using an endotoxin rat model of inflammation, Laethem *et al.* (1994) demonstrated similar changes in VER disposition. However, our study represents the first report of RA-induced changes of verapamil pharmacokinetics in man. VER enantiomers are extensively metabolized by the liver in a stereoselective manner favoring S-VER (Eichelbauem *et al.* 1979). The higher clearance S-VER has an oral bioavailabilty of 20 - 30% compared to the lower clearance R-VER with a 60 to 70 % oral bioavailabilty (Abernethy *et al.* 1993). Therefore, S-VER possesses the characteristics of a highly extracted drug while R-VER typifies an intermediately extracted drug (Wilkinson 1987). Generally, the systemic clearance of highly extracted drugs is expected to be less sensitive to changes in protein binding than a low extraction drug, however, this does not imply that the oral bioavailability of highly extracted drugs is independent of protein binding (Wilkinson 1987). Acute exacerbations of rheumatoid arthritis are associated with an increase in α1-acid-glycoprotein (AAG) and other acute phase reacting proteins (Fey and Müller

1991). Furthermore, approximately 70% of the binding variability observed in patients with arrhythmia could be accounted for by fluctuations in AAG (McGowan *et al.* 1983). In our study a 5 and 6 fold decrease in *fu* was observed with a 4 and 3 fold increase in AUC for S- and R-VER respectively. Therefore, the change in R-VER pharmacokinetics could easily be caused by an increase in protein binding since under these conditions binding could become a clearance-limiting factor. It is also possible that in the presence of increased protein binding, S-VER no longer behaves as a highly extracted drug. In a study on propranolol Walker *et al.* (1986) demonstrated a decrease in extraction ratio from 0.93 to 0.67 in arthritic rats. Furthermore, Mehvar and Reynolds (1996) using isolated perfused rat livers demonstrated that the stereoselective elimination of VER is protein-binding dependent. It has been suggested that for drugs with a high extraction ratio and extensive protein binding such as VER and propranolol that dissociation of the drug from protein may not be rapid enough to replace extracted drug within the sinusoid of the liver. Thus the fraction available within the liver may be lower than indicated by the *fu* in the hepatic portal vein (Jansen 1981). Therefore, it is possible that the observed changes in VER pharmacokinetics are primarily due to changes in protein binding.

RA-induced changes in intrinsic clearance (Cli') could also explain the observed changes in VER disposition. In the event of decreased Cli', hepatic extraction would decrease resulting in an increase in oral bioavailability. Hence, drug AUC's would rise with an apparent decrease in CL/F. Rheumatoid arthritis produces pathophysiological changes that are also capable of altering Cli'.

Inflammatory disease and individual proinflammatory cytokines have been shown to depress cytochrome P450 (CYP450) isozyme activities (Cawthorne *et al.* 1976; Descotes 1985; Moreno *et al.* 1987; Peterson & Renton 1986). RA is associated with an increase in pro-inflammatory cytokines such as interleukin-1ß (IL-1ß), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (Arend and Dayer 1990). These cytokines have been shown to induce the formation of nitric oxide which has been shown to inhibit CYP450 isozymes (Khatsenko *et al.* 1993). It is has been previously reported that NO levels are elevated in patients with active RA (Ueki *et al.* 1996). Our results demonstrate a 2-fold increase in serum nitrite a stable breakdown product of NO (Figure 5-3). Furthermore, the rise in S-VER AUC highly correlated with the rise in serum nitrite (Figure 5-4). There is a suggestion in the data that little change in S-VER $AUC_{0\rightarrow\infty}$ occurs until after serum nitrite reaches 60 μM, then a dramatic rise occurs. It is possible that inflammatory conditions that fail to achieve higher levels of nitrite would not reveal a change in VER pharmacokinetics suggesting that this effect may only occur in those patients with severe and acute disease.

In addition to changes in serum nitrite, a 7-fold increase in serum IL-6 was also observed (Figure 5-3). In animal models, IL-6 has been shown to decrease the activity of CYP3A4, CYP1A2 and CYP2C (Chen *et al.* 1994, Abdel-Rassak *et al.* 1993) the very isozymes responsible for the metabolism of VER and NOR (Kroemer *et al.* 1993, Busse *et al.* 1995). Therefore, it is possible that a RA-induced rise in cytokines and NO could decrease the metabolism of VER. Thus the combined effects of NO-mediated decrease in extraction and increased protein

binding could easily account for the observed changes in VER pharmacokinetics. Indeed, the observed decrease in propranolol extraction by Walker *et al.* (1986) could reflect a change in enzyme efficiency (hence extraction) in the presence of increased protein binding.

RA could also change hepatic blood flow (Q). The influence of reduced Q is expected to be minimal on low extraction and maximal on high extraction drugs (Pang and Rowland 1977). Hepatic and splanchnic blood flow have been shown to be regulated in disease states by many mediators including prostanoids, nitric oxide (NO) and endothelins (Robotham *et al.* 1997, Myers *et al.* 1996). Prostanoids, NO and endothelins may all be elevated in RA (Robinson 1993). NO is a potent vasodilator that could decrease resistance in the vasculature leading to increased blood flow depending on cardiac output (Robotham *et al.* 1997). The effect of vasodilatation within the hepatic and splanchnic circulatory system is variable. As has been suggested for propranolol, another highly extracted drug, both decreased and increased Q may slow down clearance of the drug (Byren *et al.* 1984, Feely *et al.* 1983). A reduced Q may limit the drug supply to the site of metabolism and an increased Q could result in first pass saturation increasing bioavailability as oberved by Wagner (1985) with propranolol. In a propranolol study by Walker *et al.* 1986, hepatic blood flow was measured using ^{57}Co-labeled microspheres. No changes in hepatic blood flow were determined. It is not know if RA in man significantly alters Q, but this is theoretically possible.

Recently is has been suggested that VER may also be metabolized in the gut wall. (Fromm *et al.* 1996, Fromm *et al.* 1998). It is, therefore, possible that

RA-induced inhibition of verapamil gut wall metabolism may also contribute to the observed reduced clearance of the drug. Furthermore, since bioavailability is a product of the amount of drug surviving intact across the gut wall and through the liver, then a change in enzyme function pre-systemically in the gut wall could significantly alter the amount of drug entering the hepatic portal vein. This is not known to occur with VER in rheumatoid arthritis patients, but this is a possibility.

The AUC $_{0\rightarrow12}$ norverapamil were also elevated in the presence of RA. However, no changes in metabolite to parent drug (NOR/VER) ratios were observed. This could indicate that clearance of NOR is decreased in a manner similar to VER clearance. Thus AUC's of both species rise, maintaining a constant NOR/VER ratio. The present data, however, does not permit a clear interpretation of the effect of RA on NOR. Given the systemic nature of rheumatoid arthritis, it is highly probable that the observed changes in VER and NOR pharmacokinetics is due at least in part to protein binding, but may have multiple causes which include decreased Cli' and altered hepatic blood flow. Furthermore, the severity of the RA could control the magnitude of alterations in protein binding, intrinsic clearance and hepatic blood flow.

