RIBONUCLEOTIDE REDUCTASE INHIBITORS FOR RESTENOSIS

MASTER’S THESIS

Presented in Partial Fulfillment of the Requirements for the Degree Master in Science in The Graduate School of The Ohio State University

By

Megan Mutchler, B.S.

* * * * *

The Ohio State University
2008

Master’s Examination Committee:

Dr. James Waldman, Adviser

Dr. Arturo J. Cardounel

Dr. Guanglong He

Approved by

Adviser

Graduate Program in Pathology
ABSTRACT

Percutaneous transluminal coronary angioplasty (PTCA) has greatly benefited patients with occluded coronary arteries, but its benefits have been undermined by a high incidence of restenosis. The introduction of coronary stents has significantly improved the short and long term outcome but restenosis still occurs in approximately 15-30 % of patients within 6 months. Research efforts are now being directed toward combination stenting and drug delivery. Among the therapeutic targets being pursued are agents which can impede smooth muscle cell (SMC) migration and proliferation as these processes are critical components of restenosis injury. We propose that inhibiting the conversion of ribonucleotides to deoxyribonucleotides will impede cell proliferation and as such limit the degree of restenosis. Therefore, we tested whether the potent Ribonucleotide Reductase (RR) inhibitors, Didox and Imidate, can limit the neointimal proliferation associated with restenosis using rat, rabbit and porcine models of vascular injury. Results demonstrated that systemic administration of the RR inhibitors Didox, Imidate and Hydroxyurea significantly reduced intimal thickening, intima/media ratio and lumen loss. Results from cell proliferation studies suggest that the mechanism of protection is inhibition of SMC proliferation and decreased number of circulating leukocytes. These
results suggest that inhibition of Ribonucleotide Reductase may provide a potent strategy
to prevent post PTCA restenosis.
ACKNOWLEDGMENTS

I wish to thank Dr. Arturo J. Cardounel for his support and guidance for the past several years. His never ending patience and dedication made this thesis possible.

I am grateful to Dr. James Waldman for always being available when I needed help or advice.

I also wish to thank Dr. Guanglong He for taking time out of his busy schedule to support me and be a part of my Master’s Examination Committee.
VITA

March 11, 1984............................................... Born – Defiance, Ohio
2003-2006....................................................... Student Research Assistant
The Ohio State University

2006............................................................... B.S. Medical Technology
The Ohio State University

2006-present.................................................... Graduate Research Associate
The Ohio State University

PUBLICATIONS

Research Publication


FIELDS OF STUDY

Major Field: Pathology
# TABLE OF CONTENTS

Abstract........................................................................................................................................... ii  
Acknowledgment.......................................................................................................................... iv  
Vita................................................................................................................................................ v  
List of Tables................................................................................................................................... viii  
List of Figures................................................................................................................................. ix  
Introduction...................................................................................................................................... 1  

Chapters:  

1. Ribonucleotide Reductase Inhibitors Using a Porcine Model of Restenosis  
   1.1 Introduction............................................................................................................................... 18  
   1.2 Methods  
      1.2.1 Materials............................................................................................................................ 20  
      1.2.2 Carotid Injury.................................................................................................................... 20  
      1.2.3 Flow cytometry................................................................................................................. 21  
      1.2.4 Intracellular dNTP quantitation...................................................................................... 21  
      1.2.5 Smooth muscle cell migration....................................................................................... 23  
   1.3 Results  
      1.3.1 Effects of Ribonucleotide Reductase Inhibitors on Restenosis Following Vascular Injury................................................................................................................................. 23  
      1.3.2 Effects of Didox, Imidate and HU on SMC proliferation...... 24  
      1.3.3 Effects of Didox, Imidate and HU on SMC migration....... 26  
   1.4 Discussion.................................................................................................................................. 27  

2. Ribonucleotide Reductase Inhibitors Reduce Atherosclerosis in a Rabbit Model of Double Injury  
   2.1 Introduction............................................................................................................................... 38  
   2.2 Methods  
      2.2.1 Surgical Procedures............................................................................................................ 40  
      2.2.2 Pharmacological Treatments............................................................................................ 41  
      2.2.3 Histological Assessment.................................................................................................... 41  
   2.3 Results  
      2.3.1 Effects of RR inhibitors on Atherosclerosis......................................................... 41  
      2.3.2 Hematological Effects of RR Inhibition....................................................... 42
3. Effects of Ribonucleotide Reductase Inhibitors Using a Porcine Model of Restenosis

  3.1 Introduction..................................................................................50
  3.2 Methods
    3.2.1 Porcine Model of In-stent Restenosis.........................52
    3.2.2 Histopathological Study.................................................53
    3.2.3 Virtual Histology...............................................................53
  3.3 Results
    3.3.1 Effects of the Ribonucleotide Reductase inhibitor Didox on in-stent restenosis..........................54
  3.4 Discussion..................................................................................55

Discussion..........................................................................................64

List of References................................................................................70
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Effects of Didox, Imidate and Hydroxyurea on smooth muscle cell daTP pools</td>
<td>32</td>
</tr>
<tr>
<td>2.1</td>
<td>Blood profiles of the rabbit groups taken at the time of euthanization</td>
<td>44</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Effects of Didox, Imidate and HU on the histopathology associated with balloon dilatation injury</td>
<td>33</td>
</tr>
<tr>
<td>1.2</td>
<td>Effects of Didox, Imidate and HU on histopathological changes following balloon injury of the rat carotid artery at 2 weeks post injury</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>1.2 A Neointimal formation</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>1.2 B Media wall thickness</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>1.2 C Intima to media ratio</td>
<td>34</td>
</tr>
<tr>
<td>1.3</td>
<td>Effects of Didox, Imidate and HU on histopathological changes following balloon injury of the rat carotid artery at 6 weeks post injury</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>1.3 A Neointimal formation</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>1.3 B Medial wall thickness</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>1.3 C Intima to media ratio</td>
<td>35</td>
</tr>
<tr>
<td>1.4</td>
<td>Effects of Didox, Imidate and HU on SMC proliferation</td>
<td>36</td>
</tr>
<tr>
<td>1.5</td>
<td>Effects of Didox, Imidate and HU on SMC migration</td>
<td>37</td>
</tr>
<tr>
<td>2.1</td>
<td>Fluoroscopy depicting a 3 mm wire guided balloon in the left common carotid artery of a New Zealand rabbit under general anesthesia</td>
<td>46</td>
</tr>
<tr>
<td>2.2</td>
<td>Histological cross sections of the common carotid artery of the control injury, Didox and Hydroxyurea rabbit group</td>
<td>47</td>
</tr>
<tr>
<td>2.3</td>
<td>Mean and standard deviation of atheroma area in the 4 rabbit groups at the end of the study</td>
<td>48</td>
</tr>
<tr>
<td>2.4</td>
<td>Mean and standard deviation of lumen area in the 4 rabbit groups at the end of the study</td>
<td>49</td>
</tr>
</tbody>
</table>
3.1 Histological cross sections of control and Didox treated left coronary arteries at 4X and 10X power…………………………………………………………... 59

3.2 Lumen area of left coronary artery in a porcine heart excised 4 weeks after stent implantation……………………………………………………… 60

3.3 Media area of left coronary artery in a porcine heart excised 4 weeks after stent implantation……………………………………………………… 61

3.4 Neointimal area of left coronary artery in a porcine heart excised 4 weeks after stent implantation……………………………………………………... 62

3.5 Intima/media ratio of left coronary artery in a porcine heart excised 4 weeks after stent implantation……………………………………………………… 63
INTRODUCTION

Cardiovascular disease (CVD) afflicts an estimated 79,400,000 people in the United States. This includes people suffering from high blood pressure, coronary heart disease, heart failure and stroke. In every year except for one since 1900, cardiovascular disease has been responsible for more deaths than any other single cause of death in the United States. Cardiovascular disease can be named as the underlying cause of death in 36.3% of the 2,398,000 deaths that occurred in 2004. Of these deaths, 147,000 victims were under the age of 65. It is estimated that close to 2,400 Americans die of cardiovascular disease each day. This averages out to about one death per 36 seconds. According to one study, a person has a 47% chance of dying from CVD versus a 22% chance of dying from cancer. In fact, CVD is responsible for killing more people per year than cancer, diabetes mellitus, chronic lower respiratory diseases and accidents combined (1).

Because of the diffuse magnitude of cardiovascular disease, the financial impact is also great. In 2004, an estimated 6,363,000 people in the United States underwent some type of inpatient cardiovascular procedure. For the year 2007, the direct and indirect financial cost of CVD was roughly $431.8 billion (1).

Of the many diseases grouped under the category of cardiovascular disease, one of the most important is coronary heart disease or ischemic heart disease (2). It is the
single largest killer of American adults. More than half of all cardiovascular events in adults under 75 years of age are due to coronary heart disease. In 2004 the prevalence of coronary heart disease in adults over 20 years of age was 15,800,000 (1). Ischemic heart disease kills an average of 500,000 Americans a year (2). In 2004, it was responsible for one in five deaths in the United States. It is estimated that every 26 seconds an American will experience some sort of coronary event and one will die from an event every minute. If the coronary event is survived, an individual has a 1.5 to 15 time’s higher chance of sickness and death versus the general public. These increased risks include heart attack, stroke, sudden death, heart failure and angina pectoris (1).

While the statistics are overwhelming, there has been improvement. Since 1963, the death rate from ischemic heart disease has been cut in half (2). This is in large part due to improved prevention through modifications of preventable risks and advances in therapeutic approaches. Drugs and procedures such as coronary artery bypass surgery, percutaneous transluminal coronary angioplasty (PTCA), and endovascular stents are just some of the advancements that have been made (2). In the United States in 2004, an estimated 1,285,000 angioplasties, 1,471,000 diagnostic catheterizations, 427,000 bypass procedures, 68,000 implantable defibrillators and 170,000 pacemaker procedures were completed. Even with the current advancements the financial cost of coronary artery disease is still high. For 2007, the estimated direct and indirect cost of the disease was $151.6 billion (1).

Several risk factors are associated with the development of the disease. These risk factors can be subdivided into nonmodifiable and modifiable risk factors (3). Common nonmodifiable risk factors include age, sex, and genetics. Age is a very
important factor in the development of coronary artery disease. Death rates associated with the disease increase with each decade that a person lives. This is most likely due to advanced atherosclerotic lesions. In addition, the risk of myocardial infarction increases by five in persons 40 to 60. In regards to a patient’s sex, males are more likely to develop the disease than females (2). In 2004, the rate of coronary heart disease was 8.9% in males and 6.1% in females (1). Genetics also contribute to disease development. Most commonly the genetic relationship is associated with other risk factors such as diabetes mellitus and hypertension which can be common in families (2).

Modifiable risk factors are those that can be altered by a conscious change in a patient’s lifestyle. These include hyperlipidemia, hypertension, cigarette smoking, diabetes mellitus, abdominal obesity, and lack of physical activity (1, 2). Of the modifiable risk factors, hyperlipidemia is a major one, especially concerning hypercholesterolemia. Elevated serum cholesterol levels can be enough to favor lesion development even if other risk factors are absent (2). Studies have shown a direct relationship between serum cholesterol levels and risk of ischemic heart disease. An individual with a total cholesterol level of 240mg/dL has twice the risk of disease development versus a person with a level of 200 mg/dL (3). The key component causing harm in the serum cholesterol is low density lipoprotein (LDL) whose role is to deliver cholesterol to peripheral tissues. In contrast, the high density lipoprotein (HDL) component of total cholesterol is protective in higher levels. It’s job is to collect and transport cholesterol to the liver so that it can be excreted in bile (2).

