1-1-2010

Induction And Regulation Of Autophagy By Novel Prenylation Inhibitors In Sts-26t Malignant Peripheral Nerve Sheath Tumor (mpnst) Cells

Komal Madhukar Sane
Wayne State University

Follow this and additional works at: http://digitalcommons.wayne.edu/oa_dissertations

Part of the Cell Biology Commons

Recommended Citation

This Open Access Dissertation is brought to you for free and open access by DigitalCommons@WayneState. It has been accepted for inclusion in Wayne State University Dissertations by an authorized administrator of DigitalCommons@WayneState.
INDUCTION AND REGULATION OF AUTOPHAGY BY NOVEL PRENYLATION INHIBITORS IN STS-26T MALIGNANT PERIPHERAL NERVE SHEATH TUMOR (MPNST) CELLS

by

KOMAL M. SANE

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2010

MAJOR: PHARMACOLOGY

Approved by:

_________________________________________________________________
Advisor Date

_________________________________________________________________

_________________________________________________________________

_________________________________________________________________

_________________________________________________________________
DEDICATION

I would like to dedicate this dissertation to my wonderful parents, Madhukar and Madhavi Sane, who have been my support, motivation and my strength throughout life. You have given me the best of everything and I owe you all of my achievements. Thank you so much and I love you.

I also dedicate this dissertation to my lovely sisters, Hemangi and Nandini, who have always been my role models. You are my friends, teachers and this long journey was so much easier because of you. Finally, this is a special dedication to my best friend, Gaurav. You gave me the confidence, support, motivation and love throughout this journey and made it a joyous experience. My graduate life may not have been as pleasant without the support I received from all of you. I thank you all with my deepest gratitude.
ACKNOWLEDGEMENTS

Firstly, I would like to express special thanks to my mentor, Dr. Raymond R. Mattingly, for offering me the best training possible and for his commitment toward moulding me into a successful scientist. His excellent guidance and support have been crucial in my dissertation as well as professional career. I truly value his sincere efforts and encouragement that gave me the needed knowledge, skills and confidence and I will always be indebted to his mentorship.

I sincerely thank the members of my dissertation committee: Drs. Russell Yamazaki, Nicholas Davis, Michael Bannon and Paul Stemmer. I appreciate their critical advice and help at every step to constantly ensure my progress. Also, I offer many thanks to Drs. Stanley Terlecky, John J. Reiners, Jr. and Roy McCauley for their advice in matters of science or otherwise.

The Mattingly lab has been a wonderful environment to work, thanks to all the past and present colleagues. I will cherish the great times together and the memories forever.

Sincere thanks to the department of Pharmacology for making this journey a memorable one.

Finally, thanks to all family and friends for the generous support and love.
# TABLE OF CONTENTS

Dedication.......................................................................................................................... ii  
Acknowledgements........................................................................................................ iii  
List of Abbreviations.......................................................................................................... ix  
List of Tables .................................................................................................................... xi  
List of Figures ................................................................................................................... xii

## CHAPTER 1 Protein Prenylation and its Role in Human Disease

1.1 Introduction.................................................................................................................... 1  
  1.1.1 Introduction to lipid modifications........................................................................ 1  
  1.1.2 Synthesis of isoprenoids........................................................................................ 1  
  1.1.3 Types of protein prenylation and prenylation targets............................................. 2  
  1.1.4 Prenylation enzymes and enzyme kinetics............................................................. 5  
1.2 Additional post-translational modifications............................................................... 9  
  1.2.1 Two-signal hypothesis............................................................................................ 9  
  1.2.2 Post-prenylation processing................................................................................... 10  
  1.2.3 Reversibility of prenylation.................................................................................... 11  
1.3 Significance of protein prenylation............................................................................. 12  
  1.3.1 Membrane targeting.............................................................................................. 12  
  1.3.2 Subcellular localization......................................................................................... 12  
  1.3.3 Protein-protein interactions.................................................................................. 13  
  1.3.4 Protein function..................................................................................................... 14
1.4 Prenylation inhibitors

1.4.1 Statins

1.4.2 Bisphosphonates

1.4.3 Icmt and Rce1 inhibitors

1.4.4 Prenyl transferase inhibitors (PTIs)

1.5 Prenylation and human disease

1.5.1 FTIs as parasitic disease therapeutics

1.5.2 Prenylation in progeria

1.5.3 Prenylation inhibitors and cancer

1.6 Specific aims

CHAPTER 2 Materials and Methods

2.1 Materials

2.1.1 Synthetic Chemistry

2.1.2 Reagents

2.2 Methods

2.2.1 Cell Culture

2.2.2 Western Blot Analysis

2.2.3 Live Cell Imaging Assays

2.2.4 Immunofluorescence Assays

2.2.5 Cell Proliferation Assay

2.2.6 MTT Assay

2.2.7 Flow Cytometric Analysis
CHAPTER 3 A Novel Geranylgeranyl Transferase Inhibitor in Combination with Lovastatin Inhibits Proliferation and Induces Autophagy in STS-26T MPNST Cells

3.1 Rationale

3.2 Results

3.2.1 Inhibition of geranylgeranylation of GTPases by GGTI-2Z and lovastatin combination

3.2.2 Combination of GGTI-2Z and lovastatin does not inhibit FTase

3.2.3 GGTI-2Z in combination with lovastatin inhibits proliferation of STS-26T cells without significant loss of cell viability

3.2.4 GGTI-2Z in combination with lovastatin arrests STS-26T MPNST cells in G_0/G_1 phase of the cell cycle

3.2.5 GGTI-2Z alone or in combination with lovastatin does not induce apoptosis in STS-26T cells

3.2.6 GGTI-2Z and lovastatin combination treatment induces autophagy in STS-26T cells

3.2.7 Inhibitors of autophagy do not affect GGTI-2Z/lovastatin induced autophagy in STS-26T cells

3.2.8 Withdrawal of treatment of STS-26T cells with GGTI-2Z/lovastatin restores their colony forming ability cancer cell lines
3.2.9 GGTI-2Z and lovastatin combination is also effective in other unrelated cancer cell lines……………………………………………………………. 53
3.2.10 GGTI-2Z/lovastatin do not induce autophagy in other MPNST cell lines…………………………………………………………………………………. 54
3.2.11 Normal immortalized rat Schwann cells (iSC) are resistant to GGTI-2Z and lovastatin co-treatment…………………………………………………………….. 54
3.3 Discussion………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………...
CHAPTER 5 Prenylation Inhibitors and Autophagy

5.1 Discussion

5.1.1 Ras and Rheb as potential targets of prenylation inhibitors........ 100
5.1.2 Role of Rabs in prenylation inhibitor induced autophagy ........... 104
5.1.3 Lysosomal protection and autophagy ...................................... 105
5.1.4 Modulation of Atg genes ....................................................... 105

5.2 Conclusion .................................................................................. 106

References .......................................................................................... 110
Abstract .............................................................................................. 144
Autobiographical Statement ............................................................... 146
LIST OF ABBREVIATIONS

2Z-GGOH: 2-Z-geranylgeraniol

AO: Acridine orange

BP: Bisphosphonate

DMAPP: Dimethylallyl diphosphate

DPI: Dual prenylation inhibitor

EAE: Experimental autoimmune encephalitis

ER: Endoplasmic reticulum

FPP: Farnesyl pyrophosphate

FTase: Farnesyl transferase

GEF: Guanine nucleotide exchange factor

GGPP: Geranylgeranyl pyrophosphate

GGTase: Geranylgeranyl transferase

GI_{50}: Inhibitor concentration at which 50% growth inhibition is observed

hVps34: Human vacuolar protein sorting 34

HGPS: Hutchinson-Gilford progeria syndrome

HMG-CoA: Hydroxymethyl glutaryl coenzyme A

iSC: normal immortalized Schwann cells

ICMT: Isocarboxyl methyltransferase

IPP: Isopentyl diphosphate

K_m: Michaelis Menten constant

LAMP: Lysosomal associated membrane protein
LC3: (Microtubule associated protein 1) light chain 3
LFA1: Leukocyte function antigen 1
LMNA: Lamin A
mTOR: Mammalian target of Rapamycin
M6P: Mannose-6-phosphate
MPNST: Malignant peripheral nerve sheath tumor
N-BP: Aminobisphosphonate
Nf: Neurofibromin
NF1: Neurofibromatosis type 1
PAS: Preautophagosomal structures
PAT: Palmitoyl acyltransferase
PE: Phosphatidylethanolamine
RabGDI: Rab guanine nucleotide dissociation inhibitor
Rep1: Rab escort protein 1
RCE1: Ras converting enzyme 1
RhoGDI: Rho guanine nucleotide dissociation inhibitor
LIST OF TABLES

1.1 Types of post-translational lipid modifications…………………………………… 2
1.2 Comparison of properties of prenylation enzymes……………………………… 4
1.3 Potential therapeutic implications of post-translational modification inhibi
   tors………………………………………………………………………………… 23
LIST OF FIGURES

1.1 The mevalonate pathway of cholesterol biosynthesis .............................................. 28
1.2 General schematic of prenylation pathways. ............................................................. 29
1.3 Prenylation reactions of GTPases ............................................................................ 30
1.4 Three-dimensional structures of prenyltransferase enzymes .................................. 32
1.5 Classes of post-translational modification inhibitors ............................................. 34
2.1 Synthesis of prodrug GGTI-2Z (compound 7), and structures of 2Z-GGMP (compound 8) and 2Z-GGPP (compound 9). ......................................................... 41
2.2 Chemical structure of lovastatin. ............................................................................. 42
3.1 The process of autophagy and autophagic flux. ..................................................... 60
3.2 Inhibition of prenylation via GGTase I and GGTase II in STS-26T cells by GGTI-2Z/lovastatin combination treatment. ......................................................... 62
3.3 Lack of effect of GGTI-2Z/lovastatin treatment on membrane localization of a farnesylated GFP construct. ................................................................. 65
3.4 Effect on proliferation and viability of STS-26T cells following GGTI-2Z/lovastatin treatment. ................................................................. 66
3.5 FACS analysis: GGTI-2Z/lovastatin treatment arrests STS-26T cells in G_0/G_1 and increases sub-G_1 DNA content. ......................................................... 68
3.6 Lack of apoptosis in STS-26T cells following GGTI-2Z/lovastatin treatment ... 69
3.7 Induction of autophagy in STS-26T cells by GGTI-2Z/lovastatin treatment.... 71
3.8 Lack of inhibition of GGTI-2Z/lovastatin induced LC3 processing by
known autophagy inhibitor, 3-methyladenine (3-MA). ........................................ 73

3.9 Effect on re-plating ability of STS-26T cells upon treatment with
GGTI-2Z/lovastatin followed by removal of the drugs. ................................. 74

3.10 Effects of GGTI-2Z/lovastatin on other human cancer cell lines............. 75

3.11 Effect of GGTI-2Z/lovastatin on other MPNST cell lines......................... 77

3.12 Lack of cytotoxicity from GGTI-2Z/lovastatin in normal immortalized
rat Schwann cells. ....................................................................................... 79

4.1 Chemical structure of FTI-1. ................................................................ 90

4.2 Effect of FTI-1/lovastatin on proliferation and viability of STS-26T cells... 91

4.3 Autophagic flux experiment. .................................................................. 92

4.4 Colony formation assay. ......................................................................... 93

4.5 Colocalization of LC3 and LAMP-1 in FTI-1 and lovastatin co-treated cultures. 94

4.6 Colocalization of LC3 and LAMP-2 in FTI-1 and lovastatin co-treated cultures. 95

4.7 Effect of FTI-1 and lovastatin co-treatment on LAMP-1 and -2 expression... 96

4.8 Effect of FTI-1/lovastatin treatment on lysosomal acidity....................... 97

4.9 Effects of FTI-1 and lovastatin co-treatment on pro-cathepin B/D processing. 98

4.10 Effect of FTI-1/lovastatin on MCF7 breast cancer cells......................... 99

5.1 Model of autophagy regulation by prenylation inhibition........................ 109
CHAPTER 1
PROTEIN PRENYLATION AND ITS ROLE IN HUMAN DISEASE

1.1 INTRODUCTION

1.1.1 Introduction to lipid modifications

Lipid modification of proteins is an essential aspect of the expression, stability as well as function of the modified proteins. The most common lipids that are added to proteins include fatty acids (myristate, palmitate etc.) and isoprenyl groups (farnesyl and geranylgeranyl) (Table 1.1). Prenylation is known to modify several hundred proteins in cells (McTaggart, 2006). These include fungal mating factors, GTPases of the Ras superfamily, subunits of trimeric G proteins, nuclear proteins such as prelamin A and lamin B, and protein kinases (Zhang and Casey, 1996). Addition of isoprenoids is thought to facilitate membrane association, subcellular localization, vesicular trafficking and protein-protein interactions of modified substrates (Konstantinopoulos et al., 2007; Zhang and Casey, 1996).

1.1.2 Synthesis of isoprenoids

Isoprenoid groups are derived from the five-carbon (C₅) unit isopentyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). These units are synthesized via the mevalonate pathway of cholesterol biosynthesis (Goldstein and Brown, 1990) (Figure 1.1). Hydroxymethyl glutaryl coenzyme A (HMG-CoA) reductase is the rate-limiting enzyme of this pathway that catalyzes the first step of conversion of acetyl CoA to
mevalonate. Further, via multiple enzymatic steps, isoprenoid units are obtained. The farnesyl isoprenoid, farnesyl pyrophosphate (FPP) is a 15-carbon lipid group, whereas geranylgeranyl pyrophosphate (GGPP) is a 20-carbon moiety. Cholesterol synthesis also utilizes FPP, and hence a tightly regulated feedback mechanism controls the activity of this pathway (Brown and Goldstein, 1980).

<table>
<thead>
<tr>
<th>1. Palmitoylation</th>
<th>Recognition sequence</th>
<th>Modifying Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>- S-palmitoylation</td>
<td>Varied sequences</td>
<td>Palmitoyl acyltransferase Rasp</td>
</tr>
<tr>
<td>- N-palmitoylation</td>
<td>(e.g. DHHC-CRD)</td>
<td></td>
</tr>
<tr>
<td>2. Myristoylation</td>
<td>MGxxxS/T</td>
<td>Myristoyl transferase</td>
</tr>
<tr>
<td>3. Prenylation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Farnesylation</td>
<td>-CaaX (X=Ser, Met, Glu)</td>
<td>Farnesyl transferase</td>
</tr>
<tr>
<td>- Geranylgeranylation</td>
<td>-CaaL</td>
<td>Geranylgeranyl transferase I</td>
</tr>
<tr>
<td></td>
<td>-CxC or -CC (Rabs)</td>
<td>Geranylgeranyl transferase II</td>
</tr>
</tbody>
</table>

Adapted from Rosh MD. Nature Chemical Biology, Nov 2006

**Table 1.1.** Types of post-translational lipid modifications.

### 1.1.3 Types of protein prenylation and prenylation targets

Prenylation was first identified to occur in mammalian proteins when the nuclear envelope protein, lamin B, was found to be modified by a metabolite of mevalonic acid in the cholesterol biosynthesis pathway (Wolda and Glomset, 1988). This metabolite was later identified as a 15-carbon farnesyl group (Farnsworth et al., 1989). Alongside
this discovery, another modifying prenyl group was also identified, the 20-carbon geranylgeranyl isoprenoid group (Farnsworth et al., 1990). About the same time, other proteins including Ras superfamily of proteins were described as targets for farnesylation, and it was discovered that farnesylation is required for transformation of cells by oncogenic Ras (Casey et al., 1989; Hancock et al., 1989; Schafer et al., 1989). Sequence comparison amongst the yeast mating factor, lamin B and Ras proteins revealed that all these proteins contain a cysteine residue at the C-terminus, followed by three other residues, in most cases, -aaX [a= aliphatic amino acid, X= a variety of amino acids] (Farnsworth et al., 1989; Powers et al., 1986). This is termed as the CaaX box, which makes a component of a variety of proteins. In the Ca₁a₂X sequence, the specificity at a₂ and X positions determines the specificity of CaaX sequence recognition by modifying enzymes. Aromatic and basic residue substitutions are tolerated at a₁, but not at a₂ (Moores et al., 1991). Aromatic residue substitution at a₂ makes a substrate that acts as a competitive inhibitor of the prenylating enzyme (Brown et al., 1992; Goldstein et al., 1991).

It is now known that there are also prenylated proteins that contain a CC or a CXC instead of a CAAX moiety (Glomset and Farnsworth, 1994). The C-terminal motif governs which isoprenoid will be attached to the protein (Figure 1.2). In case of proteins containing CaaX, when ‘X’ is serine, glutamine or methionine, farnesylation occurs, whereas when ‘X’ is a leucine, geranylgeranylation occurs (Casey et al., 1991; Yokoyama et al., 1991). Nonetheless, this is not an absolute rule, and cross-prenylation does occur. For instance, H-Ras is exclusively farnesylated, whereas K-Ras and N-Ras can be farnesylated or geranylgeranylated. Like Ras proteins, Rho GTPases also
contain CaaX sequences and undergo prenylation. However, they mostly undergo geranylgeranylation (Konstantinopoulos et al., 2007), and after modification they bind to RhoGDIs (Rho guanine nucleotide dissociation inhibitors) which keep them soluble in the cytosol and carry them to membranes where they function (Hoffman et al., 2000). There is also evidence that RhoB can be farnesylated and geranylgeranylated, and the two modified forms exert different functions (Du et al., 1999; Lebowitz et al., 1997). Interestingly, geranylgeranylation is the more commonly found prenyl modification on cellular substrate proteins (Farnsworth et al., 1990; Rilling et al., 1990). In general, CC and CXC containing proteins are geranylgeranylated (Seabra et al., 1992b). These mainly consist of Rab family of proteins, which includes more than 60 different proteins (Leung et al., 2006). The steps involved in prenylation of GTPases have been depicted in Figure 1.3.