Pharmacodynamics

The most striking observation of this study was the decreased dromotropic effect of VER despite the obvious increase in the VER and NOR concentrations. The percent prolongation in PR interval decreases from 20% in healthy controls to only 12% in RA patients (Figure 5-2). In addition, 1st-degree AV-block, a side effect associated with elevated VER concentrations (Thomas 1996), occurred in

two of the healthy control subjects and did not occur in any of the RA patients with significantly elevated VER concentration. Protein binding can also influence the pharmacodynamics of drugs. It is possible that the decrease in free fraction could be responsible for the decrease in dromotropic effect. However, the $AUCu_{0\rightarrow\infty}$ of S-VER did not change in the presence of RA. A decrease in R-VER $AUCu_{0\rightarrow\infty}$ was observed, but R-VER possesses 10 to 20 times less dromotropic activity than its antipode (Echizen *et al.* 1985). In addition, NOR serum levels were also elevated in our study. NOR also possesses pharmacological activity at the AV-node, but is approximately 50 to 100 times less potent than the parent drug (Johnson *et al.* 1991). It has been reported that NOR can displace VER from its protein binding sites (Johnson and Akers 1995), however this would lead to an increase in *fu* which could actually increase drug effect. In a study on the effects of aging on VER pharmacokinetic-pharmacodynamic relationships, Abernethy *et al.* (1993) observed reduced dromotropism with no changes in protein binding. In an adjuvant-arthritis-propranolol paper by Walker *et al.* (1986), electrically induced tachycardia was decreased in the presence of arthritis. Therefore, it appears that RA results in alterations in normal cardiac responsiveness to drugs which are independent of pharmacokinetic changes

We have shown that RA decreases the dromotropic effect of VER. Thus it appears to downregulate L-type Ca^{2+} channels in the heart. A possible mechanism involves nitric oxide. NO has been shown to have negative inotropic effects on the heart (Finkel *et al.* 1992). NO is powerful activator of guanylyl cyclase (GC) (Murad *et al.* 1993). Activation of GC leads to increased levels of cyclic 3'-5'-

guanosine monophosphate (cGMP) which has been shown to inhibit L-Type Ca^{2+} channels (Méry *et al.* 1991). Thus the extracellular Ca^{2+} current through the L-type channel ($I_{Ca\text{-}L}$) is inhibited. However, there is controversy in this area. VER as a phenylalkylamine is believed to have negative inotropic, dromotropic and chronotropic effects through binding to the intracellular portion of the L-Type Ca^{2+} channel pore decreasing $I_{Ca\text{-}L}$. (Catterall and Striessnig 1992). In our study, a strong negative correlation was observed between serum nitrite and %PR-interval prolongation ($r = -0.51$, $p = 0.02$ Figure 5-4). Therefore, the potential exists for a RA-induced rise in NO to alter the dromotropic effects of VER. However, this does not preclude a direct cytokine effect on cardiac function. For example, binding of IL-2 to cardiac IL-2 receptors causes an increase in Ca^{2+} ion flux. (Gross G 1993). Such alterations in Ca^{2+} currents could alter the normal inotropic and dromotropic responsiveness of the myocardium to drugs such as VER.

Down-regulation of β–adrenergic receptors by pro-inflammatory cytokines and inflammatory conditions has also been observed in asthma and in congestive heart failure (Meurs *et al.* 1987, Bavendiek *et al.* 1996). A decrease in receptor density is not necessary for this downregulation, but changes in receptor function due to de-coupling of the ß-receptor from guaninine nucleotide binding protein (G-protein) and changes in intracellular protein kinase C activity may be involved. (Strasser *et al.* 1988). Since VER also alters the intracellular function of protein Kinase C (DePetrillo *et al.* 1994) this could be a common pathway for both ß-adrenergic receptor and calcium channel blocker downregulation. It is important to note that treatment of the inflammation with steroids has been shown to reverse

the ß-receptor downregulation caused by inflammation in asthma. (Svedmyr 1990). Thus inflammatory disease has the potential to downregulate the responsiveness of the heart to drugs which could lead to therapeutic failure.

Disease Severity

Clinical assessment of RA severity has proven to be difficult and highly variable. The use of erythrocyte sedimentation rate (ESR) and C-reactive protein as indicators of disease severity have been unsuccessful due to variability. Serum nitrite could offer an easy measure to help quantify clinical response. In our study, arthritic index correlated strongly with serum nitrite (Figure 5-6). Thus, nitrite could provide the clinician with a measure of disease severity which could be used in the original assessment of the disease and to monitor the response to drug therapy. It is important to note that method of nitrite measurement included serum nitrate as well, since all was reduced to nitrite. It is not known if measuring nitrite alone would correlate with arthritic index in rheumatoid arthritis.

In this study, IL-6 did not correlate well with arthritic index. However, this may be due to the small sample size caused by the accidental destruction of samples. In the discriminate analysis, IL-6 was a strong predictor of the presence of RA. Nitrite was the next strongest predictor followed by S-VER AUC. Thus it appears that reactive nitrogen species are involved in the VER-inflammation disease interaction and could be used as a monitoring tool for the clinician.

In conclusion, RA results in increased drug levels of VER and NOR. These pharmacokinetic changes are likely due to changes in protein binding, but could also involve decreased Cli and altered hepatic blood flow. Despite the

elevation in drug concentration, a significant decrease in dromotropic activity was observed which may be attributed to receptor down regulation caused by increased expression of pro-inflammatory cytokines and/or NO. Implications of this observation may reach beyond verapamil and RA since other inflammatory conditions (e.g., arthritis, infection, asthma) and other receptors may prove to be involved. With increasing age, multiple disease states are more likely to occur. Treatment of cardiovascular diseases in a patient with rheumatoid arthritis, therefore, may require closer attention to prevent therapeutic failure.

Appendix 5-I

PK-PD Modeling

PK-PD relationships demonstrated a substantial, but expected amount of variability (Figures 5-A-1 and 5-A-2). Due to this variability modeling was difficult especially in the RA group where 3 patients demonstrated significant counter-clockwise hystereses loops. When these patients were excluded from the direct link sigmoid Emax modeling, (Figure 5-A-4) less variability was observed. The modeling suggests that Emax is relatively unaffected by RA (19.4% prolongation vs 19.6% in controls), however, more drug is required to obtain this effect with a right shift in EC_{50} from 10.8 ng/mL to 22. 5 ng/mL. It is interesting to note that the hystereses loops could be collapsed using an indirect link model to a theoretical effect compartment (Whiting, Holford and Sheiner 1980). Calculated keo values were very similar, 0.011, 0.012, and 0.018 min^{-1} suggesting a lag between maximum drug concentration and maximum effect of approximately 50 minutes. Changes in protein binding and or direct membrane effect could account for this delay. Membrane changes could also alter the function of the L-type Ca^{2+} channel resulting in decreased affinity or sensitivity to VER. The calculation of Emax and EC_{50} values not performed in the indirect link model since a clearly defined maximum effect was not acheived. This further supports the observed downregulation in that a very large amount of drug would be required to achieve Emax.