Hypertension is another major factor whose increased values lead to increased risk (3). A man in the age range of 45 to 62 with a blood pressure higher than 169/95
mm Hg has a five times greater risk of developing ischemic heart disease over a man in the same age range with a blood pressure of 140/90 mm Hg (2). Elevated blood pressure speeds up the course of atherosclerosis by damaging vascular endothelium and by making the vascular wall more permeable to harmful lipoproteins (3).

Other modifiable risk factors to consider are diabetes mellitus and cigarette smoking. Not only does diabetes increase the risk for atherosclerosis, it also increases the risk of potential cardiovascular events 3 to 5 fold. The suggested mechanism is related to the increased uptake of cholesterol by scavenger cells in relationship to glycation of lipoproteins (3). Smoking also possesses a significant risk. A long term, multiple pack a day smoker has a 200 percent increased risk of dying from ischemic heart disease (2). Smoking leads to atherosclerotic complications in several ways. It’s associated with oxidative modification of LDL, lower HDL levels, and endothelial dysfunction.

Several new and emerging risk factors also have to be considered. These include homocysteinemia and lipoprotein Lp(a). Homocysteinemia is an elevated serum level of the amino acid homocysteine which can lead to coronary atherosclerosis. It can result from an inborn error of metabolism or from a dietary deficiency of folate. Lipoprotein Lp(a) is form of LDL that has been linked to increased risk regardless of LDL levels (2,3).

These modifiable and nonmodifiable risk factors contribute greatly to the development and progression of atherosclerosis. Atherosclerosis itself is a general term for thickening and hardening of the walls of arteries. It is characterized by the presence of atheromas. Atheromas are lesions in the intima that weaken the adjacent media as well as project into the vessel lumen to obstruct it. Atherosclerotic lesions are divided into six
types. The first stage is the initial lesion composed of isolated macrophage foam cells (2). This is followed by the formation of fatty streaks, the earliest visible lesion in atherosclerosis (3). They are composed of lipid containing foam cells due to macrophage ingestion of LDL as well as accumulated intracellular and extracellular lipids. Grossly, fatty streaks appear as yellow areas of discoloration along the intima of the arterial wall (4). They range in size from 1 mm in diameter up to 1 cm in length. Fatty streaks are flat and therefore don’t impede the luminal blood flow (3). Independent of race, sex, geography or environmental factors, fatty streaks are present in all children by age 10. They begin by forming in areas known for plaque formation such as branch points of arteries (2). Common sites include along the ostia of abdominal aorta branches and in the proximal 6 inches of the coronary arteries (4). Because fatty streaks are present in virtually all individuals, it should be noted that not all streaks develop into plaques. However, they may be precursors (2).

The third type of lesion is the intermediate lesion. It has similar features seen in the fatty streak. It grows mostly by lipid accumulation and eventually advances to the atheroma, also called atheromatous or fibrofatty plaque. An atheroma is composed of an elevated focal lesion beginning in the intima. It is an eccentric lesion that usually only involves part of the arterial wall (2). Grossly the external surface is white and the deeper aspect is yellow (4). An atheroma has a soft core and is covered by a firm, fibrous cap. The core is composed of cell debris, cholesterol crystals, foam cells and calcium (2). The fibrous cap is made up of smooth muscle cells, macrophages, foam cells, lymphocytes, collagen, elastin and proteoglycans (2, 4).
Eventually an atheroma may advance to a fibroatheroma or type V lesion. A fibroatheroma can occur in a variety of forms. It can be composed of lipid core and fibrotic layer or it may be mostly fibrotic or mostly calcified. The final lesion is the complicated or advanced lesion. At this stage in the pathologic process, there is an increased risk for multiple changes with clinical significance such as thrombosis or rupture (2).

The pathogenesis of atherosclerotic plaque formation is complicated and includes many variables and many hypotheses. However, the basic principle behind plaque formation can be attributed to the so called response to injury hypothesis which states that atherosclerosis is an inflammatory reaction of the vessel wall in response to endothelial injury (2).

Several mechanisms play a role in this theory. One of the most important aspects to consider is endothelial injury and dysfunction. In fact, many researchers agree that endothelial injury is the main event in the pathogenesis of atherosclerosis (3). Injury to the endothelium can be due to several factors including smoking, homocysteine, viruses, and hemodynamic forces (2, 3). Of these, hemodynamic disturbances are the most significant.

The basis of hemodynamic disturbances is the pattern of blood flow. Blood flow can be either laminar or turbulent. Laminar blood flow is smooth and steady and can be further categorized into undisturbed and disturbed laminar flow. Undisturbed laminar flow is characterized by blood flowing in sync with the direction of the artery, while disturbed laminar flow is characterized by reversed flow. Turbulent flow on the other hand, is defined as a flow that is overall steady but varies in velocity. It depends on
several factors including the speed of the flow and the presence of obstructions or irregularities within the lumen. Areas of turbulent flow are common sites of plaque formation (5). These areas include ostia of vessels and at various branch points along an arterial pathway (2).

The importance of blood flow patterns stems from their effect on endothelial shear stress or ESS. Endothelial shear stress is defined as “the tangential stress derived from the friction of the flowing blood on the endothelial surface of the arterial wall and is expressed in units of force/unit area” (5). ESS is proportional to the spatial gradient of blood velocity at the wall (dv/dy) multiplied by the blood viscosity (µ). It is the nature of the blood flow as well as the overall shape of the artery that determines the ESS (5). Endothelial shear stress patterns change in relationship due to the blood flow patterns. In areas with physiological ESS there is not much, if any, change in the arteries at that site (5, 6). Physiological ESS is associated with areas having laminar blood flow and in fact, physiological ESS and laminar blood flow may hold a protective function against the formation of atherosclerotic lesions. This is due to several factors. Endothelial cells in these areas express various atheroprotective genes and enzymes. A normal laminar flow and shear force promotes the expression of enzymes that synthesize nitric oxide. Nitric oxide is protective against atherosclerosis as a vasodilator, inhibitor of platelet aggregation and an anti-inflammatory (3). Physiological ESS also enhances the expression of superoxide dismutase which is an anti-oxidant (2, 3). Overall, endothelial cells in this range achieve an overall enhanced balance that favors cell stability (6). In addition, steady laminar flow blocks inflammatory mechanisms that can contribute to dysfunction and therefore plaque formation (2).
While physiological ESS has protective functions, low ESS is associated with lesion formation. Areas with low ESS tend to coincide with lesion formation and display evidence of the molecular and cellular characteristics seen in plaque formation and progression (6). Low endothelial shear stress is associated with lesion formation for several reasons. Normally, physiological ESS stimulates nitric oxide production. When endothelial shear stress is low, the amount of nitric oxide available is decreased and the endothelium is therefore exposed to the atherogenic effects that higher levels of nitric oxide usually protect against. In addition, low ESS down regulates prostacyclin while up regulating endothelin-1. Prostacyclin acts as a vasodilator while endothelin-1 is a vasoconstrictor. This effect also contributes to the formation of an atherosclerotic lesion (6).

Low ESS also stimulates an accumulation of low density lipoprotein (LDL) in the endothelium (6). Once the endothelium becomes dysfunctional, it no longer acts as a barrier to circulating lipoproteins in the blood and LDL can accumulate in the subendothelial space. (3).

Increased levels of LDL are a known risk factor for atherosclerosis. However, it is not the accumulation itself that is most damaging, but rather the modification the LDL undergoes once it’s there. The most significant of these modifications is oxidation. LDL becomes oxidized by the actions of oxygen species and oxidizing enzymes produced by the endothelial cells, smooth muscle cells or macrophages (2, 3). Low ESS also has role in oxidation of LDL in that it enhances the formation of reactive oxygen species. Low endothelial shear stress increases gene expression of pro-oxidant enzymes such as nicotinamide adenine dinucleotide phosphate and xanthine oxidase (5). Once the LDL is
oxidized it is called modified LDL and causes damage via several mechanisms. It leads to an increased accumulation of monocytes within the forming lesion and promotes the release of various growth factors and cytokines (2). In addition, modified LDL promotes the expression of genes on the endothelial cells that encode for mediators of inflammation. These include monocyte stimulating factor, leukocyte adhesion molecules and monocyte chemoattractant protein (3). Modified LDL is engulfed by macrophages via the scavenger receptor and eventually forms foam cells (2). It also contributes to the lowered availability of nitric oxide by degrading it. This further decreases the protective effects of nitric oxide already reduced by the low ESS (5). Finally, oxidized LDL is cytotoxic to smooth muscles cells as well as endothelial cells (2). It is these mechanisms that accumulated LDL via low ESS contribute to atherosclerotic plaque formation.

Low endothelial shear stress also promotes inflammation, another key component of atherosclerosis. Normally, an endothelial cell does not have the capacity to allow the binding of white blood cells (2). However, once LDL becomes oxidized in the atherosclerotic process, endothelial cells begin to express adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) on their surfaces that are capable of binding white blood cells (2, 3). Also, low ESS promotes activation of tissue factors, especially nuclear factor kappa β (NF-κβ) which play a role in infiltration and attachment of the white blood cells (5). This occurs because inflammatory cells are recruited to the site of the forming plaque to scavenge the oxidized LDL (5). In fact, some studies suggest that oxidized LDL acts as an autoantigen in atherosclerosis and therefore recruits immune cells (7). Other studies also include heat shock proteins (HSP) as possible
autoantigens (8). The main cells recruited to the site of lesion formation are T lymphocytes and monocytes (2, 3, 7, 8).

The majority of T cells recruited to the atherosclerotic site are CD4+ T cells that express the proinflammatory cytokine interferon-γ (IFN-γ) (7, 8). The role of the CD4+ cells has been studied by using various types of knockout mice. One type of mouse used was apolipoprotein E (apoE-/-) deficient. These mice have significantly increased cholesterol levels along with faster developing atherosclerosis. The formed plaques have massive lipid deposition and are heavily infiltrated by CD4+ T cells. In addition, some of these mice had IFN-γ introduced into the system which further accelerated atherosclerotic plaque formation. These findings support the role of CD4+ T cells in inflammation. Other evidence includes the findings seen after crossbreeding apoE-/- mice with severe combined immunodeficiency (SCID) mice that lack both T and B cells. In these mice there was a significant decrease in fatty streak lesions seen in the aorta. When these same mice were reconstituted with T cells, plaque formation increased by 164% (8). A similar study used CD4/apoE double knockout (CD4/apoE dKO) mice and found reduced atherosclerosis with less advanced lesion formation. At 18 weeks old, the mice had a 70% reduced lesion size compared to apoE KO mice. In addition the expression of the activation marker I-A^b was greatly reduced in CD4/apoE dKO mice. This marker is indicative of IFN-γ signaling (7). The findings in this study also support the role of CD4+ T cells and IFN-γ in atherosclerosis.

Another key cell involved in atherosclerosis is the monocyte. Monocytes also bind to endothelial cells via adhesion molecules (2). After attaching, the monocytes migrate into the subendothelial space by traveling between the junctions of the
endothelial cells (2, 3). Once they are localized, the monocytes are further stimulated by cytokines released from T lymphocytes and morph into macrophages (2, 8). The macrophages then scavenge and engulf oxidized LDL. After engulfing the lipoproteins, the macrophages transform into foam cells. Initially this is protective, because the harmful oxidized LDL is phagocytized but the accumulation of foam cells eventually leads to lesion progression (2). In fact, foam cells are the main component of the fatty streak which is one of the earliest lesions seen in atherosclerosis (3).

In addition to forming foam cells, macrophages produce tissue factor, cytokines and other substances that contribute to lesion progression (8). They elicit TNF and IL-1 both of which increase adhesion of leukocytes. Macrophages also release multiple cytokines such as monocyte chemotactic protein-1 (MCP-1) that draw more immune cells into the plaque. Finally, macrophages produce oxygen species capable of oxidizing LDL in the area of the lesion as well synthesizing growth factors that may play a role in smooth muscle cell proliferation (2).

