

Elsamani Ismail Abdelfadiel

All Rights Reserved

INVESTIGATION OF ANTICOAGULATION PROPERTIES OF SULFATED GLYCOSAMINOGLYCAN
MIMETICS

A dissertation submitted in partial fulfillment of the requirements for the degree of Master of
Science at Virginia Commonwealth University

by

Elsamani Ismail Abdelfadiel

B.S in Radiological Science, Virginia Commonwealth University, 2008

Supervisor: DR. UMESH R. DESAI

Professor, Department of Medicinal Chemistry

Virginia Commonwealth University

Richmond, Virginia

July 2017

Acknowledgements

Over the past year and a half, I have met number of people who have been key figures in helping me obtain my Masters and shaping me into who I am today. I would like to thank all of these people specifically for their support and encouragement.

Firstly, I am very thankful to my parents Mr. Ismail M. Abdelfadiel and Mrs. Aisha A. Mohamed for all their support and sacrifice in helping me get the best education possible. I will always be grateful to them for their endless love, care and support. I am thankful to my brother, Sabir, who has always been there for me when I needed of emotional and financial support.

To my MS. advisor, Dr. Umesh Desai; I am thankful to him for taking me under his wing and helping me excel in my career. Dr. Desai has always let me express my creativity and understanding, while giving useful feedback and ideas. He has always believed in me, even when I was down. Without him, the work for this thesis would not have been possible. He is an excellent teacher and mentor.

I would also like to thank our former IMSD/ BSDP programs director Dr. Louis J. De Felice for his amazing work to improve our programs. Over the past years, our program has improved tremendously, and he is largely responsible for this improvement. I would also like to thank Dr. Jan Chlebowski the associate dean of graduate education, for his endless help and support. I will never forget his support and the help he offered me when I was down.

I would like to thank Dr. Hamid I Akbarali who interviewed me for the BSDP program. He was the only interviewer that thought I was capable of becoming a physician scientist.

I would like to thank our collaborators who have been influential in the work presented here:

1. Dr. Donald Brophy's laboratory, especially Erika J. Martin and Bassem M. Mohammed from the Department of Pharmacotherapy and Outcome Sciences, Virginia Commonwealth University – for their help in the ex vivo studies.

2. Dr. Stefano Toldo – for help with the in vivo mouse studies.

3. PhD. Daniel Afosah in the Desai Lab for help with the introductory results reported in chapter 1, which constituted his thesis work.

4. MS. Shravan Morla in the Desai Lab – For his assistance in the chromogenic enzyme assay studies performed in chapter 3 and for always having time to help others.

I appreciate the time and effort given by my committee members- Dr. Umesh Desai, Dr. Sumitra Deb, Dr Swati Deb, and Dr. Stefano; to provide their inputs with my oral proposal final dissertation.

I am thankful to all Desai Lab members (past and present) who have always been helpful and supportive. They have been more than colloquies, they have been friends.

Abstract

INVESTIGATION OF ANTICOAGULATION PROPERTIES OF SULFATED GLYCOSAMINOGLYCAN MIMETICS

By Elsamani Ismail Abdelfadiel, MS

A Dissertation submitted in partial fulfillment of the requirements for the degree of Master of
Science at Virginia Commonwealth University

Virginia Commonwealth University, 2017.

Supervisor: Umesh R Desai

Professor, Department of Medicinal Chemistry

The existence of thrombosis in numerous pathophysiological situations formed a vast necessity for anticoagulation therapy. Thrombin and factor Xa are the only two factors of the entire coagulation cascade that have been major targets for regulation of clotting via the direct and indirect mechanism of inhibition. Our recent discovery of sulfated non-saccharide glycosaminoglycan mimetics, especially G2.2, that demonstrates highly selective cancer stem-like cells (CSCs) inhibition activity. G2.2 inhibited the growth of CSCs from multiple cancer cell lines. To evaluate its in vivo anticoagulation effect, we asked a contract research organization (CRO) to produce 20 g of material, labelled as G2.2Y. Evaluation of G2.2C in HT-29 xenograft mouse model showed a significant reduction in tumor volume and CSC markers, but unexpected bleeding consequences in some animals were observed. Also in a tail bleeding experiment, G2.2Y showed a significant enhancement in bleeding volume. Comparable studies with G2.2 synthesized in our laboratory had shown no bleeding effects. To investigate the difference between the two G2.2