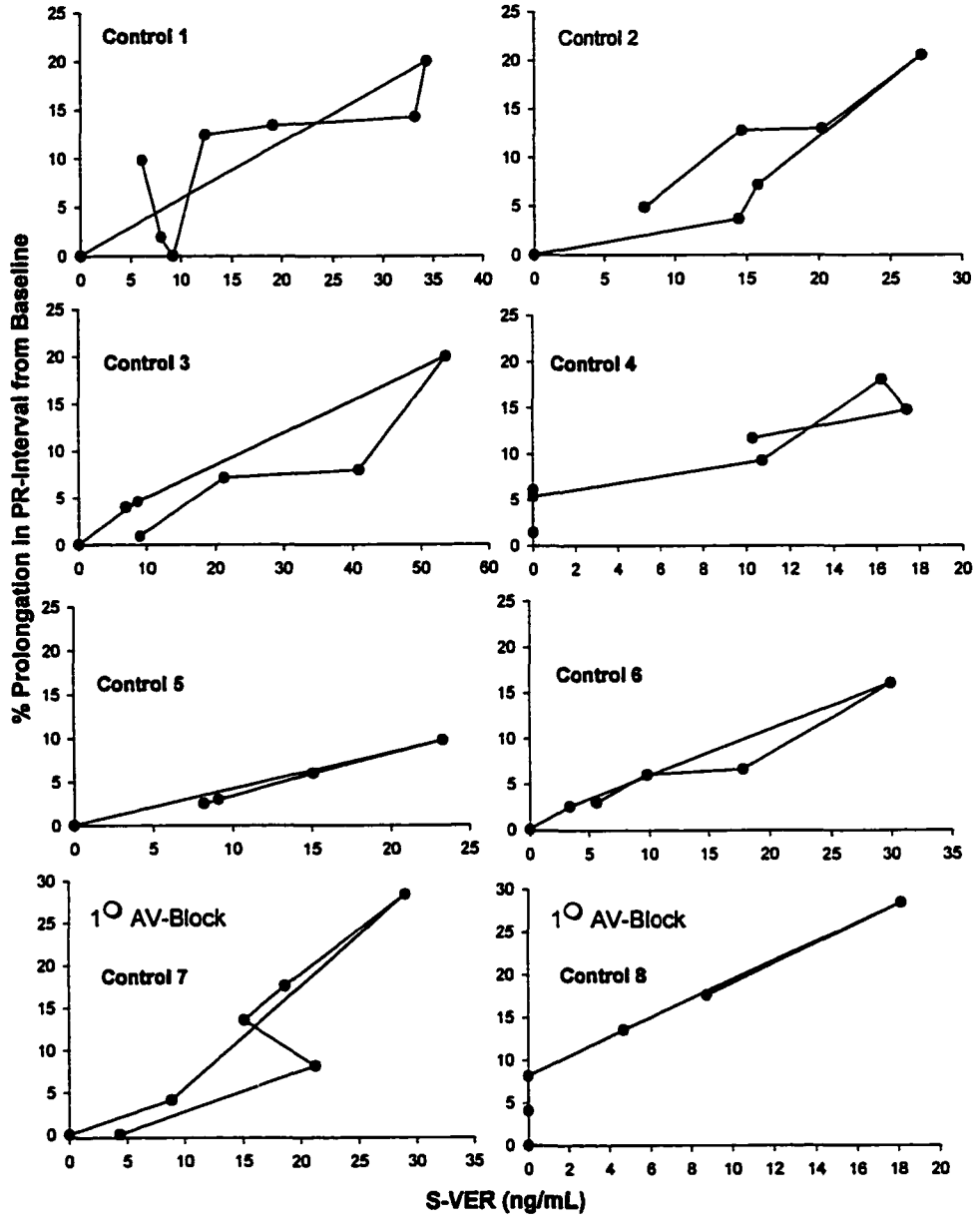


Figure 5-A-1: Control patients PK-PD summary: S-VER (ng/mL) vs. %PR-Interval Prolongation.

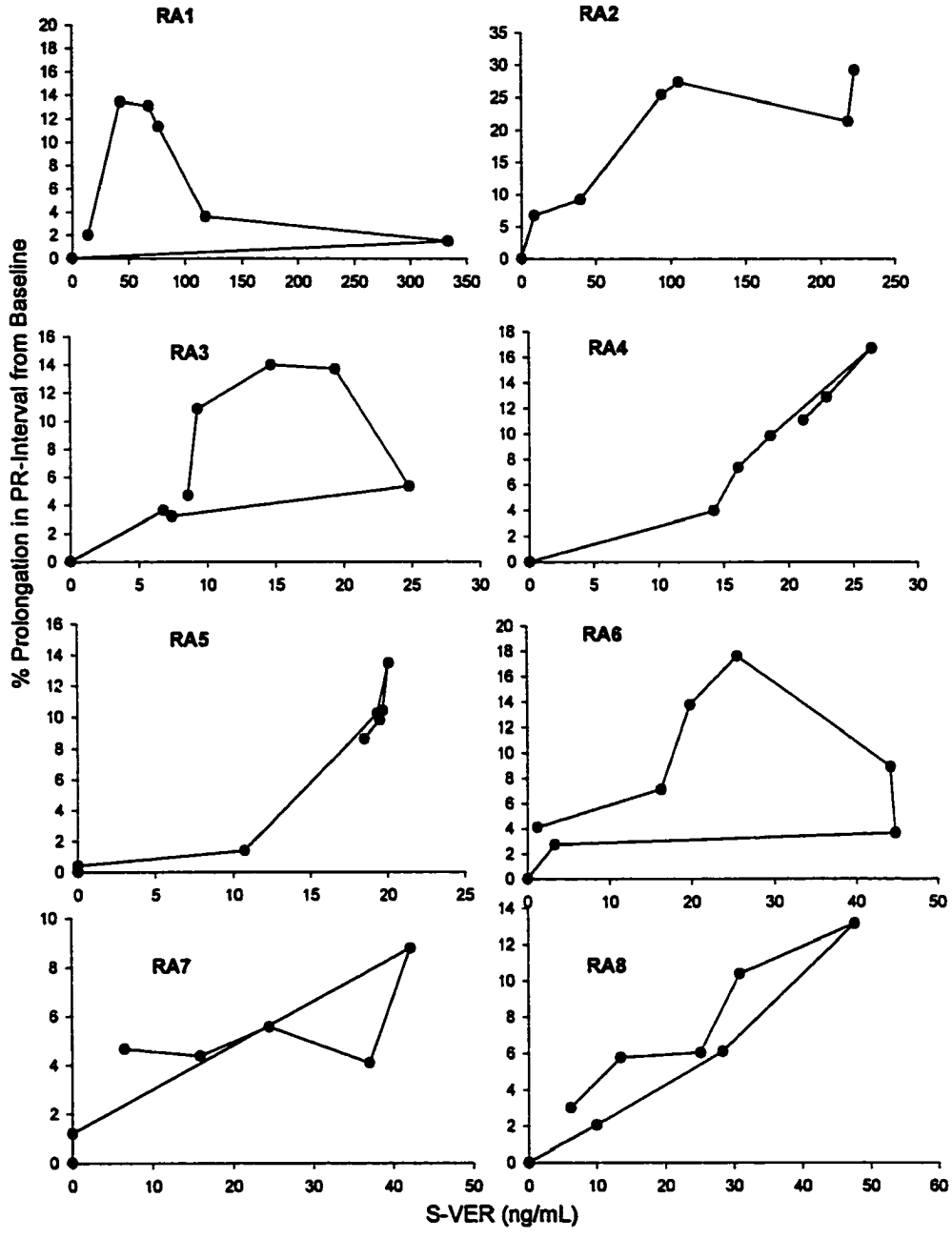


Figure 5-A-2: RA patients PK-PD summary: S-VER (ng/mL) vs. %PR-interval Prolongation.