Low endothelial shear stress not only plays a significant role in inflammation, it also contributes to smooth muscle proliferation. Proliferation of smooth muscle cells contributes to the advancement of an atherosclerotic lesion. Low ESS both promotes and prevents the expression of various substances key to smooth muscle cell proliferation. Low ESS promotes the expression of platelet derived growth factor and vascular endothelial growth factor while decreasing expression of plasminogen activator inhibitor. Plasminogen activator inhibitor functions as an inhibitor of vascular smooth muscle cell migration. The overall effect of stimulated growth promoters and under expression of growth inhibitors is vascular smooth muscle proliferation (5).
Foam cells also contribute to smooth muscle cell proliferation by producing several factors such as platelet derived growth factor which stimulates proliferation of the SMCs. Foam cells also release various growth factors and cytokines such as TNF-α, IL-1, fibroblast growth factor and transforming growth factor-β. These substances have a several functions including aiding proliferation, synthesis of extracellular matrix proteins and leukocyte activation (3).

Once the smooth muscle cells are stimulated, they migrate from the media into the intima. There the cells proliferate and release extracellular matrix components such as collagen that help stabilize the plaque (2). This process converts a fatty streak into an atheroma (2, 3). The increased number of smooth muscles cells along with the developing fibrous cap composed of extracellular matrix and the accumulated foam cells and immune cells characterizes a formed atheroma and therefore an advanced atherosclerotic lesion (3).

Low endothelial shear stress also plays an indirect role in neovascularization or angiogenesis, another component of atherosclerotic plaque formation. Low ESS promotes neovascularization in the intima indirectly by inducing intimal thickening (5). Intimal thickening leads to ischemia and neovascularization is driven partly by hypoxia (9). Low ESS also upregulates VEGF which is a main regulator of vascular permeability in detrimental angiogenesis (5, 9). In addition to low ESS, hypercholesterolemia also stimulates angiogenesis by upregulating several growth factor receptors on endothelial cells and smooth muscle cells. These receptors facilitate angiogenesis in the intima. High cholesterol levels are also associated with inflammation and inflammation is an additional stimulus for neovascularization (9).
Neovascularization is not only a part of the atherosclerotic process, it is also plays a large role in plaque instability. Normally vasa vasorum penetrate at regularly spaced intervals into the vessel wall and then bifurcate around the vessel. When hypecholesterolemia is present, a large dense network of new vessels forms in the adventia. While the vessels in the adventia are close to mature, their penetrating branches are not. As the vaso vasorum go from the adventia, through the media and into the intima the branches become more fragile and prone to leakage. This can be due to several factors such as vessel defects, proteolytic damage from inflammation, breakdowns in endothelial cell to cell contact or mechanical forces. In addition, while many of the immature vessels have endothelial cells, most do not have pericytes or smooth muscle cells. The lack of these cells also contributes to leakage (9).

The leakage of the immature vaso vasorum around the plaque and in the intima is critical to plaque instability. Red blood cells are constantly leaking into the surrounding microenvironment. The membranes of the red blood cells contain large amounts of free cholesterol. It is proposed that the free cholesterol in the leaking red blood cells accumulates in the forming plaque and along with already circulating lipids, contributes to the necrotic core. The free cholesterol contribution to the necrotic core is important because the size of the necrotic core is associated with plaque instability. The larger the core is, the greater the instability and heightened risk of rupture. This is significant because 75% of acute coronary events and 60% of carotid artery disease is associated with a disrupted plaque (9).

As mentioned above, hemodynamic factors, especially low endothelial shear stress, play a role in several of the key pathological processes in atherosclerotic plaque
formation and progression. However, several other mechanisms can cause endothelial cell dysfunction. These include cigarette smoke, hypercholesterolemia, Diabetes Mellitus and homocysteine. The common link between these causes is increased endothelial production of reactive oxygen species (2). Regardless of the cause, endothelial cell dysfunction is the initiator of atherosclerotic plaque formation and the main pathological processes of atherosclerosis.

Understanding the pathology behind atherosclerosis is crucial because of the detrimental effects of the disease. Once a plaque has reached the stage of an advanced lesion, there is great risk for several clinically significant events. The luminal surface of the plaque may undergo rupture or erosion. This leads to the induction of thrombus formation or release of debris into the blood which in turn may lead to the formation of microemboli (2). These emboli can travel through the circulation to distant sites and cause serious complications (3). Another risk associated with complicated lesions is hemorrhage due to rupture of the fibrous cap (2). Hemorrhage into the plaque further narrows the vessel lumen and contributes to the problem. A more serious and dangerous risk is thrombosis. This complication usually occurs in a ruptured or ulcerated plaque due to release of thrombogenic factors in the core out into the circulation. A thrombus can then form and lead to infarction at that site. On the other hand, the thrombus may instead incorporate itself into the plaque and help increase the size of the plaque. If this occurs, the lumen may be partially or entirely blocked. A final risk is dilation of the vessel leading to aneurysm. Dilation is due to weakened vessel walls. The growing plaque squeezes up against adjacent media and puts pressure on it. This can cause atrophy and loss of elastic tissue which in turn leads to dilation (3).
With all of the potentially dangerous risks associated with an advanced lesion, treatment to reduce the size of the plaque is crucial. The two main options most medical professionals choose is percutaneous transluminal coronary angioplasty (PTCA) or endovascular stenting (2).

In percutaneous transluminal coronary angioplasty, a balloon is inserted and inflated at the site of the plaque. This procedure increases the lumen of the sclerotic vessel by several mechanisms that lead to stretching of the lumen. A prominent mechanism is plaque fracture (2). In plaque fracture the dilation of the balloon puts pressure on the plaque and causes it to rupture from the lumen out. If the plaque splits entirely, the outer wall is able to dilate and open up the lumen (10). Other mechanisms include intimal flaps and medial dissection and stretching of the uninvolved segment of media (11).

The characteristics of the plaque affect how well these mechanisms work. Several features of the plaque can dictate how successful the procedure is. The first feature is the luminal location of the plaque. Studies have shown that fracture is more successful if the plaque is eccentric versus concentric. One study found there to be morphological success after angioplasty in 48% of eccentric plaques versus 10% of concentric ones (4). There are multiple reasons for this. First of all, in an eccentric plaque there are more likely to be thin areas in the plaque that can be more easily disrupted by the balloon (10). Similarly, an eccentric plaque is more prone to having thin media where the plaque is not present. These thinner areas are more likely to stretch out when the balloon is inflated and therefore increase the size of the lumen (11).
Another factor to consider is the composition of the plaque itself. Plaques can have varying content with different amounts of fibrin, lipids and calcifications. Several studies have found that plaques rich in lipids are more likely to rupture than a fibrous plaque (4, 11). Fibrous tissue is less apt to stretching and harder to produce cracks in. If cracks do occur, they are usually too small to make a significant difference in the dimension of the vessel lumen (4).

Calcified versus noncalcified plaques is another characteristic to consider. In one study using intravascular ultrasound imaging to observe morphological effects after coronary angioplasty it was discovered that plaques without calcification were three times less likely to fracture after dilation (10). Calcifications allow for unequal regional stresses on a plaque. This in turn leads to a greater likelihood of disruption from longitudinal tearing (4, 11).

While percutaneous transluminal coronary angioplasty (PTCA) has greatly benefited patients with occluded coronary arteries, its benefits have been undermined by a high incidence of restenosis. The introduction of coronary stents has significantly improved the short and long term outcome but restenosis still occurs in approximately 15-30 % of patients within 6 months.

Restenosis is a pathophysiological process that can occur after vascular injury with a balloon or stent. Injury to the vessel leads to excessive repair of the vessels and eventual neointimal proliferation (12). Restenosis rates in PCTA patients range from 13-53% at one month to one year after the procedure was performed (4). This is significant because restenosis can lead to coronary events such as myocardial infarction or a need for revascularization of the original lesion. In one study of 410 patients randomly assigned
to receive a Palmaz-Schatz stent or balloon angioplasty, revascularization was needed in 10.2% of the stent population and 15.4% of the angioplasty population due to recurrent myocardial infarction (13).

Although not well understood, the occurrence of restenosis is associated with several mechanisms including elastic recoil, thrombus formation mediated by platelets, smooth muscle cell proliferation and vascular remodeling (13). Its pathophysiology can be likened to a healing wound in which there is inflammation, granulation and matrix formation. Initially, there is exposure of the endothelial cells to the surrounding environment and platelet aggregation leading to thrombosis. Platelets contain proteins such as platelet derived growth factor that are released. Additional growth factors such as fibroblast growth factors are also released and initiate the reendothelialization of the damaged vessel by inflammatory cells, smooth muscle cells and endothelial cells. At this point, smooth muscle cells are also induced to proliferate (4).

While the mechanisms of restenosis may not be well understood, its clinical and financial significance is. Restenosis can cause myocardial infarcts and vessel narrowing in already fragile patients. The estimated cost of coronary heart disease in the United States for 2007 is $151.6 billion (1). Much of this cost could be eliminated if revascularization procedures as a result of restenosis could be reduced.
CHAPTER 1
RIBONUCLEOTIDE REDUCTASE INHIBITORS AND BALLOON RESTENOSIS

1.1 Introduction

The use of percutaneous transluminal coronary angioplasty (PTCA) has greatly reduced the number of fatalities in patients who suffer myocardial infarction (15-17). During PTCA, the artery walls are expanded by several times their original diameter in an attempt to increase lumen diameter and improve flow. Unfortunately, this technique is plagued by a high incidence of vessel renarrowing or restenosis occurring in 30-40% of patients within six months of the procedure (15-19). Prevention of restenosis after successful PTCA remains one of the most challenging tasks in the treatment of obstructive coronary artery disease. Attempts to ameliorate this proliferative response involve the use of coronary stents, which have significantly improved both short term and long term outcome following interventional coronary revascularization procedures. Despite a reduction in restenosis rate with stent deployment, restenosis still occurs in 15-30 % of patients within 6 months (16, 17). This incidence of in-stent restenosis is expected to increase as coronary stenting is becoming more frequent and is used in less ideal lesions. Therefore, in addition to mechanical intervention, pharmacological approaches to reduce the incidence and degree of restenosis are needed.

The vascular trauma associated with PTCA involves a cascade of molecular and cellular events occurring within the vessel wall involving the release of a variety of
vasoactive, thrombogenic, and mitogenic factors (20-22). Within this cascade, several mechanisms contribute to restenosis including elastic recoil, thrombosis, smooth muscle cell migration/proliferation and matrix formation. The result of these vascular events is intimal hyperplasia, whereby vascular smooth muscle cells (VSMC’s) migrate from the media to the intima, proliferate, and consequently form the neointima. During this proliferative response, SMC’s undergo a phenotypic modulation from a contractile to a synthetic phenotype (differentiation) (23-27). Because ultimately, the cascade of events following vascular trauma culminates in cell proliferation and neointimal hyperplasia, it would follow then that among targets being pursued would be agents which can impede smooth muscle cell migration and proliferation, as these processes are critical components of restenosis injury.

In this regard, we have tested the ability of two ribonucleotide reductase (RR) inhibitors to limit the degree of restenosis following balloon mediated dilatation injury in the rat. RR catalyzes the reductive conversion of ribonucleotides to deoxynucleotides. This reductive reaction is a prime target for impeding cellular proliferation, and therefore amenable to inhibiting VSMC replication, because it is a rate limiting step in the biochemical pathway leading to DNA synthesis and thus cell replication (19, 28-32). DNA synthesis cannot occur without invoking this reaction since the endogenous pools of dNTP in mammalian cells are inadequate to support new DNA synthesis (28, 29).

