

**Assessment of Critical Survival Mechanisms  
Exploited by *BCR-ABL1*+ Cells to Evade  
Tyrosine Kinase Inhibitor-Induced Death;  
Determination of Novel Therapeutic Targets  
in Chronic Myeloid Leukaemia**

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# Table of Contents

TABLE OF CONTENTS.....	I
TABLE OF FIGURES .....	IX
PUBLICATIONS.....	XIII
SCHOLARSHIPS AND AWARDS.....	XVI
ABBREVIATIONS .....	XVIII
ACKNOWLEDGMENTS .....	XXVI
ABSTRACT.....	XXIX
CHAPTER 1. ....	1
INTRODUCTION .....	1
1.1    HAEMATOPOIESIS.....	2
1.2    CHRONIC MYELOID LEUKAEMIA.....	3
1.2.1    History .....	3
1.2.2    Clinical Features .....	5
1.2.3    Pathogenesis and Stages of Disease .....	8
1.3    MOLECULAR BIOLOGY OF CML .....	10
1.3.1    Philadelphia Chromosome Translocation, BCR-ABL fusion gene and transcripts .....	10
1.3.2    BCR and c-Abl Structure and Function.....	10
1.3.3    Bcr-Abl Structure and Function.....	12
1.3.4    Bcr-Abl Signalling Pathways.....	15
1.4    TREATMENT OF CML.....	18
1.4.1    Historical Treatments .....	18
1.4.2    Imatinib .....	19
1.4.3    Second Generation Tyrosine Kinase Inhibitors .....	21
1.5    RESISTANCE TO TYROSINE KINASE INHIBITION .....	22

1.5.1	<i>Dynamics of TKI inhibition of Bcr-Abl kinase and cell death</i>	22
1.5.2	<i>Dependence of CML cells on BCR-ABL</i>	25
1.5.3	<i>Overexpression and targeting of STAT5</i>	27
1.5.4	<i>Persistence of leukaemic stem cells following TKI therapy</i>	28
1.5.5	<i>Haematopoietic Cytokines and their Contribution to LSC Persistence</i>	30
1.5.6	<i>Autophagy</i>	33
1.6	SUMMARY AND PROJECT AIMS	36
1.6.1	<i>Summary</i>	36
1.6.2	<i>Project Aims</i>	36
<b>CHAPTER 2.</b>		<b>38</b>
<b>MATERIALS AND METHODS</b>		<b>38</b>
2.1	COMMONLY USED REAGENTS	39
2.2	SOLUTIONS BUFFERS & MEDIA	43
2.2.1	<i>Cell culture media</i>	43
2.2.2	<i>Hanks Balanced Salt Solution (HBSS)</i>	43
2.2.3	<i>Cryoprotectant mixture</i>	43
2.2.4	<i>Binding Buffer</i>	43
2.2.5	<i>1x Laemmli's buffer (modified)</i>	43
2.2.6	<i>MACS buffer</i>	44
2.2.7	<i>2.5% Membrane blocking solution</i>	44
2.2.8	<i>MethoCult™</i>	45
2.2.9	<i>Serum deprived media (SDM)</i>	45
2.2.10	<i>SDS-Polyacrylamide Gel</i>	46
2.2.11	<i>1 x TBS</i>	47
2.2.12	<i>1xTBST</i>	47
2.2.13	<i>Thaw solution</i>	47
2.2.14	<i>White cell fluid</i>	47

2.3	INHIBITORS.....	48
2.3.1	<i>Dasatinib</i> .....	48
2.3.2	<i>Imatinib (IM)</i> .....	48
2.3.3	<i>Nilotinib (NIL)</i> .....	48
2.3.4	<i>Chloroquine</i> .....	49
2.3.5	<i>Clarithromycin</i> .....	49
2.3.6	<i>3-Methyladenine</i> .....	49
2.3.7	<i>Pimozide</i> .....	49
2.3.8	<i>N'-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide (STAT5 inhibitor)</i> .....	50
2.3.9	<i>Ruxolitinib</i> .....	50
2.3.10	<i>U0126 (MEK1/2 inhibitor)</i> .....	50
2.4	GENERAL TECHNIQUES.....	51
2.4.1	<i>Maintenance of cell lines</i> .....	51
2.4.2	<i>Cell count and viability assessment</i> .....	51
2.4.3	<i>Lymphoprep isolation of mononuclear cells (MNC)</i> .....	51
2.4.4	<i>Magnetic Cell Sorting (MACS)</i> .....	52
2.4.5	<i>Fluorescence Activated Cell Sorting (FACS)</i> .....	53
2.4.6	<i>Cryopreservation of cells</i> .....	53
2.4.7	<i>Thawing of Cells</i> .....	53
2.4.8	<i>Cell counts and viability</i> .....	54
2.5	SPECIAL TECHNIQUES .....	55
2.5.1	<i>Drug Treatments and Washing Protocols</i> .....	55
2.5.2	<i>Flow cytometry to assess the cell death</i> .....	55
2.5.3	<i>Flow cytometry to assess pSTAT5 (intracellular staining)</i> .....	57
2.5.4	<i>Western Blot following SDS-PAGE</i> .....	58
2.5.5	<i>Intracellular Uptake and Retention (IUR) assay</i> .....	60
2.5.6	<i>Analysis of cellular morphology</i> .....	60

2.5.7	<i>Full Moon BioSystems Antibody Microarray</i> .....	61
2.5.8	<i>Statistical Analyses</i> .....	62
<b>CHAPTER 3.</b> .....		<b>63</b>
<b>INDUCTION OF AUTOPHAGY BY TYROSINE KINASE INHIBITION IN <i>BCR-ABL1</i>+ CELLS PREVENTS CELL DEATH</b> .....		<b>63</b>
3.1	INTRODUCTION .....	64
3.1.1	<i>Approach</i> .....	67
3.1.2	<i>Summary and Research Contribution</i> .....	67
3.1.3	<i>Publications</i> .....	68
3.1.4	<i>Conference Presentations</i> .....	68
3.2	RESULTS .....	70
3.2.1	<i>Inhibition of Bcr-Abl with TKIs induces markers of autophagy in KU812 cells</i> .....	70
3.2.2	<i>TKI-induced loss of Bcl-2 and Bcl-x<sub>L</sub> expression in KU812 cells allows release of Beclin-1</i> 70	
3.2.3	<i>Increase in vacuoles induced by autophagy inhibitor CQ in combination with dasatinib</i> 73	
3.2.4	<i>Upregulation of autophagy-associated kinase LKB1 following dasatinib treatment</i> .....	73
3.2.5	<i>Downregulation of PIM-3, MAP3K5 and CHEK2 kinases following dasatinib treatment</i> .77	
3.2.6	<i>Blocking autophagy sensitises BCR-ABL1+ cells to TKI-induced cell death</i> .....	82
3.2.7	<i>Clarithromycin enhances dasatinib-mediated cell death</i> .....	82
3.2.8	<i>Clarithromycin has no effect on pCrkL, and therefore Bcr-Abl, sensitivity to TKI treatment</i> 83	
3.2.9	<i>Blocking late stage autophagy results in an increase in LC3-II and the accumulation of vacuoles</i> .....	89
3.2.10	<i>Clarithromycin sensitises TKI-resistant BCR-ABL1+ cells to dasatinib treatment</i> .....	94
3.3	DISCUSSION .....	96
<b>CHAPTER 4.</b> .....		<b>105</b>

<b>SIGNALLING REQUIREMENTS FOR COMMITMENT TO CELL DEATH FOLLOWING TRANSIENT HIGH OR CONTINUOUS LOW DOSE DASATINIB.....</b>	<b>105</b>
4.1 INTRODUCTION .....	106
4.1.1 Approach .....	111
4.1.2 Summary and Research Contribution.....	111
4.1.3 Publication.....	112
4.1.4 Conference Presentations.....	112
4.2 RESULTS .....	113
4.2.1 Low dose dasatinib induces cell death despite minimal inhibition of Bcr-Abl kinase activity in the KU812 CML cell line .....	113
4.2.2 Low dose dasatinib induces apoptosis despite minimal inhibition of Bcr-Abl kinase activity in newly diagnosed CP-CML patients.....	116
4.2.3 Transient exposure to 100 nM dasatinib induces cell death despite complete reactivation of Bcr-Abl kinase activity.....	119
4.2.4 Bcr-Abl signalling resumes following the removal of dasatinib by the standard washout procedure. ....	123
4.2.5 Continuous exposure to 1 nM dasatinib results in gradual inhibition of Bcr-Abl signalling and concomitant activation of pro-apoptotic signalling. ....	128
4.2.6 Residual dasatinib is undetectable by intracellular uptake and retention (IUR) assay employing <sup>14</sup> C-dasatinib.....	129
4.3 DISCUSSION .....	137
<b>CHAPTER 5. ....</b>	<b>141</b>
<b>ASSESSMENT OF THE CRITICAL FACTORS INVOLVED IN COMMITMENT OF BCR-ABL1+ CELLS TO APOPTOSIS .....</b>	<b>141</b>
5.1 INTRODUCTION .....	142
5.1.1 Approach .....	144
5.1.2 Summary and Research Contribution.....	147

5.1.3	<i>Publications</i> .....	147
5.1.4	<i>Conference Presentations</i> .....	147
5.2	RESULTS .....	149
5.2.1	<i>Equilibration of cells in drug-free media prevents apoptosis induced by 30 min treatment with 100 nM dasatinib</i> .....	149
5.2.2	<i>Reactivation of Bcr-Abl signalling and prevention of apoptosis following 30 min exposure to dasatinib and optimal washout</i> .....	151
5.2.3	<i>Extension of the initial exposure to TKI for greater than 2 h induces cell death despite the complete removal of dasatinib</i> .....	151
5.2.4	<i>Induction of apoptosis markers following 8 h 100 nM dasatinib exposure followed by optimal washout, despite reactivation of STAT5 and Erk</i> .....	159
5.2.5	<i>Inhibition of JAK1/2, MEK/ERK or autophagy survival signally does not restore cell death following 30 min exposure to 100 nM dasatinib with OPT washout</i> .....	169
5.2.6	<i>Inhibition of STAT5 activation with pimozone and STAT5i maintains cell death following 100 nM dasatinib with 30 min optimal washout in BCR-ABL1+ cell lines</i> .....	173
5.2.7	<i>Inhibition of STAT5 activation with pimozone and STAT5i maintains cell death and impair colony formation following 30 min 100 nM dasatinib OPT wash in CP-CML CD34+ patient cells</i> 180	
5.2.8	<i>Following 30 min exposure to 100 nM dasatinib and pimozone, STAT5 remains inhibited despite complete removal of dasatinib</i> .....	185
5.3	DISCUSSION .....	195
<b>CHAPTER 6. ....</b>		<b>201</b>
<b>TARGETING CYTOKINE PROTECTION FROM TKI-INDUCED CELL DEATH WITH THE PAN JAK INHIBITOR RUXOLITINIB.....</b>		<b>201</b>
6.1	INTRODUCTION .....	202
6.1.1	<i>Approach</i> .....	208
6.1.2	<i>Summary and Research Contribution</i> .....	208

6.1.3	<i>Publications</i> .....	209
6.1.4	<i>Conference Presentations</i> .....	209
6.2	RESULTS .....	210
6.2.1	<i>BCR-ABL1+ cell lines express IL-3 and GM-CSF receptors</i> .....	210
6.2.2	<i>Cytokines stimulate STAT5 activation in the presence of dasatinib</i> .....	210
6.2.3	<i>Newly diagnosed primary CP-CML CD34+ cells express IL-3 and GM-CSF receptors</i> ....	213
6.2.4	<i>Cytokines stimulate STAT5 activation in CP-CML CD34+ cells</i> .....	215
6.2.5	<i>Cytokines protect cells from cell death</i> .....	215
6.2.6	<i>Ruxolitinib inhibits JAK1 and JAK2 activation in BCR-ABL1+ cell lines</i> .....	219
6.2.7	<i>Ruxolitinib blocks cytokine-dependent activation of STAT5, but not Bcr-Abl-dependent activation of STAT5 in BCR-ABL1+ cell lines</i> .....	223
6.2.8	<i>Ruxolitinib blocks cytokine-dependent activation of STAT5, but not Bcr-Abl-dependent activation of STAT5 in CP-CML CD34+ cells</i> .....	224
6.2.9	<i>Ruxolitinib has little effect as a sole agent or in combination with dasatinib in the absence of cytokines, but abrogates cytokine protection of dasatinib-induced cell death</i> .....	224
6.2.10	<i>In primary CP-CML CD34+ cells ruxolitinib removes cytokines protection from dasatinib-induced cell death, having no additional effect to dasatinib treatment in the absence of cytokines</i> .....	228
6.2.11	<i>Ruxolitinib impairs the ability of CP-CML CD34+ cells to form colonies in the presence of 5GF</i> 231	
6.3	DISCUSSION .....	235
<b>CHAPTER 7</b> .....		<b>239</b>
<b>GENERAL DISCUSSION</b> .....		<b>239</b>
7.1	MAJOR FINDINGS .....	241
7.1.1	<i>Deactivation of STAT5 is critical for the induction of cell death in situations of low level or transient Bcr-Abl kinase inhibition</i> .....	241

7.1.2	<i>STAT5 is a critical component of the time-dependent sensitivity of BCR-ABL1+ cells to TKI treatment</i>	243
7.1.3	<i>TKI-induced autophagy occurs by inhibiting Bcr-Abl driven survival signalling and may involve energy sensing pathways</i>	246
7.1.4	<i>Tyrosine kinase inhibition of Bcr-Abl induces autophagy by activating stress response pathways</i>	246
7.1.5	<i>Combination treatment of TKI with clarithromycin targets autophagy in BCR-ABL1+ cells</i>	248
7.1.6	<i>JAK2 activation of STAT5 occurs in a cytokine-dependent, but Bcr-Abl-independent manner</i>	249
7.2	FUTURE DIRECTIONS	253
7.3	CONCLUSION	254
<b>CHAPTER 8.</b>		<b>255</b>
<b>APPENDICES</b>		<b>255</b>
APPENDIX I		256
APPENDIX II		258
APPENDIX III		260
<b>CHAPTER 9.</b>		<b>261</b>
<b>REFERENCES</b>		<b>261</b>
<b>PUBLICATIONS ARISING FROM THESIS</b>		<b>277</b>

# **Table of Figures**

<i>Figure 1.1: Haematopoiesis and the hierarchy of CML progenitors</i> .....	4
<i>Figure 1.2: History of CML</i> .....	6
<i>Figure 1.3: Progression of disease in CML</i> .....	9
<i>Figure 1.4: Philadelphia chromosome translocation</i> .....	11
<i>Figure 1.5: Structure of BCR, Abl and Bcr-Abl proteins</i> .....	13
<i>Figure 1.6: c-Abl conformation and control of activation</i> .....	14
<i>Figure 1.7: Key Bcr-Abl Signalling Pathways</i> .....	17
<i>Figure 1.8: Historical Treatments</i> .....	20
<i>Figure 1.9: Oncogenic Shock</i> .....	26
<i>Figure 1.10: Initiation of autophagy</i> .....	35
<i>Figure 2.1: Schematic of the standard and optimal washout procedures</i> .....	56
<i>Figure 3.1: Model of the autophagic process</i> .....	66
<i>Figure 3.2: Markers of Autophagy</i> .....	71
<i>Figure 3.3: Induction of Autophagy Following TKI treatment in KU812 cells</i> .....	72
<i>Figure 3.4: Imatinib and nilotinib decrease Bcl-2, preventing sequestration of Beclin-1</i> .....	74
<i>Figure 3.5: Dasatinib increases expression of Beclin-1 and decreases expression of Bcl-2 and Bcl-xL</i> .....	75
<i>Figure 3.6: CQ in combination with dasatinib results in accumulation of vacuoles</i> .....	76
<i>Figure 3.7: Kinase upregulation by dasatinib</i> .....	78
<i>Figure 3.8: LKB1 is increased following exposure to TKIs</i> .....	79
<i>Figure 3.9: Downregulation of kinases by dasatinib</i> .....	80
<i>Figure 3.10: Effect of TKI treatment on Pim3, ASK1 and Chk2</i> .....	81
<i>Figure 3.11: CQ sensitizes BCR-ABL1+ cells to TKI-induced cell death</i> .....	84
<i>Figure 3.12: CAM induces minimal cell death as a sole agent</i> .....	85
<i>Figure 3.13: CAM and CQ but not 3-MA enhances dasatinib-induced cell death in BCR-ABL1+ cells</i> .....	86
<i>Figure 3.14: Bcr-Abl sensitivity to dasatinib is not affected by CAM</i> .....	87
<i>Figure 3.15: Bcr-Abl sensitivity to imatinib is not affected by CAM</i> .....	88
<i>Figure 3.16: Addition of CAM or CQ to Dasatinib Increased LC3-II in K562 cells</i> .....	91
<i>Figure 3.17: CAM, but not 3-MA, induces vacuole formation in K562 cells treated with dasatinib</i> .....	93
<i>Figure 3.18: CAM enhances dasatinib-induced cell death in K562 Dox V299L cells</i> .....	95
<i>Figure 3.19: Inhibition of Bcr-Abl and dissociation of Beclin-1</i> .....	99

<i>Figure 3.20: Proposed mechanism of TKI-induced autophagy in CML</i> .....	102
<i>Figure 3.21: Blockade of TKI-induced autophagy by CAM</i> .....	104
<i>Figure 4.1: Balance of survival and apoptosis signals</i> .....	110
<i>Figure 4.2: KU812 viability following exposure to a range of dasatinib concentrations.</i> .....	114
<i>Figure 4.3: Bcr-Abl activity in KU812 cells as expressed as a percentage of pCrkL</i> .....	115
<i>Figure 4.4: Viability and colony forming ability of primary CP-CML CD34+ cells following exposure to a range of dasatinib concentrations.</i> .....	117
<i>Figure 4.5: Bcr-Abl activity in primary PB-MNCs CP-CML cells as measured by pCrkL/CrkL (IC50)</i> .....	118
<i>Figure 4.6: Transient (30 min) treatment with dasatinib induces cell death</i> .....	121
<i>Figure 4.7: Bcr-Abl is active following transient high and continuous low dose dasatinib treatments.</i> .....	122
<i>Figure 4.8: Dasatinib-induced transient inhibition of Bcr-Abl results in continuous inhibition of STAT5</i> .....	125
<i>Figure 4.9: Dasatinib-induced transient inhibition of Bcr-Abl is coupled with inhibition of pro-survival proteins and induction of pro-apoptotic markers.</i> .....	127
<i>Figure 4.10: Gradual inhibition of Bcr-Abl signalling by 1nM dasatinib.</i> .....	132
<i>Figure 4.11: Continuous exposure to 1 nM dasatinib induces inhibition of pro-survival proteins and induction of pro-apoptotic markers</i> .....	134
<i>Figure 4.12: Dasatinib is undetectable following standard washout</i> .....	135
<i>Figure 4.13: Dasatinib IUR limit of detection is <math>\leq 50</math>nM</i> .....	136
<i>Figure 5.1: Schematic of the standard and optimal washout procedures.</i> .....	145
<i>Figure 5.2: Optimal washout procedure prevents cell death in BCR-ABL1+ cells.</i> .....	150
<i>Figure 5.3: Reactivation of STAT5 following optimal washout</i> .....	152
<i>Figure 5.4: Optimal washout prevents loss of Bcl-2 family members and induction of apoptosis markers</i> .....	153
<i>Figure 5.5: Extension of dasatinib exposure prior to OPT washout induces cell death in BCR-ABL1+ cell lines.</i> .....	156
<i>Figure 5.6: Extension of imatinib exposure prior to OPT washout induces cell death in BCR-ABL1+ cell lines.</i> .....	158
<i>Figure 5.7: Extension of 100 nM dasatinib exposure prior to OPT washout induces cell death and impairs clonogenic potential in CP-CML CD-34+ patient cells.</i> .....	161
<i>Figure 5.8: Extension of imatinib exposure prior to OPT washout induces cell death and impairs clonogenic potential in CP-CML CD-34+ patient cells.</i> .....	163
<i>Figure 5.9: Extension of Bcr-Abl inhibition results in the inhibition of pSTAT5 and pErk prior to OPT washout.</i> .....	166

<i>Figure 5.10: Extension of Bcr-Abl inhibition induces apoptosis markers and inhibits Mcl-1 and Bcl-xL.....</i>	<i>167</i>
<i>Figure 5.11: Extension of Bcr-Abl inhibition prior to OPT washout inhibits pSTAT5.....</i>	<i>168</i>
<i>Figure 5.12: MEK/Erk inhibition had no effect on cell viability when used in combination with 30 min 100 nM dasatinib exposure followed by OPT wash in KU812 cells.....</i>	<i>170</i>
<i>Figure 5.13: Inhibition of autophagy had no effect on cell viability when used in combination with 30 min 100 nM dasatinib exposure followed by OPT wash in KU812 cells. ....</i>	<i>171</i>
<i>Figure 5.14: JAK1/2 inhibition had no effect on cell viability when used in combination with 30 min 100 nM dasatinib exposure followed by OPT wash in KU812 cells.....</i>	<i>172</i>
<i>Figure 5.15: Combination of STAT5 inhibition, with 30 min 100 nM dasatinib OPT wash, induces cell death in KU812 cells. ....</i>	<i>175</i>
<i>Figure 5.16: Combination of STAT5 inhibition, with 30 min 100 nM dasatinib OPT wash induces cell death in Meg01 cells. ....</i>	<i>177</i>
<i>Figure 5.17: Combination of STAT5 inhibition, with 30 min 100 nM dasatinib OPT wash induces cell death in K562 cells. ....</i>	<i>179</i>
<i>Figure 5.18: STAT5 inhibition with pimoziide and 30 min dasatinib 30 min OPT wash exposure induces cell death in CP-CML CD34+ patient cells.....</i>	<i>182</i>
<i>Figure 5.19: STAT5i in combination with 30 min 100 nM dasatinib OPT wash induces cell death in CP CD34+ CML cells.....</i>	<i>184</i>
<i>Figure 5.20: Pimoziide in combination with 30 min imatinib induces cell death following the OPT wash. ....</i>	<i>188</i>
<i>Figure 5.21: STAT5i in combination with 30 min imatinib induces cell death following the OPT wash. ....</i>	<i>190</i>
<i>Figure 5.22: Pimoziide in combination with 30 min 100 nM dasatinib exposure inhibits pSTAT5.....</i>	<i>191</i>
<i>Figure 5.23: Downstream targets of STAT5, Bcl-xL and Mcl-1, are inhibited in the presence of pimoziide. ....</i>	<i>192</i>
<i>Figure 5.24: Pimoziide inhibits pSTAT5 in primary CP-CML CD34+ patient cells following complete removal of dasatinib.....</i>	<i>193</i>
<i>Figure 5.25: STAT5i inhibits pSTAT5 in primary CP-CML CD34+ patient cells following complete removal of dasatinib.....</i>	<i>194</i>
<i>Figure 5.26: Proposed activation of STAT5 .....</i>	<i>200</i>
<i>Figure 6.1 Normal Haematopoietic Cytokine Signalling Pathways.....</i>	<i>207</i>
<i>Figure 6.2: Expression of IL-3R<math>\alpha</math> and GM-CSFR<math>\alpha</math> in BCR-ABL1+ cell lines. ....</i>	<i>212</i>
<i>Figure 6.3: Cytokine receptor activation of STAT5 signalling in BCR-ABL1+ cell lines .....</i>	<i>214</i>
<i>Figure 6.4: Expression of IL-3R<math>\alpha</math>, GM-CSFR<math>\alpha</math> and the <math>\beta</math> common chain in CP-CML CD34+ cells.....</i>	<i>216</i>
<i>Figure 6.5: Cytokine receptor activation of STAT5 signalling in CP-CML CD34+ cells.....</i>	<i>218</i>

<i>Figure 6.6: Cytokine protection from TKI-induced death in BCR-ABL1+ cell lines.....</i>	<i>220</i>
<i>Figure 6.7: The structure and activity of JAK inhibitor ruxolitinib in comparison to other JAK2 inhibitors..</i>	<i>221</i>
<i>Figure 6.8: Dose-dependent inhibition of JAK1, JAK2 and STAT3, but not Bcr-Abl or STAT5, with ruxolitinib in KU812 cells. ....</i>	<i>222</i>
<i>Figure 6.9: Ruxolitinib demonstrates minimal reduction of Bcr-Abl dependent pSTAT5 in KU812 cells. ....</i>	<i>225</i>
<i>Figure 6.10: Pan JAK inhibition removes cytokine stimulated pSTAT5 but not Bcr-Abl-dependent pSTAT5 in KU812 cells.....</i>	<i>226</i>
<i>Figure 6.11: Ruxolitinib reduces cytokine stimulated pSTAT5 but not Bcr-Abl-dependent pSTAT5 in CP-CML CD34+ cells. ....</i>	<i>227</i>
<i>Figure 6.12: Cytokine protection of CP-CML CD34+ cells from dasatinib-induced cell death is blocked by ruxolitinib. ....</i>	<i>229</i>
<i>Figure 6.13: Ruxolitinib abrogates cytokine-dependent protection from dasatinib-induced cell death, but does not further increase cell death compared to dasatinib alone. ....</i>	<i>230</i>
<i>Figure 6.14: JAK inhibition prevents colony formation in the presence of 5GF.....</i>	<i>233</i>
<i>Figure 6.15: Non-specific effect of ruxolitinib on clonogenic potential of normal CD34+ cells. ....</i>	<i>234</i>
<i>Figure 6.16: Proposed cytokine- and Bcr-Abl-dependent signalling in CML.....</i>	<i>238</i>
<i>Figure 7.1: Proposed STAT5 and autophagy signalling .....</i>	<i>250</i>

# **Publications**

## **Publications**

**Schafranek L**, Leclercq TM, White DL and Hughes TP. Clarithromycin enhances dasatinib-induced cell death in chronic myeloid leukemia cells, by inhibition of late stage autophagy. *Leuk Lymphoma* 2013; 54: 198–201.

**Schafranek L**, Nievergall E, Powell JA, Hiwase DK, White DL and Hughes TP. Sustained inhibition of STAT5, but not JAK2, is essential for TKI-induced cell death in chronic myeloid leukemia. *Leukemia advance online publication*, June 27, 2014; doi:10.1038/leu.2014.156; accepted article preview online May 12, 2014.

## **Conference Presentations**

### **Oral Presentations**

**Schafranek L**, Nievergall E, Powell JA, Hiwase DK, Leclercq T, Hughes TP and White DL. New evidence that transient Bcr-Abl inhibition commits cells to death in a time- and STAT5-dependent manner despite reactivation of Bcr-Abl. NDLR, March 2014, Noosa, QLD (oral presentation)

**Schafranek L**. Cannibalistic Cancer. 3 Minute Thesis Competition, July 2013, University of Adelaide, Australia (oral presentation). **Faculty Finalist**

**Schafranek L**, Leclercq TM, White DL and Hughes TP. Macrolide Antibiotic Clarithromycin targets TKI-induced autophagy in CML cells. HAA Oct, 2012 Melbourne, Australia (oral presentation).

**Schafranek L**, Leclercq TM, White DL and Hughes TP. Overcoming resistance to tyrosine kinase inhibitors in chronic myeloid leukaemia by blocking autophagy with clarithromycin. Medical Staff Society Research Prize, May, 2013, Adelaide, Australia (oral presentation).

**Schafranek L**, Hiwase H, Powell J, Melo J, White D, Hughes T. Constant Exposure to Low Dose Dasatinib Is Sufficient for Induction of Apoptosis in CML Cells. HAA Oct, 2011 Sydney, Australia (oral presentation) **Awarded HAA 2011 non-member travel grant.**

#### Poster Presentations

**Schafranek L**, Nievergall E, Hiwase H, Powell J, White D, Hughes T. Direct inhibition of STAT5 in combination with transient Bcr-Abl inhibition commits cells to apoptosis despite reactivation of Bcr-Abl. ASH Dec 2013, New Orleans, USA (poster presentation).

#### **ASH Abstract Achievement Award**

**Schafranek L**, Nievergall E, Hiwase H, Powell J, White D, Hughes T. STAT5 inhibition is critical to the commitment of chronic myeloid leukemia cells to apoptosis regardless of Bcr-Abl reactivation. CPCM Symposium, August 2013, National Wine Centre, Adelaide Australia (poster presentation)

**Schafranek L**, Nievergall E, Hiwase H, Powell J, White D, Hughes T. Inhibition of activated STAT5 sensitizes chronic myeloid leukemia cells to TKI treatment and commits cells to apoptosis despite reactivation of Bcr-Abl, independent of JAK1/2. FHS

Conference, July 2013, University of Adelaide, Australia (poster presentation). **Awarded Florey Medical Research Foundation Prize**

**Schafranek L**, Nievergall E, Hiwase H, Powell J, White D, Hughes T. Commitment of CML Cells to Apoptotic Cell Death Depends On the Length of Exposure to Das and the Level of STAT5 Activity. ASH Dec 2012, Atlanta, USA (poster presentation)

**Schafranek L**, Leclercq TM, White DL and Hughes TP. Clarithromycin increases the sensitivity of chronic myeloid leukaemia cells to dasatinib. FHS conference, August 2012, University of Adelaide, Australia (poster presentation).

**Schafranek L**, Leclercq TM, White DL and Hughes TP. Clarithromycin Enhances TKI-Induced Cell Death In CML Cells. NDLR March, 2012, Sunshine Coast, Australia (poster presentation).

**Schafranek L**, Leclercq TM, White DL and Hughes TP. Clarithromycin increases the sensitivity of chronic myeloid leukaemia cells to dasatinib. CPCM Symposium, July 2012, National Wine Centre, Adelaide, Australia (poster presentation).

**Schafranek L**, Hiwase H, Powell J, Melo J, White D, Hughes T. Blocking Cytokine Signalling Along with Intense BCR-ABL Kinase Inhibition may be necessary to Eradicate CML cells. Health Sciences Postgraduate Research Conference Aug. 2011, Adelaide, Australia (poster presentation)

# **Scholarships and Awards**

## **American Society of Hematology Abstract Achievement Award**

For the abstract entitled "Direct inhibition of STAT5 in combination with transient Bcr-Abl inhibition commits cells to apoptosis despite reactivation of Bcr-Abl. ASH Annual Conference, New Orleans, USA.

## **Florey Medical Research Foundation Prize**

For the presentation of the abstract entitled "Inhibition of activated STAT5 sensitizes chronic myeloid leukemia cells to TKI treatment and commits cells to apoptosis despite reactivation of Bcr-Abl, independent of JAK1/2", FHS conference, Adelaide, Australia

## **Faculty of Health Science, 3 Minute Thesis Finalist**

For the presentation entitled "Cannibalistic Cancer", University of Adelaide, 2013

## **Medical Staff Society Research Prize Finalist**

For the presentation entitled "Overcoming resistance to tyrosine kinase inhibitors in chronic myeloid leukaemia by blocking autophagy with clarithromycin", Royal Adelaide Hospital Medical Staff Society, Adelaide, Australia 2013.

### **Hematology Society of Australia and New Zealand non-member travel grant**

Awarded on the basis of abstract for work of exceptional novelty and significance. For the abstract entitled “Constant Exposure to Low Dose Dasatinib Is Sufficient for Induction of Apoptosis in CML Cells”, HAA Annual Conference, 2011 Sydney, Australia

### **PhD Scholarship, Leukaemia Foundation of Australia 2010-2013**

Support for the educational and professional development of researchers and other professionals undertaking a PhD. The award is to support research in Australia into the causes, treatment and care of people with leukaemia, lymphoma, myeloma and related blood disorders and is awarded on the merits of the applicant and project proposal.

# **Abbreviations**

**µg** – Microgram/s

**µL** – Microlitre/s

**µM** – Micromolar

**14-C** – Carbon-14 radioactive isotope

**3-MA** – 3-Methyladenine

**7-AAD** – 7-Aminoactinomycin D

**Ab** – Antibody

**ABL1** – Abelson murine leukaemia virus human homologue 1 gene

**ACD** – Acid Citrate Dextrose Acid

**Akt** – a serine threonine kinase also known as protein kinase B

**ALL** – Acute Lymphoblastic Leukaemia

**AMPK** – AMP-activated protein kinase

**-AP** – Alkaline Phosphatase Conjugated Antibody

**AP** – Accelerated Phase

**APS** – Ammonium Persulfate

**Ara-C** – Arabinofuranosyl Cytidine

**ATCC** – American Type Tissue Culture Collection

**ASK1** – Apoptosis signal-regulating kinase 1

**ATP** – Adenosine Triphosphate

**BAD** – Bcl-X<sub>L</sub>/Bcl-2 associated death promoter

**BAX** – Bcl-2 associated X protein

**Bcl-X<sub>L</sub>** – B-cell lymphoma extra large

**Bcl-2** – B-cell lymphoma 2

**BC** – Blast Crisis

**BCR** – Breakpoint Cluster region

**BCR-ABL1** – BCR-ABL1 oncogene

**Bcr-Abl** – Bcr-Abl oncoprotein

**Bim** – Bcl-2 interacting mediator of cell death

**BM** – Bone Marrow

**BSA** – Bovine Serum Albumin

**C** – Degrees Celsius

**CAM** – clathromycin

**CCyR** – Complete Cytogenetic Remission

**CFU** – Colony forming unit

**CFU-GM** – Colony forming unit granulocytes and macrophage

**CFC** – Colony forming cells

**Chk2** – checkpoint kinase 2

**CML** – Chronic Myeloid Leukaemia

**CP** –Chronic Phase

**CO<sub>2</sub>** – carbon dioxide

**CrkL** – C1T10 regulator of kinase like

**Ctrl** – control

**CQ** – chloroquine

**Das** – Dasatinib

**DMSO** – Dimethyl Sulphoxide

**DNA** – Deoxyribonucleic Acid

**EDTA** – Ethylenediaminetetraacetic Acid

**Erk** – Extracellular signal related kinase

**e.g.** – exempli gratia

***et al.*** – et alia

**FACS** – Fluorescence Activated Cell Sorting

**FBS** – Foetal Bovine Serum

**FDA** – Food and Drug Administration

**FSC** – Forward scatter

**FLT-3 ligand** – FMS-like tyrosine kinase 3 ligand

**Gab2** – GRB2-associated-binding protein 2

**G-CSF** – granulocyte-colony stimulating factor

**GF** – growth factor

**5GF** – five haematopoietic growth factors (G-CSF, GM-CSF, IL-3, IL-6, SCF)

**6GF** – six haematopoietic growth factors (G-CSF, IL-3, IL-6, SCF, TPO and Flt3-ligand)

**GM-CSF** – Granulocyte Macrophage Colony-Stimulating Factor

**GMP** – Granulocyte macrophage progenitors

**Grb2** – Growth factor receptor-bound protein 2

**Glut3** – glucose transporter 3

**h** – Hour/s

**HBSS** – Hanks Balanced Salt Solution

**HSC** – Haemopoietic stem cells

**HSCT** – Haemopoietic stem cell transplantation

**IC50** – Inhibitory Concentration 50

**i.e.** – id est

**IFN** – Interferon **IM** – Imatinib **IUR** –

**IL-3** – Interleukin-3

**IL-6** – Interleukin-6

**IM** – Imatinib mesylate

**IMDM** I– scove's modification of Dulbecco's medium.

**IUR** – intracellular uptake and retention assay

**kD** – kilo Dalton

**KD** – Kinase Domain

**JAK** – Janus Kinase

**JAKi** – ruxolitinib, pan JAK inhibitor

**JNK** – c-Jun N-terminal kinase

**L** – Litre/s

**LC3** – Microtubule-associated protein 1A/1B-light chain 3

**LKB1** – liver kinase B1

**M** – Molar

**mA** – mili Amp (10<sup>-3</sup> Amps)

**MACS** – Magnetic activated cell sorting

**MAPK** – Mitogen activated protein kinase

**Mcl-1** – myeloid cell leukemia sequence 1

**MFI** – Mean Fluorescence Intensity

**mg** – milligram/s

**min** – Minutes/s

**mL** – Millilitre/s

**mM** – Millimolar

**MMR** – Major Molecular Response

**MNC/s** – Mononuclear Cell/s

**mRNA** – messenger RNA

**mTOR** – mammalian target of rapamycin

**MW** – Molecular Weight

**ng** – Nanogram/s

**NIL** – Nilotinib

**nM** – Nanomolar

**O<sub>2</sub>** – oxygen

**OPT** – optimal

**p-** – Phosphorylated Form of Protein

**p62** – sequestosome 1 (SQSTM1)

**PAGE** – Polyacrylamide Gel Electrophoresis

**PARP** – Poly (ADP-ribose) polymerase

**PB** – Peripheral Blood

**PBMNC/s** – Peripheral Blood Mononuclear Cell/s

**PBS** – Phosphate Buffered Saline

**PDGFR** – Platelet-Derived Growth Factor Receptor

**PE** – Phycoerythrin

**Ph** – Philadelphia Chromosome

**PI3-K** – Phosphatidylinositol – 3-kinase

**Pim** – serine/threonine kinase

**P-loop** – Phosphate binding loop

**p-value** – Probability Value

**PVDF** – Polyvinylidene Difluoride

**Pz** – pimozide

**rcf** – Relative Centrifugal Force

**RPMI** – Roswell Park Memorial Institute (media)

**RNA** – Ribonucleic Acid

**rpm** – Revolutions Per Minute

**STAT5** – Signal Transducer and Activator of Transcription 5

**STAT5i** – inhibitor of Signal Transducer and Activator of Transcription 5 r

**SCF** – Stem cell factor

**SC** – side scatter

**SDS** – Sodium Dodecyl Sulphate

**sec** – second/s

**SEM** – Standard Error of the Mean

**SH** – Src Homology Region

**S/N** – Supernatant

**STD** – standard

**TBS** – Tris Buffered Saline

**TBST** – Tris Buffered Saline + Tween<sup>®</sup>20

**TKI/s** – Tyrosine Kinase Inhibitor/s

**Tyr** – Tyrosine

**U** – Units

**U/mL** – Units Per Millilitre

**Wash** – drug washout

**WCF** – White Cell Fluid

## Acknowledgments

One does not complete a PhD thesis without a great deal of encouragement, guidance and support, so there are a few people whom I would like to acknowledge and thank.

Thank you to my supervisors Tim Hughes, Deb White and Junia Melo. Early on in my PhD, I would often go into a meeting with my supervisors excited about my data and what it meant, only to come out overwhelmed with critiques and further experiments to perform and questions to answer. However, despite my initial misgivings to this process, I have fond memories of the challenging conversations and debates coming from those meetings. As mentioned by John Rasko in his special oration at NDLR 2012, Tim is one of the nicest guys in research: you have always challenged me to achieve my best and your clinical perspective has been invaluable. From the moment I came to this lab, Deb you have been an amazing mentor. You saw something in me, that at the time I couldn't see; the potential to be a great scientist and researcher. I finally feel like I can live up to that potential. Thank you for believing in me even when I didn't and pushing me to be a first-class researcher. You may still get that confused look when I reverse my sentence order after I get overly excited over a result or expressing a new theory, but hopefully I get it the right way around more often than not these days.

To the members of the Melissa White Lab past and present, you are a dynamic bunch, who not only supported me in my research but also in my fundraising efforts to raise over \$4000 for the Leukaemia Foundation of Australia. Thank you for your encouragement and friendship over the past years (and for putting up with my aimless humming in the lab). However, there are a few people whose influence throughout my PhD experience I would like to specifically thank.

Eva, when you came to our lab I was in the first year of my PhD and feeling hopeless about the future of my project. Talking with you about my results, good or bad, small or large, made me excited about my research again. You are an incredible researcher and an amazing friend. I feel so fortunate that you were around to enhance my research and drive me to greater things.

A special thanks also goes to Verity, not only for saving many of my samples from running off gels; for sharing your incredible technical expertise; your willingness to stop whatever you are in the middle of to help me with an experiment or give advice; your

friendship and late night conversations during/after a long day of experiments (in particular those bloody timecourses which had me in the lab at all hours).

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I must also express my significant gratitude to the Leukaemia Foundation of Australia, who provided me with a generous scholarship for the duration of my PhD. This scholarship enabled me to attend a large number of prestigious national and international conferences and also eased the financial burden of undertaking full time study while trying to support myself.

To my extended family and special friends, who never failed to express their pride and encouragement in the research which I was undertaking, even if you only follow every other word of what I'm talking about. Thank you for forgiving my unanswered text messages and cancelled dinner plans; for giving support in my fundraising for the Leukaemia Foundation; and for just lending an ear. Specific kudos goes to Suzie for being on call and for making me feel like I could achieve anything; to my aunties Lyn and Robyn who have always been a huge support, and to Joybelles and Joanie who, without fail, shared amazing weekly home cooked dinners.

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Ever since I was little, my mum has instilled in me an infallible belief that I could do anything and always encouraged me to question everything, which may be why I ended up undertaking a PhD. This may have come back to bite her, as this now includes questioning her. Without her encouragement and support throughout my life I would not have been able to accomplish all that I have to date. Additionally, I give you huge “props” for reading my entire thesis for grammar and spelling. I’m glad that you now have at least a little bit of an understanding of what I’ve been up to the past few years. I also have an amazing big brother, who constantly provides me with food for thought on concepts relevant to my PhD and thankfully a range of other topics from philosophy to computer science to anime, but additionally provides me with delicious, nutritious food. I love you both so much and thank you for all that you have contributed to my life, above and beyond my PhD.

When I told my partner Pete I was considering undertaking honours and possibly a PhD, his response was along the lines of, “You want to be a student for how long?!” Nevertheless, you have always been unbelievably supportive. I have been to a few orations now, where recipients say something like, “I couldn’t have done this without the love and understanding of my wife who takes care of my life,” to which a close friend made the comment, “Where can I get one of those?” Well, I have been lucky enough to find myself a partner who is better than just a mere “wife” and words cannot express how grateful I am to have Pete in my life. Your understanding of my crazy hours and the lack of time I had for you at various stages of my PhD, from having dinner ready when I arrived home to having my car serviced, you have been unbelievable! I promise this ends my student days (for now) and that I’ll start earning a “proper” income so that you may pursue your dreams.

## **Abstract**

Chronic myeloid leukaemia (CML) is a clonal myeloid proliferative disease that results from constitutive activation of the Bcr-Abl oncoprotein, which disrupts normal cellular signalling potentiating the survival and maintenance of *BCR-ABL1*<sup>+</sup> cells. Tyrosine kinase inhibitors (TKIs), like imatinib, have revolutionised the treatment of CML and have become the model for therapy in other cancers. Imatinib treatment also founded the paradigm that potent and continuous dosing is required for optimal patient response in patients with CML. In contrast to imatinib, the second generation TKI dasatinib has a short half-life of only 3-5 h, nevertheless a once daily dosing regime is sufficient to achieve equivalent responses to twice daily dosing suggesting that continuous and complete inhibition of Bcr-Abl may not be required for optimal response to TKI therapy.

