RELEASE KINETICS OF IMATINIB MESYLATE FROM A THERMOSENSITIVE POLYMER AND ITS EFFECT ON VASCULAR SMOOTH MUSCLE CELLS

by

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STATEMENT OF THESIS APPROVAL

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ABSTRACT

Proliferating smooth muscle cells can cause hemodialysis vascular access stenosis. Applying antiproliferative drugs like imatinib mesylate that inhibit growth factors using a delivery system that keeps these agents at the site of stenosis could inhibit these proliferating cells. The aim of the study was to understand the release kinetics of imatinib from an injectable gel and the effect of imatinib on inhibiting proliferation of vascular smooth muscle cells.

Imatinib mesylate was obtained from capsules or tablets and mixed with ReGel™. The release profile of imatinib from ReGel™ was studied in a release medium under both sink and nonsink conditions. Freebase of imatinib was also extracted and characterized by RP-HPLC and its release profile was studied under sink and nonsink conditions. The free base of imatinib was incubated with porcine smooth muscle cells to study the effect of the drug on its proliferative properties using the BrdU assay.

The results show that the release rates of imatinib mesylate or free base from ReGel™ were similar in sink and nonsink conditions. Imatinib salt or its free base released from the ReGel™ within 2-4 days. The concentrations required to inhibit 50% porcine vascular smooth muscle cells with free base was between 0.5-5 μM.

Imatinib release from ReGel™ is quick so its use for the prevention of hemodialysis vascular access stenosis would be limited. The quick release could interfere with the healing process after initial graft implantation and it would require frequent

readministrations. Imatinib release from ReGel™ therefore, needs to be prolonged by changing the delivery system to make this approach feasible before further evaluation for this application.

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NOMENCLATURE

VSMC	Vascular smooth muscle cells
ASMC	Aortic/arterial smooth muscle cells
PDGFR	Platelet derived growth factor receptor
BrdU	5-bromo-2'deoxyuridine
IC50	50% inhibitory concentration
USP	United States Pharmacopeia
NF	National Formulary
UV	Ultraviolet
PBS	Phosphate buffered saline

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CHAPTER 1

INTRODUCTION

1.1 Hemodialysis and vascular access

Hemodialysis is a common procedure performed in patients with advanced and permanent renal failure. When kidneys fail to perform the function of removing harmful wastes from the blood it leads to increases in blood pressure and decreases in the production of red blood cells in the body (1). Hemodialysis is performed with a machine known as dialyzer that replaces the function of the kidneys in patients with renal failure (Figure 1-1).

Access to the patient's blood by the dialyzer occurs through a vascular access catheter or graft that is placed in the patient's forearm. Vascular access provides an anatomical location from which the blood is removed and replaced after it has been filtered through the dialyzer and it is usually surgically prepared weeks or months before dialysis is started in patients (Figure 1-1) (1). However, vascular access dysfunction in hemodialysis patients costs approximately $1 billion per annum in United States (2-4).

Three main kinds of vascular access configuration for hemodialysis are used among patients(1): native arteriovenous fistulae (5), synthetic grafts commonly made of PTFE (polytetrafluoroethylene) (5) and dual-lumen cuffed catheters (6). Among these three access systems, synthetic PTFE grafts are most commonly used in United States (2, 7).

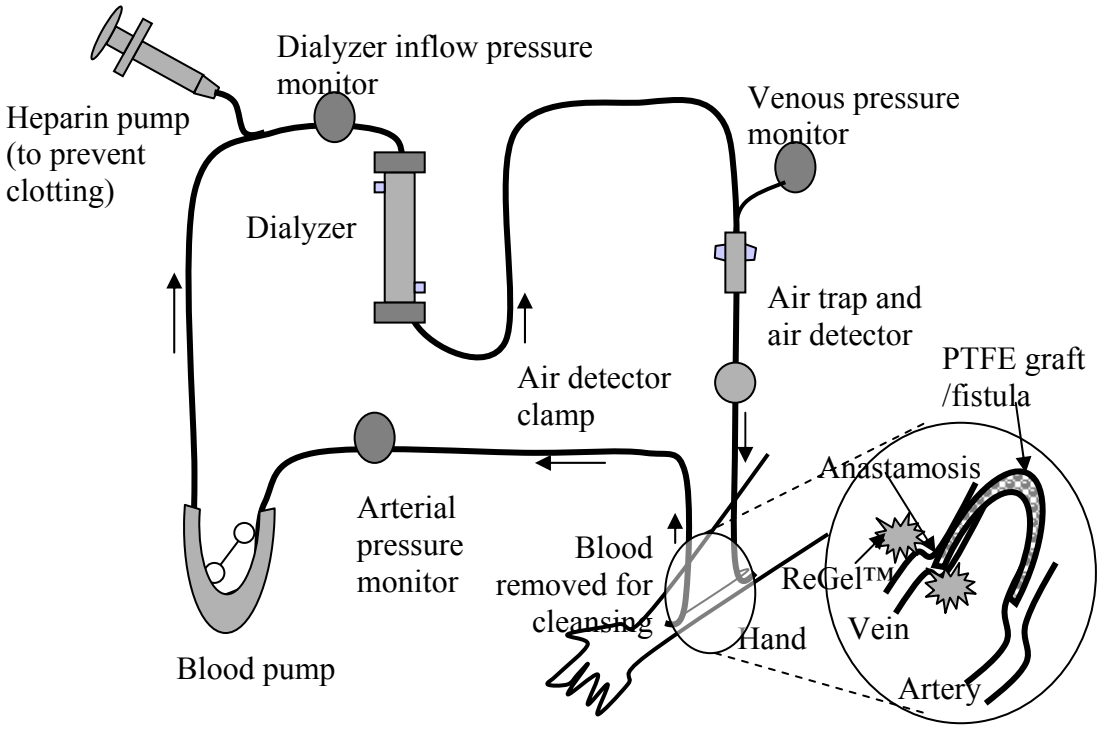


Figure 1-1: The process of hemodialysis and dialyzer

1.2 Mechanism of development of intimal hyperplasia

To understand the mechanism of development of intimal hyperplasia it is necessary to review the anatomy of the arterial wall. The arterial wall consists of three layers: intima, media and adventitia. The intima is nearest to the lumen (Figure 1-2). It contains type IV collagen, laminin and heparin sulphate proteoglycans. As shown in the Figure 1-2 the media is between the intima and the adventitia and contains vascular smooth muscle cells and type I and III collagen, fibronectin and chondroitin sulphate proteoglycans densely packed into an interstitial matrix. The outermost layer is adventitia and consists of fibroblasts in a loose connective tissue. Intimal hyperplasia results from an increase in the number of cells in the intima accompanied by increase in the extracellular matrix (8). The main reasons for formation of hyperplasia are hypothesized to be due to graft compliance mismatch, vessel stretch, surgical trauma and accumulation of various biochemical factors released from fibrin and platelets in the vessel lumen (9, 10). The major reasons for inducing hyperplasia are injury, inflammation and stress (11). Injury stimulates smooth muscle cell (SMC) proliferation by causing endothelial disruption that leads to release of intracellular mitogens. The vascular smooth muscle cells then undergo transformation from a quiescent to a proliferative phenotype (12). This proliferation leads to production of extracellular matrix. It has also been hypothesized that damage in the endothelial layer leads to platelet adhesion due to exposure of collagen. Platelet derived growth factor (PDGF) is released following aggregation of platelets (13). It has been experimentally shown that PDGF plays an important role in the migration of smooth muscle cells to the intimal surface (14).

The place where the graft is sutured to the blood vessels (artery or vein) is called

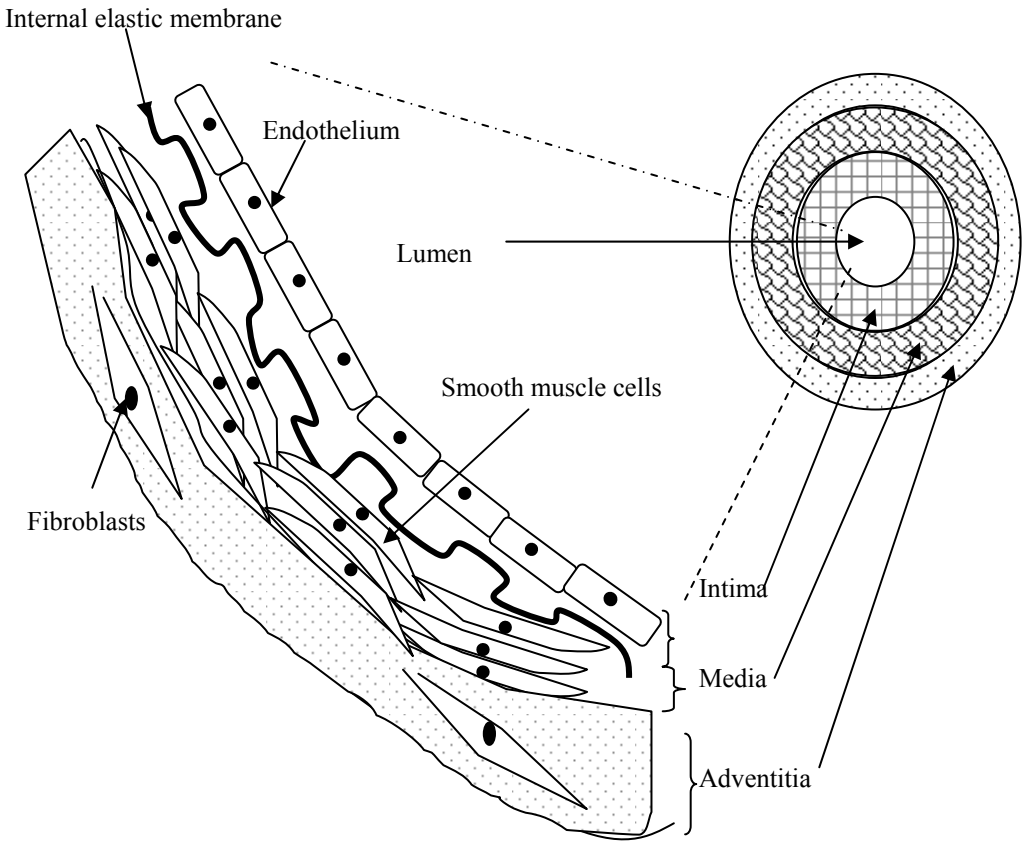


Figure 1-2: Anatomy of vessel walls

the anastomosis (Figure 1-1). Native arteriovenous (AV) fistulas and synthetic grafts used for hemodialysis commonly develop stenosis and occlusions at the site of anastomosis. Histological examinations of graft segments taken from patients have revealed extensive hyperplasia at the anastomosis (15, 16). Intimal hyperplasia is a consequence of the healing response of the normal vascular wall to injury and is caused by migration and proliferation of vascular smooth muscle cells from the media to the intima (8).

1.3 Drug delivery systems and their mechanisms

The design of a drug delivery system is an important factor to target the delivery of a chemotherapeutic agent to the site of action. Controlled drug delivery systems have advantages such as maintaining the drug levels at desired concentration range, requiring fewer administrations, and increased patient compliance (17). The important factor in controlled drug delivery systems is to achieve a desired drug level in the target tissue for as long a duration of time as possible. In the case of conventional tablets or injections, the drug level in the blood rises after each administration and decreases until the next dose is administered as shown in the Figure 1-3. This type of dosing could produce toxicity at the peak concentration and too low a drug level at the trough intervals that it is not effective. A controlled delivery systems offers a potential advantage of maintaining the drug level constant between the desired maximum and minimum value for a longer period of time depending on the application and type of formulation (17).