<table>
<thead>
<tr>
<th>Lipid donor substrate</th>
<th>FTase</th>
<th>GGTase I</th>
<th>GGTase II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Farnesyl diposphate (FPP)-15 carbon</td>
<td>Geranylgeranyl diposphate (GGPP)-20 carbon</td>
<td>Geranylgeranyl diposphate (GGPP)-20 carbon</td>
</tr>
<tr>
<td>Protein recognition motif</td>
<td>CaaX (X= S,M,Q,A,F)</td>
<td>CaaX (X= L)</td>
<td>-CC or -CxC</td>
</tr>
<tr>
<td>Select protein substrates</td>
<td>Ras, nuclear lamins, transducin γ subunit</td>
<td>Rho, Rac, Ras, Rap, G, subunit</td>
<td>Rab proteins</td>
</tr>
<tr>
<td>Subunit composition (mammalian)</td>
<td>α: 48 kDa Identical</td>
<td>α: 48 kDa</td>
<td>α: 50 kDa</td>
</tr>
<tr>
<td></td>
<td>β: 46 kDa 25% identity</td>
<td>β: 43 kDa</td>
<td>β: 38 kDa (30% identity with either counterpart)</td>
</tr>
<tr>
<td>Metal requirements</td>
<td>Zn²⁺, Mg²⁺</td>
<td>Zn²⁺</td>
<td>Mg²⁺</td>
</tr>
</tbody>
</table>

Table 1.2. Comparison of properties of prenylation enzymes.
1.1.4 Prenylation enzymes and enzyme kinetics

There are three distinct prenylation enzymes: farnesyltransferase (FTase), geranylgeranyltransferase type I (GGTase I) and geranylgeranyltransferase type II (GGTase II) (Moores et al., 1991; Reiss et al., 1990) (Table 1.2). The crystal structures of all of these enzymes are shown in Figure 1.4. Each of these is a heterodimer consisting of an α and a β subunit polypeptide (Moomaw and Casey, 1992; Reiss et al., 1990; Seabra et al., 1992a). FTase and GGTase I are closely related. They share a common α subunit (48 kDa molecular weight) (Chen et al., 1991a; Seabra et al., 1991) of almost identical structures, with slight differences in their interaction with the corresponding β subunits (Lane and Beese, 2006). The β subunits (46 kDa in FTase; 42 kDa in GGTase I) (Chen et al., 1991b; Moomaw and Casey, 1992; Yokoyama and Gelb, 1993) are distinct with ~25% sequence homology (Zhang and Casey, 1996), but share similar structures. Both α and β subunits are made up of α-helices and are arranged as a superhelical α-subunit flanking the α-α barrel β-subunit (Park et al., 1997; Taylor et al., 2003). This arrangement forms a deep, funnel-shaped cavity in the center of the barrel that is lined with several conserved aromatic residues and is hydrophobic in nature (Lane and Beese, 2006). The active sites of FTase and GGTase I lie within this cavity. The α–β interface of both enzymes is characteristic with the formation of several hydrogen bonds due to its highly polar nature (Janin et al., 1988).

FTase and GGTase I are both zinc metalloenzymes with one mole of zinc per mole of enzyme (Chen et al., 1993). The zinc ion is bound to the β subunit at the α–β interface via specific conserved residues (Lane and Beese, 2006; Park et al., 1997; Taylor et al., 2003). Zn\(^{2+}\) is not essential for binding of the isoprenoid (FPP/GGPP), but is required for
binding of the protein substrate (Reiss et al., 1992; Yokoyama et al., 1995). It directly coordinates the cysteine residue of the substrate CaaX sequence (Huang et al., 1997; Moomaw and Casey, 1992). Crystallographic studies as well as in vitro biochemical experiments suggest that the Zn$^{2+}$ cofactor is critical for catalytic activity of the enzymes (Moomaw and Casey, 1992; Yokoyama et al., 1995). FTase also requires Mg$^{2+}$ for its catalytic activity (Reiss et al., 1992), whereas Zn$^{2+}$ alone is sufficient for full GGTase I activity. FTase is thought to have distinct binding sites for its two substrates, FPP and the peptide (Reiss et al., 1991). Both binding sites lie on the β-subunit of the enzyme and either substrate can bind the enzyme independently (Ying et al., 1994). However, steady-state kinetic analyses have shown that both substrates must bind before the formation of the product (Pompliano et al., 1992). This is also true for GGTase I. Like FTase, GGTase I has two distinct substrate binding sites on its β-subunit and substrates can bind the enzyme independently (Yokoyama et al., 1995). This mechanism of substrate binding is called the random sequential mechanism, wherein either substrate can bind the enzyme first. However, with both FTase and GGTase I, isoprenoid substrate binding first to the free enzyme is thought to be the preferred mechanism (Pompliano et al., 1993; Zhang et al., 1994). Nevertheless, the precise sequence of events in the enzymatic reaction still remains undefined.

As mentioned earlier, the α subunits of FTase and GGTase I are almost identical. However, their involvement in the catalytic activity of the enzymes is not fully established. It has been suggested that some highly conserved residues in this subunit may be required for stabilizing the enzyme conformation rather than subunit interactions (Omer et al., 1993). There is also evidence that mutation of Lys164 to Asn of the
α subunit does not interfere with α-β dimerization or substrate binding, but abolishes the FTase enzymatic activity (Andres et al., 1993a), suggesting a direct role for the α-subunit in FTase catalytic activity.

Although FTase and GGTase I are generally selective for their protein substrates, their sequence and structural similarities lead to some degree of cross-reactivity (Zhang and Casey, 1996). In addition, the substrate recognition elements of their respective isoprenoid and protein substrates exhibit common features, attenuating substrate specificity. Extensive structural biological studies and sequence-based predictions have been made in order to understand what factors govern substrate specificity and catalytic mechanisms of these enzymes (Lane and Beese, 2006). Although FTase can bind both FPP and GGPP, it is not capable of transferring GGPP on to substrate proteins (Reiss et al., 1992; Yokoyama et al., 1997). In contrast, GGTase I can bind FPP and GGPP with comparable affinities, and is capable of transferring FPP to RhoB substrate with similar efficacy as GGPP (Armstrong et al., 1995; Yokoyama et al., 1995). A simple ruler hypothesis suggests that the two enzymes can distinguish between the lengths of the isoprenoid chains, which differ due to an additional isoprene unit in GGPP (Taylor et al., 2003). Crystal structures of the prenyltransferases in complexes with peptide substrates and/or isoprenoid analogs have shed light on the conformational requirements for enzyme-substrate interactions and product formation from these complexes (Lane and Beese, 2006). They indicated both substrate and prenylated peptide product bound simultaneously to the catalyzing enzyme and that the amino acid sequence of the CaaX motif may modulate product release, which was the rate-limiting step of the prenylation reaction. They also suggested that a small portion of the
isoprenoid conformation moved during catalysis. The study of the prenyltransferase complexes also confirmed that GGTase II catalysis involves a processive reaction, wherein two prenyl groups are added to its Rab substrates without dissociation of a monoprenylated intermediate. FTase and GGTase I catalyzed reactions, on the other hand, are non-processive (Lane and Beese, 2006).

The enzyme, GGTase II (also known as RabGGTase), contains both an αβ heterodimer that is required for catalytic action and an additional polypeptide, Rep1 (Rab escort protein I). GGTase II modifies proteins ending in CC or CxC that are found exclusively in the Rab protein family (Casey et al., 1991). In both cases, geranylgeranyl modification occurs at both the cysteine residues (Farnsworth et al., 1994). The α and β subunits of GGTase II identified from rat cDNA exhibit ~30% homology with their corresponding subunits in FTase and GGTase I (Armstrong et al., 1993). The GGTase II catalyzed reaction may, however, follow a distinct mechanism compared to the other prenyltransferases (Andres et al., 1993b). Purified GGTase II requires Mg$^{2+}$ for its enzymatic activity, and is actually inhibited by the presence of Zn$^{2+}$ (Seabra et al., 1992b). Unlike in FTase and GGTase I, where the catalytic activity resides mainly in the β component, the αβ dimer component of GGTase II is thought to participate in its catalytic mechanism. Rep1 (earlier known as component A) can bind to both unprenylated and prenylated Rabs and thus serve mainly to escort the substrate Rab proteins and present them to the catalytic component heterodimer. Rep1 remains bound after the geranylgeranylation reaction and the reaction is limited by the amount of Rep1 protein, which keeps the Rabs soluble in the cytosol. Finally, prenylated Rabs bind RabGDIIs (Rab guanine nucleotide dissociation inhibitors), which recognize the double
geranylgeranyl moieties and help translocate them to the plasma membrane where they can function (Andres et al., 1993b).

1.2 ADDITIONAL POST-TRANSLATIONAL MODIFICATIONS

1.2.1 Two-signal hypothesis
Most proteins that are subject to prenylation, including GTPase proteins, are required to be membrane-bound in order to function effectively. Prenyl groups, being hydrophobic in nature, are capable of assisting their substrate proteins to translocate toward their target membrane, wherein they can bind to membrane lipids and to other proteins and thereby efficiently participate in signaling pathways. The increase in hydrophobicity, however, may not be sufficient for the prenylated protein to remain membrane-bound for the required duration, especially in case of a singly farnesylated protein (Peitzsch, 1993; Silvius and l'Heureux, 1994). This observation led to the two-signal hypothesis, suggesting the requirement of a second signal, either a palmitate or a polybasic domain immediately upstream of the prenylated cysteine (Resh, 2006). Either one of these signals significantly enhances membrane affinity of an already prenylated protein and helps stabilize it in the lipid bilayer (Dunphy and Linder, 1998; Hancock et al., 1990). For instance, K-Ras4B, which contains a polybasic domain close to the C-terminus demonstrates enhanced membrane association when compared with its mutant lacking the basic residues in this region (Hancock et al., 1990). Similarly, H-Ras contains two palmitoylation sites, Cys181 and Cys184. When these residues are mutated to Ser, the percentage of membrane associated H-Ras is drastically diminished (Hancock et al.,
Since geranylgeranylation via GGTase II already attaches two lipid moieties to the protein that results in improved hydrophobicity, it leads to quite effective membrane association (Shahinian and Silvius, 1995). Moreover, CXC-containing proteins further undergo methylation, whereas CC-containing proteins do not undergo further modifications (Farnsworth et al., 1991; Smeland et al., 1994). In contrast, a farnesylated protein exhibits modest membrane association that is only short-lived, and thus requires additional signals to stabilize its interaction with membranes.

### 1.2.2 Post-prenylation processing

Following prenylation, many proteins undergo further modifications via other modifying enzymes. These additional processes can occur at different locations inside the cell along the proteins’ secretory pathway. The first step after prenylation at the cysteine residue involves cleavage of the \(-\text{aaX}\) residues by an endoprotease. This was first found to occur in yeast and mammalian Ras proteins and confirmed \textit{in vitro} (Fujiyama et al., 1987; Gutierrez et al., 1989; Ma and Rando, 1992). The enzyme responsible is known as Ras converting enzyme 1 (RCE1) and is present at the endoplasmic reticulum (ER) membrane (Boyartchuk et al., 1997; Schmidt et al., 1998).

The next step is methylation at the exposed carboxyl group of the prenylated cysteine, which also occurs at the ER (Stephenson and Clarke, 1992). All CaaX containing proteins and some Rab proteins containing CXC but not CC are methylated (Clarke, 1992; Farnsworth et al., 1994). An isocarboxyl methyltransferase (ICMT) enzyme activity present in ER fraction of cells is responsible for modification of CaaX substrates. Some of this enzyme may also be present in plasma membranes (Pillinger et al., 1994).
and its activity is thought to be stimulated by the $\gamma$-subunit of G-protein (Backlund et al., 1990). ICMT utilizes S-adenosyl-L-methionine as a methyl donor (Perez-Sala et al., 1992; Pillinger et al., 1994). Substrate recognition is highly dependent on the isoprenoid attached to the cysteine. Both farnesyl and geranylgeranyl substrates are recognized equally well (Tan et al., 1991). The kinetics of this enzymatic step have been studied (Shi and Rando, 1992). A distinct methyltransferase is required for modification of CXC-type proteins (Giner and Rando, 1994) which has not been explored in detail. Methylated proteins are further subjected to palmitoylation, when necessary, at the Golgi, which then facilitates their translocation toward plasma membranes (Konstantinopoulos et al., 2007). A family of several palmitoyl acyltransferases (PATs) can carry out this step (Feng and Davis, 2000; Mitchell et al., 2006).

1.2.3 Reversibility of prenylation

Many post-translational modifications are known to be reversible. Palmitoylation is a dynamic lipid modification due to its reversibility. Palmitoylated proteins localize in plasma membranes, including lipid rafts (Moffett et al., 2000). After it has performed its function, depalmitoylation by thioesterase enzyme helps release of the protein from the membrane and transfer back to the Golgi where it can get repalmitoylated (Rocks et al., 2005). The balance between PAT and palmitoyl thioesterase activities determines the relative abundance of palmitoylated versus non-palmitoylated protein levels (Baekkeskov and Kanaani, 2009). In contrast, prenylation is thought to be a stable, irreversible modification. Theoretically, it has been suggested that reversible prenyl group attachment could be part of a ‘prenyl switch’ to facilitate their release from the
lipid bilayer (Resh, 2006), regulating the prenylation process. This hypothesis, however, lacks experimental evidence.

1.3 SIGNIFICANCE OF PROTEIN PRENYLATION

1.3.1 Membrane targeting
As mentioned previously, the most important function of prenylation is to alter the hydrophobicity of the substrate protein and facilitate localization to the plasma membrane or other membranes, where it can associate with the membrane components and serve its functions. The efficiency of membrane targeting varies with the type of prenyl group attached, and the second signal functions to augment the efficiency when it is low. For instance, post-prenylation processing is required for proper localization of all Ras isoforms, but not necessary for Rho targeting (Michaelson et al., 2005).

1.3.2 Subcellular localization
The highly variable C-terminal domain of a prenylated protein determines its specific membrane localization (Chavrier et al., 1991). For example, lamin B contains a nuclear localization signal that targets it to the nucleus (Holtz et al., 1989). Similarly, the C-terminal polybasic region of K-Ras4B serves as a trafficking signal to direct it to plasma membranes (Hancock et al., 1990) but not to lipid rafts. This region may also direct subsequent translocation of K-Ras4B from the plasma membrane to the Golgi and endosomal membranes (Fivaz and Meyer, 2005). In comparison, H-Ras and N-Ras,
which do not contain such a polybasic domain, are targeted via the exocytic pathway to plasma membranes, and reside both inside and outside of lipid rafts (Apolloni et al., 2000). Both these isoforms are also present in endomembrane systems including endosomes, ER and the Golgi (Choy et al., 1999). The Ras proteins associated with any of these cellular locations are capable of being activated for GTP hydrolysis, although via distinct mechanisms (Bivona et al., 2003; Chiu et al., 2002; Rotblat et al., 2004).

1.3.3 Protein-protein interactions

Not only do GTPases interact with cellular membranes, but they also interact with other proteins. It is likely that their membrane association also involves protein-protein interactions. They can bind to components of lipid rafts for stable membrane binding (Simons and Toomre, 2000). Inactive H-Ras is partially localized to lipid rafts and when activated it relocates laterally to the non-raft regions of the plasma membrane for signal transduction (Prior et al., 2001). GTPases are required to cycle between membranes and cytosol, which calls for a reversible association. This is achieved by means of binding to specific proteins called guanine nucleotide dissociation inhibitors (GDIs) and guanine nucleotide exchange factors (GEFs) (Orita et al., 1993; Pfeffer et al., 1995; Steele-Mortimer et al., 1993). GEFs, such as SOS, facilitate exchange of GTP for GDP from the active proteins such as K-Ras situated at the plasma membrane, allowing their dissociation from the membrane. Similarly, binding of prenylated Rho to RhoGDI protects the isoprenoid group from the environment in the cytosol as well as facilitates dissociation from the membranes (DerMardirossian and Bokoch, 2005). GDIs are
capable of binding prenylated but not unprenylated Rab and Rho proteins, thus maintaining them in the cytosol in a GTP-dependent manner (Olofsson, 1999; Pfeffer et al., 1995). GTP binding causes dissociation of GDI to allow membrane translocation, and after GTP hydrolysis to GDP, GDI can bring the protein back into the cytosol (DerMardirossian and Bokoch, 2005).

Prenylation also contributes to formation of multisubunit complexes, e.g. the \( \beta\gamma \) complex of trimeric G proteins. Unprenylated \( \gamma \) subunit is more efficient in dimerizing with \( \beta \) than is the prenylated \( \gamma \) species. However, prenylation of \( \gamma \) subunit is required for the interaction of \( \beta\gamma \) with the \( \alpha \) subunit (Higgins and Casey, 1994). Furthermore, the interaction of these complexes with a receptor is influenced by the type of prenyl group present on the \( \gamma \) subunit (Kisselev et al., 1995).

1.3.4 Protein function

As mentioned above, prenylation is necessary for maintenance of many different phenomena related to a protein, which ultimately determine its functional efficiency. Therefore, regulation and modification of prenylation properties of proteins emerged as a critical way of manipulating their functions in the cell. Since Ras is the most commonly mutated oncoprotein in different types of cancer, and particularly because oncogenic forms of Ras require farnesylation to be able to transform cells, it was thought that targeting farnesylation would be of potential use to control Ras function and achieve anti-tumor action (Konstantinopoulos et al., 2007). Designing compounds targeted to inhibit prenylation enzymes tested this hypothesis. Several classes of such inhibitors have been designed and tested pre-clinically as well as clinically (Sebti and Hamilton,
Some of these have shown promising results and have been further developed for use in vitro and in vivo for various therapeutic applications (Gelb et al., 2006). More recently, other inhibitors of the posttranslational processing of prenylated proteins have also been tested singly or in combination with each other or with traditional anticancer therapies in order to achieve improved inhibition of prenylation (Lobell et al., 2001; Wojtkowiak et al., 2009). Among these inhibitors are statins, bisphophonates and ICMT inhibitors (Figure 1.5).