Therefore, in the present study we have examined the ability of the polyhydroxyphenolic compounds, Didox (3,4-dihydroxybenzohydraxamic acid) and Imidate (3,4,5-hydroxybenzimidate), to limit the degree of restenosis following vascular injury. Treatment with either Didox or Imidate resulted in > 60% reduction in neointimal area.
In-vitro studies demonstrated that these effects are mediated through both a reduction in SMC proliferation and migration. These results suggest that inhibition of RR may serve as a novel therapeutic target in the treatment of vasculoproliferative disorders such as restenosis.

1.2 Methods

1.2.1 Materials

Didox, Imidate and Hydroxyurea were provided by Molecules for Health Inc. (Richmond, VA). Rat vascular smooth muscle cells and culture media were purchased from ATCC (Manassas, VA). Fogarty embolectomy catheters were purchased from M & I medical (Miami, Fl.). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

1.2.2 Carotid Injury

Male Wistar rats, weighing 400-450 g (Harlan) were fed standard pellet feed and given water ad libitum. The experimental protocol was designed in accordance with Institutional ILACUC standards. Animals were anesthetized with Isoflurane (1.5-2%) in air. The right carotid artery was exposed and a 2F Fogarty balloon embolectomy catheter (Baxter) was inserted via an external carotid arteriotomy incision. The catheter was advanced to the common carotid artery, inflated to a pressure of 2 atm. and rotated in a forward and retrograde direction. The catheter was then deflated and the process repeated three times. Treatment cohorts were divided into 5 groups (n=6-8/group): Control (sham operated), Didox (200 mg/kg/d), Imidate (200 mg/kg/d), HU (200 mg/kg/d) and vehicle
Drugs were administered daily by i.p. injection for a period of 7 days after injury. At 2 weeks post-injury, rats were euthanized by pentobarbital overdose and perfused with 10% buffered formalin. Carotid arteries were removed and placed in the same fixative. Tissues were then embedded in paraffin, and 4 to 5 sections (4 µm) were cut at multiple levels. These sections were then stained with hematoxylin-eosin or elastic-van Gieson stain. Sections were examined microscopically and the cross-sectional areas of the lumen, neointima (from the internal elastic lamina to the lumen), and media were determined using digital microscopy with Spot Advanced software. The intima-to-media (I/M) ratio was then calculated from the determined mean. The data represent the mean ± SD.

1.2.3 Flow cytometry

SMC were plated on 6 well dishes at a density of 2 X 10^5 cells per well. Cells were then treated with Didox (0-200 µM), Imidate (0-200 µM) or HU (0-1000 µM) and incubated for 24 hours. Following the 24 hour incubation period, the cells were trypsinized and collected in 15 mL centrifuge tubes. The cells were then centrifuged 5 minutes at 800 x g. The supernatant was discarded and the pellet was resuspended in 5 ml PBS. The cells were then centrifuged 6 minutes at 200 x g. The supernatant was removed and the pellet was then thoroughly resuspended in 0.5 ml PBS. The cell suspension was then transferred into tubes containing 70% ethanol, keeping the cells in fixative >2 hours. The ethanol suspended cells were centrifuged 5 minutes at 200 x g, and the ethanol was decanted. The cell pellet was resuspended in 5 ml PBS and after one minute was centrifuged 5 min at 200 x g. The top layer of liquid was again removed and the cell pellet was resuspended in 1ml PI/Triton X-100 staining solution with RNAse A. This staining solution was then incubated at room temperature for 30 minutes. Flow cytometry
was then performed using a FACS Calibur (Becton Dickenson). The data are presented as
the mean.

1.2.4 Intracellular dNTP quantitation

SMC were plated in T150 flasks and treated with Didox (0-200 μM), Imidate (0-
200 μM) or HU (0-1000 μM) and incubated for 24 hours. Following the 24 hour
incubation period, the cells were trypsinized and collected in 50 mL centrifuge tubes
along with the incubation media. All the extraction steps were performed on ice.
Immediately before processing, cells were counted and viability determined using the
trypan blue exclusion method. The cells were then centrifuged 5 minutes at 800 x g. The
cell pellet was then deproteinized with the same volume of 6% TCA, vortexed for 20
seconds and incubated on ice for 10 minutes. The acid cell extracts were centrifuged 10
min at 2000 x g. The supernatants were then supplemented with an equal volume of
distilled water, vortexed for 60 seconds and neutralized by the addition of 5M K₂CO₃
prior to HPLC analysis. dNTP detection was carried out using a ESA (Chelmsford, MA)
HPLC chromatographic system with UV-Vis detection. Chromatographic separations
were performed using a Tosohaa C18 reverse phase column (ODS 80Tm 250 X 4.6
mm, 5μm pore). The mobile phase was delivered at a rate of 1.0 mL/minute during the
analysis using the following stepwise gradient elution program: A-B (80-20) at 0 min;
(40-60) at 30 min; (40-60) at 40 min and (80-20) at 45 min. Buffer A contained 10 mM
tetraethylammonium sulfate, 10mM KH₂PO₄ and 0.25% MeOH, and adjusted to pH 6.9.
Buffer B consisted of 5.6 mM tetrabutylammonium sulfate, 50 mM KH₂PO₄ and 30%
MeOH, and neutralized to pH 7.0. The injection volume for analysis was 50μL. Detection was carried out at 254 nm.

1.2.5 Smooth muscle cell migration

A wound scrape assay was performed using rat vascular SMC. The cells were grown to confluence on 60 mm dishes. The cells were then made quiescent by incubating them in media containing 0.1% serum for 24 hrs followed by treatment with Didox, Imidate (0-100 μM) or HU (0-1000 μM) in media containing 0.1% serum and platelet derived growth factor (PDGF) at concentration of 10 ng/mL. Linear wounds were made by scraping each plate with the tip of a 20 μL pipette. The ability of cells to migrate across the wound area was measured using digital microscopy. SPOT advanced software was used to measure the wound immediately, 2, 4, 12 and 24 hours following injury. The data are presented as rate of migration and represent the mean ± SD.

1.3 Results

1.3.1 Effects of Ribonucleotide Reductase Inhibitors on Restenosis Following Vascular Injury

The effects of Didox, Imidate and Hydroxyurea on the vascular remodeling process following arterial injury were determined using a rat model of balloon mediated carotid injury. Each compound (200 mg/kg/day) was delivered via i.p. injection for a period of 7 days post injury. These dosages are based on previously published reports and represent half the maximum tolerated dose in rats (33, 34). Furthermore, we and others have demonstrated that these doses are sufficient to inhibit ribonucleotide reductase (RR) activity without causing significant toxicity (35-37).
At 14 days post injury the animals were sacrificed and morphometric analysis was carried out in order to assess the histopathological changes in the vessel wall (figure 1.1). Didox treatment resulted in a 62% decrease in neointimal area and a 61% decrease in intima/media ratio (figure 1.2). Imidate treated groups demonstrated a 57% decrease in neointimal area and a 55% decrease in the intima/media ratio (figure 1.2). Because Didox and Imidate possess other chemical attributes in addition to their effects on RR (38-43) the commercially available RR inhibitor, HU (200mg/kg/day), was also tested. HU afforded similar vascular protective effects to those observed with Didox and Imidate, resulting in a 55% decrease in neointimal area and a 63% decrease in intima/media ratio (figure 1.2). These results suggest that RR inhibition can modulate the remodeling process following vascular injury. However, because the remodeling process occurs over a prolonged period of time, additional studies were performed in order to determine whether the vascular protective effects observed would be mitigated over time.

To further investigate long-term efficacy of the one week dosing regimen, we increased the duration of the study period to 6 weeks. We found that the degree of neointimal thickening at 6 weeks post-injury was increased by 57% as compared to the 2 week study paradigm (figure 1.3). Interestingly, the beneficial effect of RR inhibition persisted over the long term. Each compound (200 mg/kg/day) was given I.P. for a period of 7 days followed by a 5 week recovery period. At the end of the 6 week period, the Didox treated group exhibited a 64% decrease in neointimal area and a 71% decrease in intima/media ratio (figure 1.3). Similarly, Imidate offered a 61% reduction in neointimal area and a 62% reduction in intima/media ratio (figure 1.3). HU treatment reduced neointimal formation by 71% and decreased the intima/media ratio by 75% (figure 1.3).
These results suggest that activation of RR is important in the vascular response to injury and that inhibition of this enzyme can limit the neointimal proliferation associated with restenosis.

1.3.2 Effects of Didox, Imidate and HU on SMC proliferation.

Didox, Imidate and HU are known to be potent RR inhibitors (28, 29, 44). RR catalyzes the reductive conversion of ribonucleotides to deoxynucleotides. This reductive reaction is a prime target for impeding cellular proliferation, and therefore amenable to inhibiting VSMC replication, because it is a rate limiting step in the biochemical pathway leading to DNA synthesis (28-31). The ability of Didox and Imidate to inhibit RR activity has been well documented with published reports demonstrating a concentration at which cell division was inhibited by 50 % (IC$_{50}$) of 3-30 μM for this class of compounds (29). These values represent a > 10 fold increased effectiveness over the classical RR inhibitor HU (44). Based on this evidence, experiments were performed in order to determine whether the ability of these compounds to reduce neointimal formation was due to their ability to inhibit SMC proliferation in-vitro. Therefore, we determined the IC$_{50}$ of each compound on inhibition of smooth muscle cell growth. Cells were incubated in the presence of Didox (0-200 μM), Imidate (0-200 μM) and HU (0-1000 μM) for 24 hours. Cell numbers were then counted using flow cytometry and the concentration at which cell division was inhibited by 50 % (IC$_{50}$) was calculated. Didox yielded an IC$_{50}$ of 67 μM, Imidate was slightly less potent exhibiting an IC$_{50}$ of 82 μM while HU was the least potent with an IC$_{50}$ of 266 μM (figure 1.4). These results are consistent with RR activity
data and demonstrate that Didox and Imidate are 3-4 times more potent than HU at arresting cell division.

In order to further validate that the observed anti-proliferative properties afforded by these compounds was through inhibition of RR activity, we measured the effects of these compounds on intracellular dNTP pools, particularly dATP pools. Results demonstrated that Didox (0-200 μM), Imidate (0-200μM) and HU (0-1000 μM) dose dependently depleted the endogenous dNTP pools with maximal reductions in dATP content of 58%, 42% and 69%, respectively (table 1.1).

The concentrations at which these drugs afford their *in-vitro* biological effects are well below the range of the peak plasma levels (300-400 μM) measured following Didox and Imidate infusion (200 mg/kg/day) and would be expected to inhibit RR activity based on the published and observed Ki’s for these compounds (29). These results would suggest that part of the vascular protective effects of these compounds is due to their ability to impede SMC proliferation.

1.3.3 Effects of Didox, Imidate and HU on SMC migration.

However, SMC migration is also a critical component of neointimal proliferation. Therefore, additional studies were performed in order to determine the effects of these compounds on SMC migration. Using a wound scrape assay, SMC migration studies were carried out in the presence of Didox, Imidate and HU. VSMC’s were cultured to confluence on 60 mm dishes. The cells were made quiescent by incubating in media containing 0.1 % serum. Following 24 hours of serum deprivation, Didox, Imidate and HU were added to the wells (10-1000μM) in the presence of PDGF (10 ng/mL) and a linear wound was made across the plate. SMC migration across the wound was monitored
by digital microscopy over a 24 hour period. Results from these studies demonstrated that Didox (100 µM) and Imidate (100 µM) treatment almost completely inhibited SMC migration, decreasing the migratory rate from 15.8 µM/hour in the control to 1.7 µM/hour and 0.9 µM/hour, respectively (figure 1.5). In contrast, HU (100 µM) had little effect on SMC migration, resulting in a migratory rate of 15.1 µM/hour (figure 1.4). No further inhibition was seen with HU concentrations up to 1 mM. These results demonstrate that Didox and Imidate significantly impair SMC migration. This would be expected to contribute to the vascular protective effects afforded by these drugs. However, RR inhibition appears to be the principal mechanism as the rat arterial injury data demonstrated similar efficacy with HU.