Despite initial studies indicating that a very brief exposure to a potent dose of TKI is sufficient to induce cell death in *BCR-ABL1*<sup>+</sup> cells, recent studies have attributed this to sustained low-level inhibition of Bcr-Abl signalling due to inadequate drug washout. As reported in this thesis, experiments with low dose dasatinib treatment, which does not completely inhibit Bcr-Abl phosphorylation but is sufficient to induce cell death, demonstrated inactivation of STAT5 as a sensitive measure of Bcr-Abl activity. Here, it was also confirmed that <1 h exposure to potent TKI with adequate drug washout is insufficient to commit *BCR-ABL1*<sup>+</sup> cells to death and it is established for the first time that at least 2 h of Bcr-Abl kinase inhibition are required. Furthermore, combinations of efficient TKI washout with specific inhibitors of STAT5, JAK and ERK ascertained sustained inhibition of pSTAT5, potentially independent of JAK2, as the determinant of commitment to cell death. Together, this research established that continuous,

complete inhibition of Bcr-Abl is not required to induce cell death, but that continuous blockade of STAT5, indicative of low-level threshold Bcr-Abl inhibition, is essential, thus challenging the imatinib paradigm.

Although most CML patients respond well to imatinib, only 40% of patients achieve a complete molecular response and some patients develop resistance. Blockade of Bcr-Abl signalling can drive cells to develop new survival mechanism, and amongst others, autophagy and the acquisition of extrinsic survival signalling have been implicated in resistance to therapy and/or persistent disease.

Studies presented in this thesis define a role for the activation of autophagy in response to tyrosine kinase inhibition of Bcr-Abl. Induction of autophagy by TKI was confirmed using established markers of autophagy, such as the conversion of LC3-I to LC3-II, degradation of p62 and cellular morphology. Blockade of anti-apoptotic proteins Bcl-2 and Bcl-xL along with activation of stress response pathways were revealed as potential mechanisms of autophagy induction, however, further investigation into these pathways is required. Importantly, the data presented here also established clarithromycin as a novel inhibitor of TKI-induced autophagy, advocating combination treatment with TKI therapy in resistant patients.

Recent observations that overexpression of cytokines and their receptors may contribute to *BCR-ABL1*<sup>+</sup> cell persistence in CML patients undergoing TKI therapy. Here, the expression of IL-3 and GM-CSF cytokine receptors in *BCR-ABL1*<sup>+</sup> cell lines and chronic phase CML CD34<sup>+</sup> progenitor cells was established and signalling through those

was confirmed to maintain STAT5 survival signalling, thereby protecting cells from TKI-induced death. Inhibition of JAK2 with ruxolitinib inhibited cytokine-dependent, but not Bcr-Abl-dependent, activation of STAT5 and neutralised cytokine-induced protection from cell death while having little effect in the absence of cytokines.

Together, the findings of this thesis established the critical mechanisms in Bcr-Abl-dependent and -independent signalling that may also be targeted in combination therapeutic approaches and provides an in-depth understanding of the potential clinical effectiveness of dose reductions during dasatinib therapy. These studies will have broad implications for the ongoing development of therapeutic strategies in CML, particularly in the setting of TKI-resistance, and will aid the goal of achieving a curative treatment for patients with CML.

# **CHAPTER 1.**

## **INTRODUCTION**

## 1.1 Haematopoiesis

Haematopoiesis is the generation and turnover of the cellular components of blood, commencing during embryonic development and functional throughout adult life.<sup>1</sup> In the developing embryo, haematopoietic stem cells (HSCs) are generated in the aorta-gonad-mesonephros, where they subsequently migrate to the foetal liver and crucially, to the bone marrow where they are retained throughout adulthood.<sup>2</sup> This bone marrow microenvironment, also referred to as the HSC niche, regulate intrinsic (i.e. transcription factors)<sup>3</sup> and extrinsic factors (i.e. cytokine/chemokines<sup>4-6</sup>, extracellular matrix<sup>7</sup> and stromal interactions<sup>8</sup>) which regulate cell fate decisions (e.g. maintenance of quiescence, differentiation, cell survival and death, self-renewal and migration etc.).

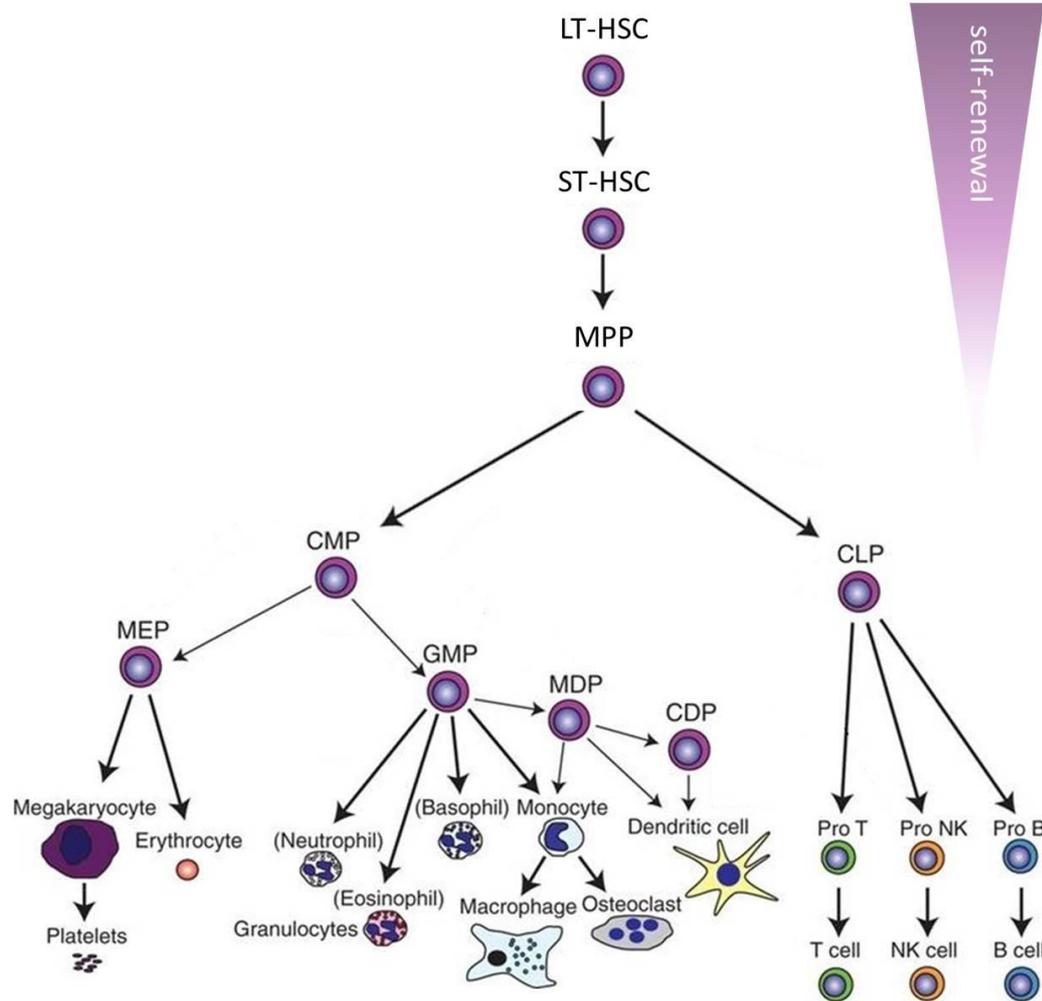
Pluripotent HSCs have the ability to produce cells with identical proliferative and long term self-renewal capacity,<sup>9</sup> they are able to sustain self-replication of approximately 50 divisions and only a small number are required to supply the haematopoietic system at any one time.<sup>10</sup> As such, they possess the potential to differentiate into multiple lineages comprising red blood cells, platelets and white blood cells (Figure 1.1).<sup>11</sup> The white blood cell compartment forms a hierarchical structure consisting of a continuum of cells which decrease in self-renewal ability and increase in proliferative potential as they mature. The first lineage commitment of HSCs is into either common myeloid progenitors (CMPs) or common lymphoid progenitors (CLPs). CLPs differentiate into T cells, B cells and natural killer (NK) cells and CMPs give rise to committed progenitors including platelets, erythrocyte, megakaryocyte, monocyte and granulocyte lineages.<sup>10</sup> In the granulocytic lineages, granulocyte macrophage progenitors (GMPs), generated from CMPs, subsequently differentiate into neutrophils, eosinophils and basophils.

## 1.2 Chronic Myeloid Leukaemia

### 1.2.1 History

Chronic myeloid leukaemia (CML) is a clonal myeloproliferative disease which arises in haematopoietic stem cells, first referred to in France in the early 1800s (Figure 1.2).<sup>12</sup> The first detailed description of CML is attributed to John Hughes Bennet who, in the *Edinburgh Medical and Surgical Journal* in 1845, comprehensively described enlargement of the spleen, liver and lymph nodes, which was attributed to suppuration of the blood as a thick pus-like substance was expressed from punctured veins.<sup>13</sup> Simultaneously in France<sup>14</sup> and Germany<sup>15</sup> additional observations of CML-like disease were described. Virchow coined the term “leukaemie” for the disorder, which roughly translates to “white blood”, referring to the expansion of white blood cells.<sup>15</sup> However, it took over a century until an abnormal “minute chromosome”, termed the Philadelphia (Ph) chromosome, was identified by Nowell and Hungerford in patients presenting with CML.<sup>16</sup>

Initially believed to be a predominantly myeloid disease, the Ph chromosome was subsequently observed in not only granulocyte lineages, but also in erythroid lineages in chronic phase CML (CP-CML). Thus, either multiple lineages had the ability to generate the Ph chromosome or the abnormality originated in, and was passed down from an HSC. Subsequent investigation into these concepts substantiated that indeed CML was of clonal and stem cell origin.<sup>17,18</sup>



**Figure 1.1: Haematopoiesis and the hierarchy of CML progenitors**

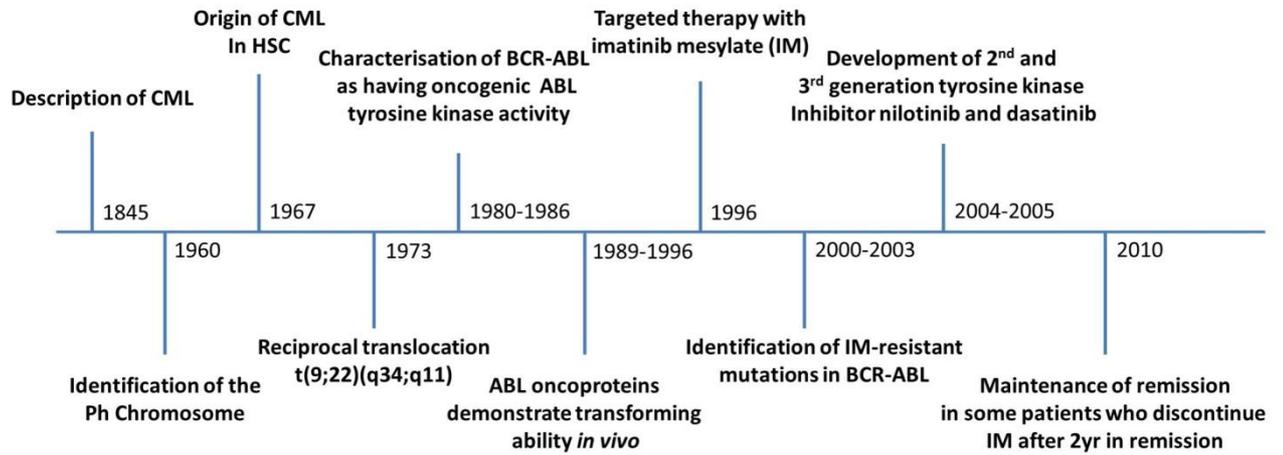
Haematopoiesis gives rise to HSCs which decrease in self-renewal ability with maturity.

As progenitors differentiate they become committed to either a myeloid (left arm) or lymphoid (right arm) lineage, thus populating the haematopoietic system with red blood cells, platelets and white blood cells. HSC = haematopoietic stem cell; LT-HSC = long term repopulating HSC; ST-HSC = short term repopulating HSC; MPP = multipotential progenitor; CMP = common myeloid progenitor; CLP = common lymphoid progenitor; MEP = megakaryocyte–erythrocyte progenitor; GMP = granulocyte–macrophage progenitor; CDP = common dendritic progenitor; MDP = monocyte–dendritic cell progenitor; NK = natural killer cell. Adapted from Rieger and Schroeder 2012.<sup>19</sup>

Although initial hypotheses suggested that the Ph chromosome was an abnormality of chromosome 21,<sup>16</sup> the establishment of banding techniques identified chromosome 22 as the aberrant Ph chromosome. Originally thought to be a deletion of the long arm of chromosome 22, vital research published by Janet Rowley in 1973,<sup>20</sup> established the Ph chromosome as a reciprocal translocation between chromosome 9 and chromosome 22 t(9;22). The isolation and description of the murine virus Abelson (*v-abl*)<sup>21</sup> and subsequent description of the translocation of *v-abl* on chromosome 9 to chromosome 22<sup>22</sup> was critical to understanding the oncogenic activity of Ph-positive cells. It was only in 1984 that the breakpoint cluster region (*bcr*) was observed on chromosome 22<sup>23</sup> and consequently a connection was identified between *BCR* and *ABL*<sup>24-26</sup> to constitute the fusion transcript responsible for pathogenesis of CML.<sup>27,28</sup>

### 1.2.2 Clinical Features

CML is asymptomatic in approximately 50% of newly diagnosed patients, presenting with nothing more distressing than fatigue, weight loss or abdominal pain; it is usually detected following routine blood tests. Clinically, CML is characterised by leukocytosis, splenomegaly (responsible for the abdominal discomfort), bone marrow granulopoietic hyperplasia and (predominantly neutrophils and myelocytes) primarily with basophilia and occasionally eosinophilia (Table 1.1).<sup>29</sup> Suppression of erythropoiesis is observed, and subsequently anaemia. In the majority of patients who present with CML, the Ph chromosome is the only lesion detected and must be detected by cytogenetic or molecular testing to confirm diagnosis.<sup>30</sup>



**Figure 1.2: History of CML**

From the first detailed descriptions in the mid-1800s, CML is now one of the most well studied malignancies and the model on which new targeted therapies are based.

Adapted from Wong and Witte 2004.<sup>31</sup>

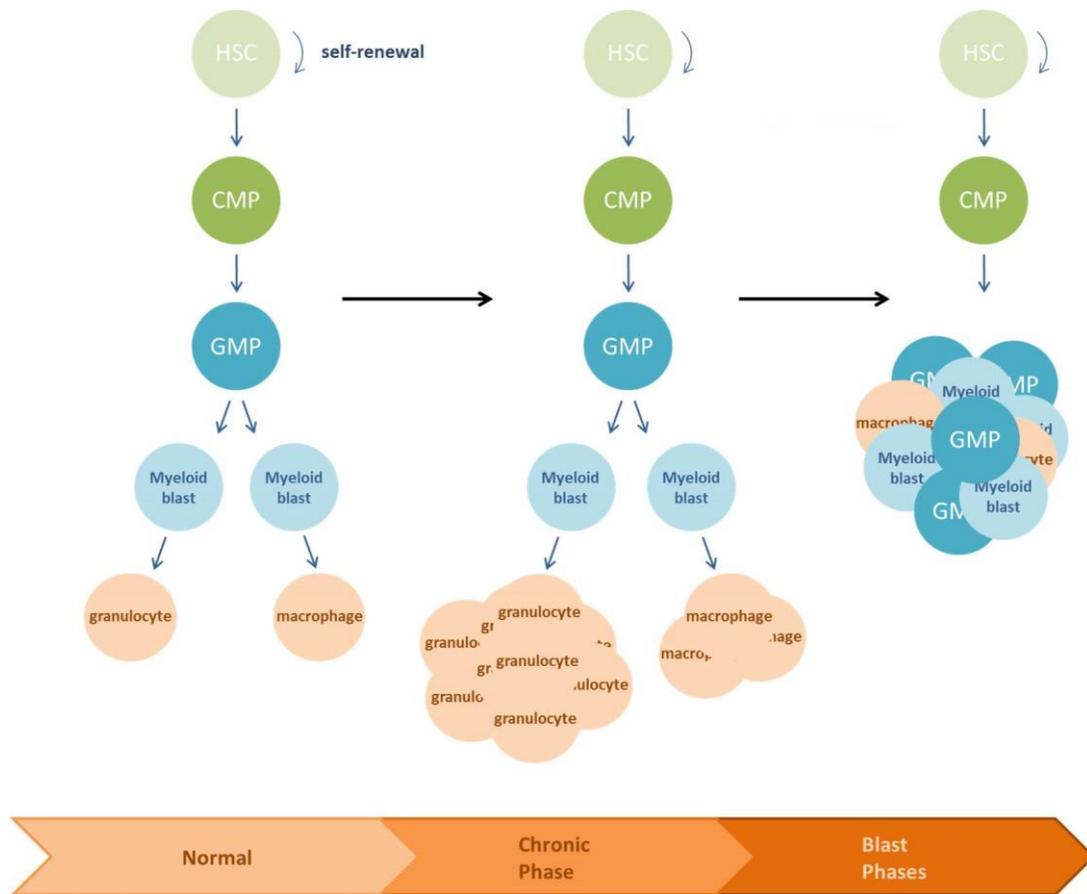
**Table 1.1: Clinical Features of CML**

(Adapted from Hasserjian 2010)

Peripheral blood findings	Bone marrow findings	Molecular genetic findings
Leukocytosis (median $100 \times 10^9/L$ )	Hypercellularity (usually 100%)	Karyotype: t(9;22)(q34;q11) translocation
Increased immature myeloid precursors	Increased myeloid:erythroid ratio (typically ~10:1)	Complex translocation involving 9q32 and 22q11
Eosinophilia	Increased myelocytes	FISH: Fusion of BCR and ABL1 loci
Basophilia	Basophilia	RT-PCR: Fusion transcript of BCR and ABL1
Often thrombocytosis	Small, hypolobated megakaryocytes	
	Lack of morphologic dysplasia of erythroid and myeloid elements	

### **1.2.3 Pathogenesis and Stages of Disease**

CML occurs in approximately 1-2 per 100,000 people and represents about 15-20% of all cases of adult leukaemias.<sup>32,33</sup> Most cases (~95%) of CML are diagnosed in the chronic phase (CP). Patients who are left untreated, would usually progress after approximately 3 to 6 years.<sup>34</sup> Of the CP patients (characterised by <15% immature progenitors, or blasts) who progress, approximately half transform directly into the blast crisis (BC) characterised by >30% blasts and the remainder do so following a period of accelerated phase (AP, 15-30% blasts).<sup>34</sup> Patients in the blastic phase have a median survival of only 3 to 9 months (Figure 1.3).<sup>34</sup>



**Figure 1.3: Progression of disease in CML**

In non-malignant systems, HSCs differentiate into multiple lineages of the blood system.

In chronic phase CML, differentiation is driven down a myeloid lineage resulting in

expansion of granulocytes. In advanced phase and blast crisis CML, differentiation is

halted at the granulocyte macrophage progenitor (GMP) resulting in rapid expansion of undifferentiated precursor cells and is more archetypal of an acute leukaemia.

## 1.3 Molecular Biology of CML

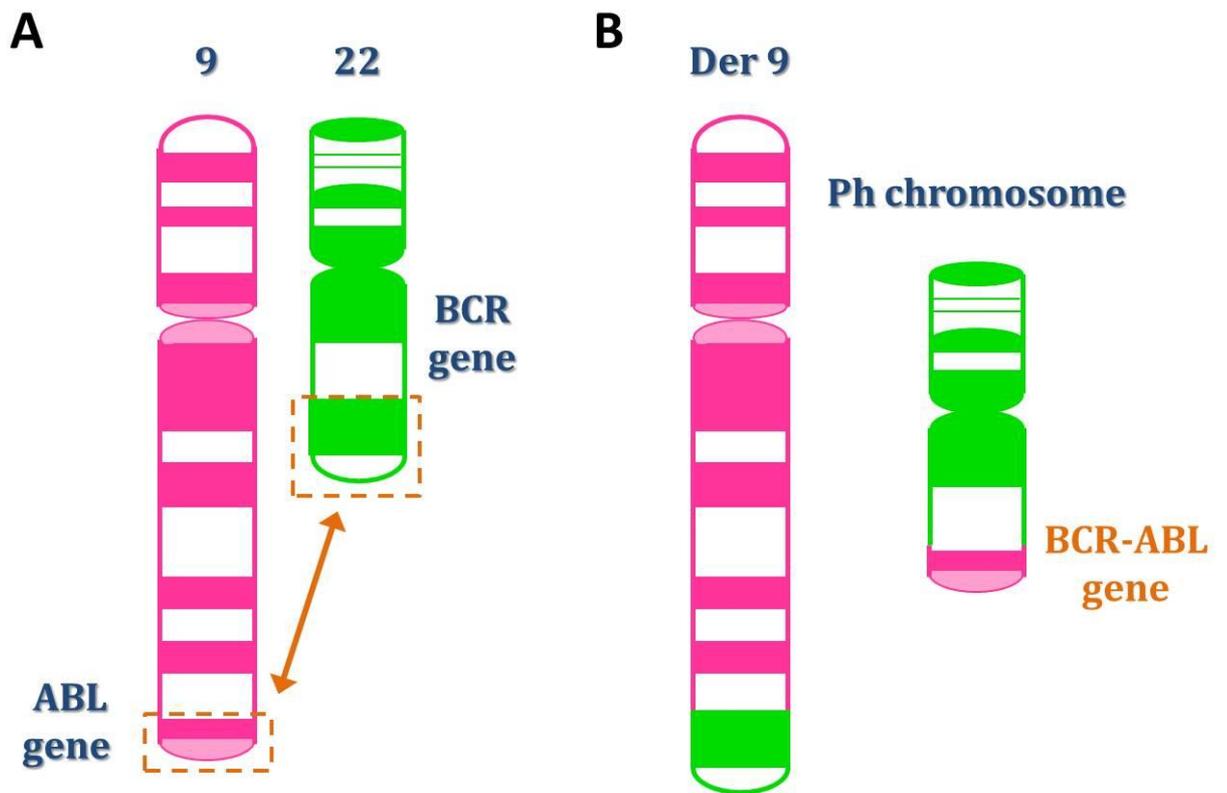
### 1.3.1 Philadelphia Chromosome Translocation, BCR-ABL fusion gene and transcripts

A single genetic alteration is essential for the pathogenesis of CML. The reciprocal translocation of the breakpoint cluster region (*BCR*) gene on chromosome 22 with the Abelson (*ABL*) gene on chromosome 9 [t(9;22)(q11;q34)] produces a shortened der(22) chromosome, termed the Philadelphia (Ph) chromosome (Figure 1.4).<sup>16</sup> The Ph chromosome encodes the *BCR-ABL* oncogene, which has been demonstrated to induce a CML-like disease in a murine model.<sup>35</sup>

### 1.3.2 *BCR* and *c-Abl* Structure and Function

#### 1.3.2.1 Breakpoint Cluster Region (*BCR*)

*BCR* is a 130 kb ubiquitously expressed gene containing 23 exons which is translated into either a 4.5 kb or a 7 kb transcript<sup>36</sup> and codes for two major cytoplasmic proteins<sup>37</sup>. Ordinarily, Bcr protein expression only occurs in early stages of myeloid differentiation, reducing as cells mature.<sup>38</sup> The N terminus features coiled-coil domain which enable *BCR* to form dimers (referred to as the oligomerization domain) and a serine threonine kinase domain (Figure 1.5A). A guanine nucleotide exchange factor domain is centrally located and a GTPase activating protein (GAP) is located at the C terminus. In non-malignant cells, G protein signalling is known to be regulated by cytoplasmic *BCR*,<sup>39</sup> however *BCR* can also associate with mitotic DNA.<sup>40</sup>



**Figure 1.4: Philadelphia chromosome translocation**

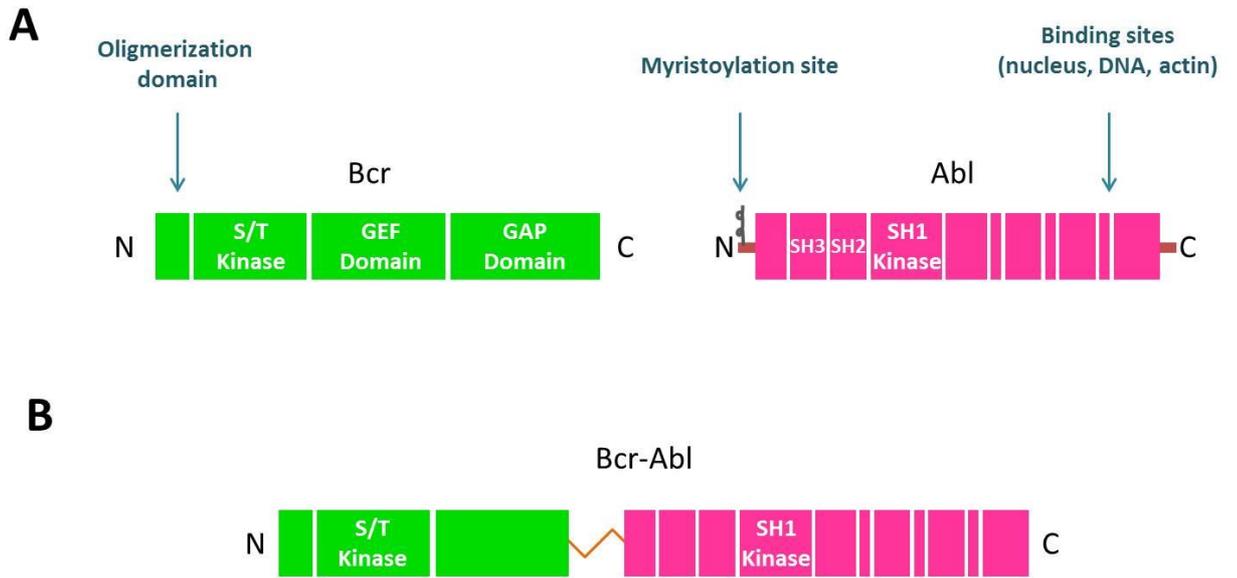
The Ph chromosome resulted from a reciprocal translocation between the long arms of chromosomes 9 and 22  $t(9;22)-(q34;q11)$ .

### **1.3.2.2 *c-Abl***

*c-Abl* (Abelson tyrosine kinase; *ABL1*) is a non-receptor tyrosine kinase which is ubiquitously expressed, however it decreases with myeloid differentiation.<sup>41</sup> *c-Abl* contains several signalling domains, a catalytic domain, Src homology 2 (SH2) and SH3 domains, an activation loop, a proline rich C terminus and an N terminal cap region (Figure 1.5A). Autoregulation of *c-Abl* occurs via SH3 binding to the SH2 and catalytic domain which renders *c-Abl* inactive, and prevents proteins from binding (Figure 1.6). Localization of *c-Abl* occurs at 3 different cellular compartments, including the plasma membrane, cytoplasmic/cyotskeletal and nuclear.<sup>42,43</sup> Signal transduction of *c-Abl* can be activated by cell surface growth factor and adhesion receptors and is known to regulate cytoskeletal structure, cell differentiation and division and responses to oxidative stress, DNA-damage and apoptosis.<sup>44</sup>

### **1.3.3 *Bcr-Abl* Structure and Function**

*Bcr-Abl* retains the N-terminal 426 amino acids of *Bcr*. Fusion of *c-Abl* to the coiled-coil domain at the N terminus of *Bcr* prevents binding of the *Abl* SH3 and SH2 domains, thus prohibiting autoregulation of the kinase. Deregulation of the kinase in this manner results in tetramerization and dimerization, thus allowing constitutive activation of downstream signalling and autophosphorylation of *Bcr-Abl*. Additionally, unlike *c-Abl*, *Bcr-Abl* no longer shuttles between compartments and remains confined to the cytoplasm due to the oligomerization domain on *Bcr* which influences binding to the cytoskeleton.<sup>45</sup> The resultant *Bcr-Abl* fusion protein is a constitutively active tyrosine kinase,<sup>26</sup> which is retained in the cytoplasm<sup>46</sup> and abnormally activates multiple downstream signalling pathways through aberrant phosphorylation of tyrosine residues. The result is uncontrolled cytokine-independent<sup>47</sup> proliferation, reduced



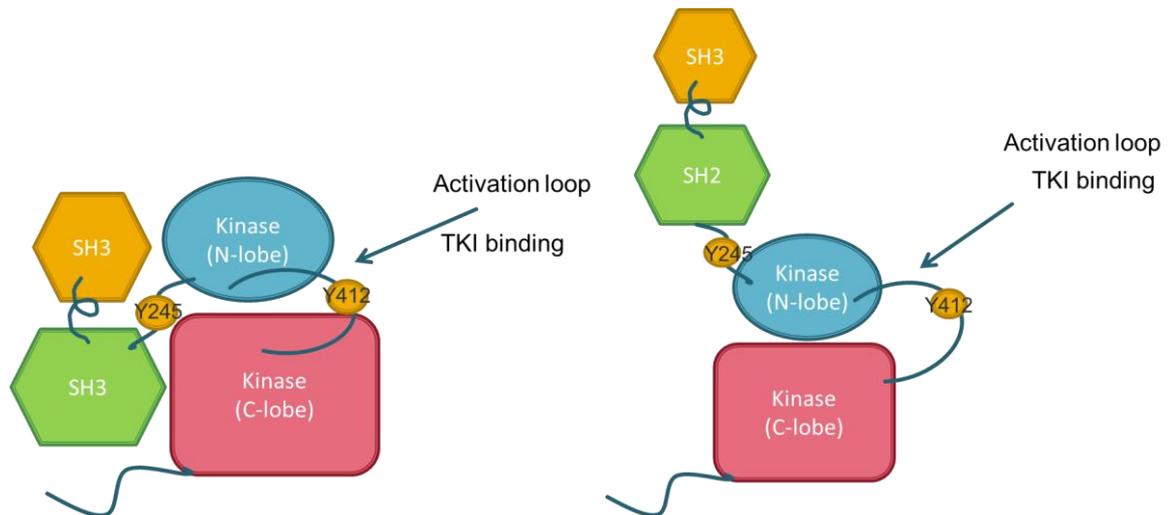
**Figure 1.5: Structure of *BCR*, *Abl* and *Bcr-Abl* proteins**

Fusion of *BCR* and *ABL* transcripts result in the constitutively active *Bcr-Abl* fusion

protein. *S/T* = serine/threonine; *GEP* = guanine nucleotide exchange factor; *GAP* =

*GTPase* activating protein. Adapted from Goldman and Melo 2003,<sup>48</sup>and Hehlmann *et al.*

2007.<sup>49</sup>



**Figure 1.6: c-Abl conformation and control of activation**

The SH3 domain of ABL binds the SH2 domain preventing activation of Y412 (left). Abl remains inactive until removal of the myristoyl group exposing the activation loop (right). Adapted from Woodring *et al.* 2003 and Wong and Witte 2004

apoptosis, aberrant adhesion and enhanced survival.<sup>50</sup>

### 1.3.4 Bcr-Abl Signalling Pathways

The disruption of the normal activity in these pathways by Bcr-Abl tyrosine kinase instigates the survival and clonal expansion of predominantly myeloid leukaemic cells. Bcr-Abl initiates leukaemic transformation by aberrant activation of signal transduction pathways including the RAS/ERK,<sup>51</sup> PI3/AKT<sup>52</sup> and JAK/STAT<sup>53</sup> pathways (Figure 1.7).<sup>54</sup> The major effects of these altered signalling pathways are reduced apoptosis, increased proliferation and altered cell adhesion properties. Inhibition of apoptosis is a key mechanism of BCR-ABL-dependent growth and accumulation of CML cells.

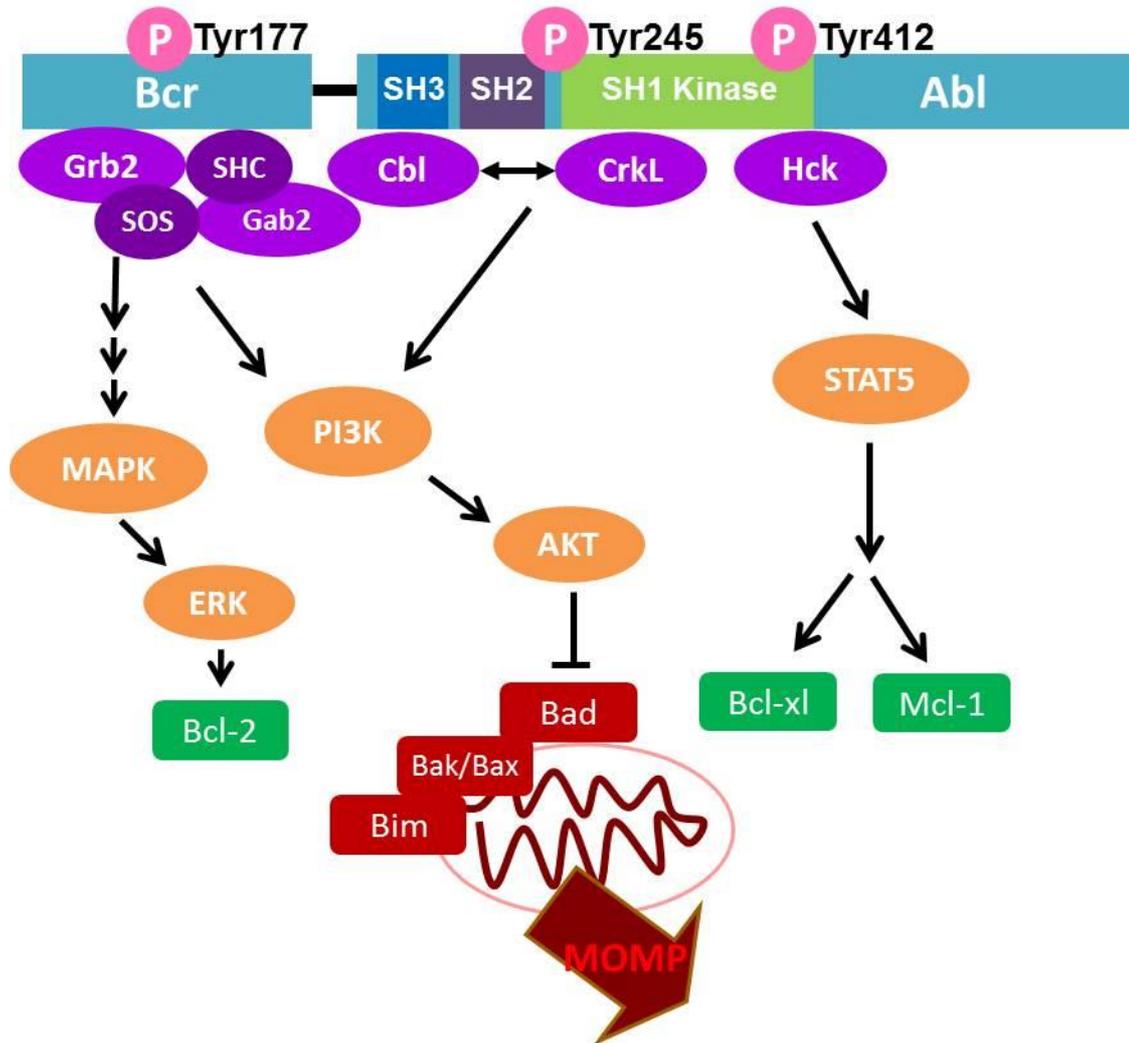
Bcr-Abl constitutively activates several downstream signalling cascades through the adaptor protein growth factor receptor bound protein 2(Grb2), which docks at the Y177 site on Bcr,<sup>51,55</sup> and recruits the adaptor proteins son of sevenless (SOS) and Grb2-associated binding protein 2 (Gab2).<sup>56</sup> Signalling can alternatively be activated through the Src homology (SH) 2 domain-containing protein (Shc)<sup>57,58</sup> and CrkL (CT10 regulator of kinase like)<sup>59</sup> at SH2 and SH3, respectively, on Abl.<sup>51</sup> These events lead to the activation of diverse intracellular signalling. The activation of multiple signalling pathways leads to the formation of multi-protein signalling complexes that initiate the process of leukaemic transformation.

Bcr-Abl causes constitutive activation of the PI3K pathway, which occurs through both the Grb2/Gab2 complex<sup>52</sup> and phospho-CrkL.<sup>60</sup> PI3K activation is then relayed through Akt<sup>84</sup> and results in a change in the balance of pro- and anti-apoptotic B cell lymphoma

gene 2 (Bcl-2) family members to favour survival. The pro-apoptotic protein Bcl-2 antagonist of death (Bad) is a substrate of Akt. Phosphorylated Bad is inactive and unable to bind and inhibit the anti-apoptotic protein Bcl-2-like protein 1 (Bcl-xL). Bcl-xL subsequently prevents Bcl-2 antagonist or killer (Bak)/Bcl-2-associated X protein (Bax) from triggering mitochondrial outer membrane permeabilisation (MOMP) and apoptosis through Cytochrome C release followed by the activation of caspase-9.<sup>61</sup>

The activation of the Ras-MAPK pathway is similar to the PI3K pathway and also requires the recruitment of the Grb2, SOS and Shc adaptor proteins by Bcr-Abl.<sup>62,63</sup> Ras constitutively signals Raf to initiate signalling of Mek1/Mek2<sup>64</sup>, Erk, Jnk/Sapk<sup>65</sup>, and p38.<sup>66</sup> These signals are then activated by a number of transcription factors that modulate apoptosis through modulation of Bcl-2 family members.<sup>67</sup> Uncontrolled MAPK signalling of this nature results in the up-regulation of the anti-apoptotic molecules myeloid cell leukaemia-1 (Mcl-1)<sup>68,69</sup> and Bcl-2.<sup>67,70</sup> Mcl-1 and Bcl-2 promote the survival of leukaemic cells by directly inhibiting pro-apoptotic Bcl-2 family members Bcl-2 interacting mediator of cell death (Bim), Bad, Bak and Bax preventing MOMP apoptosis.<sup>71</sup>

Bcr-Abl has been shown to activate the JAK2/STAT5 pathway through either direct activation of STAT5 through coupling with Hck<sup>72</sup> or activation of JAK2 via association with Gab2<sup>73,74</sup>, or both. The constitutive activation of STAT5 by Bcr-Abl<sup>75</sup> confers resistance against apoptosis by induction of Bcl-xL,<sup>76</sup> preventing apoptosis and hence promoting survival.<sup>77</sup> Defining the signalling events regulated by Bcr-Abl, and pathways which are subject to inhibition by TKIs, is important for designing better treatment strategies for patients with CML.



**Figure 1.7: Key Bcr-Abl Signalling Pathways**

Constitutive activation of Bcr-Abl harnesses growth factor signalling pathways, driving survival and proliferation and inhibiting apoptosis pathways. Pro-survival Bcl-2 family proteins are in green, and apoptotic proteins in red. Adapted from Laneville *et al.* 1995 and Goldman and Melo 2003.

## 1.4 Treatment of CML

### 1.4.1 Historical Treatments

To date, allogeneic transplant from an HLA matched donor is the only truly curative treatment for CML. Treatment consists of total body irradiation and/or myeloablative chemotherapy followed by haemopoietic stem cell infusion. Although often curative, the morbidity and mortality rates are high, and due to age and donor constraints transplant is only available to approximately 30% of patients. Historical and current CML treatment strategies are summarised in Figure 1.8.<sup>78-80</sup>

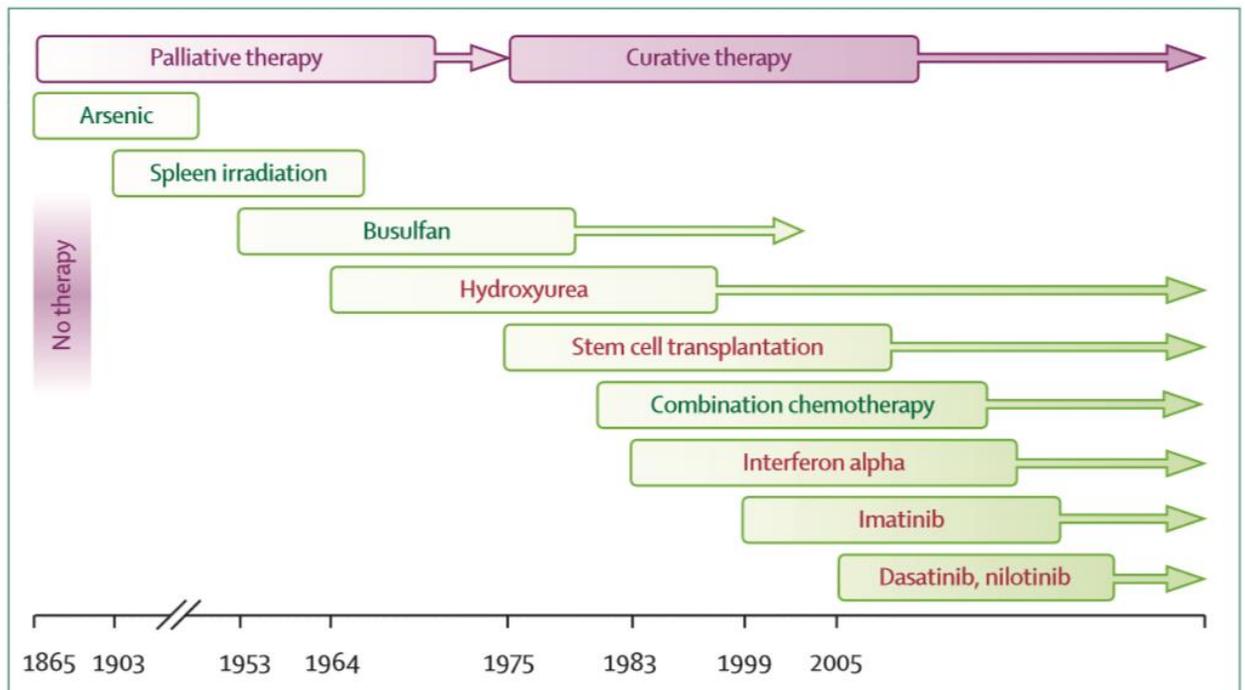
Arsenic was first used to treat leukaemic patients in the late 1800s, however due to toxicity it was largely replaced by splenic radiotherapy<sup>81,82</sup> and subsequently, radioactive phosphorus.<sup>83</sup> In the 1950's, the alkylating agent busulfan, became the preferred treatment for CML patients demonstrating a substantial benefit compared with radiation.<sup>84</sup> Historically, cytogenetic remissions in chronic phase CML were reportedly inducible using intensive chemotherapy and splenectomy.<sup>85,86</sup> However, such remissions were short-lived and were unable to deliver a substantial survival advantage.<sup>87</sup> More recently, the use of the oral chemotherapeutic drug hydroxyurea has been beneficial in reducing the high circulating levels of immature myeloid cells, neutrophils and platelets, with patients returning to normal peripheral blood cell counts and bone marrow morphology.<sup>88</sup> Although 90% of patients achieve haematologic remissions on hydroxyurea, and the absolute number of white blood cells may be reduced, reduction in the percentage of Ph chromosome positive cells is rare.<sup>88</sup>

The introduction of interferon-alpha (interferon- $\alpha$ ) produced significant improvements in overall survival and reduced progression to BC for those CP patients ineligible for transplant.<sup>89</sup> About 10-20% of patients have even achieved complete cytogenetic responses (CCyR, 0% Ph+ metaphases)<sup>90</sup> on interferon therapy alone, but some patients experienced significant side effects. Although it was thought that a small number of patients had durable complete remissions that persisted even after treatment cessation, the use of novel sensitive techniques for the presence of BCR-ABL (e.g. FISH or RQ-PCR), demonstrated that leukaemic cells were still detectable in the majority of these patients.<sup>91,92</sup>

### 1.4.2 Imatinib

Imatinib mesylate (Glivec, formerly STI571; Novartis Pharmaceuticals) was the first small molecule tyrosine kinase inhibitor (TKI) rationally designed to specifically target *BCR-ABL1*+ cells.<sup>93</sup> Imatinib also inhibits c-KIT,<sup>93,94</sup> PDGFR,<sup>94-96</sup> c-fms,<sup>97</sup> and the ABL-related ARG.<sup>98,99</sup> Imatinib inhibits Bcr-Abl by competitively binding to the ATP-binding site and preventing substrate phosphorylation and therefore its downstream effects.<sup>100</sup>

The development of imatinib proceeded quickly from *in vitro* and *in vivo* studies, to clinical trials, before being approved by the FDA in 2001.<sup>99,101</sup> The International Randomised Study of Interferon versus imatinib STI571 (IRIS) trial,<sup>102-104</sup> reported that ~90% of CP-CML patients treated with imatinib achieved CCyR by 24 months.<sup>105</sup> Furthermore, 83% of patients exhibited event-free survival at 6 years of treatment (i.e. without loss of response, disease progression, increase white blood cell count to  $>20 \times 10^9/L$ , or death).<sup>106</sup> Targeted treatment of Bcr-Abl with imatinib has revolutionised the treatment of CML, and has seen a new era in targeted cancer therapy.



