

**Haemopoiesis, Leukaemia & Imatinib:  
c-fms, a Novel Target for  
Small Molecule Inhibitor Therapy**

**Andrea L. Dewar**

Melissa White Leukaemia Research Laboratory  
Hanson Institute  
Institute of Medical & Veterinary Science

&

Department of Medicine  
The University of Adelaide  
South Australia

A thesis submitted to the University of Adelaide  
in candidature for the degree of Doctor of Philosophy  
September 2004

# TABLE OF CONTENTS

<b>DECLARATION</b> .....	<b>I</b>
<b>ACKNOWLEDGEMENTS</b> .....	<b>II</b>
<b>ABSTRACT</b> .....	<b>VI</b>
<b>ABBREVIATIONS</b> .....	<b>VII</b>
<b>PUBLICATIONS</b> .....	<b>IX</b>
<b>CHAPTER 1: GENERAL INTRODUCTION</b> .....	<b>1</b>
<b>1.1 INTRODUCTION: THE PROCESS OF DIFFERENTIATION</b> .....	<b>1</b>
<b>1.2 HAEMOPOIESIS &amp; HAEMOPOIETIC STEM CELLS</b> .....	<b>2</b>
<b>1.3 THE ORIGIN OF HAEMOPOIETIC STEM CELLS</b> .....	<b>2</b>
1.3.1 PRIMITIVE & DEFINITIVE HAEMOPOIESIS .....	2
1.3.2 FORMATION OF THE HAEMOPOIETIC SYSTEM .....	3
1.3.3 HOMING OF HAEMOPOIETIC STEM CELLS TO APPROPRIATE ENVIRONMENTS .....	4
<b>1.4 REGULATION OF STEM CELL FATE</b> .....	<b>5</b>
1.4.1 THE STOCHASTIC MODEL OF STEM CELL DECISION .....	5
1.4.2 THE DETERMINISTIC MODEL OF STEM CELL DECISION .....	6
1.4.3 STOCHASTIC & DETERMINISTIC CONTROL OF STEM CELL FATE .....	7
<b>1.5 HAEMOPOIETIC STEM CELL DIFFERENTIATION</b> .....	<b>7</b>
<b>1.6 REGULATION OF HAEMOPOIETIC STEM CELL DIFFERENTIATION</b> .....	<b>8</b>
1.6.1 EXTRINSIC REGULATION OF HAEMOPOIETIC DIFFERENTIATION .....	8
1.6.1.1 <i>Role of Tyrosine Kinases in Haemopoiesis</i> .....	10
1.6.1.2 <i>Jak-STAT Signalling</i> .....	11
1.6.1.3 <i>Growth Factors &amp; Cytokines Involved in Haemopoiesis</i> .....	11
1.6.1.3.1 Erythropoietin .....	11
1.6.1.3.2 Flt-3 Ligand .....	12
1.6.1.3.3 Granulocyte Colony Stimulating Factor.....	12
1.6.1.3.4 Granulocyte-Macrophage Colony Stimulating factor, Interleukin-3 & Interleukin-5 .....	12
1.6.1.3.5 Interleukin-6.....	14
1.6.1.3.6 Macrophage Colony Stimulating Factor & c-fms .....	15
1.6.1.3.7 Platelet Derived Growth Factor .....	16
1.6.1.3.8 Stem Cell Factor & c-kit .....	18
1.6.1.3.9 Thrombopoietin & c-Mpl.....	18
1.6.1.4 <i>Cytokine Synergism</i> .....	19
1.6.2 INTRINSIC REGULATION OF HAEMOPOIETIC DIFFERENTIATION .....	20
1.6.2.1 <i>Transcriptional Regulation of Haemopoietic Stem Cell Differentiation</i> .....	20
1.6.2.1.1 Regulation of Transcription Factor Activity .....	22
1.6.2.1.1.1 Redundancy .....	23
1.6.2.1.1.2 Cooperative or Synergistic Interactions .....	23
1.6.2.1.1.3 Antagonism.....	23
1.6.2.2 <i>Notch Receptors &amp; the Regulation of HSC Differentiation</i> .....	24
<b>1.7 DYSREGULATED HSC DIFFERENTIATION: CHRONIC MYELOID LEUKAEMIA</b> .....	<b>26</b>