1.4 Discussion

Although PTCA and coronary artery stenting have had a tremendous impact on the treatment of coronary vascular disease, these procedures are marked by a high incidence of restenosis (15-19). This process of vessel re-narrowing is characterized by neointimal hyperplasia resulting in lumen loss and impaired vascular function. The vascular response to injury is often followed by a migratory and proliferative response within the smooth muscle cells resulting in intimal thickening (15, 17, 18, 21, 24, 25, 45-49). In this regard, emphasis has been placed on developing pharmacological therapy aimed at reducing the proliferative response. Currently, two pharmacological agents have been approved for clinical use in the treatment of post-PTCA restenosis (50-52). Taxol and Rapamyacin are being delivered through the use of coated coronary stents.
Preliminary results suggest that these approaches offer significant protection against the restenosis process and validate the use of antiproliferative agents in the treatment of vascular proliferative disorders such as restenosis (53-55).

As such, we have explored the use of RR inhibition as a new therapeutic target in ameliorating balloon mediated restenosis injury. The biochemical attributes of this enzyme make it amenable for the treatment of proliferative disorders since inhibition of RR blocks DNA synthesis and thus cell replication. Therefore, using as balloon model of arterial injury we have studied the effects of RR inhibition on the restenosis process.

Our results demonstrated that following balloon injury, one week of systemic administration of the RR inhibitors, Didox and Imidate, largely inhibited neointimal formation resulting in a 60% reduction in the intima/media ratio. Morphometric analysis revealed an approximate 60% reduction in neointimal area with no significant change in the medial area between treated and untreated groups. However, there was a small but statistically significant decrease in medial area following Imidate dosing when this cohort was compared against HU and Didox. These results suggest that Imidate may have some negative effects on smooth muscle remodeling following medial injury that result in medial wall thinning.

These in-vivo studies demonstrate that inhibition of RR limits the extent of intimal hyperplasia following mechanical injury. However, these compounds possess a variety of chemical attributes which may contribute to their protective effects. Didox has been shown to inhibit nuclear factor kappa B (NFκB) and Tissue Factor while both compounds are potent free radical scavengers (38, 42, 43, 56, 57). Because of the myriad of effects elicited by these compounds, further experiments were carried out using the
commercially available RR inhibitor, HU. Following the same dosing regimen for Didox and Imidate, HU afforded similar anti-restenotic efficacy, further supporting our observation regarding the importance of RR in the vascular response to injury.

Because the vascular response to injury is a chronic process, additional studies were performed in order to assess whether the protective effects elicited by early RR inhibition are maintained throughout the remodeling period. The RR inhibitors (Didox, Imidate and HU) were administered daily for one week and the extent of injury assessed at 6 weeks after balloon dilatation. This additional recovery time resulted in a greater than 50% increase in the intima/media ratio demonstrating the progression of the lesion over time. Interestingly, Didox and HU treatment reduced the intima/media ration by greater than 70%, while slightly less efficacy was observed with Imidate. Moreover, the degree of protection afforded by these compounds was significantly increased when compared to the results of the two week study. This suggests that RR is an early target in the vascular response to injury and that inhibition of this enzyme affects the long term vascular remodeling associated with restenosis.

These results demonstrate that RR inhibition limits the degree of restenosis following arterial dilation injury. We believe these effects are mediated through an inhibition of SMC proliferation, as this process precedes neointimal formation. Therefore, studies were performed using a cell proliferation assay. Didox, Imidate and HU treatment resulted in arrest of cell division. Analysis of dNTP pools demonstrated a greater than 50% reduction in dATP levels further supporting that the observed effects of these drugs are mediated at least in part through the inhibition of ribonucleotide reductase. These effects were independent of any cytotoxic action these compounds may
posses as flow cytometry revealed < 3% apoptotic cells following the dosing regimes tested. In addition, prior to dNTP analysis, cells were counted and viability assessed using trypan blue exclusion. Cell viability was greater than 95% among all groups.

As previously stated, Didox and Imidate possess various chemical attributes in addition to RR inhibition, many of which may confer protection against restenosis (38, 42, 43, 56, 57). Among these is their ability to scavenge free radicals, which may modulate SMC migration, a critical component of neointimal proliferation (56, 57). Therefore, we tested the effects of Didox and Imidate on SMC proliferation using a wound scrape assay. Results demonstrated that both compounds almost completely inhibited SMC migration. This would suggest that inhibition of SMC proliferation may contribute to the protection afforded by these compounds. Similarly, a number of studies have demonstrated that HU, when oxidized, can release nitric oxide (NO). (58, 59). Because NO has been shown to inhibit cell migration we tested whether HU possesses anti-migratory properties which could be involved in its anti-restenotic effects (60, 61). HU at concentrations up to 1 mM had no effect on SMC migration. This is an important observation and would suggest that inhibition of RR is the principal mechanism through which these compounds afford their protection as similar efficacy was seen with all compounds. However, if the in-vivo results are interpreted on a molar basis, HU (2.6 mmoles/kg/day) doses are 2-3 fold higher than that of Didox (1.2 mmoles/kg/day) and Imidate (0.9 mmoles/kg/day) and suggests that the effects of Didox and Imidate on SMC migration may contribute to the vascular protective effects observed with these two compounds.
Taken together, the in-vivo and in-vitro data demonstrate that activation of RR is a critical, early component in the proliferative response associated with vascular injury and that inhibition of this enzyme may reduce the vascular pathology associated with restenosis injury. Although the incidence of restenosis has markedly decreased with the advent of drug coated stents, restenosis still occurs in up to 20% of patients within the first year while results on late lumen loss are still being gathered (50, 51, 62-64). In addition, because the use of coated stents may increase the risk of thrombosis there is a need for agents which can be administered systemically in patients at high risk for thrombotic events (65). We believe that inhibition of RR may be a pathway that can be therapeutically targeted through either local or systemic delivery based on the low toxicity associated with current RR inhibitor therapy using HU. Additionally, our data suggests that RR is an early target in the restenosis process and as such, early pharmacological intervention may preclude chronic therapy and its associated adverse side effects. We believe that these observations have important therapeutic potential and implicate RR as a promising therapeutic target in the treatment of vascular proliferative disorders.
Table 1.1 Effects of Didox, Imidate and Hydroxyurea on smooth muscle cell dATP pools. Didox (0-200 μM), Imidate (0-200 μM) and Hydroxyurea (0-1000 μM) were added to the SMC culture during the log phase of growth and incubated for 24 hours. dNTP’s were extracted and samples subjected to HPLC analysis. The data are presented as pmoles dATP/10^7 cells and represent the mean ± SD. *Significantly different at p<0.05 as compared to control.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Control</th>
<th>Didox (μM)</th>
<th>Imidate (μM)</th>
<th>Hydroxyurea (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose</td>
<td>NT</td>
<td>5</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>dATP levels (pmoles/10^7 cells)</td>
<td>303</td>
<td>297</td>
<td>260</td>
<td>203*</td>
</tr>
<tr>
<td>S.D.</td>
<td>50</td>
<td>35</td>
<td>26</td>
<td>20</td>
</tr>
</tbody>
</table>
Figure 1.1 Effects of Didox, Imidate and HU on the histopathology associated with balloon dilatation injury. Examples are from carotid artery sections stained with hematoxylin and eosin. Panel (A) represents a control (uninjured) section of rat carotid artery. Panel (B) demonstrates the marked neointimal hyperplasia present at 2 weeks post balloon dilatation injury. Panel (C) demonstrates the inhibitory effects of Didox (200mg/kg/day) on neointimal proliferation in response to balloon injury. Panel (D) demonstrates the inhibitory effects of Imidate (200 mg/kg/day) on neointimal proliferation in response to balloon injury. Panel (E) demonstrates the inhibitory effects of HU (200 mg/kg/day) on neointimal proliferation in response to balloon injury. Note the marked reduction in neointimal (NI) thickness following Didox, Imidate and HU treatment.
Effects of Didox, Imidate and HU on histopathological changes following balloon injury of the rat carotid artery at 2 weeks post injury. Morphometric analysis was performed at the conclusion of the study. A.) Neointima formation. B.) Medial wall thickness. C.) Intima to media ratio. (Control) represents the uninjured contralateral artery. (Injured) represent the ipsilateral balloon dilated artery. (Didox) groups were administered the drug immediately following injury followed by daily administration for 6 days. (Imidate) groups were administered the drug immediately following injury followed by daily administration for 6 days. (HU) groups were administered the drug immediately following injury followed by daily administration for 6 days. The data represent the mean ± SD. * Significantly different at p<0.05 as compared to injured (untreated). ‡ Significantly different at p<0.05 among treated groups.
Figure 1.3 Effects of Didox, Imidate and HU on histopathological changes following balloon injury of the rat carotid artery at 6 weeks post injury. Morphometric analysis was performed at the conclusion of the study. A.) Neointima formation. B.) Medial wall thickness. C.) Intima to media ratio. (Control) represents the uninjured contralateral artery. (Injured) represent the ipsilateral balloon dilated artery. (Didox) groups were administered the drug immediately following injury followed by daily administration for 6 days. (Imidate) groups were administered the drug immediately following injury followed by daily administration for 6 days. (HU) groups were administered the drug immediately following injury followed by daily administration for 6 days. The data represent the mean ± SD. * Significantly different at p<0.05 as compared to injured (untreated). ‡ Significantly different at p<0.05 among treated groups.
Figure 1.4 Effects of Didox, Imidate and HU on SMC proliferation. Didox (0-200 μM), Imidate (0-200 μM) and HU (0-1000 μM) were added to the SMC culture during the log phase of growth and incubated for 24 hours. Cells were then counted using a flow cytometer. Values represent the mean. (n = 4).
**Figure 1.5** Effects of Didox, Imidate and HU on SMC migration. Didox (0-100 μM), Imidate (0-100 μM) and HU (0-1000 μM) were added to the SMC culture in media containing 0.1 % serum and 10ng/mL PDGF. A wound scrape was then made and SMC migration was monitored for an additional 24 hours. The data represent the mean ± SD. * Significantly different at p<0.05 as compared to control.
CHAPTER 2
RIBONUCLETIDE REDUCTASE INHIBITORS REDUCE ATHEROSCLEROSIS
IN A RABBIT MODEL OF CAROTID INJURY

2.1 Introduction

Cardiovascular disease and atherosclerosis, in particular, are diseases with multiple. An atherosclerotic lesion can be elicited as a response to injury and is known to be an inflammatory process, but determining a single underlying disease mechanism leading to this vascular reaction has proven to be challenging. As such, developing a single animal model that encompasses all or even many of these processes is not possible (66).

For many years now, rabbits have been utilized for studies of atherosclerosis. There are essentially two categories of atherosclerotic rabbits: those that have heritable hyperlipidemia leading to atherosclerosis and those that are cholesterol-induced. The lesions seen in the cholesterol-induced atherosclerotic rabbits have foam cells, evidence of fibrous plaque formation, fatty streaks and are similar to the lesions seen in human atherosclerosis (67-69). The advantage of using a rabbit model of atherosclerosis is that rabbits are small, yet large enough to perform surgical experiments, relatively inexpensive and the atheromatous changes can develop within a period of 4-12 weeks (66, 70). It was for these reasons that our laboratory chose the rabbit as an intermediate animal model for our pharmacological investigation looking for alternative approaches to preventing restenosis that occurs after percutaneous transluminal coronary angioplasty (PTCA).