Recent developments in the drug dosage forms have been aimed at delivering the active molecules to the site of action in the organisms with the help of carriers that also protect active molecules from biological degradation. These systems can deliver a sustained, slow release of active molecules for longer duration of time (18). Novel

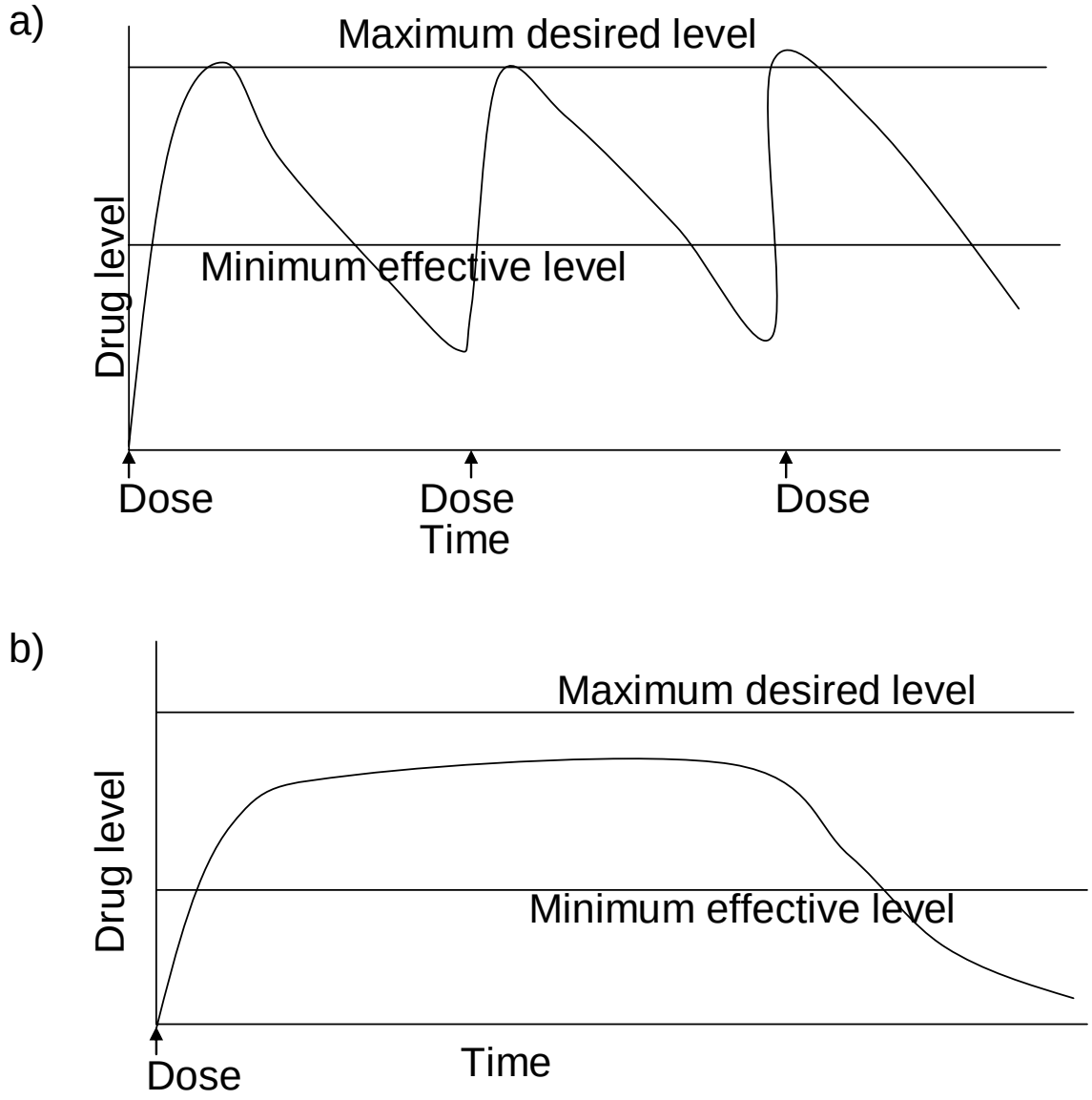


Figure 1-3: Diagram showing the advantage of drug delivery systems (b) versus conventional tablet or solution (a) in maintaining a desired drug level.

therapies for prevention of hyperplasia and vascular stenosis include drug eluting stents, perivascular delivery of therapeutic agents, and coated grafts (19).

To achieve desirable concentrations within the target tissue following the administration of a drug in a polymeric system (controlled drug delivery system), it is very important to understand the mechanism of degradation of the polymer and the process of drug release from the polymer. Different factors play an important role in biodegradation of polymers such as chemical structure, composition, presence of ionic groups, molecular weight, distribution, sterilization process, physiochemical factors (ion exchange, ionic strength, pH), mechanism of hydrolysis (enzymatic or water), site of implantation, annealing, and storage history (17).

Drug release from a polymer system can involve diffusion, degradation or swelling followed by diffusion (Figure 1-4). Diffusion occurs either on a macroscopic scale, i.e., through pores of polymer matrix or at molecular level that involves passing between polymer chains. In matrix systems, the polymer and active agent are combined to form a homogenous system. Diffusion takes place as the drug passes from the polymer matrix to the external environment. The rate of diffusion generally decreases with time in such process and requires longer time for the drug to fully release. In case of reservoir systems a therapeutic agent is in a polymeric matrix that is surrounded by film or a membrane, which is the only factor that limits the release of drug from the polymer. In such systems the delivery rate remains constant through out the release. In diffusion controlled systems the combination of bioactive agents and polymeric matrices help in diffusion of drug through the macromolecular polymeric structure of pores without causing any change in the polymer such as degradation or swelling (17).

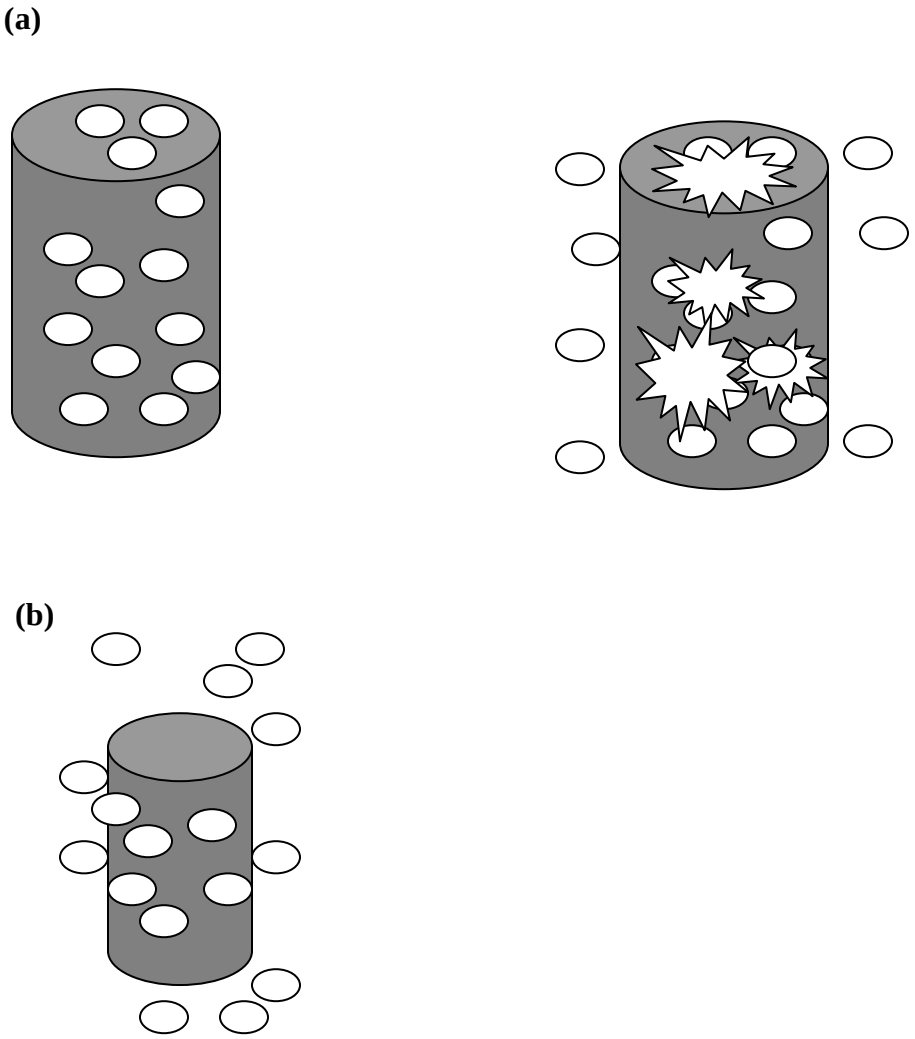


Figure 1-4: Release mechanism of drug from bio-degradable polymer: a) bulk-eroding systems b) surface eroding systems

In case of environmentally responsive polymers known as intelligent hydrogels, environmentally sensitive materials are used which are dry in general and swell when they come into contact with body fluids and water. The swelling increases polymer mesh size and aqueous content and helps the drug to diffuse from inside to the outer environment. At equilibrium, hydrogels absorb 60-90% of fluid and contain only 10-30% of polymer. These are called swelling- controlled release systems and are incapable of releasing the drug or therapeutic agent until they come in contact of appropriate biological environment. Swelling is initiated by changes in the biological environment such as pH, temperature and ionic strength. This change is generally reversible. In such systems the polymer generally swells when it comes in contact with high pH and collapses when it comes in contact with low pH. These systems can be suitable for oral drug delivery where the drug needs to be released in the upper small intestine at high pH value and requires to be protected from the biological environment with lower pH (17).

The above mentioned mechanisms are applicable to polymers that do not undergo much change in their chemical structure beyond swelling. Polymers such as polylactide (PLA), polyglycolide (PGA), poly(lactide-co-glycolides) (PLGA), polyanhydride and polyorthoesters belong to a class of biodegradable polymers that degrade into biologically inactive compounds by hydrolysis of polymer chains (17).

In some cases, polymers are broken down into lactic acid and glycolic acid and are finally converted into carbon dioxide and water by entering into metabolic pathways to be excreted from the body. The degradation of polymers occurs by two different mechanisms: bulk-eroding and surface-eroding. In bulk-eroding the degradation occurs uniformly through out the polymeric matrix by bulk-hydrolysis. In surface-eroding

polymers such as polyanhydride and polyothoesters, the degradation takes place only at the surface of polymer and the rate of degradation is proportional to the surface area of drug delivery system (17).

1.4 Vascular drug delivery

Sustained local delivery of drugs helps in reducing the need for multiple dosing and thereby helping in reducing toxic effects caused by high systemic exposure of drugs. Sustained delivery of drugs is desired for chemically unstable and rapidly degrading drugs. Sustained local delivery is achieved by incorporation of a drug into a polymeric matrix. This could be achieved at the adventitial aspect of an injured artery or by delivering the drug intraluminally, i.e., inside the artery (20). Intraluminal drug delivery consists of a stent-graft device. Drug is distributed in the intima and is effective in the treatment of thrombosis. Delivery of drug into the intima occurs through diffusion and the approach is invasive and can only be performed after surgery. In this type of drug delivery system biodegradability and biocompatibility are important issues.

One major disadvantage of drug eluting stents are increased risk for bleeding if drugs like aspirin and clopidogrel are needed after placement of stents (19). Another disadvantage of drug eluting stents is that leaching of drug from drug eluting stents could be very intensive due to continuous flow of blood through arterial segment, and this may require additional drug monitoring with the help of special devices (20). Despite these limitations, this approach has shown promising results. In a rabbit model of neointimal hyperplasia, heparin delivered locally near the anastomosis of polytetrafluoroethylene (PTFE) grafts and veins had an effect on decreasing neo-intimal hyperplasia (21). PTFE grafts coated with heparin helped in reducing formation of intimal hyperplasia at

anastomosis (22). Local delivery of drugs were effective in the prevention of intimal hyperplasia and also helped in reducing systemic toxicities of certain therapeutic agents (23).

Recently there has been an interest in the use of perivascular drug delivery systems to inhibit vascular stenosis or restenosis (19). The major advantage of perivascular drug delivery includes the application of drug directly to the adventitia surface, which helps in blocking adventitial activation and migration of fibroblasts. This also helps create a gradient with the highest level of drug concentrations in the adventitial layer and lowest level of drug concentrations in endothelial layer (19). Drug delivery takes place by diffusion along a concentration gradient. The drug passively diffuses through the vessel wall. The vasa vasorum system in the adventitia consists of a network of arterioles, venules and capillaries that helps in nourishing the vessel wall components. When the drug comes in contact with vasa vasorum the diffusion of the drug is further facilitated and the drug is evenly distributed into the inner most arterial segments such as media and intima (17).

1.5 Thermosensitive copolymers

Thermosensitive polymers show physicochemical response to temperature due to their hydrophobic-hydrophilic interactions (24). Because of these physicochemical properties, use of thermosensitive copolymers is being explored in various medical applications. One major potential for these polymers has been demonstrated in a preclinical dog and porcine models by perivascular application around the vascular access for inhibition of neointimal hyperplasia (25, 26). The thermosensitive co-polymer used in these studies was ReGel™ (BTG plc, West Valley, UT) which is a triblock co-

polymer consisting of poly-(lactide-co-glycolide) (PLGA)and poly(ethylene glycol) (PEG). It has a polydispersity of 1.3 and average molecular weight of ~ 4200 Daltons (27). Aqueous solution of ReGel™ polymer exists as a free-flowing solution at or below room temperature thus allowing easy mixing of drug with polymer and creating an easy system to inject. It spontaneously turns in to an insoluble gel depot upon raising the temperature to body temperature that releases the drug over a period of time. Drug is released initially by diffusion and at later stages by combination of polymer degradation and diffusion. ReGel™ is totally water-based (no organic solvents used) and can be sterilized by terminal filtration or γ–irradiation. In addition to its thermosensitive properties, the following advantages of ReGel™ make it more applicable for perivascular delivery of anti proliferative drugs (27).