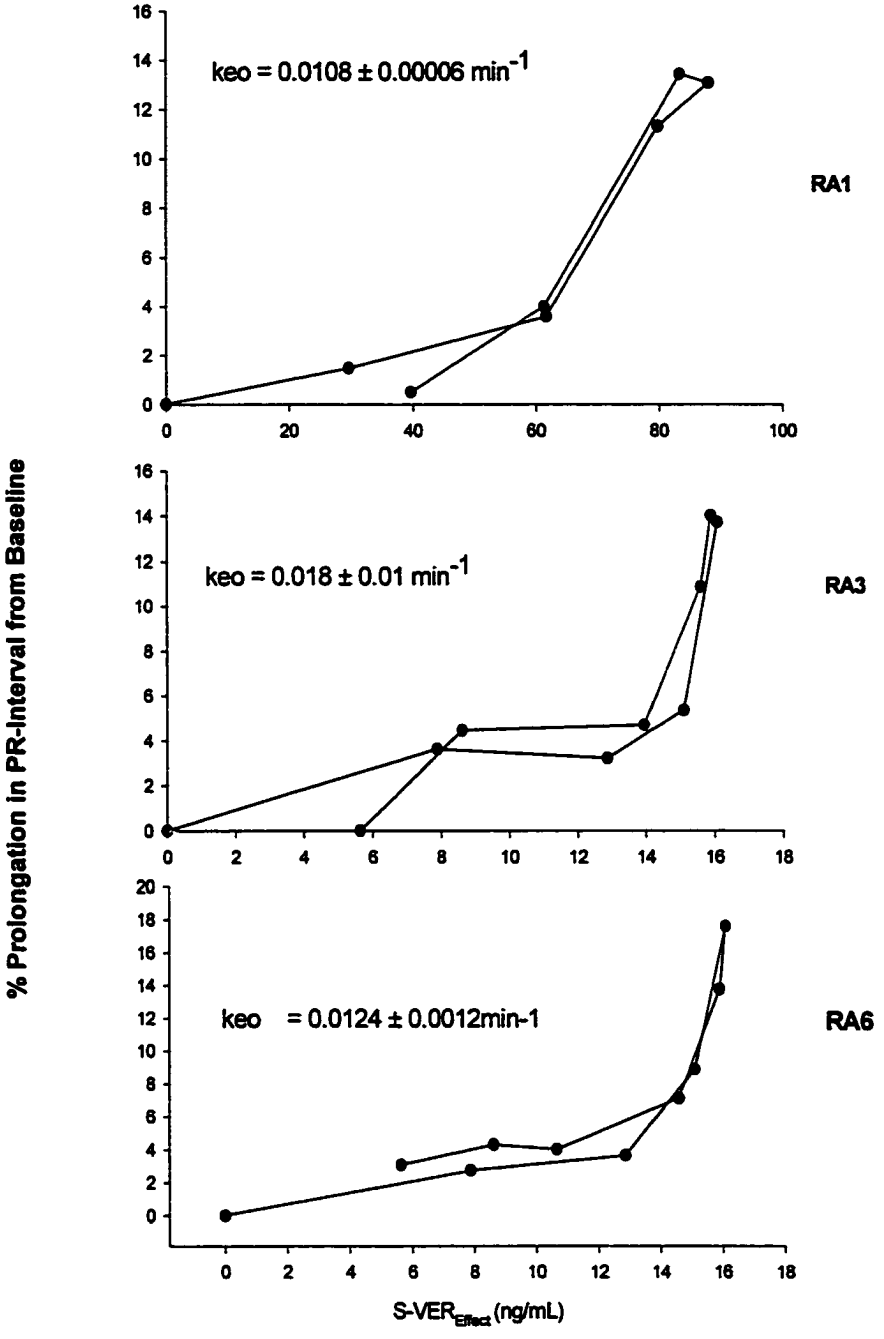
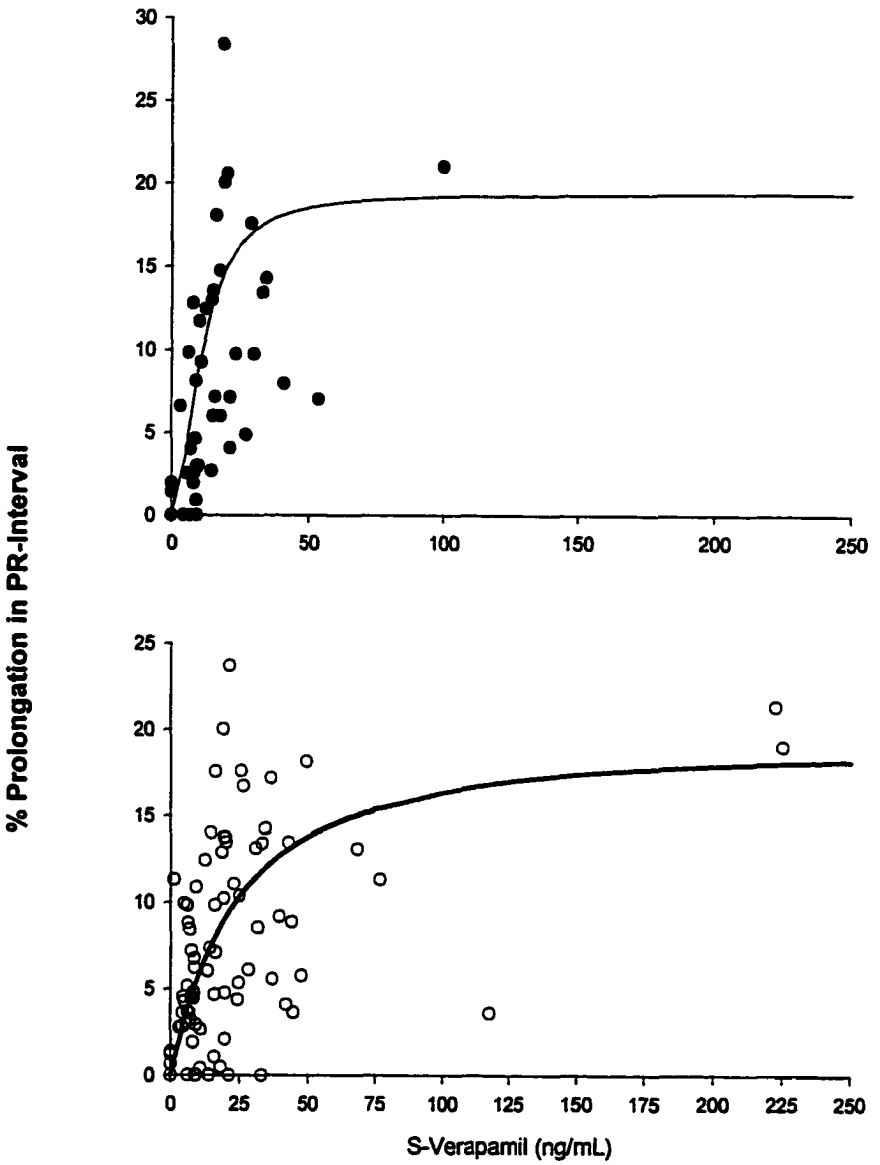


Figure 5-A-3: Rheumatoid patients with collapsed hystereses loops.



Parameter	Control	Rheumatoid	Units
Emax	19.4 (8.8)	19.6 (6.7)	%
EC50	10.8 (6.3)	22.5 (18.4)	ng/mL
Gamma	1.9 (1.4)	1.1 (0.7)	
AIC	32.3	36.4	

Figure 5-A-4: Sigmoid Emax model: Direct link for control (●) and RA patients (○).

♦♦♦

REFERENCES

Abdel-Rassak Z, Loyer P, Fautrel A, Gautier JC, Corcos L, Turlin PD, Guillouzo A. Cytokines down-regulate expression of major cytochrome P450 enzymes in adult human hepatocytes in primary culture. *Mol Pharmacol* 1993; **44**: 707-715.