**Figure 1.8: Historical Treatments**

Adapted from Hehlmann *et al.* 2007

### 1.4.3 Second Generation Tyrosine Kinase Inhibitors

Second generation Bcr-Abl inhibitors nilotinib (Tasigna; Novartis Pharmaceuticals) and dasatinib (Sprycel; Bristol-Myers Squibb) were developed to address issues associated with imatinib resistance. Nilotinib, designed to inhibit Bcr-Abl with higher affinity than imatinib, is at least 20x more potent than imatinib and has activity against most of the imatinib resistant mutants.<sup>107,108</sup> *In vivo* studies demonstrated promising results using nilotinib in imatinib resistant patients.<sup>109</sup> Nilotinib is also often successful in patients who fail to reach clinical milestones on imatinib. Recent clinical trials assessing upfront nilotinib versus imatinib in *de novo* CP-CML patients have demonstrated high rates of response and reduced rates of progression for patients treated with nilotinib.<sup>110</sup>

Dasatinib inhibits Bcr-Abl tyrosine kinase with a 300x higher potency than imatinib *in vitro*.<sup>111</sup> It is active against most imatinib-resistant mutations with the exception of T315I<sup>112</sup> and F317L, and additionally it is resistant to V299L.<sup>113</sup> Dasatinib inhibits Bcr-Abl kinase, members of the Src family kinase and other kinases including PDGFR and c-Kit.<sup>114</sup> Dasatinib is chemically different from imatinib and requires fewer critical binding residues within Bcr-Abl. Unlike imatinib, dasatinib can bind to both the catalytically active and inactive Bcr-Abl protein. In phase I and II studies of dasatinib, 90-92%, 45-50% and 35-40% of imatinib resistant/intolerant CP-CML patients achieved complete haematological response, major cytogenetic response and CCyR respectively.<sup>115-117</sup> Estimated overall survival and progression free survival at 24 months was 94% and 80% respectively.<sup>115</sup>

## 1.5 Resistance to Tyrosine Kinase Inhibition

Despite the excellent clinical results observed with imatinib treatment, there exists a cohort of patients who do not respond well to therapy<sup>118</sup> due to either primary or secondary imatinib resistance.<sup>119-121</sup> Primary resistance is defined as an inability to achieve a landmark response to imatinib treatment and is most likely due to factors intrinsic to the patient. Secondary resistance is defined as a loss of response after a successful treatment phase; progression to a more advanced disease stage may also occur.<sup>122</sup>

Factors influencing resistance range from Bcr-Abl-independent mechanisms such as CML stem cell quiescence,<sup>123-125</sup> to Bcr-Abl-dependent mechanisms of overexpression,<sup>126</sup> Bcr-Abl kinase domain point mutations,<sup>127</sup> imatinib pharmacokinetics,<sup>128,129</sup> bioavailability,<sup>99,129</sup> serum plasma levels,<sup>104,130</sup> and intracellular drug levels,<sup>131</sup> but aren't necessarily limited to one mechanism.

### 1.5.1 Dynamics of TKI inhibition of Bcr-Abl kinase and cell death

Le Coutre *et al.*<sup>132</sup> demonstrated *in vitro* that 1  $\mu$ M imatinib was required continuously for a period of 20-21 h to induce death in KU812 and MC3 CML cell lines. A shorter duration of *in vitro* exposure (6-7 h) was not adequate to trigger cell death or inhibit cell proliferation. *In vivo* experiments in mice demonstrated that daily dosing did not result in a significant reduction of tumour growth and after cessation of imatinib treatment there was a relapse of tumour growth. While administration with 1  $\mu$ M imatinib resulted in 60-70% Bcr-Abl kinase inhibition within 2 h, reactivation of Bcr-Abl kinase was restored to >70% by 8 h. Dosing which resulted in continuous Bcr-Abl kinase inhibition was demonstrated to decrease tumour growth by 98% and resulted in a longer

progression free survival and overall survival. Furthermore, White *et al.*<sup>133</sup> demonstrated that the degree of Bcr-Abl kinase inhibition achieved in CP-CML patients was predictive of patient response. Collectively, these studies suggest that optimal therapy requires continuous Bcr-Abl kinase inhibition.

Subsequent clinical pharmacokinetic and pharmacodynamic studies comparing imatinib serum trough levels, revealed better responses in patients with trough blood levels above 1000 ng/mL<sup>104,130</sup> suggesting that continuous Bcr-Abl kinase inhibition by imatinib was the optimal strategy in CML therapy.

The plasma half-life of imatinib and nilotinib provides an adequate duration of Bcr-Abl kinase inhibition which is achievable in patients on a daily dosing schedule. *In vivo*, dasatinib has a short plasma half-life of 3-5 h<sup>134</sup> and there is near-complete reactivation of Bcr-Abl kinase 8 h after drug administration corresponding with the decline in CML patient serum levels. These data demonstrate that once daily dosing with dasatinib results in transient inhibition of Bcr-Abl kinase.

However, despite the short half-life, patients on the recently approved standard dose of dasatinib (100 mg once daily) achieved similar cytogenetic and molecular responses as patients on twice daily dasatinib (50mg).<sup>111,135</sup> The once daily dose schedule was also more tolerable, resulting in fewer side effects compared with the twice daily schedule.

Recent studies have investigated a short term *in vitro* exposure of thirty minutes to a potent dose of dasatinib to mimic the *in vivo* once daily schedule. Hiwase *et al.*<sup>136</sup> and others<sup>137,138</sup> have demonstrated that 30 min dasatinib exposure to a therapeutically achievable concentration of dasatinib (100 nM) inhibited Bcr-Abl kinase by >90%. This level of inhibition is defined as intense or potent, whereas <90% Bcr-Abl kinase inhibition is referred to as partial. Following the 30 min exposure, dasatinib was 'washed out' of culture and Bcr-Abl kinase activity measured. Despite reactivation of Bcr-Abl kinase within 1-2 h of dasatinib washout, short term culture with 100 nM dasatinib still triggered cell death in *BCR-ABL1+* cell lines. Intriguingly, cell death as a result of a 30 min exposure was equivalent to cell death caused by a 10 nM dasatinib exposure for 72 h) even though inhibition of Bcr-Abl kinase was only partial.

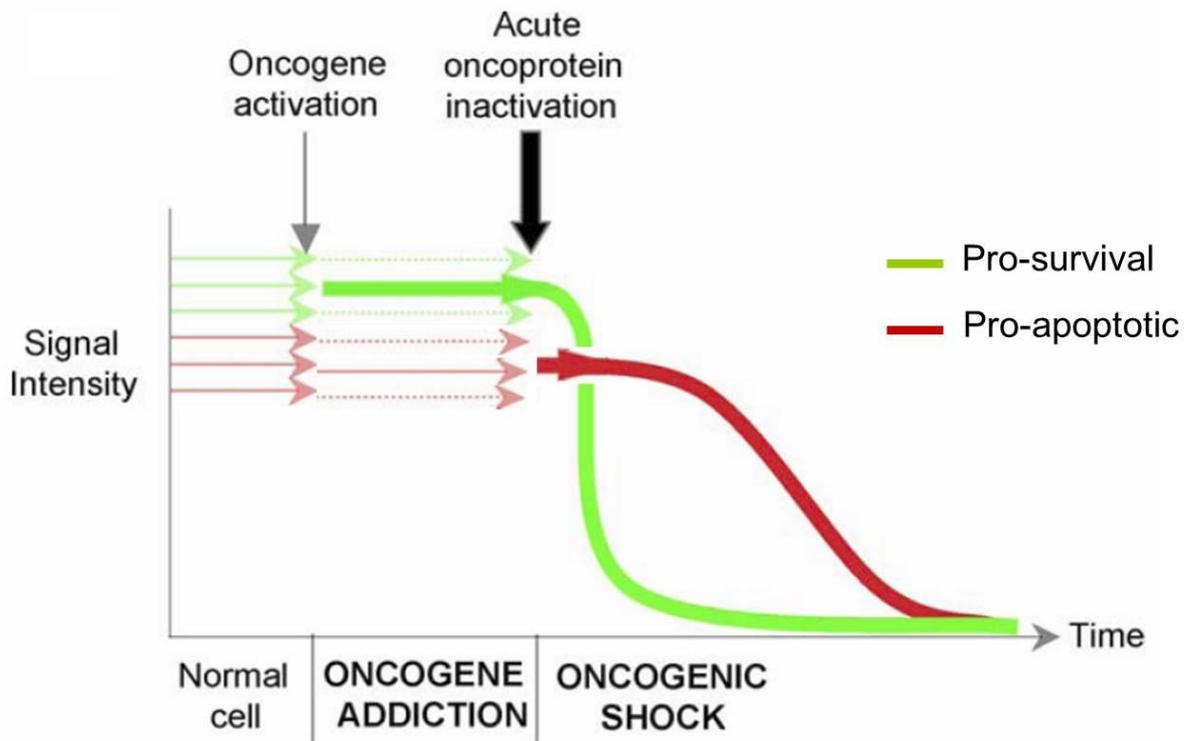
Together the clinical and *in vitro* data suggest that continuous Bcr-Abl kinase inhibition with dasatinib is not required for optimal response, which challenges the previously set paradigm of continuous Bcr-Abl kinase inhibition by imatinib for achievement of optimal response. Additionally, cell death triggered by potent 30 min Bcr-Abl kinase inhibition is not restricted to dasatinib therapy. Potent Bcr-Abl kinase inhibition with nilotinib (1-2  $\mu$ M) or imatinib (30  $\mu$ M, therapeutically unachievable) for 30 min also triggered cell death in *BCR-ABL1+* cell lines.

As drugs with a longer half-life are preferentially selected over drugs with shorter half-life and since drug dosing is matched to achieve continuous therapeutic concentration, these findings could potentially change the paradigm of drug development and change the rationale behind therapeutic regimens.

### 1.5.2 Dependence of CML cells on BCR-ABL

Recently *in vitro* studies in cell lines have demonstrated that a transient, high dose of dasatinib, imatinib or nilotinib triggers cell death despite reactivation of Bcr-Abl kinase activity.<sup>136-138</sup> A potential explanation for these observations was proposed by Weinstein *et al.*,<sup>139</sup> where cancer cells could become dependent on the activity of a single initiating oncogene (Myc, Bcr-Abl or Src)<sup>139-141</sup> when there is unbalanced expression of survival signals over apoptotic signals. Consequently, even a short interruption in oncogenic activity appears sufficient to commit these cells to death as a result of the differences in the degradation/dephosphorylation kinetics of multiple competing pro-survival and pro-apoptotic signals (Figure 1.9).<sup>142</sup> In most tumour cells, pro-survival signals outweigh pro-apoptotic signals, favouring increased cell proliferation and survival. Such an interruption has been shown to be sufficient to shift the balance toward apoptotic signals, as pro-survival signals rapidly dissipate, for long enough to induce apoptosis in CML cells.<sup>143</sup> This intriguing phenomenon may explain why cells cannot be rescued from cell death commitment even though Bcr-Abl kinase reactivated within 1-2 h of TKI washout following 30 min potent Bcr-Abl kinase inhibition.<sup>144</sup> However, further investigation is required into the changes in the balance of these signals so they can be utilised to restore cells to steady-state apoptosis.

Hence, this project will focus on unravelling the downstream effect of short-term intense Bcr-Abl kinase inhibition on pro-survival and apoptotic proteins. It will also assess the different phases of cell death commitment and whether this is reversible or irreversible. Additionally, it will investigate the point at which commitment to cell death becomes irreversible.



**Figure 1.9: Oncogenic Shock**

A short interruption in oncogenic activity commits to death due to the differences in the degradation/dephosphorylation kinetics of multiple competing pro-survival (**green**) and pro-apoptotic (**red**) signals. Sharma & Settleman 2010

### 1.5.3 Overexpression and targeting of STAT5

STAT5 is an ubiquitously expressed transcription factor with established growth factor survival signalling in non-malignant cells.<sup>145</sup> JAK kinases are non-receptor tyrosine kinases which are activated following ligand binding to cytokine receptors. Activated JAK kinases then provide a docking site for the SH2 domain of STAT5, allowing phosphorylation of the C-terminal domain of STAT5 upon growth factor signalling.<sup>146</sup> Activated STAT5 (pSTAT5), then dimerises and migrates to the nucleus to regulate gene transcription.

STAT5 is constitutively expressed in *BCR-ABL1*+ cells and recognised to have an important role in the survival and proliferation of CML cells,<sup>72</sup> however it is unclear whether JAK2 contributes to Bcr-Abl dependent phosphorylation of STAT5 in CML.<sup>147</sup> Although there is evidence to suggest that JAK2 interacts directly with Bcr-Abl,<sup>148,149</sup> and that inhibition of JAK kinases with TG101209 induced minimal apoptosis in CML cells, subsequent reports have demonstrated that JAK inhibition targets extrinsic cytokine-mediated survival signalling rather than Bcr-Abl-dependent signalling.<sup>150</sup> Moreover, experiments performed in the genetic absence of JAK2 did not affect leukaemia maintenance in a CML-like murine model,<sup>151</sup> however STAT5 deletion resulted in failure to maintain leukaemic haematopoiesis.<sup>152</sup>

Elevated expression of total STAT5 levels have recently been observed to reduce the responsiveness of CML cells to TKIs and additionally contribute to an increased *BCR-ABL* mutation rate.<sup>153</sup> Interestingly, the attenuation of STAT5A with RNAi was unable to induce apoptosis, but resulted in an increased stress response to TKIs,<sup>154</sup> thus establishing increased STAT5 as an important mechanism of resistance to TKI

treatment. Following a screen of compounds known to be safe in humans drugs, the anti-psychotic drug pimozide, a calcium channel<sup>155</sup> and dopamine D<sub>2</sub> receptor antagonist<sup>156</sup> was recently identified as an inhibitor of constitutive STAT5 activation in CML.<sup>157</sup> As STAT5 has been implicated in TKI resistance and has an essential role in Bcr-Abl-dependent leukaemogenesis, STAT5 has subsequently become an attractive drug target.

#### **1.5.4 Persistence of leukaemic stem cells following TKI therapy**

Despite the CCyR achieved in 87% of newly diagnosed CP-CML patients after 60 months of imatinib treatment, only a minority of patients achieve complete molecular response (CMR).<sup>103</sup> Moreover, Rousselot *et al.*<sup>158</sup> recently reported that discontinuation of imatinib in patients who had sustained a CMR for at least 2 years resulted in relapse rate of 50% within 6 months, suggesting that the majority of the CP-CML patients are not cured by imatinib treatment.

Recent studies demonstrated the persistence of leukaemic progenitor cells in patients who had achieved CMR with imatinib treatment.<sup>124,159,160</sup> *In vitro* data demonstrated that while imatinib and the more potent second generation tyrosine kinase inhibitors nilotinib and dasatinib all had antiproliferative effects, without inducing apoptosis, on CD34+ leukaemic progenitor cells,<sup>125,161</sup> or in the more primitive CD34+38-<sup>162</sup> leukaemic progenitor population. Thus, both clinical and *in vitro* studies demonstrated that leukaemic early progenitors were refractory to TKI and were potentially responsible for long-term disease persistence and resistance development during imatinib treatment.

Refractoriness of leukaemic progenitor cells to imatinib has been postulated to be due to inadequate Bcr-Abl kinase inhibition within these cells.<sup>162</sup> As treatment of quiescent CML progenitors with the more potent second generation TKIs (nilotinib and dasatinib), which inhibit Bcr-Abl kinase more effectively, does not result in increased levels of apoptosis,<sup>162,163</sup> it is therefore likely that the resistance of these progenitor cells to TKIs is Bcr-Abl independent.

Leukaemic stem cells and HSCs reside in the bone marrow niche which provides a cytokine rich microenvironment for the maintenance of both normal and CML HSCs. Under normal conditions there exists a balance of pro-survival and pro-apoptotic signals in haematopoietic cells. However in CML, Bcr-Abl driven signalling pathways result in this balance tipping in the favour of pro-survival signals. The inactivation of the Bcr-Abl oncogenic activity by TKI therapy results in a reduction of pro-survival signals and activation of pro-apoptotic signals consequently inducing cell death.<sup>93,142,164</sup>

It has recently been demonstrated that short exposure and high dose treatment of CD34+ progenitor cells with dasatinib and imatinib is sufficient to commit cells to apoptotic death.<sup>136,137</sup> However, *in vitro* experiments may not accurately represent the *in vivo* situation given that these leukaemic progenitors normally exist in a cytokine rich microenvironment and Bcr-Abl shares survival pathways with cytokine receptors. It is therefore possible that the presence of cytokines in the bone marrow niche may provide protection for CML progenitor cells from the cytotoxic effect of TKI therapy.

### 1.5.5 Haematopoietic Cytokines and their Contribution to LSC Persistence

Although the role of cytokines in CML pathogenesis is controversial, there is increasing evidence that cytokines may play a role in TKI resistance. Under non-pathological circumstances HSCs reside in the bone marrow which provides a microenvironment that supports survival and self-renewal. This cytokine rich microenvironment supports the maintenance of both normal and CML HSCs. In CML, cytokine pathways including interleukin 3 (IL-3) and granulocyte macrophage colony stimulating factor (GM-CSF) are utilised by Bcr-Abl in mature cells to maintain survival and proliferation.<sup>54</sup> There also appears to be cross talk between the beta common ( $\beta$ c) receptor<sup>165</sup> and Bcr-Abl,<sup>56</sup> additionally, Bcr-Abl has been reported to use the  $\beta$ c subunit and also the c-Kit receptor growth factor machinery to send its survival signals.

Several studies have now demonstrated the capability of cytokines to provide protection against TKI-induced cell death. Initial studies in Bcr-Abl-transformed 32Dp210BCR-ABL cells, which are IL-3 responsive, are partially rescued from imatinib-mediated cell death in the presence of IL-3.<sup>93,166</sup> Abnormal activation of the autocrine production of IL-3,<sup>167-169</sup> granulocyte colony stimulating factor (G-CSF)<sup>168</sup> and GM-CSF<sup>170,171</sup> is likely associated with autonomous growth in cell lines<sup>167,171</sup> and CD34+ CML progenitors.<sup>168,172</sup> Dorsey *et al.*<sup>173</sup> also found that IL-3 protected Bcr-Abl-transformed cell lines (Baf3p210BCR-ABL and 32Dp210BCR-ABL) from TKI-mediated apoptosis. In conjunction with previous findings of IL-3 autocrine secretion,<sup>168</sup> these results suggest that CD34+ progenitor cells may escape complete eradication by this mechanism.

Liu *et al.*<sup>174</sup> recently demonstrated that conditioned media from culture of resistant Bcr-Abl-expressing DA1-3b cells, which over-express IL-3, induced TKI resistance in sensitive (non-mutated) DA1-3b cells by activation of STAT5 and MEK/ERK pathways. Inhibition of JAK2 or MEK1/2 abrogated the protective effect of conditioned media on TKI-induced apoptosis in these cells.

In order to investigate whether sensitivity to TKI increased with cell maturity, Jiang *et al.*<sup>175</sup> examined the effect of cytokines on CML progenitor cells. Autocrine secretion of IL-3 and G-CSF was found to induce TKI resistance in CML CD34+/CD38- even in the absence of exogenous growth factors; whereas in the absence of exogenous growth factors, more mature CML CD34+/CD38+ cells were more sensitive to imatinib. This variation in TKI sensitivity could be explained on the basis of IL-3 and G-CSF expression where the levels of G-CSF and IL-3 transcripts peaked in the CML stem cells and then appeared to be switched off when the cells began to differentiate.<sup>168,175</sup>

Wang *et al.*<sup>171</sup> recently demonstrated that adaptive autocrine secretion of GM-CSF could instigate Bcr-Abl-independent survival signalling in the presence of imatinib and nilotinib. This resistance was mediated by the activation of the anti-apoptotic Jak-2/STAT5 pathway. Signalling of stem cell factor (SCF) through c-KIT has also been demonstrated to circumvent Bcr-Abl inhibition and thereby rescue cells from TKI-induced apoptosis.<sup>176</sup> Such data suggests that CML progenitor cells may not completely rely on Bcr-Abl for survival.

The IL-3 receptor  $\alpha$ -chain (IL-3R $\alpha$ ) has been described as a unique marker in the identification of acute myeloid leukaemia (AML) stem cells.<sup>177,178</sup> A recent murine study demonstrated that selectively targeting IL-3R $\alpha$  in AML with a specific monoclonal antibody resulted in reduced AML burden in bone marrow and improved survival.<sup>179</sup> The co-expression of IL-3R $\alpha$  on CD34<sup>+</sup>CD38<sup>-</sup> cells has also been demonstrated in CML patients.<sup>180</sup> Collectively, this data suggests that IL-3 could be implicated in the protection of CML progenitors from TKI-induced cell death.

Hiwase *et al.*<sup>144</sup> recently reported that CML progenitor cells are rescued from dasatinib-induced apoptosis in the presence of GM-CSF. This study demonstrated that STAT5 is phosphorylated in CML-CD34<sup>+</sup> cells and supplementation of GM-CSF or a cocktail of six growth factors (6-GF containing G-CSF, IL-3, IL-6, SCF, FLT3-ligand and TPO) increased the level of phosphorylated STAT5 (p-STAT5). In the absence of GM-CSF or 6-GF, decreased levels of p-STAT5 were observed in CML-CD34<sup>+</sup> cells when treated with dasatinib. Furthermore, inhibition of both Bcr-Abl and JAK kinases, by combination treatment of a JAK inhibitor with dasatinib in the presence of GM-CSF or a 6-GF, dramatically reduced the phosphorylation of STAT5. This suggests that cytokines mediate the activation of the JAK2-STAT5 pathway in CML-CD34<sup>+</sup> cells which renders them refractory to dasatinib. Consistent with these findings, Konig *et al.* also reported that nilotinib<sup>163</sup> and dasatinib<sup>181</sup> inhibited MAPK, Akt and STAT5 phosphorylation in CML-CD34<sup>+</sup> cells in the absence, but not in the presence, of growth factors.

Therefore, the maintenance of survival signals in leukaemic progenitors by cytokines is likely to contribute significantly to their persistence during TKI therapy.

An understanding of this cytokine-mediated TKI resistance is essential for development of therapeutic strategies to target and eliminate these leukaemic progenitors and potentially provide a cure for CML patients.

### **1.5.6 Autophagy**

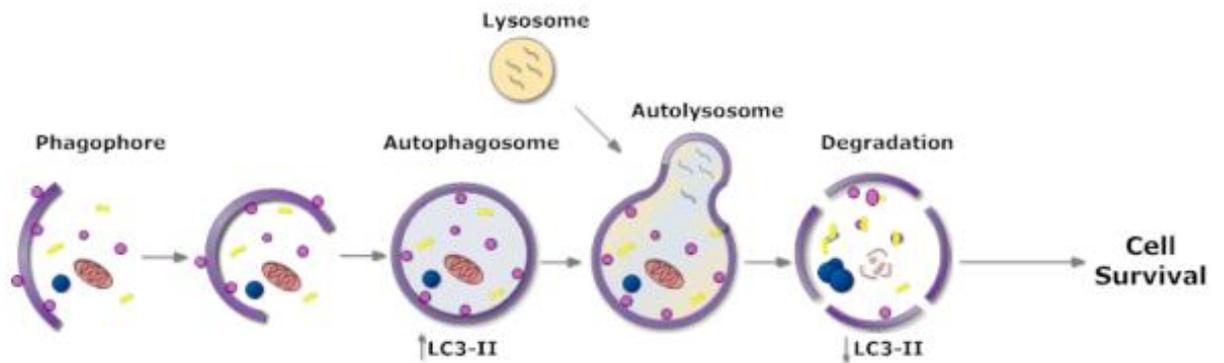
Non-malignant cells rely on growth factor signalling to regulate growth and proliferation and survival as well as maintain nutrient uptake and intracellular homeostasis.<sup>182</sup> The absence of growth factors or the inhibition of these signalling pathways leads to organelle and metabolic stress, cells subsequently display a diminished capacity to uptake and metabolize extracellular nutrients and eventually undergo apoptosis. Cancerous cells gain the ability to maintain growth and metabolism in the absence of growth factors while evading growth-factor withdrawal-induced apoptosis. In CML, Bcr-Abl mimics growth factor signal transduction pathways including the RAS/ERK,<sup>51</sup> PI3/AKT<sup>52</sup> and JAK/STAT<sup>53</sup> pathways.<sup>54</sup>

Utilised as an adaptive response to starvation induced stress the induction of macroautophagy, hereafter referred to as autophagy, is a mechanism by which cells survive in growth factor deprived environments.<sup>183</sup> Initiation of autophagy instigates the formation of phagophores, which sequester proteins and whole organelles to form double-membrane autophagosomes, a morphological characteristic of autophagic cells (Figure 1.10).

Several studies have now demonstrated that inhibition of Bcr-Abl induces autophagy,<sup>184-</sup><sup>186</sup> thus inhibition of autophagy was proposed to have a negative impact on cell survival.

Chloroquine (CQ) is an anti-malarial agent which has been demonstrated to inhibit autolysosomal degradation thereby blocking late stage autophagy.<sup>187</sup> TKI-induced blockade of Bcr-Abl driven survival pathways combined with inhibition of autophagy by CQ has now been demonstrated to restore sensitivity of resistant CML cells to TKI.<sup>184,185</sup>

Recently, Carella *et al.*<sup>188</sup> reported rapid reductions in the BCR-ABL transcript level in TKI-resistant CML patients where clarithromycin (CAM) was combined with ongoing TKI therapy. This research highlighted CAM as a candidate for combination therapy in CML patients, in particular those with advanced and resistant disease. CAM is one of several macrolide antibiotics demonstrated to inhibit cancer cell growth. In multiple myeloma cells, CAM induces cell death through inhibition of autophagy at the clinically relevant concentrations of 6-50 µg/mL<sup>189</sup> and thus Carella *et al.*<sup>188</sup> hypothesised that CAM was acting as an inhibitor of autophagy in resistant CML cells, however their study did not demonstrate any supporting experimental evidence.



**Figure 1.10: Initiation of autophagy**

Upon induction of autophagy signals, elongation of the phagophore engulfs long-lived proteins and whole organelles to form double-membrane autophagosomes, which can be experimentally identified as LC3-positive vacuoles. Fusion of the autophagosome with the lysosome results in the formation of an autophagolysosome which degrades the sequestered components supplying energy and thus allowing cell survival.

## 1.6 Summary and project aims

### 1.6.1 Summary

Long term follow up of CP-CML patients demonstrated that imatinib was unable to eradicate leukaemic progenitor cells. The majority of CP-CML patients are not cured by imatinib treatment and most patients exhibit persistent disease. As TKI therapy deprives *BCR-ABL1+* cells of survival signalling and puts cells under metabolic stress, autophagy was assessed as a critical resistance mechanism.

Here, the critical factors involved in commitment of cells to TKI-induced cell death are evaluated in the setting of transient potent Bcr-Abl kinase inhibition and continuous partial Bcr-Abl kinase inhibition. Subsequently, STAT5 was assessed as a critical factor, in particular, in relation to JAK2. It is proposed that STAT5 activation, as a result of Bcr-Abl, does not occur through JAK2, but instead the inhibition of JAK2 is only relevant in the extrinsic survival signalling from cytokines. The findings presented here will help establish critical CML signalling components that may be targeted by combination therapeutic approaches. These studies will have broad implications for ongoing therapeutic strategies in resistant cases of CML and in providing a curative treatment.

### 1.6.2 Project Aims

1. Assess the mechanism by which TKIs induce autophagy on cell death and subsequently assess combination therapy with inhibitors of autophagy.

2. Compare the effects of treatment with continuous low dose dasatinib and transient high dose dasatinib on Bcr-Abl kinase inhibition and the kinetics of downstream survival and apoptotic proteins.
  
3. Elucidate the critical pathways which allow leukaemic progenitor cells to escape TKI-induced cell death.
  
4. Assess the effect of combination TKI and JAK inhibitors on CML progenitor cells *in vitro*.

## **CHAPTER 2.**

# **MATERIALS AND METHODS**

## 2.1 Commonly used reagents

**Table 2.1: Suppliers and catalogue numbers of commonly used reagents**

Reagents	Company/Supplier	Catalogue No.
0.1% bromophenol blue	Sigma-Aldrich	114391
3-Methyladenine (3MA)	Sigma-Aldrich	M9281
7-Aminoactinomycin D (7-AAD)	Life Technologies	A-1310
Acrylamide/Bis Solution (40%,37.5:1 ratio)	Bio-Rad	161-0148
Ammonium Persulfate (APS)	Sigma	A9164
Annexin-V (PE-conjugated)	Becton Dickinson	556421
Acid Citrate Dextrose (ACD; 5%)	Aurora Bioscences	AHB7898
Benchmark prestained protein standard	Life Technologies	10748-010
$\beta$ -Mercaptoethanol	Sigma-Aldrich	M-6250
Bovine serum albumin (BSA)	Sigma-Aldrich	A9418-100G
Calcium Chloride (CaCl <sub>2</sub> )	Sigma	449709
CD34 (8G12) PE	Becton Dickinson	348057
Chloroform	Merck	100776B
Chloroquine	Sigma-Aldrich	C6628
Clarithromycin	Sigma-Aldrich	C9742
Criterion Precast TGX Gel (4-15%)	Bio-Rad	567-1084
DEPC water	MP Biomedicals Inc.	821739
Dimethyl sulphoxide (DMSO)	Merck	102952-2500
dNTP set (N = A, C, G, T)	GE Healthcare Lifesciences	27-2035-02
ECF substrate Attaphos	GE Healthcare Lifesciences	RPN5785
Erk	Cell Signalling Technology	9102
Erythropoietin (Eprex)	Janssen	113427-24-0
Ethanol	Merck	6-10107-2511
Ethylenediaminetetraacetic acid (EDTA)	APS	180-500G
FcR Blocking Reagent	Miltenyi Biotec Germany	130-046-702
FLT3 Ligand	PeproTech	300-19

Foetal Bovine Serum (FBS)	SAFC Biosciences	12103-500ml
40% w/v Formaldehyde	BDH	10113
Glycine	Sigma-Aldrich	G8898
Glycogen	Roche	901393
G-CSF	PeproTech	300-23
GM-CSF	PeproTech	300-03
Hanks Balanced Salt Solution (HBSS) Ca <sup>2+</sup> Mg <sup>2+</sup> free	SAFC Biosciences	55021C-500ML
HEPES 1M	SAFC Biosciences	59205C-100ml
Hydrocortisone (Solu-Cortef)	Pfizer	R12264
Isopropanol	Merck	6-10224.2511
IL-3	PeproTech	200-03
IL-6	PeproTech	200-06
Insulin (Actrapid)	NovoNordiskPharmaceuticals	R169625
Iscove Modification of Dulbecco Medium (IMDM)	Sigma-Aldrich	13390
Kaleidoscope prestained protein standard	Bio-Rad	161-0375
L-Glutamine 200mM	Sigma-Aldrich	G-709
Low density Lipoprotein	Sigma-Aldrich	L5402
Lymphoprep	Axis Shield. Oslo Norway	1114547
MACS CD34 MicroBeads	Miltenyi Biotec Germany	130-046-702
Membrane blocking agent	GE Healthcare Lifesciences	RPN 2125V
Methanol	Chem Supply	MA004-P
MethoCult™ H4230	Stemcell Technologies	H4230
MICROSCINT-20 scintillation fluid	PerkinElmer	6013621
Mouse IgG1 RPE-conjugated	Dako Cytomation Denmark	0928
Mouse IgG2b RPE-conjugated	Dako Cytomation Denmark	X0951
MyeloCult™ H5100	Stemcell Technologies	05150
N'-((4-Oxo-4H-chromen-3-yl)methylene) nicotinohydrazide (STAT5 inhibitor)	Merck	573108
Paraformaldehyde (PFA) 16%	ProSciTech	15710
Penicillin 5000U/ml Streptomycin 5mg/ml	Sigma-Aldrich	P4458
Phosphate Buffered Saline (PBS)	SAFC Biosciences	59331-500mls
Pimozide	Sigma-Aldrich	P1793
PVDF Membrane	GE Healthcare Lifesciences	PRN 303F
PVDF membrane, LF Trans-Blot® Turbo™ RTA Midi Transfer Pack kit	Bio-Rad	170-4275
Random Hexamer Primer (100mg stock)	Geneworks	RP-6

RPMI-1640 Medium w/o L-Glutamine Ca <sup>2+</sup> Mg <sup>2+</sup> free	SAFC Biosciences	51502C-500ML
See Blue Plus 2 Protein Standard	Life Technologies	LC5925
Sodium Dodecyl Sulfate (SDS)	Sigma-Aldrich	L-4509-1KG
Sodium Chloride (NaCl)	Ajax Finechem Pty Ltd	465-2.5 kg
Sodium (ortho) Vanadate (Na <sub>3</sub> VO <sub>4</sub> )	Sigma-Aldrich	S6508
Sodium Fluoride	Sigma-Aldrich	S7920
Stem Cell Factor (SCF)	PeptoTech	300-07
SuperScript II Reverse Transcriptase	Life Technologies	18064-014
SYBR Green Supermix	Bio-Rad	PA-012-24
Taqman Master Mix	Applied Biosystems	4318157
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich	87687
Thrombopoietin	PeptoTech	300-18
Transferrin	Sigma-Aldrich	T-0665
TRIzol® reagent	Life Technologies	15596-018
Tris	Merck	6.10315.0500
Trypan Blue Solution, 0.4%	Sigma-Aldrich	T8154
Tween-20	Sigma-Aldrich	P9416

Table 2.2: Western Antibody List

Antibodies	Use @	Size (kDa)	Company/Supplier	Catalogue #
AP-conjugated $\alpha$ -rabbit Ig	1:20000		Santa Cruz Biotechnology	SC2007
ASK1	1:1000	155	Cell Signaling Technology	3762
Bcl-xl	1:1000	30	Cell Signaling Technology	2762
Bcl-2	1:1000	26	Cell Signaling Technology	2870
Beclin-1	1:1000	60	Cell Signaling Technology	3738
Bim	1:1000	23	Cell Signaling Technology	2933
c-Abl	1:1000	135/210	Cell Signaling Technology	2862
Chk2	1:1000	62	Cell Signaling Technology	2662
LKB1	1:1000	54	Cell Signaling Technology	3050
LC3B	1:1000	14,16	Cell Signaling Technology	3868
Mcl1	1:1000	40	Cell Signaling Technology	4572
Phospho-Bcr (Y245)	1:1000	210	Cell Signaling Technology	3901
Phospho-Erk	1:1000	42,44	Cell signaling Technology	9101
Phospho-JAK1	1:1000	130	Cell Signaling Technology	3331
Phospho-JAK2	1:1000	125	Cell Signaling Technology	3771
Phospho-STAT3	1:1000	79,86	Cell Signaling Technology	9145
Phospho-STAT5	1:1000	90	Cell Signaling Technology	9359
JAK1	1:1000	130	Cell Signaling Technology	3332
JAK2	1:1000	125	Cell Signaling Technology	3230
p62/SQM	1:1000	62	Cell Signaling Technology	8025
Pim3	1:1000	35	Cell Signaling Technology	4165
STAT3	1:1000	79,86	Cell Signaling Technology	9139
STAT5	1:1000	90	Cell Signaling Technology	9363

## **2.2 Solutions Buffers & Media**

### **2.2.1 Cell culture media**

RPMI-1640 medium

2 mM L-Glutamine

50 units/mL Penicillin

50 µg/mL Streptomycin

10% FBS

Store at 4°C, and preheat to 37°C in a water bath prior to use.

### **2.2.2 Hanks Balanced Salt Solution (HBSS)**

Ca<sup>++</sup> and Mg<sup>++</sup> Free. Add 10mM HEPES 1M prior to use.

### **2.2.3 Cryoprotectant mixture**

70% HBSS with 10 mM HEPES

20% FBS

10% DMSO

This is made up fresh for each batch of samples to be cryopreserved.

### **2.2.4 Binding Buffer**

5 mM CaCl in 500mL HBSS

### **2.2.5 1x Laemmli's buffer (modified)**

50 mM Tris-HCL (pH 6.8)

10% glycerol

2% SDS

5%  $\beta$ -mercaptoethanol

0.1% bromophenol blue

1 mM NaVanadate

10 mM NaFluoride

### **2.2.6 MACS buffer**

1x PBS

0.5% BSA

2 mM EDTA

BSA was dissolved slowly in PBS using a rolling mixer. The EDTA was added to the buffer, and the solution sterilised using a 0.2 $\mu$ m bottle top filter. The buffer was stored at 4°C.

### **2.2.7 2.5% Membrane blocking solution**

#### **For milk optimised antibodies**

2.5% Membrane Blocking Agent in 1xTBST

Solution was dissolved on a rolling mixer and stored at 4°C.

#### **For BSA optimised antibodies**

3% BSA in 1xTBST

Solution was dissolved on a rolling mixer and stored at 4°C.

### 2.2.8 MethoCult™

MethoCult™, methylcellulose based media (100 ml, Catalogue 4230; Stemcell Technologies, Tullamarine, VIC). MethoCult was stored at -20°C and was thawed at 4°C (overnight) or at room temperature (over few hours). After thoroughly mixing, 3.5 mL was aliquoted in to sterile 12 mL tubes and stored at -20°C until required.

### 2.2.9 Serum deprived media (SDM)

CML-CD34+ cells were cultured in serum deprived media (SDM), which contains Iscove's Modified Dulbecco's Medium (IMDM) supplemented with;

1% bovine serum albumin (BSA)

1 U/mL insulin (Actrapid)

200 µg/mL transferrin

10 µg/mL low-density lipoproteins

0.1 µM β-mercaptoethanol

### 6 Growth Factor (GF) cocktail

10 ng/mL Flt3-ligand

10 ng/mL stem cell factor (SCF),

10 ng/mL interleukin-3 (IL-3),

10 ng/mL interleukin-6 (IL-6),

10 ng/mL granulocyte colony-stimulating factor (G-CSF)

10 ng/ml thrombopoietin (TPO).

**5GF cocktail**

1 ng/mL G-CSF

0.2 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF)

0.2 ng/mL SCF

1 ng/mL IL-3

1 ng/mL IL-6

**2.2.10 SDS-Polyacrylamide Gel**

**12% Resolving gel (for 30 mL)**

12.9mL H<sub>2</sub>O

9 mL 40% Acrylamide/Bis solution, 37.5:1

7.5 mL 1.5M Tris-HCL, pH 8.8

300 µL 10% SDS

300 µL 10% APS

8 µL TEMED

**5% Stacking gel (for 10 mL)**

6 mLH<sub>2</sub>O

1.26 mL 40% Acrylamide/Bis solution, 37.5:1

2.52 mL 0.5M Tris-HCL, pH 6.8

100 µL 10% SDS

100 µL 10% APS

10 µL TEMED

### **2.2.11 1 x TBS**

20 mM Tris-HCL (pH 7.5)

150 mM NaCl

### **2.2.12 1xTBST**

20 mM Tris-HCL (pH 7.5)

150 mM NaCl

0.1% Tween20

### **2.2.13 Thaw solution**

Hanks Buffered Salt Solution

10 mM HEPES

5% FBS

5% ACD

The solution (without ACD) was stored at 4°C. ACD was added immediately prior to use and the solution was heated to 37°C in a water bath.

### **2.2.14 White cell fluid**

2% Glacial acetic acid in Milli-Q® Water with a few crystals of Methyl Violet

Acetic acid was slowly added to Milli-Q® water. The methyl violet (Gurr®, BDH) was then added, and the solution mixed well to ensure that the crystals completely dissolved.

The solution was filtered using a 0.2 µm bottle top filter, and stored at room temperature.

## 2.3 Inhibitors

### 2.3.1 Dasatinib

MW = 506.2

Dasatinib (Das) was kindly provided by Bristol-Myers Squibb (New Brunswick, USA) and a 10 mM stock solution was prepared in dimethyl sulfoxide (DMSO, Merck KGaA, Darmstadt, Germany) and stored at 4°C.