Currently PTCA with stent deployment is the mainstay of coronary artery disease (CAD) therapy; however, this procedure is plagued by a high incidence of restenosis which is
responsible for 10-20% of long term failure following coronary revascularization (15-18). Recently, drug-eluting stents have come to the forefront as showing promising results in limiting restenosis; however, there is developing data which suggests that the clinical benefits may be overestimated and that late-developing fatal thrombosis may be an unfortunate complication (71, 72). Therefore, prevention of restenosis after successful PTCA still remains one of the most challenging tasks in the treatment of obstructive coronary artery disease (CAD) and as such alternative pharmacological approaches are currently being pursued.

The vascular trauma associated with PTCA initiates the vascular smooth muscle cells (VSMC) to undergo a modulation from a contractile to a synthetic phenotype. They then proliferate in the tunica media, migrate to the tunica intima and consequently form the neointima(15, 20, 25). Thus pharmacological agents being investigated to ameliorate this response include those which may impede VSMC proliferation and migration.

Ribonucleotide reductase (RR) when activated by a free radical intermediate catalyzes the conversion of ribonucleotides to deoxynucleotides. This reductive reaction is not only the rate limiting step in the biochemical pathway leading to DNA synthesis and cell replication, but, in addition, DNA synthesis cannot occur without invoking this reaction since the endogenous pools of dNTP in mammalian cells is inadequate to support new DNA synthesis (28, 29, 44). Our research has demonstrated that inhibition of this critical enzyme in a rat model of balloon mediated carotid artery injury resulted in a reduction of restenosis and a significant amount of cell cycle arrest. Because the previous studies were performed in an otherwise healthy vessel, we chose the rabbit hypercholesterolemia model which more closely mimics the clinical setting of intervention on a diseased vessel. Therefore, in the
present study we investigated the role of the ribonucelotide reductase (RR) inhibitors in preventing the incidence of atheroproliferative disorders such as atherosclerosis/restenosis using a rabbit cholesterol-induced atherosclerosis model.

2.2 Methods

2.2.1 Surgical Procedures

The experimental protocol was designed and conducted in accordance with ILACUC standards. New Zealand rabbits (2-3 kg) were placed under general anesthesia and underwent angioplasty using sterile surgical technique. A 3 mm, wire-guided balloon catheter was inserted into the right common carotid artery via an ateriotomy in either the right or left femoral artery. Progress of the catheter and the location of the injury was monitored using fluoroscopy (figure 2.1). Endothelial injury of the carotid was initiated by inflating the balloon to 12 atm and moving the inflated balloon in a forward and retrograde direction. The catheter was removed, the ateriotomy closed and the animals were allowed to recover. The rabbits were then placed on a high fat diet (2% cholesterol, 1% peanut oil) for the duration of the study. At 4-weeks post initial injury, balloon angioplasty of the developing atherosclerotic lesion was performed. A 3 mm X 25 mm, wire-guided balloon catheter was inserted into the injured common carotid artery via an ateriotomy in the non-accessed femoral artery. Lesion formation was verified by IVUS and the balloon was inflated at the site of injury to 12 atm, 3 times for 5 seconds. Following angioplasty, the catheter was removed and the femoral ligated. The animals were recovered and the high fat diet continued for an additional 4-weeks. At the conclusion of the study, the animals were euthanized and the carotids removed for histological evaluation.
2.2.2 Pharmacological Treatments

Following the initial injury, rabbits were given a subcutaneous (SC) injection of either Didox (a novel RR inhibitor) at 200 mg/kg or Hydroxyurea (HU) at 400 mg/kg (a commercially available RR inhibitor). Didox and Hydroxyurea were both provided by Molecules for Health Inc. (Richmond, VA). Injections were given SC, 3 days a week throughout the duration of the study. Treatment groups consisted of control (no balloon injury), injured (balloon injury only), Didox (balloon injury + Didox) and HU (balloon injury + Hydroxyurea) n=4-6 animals/group.

2.2.3 Histological Assessment

Injured and contralateral carotid arteries were perfusion fixed and paraffin embedded. The tissues were then sectioned at 8 µm and stained with hemotoxin-eosin, trichrome or elastic-van Gieson stain. Alternating sections were left unstained for immunohistochemical analysis. Morphometric analysis of the cross section of the arteries was performed using Spot Advanced software.

2.3 Results

2.3.1 Effects of RR inhibitors on Atherosclerosis

Using the rabbit model of double injury with hypercholesteremia, we were able to generate an atherosclerotic lesion with pathological characteristics similar to those seen in the human atheromatous plaque (i.e. foam cell formation, lipid accumulation, acellular core, fibrous cap and thin shoulder regions) (figure 2.2). As such, this model was employed to assess the athero-protective properties of Ribonucleotide Reductase inhibition. The effects of two RR inhibitors, hydroxyurea (HU) and Didox, on both atheroma development and vascular...
remodeling processes were determined. Lesion sizes were measured and results demonstrated an atheroma area of 1.13 mm$^3$ in control (untreated rabbits). Treatment with either RR inhibitor resulted in significantly reduced lesion area with Didox (200mg/kg) reducing the lesion area to 0.60 mm$^3$ and HU (400 mg/kg) groups to 0.57 mm$^3$ (figure 2.3). Moreover, RR inhibition completely prevented lumen loss (43 % in control-untreated) associated with the atherosclerotic lesion (figure 2.4). The dosages of Didox and HU used for these studies were based on previously published reports and are sufficient to inhibit RR activity without causing significant toxicity (39, 73, 74).

2.3.2 Hematological Effects of RR Inhibition

Measurements of total plasma cholesterol were performed at sacrifice in order to confirm that the cholesterol burden was similar among the treatment groups. Results demonstrated that all groups had an average plasma cholesterol level above 1200 mg/dL and the differences in levels were not statistically significant among the groups (table 2.1). Hematological results demonstrated that the RR treated groups had significantly reduced numbers of circulating leukocytes as compared to non-treated controls. Didox treatment resulted in a 74% decrease in leukocyte count as compared to control (6.1 x 10$^3$/μL vs 1.6 x 10$^3$/μL) while HU treatment reduced leukocyte counts by 59% (6.1 x 10$^3$/μL vs 2.5 x 10$^3$/μL) (table 2.1).

2.4 Discussion

It has been proposed that endothelial dysfunction is the initiating step in the cascade of events leading to an atherosclerotic plaque (15, 20, 75, 76). As such, the double injury rabbit model of atherosclerosis which involves an initial injury of the endothelium is an
important and appropriate representation of the potential underlying cellular mechanisms of this disease. In addition, this approach creates a complex lesion which closely mimics human atherosclerotic lesions in that histologically there is evidence of neointimal formation, smooth muscle cell proliferation and migration, and increasing numbers of lipid-laden macrophages (foam cells) resulting in luminal stenosis. Although the human lesion tends to also have other attributes, such as areas of fibrocalcification and necrosis, much of the research done today is aimed primarily at the arterial response to injury, and the vascular response in these animals, as seen by ultrasound and histology, does exhibit similar pathology to that seen in humans in vivo and at necropsy (77).

In the present study we examined the role of ribonucleotide reductase (RR) in neointimal hyperplasia, atheroma production and vascular remodeling following balloon injury. As the vascular response to injury triggers a migratory and proliferative response from the smooth muscle cells, emphasis has been placed on developing pharmacological therapy aimed at reducing the proliferative response. Inhibition of RR blocks DNA synthesis and thus cell replication making it an enzyme of interest for the treatment of atheroproliferative disorders. In this regard, we have demonstrated that treatment of rats with RR inhibitors significantly reduces intimal hyperplasia in a rat model of balloon injury (78). These studies, however, were performed in an otherwise healthy vessel and do not mimic the clinical setting wherein dilation injury is occurring in a diseased vessel. As such, the current studies were performed using a double injury model with superimposed hypercholesterolemia, which more closely mimics the clinical setting. Results from these studies demonstrate an almost 50% reduction in atheroma area following RR inhibition. Moreover, the 43% lumen
loss observed in the control untreated animals was completely prevented with Didox and HU treatment.

The athero-protective effects of Didox and HU were independent of cholesterol lowering effects as there was no statistically significant difference between the cholesterol levels in the control and treated animals. However, hematologic results demonstrated greatly reduced white blood counts in the RR treated groups. Because atherosclerosis is ultimately an inflammatory disease characterized by leukocyte infiltration and foam cell formation, reducing circulating leukocytes may be beneficial in protecting the vasculature. In this regard, immunohistochemical studies demonstrated reduced leukocyte infiltration in the plaques of Didox and HU treated animals.

These results implicate Ribonucleotide Redutase as a potential therapeutic target for the development of anti-atherosclerotic therapy and support a role for anti-proliferative therapy in the treatment of vasculoproliferative disorders.
<table>
<thead>
<tr>
<th>Group</th>
<th>Cholesterol (mg/dL)</th>
<th>RBC (x10³/μL)</th>
<th>WBC (x10³/μL)</th>
<th>Platelets (x10³/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (HC)</td>
<td>1412 ± 587</td>
<td>5.1 ± 0.8</td>
<td>6.1 ± 1.7</td>
<td>322 ± 122</td>
</tr>
<tr>
<td>Didox (HC)</td>
<td>1255 ± 481</td>
<td>3.93 ± 0.6</td>
<td>1.6 ± 0.9</td>
<td>224 ± 60</td>
</tr>
<tr>
<td>HU (HC)</td>
<td>1540 ± 617</td>
<td>4.55 ± 0.8</td>
<td>2.5 ± 1.3</td>
<td>262 ± 78</td>
</tr>
</tbody>
</table>

Table 2.1 Blood profiles of the rabbit groups taken at the time of euthanasia.
Figure 2.1 Fluoroscopy depicting a 3 mm wire guided balloon in the left common carotid artery of a New Zealand rabbit under general anesthesia. Balloons were inserted via the left or right femoral artery and inflated to 12 atm. The inflated balloons were moved in a forward and retrograde direction to produce endothelial injury.
Figure 2.2 Rabbits were split into 4 groups (4-6 per group). The groups consisted of a control group that was not injured, an injury group, a Didox treated group (Rabbits were injured and injected with 200 mg/kg Didox subcutaneously 3 days a week for the duration of the study) and a Hydroxyurea treated group (Rabbits were injured and injected with 400 mg/kg Hydroxyurea subcutaneously 3 days a week for the duration of the study). Endothelial cells of the carotid were injured by moving a 3 mm balloon in a forward and retrograde direction. Animals were then placed on a high fat diet (2% cholesterol, 1% peanut oil) for the duration of the study. At 4 weeks post initial injury, balloon angioplasty of the developing atherosclerotic lesion was performed. Lesion formation was verified using IVUS and the balloon was inflated at the site of injury to 12 atm 3 times for 5 seconds. Animals were put back on a high fat diet for an additional 4 weeks. At the conclusion of the study they were euthanized and carotids were removed for histological examination.
Figure 2.3  Mean and standard deviation of atheroma area in the 4 rabbit groups at the end of the study. Measurements were taken using Spot Advanced software.
Figure 2.4 Mean and standard deviation of lumen area in the 4 rabbit groups at the end of the study. Measurements were taken using Spot Advanced software.
CHAPTER 3
EFFECTS OF RIBONUCLEOTIDE REDUCTASE INHIBITORS USING A PORCINE MODEL OF RESTENOSIS

3.1 Introduction

Prevention of restenosis after successful PTCA remains one of the most challenging tasks in the treatment of obstructive coronary artery disease. However the introduction of coronary stents has significantly improved both short term and long term outcome following interventional coronary revascularization procedures. Despite a reduction in restenosis rate with stent deployment, restenosis still occurs in 15-30 % of patients within 6 months (16, 17). This incidence of in-stent restenosis is expected to increase as coronary stenting is becoming more frequent and is used in less ideal lesions (45). Therefore, in addition to mechanical intervention, pharmacological approaches to reduce the incidence and degree of restenosis are being pursued.