Abernethy D, Wainer I, Longstreth J, Adrawis N. Stereoselective verapamil disposition and dynamics in aging during racemic verapamil administration. *J Pharmacol Exp Ther* 1993; **266**: 904 - 911.

Arend W, Dayer J. Cytokines and cytokine inhibitors or antagonists in rheumatoid arthritis. *Arthritis Rheum* 1990; **33**: 305 - 315.

Bavendiek U, Brixius K, Frank K, Reuter H, Pietsch M, Gross A, Muller-Ehmsen J, Edmann E, Schwinger R. Altered inotropism in the failing human myocardium. *Basic Res Cardiol* 1996; **91**: 9 - 16.

Busse D, Cosme J, Beune P, Kroemer H, Eichelbaum M. Cytochromes of the P450 2C subfamily are the major enzymes involved in the O-demethylation of verapamil in humans. *Naunyn-Schmiederberg's Arch Pharmacol* 1995; **353**: 116 - 121.

Byrne A, McNeil J, Harrison P, Louis W, Tonkin A, McLean AJ. Stable oral availability of sustained release propranolol when co-administered with hydralazine or food: evidence implicating substrate delivery rate as a determinant of presystemic drug interactions. *Br J Clin Pharmacol* 1984; **17**: Supp 1:45S-50S.

Catterall WA, Striessnig J. Receptor sites for Ca2+ channel antagonists. *TiPS* 1992; **13**: 256 - 262.

Cawthorne M, Palmer E, Green J. Adjuvant induced arthritis and drug-metabolizing enzymes. *Biochem Pharmacol* 1976; **25**: 2683 - 2688.

Chen YL, Vraux VL, Leneveua A, *et al.*, Acute-phase reponse, IL-6 and alteration of cyclosporine pharmacokinetics. *Clin Pharmacol Ther* 1994; **55**: 649 - 660.

De Belder A, Radomski M. Nitirc oxide in the clinical area. J Hyperten 1994; **12**: 617 - 624.

DePitrillo P, Abernethy D, Wainer I, Andrawis N. Verapamil decreases lymphocyte protein kinase C activity in humans. *Clin Pharmacol Thera* 199455; 44-50.

Descotes J. Immunomodulating agents and hepatic drug metabolizing enzymes. *Drug Metab Rev* 1985; **16**: 175 - 185.

Eichelbaum M, Ende M, Rember G, Schomerus M, Dengler H. The metabolism of ^{14}C-D,L-verapamil in man. *Drug Metab Disp* 1979; **7**: 145 - 148.

Echizen H, Brecht T, Niedergesäss S, Vogelgesang B, Eichelbaum M. The effect of dextro-, levo-, and racemic verapamil on atrioventricular conduction in humans. *Am Heart J* 1985; **109**: 210 - 217.

Ferrari L, Herber R, Batt A, Siest G. Differential effects of human recombinant IL-1ß and dexamethasone on hepatic drug-metabolizing enzymes in male and female rats. *Biochem Pharmacol* 1993; 45: 2269 - 2277a.

Ferrari L, Jouzeau JY, Gillet P, Herber R, Fener P, Batt AM, Netter P. IL-1ß differentially represses drug-metabolizing enzymes in arthritic female rats. *J Pharmacol Exp Ther* 1993; **264**: 1012 - 1020b.

Feely J, Nadeau J, Wood A. Effects of feeding on the systemic clearance of indocyanine green and propranolol blood concentrations and plasma binding. *Br J Clin Pharmacol* 1983; **15**: 383 - 385.

Fey GF, Fuller GM. Regulation of acute phase gene expression by inflammatory mediators. *Mol Biol Med* 1987; **4**: 323 - 338.

Finkel M, Oddis C, Jacob T, Watkins S, Hattler B, Simmons R. Negative inotropic effects of cytokines on the heart mediated by nitric oxide. *Science* 1992; **257**: 387 - 389.

Fromm M, Busse D, Kroemer H, Eichelbaum M. Differential Induction of prehepatic and hepatic metabolism of verapamil by rifampin. *Hepatology* 1996; **24**: 796 - 801.

Fromm M, Dilger K, Busse D, Kroemer H, Eichelbaum M, Klotz U. Gut wall metabolism of verapamil in older people: Effects of rifampicin-mediated enzyme induction. *Br J Clin Pharmacol* 1998; **45**: 247 - 255.

Grisham M, Johnson G, Gautreaux M, Berg R. Measurement of nitrate and nitrite in extracellular fluids: A window to systemic nitric oxide metabolism. . *Methods* 1990; **7**: 84 - 90.

Gross AS, Mikus G, Morkike K, Eichelbaum M. Pharmacokinetics and pharmacodynamics of the enantiomers of gallopamil. *Eur J Pharmacol* 1990; 183: 1651 - 1652.

Gross G. Inflammatory Mediators and the Stunned Myocardium Chapter 8 in Immunopharmacology of the Heart. Academic Press Ltd. 1993.

Ishikawa M, Sasakiu K, Ozaki M, Watanabe K, Takayanagi Y, Sasaki K. Hepatic drug metabolizing activity in rats with carageenan-induced inflammation. *J Pharmacobio Dyn* 1991; **14**: 132 - 138.

Jansen J. Influence of plasma protein binding on hepatic clearance assessed from a "tube" model and a "well-stirred" model. *J Pharmacokinet Biopharm* 1981; **9**: 15 - 26.

Johnson K, Balderston S, Piper J, Mann D, Reiter M. Electrophysiological effects of verapamil metabolites in the isolated heart. *J Cardiovasc Pharmacol* 1991; **17**: 830 - 837.

Johnson J, Akers W. Influence of metabolites on protein binding of verapamil. *Br J Clin Pharmacol* 1995; **39**: 536 - 538.

Khatsenko O, Gross S, Rifkind A, Vane J. Nitric oxide is a mediator of the decrease in CYP450-dependent metabolism caused by immunostimulants. *Proc Nat Acad Sci USA* 1993; **90**: 11147 – 11151.

Kroemer H, Gautier J, Beaune P, Henderson C, Wolf C, Eichelbaum M. Identification of P450 enzymes involved in metabolism of verpamil in humans. *Naunyn-Schmiedeberg's Arch Pharmacol* 1993; **348**: 332 - 337.

Kroemer H, Echizen H, Heidemann H, Eichelbaum M. Predictability of the in vivo metabolism of verapamil from in vitro data: contribution of individual metabolic pathways and stereoselective aspects. *J Pharmacol Exp Ther* 1992; **260**: 1052 - 1057.

Laethem ME, Belpaire FM, Wijnant P, Rosseel MT, Bogaert MC. Influence of endotoxin on the steroselective pharmacokinetics of oxprenolol, propranolol, and verapamil in the rat. *Chirality* 1994; **6**: 405 – 410.

Lyle WH. Penicillamine. *Clin Rheum Disease* 1979; **5**: 569 - 601.

McGowan F, Reiter M, Pritchett E, Shand D. Verapamil plasma binding: Relationship to α1-acid glycoprotein and drug efficacy. *Clin Pharmacol Ther* 1993; **33**: 485 - 490.

Mehvar R, Reynold J. Reversal of stereoselectivity in the hepatic availability of verapamil in isolated perfused rat livers. *Drug Metab Disp* 1996; **24**: 1088 - 1094.

Mery P, Lohmann S, Wlater U, Fischmeister R. Ca^{2+} current is regulated by cGMP-dependent protein kinase in mammalian cardiac myocytes. *Proc Nat Acad Sci USA* 1991; **88**: 1197 – 1201.