[<sup>14</sup>C]-dasatinib (Bristol-Myers Squibb, New Brunswick, USA) was dissolved in ethanol at 1 mg/mL (2049  $\mu$ M) and stored at -80°C. Specific Activity was 1 mBq/mL (31.9  $\mu$ Ci/mL). A 50% mixture of <sup>14</sup>C-labelled and unlabelled dasatinib, 100  $\mu$ M final concentration, was prepared fresh prior to use. For one millilitre of working solution 24.4  $\mu$ l of 2049  $\mu$ M <sup>14</sup>C-dasatinib and 5  $\mu$ L of 10 mM unlabelled dasatinib were added to 970.6  $\mu$ l of RPMI-1640 media.

### 2.3.2 Imatinib (IM)

MW = 589.72

Imatinib mesylate (imatinib; Glivec; formerly STI-571) was provided by Novartis Pharmaceuticals (Basel, Switzerland). Stock solutions of this compound were prepared at 10 mM with distilled water, sterile filtered and stored at -70°C.

### 2.3.3 Nilotinib (NIL)

MW = 529.5

Nilotinib (formerly AMN107) was provided by Novartis Pharmaceuticals. Stock solutions were prepared at 10 mM in DMSO and stored at 4°C.

### 2.3.4 Chloroquine

MW = 515.9

Chloroquine (CQ) was purchased from Sigma-Aldrich. Stock solutions were prepared fresh at 10 mM with distilled water.

### 2.3.5 Clarithromycin

MW = 747.95

Clarithromycin (CAM) was purchased from Sigma-Aldrich. Stock solutions were prepared fresh at 5 mg/mL in acetone.

### 2.3.6 3-Methyladenine

MW = 149.2

3-Methyladenine (3MA) was purchased from Sigma-Aldrich. Stock solutions were prepared at 10 mM in DMSO and stored at 4°C.

### 2.3.7 Pimozide

MW = 461.55

Pimozide (Pz) was purchased from Sigma-Aldrich. Stock solutions were prepared fresh at 10 mM in DMSO.

**2.3.8 N'-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide (STAT5 inhibitor)**

MW = 293.3

N'-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide (STAT5i) was purchased from Merck. Stock solutions were prepared at 10 mM in DMSO and stored at 20°C in the dark.

**2.3.9 Ruxolitinib**

MW = 306.37

Ruxolitinib (formerly INCB-018424) was purchased from Active Biochemicals (Wanchai, Hong Kong). Stock solutions were prepared at 10 mM in DMSO and stored at 20°C.

**2.3.10 U0126 (MEK1/2 inhibitor)**

MW = 380.5

U0126 was purchased from Cell Signaling Technology. Stock solutions were prepared at 10 mM in DMSO and stored at 20°C.

## 2.4 General Techniques

### 2.4.1 Maintenance of cell lines

The *BCR-ABL1* expressing human K562, KU812 and Meg01 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). They were cultured in pre-warmed (37°C) culture media (RPMI-1640 + 10% FBS) in 25 or 75 cm<sup>2</sup> flask and were maintained in 37°C/5%CO<sub>2</sub> incubator. Cell lines were sub-cultured in a class two biohazard safety cabinet (Gelman Sciences) on Monday, Wednesday and Friday every week and were maintained at cell density between 1x10<sup>5</sup> to 1x10<sup>6</sup>/mL.

### 2.4.2 Cell count and viability assessment

Live cells exclude trypan blue efficiently, however dead cells cannot. This discriminatory feature of trypan blue dye is exploited to assess the cell viability. Cells were mixed with an equal volume of 0.4% trypan blue solution and 10 µl of suspension was loaded beneath a coverslip of a haemocytometer counting chamber (Neubauer Improved, Assistant, Germany) and cell concentration and viability were assessed. To assess the cell concentration only (in highly concentrated cells), cells were diluted in white cell fluid (WCF) and loaded onto the haemocytometer to determine total cell count.

### 2.4.3 Lymphoprep isolation of mononuclear cells (MNC)

Peripheral blood (PB, 40-60 mL) was collected into Lithium Heparin tubes from patients with CML or from normal donors. All samples were collected with informed consent in accordance with the Institutional Ethics approved protocols and with reference to the Declaration of Helsinki. A white cell count was performed using white cell fluid and a maximum of 1x10<sup>8</sup> cells (or maximum of 15 ml of blood) were transferred into a 50 mL

polypropylene conical tube. The blood volume was brought to 35 mL using hanks balances salt solution with 10 mM HEPES (HBSS) and underlain with 15 ml of lymphoprep. Tubes were then centrifuged at 1,200 rpm for 30 min with no brake. The interface containing the mononuclear cells (MNC) was then transferred to another 50 ml tube and washed once in HBSS.

#### **2.4.4 Magnetic Cell Sorting (MACS)**

MicroBeads for CD34 (progenitor cells) were obtained from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). Cells were suspended in MACS buffer and CD34 MicroBeads and FcR Blocking Reagent were added in equal volumes according to the manufacturer's recommendations. Samples were incubated at 4°C with continuous mixing for 30 min.

##### **2.1.1.1 Magnetic Separation**

A pre-cooled MACS LS column was placed in a midi-MACS separator magnet and rinsed with 3 mL of degassed MACS buffer. The magnetically labelled cell suspension was then placed onto the column, and the effluent collected as the negative fraction. The column was washed thrice with 3 mL of buffer, then removed from the magnet and flushed with 5 mL of buffer to collect the positive fraction. In some cases the positive fraction was passed over a new freshly prepared column to increase the purity of positively labelled cells. The purity of cells following the isolation procedure was assessed by staining with immunofluorescent antibodies and measured.

#### **2.4.5 Fluorescence Activated Cell Sorting (FACS)**

MACS sorted CML CD34<sup>+</sup> cells were aliquoted into 5 mL FACS tubes as described in Table 2.4. Test cells were sorted for CD34<sup>+</sup>38<sup>+</sup> and CD34<sup>+</sup>38<sup>-</sup> fractions on a Becton Dickinson Aria, using FACS Diva Software version 6.1.3 (BD Biosciences, San Diego, CA). The CD34<sup>+</sup>38<sup>-</sup> fraction was routinely <5% of the total CD34<sup>+</sup> population.

#### **2.4.6 Cryopreservation of cells**

Cells were resuspended slowly in a cryoprotectant mixture of 70% HBSS with 10 mM HEPES, 20% FBS and 10% cryoprotectant dimethylsulphoxide (DMSO) at concentration of  $1 \times 10^7$ /mL. Resuspended cells were immediately transferred to pre-labelled ice-cold cryogenic vials and cryopreserved using a Planer KRY010 Series 2 controlled-rate freezer. Cryopreserved samples were stored in the vapour phase of liquid nitrogen (-196°C).

#### **2.4.7 Thawing of Cells**

Cryogenic samples were removed from liquid nitrogen and thawed rapidly by immersion in a 37°C water bath with manual agitation. The cell suspension was quickly transferred to a 50 mL tube, and approximately 20 ml of thaw solution (pre-warmed to 37°C) was added drop-wise with continuous gentle mixing. The sample volume was then increased to 30 mL with thaw solution and the cells pelleted by centrifugation at 1,200 rpm for 10 min. The supernatant was aspirated and the procedure was repeated to remove all residual DMSO.

#### **2.4.8 Cell counts and viability**

Cell concentration was determined by diluting the cell suspension 1:9 in white cell fluid (WCF) and cell viability was assessed by diluting samples 1:1 with 0.4% trypan blue solution. 10 $\mu$ l of these suspensions were transferred to a haemocytometer counting chamber (Neubauer Improved, Assistant, Germany) and cell concentration and viability calculated accordingly.

## 2.5 Special techniques

### 2.5.1 Drug Treatments and Washing Protocols

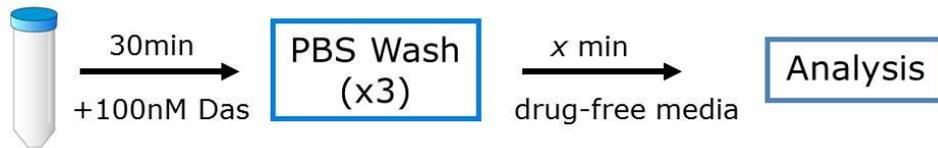
Cells were cultured with either 100 nM dasatinib or 32.5  $\mu$ M imatinib for a minimum of 30 min to a maximum of 24 h followed by the standard (STD) wash or optimal (OPT) wash protocols (Figure 2.1). In the standard wash protocol, following incubation with TKI, cells were centrifuged at 1400 rpm for 5 min, the supernatant was aspirated and the cells were washed with 37°C 1xPBS three consecutive times before being suspended in TKI-free media for the remainder of the 72 h at 37°C/5% CO<sub>2</sub>. The optimal washout protocol is similar to the standard wash protocol, with the exception that cells are allowed to equilibrate for 1 h at 37°C/5% CO<sub>2</sub> in drug-free media between each wash.

### 2.5.2 Flow cytometry to assess the cell death

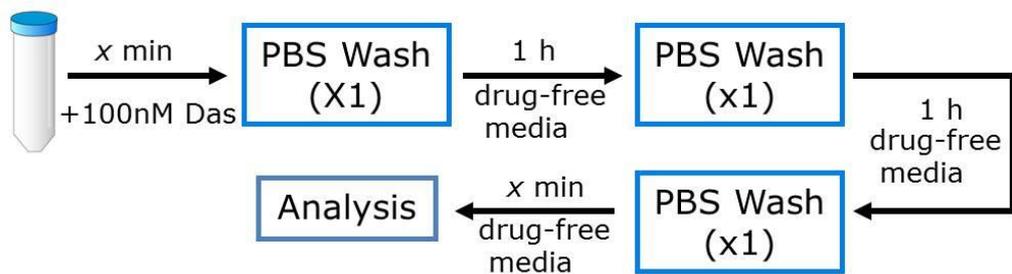
*BCR-ABL1*<sup>+</sup> cell lines ( $2 \times 10^5$  cells) were cultured with TKIs under the specified conditions. KU812 and Meg01 cells were cultured in RPMI-1640 + 10% FBS. K562 cells were culture in IMDM + 5% BSA. Primary patient CD34<sup>+</sup> cells were cultured in SDM in the presence or absence of a 5GF cytokine mix (refer to Materials and Methods 2.2.9). After 72 h cell viability was assessed by trypan blue exclusion. Cell death was assessed by flow cytometry using PE Annexin V (BD Biosciences, USA), 7-AAD and trypan blue staining at 24 h, 48 h or 72 h.

A working solution of Annexin V and 7AAD was prepared depending on the number of samples to be processed. 3  $\mu$ l of Annexin V stock solution was diluted in the 10  $\mu$ l of binding buffer per sample and 1  $\mu$ l of 7-AAD stock solution was diluted in 40  $\mu$ l of binding buffer. The positive control for Annexin V was prepared by incubating cells with

### Standard (STD) washout



### Optimal (OPT) washout



**Figure 2.1: Schematic of the standard and optimal washout procedures.**

Standard washout (STD) refers to transient exposure to dasatinib (Das) for 30min, followed by 3 immediate washes in 10 ml pre-warmed PBS. Optimal washout (OPT) refers to x 3 washes in 10 ml pre-warmed PBS, with equilibration of cells in drug-free media for 1 h in between washes.

50% DMSO for 10 min at room temperature and positive control for 7-AAD was prepared by incubating cells with 70% cold ethanol on ice for 10 min. After incubation with DMSO or ethanol, cells were washed three times with 5 ml of binding buffer.

Cells were harvested in labelled tubes at specified time; 5 ml of binding buffer was added and centrifuged at 2700 rpm for 2 min. Supernatant was removed without disturbing the cell pellet and 40  $\mu$ l 7-AAD and 10  $\mu$ l Annexin V working solution was added in all the samples and respective positive control tubes. After gentle mixing, cells were incubated on ice, in the dark for 30 min. After incubation, 250  $\mu$ l of binding buffer was added to each tube and tubes were kept on ice until flow cytometry could be performed.

Samples were analysed on a FC500 flow cytometer (Beckman Coulter, Fullerton, CA 92831, USA). Forward scatter, side scatter, voltage and compensation were set from the negative and positive control tubes. The Annexin V-PE positive events were collected in FL2 while 7-AAD positive events were collected in FL-4. After setting the voltage and compensation, samples were run and data on 30,000 events were collected. The data were analysed using FCS Express 4 Research Edition (De Novo Software).

### **2.5.3 Flow cytometry to assess pSTAT5 (intracellular staining)**

Following indicated treatment, cells were harvested in labelled tubes and fixed with 1.5% paraformaldehyde (PFA) by incubating for 15 min at room temperature. Following fixation, the cells were centrifuged and washed once with 1x PBS. After removing supernatant, cells were permeabilised by the drop-wise addition of 1 ml ice-cold 100%

methanol while gently vortexing. The sample was incubated at 4°C for at least 20 min, or alternatively, cells can be temporarily stored in 100% methanol at -20°C for up to a week. The cells were washed three times with 1x PBS (1600 rpm for 5 min). After removing the supernatant, 50 µl of diluted p-STAT5 Alexa 488 (BD) antibody was added to each tube and the sample was vortexed and incubated at room temperature in the dark for 30 min. After incubation the sample was washed and then resuspended in 500 µl of 1xPBS. The samples were analysed on the FC500 flow cytometer and the data collected in FL-1 were analysed by using FCS Express software.

#### **2.5.4 Western Blot following SDS-PAGE**

Following a specific treatment  $2 \times 10^5$  cells were placed into a 1.5 mL microfuge tube, and pelleted by centrifugation for 5 min at 6,500 rpm. All supernatant was removed and cells were washed once in cold 1x PBS before lysing in 20 µL of modified 1x Laemmli's buffer by boiling in a 100°C heat block for 12 min. Cell lysates were stored at -20°C. Protein lysates corresponding to  $1 \times 10^5$  cells/10 µL were resolved on a 4-15% Criterion Precast TGX Gel (Bio-Rad Laboratories, Hercules, CA), and the protein electrophoretically transferred to low-fluorescence PVDF membrane (Bio-Rad) using a Bio-Rad Trans-Blot® Turbo™ Blotting System.

The membrane was incubated for 1 h at room temperature with appropriate blocking solution (either 2.5% membrane blocking buffer in 1xTBST or 3% BSA in 1xTBST). The membrane was then probed for 2 h at room temperature with Santa Cruz anti-CrkL C20 antibody, 1:1000 in 2.5% blocking solution, or overnight at 4°C for Cell Signaling Technology primary antibodies. Following primary antibody incubation, the membrane was rinsed twice in 1xTBST buffer, and then washed for 3x5 min with 1xTBST buffer

before incubating with 1:2000 alkaline-phosphatase (AP) conjugated anti-rabbit immunoglobulin (Santa Cruz Biotechnology) in 2.5% blocking solution for 1 hour at room temperature. The membrane was then rinsed twice in 1x TBST buffer, 3x5 min in 1xTBST buffer and then 3x5 min in 1xTBS buffer. Bound antibodies were detected using ECF attaphos substrate (GE Healthcare Lifesciences, Uppsala, Sweden) on a Typhoon FLA 9000 fluorimager (GE Healthcare Lifesciences). Phosphorylation was then quantified using ImageQuant-TL software for densitometric analysis (Molecular Dynamics, Sunnyvale, CA).

To detect total protein, the membrane was incubated in 1x Western Stripping Buffer (Alpha Diagnostic) at room temperature for 15 min and washed in the appropriate blocking solution 2x 20 minutes. Membrane was then re-probed with Total Protein antibodies (1:1000) overnight at 4°C. The membrane was then rinsed twice in 1x TBST buffer and washed 3x5 min in 1xTBST buffer before incubation with AP-conjugated anti-rabbit immunoglobulin in 2.5% blocking solution for 1 hour at room temperature and then washed as above. Bound antibodies were detected with ECF attaphos substrate on a Typhoon FLA 9000 fluorimager. Total proteins were quantified using ImageQuant-TL software.

Phosphorylated CrkL (p-CrkL) was determined as a % of the total CrkL protein.

*Volume phospho-protein / volume of Total protein = relative ratio at given time point*

*Relative ratio at any time point / relative ratio of untreated levels x 100*

*= % phospho-protein / Total protein for each time point*

### **2.5.5 Intracellular Uptake and Retention (IUR) assay**

The IUR assays were performed as previously described by White *et al.* All assay points were performed in triplicate.  $2 \times 10^5$  cells were incubated in 2 mL of RPMI-1640 media + 10% FBS in the presence or absence of 2  $\mu$ M [ $^{14}$ C]-labelled dasatinib for 2 h at 37°C/5% CO<sub>2</sub>. Following this time tubes were centrifuged at 6,800 rpm for 5 min, then pulse spun to 13,000 rpm for 30 seconds. A 20  $\mu$ L aliquot of supernatant (S/N) from each tube was then added to 100  $\mu$ L of Microscint-20 scintillation fluid (PerkinElmer, Waltham, Massachusetts) in a white 96-well flat bottomed plate (OptiPlate, PerkinElmer). The remaining supernatant was then aspirated from the tubes and 50  $\mu$ L of Microscint-20 was added. The tube was vortexed well, and pulse spun for 15 seconds at 13,000 rpm. The lysed cells were then transferred to the 96- well plate with wells containing 50  $\mu$ l of Microscint-20. The plate was covered with an adhesive plastic seal and counted on a TopCount beta scintillation counter (PerkinElmer) as counts per minute (cpm). The incorporation of [ $^{14}$ C]-dasatinib in the cells and supernatant was determined and quantity of dasatinib expressed as cpm.

### **2.5.6 Analysis of cellular morphology**

The cellular morphology of *BCR-ABL1*<sup>+</sup> cell lines was examined by May-Grunwald/Giemsa staining following cyospin of cells. To prepare cyospins, *BCR-ABL1*<sup>+</sup> cells were harvested from culture and resuspended at  $3 \times 10^5$  cells/ml. The cell suspension (100  $\mu$ l) was deposited onto microscope slides and spun for 5 min at 500 rpm. Slides were allowed to dry and fixed for 1 min in 100% methanol before staining with May-Grunwald (Sigma-Aldrich) for 5 min. The slides were rinsed briefly in distilled water and placed in 1:20 dilution of Giemsa for 20 minutes. The slides were dipped in water again and allowed to dry prior to mounting. Cells were evaluated under 20x or

40x magnification on a light microscope (BX51, Olympus, Japan). Photographs were taken with a DP70 camera (Olympus, Japan) and DP analysis software (Olympus Imaging Systems, Germany).

### **2.5.7 Full Moon BioSystems Antibody Microarray**

Antibody microarrays (Full Moon BioSystems, Sunnyvale, CA) were utilised for fluorescent detection of proteins, providing a high-throughput system for expression profiling and comparison of treated and untreated samples. For full details of the proteins contained in each array see Appendix I-II. The arrays were carried out according to the manufacturer's instructions. Briefly,  $2.5 \times 10^6$  K562 or KU812 cells were harvested following 30 min exposure to 100 nM dasatinib and placed in a 50 ml tubes for centrifugation (5 min at 1400 rpm). The supernatant was aspirated and the cells were washed 3× in cold PBS. Cells were transferred to clean 1.7 ml tubes before addition of lysis beads and 200 µl extraction buffer. Cells were vortexed vigorously for 1 min before incubation on ice for 10 min. The vortexing step was repeated at 10 min intervals for the next 60 min. Subsequently, cells were centrifuged at 14,000 rpm for 20 min at 4°C and transferred to clean tubes. The supernatant was applied to spin columns 100 µl at a time, the columns placed in collection tubes and tubes centrifuged for 2 min at 750 g. The UV absorptions of resultant protein lysates were measured using a NanoDrop Spectrophotometer (Thermo scientific). Subject to satisfactory absorbance and concentration readings in line with the manufacturer's instructions, 45 µg of lysates were mixed with labelling buffer and 3 µl Biotin/DMF solution. The mixture was incubated at room temperature for 2 h before addition of 35 µl stop reagent. After incubation at room temperature for 30 min, lysates were combined with 6 ml coupling solution and poured over pre-blocked microarray slides. Slides were incubated for 2 h at

room temperature on an orbital shaker before washing twice for 10 min in wash solution. Slides were rinsed extensively in Milli-Q® H<sub>2</sub>O before detection with detection buffer containing 60 µl Cy3-Streptavidin (0.5 mg/ml). Completed slides were sent to Full Moon BioSystems Array Scanning Service for analysis.

### **2.5.8 Statistical Analyses**

Figures were constructed using GraphPad Prism 5.01 © software (GraphPad Software Inc. La Jolla, CA). Column graphs represent the mean plus the standard error of the mean (SEM). Student's T-test was used to determine differences between experimental groups. Differences were considered to be statistically significant when the probability value (p value) was <0.05.

## **CHAPTER 3.**

# **INDUCTION OF AUTOPHAGY BY TYROSINE KINASE INHIBITION IN *BCR- ABL1*+ CELLS PREVENTS CELL DEATH**

### 3.1 Introduction

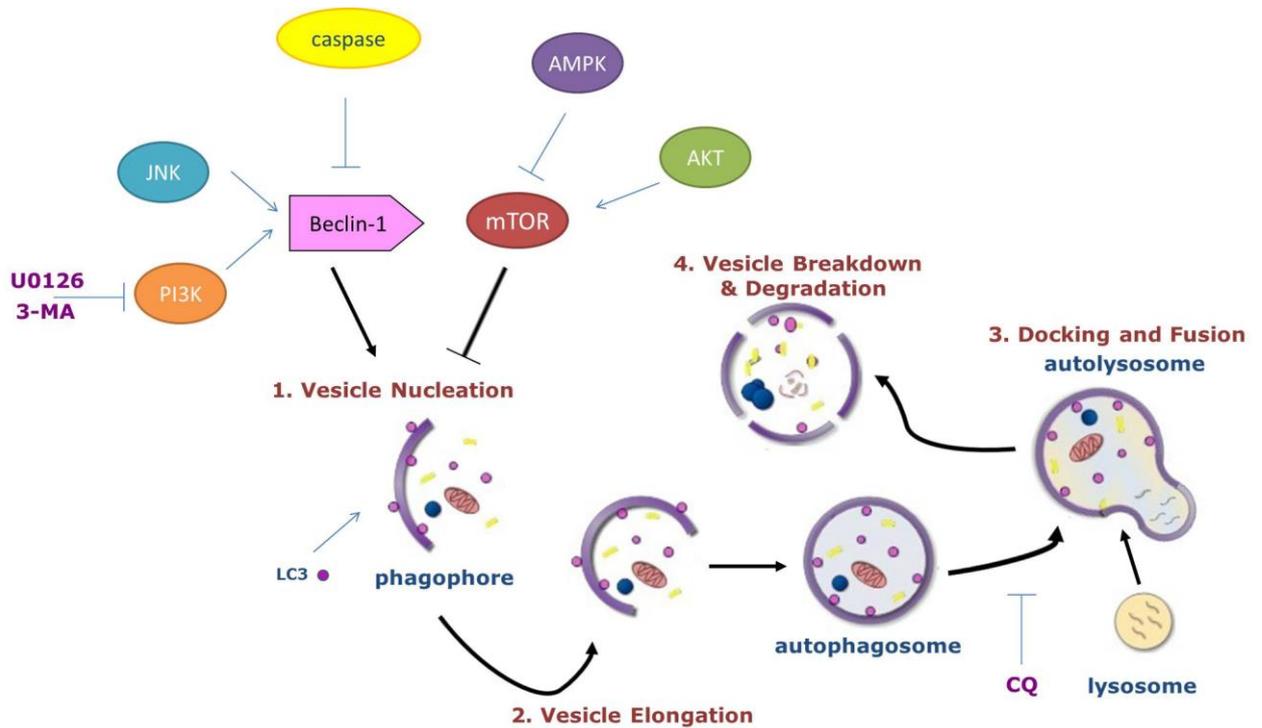
CML is characterised by the presence of Bcr-Abl, a constitutively active tyrosine kinase.<sup>35</sup> Although the therapeutic inhibition of Bcr-Abl with TKIs has been largely successful, some patients develop resistance to TKI-therapy.<sup>106</sup> Mechanisms of acquired TKI resistance include BCR-ABL kinase domain mutations,<sup>127</sup> BCR-ABL amplification,<sup>126</sup> and most recently, the induction of macroautophagy have been proposed as possible mechanisms of TKI-resistance.<sup>185</sup>

Macroautophagy, hereafter referred to as autophagy, has historically been considered an adaptive response to starvation induced stress, allowing cell survival in growth factor deprived environments.<sup>183</sup> Initiation of autophagy instigates the formation of phagophores, which sequester proteins and whole organelles to form double-membrane autophagosomes, a morphological characteristic of autophagic cells (Figure 3.1). The autophagosomes then fuse with lysosomes resulting in the degradation of cellular components by lysosomal activity in the newly formed autolysosome, providing energy for the cell to sustain itself through a time of low nutrient availability.

Not surprisingly, inhibition of autophagy has a negative impact on cell survival. Inhibition of early stage autophagy results in classical apoptosis, whereas inhibition of late stage autophagy results in the accumulation of autophagic vacuoles, followed by apoptosis.<sup>190</sup> TKI-induced blockade of Bcr-Abl-driven survival pathways combined with inhibition of autophagy by chloroquine (CQ), a late stage autophagy inhibitor, has previously been shown to restore sensitivity of resistant *BCR-ABL1*+ cells to TKI.<sup>184,185</sup> However, information concerning how inhibition of Bcr-Abl induces autophagy is scarce,

thus the first part of this chapter aims to determine the important events that follow inhibition of Bcr-Abl blockade which lead to the induction of autophagy.

Recently, rapid reductions in the BCR-ABL transcript level in TKI-resistant CML patients were reported where clarithromycin (CAM) was added in combination to their TKI therapy.<sup>191</sup> This highlighted CAM as a candidate for combination therapy in CML patients, in particular those with advanced and resistant disease. CAM is one of several macrolide antibiotics demonstrated to inhibit cancer cell growth. In multiple myeloma cells, CAM induces cell death through inhibition of autophagy at the clinically relevant concentrations of 6-50 µg/ml.<sup>189</sup> Carella *et al.*<sup>188</sup> hypothesised that CAM was acting as an inhibitor of autophagy in resistant CML cells, however the study did not demonstrate any experimental evidence to support this. Therefore the major aims of the second part of this chapter were to investigate the effect of CAM on TKI-induced cell death *in vitro*, and to determine the effect of CAM on the inhibition of autophagy.



**Figure 3.1: Model of the autophagic process**

Upon induction of autophagy signals, elongation of the phagophore engulfs long-lived proteins and whole organelles to form double-membrane autophagosomes. Fusion of the autophagosome with the lysosome results in the formation of an autolysosome which degrades the sequestered components supplying energy and thus allowing cell survival.

### 3.1.1 Approach

Induction of autophagy was assessed with the markers LC3 (converted from LC3-I to LC3-II upon formation of the autophagosome) and p62 (degraded in the autolysosome). The expression of Beclin-1 (required for induction of autophagy) was assessed in context of the expression of its interacting partners Bcl-2 and Bcl-xL (which sequester Beclin-1 thereby preventing induction of autophagy). A protein kinase assay was utilised to identify additional pathways which may be involved.

The effect of CAM on autophagy in *BCR-ABL1+* cells has not previously been examined *in vitro*, thus the effects of CAM in combination with dasatinib and imatinib were investigated in the *BCR-ABL1+* cell lines, K562 and KU812. Cell death was determined by flow cytometry following Annexin V and 7AAD staining. Bcr-Abl activity was assessed using the Bcr-Abl substrate CrkL, a surrogate for Bcr-Abl activity. To detect additional effects of combination therapy dasatinib was used at a concentration that induced approximately 50% reduction in live cells. May-Grunwald/Giemsa staining was used to assess vacuole formation in the presence of inhibitors.

### 3.1.2 Summary and Research Contribution

This study demonstrates a role for autophagy in the resistance of *BCR-ABL1+* cells to TKI therapy. Induction of autophagy is demonstrated by increased LC3-II and decreased p62, with novel information demonstrating the availability of Beclin-1 corresponding with Bcl-2 and Bcl-xL. The energy sensing LKB1/AMPK pathway, in conjunction with Pim3 kinases, is presented as a possible mechanism for TKI-induced autophagy, highlighting these pathways for further investigation. For the first time, CAM is shown to

effectively increase the sensitivity of *BCR-ABL1*+ cells to TKI-induced cell death *in vitro*, whilst having little or no effect on cell death as a sole agent. The mechanism of induction of cell death by CAM combined with dasatinib appears to be via inhibition of late stage autophagy.

### 3.1.3 Publications

**Schafranek L**, Leclercq TM, White DL and Hughes TP. Clarithromycin enhances dasatinib-induced cell death in chronic myeloid leukemia cells, by inhibition of late stage autophagy. *Leuk Lymphoma* 2013; 54: 198–201.

### 3.1.4 Conference Presentations

**Schafranek L**, Leclercq TM, White DL and Hughes TP. Macrolide Antibiotic Clarithromycin targets TKI-induced autophagy in CML cells. HAA Oct, 2012 Melbourne, Australia (oral presentation).

**Schafranek L**. Cannibalistic Cancer. 3 Minute Thesis Competition, July 2013, University of Adelaide, Australia (oral presentation). **Faculty Finalist**

**Schafranek L**, Leclercq TM, White DL and Hughes TP. Overcoming resistance to tyrosine kinase inhibitors in chronic myeloid leukaemia by blocking autophagy with clarithromycin. Medical Staff Society Research Prize, May, 2013, Adelaide, Australia (oral presentation).

**Schafranek L**, Leclercq TM, White DL and Hughes TP. Clarithromycin Enhances TKI-Induced Cell Death In CML Cells. NDLR March, 2012, Sunshine Coast, Australia (poster presentation).

**Schafranek L**, Leclercq TM, White DL and Hughes TP. Clarithromycin increases the sensitivity of chronic myeloid leukaemia cells to dasatinib. CPCM Symposium, July 2012, National Wine Centre, Adelaide, Australia (poster presentation).

**Schafranek L**, Leclercq TM, White DL and Hughes TP. Clarithromycin increases the sensitivity of chronic myeloid leukaemia cells to dasatinib. FHS conference, August 2012, University of Adelaide, Australia (poster presentation).

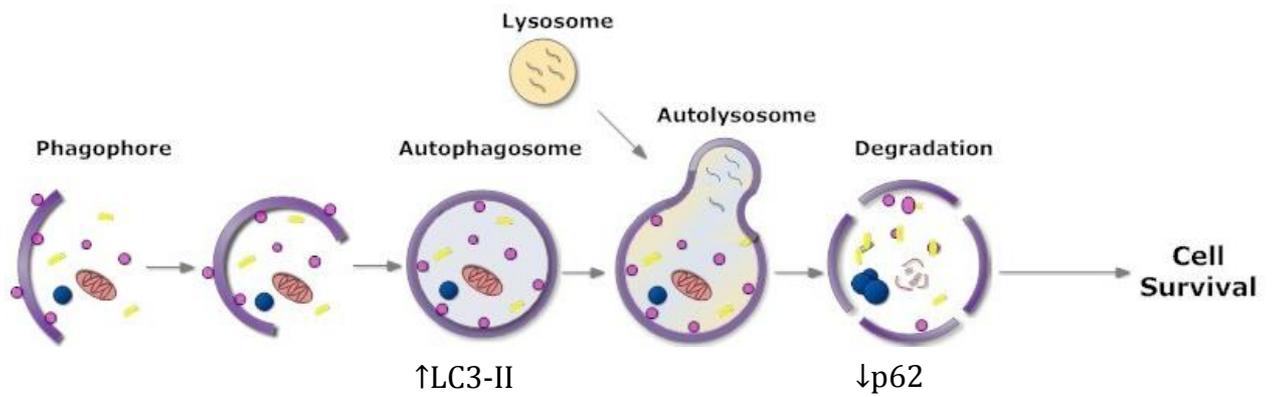
## 3.2 Results

### 3.2.1 Inhibition of Bcr-Abl with TKIs induces markers of autophagy in KU812 cells

Emerging evidence suggests that cancer cells escape chemotherapeutic induced death through activation of autophagy.<sup>19,192</sup> To determine if autophagy is activated as a protective response to TKI therapy in *BCR-ABL1*+ cells, markers of autophagy were evaluated in the KU812 cell line. The LC3 protein is converted from LC3-I to LC3-II during the formation of the autophagosome,<sup>193</sup> therefore an increase in LC3-II signifies the induction of autophagy (Figure 3.2). Additionally, autophagy can be measured by p62 protein which is decreased upon autolysosome degradation (Figure 3.2). Cells were treated with 2  $\mu$ M imatinib, 1000 nM nilotinib or 100 nM dasatinib and increased LC3-II (and subsequent decreased LC3-I) were observed (Figure 3.3). In parallel, each of the three TKIs induced a decrease in p62 (Figure 3.3). These results indicate that Bcr-Abl tyrosine kinase inhibition with imatinib, nilotinib or dasatinib induce autophagy.

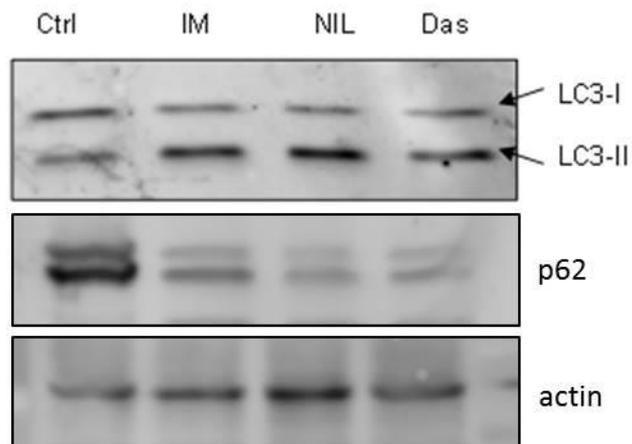
### 3.2.2 TKI-induced loss of Bcl-2 and Bcl-x<sub>L</sub> expression in KU812 cells allows release of Beclin-1

Expression of Beclin-1, which is required for the induction of autophagy,<sup>194</sup> was assessed in *BCR-ABL1*+ KU812 cells treated with increasing concentrations of imatinib, nilotinib or dasatinib, to ascertain if TKI treatment induces upregulation of Beclin-1. Exposure to either imatinib or nilotinib had no effect on the expression of Beclin-1 (Figure 3.4). However, Beclin-1 can be sequestered by Bcl-2 and Bcl-x<sub>L</sub>, which are overexpressed due to the presence of Bcr-Abl.<sup>47,76,195</sup> In cells treated with imatinib and nilotinib, a dose dependent decrease in Bcl-2 was observed (Figure 3.4),



**Figure 3.2: Markers of Autophagy**

Induction of autophagy results in the metabolism of proteins and organelles, allowing cell survival during environmental stress. There is an increase in conversion of LC3-I to LC3-II upon formation of the autophagosome, and a decrease in p62 upon degradation of the autolysosome.



**Figure 3.3: Induction of Autophagy Following TKI treatment in KU812 cells**

KU812 cells were treated with 2  $\mu$ M imatinib (IM), 1000 nM nilotinib (NIL) or 100 nM dasatinib (Das) for 24 h. Lysates were analysed by western blotting for LC3 and p62. LC3-I is converted to LC3-II upon formation of the autophagosome. p62 is degraded following fusion of the autophagosome with the lysosome. Data is representative of three independent experiments.

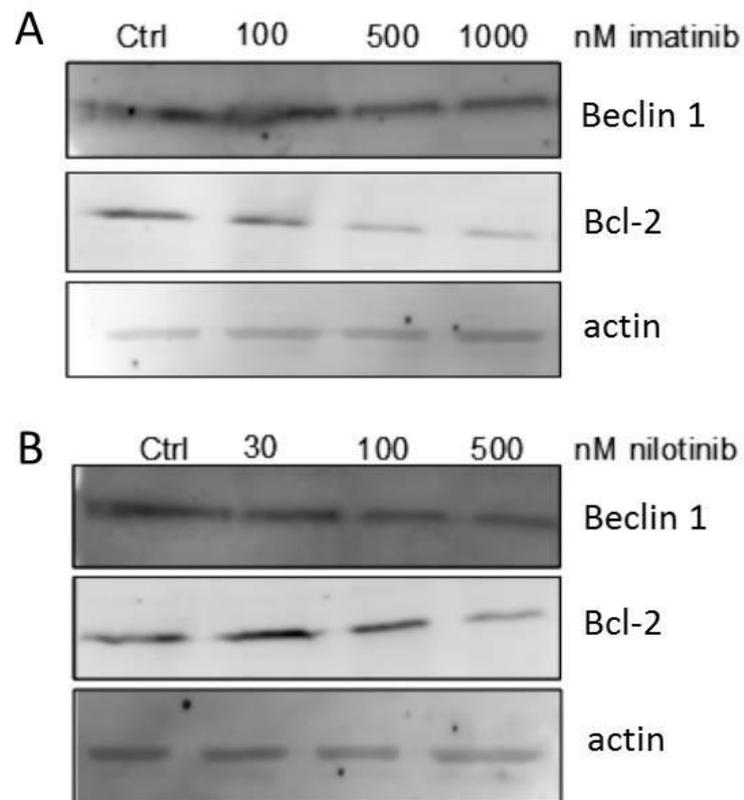
indicating the release of Beclin-1 for the induction of autophagy. Interestingly, dasatinib resulted in an increase in Beclin-1, in addition to a dose dependent decrease in Bcl-2 and Bcl-x<sub>L</sub> (Figure 3.5).

### **3.2.3 Increase in vacuoles induced by autophagy inhibitor CQ in combination with dasatinib**

The morphology of K562 cells was examined by May-Grunwald/Giemsa staining (**Methods 2.5.6**) for the presence of vacuoles (phenotypic marker of autophagy) following treatment with or without CQ in the presence or absence of dasatinib for 24 h. Exposure to 100 nM dasatinib resulted in the presence of vacuoles in some cells, coupled with cell membrane blebbing (Figure 3.6). No obvious difference was observed in the percentage of cells with vacuoles between untreated cells and cells treated with 10  $\mu$ M CQ. However the combination of CQ with dasatinib demonstrated an evident accumulation in vacuoles, indicating the inhibition of Bcr-Abl with dasatinib induced autophagy as the vacuoles are being prevented from fusion with the lysosome.

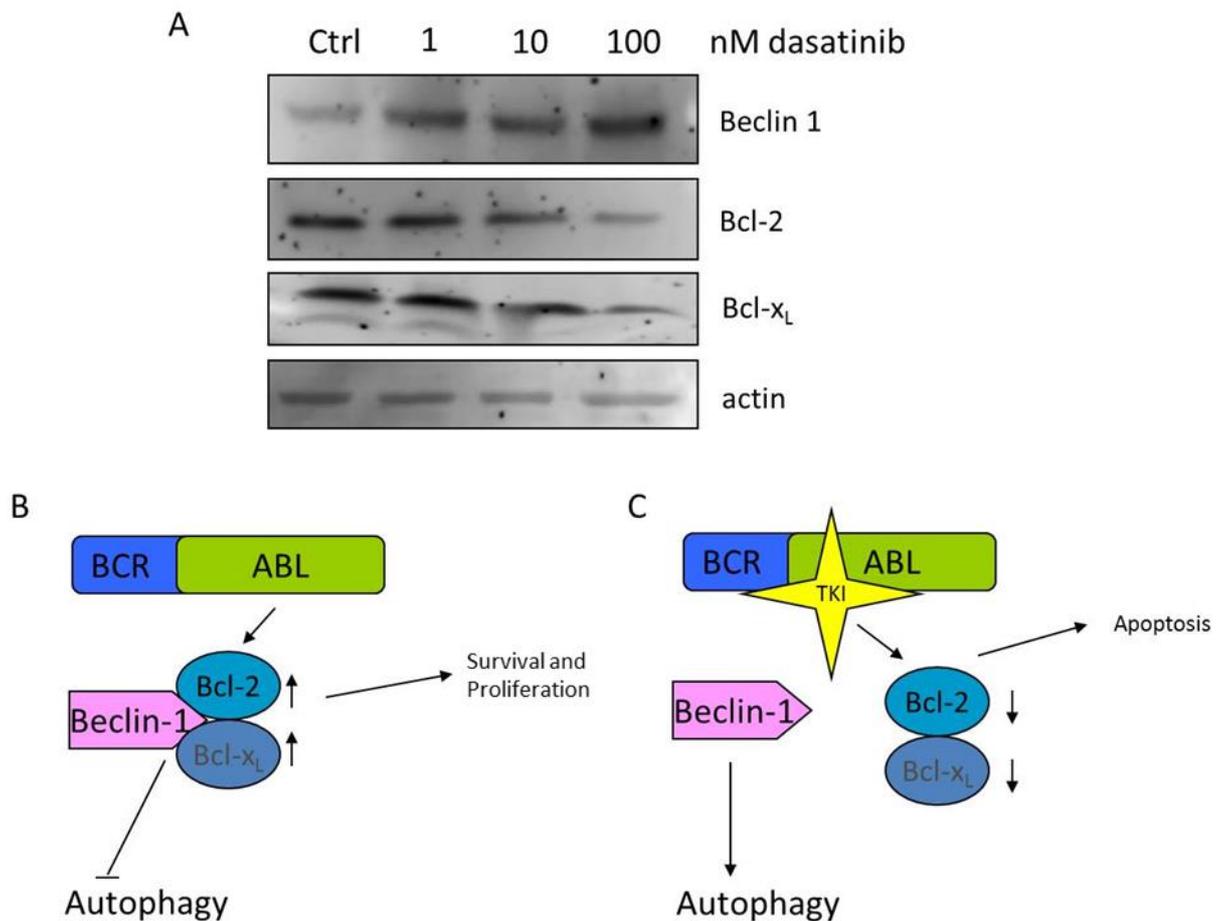
### **3.2.4 Upregulation of autophagy-associated kinase LKB1 following dasatinib treatment.**

Having identified the presence of Beclin-1 following treatment with TKIs, a protein microarray (**Methods 2.5.7**) was utilised to assess 276 potential kinase targets of dasatinib. KU812 cells were exposed to 100 nM dasatinib for 30 min and lysates were prepared and signals were analysed by Full Moon BioSystems (Appendix III). The most upregulated kinase in this analysis was serine/threonine kinase 11 (STK11) which increased from an intensity of 284 (mean signal intensity (MSI)) in untreated KU812 cells to 1080 MSI in KU812 cells treated with dasatinib (Figure 3.7).



**Figure 3.4: Imatinib and nilotinib decrease Bcl-2, preventing sequestration of Beclin-1.**

KU812 cells were transiently exposed to increasing concentrations of **(A)** imatinib or **(B)** nilotinib. Lysates were analysed by western blotting for Beclin-1 and Bcl-2. Actin was used as the loading control. Data is representative of three independent experiments.



