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Effects of Inflammation and the Severity of Disease on the Action and Disposition of Drugs

By

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Dedications

To my lovely mother who was my first teacher, and my dear father, Reza, who always encouraged me.

To my dear Alireza, for being my other half and his continuous support.

To my lovely daughter Farnia, who gave me a real sense of life.

To Dr Jamali, for his support and providing scientific inspiration.

Abstract

Inflammation is involved in the pathogenesis of some cardiovascular diseases and contributes to the observed increased overall mortality rate. It also influences the action and disposition of drugs including some of the cardiovascular agents that are commonly used by patients with inflammatory conditions. This may result in reduced response to pharmacotherapy.

Previous studies have demonstrated that inflammatory conditions such as rheumatoid arthritis, old age and obesity result in altered response to verapamil as measured by the PR prolongation despite increased plasma drug concentration. Interestingly, the action and disposition of verapamil are restored in patients, whose rheumatoid arthritis is in remission, pointing to the possibility that the degree of disease severity may have a role in the above observation.

Both the reduced clearance, hence, increased plasma drug concentration and diminished pharmacological response caused by inflammatory conditions have been attributed to down-regulations of target proteins, i.e., drug metabolizing enzymes and receptors, respectively.

As our first objective, we investigated the effect of disease severity on the pharmacokinetic and pharmacodynamics of verapamil, a well-studied representative of the calcium channel blockers. As a model of inflammation, we chose Crohn’s disease. We observed that increased disease severity decreased response to the drug while elevating its plasma concentration.

As our second objective, we studied the action and disposition of nebivolol in rat adjuvant arthritis. Nebivolol is a third generation β-blocker and is thought to be exclusively metabolized in the liver. It is administered as a racemate of equal ratios of the D- (S,R,R,R) and the L- (R,S,S,S) isomers. It has some unique mechanisms of action including a high selectivity for β1 and β3-adrenoceptors. Nebivolol has the ability to release nitric oxide from the cardiovascular endothelium and possesses antioxidant properties which can increase the level of NO by reducing its oxidative inactivation. These benefits suggest nebivolol as the drug of choice in patients with cardiovascular complications when inflammatory conditions exist.

As our third objective, we investigated the pharmacokinetics of nebivolol with the aim of finding whether its intestinal metabolism contributes to its first-pass metabolism in the rat. We also investigated the stereospecific pharmacokinetics of nebivolol to determine factors which cause stereoselectivity in its disposition. Our results revealed that the pharmacological response to nebivolol remained unchanged in the presence of inflammation and the gut rather than liver is responsible for drug's first pass metabolism. The drug's pharmacokinetic profile is stereoselective due to its stereoselective binding to plasma proteins. This study revealed that neither the pharmacokinetics nor pharmacodynamics of nebivolol are influenced by inflammation, making it unique among the thus far investigated β-blockers such as propranolol and sotalol.

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List of Abbreviations

AA	adjuvant arthritis
ACE	angiotensin converting enzyme
AI	arthritis index
AR	adrenergic receptor
ARB	angiotensin II receptor blocker
ATR	angiotensin converting enzyme receptor
AUC	area under the curve
AUEC	area under the effect curve
BCRP	breast cancer resistance protein
BSEP	bile salt export pump
CCBs	calcium channel blockers
CD	Crohn's disease
Cmax	maximum drug concentration
CRP	C-reactive protein
CYP 450	cytochrome p 450
ECG	electrocardiogram
FAD	flavin adenine dinucleotide
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte macrophage colony stimulating factor
HBI	Harvey Bradshaw Index

HF	heart failure
HPLC	high performance liquid chromatography
IBD	inflammatory bowel disease
IFN	interferon
IL	interleukin
MDR	multidrug resistance transporter
MMP	matrix metalloproteinase
NADPH	nicotinamid adenine dinucleotide phosphate
NE	norepinephrine
NO	nitric oxide
OAT	organic anion transporter
OATP	organic anion transporter protein
OCT	organic cation transporter
OCTN	organic cation/carnitine transporter
P-gp	P-glycoprotein
PKA	protein kinase A
PKC	protein kinase C
Pre-AA	pre-adjuvant arthritis
RA	rheumatoid arthritis
RAS	renin angiotensin system
ROS	reactive oxygen species
SA node	sinoatrial node
SD	standard deviation

SEM	standard error of the mean
Th	helper-inducer T lymphocyte
TNF	tumor necrosis factor

Chapter 1 Introduction

1.1. Inflammation

Inflammation is a complex process which is initiated by various factors, including bacterial infection, chemical injury and environmental pollution, all of which can result in cell injury or death (1). It is usually a localized protective reaction of cells/ tissues of the body to bacteria, allergens or chemical irritation. Dilation of blood vessels due to inflammation leads to increased blood supply and appearance of the symptoms of inflammation, including pain, heat, redness, swelling and loss of function (2-4). Increased intracellular space can result in the release of inflammatory mediators and the movement of leukocytes, protein and fluids into the inflamed regions (2); apoptosis is then initiated, and finally, cell replacement and re-vascularization occur (5). A controlled inflammatory response is thought to be beneficial, although it can become detrimental (6).

1.1.1 Mechanisms of inflammatory response

The immune system provides defense in two ways: innate (nonspecific) and adaptive (specific) immunity. Innate immunity commonly refers to the first line of defense against pathogens and broadly distributed varieties of myeloid and lymphoid cells such as monocytes, tissue macrophages and neutrophils, which can exert a rapid effective function through a limited repertoire of germline-encoded

receptors (4,7). In addition the innate immune response activates and amplifies acquired immunity. This effect is mediated by IL-12, which results in activation of T cells and differentiates naive T cells into the Th1 phenotype (8).

Adaptive immunity, on the other hand, can be differentiated from innate immunity based on four main features including: antigenic specificity, variety of challenges, immunologic memory and recognition of self from non-self. The adaptive immune system is characterized by two types of lymphocytes, T and B cells, and by antigen presenting cells. Naive T and B cells undergo a process of cell division and need to mature before exerting their effector function (8).

In the present of foreign antigens, the adaptive immune response is induced primarily in CD4+and CD8+ T cells. The activation of CD4+ T cells causes further cytokine production and amplifies the innate and adaptive immune systems. Depending on the cytokines profile that CD4+ cells are exposed to, Th1 or Th2 cells are differentiated (8,9). The primary cytokine produced by Th1 cells is IFN-γ, which amplifies the pro-inflammatory response by activating macrophages and stimulating the cytolytic functions of CD8+ T cells.

Macrophages and dendritic cells produce IL-12, which promotes the Th1 response. Some parasites, intracellular bacteria and bacterial products stimulate IL-12 production. IL-12 results in the activation of the transcription factor STAT4 (signal transducers and activators of transcription) in activated T cells and promotes Th1 differentiation (10). T-bet, another transcription factor that is important in Th1 differentiation is induced by IFN-γ, thereby providing an

amplification mechanism for Th1 development (8,11). Th2 cells secrete anti-inflammatory mediators including IL-4, IL-5, IL-10 and IL-13, which aid in the formation and response of B cells (12-15). IL-4, IL-10 and IL-13 cause the production of IgG1 and IgE antibodies and also result in macrophage suppression. In addition, IL-5 causes activation of eosinophil (8). The balance between Th1 and Th2 is very important. Th1 and Th2 cytokines may suppress each other which results in Th1 or Th2 cell dominant inflammatory disorders. In the presence of some disease such as rheumatoid arthritis and asthma an imbalance Th1 and Th2 immune cells has been observed (9,14,16).

1.1.2 Types of inflammatory conditions

Inflammatory responses are divided into two types, acute and chronic, each of which may be either detrimental or beneficial (6).

Acute inflammation has a rapid onset and a short duration. It is characterized by the leakage of plasma proteins and fluids and the migration of leukocytes, mostly neutrophils, into the injured area. The acute response is thought to be a defense mechanism which results in the elimination of inflammatory factors. This can be considered beneficial.

Chronic inflammation, on the other hand, is a more prolonged condition due to the persistent presence of lymphocytes and macrophages, tissue necrosis and fibrosis in the tissue. Persistent chronic inflammation is associated with an increased risk of developing degenerative diseases, including rheumatoid

arthritis, diabetes, inflammatory bowel disease, Alzheimer's disease, atherosclerosis, multiple sclerosis , acquired immunodeficiency disorder , heart disease, asthma, cancer, congestive heart failure , infections (bacteria, fungi, parasites) and gout (1,17).

Inflammatory conditions are modulated by cytokines, which are secreted from lymphocytes, macrophages, glial and endothelial cells (18). More than 100 cytokines have been discovered which have various and important roles in the immune system. Table 1-1 describes the biological activity of different cytokines (18-21).

Under normal conditions, the concentration of cytokines is very low; however, pathophysiological modification can result in an elevated concentration of cytokines. This increased concentration is associated with various inflammatory conditions, as listed in Table 1-2 (16).

1.1.2.1 Inflammatory bowel disease

Idiopathic inflammatory bowel disease includes Crohn's disease (CD) and ulcerative colitis. Both conditions are thought to be the result of an inappropriate inflammatory response to intestinal microbes in capable hosts (22-25).

The incidence of inflammatory bowel disease is higher in more developed countries, including those in Scandinavia, northern Europe, and North America. The rate of disease is lower in Asia, Africa, and South America (26).

Inflammatory bowel disease affects approximately 1.4 million Americans, with a peak onset in people aged 15- 30 (27).

CD may affect any part of the gastrointestinal tract, especially the ileum and colon. Ulcerative colitis may affect part of the colon or the entire colon in a connected pattern. Inflammation is often transmural in CD, and is limited to mucosa in ulcerative colitis. Genetic factors have a role in the pathogenesis of both diseases, playing a more prominent role in CD (25). As both of these diseases may be observed within the same family, the involvement of some common genes in both diseases is suggested (22,23). Cigarette smoking increases the risk of CD and elevates its severity, whereas non-smokers are at an elevated risk for ulcerative colitis in compare to smokers (22,23,25). The differentiation between ulcerative colitis and CD is not easy.

Table 1-1. Selected important cytokines, their source and clinical implication. Adapted from references [18-21].

Cytokine	Source	Biological function and clinical role
Interleukins (IL)		
IL-1 (IL 1α &1β)	Macrophages	Activates T-cells and macrophages Implicated in pathogenesis of RA, septic shock and atherosclerosis
IL-1ra	Macrophages	IL-1 antagonist
IL-2	Th1	Activates natural killer cell, macrophages, lymphocytes use in the treatment of metastatic renal carcinoma and melanoma
IL-4	Th2, NKC, mast cells	Stimulates IgE production Activates lymphocytes, monocytes have role in allergy
IL-6	Macrophages	Activates lymphocytes and B cells involved in acute phase protein production involved in the myeloma and mesangial pathogenesis
IL-10	Th2	Inhibits cell mediated inflammatory responses
IL-12	Dendritic B cells and macrophages	Stimulates production on IFNγ Stimulates Th1 response
Tumor Necrosis Factors (TNF)		
TNF-α	B cells, Th1, NKC, mast cells	Antibodies against TNF-α used in the treatment of autoimmune disease

Interferons (IFN)		
IFN-α & IFN-β	Cells infected with viruses	Promotes resistance to viral infections Used for treatment of Hepatitis B and C infections
IFN-γ	Th1, NKC	Main macrophage activator Inhibitor of Th2 response
Colony Stimulating Factors (CSF)		
GM-CSF	B cells, endothelial cells, macrophages, fibroblast, NKC, T cells	Promotes growth of granulocytes and macrophage precursors Used in the treatment of neutropenia Stimulates cell production after bone marrow transplants
G-CSF	Monocytes, granulocytes, fibroblasts, some tumor cells	Promotes growth of granulocytes
Chemokines		
MCP-1	Macrophages, endothelial cells	Promotes monocytes and T cells chemotaxis to site of inflammation

Table 1-2. Pro-inflammatory mediators expression change in human inflammatory conditions. Adapted from reference [16].

Inflammatory condition	Inflammatory mediator expression
Human Immunodeficiency Virus	Increased secretion of TNF-α, IL-1 and IL-6 from macrophages and monocytes by increased viral load, overexpression ofIL-10 and B-cell hyperactivitywhich increased risk of AIDS-lymphoma.
Infection	Increased secretion of myeloperoxidase and IL-6 in severe infections.
Acute myocardial infarction	Increased plasma concentration of IL-6, IL-1β, TNF-α and CRP in infarcted heart tissue.
Hypertension	Elevated concentration of IL-1ra patients with essential hypertension.
Behcet's disease	Active form of disease is associated with increased IL-6, -10, -17, -18 and IFN-γ.
Atopic diseases	Increased eosinophils and cytokines which regulate IgE in asthma, allergic rhinitis and atopic dermatitis and elevated IL-5 concentrations in asthma.
Cancer	Correlated level of IL-6 and IL-6s receptors with progression and metastasis of prostate cancer and elevated level of IL-6 in multiple myeloma.
Atherosclerosis	Increased endothelium concentrations of IL-1, P-selectin, eselectin, VCAM-1, ICAM-1 in atherosclerotic tissue, endothelial dysfunction due to altered NO bioavailability; increased CRP levels.
Congestive heart failure	Increased concentrations of TNF-α and IL-6.
Unstable angina	Imbalance between TNF-α and IL-10, increased concentrations of CRP, MC-SF and IL-6; elevated serum level of IL-6 results in higher risk of mortality.
Stroke	Reduced IL-10 concentrations results in increased severity of neurological disorders.
Elderly	Changes in T-cell chemokine expression in the elderly. Elevated concentrations of IL-1, TNF-α (associated with more risk of atherosclerosis), IL-6 (associated with more mortality),and CRP (associated with more mortality and increased incidence of diabetes mellitus).
Fever	Fever is associated with increased concentrations of IL-1α, 1β, TNF-α and IL-6.

Crohn’s disease	Increased activity of Th1 cell.
Peptic ulcer	Increased activity of IL-1, IL-8 and TNF-α
Liver disease	Individuals afflicted with non-alcoholic steatohepatitis have increased level of TNF-α, IL-18 and IL-18 binding protein which is correlated with severity of disease.
Alzheimer’s disease	Increased expression of IL-1, TNF-α, IL-1β and IL-6.
Cerebral ischemia	Increased concentration of IL-1, TNF-α, TNF-β and IL-6.
Down’s syndrome	Overexpression of IL-1.
Multiple sclerosis	Increased concentration of TNF-α in serum and cerebral spinal fluid; increased concentrations of adhesion molecules in the peripheral and central nervous system.
Obesity	Elevated plasma concentration of CRP, TNF-α and soluble receptors, IL-6.
Diabetes	Increased inflammatory mediators such as CRP levels are related to development of type 2 diabetes and insulin resistance.
Pain	Pain is associated with changes in level of many inflammatory mediators including TNF-α, IL-1 and IL-6 in the peripheral and central system.
Parasitic infections	Increased TNF-α.
Dementia	Increased IFN-α and decreased TGFβ-1 which are correlated with excessive neurocognitive dysfunction.
Depression	Increased expression of IL-1β in cerebrospinal fluid, IL-6, IFN-γ, IL-1ra, sIL-6r and TNF-α.
Obsessive compulsive disorder	Decreased plasma concentrations of IL-1β and TNF-α and increased cortisol concentrations.
Schizophrenia	Increased concentrations of IL-6, TNF-α, IL-1β polymorphism, IL-2 and IFN-γ.
Stress	Increased concentration of TNF-α, IL-1, IL-1ra, IFN-γ and decreased level of IL-4 and IL-10.
Rheumatoid arthritis	Increased concentrations of IL-1, IL-6, TNF-α, GM-CSF, and IL-8.
Thyrotoxicosis	Increased concentrations of IL-6 and IL-8.
Tuberculosis	Elevated concentration of IL-6.
Burn	Increased concentration of IL-1, IL-1β and IL-6.
Organ transplant	Increased concentrations of TNF- α in liver, IFN-γ in kidney and IL-1ra in lung of transplant patients are associated with poor prognosis.

To confirm the diagnosis and to determine location of CD, colonoscopy and a small-bowel x-ray are used. It is believed that genetics, environmental factors and immunologic abnormalities play a major role in the pathogenesis of the disease (26).

1.1.2.1.1 Crohn's disease (CD)

CD is a progressive, heterogeneous (28) chronic inflammation of the gastrointestinal tract (26). CD starts with an inflammatory condition. Failure to control the inflammation results in a penetrating or stricturing complication (28). It is a relapsing condition with frequent periods of remission. CD may involve any part of the gastrointestinal tract from the mouth to the anus, but in two-thirds of patients, the terminal ileum is mainly affected (26,28). The disease was named after a gastroenterologist, Dr Burrill Bernard Crohn, who in 1932 described inflammation of the terminal ileum in patients (29).

The clinical symptoms of CD differ based on the location of the disease; however, abdominal pain and diarrhea (in more than 70% of patients) (30), fever, weight loss, bloody stools, strictures, and fistula are common symptoms. The incidence of esophageal involvement with signs of dysphagia, odynophagia, heartburn, or chest pain is rare (0.2%) (31). The incidence of gastroduodenal involvement has been reported in 0.5-4% of patients, who show signs of nausea, vomiting, upper abdominal pain and weight loss. Extra-intestinal signs may also occur in other parts of the body including the dermatologic system (erythema

nodosum and pyoderma gangrenosum), ocular system (uveitis and episcleritis), joints (ankylosing spondylitis, sacral ileitis, and peripheral polyarthropathy), or the hepatobiliary system (primary sclerosing cholangitis) (26).

Assessing the severity of CD is not easy due to the heterogeneous nature of the disease and the possibility of involvement in different locations. To assess the severity of disease physicians typically use a combination of blood tests, clinical evaluation, endoscopic and radiographic imaging (32).

To assess the severity of CD, no single indicator is preferred; however, the Harvey-Bradshaw Index (HBI) is one measurement scale that is a simple index of CD activity. This method has been established based on five items. The HBI is a cumulative score of the domains of general well-being, abdominal pain, number of liquid or soft bowel motions and extra-intestinal manifestations (33). An HBI score of 0 to 4 indicates clinical remission, 5 to 7 mild disease, 8 to 16 moderate disease and above 16 severe disease (34).

Cytokines are important in the pathogenesis of CD, and they are key signals in the intestinal immune system for maintaining normal gut homeostasis. In addition, it has been proven that a balance between pro- and anti-inflammatory cytokines has successfully reduced the severity of disease and maintained remission (35,36). In active cases of CD, the level of pro-inflammatory cytokines including IL-1 family, TNF superfamily, IL-6 family, IL-12 family, IL-17 family and IL-21, are highly elevated , while the level of anti-inflammatory cytokines

such as IL-10 family and TGF-β (transforming growth factor β) are down-regulated in human mucosal tissues (35,36).

Autonomic regulation in CD is an important consideration. In CD, norepinephrine fibers are reduced in both inflamed and non-inflamed tissue and an elevated sympathetic nerve tone is observed. Although the concentration of circulating norepinephrine is increased two- to three-fold, the local concentration of norepinephrine is not sufficient to show anti-inflammatory activity (37). Biopsies taken from the sigmoid colonic mucosa from patients with CD or ulcerative colitis show significant changes in the levels of the α2-adrenoceptor transcript; however, the expression of the norepinephrine transporter was down regulated in CD patients. In a study involving mice with CD, the decrease of norepinephrine transporters was found to be the likely consequence of the loss of sympathetic nerve fibers in the mucosa and submucosa (38).

1.1.2.1.1.1 Effects of CD on the cardiovascular system

The association between inflammation and cardiovascular events is well established. Indeed, the degree of inflammation measured as the concentrations of pro-inflammatory mediators such as C-reactive protein (39) and interleukin-6 (40) is correlated with the risk of cardiovascular events. Although the prevalence of cardiovascular complications is much greater in patients with rheumatoid arthritis than in the general population (41), the involvement of CD in cardiovascular events is controversial. Some studies indicate that inflammatory bowel disease,

including CD, is not associated with increased cardiovascular events (42,43), while in other studies, CD appears to influence the cardiovascular system by increasing carotid intima media thickness (44), producing elevated levels of tumor necrosis factor-α, raising oxidative stress(45), increasing coagulation cascade activation (46), and significantly elevating fibrinogen (47), all of which lead to an increased risk of vasculitis (48).

1.1.2.1.1.2 Animal models of Crohn's disease

a. Murine models of Crohn's disease

Animal models of intestinal inflammation have been developed through the use of chemical induction, immune cell transfer and genetic manipulations (49). Chemical agents, including 2,4,6-trinitrobenzene sulfonic acid (50), dextran sodium sulfate (51) and oxazolone (52), can be used for the induction of colitis. These models help us to understand the pathways of inflammation and to prove the efficacy of drug therapy in a simple and inexpensive manner. In addition, these models are valuable in enhancing our understanding of specific events involved in intestinal inflammation. For example, dextran sodium sulfate -induced colitis does not represent the chronic phases of disease. Chemically induced models of colitis, lead to acute mucosal injury, which clearly differs from human inflammatory bowel disease (53). Nevertheless, these models can provide important information about the pathogenic role of specific cytokines in experimental colitis (54).

Immunological models in fact are models of selective T cells or bone marrow precursors, transferred into immunodeficient recipient mice. The CD45RB (55) and bone marrow chimera transfer models (56) are two well-known immunological models. Using these models, the role of pathogenic and regulatory T cells in controlling mucosal immunity and intestinal inflammation, and also the role of Th1 as a key factor in the pathogenesis of CD, are clarified (57). These models, however, do not represent innate factors in human CD response, because there are significant abnormalities in the immune system of recipients. Genetic models, including transgenic and knockout models, are models to study inflammatory bowel disease. Most of the models, with the exception of that for E-cadherin in transgenic mice, are gene knockouts including the IL-2 (58), T cell receptor a/b (59), IL-10 (60), and Gi2-α (61) knockout models. However, it seems that the imposed genetic mutations do not represent human inflammatory bowel disease.

b. Rat model of Crohn's disease

Lesions similar to those seen in CD can be induced through subserosal injection of peptidoglycan polysaccharide (the components of bacterial cell walls) into the rat caecum. In Lewis rats, which are a genetically susceptible species, peptidoglycan polysaccharide injection results in chronic transmural inflammation in the form of lymphoid follicles and multiple hepatic granulomas (62). This model also reveals fat wrapping, which is a unique feature of CD in humans (63). Spontaneous colitis may develop in transgenic rats with HLA B27 (64). CD may

also be induced in rats through intraluminal instillations of 2,4,6-trinitrobenzenesulfonic acid and 50% ethanol, which seems to be similar to characteristic of human CD, including its pathological characteristics and chronic inflammation (50,65).

1.1.2.1.2 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a systemic autoimmune chronic inflammatory and destructive joint disease characterized by joint swelling and joint tenderness which leads to severe disability and premature mortality (66-71).

RA affects 0.5–1% of the population in the industrialized world; it leads to significant disability and reduces the quality of life (72,73) . The incidence of the disease in women is two to three times more frequent than in men at any age, with a peak incidence between ages 40-60. RA in the early stages can affect one or a few joints, but because it is a polyarthritic condition, many joints, including all peripheral joints, may be involved; however, hands, feet and knees are at a higher risk of involvement (74,75). Another feature of RA is extra-articular involvement (74) including rheumatoid nodules, ocular inflammation, cardiovascular disease, pulmonary disease, neuropathy, alteration of thyroid hormones and splenomegaly (76).

RA is associated with high care cost and suboptimal treatment of the disease results in reduced life expectancy (77-79).

The pathogenesis of RA is not well understood; however, it is thought that RA is a disease of the immune and inflammatory systems which leads to the destruction of cartilage and bone. The cause of RA remains unknown, but many pathways are thought to be involved in the generation of the disease. Some studies show that RA could be the result of infectious agents (80-82), as RA patients demonstrate favorable response to antibiotic derivatives or drugs with antibiotic activity (83-85). However, most studies have suggested that RA is a result of the loss of immune tolerance by the B cell system toward self-antigens. Activated B cells produce antibodies against joint tissues, leading to the activation of the complement system, the recruitment of polymorphonuclear leukocytes, the release of compounds from renin-angiotensin system and other cytotoxic substances, phagocytosis and T cell activation due to antigen presentation (76,86). T lymphocytes, one of the major immune system cell types, are located in the inflamed synovium of patients with RA (87). T cells undergo polarization into either Th1 or Th2 cells (88). In RA conditions, the release of Th1, a pro-inflammatory cytokine which leads to the release IFN-γ, IL-2, TNF-α and IL-1, is predominant over the release of Th2, an anti-inflammatory cytokine which leads to the release of IL-4 and IL-5 (87).

The polarity of Th cells is crucial in determining the type of B cell activation. While Th1 cells with pro-inflammatory activities promote certain humoral responses, Th2 cells with anti-inflammatory potential promote other types of humoral responses, including immunoglobulin (Ig) E production (89). In RA in fact, there is a shift in the balance of Th1/Th2 toward the Th1 (14). Given that

RA is an autoimmune disease, there is a strong association between RA and several autoantibodies (90), including rheumatoid factor, the most important autoantibody. RA is associated with changes in bone structure. Excessive signaling of cytokines due to infiltration of B cells, T cells and macrophages result in the activation of osteoclasts (directly) and an elevated density of the receptor activator for nuclear factor-kB ligand (indirectly) in inflamed joints, which leads to the acceleration of bone degradation. On the other hand in RA, matrix metalloproteinase activation occurs, resulting in degradation of connective tissue such as collagen or other extracellular matrix (87,91).