1) ReGel™ is biodegradable and its degradation products are polyethylene glycol, lactic acid and glycolic acid that are generally regarded as safe.
2) Biodegradation of ReGel™ eliminates the need of depot retrieval after the drug has been delivered; hence, it is suitable for repeat injections.
3) ReGel™ has domains that could dissolve both hydrophilic and hydrophobic drugs and most commonly used antiproliferative drugs are hydrophobic.
4) ReGel™ has an advantage of remaining at the site of injection up to one month. As neointimal hyperplasia develops over the time, this property of ReGel™ makes it applicable for perivascular delivery of anti proliferative drugs.
5) The rate of drug release from ReGel™ could be controlled by changing the lactide to glycolide ratio in the polymer composition.

The aim of these studies was to investigate the release profiles of a potential antiproliferative drug from ReGel™ so that it can be used for inhibiting vascular smooth muscle cells in future studies using an animal model of neointimal hyperplasia.

1.6 Antiproliferative drugs

Various studies have tried the use of chemotherapeutic agents for prevention of neo-intimal hyperplasia including heparin (28), tranilast (29), perindopril (an angiotensin-converting enzyme inhibitor) (30) and calcium channel blockers like verapamil and diltiazem (31). These have shown limited success.

Rapamycin is a Food and Drug Administration (FDA) approved immuno suppressive agent used to prevent rejection after organ transplantation. It acts by specifically binding to immunophilins (32). Gregory et al. have shown its dose dependent inhibition of arterial intimal thickening in a rat model (33, 34). Later studies demonstrated its effect on inhibiting proliferation of vascular smooth muscle cells in vitro (35). It has recently been shown that rapamycin significantly reduces restenosis in porcine coronary models (36). Dipyridamole is a weak base (37) and an antiproliferative drug which acts by inhibiting phosphodiesterases and thereby increasing intracellular cyclic nucleotide levels (38). Various studies have shown its efficacy in inhibiting PDGF and bFGF- induced vascular smooth muscle cell proliferation (39, 40). The mechanism of action of dipyridamole is related to inhibiting cyclic nucleotide phosphodiesterases that lead to increase in the cAMP and cGMP levels (41, 42). Paclitaxel is a chemotherapeutic agent that is effective at inhibiting various cancer cell lines and tumors. Intravenous administration with Cremophor EL has been unsuccessful due to the allergic reactions. However, it could be used for local administration in the prevention of neo- intimal

hyperplasia. Recent studies have shown that paclitaxel could inhibit neo-intimal hyperplasia in arteriovenous hemodialysis grafts (25, 43).

Most of the above mentioned drugs have been shown to inhibit VSMC proliferation in different in vitro test conditions but these have not been successful in vivo. Therefore, our group is investigating and exploring the potential application of imatinib mesylate for inhibiting neo-intimal hyperplasia both in vitro and in vivo.

1.7 Imatinib mesylate

Imatinib mesylate is a synthetic, 2-phenylamino-pyrimidine derivative chemically recognized as 4-[(4-methyl-1-piperazinyl)methyl]-N-[4-methyl-3-[4-(3-pyridinyl)-2-pyrimidinyl]amino]-phenyl] benzamide methanesulfonate (Figure 1-5) (44). Its commercial trade name is Gleevec™ (Novartis Pharmaceuticals, Basel, Switzerland) (44). It is a potent tyrosine kinase inhibitor approved for the treatment of gastrointestinal tumors and chronic myelogenous leukemia (44). Imatinib mesylate has a low molecular weight (589.7 Da) and shows good aqueous solubility at low pH (<5.5) but is poorly soluble or insoluble at neutral and alkaline pH. In nonaqueous solvents, the drug substance is freely soluble to very slightly soluble in DMSO, methanol, and ethanol but is insoluble in n-octanol, acetone, and acetonitrile (45). It has also been found that standard doses of imatinib can be safely administered to patients on hemodialysis with renal failure at any stage (46). Pharmacokinetic studies of imatinib show that it has excellent bioavailability following oral administration in humans and is mainly metabolized by cytochrome P450 3A4 (47). The currently available oral formulation has other inactive ingredients like crospovidone, colloidal silicon dioxide, hydroxypropyl methyl cellulose, microcrystalline cellulose, and magnesium stearate (44). It is supplied as 100 mg of

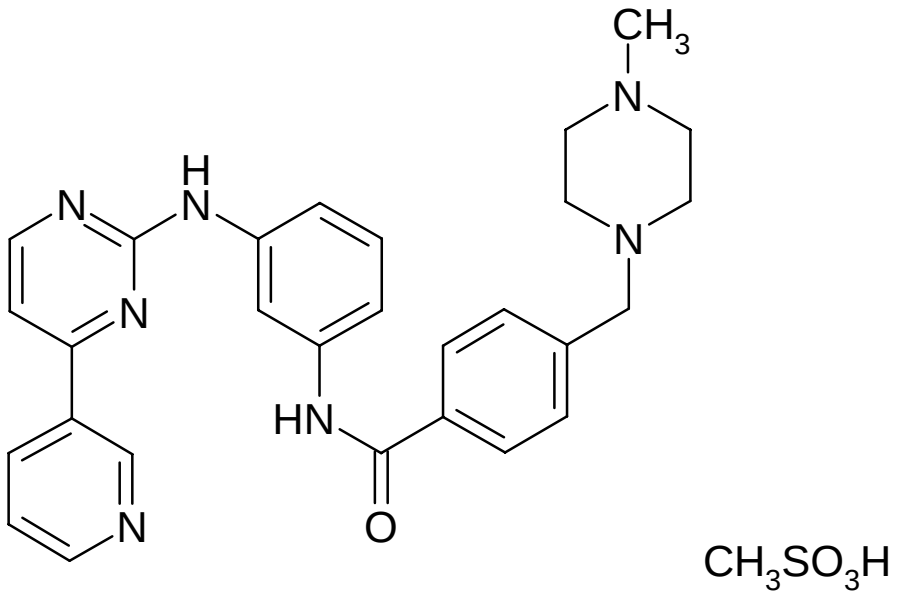


Figure 1-5: Chemical structure of imatinib mesylate.

imatinib mesylate equivalent. Li et al. (48), have recently showed the potential application of imatinib mesylate in inhibiting venous smooth muscle cell (VSMC) proliferation. This evidence suggested that imatinib acts on the PDGF receptor (PDGFR) tyrosine kinase inhibitor that leads to the inhibition of the vascular smooth muscle cells proliferation. The concentrations of imatinib mesylate salt required to inhibit 50% of the proliferative vascular smooth muscle cells (IC_{50}) in human smooth cells was found to be 0.5μM (48). Therefore, this study was aimed at exploring whether it was feasible to incorporate imatinib in a local delivery system using a thermosensitive copolymer in order to inhibit proliferative smooth muscle cells in the context of AV fistula anastomotic hyperplasia.

1.8 Significance of this work

Before further application of imatinib mesylate for prevention of hemodialysis vascular access stenosis, we need to characterize the release profile of the drug from the delivery system and explore factors that impact this release. The duration of drug release from the polymer is an important factor to achieve therapeutic concentrations at the target tissue and defines the frequency of administration of the drug/ polymer system for the complete prevention of intimal hyperplasia. This study focuses on the release profiles of imatinib release from ReGel™ using several in vitro release experiments. Inhibitory concentrations of imatinib needed to prevent vascular smooth muscle cell proliferation (IC_{50}) are evaluated using these in vitro cell culture experiments. Characterization of the imatinib/ReGel™ system to attain desired release profiles in the target tissue have also been proposed and discussed.

The main objectives of this study are:

1) To determine the effect of the free base of the drug on the proliferation of vascular smooth muscles cells and compare the IC_{50} values from the literature.
2) To determine the duration of drug release from a thermosensitive co-polymer under in vitro conditions.
3) To assess imatinib/ReGel™ system for the sustained release of the drug from the thermosensitive polymer in preventing vascular access stenosis.

CHAPTER 2

RELEASE PROFILE OF IMATINIB MESYLATE FROM REGEL™

2.1 Introduction

In a solid dosage form there are two main ingredients. One is the active substance or the drug that elicits a pharmacological response and the others are the inactive substances and often known as excipients that do no have any effect on the pharmacological response (49). Imatinib mesylate is commercially available in two solid dosage forms: tablet or capsule (44, 48). Gleevec™ film – coated tablets consist of 100 mg of imatinib mesylate along with other inactive ingredients such as colloidal silicon dioxide (NF), crospovidone (NF), hydroxypropyl methyl cellulose (USP), magnesium stearate (NF) and microcrystalline cellulose (NF). Tablet coating is made up of ferric oxide, red (NF); ferric oxide, yellow (NF); hydroxyl propyl methylcellulose (USP); polyethylene glycol (NF) and talc (USP) (44). Gleevec capsules consists of 100 mg equivalent of imatinib mesylate along with other inactive ingredients like colloidal silicon dioxide (NF), crospovidone (NF), magnesium stearate (NF) and microcrystalline cellulose (NF) (50). Li et al. (48), have recently showed the potential application of imatinib mesylate in inhibiting venous smooth muscle cell (VSMC) proliferation which is responsible for the formation of neointimal hyperplasia.

In order for a drug to inhibit neointimal hyperplasia around the hemodialysis vascular access it is also important to look at the rate at which the drug is getting released from the drug delivery system like ReGel™. An ideal drug delivery system that would be suitable for inhibiting hyperplasia should not have too quick release rates of the drug as this could interfere with the healing process. On the other hand a prolonged rate may not provide adequate concentrations in the tissue for inhibiting stenosis. Imatinib mesylate was not commercially available in purest form as only active ingredient, but available in marketed forms of tables and capsules with active ingredient and other excipients as mentioned above. Since these excipients could affect the release rates of imatinib mesylate from ReGel™, it was essential to study the release rates of both tablet and capsule. The one with a prolonged and sustained release profiles would be more desirable for further investigation in preventing neointimal hyperplasia.

2.2 Methods

2.2.1 Materials. Imatinib mesylate manufactured by Novartis Pharmaceuticals (Basel, Switzerland) was obtained from the Veterans Affairs Medical Center Pharmacy as 100 mg tablets and 100 mg capsules. ReGel™ was obtained from BTG Inc. (West Valley, UT). All other chemicals were purchased from Sigma Aldrich Ltd (ST. Louis, MO).

2.2.2 Standard curve of imatinib mesylate. Tablets were ground into powder with the help of mortar and pestle. Standard solutions were prepared in the concentration range from 2.5 to 60 ug/mL in 5% ethanol in phosphate buffered saline (PBS, pH 7.4). The standard solutions were measured by absorbance at 265nm using UV-spectrophotometry by subtracting the absorbance of 5% ethanol in phosphate buffered saline (PBS, pH 7.4).

2.2.2 Release kinetics of imatinib mesylate tablet. Two mg of imatinib mesylate (actual amount of drug in the powder) were suspended in 2 mL of ReGel™ under cold conditions (4 ^{o}C) in a scintillation vial. The drug-ReGel™ mix was allowed to solidify at 25 ^{o}C. Following the solidification of the drug-ReGel™ mix, 15 mL of release medium consisting of 5% ethanol in phosphate buffered saline (PBS, pH 7.4) was added into the vial. The vial was then placed in the Gyratory water bath shaker (G76D, New Brunswick Scientific Co Inc, NJ) and agitated at 50 rpm while the temperature was maintained at 37^{o}C and covered to protect from light. Release profiles in two procedures were determined in sink and nonsink conditions. For sink conditions, 50μL of the release medium were taken from the scintillation vial at specific time points for further analysis of imatinib mesylate using a UV-spectrophotometer. The rest of the release medium was discarded in the scintillation vial and replaced by fresh release medium at each sampling time point. In case of nonsink conditions, 50μL of the release medium were taken from the scintillation vial at specific time points for further analysis of imatinib mesylate using the UV-spectrophotometer method but only 50uL of fresh release medium was replaced in the scintillation vial.

2.2.3 Release kinetics of imatinib mesylate capsule. The imatinib mesylate powder was removed from the capsule and 2 mg (actual amount of drug in the powder) was suspended in 2 mL of ReGel™ under cold conditions (4 ^{o}C) in a scintillation vial. The rest of the procedure was similar to that of the imatinib mesylate tablet. The release kinetics were obtained for both sink and nonsink conditions.