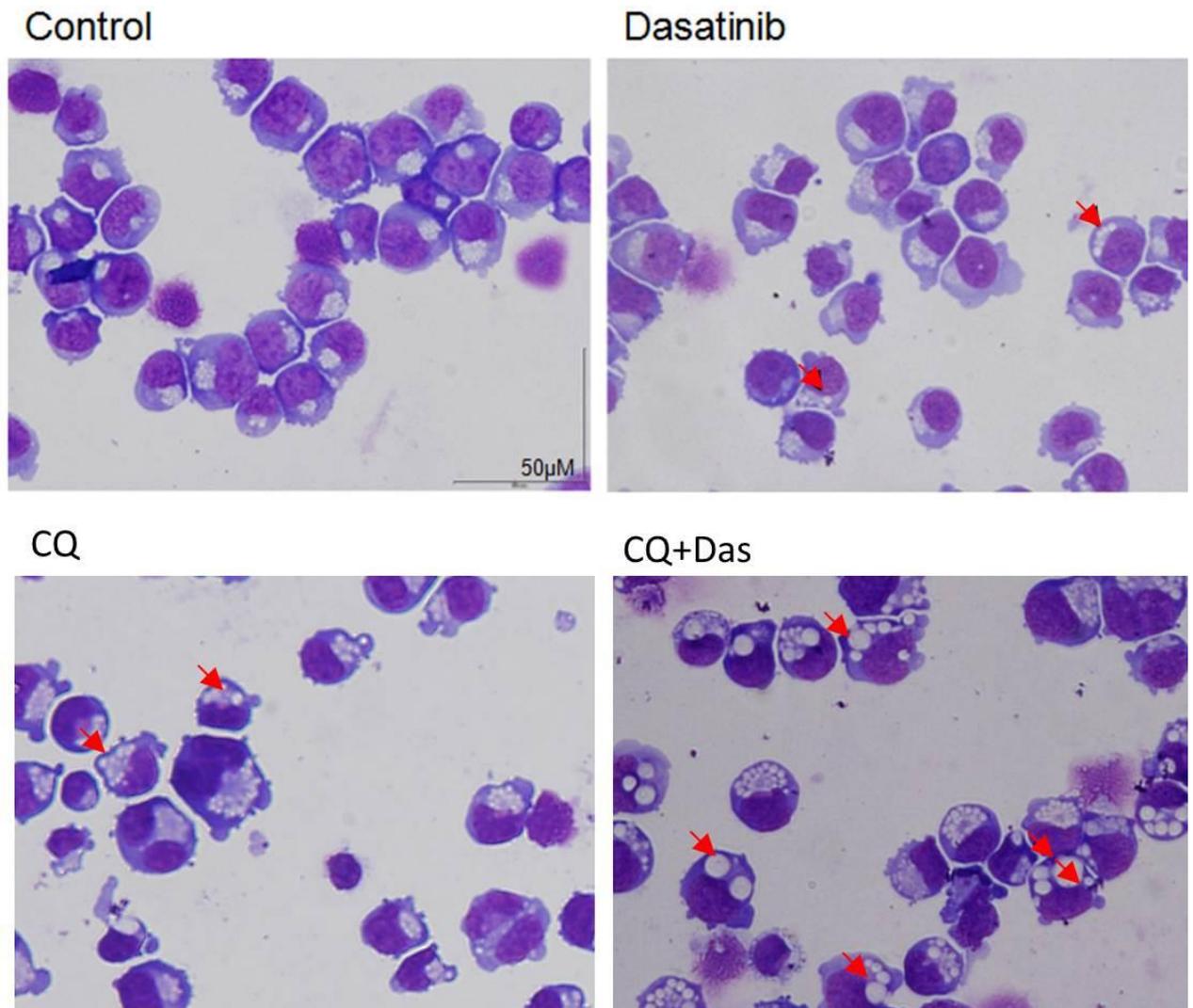
**Figure 3.5: Dasatinib increases expression of Beclin-1 and decreases expression of Bcl-2 and Bcl-x<sub>L</sub>.**

**(A)** KU812 cells were transiently exposed to increasing concentrations of dasatinib.

Lysates were analysed by western blotting for Beclin-1, Bcl-2 and Bcl-x<sub>L</sub>. Actin was used as loading control. Data is representative of three independent experiments. **(B)** Beclin-

1 and Bcl-2 family interaction. Bcl-2 family members bind Beclin-1 preventing the

initiation of autophagy. **(C)** Bcl-2 and Bcl-x<sub>L</sub> are reduced upon tyrosine kinase inhibition releasing Beclin-1 for the initiation of autophagy.

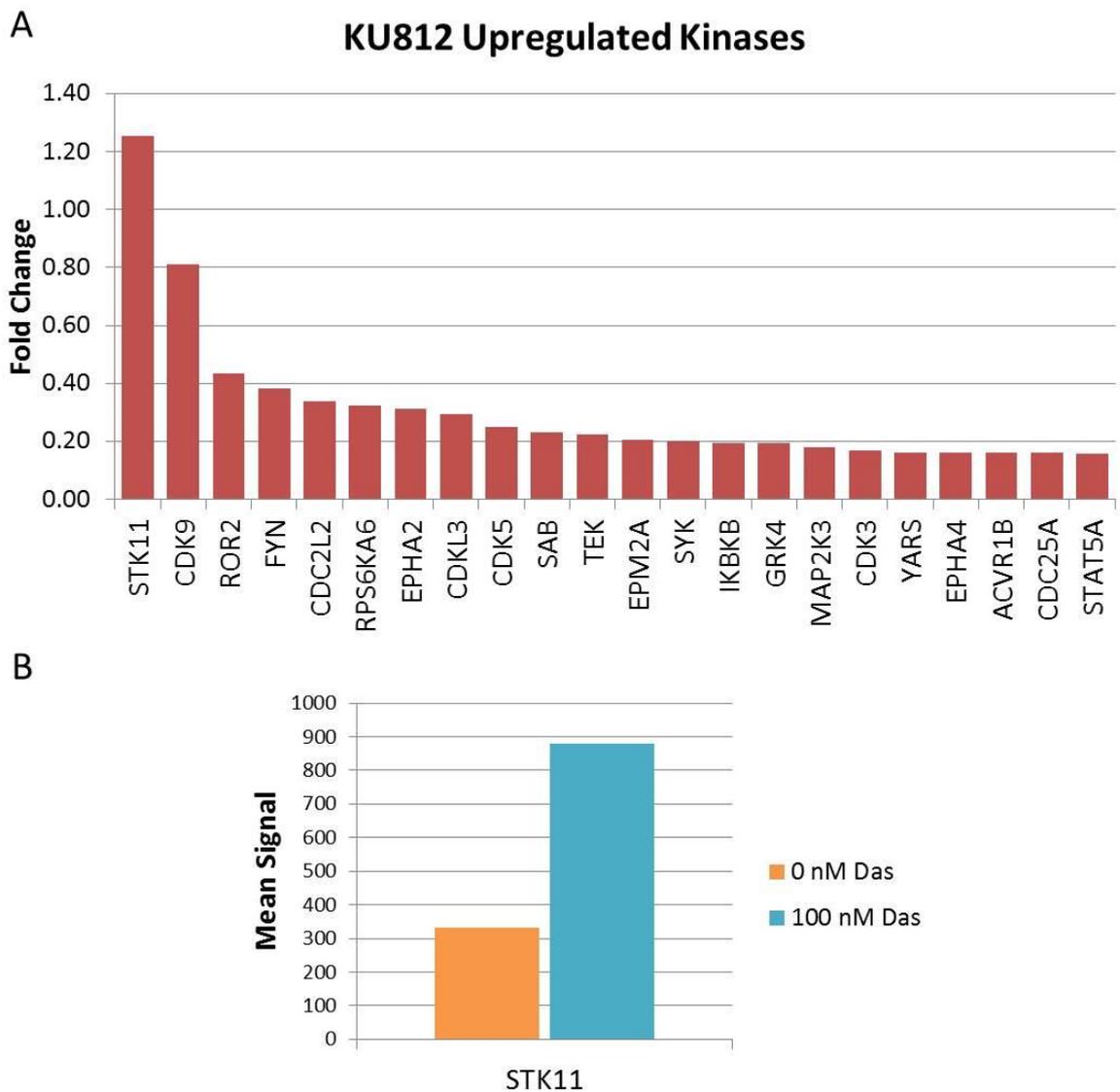


**Figure 3.6: CQ in combination with dasatinib results in accumulation of vacuoles**  
Vacuole morphology was assessed by May-Grunwald/Giemsa stain following cytopspin of K562 cells cultured with or without 5 nM dasatinib (Das), 10 μM CQ, or a combination of Das with CQ for 24 hrs. Arrows (▶) indicate vacuoles.

STK11, also known as liver kinase B1 (LKB1), is an energy sensing tumour suppressor that induces autophagy in conjunction with AMPK through the inhibition of mTOR.<sup>196,197</sup> The upregulation of LKB1 as a result of Bcr-Abl inhibition was then validated in KU812 cells exposed to 2  $\mu$ M imatinib, 1000 nM nilotinib or 100 nM dasatinib for 3 h. A dose dependent increase was observed in cells exposed to each of the three TKIs (Figure 3.8).

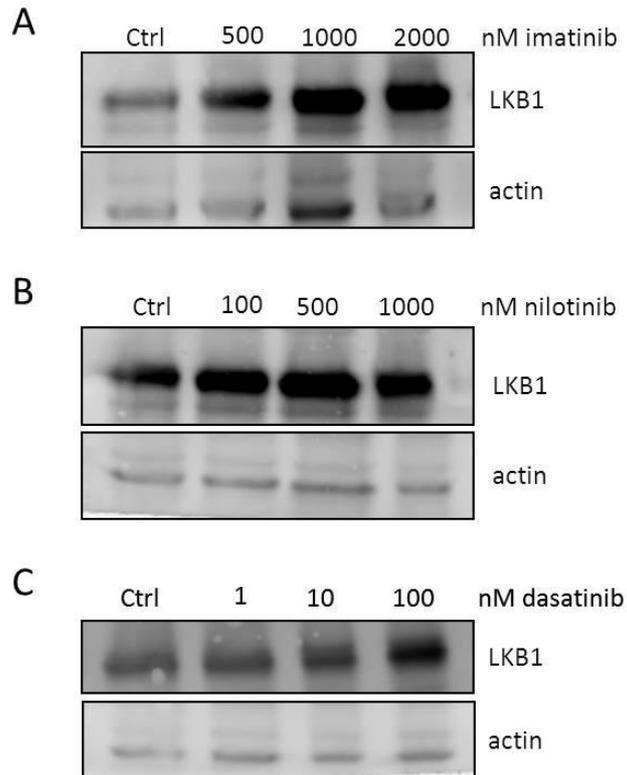
### **3.2.5 Downregulation of PIM-3, MAP3K5 and CHEK2 kinases following dasatinib treatment.**

Utilising the same protein microarray approach, a number of downregulated kinases were also observed (Figure 3.9). Mitogen-activated protein kinase kinase kinase 5 (MAP3K5), checkpoint kinase 2 (CHECK2), and PIM3 were chosen to be validated with imatinib and nilotinib in addition to dasatinib treatment. MAP3K5, also known as apoptosis signalling kinase 1 (ASK1), is involved in stress-related apoptosis,<sup>198</sup> was reduced from 1755 MSI in untreated KU812 cells to 165 MSI in KU812 cells treated with 100 nM dasatinib. CHECK2, from now on referred to as Chk2, which is reported to phosphorylate p53 as a DNA damage response,<sup>199</sup> demonstrated a reduction from 2820 MSI in untreated KU812 cells to 1491 MSI in KU812 cells treated with dasatinib. PIM3, a serine/threonine kinase, shown to negatively regulate LKB1,<sup>200</sup> was reduced from 12527 MSI in untreated KU812 cells to 11466 MSI in KU812 cells treated with dasatinib. To confirm these results, KU812 cells were exposed to 2  $\mu$ M imatinib, 1000 nM nilotinib or 100 nM dasatinib. Only dasatinib treatments resulted in inhibition of ASK1 and Chk2 expression, whereas Pim3 expression was decreased by imatinib, and nilotinib, as well as dasatinib treatment (Figure 3.10).



**Figure 3.7: Kinase upregulation by dasatinib.**

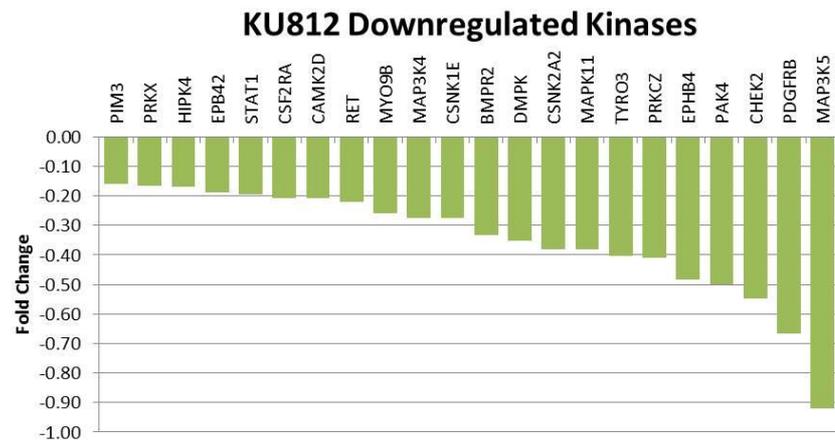
KU812 cells were treated with 100 nM dasatinib for 30 min. Cells were lysed and analysed according to Full Moon BioSystems protocol (refer to Materials and Methods 2.5.7). **(A)** Mean fold change of top upregulated kinases. **(B)** Mean signal intensity of STK11 with and without dasatinib treatment.



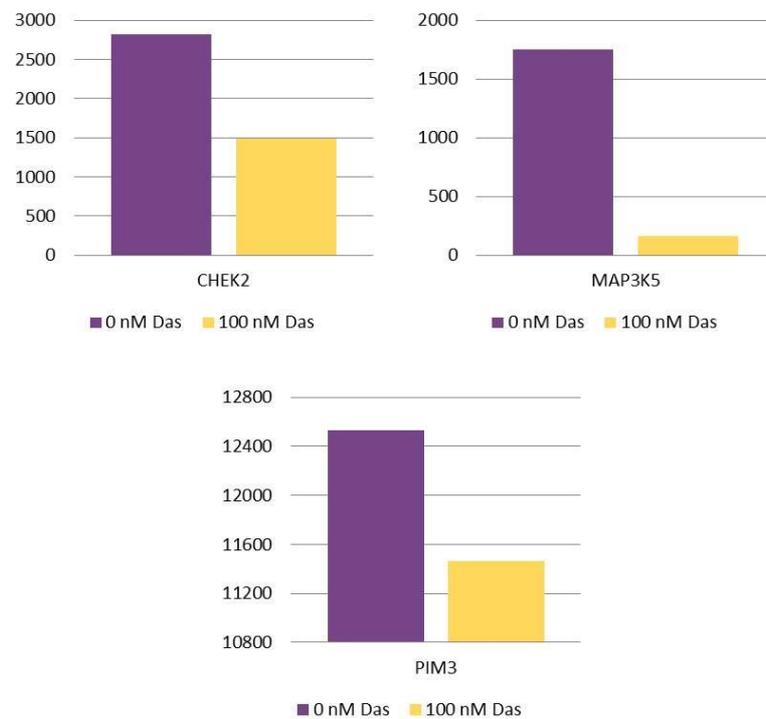
**Figure 3.8: LKB1 is increased following exposure to TKIs**

KU812 cells were transiently exposed to increasing concentrations of **(A)** imatinib, **(B)** nilotinib and **(C)** dasatinib. Lysates were analysed by western blotting for LKB1. Actin was used as the loading control. Data is representative of three independent experiments.

A

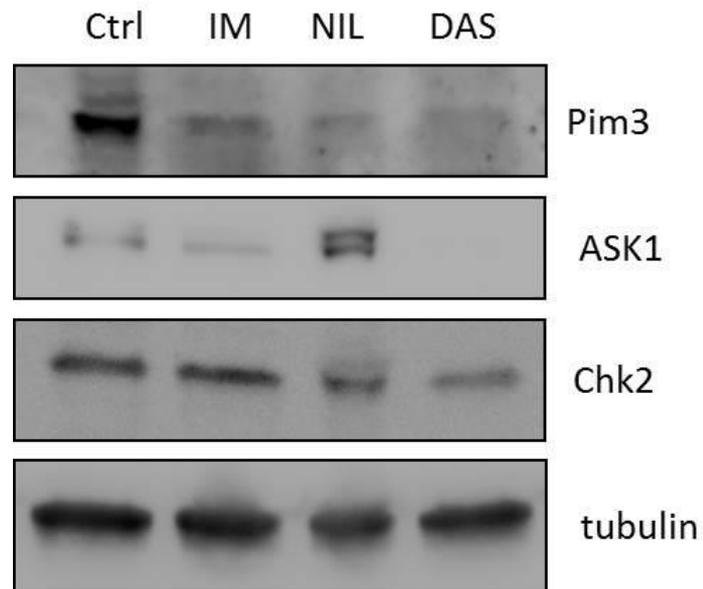


B



**Figure 3.9: Downregulation of kinases by dasatinib.**

KU812 cells were treated with 100 nM dasatinib for 30 min. Cells were lysed and analysed according to Full Moon BioSystems (Methods Section 2.5.9) **(A)** Mean fold change of top downregulated kinases. **(B)** Mean signal intensity of CHEK2, MAP3K5 and PIM3 before and after treatment.



**Figure 3.10: Effect of TKI treatment on Pim3, ASK1 and Chk2**

KU812 cells were transiently exposed to 2  $\mu$ M imatinib, 1  $\mu$ M nilotinib or 100 nM dasatinib. Lysates were analysed by western blotting for Pim3, ASK1 and Chk2. Lysates were analysed by western blotting for Pim3, ASK1 and Chk2. Tubulin was used as the loading control. Data is representative of three independent experiments.

### 3.2.6 Blocking autophagy sensitises *BCR-ABL1*+ cells to TKI-induced cell death

Having provided evidence that TKIs induce autophagy, the autophagy inhibitor chloroquine (CQ) was used as a co-treatment with dasatinib to determine its effect on autophagy in *BCR-ABL1*+ cells. Treatment with 10  $\mu$ M CQ was demonstrated to have a minimal effect on the viability of K562 (75.6% live) and KU812 cells (72.5% live) as a sole agent (Figure 3.11). Treatment of K562 cells with 5 nM dasatinib induced cell death (24.5% live) which was further reduced in combination with CQ (4.1% live). Combination of CQ with 0.5 nM dasatinib also reduced viability in KU812 cells (13.5% live) compared to dasatinib alone (29.3% live).

### 3.2.7 Clarithromycin enhances dasatinib-mediated cell death

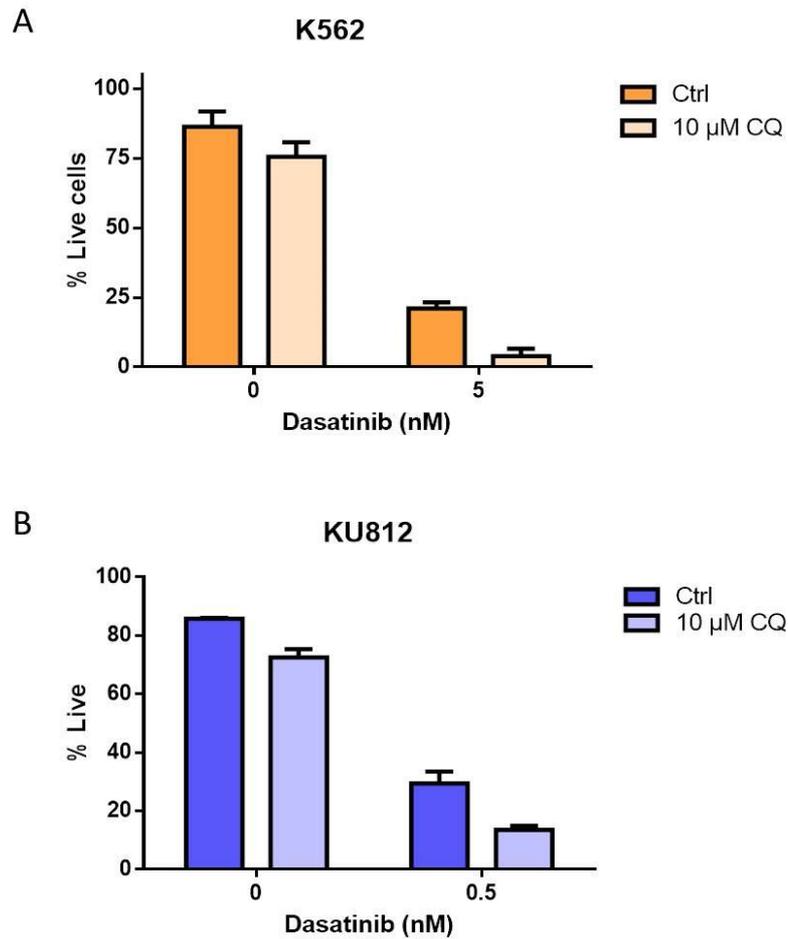
Rapid reductions in the *BCR-ABL1* transcript level were recently reported in the leukaemic cells of TKI-resistant CML patients where CAM was added in combination to their TKI therapy.<sup>191</sup> To determine whether this was a real effect against *BCR-ABL1*+ cells, the effect of CAM on cell death was examined alone or in combination with dasatinib. Exposure to the clinically relevant dose of 50  $\mu$ g/ml CAM<sup>189</sup> had minimal effect on cell viability in either K562 cells (80.1% live) or KU812 cells (70.4% live) (Figure 3.12).

However, CAM significantly enhanced cell death when culture in combination with 5 nM dasatinib in K562 cells (2.9% live,  $p=0.002$ ) or 0.5 nM dasatinib in KU812 cells (10.5% live,  $p=0.01$ ) compared to dasatinib alone (K562 21.4% live and KU812 29.5% live) (Figure 3.13). Increased sensitivity to dasatinib-induced cell death was comparable to CQ alone in both K562 (4% live,  $p<0.01$ ) and KU812 cells (13.5% live,  $p<0.01$ ) (Figure 3.13). Interestingly, co-culture with an early stage autophagy inhibitor, 3-methyladenine

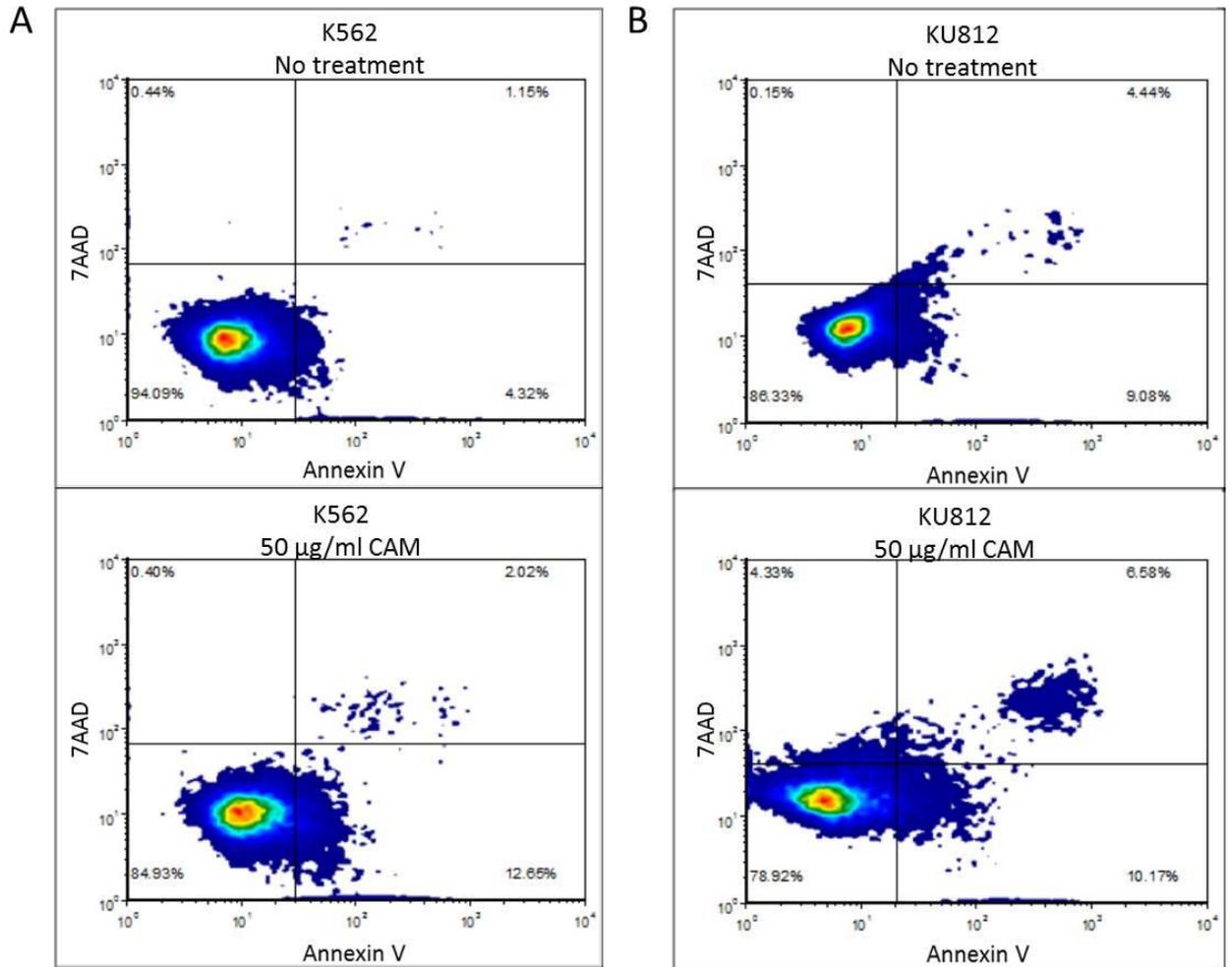
(3-MA) did not enhance dasatinib induced cell death in either K562 (28.6% live,  $p=0.18$ ) and KU812 cells (34.5% live,  $p=0.47$ ) compared to dasatinib alone (Figure 3.13).

### **3.2.8 Clarithromycin has no effect on pCrkL, and therefore Bcr-Abl, sensitivity to TKI treatment**

To determine if the effect of CAM on cell death was by increasing the sensitivity of Bcr-Abl kinase to TKI treatment, the Bcr-Abl substrate CrkL was utilised, the activation of which is commonly used as a surrogate for Bcr-Abl activity. K562 and KU812 cells were cultured in increasing concentrations of either dasatinib or imatinib in the presence or absence of CAM. In the absence of CAM, a dasatinib IC<sub>50</sub> of 3.8 nM in K562 cells and 10.1 nM in KU812 cells were observed (Figure 3.14). The combined culture of CAM with dasatinib did not significantly increase the sensitivity of K562 cells (IC<sub>50</sub> 4.5 nM) or KU812 cells (IC<sub>50</sub> 9.5 nM) to dasatinib (Figure 3.14). Similarly, there was no significant change in the sensitivity of pCrkL to imatinib in the absence or presence of CAM in K562 cells (IC<sub>50</sub> range 2.5-5  $\mu$ M) or KU812 cells (IC<sub>50</sub> range 5-7.5  $\mu$ M) (Figure 3.15). This data suggests that the ability of CAM to increase the sensitivity of *BCR-ABL1*<sup>+</sup> cells to TKI-induced cell death is not due to increased sensitivity to Bcr-Abl inhibition.

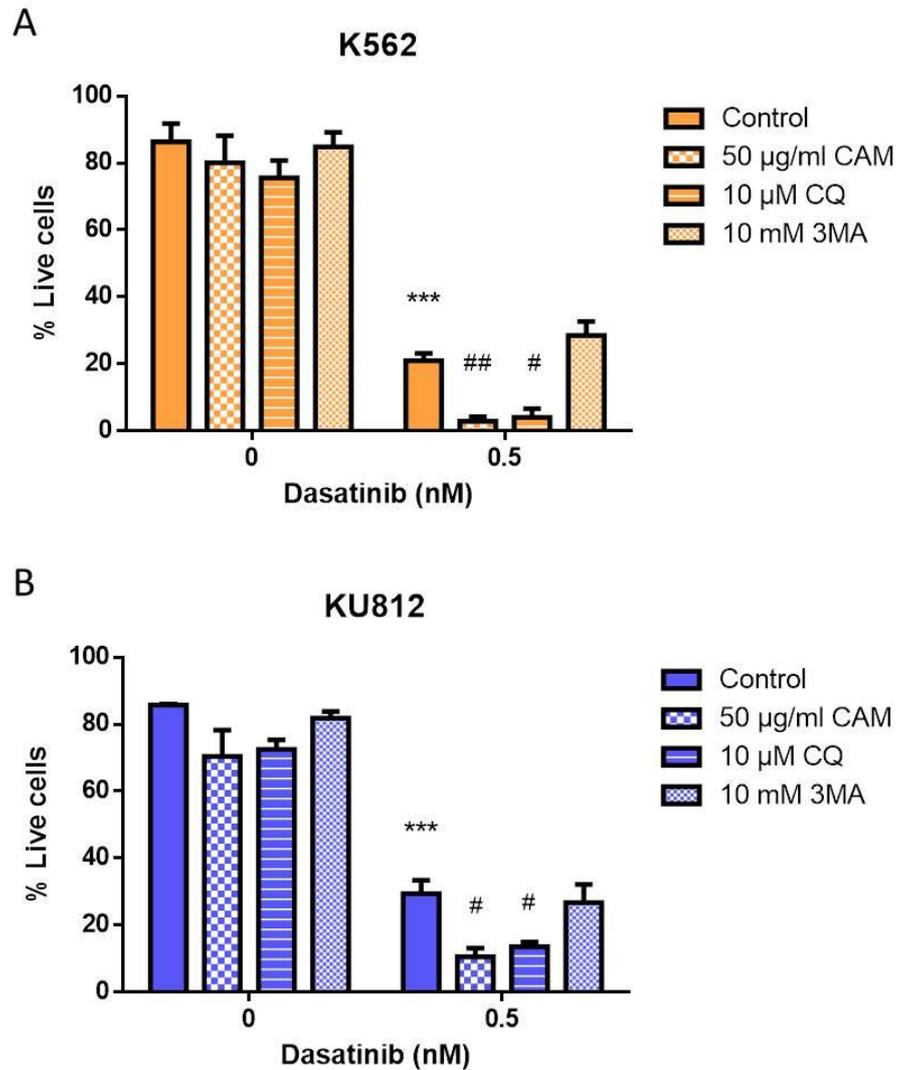


**Figure 3.11: CQ sensitizes *BCR-ABL1*+ cells to TKI-induced cell death**  
**(A)** K562 and **(B)** KU812 cells were treated with dasatinib with or without 10 μM chloroquine (CQ) for 72 h. Cells were then analysed by Annexin V/7-AAD staining for viability. Results are mean + SEM of three individual experiments.



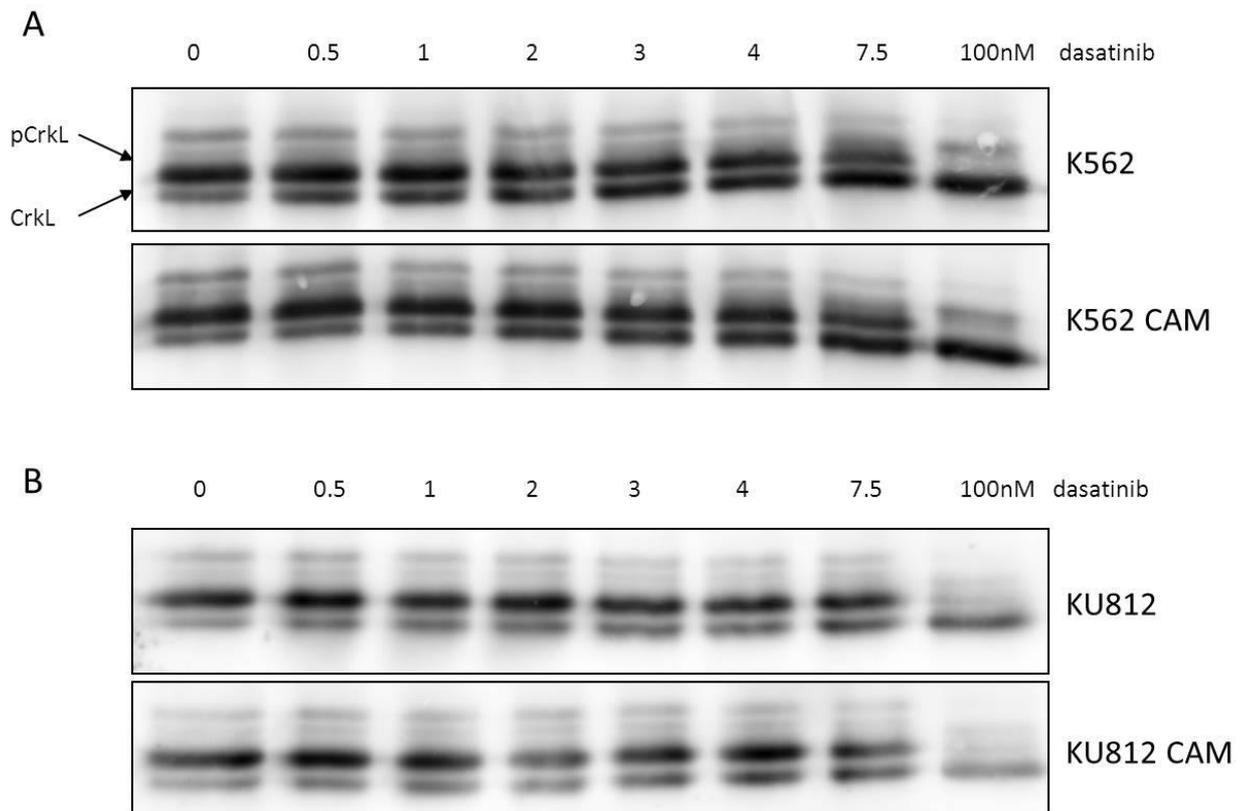
**Figure 3.12: CAM induces minimal cell death as a sole agent**

**(A)** K562 and **(B)** KU812 cells were incubated in the absence (top panel ) or presence (bottom panel) or 50 µg/ml clarithromycin (CAM) for 72 h. Cells were then analysed by flow cytometry for Annexin V/7-AAD staining. Data are representative of at least 3 independent experiments.



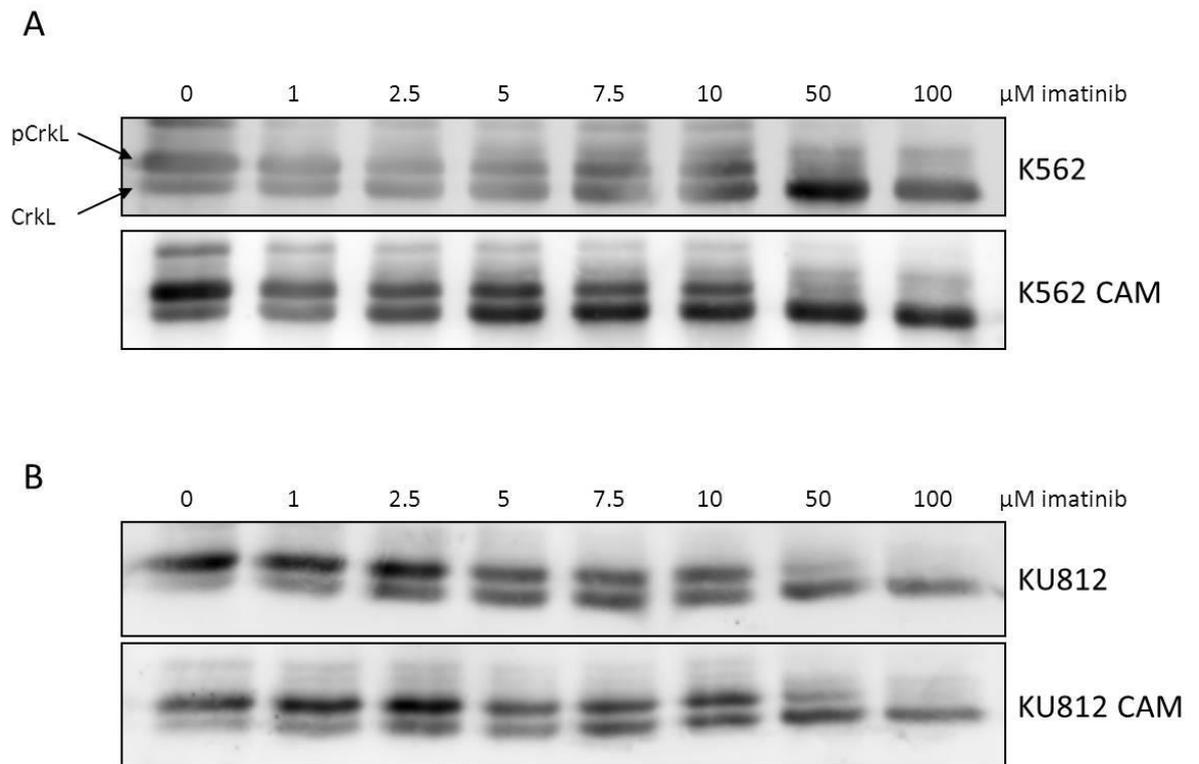
**Figure 3.13: CAM and CQ but not 3-MA enhances dasatinib-induced cell death in *BCR-ABL1*+ cells**

**(A)** K562 and **(B)** KU812 cells were cultured with or without dasatinib, in the presence or absence of 50 µg/ml clarithromycin (CAM), 10 µM chloroquine (CQ), or 10 mM 3-methyladenine (3-MA) for 72 h and cell death was determined by Annexin V and 7AAD staining. Results are mean + SEM of three individual experiments. \*\*\* $p < 0.0001$  vs. 0 nM dasatinib control and # $p < 0.01$  or ## $p < 0.005$  vs 0.5 nM dasatinib control.



**Figure 3.14: Bcr-Abl sensitivity to dasatinib is not affected by CAM**

(A) K562 and (B) KU812 cells were cultured with increasing concentrations of dasatinib, in the presence or absence of 50 µg/ml clarithromycin (CAM). Lysates were analysed for phosphorylation of CrkL (a surrogate for Bcr-Abl activity). Blots are representative of 3 independent experiments.



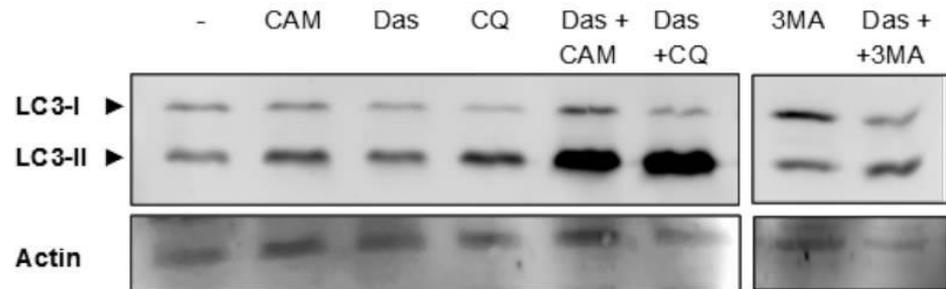
**Figure 3.15: Bcr-Abl sensitivity to imatinib is not affected by CAM**

**(A)** K562 and **(B)** KU812 cells were cultured with increasing concentrations of imatinib, in the presence or absence of 50  $\mu\text{g}/\text{ml}$  clarithromycin (CAM). Lysates were analysed for phosphorylation of CrkL (a surrogate for Bcr-Abl activity). Blots are representative of 3 independent experiments.

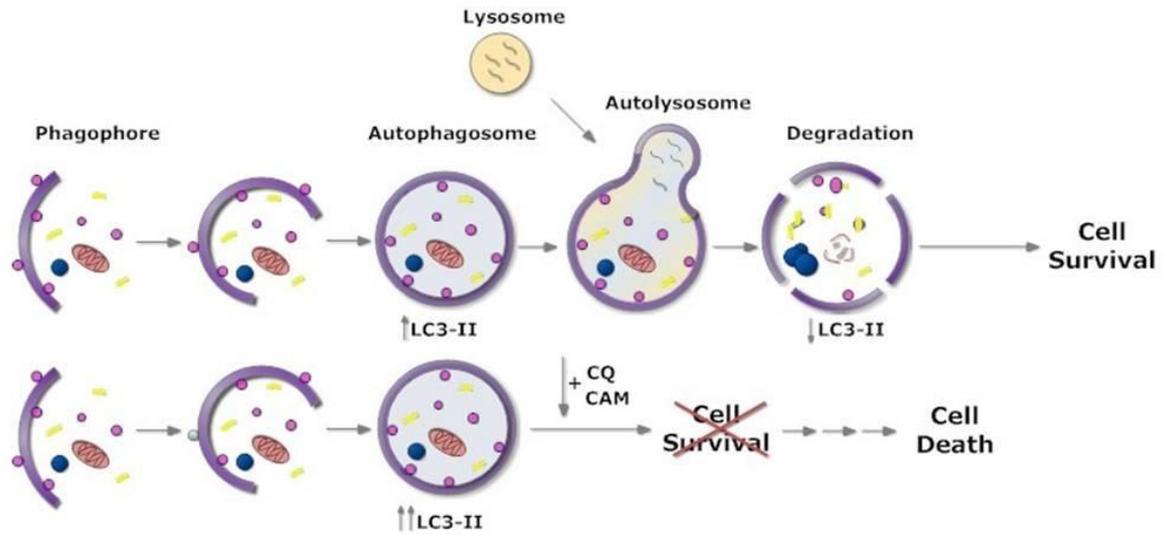
### 3.2.9 Blocking late stage autophagy results in an increase in LC3-II and the accumulation of vacuoles

Carella *et al.*<sup>188</sup> hypothesised that CAM was acting as an inhibitor of autophagy in resistant patient CML cells, however the study did not demonstrate any experimental evidence to support this. To establish the role of CAM on autophagy in *BCR-ABL1*+ cells, the protein marker of autophagy LC3 was observed along with cellular morphology. Bellodi *et al.*<sup>185</sup> previously reported that culture of *BCR-ABL1*+ cells with imatinib resulted in increased LC3-II protein levels due to induction of autophagy. In agreement with this, the culture of K562 cells for 24 h with dasatinib alone resulted in an increase in LC3-II (Figure 3.16A). However, the conversion of LC3-I to LC3-II has been reported as both a marker of autophagy induction and autophagy inhibition.<sup>184</sup> LC3-II is formed during the autophagy process, but also accumulates when autophagy is inhibited at a late stage, as observed using the inhibitor CQ (Figure 3.16B). Where late stage inhibition occurs, LC3-II is formed but not removed from the cell, thus exposure to CQ, which blocks late stage autophagy, resulted in an increased level of LC3-II (Figure 3.16A). In addition, the combination of dasatinib with CQ resulted in a further increase in LC3-II protein, in agreement with previous reports for the combination of imatinib with CQ.<sup>184,185</sup> An increase in LC3-II was observed in the presence of CAM alone, and a further increase in LC3-II was observed when *BCR-ABL1*+ cells were cultured in a combination of dasatinib and CAM (Figure 3.16A). As the CAM-induced effects on LC3 are similar to CQ, this provides evidence that CAM may also act as a late stage inhibitor of autophagy, providing experimental evidence for the hypothesis of Carella *et al.*<sup>188</sup>

A



B



**Figure 3.16: Addition of CAM or CQ to Dasatinib Increased LC3-II in K562 cells**

**(A)** K562 cells were treated with or without 100 nM dasatinib (Das), in the presence or absence of 50 µg/ml clarithromycin (CAM), 10µM chloroquine (CQ) or 10mM 3-methyladenine (3-MA) for 24 h. Lysates were analysed by western blotting for LC3-1 and LC3-II protein. Actin was used as a loading control. Blots are representative of at least 3 individual experiments.