Until the 1990s aspirin and other non-steroidal anti-inflammatory drugs were used to treat patients with rheumatoid arthritis. Then diseases modifying anti-rheumatic drugs such as methotrexate were introduced for disease reduction. Patients with the most severe disease were candidates to receive combined treatment with more than one disease modifying anti-rheumatic drugs. Now the RA treatment goal is to achieve remission with limited damage and to prevent deterioration (92).

Recently, the effectiveness and safety of biologics including anti-TNF-α (etanercept, adalimumab, infliximab), IL-1 antagonist and anakinra have been proven (91,93).

Various methods are used to assess the activity of the disease, including physical examination, acute phase reactants measurement, global assessments of

disease activity by the patient and physician, and the assessment of pain and fatigue by patients.

The American College of Rheumatology, the European League Against Rheumatism and the World Health Organization/International League Against Rheumatism have standardized these methods. These multiplex indices, including the disease activity score (which involves a 28-joint count) (94), the simplified disease activity index (SDAI), and the clinical disease activity index (CDAI) (95), are used to categories disease activity into specific degree, such as high, moderate and low (remission) disease activity . The CDAI is a more practical method of assessing RA activity in patients and can be calculated quickly by disregarding the CRP level, while the SDAI considers CRP level. A comparison of SDAI and CDAI is presented in Table 1-3 (96).

1.1.2.1.2.1 Effects of rheumatoid arthritis on the cardiovascular system

There is a growing body of evidence showing that patients suffering from RA are at a higher risk of cardiovascular events (97-99). In fact, rheumatoid arthritis patients are at a higher risk of death due to cardiovascular disease (81,100,101). This occurs as a result of reduced physical activity and conventional risk factors including hypertension, abnormal body mass index, smoking, accelerated atherosclerosis, arterial thickening and autonomic dysfunction (102-105). On the other hand independent associations, exist between cardiovascular risk and non-specific pro-inflammatory mediators like CRP, TNF-α, IFN-γ and IL-6 (81,106-108). RA also is associated with sympathetic nervous system activation, which

may result in a higher risk of arrhythmia-related sudden cardiac death (109). The autonomic nervous system is one of the neuronal pathways that have an essential role in inflammatory conditions (103).

The cholinergic pathway via the vagus nerve can control the release of cytokines, which can lead to reduced pro-inflammatory cytokine production. This has been well established as a cholinergic anti-inflammatory pathway (103-105). Autonomic impairment in RA patients may lead to arrhythmias and increases cardiovascular mortality (110). The prevalence of atherosclerosis is significantly higher in RA patients due to endothelial dysfunction. In the blood vessels of such patients endothelial dysfunction occurs as a result of poor nitric oxide (NO) signaling due to less NO availability. A lack of sufficient amounts of available NO results in improper vessel dilation and in the adhesion of platelets and leukocytes to the endothelium; therefore, it enhances the risk of cardiovascular events.

RA is associated with increased risk of congestive heart failure, mostly left ventricular dysfunction at any stage of disease (111-113). Patients with more sever inflammatory conditions who have a higher level of pro-inflammatory mediators such as CRP, IL-6 and TNF-α are at higher risk of congestive heart failure (114). In addition, in RA, some acute phase proteins, including amyloid-A and phospholipase A2, can affect high-density lipoprotein cholesterol composition and increase its risk of oxidation.

Table 1-3. Comparison of Simplified Disease Activity Index (SDAI) and Clinical Disease Activity Index (CDAI). Adapted from reference [96].

<u>**SDAI**</u>	<u>**CDAI**</u>
Tender-joint count	Tender-joint count
Tender-joint count	Tender-joint count
Swollen-joint count	Swollen-joint count
C-reactive protein	Visual assessment of patient disease activity
Visual assessment of patient disease activity	Global assessment of disease activity based on visual scale
Global assessment of disease activity based on visual scale	CDAI: The numerical sum of the above components (range: 0-76)
SDAI: The numerical sum of the above components (range: 0-86)	
Classification	**Classification**
Remission≤ 3.3	Remission≤ 2.8
Low disease activity <3.3≤20	Low disease activity <2.8≤10
Moderate disease activity <20≤40	Moderate disease activity <10≤22

Some clinical trials indicate that patients with active RA are at a higher risk of abnormal systolic blood pressure, total cholesterol, arterial stiffness, and carotid intima-media thickness with higher carotid intima plaques (98,113,115-117). RA patients have also demonstrated an increased platelet count and more platelet activation markers (118) and decreased elasticity of the aorta in both genders (119). Other studies show that RA patients experience increased activation of coagulation/ fibrinolysis cascade including increased levels of fibrinogen, von Willebrand factor, plasminogen activator inhibitor-1 , tissue plasminogen activator antigen, D-dimer, and prothrombin fragment F1 + 2 (a marker of thrombin generation), all of which are correlated with disease activity (120,121). According to a meta-analysis of 24 observational studies, the incidence of death in RA patients due to cardiovascular disease, ischemic heart disease and cerebrovascular accidents were 50%, 59% and 52% higher than that of the general population respectively (122). RA patients experience unrecognized myocardial infarction and sudden death twice as frequently as do controls (100). In addition, young seropositive patients (<55 years old) and patients with an active form of the disease are at a higher risk of cardiovascular death than clinically quiescent subjects (100,123).

1.1.2.1.2.2 Experimental arthritis

Animal models of RA are useful tools and have been used extensively to study the fundamental mechanisms of disease pathogenesis (124-126).

In the rat, in both the streptococcal cell wall arthritis model (124,127) and the collagen-induced arthritis model (124-128), innate and adaptive immune mechanisms are involved (124). Transgenic models such as K/BxN (124) and adjuvant arthritis (127) are common examples of experimental arthritis.

The selected model should be easy to perform, have the ability to reproduce data, feature a reasonable duration of test period, and have pathology similar to that of RA in humans. The most important differences between animal models and those of humans are that there is a much more rapid progress of RA in animals than in humans, and that there is a tendency to bone resorption and formation in rats in response to joint inflammation (126).

1.1.2.1.2.2.1 Adjuvant Arthritis

Adjuvant arthritis is an experimental model of polyarthritis which is similar to human disease due to its ongoing joint inflammation, synovial hyperplasia and the infiltration of inflammatory cells (129). However adjuvant arthritis features some differences from RA, including the presence of extra-articular effects and the absence of rheumatoid factor which are observed in humans (129).

The disease is induced by the tail base injection of adjuvant, which may be either Freunds complete containing desiccated mycobacterium or synthetic adjuvant N, N dioctylddecyl- N', N-bis (2-hydroxy-ethyl) propanediamine (130). Adjuvant also can be injected in one of the foot pads, which allows researchers to study the immunological reaction and the acute inflammatory reaction (126).

Symptoms of adjuvant arthritis can be observed about 14 days after adjuvant injection (127). Adjuvant arthritis rats are often relatively immobile due to the severity of paw swelling, therefore the special care are needed to ensure that animals receive enough water and food (126).

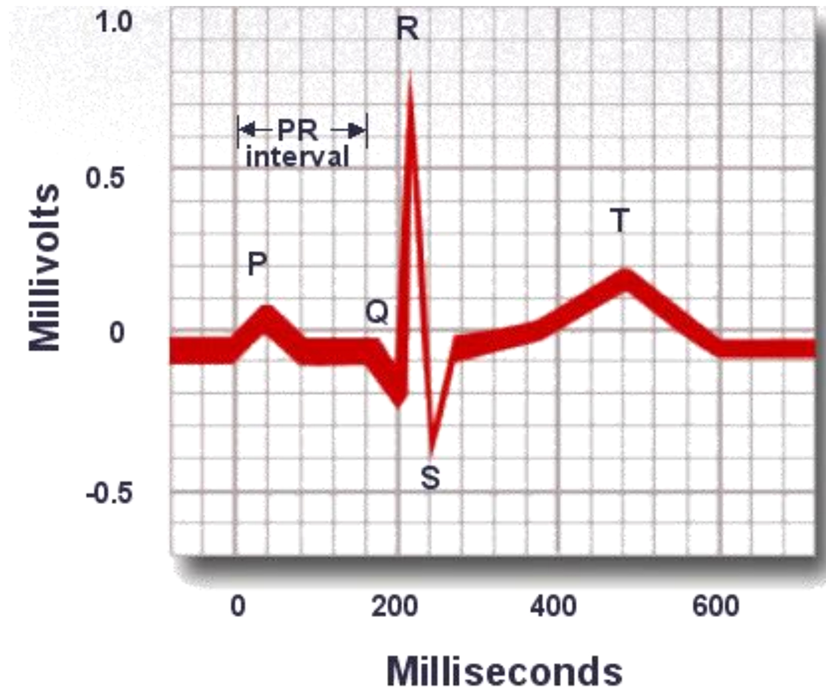
To assess disease progression, various clinical signs of disease are monitored during the experiment, including caliper measurements of ankle joint width or volume using a water displacement device to measure increased paw thickness, weight gain, erythema and formation of eye nodes in order to calculate arthritis index. Arthritis index is calculated according to the method previously described. A maximum score of 14 can be assigned to each animal as follows: hind paws are scored on a 0–4 scale, where zero is no virtual sign of arthritis, one is involvement of a single joint, two is involvement of more than one joint and/or ankle, three is involvement of several joints and ankle with moderate swelling, and four is involvement of several joints and ankle with severe swelling. Each forepaw is scored on a 0–3, where 0 is no sign of arthritis, one is involvement of single joint, two is involvement of more than one joint and/or wrist, and three is involvement of wrists and joints with moderate-to-severe swelling (131). The levels of inflammatory mediators are changed within one day post adjuvant injection. An enhancement in CRP concentration is observed after one day, and by day 3 the nitrite level is increased, while the concentration of circulating TNF-α is elevated on day 6 (127,132,133).

Pre-adjuvant arthritis (Pre-AA) is another model of animal arthritis used to study rates. Arthritis index causes excessive pain and stress because animals

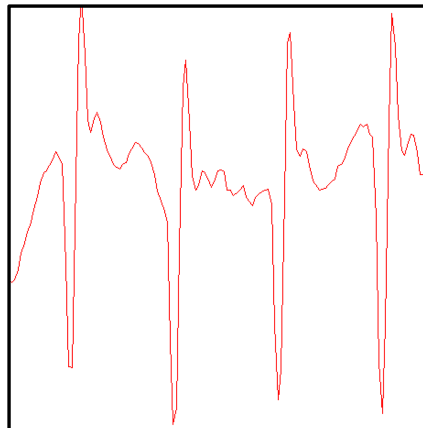
suffer from acute periarticular inflammation. Pre-AA, on the other hand, is a desirable feature of the animal model which is associated with elevated pro-inflammatory mediators and allows studies under systemic inflammatory conditions in the absence of pain and stress while still establishing an alteration in the cardiovascular system (127). Pre-AA has all three features of rheumatoid arthritis and adjuvant arthritis. First, pharmacokinetic changes in Pre-AA are the same as in adjuvant arthritis and RA; they include: (a) down-regulation of hepatic cytochrome P450: 1A and 3A and (b) reduced the levels of verapamil, an L-type calcium channel blocker, the free fraction of which results in a significantly higher verapamil blood concentration (127). Second, it is related to a diminished response to drugs. For example, both verapamil and propranolol have demonstrated significantly less ability to prolong PR intervals in pre-AA rats. Third, similar to RA and adjuvant arthritis, Pre-AA is associated with Th1 immune system response (134). The PR interval is determined by the conduction time from the sinus node to the ventricles (Figure1-1). Prolongation of the electrocardiogram PR interval is as a result of a first degree atrioventricular block, which may result from a conduction delay in the atrium, atrioventricular node, and/or His-Purkinje system (135,136).

Figure 1- 1. Electrocardiographic representation of the PR interval in human (a) (Available at: http://www.ambulancetechnicianstudy.co.uk/rules.html#.UzD0C85OVok) and rat (b).

(a) Human



(b) Rat



1.2 Effects of inflammation on pharmacokinetic indices of drugs

Inflammation changes the pharmacokinetic indices of drugs including absorption, distribution, metabolism and elimination.

1.2.1 Absorption

The oral dose is most preferred for drug administration. Convenience for patients and lower costs of therapy are the most important reasons for the superiority of oral dosing. The rate and the extent of absorption can be affected by different variables, including the physiology of the gastrointestinal tract and the physicochemical and formulation properties of drugs. This phenomenon leads to an unpredictable blood-concentration profile. Disintegration, solubility, dissolution and drug stability in the gastric and stomach fluid affect drug absorption. In addition, factors including gastrointestinal pH, gastrointestinal transit rate, permeability and transporter function affect drug absorption (137,138). In order to reach systemic circulation, drug molecules must pass through the intestinal epithelial membranes, mostly through passive or active methods (139). Active transport system, a highly specific means of movement, does require energy and moves drugs against a concentration gradient. This form of transportation is saturable and tends to have highly variable plasma concentration.

Inflammation is known to change the rate and/or the extent of drug absorption. Intestinal inflammation observed in Crohn's disease, which results in elevated IL-12 concentration and enhances the presence of mast cells in the intestine, affects the permeability of macromolecules and reducing gastrointestinal -transit time, may decrease drug absorption (140,141). Inflammatory mediators also can have an effect on the expression of intestinal drug transporters that can alter drug absorption (127).

1.2.2 Distribution and protein binding

When a drug gets access to the general circulation, it is distributed into the tissues. Drugs may bind to plasma and /or tissue proteins. Only the free fraction (unbound portion) of the drug can diffuse into/from peripheral tissues and has access to target proteins and drug-metabolizing enzymes. Then the free fraction of the drug equilibrates between the central and peripheral compartments (139). Many factors, including the physicochemical properties of the drug, the affinity of the drug for binding to plasma, tissue proteins and blood cells, affect drug distribution. α-1 acid glycoprotein, albumin, immunoglobulins and lipoproteins are the main proteins which drugs bind to.

Weakly acidic drugs bind mainly to albumin, while cationic basic drugs preferentially bind to α-1 acid glycoprotein, which has been more frequently subjected to saturable binding (139). Some diseases and conditions, including inflammatory conditions and hepatic impairment, affect protein binding.

The highly protein-bound drugs are more subject to pharmacokinetic changes during inflammatory conditions (16). Inflammation is known to alter the level of plasma proteins. Increased level of α-1 acid glycoprotein in conditions such as rheumatoid arthritis, surgery, pain, infection, myocardial infarction, renal and hepatic disease results in a reduced free fraction of the basic drug. This phenomenon leads to lower drug distribution and elimination (142). Verapamil, propranolol and oxpranolol are examples of drugs affected by inflammatory conditions in this way (127,143,144). On the other hand, the decreased level of albumin in the same situation results in increased levels of free fractions within acidic drugs. The free fractions of some drugs such as hydroxychloroquine can be taken up by blood cells, which results in higher drug concentrations in blood cells than in plasma (145).

Drug metabolism and elimination may also be affected by protein binding. A highly protein-bound drug is prevented from entering the hepatocyte, the place where metabolism happens, and glomeruli where the excretion of drug occurs (139). However, some highly protein-bound drugs such as propranolol escape from restricted elimination because the drug equilibrates off fast from the protein and can be metabolized (139).

1.2.3 Metabolism

Drug metabolism is the process which changes drugs into more polar metabolites with higher water solubility, which allows them to get eliminated through either urine or bile.

The liver is the most important organ for drug metabolism. Drug metabolism is a two-phase process. Phase I refers to a structural alteration of a drug molecule, while in phase II (conjugation) a water-soluble group is attached to the drug to make it ready to be eliminated. Several drug-metabolizing enzymes are present in the hepatocyte and are involved in drug metabolism. Phase I metabolism is catalyzed mainly by members of the P450 enzymes superfamily, which includes 57 members in the human genome. 70-80% of phase I metabolism, are mediated by fifteen P450 enzymes belonging to the CYP1, 2 and 3 gene families (Table 1-4) (146-150). Metabolites formed in phase I are either sufficiently water soluble that they can be eliminated directly or may be considered as substrates for phase II enzymes. Phase II metabolism involves the conjugation of a hydrophilic group to a drug molecule in the presence of transferase enzymes which catalyze the transfer of a hydrophilic group to the drug recipient. Sometimes the product of phase I metabolism is a toxic compound; in such cases phase II enzymes detoxify reactive molecules (151). In addition to the CYP 450 superfamily, flavin monooxygenase (152), UDP- glucuronosyltransferase (153), sulfotransferase (154), and glutathione S-transferase (155) families have important roles in drug metabolism.

The liver has the highest capacity for drug metabolism in both phase I and phase II; however, other organs, such as the gastrointestinal tract, brain, kidney, placenta, lung and white blood cells, also have a role in drug metabolism (156). The small intestine on the other hand plays an important role in first-pass metabolism of some drugs (157).

Table 1-4. Some important drug-metabolizing cytochrome P450s and their responsible receptors in human. Adapted from reference [146-150].

Human CYP450 family	Member	Responsible receptor
CYP1	CYP1A2	AhR
CYP2	CYP2A6	CAR
	CYP2C9	CAR, PXR
	CYP2C19	PXR, CAR
	CYP2D6	
CYP3	CYP3A4	PXR, CAR
	CYP3A5	PXR, CAR

AhR: aryl hydrocarbon receptor

CAR: constitutive androstane receptor

PXR: pregnane X receptor

Metabolizing enzymes can be affected by many conditions. For example, genetic polymorphism, drugs, burns, and the presence of some diseases such as those of cancer and auto-immune disease, the expression, stability and activity of metabolizing enzymes can be changed (156,167). Various studies have shown that in rheumatic diseases and in rats with adjuvant arthritis, the gene expressions of some metabolizing enzymes have been altered (168,169). The activities of hepatic CYP3A were significantly decreased in adjuvant arthritis rats (170), which is comparable with the observed results in RA (171).

Inflammatory conditions are associated with an increased level of several cytokines, including IL-1, IL-6, IFN-γ and TNF-α. High concentration of cytokines may down-regulate hepatic metabolizing enzymes, especially cytochrome P450s, at both transcriptional and translational levels (172). In addition, it is thought that regulation of CYP enzymes could play a role in the inflammatory response (173). CYP enzymes produce reactive oxygen species (174), and the overexpression of CYPs can cause endoplasmic reticulum stress (175). Therefore, in inflammatory conditions, CYP enzymes could be down-regulated to avoid this stress. However, the up-regulation of CYP2A6 following some disease such as renal failure and hepatitis B and C andCYP4F16 following lipopolysaccharide administration are some exceptions (176).

Drugs with high hepatic extraction ratio are at the highest risk of pharmacokinetic changes during inflammatory conditions, especially following

oral ingestion, due to the role of the hepatic first pass effect; however, this condition can also affect low-hepatic extraction ratio drugs. In fact, an inflammatory condition converts high extraction ratio drugs into intermediate or even low extraction ratio drugs due either to a decrease in intrinsic clearance or a decrease in the drug-free fraction. Decreased drug clearance is not limited to the oral route and can be observed following application of drugs via other routes such an intravenous. Under inflammatory conditions, drugs with a narrow therapeutic window or highly metabolized drugs may cause toxicity as a result of decreased drug clearance (167,173,176-178).

1.2.4 Effect of inflammation on drug transporters

Passage of drugs through lipid bilayers is often necessary for their access to target proteins, and for getting eliminated from the body, so membrane flux is an important modifier for pharmacokinetic indices including absorption, distribution, metabolism, and excretion. This movement sometimes is a simple diffusion (passive) which allows drugs to enter and exit from cells, in such cases, physicochemical properties such as size, charge, and hydrophilicity play a key role in the process by which drugs cross membranes.

Transporters are membrane proteins; they facilitate the translocation of drugs by using either active and/or passive mechanisms into and out of cells.

In active transport, drug molecules can pass across biological membranes against a concentration gradient by consuming energy (179).

In humans, hepatic transport systems can play an important role in drug metabolism and disposition (180). There are three types of hepatic elimination: basolateral uptake of drugs from the blood side into the hepatocytes; basolateral efflux, in which the drug goes from hepatocytes into the blood; and biliary excretion (181). This hepatic uptake process is controlled by both passive and active transporters. Solute carrier transporters, such as organic anion transporting polypeptide 1B1/1B3, organic anion transporter 2, and Na+-taurocholate co-transporting polypeptide, are involved in active transport while the diffusion process is mediated by organic cation transporter 1. The drugs and/or their metabolites may finally be excreted into the bile by ATP-binding cassette transporters including P-glycoprotein, multidrug resistance 1, multidrug resistance–associated protein 2, breast cancer resistance protein and bile salt export pump. In addition, multidrug resistance associated protein 3 and 4 transporters, located at the basolateral membrane, are involved in active sinusoidal efflux of some drugs (182).

In humans, intestinal transport systems play a critical role in the bioavailability of drugs. It has been well known that intestinal barrier, metabolism, and active transport system can have a major role in bioavailability of drugs. The transcellular transport of some drugs via intestinal epithelial cells is

limited due to intestinal metabolism (mainly by CYP family) and the active efflux transporters (P-glycoprotein). It has been shown that P-glycoprotein and CYP enzymes serve as an efficient barrier to limit the absorption of some drugs, as they have similar substrate specificities and often work together (183). Many important drugs, including irinotecan, doxorubicin, vinblastine, paclitaxel, digoxin, loperamide, berberine and fexofenadine, are substrates for P-glycoprotein.

Recently it has been suggested that breast cancer resistance protein plays an important role in the intestinal absorption of a wide range of drugs including irinotecan, SN-38, methotrexate, imatinib, gefitinib, erlotinib, zidovudine, lamivudine, abacavir, rosuvastatin, pitavastatin, ciprofloxacin and nitrofurantoin (184).

In addition, basolateral efflux and apical uptake transporters have an important role in drug absorption and bioavailability. Di and tri peptides, β-lactam antibiotics and angiotensin-converting-enzyme inhibitors are substrates for peptide transporter 1 (185). The apical basal membranes, including sodium-dependent bile acid transporter and organic solute transporter α/β, have an important role in maintaining bile acids enterohepatic circulation by transportation of bile acids from the intestinal lumen to the portal vein (186). Figure 1-2 shows transporters expressed in the human liver and intestine (182).

Inflammatory conditions such as lipopolysaccharide-induced inflammation are known to reduce the mRNA levels of hepatic transporters in rats and mice. These include Ntcp; Bsep; Oatps 1, 2, and 4; Mrp2; and Mrp3 (128,187-195).

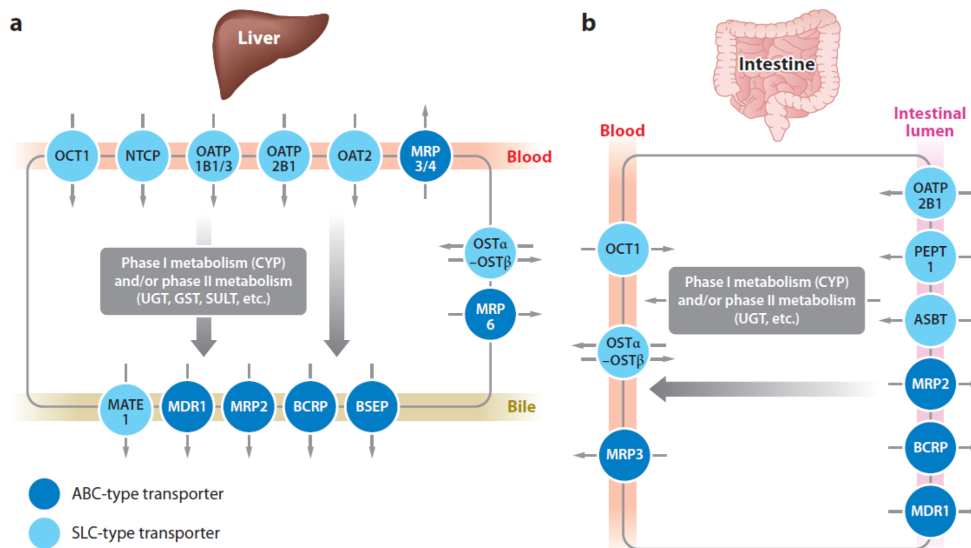
In the adjuvant arthritis rat model, the expression of the OCT1, OATP4A1 and MRP1 genes was significantly down-regulated in the liver, as was OATP2B1, MRP6 and BSEP gene expression in the kidney; however, OATP4A1 and MDR1A were found to be significantly up-regulated in the heart (196).

Cytokines are thought to have a significant role in transporter down-regulation. In the rodent model, administration of IL1β, IL6, or TNFα resulted in decreases in the hepatic mRNA levels of Ntcp, Bsep, Oatp1 and 2, Mrp 2 and 3, Mdr1a, 1b, and 2 (190).