2.2.4 Data analysis. All in vitro experiments were performed with n=5 and each of the release rate profiles (% cumulative release versus days) obtained from in vitro

experiments were fitted to a monoexponential equation (Equation 1) using Sigmaplot (San Jose, CA).

$$\%\text{Cumulative release} = 100*(1-\exp^{-kt}) \qquad \text{Equation 1}$$

where k is the rate constant (day^{-1}) and t is the time in days. The rate constants of each experiment were expressed as the mean and standard deviation (SD). Release rates of sink and nonsink conditions and release rates of capsule versus tablets were compared using analysis of variance (ANOVA) with correction for multiple comparisons, where $p < 0.05$ was considered to be statistically significant. Number of days to reach 100% release was calculated from the inverse of mean rate constants of each profile.

2.2.5 Paclitaxel and dipyridamole release from ReGel™. The release data of dipyridamole from ReGel™ were obtained from Figure 4 of the study of Zhu et al. (39). The results in this study showed that it takes approximately 18 days for dipyridamole to be 100% released from ReGel™ (39). Similarly paclitaxel release from ReGel™ was obtained from figure 5 of Zentner et al's study which showed that it takes approximately 45 days for paclitaxel to be 100% released from ReGel™. This information was used to the plot the relationship between log P of drugs and the time of 100% drug release (27).

2.3 Results

A linear standard curve was obtained for imatinib mesylate concentrations and absorbance with an r^2 value of 0.99, with a slope of 0.0515 (Figure 2-1). The standard curve of imatinib mesylate was reproducible for either capsule or tablet. The release rates of imatinib mesylate in sink and nonsink conditions obtained from tablet or capsule are shown in Table 2.1. There was no statistically significant differences in release rates

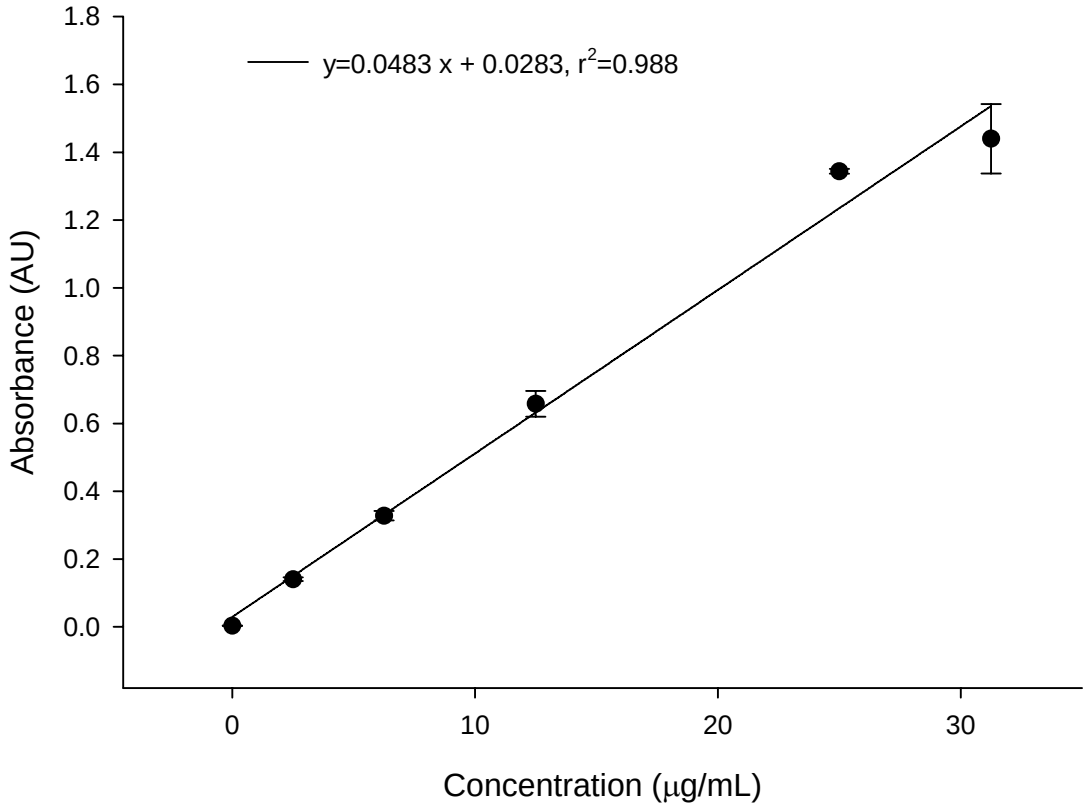


Figure 2-1: Standard curve of imatinib mesylate in 5% ethanol in phosphate buffered saline (PBS, pH 7.4) (n=5).

Table 2.1: Release rates of imatinib mesylate from tablet or capsule in sink and nonsink conditions. Comparison by ANOVA with correction for multiple comparisons was not statistically significant (p>0.05).

Experiment	Release rate (per day)		Number of days for 100% release
	Mean	S.D.	
Nonsink tablet	0.3224	0.0762	3
Nonsink capsule	0.216	0.0916	4
Sink Tablet	0.3191	0.0623	3
Sink Capsule	0.2816	0.0724	4

between sink and nonsink conditions for imatinib mesylate obtained from tablet ($p>0.05$) (Table 2.1, Figure 2-2). There were no significant differences in release rates between sink and nonsink conditions for imatinib mesylate obtained from capsule (Table 2.1, Figure 2-3). Within 3-4 days approximately 100% of the imatinib mesylate obtained from either capsule or tablet was released in both sink and nonsink conditions (Figure 2-4). When log P (octanol-water partition coefficient) values are taken into consideration there is a correlation between the log P value of the drug and its release from ReGel™ (Figure 2-5) (47, 51, 52). With a decrease in the log P value there is a faster release of the drug from ReGel™. Figure 2-5 illustrates the relation between the log P of drug value and the duration of the drug release from the thermosensitive polymer ReGel™.

2.4 Discussion

In this study, we found that the drug release from imatinib mesylate occurs within a week. The results also showed that the sink and nonsink conditions did not influence the release of the drug from the ReGel™. This shows that even after in vivo administration where conditions are likely to be nonsink the release of imatinib is likely to be rapid. The results showed that there was no significant difference in release profiles between results either obtained from capsule or tablet. Since, imatinib tablets were subjected to grinding using mortar and pestle, there is a possibility of differences in the particle size and particle size distribution of tablets. Therefore, in order to decrease this variability that could affect the release profiles, imatinib obtained from the capsule is preferable over tablet for further in vivo studies.

The results show a correlation between log P value of the drug and duration of the drug release. With the decrease in the log P value and increase in the hydrophilicity, there

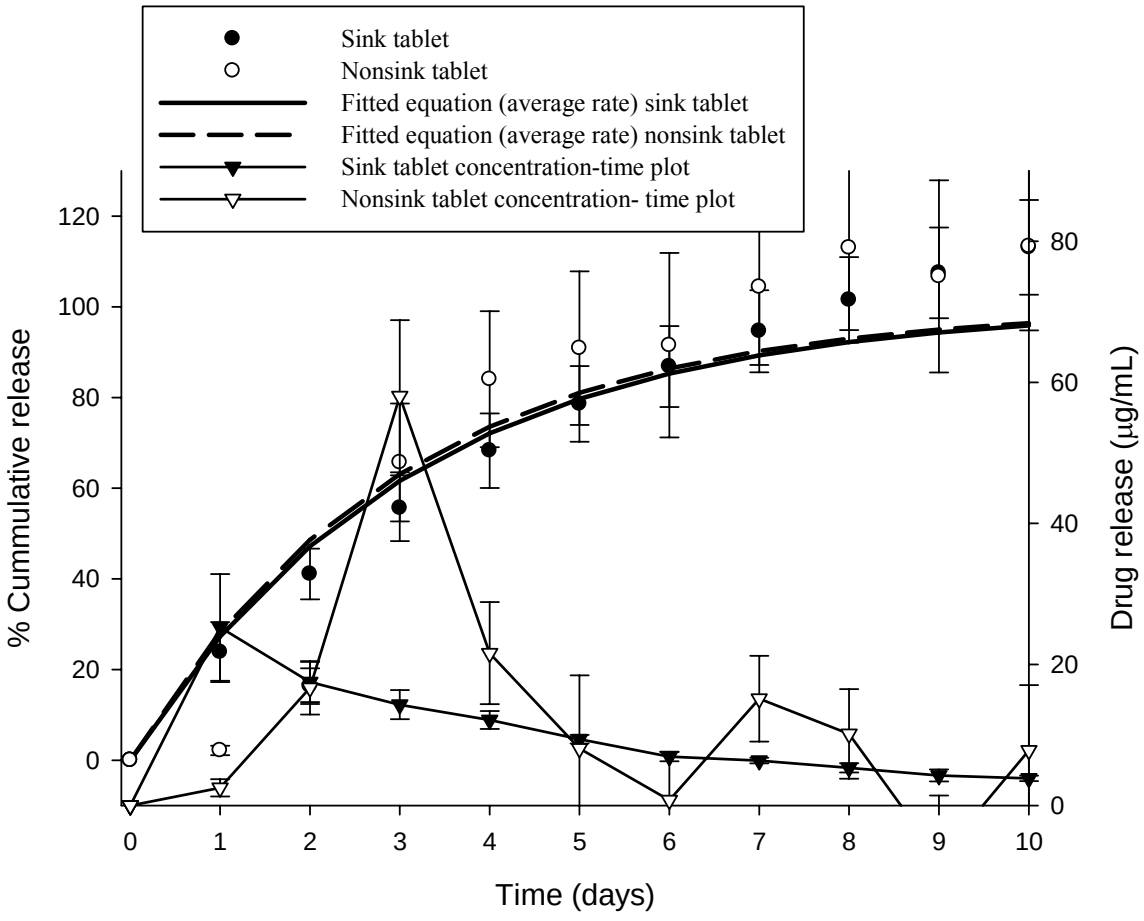


Figure 2-2: Release profiles of imatinib mesylate obtained from tablet in sink and nonsink conditions. The release rates between sink and nonsink conditions were not statistically significant.

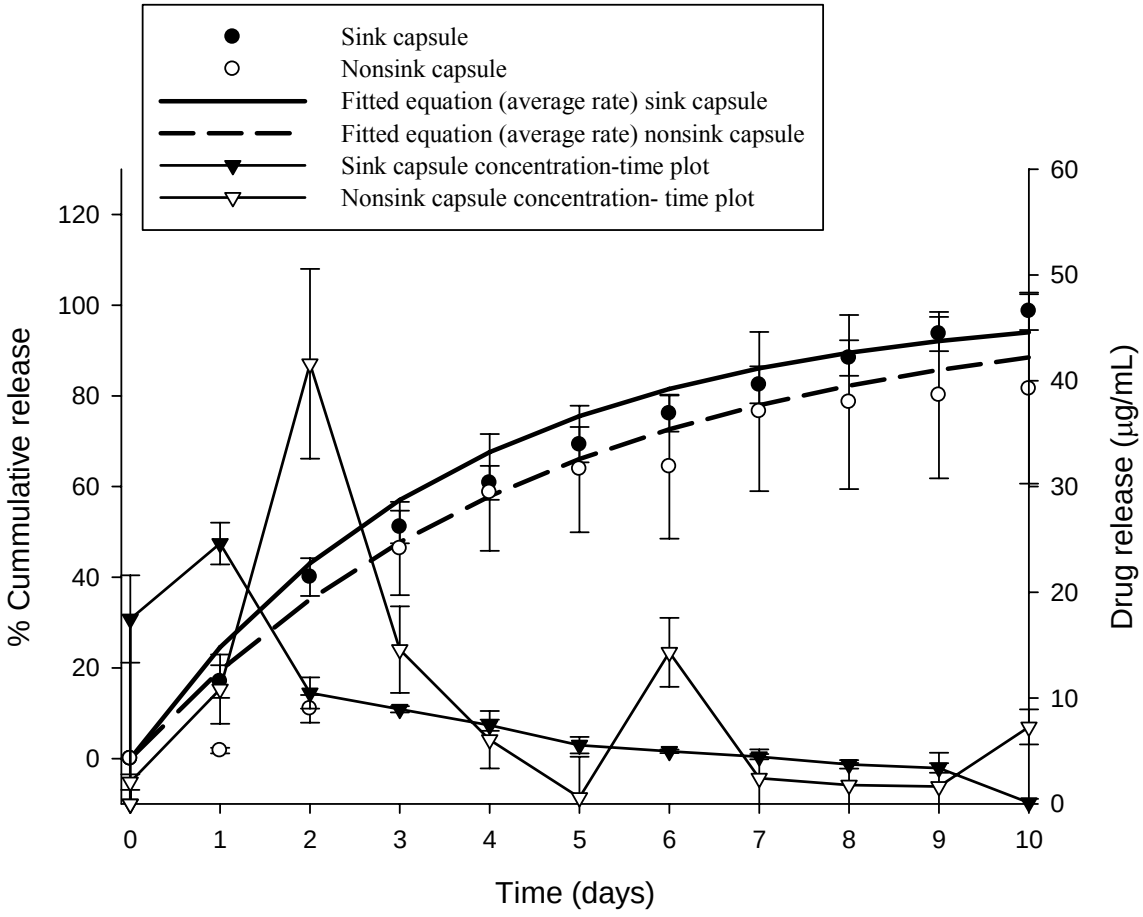


Figure 2-3: Release profiles of imatinib mesylate obtained from capsule in sink and nonsink conditions. The release rates between sink and nonsink conditions were not statistically significant.