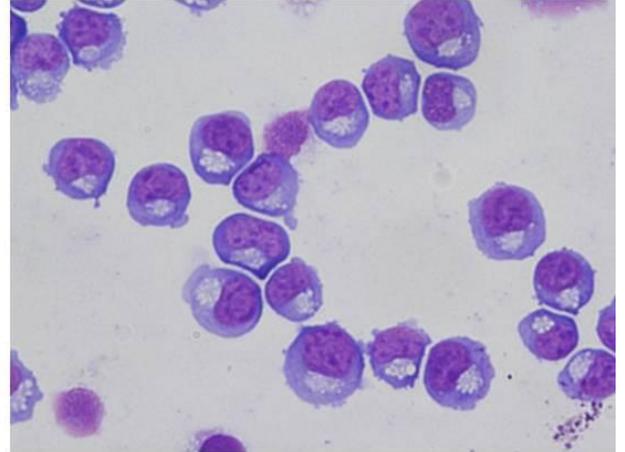
**(B)** Schematic model of CAM method of action. CAM inhibits late stage autophagy resulting in an accumulation of cytoplasmic vacuoles and induction of cell death.

Induction of autophagy, following the loss of survival signals, results in the metabolism of proteins and organelles allowing the cell to survive. There is an increase in conversion of LC3-I to LC3-II (open circles) upon formation of the autophagosome and a decrease in LC3-II upon degradation of the autolysosome. CQ and CAM cultured in combination with Das prevent formation of the autolysosome and therefore degradation, resulting in an accumulation of autophagosomes (visualised morphologically as cytoplasmic vacuoles) and an increase in levels of LC3-II, which is prevented from being degraded.

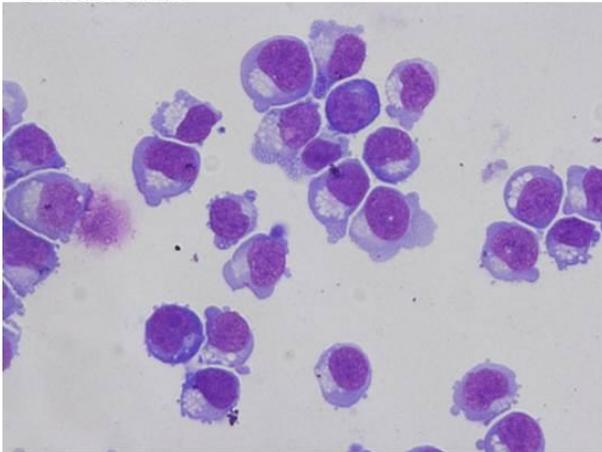
Control



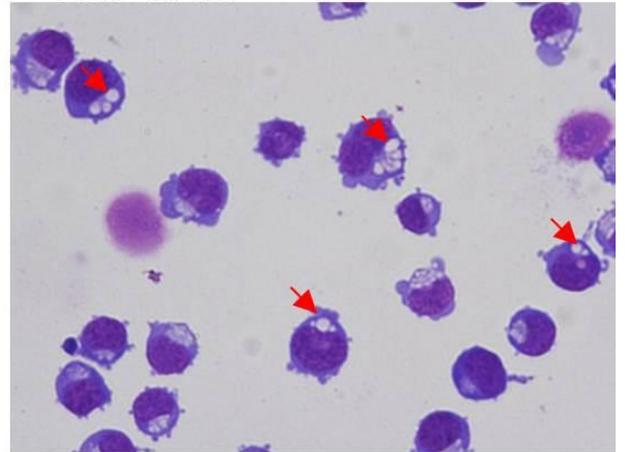
CAM



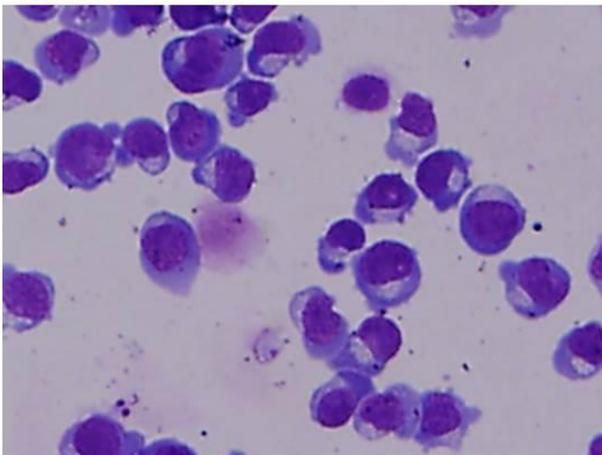
Dasatinib



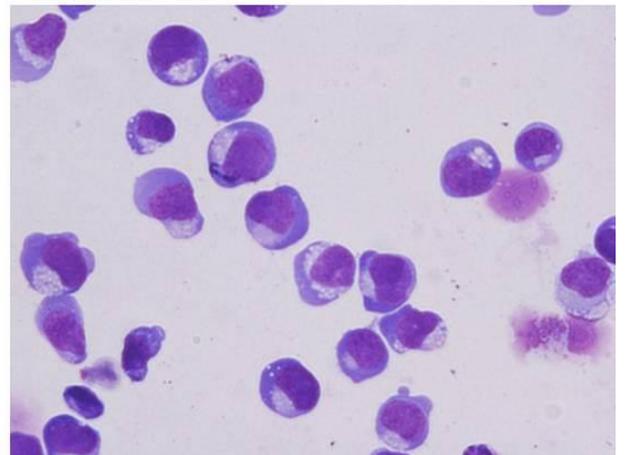
Das+CAM



3-MA



Das+3-MA

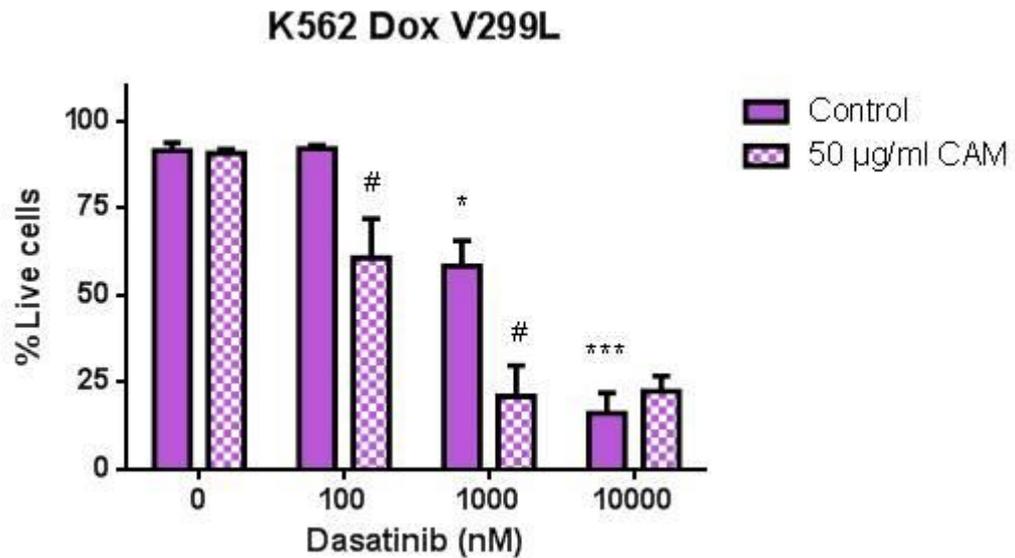


**Figure 3.17: CAM, but not 3-MA, induces vacuole formation in K562 cells treated with dasatinib**

Vacuole morphology was assessed by May-Grunwald/Giemsa stain following cytopsin of K562 cells cultured with or without 5 nM Dasatinib (Das) together with 50 µg/ml clarithromycin (CAM) or 10 mM 3-MA alone, or in combination with Das for 24 h. Red arrows indicate vacuoles.

### 3.2.10 Clarithromycin sensitises TKI-resistant *BCR-ABL1*+ cells to dasatinib treatment

In the study presented by Carella *et al.*<sup>188</sup> one of the patients had acquired a TKI-resistant BCR-ABL kinase domain mutation (E255V), and was not responding to treatment with dasatinib. Interestingly when CAM was added to the treatment regime this patient demonstrated a rapid decline in their percentage of BCR-ABL1 transcripts from 140% to 30% in just 2 weeks, and to 0.096% within 2 months. With this in mind, the ability of CAM to increase the sensitivity to dasatinib of the TKI-resistant K562 Dox cell line, harbouring the V299L kinase domain mutation<sup>201</sup> (a mutation clinically resistant to dasatinib)<sup>202</sup> was investigated. A concentration of 1000 nM dasatinib was required to significantly reduce cell viability (58.3% live) compared to untreated control (91.5% live,  $p=0.012$ ) (Figure 3.18). The addition of CAM significantly increased the sensitivity of this line to dasatinib, resulting in a significant reduction in viable cells (21% live) in combination with 1000 nM dasatinib. Importantly we show that while a clinically relevant concentration of 100 nM dasatinib alone had no measurable effect on cell death, the addition of CAM to 100 nM dasatinib resulted in significantly reduced cell viability (60.8% live,  $p<0.05$ ) (Figure 3.18), suggesting that CAM is able to potentiate the effect of dasatinib on cells harbouring a dasatinib-resistant *BCR-ABL1* mutation.



**Figure 3.18: CAM enhances dasatinib-induced cell death in K562 Dox V299L cells**  
K562 Dox cells harbouring the V299L mutation, were cultured with or without the indicated concentrations of dasatinib and 50µg/ml CAM for 72 h. \* $p < 0.05$ , \*\*\* $p < 0.001$  vs. 0 nM dasatinib control and # $p < 0.05$  vs. dasatinib alone (at the relevant concentrations).

### 3.3 Discussion

Autophagy is a process which is present in all cells at basal levels<sup>203</sup> and is increased in response to stress and loss of survival signals.<sup>204</sup> Autophagy is often implicated in resistance to chemotherapy, emphasizing that combination treatments with inhibitors of autophagy is a viable therapeutic strategy.<sup>205-208</sup> TKI-induced blockade of Bcr-Abl driven survival signalling pathways is proposed to induce autophagy, with recent evidence suggesting that blocking autophagy at a late stage sensitises *BCR-ABL1*+ cells to TKI-induced cell death.<sup>184,185</sup> However, these preliminary investigations have provided little insight into how inhibition of Bcr-Abl drives this induction of autophagy.

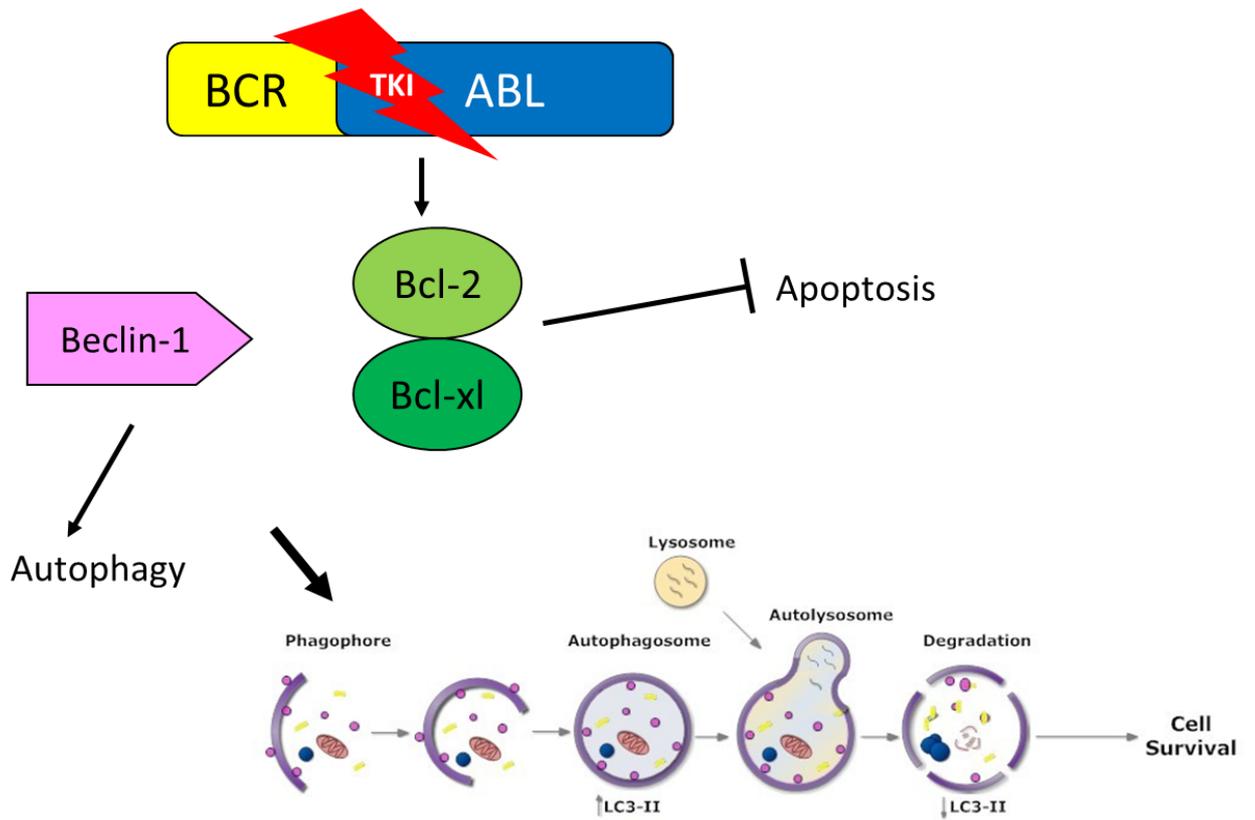
The primary assay for detection of autophagy induction is the conversion of LC3-I to LC3-II.<sup>209</sup> Imatinib induced autophagy demonstrated an increase in LC3-II in KU812 cells, confirming previous observations in 32Dp210<sup>Bcr-Abl</sup> and K562 cells,<sup>184,185</sup> further evidenced by the increased conversion to LC3-II by nilotinib and dasatinib in conjunction with the loss of p62 (Figure 3.3). These data reveal that autophagy is activated in response to Bcr-Abl inhibition with TKIs which may act as a protective mechanism, rendering the cells TKI-resistant.

Bcr-Abl is known to result in the overexpression of Bcl-2, an anti-apoptotic multi-domain protein which protects *BCR-ABL1*+ cells from induction of apoptosis.<sup>47,67</sup> Beclin1 is a Bcl-2-interacting protein which constitutively couples with Bcl-2 family members Bcl-2 and Bcl-xL.<sup>210-212</sup> Beclin1, also referred to as Atg6, is essential for the induction of autophagy, forming a complex with Vsp34 and p150 (Vsp15) to initiate nucleation during elongation of the phagophore,<sup>213,214</sup> thus disassociation from Bcl-2 family

members is required for induction of autophagy. Although Beclin1 is present over 24 h exposure to either imatinib or nilotinib in KU812 cells, increased Beclin1 was not observed (Figure 3.4). These results oppose recent observations which detected an increase in Beclin-1 gene expression in K562 cells treated with imatinib for 72 h,<sup>215</sup> which may be due to the extended time course employed in this investigation. Importantly, an imatinib- and nilotinib-induced reduction of Bcl-2 was observed, permitting disassociation of Beclin1, now available for induction of autophagy (Figure 3.4). For the first time, an increase in Beclin1 with a concomitant decrease in Bcl-2 and Bcl-XL was demonstrated following a 24 h exposure to 100 nM dasatinib (Figure 3.5). This may be attributed to off target effects on Src kinases which are inhibited by dasatinib, but not nilotinib or imatinib. Together this data emphasizes the importance of Beclin1-Bcl-2 family interaction in the induction of TKI-induced autophagy in *BCR-ABL1*+ cells (Figure 3.19), however further study into the interaction between Bcl-2 and Beclin1 is required in CML.

KU812 cells were screened for candidate kinases to be investigated for a role in dasatinib-induced autophagy. LKB1 was found to be highly upregulated by dasatinib (Figure 3.7), an observation further confirmed by western blotting following exposure to imatinib and nilotinib, in addition to dasatinib. LKB1/AMPK regulates glucose and lipid metabolism, essentially sensing metabolic stress to decide whether to induce autophagy in low energy environments.<sup>196</sup> Activation of AMPK has previously been demonstrated to induce autophagic cell death in CML cells,<sup>216</sup> however changes in LKB1 have not previously been observed. For the first time here, it was subsequently demonstrated that increased LKB1 was evident following exposure to imatinib and

nilotinib in addition to treatment with dasatinib (Figure 3.8). A concomitant reduction in Pim3 kinase was also observed in the screen of kinases following treatment



**Figure 3.19: Inhibition of Bcr-Abl and dissociation of Beclin-1**

Depiction of the interaction between Beclin-1 and members of the Bcl-2 family. Bcl-2 and Bcl-x<sub>L</sub> sequester Beclin-1 preventing the initiation of autophagy. Upon inhibition of Bcr-Abl with TKIs, Bcl-2 and Bcl-x<sub>L</sub> are reduced releasing Beclin-1 for the initiation of autophagy, thus permitting cell survival.

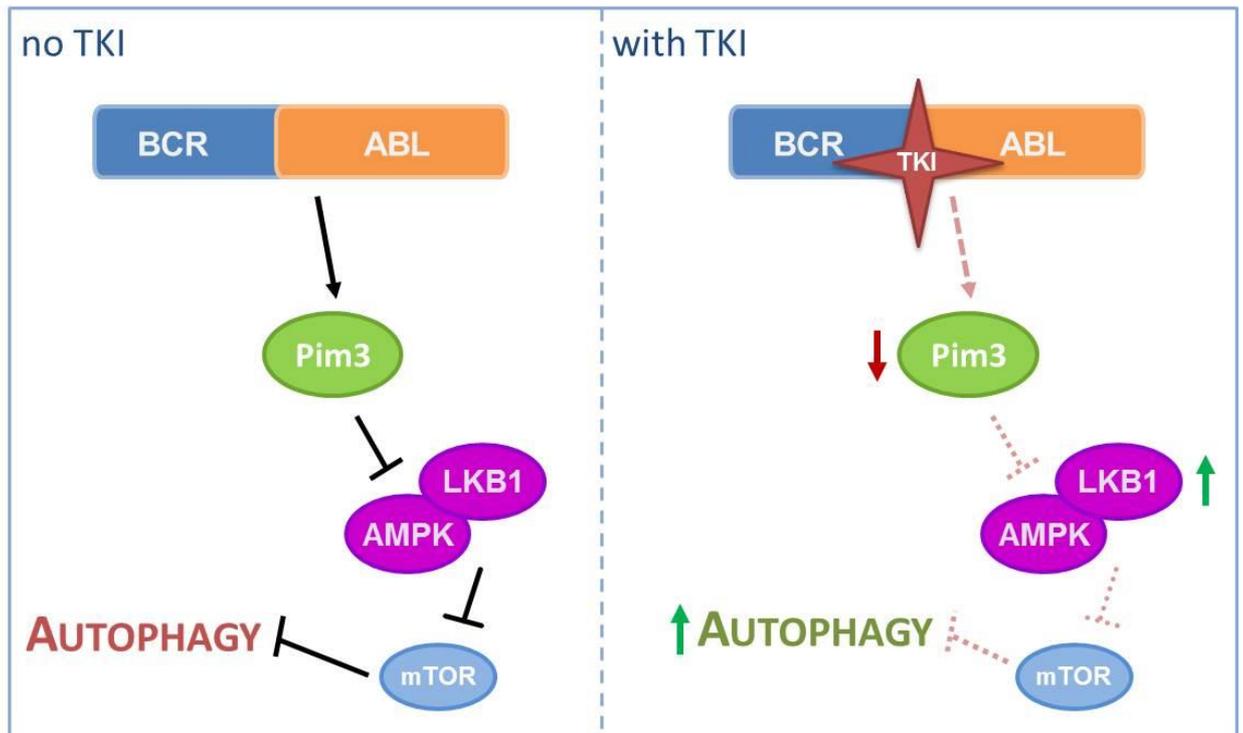
with dasatinib (Figure 3.9), and subsequently confirmed by western blotting following TKI treatment (Figure 3.10). Interestingly, Pim3 has previously been demonstrated to reduce activation of AMP activated protein kinase (AMPK),<sup>200</sup> which is suggested to be via suppression LKB1,<sup>217</sup> with the subsequent activation of mTOR resulting in the suppression of autophagy.<sup>205</sup> These data suggest that Bcr-Abl activation of Pim3 may suppress LKB1/AMPK inhibition of mTOR, thus inactivation of Bcr-Abl with TKIs may activate autophagy through the Pim3-LKB1/AMPK-mTOR axis (Figure 3.20). However, as this is only the preliminary evaluation into what drives Bcr-Abl-dependent blockade and subsequent TKI-induced autophagy, further investigation is required and is the focus of ongoing work.

Imatinib blockade of Bcr-Abl driven survival pathways combined with inhibition of autophagy by chloroquine (CQ), a late stage autophagy inhibitor, has previously been shown to restore sensitivity of resistant CML cells to TKI<sup>184,185</sup> and here has been confirmed in K562 and KU812 cells (Figure 3.11 and Figure 3.6). As previously discussed, CAM has been proposed to rapidly reduce *BCR-ABL1*+ cells in TKI-resistant CML patients by blockage of TKI-induced autophagy.<sup>188</sup> CAM and other macrolide antibiotics have been demonstrated to inhibit cancer cell growth and in multiple myeloma cells, and induce cell death through inhibition of autophagy.<sup>189</sup> This case study highlighted CAM as a candidate for combination therapy in CML patients, in particular those with advanced and resistant disease.

In this chapter, the effects of CAM in combination with TKIs were investigated in *BCR-ABL1*+ cells. CAM is effective at increasing the sensitivity of *BCR-ABL1*+ cells to dasatinib, whilst having little or no effect on cell death as a sole agent (Figure 3.12-13). The mechanism of induction of cell death by CAM combined with dasatinib appears to be via

inhibition of late stage autophagy, in a similar way to the late stage autophagy inhibitor CQ. This is clearly demonstrated by an increase in LC3-II and a concomitant increase in cellular vacuole formation (Figure 3.16-17).

Interestingly, there was no measurable effect on cell survival of the early stage autophagy inhibitor 3-MA (Figure 3.13). Based on this work, and that of others,<sup>186,218,219</sup> early stage autophagy is a process involving AKT/PI3K which when inhibited (by 3-MA) reverts the cells to "normal" death pathways, in this case mediated by dasatinib treatment. At an early, and likely reversible stage, autophagy blockade will result in failure of the cells to significantly accumulate autophagosomes (a PI3K/AKT dependent process). Blocking of late stage autophagy, when the autophagosomes are already formed, results in failure of lysosome fusion and therefore accumulation of autophagosomes.<sup>206</sup> It was also interesting to observe that the combination of 3MA with dasatinib did not result in enhanced cell death. However, studying the diverse descriptions of autophagy in the current literature reveals that others have also experienced the phenomenon where specific inhibition of only late-stage autophagy is able to result in cell death.<sup>186,218,220</sup>

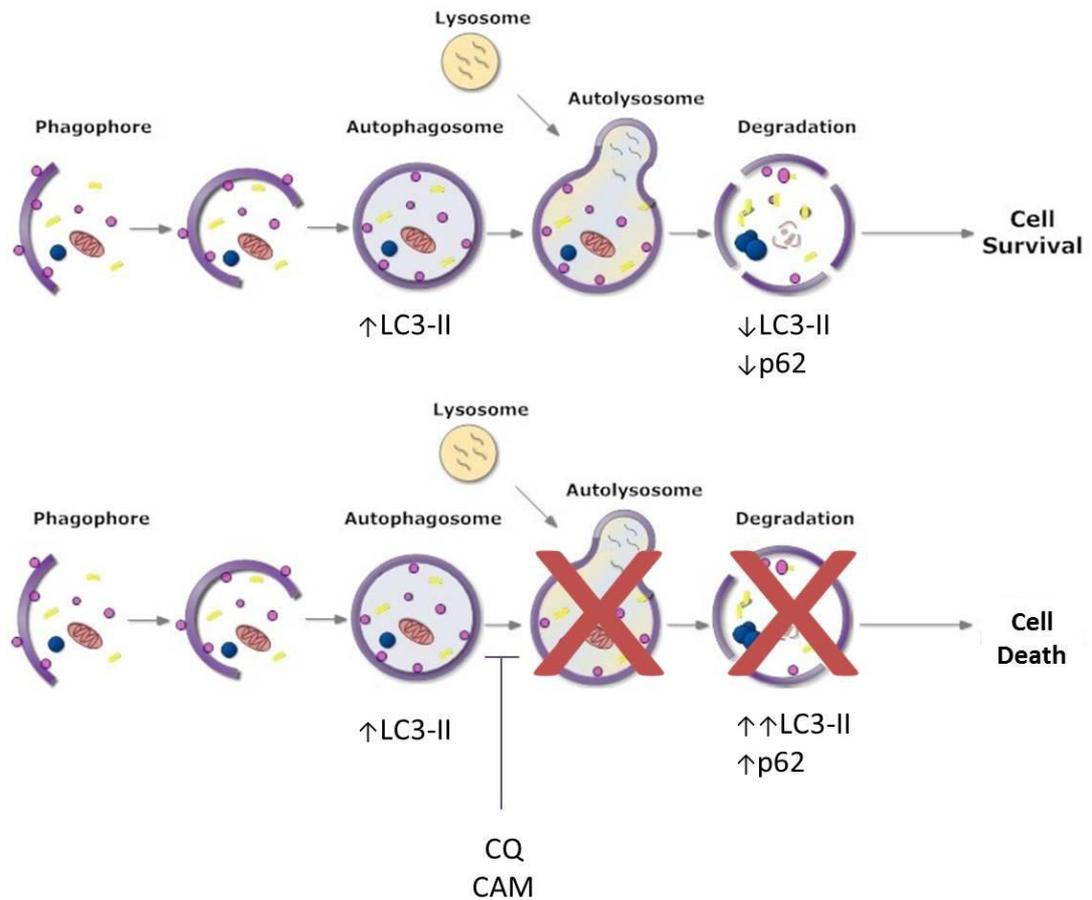


**Figure 3.20: Proposed mechanism of TKI-induced autophagy in CML**

Bcr-Abl constitutively activates several survival pathways, including but not limited to, those involving Pim kinases.<sup>205</sup> Pim kinases inhibit the activation of AMPK,<sup>200</sup> thereby preventing LKB1/AMPK inhibition of mTOR and thus autophagy. Inhibition of Bcr-Abl with TKIs inhibits Pim3, subsequently increasing LKB1 and the activation of AMPK. AMPK inhibits mTOR, allowing the induction of autophagy.

At an early, and likely reversible stage, autophagy blockade will result in failure of the cells to significantly accumulate autophagosomes (a PI3K/AKT dependent process). Blocking of late stage autophagy, when the autophagosomes are already formed, results in failure of lysosome fusion and therefore accumulation of autophagosomes. It is therefore hypothesised that cell death here is due to the accumulation of the toxic contents of the autophagosomes being leaked into the cell upon CQ or CAM treatment (Figure 3.21).<sup>221</sup>

This chapter highlights a role for TKI-induced autophagy in the resistance of *BCR-ABL1*<sup>+</sup> cells to TKI therapy, demonstrating a Beclin1-Bcl-2 switch between apoptosis and autophagy and suggesting a potential role for the Pim3-LKB1/AMPK-mTOR pathway in the induction of autophagy. The data presented here provides the first *in vitro* evidence that CAM inhibits late stage autophagy to sensitise *BCR-ABL1*<sup>+</sup> cells to TKIs and thus presents the combination of CAM with TKIs as a plausible therapy for the treatment of TKI-resistant CML.



**Figure 3.21: Blockade of TKI-induced autophagy by CAM**

CAM inhibits late stage autophagy resulting in an accumulation of cytoplasmic vacuoles (autophagosomes) and induction of cell death. Induction of autophagy following the loss of survival signals, results in the metabolism of proteins and organelles, allowing the cell to survive. There is an increase in conversion of LC3-I to LC3-II upon formation of the autophagosome and a decrease in LC3-II upon degradation of the autolysosome. CQ and CAM cultured in combination with dasatinib prevents formation of the autolysosome and therefore degradation, resulting in an accumulation of autophagosomes (visualised morphologically as cytoplasmic vacuoles) and an increase in the levels of LC3-II which is prevented from being degraded.

## **CHAPTER 4.**

# **SIGNALLING REQUIREMENTS FOR COMMITMENT TO CELL DEATH FOLLOWING TRANSIENT HIGH OR CONTINUOUS LOW DOSE DASATINIB**

## 4.1 Introduction

Chronic myeloid leukaemia (CML) is driven by the oncogenic fusion protein Bcr-Abl, which activates multiple signalling pathways promoting leukaemia cell survival and proliferation.<sup>222</sup> Bcr-Abl initiates leukaemic transformation through aberrant activation of signal transduction pathways, including the RAS/ERK, PI3K/AKT and JAK/STAT pathways.<sup>54</sup> Signal transducer and activator of transcription 5 (STAT5) was observed to be constitutively activated by Bcr-Abl in CML cells<sup>72</sup> and is critical for maintenance of the disease.<sup>4</sup> Bcr-Abl autophosphorylation results in the activation of Ras, which subsequently activates MAPK/Erk resulting in the enhanced proliferation in CML cells.<sup>51</sup> PI3K can be activated either directly by Bcr-Abl, in addition to Bcr-Abl activation of MAPK and is essential for leukaemogenesis.<sup>223</sup>

Imatinib is a tyrosine kinase inhibitor (TKI) which inhibits Bcr-Abl by competitively binding to the ATP-binding site and preventing substrate phosphorylation and therefore downstream oncogenic signalling.<sup>93</sup> TKI therapy has been developed on the premise that continuous Bcr-Abl kinase inhibition is required for sustained disease control. Le Coutre *et al.*<sup>132</sup> demonstrated *in vitro* that 1  $\mu$ M imatinib was required for a period of 20-21 h to induce cell death in cultured KU812 and MC3 CML cells. While administration with 1  $\mu$ M imatinib results in 60-70% Bcr-Abl kinase inhibition within 2 h, a short *in vitro* exposure of 6-7h was not adequate to trigger cell death or inhibit cell proliferation. Dosing which resulted in continuous Bcr-Abl kinase inhibition was demonstrated to decrease tumour growth by 98% and result in longer progression free survival and overall survival.<sup>132</sup> Furthermore, White *et al.*<sup>133</sup> demonstrated that the degree of *in vitro* Bcr-Abl kinase inhibition achieved by imatinib in *de novo* CP-CML patient samples was predictive of response, where a low inhibitory concentration of the adaptor protein CrkL (CT10

regulator of kinase like), an immediate downstream phosphorylation partner of Bcr-Abl, correlates with optimal patient outcomes. Collectively, these studies suggest that optimal therapy requires continuous Bcr-Abl kinase inhibition.

Clinical pharmacokinetic and pharmacodynamic studies of imatinib revealed excellent responses associated with trough drug levels above 1000 ng/ml,<sup>104,130</sup> providing evidence that continuous Bcr-Abl kinase inhibition by imatinib was the optimal strategy for CML therapy. The plasma half-life of both imatinib and nilotinib provide an adequate duration of Bcr-Abl kinase inhibition, achievable in patients on a once daily dosing schedule. Dasatinib has an *in vivo* plasma half-life of 3-5 h<sup>134</sup> and there is near-complete reactivation of Bcr-Abl kinase by 8 h following drug administration as serum dasatinib concentrations rapidly decline<sup>137</sup>, indicating that dasatinib inhibition of Bcr-Abl kinase is transient when given once daily. Despite the short half-life of dasatinib, patients on a standard dose of once daily dasatinib (100 mg QD) achieved similar cytogenetic and molecular responses as patients receiving twice daily dasatinib (50 mg BID).<sup>111,135</sup> Additionally, and of great value clinically, the once daily dose schedule was more tolerable and resulted in less side effects compared to the twice a day schedule.<sup>224</sup>

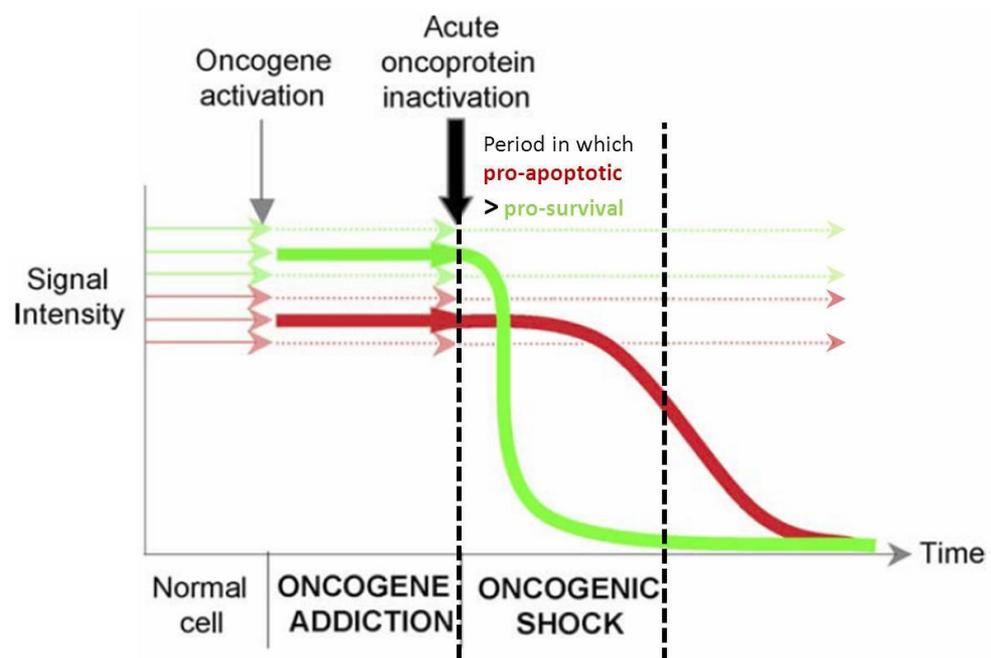
Recent studies have investigated a range of short exposures to dasatinib and their ability to induce apoptosis. Hiwase *et al.*<sup>136</sup> and others<sup>137,138</sup> have demonstrated that a 30 min exposure of CML cell cultures, defined as short term exposure, to a therapeutically achievable concentration of dasatinib (100 nM) inhibited Bcr-Abl kinase by >90% . This level of inhibition is defined as intense or potent, whereas <90% Bcr-Abl kinase inhibition is referred to as partial. Short-term (30 min) intense (100 nM) inhibition of

Bcr-Abl with dasatinib induced equivalent cell death to long-term (72 h), partial inhibition of Bcr-Abl.

Together the clinical and *in vitro* data suggests that continuous Bcr-Abl kinase inhibition with dasatinib is not required for optimal response, which challenges the previously established paradigm of continuous Bcr-Abl kinase inhibition for achievement of optimal response. Additionally, cell death triggered by short term intense Bcr-Abl kinase inhibition is not restricted to dasatinib therapy. Short-term, intense TKI treatment with nilotinib (1-2  $\mu\text{M}$ ) or imatinib (32.5  $\mu\text{M}$ ) also induced cell death in *BCR-ABL1*-positive cell lines.<sup>136-138</sup> The paradigm established by imatinib therapy for continuous inhibition of Bcr-Abl, directed drug development to 1) preferentially select compounds with a longer half-life over drugs with a shorter half-life, and 2) match drug dosing to achieve optimal therapeutic concentration with proven continuous inhibition of their targets. As such, these findings can potentially change the paradigm of drug development established on imatinib therapy and questions the requirement for continuous inhibition of targets.

Evidence of cytotoxicity in oncogenic cells following only transient targeted drug exposure is not restricted to CML. Weinstein *et al.*<sup>139</sup> proposed that cancer cells could become dependent on the activity of a single oncogene (an initiating oncogene such as Myc, Bcr-Abl or Src)<sup>139,141,225</sup> where there is unbalanced expression of survival proteins over apoptotic proteins. Consequently, even a short interruption to the dominant oncogenic signalling pathways appears sufficient to commit these cells to death, primarily due to the variation in the degradation/dephosphorylation kinetics of multiple competing pro-survival and pro-apoptotic proteins (Figure 4.1).<sup>142</sup> In most tumour cells,

pro-survival signals outweigh pro-apoptotic signals, favouring increased cell proliferation and survival and decreased cell death. Subsequently, an interruption in oncogenic signalling, termed “oncogenic shock” is sufficient to shift the balance toward apoptotic signals, as pro-survival signals rapidly dissipate, for a period extensive enough to induce cell death in CML cells.<sup>143</sup> This intriguing phenomenon may explain why cells cannot be rescued from commitment to cell death even though Bcr-Abl kinase reactivates within 1-2 hours of TKI washout following short-term intense Bcr-Abl kinase inhibition.<sup>144</sup> However, further investigation into the essential signals required to restore steady-state apoptosis in oncogenic cells is necessary to direct optimal treatment strategies.



**Figure 4.1: Balance of survival and apoptosis signals.**

Model of oncogenic shock. The period of oncoprotein signalling interruption in which the pro-survival signalling (represented in green) falls below the pro-apoptotic signalling (represented in red), allowing a cell to die despite reactivation of survival signalling. Adapted from Sharma *et al.* (2010).<sup>226</sup>

### 4.1.1 Approach

Here we investigate the effect of transient high dose and continuous low dose dasatinib on the inhibition of Bcr-Abl, and the corresponding effects on downstream signalling pathways. This project will focus on revealing the effects of partial Bcr-Abl inhibition with a continuous low dose of dasatinib in comparison to a transient, potent Bcr-Abl kinase inhibition (short-term high dose dasatinib) on survival and apoptotic signalling.

KU812, Meg01 cell lines and CP-CML CD34+ cells were used to assess Bcr-Abl survival signalling, cell death and colony formation following transient treatment with dasatinib. Bcr-Abl signalling was assessed by western blotting for pBcr-Abl (Y245), pCrkL, pSTAT5 and pErk over a 24 h period. Bcl-2 family members Mcl-1, Bcl-xL and Bcl-2, along with Bim and cleaved PARP were measured by western blotting following 8-48 h dasatinib treatment to investigate apoptotic signalling. Cell death was measured by Annexin V and 7AAD staining at 72 h with flow cytometric analysis. Clonogenic potential was assessed using CD34+ progenitor cells, cultured with or without inhibitors for 72 h prior to plating in methycellulose based media containing 5 growth factor mix and enumeration at 2 weeks.

### 4.1.2 Summary and Research Contribution

This research highlights the importance of low level inhibition of Bcr-Abl kinase and the subsequent commitment of cells to apoptosis. Observations of low dose and transient treatments emphasise STAT5 activation as a sensitive measure of Bcr-Abl kinase activity which result in apoptosis of CML cells. These findings challenge the imatinib paradigm that continuous availability of tyrosine kinase inhibitors and complete inhibition of Bcr-Abl is required for optimal responses in patients.

#### 4.1.3 Publication

**Schafranek L**, Nievergall E, Powell JA, Hiwase DK, White DL and Hughes TP. Sustained inhibition of STAT5, but not JAK2, is essential for TKI-induced cell death in chronic myeloid leukemia. *Leukemia advance online publication*, June 27, 2014; doi:10.1038/leu.2014.156; accepted article preview online May 12, 2014.

#### 4.1.4 Conference Presentations

**Schafranek L**, Nievergall E, Hiwase H, Powell J, White D, Hughes T. Commitment of CML Cells to Apoptotic Cell Death Depends On the Length of Exposure to Das and the Level of STAT5 Activity. ASH Dec 2012, Atlanta, USA (poster presentation).