Meurs H, Kauffman HF, Koeter GH, Timmermans A, de Vries K. Regulation of the beta-receptor-adenylate cyclase system in lymphocytes of allergic patients with asthma: possible role for protein kinase C in allergen-induced nonspecific refractoriness of adenylate cyclase. *J Allergy Clin Immunol* 1987; **80**: 326 - 39.

Moreno JJ, Escofet A, Castell M, Castellote C, Queralt J. Hepatic cytochrome P-450 activities and serum biochemical changes in adjuvant arthritis. *Med Sci Res* 1987; **15**: 1469 - 1470.

Morton D, Chatfield D. The effects of adjuvant induced arthritis on the rat liver metabolism of drugs in rats. *Biochem Pharmacol* 1970; **19**: 473 - 481.

Murad F, Forstermann U, Nakane M, Pollock J, Tracey R, Matsumoto T, Buechler W. The nitric oxide-cGMP signal transduction system for intracellular and intercellular communication. *Advance Second Messenger Phosphoprotein Res* 1993; 28: 101 - 109.

Myers S, Turnage R, Hernandez R, Castenada A, Valentine R. Autoregulation of renal and splanchnic blood flow following infra-renal aortic clamping is mediated by nitric oxide and vasodilator prostanoids. *J Cardiovascular Surgery* 1996; **37**: 97 - 103.

Nelson W, Olson L, Beitner D, Pallow J. Regiochemistry and substrate specificity of O-demethylation of verpamil in the presence of the microsomal fraction from rat and human liver. *Drug Metab Dis* 1988; **16**: 184 - 188.

Pang KS, Rowland M. Hepatic clearance of drugs. I. Theoretical considerations of a well stirred model and a parallel tube model. Influence of hepatic blood flow, plasma and blood cell binding, and the hepatocellular enzymatic activity on hepatic drug clearance. *J Pharmacokin Biopharm* 1977; **5**: 625 - 653.

Peterson TC, Renton KW. Kupfer cell factor mediated depression of hepatic parenchymal cell cytochrome P-450. *Biochem Pharmacol* 1986; **35**: 1491 - 1497.

Piquette-Miller M, Jamali F. Selective effect of adjuvant arthritis on the disposition of propranolol enantiomers in rats detected using a stereospecific HPLC assay. *Pharm Res* 1993; 10: 294 - 299.

Poulton K, Griffith S, Thomson W, Mattey D, Fischer J,Clarke S, Dawes P, Illier W. Adverse drug reactions in patients with rheumatoid arthritis and HLA-DR3. *Eur J Immunogen* 1998; **25**: 62 – 66.

Robinson D. Lipid Mediators, Acitve Oxygen, Amines, Nitric Oxide, Kinins and Clotting Factors in Rheumatoid Arthritis. In Primer on the Rheumatic Diseases, 10th Ed. Schmacher H, Klippel J and Koopman W (eds). Arthritis Foundation:Willam Byrd Press Richmond. 1993. 46 - 49

Robotham J, Clemens M, Geiger K. Regulation of hepatic blood flow during resuscitation from hemorrhagic shock. Role of NO and endothelins. *Am J Physiology* 1997; **272**: (6 41-6) H2736 - H2745.

Sasaki K, Ishikawa Y. The effect of phenobarbital on the impairment of drug metabolism by the carrageenan-induced inflammation in male rat. *Res Comm Chem Pathol Pharmacol* 1990; **69**: 377 - 380.

Schneider RE, Bishop H, Kendall MJ, Quarterman CP. Effect of inflammatory disease on plasma concentrations of three ß -adrenoreceptor blocking agents. *Int J Clin Pharmacol Ther Toxicol* 1981; **19**: 158 - 162.

Schumacher H, Klippel J, Koopman W (eds). Primer of the Rheumatic Diseases, 10th ed Atlanta ,GA, The Arthritis Foundation, 1993.

Schwartz J, Troconiz I, Verotte D, Liu S and Capili H. Aging Effects on stereoselective pharmacokinetics and pharmacdynamics of verapamil. *J Pharmcol Exp Ther* 1993; **265**: 690 - 697.

Shibukawa A, Wainer I. Simultaneous direct determination of the enantiomers of verapamil and norverapamil in plasma using a derivatized amylose HPLC chiral stationary phase. *J Chrom Bio App* 1992; **574**: 85 - 92.

Strasser R, Benovic J, Lefkowitz R, Caron M. The beta-arendergic receptor kinase: role in homologous desensitization in S49 lymphoma cells. *Adv Exp Med Bio* 1988; **232**: 503 - 517.

Svedmyr N. Action of corticosteroids on beta-adrenergic receptors: Clinical Aspects. *Am Rev Resp Disease* 1990; 141: S31- S38.

Tatsuoka M. Selected Topics in Advanced Statistics: An Elementary Approach #6. Discriminant Analysis: The Study of Group Differences. Institute for Personality and Ability Testing. Champaign Illinois, 1970.

Thomas S, Stone C, Koury S. Cardiac dysrhythmias in severe verapamil overdose: characterization with a canine model. *Eur J Emerg Med* 1996; **3**: 9 - 13.

Ueki Y, Miyake S, Tominaga Y. Eguchi K. Increased nitric oxide levels in patients with rheumatoid arthritis. *J Rheumatol* 1996; **23**: 230 - 236.

Wagner J. Kinetics of pharmacological response I. Proposed relationships between response and drug concentration in the intact animal and man. *J Theor Biol* 1968; **20**: 171 - 177.

Wagner JG. Propranolol: pooled Michaelis-Menten parameters and the effect of input rate on bioavailability. *Clin Pharm Ther* 1985;**37**:481-487.

Walker K, Barber H, Hawksworth G. Mechanism responsible for altered propranolol disposition in adjuvant-induced arthritis in the rat. *Drug Metab Dispos* 1986; **14**: 482 - 486.

Wilkinson G. Clearance approaches in pharmacology. *Pharmacol Rev* 1987; 39: 1 - 47.

Walshe JM, Chairman. Round Table Discussion-Proper use of penicillamine. *Post Grad Med J* 1974 SUPP; 80 - 83.

Whitehouse FM. Adjuvant-induced polyarthritis in rats. In: Handbook of animal models of the rheumatic diseases. R. Greenwald and H. Diamond eds. Vol I, pp 3 - 16.CRC Press, Boca Raton Fl, 1988.

Wijnands MJH, Van 'thof MA, Van De Putte LBA, Van Riel PLCM. Rheumatoid arthritis: a risk factor for sulfasalazine toxicity? A meta analysis *Br J Rheumatol* 1993; 32: 313 - 318.

Whiting B, Holford N, Sheiner L. Quantitative analysis of the disopyramide concentration-effect relationship. *Br J Clin Pharmac* 1980; **9**: 67 - 75.

Wolfe F. Adverse drug reactions of DMARR's and DC-ARTs in rhematoid arthitis. *Clin Exp Rheumatol* 1997; **15**: (SUPP 17); S75-S81.

Yamaoka K, Nakagawa T, Uno T. Application of Akaike's information criterion (AIC) in the evaluation of linear pharmacokinetic equations. *J Pharmacokin Biopharm* 1978; **6**: 165 - 175.

Chapter 6

6.0 General Conclusions

Systemic inflammatory diseases do not merely reflect an absence of normal health, but a profoundly altered physiological state. Therefore, the patient with rheumatoid arthritis may react to pharmacological intervention differently than the patient without systemic inflammation. The focus of this work was to evaluate the effects of systemic inflammation and inflammatory mediators on the pharmacokinetics and pharmacodynamics of the L-type calcium channel blocker, verapamil. Thus the effects of inflammation on the pharmacokinetics and pharmacodynamics were evaluated in three different experiments.