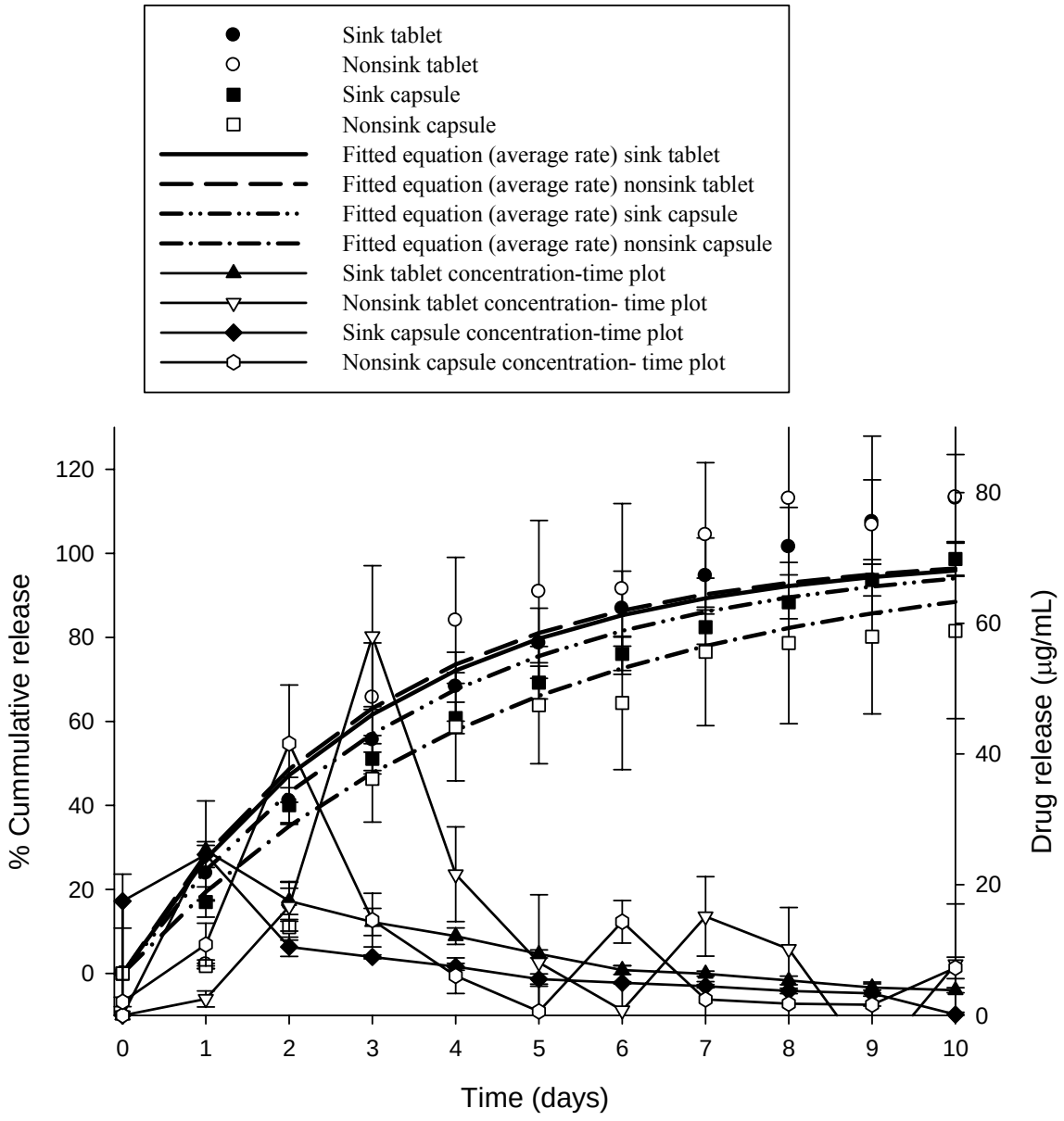


Figure 2-4: Release profiles of imatinib mesylate obtained from capsule and tablet in sink and nonsink conditions. The release rates between sink and nonsink conditions were not statistically significant.

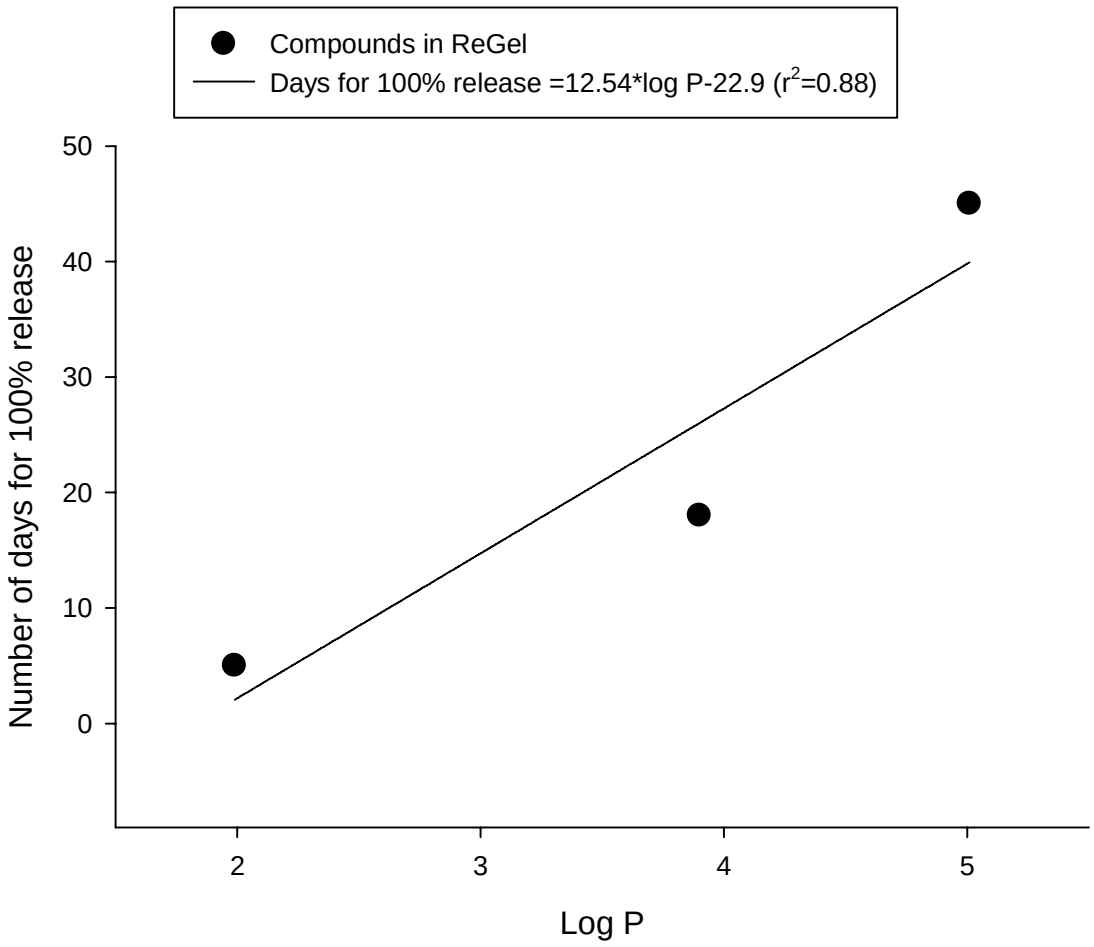


Figure 2-5: Relation between log P and duration of drug release from ReGel™

was a faster release of the drug from ReGel™. The possible mechanism behind the different release rates of the drug could be due to their partitioning in the hydrophilic domain and the hydrophobic domain of the ReGel™. In case of imatinib mesylate that has a log P value of 1.99 the drug was concentrated in the hydrophilic domain of ReGel™ and was released by diffusion from the hydrophilic domain of ReGel™. This result in monophasic release profiles of imatinib mesylate from the ReGel™ (Figure 2-2, 2-3 and 2-4). The most probable mechanism of imatinib release from the polymer occurs by diffusion and some matrix degradation. In the case of dipyridamole, which has a log P value of 3.9, the drug partitions between both the hydrophilic and hydrophobic domains of ReGel™ and results in a two stage release pattern (39). The first stage of the release profile is due to the burst effect from the hydrophilic domain and the later stage is the release from the hydrophobic domain (39). On the other hand, in case of highly hydrophobic drugs like paclitaxel, the drug is mainly in the hydrophobic domain, leading to the sustained release of drug from the hydrophobic core of ReGel™ over time (27).

In order to inhibit neointimal hyperplasia, these results suggest that imatinib ReGel™ mixture would need to be replenished once every week. Although the drug-ReGel™ mixture can be administered even after surgery, this administration would likely be too frequent compared to other antiproliferative drugs. The fast release rates of the drug could hinder the healing process at the anastomosis which occurs during the first week. An ideal system should have an optimal release rate such that release profiles are greater than 3-4 days, ideally lasting for months without need for readministration.

CHAPTER 3

EXTRACTION OF FREE BASE OF IMATINIB MESYLATE AND ITS RELEASE KINETICS FROM REGEL™

3.1 Introduction

Imatinib mesylate is chemically known as 4-[(4-methylpiperazin-1-yl)methyl]-N-[4-methyl-3-[(4-pyridin-3-ylpyrimidin-2-yl)amino]-phenyl]- benzamide methane sulfonate. The molecular formula is C_{29} H_{31} $N_{7O}.CH_4SO_3$ and molecular weight is 589.7 with a pKa ranging from 3-8 (44). It is soluble in aqueous buffers of pH less than or equal to 5.5. The piperazinylmethyl group of the molecule provides the water solubility and oral bioavailability (53). Based on pH-dependent capillary zone electrophoresis, it was claimed that protonation of neutral imatinib starts below pH 5 (54, 55). Studies showed that imatinib is predominantly neutral, monocationic and tricationic at intestinal, blood and gastric pH, respectively (55). Therefore, this suggests that imatinib will be in a monocationic phase at neutral pH 7.4. Knowing the pKa value of imatinib and its protonation state at various pH values, the salt form can be converted to its free base. This free base of imatinib is expected to be more lipophilic than its salt form. Higher lipophilicity of free base over its salt form could potentially help prolong the release of imatinib from ReGel™. We were therefore, interested in extracting a free base of the salt form of imatinib to investigate its release rate from ReGel™.

3.2 Methods

3.2.1 Materials. Imatinib mesylate manufactured by Novartis Pharmaceuticals (Basel, Switzerland) was obtained from the Veterans Affairs Medical Center Pharmacy as 100 mg of imatinib mesylate along with other excipients. ReGel™ was obtained from BTG Inc. (West Valley, UT). All other chemicals were purchased from Sigma Aldrich Ltd (St. Louis, MO).

3.2.2 Extraction of free base. Imatinib mesylate tablets were ground to a fine powder with the help of mortar and pestle. The powder was added to water whose pH was changed to 3.14 by 1N HCl followed by continuous stirring for 5 minutes in order to solubilize imatinib mesylate. After the dissolution, the solution was filtered to remove undissolved excipients. This mixture was further treated by changing to pH 12 by adding 1N NaOH. This resulted in the precipitation of the free base of imatinib. The solution was further centrifuged using an ultracentrifuge (Allegra 6R centrifuge, Beckman Coulter Inc.) and the supernatant was discarded. The remaining precipitate was lyophilized using a lyophilizer (Freezone 6 freeze dry system, Labconco Corp). The presence of free base of imatinib was confirmed with the help of reverse phase high performance liquid chromatography (RP-HPLC) method (Agilent Technologies, CA) using a C-8 column (Waters, MA) (250 mm x 4.6 mm id; particle size 5 μm) with UV-Detection (Agilent Technologies, CA). The samples were eluted in a mobile phase of 0.02M potassium dihydrogen phosphate – acetonitrile (7:3,v/v) at a flow rate of 1ml/min. The mobile phase was filtered through a 0.22μm filter (Millipore, MA) and was degassed under vacuum prior to use. The detector was set at a wavelength of 265 nm. Both the free base and the

salt form (mesylate) of imatinib samples were analyzed using the above mentioned chromatographic conditions.