1.4 PRENYLATION INHIBITORS

1.4.1 Statins
Statins are the most widely prescribed drugs for hypercholesterolemia associated with coronary artery disease. They inhibit the rate-limiting enzyme HMG-CoA reductase of the mevalonate pathway, which catalyzes the initial step in the formation of mevalonate for biosynthesis of cholesterol. Statins not only inhibit the synthesis of cholesterol, but also interfere with the formation of the intermediate isoprenoids, FPP and GGPP, that are required for prenylation. Due to this action, statins have been used for anticancer effects in a number of different types of cancer (Sassano and Platanias, 2008). Apart from the above-mentioned effects, they are also useful as immunomodulators. They have been shown to bind to the integrin, leukocyte function antigen 1 (LFA1) and reduce invasion and migration of pro-inflammatory leukocytes, although in low micromolar concentrations (Weitz-Schmidt et al., 2001). Extrapolating this observation, they have been explored for use in several autoimmune disease models, e.g.

Lovastatin was the first to be identified among statins and it is a naturally occurring compound. The main mechanism of action of lovastatin and its other family members including simvastatin is believed to be inhibition of RhoA and Rac1 geranylgeranylation (Nakagami et al., 2003; Zhong et al., 2003). Lovastatin was also recently found to inhibit Ras farnesylation and induce apoptosis in K-Ras transformed thyroid cells (Laezza et al., 2008). Many statins have been shown to exert their effect on cell cycle progression by altering p21 and/or p27Kip1 levels, leading to G1 cell cycle arrest in a p53-dependent or -independent manner (Naderi et al., 1999; Saito et al., 2008). Statins exert pleiotropic effects on cancers, some of which may be undesirable (Gonyeau and Yuen). They can aid antitumor therapy via inhibition of cancer cell growth and/or angiogenesis or via induction of apoptosis.

Statins are well-tolerated in patients, although cancer monotherapy with statins in clinical trials exhibited dose-limiting toxicities including hepatotoxicity, nephrotoxicity, gastrointestinal toxicity, myelotoxicity and rhabdomyolysis (Konstantinopoulos et al., 2007). Cerivastatin was withdrawn from the market in 2001 due to high incidence of rhabdomyolytic toxicity in patients. Several statins (eg. fluvastatin, simvastatin) are being evaluated currently as chemopreventive agents in clinical trials for cancers including breast, colorectal, non-small cell lung cancer (NSCLC) and so on (Sleijfer et al., 2005). Lovastatin trials are being set up for testing its safety and efficacy in neurofibromatosis type 1 (NF1) patients. In most ongoing clinical trials, statins are being
used as adjuvants with other chemotherapeutics, or for chemoprevention in cases of recurrence after chemotherapy, surgery and radiation therapy (www.clinicaltrials.gov).

1.4.2 Bisphosphonates

Bisphosphonates (BPs) are compounds that inhibit two different enzymes in the mevalonate pathway, isopentenyl diphosphate (IPP) isomerase and FPP synthase. Both these enzymes catalyze steps upstream of FPP and GGPP synthesis (Figure 1.1) and hence carry potential to inhibit all routes of protein prenylation. BPs are divided into two different classes of compounds. It is the nitrogen-containing (aminobisphosphonates) class (N-BPs) that inhibits prenylation, unlike the class of compounds lacking nitrogen, which have a different mode of action (Rogers et al., 2000). In addition, the N-BPs are more potent.

N-BPs (zoledronic acid, pamidronate, ibandronate etc.) are used primarily for the prevention and treatment of osteoporosis and are also indicated for bone-related complications associated with bone metastases arising from several neoplasms including breast cancer (Caraglia et al., 2006). These drugs directly inhibit the activity of osteoclasts, the cells that are responsible for bone resorption, owing to their high affinity for calcium (Papapoulos, 2008). Their uptake by osteoclasts occurs via bone resorption facilitated by the P-C-P backbone of their chemical structure. There is in vitro and in several cases in vivo evidence that the mechanisms of action of N-BPs include G₁ and S-phase cell cycle arrest and induction of apoptosis in osteoclasts, inhibition of tumor cell adhesion and invasion into the extracellular matrix as well as inhibition of angiogenesis (Green, 2003). It has been suggested that the main actions of N-BPs on
osteolysis may be due to blockade of geranylgeranylation of Rho and Rab family proteins (Coxon et al., 2005; Fisher et al., 1999). Most recently, a class of lipophilic BPs has emerged as a group of potential anticancer agents with enhanced activity and improved potency attributed to increased lipophilicity and hence attenuated affinity for the bone (Zhang et al., 2009). Zoledronic acid is the most studied BP and like several other BPs of its class, it was thought to be promising for use in prevention of bone loss in breast cancer and prostate cancer (Doggrell, 2009; Lyseng-Williamson, 2008). It was also thought that these bisphosphonates could be beneficial in treatment of metastatic bone disease associated with breast, prostate, lung cancers as well as multiple myeloma. Initial clinical trials found that zoledronic acid, ibandronate and pamidronate may be useful in improving the outcome of such patients, whereas clodronate did not show significant effects of prolonged survival and also exhibited some toxicity (Bauss and Bergstrom, 2008; Body and Mancini, 2002; Gnant, 2009). Several clinical trials are currently being undertaken to evaluate the performance of BPs particularly as adjuvants with chemotherapy or radiation therapy (Bauss and Bergstrom, 2008) (www.clinicaltrials.gov).

1.4.3 Icmt and Rce1 inhibitors
Isocarboxymethyl transferase (Icmt) and Ras converting enzyme 1 (Rce1) inhibitors prevent post-prenylation modification of proteins via carboxymethylation. Effects of disruption of Icmt and Rce1 genes have been studied in vitro and in vivo. Complete ablation of the Icmt gene in mice is embryonically lethal (Bergo et al., 2004), whereas Rce1 genetic ablation was much more tolerable (Bergo et al., 2002). Inhibition of Icmt
activity resulted in impaired transforming capability of oncogenic Ras (Wahlstrom et al., 2008). In contrast, inhibition of Rce1 activity worsened the myeloproliferative disease caused by oncogenic K-Ras (Wahlstrom et al., 2007). Both peptide and non-peptide Rce1 inhibitors have been developed (Schlitzer et al., 2001; Winter-Vann and Casey, 2005). Icmt inhibitors were first made as S-adenosylhomocysteine (AdoHcy) analogs, which exhibited anti-tumor activity (Wnuk et al., 1997). Subsequently, analogs of the substrate N-acetyl-S-farnesylcysteine were synthesized and evaluated, but were found to be weak inhibitors (Henriksen et al., 2005). Indole-based small molecule inhibitors followed these and also showed anti-tumor activity in human colon cancer cells (Winter-Vann et al., 2005). One such compound, cysmethynil, when used in the MDA-MB-231 breast cancer cells, impairs cell adhesion and cell spreading via inhibition of RhoA and Rac1 activity (Cushman and Casey, 2009). Spermatinamine, a natural Icmt inhibitor, was also discovered and can potentially be a useful inhibitor in the future (Buchanan et al., 2007).

Despite these efforts, this class of inhibitors has not moved into the clinic due to anticipated issues. These include the concern that Icmt and Rce1 modify many more target proteins than FTase or GGTase, thus their inhibition may concur higher toxicity. The toxicity observed that is of highest concern is atherosclerotic vascular injury associated with endothelial cell apoptosis (Kramer et al., 2003).

1.4.4 Prenyl transferase inhibitors (PTIs)

This class consists of farnesyl transferase inhibitors (FTIs) and geranylgeranyl transferase inhibitors (GGTIs). They target the enzymatic activity of FTase or GGTases
to achieve desired effects. Several strategies have been developed over the years to synthesize drugs for this purpose. Our existing knowledge of enzyme kinetics as discussed earlier has served as an invaluable tool in the design of various competitive inhibitors including peptidomimetics (CaaX peptide analogs), small molecule inhibitors, and isoprenoid substrate analogs (FPP or GGPP analogs). Many of these have been studied for their potential benefits in various types of cell and animal models of cancer and further evaluated in human clinical trials as well (Sebti and Hamilton, 2000).

FTIs were initially designed with the intention of targeting Ras farnesylation, since it was known that oncogenic Ras is involved in over 30% of all human cancers. CaaX peptide inhibitors of FTase were the first ones in this category. This approach was initially hindered by the fact that the peptides were not taken up by the cells efficiently and underwent rapid degradation (Zhang et al., 1994). However, CaaX tetrapeptide derivatives that were more effective also evolved (Stradley et al., 1993). Treatment of transgenic H-Ras, but not K-Ras and N-Ras, mouse models with the CaaX mimetic, L-744832, resulted in drastic regression of salivary and mammary carcinomas (Kohl et al., 1995). Further, high throughput screening methods followed by structural optimization techniques allowed identification of small molecule inhibitors of FTase. These compounds, e.g. lonafarnib and tipifarnib, exhibited potent activity in vitro and emerged as promising potential anticancer agents in preclinical studies with negligible toxicity [reviewed by (Basso et al., 2006)]. In several clinical trials, however, when used as monotherapy, FTIs failed to show significant benefits and also caused considerable toxicity, particularly nephrotoxicity (Konstantinopoulos et al., 2007). It was later thought that the cause for this unexpected toxicity may be that there are numerous farnesylated
proteins in the cell that may be affected by FTase inhibition. Other Ras family proteins, such as Rho, Rheb proteins and nuclear lamins surfaced as potential targets of FTIs in their FTase inhibitory action (Basso et al., 2006). Many of these other Ras proteins were also identified to be aberrantly regulated in certain tumors. For instance, Rheb is upregulated in transformed cells and human tumor cells (Jiang and Vogt, 2008; Lu et al.), while two different isoforms of Rho, i.e. RhoA and RhoB, are differentially involved in tumor cell invasion, adhesion and survival (Karlsson et al., 2009). These proteins may, therefore, be important FTI targets and may play key roles in their anti-tumor effect.

The mechanisms of action of the current classes of known FTIs encompass multiple different pathways including interference with cell cycle progression via G1 phase arrest, induction of apoptosis and cell death, and most recently, induction of autophagic pathway (Pan et al., 2008; Qiu et al., 2007; Tamanoi et al., 2001). In addition, researchers observed that upon treatment with FTI, some proteins including K-Ras and N-Ras can undergo alternative geranylgeranylation via GGTase enzymes (Lerner et al., 1997). On the other hand, more extensive screening of possible FTI targets also revealed that the geranylgeranylation of certain Rab proteins can be blocked by FTIs (Lackner et al., 2005). These studies suggested that the inhibition of several prenylated proteins (such as Ras, Rho, Rab etc.) may contribute to the effects exerted by FTIs on cells. It also eluded to the possibility of a major role for geranylgeranylation of proteins in their signaling mechanisms. This thought has led to a rise in the search for effective GGTI compounds.
Similar to FTIs, various classes of GGTIs have been developed and tested pre-clinically, including peptide mimetics, small molecules and GGPP analogs. Since a much higher number of proteins in the cell undergo geranylgeranylation compared to farnesylation, GGTIs were initially expected to be less selective, and hence more toxic to cells. However, a small molecule inhibitor, GGTI-298, was shown to inhibit cell cycle progression via G1 phase arrest in a wide variety of cancer cell lines such as A549 lung adenocarcinoma cells (Miquel et al., 1997). This inhibition was accompanied by p53-independent induction of the cyclin-dependent kinase inhibitor, p21 (Vogt et al., 1997). It was also suggested that the geranylgeranylated protein, RhoA, might be the target of inhibition by GGTI-298 (Adnane et al., 1998). In addition, based on the literature, current GGTIs show promise as effective antitumor agents in leukemic, pancreatic and breast cancer cell lines, as well as in breast tumor xenograft models, with reasonable toxicity (Kazi et al., 2009; Watanabe et al., 2008). Despite great efforts with GGTI compounds, their dose-limiting toxicities seem to decelerate their advancement through later phases of drug development. In an attempt to reduce toxicities associated with FTIs and GGTIs, Lobell et al used combinations of both these drugs in cancer cell lines and in tumor xenografts. They showed that the dual prenylation inhibitors (DPIs) or a combination of an FTI and a GGTI was capable of inhibiting K-Ras prenylation, however, continuous infusion of GGTI alone or in combination with FTI was lethal to mice (Lobell et al., 2001). These data suggest that the FTI-GGTI combination treatment regimen should not be pursued. Nevertheless, combination of different inhibitors with conventional chemotherapeutics or radiation therapy is considered to be the most promising regimen (Sebti and Hamilton, 2000). The Sebti group has attempted to substantiate this idea.
They have shown that GGTI-2154 when combined with cisplatin, gemcitabine or taxol is more efficacious than monotherapy (Sun et al., 1999). Even so, additional extensive testing is required to discover better combination regimens that will prove to be successful in future clinical trials.

<table>
<thead>
<tr>
<th></th>
<th>Existing/potential disease implications</th>
<th>Number of clinical trials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Statins</td>
<td>Hyperlipidemia, myeloma, lymphoma, hepatocellular carcinoma, NSCLC, etc.</td>
<td>979</td>
</tr>
<tr>
<td>Bisphosphonates</td>
<td>Osteoporosis, prostate and breast cancers, myeloma, etc.</td>
<td>244</td>
</tr>
<tr>
<td>Icmt and Rce1 inhibitors</td>
<td>Prostate cancer, colon cancer</td>
<td>None</td>
</tr>
<tr>
<td>FTIs</td>
<td>Progeria, parasitic diseases, HNSCC, breast cancer, neurofibromatosis, etc.</td>
<td>51</td>
</tr>
<tr>
<td>GGTIs</td>
<td>Melanoma, breast cancer</td>
<td>None</td>
</tr>
</tbody>
</table>

Table 1.3. Potential therapeutic implications of post-translational modification inhibitors.

1.5 PRENYLATION INHIBITION AND HUMAN DISEASE

1.5.1 FTIs as parasitic disease therapeutics

FTIs have been shown to be useful in the treatment of parasitic diseases. *Trypanosoma brucei* that causes African sleeping sickness and the malaria-causing parasite *Plasmodium falciparum* are both susceptible to FTI treatment (Eastman et al., 2006).
Growth of these protozoa is severely impaired under the influence of FTI as antiparasitic agents, (Wiesner et al., 2004; Yokoyama et al., 1998). One of the reasons for this activity of FTIs is thought to be abolishment of host enzyme activity, since these protozoa lack GGTase I but express FTase. Several existing FTI compounds have been tested for their antiprotozoal activity and the tetrahydroquinoline containing FTIs synthesized by Bristol-Myers Squibb demonstrated the most potent activity (Eastman et al., 2006). This application has broadened the range of usefulness of FTIs and may further assist in the development of improved FTI drug moieties.

1.5.2 Prenylation in progeria

Hutchinson-Gilford progeria syndrome (HGPS) is a rare premature aging disease characterized by growth retardation, hair loss, accelerated aging and early mortality that occurs due to atherosclerotic complications in most cases. Highly misshapen, blebbled fibroblastic nuclei are pathological hallmarks of this condition (Goldman et al., 2004). HGPS is commonly caused by a de novo mutation in a nuclear protein called lamin A (LMNA), resulting in an aberrantly spliced 50 amino acid-deleted form of LMNA (or progerin). This deletion encompasses a zinc metalloproteolytic site called Zmpste24 that facilitates CaaX processing. Thus, progerin cannot undergo endoproteolytic cleavage and remains constitutively farnesylated (Glynn and Glover, 2005). In recent years, FTIs have been shown to block and possibly reverse the nuclear morphology defects in fibroblasts derived from HGPS patients and improved the progeroid phenotype in HGPS mouse models (Fong et al., 2006; Gelb et al., 2006). Clinical trials
are being set up to develop the FTI, lonafarnib, as a progeria drug (www.clinicaltrials.gov).

1.5.3 Prenylation inhibitors and cancer

As discussed earlier, FTIs and GGTIs have been very promising in numerous cancer cell models. They may serve as better anticancer drugs, however, in combination with other therapies (Konstantinopoulos et al., 2007). Our group and others have shown that prenyl transferase inhibitors and statins or bisphosphonates act synergistically to exert cytostatic and/or cytotoxic effects on different types of tumor cells (Andela et al., 2002; Morgan et al., 2005; Wojtkowiak et al., 2008).

The primary goal of our laboratory is to target GTPases of the Ras superfamily of proteins, many of which are known to be aberrantly regulated in several types of human tumors. We achieve this by pharmacological intervention in the form of agents that inhibit prenylation of these proteins. Thus, we have been actively evaluating novel prenylation inhibitors, synthesized as isoprenoid analogs, for their potential antitumor activity. We combine these compounds with low doses of the HMG-CoA reductase inhibitor, lovastatin, in an attempt to potentiate their activity while minimizing side-effects (Wojtkowiak et al, 2009). The rationale underlying our approach is that the statin would inhibit early steps in the isoprenoid synthesis pathway and reduce the endogenous FPP/GGPP pools, such that the FPP/GGPP analog-based inhibitor could better compete out the remaining pyrophosphate (Wojtkowiak et al., 2009). Malignant peripheral nerve sheath tumors (MPNSTs) have served as a very useful model to conduct these studies. We study three distinct MPNST cell lines. Of these, NF90-8 and
ST88-14 were derived from neurofibromatosis type 1 (NF1) patients who carry a mutation in the neurofibromin (Nf) gene. This mutation leads to constitutive Ras activation in the cells and results in schwannomas (now called MPNSTs). Therefore, blocking Ras prenylation may inhibit Ras function, which may further prohibit Ras-induced proliferation of these malignant cells. We have previously shown that a novel FTase inhibitor, FTI-1, when combined with lovastatin induces apoptosis in the NF1 MPNST cell lines, NF90-8 and ST88-14 (Wojtkowiak et al., 2008). More recently, some groups have shown that other post-translational modification inhibitors induce autophagy, which may result in non-apoptotic cell death (type II cell death) (Araki and Motojima, 2008; Pan et al., 2008; Wang et al., 2008b).