1. The effect of a single pro-inflammatory cytokine, IFN-α2a, on VER disposition and pharmacodynamics in rats (acute).
2. The effect of inflammation induced by a transgenic rat model of human HLAB27/HBM2 genes on VER disposition and pharmacodynamics using a
3. The effect of systemic rheumatoid arthritis on the disposition and pharmacodynamics of VER in humans (acute).

6.1 Pharmacodynamics

A universal finding of all three studies was the observed down regulation of the calcium channel blocking effect of verapamil. This occurred in the presence or absence of pharmacokinetic changes or increased reactive nitrogen species production. This suggests that down regulation of L-type Ca^{2+} occurs independently of changes in pharmacokinetics and is likely due to the inflammatory process itself. It has been suggested that L-type Ca^{2+} channels

represent the major pathway for voltage-gated Ca^{2+} entry into human cardiac myocytes (Quinard *et al.* 1997). Animal studies have shown that the L-type Ca^{2+} channel current (I_{Ca}^{2+}) is enhanced by a rise in intracellular cAMP levels and suppressed by a rise in intracellular cGMP levels (Taguchi *et al.* 1997). Thus a NO-mediated rise in cGMP would be expected to inhibit L-Type Ca^{2+} channels. Considerable controversy exists within the scientific literature on the importance of this type of regulation. I_{Ca} can be inhibited by NO stimulating or releasing agents with or without an increase in cGMP (Quinard *et al.* 1997, Hu *et al.* 1997). It seems reasonable that systemic inflammatory disease through release of cytokines and NO could have a direct effect on L-type Ca^{2+} channels. The pharmacological activity of verapamil could be altered under these conditions. Studies in basilar artery smooth muscle cells using an NO-donating compound have suggested that NO decreases the frequency of Ca^{2+} channel openings without an alteration in Ca^{2+} channel properties. The mechanism likely involves cGMP-dependent protein kinase (Tewari and Simard 1997). Since it has been shown that verapamil activity requires binding to an intracellular site, a decrease in channel opening would be expected to decrease the activity of verapamil. Both the RA and the individual cytokine studies demonstrated a rise in reactive nitrogen species as measured by nitrite. However, the transgenic studies demonstrated a similar down-regulation in dromotropic effect without a statistically significant rise in serum nitrite. This could suggest an NO-independent mechanism such as a direct cytokine effect on calcium translocation (Gross *et al.* 1993). However, the transgenic model could reflect a less severe, chronic state in which the animal has

adapted with enhanced free radical scavenging. It is also possible, that serum nitrite measurements lack the sensitivity and precision to completely explore the role of reactive nitrogen species.

In addition to changes in the pharmacological properties of the L-type Ca^{2+} channel, changes in protein binding could also alter the pharmacodynamics of VER. Assuming that AAG binding is reversible, and does not transport the drug out of the systemic circulation, a new equilibrium would be achieved at steady state. Availability of the drug to the site of action, namely the L-type Ca^{2+} channel, could be diminished or delayed. A delay could account for the hystereses loops observed in the rheumatoid arthritis group. It is important to note that the $AUCu_{(0-\infty)}$ for the more potent S-VER, was not changed. This suggests that the decrease in dromotropic effect was not due to an increase in protein binding. This observation appears to be in agreement with IFNα2a animal studies where a decrease in dromotropic effect was observed without any changes in protein binding.

6.2 Pharmacokinetics

The pharmacokinetics of verapamil were not uniformly effected in the three studies. Oral clearance was decreased in both the RA and cytokine experiments. RA patients also demonstrated a reduction in *fu* of the drug, however this was not observed in the cytokine treated animals. Oral clearance was not altered in the transgenic experiments. Therefore, pharmacokinetic differences were observed in the more acute forms of inflammation, but not in the

more chronic transgenic model. This suggests that the acuity and severity of the inflammation can determine the effect on pharmacokinetics.

The first-pass pharmacokinetics of VER could be potentially altered by changes in hepatic blood flow, intrinsic clearance and alterations in protein binding. The human RA study clearly demonstrated increased protein binding. Systemic inflammatory disease has been shown to increase levels of acute phase reacting proteins such as C-reactive protein and alpha-1-acid-glycoprotein (AAG) (Fey and Müller 1991). In addition, much of the variability in verapamil levels can be accounted for by changes in AAG (McGowan *et al.* 1983). Thus it is possible that the decrease in *fu* could be due to increased AAG-binding of VER. For a moderately to poorly extracted drug, an increase in protein binding could easily decrease first-pass clearance since under these conditions protein binding could become a clearance-limiting factor. VER enantiomers behave as a highly and a moderately extracted drug which has been shown to be species-dependent. In general terms, increased protein binding would be expected to have a greater effect on the moderately extracted enantiomer. The highly extracted enantiomer would be expected to be more sensitive to changes in hepatic blood flow, however bioavailability could be altered. It is also possible that in the presence of inflammation the highly extracted enantiomer no longer behaves as a highly extracted drug. In a study on propranolol Walker *et al.* (1986) demonstrated a decrease in extraction ratio from 0.93 to 0.67 in arthritic rats. Thus a decrease in intrinsic clearance due to inflammation could decrease the extraction efficiency making the first-pass metabolism more sensitive to the effects of protein binding

for both enantiomers. It has also been suggested for drugs with a high extraction ratio and extensive protein binding such as VER and propranolol that dissociation of the drug from protein may not be rapid enough to replace extracted drug within the sinusoid of the liver. Thus the fraction available within the liver may be lower than indicated by the *fu* in the hepatic portal vein (Jansen 1981). Therefore, it is possible that the observed changes in VER pharmacokinetics are primarily due to changes in protein binding for the human RA patients. However, a similar change in oral clearance was observed for the IFNα2a-treated rats in the absence of any change in protein binding. This suggests that changes in intrinsic clearance (Cli') or hepatic blood flow may also be involved. In the event of decreased Cli', hepatic extraction would decrease resulting in an increase in oral bioavailability. Hence, drug AUC's would rise with an apparent decrease in CL/F. It is clear from in vitro studies that pro-inflammatory cytokines are capable of altering Cli' through a reduction in cytochrome P450 (CYP450) isozyme activities (Cawthorne *et al.* 1976; Descotes 1985; Moreno *et al.* 1987; Peterson & Renton 1986). These cytokines have also been shown to induce the formation of nitric oxide, which has been shown to inhibit CYP450 isozymes (Khatsenko *et al.* 1993). In these studies the human RA and the IFNα2a-treated animals demonstrated an increase in serum nitrite a stable breakdown product of NO which correlated with a rise in VER AUC. In the transgenic study no significant rise in serum nitrite or change in pharmacokinetics was observed. Thus it appears that a rise in serum nitrite was necessary for a change in VER disposition suggesting that reactive nitrogen species are involved.