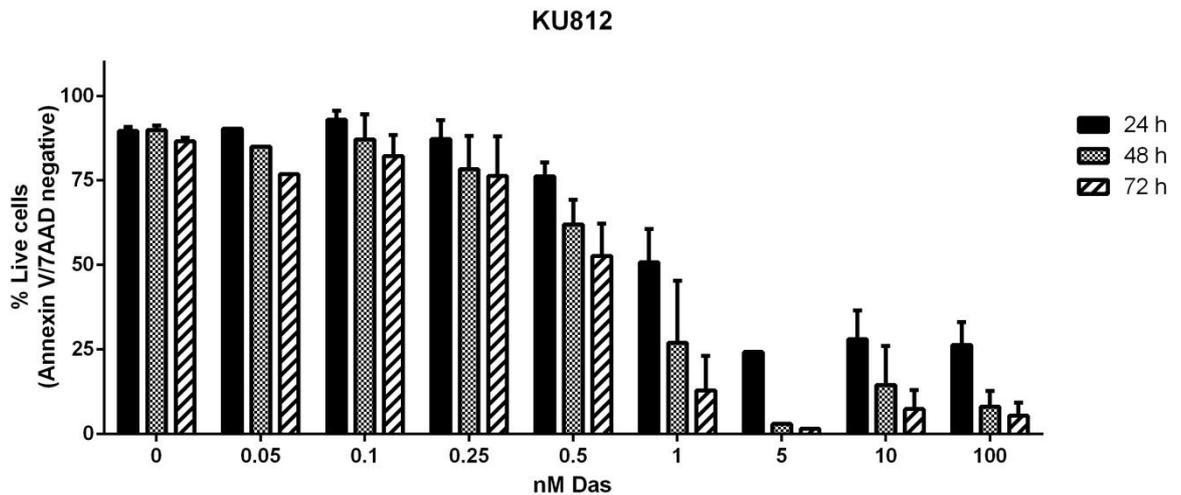
**Schafranek L**, Hiwase H, Powell J, Melo J, White D, Hughes T. Constant Exposure to Low Dose Dasatinib Is Sufficient for Induction of Apoptosis in CML Cells. HAA Oct, 2011 Sydney, Australia (oral presentation) **Awarded HAA 2011 non-member travel grant**

## 4.2 Results

### 4.2.1 Low dose dasatinib induces cell death despite minimal inhibition of Bcr-Abl kinase activity in the KU812 CML cell line

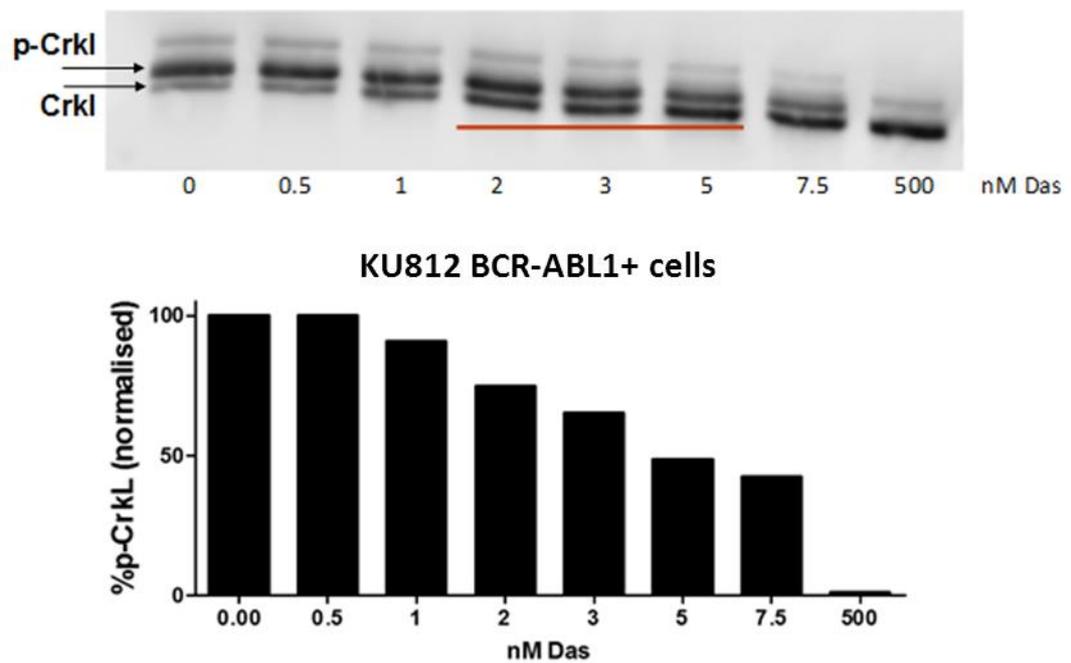
The underlying mechanism of optimal patient response in the setting of transient Bcr-Abl kinase inhibition afforded by dasatinib therapy is unclear. To interrogate this observation, the survival response of KU812 cells was investigated over a range of dasatinib concentrations in relation to Bcr-Abl activity. Cell viability was measured at 24, 48 and 72 h following exposure to 0.05 nM to 100 nM dasatinib. Treatment with 0.5 nM dasatinib resulted in a significant reduction in the viability of KU812 cells by 72 h (52.7% viable,  $p < 0.05$  relative to untreated control,  $n = 3$ ). By 24 h, 1 nM dasatinib had induced cell death in a major proportion of the cells (50.7% viable), and maximal cell death was observed at 72 h (12.9% viable) (Figure 4.2). Cell death induced by 1 nM dasatinib (12.9% viable) was not significantly different from assessment at 72 h for either 10 nM (7.4% viable,  $p = 0.68$ ) or 100 nM (5.4% viable,  $p = 0.56$ ) treatments with dasatinib (Figure 4.2).

To interrogate the correlation between cell death and the extent of Bcr-Abl inhibition following exposure to dasatinib, the phosphorylation status of CrkL (a well characterised surrogate for Bcr-Abl activity<sup>60,133</sup>) was measured, and the inhibitory concentration of dasatinib required to reduce the percentage of pCrkL by 50% (IC<sub>50</sub>) was determined. If complete Bcr-Abl kinase inhibition was required to induce cell death, an IC<sub>50</sub> of approximately 0.5-1 nM dasatinib would be expected. Surprisingly, a much higher IC<sub>50</sub> of 2-5 nM dasatinib was observed. Additionally, 2 h exposure to 1 nM dasatinib resulted in only a 10% reduction in %pCrkL (Figure 4.3).



**Figure 4.2: KU812 viability following exposure to a range of dasatinib concentrations.**

KU812 cells were treated with increasing concentrations of dasatinib and cell viability was measured at 24, 48 and 72 h. Cell viability was measured by Annexin V/7AAD staining. Cell death was achieved following treatment with 1 nM for 72 h. Data are mean + SEM of three independent experiments.

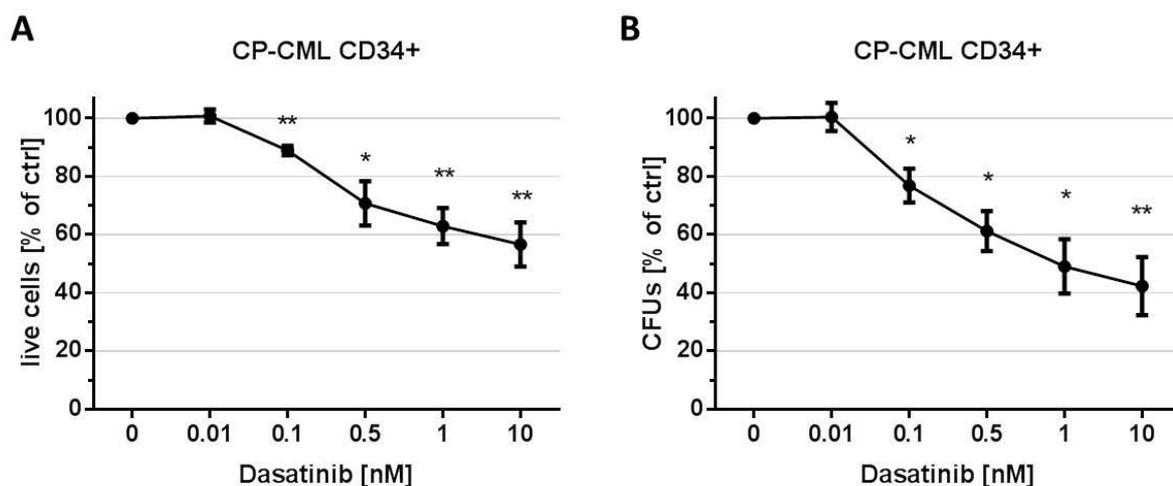


**Figure 4.3: Bcr-Abl activity in KU812 cells as expressed as a percentage of pCrkL.** KU812 cells were treated with increasing concentrations of dasatinib (nM Das) and Bcr-Abl activity was measured at 2 h by assessment of the phosphorylation of the Bcr-Abl substrate pCrkL/CrkL. CrkL phosphorylation was assessed by western blotting analyses. Data is representative of three independent experiments. Quantitative graph is representative of the western blot presented here.

### 4.2.2 Low dose dasatinib induces apoptosis despite minimal inhibition of Bcr-Abl kinase activity in newly diagnosed CP-CML patients

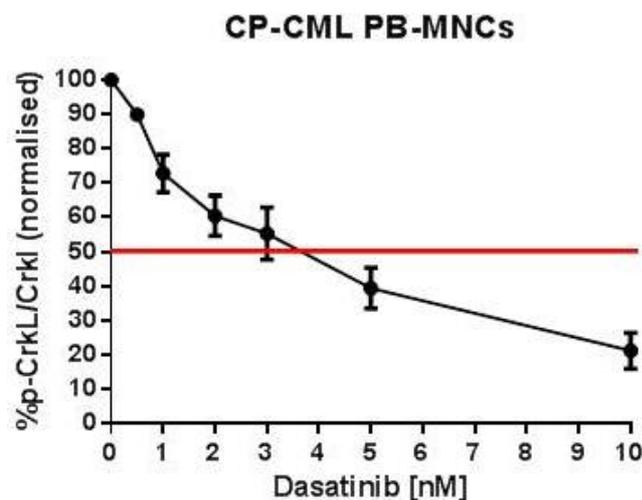
To determine whether the observed phenomenon was restricted to cell lines, cell death and Bcr-Abl activity were investigated in CD34<sup>+</sup> cells from the peripheral blood mononuclear cells (PM-MNCs) of newly diagnosed CP-CML patients. Cells were treated with increasing concentrations of dasatinib and it was observed that as little as 0.1 nM dasatinib was able to induce cell death at 72 h (88.9% viable,  $p < 0.001$  relative to untreated control,  $n = 3$ ). Furthermore, there was no significant difference between 10 nM dasatinib (56.6% viable) and 0.5 nM (70.7% viable,  $p = 0.24$ ) or 1 nM dasatinib (62.9% viable,  $p = 0.54$ ) (Figure 4.4A). The viability of the cells was also reflected by the ability of this primitive population to form colonies, as there was no significant difference between the colony forming ability of the cells following treatment with 0.5 nM (61.2% colony forming units, CFUs,  $p < 0.05$  normalised to untreated control,  $n = 3$ ) and 1 nM dasatinib (49.1% CFUs,  $p = 0.34$ ), or 10 nM dasatinib (42.4% CFUs,  $p = 0.19$ ) (Figure 4.4B).

Subsequent analysis of Bcr-Abl activity in PB-MNCs from these patients determined the pCrkL IC<sub>50</sub> to be approximately 4 nM dasatinib (Figure 4.5), much higher than the cell death IC<sub>50</sub> (Figure 4.4). This confirms the result in the KU812 cell line that there is a disparity between the concentration of dasatinib required to inhibit Bcr-Abl and the concentration required to induce cell death, suggesting that complete inhibition of Bcr-Abl is not required to induce cell death.



**Figure 4.4: Viability and colony forming ability of primary CP-CML CD34+ cells following exposure to a range of dasatinib concentrations.**

**(A)** CD34+ cells collected from newly diagnosed CP-CML patients were treated with increasing concentrations of dasatinib and cell viability was measured at 72 h. Maximal cell death was achieved at 10 nM dasatinib. Treatment with 1 nM dasatinib did not induce cell death significantly less from than 10 nM exposure. **(B)** Cells were then enumerated for clonogenic potential after 14 days employing the CFU-GM assay. Data are mean  $\pm$  SEM of three independent experiments. \*  $p < 0.05$ , \*\*  $p < 0.01$  compared to 0 nM dasatinib. Assays were obtained with the assistance of Eva Nievergall.



**Figure 4.5: Bcr-Abl activity in primary PB-MNCs CP-CML cells as measured by pCrkL/CrkL (IC<sub>50</sub>).**

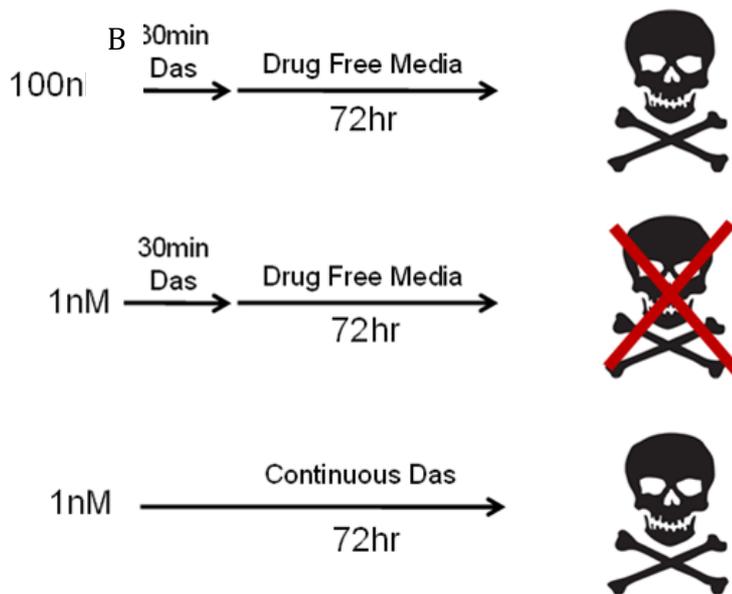
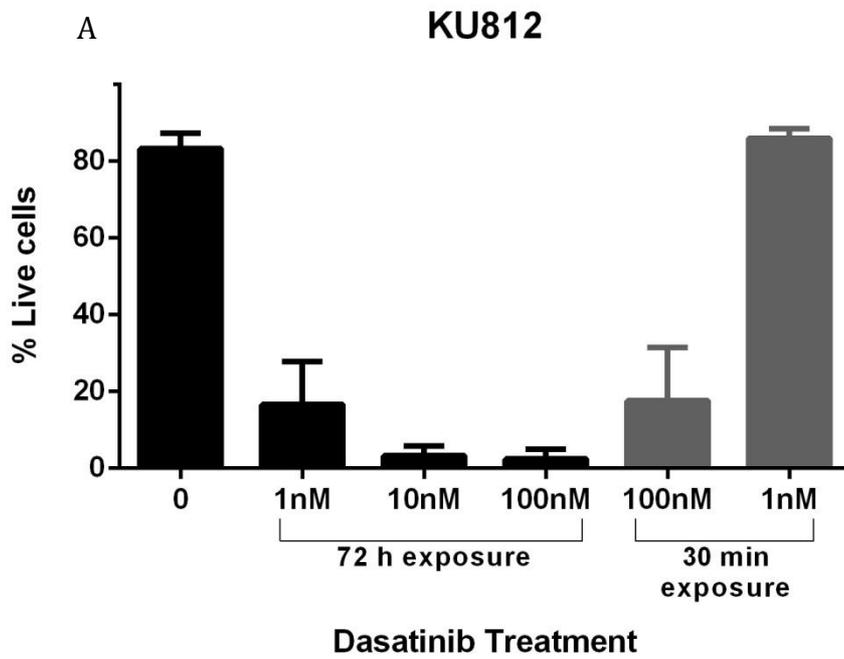
Peripheral blood mononuclear cells (PB-MNCs) collected from newly diagnosed CP-CML patients were treated with increasing concentrations of dasatinib and Bcr-Abl activity was measured at 2 h by assessment of phosphorylation of the Bcr-Abl substrate CrkL. The pCrkL/CrkL IC<sub>50</sub> was 3-5nM (demarcated by the red line), up to 10-times higher than the concentration of dasatinib required to induce cell death (Figure 4.4). Data are mean  $\pm$  SEM of six individual patients. Assays were obtained with the assistance of Verity Saunders.

### 4.2.3 Transient exposure to 100 nM dasatinib induces cell death despite complete reactivation of Bcr-Abl kinase activity

With a short half-life of 3-5 h, clinical success of once daily dasatinib was surprising, therefore the kinetics of transient Bcr-Abl inhibition was assessed. The effect of transient exposures (30 min) to 100 nM dasatinib (a potent dose at which Bcr-Abl phosphorylation of CrkL is completely inhibited) was observed *in vitro* following the removal of dasatinib by 3 consecutive washes in 37°C pre-warmed drug-free media, termed the standard washout (STD wash).

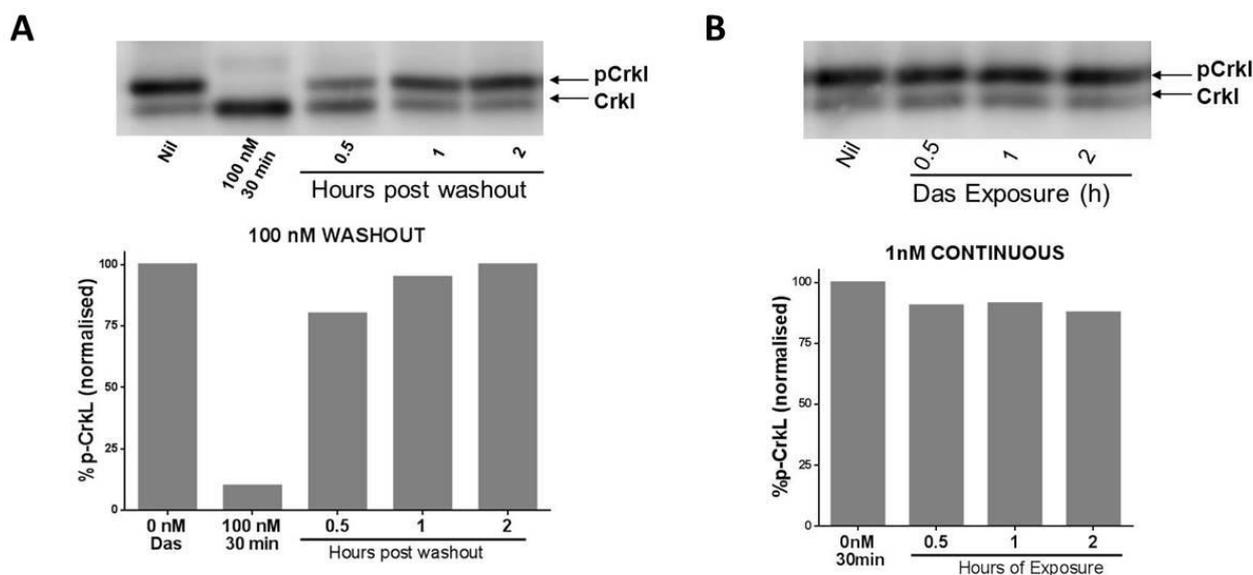
In comparison to continuous treatments with 100 nM dasatinib (5.3% live cells), induction of cell death in KU812 cells was demonstrated despite the removal of 100 nM dasatinib by the STD wash (13.6% live) (Figure 4.6). The cell death observed following this 30 min exposure to 100 nM dasatinib was not significantly different following exposure to 1 nM dasatinib (14.6% viable,  $p=0.88$ ) or 100 nM ( $p=0.12$ ) continuous dasatinib treatments. Notably, transient treatment with low dose (1 nM) dasatinib was unable to induce cell death following washout (86% viable), despite the ability to induce cell death when continuously available (Figure 4.6).

Therefore the Bcr-Abl kinase activity following the standard washout of dasatinib was investigated to determine the mechanism of cell death under incomplete Bcr-Abl inhibition. Surprisingly, pCrkL began to recover within 30 min following dasatinib washout and was completely restored by 2 h (Figure 4.7A). Additionally, continuous 1 nM dasatinib only inhibited pCrkL by approximately 10% over the 2 h experimental course (Figure 4.7B).



**Figure 4.6: Transient (30 min) treatment with dasatinib induces cell death**

**(A)** KU812 cells were either continuously treated with 1, 10 or 100 nM dasatinib for 72 h, or transiently treated with 1 or 100 nM dasatinib for 30 min which was removed by 3 consecutive washes followed by culture in drug-free media for 72 h (washout). Data are mean + SEM of three independent experiments. **(B)** 30 min exposure to 100 nM dasatinib followed by washout or continuous exposure to 1 nM dasatinib induce comparable cell death.



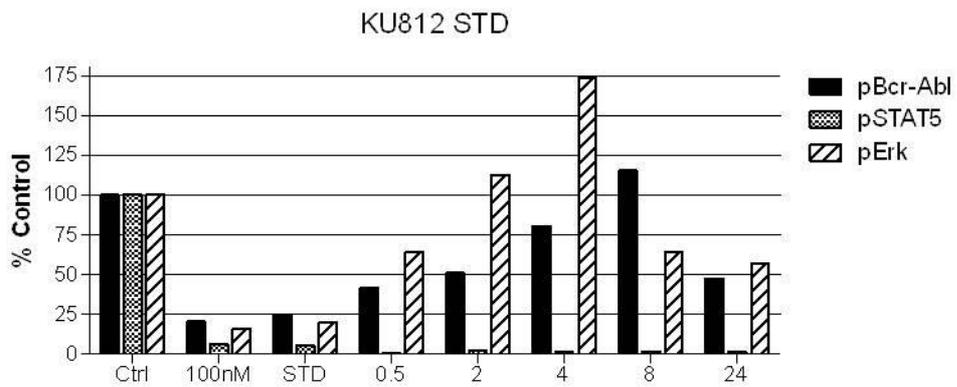
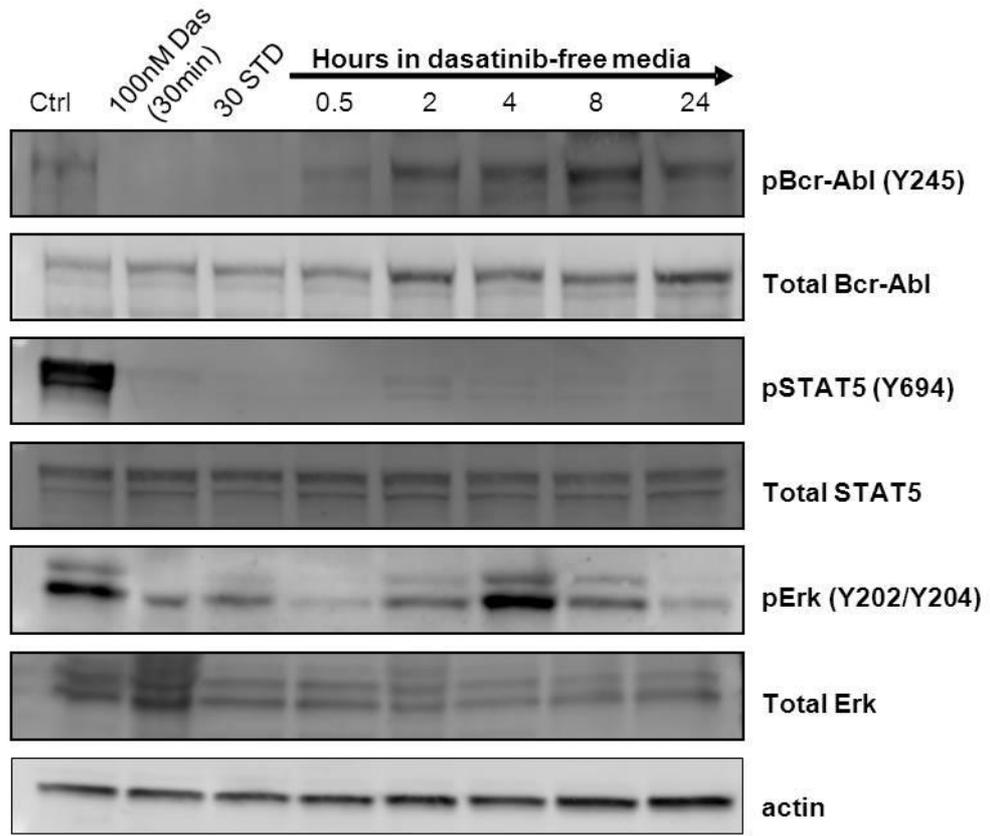
**Figure 4.7: Bcr-Abl is active following transient high and continuous low dose dasatinib treatments.**

Bcr-Abl activity was measured at 30 min, 1 h and 2 h for changes in pCrkL. KU812 cells were either **(A)** transiently treated with 100 nM dasatinib for 30 min followed by 3 consecutive washes or **(B)** continuously treated with 1 nM dasatinib. CrkL phosphorylation was assessed by western blotting analyses. Data is representative of three independent experiments. Quantitative graph is representative of western blots presented here.

Therefore, cell death was induced despite reactivation of Bcr-Abl, suggesting that there is potentially persistent inhibition of pro-survival signalling downstream of Bcr-Abl which is responsible for induction of cell death.

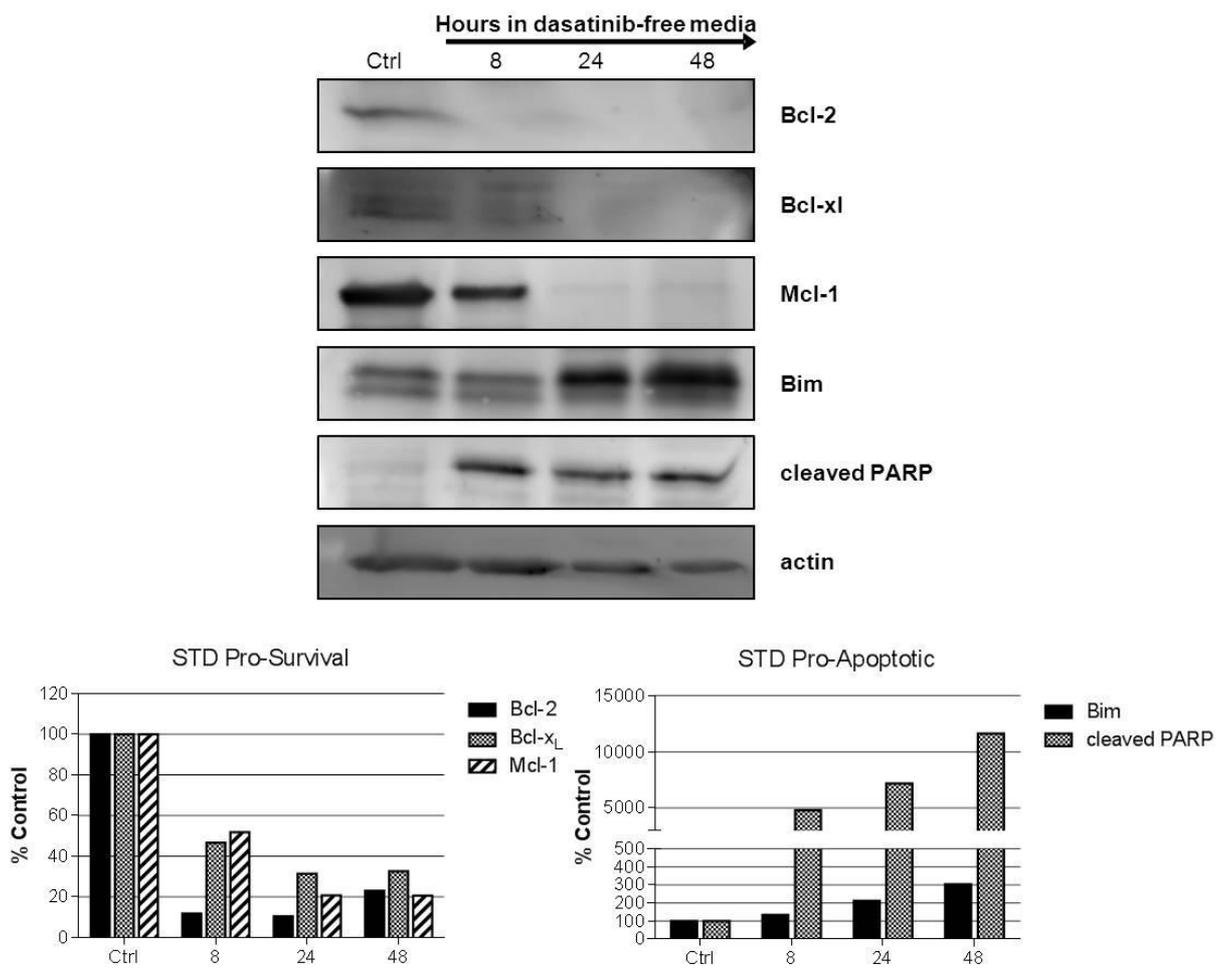
### **4.2.4 Bcr-Abl signalling resumes following the removal of dasatinib by the standard washout procedure.**

CrkL is a well characterised surrogate marker of Bcr-Abl activity, where inhibition of pCrkL by TKIs correlates to response to therapy.<sup>60,133</sup> To determine the critical signals involved in the commitment of CML cells to cell death, the effects of transient high and continuous low dose dasatinib exposures on Bcr-Abl-dependent signalling were investigated. STAT5 and Erk are also established targets of Bcr-Abl,<sup>51,72</sup> therefore the phosphorylation status of STAT5, Erk was assessed in addition to Bcr-Abl itself. In KU812 cells, following transient exposure to 100 nM dasatinib, there was rapid reactivation of Bcr-Abl (pY245) within 2 h of the STD wash (Figure 4.8). This reactivation profile was reflected by phosphorylation of Erk, a downstream signalling partner of Bcr-Abl. Interestingly, STAT5 phosphorylation was continuously inhibited for 24 h following STD wash, indicating that STAT5 could either be a sensitive measure of Bcr-Abl activity, or that STAT5 inhibition is critical to the induction of apoptosis, or both. In order to investigate the role of pro-apoptotic signalling in these experimental observations, the expression of the anti-apoptotic signalling proteins Mcl-1, Bcl-x<sub>L</sub> and Bcl-2 were interrogated along with markers of apoptosis induction Bim and cleaved PARP. In agreement with the cell viability findings (Figure 4.6), decreased expression of Mcl-1, Bcl-x<sub>L</sub> and Bcl-2 and a corresponding induction of Bim and cleaved PARP was observed (Figure 4.9).



**Figure 4.8: Dasatinib-induced transient inhibition of Bcr-Abl results in continuous inhibition of STAT5.**

KU812 cells were treated with 100 nM dasatinib (Das) for 30 min followed by the STD wash and reculturing for 24 h in dasatinib-free media. Bcr-Abl signalling was assessed by western blotting analyses. Bcr-Abl signalling was reactivated within 2 h of standard washout but STAT5 remained inhibited. Data is representative of three independent experiments. Quantitative graph is representative of western blots presented here.



**Figure 4.9: Dasatinib-induced transient inhibition of Bcr-Abl is coupled with inhibition of pro-survival proteins and induction of pro-apoptotic markers.**

KU812 cells were treated with 100 nM dasatinib for 30 min followed by the STD wash and reculturing for 48 h in dasatinib-free media. Apoptotic signalling was assessed by western blot analyses. Bcl-2 family members were inhibited and the pro-apoptotic proteins Bim and cleaved PARP were induced. Data is representative of three independent experiments. Quantitative plots are representative of western blots presented here.

**4.2.5 Continuous exposure to 1 nM dasatinib results in gradual inhibition of Bcr-Abl signalling and concomitant activation of pro-apoptotic signalling.**

Simara *et al.*<sup>227</sup> revealed that following removal of 100 nM dasatinib using an STD wash protocol, a residual amount of dasatinib remains that is similar to 1 nM continuous dasatinib for 72 h. It is possible that prolonged inhibition of STAT5 following the STD wash is due to residual dasatinib which has not been removed by the washout procedure. Examination of continuous exposure to 1 nM dasatinib revealed that Bcr-Abl activity, as measure by pCrkL, is only inhibited by 10% at 2 h (Figure 4.7B) and therefore residual dasatinib could remain following STD wash that could not be detected by measuring inhibition of pCrkL.

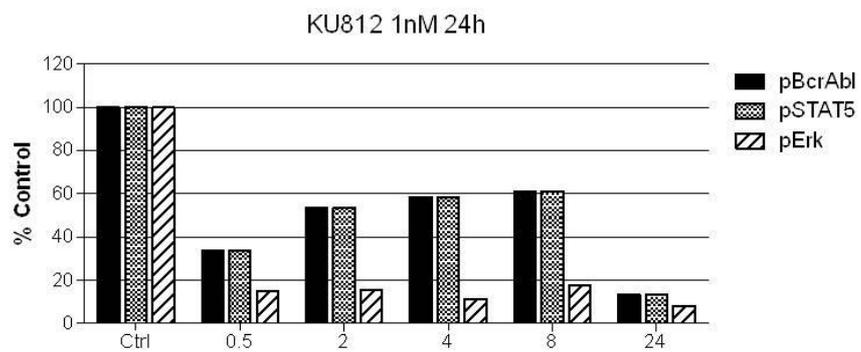
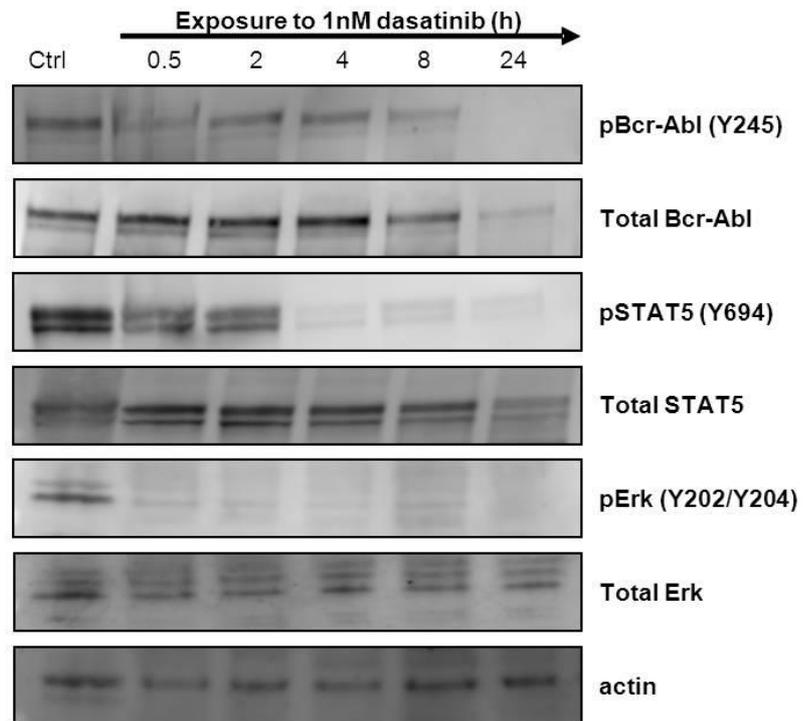
Subsequently, the phosphorylation status of Bcr-Abl, pErk and STAT5 were investigated over a 24 h exposure to 1 nM dasatinib. Phosphorylation of Erk is inhibited by 30 min (Figure 4.10), however there was only gradual inhibition of pSTAT5 over the first 2 h of the time course, followed by complete inhibition at 4 h. Bcr-Abl phosphorylation was partially inhibited following exposure to 1 nM dasatinib and total Bcr-Abl began to decline after 8 h (Figure 4.10) in contrast to 100 nM 30 min dasatinib treatment. The loss of these Bcr-Abl survival signals resulted in the inhibition of Mcl-1, Bcl-x<sub>L</sub> and Bcl-2 and the induction of Bim and cleaved PARP following treatment with 1 nM dasatinib (Figure 4.11), similar to what had been observed with the 30 min STD wash following transient exposure to 100nM dasatinib (Figure 4.9).

Although Bcr-Abl and Erk phosphorylation remained inhibited following continuous exposure to 1 nM dasatinib, the signals returned in 100 nM cultures followed by the STD wash. These results suggest that if residual dasatinib remains following washout, it must be below 1 nM. Conversely, STAT5 remained inhibited in both treatments highlighting a possible connection between inhibition of STAT5 and induction of apoptosis.

### **4.2.6 Residual dasatinib is undetectable by intracellular uptake and retention (IUR) assay employing <sup>14</sup>C-dasatinib**

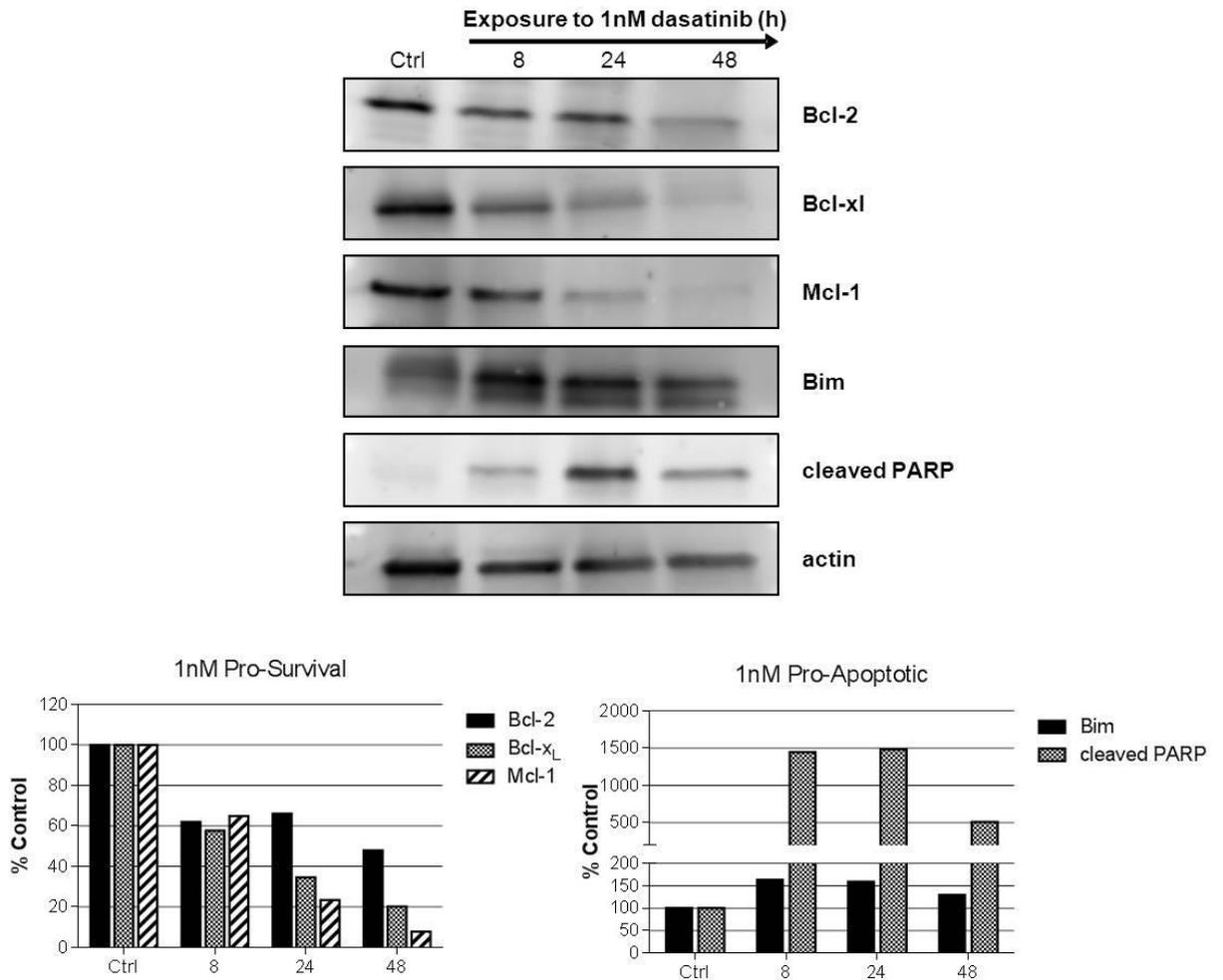
The sensitive inhibition of STAT5 due to 1 nM dasatinib raises the question of whether residual amounts of dasatinib remain in cells following the STD wash protocol, and whether it is this residual dasatinib that commits the cells to death. To interrogate this possibility, <sup>14</sup>C radiolabelled dasatinib was utilised to assess *in vitro* uptake and retention of dasatinib before and after the standard washout protocol (Figure 4.12). Exposure to 100nM <sup>14</sup>-C dasatinib for 30 min generated 54 counts per minute (cpm) in KU812 cells and 59 cpm in Meg01 cells compared to a baseline of 14 cpm and 13 cpm in untreated KU812 and Meg01 respectively (Figure 4.12). Exposure to 100 nM dasatinib resulted in 74.5 cpm in KU812 and 48.7 cpm in Meg01 cells which were similarly reduced to undetectable levels immediately following washout in KU812 (13-20 cpm) and Meg01 cells (10-21 cpm) (Figure 4.12). Over a 0.5-48 h period following the washout, counts were not significantly different from untreated cells (range 16-20 cpm in KU812 and 11-15 cpm Meg01 cells), suggesting effective removal of intracellular dasatinib from the cells.

However, subsequent investigation into the limit of detection of this system demonstrated that concentrations below 50nM dasatinib were not distinguishable from untreated KU812 cells (Figure 4.13). Concentrations of 1nM (10.5 cpm) and 10nM (13.5 cpm) dasatinib, which are sufficient to induce cell death, were not significantly different from untreated cells (15 cpm,  $p=0.45$  and  $p=0.75$  respectively) when measure by this experimental method (Figure 4.13).



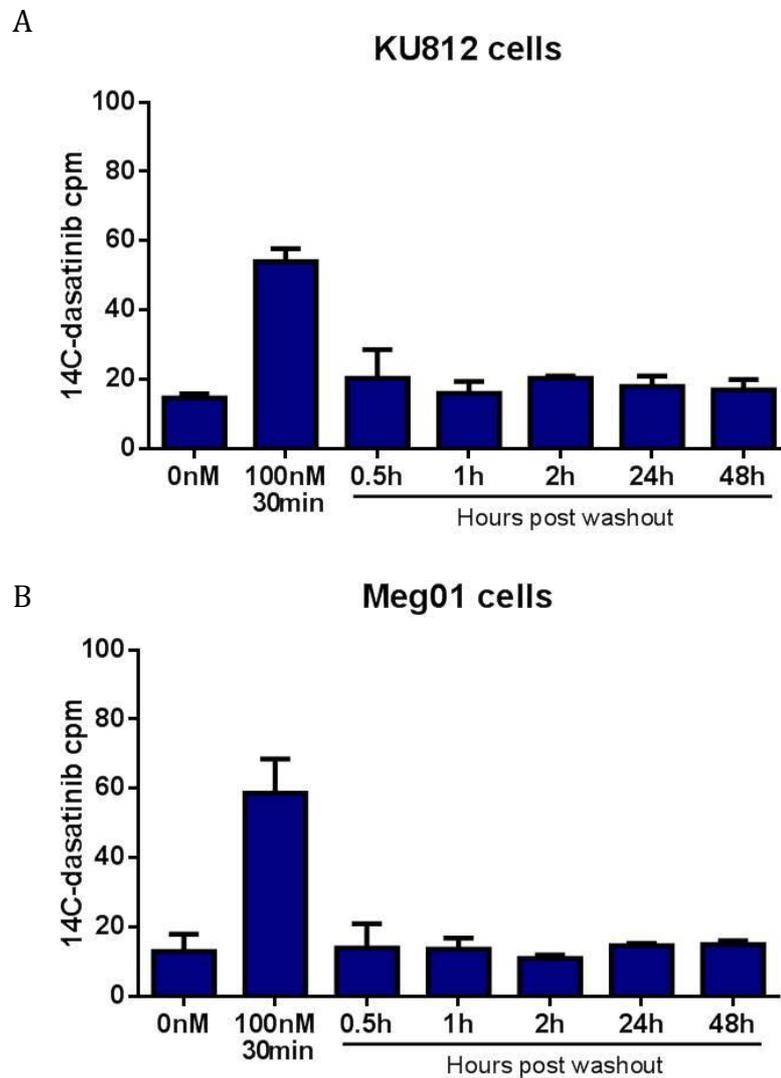
**Figure 4.10: Gradual inhibition of Bcr-Abl signalling by 1nM dasatinib.**

KU812 cells were treated continuously with 1nM dasatinib for 24 h. Bcr-Abl signalling was assessed by western blotting analyses. Sensitive inhibition of STAT5 and Erk signals by 1 nM dasatinib was observed. Data is representative of three independent experiments. Quantitative plot is representative of western blots presented here.