1.7.1	THE PHILADELPHIA CHROMOSOME .....	27
1.7.2	SELECTIVE ADVANTAGE OF CML PROGENITORS .....	28
1.7.3	IDENTIFICATION OF QUIESCENT CML PROGENITOR CELLS.....	29
1.7.4	TREATMENT OF CML .....	30
1.7.4.1	<i>Interferon</i> .....	30
1.7.4.2	<i>Imatinib</i> .....	31
1.7.4.2.1	Resistance to Imatinib.....	32
1.7.4.2.2	Effect of Imatinib on Normal Haemopoiesis.....	34
<b>1.8</b>	<b>SUMMARY &amp; PROJECT AIMS.....</b>	<b>35</b>

## **CHAPTER 2: MATERIALS & METHODS.....37**

### **2.1 SUPPLIERS OF COMMONLY USED REAGENTS ..... 37**

### **2.2 SOLUTIONS, BUFFERS & MEDIA FOR CELL CULTURE ..... 39**

2.2.1	0.66% AGAR .....	39
2.2.2	AGAR- ISCOVE'S MODIFIED DULBECCO'S MEDIUM (A-IMDM).....	39
2.2.3	ASSAY BUFFER FOR IL-3/IL-6 ELISA (PBS/0.5% BSA/0.1% TWEEN-20) .....	39
2.2.4	ASSAY BUFFER FOR M-CSF ELISA (PBS/1.0% BSA).....	40
2.2.5	BLOCKING BUFFER (HBSS/0.8% BSA/ 4% NHS/ 5% FCS) .....	40
2.2.6	DOUBLE-STRENGTH ISCOVE'S MODIFIED DULBECCO'S MEDIUM (2X-IMDM).....	40
2.2.7	DULBECCO'S MODIFICATION OF EAGLE'S MEDIUM <sup>+</sup> (DMEM <sup>+</sup> ) .....	41
2.2.8	FLOW CYTOMETRY FIXATIVE (FACS FIXATIVE) .....	41
2.2.9	3% GLUTARALDEHYDE FIXATIVE.....	41
2.2.10	HHF (HBSS/10MM HEPES/5% FCS) .....	42
2.2.11	IMATINIB (10MM STOCK).....	42
2.2.12	IMDM/1% BSA.....	42
2.2.13	ISOLATION BUFFER (IB) (HBSS/2% BSA/DNASE*) .....	43
2.2.14	MACS CD34 <sup>+</sup> BUFFER (HBSS/0.5% BSA/2MM EDTA).....	43
2.2.15	MACS MONOCYTE BUFFER (HBSS/0.5% BSA/10% FCS/2MM EDTA).....	43
2.2.16	2-MERCAPTOETHANOL (1M).....	44
2.2.17	NORMAL HUMAN SERUM .....	44
2.2.18	PERCOLL ( $\rho = 0.99\text{G/ML}$ ) .....	44
2.2.19	0.067M PHOSPHATE BUFFER, PH 6.3.....	44
2.2.20	0.067M PHOSPHATE BUFFER, PH 7.4.....	45
2.2.21	SERUM DEPRIVED MEDIUM (SDM).....	45
2.2.22	THAW SOLUTION.....	45
2.2.23	UREA SATURATED ETHANOL .....	46
2.2.24	WHITE CELL FLUID .....	46

### **2.3 CELL CULTURE TECHNIQUES ..... 46**

2.3.1	MAINTENANCE OF CELL LINES .....	46
2.3.1.1	<i>FDC-P1</i> .....	47
2.3.1.2	<i>Psi-2 (<math>\psi</math>2)</i> .....	47
2.3.2	CRYOPRESERVATION OF CELLS .....	47
2.3.3	THAWING OF CRYOPRESERVED CELLS .....	47
2.3.4	CELL WASHING .....	47
2.3.5	CELL COUNTS AND VIABILITY.....	48
2.3.6	IMMUNOFLUORESCENT STAINING & FLOW CYTOMETRIC ANALYSIS .....	48
2.3.6.1	<i>Antibody Staining for Flow Cytometric Analysis</i> .....	48
2.3.6.2	<i>Carboxyfluorescein Diacetate Succinimidyl Ester Labelling of Cells</i> .....	48
2.3.7	PREPARATION OF MONONUCLEAR CELLS (MNC) .....	50
2.3.7.1	<i>Isolation of CD34<sup>+</sup> Cells: Dynabead Separation</i> .....	50
2.3.7.2	<i>Isolation of CD34<sup>+</sup> Cells: MACS Separation</i> .....	51
2.3.8	CFSE CD34 <sup>+</sup> CELL ASSAYS.....	52