We have now evaluated a novel GGPP analog-based GGTase inhibitor, GGTI-2Z, in the STS-26T MPNST cell line. STS-26T cells were derived from a patient with a sporadic case of MPNST, who had no known mutations in the Nf gene. All three MPNST cell lines mentioned above express detectable levels of predominantly N-Ras and some K-Ras, while they lack H-Ras expression (Mattingly et al., 2006).

1.6 SPECIFIC AIMS

Aim 1: Characterize a novel GGPP analog-based geranylgeranyl transferase inhibitor, GGTI-2Z, and study the effect of its combination with potent doses of lovastatin on STS-26T MPNST cell line.

Our collaborator’s group had recently synthesized a novel class of GGPP analogs based on the structure of GGPP. These consisted of analogs with different substitutions
at positions 3- and 7- with either allyl or vinyl groups (Maynor et al., 2008). Of all these substitutions, 7-allyl yielded the most potent inhibitor of GGTase I enzyme \textit{in vitro} with a \(K_m\) value of \(\sim 27\)nM without any significant binding to FTase. My working hypothesis for this aim is that GGTI-2Z, the novel compound derived from 7-allyl GGPP, in combination with low doses of lovastatin may potentially be effective as an antitumor agent in a culture model of STS-26T MPNST cells. Its mechanism of action may be similar to or distinct from that of the related inhibitor, FTI-1, as tested previously in our laboratory.

\textbf{Aim 2: Delineate the mechanism of induction of an aborted autophagic program by FTI-1/lovastatin combination in STS-26T MPNST cell line.}  

As mentioned above, FTI-1/lovastatin therapy induced apoptosis in NF1 MPNST cell lines, NF90-8 and ST88-14. When Wojtkowiak J.W. in the laboratory tested this combination in STS-26T cells, he observed a mechanistically distinct effect on these cells. He observed that there was no induction of apoptosis in these cells, however, there was a robust induction of the autophagic pathway. Moreover, this autophagy did not undergo completion and resulted in a non-apoptotic cell death (type II cell death) along with procathepsin trafficking defects. My working hypothesis for this aim is that besides induction of autophagy, the compounds may exert a distinct effect on the cellular proteolytic machinery that serves as the underlying mechanism of this aborted autophagic program and non-conventional cell death.
Figure 1.1. The mevalonate pathway of cholesterol biosynthesis. Acetyl-CoA is first converted to mevalonate via the rate-limiting enzyme, HMG-CoA reductase. Isopentyl-PP (IPP) is obtained, which is further modified to farnesyl pyrophosphate (FPP). IPP isomerase and FPP synthase enzymes catalyze intermediate steps in this pathway. FPP can be utilized by farnesyl transferase (FTase) to modify proteins or can be converted to geranylgeranyl pyrophosphate (GGPP). GGPP prenylates substrate proteins via geranylgeranyl transferase (GGTase). Finally, FPP may also alternatively be converted to cholesterol, which provides a positive feedback to regulate the rate-limiting step of the pathway.
Figure 1.2. General schematic of prenylation pathways. G-proteins of the Ras family containing C-terminal CaaX moieties (where X=methionine, M or serine, S) are farnesylated via FTase and undergo additional modifications by other enzymes and translocate to the plasma membrane. Some proteins, such as K-Ras and N-Ras (where X=leucine, L) or other proteins containing CC or CXC at the C-terminus are geranylgeranylated via GGTase I or GGTase II followed by additional modifications and membrane translocation.
**Figure 1.3.** Prenylation reactions of GTPases. A. H-Ras, N-Ras and K-Ras are prenylated (H-Ras is only farnesylated, whereas N-Ras and K-Ras can be farnesylated or geranylgeranylated) followed by proteolytic removal of the AAX tripeptide by RAS converting enzyme 1 (RCE1) and carboxymethylation by isoprenylcysteine carboxymethyltransferase (ICMT) in the endoplasmic reticulum. Subsequently, they undergo palmitoylation in the Golgi and translocate to the plasma membrane to which they attach through their farnesyl (F) or geranylgeranyl (GG), and palmitoyl moieties (P).
Figure 1.3 contd. B. Rho GTPases are prenylated followed by proteolytic removal of the AAX tripeptide by RCE1 and carboxymethylation by ICMT. Subsequently, they bind to RhoGDIs, which deliver them to various membrane locations where they function. C. Most Rab GTPases contain C-terminal C-X-C or C-C residues (where C=cysteine and X=another amino acid). They are doubly geranylgeranylated as shown by GGTase II. Unprenylated Rab GTPases are presented by REP1 (RAB escort protein 1) to GGTase II. Subsequently only Rabs ending in C-X-C undergo carboxymethylation by ICMT. They attach to various membranes through their two GG moieties.
Figure 1.4. Three-dimensional structures of prenyltransferase enzymes. Overall structures of A. FTase, and B. GGTase-I, with the α subunit shown in red, the β subunit in blue and yellow, respectively, and the catalytic zinc ion in magenta. C. Superposition of FTase (blue) and GGTase-I (yellow) demonstrates the structural homology of the β subunit of these enzymes.
Figure 1.4 contd. D. Overall structure of GGTase II with the $\alpha$ subunit shown in pink, the $\beta$ subunit in blue, and the catalytic zinc ion in brown.
Figure 1.5. Classes of post-translational modification inhibitors. The mevalonate pathway has been targeted by several different classes of inhibitors. Statins inhibit HMG-CoA reductase and, therefore, are capable of inhibiting both the cholesterol synthesis and protein prenylation arms of the pathway. Bisphosphonates (BPH) inhibit IPP and FPP synthase. FTase inhibitors (FTIs) block FTase, while GGTase inhibitors (GGTIs) inhibit GGTase I and/or II. There are other post-translational modification inhibitors including Icmt inhibitors and RCE1 inhibitors.
CHAPTER 2
MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Synthetic Chemistry

The synthetic chemistry to produce novel compounds used in these studies was performed in the Gibbs laboratory at Purdue University. Prodrug GGTI-2Z is a derivative of 7-allyl GGPP (referred to as compound 2 in Figure 2.1), which was the most potent of all the GGPP analogs tested in previous in vitro studies. Synthesis of 7-allyl GGPP was described previously (Maynor et al., 2008). 2-Z-geranylgeraniol (2-Z-GGOH) was synthesized from 7-allyl GGPP via a multi-step chemical reaction. It was then converted by phosphoramidation to prodrug GGTI-2Z. The schematic synthesis is described in Figure 2.1.

2.1.2 Reagents

GGTI-2Z and FTI-1 (previously described in (Clark et al., 2007)) aliquots were prepared in dimethyl sulfoxide (DMSO) and stored at -80°C. HA14-1 (Ryan Scientific Inc., Isle of Palms, SC) and lovastatin (Sigma-Aldrich, St. Louis, MO) aliquots were prepared and stored similarly. Bafilomycin A1 (Calbiochem, Gibbstown, NJ), 3-methyladenine and wortmannin (Sigma-Aldrich, St. Louis, MO) aliquots were stored at -20°C. A plasmid (pRK7.GFP.H-Ras.CaaX) encoding green fluorescent protein (GFP) fused to the C-terminal 10 amino acids of rat H-Ras sequence, which encompasses its CaaX sequence, was constructed by subcloning into the pRK7.GFP plasmid (Yang and
Mattingly, 2006), a forward primer with the sequence 5’GATCCGGCTGCATGAGCTGCAAATGTGTGCTGTCCTG3’ and a reverse primer with the sequence 5’AATTCAAGGACAGCACACACTTTGCAGCTCATGCAGCCG3’ using the sticky-end ligation method.

2.2 METHODS

2.2.1 Cell Culture

STS-26T cells and normal, spontaneously immortalized rat Schwann cells (iSC) were obtained and maintained as described previously (Wojtkowiak et al., 2008). HEK293 cells were cultured and transfected as previously described (Norum et al., 2005). The murine hepatoma 1c1c7 cell line was obtained from Dr. J. Whitlock, Jr. (Stanford University, Palo Alto, CA) and cultured in minimal essential medium containing 5% fetal bovine serum with 100 units/ml penicillin and 100 mg/ml streptomycin. Derivatives of 1c1c7 cells that stably expressed GFP-LC3 were generated by transfection of an expression plasmid obtained from N. Mizushima (Tokyo Medical and Dental University, Tokyo, Japan). The MCF7 and MCF10.DCIS cell lines were obtained from the Cell Lines Resource (Karmanos Center Institute, Detroit, MI). MCF10.DCIS cells were maintained as a monolayer in DMEM/F12 containing 5% horse serum, 20 ng/ml EGF, 0.5 µg/ml hydrocortisone, 10 µg/ml insulin, 50 U/ml penicillin, and 50 µg/ml streptomycin at 37°C and 5% CO₂. MCF7 cells were maintained in DMEM F12 (1:1).

2.2.2 Western Blot Analysis
Lysates were prepared from monolayers of cells in 2x Laemmli sample buffer by boiling for 5 min and cleared by centrifugation (Mattingly et al., 2001). Samples were then separated on SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose. Membranes were then probed with 1:200 dilutions of anti-RhoA and anti-pan Ras, 1:500 dilution of anti-Rab5 and 1:600 dilution of anti-unprenylated Rap1 antibodies, and caspase-3 antibody at 1:1000 dilution (Santa Cruz Biotechnology). LC3-I and LC3-II were detected using 1:2000 dilution of anti-LC3 antibody (gift from Dr. David Kessel, Wayne State University). Cathepsin-B antibody (gift from Dr. Bonnie Sloane, Wayne State University) was used at 1:3000 dilution.

2.2.3 Live Cell Imaging Assays

HEK293 cells were plated into 35-mm culture plates 24 h prior to transfection with pRK7.GFP.H-Ras.CaaX using lipofectamine 2000 reagent (Invitrogen) as previously described (Norum et al., 2005). Four hours after transfection, fresh media were added along with vehicle or drug at appropriate concentrations as stated in the figure legend. At the end of treatment, nuclei were stained using a live cell nuclear stain, Hoechst 33342, followed by confocal live-cell imaging on the LSM-510 at 40x magnification. A similar protocol was used to study nuclear morphology of STS-26T cells with or without drug treatment. For cellular morphology assays, STS-26T cells and iSC were treated as indicated in the figure and differential interference contrast (DIC) images were captured on the LSM-510 at 40x magnification.

2.2.4 Immunofluorescence Assays
STS-26T cells were plated onto glass coverslips and treated as indicated in the figure legends. The cells were fixed and processed for confocal immunofluorescence analysis using anti-Lamp-1 or anti-LAMP-2 mouse monoclonal (BD Biosciences), and anti-LC3 (Abgent, San Diego, CA) rabbit polyclonal antibodies at 1:50 dilution followed by appropriate fluorescently-coupled secondary antibodies. The number of LC3-positive puncta was quantified using Volocity software 5.2.1 (Perkin Elmer, Waltham, MA).

2.2.5 Cell Proliferation Assay

STS-26T cells, ST88-14 cells and iSC were plated at ~20,000 cells per 35-mm dish 24 h before drug treatment. At appropriate time points, attached cells were trypsinized and combined with media containing detached cells. The cells were collected by centrifugation for 5 min at 50g and counted via a hemacytometer.

2.2.6 MTT Assay

Cells were passaged into 96-well plates at a density of 2500 cells per well containing 200 µl of growth media with inhibitors or vehicle and cultured for 72 h at 37°C. 20 µl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Invitrogen, Eugene, Oregon) stock solution (5 mg/ml in PBS) was then added and the plates were incubated for 4 h. The medium was removed and the formazan precipitate formed was dissolved in 150 µl of DMSO. Absorbance values were measured using a plate reader (SpectraFluor Plus, Tecan, Salzburg, Australia) at 485 nm wavelength. After normalizing the absorbance values for media and vehicle controls, the data were analyzed using GraphPad Prism version 4.0c (GraphPad Software Inc., San Diego, CA)
by non-linear regression (curve fit) and plotting sigmoidal dose-response to obtain GI<sub>50</sub> values, which were further plotted on an isobologram for synergy analysis (Zhao et al., 2004).

### 2.2.7 Flow Cytometric Analysis

STS-26T cells were treated and collected for DNA analysis as described previously (Mattingly et al., 2006). DNA content was analyzed using a FACScalibur instrument (BD Biosciences, San Jose, CA). A minimum of 10<sup>4</sup> cells per sample was analyzed to determine the percentage of apoptotic cells and cells in G<sub>1</sub>, S and G<sub>2</sub>/M phases (Modfit; Variety Software, Topsham, ME).

### 2.2.8 DEVDase Activity Assay

Lysates of STS-26T cells were prepared and used for DEVDase assays as described previously (Wojtkowiak et al., 2008). Changes in fluorescence over time were converted to picomoles of product by comparison with a standard curve made with 7-amino-4-methylcoumarin. DEVDase specific activities are reported as nanomoles of product made per minute per milligram of protein. The bicinchoninic acid assay, using bovine serum albumin as a standard, was used to estimate protein concentrations.

### 2.2.9 Colony Formation Assay

STS-26T cultures were plated at a density of 2 x 10<sup>4</sup> per 35-mm culture plate ~24 h prior to drug treatment. They were then treated with either DMSO, 1mM rapamycin or with the combination drugs with or without rapamycin for 48 h. At the end of the
treatment, cells were collected by trypsinization and centrifugation and $3 \times 10^3$ treated cells were sub-cultured in triplicate in 60-mm plates containing fresh media without drugs. Colonies containing 4 or more cells after 48 h and 72 h of growth were counted in 10 randomly selected fields per plate. Data were plotted as number of colonies against drug treatment.

2.2.10 Lysosomal pH determination using acridine orange (AO)

STS-26T cells were treated with DMSO, 500 nM lovastatin or 500 nM FTI-1 either alone or in combination. At the end of the treatment, media were replaced with media containing 200 nM AO followed by incubation at 37°C for 15 min with the addition of Hoechst 33342 during the final 5 min. Cells were quickly rinsed with 1X PBS three times and immediately imaged using a Carl Zeiss Axiovert 200M fluorescent microscope at 40x magnification. The filter cubes used for capture of red and green fluorescence employed exciter/emitter/beam splitter wavelengths of 365/420/395 nm and 500/535/515 nm respectively.
Figure 2.1: Synthesis of prodrug GGTI-2Z (compound 7), and structures of 2Z-GGMP (compound 8) and 2Z-GGPP (compound 9). GGTI-2Z is based on the 7-allyl analog of GGPP (compound 2; Maynor et al, 2008). 2-Z-geranylgeraniol (2-Z-GGOH) was synthesized from compound 2. It was then converted by phosphoramidation to prodrug GGTI-2Z. All synthetic chemistry was performed by the Gibbs laboratory at Purdue University.
Figure 2.2. Chemical structure of lovastatin. Lovastatin is a naturally occurring compound of the statins class of inhibitors of HMG-CoA reductase enzyme.
CHAPTER 3

A NOVEL GERANYLGERANYL TRANSFERASE INHIBITOR IN COMBINATION WITH LOVASTATIN INHIBITS PROLIFERATION AND INDUCES AUTOPHAGY IN STS-26T MPNST CELLS

3.1 RATIONALE

In the past our group has tested prenylation inhibitors including FTIs and statins as potential therapies for Type I neurofibromatosis (NF1) (Wojtkowiak et al., 2008) and other hyperproliferative disorders (Mattingly et al., 2002). For example, a novel FTI compound, FTI-1, in combination with lovastatin induces apoptosis in two different NF1 MPNST cell lines (Wojtkowiak et al., 2008). Moreover, we observed little to no detectable toxicity of the treatment in normal iSC, indicating the potential use of this combination treatment for NF1 MPNSTs. The FTase substrate(s) that are affected by FTI treatment to produce inhibition of cell proliferation and survival are still unclear. However, an interesting study involving a chemical genetics approach revealed RabGGTase or GGTase II as a target of FTIs (Lackner et al., 2005). This finding supported the idea that FTIs have many different targets that may be responsible for their activity and side effects, and also identified a potential role for Rab proteins and RabGGTase in p53-independent apoptosis induced by FTIs. It also emphasized the potential of GGTIs as an alternative to FTIs. More recently, GGTase I inhibition was shown to reduce tumor formation and improve survival in mice with K-Ras induced lung cancer (Sjogren et al., 2007).
One of our aims in developing prenylation inhibitors is to identify their target substrates and their mechanisms of action in the subject cells. Recently, autophagy was suggested to be one of the mechanisms by which FTIs may exert their effects on tumor cells (Pan et al., 2008). Autophagy is generally thought of as a pro-survival mechanism for cells (Glick et al.). The molecular machinery involved in the autophagic process was initially identified and characterized in yeast. Most of the autophagy genes are conserved in mammals and are regulated similarly (Rubinsztein et al., 2007). There are three known types of autophagy: microautophagy, macroautophagy and chaperone-mediated autophagy (Scarlatti et al., 2009). Microautophagy involves direct invagination of cytoplasm in the form of vesicles at the lysosomal membrane. Chaperone-mediated autophagy involves chaperone-assisted translocation of cytosolic proteins containing a particular peptide motif across the lysosomal membrane. Macroautophagy, which is likely to be most relevant to this study, is a mechanism that involves formation of double-membrane vesicles, called autophagosomes that encase cytosol as well as whole organelles and ultimately fuse with lysosomes for degradation and recycling of contents (Figure 3.1A). A basal rate of macroautophagy (hereafter referred to as autophagy) occurs in most cells in order to eliminate damaged organelles or aggregated proteins (Scarlatti et al., 2009) and, as suggested most recently, is a mechanism for lipid metabolism (Singh et al., 2009). In conditions of nutrient starvation or other stress, the process is upregulated and thus serves as a survival mechanism. Owing to its cytoprotective nature, autophagy has been implicated as a mechanism to combat proteinopathies, including Huntington’s disease and Alzheimer’s disease (Rubinsztein et al., 2007). More recently, however, it was suggested that autophagy might in fact result
in cell death. This is termed as autophagic cell death and its underlying mechanism is yet unknown (Kroemer et al., 2009). Both the cytoprotective and cytotoxic effects of autophagy are now being exploited for potential antitumor activity (Turcotte and Giaccia).