1.2.5 Lack of response to cardiovascular drugs

There is evidence demonstrating the efficacy of some cardiovascular drugs decreases in different populations. Decreased pharmacological effects of traditional β-blockers in experimental arthritis (3,134,197), elderly and black patients (198), L-type calcium channel blockers in experimental arthritis (143,199), rheumatoid arthritis (200,201), CD (202) and pediatric obese patients (203) has been demonstrated.

Figure 1- 2. Expression of transporters in human (a) the liver (b) the intestine, adapted (with permission) from reference [182].



1.2.6 Effects of inflammation on pharmacodynamics of drugs

Inflammation down-regulates some cardiovascular receptors, such as L-type calcium channels and β-adrenoreceptors in the heart. This phenomenon results in the reduced efficacy of drugs, while plasma drug concentration is significantly increased (3,196,197,201). This effect is associated with an increased level of C-reactive protein and inflammatory cytokines. The down-regulated effect can be reversible following treatment with statins (134). However, inflammatory conditions do not reduce response to all cardiovascular drugs, as the efficacy and disposition of angiotensin II receptor type I blockers, valsartan and losartan, are not down-regulated in patients with rheumatoid arthritis (204,205). The pharmacodynamic effect of cardiovascular drugs has been shown by measuring the prolongation of the PR interval in electrocardiogram waveform.

1.2.6.1 Angiotensin II type 1 receptor antagonists (AT1-blockers)

Angiotensin II receptor blockers selectively inhibit the binding of angiotensin II (Ang II), the major end-product of the renin-angiotensin system, to the AT1 receptor (206-208). This class of antihypertensive drug can be used as an alternative, instead of ACE inhibitors. Following blocking AT1, angiotensin II receptor blockers reduce glomerular pressure through their vasodilatory effect on the efferent arterial. Angiotensin II receptor blockers do not inhibit the degradation of bradykinin therefore they do not cause dry cough. Clinical evidence suggests that in addition to controlling blood pressure, n II receptor

blockers have protective effects on the cardiovascular, cerebral, and renal systems (29,209-211). Several orally active, selective AT1 receptor antagonists are available in the market. Drugs in this family have a high affinity for AT1 receptors without affinity for AT2 receptors, and have a very high level of protein binding (212,213). Daneshtalab et al. have shown that the efficacy of AT1R antagonists does not appear to be reduced by inflammation. They evaluated the effect of rheumatoid arthritis on the pharmacokinetics and pharmacodynamics of valsartan and losartan, two AT1R antagonists. They noted that rheumatoid arthritis did not significantly alter either the pharmacokinetics or pharmacodynamic indices of valsartan. There was no significant difference between rheumatoid arthritis patients and healthy subjects in terms of valsartan pharmacokinetics and pharmacodynamics (204). They found the same results following the administration of losartan to rheumatoid arthritis patients and control subjects. Losartan, another AT1R antagonist, is a pro-drug; the pharmacologic activity of losartan depends mainly on its metabolite, EXP 3174 (214), which is formed in the liver. In this study authors found that rheumatoid arthritis did not change the pharmacokinetic parameters of losartan, but the disease significantly decreased the AUC of EXP 3174, the pharmacologically active metabolite. They noticed that the ratio of EXP 3174 AUC to losartan AUC was significantly correlated with disease severity. However a decrease in the AUC of EXP 3174, most likely due to the inhibition of the metabolizing enzymes CYP2C9 and CYP3A4, did not result in a decrease in response to the drug in

patients, probably because of the pharmacologic effect of the parent drug and/or the AT1R up-regulation in the presence of inflammatory mediators (205).

1.2.6.2 Calcium channel blockers (CCBs)

Voltage-gated calcium channels, like voltage-gated potassium and sodium channels, are members of the transmembrane ion channel proteins superfamily. More than four of its subunits play a critical role in calcium transmission. When membrane depolarization occurs, they mediate calcium influx, which is a key regulator of contraction, secretion and neurotransmission (215).

CCBs are a heterogeneous group of drugs. CCBs are classified as dihydropyridines and nondihydropyridines, or according to their duration of action, into short or long-acting CCBs (216). These agents are used for the treatment of cardiovascular diseases, including hypertension, angina, and/or supraventricular arrhythmias (217).

They block L-type calcium channel receptors and so inhibit the influx of calcium across membranes. Several in-vitro studies have shown that CCBs also suppress the activation of several immune system components, including T cells (218,219), mast cells (220) and macrophages (221,222).

1.2.6.2.1 Verapamil

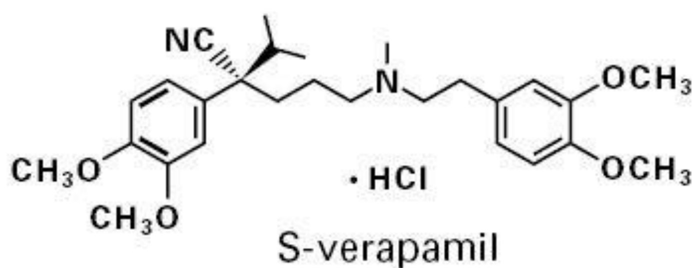
Verapamil (Figure 1-3), is an L-type calcium channel blocker, a basic drug and a racemate of two enantiomers. S-verapamil is the more pharmacologically active enantiomer which has a 2.3 fold higher volume of distribution than the R

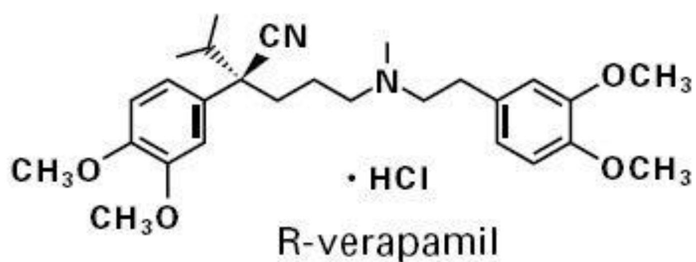
enantiomer (223). In humans, the R enantiomer has higher plasma protein binding, while the S enantiomer clears faster and causes higher plasma concentrations of R enantiomer (223). Verapamil is highly bound to the acute-phase protein α-1 acid glycoprotein and is efficiently cleared in the liver. Inflammatory conditions, which are associated with increased level of α-1 acid glycoprotein (224), result in a greater extent of binding and a decrease in verapamil free fractions (144). Rheumatoid arthritis is associated with increased plasma verapamil concentrations. This pharmacokinetic change is most likely due to increased verapamil protein binding, decreased drug clearance and/or altered hepatic blood flow. On the other hand RA subjects have shown a significant decrease in the dromotropic effect, due to receptor down-regulation caused by elevated concentrations of pro-inflammatory cytokines and/or NO (201).

On the other hand endotoxin-induced inflammation reduces the unbound fraction of verapamil, while interferon-induced inflammation does not affect protein binding while significantly reduces the dromotropic response to the drug (143).

Pre-AA, however, is associated with a significant decrease in the unbound fraction of both enantiomers of verapamil and with reduced verapamil clearance. Pre-AA is also associated with significant down-regulation of CYP3A/1A content (127). This suggests that altered protein levels may be dependent on the type of inflammation.

Figure 1- 3. Chemical structures of verapamil enantiomers (Adapted from: http://dailymed.nlm.nih.gov/dailymed/archives/fdaDrugInfo.cfm?archiveid=28057).





$C_{27}H_{38}N_2O_4 \cdot HCl$ M.W. = 491.07

1.2.6.3 β-Adrenoceptor antagonists

β-adrenoceptor (β-AR) antagonists (β-blockers) are one of the most widely used classes of drugs; they are frequently used by patients suffering from hypertension, ischemic heart disease and heart failure. They also are used in controlling anxiety, migraine and glaucoma (225).

β-blockers antagonize beta adrenergic receptor subtypes in a competitive way (competition of agonists and antagonists for the same binding sites) based on their structures. The binding is reversible therefore in the present of large amounts of the agonist; the antagonist may be displaced from the receptor (226).

β-blockers are a heterogeneous group which competitively antagonizes the action of β-AR subtypes including β1, β2 and β3. Receptor selectivity, intrinsic sympathomimetic activity, vasodilatory properties and metabolic profile differ among the β-blockers (227).

Water-soluble β-blockers such as atenolol have shorter half-lives than lipid-soluble β-blockers and are eliminated mainly via the kidney. Lipid-soluble β-blockers such as propranolol, in contrast, have longer half-lives and are mainly metabolized in the liver (228). The lipophilicity of each β-blocker is important to

determine its bioavailability and side-effects. High lipophilic β-blockers are able to penetrate the blood-brain barrier and cause central nervous system side effects including lethargy, nightmares, confusion, while water-soluble β-blockers have less ability to pass through the blood-brain barrier and cause fewer central nervous system side effects (229).

Three types of β receptors, β1, β2, β3, are distributed in different tissues (230). While β1 receptors are mainly found in the heart, β2 receptors are mainly located in vascular and bronchial smooth muscle. β3 receptors, on the other hand, can be found in both the adipocytes and the heart (231). First-generation β-blockers are nonselective; propranolol, pindolol and labetalol, for example, antagonize both β1 and β2 receptors. Second-generation β-blockers, β1 selective adrenoceptor blockers such as metoprolol and atenolol are known as cardioselective β-blockers. This group show greater affinity for β1 versus β2 receptors; however, when the dose of drug increases, the selectivity decreases. Third-generation β-blockers like carvedilol and nebivolol are nonselective; they exhibit vasodilatory activity (232-234) either via antagonism of the α-1 receptor such as labetalol and carvedilol, or via enhanced availability of NO like nebivolol (235-237).

β-blockers effectively reduce both systolic and diastolic blood pressure (226,238,239) most likely due to: a) reducing heart rate and cardiac output through preventing activation of beta-1 receptors in the heart by catecholamines (240) , b) inhibiting renin release which is mediated through the sympathetic nervous system via beta-1 receptors on the juxtaglomerular cells of the kidney (241) c) inhibiting central nervous sympathetic outflow, which reduces the release of catecholamines d) reducing venous return and plasma volume e) increasing availability of NO, which reduces peripheral vascular resistance (237).

β receptors belong to the G-protein coupled adenyl cyclase system. These receptors are activated in the present of catecholamines. The stimulation of receptors causes Gs protein to couple the activated receptor to adenyl cyclase and generates cAMP. cAMP is a second messenger which activates protein kinase A. Protein kinase A then phosphorylates the membrane calcium channel and enhances calcium entry into the cytosol. In addition, protein kinase A increases calcium release from the sarcoplasmic reticulum and exhibits a positive inotropic effect. Protein kinase A also has role in positive chronotropic and dromotropic effects (242). Based on available data, the interaction of β-blockers with target proteins appears to be stereoselective (243-252).

For the racemic β-blockers, while the S (-) enantiomer antagonizes β1-AR (243-248), the R (+) enantiomer maintains relatively strong activity in blocking β2 receptors (245).

In some disease conditions, the relative density of β1, β2 and β3 receptors are changed. In heart failure, β1 receptors are down-regulated (232,233). Tests on diabetic rats have revealed that the mRNA and protein levels of β1-ARs are decreased, while the protein levels of β3 receptors and the mRNA levels of both β2 and β3-ARs are increased (253). Human failing myocardium (254), sepsis (255) and chronic β adrenergic stimulation (256) are associated with the cross-regulation of β-ARs.

1.2.6.3.1 Structure and characteristics of β3-ARs

β3-ARs are related to the metabolic effects of sympathetic stimulation; these include lipolysis in adipocytes and insulin sensitivity (257). In 1989, the gene encoding of human β3-AR was cloned (258). Later β3-AR gene was cloned in rats, mice, monkeys, dogs, sheep, bovines, and goats (259).

Like β1- and β2-ARs, the β3-AR belongs to the family of G protein-coupled receptors, which is characterized by seven trans-membrane domains and approximately 40 to 50% homology between the β1-, β2- and β3-ARs amino acid sequences. In contrast with β1- and β2-ARs, the β3-AR is activated at higher catecholamine concentrations (260). As a result, prolonged activation of the sympathetic nervous system results in a preserved response to β3-ARs, whereas the response to the β1- and β2-ARs is diminished. β3-ARs are resistant to long-term down-regulation (261,262). The efficacy and affinity of conventional β-AR antagonists such as propranolol and nadolol for β3-ARs is low. This receptor has

inter-species variability and is blocked by nonselective β-AR antagonists such as bupranolol (263-265).

The expression of β3 receptors has been shown to be change under several conditions. Increased expression of β3-AR, mRNA and protein levels has been observed in diabetic rats while β1-AR, mRNA and protein levels are significantly down-regulated (253). During chronic heart failure, the cardiac β1-adrenergic receptors are down-regulated, leading to decreased contractility, while the overexpression of β3-ARs remains quiescent until it is stimulated in the presence of a selective agonist, which results in marked increment in left ventricular contractility. β3-ARs are insensitive to catecholamines, and endogenous catecholamines can activate them minimally. This may have an important therapeutic potential in patients with heart failure. In these patients delivery of the human β3-AR by gene therapy could provide a functionally inactive signaling protein that becomes activated in the presence of a highly selective agonist (266).

It has been suggested that activation of β3-AR inhibits L-type calcium channels in both normal and heart failure myocytes. In heart failure, inhibition of L-type calcium channel is increased in the presence of β3-AR stimulation, and this is coupled with PTX-sensitive G-protein through a NO synthase-dependent pathway (267). It has been shown that during both chronic stimulation with noradrenaline (268) and sepsis, β3-ARs are up-regulated.

1.2.6.3.2 Propranolol

Propranolol is a non-selective, first generation β-AR blocker. It has been used in the treatment of hypertension, ischemic heart disease, angina, acute myocardial infarction, ventricular and super ventricular tachyarrhythmia and heart failure as well as in the control of anxiety, migraine and glaucoma. It is a racemate of R- and S-enantiomers. The β-blocking activity of propranolol is mostly due to the S-enantiomer (248). Propranolol is highly bound to α-1 acid glycoprotein and undergoes extensive metabolism in the liver. In patients with inflammatory diseases (269-271) and in the rat adjuvant arthritis model of inflammation, following oral administration of propranolol, a significant increase in plasma concentration of propranolol enantiomers has been observed (272). However this increase was not observed following i.v. administration in adjuvant arthritis rats. This means that disposition changes are more likely due to the effects of pre-systemic rather than systemic clearance (272). An elevated concentration of propranolol enantiomers, however, fails to exhibit efficacy that is higher than or the same as that in control rats. Despite the elevated propranolol concentrations, the prolongation of PR interval is significantly reduced in adjuvant arthritis and pre-adjuvant arthritis rats, as compared with controls (197). The reduced efficacy of propranolol in inflammatory conditions is due to the down-regulation of β1-ARs (134).

1.2.6.3.3 Sotalol

Sotalol is a chiral nonselective water-soluble β-AR antagonist without intrinsic sympathomimetic or membrane-stabilizing activity. It is a unique β-blocker that prolongs cardiac action potential duration and cardiac refractoriness without altering conduction velocity or the slope of phase 0 depolarization due to its ability to block potassium channels (Class III antiarrhythmic activity). Two enantiomers of sotalol have equal Class III antiarrhythmic activity, while the R-enantiomer is responsible for its β-blocking activity. The absorption of sotalol is almost complete; therefore, its bioavailability is close to 100%. Age and food have negligible effects on its bioavailability. The Tmax of sotalol is 2 to 3 hours, and its terminal half-life varies between 7 and 15 hours. Excretion of sotalol occurs mainly through the kidneys without hepatic metabolism; therefore, sotalol is not subject to the hepatic first-pass effect (273,274).

In contrast with the cases of verapamil and propranolol, the ratio of AUC of the two enantiomers of sotalol, (R and S) is approximately 1, even in the presence of inflammation (3,223). To assess the effect of inflammation on sotalol pharmacokinetics and pharmacodynamics, two studies were undertaken by Kulmatycki et al. They investigated the effect of both acute and chronic inflammation on sotalol action and disposition. They administrated interferon α-2a to rats and induced acute inflammation. Then, 3 and 12 hours post interferon administration, racemate sotalol was administered, and the response to the drug was measured. Chronic inflammation was induced by injection of Mycobacterium butyricum in squalene into the tail base of rats. The response to the drug was

evaluated by measuring QT prolongation (potassium channel blockade) and PR prolongation (β-receptor blockade). In both the acute and chronic inflammation, response to sotalol (prolongation of QT and PR interval), was reduced following administration of the racemate and R-enantiomer. On the other hand the pharmacokinetic indices of sotalol were not affected by inflammation. They noticed that the treatment by infliximab, an anti-TNF antibody, reversed the down-regulated effect of sotalol in the inflamed rats, probably because of the reduced nitrite and TNF-α concentrations in the inflamed rats (3,275). The similar disposition of sotalol enantiomers in the healthy and inflamed rats was probably due to low sotalol plasma protein binding and complete elimination of drug through the renal route (276). Therefore two major phenomena which happen during inflammatory conditions and have a significant effect on drug disposition, the elevation of serum α1-acid glycoproteins and decreased intrinsic hepatic clearance (277,278), do not play any role in the disposition of sotalol (3).

1.2.6.3.4 Nebivolol

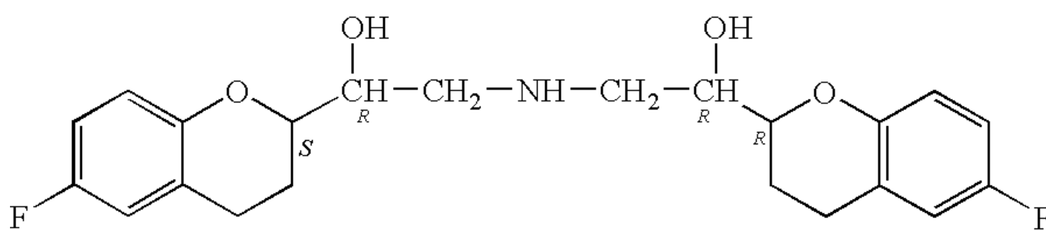
Nebivolol (Figure 1-4), is a highly lipophilic, third-generation β-blocker (279) containing 4 asymmetric carbon atoms and 10 stereoisomers (280). Clinically, nebivolol (DL-nebivolol) is administered as a racemate of equal ratios of the D-(S,R,R,R) and the L-(R,S,S,S) isomers of the drug (281-285). Nebivolol affects β-ARs and interferes with some vasoactive factors such as NO. The unique mix of β1-selectivity and NO-mediated vasodilatation offer nebivolol its typical

hemodynamic profile, which distinguishes it from other β-adrenergic antagonists (279,286).

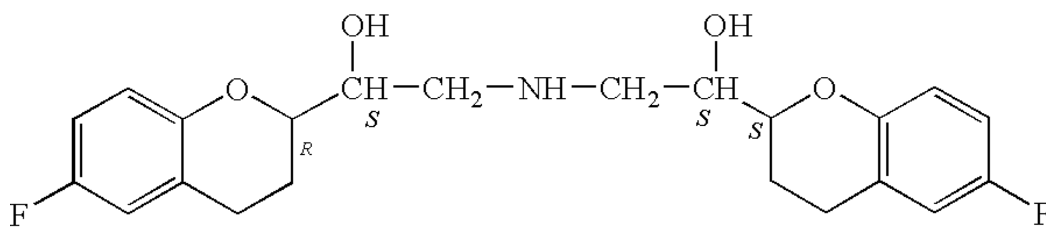
Nebivolol is a highly selective β1-adrenergic blocker. The D-isomer exerts β-blocking effect; and it seems to have vasodilatory capacities. Like all β-blockers, nebivolol blocks β2-ARs; however in therapeutic doses it is highly β1 selective, and in human myocardium, its β1-AR to β2-AR affinity is 300-fold (281), which is higher than that of bisoprolol, metoprolol, carvedilol and bucindolol (279). Its affinity for α (1,2)-ARs is very low. This high degree of selectivity causes unique and favorable effects on metabolism and its safety profile (281).

Vascular tone is regulated by cardiovascular endothelium. Cardiovascular endothelium releases different vasoactive compounds such as NO, which produces vasodilation by enhancing cyclic guanosine monophosphate, inhibits platelet aggregation and proliferates smooth-muscle cell proliferation.

Figure 1- 4. Chemical structures of nebivolol enantiomers**.** (Adapted from Compositions comprising nebivolol US 7838552 B2 (http://www.google.com.tr/patents/US20050272810))



d-nebivolol



l-nebivolol

Cardiovascular complications such as hypertension, atherosclerosis, stroke, and heart failure can destroy the protective effects of endothelium and can disrupt the functioning of NO within the vasculature. Low levels of NO s may lead to decreased arterial elasticity and enhance peripheral vascular resistance (285).

The second mechanism of nebivolol action is NO-mediated vasodilatation. The stimulation of β3-adrenergic receptors seems to mediate vasodilation. A high expression of β3-adrenergic receptors in peripheral vessels and in the heart has been reported. In peripheral vessels, β3-adrenergic receptors are located in the endothelial cells; they stimulate endothelial NO synthase which leads to increase NO release. β3-adrenergic receptors, which are located in the cardiomyocytes, have negative inotropic effects through the coupling of G-α-I with NO synthase. Therefore increased NO release following nebivolol administration results in peripheral vasodilatation and improves endothelial function (279). Other studies have suggested that nebivolol expresses vasodilatory effects via the Larginine/ NO pathway (283,287,288).

In humans, nebivolol induces vasodilation in both veins and arteries. In fact, consequent stimulation of P2Y-purinoceptors causes NO release, which leads to ATP efflux and vasodilation. It is important to note that beneficial effect of nebivolol on NO occurs via the β2-ARs and the β3-ARs, not via the β1-ARs (281).

Studies in animals, healthy volunteers and hypertensive patients have revealed that nebivolol causes the production and release of NO by inducing NO synthase expression, which leads to vasodilatory action. On the other hand nebivolol has a complementary anti-oxidative stress activity, which results in a decrease in NO degradation (289). This vasodilatory effect has been observed following the administration of nebivolol, but not after that of atenolol. Cockcroft et al. assessed the vasodilatory effects of nebivolol as compared to those of atenolol. The results indicated that infusion of nebivolol into the brachial artery in a dose-dependent manner could significantly increase forearm blood flow, while atenolol did not express any vasodilatory effects (288).

To evaluate whether nebivolol has any α-adrenergic antagonist activity, the effects of nebivolol on prostaglandin F2a-preconstricted veins were determined. The results supported the hypothesis that the vasodilatory effects of nebivolol are mediated by NO, not by antagonist effects at α-adrenergic receptors (285).

Nebivolol may inhibit 5-HT1A-receptors (290). The stimulation of 5-HT1A-receptors results in the inhibition of the contraction stimulated by cholinergic nerves in the intestinal tissues of guinea-pig.

High doses of nebivolol demonstrated inhibitory effects on the muscle response to nerve stimulation, while a serotoninergic receptor antagonist, methiotepin, could not antagonize this effect (289).

Nebivolol does not have effect on other neurotransmitter receptors, with the exception of β-adrenergic and 5-HT1A receptors. Nebivolol at micromolar

concentrations has an effect on Ca2+ induced or prostaglandin F2a-induced vasoconstriction (286). Nebivolol also interacts with estrogen receptor-mediated pathways (289).

In humans, pharmacokinetic studies have shown that nebivolol is well and rapidly absorbed following oral administration, reaching peak plasma concentrations in 0.5 – 4.0 h. The rate or extent of absorption is not affected by food and age (281,282,285,286,289,291,292). Its absolute bioavailability after metabolism is 12%. Kamali et al. have shown in healthy extensive metabolizer volunteers, after single oral dose of nebivolol (5mg), the mean peak plasma drug concentration (Cmax) for unchanged racemate nebivolol was 1.48 μg/L (293). The steady-state plasma concentrations are achieved for nebivolol within 1 day and for active metabolites within a few days in most individuals (282). Both enantiomers of nebivolol are highly bound (≈98%) to plasma proteins (mainly albumin) (285,292). The nebivolol volume of distribution is approximately 10 L/kg and despite having high lipophilicity, its volume of distribution is not changed in obese patients (282,285). The bounding of nebivolol to plasma proteins is in a non- stereospecific manner (98.1±0.2% for D-nebivolol; 98.0±0.3% for L-nebivolol). In addition, distribution of nebivolol in red blood cells is non-stereoselective (46% of D-nebivolol and 51% of L-nebivolol) (286).

Nebivolol is subject to an extensive first-pass effect via hepatic oxidative metabolism, primarily mediated by CYP450 2D6 enzymes, which results in the formation of numerous metabolites, some of which are active (280,286,289). Because there is a significant variability in CYP2D6 activity throughout

population , remarkable differences is exist between individual patients in terms of bioavailability, volume of distribution, half-life and clearance. Half-life of nebivolol varies as well: 13 h in extensive metabolizers and 56 h in poor metabolizers. Hepatic impairment may decrease the elimination of nebivolol (280,281). The bioavailability of nebivolol in individuals varies from 12% in extensive metabolizers to 96% in poor metabolizers, but the formation of active hydroxyl metabolites results in little clinical difference between poor and extensive metabolizers.

Following a single oral dose of nebivolol (5mg), the clearance rate was 30 L/h in poor metabolizers and 111 L/h in extensive metabolizers, and the terminal half-life values were 27 hours in poor and 8 hours in fast metabolizers (280,282,285,286,289,291,292).