2.3.8.1	<i>Establishment of Cultures</i> .....	52
2.3.8.2	<i>Analysis of CFSE CD34<sup>+</sup> Cell Cultures</i> .....	53
2.3.9	HAEMOPOIETIC COLONY ASSAYS.....	53
2.3.9.1	<i>Establishment of Colony Assay Cultures</i> .....	53
2.3.9.2	<i>Fixation of Colony Assay Cultures</i> .....	54
2.3.9.3	<i>Tri-staining of Colony Assay Cultures</i> .....	54
2.3.9.3.1	Naphthol acetate esterase staining: .....	55
2.3.9.3.2	Chloroacetate esterase staining: .....	55
2.3.9.3.3	Luxol fast blue dye: .....	55
2.3.10	CD34 <sup>+</sup> LIQUID CULTURE ASSAYS.....	55
2.3.11	MONOCYTE CULTURES .....	56
2.3.11.1	<i>Isolation of Monocytes</i> .....	56
2.3.11.2	<i>Establishment of Monocyte Cultures</i> .....	57
2.3.12	MONOCYTE/MACROPHAGE FUNCTIONAL ASSAYS.....	58
2.3.12.1	<i>Morphologic Response to Activation with Lipopolysaccharide</i> .....	58
2.3.12.2	<i>ELISAs to Measure IL-6 &amp; TNF-<math>\alpha</math> Production</i> .....	58
2.3.12.3	<i>ELISA to Measure M-CSF Production</i> .....	59
2.3.12.4	<i>Antigen Uptake Assay: Zymosan Bioparticles<sup>®</sup></i> .....	60
2.3.12.5	<i>Mixed Lymphocyte Reaction (MLR)</i> .....	60
2.3.13	PROLIFERATION ASSAY: FLOW BEADS .....	62
<b>2.4</b>	<b>MOLECULAR BIOLOGY REAGENTS.....</b>	<b>62</b>
2.4.1	LOADING BUFFER, 2X DENATURING .....	62
2.4.2	LOADING BUFFER, 2X NON-REDUCING .....	63
2.4.3	LURIA BROTH(LB).....	63
2.4.4	LB AGAR .....	63
2.4.5	LYSIS BUFFER .....	64
2.4.6	MEMBRANE BLOCKING SOLUTION .....	64
2.4.7	5% POLYACRYLAMIDE GEL (STACKING GEL).....	64
2.4.8	8% POLYACRYLAMIDE GEL (RESOLVING GEL).....	65
2.4.9	SDS-PAGE ELECTRODE BUFFER (25MM TRIS/ 192MM GLYCINE/ 0.1% SDS).....	65
2.4.10	TBS STOCK (10X).....	65
2.4.11	1X TBS.....	66
2.4.12	TBS-T (1X TBS / 0.1% TWEEN).....	66
2.4.13	TE BUFFER (10X).....	66
2.4.14	TRANSFER BUFFER (25MM TRIS, 192MM GLYCINE, 20% METHANOL).....	66
2.4.15	1.5M TRIS (PH 8.8) .....	67
2.4.16	0.5M TRIS (PH 6.8) .....	67
2.4.17	TSE BUFFER (50MM TRIS,100MM NaCl, 1mM EDTA, pH 8.0).....	67
<b>2.5</b>	<b>MOLECULAR BIOLOGY TECHNIQUES .....</b>	<b>67</b>
2.5.1	TRANSFORMATION OF COMPETENT CELLS .....	67
2.5.2	PURIFICATION OF PLASMID DNA FROM BACTERIAL CULTURES .....	68
2.5.2.1	<i>Small Scale Plasmid DNA Extraction (Mini-Prep Protocol)</i> .....	68
2.5.2.2	<i>Medium Scale Plasmid DNA Extraction (Midi-Prep Protocol)</i> .....	69
2.5.3	MANIPULATION OF DNA PRODUCTS.....	70
2.5.3.1	<i>Quantitation of DNA</i> .....	70
2.5.3.2	<i>Electrophoresis of DNA</i> .....	70
2.5.3.3	<i>Restriction Digest to Check Presence of Insert in Plasmid DNA</i> .....	70
2.5.3.4	<i>Size Determination of DNA Fragments</i> .....	70
2.5.4	RETROVIRAL TRANSFECTION OF THE ECOTROPIC PACKAGING CELL LINE, $\Psi_2$ .....	70
2.5.5	RETROVIRAL INFECTION OF THE SUSPENSION CELL LINE, FDC-P1, BY CO-CULTIVATION .....	71
2.5.6	IMMUNOPRECIPITATION OF C-FMS PROTEIN.....	72
2.5.7	PREPARATION OF SAMPLES FOR SDS-PAGE.....	72
2.5.8	SDS-PAGE GEL PREPARATION .....	72
2.5.9	LOADING & RUNNING OF SDS-PAGE GEL.....	73
2.5.10	TRANSFER OF PROTEIN TO PVDF USING A SEMI-DRY BLOTTING APPARATUS .....	73
2.5.11	PROTEIN DETECTION .....	74