The vascular trauma associated with PTCA involves a cascade of molecular and cellular events occurring within the vessel wall involving the release of a variety of vasoactive, thrombogenic, and mitogenic factors. Within this cascade, several mechanisms contribute to restenosis including elastic recoiling, thrombosis, smooth muscle cell migration, re-endothelialization, matrix formation and neointimal hyperplasia (18, 20-22, 49, 75, 79-83). Neointimal hyperplasia resulting from smooth muscle cell migration and proliferation represents a plausible target in the development of therapeutic agents aimed at modulating the vascular remodeling process associated with restenosis.
In response to injury, vascular smooth muscle cells (VSMC’s) undergo a phenotypic modulation from a contractile to a synthetic phenotype (differentiation). They then proliferate in the media, migrate from the media to the intima and consequently form the neointima (23-26). An increasing body of evidence suggests that inflammation plays a pivotal role in linking early vascular injury to subsequent neointimal proliferation (21, 24, 82). Several studies have suggested that platelet/fibrin deposition and activation of inflammatory genes, followed by increased leukocyte trafficking into the injured vessel wall, may provide a key stimulus for subsequent neointima formation (27, 84).

Because ultimately the cascade of molecular and cellular events following vascular trauma culminates in a proliferative response, it would follow then that among targets being pursued would be agents which can impede smooth muscle cell migration and proliferation, as these processes are critical components of restenosis injury. In this regard, we propose that inhibiting the conversion of ribonucleotides to deoxyribonucleotides is an attractive approach to the problem of restenosis.. Therefore, inhibition of RR, may serve as a novel therapeutic target in the treatment of vascular proliferative disorders, specifically restenosis.

The purpose of this research is to investigate the potential effects of the novel drug Didox to limit the degree of coronary restenosis in the pig using a stent based delivery platform. The pig is an ideal model due to increased vessel size as compared to a rat or rabbit model. In pigs, the LAD can be injured, which is more similar to a typical human lesion.
3.2 Methods

3.2.1 Porcine Model of In-stent Restenosis

Male and female pigs weighing 35 to 45 kg were used to perform the studies. The animals were sedated with ketamine (20 mg/kg IM) and acepromazine (0.22 mg/kg IM) and given sodium pentobarbital (4 mg/kg IV) to facilitate supine positioning and endotracheal intubation. Anesthesia was maintained with 1% to 2% isoflurane in oxygen flowing at 2 L/min. A 6F arterial sheath was inserted into the right femoral artery under sterile surgical technique, and heparin (5000 U) administered as an intra-arterial bolus.

An 18 mm Velocity RX stent (Cordis Corp., Miami Lakes, FL) was advanced to the left anterior descending coronary artery (LAD) through an 8F IM guiding catheter and deployed by two 30-second balloon inflations at 8 atm. The segment of artery that was stented was selected to allow 1.2 times oversizing by visual estimation using the guide catheter as a reference. Coronary angiography was performed in 2 views (generally anteroposterior and 30° left anterior oblique) immediately before and after stent implantation. At 14 days post-stent deployment, IVUS measurements were performed on the stented arterial segment in order to evaluate lesion progression. IVUS evaluations were performed using a mechanical IVUS system (ClearView, Boston Scientific). The arterial wall at maximum stenosis was specifically evaluated using a 40 MHz Atlantis™ SR Pro IVUS imaging catheter (Boston Scientific). The sonograms were graded by 2 independent observers, (Cardiology Fellows, UFl Medical Center) using computerized planimetry (TapeMeasure, Indec Systems). Media thickness can not be measured accurately by IVUS; therefore, plaque cross sectional area (CSA) was measured as external elastic membrane (vessel) CSA minus the lumen CSA.
Left coronary angiography was performed before, immediately after, and 4 weeks after the stent implantation. A preshaped IM catheter was inserted into the right or left femoral artery, and coronary angiography in a left anterior oblique view was performed. Arterial pressure, heart rate, and ECG was continuously monitored and recorded.

3.2.2 Histopathological Study

Morphometric analysis was performed to quantify the effects of RR inhibition on the vascular remodeling that occurs following stent deployment. For histological analysis, the heart was excised 4 weeks after stent implantation; the left coronary artery was perfused with 10% formalin at 120 mm Hg and fixed for 24 hours. The dissected whole artery was then embedded in methylmethacrylate, leaving the stent wires intact to minimize potential artifacts from cutting the wires. Morphometric analysis was then carried out as described below.

3.2.3 Virtual Histology

Images of the histological slides were taken using ImageScope. The images were taken at 4x and 20x magnification. The images were then downloaded into MetaMorph 7.1 by Molecular Devices. The thickness of the intima in the control vessels and Didox vessels was measured by using the program to draw a linear line from the lumen to the internal elastic lamina. The length of the line was then generated in micrometers by Metamorph. The length of the media for the same vessels was generated by drawing a line from the internal elastic lamina to the external elastic lamina. Metamorph was also used to measure the area of the intima and media of the control vessels and the Didox vessels. A circle was drawn around the lumen to find the area inside the lumen. Similar circles were drawn around the internal elastic lamina and the external elastic lamina. The
area inside the circles was generated by Meta morph in micrometers squared. The area of the intima was calculated by subtracting the area inside the lumen from the area inside the internal elastic lamina. The area of the media was calculated by subtracting the area inside the internal elastic lamina from the area inside the external elastic lamina.

### 3.3 Results

#### 3.3.1 Effects of the Ribonucleotide Reductase inhibitor Didox on in-stent restenosis.

Results indicated that at 28 days post-stent deployment, stented coronary arteries exhibited a large degree of intimal hyperplasia, significant lumen loss and leukocyte infiltration (figure 3.1). Specifically, morphometric analysis revealed a significant decrease in lumen area between the control (3.48 ± 0.2 mm$^2$) and the bare metal stent group (1.49 ± 1.13 mm$^2$) (figure 3.2). In addition, there was a significant increase in medial area (1.18 ± 0.16 mm$^2$ vs. 1.67 ± 0.86 mm$^2$) and neointimal area (0.08 ± 0.01 mm$^2$ vs. 0.59 ± 0.67 mm$^2$) between the control and bare metal stent groups, respectively (figures 3.3-3.4). The stented segments also exhibited robust leukocyte infiltration surrounding the strut areas (figure 3.1). These results demonstrate the feasibility of the studies and demonstrate a proliferative restenosis injury with a significant inflammatory component. Subsequent morphometric assessment of the anti-restenotic potential of Didox revealed an exacerbation of the injury and an increased inflammatory response. When compared to the bare metal stent group, Didox drug eluting stents worsened the outcome of all morphometric measurements performed. Specifically, lumen area decreased to 1.04 ± 0.68 mm$^2$, medial area increased to 3.06 ± 1.34 mm$^2$ and neointimal area increased to 1.89 ± 1.53 mm$^2$ (figures 3.1-3.4). Further confirmation of the
worsened outcome with Didox drug eluting stents is the significant increase in intima/media ratio (I/M). The I/M ratio is the gold standard morphometric assessment for evaluating the anti-restenotic effects of potential therapeutic compounds. Results demonstrated that bare metal stents result in an increase in the I/M ratio from $0.07 \pm 0.01$ mm$^2$ in control groups to $0.29 \pm 0.18$ mm$^2$ in bare metal stent groups and this indices of injury was significantly worse in the Didox group ($0.53 \pm 0.35$ mm$^2$) (figure 3.5). These results demonstrate that the use of Didox drug eluting stents resulted in a greater than 3 fold increase in neointima formation and an almost two fold increase in the I/M ratio. Histopathological evaluation of the stented segments demonstrated a significant increase in the number of inflammatory cells in the Didox group and would suggest that the polymer used for drug elution may contribute to the inflammatory response observed with mechanical injury of the vessel wall.

3.4 Discussion

Our previous studies have demonstrated the anti-proliferative and anti-restenotic effects of ribonucleotide reductase inhibition in several animal models including the balloon injured rat and hypercholesterolemic rabbit. These animal models provide important preclinical data supporting the further pursuit of ribonucleotide reductase inhibitors for treating or preventing restenosis. However, each of these models has inherent weaknesses which dictate the use of additional models for preclinical development. The rat model is often used for proof of concept and mechanistic studies due to accessibility and cost; however the rat balloon model of injury does not mimic the clinical setting since injury is performed on an otherwise healthy vessel and may mitigate
the effects of chronic inflammation present in diseased vessels. As such, the rabbit hypercholesterolemic model is often used wherein endothelial injury and atherosclerosis are induced prior to balloon dilatation injury \((78)\). This model more closely mimics the clinical setting of intervention on a diseased vessel but does not employ the use of stents, which is the current standard of therapy. Recent clinical studies reveal that >90% of all coronary interventions employ the use of drug eluting stents and this has become the gold standard of treatment as drug-eluting stents have a greatly reduced incidence of target lesion restenosis \((71, 72)\). Currently, Taxol and Rapamycin are the only two drugs FDA approved for stent use \((71, 72)\). Stent delivery of these drugs was originally developed to overcome the toxicity concerns of these agents when used systemically. This technology has reduced the incidence of restenosis, however, thrombotic events have increased and thus called into question the efficacy of drug eluting stents \((85)\). Nevertheless, this technology has shifted the pharmaceutical pipeline towards the development of novel anti-proliferatives which can be used on a stent platform. Novel target agents sought are those which have similar or better anti-proliferative properties than current agents and devoid of the pro-thrombotic effects of current therapy. In this regard, the current study was designed to investigate the anti-restenotic effects of a Didox drug eluting stent following implantation into a porcine coronary artery. This model most closely mimics the clinical setting and is a requirement for final preclinical development of new pharmacological agents to treat in-stent restenosis.

Initial studies were performed in order to validate the method and assess the time course and degree of restenosis injury following stent deployment in the coronary artery. In the study, 2 mm Velocity stents were percutaneously advanced into the
proximal LAD and deployed. Luminal patency and stent positioning was confirmed by angiography. At 28 days post-injury, the animals were sacrificed and the stented segment of the LAD was removed and fixed for histological assessment. Morphometric analysis revealed significant lumen loss and neointimal hyperplasia providing critical evidence as to the suitability of the model. Subsequent studies were carried out using a Didox drug eluting stent platform employing polycaprolactone as the drug releasing polymer. Polycaprolactone is a biologically inert, biodegradable polymer which has been used in the biomedical engineering field as a matrix for tissue engineering (86).

Results demonstrated that the use of Didox resulted in an exacerbation of the injury and an increased inflammatory response. When compared to the bare metal stent group, Didox drug eluting stents worsened the outcome of all morphometric measurements performed. Specifically, lumen area decreased by nearly 50%, medial area increased to almost two fold and the neointimal area increased 3.2 fold. Further confirmation of the worsened outcome with Didox drug eluting stents is the significant increase in intima/media ratio (I/M). The I/M ratio is the gold standard morphometric assessment for evaluating the anti-restenotic effects of potential therapeutic compounds. Results demonstrated that when compared to bare metal stents Didox drug eluting stents increased the I/M ratio by 82%. Histopathological evaluation of the stented segments demonstrated a significant increase in the number of inflammatory cells in the Didox group and would suggest that the polymer used for drug elution may be contribute to the inflammatory response observed with mechanical injury of the vessel wall.