3.2.3 Standard curve of free base of imatinib. The lyophilized powder of free base of imatinib obtained by this separation was used to prepare standard solutions in the concentration range from 0.6 to 30 ug/mL in 5% ethanol in phosphate buffered saline (PBS, pH 7.4) with 1% (v/v) Cremophor EL and 1% (v/v) Tween 80. The standard solutions of free base of imatinib were measured by absorbance at 265nm using a UV-spectrophotometer method by subtracting the absorbance of 5% ethanol in phosphate buffered saline (PBS, pH 7.4) with 1% (v/v) Cremophor EL and 1% (v/v) Tween 80.

3.2.4 Release kinetics of free base of imatinib. Two mg equivalents of extracted free base of imatinib was suspended in 2 mL of ReGel™ under cold conditions (4 ^{o}C) in a scintillation vial. The drug-ReGel™ mix was allowed to solidify at 25 ^{o}C. Following the solidification of the drug-ReGel™ mix, 15 mL of release medium consisting of 5% ethanol in phosphate buffered saline (PBS, pH 7.4) with 1% (v/v) Cremophor EL and 1% (v/v) Tween 80 was added into the vial. The vial was then placed in the Gyratory water bath shaker (G76D, New Brunswick Scientific Co Inc, NJ) and agitated at 50 rpm with the temperature maintained at 37^{o}C and covered to protect from the light. Release profiles in two procedures were followed that created sink and nonsink conditions. For sink conditions, 50μL of the release medium were taken from the scintillation vial at specific time points for further analysis of free base of imatinib using a UV-spectrophotometer method. The rest of the release medium was discarded in the scintillation vial and replaced by fresh release medium at each sampling time points. In case of nonsink conditions, 50μL of the release medium were taken from the scintillation

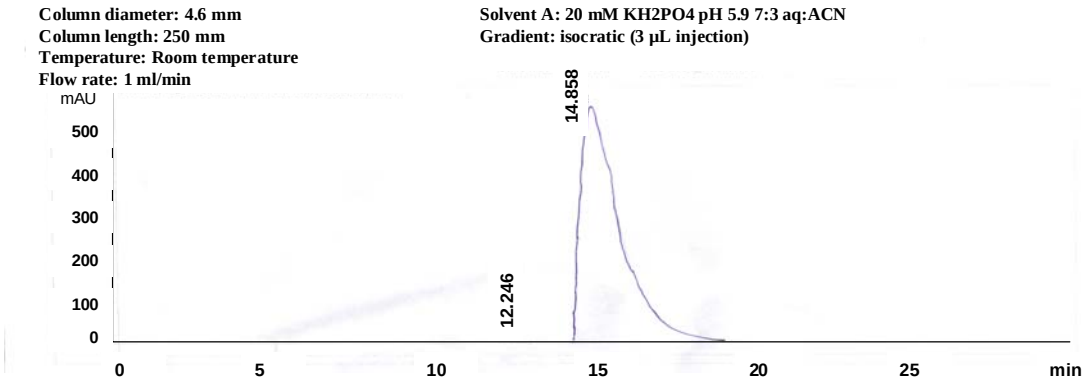
vial at specific time points for further analysis of free base of imatinib using the UV-spectrophotometer method but only 50uL of fresh release medium was replaced in the scintillation vial.

3.2.5 Data analysis. All in vitro experiments were performed with n=5 and each of the release rate profiles (% cumulative release versus days) obtained from in vitro experiments were fitted to a monoexponential equation using SigmaPlot (San Jose, CA). The rate constants of each experiment were expressed as the mean and standard deviation (SD). Release rates of sink and nonsink conditions and release rates of free base versus its mesylate form (tablet) were compared using one way analysis of variance (ANOVA), where $p < 0.05$ was considered to be statistically significant. Number of days for 100% release was calculated from the inverse of mean rate constants of each profile.

3.3 Results

Under the chromatographic conditions the free base of imatinib and imatinib mesylate eluted at a retention time of 14.85 and 13.6 minutes respectively (Figure 3-1). Since the separation was performed in RP-HPLC, this suggests that free base is slightly hydrophobic to the mesylate salt form because compounds with greater retention times will elute slowly because of increase interactions with the hydrophobic column. Linearity was obtained for the standard curve of free base of imatinib versus absorbance with an r^2 value of 0.99 and a slope of 0.227 (Figure 3-2). The release rates of free base of imatinib under sink and nonsink conditions were similar without any significant difference (Figure 3-3) with 100% of the drug released within ~2-3 days (Table 3.1). The release rates of free base of imatinib was not statistically different than the release rates of imatinib mesylate (obtained from the tablet in chapter 2) from the ReGel™ ($p > 0.05$) (Figure 3-4,

Free Base of Imatinib mesylate: Retention time 14.85 min



Imatinib mesylate: Retention time 13.6 min

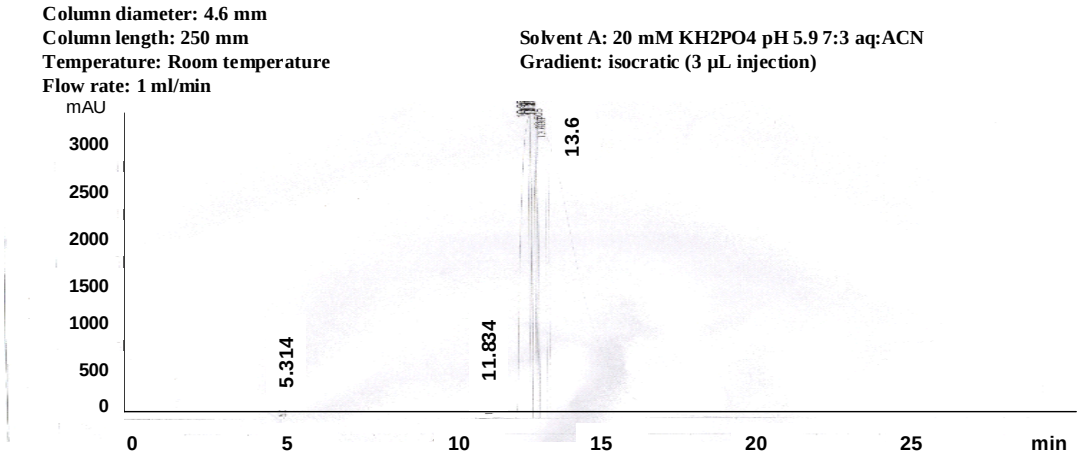


Figure 3-1: Retention times of imatinib mesylate and its free base using RP-HPLC.

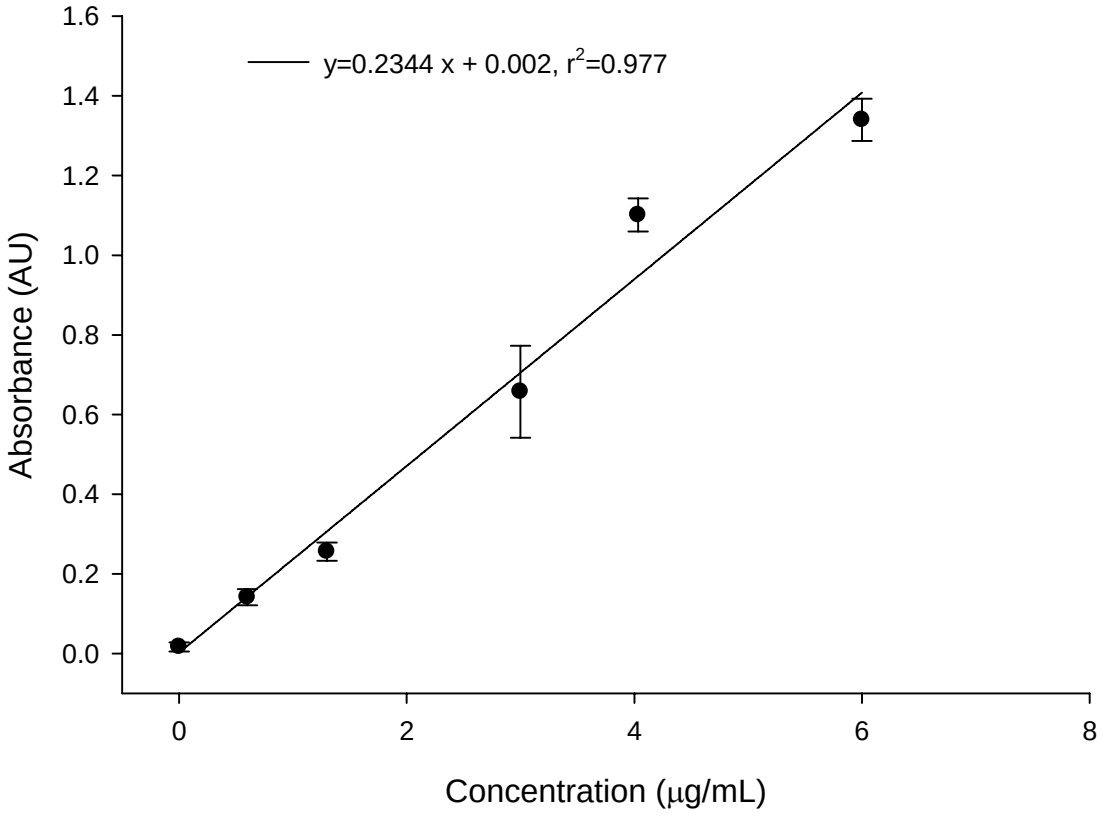


Figure 3-2: Standard curve of free base of imatinib in 5% ethanol in phosphate buffered saline (PBS, pH 7.4) with 1% (v/v) Cremophor EL and 1% (v/v) Tween 80 (n=5).

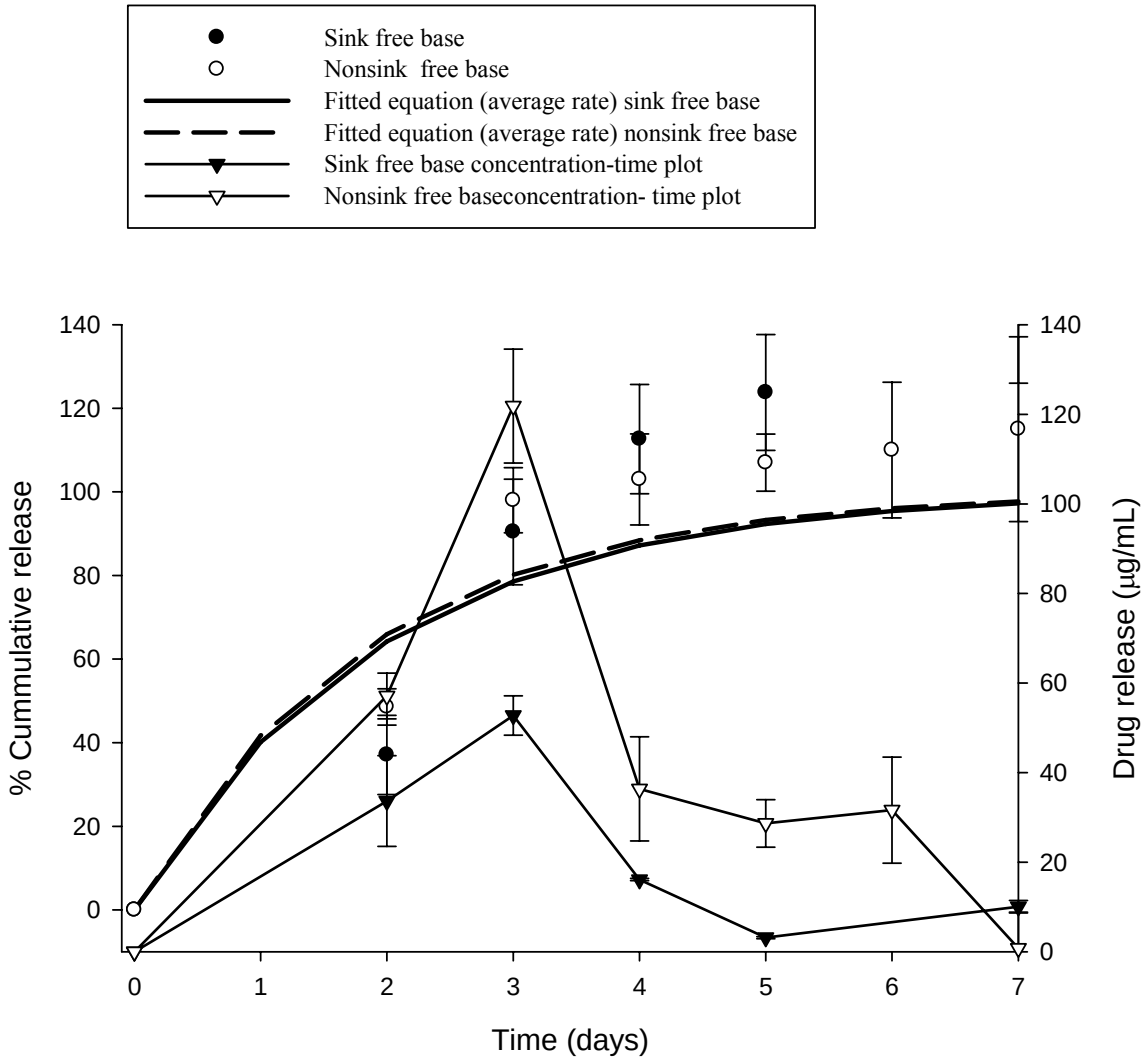


Figure 3-3: Release profile of free base of imatinib under sink and nonsink conditions.