2.5.11.1	Western Blotting for Anti-Phosphotyrosine.....	74
2.5.11.2	Western Blotting for Anti-c-fms Protein.....	74
<b>2.6</b>	<b>STATISTICAL &amp; PHARMACOKINETIC DATA ANALYSES .....</b>	<b>75</b>

## **CHAPTER 3: DIFFERENTIATION OF NORMAL & CML CD34<sup>+</sup> CELLS.....76**

<b>3.1</b>	<b>INTRODUCTION .....</b>	<b>76</b>
<b>3.2</b>	<b>RESULTS.....</b>	<b>79</b>
3.2.1	COMPARING THE PROLIFERATION OF NORMAL & CML CD34 <sup>+</sup> CELLS .....	79
3.2.1.1	Comparison of the Percentage of Starting Cells Entering Division in Normal Donors & CML Patients .....	79
3.2.1.2	Comparison of the Proliferation Index of Normal & CML CD34 <sup>+</sup> Cells.....	80
3.2.1.3	Comparison of the Viability of Cultured Cells from Normal Donors & CML Patients .....	80
3.2.1.4	Comparison of the Distribution of Cells Across Cell Divisions in Normal Donors & CML Patients.....	81
3.2.2	COMPARISON OF THE DIFFERENTIATION PROGRAM OF NORMAL AND CML CD34 <sup>+</sup> CELLS USING DYE DILUTION.....	81
3.2.2.1	Comparison of the Phenotype of Freshly Isolated Normal & CML CD34 <sup>+</sup> Cells.....	82
3.2.2.2	CD34 Expression .....	83
3.2.2.3	CD38 Expression .....	84
3.2.2.4	HLA-DR Expression.....	85
3.2.2.4.1	HLA-DR <sup>high</sup> Expression .....	86
3.2.2.4.2	HLA-DR <sup>medium</sup> Expression .....	86
3.2.2.5	CD33 Expression .....	87
3.2.2.6	CD13 Expression .....	88
3.2.2.7	CD36 Expression .....	90
3.2.2.8	CD14 Expression .....	91
3.2.2.9	CD11c Expression.....	92
3.2.2.10	CD15 Expression .....	93
3.2.3	EFFECT OF CELL DIVISION ON THE LEVEL OF SURFACE MARKER EXPRESSION .....	93
3.2.3.1	Changes in the Level of CD38 Expression.....	93
3.2.3.2	Changes in the Level of HLA-DR Expression .....	94
3.2.3.3	Changes in the Level of CD33 Expression.....	95
3.2.3.4	Changes in the Level of CD13 Expression.....	96
<b>3.3</b>	<b>DISCUSSION.....</b>	<b>98</b>

## **CHAPTER 4: EFFECT OF IMATINIB ON NORMAL HAEMOPOIESIS.....105**

<b>4.1</b>	<b>INTRODUCTION .....</b>	<b>105</b>
<b>4.2</b>	<b>RESULTS.....</b>	<b>107</b>
4.2.1	EFFECT OF IMATINIB ON NORMAL COLONY FORMATION .....	107
4.2.1.1	Identification of Colony Types .....	107
4.2.1.2	Effect of Imatinib on Total Colony Formation.....	107
4.2.1.3	Effect of Imatinib on Monocyte/Macrophage Colony Formation .....	108
4.2.1.4	Effect of Imatinib on Eosinophil Colony Formation .....	108
4.2.1.5	Effect of Imatinib on Neutrophil Colony Formation .....	108
4.2.1.6	Dose Response of Colony Cultures Treated with Imatinib.....	108
4.2.1.7	Effect of Imatinib on Colony Growth Following Stimulation with a Combination of M-CSF & GM-CSF.....	109