Finally, inflammation could also change hepatic blood flow (Q). The influence of reduced Q is expected to be minimal on low extraction and maximal on high extraction drugs (Pang and Rowland 1977). Hepatic and splanchnic blood flow have been shown to be regulated in disease states by many mediators including prostanoids, nitric oxide (NO) and endothelins (Robotham *et al.* 1997, Myers *et al.* 1996). NO is a potent vasodilator that could decrease resistance in the vasculature leading to increased blood flow depending on cardiac output (Robotham *et al.* 1997). The effect of vasodilatation within the hepatic and splanchnic circulatory system is variable. As has been suggested for propranolol, another highly extracted drug, both decreased and increased Q may slow down clearance of the drug (Byren *et al.* 1984, Feely *et al.* 1983). A reduced Q may limit the drug supply to the site of metabolism and an increased Q may saturate first pass metabolism reduce the extent of metabolism and increasing bioavailability. In the propranolol study by Walker *et al.* 1986, hepatic blood flow was measured using ^{57}Co-labeled microspheres. No changes in hepatic blood flow were determined. Furthermore, given the extent of the increase in VER AUC due to inflammation, blood flow changes would have to be significant. It is not know if inflammation significantly alters Q, but this is theoretically possible.

6.3 Implications

These data may suggest that any condition which involves the release of pro-inflammatory cytokines or other mediators of inflammatory diseases could alter the normal pharmacokinetic-pharmacodynamic relationship of drugs. For example, it has been demonstrated clinically that patients on non-steroidal anti-

inflammatory drugs do not respond normally to the antihypertensive effects of beta-blockers and diuretics. This has traditionally been assumed to be a drug interaction. However, it is possible the underlying inflammation could also be responsible for this lack of efficacy. Down-regulation of α and β adrenergic receptors by pro-inflammatory cytokines and inflammatory conditions has also been observed in asthma and in congestive heart failure (Meurs *et al.* 1987, Bavendiek *et al.* 1996). This work suggests that both the pharmacokinetics and pharmacodynamics of drugs may be highly regulated by the immune system. Clinically, this suggests that therapeutic failures could occur using standard drug doses in patients with pro-inflammatory conditions. Disease markers such as serum nitrite or individual cytokines could prove useful in determining patients at risk for these alterations.

These findings also have implications for the basic scientist. For example, non-sterile surgery has been shown to increase NO production through bacterial exposure and an increase in endotoxin. Isolated organ preparations such as working heart or liver could be confounded by the presence of endotoxin and the production of NO within the system. While efforts are made to minimize the possible effects of endotoxin in isolated working hearts through the use of endotoxin free bovine serum albumin or the addition of polymixin B or D-actinomycin, it is not currently the practice to do this in isolated perfused liver preparations. It is possible the presence of inflammatory mediators such as endotoxin could confound the results from these experiments. Further work in this area should explore the precise mechanisms of pharmacokinetic and

pharmacodynamic changes in order to predict and possibly design better drug therapy.

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REFERENCES

Bavendiek U, Brixius K, Frank K, Reuter H, Pietsch M, Gross A, Muller-Ehmsen J, Edmann E, Schwinger R. Altered inotropism in the failing human myocardium. *Basic Research Cardiol* 1996; **91**: (SUPPL 2) 9 - 16.

Byrne A, McNeil J, Harrison P, Louis W, Tonkin A, McLean AJ. Stable oral availability of sustained release propranolol when co-administered with hydralazine or food: evidence implicating substrate delivery rate as a determinant of presystemic drug interactions. *Br J Clin Pharmacol* 1984; **17**: 45S - 50S.

Cawthorne M, Palmer E, Green J. Adjuvant induced arthritis and drug-metabolizing enzymes. *Biochem Pharmacol* 1976; **25**: 2683 - 2688.

Descotes J. Immunomodulating agents and hepatic drug metabolizing enzymes. *Drug Metab Rev* 1985; **16**: 175 - 185.

Feely J, Nadeau J, Wood A. Effects of feeding on the systemic clearance of indocyanine green and propranolol blood concentrations and plasma binding. *Br J Clin Pharmacol* 1983; **15**: 383 - 385.

Fey GF, Fuller GM. Regulation of acute phase gene expression by inflammatory mediators. *Mol Biol Med* 1987; **4**: 323 - 338.

Gross G. Inflammatory Mediators and the Stunned Myocardium Chapter 8 in Immunopharmacology of the Heart. Academic Press Ltd. 1993.

Hu H, Chiamvimonvat N, Yamagishi T, Marban E. Direct inhibition of expressed cardiac L-type Ca^{2+} channels by S-nitrosothiol nitric oxide donors. *Circulation Research* 1997; **81**:742-52.

Jansen J. Influence of plasma protein binding on hepatic clearance assessed from a "tube" model and a "well-stirred" model. *J Pharmacokinet Biopharm* 1981; **9**: 15 - 26.

Khatsenko O, Gross S, Rifkind A,Vane J. Nitric oxide is a mediator of the decrease in CYP450-dependent metabolism caused by immunostimulants. *Proc Nat Acad Sci USA* 1993; 90: 11147 – 11151.

McGowan F, Reiter M, Pritchett E, Shand D. Verapamil plasma binding: Relationship to α1-acid glycoprotein and drug efficacy. *Clin Pharmacol Ther* 1993; **33**: 485 - 490.

Meurs H, Kauffman HF, Koeter GH, Timmermans A, de Vries K. Regulation of the beta-receptor-adenylate cyclase system in lymphocytes of allergic patients with asthma: possible role for protein kinase C in allergen-induced nonspecific refractoriness of adenylate cyclase. *J Allergy Clin Immunol* 1987; **80**: 326 - 39.

Moreno JJ, Escofet A, Castell M, Castellote C, Queralt J. Hepatic cytochrome P-450 activities and serum biochemical changes in adjuvant arthritis. *Med Sci Res* 1987;**15**: 1469 - 1470.

Myers S, Turnage R, Hernandez R, Castenada A, Valentine R. Autoregulation of renal and splanchnic blood flow following infra-renal aortic clamping is mediated by nitric oxide and vasodilator prostanoids. *J Cardiovascular Surgery* 1996; **37**: 97 – 103.

Pang KS, Rowland M. Hepatic clearance of drugs. I. Theoretical considerations of a well stirred model and a parallel tube model. Influence of hepatic blood flow, plasma and blood cell binding, and the hepatocellular enzymatic activity on hepatic drug clearance. *J Pharmacokin Biopharm* 1977; **5**: 625 - 653.

Peterson TC, Renton KW. Kupfer cell factor mediated depression of hepatic parenchymal cell cytochrome P-450. *Biochem Pharmacol* 1986; **35**: 1491 - 1497.

Quignard JF, Frapier JM, Harricane M, Albat B, Nargeot J, Richard S. Voltage-gated calcium channel currents in human coronary myocytes. Regulation by cyclic GMP and nitric oxide. *J Clin Invest* 1997; **99**: 185 - 93.

Robotham J, Clemens M, Geiger K. Regulation of hepatic blood flow during resuscitation from hemorrhagic shock. Role of NO and endothelins. *Am J Physiology* 1997; **272**: H2736 - H2745.

Taguchi K, Ueda M, Kubo T. Effects of cAMP and cGMP on L-type calcium channel currents in rat mesenteric artery cells. *Japanese J Pharmacol* 1997; **74**: 179 - 86.

Tewari K, Simard JM. Sodium nitroprusside and cGMP decrease Ca2+ channel availability in basilar artery smooth muscle cells. *Pflugers Archiv Eur J Physiology* 1997; **433**: 304 - 311.

Walker K, Barber H, Hawksworth G. Mechanism responsible for altered propranolol disposition in adjuvant-induced arthritis in the rat. *Drug Metab Dispos* 1986; **14**: 482- 486.

Wilkinson G. Clearance Approaches in Pharmacology. *Pharmacol Rev* 1987; **39**: 1 - 47.