The major pathway for nebivolol metabolism in extensive metabolizers is aromatic hydroxylation, whereas in poor metabolizers, glucuronidation and alicyclic hydroxylation are the predominant metabolizing pathways. In both groups, alicyclic hydroxylation, glucuronidation and hydroxylated metabolites are major pathways of metabolism, while N-dealkylation is less important (280,286). The aromatic hydroxylated and alicyclic oxidized metabolites of nebivolol are active, while the N-dealkylated metabolites and glucuronides are pharmacologically inactive or exhibit little activity (280,285,292). Poor metabolisers cannot hydroxylate the aromatic rings adequately therefore the unchanged drug concentrations remain high. In extensive metabolizers, by contrast, there is remarkable formation of the active hydroxy metabolites, which

compensates for the low concentration of unchanged nebivolol in extensive metabolizer patients. Therefore, there is no difference in the antihypertensive activity of nebivolol between poor and extensive metabolizers (282).

A study under fasting conditions found that the relative bioavailability of nebivolol (tablet/ oral solution) was approximately 88% and 112% for extensive and poor metabolizers respectively. Another study revealed a similar linearity in pharmacokinetic indices following the oral administration of nebivolol (dosing range of 2.5 to 20 mg daily) in extensive and poor metabolizers (280,294,295).

Less than 1% of the nebivolol is excreted unchanged in the urine; as a result, there is no need for dose adjustment in patients with chronic kidney disease (285,286,291).

Nebivolol is a highly lipophilic drug; one would therefore expect to observe differences in pharmacokinetic indices between obese and non-obese patients. A study in obese individuals reported that the clearance and volume of distribution of unchanged nebivolol at steady state were higher in obese patients than in non-obese patients (unchanged nebivolol; the racemate and each enantiomer were about 30 to 40% higher in obese patients); however, when the weight differences were considered (per kilogram bodyweight), no significant differences were observed (282,289). The authors therefore concluded that there was no difference between the tissue distribution of the drug in obese and lean individuals (282,289).

A study of eight patients with mild to moderate hepatic impairment showed that, following a single oral dose of 5 mg of nebivolol, the mean AUC was 48.99 ng • h/mL as compared with 11.18 ng • h/mL in the same number of healthy volunteers (289,292). However, the effects of other degrees of hepatic impairment on the pharmacokinetic profile of nebivolol have not been reported (292).

Mild renal impairment did not affect nebivolol clearance, but in patients with severe renal impairment nebivolol clearance was reduced by 53% (289,292). There are no published data to identify concern in use of nebivolol in patients undergoing dialysis (292).

The data from a clinical trial including 478 patients over 65 years old receiving nebivolol show no overall difference in the efficacy or tolerability of nebivolol in this population compared with younger patients (292). Although age does not influence the pharmacokinetics of nebivolol, a reduction in nebivolol starting dosage is recommended for elderly patients (289).

There are no published reports on the safety profile and efficacy of nebivolol in the pediatric population (292).

To evaluate the efficacy and tolerability of nebivolol in black patients with high blood pressure, a randomized, placebo-controlled, multicenter trial in Europe and the US was conducted. The efficacy of the drug in black and white hypertensive patients was same with no significant differences (response rates: 58% and 62%, respectively). Tayler et al. have shown in black patients the role of NO is very important. They conducted a study in black patients suffering from

congestive heart failure, in which they found that a combination of hydralazine and nitrates in compare to an ACE inhibitor and a β-blocker (a standard regimen), can decrease the rate of mortality by 43%. The efficacy of nebivolol in black patients may in part be due to increase NO levels (296). In black patients, nebivolol, independent of its β1-selective blocking activity, improves NO availability on the same level as seen in the white patients through increasing the release of NO, and by reducing the formation of peroxynitrite in the vascular endothelium (297). Based on these findings, it has been suggested that in hypertensive black patients nebivolol, as compare to older β-blockers, is well tolerated and may be a more effective drug in this population (298).

Nebivolol is metabolized by CYP2D6 isoenzymes. Medications that inhibit or induce CYP2D6 can be expected to increase or decrease plasma levels of nebivolol, including amiodarone, buproprion, celecoxib, chlorpheniramine, chlorpromazine, cimetidine, citalopram, cocaine, desipramine, diphenhydramine, doxepin, doxorubicin, escitalopram, fluoxetine, fluphenazine, haloperidol, methadone, metoclopramide, midodrine, paroxetine, propafenone, quinidine, ranitidine, ritonavir, sertraline, and ticlopidine. In these conditions, patients should be monitored closely, and a dose adjustment may be needed (292). It is very important to evaluate the administration of β-blockers, which often are used with diuretics, ACE inhibitors, spironolactone or digoxin for the treatment of CHD and HF. Nebivolol has no effect on the pharmacokinetics of digoxin, warfarin, losartan, spironolactone, ramipril or furosemide; on the other hand, losartan, ramipril, furosemide and hydrochlorothiazide have no effect on the

pharmacokinetics of nebivolol (289,299-301). In a study following the co-administration of fluoxetine (repeat administration) or cimetidine, the plasma concentration of nebivolol increased (280,289,299). Co-administration of nebivolol with nicardipine results in slight increases in the plasma concentrations of both drugs (280,289). Altogether, available data showing the metabolism and distribution of nebivolol on various populations and data indicating nebivolol's interactions with other drugs, do not limit the use of nebivolol in most hypertensive patients. However, co-administration of nebivolol with strong CYP2D6 inhibitors are needed to be evaluated (280,285).

Like other β-adrenergic blockers, caution should be taken in administering nebivolol to patients with severe bronchospastic disease, and in the co-administration of nebivolol with other drugs that result in negative inotropic and chronotropic effects such as verapamil, diltiazem, digoxin or amiodarone (285).

Only a few studies have examined the pharmacokinetics of nebivolol in animals. Facundo et al. have shown the pharmacokinetic indices of D-nebivolol and L-nebivolol in control (Wistar Kyoto (WKY), n=12) and spontaneously hypertensive (SHR, n=12) rats after intravenous administration. After a single dose of 3 or 10 mg kg−1 of nebivolol, a pharmacokinetic two-compartment model was considered. The pharmacokinetic parameters showed no differences in the distribution, the Vdss or the Cmax of both D- and L-nebivolol in SHR rats compared with those in WKY rats. A significantly lower clearance of L-nebivolol was observed in SHR animals than in WKY animals at both dose levels. On the other hand a non-significant reduction in clearance of d-nebivolol was obtained in

SHR rats compared with the control group. Nebivolol showed enantioselective pharmacokinetics; the clearance of L-nebivolol in both experimental groups was significantly higher than that of D-nebivolol. The AUC of D-nebivolol was greater than the L-nebivolol in SHR and WKY rats. Both nebivolol enantiomers showed linear pharmacokinetics; both Cmax and AUC increased in a dose-dependent manner in both experimental groups (302).

1.3 Rationale

- Patients who are aged, obese, or who have rheumatoid arthritis are at higher risk of cardiovascular morbidity and mortality. These patients need cardiovascular therapy to decrease the risk of cardiovascular complications. However, inflammation may cause the therapy to fail.
- Verapamil is a calcium channel blocker. In RA patients and pre-adjuvant arthritis rats, the cardiovascular response to verapamil is diminished even though the concentration of verapamil enantiomers is elevated.
- The action and disposition of verapamil in patients whose rheumatoid arthritis is in remission is restored, which suggests that the degree of the disease severity plays an important role in the action and disposition of verapamil.
- The pharmacodynamics of propranolol are diminished in pre-adjuvant arthritis rats even in the presence of significantly higher plasma concentration of propranolol enantiomers.
- Not all cardiovascular drugs decrease the response to cardiovascular therapy and elevate the concentration of drugs during inflammatory conditions. Inflammation does not appear to reduce the efficacy of AT1R antagonists like losartan and valsartan.
- Nebivolol is a third generation β-AR blocker with high selectivity for blocking β1 and β3-agonistic properties. Activation of β3-ARs enhances endothelial NO bioavailability and causes a vasodilatory effect. Nebivolol also has inhibitory effects on oxidative stress and vascular inflammation. Its favorable

metabolic profile on both carbohydrates and lipids, beneficial effects on arterial stiffness and antioxidant properties, has made it a unique β blocker, different from other β blockers.

1.4 Hypotheses

- The concentration of verapamil enantiomers are elevated in the active form of CD.
- The active form of CD is associated with a decrease in the verapamil response, including PR interval prolongation, heart rate, systolic and diastolic blood pressure.
- The remission form of CD is associated with normal pharmacokinetic and pharmacodynamics of verapamil.
- Unlike propranolol, the nebivolol plasma concentration is not elevated in the pre-adjuvant arthritis model of inflammation.
- In contrast with propranolol, the dromotropic response to nebivolol is not down-regulated in the presence of inflammatory conditions.
- The diminished response to propranolol in the presence of inflammation is due to the reduced β1-ARs' target proteins while the inflammation does not down-regulate the density of β3-ARs.
- Similar to most beta blockers, nebivolol pharmacokinetics are stereoselective.

1.5 Objectives

- To determine the effects of the severity of CD on pharmacokinetic indices of verapamil.
- To determine whether the severity of CD can affect the pharmacodynamic properties (PR prolongation, heart rate reduction) of verapamil similar to what has been observed in rheumatoid arthritis patients.
- To determine the effects of the pre-adjuvant arthritis model of inflammation on nebivolol pharmacokinetic indices.
- To investigate the possibility of stereoselectivity in the pharmacokinetics of nebivolol in control and pre-adjuvant arthritis rats.
- To determine the effect of inflammation on the pharmacodynamic effects (PR-interval prolongation and heart rate reduction) of nebivolol.
- To determine the effect of inflammation on the expression and density of β-AR subtype proteins (β1, β2, β3) in the pre-adjuvant arthritis model of inflammation in rats.

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Chapter 2 Drug-disease interaction: Crohn's disease elevates verapamil plasma concentrations but reduces response to the drug proportional to disease activity[1]

2.1 Introduction

Inflammation is associated with cardiovascular complications. Indeed, the survival of the post-myocardial infarction patients and the risk of in-hospital coronary events for unstable angina correlate well with the degree of inflammation measured as the concentrations of pro-inflammatory mediators such as C-reactive protein (1) and interleukin-6 (2). Furthermore, the prevalence of cardiovascular complications is much greater in patients with rheumatoid arthritis than in the general population (3). Similarly, inflammatory bowel disease (IBD) including Crohn's disease (CD) appears to influence the cardiovascular system, although an increased risk has not been consistently demonstrated (4-10). This effect of inflammation which has not been fully investigated but may be associated with altered effectiveness of cardiovascular therapy as a consequence of a disease-drug interaction.

[1] A version of this chapter has been published. Forough Sanaee, John D.Clements, Alistair W.G. Waugh, Richard N. Fedorak, Richard Lewanczuk, Fakhreddin Jamali. Br J Clin Pharmacol 2011, 72:5, 787-797

Inflammation also influences action and disposition of drugs including some of the cardiovascular agents that are commonly used by patients with inflammatory conditions. It is known to result in elevated plasma concentrations of drugs that are efficiently metabolized in the liver (11-13). Verapamil is such a drug with low bioavailability due to its efficient first pass hepatic metabolism.

It is, therefore, expected for plasma verapamil concentrations to be greater in patients with inflammatory conditions when compared with otherwise healthy individuals. Such elevated concentrations may have both therapeutic and toxic consequences. However, previous studies have demonstrated that inflammatory conditions such as rheumatoid arthritis (14), old age (15) and obesity (16,17) result in altered response to verapamil despite substantially increased plasma concentrations. Furthermore, it has been shown in humans and animal models that the pharmacological response to calcium (14,18) and potassium (19) channel blockers as well as β-adrenergic antagonists (20,21), but not angiotensin II inhibitors (22), is reduced by inflammation. This reduced response has not been investigated in IBD which, although an inflammatory condition, has its own etiology.

The dual effect of inflammation does not seem to be limited to cardiovascular drugs since an elevated drug concentration that is not accompanied with increased effect has very recently been reported for midazolam in critically ill children (23) . This observation has also been linked to inflammation.

Interestingly, the action and disposition of verapamil is restored in patients whose rheumatoid arthritis is in the remission (24) (25) pointing to the possibility that the degree of the disease severity may have a role in this observation. Herein, we report the pharmacokinetics and pharmacodynamics of verapamil in IBD (CD), a condition for which a convenient and relatively robust method exists for grading of the disease severity (26). Due to its inflammatory nature, CD is expected to alter both plasma protein binding and the hepatic metabolism of certain drugs, resulting in reduced clearance (27-29). The effect of the disease on response to cardiovascular drugs is, however, unknown.

Following therapeutic doses, verapamil causes negative dromotropic effects reflected as a plasma concentration-dependent prolongation of PR interval and AV node block (30), indicative of L-type calcium channels function. The PR interval prolongation is conveniently detectable even after small single doses (31). Hence, the response to the drug, as a typical reflection of the blockade of L-type calcium channels, is readily measurable even in normal subjects. The main objective of the study was to examine the effect of disease severity on the action and disposition of an efficiently metabolized drug.

2.2 Material and methods

2.2.1 Chemicals

Apo-verapamil 80-mg tablets were purchased from the University of Alberta Hospital Pharmacy (Edmonton, Alberta, Canada). Verapamil hydrochloride, aspergillus nitrate reductase (10 U/mL), heptafluorobutanol, FAD, NADPH,

pyruvic acid, sulfanilamide, (+) glaucine, lactic dehydrogenase (1500U/mL) and naphthyl ethylenediamine dihydrochloride were purchased from Sigma-Aldrich Chemical Canada (Oakville, ON, Canada). High-performance liquid chromatography (HPLC) grade hexane and HPLC grade isopropanol, triethylamine and 98% ethanol were purchased from Caledon Laboratories (Georgetown, Canada). HEPES was purchased from Fisher Biotech (Edmonton, AB, Canada).

2.2.2 Study Design and Patients

This was a single center, single treatment, healthy volunteer-controlled study. The protocol was approved by the University of Alberta Health Research Ethics Board and was conducted in accordance with the Declaration of Helsinki. The study was registered in the Northern Alberta Clinical Trials and Research Centre. All participants provided written informed consent. A baseline electrocardiogram was performed to ensure that there were no underlying cardiac abnormalities. Three groups of subjects were enrolled: 1) healthy volunteers (Control, n=9), 2) patients with quiescent Crohn's disease (CD remission, n=22), and 3) patients with active Crohn's disease (CD active, n=14).

Subjects were 18 to 69 years of age, male or female of non-childbearing potential and non-smokers for at least 6 months before entry into the study. Subjects with illnesses other that CD within 30 days prior to the study were excluded. Subjects were informed that they should not ingest grapefruit juice or any new prescription or over-the-counter medications for at least 7 days prior to the study. They were, however, permitted to continue, unchanged, their CD

medications. All patients completed a HIB questionnaire (26) as a validated clinical assessment of CD activity. The HIB is cumulative score of the domains of general well-being, abdominal pain, number of liquid or soft bowel motions and extra-intestinal manifestations. A HIB score of zero to 4 indicates clinical remission, 5 to 7, mild disease, 8 to 16, moderate disease and above 16, severe disease. The disease category of the subjects was kept from workers who carried out the pharmacokinetic and pharmacodynamics experiments.

2.2.3 Drug administration and pharmacokinetic/pharmacodynamic sampling

Subjects fasted overnight for at least 8 h prior to taking a single 80 mg verapamil tablet by mouth with 200 mL of water. An intravenous line from an antecubital vein was inserted for blood sampling, and the patient was allowed to rest recumbent for 30 minutes prior to measuring baseline physiologic variables. A standard meal was provided 3 h post dose and subjects were free to drink water throughout the study. Blood samples (3 mL) were taken at 0 (pre dose), 20, 40 min and 1, 2, 3, 5, 6.5 and 8 h for verapamil concentration analysis.

For electrocardiogram recording, a standard lead I and aV5 electrocardiogram was recorded using a Holter monitor (Hewlett-Packard, Avondale, PA). The mean of five PR intervals and heart rate measurements were recorded during the minute before blood samples collection for the pharmacokinetic analysis. Mean arterial pressure (MAP), systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured using an HDI/Pulsewave Cardiovascular Profiling

Instrument CR-2000 (Hypertension Diagnostics Inc, Minneapolis, Minn). The experimentally observed maximum percentage effects were recorded.

Two patients with active disease were brought back after approximately 4 months and re-examined for their HBI score and response to verapamil. Our aim was to test whether a change in the disease activity resulted in a proportionally altered responsiveness to the drug. Only the results from the first examination were included in our statistical evaluations.

2.2.4 Protein binding

Serum for the protein binding study was pooled from the time zero h blood sample of all three groups (n = 5/group). The pooled serum was adjusted to pH 7.4 with 0.1 M HCl. The serum was then spiked with 100 ng/mL of racemic verapamil. Samples were incubated at 37°C for 1 h and then transferred to micropartition chambers (Amicon Division of W.R. Grace & Co, Danvers MA) for ultrafiltration. The chambers were centrifuged at 2000 g for 1 h. In addition, 8 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 non-filtrate concentrations were measured and the fraction unbound (fu) determined as the unbound concentration divided by total concentration. To ensure concentrations were above the minimum quantifiable limit for the HPLC assay, 4 micropartitions of the 12 chambers were pooled allowing for a total of 3 measurements per group.

2.2.5 Stereospecific verapamil assay

A previously described stereospecific HPLC method (32), as later modified (33) was used to determine plasma concentrations of R- and S-verapamil. The assay was precise (CV<10%) and accurate (error <10%) at the minimum quantifiable enantiomers concentrations of 5 ng/mL. We did not have access to norverapamil, a major metabolite of verapamil found in plasma with much less pharmacological activity than the parent compound (34). Instead of norverapamil concentrations, therefore, we examined the area of the eluted HPLC peaks. The order of appearance and retention time of norverapamil enantiomers were known to us from previous studies (14).

2.2.6 Inflammatory biomarker assays

Nitrites were analyzed in the pre dose serum samples indirectly by measuring the concentration of its stable breakdown product, nitrite through using a previously described method (35,36). Serum C-reactive protein was determined in the pre dose blood sample at the University of Alberta Hospital using the Dade-Behring (Deerfield, Ill) assay kit.

2.3 Data analysis and statistics

Data are presented as the mean± standard deviation. Pharmacodynamic responses include changes in systolic and diastolic blood pressure, heart rate and PR interval

were measured based on observed differences between baseline and post-treatment values. The area under the percentage effect-time curve (AUEC) was measured using the linear trapezoidal rule.

Pharmacokinetic metrics included the observed maximum plasma concentration (Cmax) and the time of its attainment (Tmax) as well as the area under the plasma drug concentration-time curve until the last experimental data point (AUC) calculated using the linear trapezoidal rule. The terminal elimination rate constant was calculated for those subjects who exhibited a log-linear terminal phase with at least 3 usable experimental points; a non-weighted nonlinear least-squares regression was used. The relationship between the PR interval and plasma S-verapamil concentrations were explored using WinNonlin 4.1 (Pharsight). Mean S-verapamil concentration and percent change from baseline in PR interval data were fitted to a series of PK/PD models. The final exposure-response plot was created using S-Plus 8.1 (TIBCO Spotfire, Somerville, MA). The significance of the differences was tested using the one way ANOVA with the Duncan Multiple Range ad-hoc test at $\alpha=0.05$. Nonparametric Mann-Whitney was used to evaluate the HBI differences between the two groups (CD remission; CD active).

2.4 Results

2.4.1 Subjects' characteristics

The baseline demographic information of subjects is shown in Table 2-1. Subjects in the CD remission group were on the following medications: infliximab (n=6), methotrexate (n=2), azathioprine (n=7), lorazepam (n=1), 5-ASA (n=4), oral contraceptive (n=1), felodipine (n=1), prednisone (n=1) and amitriptyline (n=1). Patients in CD active group were taking methotrexate (n=2), azathioprine (n=6), levothyroxine (n=1), 5-ASA (n=1), hyocine (n=1), metronidazole (n=2), ciprofloxacin (n=4), morphine (n=1), citalopram (n=1), folic acid (n=1), cholestyramine (n=1), prednisone (n=1), premarine (n=1), topiramate (n=1), infliximab (n=1) and certilizumab (n=1). Patients in the control group were on no medications. There were no significant differences between the three groups in terms of age, weight, height, baseline blood pressure, heart rate or PR interval.

2.4.2 Inflammatory biomarkers

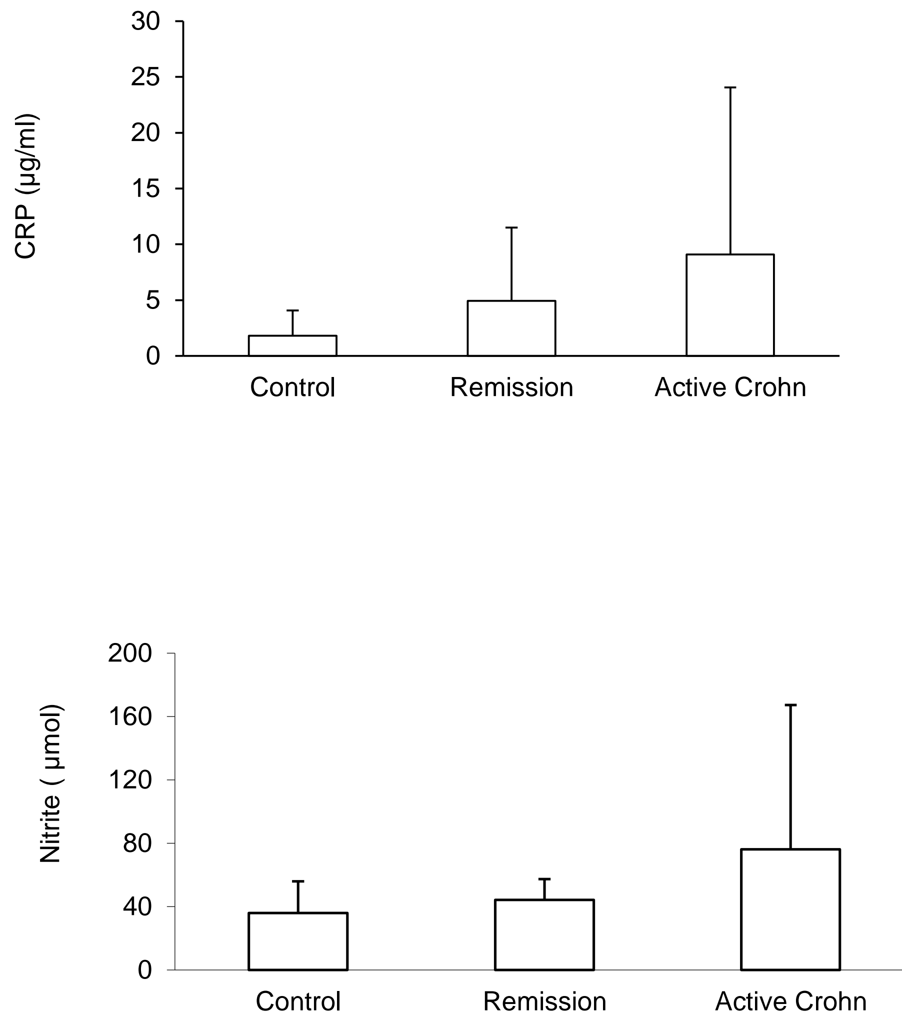
The concentrations of serum nitrite and C-reactive protein were numerically higher in the patients with active CD compared with patients with CD in remission and healthy controls. The differences, however, did not reach statistical significance due to a great inter-subject variability particularly among the patients with active disease (Figure 2-1).

Table 2- 1. Patients' characteristics.

	Control (n= 9)	Crohn's Disease	
		Remission (n=22)	Active (n=14)
Age, yr	34.9±18.6	38.4±14.9	33.7±11.7
Sex, M/F	3/6	9/13	5/9
Height, cm	167±8	171±8	169±8
Weight, kg	69.5±12.8	71.2±16.8	74.9±17.5
Baseline SBP, mmHg	117±14	113±11	109±10
Baseline DBP, mmHg	66±13	66±9	63±7
Baseline HR, bpm	63±9	61±9	67±9
HBI score	0.00	2.16±2.09	10.79±6.48*
PR interval, ms	159±20	155±20	136±33

*significantly different from other groups ($p<0.05$); SBP, systolic blood pressure; DBP diastolic blood pressure,; HR, heart rate; HBI, Harvey Bradshaw Index.

Figure 2- 1. Mean of pro-inflammatory mediators measured in the three groups of subjects (Control, n=9; Remission, n=22; Active, n=14). The means were not significantly different**.**



2.4.3 Verapamil pharmacokinetics

The effect of active disease on pharmacokinetics of verapamil was stereoselective. Plasma S-verapamil concentration in patients with active CD was significantly higher than in both healthy controls and patients in CD in remission (Figure 2-2, Table 2-2). AUC was increased 9- and 14-fold in patients with active CD when compared with CD patients in remission and controls, respectively ($p<0.001$). With respect to R-verapamil, although both mean Cmax and AUC values were greater in patients with active CD as compared with the other groups, the differences did not reach statistical significance.