3.2 RESULTS

3.2.1 Inhibition of geranylgeranylation of GTPases by GGTI-2Z and lovastatin combination

In collaboration with the Gibbs laboratory, previous students from our laboratory have recently demonstrated that the monophosphate derivatives of certain FPP analogs are potent FTIs, and that prodrugs derived from these analogs block protein farnesylation (Clark et al., 2007; Wojtkowiak et al., 2008). The Gibbs laboratory has also synthesized and evaluated novel GGPP analogs, and found several analogs that are in vitro inhibitors of GGTase I (Gibbs et al., 1999; Maynor et al., 2008; Zahn et al., 2001). In particular, the 2Z GGPP analog, compound 9, from which the prodrug GGTI-2Z was synthesized, is an excellent inhibitor of geranylgeranylation of dansyl-GCVLL peptide by GGTase I (Zahn et al., 2001), and that the corresponding monophosphate compound 8 is the most potent GGPP based GGTI yet reported (IC$_{50}$ = 21 nM).

In this dissertation, I sought to undertake the first cellular evaluation of GGTI-2Z to confirm whether the compound also inhibits geranylgeranylation in vivo. We first tested whether this novel GGTI could inhibit geranylgeranylation of Rap1A via GGTase I. STS-26T cells were treated with 3 µM GGTI-2Z either alone or in combination with 500 nM
lovastatin. DMSO treatment was used as a vehicle control. We performed western blotting with an antibody that only recognizes the unprenylated form of Rap1A. GGTI-2Z alone was unable to inhibit Rap1A geranylgeranylation even after 48 hours of treatment. A distinct band representing unprenylated Rap1A appeared within 24 h in whole cell lysates treated with lovastatin alone and this unprenylated Rap1A was strikingly increased upon treatment with a combination of GGTI-2Z and lovastatin (Figure 3.2A; upper panel). The amount of unprenylated Rap1A in comparison to the total Rap1 levels (Fig. 3.2A; lower panel) increased over time with the combination treatment. This result indicates that GGTI-2Z, when combined with lovastatin, inhibits Rap1A geranylgeranylation.

Another geranylgeranylated protein that has been inhibited in the past by laboratories using other GGTI compounds is RhoA. Sebti and colleagues have shown that GGTI treatment of pancreatic cancer cells results in an increase in RhoA expression levels (Delarue et al., 2007). In our study with GGTI-2Z and lovastatin combination, we saw a similar marked increase in the expression level of RhoA within 24 h compared to vehicle control, and this increase was maintained even at 48 h of treatment (Figure 3.2B, lanes 4 and 8).

We also tested for inhibition of RabGGTase or GGTase II by looking for reduced prenylation of Rab5. Strikingly, we observed a clear up-shift due to appearance of unprenylated Rab5 upon combination treatment (Figure 3.2C, lane 4) as opposed to vehicle or single compound treatments. Additionally, we observed a dose-dependent increase in inhibition of Rab5 prenylation. As little as 1 µM GGTI-2Z synergized with 500 nM lovastatin to effectively inhibit Rab5 prenylation (Figure 3.2D; lane 8). When used
singly, at least 1 μM lovastatin was required to observe an inhibition to a similar extent (Figure 3.2D; lane 4). Conversely, as much as 10 μM GGTI-2Z alone failed to have any effect on Rab5 prenylation (Figure 3.2D; lane 7).

In addition to the above proteins, GGTI-2Z and lovastatin were also found to inhibit prenylation of Ras and Rab6. There was a distinct band of unprenylated Ras with combination treatment, which could include all three isoforms of Ras: H-Ras, N-Ras and K-Ras, although our previous analysis of these cells suggests that H-Ras is not expressed (Mattingly et al., 2006). When probed for Rab6, there appeared to be a band shift, which may imply that Rab6 prenylation was reduced (Figure 3.2E).

### 3.2.2 Combination of GGTI-2Z and Lovastatin does not inhibit FTase

Prenylation of Ras proteins helps target them to the plasma membrane where their site of action lies. These membrane proteins can be fluorescently tagged to visualize their cellular localization patterns in the presence or absence of prenylation inhibitors (Maurer-Stroh et al., 2007). We transfected HEK293 cells with a construct that encodes GFP fused to the CaaX motif of H-Ras (an exclusively farnesylated protein) and then treated the cells with our compounds alone or in combination. The nuclei were then stained followed by live-cell imaging via confocal microscopy for localization of GFP. As seen in Figure 3.3, we observed that in the case of vehicle-treated cells, GFP.H-Ras.CaaX localizes to the plasma membrane along with some intracellular expression that may represent the Golgi (Choy et al., 1999). Treatment with a low dose combination of lovastatin and a FTI that we have previously shown to block farnesylation (Wojtkowiak et al., 2008), inhibits the membrane localization and induces
a diffuse cytosolic distribution of GFP.H-Ras.CaaX. In contrast, as high as 6 µM GGTI-2Z plus 500 nM lovastatin combination failed to prevent membrane localization of the GFP.H-Ras.CaaX protein. These data indicate that GGTase inhibition by GGTI-2Z and lovastatin does not inhibit prenylation of the exclusively farnesylated GFP.H-Ras.CaaX protein (Figure 3.3).

3.2.3 GGTI-2Z in combination with lovastatin inhibits proliferation of STS-26T cells without significant loss of cell viability

Next we sought to test the effect of inhibition of geranylgeranylation by the two compounds on growth and proliferation of STS-26T cells. We treated the cells with the compounds alone or in combination and found that 1 µM concentration of GGTI-2Z or lovastatin alone had little effect on proliferation (Figure 3.4A). However, 45 h of exposure of the cells to a combination of 3 µM GGTI-2Z and 1 µM lovastatin caused a significant inhibition of proliferation of the cells, and this inhibition was similar to the extent of 10 mM GGTI-2Z treatment alone (Figure 3.4B). In addition, we characterized dose-dependent as well as time-dependent inhibition of proliferation of these cells by the combination treatment (Figure 3.4A, 3.4B). In addition to proliferation, we examined the percent viability of the cells. At all the time points tested, there was little effect on cell viability with single or combination treatments (Figure 3.4D). The inhibition of proliferation and lack of significant effect on cell viability was also confirmed via a live cell morphology assay (Figure 3.4C). We further tested whether there was synergy between the two compounds when used in combination, via an MTT assay. After 72 h of treatment, the data analysis showed that these compounds were indeed synergistic
in their growth inhibitory effect (Figure 3.4E). The synergistic inhibition was indicated by the GI\textsubscript{50} (concentration of drug required for 50% inhibition of cell growth) values for the combination treatment lying below the theoretical line connecting the GI\textsubscript{50} values for GGTI-2Z and lovastatin alone.

3.2.4 GGTI-2Z in combination with lovastatin arrests STS-26T MPNST cells in G\textsubscript{0}/G\textsubscript{1} phase of the cell cycle

We observed significant inhibition of proliferation of STS-26T cells by co-treatment with GGTI-2Z andLovastatin. We therefore next determined which point of the cell cycle these compounds targeted in order to inhibit proliferation. We performed flow cytometry analysis of STS-26T cells treated with GGTI-2Z and lovastatin singly or in combination (Figure 3.5). Our results showed that treatment with 3 µM GGTI-2Z or 1 µM lovastatin alone did not affect cell cycle progression. Interestingly, the same concentrations of the drugs, when used in combination, induced an accumulation of cells in the G\textsubscript{0}/G\textsubscript{1} cell cycle phase and a simultaneous reduction in percentage of cells in G\textsubscript{2}/M and S phases. These data are consistent with a G1 cell cycle arrest.

3.2.5 GGTI-2Z alone or in combination with Lovastatin does not induce apoptosis in STS-26T cells

Although analyses of cell viability by trypan blue exclusion assay suggested no cytotoxicity by combined GGTI-2Z and Lovastatin treatment, we wanted to re-examine if the treatment induced any apoptosis in the cells. For this purpose, we used N-acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin to assay the activity of caspases-3 and -7
(Wojtkowiak et al., 2008). HA14-1, a known inducer of apoptosis through inhibition of Bcl-2, was used as a positive control for this assay. When treated with HA14-1 for 2 h, STS-26T cells showed significant induction of apoptosis as demonstrated by a strong increase in DEVDase activation compared to untreated control cells (Figure 3.6A). There was even stronger induction of caspase-like activity after 4 h of treatment with HA14-1. In contrast, treatment with the prenylation inhibitors alone or in combination did not yield any detectable DEVDase activation (Figure 3.6A). As an additional test for caspase activation in these cells, we probed lysates of cells treated with the drugs alone or in combination, for expression of cleaved caspase-3. As shown in Figure 3.6B, procaspase-3, which is the uncleaved form of caspase-3, was evident in all the cell lysates at 35 kDa. However, in contrast to HA14-1, the prenylation inhibitors did not induce any cleaved caspase-3, indicating lack of detectable apoptosis in these cells by this measure. Finally, we also performed nuclear morphology assays using Hoechst 33342 dye to monitor chromatin condensation as an indicator of apoptosis. Within 45 min of HA14-1 treatment, we observed nuclear condensation in the form of bright blue spots as seen in Figure 3.6C. Conversely, with GGTI and/or lovastatin treatment, neither did we see any nuclear morphological changes (Figure 3.6C), nor did we see any appearance of DNA laddering (data not shown), which further confirms lack of apoptosis in the cells.

3.2.6 GGTI-2Z and lovastatin combination treatment induces autophagy in STS-26T cells
We further investigated whether autophagy was involved in determining the response of the cells following prenylation inhibition. This was achieved by assaying LC3, the classical marker of autophagy (Klionsky et al., 2008). During the formation of autophagosomes, LC3-I is processed to LC3-II via phosphatidylyethanolamine (PE) attachment. Presence of LC3-II is, therefore, associated with occurrence of autophagy. We observed a subtle increase with single treatments and a marked increase with combination treatment in the appearance of LC3-II in STS-26T cells within 24 h when compared with vehicle treatment (Figure 3.7A). This increase was sustained at 48 h. This result suggests that the drugs may be inducing or up-regulating the autophagic process in these cells.

Increase in LC3-II levels may be associated with either of two possible mechanisms: an increase in formation of autophagosomes or a decrease in processing/degradation of LC3-II due to an absence of autophagosome/lysosome fusion or depression of lysosomal protease activities (Figure 3.1B). In order to distinguish between effects on synthesis and on degradation, we pretreated the cells with protease inhibitors, E64D and pepstatin A. These compounds inhibit lysosomal proteases, which would prevent degradation of LC3-II in the autophagolysosome. The results showed that the protease inhibitors did induce a further increase in LC3-II levels (Figure 3.7B), which is consistent with there being autophagosome and lysosome fusion, and subsequent proteolytic processing in the autophagolysosome. We also used bafilomycin A1, which blocks the maturation of autophagosomes by its inhibition of the vacuolar ATPase. In this case, we treated the cells with the vehicle, single compounds, or combination treatment for 48 h, with bafilomycin A1 also present for the final 2 h of the incubation. The results show
that the single treatments do not have much effect on autophagic flux compared to vehicle control, whereas the combination treatment again increases LC3-II accumulation either in the presence or absence of bafilomycin A1 (Figure 3.7C). Furthermore, immunocytochemical staining of LC3 along with the lysosomal marker, LAMP-2, was also performed following treatments with the compounds as indicated (Figure 3.7D). Vehicle or single compound treatments did not result in co-localization of the two proteins. In contrast, a very distinct punctate co-localization of LC3 and LAMP-2 was observed in cells following the combination treatment. Quantitative analysis of LC3-positive puncta, using two independent LC3 antibodies, revealed an approximately five-fold increase in the number of punctate structures upon combination treatment as compared to DMSO treatment (Figure 3.7E). These results indicate that the autophagic process is both induced and proceeds to completion in STS-26T cells co-treated with GGTI-2Z and lovastatin.

3.2.7 Inhibitors of autophagy do not affect GGTI-2Z/lovastatin induced autophagy in STS-26T cells

To determine whether GGTI-2Z/lovastatin induce autophagy via the canonical pathway involving PI3K and beclin (Figure 3.8C), we used PI3K inhibitors, 3-MA and wortmannin, to test their ability to block the induction of autophagy. Upon pre-treatment with these inhibitors, there was no blockade of autophagy. In contrast, LC3-II processing was enhanced, suggesting an upregulation of autophagy (Figure 3.8A and 3.8B).
3.2.8 Withdrawal of treatment of STS-26T cells with GGTI-2Z/lovastatin restores their colony forming ability

Since there was no loss of cell viability upon combination treatment, we investigated whether the colony forming ability of the cells was restored upon withdrawal of the drugs and replating in fresh media. Rapamycin, a known autophagy inducer, was used as a positive control for this assay. We observed that similar to DMSO and rapamycin treatment, cells treated with GGTI-2Z/lovastatin, upon withdrawal of the inhibitors and replating in fresh media, were able to rescue growth and form colonies (Figure 3.9).

3.2.9 GGTI-2Z and lovastatin combination is also effective in other unrelated cancer cell lines

In order to test the effects of the drugs in cell lines that model other cancers, we performed cell proliferation and viability assays in a murine hepatoma cell line, 1c1c7. We found that GGTI-2Z and lovastatin combination also inhibits proliferation of these cells without significantly affecting their viability (Figure 3.10A and 3.10B). This cell line stably expresses a GFP fusion construct of LC3. Upon combination treatment, there was increased appearance of LC3-positive vesicles, indicating induction of autophagy (Figure 3.10C).

We also treated cultures of MCF10.DCIS cells with the drugs and assayed for LC3-II accumulation by western blotting. The results showed increased LC3 processing following dual treatment, confirming autophagic induction in these cells as well (Figure 3.10D). The above results confirmed autophagy induction by two independent techniques in two separate cancer cell lines.
3.2.10 GGTI-2Z/lovastatin do not induce autophagy in other MPNST cell lines

It was an interesting question whether GGTI-2Z/lovastatin exert similar effect of autophagy induction on other related MPNST cell lines. To test this, we checked proliferation of ST88-14 cells under the influence of the inhibitors and found that similar to STS-26T cells, their proliferation is also inhibited significantly by combination treatment (Figure 3.11A). Furthermore, we tested the effect on LC3 processing to assess autophagy. In contrast to STS-26T cells, however, ST88-14 as well as NF90-8 cell lines did not exhibit LC3 processing characteristic of autophagic induction upon combination treatment (Figure 3.11B).

3.2.11 Normal immortalized rat Schwann cells (iSC) are resistant to GGTI-2Z and lovastatin co-treatment

An effective anti-tumor agent is expected to be highly selective for tumor cells without affecting normal cells, such that it is minimally toxic. We, therefore, tested whether GGTI-2Z and lovastatin in combination affected proliferation of normal iSC as a measure of therapeutic safety. As shown in Figure 3.12A, the compounds either alone or in combination had little to no effect on the proliferation of iSC. In addition, we found no detectable morphological changes in these cells upon treatment with the compounds (Figure 3.12B).

3.3 DISCUSSION
In this study, we have developed a novel GGTI whose action is potentiated by co-treatment with lovastatin, resulting in inhibition of proliferation and cell cycle arrest associated with induction of autophagy in STS-26T MPNST cell line. The strategy for development of the GGPP-based inhibitor compound, GGTI-2Z, is analogous to that previously described for the development of FTIs (Maynor et al., 2008). Of all the GGPP analogs evaluated in prior studies, 2Z geranylgeranyl monophosphate compound served as the most efficacious inhibitor with an impressive IC\textsubscript{50} value of approximately 21 nM for GGTase I enzyme \textit{in vitro} (Maynor et al., 2008). Encouragingly, this analog also exhibited no significant binding to mammalian FTase \textit{in vitro}, further confirming its promise as a tool to evaluate cellular GGTase I inhibition. Additionally, in the first cellular evaluation of this compound in this study, as seen by immunocytochemistry, GGTI-2Z did not affect the membrane localization of a GFP construct that is exclusively farnesylated, suggesting that it acts to solely inhibit geranylgeranylation while farnesylation remains unaffected. Furthermore, the fact that it did not have any effect on either morphology or proliferation of normal iSC implies lower risk of toxicity to normal cells.

Our rationale for the combinatorial approach was to achieve more efficient GGTase I inhibition via GGTI-2Z by simultaneously depleting endogenous GGPP pools. Rap1A is a Ras family GTPase that is known to be solely geranylgeranylated presumably by GGTase I (Casey et al., 1991). We observed inhibition of Rap1A prenylation, thus confirming the previous \textit{in vitro} result that GGTI-2Z effectively inhibits GGTase I, although it does so only when combined with lovastatin. Conversely, lovastatin alone is sufficient to modestly inhibit Rap1A geranylgeranylation. These data suggest that
Rap1A may not be a critical target for the inhibition of STS-26T cell proliferation, as even higher levels of lovastatin (up to 1 µM) do not affect cell cycle distribution. We also observed an inhibition of Ras prenylation, however, the cells express detectable levels of both N- and K-Ras, warranting further investigation of the isoform(s) involved.

The combination of GGTI-2Z/lovastatin synergistically inhibits proliferation of STS-26T cells. This anti-proliferative activity is consistent with induction of cell cycle arrest in the G₁ phase. Other GGTIs have also been shown to block cell cycle progression of several tumor cell lines and subsequently induce apoptosis (Vogt et al., 1997). However, with GGTI-2Z/lovastatin, there was little effect on cell viability and we did not observe the classical apoptotic morphology or apoptotic markers in STS-26T cells.