Table 3.1: Release rates of imatinib salt (mesylate) and free base from ReGel™. Comparison by ANOVA with correction for multiple comparisons was not statistically significant (p>0.05).

Experiment	Release rate (per day)		Number of days for 100% release
	Mean	S.D.	
Nonsink Free Base	0.5349	0.1424	2
Nonsink Salt	0.3224	0.0762	3
Sink Free Base	0.5136	0.117	2
Sink Salt	0.3191	0.0623	3

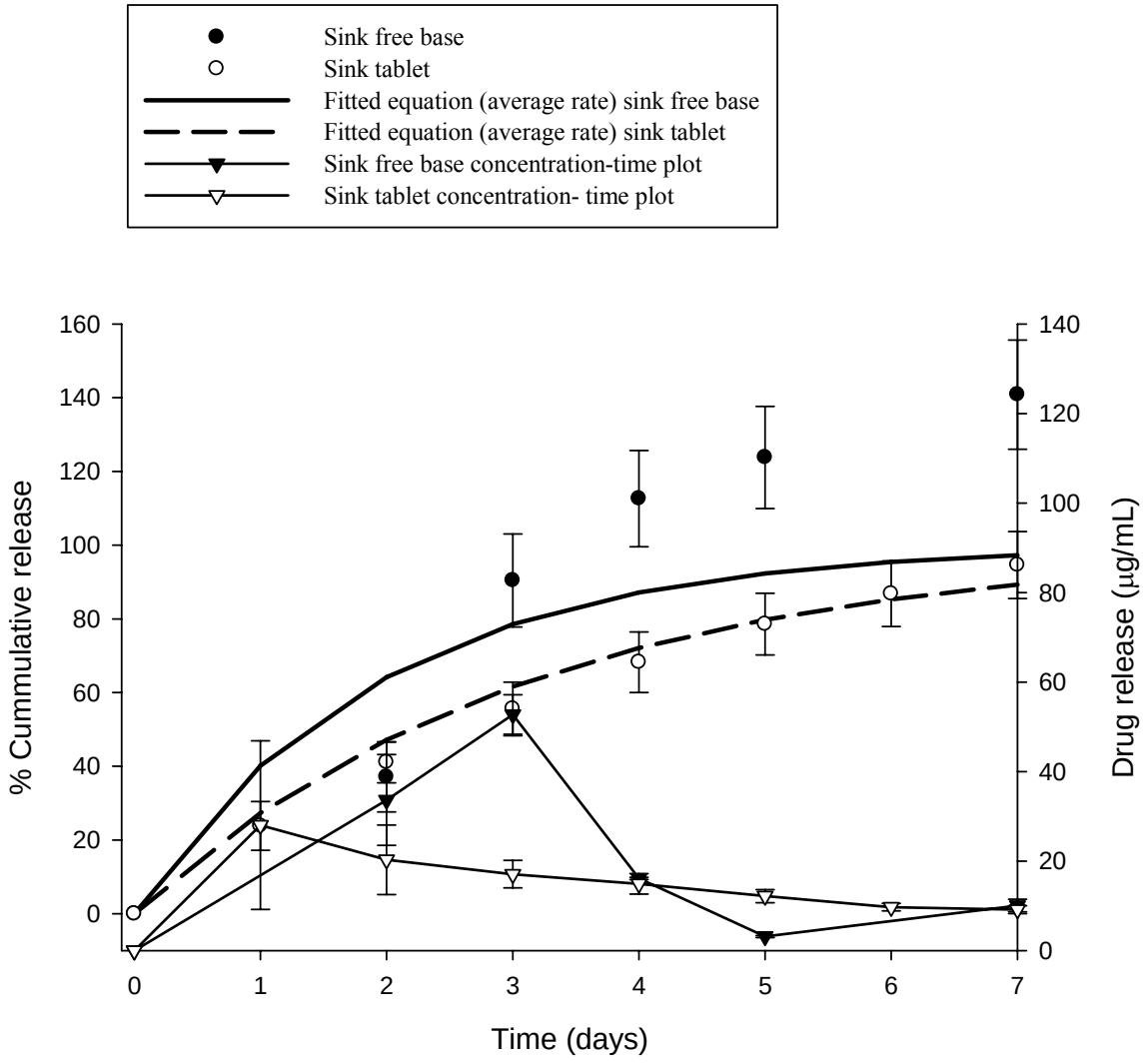


Figure 3-4: Release profile of free base of imatinib and imatinib mesylate (tablet) from ReGel™ under sink conditions.

Figure 3-5).

3.4 Discussion

The data show that the free base of imatinib eluted at a later time point in the reverse phase HPLC suggesting that the base is more hydrophobic than the salt. However, the retention times on the RP-HPLC of free base and its salt were fairly similar (14.8 vs 13.6 min), suggesting that the free base is only slightly hydrophobic than its salt form. Based on these results free base of imatinib would not have affected the release rate from ReGel™ significantly compared to its salt form. This was shown by the release profiles of the free base of imatinib from ReGel™ compared to the imatinib mesylate salt.

In order for imatinib concentration to maintain inhibition of neointimal hyperplasia, the results suggest that imatinib free base ReGel™ mixture would need to be replenished every 3 days. Thus neither imatinib free base nor its salt can be used successfully in this system because the release of imatinib is too quick (less than a week). This modified ReGel™ or microsphere/ReGel™ delivery systems could potentially extend the release of free base from ReGel™ which would help in inhibiting hemodialysis vascular access stenosis.

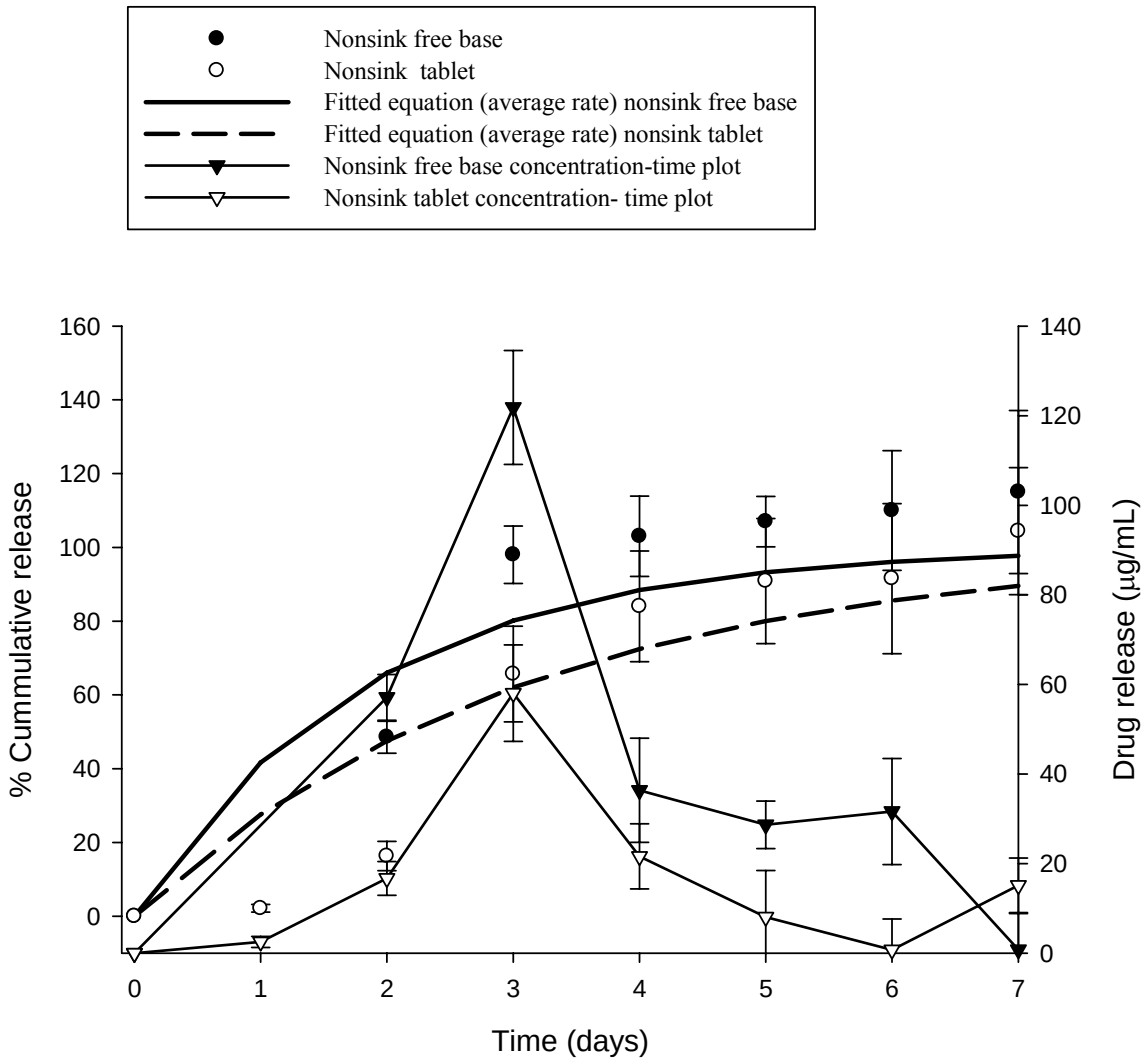


Figure 3-5: Release profile of free base of imatinib and imatinib mesylate (tablet) from ReGel™ under nonsink conditions.

CHAPTER 4

EFFECT OF IMATINIB ON SMOOTH MUSCLE CELLS

4.1 Introduction

Naturally occurring proteins like growth factors are responsible for stimulating cellular proliferation and cellular differentiation (56). One growth factor, platelet-derived growth factor (PDGF) is dimeric glycoprotein composed of two A (-AA) or two B (-BB) chains or a combination of the two (-AB). PDGF-AB released from activated platelets, SMC and monocytes has been found to be an important mediator of SMC proliferation (56). The major functions of PDGF includes embryonic development, cell proliferation, cell migration, and angiogenesis (56). It has been shown that PDGF released from activated platelets, smooth muscle cells (SMC) and monocytes are important factors for SMC proliferation (57, 58). PDGF activates cellular response through a cell surface receptor known as the PDGF receptor (PDGFR). In vivo studies in various animal models have shown that inhibiting PDGFR causes antiproliferative effects (59, 60).

Imatinib mesylate (Gleevec) has been shown to be an effective tyrosine kinase inhibitor used in the treatment of chronic myelogenous leukemia and has specificity towards c-Abi, c-kit and PDGFR (44). Antiproliferative effects of imatinib have been demonstrated using in vivo restenosis models (61). A recent study by Li et al. has shown the efficacy of imatinib mesylate salt on the PDGF-AB induced proliferation of human

arterial and venous smooth muscle cells (48). In this study, we investigated the antiproliferative effects of free base of imatinib on vascular smooth muscle cells using the Brdu(5-Bromo, 2- deoxy uridine) assay which measures the DNA synthesis of proliferating cells (48).

4.2 Methods

4.2.1 Materials. All cell culture reagents were purchased from Cascade Biologics (Portland, OR), except fetal bovine serum (FBS), which was from Atlanta Biologics (Lawrenceville, GA). Recombinant PDGF-AB was purchased from R&D Systems (Minneapolis, MN). Imatinib mesylate manufactured by Novartis Pharmaceuticals (Basel, Switzerland) was obtained from Veterans Affairs Medical Center Pharmacy as 100 mg of imatinib mesylate tablet. Imatinib free base was obtained using the procedure described in Chapter 3. All other chemicals were purchased from Sigma Aldrich Ltd (St.Louis, MO). The DNA synthesis of SMC in culture was assessed using the 5-bromo-2'deoxyuridine (BrdU) incorporation Cell proliferation Biotrak ELISA kit purchased from Amersham Biosciences (Piscataway, NJ).