The ratios of HPLC peak areas of both S and R norverapamil over their corresponding verapamil enantiomers were not significantly different among the three groups of subjects at any measured time points.

There was no significant difference in the fraction of protein unbound drug between the controls (S: 0.11±0.02; R: 0.05±0.01) and patients with CD in remission (S: 0.07±0.02; R: 0.04±0.01). However, as compared to controls, patients with active CD had significantly reduced unbound fraction of S and R enantiomers (0.05±0.01 and 0.02±0.01, respectively).

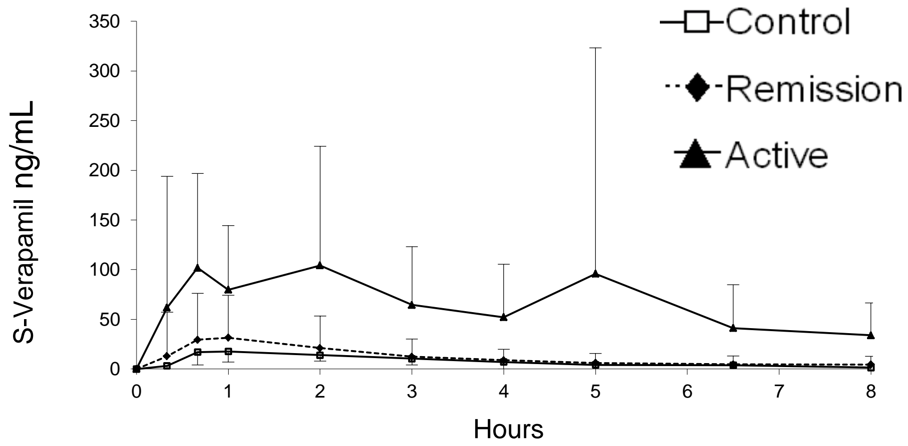
Table 2- 2. Pharmacokinetics of S and R verapamil, PR prolongation and changes in blood pressure following single doses of 80 mg to healthy control and Crohn's patients in remission or with active disease.

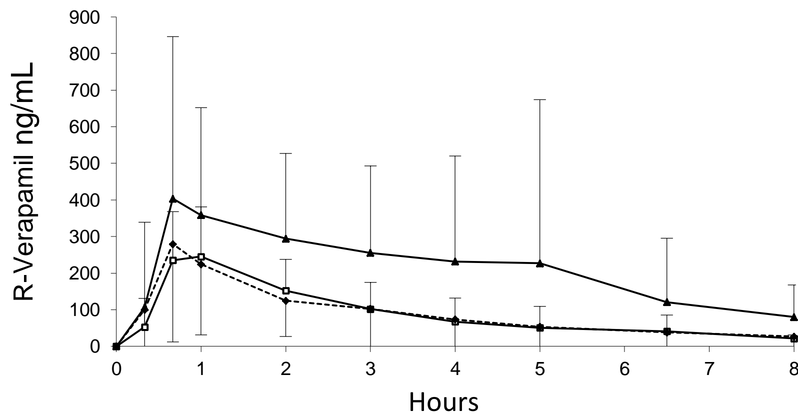
	Verapamil enantiomer	**Control (n=9)**	**Crohn's Disease Remission (n=22)**	**Active (n=14)**
Tmax, h	S	60 (40-60)[a]	60 (40-60)[a]	60 (40-60)[a]
	95% CI	0.74-1.46	0.81-1.33	0.57-1.83
	R	60 (20-60)[a]	60 (40-60)[a]	60 (20-60)[a]
	95% CI	0.60-0.88	0.79-1.21	0.67-1.93
Cmax, ng/mL	S	19.9±10.5[a]	39.3±51.6[a]	189.7±243.1[b]
	95% CI	13.0-26.8	17.7-60.9	62.0-317
	R	278±127[a]	353±313[a]	558±538[b]
	95% CI	195-361	222-484	271-840
t½, h	S	4.33±1.93[a] (n=4)	3.56±1.55[a] (n=11)	17.0±22.7[b] (n=11)
	95% CI	2.44-6.22	2.64-4.48	3.59-30.41
	R	3.16±1.04[a] (n=9)	3.13±1.23[a] (n=22)	3.13±1.54[a] (n=13)
	95% CI	2.48-3.84	2.62-3.64	2.29-3.97
AUC, ng.h/mL	S	61.7±46.8[a]	95.5±140.7[a]	537±598[b]
	95% CI	31.1-92.3	36.71-154.3	222-849
	R	723±453[a]	720±755[a]	1714±1693[a]
	95% CI	427-1019	396-1044	827-2601

PR interval prolongation	% max/baseline	21.4±14.9[a]	14.8±9.8[a]	17.0±17.0[a]
	95% CI	11.7-31.1	10.7-18.9	2.86-31.1
	AUEC, %.h	44.3±45.1[a]	36.0±41.4[a]	32.2±67.8[a]
	95% CI	14.8-73.8	18.7-53.3	-3.32-67.7
Blood pressure, %	Systolic	13.4±10.0[a]	10.8±4.4[a]	12.2±7.5[a]
	95% CI	6.87-19.9	8.96-12.6	8.17-16.2
	Diastolic	12.6±3.5[a]	9.4±3.0[a]	9.9±3.6[a]
	95% CI	10.3-14.9	8.15-10.7	8.01-11.8
Heart rate. %		11.3±5.8[a]	10.9±5.3[a]	9.6±5.0[a]
	95% CI	7.51-15.1	8.69-13.1	6.98-12.2

Different characters in the row indicate significant difference at α=0.05. Blood pressure (mmHg) and heart rates are changes from baseline. Experimental points from fewer patients were used to calculate t1/2 due to fluctuation.

Figure 2- 2. Plasma verapamil enantiomers concentration-time curves following single oral doses of 80 mg racemic verapamil in three groups of subjects (Control, n=9; Remission, n=22; Active, n=14).





The t½ of the log-linear portion of the plasma drug concentration curve was not calculated for all subjects due to fluctuations in concentration (Figure. 2-2). Nevertheless, while the t½ was significantly and substantially prolonged for S-verapamil, that of R-verapamil remained unchanged (Table 2-2).

2.4.4. Verapamil pharmacodynamics

Except for the patients with high HBI, all subjects responded to verapamil by demonstrating reduced systolic and diastolic blood pressure, heart rate and PR prolongation (Figure 2-3, Table 2-2). The observed marked increased S-verapamil concentrations in the patients with active disease did not result in greater response as the mean pharmacodynamic parameters remained at the same level as those calculated for control and remission groups (Table 2-2). Furthermore, with regard to the PR interval, there was a strong significant negative correlation between the maximum percent change from baseline ($p=0.0006$) or AUEC ($p=0.035$) and HBI (Figure 2-4). Patients with high HBI demonstrated little or no response. A significant association between the disease activity and response to verapamil was absent in CD remission group. No other pharmacokinetic or pharmacodynamics indices demonstrated significant correlation with disease severity.

Figure 2- 3. PR interval prolongation-time profiles following single oral doses of 80 mg racemic verapamil in the three groups of subjects (Control, n=9; Remission, n=22; Active, n=14). Error bars represent standard deviation.

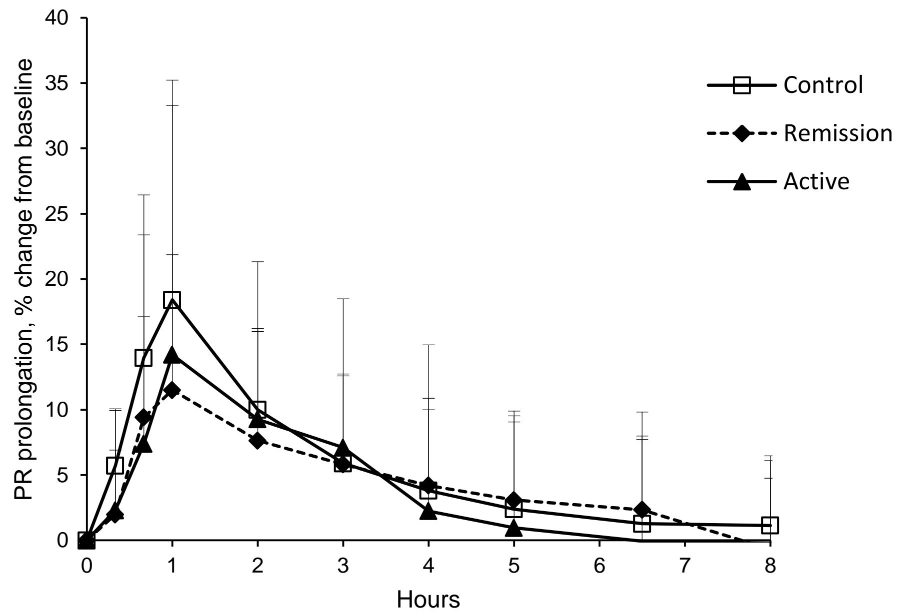
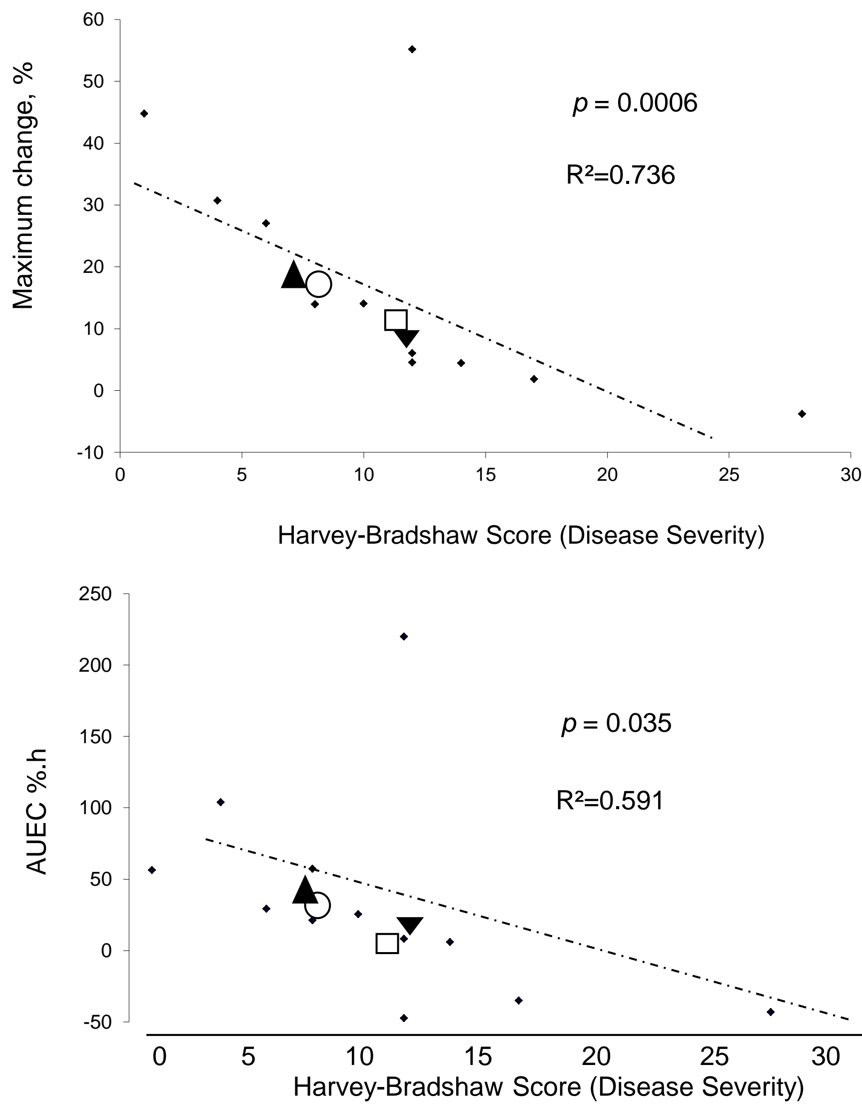


Figure 2- 4. The effect of disease severity on PR interval prolongation in response to single oral doses of 80 mg racemic verapamil administration to patients with active CD. The data present the observed maximum values (top) or the area under effect-time curves (AUEC) (bottom). Patients who were tested twice in 4 months are shown with symbols that are different from others. The first test is donated by ▲ and ▼ while the second tests are denoted by ○ and □, respectively. The data from the second evaluations were not included in the statistical analysis.



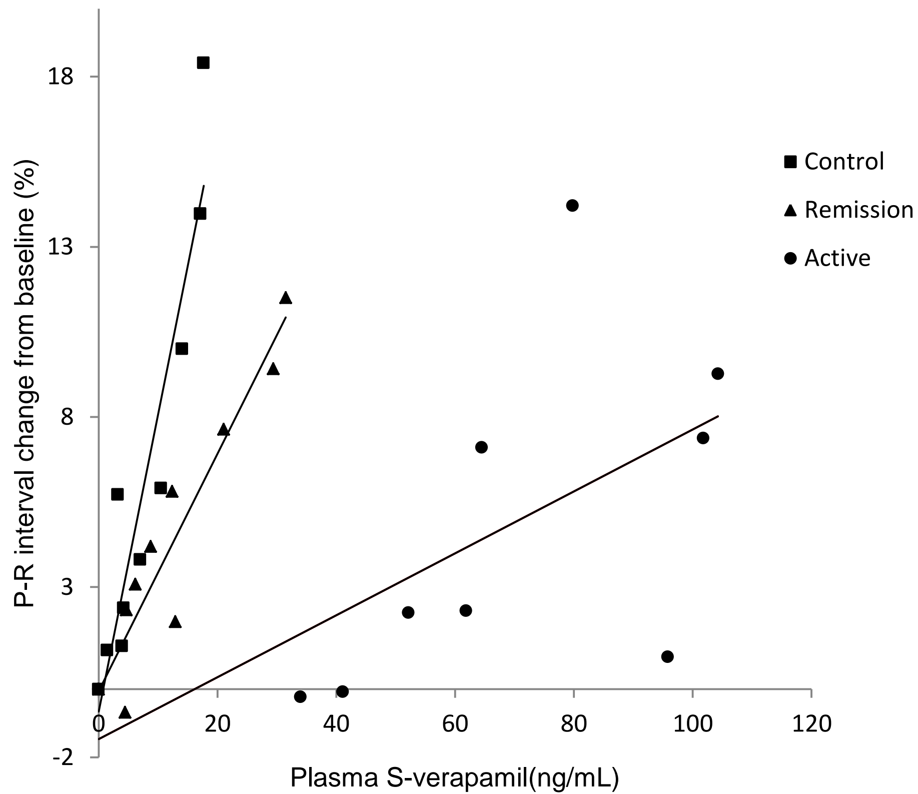
Counter-clockwise hystereses were observed for the plasma S-verapamil concentration-PR interval prolongation relationship in some but not all individual subjects, indicating a time delay between the plasma concentrations and cardiovascular response. When the concentration-effect data from each group were pooled, the relationship between PR interval and verapamil concentration was best described by a simple linear relationship (Figure 2-5). Interestingly, the associations between the mean values were significant for all groups ($P < 0.05$) with a slope for each group distinguishable from others. The group with active disease had the least steep slope and the greatest variability as compared with other two groups. Our attempt to calculate EC50 failed as the estimated values were much greater than the highest observed experimental data points.

The HBI in the two patients that were re-examined in 4 months had not substantially changed (HBI: from 7 to 8 and from 12 to 11). However, their disease activities vs verapamil responsiveness data fitted the general plot remarkably well (Figure 2-4).

2.5 Discussion

The effect of inflammatory conditions (12) including CD on the disposition of drugs has been widely reported since the 1970s (28). It is generally believed that inflammatory conditions result in reduced drug clearance, thereby, elevating concentrations of efficiently metabolized drugs (12,28). An increased

Figure 2- 5. The relationship between mean PR interval prolongation and mean S verapamil plasma concentrations. Regression lines are the best estimate of the relationship between two variables. All relationships were significant ($P < 0.05$). Control, $r = 0.9271$ (□); Remission, $r = 0.926$ (Δ); Active, $r = 0.580$ (o)



drug concentration is alarming due to the possibility of increased undesirable effects. However, most studies have only examined the pharmacokinetics of drugs, hence; their cautionary conclusions are usually without supporting pharmacodynamic data. The present data highlight, a few important points: First, affliction with IBD does not necessarily result in elevated drug concentrations. Indeed, plasma concentrations of verapamil, a typical drug with efficient hepatic metabolism, are not elevated in Crohn's patients whose disease has subsided to remission (Figure 2-2, Table 2-2). Verapamil concentrations increase only when the disease is active. The second important point is that an elevated verapamil concentration does not necessary result in increased efficacy or toxicity. This is contrary to the general belief that a high drug concentration may result in increased efficacy. The third point is the interesting observation that reveals, for the first time, a strong association between the severity of an inflammatory condition and response to a cardiovascular drug; i.e., the greater the disease activity, the lower the response to verapamil (Figure 2-4). Indeed, the patients with HBI of greater than 12 demonstrated no or negligible PR prolongations in response to verapamil. This is while those with less disease activity still showed some response and patients in remission yielded the same pharmacodynamic and pharmacokinetic indices as did the healthy volunteers.

It is important to note that HBI reflects the patient's well-being and is not a direct measure of the severity of inflammation as its components include various symptoms such as pain, nature of stool and use of drugs. Hence, the observed reduced response to verapamil in patients with high that HBI cannot be

unequivocally attributed to the inflammation associated with the disease. Although the mean pharmacological responses to verapamil were not significantly different among the examined groups, the slope of the mean changes in PR prolongations vs. S-verapamil concentrations was substantially steeper for healthy volunteers than those of other groups with that of the group in remission in between (Figure 2-5). This suggests yet another sign of the effect of disease severity that due to the associated great variability cannot be readily quantified.

The observed reduction in both clearance and pharmacological activity of verapamil is attributed to down- regulation of target proteins, i.e., cytochrome P450 enzymes (35) and calcium channels (37), respectively. While the latter results in elevated drug plasma concentrations, the former reduces the drug-receptor binding (18) to yield a reduced response. Since verapamil is a substrate for P-glycoprotein (38) the possibility of increased bioavailability stemming from an inflammation-induced down regulation of efflux protein (39,40) also exists. Previous reports dealing with other forms of inflammatory conditions, have ruled out an association between the diminished response and a reduced unbound drug concentration secondary to an inflammation-induced elevated plasma protein responsible for verapamil binding (19). Indeed, the present study confirmed that despite the observed 50% reduction in the fraction of the unbound drug caused by active CD, the free drug concentration remains still substantially higher than normal due to the several fold increase in the total drug concentration. Nevertheless, in light of the observed reduced response in the tested patients reported herein one may conclude that the therapeutic consequence of the elevated

concentration of certain cardiovascular drugs becomes moot or, at least, questionable. However, other effects, unrelated to the blocking of the calcium channels, may emerge in response to high concentrations.

The mechanism involved in the down regulation of these target proteins is not quite clear. However, elevated expression of pro-inflammatory mediators such as interlukin-6, tumor necrosis factor, angiotensin II and NO can, at least in part, be involved (41). According to a recent report in an animal model of inflammation, an observed reduced responsiveness to the β-adrenergic blocker propranolol is associated with lower cardiac norepinephrine transporter concentration (42). A reduction in the transporter, the primary component of norepinephrine uptake-1 that removes norepinephrine from neuronal junctions is closely related to a reduced β-adrenergic target protein. Excessive sympathetic nervous system innervation is a well-established component of inflammatory conditions (10,43-45) that may be involved in down regulation of the receptor. An altered sympathetic nervous system modulation in intestinal inflammation has been discussed in the literature, e.g., Straub et al (46).

The changes in the disposition of norverapamil followed those of the parent drug as the ratios of HPLC response of the metabolite enantiomers over those of respective verapamil enantiomers remained unaffected by the disease. This indicates that the increased norverapamil concentration in the patients with active CD as compared with other groups is secondary to the corresponding increase in verapamil concentration.

Increased NO is a reliable marker of inflammation in active rheumatoid arthritis (14). We observed an increased mean concentration of nitrite, a stable metabolite of NO in our group with active CD as compare with those in remission as well as the healthy control group. The differences among the three groups, however, did not reach the set significant level due, probably to a great inter-subject variability associated with the latter group (Figure 2-1). This was expected due to the substantial variability in the disease severity (Figure 2-4). A similar observation was made for C-reactive protein, another marker of inflammation in human which increases endothelial dysfunction and plays a role in the development coronary artery disease (2) (Figure 2-1).

Our data suggest that in assessing the effectiveness of cardiovascular drugs which are affected by inflammation, the severity of the condition must be considered regardless of the observed or expected plasma drug concentration. The diseases severity can be controlled for CD or rheumatoid arthritis. This will result in restoration of the calcium channels function. In obesity and old age, however, control of inflammation is not achieved as readily as in the mentioned diseases. Animal studies suggest that even under active conditions HMG-CoA reductase inhibitors (21) and angiotensin II receptor blockers (37) restore the diminishing effect of inflammation on response to verapamil and the β-AR antagonist, propranolol, respectively. In addition, angiotnesin II blockers appear to maintain their effectiveness in active rheumatoid arthritis (22,47) due, perhaps, to their anti-inflammatory effects (41). These observations remain to be made in obese and elderly humans.

Patients with IBD are at least as prone to cardiovascular conditions as the general population (4-10). The effectiveness of common cardiovascular classes of drug is, therefore, important when treating this category of patients. Accordingly, the observation that the pharmacodynamics and pharmacokinetics of verapamil, a reprehensive of the L-type calcium channel antagonist class, has therapeutic relevance that extends beyond the drug used and the disease investigated herein.

Interestingly, greater than usual doses of alfentanil, a supplement to patients under nitrous oxide anesthesia, are needed for patients with CD due to lower threshold response (48). The mechanism of this lower than usual response to alfentanil is unknown but is shown to be, similar to verapamil, at the level of pharmacodynamics.

The limitations of the present study include the fact that our subjects were normotensive. Nevertheless, we have shown that, indeed, severe CD may result in altered calcium channel function. It would be most interesting to examine the same effect in patients who actually need cardiovascular therapy. Our second limitation may be the fact that we made our observation only after single doses of verapamil. Our objective, however, was to see whether the response to verapamil as a reflection of the function of the L-type calcium channels, was altered. This was achieved even after single doses by measuring PR interval prolongation caused by verapamil. The prolonging effect is a sensitive and concentration-dependent marker of calcium channel function and administration of single doses provides the necessary information (15,16).

3.6 Conclusions

In conclusion, in patients with clinically active CD, the drug efficacy is decreased despite an increase in the verapamil enantiomers concentration. However, in patients who are in clinical remission, the pharmacokinetics and pharmacodynamics of verapamil return to normal. In addition, in the treatment of cardiovascular disease in patients with inflammatory conditions such as CD, consideration should be paid to the activity of the disease and the degree of inflammation. It appears that for an effective control of cardiovascular conditions in patients with severe inflammatory diseases, curtailing of the inflammation is essential; treatments that include HMG-CoA reductase inhibitors and/or angiotensin interruption may be found useful. The present observation may have clinical implications beyond the tested drug and the selected disease as it may be extrapolated to other inflammatory diseases. Evidence of similar observations involving other drugs or different diseases have started to emerge (23,48).

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Chapter 3 Action and disposition of the β3-agonist nebivolol in presence of inflammation; an alternative to conventional β1-blockers [2]

3.1 Introduction

Inflammation, a complex process in response to tissue or cell damage, is associated with many diseases and conditions such as arthritis, cancer, infection, diabetes, obesity, old age and cardiovascular diseases (1). A rise in the inflammatory mediators expression is linked to cardiovascular complications so that patients with inflammatory diseases such as rheumatoid arthritis exhibit greater incidents of cardiovascular problems than the general population (2,3). In addition, inflammation causes reduced clearance of drugs that are efficiently metabolized in the liver, thereby, increases plasma drug concentration (4-10). Interestingly, this rise in concentration is associated with reduced rather than increased response to some cardiovascular drugs such as the β-blocker propranolol and calcium channel blocker verapamil (5,7,8,10). These paradoxical changes are attributed to reduced target protein levels at the metabolic enzymes and β1-adrenergic as well as calcium channel receptor (11-13). The use of β-blockers is not necessarily beneficial across the general population due, likely, to the presence of various contributing factors in certain patients particularly the elderly (14).

In this context, a reduced response to cardiovascular pharmacotherapy may be a contributing factor in the elderly (15) and obese patients (16) as both conditions

[2] A version of this chapter has been published. Forough Sanaee and Fakhreddin Jamali1. Current pharmaceutical design, July 25, 2013

are associated with inflammation. In these patients alternative approaches are timely.

The main objectives of this study were, first, to investigate the effect of inflammation on the action and disposition of nebivolol, a relatively new β1- β2-adrenergic (AR) blocker with β3-adrenergic agonistic properties (17-20). In human, nebivolol undergoes significant first-pass metabolism, hence, we expected its clearance to be reduced under inflammatory conditions as has been reported for propranolol (7-9). Second, for its pharmacological effects, we examined whether due to the involvement of β2- and β3-AR, nebivolol is an alternative to other β-ARs, e.g., propranolol, which their target receptor proteins are down-regulated in the presence of inflammation. The effect of inflammation on β2- and β3- AR has been unknown. The outcome of this study may suggest an alternative approach to the presence β-blocker pharmacotherapy.