Another potential determinant of cell survival is the phenomenon of autophagy. In recent years, autophagy has been discovered to be an important mechanism adopted by many different cell lines for determining their fate, and it is still a topic of debate whether autophagy is a cell survival or a cell death mechanism (Apel et al., 2009). Interestingly, there is increasing evidence suggesting that several cancer cells show up-regulation of the process leading to cell survival and cancer progression (Rubinsztein et al., 2007).

Recently, three FTIs were found to induce autophagy in two different human cancer cell lines (Pan et al., 2008). In addition, some statins can induce autophagy in a cell-type specific manner owing to their ability to inhibit protein prenylation rather than cholesterol synthesis (Araki and Motojima, 2008). For instance, cerivastatin or simvastatin are capable of inducing autophagy in rhabdomyosarcoma cells (Araki and Motojima, 2008), whereas lovastatin or simvastatin fail to do so in hepatocytes (Samari and Seglen, 1998). We examined our GGTI/lovastatin combination treated cultures to see if
autophagy occurred. We also performed autophagic flux experiments using pre-treatment with protease inhibitors, E64D and pepstatin A, or with bafilomycin A1 treatment. Based on the analysis of LC3-I conversion to LC3-II via western blot, and the co-localization of LC3 with the lysosomal protein LAMP-2 via immunocytochemistry, we confirmed that autophagy was induced and driven to completion in STS-26T cultures co-treated with GGTI-2Z and lovastatin. The lack of inhibition of autophagy by the common PI3K inhibitor, 3-MA, suggests that the autophagic response seen in STS-26T cells may be restricted to the non-canonical pathway of autophagy. There is not much known about this alternative route of autophagy despite the thought that it may share some of its key players with the canonical pathway (Scarlatti et al., 2008b). Albeit, analysis of the key initiator of canonical autophagy, beclin1, which is not involved in the non-canonical pathway, may assist in dissecting this hypothesis.

One of our observations shows that the compounds cause dose- and time-dependent inhibition of prenylation of Rab5, a GGTase II or RabGGTase substrate. Moreover, another Rab protein, Rab6, undergoes a prenylation shift. These data indicate that the compounds not only inhibit GGTase I, but also serve as substrates for GGTase II. This was not surprising since the two enzymes share strikingly similar active sites (Lackner et al., 2005) and hence, a compound designed to bind the GGPP binding pocket of either of the two can be expected to bind similarly to the other. The preference of binding in that case will be determined by relative affinity of the compound for the enzymes. We have not yet tested the in vitro ability of GGTI-2Z to bind to GGTase II to determine the $K_M$ value. An alternative explanation for the dual inhibition of GGTase I and RabGGTase-mediated prenylation is that GGTI-2Z inhibits GGPP synthase,
blocking the production of the GGPP substrate needed for both processes (Wiemer et al., 2007). In addition, there is a noticeable decrease in the expression level of Rab5 upon GGTI-2Z/lovastatin treatment. Potentially, this RabGGTase inhibition and/or enhanced Rab5 turnover may contribute to the effects of the compounds on MPNSTs.

Rab proteins have been shown to play an important role in carcinogenesis (Cheng et al., 2004). Originally, Rab5 was known to have a well-established role in endocytosis and vesicular transport of proteins (Bucci et al., 1992). More recently, however, an interesting study in cell culture and fly models of Huntington’s disease suggested a role for Rab5 in the early stages of the process of macroautophagy that is independent of its endocytic function (Ravikumar et al., 2008). This study showed that Rab5 inhibition via expression of dominant-negative Rab5 results in a decrease in LC3-positive autophagic vacuoles, and also enhances polyglutamine toxicity. In the case of our study we saw that inhibition of Rab5 prenylation via GGTase inhibition is correlated with an increase in LC3-II levels. Prenylation inhibitors may be capable of only partially blocking Rab5 activity, and thus, the partially prenylated and active Rab5 may still be sufficient for autophagic progression. Additional evidence that may support such a connection is that fluvastatin and pravastatin-induced RabGGTase inhibition causes vacuolation in rat skeletal myofibers (Sakamoto et al., 2007). Alternatively, the GGTI may target one or more other protein(s) that may potentially contribute to its action. Further studies would be required to better elucidate the role of Rab5 and other proteins in GGTI/lovastatin induced autophagy in MPNSTs.

In conclusion, we have developed a novel compound GGTI-2Z that blocks prenylation mediated by both GGTase I and GGTase II, and exerts cytostatic activities in STS-26T
MPNST cells in a caspase-3-independent manner. The action of GGTI-2Z is potentiated by low-dose statin combination treatment and strongly correlates with induction of autophagy. This combination treatment does not block proliferation of, or induce toxicity in normal, immortalized Schwann cells, but does have inhibitory activity against two other transformed cell lines: 1c1c7 murine hepatoma cells and MCF10.DCIS cells that model human breast ductal carcinoma in situ. Further studies toward preclinical development of GGTI-2Z may serve to develop better understanding of geranylgeranylation inhibitors and to evaluate their potential in the context of cancer therapy as well as some Rab-associated protein-trafficking disorders.
Figure 3.1: A. The process of autophagy. Initiation of autophagy begins with the formation of small sections of membranes called phagophores/phagosomes. These phagophores undergo elongation to formed double membrane vesicles called autophagophores/autophagosomes. During this process, a protein named as LC3 undergoes cleavage and modification via phosphatidylethanolamine (PE) attachment to form LC3-II, which is localized on the inner membrane of the autophagosomes. The autophagosomes eventually fuse with lysosomes to form autophagolysosomes, where the contents including LC3-II are degraded and recycled. Autophagy genes, such as Atg7 and Atg3 play distinct roles at several steps throughout this entire process.
Figure 3.1: B. Autophagic flux. Induction of autophagy coincides with LC3-II processing. However, increase in LC3-II may occur due to two different reasons. One is via induction of autophagy. Two is when the last step involving fusion of autophagosomes and lysosomes is blocked, leading to reduced degradation and increased accumulation of LC3-II in autophagosomes.
Figure 3.2: Inhibition of prenylation via GGTase I in STS-26T cells by GGTI-2Z/lovastatin combination treatment. STS-26T cultures were treated as indicated for 24 h or 48 h. Whole-cell lysates were probed for prenylation status of Ras superfamily GTPases via Western analysis. A. Detection of Rap1A via an antibody directed toward the unprenylated form of Rap1A (middle panel), and detection of total Rap1 (lower panel). B. Detection of RhoA, which has been reported to be over-expressed following block of GGTase (Falsetti et al., 2007).
Figure 3.2 contd: Inhibition of prenylation via GGTase II in STS-26T cells by GGTI-2Z/lovastatin combination treatment. STS-26T cultures were treated as indicated. Whole-cell lysates were probed for prenylation status of Rab5 via Western analysis. 

C. Detection of Rab5. Unprenylated GTPases migrate more slowly on SDS-PAGE gels. β-tubulin was used as a loading control in all western blots. 

D. Dose-response of Rab5 prenylation status. Data shown are representative of at least three independent experiments.
Figure 3.2 contd: Inhibition of geranylgeranylation of additional proteins by GGTI-2Z/lovastatin. E. Whole cell lysates of treated STS-26T cells were probed for pan-Ras and Rab6.
Figure 3.3: Lack of effect of GGTI-2Z/lovastatin treatment on membrane localization of a farnesylated GFP construct. HEK293 cells were transiently transfected with pRK7.GFP.H-Ras.CaaX plasmid, followed by treatment with prenylation inhibitors as shown for 16 h. FTI-1/lovastatin treatment inhibited H-Ras.CaaX localization at the plasma membrane, whereas GGTI-2Z/lovastatin treatment did not affect the localization even at 6 µM GGTI-2Z concentration. Results are representative of three independent experiments.
Figure 3.4: Effect on proliferation and viability of STS-26T cells following GGTI-2Z/lovastatin treatment. A and B. STS-26T cells were subjected to treatment as shown. Samples were collected at the time of treatment and every 24 h post-treatment for analysis of cell number. Data represent means ± S.D. of three independent experiments. C. Cells were treated as indicated above. After 72 h of treatment, live cell differential interference contrast (DIC) images were taken on a LSM-510 at 40x magnification. Data are representative of three independent experiments.
Figure 3.4 contd.: D. Number of non-viable cells was analyzed with respect to total number of cells to calculate percent viability at the given time points. E. Cells were treated with the compounds at several different concentrations as shown, alone or in combination and the data were tested for synergy using isobologram analysis. Data represent means ± S.D. of two independent experiments.
Figure 3.5: FACS analysis: GGTI-2Z/lovastatin treatment arrests STS-26T cells in G₀/G₁ and increases sub-G₁ DNA content. STS-26T cells were treated as shown. Cultures were harvested 48 h post-treatment for DNA content by staining with propidium iodide. Histograms represent 10⁴ events, and the cell cycle profile was determined using MODFIT. Results are representative of two independent experiments.
Figure 3.6: Lack of apoptosis in STS-26T cells following GGTL-2Z/lovastatin treatment. HA14-1 treated cells were used as a positive control. A. STS-26T cells were treated as indicated in the figure. Data represent means of triplicate samples and are representative of two independent experiments. B. STS-26T cells were treated as indicated in the figure. Attached as well as detached cells were pooled, and whole-cell lysates were separated and probed for cleaved caspase-3.
Figure 3.6 contd: C. STS-26T cells were treated for 48 h with the indicated concentrations of lovastatin and/or GGTI-2Z or for 30 min with HA14-1. Nuclei were stained using Hoechst 33342 and live-cell imaging was performed on an LSM510 confocal microscope at 40x magnification. Data are representative of results from two independent experiments.
Figure 3.7: Induction of autophagy in STS-26T cells by GGTI-2Z/lovastatin treatment. 

A. STS-26T cells were treated with indicated concentrations of GGTI-2Z and lovastatin alone or in combination. B. Cells were subjected to 2 h pretreatment with protease inhibitors, 10 µM pepstatin A and 10 µM E64D, followed by the prenylation inhibitors for 48 h as indicated. C. Cells were subjected to 48 h drug treatment, with addition of 50 nM bafilomycin A1 for the last 2 h of the incubation. At the end of all treatments, whole cell lysates were then probed for LC3 and β-tubulin. Results are representative of three independent experiments.
Figure 3.7 contd: D. STS-26T cells were treated as indicated for 48 h followed by methanol fixation. Cells were then stained for LC3 and LAMP-2. Nuclei were stained using DAPI and cells were visualized under the LSM-510 at 40x magnification. E. Quantitative analysis of LC3-positive puncta treated with either DMSO or the drug combination for 48 h. Data are representative of at least two independent experiments.
**Figure 3.8:** Lack of inhibition of GGTI-2Z/lovastatin induced LC3 processing by known autophagy inhibitor, 3-methyladenine (3-MA) (Seglen and Gordon, 1982). STS-26T cells were pretreated with 3-MA for 2 h followed by addition of the prenylation inhibitors as shown for 48 h. Whole cell lysates were then probed for LC3 and β-tubulin. Data are representative of at least two independent experiments.
**Figure 3.9:** Effect on re-plating ability of STS-26T cells upon treatment with GGTI-2Z/lovastatin followed by removal of the drugs. STS-26T cells were treated as depicted on the X-axis for 48 h. At the end of treatment $3 \times 10^3$ treated cells were re-plated in fresh growth medium without inhibitors. Colonies containing 4 or more cells from 10 randomly selected fields were counted at 48 and 72 h. Data represent mean ± S.D. of three independent experiments each counted in triplicates.
Figure 3.10: Effects of GGTI-2Z/lovastatin on other human cancer cell lines. A, B. Cultures of murine hepatoma 1c1c7 cells were treated one day after plating with varied concentrations of either lovastatin and/or GGTI-2Z. Cultures were harvested 48 h after treatment for assessment of total cell number (A) and viability (B), as assessed by ability to exclude trypan blue. Data represent means ± SD of analyses of three culture dishes per treatment. Dashed line in panel A represents cell number at time of treatment.
Figure 3.10 contd: C. Cultures of murine hepatoma 1c1c7 cells that stably expressed GFP-LC3 were treated as indicated above for 48 h prior to being analyzed for the formation of punctate GFP-LC3 spots (i.e. autophagosomes) by fluorescence microscopy. Co-treatment with lovastatin and GGTI-2Z resulted in marked accumulation of autophagosomes. D. Cultures of MCF10.DCIS (ductal carcinoma in situ) were treated as indicated and lysates were subjected to immunoblotting for LC3.
Figure 3.11. Effect of GGTI-2Z/lovastatin on other MPNST cell lines. A. ST88-14 cells were treated as indicated. Samples were collected at time points shown and total cell number was analyzed using a trypan blue exclusion assay. All treatments were unable to affect proliferation of iSC. Data represent mean ± S.D. of three independent experiments.
Figure 3.11 contd: B. Effect of GGTI-2Z/lovastatin on LC3 processing in NF1 MPNST cell lines, ST88-14 and NF90-8. Cells were treated as indicated with the prenylation inhibitors for 48 h with or without a 2 h pre-treatment with protease inhibitors, 10µM E64D and 10µM pepstatin A, and whole cell lysates were probed for LC3 and β-tubulin. Data represent results from at least two independent experiments.
Figure 3.12: Lack of cytotoxicity from GGTI-2Z/lovastatin in normal immortalized rat Schwann cells. A. iSC were treated as indicated. Samples were collected at times shown, and total cell number was analyzed. All treatments were unable to affect proliferation of iSC. Data represent mean ± S.D. of three independent experiments. B. iSC were treated as shown in the figure and observed every 24h post-treatment. The figure represents the 72 h time point and shows a lack of observable toxicity.
CHAPTER 4

INDUCTION OF AN ABORTED AUTOPHAGIC PROGRAM BY COTREATMENT WITH A NOVEL FARNESYL TRANSFERASE INHIBITOR AND LOVASTATIN LEADS TO NON-APOPTOTIC DEATH IN STS-26T MPNST CELLS

4.1 RATIONALE

In parallel to testing GGTI-2Z, our group has also characterized a related prodrug FTase inhibitor called FTI-1 (Figure 4.1) in combination with lovastatin for its antitumor effects on MPNST cell lines. Our laboratory has previously published that FTI-1 and lovastatin co-treatment induces apoptosis in two different NF1 MPNST cell lines, NF90-8 and ST88-14 (Wojtkowiak et al., 2008). Interestingly, when tested in the non-NF1 MPNST cells, STS-26T, the compounds failed to induce apoptosis. We found that there was significant inhibition of proliferation that ultimately resulted in cell death; however, the typical apoptosis markers were absent. There was no induction of caspase-3/7 activity or caspase-3 cleavage products.

Some initial work by a previous student in the laboratory included testing this inhibitor combination in the STS-26T cell line and investigating whether the cells may be undergoing autophagic cell death (type II cell death). There was indeed a massive upregulation of LC3 processing, more so than that with GGTI-2Z/lovastatin co-treatment. These results suggested that FTI-1/lovastatin also caused induction of autophagy. Further autophagic flux measurements using pre-treatment with protease inhibitors, however, indicated that this autophagic process does not undergo
completion. This aborted autophagy may be a consequence of a blockade of autophagosome-lysosome fusion or impaired lysosomal proteolysis. I sought to determine which of these possibilities was more likely to occur and the underlying mechanism by which the inhibitors may affect lysosomal function.

4.2 RESULTS

4.2.1 FTI-1 plus lovastatin treatment is cytotoxic to and induces aborted autophagy in STS-26T cells

We have previously found that sub-micromolar concentrations of FTI-1 and lovastatin in combination disrupt Ras, Rab5 and Rheb prenylation in STS-26T cells (Wojtkowiak, J.W.; Thesis, 2009). Measurement of viable cell numbers via a trypan blue exclusion assay revealed that this treatment also inhibits proliferation significantly compared to vehicle or single treatments (Figure 4.2A). This inhibition occurred with a significant, although delayed, loss of cell viability (Figure 4.2B). There was no indication of occurrence of apoptosis (not shown), whereas the compounds were found to induce autophagy. As mentioned above, autophagic flux experiments with protease inhibitors suggested an aborted autophagic phenomenon (not shown). Subsequent flux measurement with bafilomycin A1 co-treatment was performed. All the inhibitor treatments induced an increase in LC3-II levels when compared with DMSO control. Starvation of the cells in leucine-free media and GGTI-2Z/lovastatin, which are known to induce autophagy in this cell line were used as positive measures of autophagic flux. Bafilomycin A1 was able to induce further increase in LC3-II levels in case of both these
treatments. In contrast, there was no further increase in LC3-II expression upon FTI-1/lovastatin and bafilomycin A1 treatment. (Figure 4.3). This result confirmed aborted autophagy in the cells.

The autophagic pathway is regulated by a protein called mTOR (mammalian target of rapamycin (Sarbassov et al., 2005). mTOR is a negative regulator of induction of autophagy. Rapamycin is an agent that inhibits mTOR and thus is known to induce the autophagic pathway. Treatment of STS-26T cells with this agent depicted a dose- and time-dependent inhibition of proliferation similar to that with FTI-1/lovastatin treatment (not shown). This inhibition was accompanied by autophagic induction as expected, however, autophagic flux indicated completion of autophagy in contrast to FTI-1/lovastatin treatment (not shown). Colony formation assays were performed to measure the re-plating ability of cells treated with FTI-1/lovastatin versus rapamycin (Figure 4.4). The re-plating ability of cells was inhibited by FTI-1/lovastatin treatment whereas it remained unperturbed by rapamycin treatment. Furthermore, rapamycin was unable to protect the cells from FTI-1/lovastatin induced cytotoxicity. These results showed that rapamycin had a cytostatic effect whereas FTI-1/lovastatin exhibited a cytotoxic effect on STS-26T cells despite the fact that both treatments induced autophagy in the cells.