samples (G2.2W (white) and G2.2Y (Yellow) that were performed using UPLC-MS characterization, we were able to determine that the G2.2Y sample was an 85:15 blend of two compounds. Elemental, NMR and MS data revealed that G2.2W was fully sulfated flavonoid derivative, as expected, but G2.2Y contained one less sulfate group. We tested both agents for their inhibition of various coagulation factors and revealed that G2.2Y inhibited fXIa nearly 2-fold better in comparison to G2.2W. Furthermore, activated partial thromboplastin time assay (APTT) indicated that G2.2W exhibited almost 3-4-fold less anticoagulant activity compared to G2.2Y. This indicates that the loss of just one sulfate group could induce substantial side effects and lead to a discovery of new anticoagulant agent. Such structure–activity relationship is important to understand if the in vivo metabolism of the agents leads to accumulation of de-sulfated products.

Table of Contents

| | Page |
|---|------|
| Acknowledgements----- | ii |
| List of Tables----- | xi |
| List of Figures----- | xii |
| Chapter | |
| 1 Introduction----- | 1 |
| 1.1 Blood, Coagulation and Haemostasis----- | 1 |
| 1.2 The Coagulation Cascade----- | 3 |
| 1.3 The Platelets----- | 5 |
| 1.4 Synthesis and Structure of Thrombin----- | 7 |
| 1.5 Thrombin Catalytic Triad and Mechanism----- | 8 |
| 1.6 Thrombin active site Structure----- | 11 |
| The sodium Binding Site----- | 13 |
| Exosite 1----- | 15 |
| Exosite 2----- | 15 |
| 1.7 Thrombin Allostery----- | 17 |
| 1.8 Structure and Function of GPIb α ----- | 17 |
| 1.9 Thrombin Interaction with GPIb α ----- | 19 |
| 1.10 Structure and Function of Factor XIa- as an Evolving Target for Prophylactic Anticoagulation----- | 19 |
| 1.11 Glycosaminoglycans----- | 22 |

| | |
|--|----|
| Classification of GAGs----- | 22 |
| 1.12 GAG Biosynthesis----- | 23 |
| 1.13 GAGs Biological Functions----- | 23 |
| 1.14 GAG Interaction with Proteins----- | 24 |
| 1.15 Serine Protease Inhibitors (Serpins)----- | 25 |
| 1.16 Antithrombin (ATIII)----- | 25 |
| 1.17 Glycosaminoglycan Mimetics----- | 26 |
| 1.18 Non-Saccharide GAG Mimetics (NSGMs)----- | 27 |
| 1.19 The Role of NSGMs----- | 28 |
| 2 Rationale----- | 30 |
| 2.1 Background----- | 30 |
| 2.2 SPGG Structure----- | 32 |
| 2.3 G2.2 Structure----- | 33 |
| 2.4 UPLC-MS----- | 34 |
| 2.5 UPLC-MS Characterization of G2.2W and G2.2Y----- | 35 |
| 3 Characterization of the anticoagulation Profile of G2.2 in Plasma and Blood----- | 38 |
| 3.1 Introduction to Different Assays----- | 40 |
| Activated Partial Thromboplastin Time (APTT)----- | 40 |
| Tail Bleeding Assay----- | 42 |
| Enzyme Substrate Assay----- | 42 |
| Thromboelastometry and Thromboelastography----- | 43 |
| 3.2 Experimental ----- | 45 |

| | |
|--|----|
| Material and Methods----- | 45 |
| Chromogenic substrate Hydrolysis Assay----- | 46 |
| Activated Partial Thromboplastin Time (APPT)----- | 48 |
| Rotational Thromboelastometry (ROTEM)----- | 48 |
| Tail Bleeding Time Studies----- | 49 |
| 3.3 Results and Discussion----- | 50 |
| G2.2 Analogs are Inhibitors of Factor XIa----- | 50 |
| G2.2 is an effective Anticoagulant in Human Plasma----- | 52 |
| G2.2 is an Effective anticoagulant in Human Whole Blood as Indicated by Thromboelastometry----- | 54 |
| G2.2W Does Not Increase Tail Bleeding----- | 57 |
| G2.2 Coagulation Assay for Factors Deficient Plasma----- | 59 |
| Conclusions----- | 64 |
| 4 Advanced Level Characterization of Antithrombotics Potential of SMI----- | 65 |
| 4.1 Introduction----- | 65 |
| 4.2 Experimental Procedures----- | 65 |
| Material and Methods----- | 65 |
| Activated Partial Thromboplastin Time (APTT)----- | 66 |
| Rotational Thromboelastometry (ROTEM)----- | 66 |
| Tail Bleeding Time Studies----- | 67 |
| 4.3 Results and Discussion----- | 67 |
| SMI is an Effective Anticoagulation in Human Plasma----- | 67 |