We hypothesized inflammation affects pharmacokinetics and pharmacodynamics of nebivolol in pre-AA model rats. Our main pharmacodynamics endpoint was the prolongation of RR and PR intervals. We used the pre-adjuvant arthritis as a model of inflammation as it is associated with elevated pro-inflammatory mediators and allows studies under systemic inflammatory conditions in the absence of pain and stress associated with the experimental disease (11). In addition, we used propranolol as a positive control.

3.2 Material and methods

3.2.1 Chemicals

Following materials were acquired: Racemic propranolol HCL and racemic verapamil HCL (Sigma Chemical Co., St. Louis, MO); racemic nebivolol HCL (Tocris bioscience, Ellisville, Missouri USA); killed, desiccated Mycobacterium butyricum (Difco Laboratories, Detroit, MI); HPLC grade hexane, chloroform, methanol, diethylether, isopropanol and acetonitril (Caledon Laboratories, Georgetown, Canada); HPLC grade dichloromethane (Fisher Scientific, Nepean, Ontario); Teflon-coated wire used for the ECG electrodes (Biomed Wire, Chatsworth, CA); PE-50 polyethylene (Clay Adams, Parsippany, NY); silastic tubing (Dow Corning Co., Midland, MI); Protease inhibitor cocktail for mammalian tissues (Sigma-Aldrich, St. Louis, MO); tris (0.025M)/glycine (0.192 M) buffer, and tris (0.025 M)/glycine (0.192 M)/SDS (0.1%) buffer (ICN Biomedicals, Aurora, Ohio); Immun-Star horseradish peroxidase Chemiluminescent kit, Precision Plus Protein Standards Dual Color, Bio- Rad DC Protein Assay Sodium, dodecyl sulfate, TEMED, 10% Tween 20, and 40% acrylamide/Bis 29:1 solution (Bio-Rad, Hercules, CA); Labor LDN mouse monoclonal to β-actin loading control (Abcam Inc., Cambridge, MA); rabbit polyclonal to β1-AR, rabbit polyclonal to β2-AR and rabbit polyclonal to β3-AR (Abcam Incorporated, Cambridge, MA).

3.2.2 Animals

The study was approved by the Health Sciences Animal Policy and Welfare Committee of the University of Alberta. Adult male Sprague–Dawley rats were used. Animals were housed in a 12 h light/dark cycle with free access to water, though food was withheld for 12 h prior to the drugs dosing and for 6 h afterwards.

Rats were randomly divided into 2 groups, pre-adjuvant (Inflamed) and Control. On day 0, under anesthesia, Inflamed rats were injected with 0.2 mL of 50 mg/mL Mycobacterium butyricum suspended in squalene (adjuvant) and Control rats received normal saline into the base of the tail. The pharmacodynamic and pharmacokinetic experiments were conducted within 15 days post adjuvant injection and before the emergence of the physical signs of adjuvant arthritis (11). Each of the two groups was further divided into 2 subgroups: Inflamed/propranolol (n=8), Control/propranolol (n=7), Inflamed/nebivolol (n=5) and Control/nebivolol (n=5).

3.2.3 Pharmacodynamics

On day 15, rats were anesthetized and teflon-coated ECG electrodes were implanted subcutaneously into the left and right axial regions. On the following day the baseline (pre-propranolol/nebivolol) PR and RR interval were recorded using Acknowledge 3.01 data acquisition system (BIOPAC Systems Inc. Santa Barbara, CA). Subsequently, either oral doses of 25 mg/kg racemic propranolol

HCL(in water) or 2 mg/kg nebivolol HCL(in polyethylene glycol 400) were administered via gavage and PR and RR intervals recorded at 15, 30, 45, 60, 90, 120, 150, 180, 210 and 240 min. For the nebivolol group, an additional recording was made at 360 min. ECG response was recorded as the mean of 6 consecutive cycles for both PR and RR intervals. PR interval was considered from the beginning of the P-wave to the beginning of QRS complex.

3.2.4 Pharmacokinetics

On day 16, rats were anesthetized and fitted right side with a jugular vein polyethylene catheter tipped with silastic tubing that was externalized dorsally in the interscapular region. On day 17 and after 12 h fasting, rats received either 25 mg/kg of oral racemic propranolol HCL or 2 mg/kg oral racemic nebivolol HCL. Serial blood samples (200 μL) were collected at 0, 30, 60, 120, 180, and 240 min post propranolol dosing and up to 24 h post nebivolol dosing. Catheters were flushed with 10 IU/mL heparinized saline after sampling. The plasma was separated and stored at −80° C until they were analyzed for propranolol and nebivolol.

3.2.5 Target Proteins density

Another two groups of rats (Control; Inflamed; n=4/group) were used to evaluate the level of myocardial β1, β2 and β3-AR in control and inflamed rats. On day 14

post adjuvant/normal saline injection and before the emergence of the physical signs of adjuvant arthritis, under anesthesia, incisions were made; hearts collected and immediately were frozen in liquid nitrogen and stored at -80° C until analyzed.

3.2.6 Propranolol HPLC assay

A previously published stereospecific assay was used to assess rat plasma propranolol concentrations (21). To 100 µL of plasma, 25 µL of internal standard (bupranolol HCL, 10 µg/mL in distilled water) and 250 µL of 0.2 M NaOH were added. The mixture was vortexed for 30 s using 5 mL of diethyl ether. Test tubes were dipped into acetone in dry ice and the organic layer was poured into clean test tubes. The organic layer was evaporated to dryness using a Savant Speed Vac concentrator–evaporator (Emerston Instruments, Scarborough, Canada). The residue was derivatized with 185 µL of 0.02% (S)-(+)-1-(1-Naphthyl)-ethyl isocyanate (Sigma– Aldrich, USA) in 50:50 hexane: chloroform followed by vortex-mixing for 60 s. The diastereomeric samples were separated using HPLC. The mobile phase, hexane: chloroform: methanol 75:25:0.45 was pumped at 2 mL/min through a 25 cm×4.6 mm stainless steel Partisil 5 (Phenomenex) silica column. The HPLC system consisted of Waters 501 pump (Millipore-Waters, Mississauga, Canada), a Shimadzu SIL 9A auto injector (Shimadzu, Japan), and a Shimadzu CR601 Chromatopac integrator (Shimadzu, Japan). The detector was a Waters 474 scanning fluorescence detector (Millipore-Waters, Mississauga, Canada) and was set at 280 nm for excitation and 340 nm for emission.

Calibration curves were linear for the propranolol enantiomers over the 12.5–10000 ng/mL range (r2>0.99; coefficient of variation <20%).

3.2.7 Nebivolol assay

All experiments were conducted using an achiral assay. However, near the completion of the work we developed a chiral assay that was used briefly to qualitatively examine the possibility of stereoselectivity in the effect of inflammation on nebivolol pharmacokinetics. Nebivolol is available as the racemate consisting of D- (+SRRR) and L- (–RSSS) isomers.

For both procedures, to 100 µL plasma, 150 µL of internal standard (verapamil HCL 1µg/mL) and 50 µL NaOH (0.1M) were added. The mixture was vortexed for 30 second and then 4 mL mixture of diethylether and dichloromethan (70/30) were added and vortexed for 3 min. The samples were centrifuged at 2000g for 10 min and the organic phase layers were separated by using acetone in dry ice and transferred to clean tubes and evaporated to dryness. The residual was used to analyze nebivolol using one of the assays described below.

3.2.7.1 Achiral

The residues were reconstituted in120 µL mixture of the starting mobile phase and 100 µL injected into an HPLC equipped with a Phenomenex C18 (100mm X 4.6 mm, id 3µm) column and a fluorescent detector that was set at excitation and emission wavelengths of 284 and 310 nm, respectively. The mobile phase

consisted of 0.1% glacial acetic acid in HPLC-grade water (A) and acetonitrile (B). A gradient elution was programmed to commence with 40% B for 25 min post-injection followed by gradual increase in 5 min of B to 90%. The composition was maintained for 5 min before it was decreased back to 40% of B in 5 min. The flow rate was 1 mL/min. Standard curves were linear over the range of 5–1000 ng/mL ($r2>0.99$; coefficient of variation <20%).

3.2.7.2 Chiral

The residues were reconstituted in120 μL mixture of IPA/Hexane (15/85) and injected 100 μL into the HPLC at flow rate of 0.5 mL/min. Peaks were resolved using a Phenomenex Lux 3u Amylose-2 (150 mm X 4.6 mm) column and detected at excitation and emission wavelengths of 284 and 309 nm, respectively. Standard curves were linear over the range of 2–1000 ng/ mL. Only sample of control and inflamed groups containing the maximum nebivolol concentrations as determined by our achiral assay were tested.

3.2.8 Western blot analysis

Western immunoblot was used to determine the β1, β2 and β3 subunits of β-AR protein levels in the heart. β-actin was used as a loading control. Whole heart was triturated in the presence of liquid nitrogen. Approximately 75 mg of ground heart was diluted 1 in 5 with a buffer (0.05 M tris buffer pH 7.4, 2% protease cocktail inhibitor) followed by 1 min of trituration on ice. Following centrifugation, the

supernatant was kept and assessed for protein concentration using the Lowry method. Heart protein samples (loading, 50 μg) were prepared with Lane Marker Reducing Sample Buffer and put into boiling water for 5 min. Proteins were separated using SDS-polyacrylamide electrophoresis on a 7.5% gel for 1 h at 200V. The resultant separation was transferred to a nitrocellulose membrane. To block the nonspecific binding, the membrane was incubated with 2% bovine serum albumin, 5% skim milk and 0.05% Tween 20 in Tris-buffered saline for 2 h at room temperature. Subsequently, the membranes were incubated with the primary antibody diluted with the blocking solution at 1:1000, 1:100 and 1:250 for β1-AR, β2-AR and β3-AR, respectively. The dilution of 1:1000 in 0.05% Tween 20 in tris-buffered saline was applied for mouse monoclonal to β-actin (Abcam Inc., Cambridge, MA, USA). Horse radish peroxidise-conjugated secondary antibodies (goat anti-mouse IgG antibody; Bio-Rad Laboratories), were diluted 1:15000 in 0.05% Tween in tris-buffered saline and incubated at room temperature for 1 h. Secondary antibodies were visualized using chemiluminescense (ECL Western Blotting Detection reagents; Bio-Rad Laboratories) and captured using Kodak BioMax Light Film (Sigma-Aldrich, St. Louis). Bands were assessed by densitometry using ImageJ software (National Institute of Health, Bethesda, MD). The level of the proteins of interest was corrected for loading variations using β-actin.

3.3 Data analysis

All data are presented as mean±standard deviation. Plasma propranolol enantiomer concentrations and nebivolol were plotted vs. time and the area under plasma concentration– time curve (AUC) was measured using the trapezoidal rule. Maximum plasma concentration (Cmax), the highest observed value and the time of its attainment (Tmax) were recorded.

For PR and RR intervals, the percent changes from the baseline were calculated and plotted vs. time. The area under the % change–time (AUEC, % min) was calculated using the trapezoidal rule. Percent maximum effect from baseline was the highest observed value.

Data were evaluated for homogeneity of variances using an F-test before using the two-tailed Student's t-test at $p<0.05$.

The observed percent changes in PR and RR interval values were plotted versus corresponding plasma nebivolol concentrations. The two measurements were made using the same animal but one day apart as stated in the protocol.

3.4 Results

3.4.1 The emergence of inflammation and confirmation of its effect on propranolol

Rats that received Mycobacterium butyricum exhibited significantly increased paw thickness in 8-10 days post-adjuvant injection. The emergence of pre-adjuvant arthritis was associated with erythema and reduced weight gain (11).

As expected (8,9), inflammation caused a slow-down of propranolol clearance and resulted in increased plasma concentrations of both S and R propranolol (Figure 3-1). The AUC values increased 3.3- and 7.6-fold for S- and R-propranolol, respectively (Table 3-1). Propranolol significantly prolonged PR interval and reduced heart rate (prolonged RR) in both control and inflamed rats. However, inflammation resulted in a significant reduction in response (Figure 3-2-3-4). The reduced response was associated with a down-regulation of the β1 target protein (Figure 3-5).

3.4.2 Nebivolol pharmacokinetics and pharmacodynamics

Inflammation failed to influence pharmacokinetics of nebivolol (Figure 3-1, Table 3-1). The chiral assay also suggests no significant effect of inflammation on the enantiomers as the ratio of the peak representing the first enantiomer over that of the second one was 1.23±0.61 and 0.95 ±0.50 for the control and inflamed groups, respectively.

The drug prolonged PR interval and reduced heart beats in all animals. In response to nebivolol, there was no significant difference in the measured ECG metrics between control and inflamed groups (Figures 3-2-3-4).

Figure 3- 1. Mean plasma concentration versus time curves following single oral doses of 25 mg/kg propranolol (n=7-8) or 2 mg/kg nebivolol (n=5) in control and inflamed rats. Error bars are standard deviation and are shown one-sided for clarity.

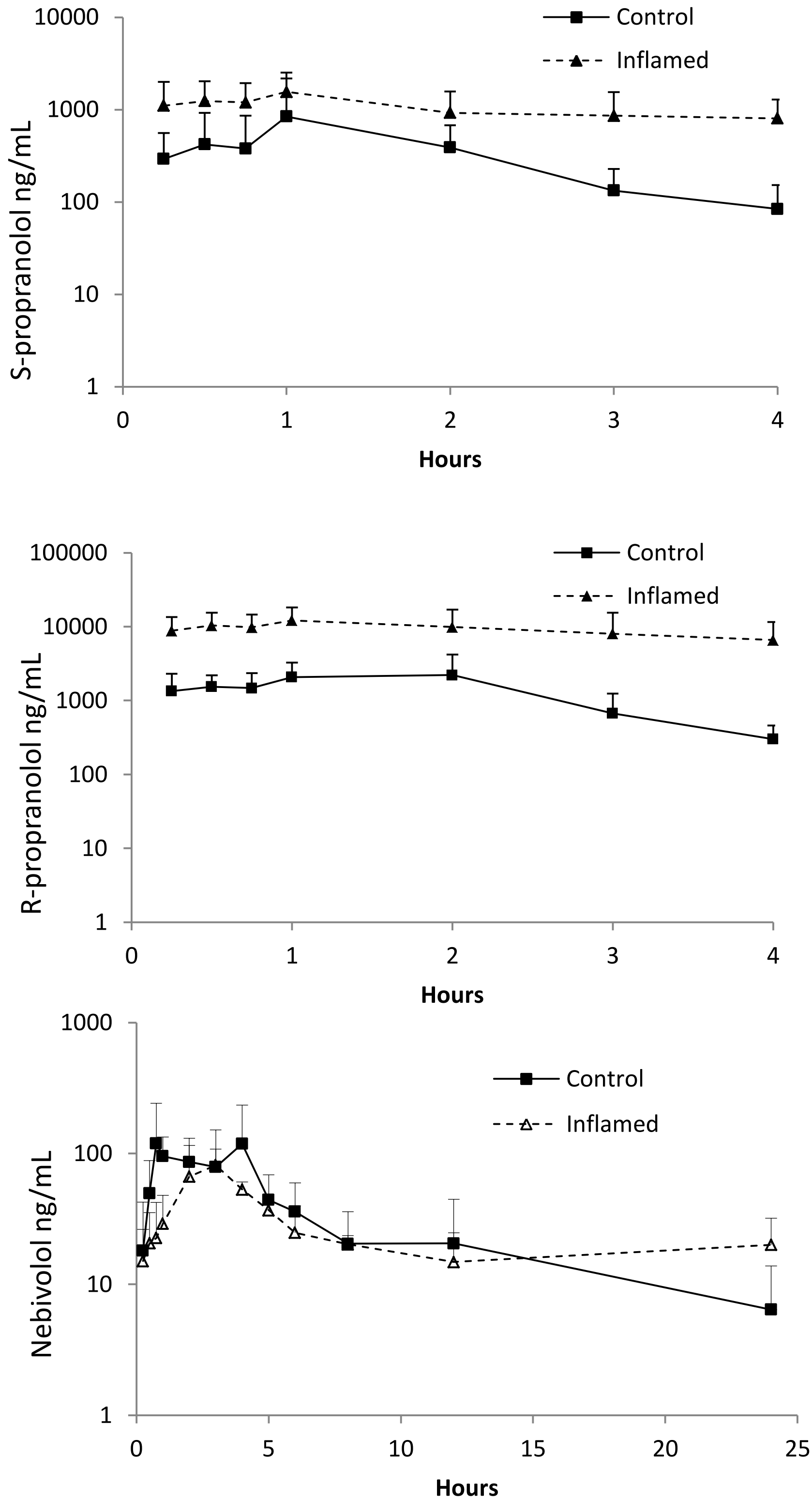


Table 3- 1. Pharmacokinetic and pharmacodynamic parameters after oral doses of 2 mg/kg nebivolol (n=5) or 25 mg/kg propranolol (n=7-8).

	Nebivolol		Propranolol			
	Control	Inflamed	Control		Inflamed	
n	5	5	7		8	
			S	R	S	R
Tmax, h	3.4±2.8	2.8±1.3	0.9±0.8	1.0±0.6	0.8±0.2	0.8±0.2
Cmax, ng/mL	176±69	96±53	390±200	3016±1464	1705±907*	13971±5485*
AUC, ng.h/mL	717±296	577±221	1175±1395	4682±2578	3986±2515*	35580±22151*
PR interval, Max% change	22.9±12.6	27.4±6.2	26.5±16.5		12±10.9*	
Heart beat, Max %change	9.9±7.8	19.4±4.3	14±8.6		4.3±3.4*	

*Significantly different from Control at α=0.05

Figure 3- 2. PR prolongation in control and inflamed rats following single oral doses of 25 mg/kg propranolol (n=7-8/group) or 2 mg/kg nebivolol (n=5/group). Error bars are standard deviation and are shown one-sided for clarity.

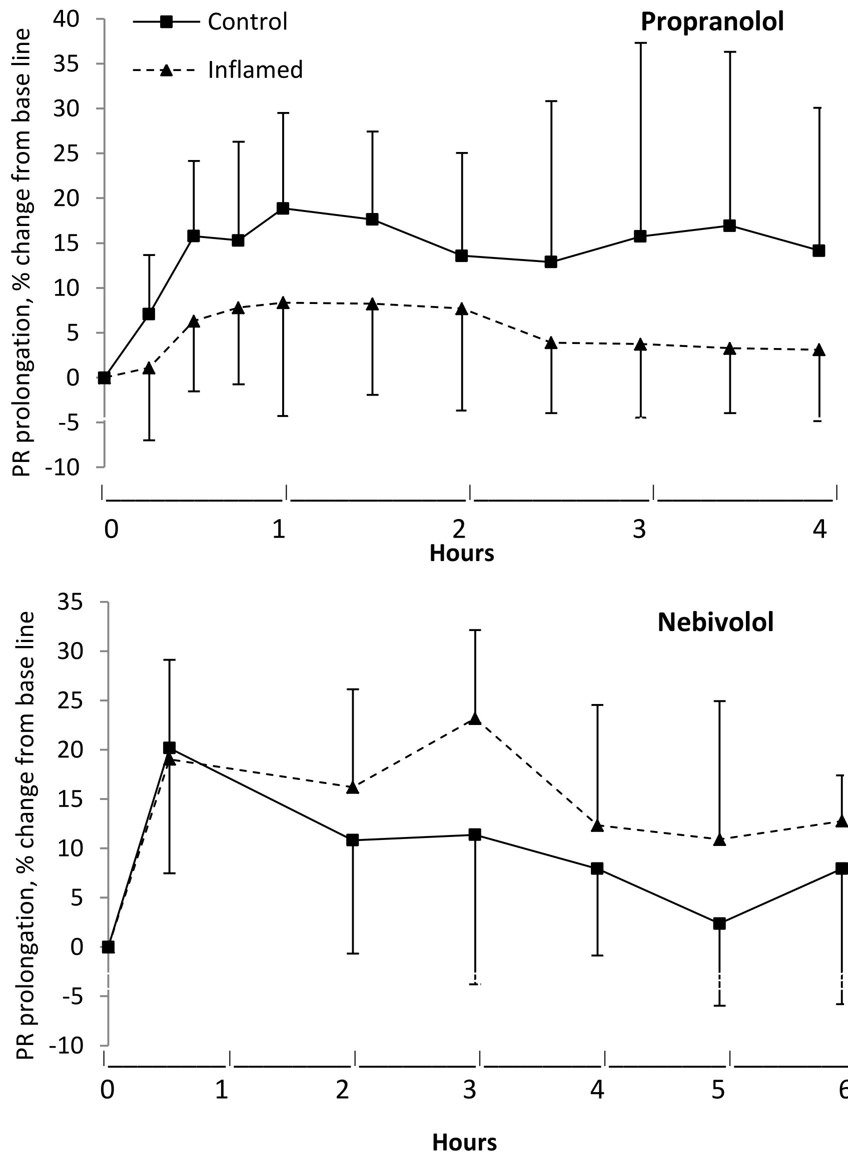


Figure 3- 3. Reduced heart rate in control and inflamed rats following single oral doses of 25 mg/kg propranolol, (n=7-8/group) or 2 mg/kg nebivolol (n=5/group). Error bars are standard deviation and are shown one-sided for clarity.

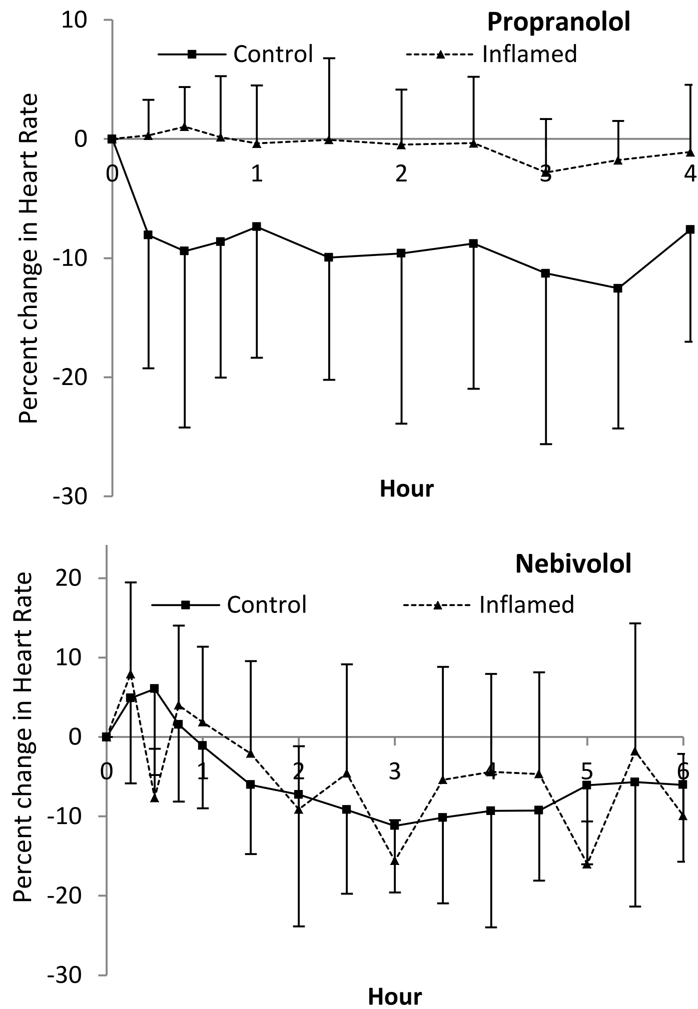


Figure 3- 4. The area under the effect curve (AUEC, %.min from baseline) following single oral dose of 2 mg/kg nebivolol and 25 mg/kg propranolol. Error bars are standard d of means (n=7-8/group for propranolol and 5 for nebivolol); *P<0.05vs Control.