4.2.2 Vesicle fusion events and lysosomal proteins are perturbed by FTI-1/lovastatin co-treatment

The aborted autophagy may be a consequence of the effects of the inhibitors on autophagosome-lysosome fusion. To investigate this possibility, we performed co-
immunofluorescence assays by probing for LC3 and LAMP-1 (late endosomal marker) or LAMP-2. As seen in the image overlays, single cell magnified images and co-localization profiles, cells treated with DMSO, single drugs or leucine-free media showed substantial co-localization of LC3 with both LAMP-1 and LAMP-2 (Figures 4.5, 4.6). On the other hand, the extent of co-localization was reduced in cells co-treated with FTI-1 and lovastatin. These results suggest that the vesicle fusion events may be inhibited, leading to LC3-II accumulation.

Surprisingly, as suggested by decreased intensity of fluorescence detection of LAMP-2 (Figure 4.6) western blotting indicated markedly reduced level of LAMP-2 in the co-treated cells. While there was no effect on LAMP-1 expression upon treatment, the combination treatment resulted in dramatic attenuation of LAMP-2 coupled with the appearance of a higher mobility form of the protein, which may depict a LAMP-2 degradation product (Figure 4.7). LAMP-2 depletion has previously been reported in a rat model of acute pancreatitis as a consequence of lack of autophagosome-lysosome fusion (Fortunato and Kroemer, 2009) as well as in transformed cells with high cysteine cathepsin levels and activity (Fehrenbacher et al., 2008).

Loss of LAMP-2 expression as well as lack of fusion could be a consequence of lysosomal disruption or loss of lysosomal acidity. Lysosomal pH was inspected using acridine orange (AO) dye, which fluoresces orange-red when present in the acidic environment of intact lysosomes (Moriyama et al., 1982). AO staining indicated that at the early 24 h time point, distinct lysosomal structures appeared as depicted by orange-red staining under all treatment conditions (data not shown). However, at 36 h there was some diffuse staining indicating lysosomal dispersion and a further conspicuous
dispersion at 48 h with combination treatment as opposed to other treatments. Thus, although lysosomes were present and acidic, they were mislocalized (Figure 4.8).

4.2.3 Procathepsin trafficking defects and subsequent impairment of cathepsin activity following prenylation inhibitor treatment

Next, we investigated the latter possibility of proteolysis impairment by the drugs. This mechanism was explored by evaluating the expression and activity of lysosomal cathepsin proteases that are known to contribute to the degradation of autophagolysosome contents, including cathepsins B and D. These cathepsins are cysteine proteases that are post-translationally modified in the ER and Golgi followed by trafficking through the endocytic pathway (Victor and Sloane, 2007). Protein expression analysis and activity assays indicated lack of mature, active proteases and concurrent accumulation of their consecutive inactive pro-forms. In cultures treated with DMSO, 500 nM lovastatin or 500 nM FTI-1, three forms of cathepsin B (pro-form; mature single chain form; and the heavy chain of mature, double-chain cathepsin B), and the pro-form and the heavy chain of mature, double-chain cathepsin D appeared. Co-treatment with FTI-1/lovastatin led to dramatic accumulations of procathepsins B and D, along with loss of the mature cathepsins (Figure 4.9). One possibility for loss of mature cathepsins was their secretion into the extracellular media. Nevertheless, western blot analysis did not show any detectable mature cathepsin B in concentrated extracellular media (not shown), refuting this idea. Moreover, when assayed for cathepsin B activity via a substrate-based activity assay as well as by using an activity-based probe, there was
substantially less activity in combination treated cells compared to vehicle control (data not shown). These observations confirm that FTI-1/lovastatin co-treatment of STS-26T cells led to impaired procathepsin trafficking.

4.2.4 MCF7 cells exhibit aborted autophagic program and lysosomal dysfunction upon co-treatment

One important question in this study was whether this aborted autophagic phenomenon occurs in any other tumor cell line, particularly considering that the other MPNST cell lines tested failed to undergo apoptosis upon treatment with the inhibitors. MCF7 breast cancer cells were treated with the compounds and lysates were analyzed for LC3 expression. We observed that similar to STS-26T cells, MCF7 cells showed an induction of autophagy as depicted by an increase in LC3-II expression levels (Figure 4.10A). Additionally, there was no further increase in LC3-II level upon bafilomycin A1 co-treatment, confirming that these cells also undergo aborted autophagy upon FTI-1/lovastatin treatment. This result was further substantiated by appearance of potential LAMP-2 degradation products via western blot analyses (Figure 4.10B).

4.3 DISCUSSION

FTIs have previously been shown to act on tumors via several different mechanisms including cell cycle inhibition, proteasome inhibition, apoptosis and so on (Efuet and Keyomarsi, 2006; Tamanoi et al., 2001; Wojtkowiak et al., 2008). A recent advancement in this field was when Pan et al showed that FTIs are capable of inducing autophagy in
tumor cells (Pan et al., 2008). Statins were also found to induce autophagy in some tumor cell types (Araki and Motojima, 2008). In fact, before these studies were published, statins were indirectly implied to induce autophagy-like phenotype (then termed as vacuolation) in rat skeletal myofibers followed by apoptotic cell death (Sakamoto et al., 2007). However, the mechanism of autophagic induction and its consequences by these inhibitors remained a mystery. In the MPNST cell line tested in this study, STS-26T, we observe a similar phenomenon of autophagy induction, but there was a distinct difference in the drug-induced phenotype in the form of aborted autophagy and non-apoptotic cell death. This type of cell death (also termed autophagic cell death) has also been observed in some other studies, particularly in apoptosis-resistant cell lines. For instance, rapamycin was reported to induce autophagic cell death in malignant glioma cell lines, U87-MG and T98G (Takeuchi et al., 2005). There has been a missing link and an unexplained association of autophagy and autophagic cell death in this and other such reports. In our study, the investigation of the underlying mechanism of this phenomenon in STS-26T cells has led us to reveal important mechanistic aspects of ‘autophagic cell death’ observed in our case and perhaps many other cases. It also raises an important issue as to whether autophagic cell death occurs as a consequence of aborted autophagy versus the previous assumption of excessive autophagy.

The autophagic pathway often involves Rheb/mTOR signaling cascades. The mTOR complex 1 (mTORC1) activity when suppressed, induces autophagy (Meijer and Codogno, 2009). In addition, farnesylation of Rheb, a small GTPase that stimulates mTORC1, has been shown to be necessary for autophagy signaling (Buerger et al.,
2006; Castro et al., 2003). These studies support our idea that Rheb may serve as a critical target of the inhibitors. On the other hand rapamycin, which inhibits mTOR, did induce autophagy but was not cytotoxic. Based on this result, the involvement of mTOR signaling in aborting autophagy and interfering with endocytic trafficking may be discounted, but it may still be valid to consider it as a player in the induction of the autophagic process.

The lack of colocalization of LC3 with LAMP-1 and LAMP-2 clearly depicted a fusion defect in co-treated cells. Switching cells to leucine-free media, which is known to induce autophagy and drive autophagosome-lysosome fusion, showed distinct punctate vesicular structures positive for both proteins being detected, presumably autophagolysosomes. Apparently similar structures were diminished in cotreated cultures. Further confirmation of this defect was the dramatic loss of LAMP-2 expression in these cultures. Although LAMP-1 and LAMP-2 are both heavily glycosylated transmembrane proteins associated with lysosomes, the deficiency of LAMP-2 in mice exhibits a more severe phenotype than LAMP-1 deficiency (Tanaka et al., 2000). Therefore, despite sharing common functions with LAMP-1, LAMP-2 has been suggested to play a more critical role in lysosomal function (Eskelinen et al., 2002). It is thought to act as a selective receptor on lysosomal membranes for import and degradation of cytosolic proteins inside the organelle (Cuervo and Dice, 1996, 1998). Furthermore LAMP-2, but not LAMP-1, is required for proper fusion of autophagosomes and lysosomes (Gonzalez-Polo et al., 2005). An interesting observation is that the phenotype seen in our double treatment exposed cells is very similar to that seen in the cells of patients suffering from a lysosomal storage disorder, called Danon disease.
Danon disease is caused by a primary deficiency of LAMP-2 protein due to mutations or aberrant splicing of the LAMP-2 gene (Di Blasi et al., 2008; Nishino, 2006). The most striking pathological feature associated with this deficiency is the accumulation of autophagosomes in myofibers (Saftig et al., 2008). Based on this knowledge, we believe that LAMP-2 may be a critical player in the effects of FTI-1/lovastatin on STS-26T cells.

An investigation of whether the lysosomal fusion defects arose from disruption of lysosomes or from other lysosomal defects such as lack of acidity clarified that lysosomal occurrence and their pH remained unaffected even at later time points after treatment when loss of cell viability had already occurred. This result showed that intact lysosomes were still present after co-treatment and their pH rendered acidic. These studies were very important since they eluded to an alternate mechanism of lysosomal dysfunction in the cells.

Since there was no obvious physical defect in the lysosomes upon treatment, we questioned the integrity of the lysosomal enzymes. Lysosomes are composed of several cathepsin proteinases that digest lysosomal contents. Most cathepsins are synthesized in the ER and then trafficked inside vesicles to the lysosomes via the trans-Golgi network via mannose-6-phosphate (M6P) receptors (Victor and Sloane, 2007). Cathepsins B and D are lysosomal cysteine proteases that are commonly found in the lysosomes. Cathepsin D deficiency as well as double deficiency of cathepsins B and L have been associated with a lysosomal storage disorder called lipofuscinosis in murine CNS neuronal and Drosophila models (Koike et al., 2000; Koike et al., 2005; Kuronen et al., 2009). Additionally, the neuronal model also exhibited autophagosome accumulation
in the cells. In our study, the lack of procathepsin processing and cathepsin activity in FTI-1/lovastatin treated cells confirmed impairment of trafficking of active cathepsins to lysosomes. This result explains why lysosomes, although intact, do not function and cause the autophagosome fusion defect. The loss of LAMP-2 may also be partly due to its trafficking impairment, albeit the LAMP-2 trafficking mechanism is thought to be separate from the cathepsin trafficking mechanism (Hasilik et al., 2009; Karlsson and Carlsson, 1998). One interesting theory behind the impaired lysosomal enzyme trafficking that could be worth investigating is the loss of function or number of M6P receptors.

To summarize, our findings indicate that although STS-26T cells are capable of undergoing apoptosis like with HA14-1 treatment, prenylation inhibition by FTI-1/lovastatin fails to do so and rather stimulates the autophagic route. MCF7 cells, which lack caspase-3, are apoptosis-incompetent and thus exhibit similar consequences of co-treatment. Hence we conclude that autophagy is a predominantly active pathway in STS-26T cells and that the cells are highly dependent on autophagy. Drug-induced interference with this pathway coupled with cell cycle arrest leads to cell death.

Overall, we believe that the aborted autophagic program induced by FTI-1/lovastatin in STS-26T cells is a consequence of a procathepsin trafficking defect, which coupled with LAMP-2 deficiency, renders the lysosomes dysfunctional. The resulting accumulation of autophagosomes impairs protein turnover and ultimately causes cell death.
Figure 4.1: Chemical structure of FTI-1. The synthesis of prodrug FTI-1, which was earlier reported as compound 5c, has been previously described in Clark et al, 2007. FTI-1 was synthesized by our collaborator, Dr. Richard Gibbs’ group. Upon entry inside the cell, the prodrug moiety is cleaved while releasing the FPP analog, which can act as a competitive inhibitor of FPP.
Figure 4.2: Effect of FTI-1/lovastatin on proliferation and viability of STS-26T cells. STS-26T cells were plated 24 h prior to being treated with DMSO, lovastatin, FTI-1, or a combination of FTI-1 plus lovastatin. Cultures were harvested at various times after treatment as shown for estimates of cell numbers (A) and viability (B) by Trypan blue exclusion assays.
**Figure 4.3:** Autophagic flux experiment. STS-26T cells were subjected to 4 h in leucine free media, or 48 h of DMSO, or 500 nM GGTI-2Z or 500 nM FTI-1 either alone or in combination with 500 nMLovastatin as indicated, without or with addition of 50 nM bafilomycin A1 for the final 2 h of the culture. Whole-cell lysates were then separated on SDS-PAGE gels and analyzed for LC3 and β-tubulin expression.
Figure 4.4: Colony formation assay. STS-26T cultures were treated as described in the figure for 48 h. At the end of the treatment, 3x10^3 treated cells were replated in fresh growth medium without inhibitors. Colonies containing 4 or more cells from 10 randomly selected fields were counted at 48 and 72 h post-replating. Rapamycin treated cells formed colonies following 48 h of treatment. Data represent mean ± S.D. of 3 independent experiments counted in triplicates.
Figure 4.5: Colocalization of LC3 and LAMP-1 in FTI-1 and lovastatin co-treated cultures. STS-26T cultures were treated with DMSO, 500 nM lovastatin, 500 nM FTI-1 or FTI-1 plus lovastatin for 48 h prior to processing of cultures to analyze colocalization of LC3 with LAMP-1. Nuclei were stained with DAPI. Parallel cultures were shifted to a leucine-free media for 6 h in order to intentionally induce autophagy. Co-localization of LC3 with LAMP-1 is indicated by punctate orange/yellow fluorescence in overlaid images, or congruence of overlaid scans of the red and green channels. Magnified images show the cells that were analyzed to generate colocalization profiles. Colocalization profiles were generated by analysis of a random line drawn through a single cell per field and plotting fluorescence intensity against distance along the line in microns.
Figure 4.6: Colocalization of LC3 and LAMP-2 in FTI-1 and lovastatin co-treated cultures. STS-26T cultures were treated with DMSO, 500 nM lovastatin, 500 nM FTI-1 or FTI-1 plus lovastatin for 48 h prior to processing of cultures to analyze colocalization of LC3 with LAMP-2. Nuclei were stained with DAPI. Parallel cultures were shifted to a leucine-free media for 6 h in order to intentionally induce autophagy. Co-localization of LC3 with LAMP-2 is indicated by punctate orange/yellow fluorescence in overlaid images, or congruence of overlaid scans of the red and green channels. Magnified images show the cells that were analyzed to generate colocalization profiles. Colocalization profiles were generated by analysis of a random line drawn through a single cell per field and plotting fluorescence intensity against distance along the line in microns.
Figure 4.7: Effects of FTI-1 and lovastatin co-treatment on LAMP-1 and -2 expression. STS-26T cultures were treated with lovastatin, FTI-1 or lovastatin plus FTI-1 for either 24 or 48 h prior to being processed for quantification of LAMP-1 and LAMP-2 expression by western blot analysis. Parallel cultures were shifted to a leucine-free media for 6 h in order to intentionally induce autophagy. Analyses are of 25 µg of protein per lane.
Figure 4.8: Effect of FTI-1/lovastatin treatment on lysosomal acidity. STS-26T cells were treated with DMSO, lovastatin or GGTI-2Z either alone or in combination for 36 and 48 h as indicated. At the end of the treatment, media were replaced followed by AO treatment at 200 nM concentration for 2 min. Cells were then quickly rinsed and fresh media added followed by imaging by fluorescence microscopy.
Figure 4.9: Effects of FTI-1 and lovastatin co-treatment on pro-cathepsin B/D processing. A. STS-26T cultures were treated with solvent, lovastatin, FTI-1, or lovastatin + FTI-1 for 16-62 h prior to the preparation of whole cell lysates and analyses of pro-cathepsin B/D processing by western blot analyses. Analyses presented in panels A and B utilized 25 µg protein per lane. Blots are representative of data obtained in a minimum of 3 independent experiments. B. STS-26T cultures were treated as described in panel B, but processed for measurements of cathepsin B activity. C. STS-26T cells were treated with DMSO or FTI-1 + lovastatin for 48 h prior to localizing active cysteine cathepsins with GB117, a quenched activity based probe. Size bar, 10 µm.
Figure 4.10: Effect of FTI-1/lovastatin on MCF7 breast cancer cells. MCF7 cells were treated with DMSO, 500 nM GGTI-2Z or 500 nM FTI-1 either alone or in combination with 500 nM lovastatin as indicated for 48 h without or with 50 nM bafilomycin A1 for the final 2 h of the culture. Whole-cell lysates were then separated on SDS-PAGE gels and analyzed for (A) LC3 and (B) LAMP-2 expression. β-tubulin was used as a loading control.
CHAPTER 5
PRENYLATION INHIBITORS AND AUTOPHAGY

5.1 DISCUSSION

Prenylation inhibitors and their anti-tumor potential have been an interesting topic of investigation for years. Despite continual efforts, however, their use in the clinic as anti-tumor agents has not yet been approved for this purpose. We are still in an urgent need of highly effective agents of this class of therapeutics in order to exploit their benefits and for their translation from the bench to bedside (Wojtkowiak et al., 2008). Addressing the most critical issues that hamper the progress of existing agents would be one method to achieve significant success in this area. Our approach of combining novel analog-based prenyl transferase inhibitors with lovastatin was an effort in this direction. As discussed in Chapter 1, our combinatorial strategy may help scale down the physiological doses and alleviate toxic side effects at the same time. This study has shown that our novel compounds, GGTI-2Z and FTI-1, may be beneficial in MPNSTs as well as many other tumor cell lines broadening their range of application in cancer. Future studies in different animal models of cancer will be required to address their potential as antitumor therapeutics.