4.2.1.8	<i>Effect of Imatinib on Colony Growth Following Stimulation with either M-CSF or GM-CSF.....</i>	110
4.2.2	EFFECT OF IMATINIB ON THE NORMAL CD34 <sup>+</sup> CELLS IN LIQUID CULTURE .....	111
4.2.2.1	<i>Effect of Imatinib on Proliferation .....</i>	111
4.2.2.2	<i>Effect of Imatinib on Cell Phenotype: Flow Cytometric Analysis .....</i>	111
4.2.2.3	<i>Effect of Imatinib on Cell Phenotype: Jenner-Giemsa Staining.....</i>	112
4.2.3	EFFECT OF IMATINIB ON THE DIFFERENTIATION OF MONOCYTES IN VITRO .....	113
4.2.3.1	<i>Morphological Analysis of M-CSF Stimulated Cultures.....</i>	113
4.2.3.2	<i>Effect of Imatinib on the Immunophenotype of M-CSF Stimulated Monocyte Cultures...</i>	114
4.2.3.3	<i>Morphological Analysis of GM-CSF Stimulated Cultures .....</i>	115
4.2.3.4	<i>Effect of Imatinib on the Immunophenotype of GM-CSF Stimulated Monocyte Cultures</i>	115
4.2.3.5	<i>Effect of Imatinib on the Viability of Cultured Monocytes Stimulated with M-CSF or GM-CSF.....</i>	116
4.3	DISCUSSION.....	117

## **CHAPTER 5: EFFECT OF IMATINIB ON THE FUNCTION OF CULTURED MONOCYTES.....121**

5.1	INTRODUCTION .....	121
5.2	RESULTS.....	124
5.2.1	EFFECT OF IMATINIB ON THE ACTIVATION OF CULTURED MONOCYTES BY LPS: MORPHOLOGICAL ASSESSMENT.....	124
5.2.1.1	<i>M-CSF Stimulated Monocytes.....</i>	124
5.2.1.2	<i>GM-CSF Stimulated Monocytes.....</i>	124
5.2.1.3	<i>M-CSF &amp; GM-CSF Stimulated Monocytes.....</i>	125
5.2.2	EFFECT OF IMATINIB ON THE PRODUCTION OF IL-6 & TNF- $\alpha$ IN RESPONSE TO LPS ACTIVATION ..	125
5.2.2.1	<i>IL-6 Production.....</i>	125
5.2.2.2	<i>TNF-<math>\alpha</math> Production.....</i>	126
5.2.3	EFFECT OF IMATINIB ON THE PHAGOCYTOSIS OF ZYMOBAN PARTICLES BY CULTURED MONOCYTES .....	127
5.2.3.1	<i>Non-Opsonised Zymosan.....</i>	127
5.2.3.2	<i>Opsonised Zymosan .....</i>	129
5.2.4	EFFECT OF IMATINIB ON THE ABILITY OF CULTURED MONOCYTES TO STIMULATE A MIXED LYMPHOCYTE REACTION .....	131
5.2.4.1	<i>M-CSF Stimulated Monocyte Cultures.....</i>	132
5.2.4.2	<i>GM-CSF Stimulated Monocyte Cultures.....</i>	132
5.2.5	EFFECT OF IMATINIB ON CD80 & CD86 EXPRESSION.....	133
5.3	DISCUSSION.....	134

## **CHAPTER 6: MECHANISM OF IMATINIB SUPPRESSION OF MONOCYTE/MACROPHAGES .....138**

6.1	INTRODUCTION .....	138
6.2	RESULTS.....	141
6.2.1	EFFECT OF ANTI-C-KIT ON HAEMOPOIETIC COLONY FORMATION.....	141
6.2.2	EFFECT OF ANTI-C-FMS ON MONOCYTE/MACROPHAGE COLONY FORMATION .....	142
6.2.3	ANALYSIS OF AUTOCRINE M-CSF PRODUCTION.....	142
6.2.4	EFFECT OF IMATINIB ON AN M-CSF-DEPENDENT CELL LINE.....	143

6.2.4.1	<i>Effect of Imatinib on the Proliferation of FDC-cfms Cells</i> .....	143
6.2.4.1.1	Calculation of the IC <sub>50</sub> for Imatinib Inhibition of FDC-cfms Proliferation .....	144
6.2.4.2	<i>Effect of Imatinib on the Phosphorylation &amp; Expression of c-fms</i> .....	144
6.2.4.2.1	Phosphorylation of c-fms .....	144
6.2.4.2.2	Expression of c-fms: Western Blot Analysis .....	145
6.2.4.2.3	Expression of c-fms: Flow Cytometric Analysis .....	145
<b>6.3</b>	<b>DISCUSSION</b> .....	<b>146</b>
 <b>CHAPTER 7: GENERAL DISCUSSION</b> .....		<b>148</b>
 <b>CHAPTER 8: REFERENCES</b> .....		<b>157</b>

## **DECLARATION**

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Andrea Dewar



## ACKNOWLEDGEMENTS

Thanking everyone for all their help during my PhD isn't an easy task, as there have been many people over the course of my studies that have gone out of their way to help. And I know that without their encouragement and support, I wouldn't have got this far.