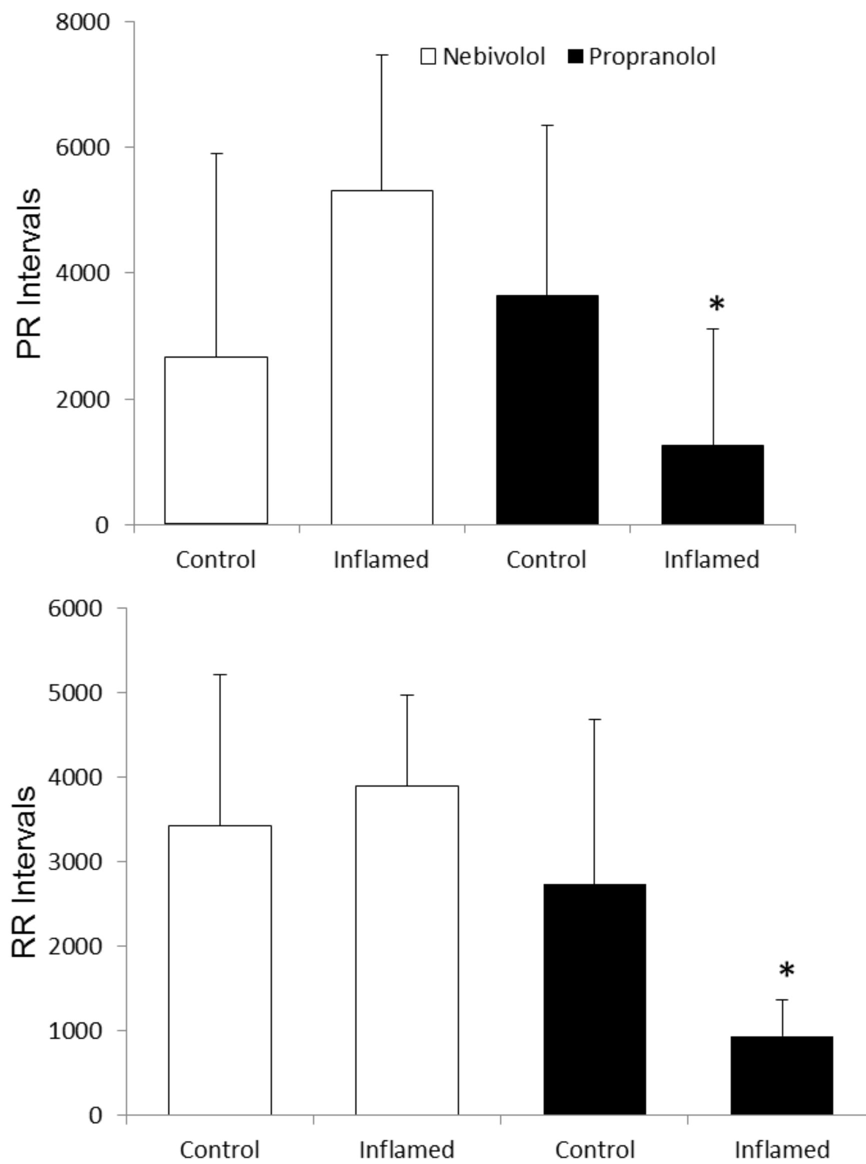
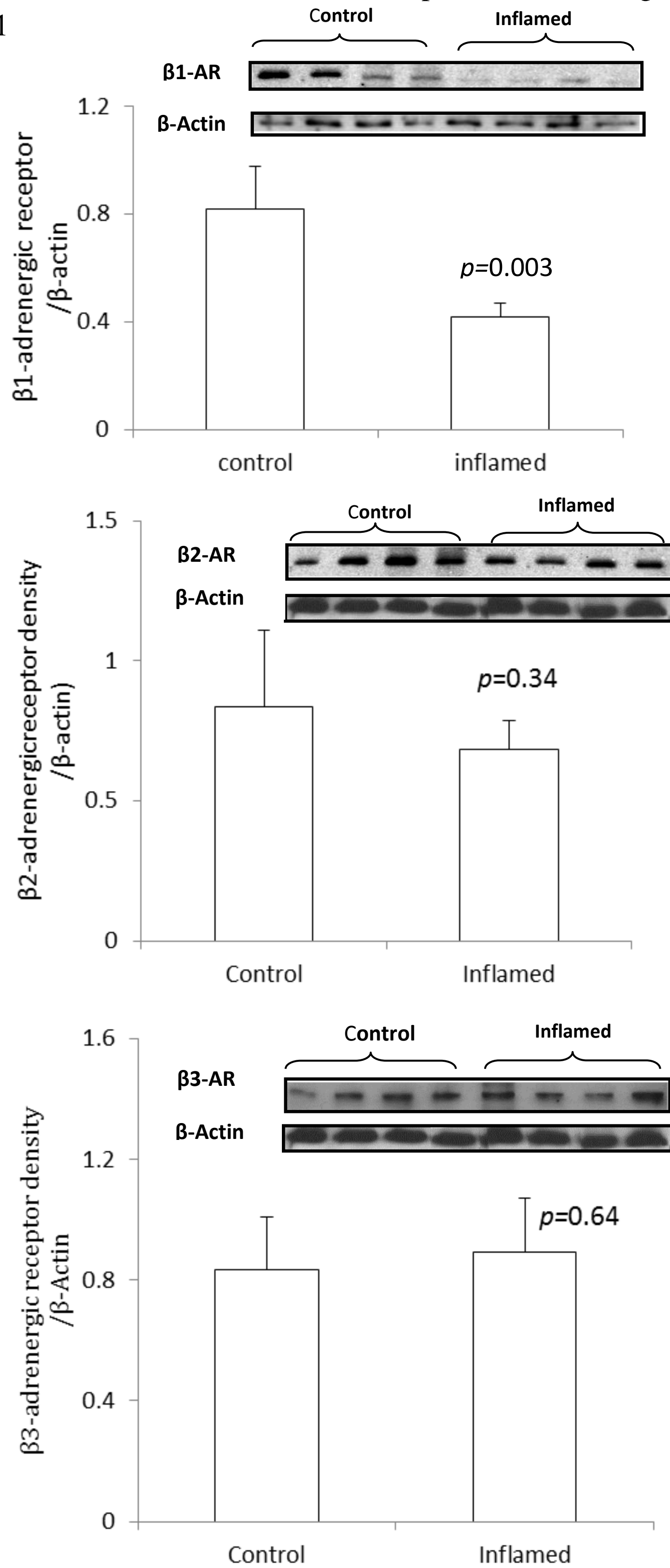


Figure 3- 5. Mean photometric densities of the β1- (51 kDa), β2- (55 kDa) and β3- (43 kDa) adrenoceptors relative to β-actin (42 kDa) in the rat heart. Error bars are standard deviation of means (n=4/group); Inflamed significantly different only for β1



3.4.3 Target proteins

As depicted in Figure 3-5, the relative density of β1-AR protein in the rat hearts was significantly reduced in inflamed animals as compared with the control rats. The β2-AR and β3-AR, on the other hand, were not significantly influenced by inflammation.

3.5 Discussion

We used propranolol as a positive control and, as expected, it confirmed that inflammation alters both the pharmacodynamics and pharmacokinetics of the drug (Figures 3-1-3-4; Table 3-1). This has been reported to be due to down-regulation of both the myocardial β1-AR level (7,8) and cytochrome P450, respectively (11); i.e., reduced pharmacological response despite increased concentration. Interestingly, however, the observed down-regulation of β1-AR was not associated with a reduced response to nebivolol (Figures 3-2-3-4; Table3-1), another β1-AR blocker (17-20). This can be explained by the fact that, in addition to the blocking of β1-AR, nebivolol is reported to exert its vasodilatory effect through endothelial β2-adrenergic receptor–mediated NO production and also inhibition of NO synthase uncoupling (22). It also possesses an agonistic effect on a relatively newly discovered receptor, β3-AR (17,18,20). β3-ARs which are located in the cardiomyocytes are involved in the metabolic effects of sympathetic stimulation (23). The stimulation of β3-ARs in human endomyocardial and

ventricle results in negative inotropic effect through activation of Gi/0 proteins and stimulation of the NO pathway. Furthermore, a stimulation of β3-AR in the peripheral vessels and in the heart seems to enhance endothelial NO synthase and increased NO causing vasodilatation (24-30). It has also been suggested that a metabolite of nebivolol may have NO-generating properties through activation of β2-AR (31). In addition, nebivolol has a complementary anti-oxidative stress activity which results in decreased NO degradation (25-32).

The observed consistency of the response to nebivolol despite the presence of inflammation and down-regulation of β1-AR may be suggestive of the predominance of β2 and β3-AR in the overall effect of the drug and/or a compensation mechanism that shifts the function from one receptor to another. Indeed, we are reporting, for the first time, that, under our experimental conditions, the level of β2- and β3-AR remains unaffected by inflammation despite the observed down-regulation of β1-AR (Figure 3-5). Our data present a complicated scenario for a drug that blocks two β-ARs and stimulates another one.

Despite the observed down-regulation of one of the three target proteins the measured pharmacodynamic outcome remains unaffected by inflammation. This highlights the importance of mechanisms other than β1-AR that are involved in the action of nebivolol. It is worthy of mentioning that, it has been reported that in

some inflammatory conditions such as diabetes (33), sepsis (34), human failing myocardium (34,35) and human with heart failure (27), β3-ARs are up-regulated.

In the treatment of cardiovascular conditions, nebivolol may have a unique position as its effects are not influenced by inflammation based on the present observation in the rat and that reported earlier in obese humans. Cheymol et al (36) have reported that in humans, obesity has no significant effect on the pharmacokinetics and pharmacodynamic of the drug except for a minor influence on the volume of distribution. Considering the association of obesity and inflammation, and the fact that the condition results in reduced response to drugs such as calcium channel blockers (15,16), the finding of Cheymol et al that is in line with the present data, gains a more general significant therapeutic relevance; i.e., nebivolol seems to be effective even in the presence of inflammatory conditions. This is important because inflammatory conditions such as arthritis increase the risk of cardiovascular complications (1,2).

It has been shown that fasting can influence adrenergic activity of human adipocytes (37). To obviate the potential effect of food on the absorption of the administered drugs, we withheld food for 12 h pre-dose and 6 h post-dose. Although the effect of food withdrawal on the cardiac β-adrenergic target protein is unknown, in the present study we included control rats in all of our studies to account for any potential interfering issue. In addition, we have observed the reduction of response to propranolol due to inflammation under both fasted (present work and (9) and fed (8) conditions.

Inflammation is known to slow-down clearance of drugs such as propranolol that are efficiently metabolized by the liver (Table 3-1, Figure 3-1).There is no report of nebivolol metabolism in the rat. In humans, however, the CYP2D6 system appears to be involved in nebivolol metabolism and extensive first-pass metabolism has been speculated (38). Metabolism of propranolol in both human and rats also involves CYP2D6 (39). Inflammation reduces clearance of propranolol as we have confirmed herein. Interestingly, on the other hand, pharmacokinetics of nebivolol appear unaffected by inflammation. This suggests that, at least in the Sprague-Dawley rat, nebivolol mode of clearance is different from those drugs that are known to be influenced by inflammation. Our data in the rat is in agreement with those that suggest human obesity, a condition that is associated with inflammation (14,15), does not influence nebivolol pharmacokinetics (36). This will only be clarified with more detail pharmacokinetic studies.

The assay used to delineate the effect of inflammation on the pharmacokinetics of nebivolol was achiral despite the fact that racemic drug was used. In the absence of stereospecific data, however, one cannot rule out the possibility of a stereoselective effect that may be masked when the assay does not differentiate between the enantiomers (40). Our brief chiral assay carried out on only one sample per rat rules out the possibility of a stereoselective effect of inflammation on nebivolol plasma concentrations. It should be mentioned that the chiral column used in our assay exhibited a short functional life as the peaks lost their resolutions upon a change in the mobile phase.

When the percentage changes of PR intervals were plotted against the nebivolol plasma concentration, a trend towards a positive relationship between the two parameters was observed. We could, however, make no equivocal conclusion as to the nature of the relationship except that, in some cases, it appeared as clockwise hysteresis; i.e., a loss of efficacy with time an indicative of acute tolerance as has been reported for the β2-AR efficacy of broncodilators (41). This needs further investigation.

Limitations of the present study include: 1) the uncertainty of the suitability of the animal model used for the pharmacokinetic studies of nebivolol; 2) the lack of complete stereochemical analysis of nebivolol pharmacokinetics; and 3) the single dose nature of the pharmacodynamic experiments.

3.6 Conclusions

Pharmacodynamics of nebivolol are not influenced by inflammation despite a significant down-regulation of β1-adrenergic target protein; a characteristic different from other reported β-adrenergic receptor and calcium channel antagonists. This may suggest predominance of β3 adrenergic target proteins under the examined conditions. Pharmacokinetics are also unaffected by inflammation despite its poor bioavailability suggesting mechanisms other than first pass metabolism for its loss following oral doses. If our observations are translated to human inflammatory diseases, nebivolol presents an alternative to

β1-blockers for which reduced response and lower oral clearance under inflammatory conditions have been suggested.

3.7 Post submission notes

Bertera et al (42) have reported stereoselectivity in the pharmacokinetics of nebivolol in simultaneously hypertensive and Wistar Kyoto rats. They also have reported that former has a lower clearance for both enantiomers than the latter strain of rats.

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Chapter 4 Pharmacokinetics of Nebivolol in the Rat: Low Oral Absorption, Loss in the Gut and Systemic Stereoselectivity [3]

4.1 Introduction

Infection or tissue damage may cause activation of the innate immune system and lead to inflammation. This phenomenon is a cascade of biochemical events and is related to release of pro-inflammatory cytokines, tumor necrosis factor alpha (TNFα) and interferons (IFN) as well as NO (1-3). Inflammatory conditions are associated with pathophysiological changes such as hypoalbuminemia (4), α1-acid glycoprotein elevation (5) and hepatic drug metabolism reduction (6,7). Inflammation increases binding of basic drugs to α1 acid glycoprotein (5), downregulates the majority of liver cytochrome P-450 enzymes and reduces hepatic blood flow. These changes can lead to decreased drug clearance and increase plasma drug concentration as well as drug toxicity (1,7,8). Altered drug disposition during some inflammatory conditions due to changes in drug metabolism and protein binding has been reported (9).

Previous studies have shown that inflammation alters pharmacokinetics of some drugs which are efficiently metabolized by the liver such as β-AR antagonists (10-12) and calcium channel blockers (13-15).

[3] A version of this chapter has been published. Forugh Sanaee, Daniel Valente Neves, Vera Lucia Lanchote and Fakhreddin Jamali. Biopharm Drug Dispos. 2013 Sep;34(6):312-20

Hence, we were surprised to find out that the oral clearance of nebivolol, a third-generation beta-blocker that is suggested to undergo extensive hepatic first-pass metabolism, was not influenced by inflammation (16). We, therefore, hypothesized that the loss of nebivolol after oral administration is due to mechanisms other than those reported for other drugs with extensive hepatic first-pass metabolism.

Accordingly, the objectives of this work were to first evaluate the pharmacokinetics of nebivolol following various routes of administration and to investigate whether intestinal metabolism also contributes to the first-pass metabolism of the drug; and second, to investigate the possibility of stereoselectivity in the pharmacokinetics of nebivolol. Nebivolol, a racemate of (+) and (-) enantiomers (also known as d and l, respectively), is a highly selective β1-AR antagonist and β3-AR agonist (17-19).

4.2 Material and methods

4.2.1 Chemicals

Racemic and R (+) verapamil HCL were purchased from Sigma Chemical Co. (St. Louis, MO). Racemic nebivolol HCL was gift from CIP Global Co, LTD (Jinana, China). High performance liquid chromatography (HPLC) grade diethylether, triethylamine and acetonitrile were purchased from Caledon Laboratories (Georgetown, Canada). HPLC grade dichloromethane was purchased from Fisher

Scientific (Nepean, Ontario). Jugular vein catheters were constructed from PE-50 polyethylene (0.58 mm i.d. x 0.965 mm o.d.) purchased from Clay Adams (Parsippany, NY) and silastic tubing purchased from Dow Corning Corporation (Midland, MI).

4.2.2 Animals

The study was approved by the Health Sciences Animal Policy and Welfare Committee of the University of Alberta. Adult male Sprague–Dawley rats were used for pharmacokinetic experiments. The animals were housed in a 12 h light/dark cycle and always had free access to water, although food was withheld for 12 h prior to drug dosing and 6 h afterwards.

Rats were divided into 3 groups (n=6-10/ group). For i.v. drug administration and blood sampling, animals were cannulated in the right jugular vein and silastic catheters were implanted under anesthesia (20,21).

4.2.3 Effect of routes of administration on pharmacokinetics

Single doses of 1 mg/kg i.v. or intraperitoneal (i.p.) (n=6-8/group) or 2 mg/kg oral (n=10, via gastric gavage) were administered. Following i.v. injection, the cannula was washed with normal saline before the first blood sample collection. Serial blood samples (200 μL) were collected prior and at 0.08, 0.25, 0.5, 0.75, 1, 3, 6, 12 and 24 h post-dose. Plasma was separated and kept at -20° C until it was analyzed for drug content. Individual samples were analyzed for nebivolol

concentration using an achiral HPLC method. For the analysis of individual enantiomers, equal aliquots of the remaining samples collected at any given time were pooled and assayed using a chiral assay. The pooling was necessitated due to the limitation of the sample size used for the chiral assay.

4.2.4 Transport study in everted intestinal sacs

As described previously (22), rats (n=3) were anesthetized and abdominal incisions were made. Three 10 cm segments of intestine below the pyloric sphincter were removed. Subsequently the animals were euthanized. The segments were everted using a glass rod after rinsing with cold Krebs-Henseleit bicarbonate buffer (119 mM NaCl, 4.75 mM KCl, 2.54 mM CaCl2, 1.19 mM KH2PO4, 1.19 mM MgSO4, 25 mM NaHCO3, and 11 mM Dextrose, pH 7.4). One end of the everted sac was ligated with silk thread and a silastic catheter (0.58 mm i.d. x 0.965 mm o.d.) was inserted into the other end and tied. The segments were filled with Krebs-Henseleit bicarbonate buffer and placed in perfusion chambers containing 10 μg/mL of nebivolol HCl in the same buffer (total volume 40 mL) which was continuously aerated with O2/CO2 (95/5) at 37°C. Aliquots of 0.5 mL from both serosal and mucosal solutions were collected at 0, 15, 30, 45, 60 and 90 min and the fluid lost was replaced by the same volume of buffer. Samples were analyzed using both chiral and achiral assays.

4.2.5 Nebivolol protein binding

One milliliter of serum from rats was adjusted to pH 7.4 with 0.1 N HCl. The serum was spiked with 900 ng/mL racemic nebivolol to approximate the serum concentrations of nebivolol in rats after i.v. dosing. The serum was incubated at 37°C for 1 h before being transferred to Centrifree Ultrafiltration Devices (Merck Millipore Ltd, Germany) for ultrafiltration at 2000g for 1 h. Filtrate and nonfiltrate nebivolol enantiomer concentrations were measured by HPLC. The fraction unbound, *fu*, was determined as *fu* = Cu/Ct, where Cu is the unbound concentration and Ct is the total concentration.

4.2.6 Nebivolol HPLC assay

A Shimadzu Prominence HPLC system (Mandel Scientific, Guelph, ON Canada) consisting of a DGU-20A5 degasser, an LC-20AT pump, a SIL-20A autosampler, a CTO-20AC column oven, an RF-10AxL fluorescence detector and a CBM-20A communication bus module was used. The integration was performed using Shimadzu Class- VP 7.4 software.

4.2.6.1 Achiral method

In 15 mL dry tubes, to 100 µL plasma, 150 µL of internal standard (racemic verapamil HCl, 1µg/mL) and 50 µL NaOH (0.1M) were added. The mixture was vortexed for 30 second followed by addition of 4 mL of a mixture of diethylether

and dichloromethane (70/30). Tubes were vortexed for 3 min and centrifuged at 2000 g for 10 min. Using acetone/dry ice, the aqueous layer was frozen and the organic phase was separated, transferred to clean tubes and evaporated to dryness. The residues were reconstituted in a 120 µL mixture of water/acetonitrile (50/50) and aliquots of 100 µL were injected into the HPLC system with a mobile phase flow rate of 1 mL/min. Peaks were resolved using a Phenomenex C18 (100 mm X 4.6 mm, id 3 µm) column guarded with a Phenomenex Security Guard Cartridge C18 (4mm x 3 mm) column. (both purchased from Phenomenex) and detected at excitation and emission wavelengths of 284 and 310 nm, respectively. The mobile phase consisted of 0.1% glacial acetic acid in HPLC-grade water (A) and acetonitrile (B). A gradient elution was programmed to commence with 40% B for 25 min post-injection followed by a gradual (5 min) increase of B to 90%. The composition was maintained for 5 min before it was decreased back to 40% B over 5 min. R-verapamil and nebivolol appeared 11.1 and 12.5 min post-injection, respectively. Standard curves were linear over the range of 5–1000 ng/mL ($r2>0.99$; coefficient of variation $<20\%$); stability after freezing twice was $>95\%$.

4.2.6.2 Chiral method

To 100 µL pooled plasma, 25 µL internal standards (R-(+) verapamil 400 ng/mL in methanol), 50 µL NaOH (0.1M) and a 4 mL mixture of diethylether and dichloromethane (70/30) were added and vortexed for 3 min. Samples were

centrifuged at 2000 g for 10 min and immersed into acetone/dry ice. The organic layers were separated, transferred into clean tubes and evaporated to dryness. The residues were reconstituted in 120 µL of mobile phase and aliquots of 100 µL were injected into the HPLC system with a mobile phase flow rate of 1 mL/min. Peaks were resolved using a Chirobiotic V (25 cm X 4.6 mm 5 µm) column and detected at excitation and emission wavelengths of 290 and 304 nm, respectively. The mobile phase consisted of methanol 99.9%, 0.075% glacial acetic acid and 0.025% triethylamine. Standard curves were linear over the range of 10–1000 ng/mL/enantiomer ($r2>0.99$; coefficient of variation <20). The retention times of the enantiomers were recorded by injecting stereochemically pure compounds. (-)-nebivolol, (+)-nebivolol and R-verapamil typically appeared 11.4, 12.3 and 15.2 min post-injection, respectively.

4.3 Data analysis

Data are expressed as mean±SD. The maximum plasma concentration (Cmax) and the time of its attainment (Tmax) were the experimentally observed values. The non-compartmental method was used to calculate pharmacokinetic indices. The area under the plasma drug concentration-time curve until the last experimental data point (AUC) was calculated using the linear trapezoidal rule. The terminal elimination rate constant was calculated for those rats that exhibited a log-linear terminal phase with at least 3 usable experimental points.

Despite a high level of fluctuation attempts were made to fit plasma concentration-time data into various compartmental models. Model discrimination was determined using Akaike's Information Criterion and goodness of fit (r2). The systemic availabilities were determined by dividing the area under the plasma concentration-time curves (AUC 0-24) calculated for the i.p. and p.o. over that of the i.v. route. For each animal, the mean residence time was obtained by dividing AUMC over AUC following different routes.

With a p-value of less than 0.05, the Student's t-test and the one-way ANOVA followed by Bonferroni as the post hoc were used to assess the statistical significance between two or more groups, respectively.

4.4 Results

The nebivolol bioavailability measured as AUC was significantly lower after p.o. but not following i.p. doses as compared with that observed following the i.v. route (Table 4-1 Figure 4-1, 4-2). There was a great level of fluctuation in most plasma concentration-curve patterns so that the slope of the log-linear portion of the plasma drug concentration curve was not calculated for all of the animals. Although we measured plasma concentrations up to 24 h, for some animals the values fell below the minimum quantifiable concentration (Figures 4-1 and 4-2) further rendering the estimation of pharmacokinetic parameters difficult. Nevertheless, for those where the slope was reliably estimated (n= 4-5/group),

Table 4- 1 . Pharmacokinetic parameters following different routes of racemic nebivolol administration estimated using an achiral assay.

	Tmax, h	Cmax, ng/mL	AUC, ng.h/mL	F	Vdss L/Kg	AUMC h^2.µg/mL	MRT h
		Corrected for 1 mg/kg dose					
i.v. (n=6)	nd	nd	869±456	1.00	2.02±1.13	2.04±0.56	4.74±2.51
i.p. (n=8)	3.2±2.7	239±257	726±675	0.75±0.7	nd	1.74±0.81	8.61±3.96
p.o. (n=8)	3.2±2.1	72.2±35.3*	330±132*	0.34±0.13*	nd	4.43±2.86	7.12±3.65

nd, not determined; *, significantly difference from other routes.

Figure 4- 1. Mean plasma concentration versus time curves of nebivolol following administration of 1 mg/kg i.v. or i.p. and 2 mg/kg p.o. racemic nebivolol. Error bars represent SD of the mean (n=8-10).