| | |
|---|----|
| SMI is an Effective Anticoagulant in Human Whole blood as Indicated | |
| by Thromboelastometry----- | 69 |
| SMI Does Increase Tail Bleeding----- | 70 |
| SMI Coagulation assay for Factors Deficient Plasma----- | 71 |
| 4.4 SMI Conclusion----- | 75 |
| 5 Investigation of Anticoagulation Prosperities of Sulfated Glycosaminoglycan | |
| Mimetics----- | 76 |
| 5.1 Conclusions and Future Directions----- | 76 |
| References----- | 78 |
| Appendix A. Abbreviations----- | 94 |

List of Tables

Table 1: Enzyme substrate assay and IC₅₀ for G2.2W and G2.2Y with fXa, fXIa, and thrombin in the presence and absence of antithrombin-----50

Table 2: Thromboelastometry parameters for G2.2W in comparison to G2.2Y in human whole blood-----58

Table 3: Activated partial thromboplastin time(APTT) of G2.2W in comparison to G2.2Y with different plasma deficient factors and different concentrations-----61

Table 4: Thromboelastometry parameters for SMI in human whole blood-----69

Table 5: Activated partial thromboplastin time(APTT) of SMI with different plasma deficient factors and different concentrations-----77

List of Figures

| | |
|--|----|
| Figure 1: The coagulation cascade of blood----- | 2 |
| Figure 2: The role of platelet during injury----- | 6 |
| Figure 3: Structure of human thrombin----- | 9 |
| Figure 4: Serine protease catalysis----- | 11 |
| Figure 5: The active site of thrombin----- | 13 |
| Figure 6: Structure different due to sodium binding relays into the catalytic triad----- | 15 |
| Figure 7: Structure of human thrombin showing all electropositive residues (arginine and lysine) present at exosite 1----- | 17 |
| Figure 8: Structure of human thrombin showing all electropositive residues (arginine and lysine) present at exosite 2----- | 17 |
| Figure 9: The GPIb-IX-V complex----- | 19 |
| Figure 10: Structure of factor XI and factor Xia----- | 21 |
| Figure 11: Structure of heparin----- | 24 |
| Figure 12: Structure of SPGG----- | 32 |
| Figure 13: Structure of G2.2----- | 33 |
| Figure 14: UPLC analysis both G2.2W and G2.2Y----- | 34 |
| Figure 15: UPLC analysis of G2.2W showing a single peak----- | 35 |
| Figure 16: UPLC analysis of G2.2Y shows two peaks indicating a mixture of compounds G2.2W and G2.2Y----- | 36 |
| Figure 17: Mass spectrum of peak eluting at 3.852sec----- | 36 |
| Figure 18: Mass spectrum of peak eluting at 3.762 sec----- | 37 |

| | |
|--|----|
| Figure 19: A schematic of the activated partial thromboplastin time(APTT)----- | 41 |
| Figure 20: Hemostatic effect in mouse tail transection bleeding model----- | 42 |
| Figure 21: Illustration of Enzyme and substrate interaction----- | 42 |
| Figure 22: Working principle of TEG and ROTEM----- | 44 |
| Figure 23: Chromogenic substrate hydrolysis assay----- | 51 |
| Figure 24: Concentration of G2.2 required to double APTT----- | 53 |
| Figure 25: Effect of G2.2 on whole blood hemostasis using Thromboelastometry(ROTEM)----- | 56 |
| Figure 26: In vivo anticoagulant effect of G2.2 in mice----- | 60 |
| Figure 27: Displays different concentration of G2.2W and G2.2Y with human plasma deficient factors----- | 65 |
| Figure 28: Concentration of SMI required to double clotting time----- | 70 |
| Figure 29: Effect of SMI on whole blood hemostasis using Thromboelastometry(ROTEM)----- | 72 |
| Figure 30: In vivo anticoagulant effect of SMI in mice----- | 74 |
| Figure 31: Displays different concentration of SMI with human plasma deficient factors----- | 77 |