One of the major questions that we addressed in this study was to identify the mechanism(s) of action of the drugs in STS-26T MPNSTs. We found that there was a cell cycle arrest in the G1 phase and there was no apoptosis, but an induction of autophagy. Both FTI-1 and GGTI-2Z in combination with lovastatin induced autophagy, but they regulated the pathway in different ways. This observation was very interesting
since there have been a few direct reports of autophagic induction by prenylation inhibitors and none of those studies used GGTIs. Additionally, these earlier reports did not clarify the exact mechanism(s) of autophagy. Specifically, Pan et al showed that the FTI lonafarnib induced autophagy in U2OS osteosarcoma cells and the naturally occurring FTI manumycin A showed a similar effect in Panc-1 pancreatic cancer as well as U2OS cell lines. These cell lines were in fact undergoing both autophagy and apoptosis since lonafarnib-induced autophagy was potentiated by the presence of a pan-caspase inhibitor, z-vad.fmk (Pan et al., 2008). In contrast, STS-26T cells solely undergo autophagy under the influence of our inhibitors and seem to be highly dependent on this pathway. Some statins were also found to induce autophagy in a few of the tested cancer cell lines (Araki and Motojima, 2008). In addition, a small molecule inhibitor of lcnmt induced autophagic cell death in prostate cancer cells (Wang et al., 2008b). However, the mechanism of autophagic cell death in this case is unclear. In our study, we have been able to address many of the questions regarding cell death as a consequence of therapy-induced autophagy.

Based on the results presented, we describe a model (Figure 5.1). In STS-26T cells, GGTI-2Z/lovastatin inhibits prenylation of its target substrate proteins, which are yet unknown despite some clues. This inhibition leads to a complete autophagic induction response in the cells. In the same cell line, FTI-1/lovastatin also inhibits prenylation of its unidentified target proteins, which also induces an autophagic response. However, this autophagy is aborted owing to a secondary effect wherein autophagosome-lysosome fusion is prevented by the treatment, coupled with impairment of procathepsin trafficking to lysosomes and LAMP-2 degradation (Figure 5.1). There still remain a few
unanswered queries in this model. Some of those are listed below and ways to address them have also been discussed.

Autophagy is often initiated by a protein called Beclin 1, which forms a multiprotein complex with hVps34 (human vacuolar protein sorting 34) or phosphatidylinositol-3-kinase (PI3K), to initiate the formation of preautophagosomal structures [reviewed in (Scarlatti et al., 2009)]. This is the classical or canonical autophagy pathway. In contrast, non-canonical autophagy is thought of as a process that does not require the entire set of autophagy-related (Atg) proteins in particular Beclin 1, to form the autophagosome. This was shown by the lack of effect on non-canonical autophagy by the knockdown of Beclin 1 or of its binding partner hVps34 (Scarlatti et al., 2008b). In addition, it is suggested that PI3K inhibitors such as 3-MA do not inhibit non-canonical autophagy (Scarlatti et al., 2008a). The lack of inhibition of FTI-1/lovastatin or GGTI-2Z/lovastatin induced autophagy by 3-MA implies that the co-treated STS-26T cells may be undergoing non-canonical autophagy. This idea needs further scrutiny by determination of effect of inhibitor treatment on beclin-1 expression, since upregulation of beclin-1 is essential for induction of PI3K-induced autophagy (Wang et al., 2008a). On the other hand, both canonical and non-canonical forms of autophagy occur with mTOR inhibition (Scarlatti et al., 2009). To further identify the role of mTOR complex in the observed autophagy, the downstream phosphorylation substrates of mTOR, namely S6kinase and 4EBP1, can also be evaluated. Reduced phosphorylation of these kinases would imply mTOR inhibition and involvement of mTOR signaling in therapy-induced autophagy. Additionally, the compounds also inhibit another player that is part of the mTOR complex 1, thus emphasizing the role of this complex in autophagy
induction.

Two different prenylation inhibitor compounds (FTI-1 and GGTI-2Z) in combination with lovastatin have part similar and part varied effects on STS-26T cells. GGTI-2Z/lovastatin induces a complete autophagic response in the cells accompanied by growth arrest, whereas FTI-1/lovastatin induces an aborted autophagic pathway followed by cell death. The cause(s) for observing similar initial responses but different endpoints in the same cell line are yet to be determined. There are multiple hypotheses that could explain this discrepancy. One, the difference could be attributed to separate cellular targets of the two compounds. Two, the autophagic pathway may be influenced by the two compounds at different points, driving the pathway in distinct directions. Three, autophagy may not be a direct response to prenylation inhibition and an unknown mechanism may be the missing link driving the cellular response to these inhibitors.

A thorough consideration of each of these possibilities may not necessarily be feasible. For instance, in case of the first one, there are hundreds of potential target proteins in the cell for both compounds. The modifying enzymes being common for a host of these target proteins, the compounds presumably modify prenylation of multiple proteins at any point of time in any given cellular system. Additionally, alternative prenylation occurs more commonly than thought. Although there may be clues indicating the involvement of certain specific proteins, it is more than likely that more than one of these is critical for the compounds to exhibit their effects. For this reason, it may be challenging to assertively identify the target proteins. Nonetheless, our observations do point towards some GTPases that may potentially be evaluated for their involvement.
5.1.1 Ras and Rheb as potential targets of prenylation inhibitors

STS-26T cells express predominantly N-Ras and some K-Ras (Mattingly et al., 2006). Both of these isoforms can be farnesylated or geranylgeranylated. Although there was inhibition of Ras prenylation with cotreatment, it is not clear which isoform(s) were affected. Moreover, the different isoforms may signal via distinct membranes including the ER, Golgi, endosomal and plasma membranes (Silvius, 2002). Kaul et al have shown that activated H-Ras and K-Ras are capable of stimulating type II cell death (non-apoptotic cell death) in glioblastoma cells, and Ras farnesylation and membrane association are integral events in this process. They also indicate that modified and activated Ras may signal via a unique effector pathway to induce autophagy (referred to as vacuolation) (Kaul et al., 2007). If this holds true in STS-26T cells, Ras may not be a critical target of the prenylation inhibitors for inducing autophagy. Nevertheless, downregulating specific isoforms of Ras to check if there is an effect on the autophagic response may reveal the importance of Ras as a target for the compounds.

Rheb is a small GTPase that is exclusively farnesylated and interacts with mTORC1 (Bai et al., 2007). A previous study suggested that Rheb is a potential target of the FTI lonafarnib (Basso et al., 2005). Our study partially agrees with this idea since FTI-1/lovastatin was found to substantially inhibit Rheb farnesylation at a time point prior to autophagic induction. On the other hand, GGTI-2Z/lovastatin also induces autophagy but may not inhibit Rheb farnesylation. Again, silencing Rheb expression prior to inhibitor treatment and evaluating effects on autophagic induction may help solve this mystery.
5.1.2 Role of Rabs in prenylation inhibitor induced autophagy

Rab proteins were originally thought to be solely involved in endocytosis and vesicular trafficking (Bucci et al., 1992). More recently, some Rabs have been found to be key regulators of autophagic events. For instance, Rab5 is required for the initiation of autophagosome formation (Ravikumar et al., 2008). Also, Rab7 is not only implicated in transport from early to late endosomes, but also is a key factor in lysosome biogenesis (Bucci et al., 2000). Rab7 mutation leads to an accumulation of procathepsin D and cation-independent M6P receptors in early endosomes (Press et al., 1998). We have shown in the present study that the compounds inhibit Rab5 prenylation. Thus, we suggest that Rab5 and possibly Rab7 or other Rab proteins involved in vesicular trafficking may be important targets. Silencing Rab5 or Rab7 may interfere with either the induction or fusion steps in the autophagic pathway.

The second hypothesis to explain why the two compounds exert different effects on STS-26T cells is already strengthened by the fact that FTI-1/lovastatin induces the autophagic pathway as well as inhibits autophagosome-lysosome fusion, whereas GGTI-2Z/lovastatin does not show the latter effect. One method to evaluate this hypothesis is to protect the lysosomes from FTI-1/lovastatin induced insult and determine whether autophagy is no longer aborted.

5.1.3 Lysosomal protection and autophagy

FTase is a zinc metalloenzyme and its activity in vitro is potentiated by Mg2+. Hence, FTase inhibition may lead to zinc imbalance affecting oxidation status of the cells. There
is evidence that accumulation of zinc potentiates oxidant-induced lysosomal membrane permeabilization and autophagy in astrocytes and cultured hippocampal neurons (Hwang et al., 2008; Lee et al., 2009). This may be the cause of the lysosomal toxicity we observed upon FTI-1/lovastatin treatment. Therefore, if the free zinc released by treatment is chelated using a cell-permeable zinc chelator such as 1,10-phenanthroline, it may partly rescue the lysosomes and prevent the aborted autophagy. Consequently, there may be a complete autophagic response similar to that with GGTI-2Z/lovastatin. One of the issues with this idea is that it is not clear whether FTI treatment destabilizes FTase and causes zinc to be released. Furthermore, it is also not known if there would be a substantial alteration of zinc levels to lead to a significant downstream effect. If future kinetic studies reveal answers to these queries, it may be worthwhile to investigate our hypothesis.

Finally, it is not yet clear whether autophagy is a direct consequence of prenylation inhibition in these cells, which leads to investigation of the third possible mechanism. One feasible way to work toward this aim is to inhibit the induction of autophagy and observe the effects on protein prenylation as well as on the fate of STS-26T cells.

5.1.4 Modulation of Atg genes

The Atg (autophagy) gene family consists of several genes involved in the autophagic process. These genes play different roles in the induction and implementation of the autophagic pathway in the cell. Atg5 forms a complex with Atg12 in a reaction that requires Atg7 and Atg10. Atg5-Atg12 conjugates form the pre-autophagosomal
structures (PAS), initiate the elongation process and dissociate upon autophagosome formation. Thus, Atg5 is required for the very initial steps in the formation of autophagosomes. Atg5-knockout (Atg5−/−) mouse embryonic stem (ES) cells were found to be deficient in autophagosome formation (Mizushima et al, 2001). Atg5-deficient mice are born normally, but die neonatally (Kuma et al, 2004). Hence, preventing autophagic induction in STS-26T cells via a knockdown of Atg5 may be a useful tool to evaluate the role of autophagy in the effect of prenylation inhibitors on these cells.

Atg5 knockdown is expected to inhibit the induction of autophagy. Since both FTI-1 and GGTI-2Z were found to induce autophagy, the knockdown may prevent this effect. This inhibition may compromise the cell's innate ability to program its survival or death, and may lead to upregulation of compensatory pathways including apoptosis and necrosis. This is especially likely with GGTI-2Z/lovastatin treatment, which would mean that the role of autophagy under the influence of GGTI-2Z/lovastatin is cytoprotective in these cells. Most likely, though, there may be a host of other alternative consequences, especially considering that along with autophagic induction, FTI-1 treatment in Atg5 wild-type cells also leads to a mysterious non-apoptotic cell death.

FTI-1/lovastatin not only induces autophagy but also causes compromised lysosomal activity via altered procathepsin trafficking, LAMP-2 deficiency and impaired autophagosome-lysosome fusion, and an aborted autophagic program. Atg5 knockdown in this case will completely abolish the autophagic pathway of cell content recycling. This may result in toxic accumulation of cellular contents and massive cell death.
In case of GGTI-2Z/lovastatin treatment, we may alternatively observe reversion of effect. The cells may eventually overcome the cell cycle arrested state after sufficient period of time when the drugs are metabolized and no longer effective. The ubiquitin/proteasome pathway may compensate for loss of autophagic lysosomal degradation.

5.2 CONCLUSION

In conclusion, we have characterized novel prenylation inhibitors, GGTI-2Z and FTI-1, which in combination with lovastatin inhibit proliferation of STS-26T MPNST cells. They induce cell cycle arrest in the G1 phase and act synergistically with low dose lovastatin without affecting normal immortalized Schwann cells. GGTI-2Z induces autophagy and is cytostatic while FTI-1 induces an aborted autophagic phenotype and is cytotoxic. This is the first ever study showing antitumor potential of a GGTI compound via the mechanism of autophagic induction. This is also a novel study showing autophagic cell death as a consequence of an aborted autophagic program. In addition, our findings emphasize the importance of autophagy and lysosomal function in the action of prenylation inhibitors as antitumor agents. The strategy of combination therapy with prenyl transferase inhibitors and statin and their novel mechanisms of action may be potentially useful to develop better therapeutic regimen for cancer treatment as well as certain Rab-associated protein trafficking disorders.
Figure 5.1. Model of autophagy regulation by prenylation inhibition. The compounds once intracellular are activated by prodrug cleavage. GGTL-2Z/lovastatin can inhibit prenylation of yet unidentified target GTPases, e.g. Rabs, and this serves as a positive stimulus for autophagy induction and completion. FTI-1/lovastatin also inhibits prenylation of unknown targets and induces autophagy. In addition to this effect, however, it also affects the lysosomal function via blockade of procathepsin trafficking to lysosomes and LAMP-2 degradation. This lysosomal dysfunction presumably leads to accumulation of toxic material in autophagosomes and ultimately cell death.


transferase disrupt the prenylation and membrane localization of Rab proteins in osteoclasts in vitro and in vivo. Bone 37, 349-358.


Glick, D., Barth, S., and Macleod, K.F. Autophagy: cellular and molecular mechanisms. J Pathol 221, 3-12.


for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. Autophagy 4, 151-175.


binding, and functional homology with yeast prenyl-protein transferases.

Biochemistry 32, 5167-5176.


geranylgeranyltransferase I inhibitors and cancer therapy: lessons from
mechanism and bench-to-bedside translational studies. Oncogene 19,
6584-6593.

Seglen, P.O., and Gordon, P.B. (1982). 3-Methyladenine: specific inhibitor of
autophagic/lysosomal protein degradation in isolated rat hepatocytes. Proc
Natl Acad Sci U S A 79, 1889-1892.

motifs exhibit long-lived anchorage to lipid bilayer membranes.
Biochemistry 34, 3813-3822.


Membr Biol 190, 83-92.

Silvius, J.R., and l'Heureux, F. (1994). Fluorimetric evaluation of the affinities of
isoprenylated peptides for lipid bilayers. Biochemistry 33, 3014-3022.


Singh, R., Kaushik, S., Wang, Y., Xiang, Y., Novak, I., Komatsu, M., Tanaka, K.,


ABSTRACT

INDUCTION AND REGULATION OF AUTOPHAGY BY NOVEL PRENYLATION INHIBITORS IN STS-26T MALIGNANT PERIPHERAL NERVE SHEATH TUMOR (MPNST) CELLS

by

KOMAL M. SANE

December 2010

Advisor: Dr. Raymond R. Mattingly

Major: Pharmacology

Degree: Doctor of Philosophy

Prenylation pathways have been targeted via several different compounds that inhibit farnesyl transferase (FTase) and/or geranylgeranyl transferase (GGTase) enzymes in many cellular and animal models of cancer. Some of these have also been evaluated in clinical trials with limited success. Multiple mechanisms of action have been elucidated for such compounds, including cell cycle arrest, proteasome inhibition, apoptosis and most recently, autophagy. However, there is still an urgent need of effective agents of this class of anti-tumor therapeutics. In this dissertation, I sought to delve into this issue by characterizing our novel prenylation inhibitors and their potential as anti-tumor agents. Novel compounds, GGTI-2Z and FTI-1, were used in combination with lovastatin in STS-26T malignant peripheral nerve sheath tumor (MPNST) cells. We found that GGTI-2Z/lovastatin inhibit proliferation, cause cell cycle arrest in the G1
phase and induce autophagy in STS-26T MPNST cells. FTI-1/lovastatin not only inhibit proliferation and cause cell cycle arrest, but also induce an aborted autophagic program followed by non-apoptotic cell death in STS-26T cells. This distinct phenotype observed with FTI-1/lovastatin is the consequence of their action on the lysosomal trafficking of proteins. The compounds impaired procathepsin trafficking via the endocytic pathway along with degradation of the lysosomal protein, LAMP-2, which is required for autophagosome-lysosome fusion. These effects consequently lead to altered protein turnover and hence non-apoptotic cell death. Our observations identify a novel mechanism of action of GGTIs. We also show that autophagic cell death can be a consequence of an aborted autophagic program versus excessive autophagy. This mechanism also suggests that prenylated proteins may play an important role in a complete autophagic response and blocking their prenylation may interfere with this function of these proteins. Finally, the strategy of combination therapy with low doses of a statin and an FTI or a GGTI compound may serve as a useful tool to develop better therapeutic regimen for many cancers and other Rab-associated trafficking disorders.
AUTOBIOGRAPHICAL STATEMENT

KOMAL M. SANE

EDUCATION

- Ph.D. Pharmacology
  Sept 2004 – May 2010
  Wayne State University, School of Medicine, Detroit, MI

- B.S. Pharmaceutical Sciences
  June 1999 – May 2003
  Mumbai University Institute of Chemical Technology (Formerly UDCT), Mumbai, India

BIBLIOGRAPHY

- A novel geranylgeranyl transferase inhibitor in combination with lovastatin inhibits proliferation and induces autophagy in STS-26T MPNST cells
  Sane KM, Mynderse M, LaLonde DT, Dean IS, Wojtkowiak JW, Fouad F, Borch RF, Reiners, JJ, Jr., Gibbs RA, Mattingly RR (Journal of Pharmacology and Experimental Therapeutics, Apr 2010)

- Aborted autophagy and non-apoptotic death induced by farnesyl transferase inhibitor and lovastatin
  * These authors contributed equally to this manuscript.

- Liposomal delivery: defying conventional systems?
  Dandekar PP, Sane KM, Joshi SR (Pharma Research, A special supplement to Indian Express Pharma Pulse, India, Sept 2001)

SELECTED PRESENTATIONS

- “Potential role of the serine745 phosphorylation residue of Ras-GRF1 in regulation of interaction with NMDA-R2B and activation of Rac” - Poster presentation*
  *Travel Award Recipient
  - Pharmacology Colloquium, University of Michigan, Ann Arbor, MI, June 2008

- “A novel geranylgeranyl transferase inhibitor in combination with lovastatin inhibits proliferation and induces autophagy in STS-26T MPNST cells” – Talk
  - Graduate Student Research Day, Wayne State University, Sept 2009
  - Summer Undergraduate Research Program Seminar Series, Wayne State University, MI, June 2009