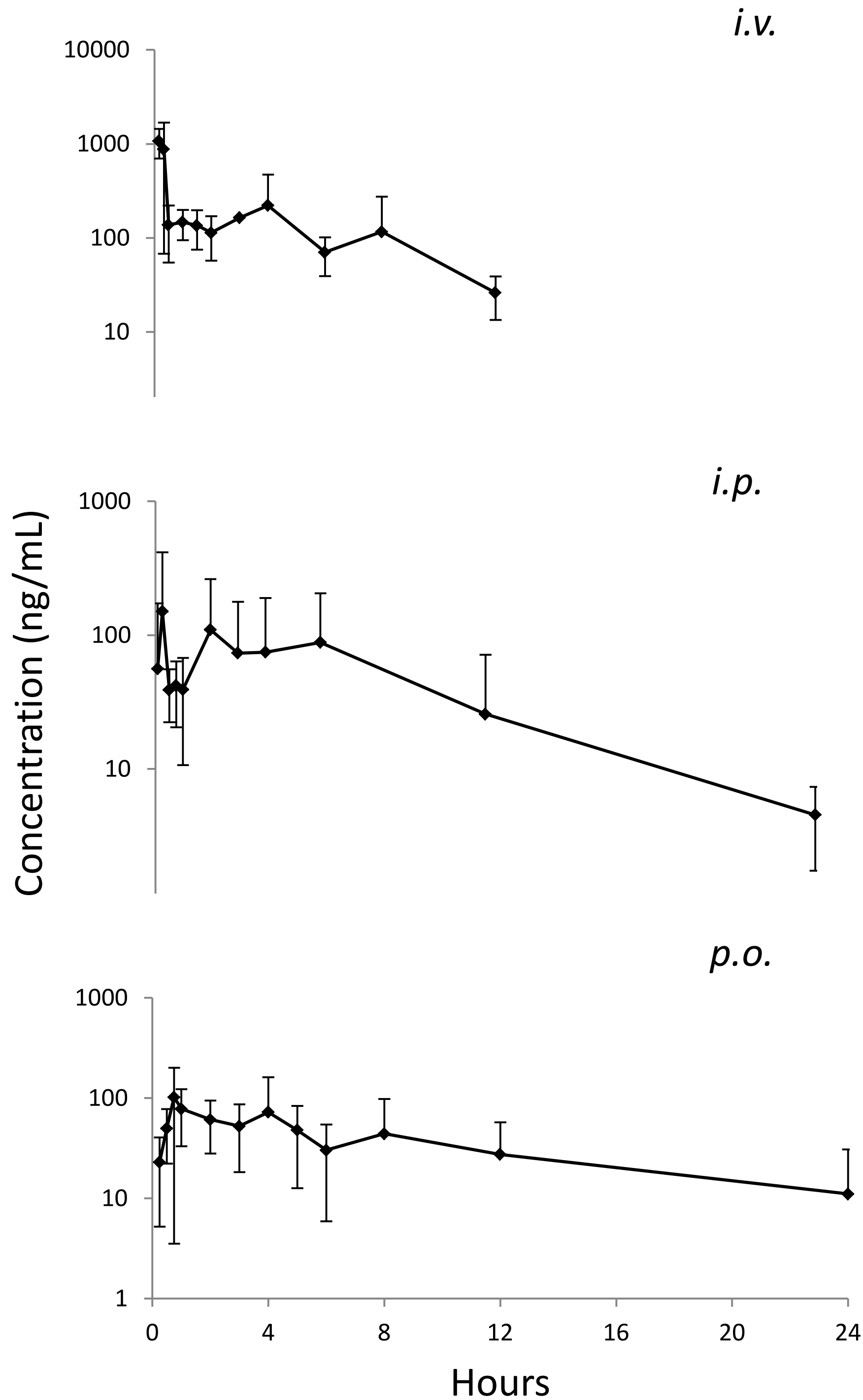
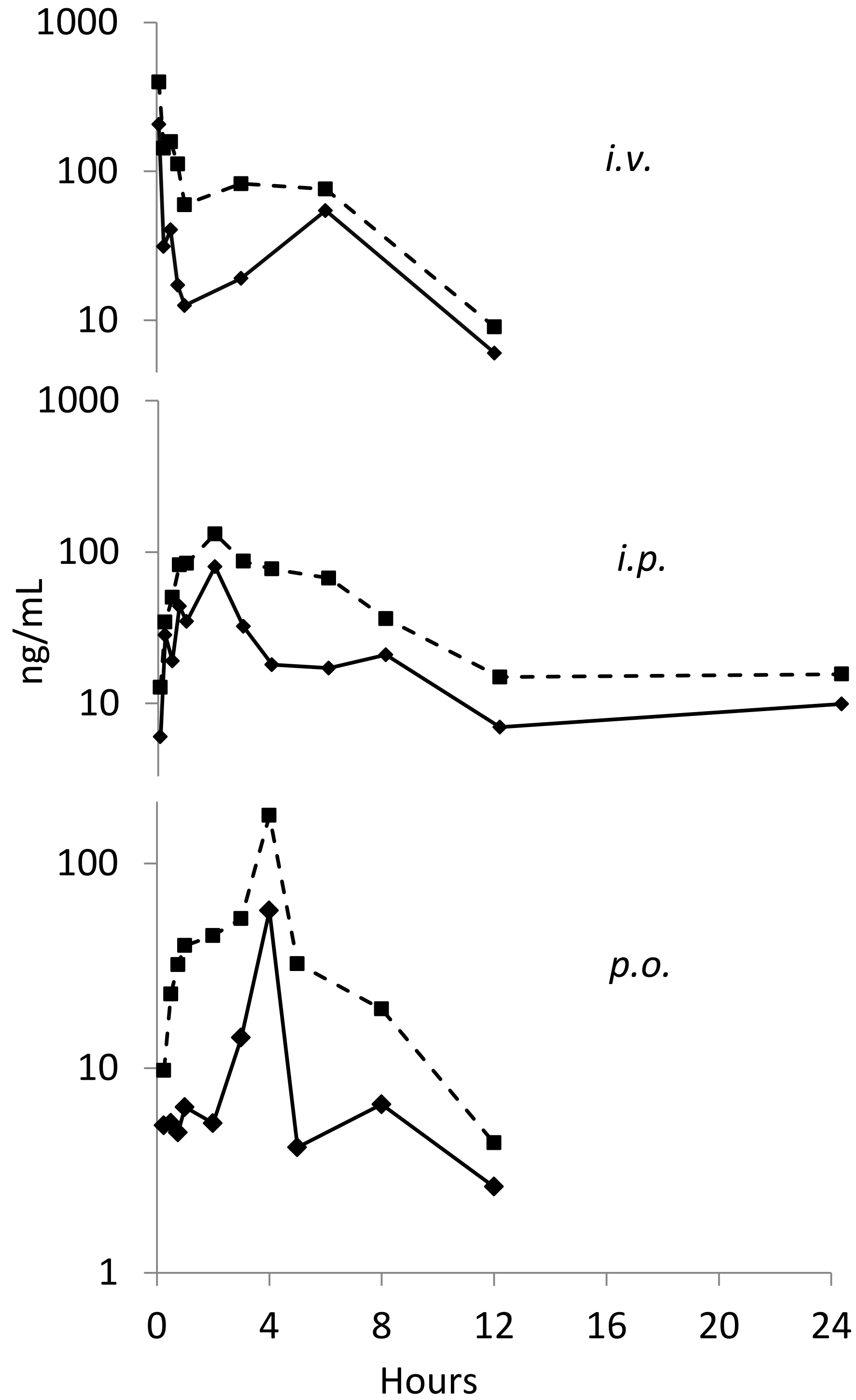


Figure 4- 2. Pooled mean plasma concentration versus time curves of (+)-(dashed lines) and (-)-(solid lines) nebivolol following administration of 1 mg/kg i.v. or i.p. and 2 mg/kg p.o. racemic nebivolol. Data generated by analyzing pooled data, hence, no variance is listed.



there was no significant difference in the t½ values following different routes (4.2±2.8 h for i.v., 7.0±4.3 h for i.p. and 4.0±3.2 h for oral).

Although Cmax values were greater following i.p. doses as compared with the oral doses, the differences did not reach statistical significance due to a high inter-animal variability (p=0.08; Table 4-1).

Nebivolol pharmacokinetics were reliably described by a two-compartment open model for the i.v. data. High levels of fluctuation observed following other routes of administration rendered modeling unreliable. The mean Nebivolol clearance (dose/AUC) following i.v. doses was 19mL/min/kg.

Following i.v., i.p. and oral doses, nebivolol pharmacokinetics were stereoselective as the AUC of (+)-nebivolol was higher than (-)-nebivolol (Table 4-2 Figure 4-2). In addition, the pooled mean values were consistently greater for (+) than (-) enantiomer further confirming the predominance of (+) over (-) in plasma.

Nebivolol concentration in serosal side and percent remaining in mucosal side over time are depicted in Figure 4-3.

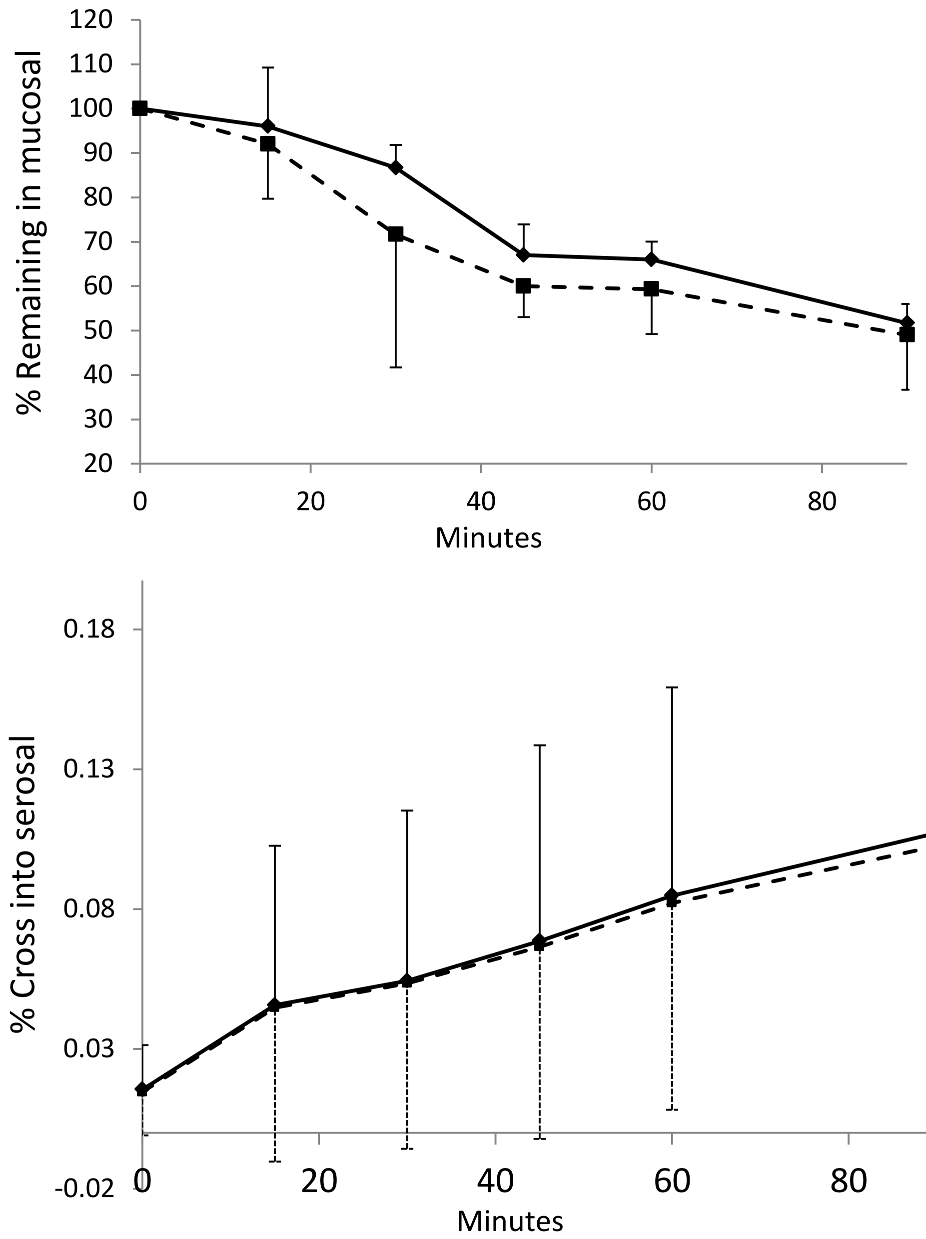
Upon 90 min incubation of racemic nebivolol in the presence of everted rat gut, only 49.0±4.4% of (+) and 51.7±12.3% of (-)-enantiomers remained in the mucosal side. This was accompanied by approximately 0.1% of nebivolol enantiomers crossing the 10 cm intestinal sacs (Figure 4-3). The concentration of nebivolol enantiomers did not demonstrate stereoselectivity over the examined time in either the serosal or the mucosal side. In contrast, the serum binding of

Table 4- 2. Stereoselective pharmacokinetic parameters following different routes of racemic nebivolol administration.

	(-)- nebivolol			(+)-nebivolol		
	Tmax h	Cmax ng/mL	AUC ng.h/mL	Tmax h	Cmax ng/mL	AUC ng.h/mL
i.v.(n=6)	nd	nd	448	nd	nd	909
i.p.(n=8)	2	79.9	395	2	132	885
p.o.(n=8)	5	29.4	58	5	85.7	205

Data generated by analyzing pooled data, hence, no variance is shown; doses: 1 mg/kg i.v. and i.p.; 2 mg/kg p.o.; AUC and Cmax corrected for 1 mg/kg dose.

Figure 4- 3. The time course of (+)- (solid lines) and (-)- (dashed lines) nebivolol in the rat serosal and mucosal everted intestinal sacs; (n=3). Error bars represent SD and, for clarity, are presented only on one side of the mean.

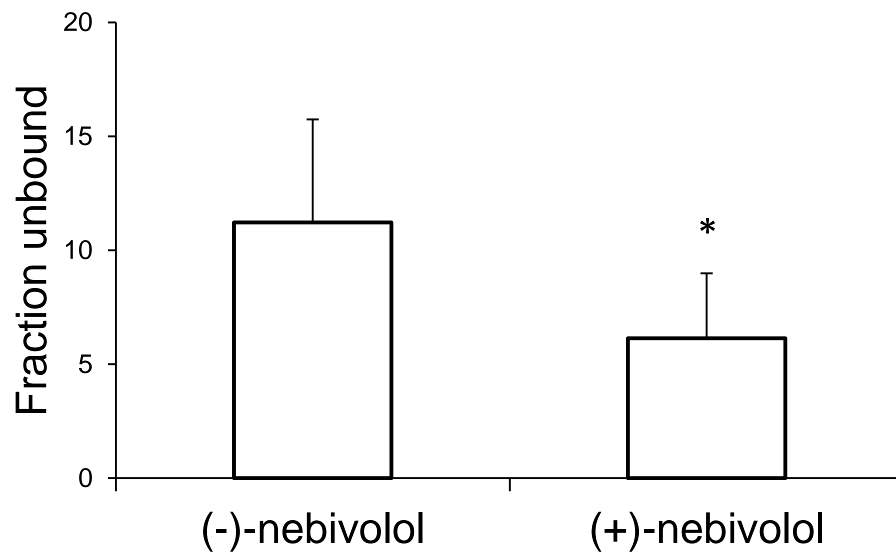


nebivolol was found to be significantly stereoselective with (-)-nebivolol demonstrating a greater unbound fraction (11.22± 4.52) as compare with (+)-nebivolol (6.13± 2.86) with a (-)/(+) average of 1.84±0.19 (Figure 4-4).

4.5 Discussion

In humans orally administered nebivolol is rapidly absorbed following extensive first-pass metabolism (23). There is evidence suggestive of CYP2D6 (23) and CYP3A4 (24,25) involvement which is assumed to indicate hepatic metabolism (23). In addition, the oral bioavailability of nebivolol (17) and its efficacy (26) are subject to polymorphism: It is 12% and 96% in extensive and poor metabolisers, respectively. However, there is no direct evidence for the involvement of the liver in nebivolol first-pass metabolism. The metabolic pathways of the drug in the rat are unknown and the suitability of the rat as a model of nebivolol clearance remains to be proven. Nevertheless, the present data points to the gut rather than the liver as the site of loss of nebivolol following oral administration. Our data clearly indicate a low nebivolol oral bioavailability (Tables 4-1 and 4-2) similar to what has been reported for humans (17). This can have various causes including low absorption and pre-systemic metabolism. The fact that i.p. doses are fully available excludes the liver as the site of first-pass loss of the drug. Hence, the involvement of the gut is strongly suspected. The present everted gut data support the latter notion. Incubation of the drug in the presence of the rat gut resulted in substantive loss of nebivolol (Figure 4-3). This unequivocally identifies the pre-systemic site of the loss of a high portion of orally administered nebivolol doses

Figure 4- 4. Percent of the serum unbound nebivolol enantiomers; *Significantly different from (-)-nebivolol.



to be the gut due to metabolism or chemical decomposition. The gastrointestinal tract is rich with enzymes with the same origin as those in the liver. However, contrary to the liver, the enzymes are not concentrated in a small area but are found throughout the length of the gut; hence, the drug can escape extensive the metabolism therein if absorbed quickly. In such cases the effect of the gut metabolism is not expected to be substantial. For nebivolol, it seems that another barrier is at work as well. We noticed that the metabolism in the presence of the everted gut was accompanied by only 0.1% passage of the drug through the wall of intestine. This was despite a relatively high concentration of intact drug still present in the mucosal side (Figure 4-3), which is suggestive of an absorption obstacle, e.g., low solubility and/or transporter-dependence absorption.

In addition, nebivolol with a systemic clearance of 19 mL/min/kg after i.v. doses to the rat is considered a relatively low extraction drug (clearance much lower than hepatic blood flow of 85 mL/min/kg). This suggests that, indeed, the hepatic clearance of the drug is not so efficient to result in substantial hepatic first-pass metabolism. Our observation does not contradict an involvement of hepatic enzymes as has been suggested for humans (23) as these enzymes may be responsible for the clearance of nebivolol after it reaches the systemic circulation.

After all routes of administration, the plasma nebivolol concentration curves gave multiple peaks. Our data cannot pinpoint the mechanism behind the multiple peaking but since it happens after all routes of administration hepato-biliary recirculation is suspected (27).

Inflammation is known to supress the first-pass metabolism of drugs with efficient hepatic metabolism by reducing their intrinsic clearance (10-15). The fact that the pre-systemic loss of nebivolol does not take place in the liver likely explains why clearance of the drug is not influenced by inflammation (16). The potential suppressing effect of inflammation on GI metabolism is unknown.

We were planning to carry out the objectives of this study using a stereospecific assay. Initially we did not succeed due to a column stability problem (16), so we used an achiral method instead. Near the completion of the initial work, we found a suitable chiral column and used it to look into the stereochemical aspects of our findings. At that stage we had a limited volume of samples left; hence, we pooled the samples and generated data sets based on single values that each represented the pooled mean of samples collected at each time with no variance. In addition, approximation of means from pooled and unpooled data are not expected to yield exactly the same values but the former will be within the variability of the latter. Nevertheless, the approach provided a valuable insight into the stereochemical aspects of nebivolol pharmacokinetics.

Pharmacokinetics of nebivolol are stereoselective in favor of the (+)-enantiomer after all routes of administration. However, the ratio of the enantiomers in the rat is opposite to what has been reported in humans (17). For other chiral drugs such as verapamil (13,28) and propranolol (11,12) such inter-species difference has also been observed. This has to be taken into consideration when Sprague-Dawley rats are used as an animal model for nebivolol pharmacokinetic studies.

Various mechanisms can be responsible for such a significant stereoselectivity observed for nebivolol. They included stereoselectivity in the first-pass and/or systemic metabolism (29), absorption (30), protein binding and chiral inversion (31,32). Interestingly, the metabolism of nebivolol in the presence of everted rat gut was not stereoselective, ruling out the possibility of a pre-systemic stereoselective mechanism. In contrast we noticed significant stereoselectivity in serum protein binding of nebivolol. The free fraction of (-)-nebivolol was significantly higher than (+)-nebivolol. This can explain the cause of the stereoselective differences in metabolism and clearance as only the free fraction of drug in plasma is available for metabolism and excretion. Indeed, for drugs with low extraction ratio, as is the case for nebivolol following i.v. doses, clearance depends on protein binding (33). Our observation suggests that stereoselectivity in nebivolol pharmacokinetics occurs after the drug is absorbed and at the level of plasma protein binding. This process may be more important in conditions associated with markedly decreased plasma levels of albumin as nebivolol is bound to albumin at 98% (32).

A knowledge of stereochemistry of nebivolol is important because both its enantiomers are involved in the beneficial effect of the drug. The (+)-isomer provides the β1 blocking property, and both (+)- and mainly (-)-nebivolol have an endothelial NO-dependent vasodilating effect by the L-arginine/NO pathway (23,25,34,35). (-)-nebivolol increases the activity of the endothelial isoform of NO synthase through direct β3-AR agonistic effect (36).

There are a few limitations to this work. First, it is not known whether the experimental animal used, indeed, represents a suitable model. Second, the in vivo stereospecific data are presented without an indication of their variance. Nevertheless, the combination of both chiral and achiral data and the fact that in vitro data are presented with the associated variance render our stereochemical data worthy of reporting.

4.6 Conclusions

Nebivolol has a low bioavailability following p.o. but not after i.p. doses suggesting the involvement of the gut in drug first-pass loss rather than the liver. The crossing of nebivolol enantiomers through the gut wall is minimal, implying another reason for their low bioavailability. Stereoselective binding to serum proteins (rather than selective first-pass loss of nebivolol enantiomers) is the main reason behind the observed stereoselectivity in drug pharmacokinetics.

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Chapter 5 General conclusions

Inflammation is a physiological response to non-specific events stimulated by infection or tissue damage, resulting in cell injury or death (1). It is usually a localized protective reaction which leads to increased blood supply and appearance of pain, heat, redness, swelling and loss of function due to immune system response including innate and adaptive immunity (2-4). It is associated with the release of pro and anti-inflammatory mediators such as cytokines which, in fact, have a critical role in the pathogenesis of disease (1,5-7). In addition to pathophysiological effects, these inflammatory mediators affect the target proteins' density (8-10), drug metabolizing enzymes' availability (3,8,11,12), and drug transporters (13). As a result, the disposition and effectiveness of drugs are altered. It is generally believed that inflammatory conditions result in reduced drug clearance, thereby, elevating concentrations of efficiently metabolized drugs (3,11,12,14-17).

The pharmacological effects of traditional beta blockers were decreased in elderly (18) and black patients (19)and in animals with experimental arthritis (11,14). The pharmacological effects of L-type calcium channel blockers were decreased in animals with experimental arthritis (9,12,15)and in people with rheumatoid arthritis (12,20), and obese patients (21) .

In the present study we have focused on the effects of Crohn's disease and experimental arthritis, two well-known inflammatory conditions, on pharmacokinetic indices and pharmacodynamics of two cardiovascular drugs, verapamil (an L-type calcium channel blocker) and nebivolol (a third generation beta blocker).

In the clinical arm of the study, we observed that in patients with clinically active Crohn's disease, the efficacy of verapamil is decreased despite an increase in the drug enantiomers concentration. However, in patients who are in clinical rebate, the pharmacokinetics and pharmacodynamics of verapamil return to normal. An elevated verapamil concentration does not necessary result in increased efficacy or toxicity, which is contrary to the general belief that a high drug concentration may result in increased efficacy. It is interesting to note that there is a strong association between the severity of an inflammatory condition and the response to a cardiovascular drug. The observed reduction in the clearance and pharmacological activity of verapamil is attributed to the down-regulation of target proteins, i.e., cytochrome P450 enzymes and calcium channels receptors, respectively. Therefore, in the treatment of cardiovascular disease in patients with inflammatory conditions such as Crohn's disease, consideration should be paid to the activity of the disease and the degree of inflammation.

In the animal arm of the study we observed that in pre-adjuvant arthritis rats, neither the effectiveness of nebivolol nor its pharmacokinetics is changed. The observed consistency of the response to nebivolol despite the presence of inflammation and down-regulation of β1-AR may indicate the predominance of

β2 and β3-AR in the overall effect of the drug and/or a compensation mechanism that shifts the function from one receptor to another. Under our experimental conditions, the levels of β2- and β3-AR remain unaffected by inflammation despite the observed down-regulation of β1-AR. This highlights the importance of mechanisms other than β1-AR that are involved in the action of nebivolol.

Inflammation reduces the clearance of propranolol, as we have confirmed. However the pharmacokinetics of nebivolol appear unaffected by inflammation. This suggests that the nebivolol mode of clearance is different from that of those drugs that are known to be influenced by inflammation.

To determine the reason for nebivolol's low bioavailability and its escaping from pharmacokinetics' changes in the presence of inflammation, we used different routes of administration to deliver nebivolol including p.o., i.v., and i.p. Nebivolol has a low bioavailability following p.o. but not after i.p. doses, which suggests the involvement of the gut rather than the liver in drug first-pass loss. There is a minimal crossing of nebivolol enantiomers through the gut wall, implying another reason for their low bioavailability. Inflammation is known to suppress the first-pass metabolism of drugs that have an efficient hepatic metabolism by reducing their intrinsic clearance. The fact that the pre-systemic loss of nebivolol does not take place in the liver likely explains why inflammation does not influence its clearance.

We also noticed stereoselectivity in the pharmacokinetics of nebivolol following different methods of drug administration. Various mechanisms can be

responsible for stereoselectivity, including stereoselectivity in the first-pass and/or systemic metabolism, absorption, protein binding and chiral inversion. Stereoselective binding to serum proteins, rather than selective first-pass loss of nebivolol enantiomers is the main reason for the observed stereoselectivity in drug pharmacokinetics.

Traditional β1-blockers have a reduced response and lower oral clearance under inflammatory conditions (11,14). If our observations are translated to human inflammatory diseases, nebivolol presents an alternative to those traditional β1-blockers.

In the treatment of cardiovascular complications in patients with inflammatory conditions, severity of inflammation and type of cardiovascular medication should be considered. Controlling inflammation and choosing a unique cardiovascular drug such as nebivolol, the effect of which is not altered in the presence of inflammation, will be important for avoiding failure in treatment.

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