First of all, I'd like to thank my supervisors Dr Kathleen Doherty and Dr Bruce Lyons. I don't doubt that by being a determined student who was keen to get finished two weeks after starting has driven you crazy at times! Thank you for your ideas, patience, guidance and stories of the good old days. As a lasting legacy of your supervision, I'm sure the terms "proactive" and "interesting" will be sounding in my ears forever.

Thank you to Associate Professor Tim Hughes for all your valuable ideas during meetings and for allowing me to carry out my PhD in your lab. Thank you also to other members of the Melissa White Laboratory - Deb White, Dr Pongtep Viboonjuntra and Verity Saunders - for all your willing help in the lab (even when you've already told me once before, Verity!) and for putting up with my rants and raves when experiments went pear-shaped. A special thank you goes to Bernadette Miller for keeping me entertained and sane, for being an eager and enthusiastic assistant to a bossy boss, and for always being there to help me out and listen when I needed it the most. Thank you also to Dr Rachel Gibson who left the MWL mid-way through my studies, but still went out of her way to do anything for me and dropped by regularly to keep me inspired and say hello.

When all else failed, or even when I didn't have an excuse, I always managed to find smiling faces, help and a sounding board around the corner in the Matthew Roberts Laboratory. Thank you to Dr Andrew Zannettino and Dr Stan Gronthos for always having time to help me out and for providing me with invaluable suggestions, advice and humour. Thank you to Amanda Farrugia, Angela Kortesidis and Dr Betty Pan for always having a spare chair for me in the lab or office, for allowing me to run off with equipment and for listening to all my stories. Your friendships have meant a lot to me and have made the past three years a lot easier than what they would otherwise have been.

Members of the Molecular Regulation Laboratory have also provided me with an enormous amount of advice and support, given answers to my endless questions, and regularly dropped by to check that things were going ok. Thank you in particular to Andrew Fotia, Dr Stuart Read, Dr Dimitrious Kacourous and Dr Linda Sherwin. An extra thank you also goes to Andrew Fotia for keeping me laughing with all his office humour, and for telling stories that were always very believable.

Thank you to Tony Cambareri for providing me with an enormous amount of advice, happily answering all my questions and never complaining when I came back to ask even more! Your patience and willingness to help is greatly appreciated.

There are many other people in the Hanson Institute that have helped me during the course of my studies - thank you to Dr Haylee Ramshaw for help with colony assays, and Ghafar xxxx for confocal microscopy. Thank you to Alan Bishop and Sandy Macintyre for your expertise in flow cytometric cell sorting, happily finding spare time to squeeze in a last-minute experiment and for dropping by to see how things were going. I would also like to thank the Australian Red Cross Blood Service, South Australia for providing me with buffy coats whenever I needed them.

Endless support for my studies has also come from my family and friends - even though they never understood what I was up to, they asked, listened and cared. Some even went the extra distance and attempted to pick up some science jargon - so to them, I give another aliquot of thanks.

Thank you to Mum and Dad who aside from being great parents, are also great friends. I am very fortunate to be part of such a supportive and loving family, and am extremely grateful for all your encouragement, for being there whenever I needed you and for your phone calls to break up the tedium of writing. Thank you also to my brother Mark, who gets a mention simply because he wanted to be mentioned.

The biggest thank you goes to my husband, Michael. You have provided me with an endless amount of love, encouragement and support, and my PhD would have been even

tougher without you by my side. You are an extremely wise person, and your perspective has always provided me with guidance and the inspiration to keep going with a smile on my face. And as a well known song sums it up, “I want to thank you for giving me the best day of my life.... to be with you is having the best day of my life”.

Lastly, I would like to thank Professor To for allowing me to undertake my studies in the department of haematology at the Hanson Institute, and the University of Adelaide and Lions Medical Research Foundation for providing financial support during my studies.