4.2.2 Cell culture. Porcine venous smooth muscle cells (SMC) were isolated from femoral veins of Yorkshire cross domestic pigs by collagenase and elastase digestion. Cells were grown to 70-80% confluence and then rendered quiescent by incubation with medium containing 0.5% FBS without any additional growth factors. All cells were in culture medium at a temperature of 37^0C and humidified in 5% CO_2 incubator. Cells from passages 3 to 7 were used for the following experiments.

4.2.3 Cell proliferation assay. Venous smooth muscle cells were seeded in culture medium at a density of $1x10^4$ cells/well on 96 well plates for 48 hours and then

were treated with various concentrations of imatinib free base in dimethyl sulfoxide (0 (control), 0.05, 0.5, 5μM) for 90 minutes followed by stimulation with 50 ng/mL of PDGF-AB for the cells to proliferate. After this, BrdU labeling solution was added at a concentration of 10μM and incubated for 12 hours. The culture medium was then removed, the cells fixed and the DNA was denatured with the help of a fixative, which was removed after 30 minutes incubation. A peroxidase labeled anti BrdU monoclonal antibody Amersham Biosciences (Piscataway, NJ) was then added and the plate was incubated for 90 min at room temperature. A Microplate reader (Multiskan Ascent, Thermoelectron corp, San Jose, CA) was used to detect the BrdU-antibody complexes using colorimetric reaction at an optical density of 450 nM and the dynamic linear range was found to be up to 200 ng/mL of PDGF-AB (48).

4.2.4 Statistical analysis. The results are reported as mean ± SD and the comparison between drug-treated and control groups were performed using the two tailed student's *t*-test performed in Excel (Microsoft, Seattle, WA). Statistically significant results are defined as $p < 0.05$.

4.3 Results

The results (mean ± SD) show that in the presence of free base of imatinib there was inhibition of proliferation of porcine venous smooth muscle cells compared to control (Figure 4-1). At 0.05 μM of the free base of imatinib only 69.6±8.4 % of the cells proliferated compared to 100% of the control. At 0.5 μM of the free base of imatinib only 70.1±8.4 % of the cells proliferated compared to 100% of the control. At 5 μM of the free base of imatinib only 48.2±13 % of the cells proliferated compared to 100% of the

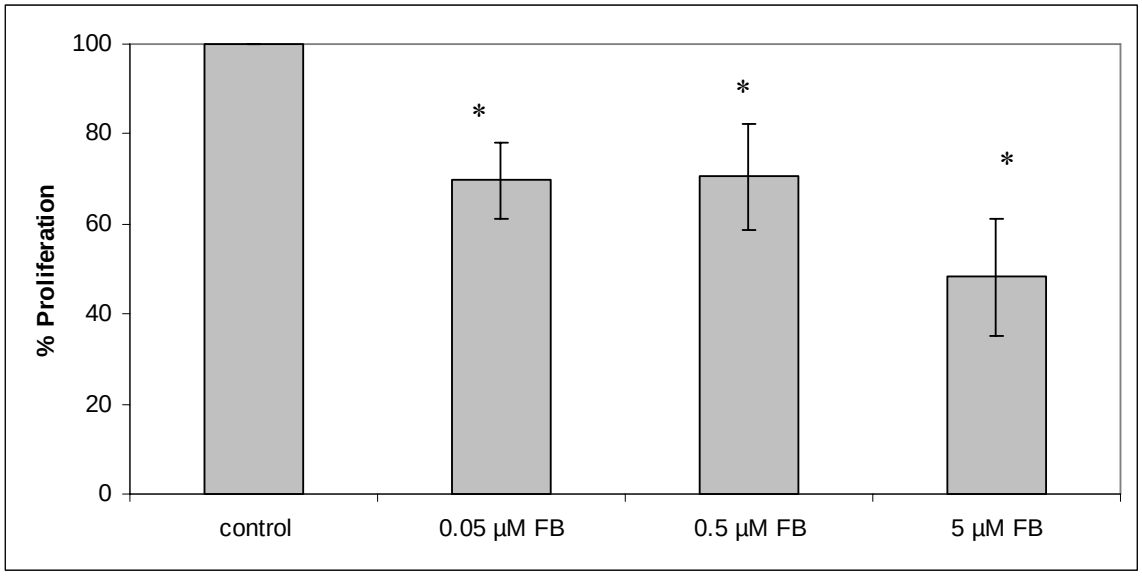


Figure 4-1: Cell proliferation assay of venous smooth muscle cells using imatinib free base (FB) of imatinib stimulated with 50 ng/mL of PDGF-AB. Results represent means ± S.D of 6 experiments. * $p<0.05$, control vs treated group.

control. An exact IC_{50} could not be determined because the dose response relationship could not be established due to the limited number of concentrations used in this study. To achieve ~50% inhibitory concentrations in cell proliferation the concentrations of imatinib free base would likely have to be between 0.5-5 μM (Figure 4-1).

4.4 Discussion

A complete dose-response was not available hence an accurate estimate of IC_{50} values of free base could not be established for our study. However, based on the concentrations studied, the IC_{50} is expected to be between 0.5-5 μM and is likely close to 5 μM since this stopped the cell proliferation by 50%. The literature reported 50% inhibitory concentration value for human vascular smooth muscle cell proliferation was 0.5 μM using imatinib mesylate salt (48). One difference in these IC_{50} values is the difference in species, with free base experiments porcine cells were used whereas the studies conducted by Li et al. (48) were carried out in human smooth muscle cells. A complete dose response of free base and the human smooth muscle cells would be needed for proper comparison of the IC_{50}.

It has been noted that antiproliferative effects could differ from the toxic effects as in the case of dipyridamole were it was found that the concentrations required for toxic effects could be 5-10 fold higher than the concentrations required for antiproliferative effects (39). The clinical end point whether it is the antiproliferative dose or the dose that induces toxicity has to be further evaluated for imatinib salt and its free base in both in vitro and the in vivo experiments. These preliminary results will be helpful in designing appropriate drug delivery systems for the prevention of hemodialysis vascular access

stenosis for imatinib mesylate salt or its free base. With the current ReGel™/imatinib delivery system, to achieve these concentrations in vascular tissue either with free base or its salt is feasible but the since the release of imatinib would be quick, further modifications of release system as mentioned in Chapter 2 would be needed to see a sustained effect in the inhibition of proliferation of vascular smooth muscle cells.

In the presence of PDGF-AB at 50 ng/mL without any imatinib there was proliferation of VSMC (48). This concentration of PDGF -AB was in the same range used in other PDGF-stimulated cell proliferation studies and in the range of serum concentrations of variety of patient populations (48). However, a reduced concentration of PDGF-AB may have less proliferative effect on the VSMCs and as result more inhibition by imatinib mesylate. These can be explored in further studies depending on the variations in the serum concentration of PDGF-AB in patient populations to understand the correlation between PDGF-AB and imatinib mesylate.

CHAPTER 5

SUMMARY

Hemodialysis vascular access dysfunction is a major problem and one of the major causes is vascular access stenosis (25, 26). One of the ways to reduce the vascular access stenosis is by perivascular delivery using polymeric systems that release antiproliferative drugs (25, 26). ReGel™, which is a novel thermosensitive polymer, can be used as a polymeric system for perivascular delivery (25, 26). The proper choice of antiproliferative drugs with ReGel™, however, needs to be explored and evaluated before such a use. In this study we evaluated the use of imatinib mesylate with ReGel™ for perivascular delivery using in vitro studies.

Imatinib mesylate was commercially available in capsule and tablets (44, 50). The in vitro release studies show that 100% of imatinib mesylate would release from imatinib mesylate-ReGel™ mixture in 2-4 days both in sink and nonsink conditions. Use of free base of imatinib did not prolong the release from ReGel™ beyond 3 days. Therefore, in order to inhibit hyperplasia imatinib must be replenished every 2-4 days in the ReGel™, which is not a feasible solution. Therefore, further investigation to prolong the release rate of imatinib from ReGel™ needs to be explored if imatinib is to be used in this application. One of the methods to achieve this is by modifying the drug delivery system.

For instance, Zhu et al. showed that by incorporating drug in microspheres within ReGel™ can prolong the release of the drug substance (62). Other researchers have shown that by modifying PEG on polymer matrices they were able to decrease the burst effect for paclitaxel in a different delivery system than ReGel™ for perivascular applications (63, 64). Also changing the ratio of lactide to glycolide ratio from 3:1 (ReGel™) to 4:1 (ReGel-2™) there was a suppression of the burst effect and sustained release of insulin (Figure 5-1) (27). Further work is needed to prolong the release profile from ReGel™ and all the above studies can be used for improving the release profile of imatinib from ReGel™.

The cell culture studies showed that inhibitory concentrations required to inhibit 50% of vascular smooth muscle cell proliferation in porcine smooth muscle cells is between 0.5-5 μM for the free base of imatinib. Further evaluation is needed to determine the concentrations required for 50% or 80% of inhibition on vascular smooth muscle cells. Additional experiments can be performed on the relationship between PDGF-AB levels and the imatinib concentrations that are required for antiproliferative effect.

Thus, a system that can sustain > 5 μM locally for up to 2-3 weeks would be needed to make this delivery system clinically feasible.

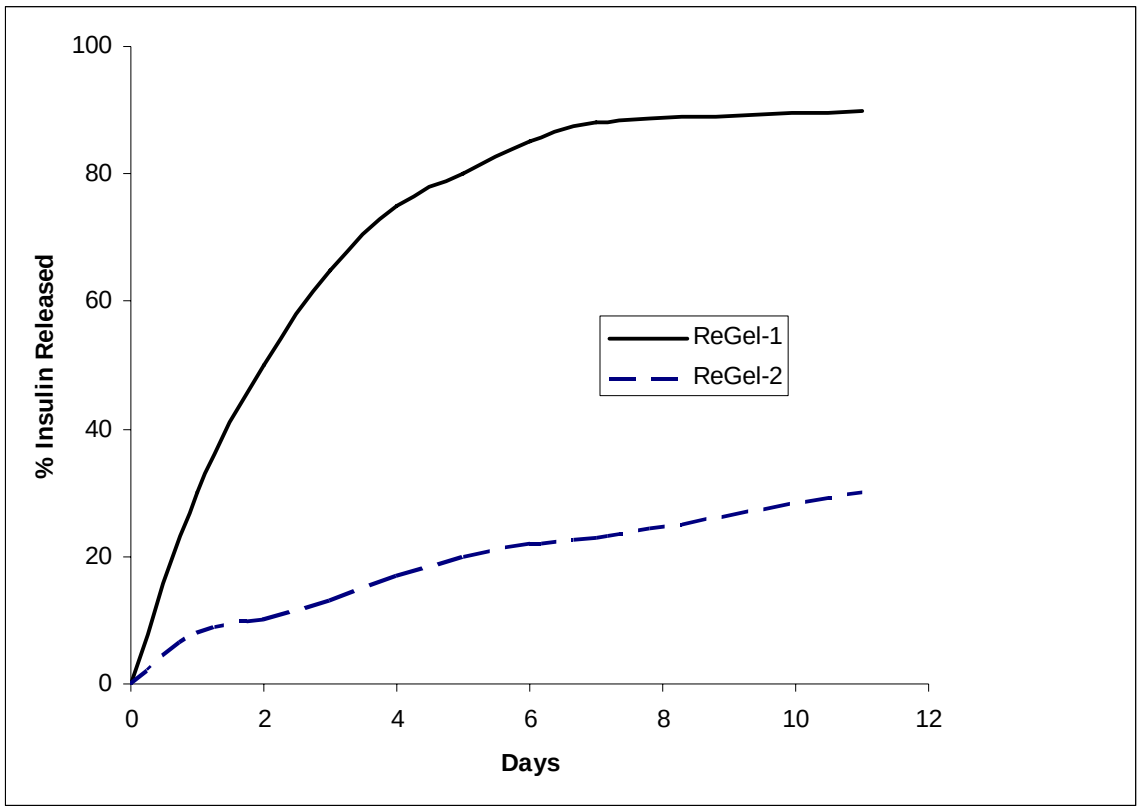


Figure 5-1: Release profile of insulin from ReGel™ versus ReGel-2™ with lactide to glycolide ratio of 3:1 and 4:1 respectively.

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