Based on our initial results using ribonucleotide reductase inhibitors which demonstrated a significant reduction in both restenosis and atherosclerosis in the rat and
rabbit models, we predicted that Didox delivery via a drug eluting stent platform would provide similar efficacy in the porcine model. In retrospect we see that this was a gross oversimplification and there are several possibilities which may explain the exacerbation of injury observed with the Didox stent. Histological assessment demonstrates a significantly increased inflammatory response characterized by granuloma formation and giant cell infiltration. This is likely due to an inflammatory response of the polymer coating which was not present on our stented controls. Additional studies will be required to evaluate the vascular response to polymer alone coated stents and more biologically inert materials may need to be selected. In addition to possible polymer mediated effects, the studies performed in chapters 1 and 2 were carried out with systemic drug delivery while the porcine studies employed a local stent delivery modality. Given that the systemic treated animals exhibited significantly decreased leukocyte counts, it is possible that the beneficial effects observed in the systemic studies are a direct consequence of the reduction in both circulating and infiltrating leukocytes. The use of a local drug delivery platform would be expected to eliminate the systemic effects on WBC’s and may therefore eliminate an important therapeutic pathway through which these agents reduce restenosis. Future studies will need to be designed to specifically address these concerns in order to appropriately examine the potential therapeutic benefits afforded by ribonucleotide reductase inhibitors.
Figure 3.1 A.) 4X control bare metal stent; B.) 20X control bare metal stent, area around stent; C.) 4X Didox treated; D.) 10X Didox treated, area around stent. Porcine hearts were excised 4 weeks after stent implantation. The left coronary artery was perfused with 10% formalin at 120 mm Hg and fixed for 24 hours. The whole artery was embedded in methylmethacrylate and the stent wires left intact to minimize potential artifact.
Porcine hearts were excised 4 weeks after stent implantation. The left coronary artery was perfused with 10% formalin at 120 mm Hg and fixed for 24 hours. The whole artery was embedded in methylmethacrylate and the stent wires left intact to minimize potential artifact. Lumen area was obtained using Metamorph 7.1.

Figure 3.2
Figure 3.3 Porcine hearts were excised 4 weeks after stent implantation. The left coronary artery was perfused with 10% formalin at 120 mm Hg and fixed for 24 hours. The whole artery was embedded in methylmethacrylate and the stent wires left intact to minimize potential artifact. Media area was obtained using Metamorph 7.1.
Figure 3.4 Porcine hearts were excised 4 weeks after stent implantation. The left coronary artery was perfused with 10% formalin at 120 mm Hg and fixed for 24 hours. The whole artery was embedded in methylmethacrylate and the stent wires left intact to minimize potential artifact. Neointimal area was obtained using Metamorph 7.1.
Figure 3.5 Porcine hearts were excised 4 weeks after stent implantation. The left coronary artery was perfused with 10% formalin at 120 mm Hg and fixed for 24 hours. The whole artery was embedded in methylmethacrylate and the stent wires left intact to minimize potential artifact. Intima/Media ratio was obtained using Metamorph 7.1.
DISCUSSION

The introduction of percutaneous transluminal coronary angioplasty (PTCA) revolutionized the surgical treatment of coronary artery disease. However, despite increased surgical experience and technical breakthroughs, restenosis occurs in 30%-40% of patients undergoing simple balloon angioplasty and in 15%-30% of patients who receive an intravascular stent. The pathophysiology of restenosis is complex and incompletely understood. Current evidence suggests that restenosis is a maladaptive response of the coronary artery to trauma induced during angioplasty consisting of thrombosis, inflammation, cellular proliferation, and extracellular matrix production that together contribute to postprocedural lumen loss over approximately 6 months. Lumen loss after balloon angioplasty can be separated into 3 stages: early loss associated with elastic recoil, late loss due to negative remodeling, and neointimal hyperplasia (16,19).

The need for reintervention in a high percentage of patients due to restenosis remains an important limitation to the long-term success of PTCA. Stenting reduces initial elastic recoil and limits negative arterial remodeling; however, bare-metal stents may promote intimal hyperplasia by eliciting an immune and proliferative response. Consistent with these data, clinical studies suggest that drug-eluting stents, coated with anti-inflammatory or antiproliferative agents, reduce the risk for restenosis (51, 62, 87).

Multiple randomized trials have demonstrated that drug-eluting stents (DES) can significantly reduce rates of restenosis by 60–75% across both lesion and patient subsets.
In recent years there has been an exponential increase in the worldwide use of DES. This expansion has occurred as a result of an enthusiastic extrapolation of results from randomized trials leading to use of DES in anatomical or clinically high-risk scenarios for which stent use may be contraindicated. However, emerging long-term follow-up data have raised concerns about the safety of drug eluting stents. A number of meta-analyses studies have shown increased rates of late stent thrombosis in patients receiving DES.

Although late stent thrombosis and non-cardiac mortality are both rare events (affecting between 0.5 and 1.0% of patients), their importance is only apparent when placed into the perspective of current interventional practice. Considering that around one million drug eluting stents are deployed annually, an adverse event rate of 1% could potentially account for 10,000 events a year, a figure which cannot be ignored. Emerging data clearly indicates that the original clinical trials for DES and their subsequent meta-analysis have been underpowered and likely do not detect rare adverse events. Furthermore, the conditions of patients recruited to these studies were also significantly less complex than the current real-world practice, hence further decreasing the chance of detecting rare adverse events.

Despite major concerns over late adverse events, there is no question that Drug-eluting stents have dramatically reduced the incidence of restenosis in patients undergoing PTCA. However, stenting represents a considerable cost burden and even with drug-eluting stents, a significant percentage of higher-risk patients develop in-stent restenosis. Treatment strategies should focus on selective use of expensive drug-eluting stents in populations where they have been found to be more clinically effective than
bare-metal stents (ie. patients who are at high risk for restenosis or who develop restenosis with bare-metal stents). Recent studies suggest that the pharmacologic management of restenosis is now feasible (88). Together, the use of stents and oral pharmacotherapy promise to reduce the risk for restenosis, even among high-risk patients. These data suggest that a role remains for effective, well-tolerated systemic pharmacologic therapies to further reduce the rate of restenosis. Among the therapeutic targets being pursued are agents which can impede smooth muscle cell migration and proliferation as these processes are critical components of restenosis injury. We propose that inhibiting the conversion of ribonucleotides to deoxyribonucleotides will impede cell proliferation and as such limit the degree of restenosis. Therefore, we tested whether the potent Ribonucleotide Reductase inhibitors, Didox and Imidate, can limit the neointimal proliferation associated with restenosis using a rat carotid model of balloon dilatation injury. Results demonstrated that both Didox and Imidate significantly reduced intimal thickening, resulting in a 71% and 62% decrease in the intima/media ratio, respectively. Similar efficacy was seen with the commercially available RR inhibitor, Hydroxyurea, demonstrating the importance of this enzyme in vascular remodeling. Results from cell proliferation studies suggest that the mechanism of protection is inhibition of SMC proliferation. In addition, Didox and Imidate (100 microM) are potent inhibitors of SMC migration, which may also contribute to their vascular protective effects. These results suggest that inhibition of Ribonucleotide Reductase may provide a potent strategy to prevent in-stent restenosis.

Based on the robust anti-restenotic effects exhibited by ribonucleotide reductase inhibition in the rat balloon injury model, we carried out additional studies using the rabbit
A hypercholesterolemic model which more closely mimics the clinical setting in which balloon injury is performed on a diseased vessel. Studies were carried out using a rabbit double model of carotid injury in which an initial percutaneous endothelial denudation is performed followed by the administration of a high cholesterol diet. At 4-weeks post-injury, the developing atherosclerotic lesion was subjected to percutaneous transluminal angioplasty and the degree of restenosis and atheroproliferation was assessed at 8-weeks. Results demonstrated that treatment with the ribonucleotide reductase inhibitors Didox and Hydroxyurea resulted in a significant decrease in lesion area and reduced lumen loss. Histological evaluation of the lesion demonstrated decreased leukocyte infiltration and reduced circulating leukocytes in the Didox and HU treated cohorts. These results implicate Ribonucleotide Reductase inhibition as a novel therapy in the treatment of atheroproliferative disorders through its effects on vascular and immune cell proliferation. Together with drug-eluting stents, these therapies may for the first time reduce the rate of restenosis, even in high-risk patients, such as individuals with diabetes mellitus.

Despite the promising data we obtained with systemic administration of ribonucleotide reductase inhibitors, drug eluting stents continue to remain an important part of the interventional cardiology arsenal. As outlined above, there is a major concerted drive by scientists and manufacturers to develop the next generation of agents which can offer the robust anti-proliferative properties of current therapy without the increased risk for thrombosis and late coronary events. Therefore, we assessed whether the therapeutic benefits observed with systemic administration of the RR inhibitor Didox, could be extrapolated to a drug eluting stent platform.
Results demonstrated that the use of Didox resulted in an exacerbation of the injury and an increased inflammatory response. When compared to the bare metal stent group, Didox drug eluting stents worsened the outcome of all morphometric measurements performed. Specifically, lumen area decreased by nearly 50%, medial area increased to almost two fold and the neointimal area increased 3.2 fold. Further confirmation of the worsened outcome with Didox drug eluting stents is the significant increase in intima/media ratio (I/M). Overall, results demonstrated that when compared to bare metal stents, Didox drug eluting stents increased the I/M ratio by 82%. Histopathological evaluation of the stented segments demonstrated a significant increase in the number of inflammatory cells in the Didox group and would suggest that the polymer used for drug elution may be contribute to the inflammatory response observed with mechanical injury of the vessel wall.

Polymer selection is critical to development of an efficacious drug eluting stent and the porcine studies undertaken were likely premature in that proper polymer evaluation was not performed. Our polymer platform was based on in-vitro pharmacokinetic studies demonstrating that polycaprolactone possesses biological breakdown properties amenable to use on a drug eluting stent platform in terms of sustainable drug release over a prolonged (several weeks) period (86). This is an exponentially growing area of research interest and recent studies have provided new insight into biomaterial well suited for drug eluting stents. Phosphorylcholine, a major component of the outer layer of the cell membrane, seems a natural choice for a biomimetic polymer. Although phosphorylcholine-based polymers are properly categorized as nonbiodegradable, they are considered less thrombogenic because of their
biological properties. Because the phosphorylcholine coating is able to retain and release drugs, it can be used as a DES elution platform. Recent clinical trials of the Endeavor ZES, which uses this technology, have shown excellent midterm clinical results, with a low frequency of stent thrombosis (89).

Because of safety concerns over longer dwelling polymeric materials, modifications to the design of the stent surface may lead to a theoretically safer “no-polymer” approach for drug delivery. These efforts include the use of porous stent surfaces, designed to function as reservoirs for the continuous release of antirestenotic agents. Pore sizes range from 1–100 nm in a nanoporous stent prototype (Setagon, Inc., Charlottesville, VA) and 1–100 μm in a microporous stent system (Yukon stent; Translumina GmbH, Hechingen, Germany). This technology allows “programmable” drug elution without the use of polymers. In a recent randomized clinical trial, efficacy evaluated as late luminal loss was comparable for a nonpolymer-based rapamycin-eluting stent versus a polymer-based stent delivery system (90).

Based on our study, ribonucleotide reductase inhibitors impede restenosis by limiting SMC proliferation. It is our hope that in the future, this will be a widely used and successful treatment option for patients that develop restenosis.
REFERENCES

(3) (2003) Pathophysiology of Heart Disease A Collaborative Project of Medical Students and Faculty, 3rd edition ed., Lippincott Williams & Wilkins.


(65) Morice, M. (2005) Eight-Month Outcome of the Reality Study: A Prospective Randomized Multi-Center Head-to-Head Comparison of the Sirolimus-Eluting Stent (Cypher) and the Paclitaxel-Eluting Stent (Taxus). *JACC* 45.