**“Obstacles cannot crush me;  
every obstacle yields to stern resolve”**

Leonardo da Vinci

**“Don’t wait for a light to appear at the end of the tunnel;  
stride down there and light the bloody thing yourself!”**

Sara Henderson

## ABSTRACT

Understanding the factors that regulate the growth and differentiation of haemopoietic stem cells (HSC) remains a major challenge. In this study, the proliferation and differentiation of CD34<sup>+</sup> cells from normal donors and chronic myeloid leukaemia (CML) patients was compared. The proliferation and entry of CML cells into the cell cycle was decreased relative to cells from normal donors, and greater heterogeneity in the phenotype of CML cells at the initiation of culture was observed. Analysis of phenotype concomitant with cell division also demonstrated that the differentiation of normal CD34<sup>+</sup> cells was consistent between donors, while marked variability was observed in the differentiation of CD34<sup>+</sup> cells from CML patients. This included expression of CD13, CD33, CD38 and HLA-DR, which were linked to cell division in normal but not CML cells.

The tyrosine kinase inhibitor, imatinib, is a novel drug displaying promising results in the treatment of CML by specifically inhibiting the growth of leukaemic cells. To examine whether myelosuppression observed in patients treated with imatinib may arise from inhibition of normal haemopoiesis, imatinib was added to colony assays established using cells from normal bone marrow. Suppression of monocyte/macrophage growth, but not that of eosinophils or neutrophils, was observed at therapeutic concentrations of imatinib. Inhibition of monocytic differentiation to macrophages was also observed and was associated with decreased functional capacity such as altered antigen uptake, production of proinflammatory cytokines and stimulation of responder cells.

The specific suppression of monocyte/macrophage differentiation and function was not due to blockade of tyrosine kinases known to be inhibited by imatinib and was consistent with an inhibition of the M-CSF/c-fms signalling pathway. This hypothesis was tested using a cell line that was dependent on M-CSF for growth and survival. Cell proliferation and phosphorylation of c-fms were inhibited at an IC<sub>50</sub> of 1.9µM and 1.4µM imatinib respectively and this was not attributable to decreased c-fms expression. These important findings therefore identify c-fms as a further target of imatinib, and suggest that imatinib should be considered for treatment of diseases where c-fms is implicated. This includes breast and ovarian cancer and inflammatory conditions such as rheumatoid arthritis. Potential side effects resulting from imatinib treatment must also be considered.

## ABBREVIATIONS

µg	Micro gram
µm	Micro metre
µM	Micro molar
4HGF	Four haemopoietic growth factors (IL-3, IL-6, G-CSF, GM-CSF)
5HGF	Five haemopoietic growth factors (IL-3, IL-6, G-CSF, GM-CSF, SCF)
ATP	Adenosine triphosphate
BM	Bone marrow
BMMNC	Bone marrow mononuclear cell
BSA	Bovine serum albumin
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CM	Cultured monocyte
CML	Chronic myeloid leukaemia
CSF	Colony stimulating factor
DMEM	Dulbecco's modification of Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra acetic acid
ELISA	Enzyme linked immunosorbent assay
EPO	Erythropoietin
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
g	Gram
G-CSF	Granulocyte colony stimulating factor
GIST	Gastrointestinal stromal tumour
GM-CSF	Granulocyte-macrophage colony stimulating factor

HBSS	Hank's balanced salt solution
HLA	Human leukocyte antigen
HSC	Haemopoietic stem cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove's modification of Dulbecco's medium
LPS	Lipopolysaccharide
M	Molar
MACS	Magnetic activated cell sorting
M-CSF	Macrophage colony stimulating factor
MFI	Mean fluorescence intensity
m	Murine
mg	Milli-gram
mL	Millilitre
MLR	Mixed lymphocyte reaction
mM	Milli molar
MNC	Mononuclear cell
PB	Peripheral blood
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PE	Phycoerythrin
Ph chromosome	Philadelphia chromosome
PI	Proliferation index
Rh	Recombinant human
RT	Room temperature
SCF	Stem cell factor
SDM	Serum deprived medium
TNF	Tumour necrosis factor
TPO	Thrombopoietin
v/v	Volume per volume
w/v	Weight per volume

## PUBLICATIONS

1. Imatinib Inhibits the Tyrosine Kinase Activity of the Macrophage Colony Stimulating Factor Receptor c-fms at Clinically Relevant Concentrations. **AL Dewar**, AC Cambareri, AC Zannettino, BL Miller, KV Doherty, TP Hughes, AB Lyons. *Manuscript in preparation*.
2. Imatinib inhibits the functional capacity of cultured human monocytes. **AL Dewar**, KV Doherty, TP Hughes, AB Lyons. *Journal of Immunology & Cell Biology* (2004). *In Press*.
3. Imatinib inhibits the *in vitro* development of the monocyte/macrophage lineage from normal human bone marrow progenitors. **AL Dewar**, RM Domaschenz, KV Doherty, TP Hughes, AB Lyons. *Leukemia* (2003). 17(9):1713-1721.
4. Acquisition of immune function during the development of the Langerhans cell network in neonatal mice. **AL Dewar**, KV Doherty, GM. Woods, AB. Lyons, HK Muller. *Immunology* (2001). 103: 61-69.
5. Prevention of autoimmunity by induction of cutaneous tolerance. GM Woods, YP Chen, **AL Dewar**, KV Doherty, BH Toh, HK Muller. *Cell Immunol* (2001). 207(1):1-5.



# THESIS AMENDMENTS

## ***Corrections Relevant to Entire Thesis***

- Error bars represent the SEM, and were derived from triplicate data points unless otherwise stated.
- Human genes should be written in capitals and italics. Proteins encoded by these genes should be written with the first letter in capital case, and should not be preceded by the “c-” prefix.

## ***Chapter 1: Introduction***

- Page 11, paragraph 1: the final sentence should be deleted as it does not follow.
- Page 14, paragraph 3: RAS is part of the MAPK pathway. The MAPK pathway is therefore not a further example of signal transduction pathways activated following ligand binding to the GM-CSF, IL-3 and IL-5 receptors.
- Page 26: The word “invariably” should be changed to read “usually”, as there are many cases where CML patients remain in chronic phase for longer than 5 years.
- Page 28, first paragraph, final sentence: *Heistercamp et al (1990)* reference is incorrect. The reference should be *Daley et al (1990) Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome; Science. Feb 16; 247(4944): 824-30.*
- Page 31, 2<sup>nd</sup> paragraph: Reference to *Deininger et al (1997)* in statements that imatinib inhibits the growth of CML cells with minimal effects on normal haemopoiesis were omitted.
- Figure 1.1: LT-HSC is a “long-term” HSC. ST-HSC is a “short-term” HSC.

## ***Chapter 2: Materials and Methods***

- Page 46, 2.3.1: The final sentence in paragraph one should read: “Media were prewarmed...”.
- Page 47, 2.3.1.1 & page 71, 2.5.5: The dilutions of IL-3 and GM-CSF that provided maximal cell growth were determined by titration using a factor dependent cell line.
- Page 71, 2.5.5: Use of the term selected means that the cells were selected for the ability to grow in M-CSF.

## ***Chapter 3: Results Chapter 1***

- Viability was determined following calculation of the percentage of cells with decreased FS/SS values.
- Surface markers were chosen which were expressed on specific myeloid lineages.
- Table 3.2: CML values were deemed significantly different from normal controls when *p* values calculated using ANOVA were less than 0.05.
- The data in Figures 3.13-16 are derived from the same experiment as Figures 3.5-3.8.
- Page 99, paragraph 1: “proliferative potential” should read “proliferation”.

## ***Chapter 4: Results Chapter 2***

- Figure 4.2: The data was normalised relative to the 5HGF control, not the 4HGF control.
- Figure 4.9A (and elsewhere): Contrary to the statement in the legend, the marker tool was set on the 95<sup>th</sup> percentile of the fluorescence for the negative control.
- Figure 4.10: The morphology of the cells is not that of promyelocytes. The cells display an atypical morphology (excentric nucleus and a large, clear, vacuolated cytoplasm), however trisaining suggests the cells are immature myeloid cells.

## ***Chapter 5: Results Chapter 3***

Page 129, 5.2.3.2: The opsonin used was derived from purified rabbit polyclonal IgG antibodies that are specific for the zymosan particles. Phagocytosis of opsonised zymosan particles was mediated by Fc and/or complement receptors.

## ***Chapter 6: Results Chapter 4***

Figures 6.6 and 6.7: “...infected with human c-fms...” should read “infected with a retrovirus encoding human FMS...”.

## ***Chapter 7: General Discussion***

Page 152, paragraph 2: Abl is an intracellular tyrosine kinase, not a receptor tyrosine kinase.