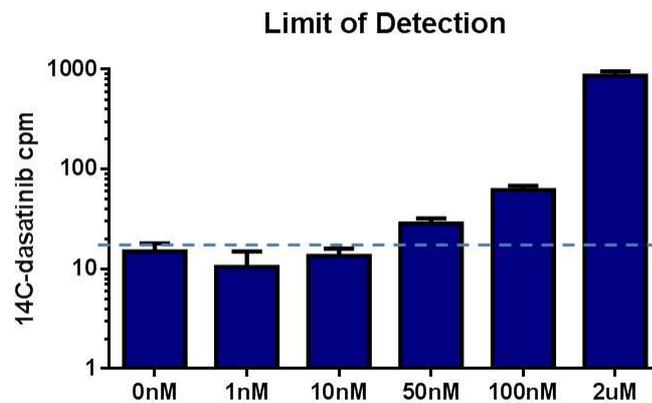
**Figure 4.11: Continuous exposure to 1 nM dasatinib induces inhibition of pro-survival proteins and induction of pro-apoptotic markers.**

KU812 cells were treated continuously with 1 nM dasatinib for 48 h. Apoptosis signalling was assessed by western blotting analyses. Treatment inhibited Bcl-2 family members and induced Bim and cleaved PARP. Data is representative of three independent experiments. Quantitative plot is representative of western blots presented here.



**Figure 4.12: Dasatinib is undetectable following standard washout.**

KU812 cells (**A**) and Meg01 cells (**B**) were treated with 100 nM dasatinib for 30 min followed by the STD washout. <sup>14</sup>C dasatinib was measured by scintillation in the cells. Dasatinib was undetectable in the cells following standard washout protocol. Data are mean + SEM of three independent experiments.



**Figure 4.13: Dasatinib IUR limit of detection is  $\leq 50\text{nM}$ .**

KU812 cells were treated 1nM-2 $\mu\text{M}$  dasatinib for 2 h.  $^{14}\text{C}$  labelled dasatinib was measured by scintillation. Dasatinib was undetectable in the cells at levels  $<50\text{nM}$ . Data are mean + SEM of three independent experiments.

## 4.3 Discussion

The treatment paradigm, established with the clinical development of imatinib, was based on the belief that continuous TKI availability, and therefore continuous Bcr-Abl kinase inhibition, is required for induction of apoptosis in CML cells.<sup>132</sup> This hypothesis fails to account for the clinical efficacy of once daily dasatinib therapy<sup>111,135</sup> which only transiently inhibits Bcr-Abl *in vivo* due to the short half-life of dasatinib in serum.<sup>134</sup>

Observations in cultured *BCR-ABL1*+ cell lines and CP-CML CD34+ patient cells, treated with transient or continuous dasatinib, revealed a disparity between the dose of dasatinib required to inhibit Bcr-Abl kinase activity versus the dose required to induce cell death. Whilst 2-5 nM dasatinib reduced Bcr-Abl activity by 50%, only 1 nM was required for significant effect on cell viability (Figure 4.2). This result indicates that a lower concentration of dasatinib is able to induce cell death whilst not resulting in complete Bcr-Abl inhibition. Therefore, it was hypothesised that low dose dasatinib which provides only partial inhibition of Bcr-Abl, is sufficient to induce apoptosis and thereby provide an explanation for the efficacy of once daily dasatinib therapy. Subsequent analyses in this chapter considered the means by which the partial inhibition of the Bcr-Abl oncoprotein commits cells to death, by investigating the signalling dynamics of *BCR-ABL1*+ cells exposed to continuous low dose and transient high dose dasatinib.

The survival of *BCR-ABL1*+ cells is driven by the constitutive activation of the oncogenic fusion protein Bcr-Abl.<sup>228</sup> As a consequence, cells become reliant on Bcr-Abl-dependent

survival signalling and this phenomenon provides opportunity for inhibitors to target the required signalling proteins.<sup>139</sup> This “addiction” of tumour cells to a dominant oncoprotein and its associated signalling is not restricted to Bcr-Abl, and there is increasing evidence to suggest that oncoproteins, such as MYC<sup>229</sup> and EGFR<sup>230</sup>, drive the maintenance of cancer cell signalling and subsequently the cancer cells become dependent on these survival signals.<sup>139</sup>

These data are in accordance with previous *in vitro* observations in our laboratory that 100 nM dasatinib treatments for 30 min followed by a standard drug washout induces cell death,<sup>144</sup> and support clinical evidence that continuous Bcr-Abl kinase inhibition is not required for optimal response to therapy.<sup>135,231</sup> This is in agreement with theory of oncogenic shock,<sup>226</sup> suggesting that the disruption of survival signalling by transient dasatinib treatment is enough to commit cells to death. Analysis of Bcr-Abl-dependent signalling in these studies was restricted to observations of CrkL phosphorylation, however as CrkL was reactivated following dasatinib washout, it was unclear what committed the cells to death and so other Bcr-Abl dependent signalling was investigated in this chapter.

Following the standard washout, the reactivation of Bcr-Abl and Erk (as indicated by the presence of pBcr-Abl and pERK) was observed, but importantly continuous inhibition of STAT5 (indicated by the lack of pSTAT5) was observed (Figure 4.8). These findings were comparable to those observed following exposure to continuous 1 nM dasatinib which also resulted in the inhibition of STAT5 together with Erk signalling (Figure 4.10). These results indicated that low levels of dasatinib may remain in the cells following the

standard washout procedure which, whilst allowing reactivation of Bcr-Abl, was still sufficient to induce cell death.

To determine whether residual dasatinib remained in these cells following the standard washout protocol, <sup>14</sup>C radiolabelled dasatinib was utilised in an IUR assay. No difference between intracellular dasatinib levels were observed in untreated cells compared to cells following standard washout of dasatinib (Figure 4.12). Additionally, dasatinib was undetectable in the washout medium of these cells indicating that dasatinib was completely removed by the washout protocol. However, this assay was unable to distinguish dasatinib levels less than 50 nM from untreated cells, and therefore this particular assay was not sensitive enough to definitively determine if any residual dasatinib (< 50 nM) remained in the cells following washout.

Recent reports suggest that low levels of TKI remain following the standard washout after a 30 min exposure to high dose TKI.<sup>227,232-234</sup> Lipka and colleagues<sup>232,234</sup> detected residual levels of dasatinib, imatinib and nilotinib in K562 and BaF3-*BCR-ABL1*<sup>+</sup> cells following this standard washout procedure, using a HPLC-based method for TKI detection. The observations of Simara *et al.*<sup>235</sup> suggest that following standard washout of 100 nM dasatinib, the residual concentration of TKI is similar to that achieved by 24 h continuous treatment with 1 nM dasatinib. These previously reported results support the hypothesis that low levels of dasatinib remain in cells following the standard washout procedure and that this is sufficient to induce cell death, despite approximately 90% reactivation of Bcr-Abl, as measured by the presence of pCrkL.

Additionally, these studies highlight STAT5 activity as a sensitive measure of *low level* Bcr-Abl kinase inhibition; however the measurement of CrkL phosphorylation remains an important and specific measure of the *degree* of Bcr-Abl kinase inhibition. Unlike pSTAT5, changes in pCrkL following TKI treatment can ascertain intrinsic factors which determine response to therapy with strong predictive power,<sup>133,236</sup> and would be missed due to the sensitivity of pSTAT5 to TKI. Thus, the measurement of multiple Bcr-Abl dependent signalling components should be considered to provide a complete picture of response to TKI therapy, depending on the investigation being undertaken.

The research presented in this chapter highlights the importance of low level inhibition of Bcr-Abl kinase and the subsequent commitment of cells to death. Observations of low dose and transient dasatinib treatments indicate that STAT5 activity may be a sensitive measure of Bcr-Abl kinase activity and highlights STAT5 as a potentially important target for the induction of cell death in *BCR-ABL1+* cells. These findings challenge the imatinib paradigm that continuous availability of tyrosine kinase inhibitors, and therefore complete inhibition of Bcr-Abl, is required for optimal clinical responses in patients. Rather, taken together, the results presented here indicate that the clinical efficacy of dasatinib does not require continuous inhibition of Bcr-Abl and thus only sustained, partial inhibition of Bcr-Abl may be required.

## **CHAPTER 5.**

# **ASSESSMENT OF THE CRITICAL FACTORS INVOLVED IN COMMITMENT OF *BCR- ABL1+* CELLS TO APOPTOSIS**

## 5.1 Introduction

Dasatinib is a potent second generation TKI which, despite a short *in vivo* half-life of 3-5 h,<sup>134</sup> achieves optimal cytogenetic and molecular responses in patients when administered once daily.<sup>111</sup> A single daily dose has been shown to reduce the incidence of adverse effects compared to the initial twice daily regime.<sup>231</sup> Dasatinib plasma levels in CML patients reach 150-200 nM by 30 min, but quickly drop over 4-24 h post-administration, at which time there is concomitant reactivation of Bcr-Abl kinase activity.<sup>137</sup> As such, once daily dasatinib therapy challenges the accepted therapeutic rationale of TKI therapy based on the requirement for continuous Bcr-Abl inhibition by imatinib for sustained disease control.

We and others<sup>136-138</sup> have previously reported that high dose, short-term dasatinib exposure (100 nM for 30 min) induces cell death in *BCR-ABL1*+ cells, despite restoration of Bcr-Abl signaling 2 h post drug washout.<sup>136</sup> However, recent observations by Lipka and colleagues<sup>232,234</sup> demonstrated that the standard washout procedure established by Shah *et al.*<sup>137</sup> is ineffective at completely removing TKI. Residual levels of dasatinib, imatinib and nilotinib, determined using a HPLC-based method of TKI detection, were detected in K562 and Ba/F3-BCR-ABL1+ cells following this standard washout (STD wash) procedure.<sup>232,234</sup> Equilibration of cells in drug-free media between washes (optimal washout, OPT wash) removed the residual TKI (dasatinib, imatinib or nilotinib). In the setting of effective dasatinib washout, short term exposure was no longer capable of inducing apoptosis in CML cells.<sup>232,234</sup> This has subsequently been confirmed by other groups who have suggested that reduced phosphorylation of STAT5 is a more sensitive marker of residual low concentrations of TKI, than reduced CrkL phosphorylation.<sup>227,237</sup>

Conventionally, STAT5 activation occurs in response to cytokine signalling through JAK kinases<sup>145</sup>. In CML, it is currently controversial whether JAK2 is required for the Bcr-Abl-dependent activation of STAT5.<sup>147</sup> Although there is evidence to suggest that JAK2 interacts directly with Bcr-Abl,<sup>148,149</sup> JAK inhibition with TG101209 induced minimal apoptosis in CML cells and appeared to target extrinsic cytokine-mediated survival signalling rather than Bcr-Abl-dependent signalling.<sup>150</sup> Moreover, the absence of JAK2 did not affect leukaemia maintenance in a CML-like murine model,<sup>151</sup> however STAT5 deletion resulted in failure to maintain leukaemic haematopoiesis.<sup>152</sup>

High levels of total STAT5 protein has recently been demonstrated as an important mechanism of resistance to TKI treatment,<sup>153</sup> and highlighted as a target in CML CD34+ patient cells with acquired resistance to imatinib, where STAT5A protects cells from oxidative stress.<sup>154</sup> Concurrently, the calcium channel<sup>155</sup> and dopamine D<sub>2</sub> receptor antagonist<sup>156</sup> pimozide was recently identified as an inhibitor of constitutive STAT5 activation in CML.<sup>157</sup> As STAT5 has been implicated in TKI resistance and has an essential role in Bcr-Abl-dependent leukaemogenesis, STAT5 has subsequently become an attractive drug target.

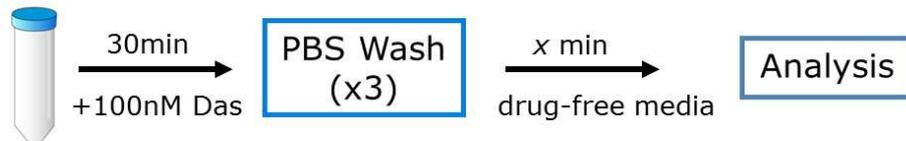
This chapter evaluates the critical factors involved in commitment of cells to TKI-induced cell death in the setting of transient Bcr-Abl kinase inhibition. The duration of potent TKI exposure required to commit *BCR-ABL1*+ cells to apoptosis was subsequently investigated. This study postulates that continued inhibition of STAT5 activation is one

of the most critical factors for commitment to cell death, in the setting of transient or incomplete Bcr-Abl inhibition, and that STAT5 activation is independent of JAK2.

### **5.1.1 Approach**

The aim of the research presented in this chapter was to determine the critical signalling required for commitment to cell death following transient inhibition of Bcr-Abl. An optimal washout procedure was developed to allow equilibration of cells in drug-free media between washes, as this is thought to result in complete removal of TKI from cells (Figure 5.1). KU812, Meg01 and K562 *BCR-ABL1+* cell lines and CP-CML CD34+ primary patient cells were used to assess Bcr-Abl signalling, apoptosis and colony formation following transient exposure to potent doses of either dasatinib or imatinib. Bcr-Abl signalling was assessed by western blot for pBcr-Abl (Y245), pSTAT5 and pErk over a 24 h period. Bcl-2 family members Mcl-1, Bcl-xL and Bcl-2, along with Bim and cleaved PARP were measured by western blot after 8-48 h TKI exposure. Cell viability was measured by Annexin V and 7AAD staining following 72 h treatment. Clonogenic potential was measured 2 weeks after the cell viability assay using the CFU-GM assay. Known pSTAT5 inhibitors pimozide (also a calcium channel<sup>155</sup> and dopamine D<sub>2</sub> receptor antagonist<sup>156</sup>) and N'-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide (STAT5i), which targets the SH2 domain of STAT5<sup>12</sup>, along with the autophagy inhibitor chloroquine, the MEK inhibitor U0126 and the JAK1/2 inhibitor ruxolitinib were used to interrogate Bcr-Abl-independent signalling.

### Standard (STD) washout



### Optimal (OPT) washout

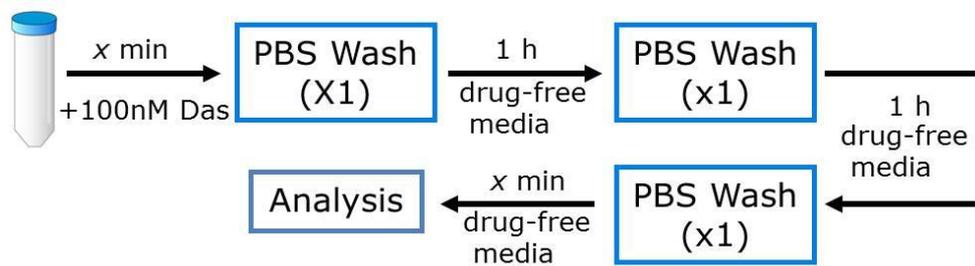


Figure 5.1: Schematic of the standard and optimal washout procedures.

Standard washout (STD) refers to transient exposure to dasatinib (Das) for 30min, followed by 3 immediate washes in 10 ml pre-warmed PBS. Optimal washout (OPT) refers to x 3 washes in 10 ml pre-warmed PBS, with equilibration of cells in drug-free media for 1 h in between washes.

### **5.1.2 Summary and Research Contribution**

Using an optimal washout technique to completely remove residual dasatinib, prevention of apoptosis in KU812 and Meg01 cells is confirmed. Additionally the inhibition of STAT5 and Erk activation are highlighted as key indicators of low level Bcr-Abl signalling inhibition. This data supports existing evidence that JAK kinase signalling is only relevant in Bcr-Abl-independent, extrinsic activation of STAT5, as JAK1/2 inhibition had no effect on Bcr-Abl-driven STAT5 signalling or cell death as a sole agent in the absence of cytokines. This study demonstrates that continuous inhibition of Bcr-Abl activity is not required to induce cell death in *BCR-ABL1+* cells, rather the continuous inhibition of its downstream partner STAT5 is essential. Critically, additional STAT5 inhibition together with dasatinib pulse treatment results in increased cell death in primary CP-CML CD34+ cells. The findings presented here will help establish critical CML signalling components that may be targeted in combination therapeutic approaches, to further improve patient clinical outcomes.

### **5.1.3 Publications**

**Schafranek L**, Nievergall E, Powell JA, Hiwase DK, Leclercq T, Hughes TP and White DL. Sustained inhibition of STAT5, but not JAK2, is essential for TKI-induced cell death in chronic myeloid leukemia. *Leukemia advance online publication*, June 27, 2014; doi:10.1038/leu.2014.156; accepted article preview online May 12, 2014.

### **5.1.4 Conference Presentations**

**Schafranek L**, Nievergall E, Powell JA, Hiwase DK, Leclercq T, Hughes TP and White DL. New evidence that transient Bcr-Abl inhibition commits cells to death in a time- and

## **Chapter 5 | Critical factors involved in commitment of CML cells to apoptosis**

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STAT5-dependent manner despite reactivation of Bcr-Abl. NDLR, March 2014, Noosa, QLD (oral presentation).

**Schafranek L**, Nievergall E, Hiwase H, Powell J, White D, Hughes T. Direct inhibition of STAT5 in combination with transient Bcr-Abl inhibition commits cells to apoptosis despite reactivation of Bcr-Abl. ASH Dec 2013, New Orleans, USA (poster presentation).

**Schafranek L**, Nievergall E, Hiwase H, Powell J, White D, Hughes T. Inhibition of activated STAT5 sensitizes chronic myeloid leukemia cells to TKI treatment and commits cells to apoptosis despite reactivation of Bcr-Abl, independent of JAK1/2. FHS conference, July 2013, University of Adelaide, Australia (poster presentation). **Awarded Florey Medical Research Foundation Prize**

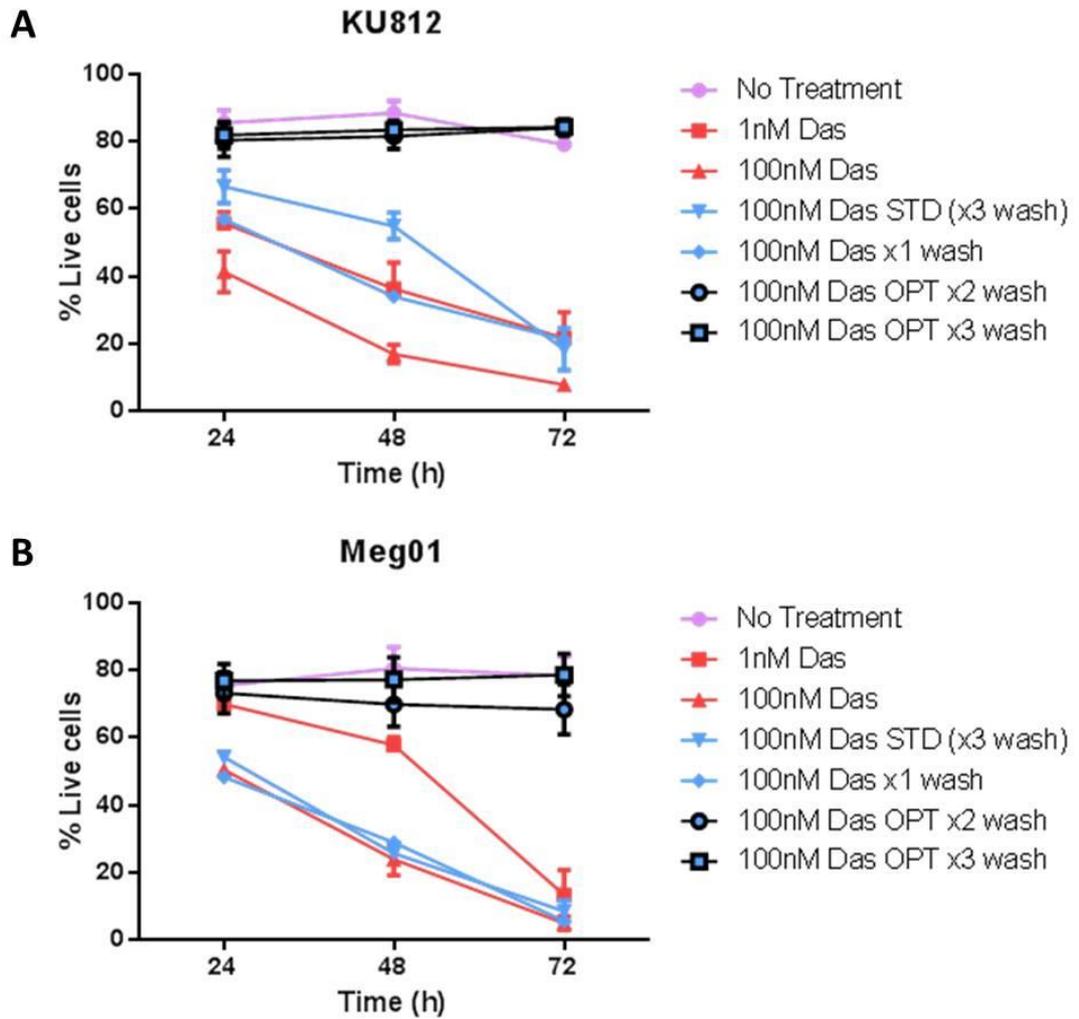
**Schafranek L**, Nievergall E, Hiwase H, Powell J, White D, Hughes T. STAT5 inhibition is critical to the commitment of chronic myeloid leukemia cells to apoptosis regardless of Bcr-Abl reactivation. CPCM Symposium, August 2013, National Wine Centre, Adelaide Australia (poster presentation).

**Schafranek L**, Nievergall E, Hiwase H, Powell J, White D, Hughes T. Commitment of CML Cells to Apoptotic Cell Death Depends On the Length of Exposure to Das and the Level of STAT5 Activity. ASH Dec 2012, Atlanta, USA (poster presentation)

## 5.2 Results

### 5.2.1 Equilibration of cells in drug-free media prevents apoptosis induced by 30 min treatment with 100 nM dasatinib

To interrogate whether complete removal of dasatinib using an optimal washout procedure would prevent cell death, as previously observed,<sup>227,232,234</sup> KU812 and Meg01 cells exposed to 1 nM dasatinib for 72 h or 100 nM dasatinib for 30 min, followed by either the standard washout procedure (3 consecutive washes) or an optimal washout procedure (OPT wash) in which cells were allowed to equilibrate for an hour at 37°C/5% CO<sub>2</sub> in drug-free media between washes. In KU812 cells, continuous 1 nM dasatinib is sufficient to induce cell death by 24 h (55% viable), with a maximum reduction to 21.7% viable cells reached by 72 h (Figure 5.2A). Exposure to a continuous dose of 100 nM dasatinib results in a substantial decrease in viable cells by 72 h (7.9% viable). Similar observations were made in Meg01 cells where 1 nM dasatinib induced reduced cell viability (13.4%) which was further reduced by 100 nM dasatinib (4.9% viable) at 72 h (Figure 5.2B). Treatment for 30 min with 100 nM dasatinib, followed by the standard (STD) washout procedure resulted in a reduction in live cells in both KU812 (18.4% viable, p=0.75) and Meg01 (8.5% viable, p=0.6) cells at 72 h that was not significantly different from cells treated with continuous 1 nM dasatinib. Permitting equilibration of cells between each wash (wash, equilibration, wash, equilibration, wash, the optimal washout protocol, OPT x 3 wash) resulted in a significantly higher proportion of live cells in KU812 (82.6% viable cells, p<0.005) and Meg01 (78.7% viable, p<0.05) cells compared to the STD wash (16.1% and 8.5% viable respectively) (Figure 5.2). Application of only one period of equilibration (wash, equilibrate, wash, OPT x 2) was also able to prevent apoptosis and was not significantly different from the



**Figure 5.2: Optimal washout procedure prevents cell death in *BCR-ABL1*+ cells.** Optimal washout (OPT x3) prevents induction of cell death induced by transient exposure to dasatinib in KU812 (A) and Meg01 (B) cells. Cells were incubated either continuously with 1 nM dasatinib for 72 h, 100 nM dasatinib for 72 h or transiently exposed to 100 nM dasatinib for 30 min followed either by standard washout (STD) or optimal washout (OPT) and culturing for 72 h in drug-free media. Cells were then analysed by Annexin V/7-AAD staining (n=3, data are mean  $\pm$  SEM).

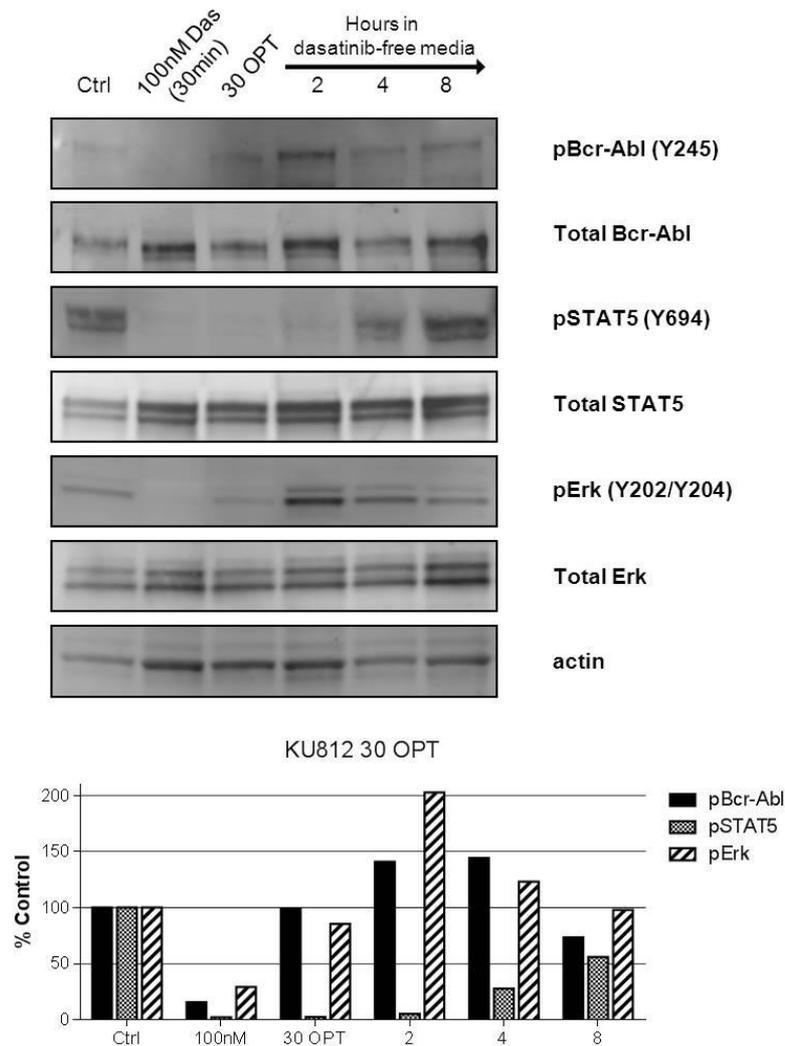
OPT x 3 wash in KU812 (84.2%, p=0.43) or Meg01 (84.4%, p=0.39) cells at 72 h (Figure 5.2).

### **5.2.2 Reactivation of Bcr-Abl signalling and prevention of apoptosis following 30 min exposure to dasatinib and optimal washout.**

To determine the critical factors required for TKI-induced cell death following transient treatment with dasatinib, the active signalling pathways following 30 min treatment with 100 nM dasatinib and the OPT washout were interrogated. In contrast to the 30 min STD wash (Figure 4.9), Bcr-Abl phosphorylation recovered immediately following the 30 min optimal washout (OPT wash) and was fully restored within 8 h of the OPT wash (Figure 5.3). Notably, there was also reactivation of both STAT5 and Erk following the OPT wash, where phosphorylation was fully restored by 2 h for Erk and 8 h for STAT5. The complete removal of dasatinib after 30 min of 100 nM dasatinib exposure prevented the loss of anti-apoptotic Mcl-1, Bcl-x<sub>L</sub> and Bcl-2 and was reflected by no significant increase in Bim or cleaved PARP, thereby indicating no induction of apoptosis (Figure 5.4).

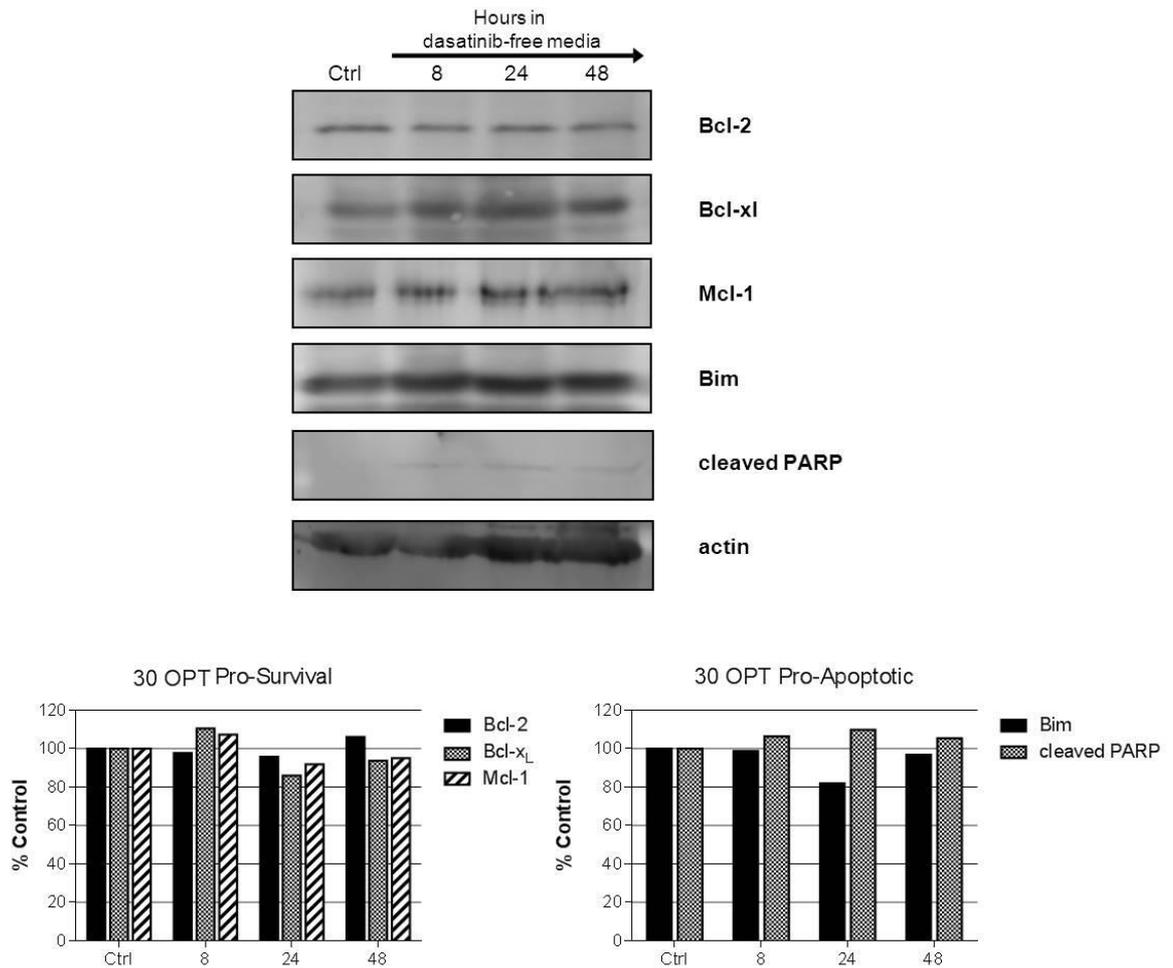
### **5.2.3 Extension of the initial exposure to TKI for greater than 2 h induces cell death despite the complete removal of dasatinib**

Original standard washout experiments assessed exposures to TKI between 20 min and 24 h prior to washout, however recent reports have only investigated exposures of less than or equal to 2 h.<sup>137,138</sup> Peak plasma levels of dasatinib occur up to 6 h following administration<sup>134</sup> and can be available at decreasing, but adequate levels to induce kinase inhibition (>10 nM) for 24 h.<sup>137</sup> Subsequently, the length of dasatinib exposure



**Figure 5.3: Reactivation of STAT5 following optimal washout.**

KU812 cells were transiently exposed to 100 nM dasatinib (Das) for 30 min followed by optimal washout (OPT) and culturing for up to 8 h in drug-free media. Lysates were analysed by western blotting for Bcr-Abl survival signalling. Data is representative of three independent experiments. Quantitation of protein phosphorylation normalised to total protein and actin (loading control). Percentage is relative to control (Ctrl). Graph is representative of western blots presented here.



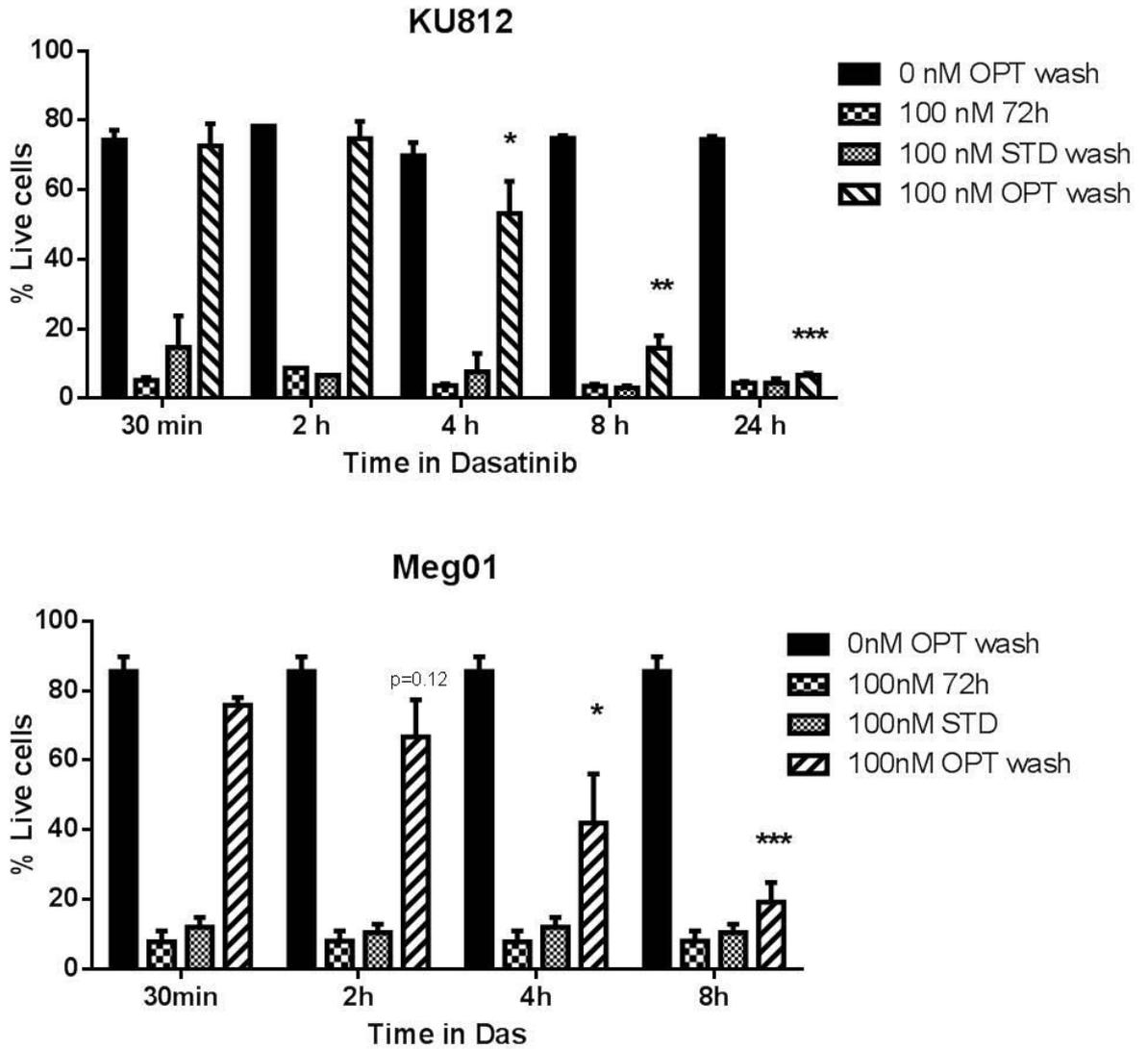
**Figure 5.4: Optimal washout prevents loss of Bcl-2 family members and induction of apoptosis markers.**

KU812 cells were transiently exposed to 100 nM dasatinib (Das) for 8 h followed by OPT washout, and then cultured for up to 48 h in drug-free media. Lysates were analysed by western blotting for apoptotic signalling. Data is representative of three independent experiments. Quantitation of protein normalised to actin (loading control) and percentage is relative to control (Ctrl). Graph is representative of western blots presented here.

## **Chapter 5 | Critical factors involved in commitment of CML cells to apoptosis**

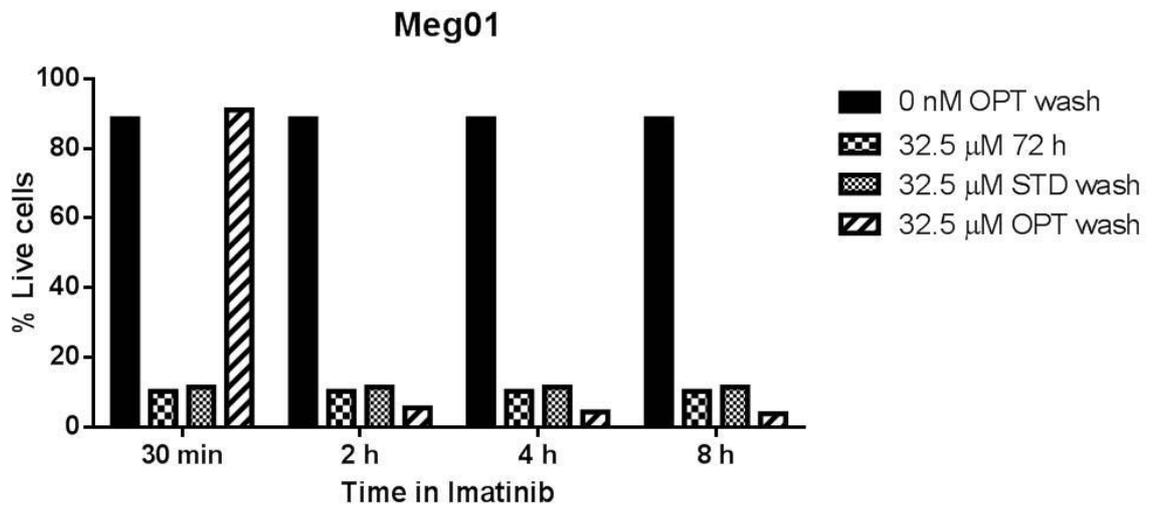
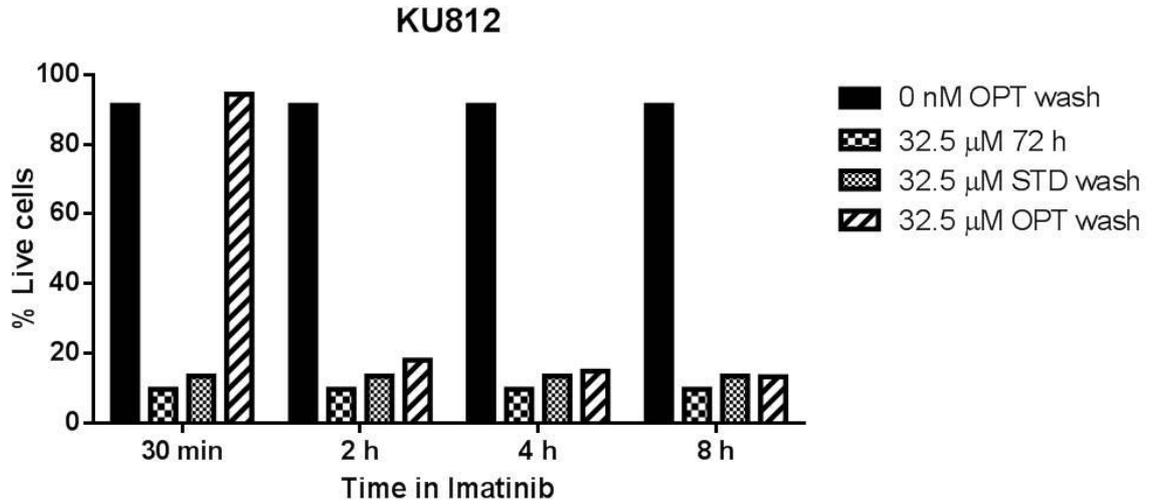
prior to washout that was sufficient to induce cell death was determined. Whilst continuous treatment with 100 nM dasatinib reduced the percentage of viable cells to 4.5% in KU812 and 5.3% in Meg01 cells (Figure 5.5), prolonging transient exposure of 100 nM dasatinib from 30 min to 4 h and 8 h prior to OPT wash resulted in significant decreases in cell viability in KU812 (53% and 14% viable cells respectively) and Meg01 (42% and 19% viable cells respectively) cells compared to 30 min dasatinib treatment (72.6% viable KU812, 75.8% viable Meg01) (Figure 5.5). Importantly, cell death was also induced following extended transient exposures to potent imatinib treatments of 32.5 $\mu$ M (Figure 5.6), indicating that this observation is not TKI-specific (KU812, 18.9% viable at 2 h, 14.9% at 4 h and 13.3% at 8 h)(Meg01, 5.6% viable at 2 h , 4.4% at 4 h and 3.9% at 8 h).

To determine the relevance of these findings in primary CML, CD34+ cells from *de novo* CP-CML patients were treated with 100 nM dasatinib or 32.5  $\mu$ M imatinib for 30 min, 2 h, 4 h or 8 h and assessed for cell death and subsequent colony forming ability. A 30 min exposure to 100 nM dasatinib followed by the STD wash induced cell death (83% viable cells), however application of the OPT wash following the 30 min treatment with dasatinib prevented cell death (94.6% viable cells) (Figure 5.7A). As such, the 30 min dasatinib treatment in conjunction with the STD wash significantly impaired clonogenic potential (55.8% CFU normalised to control,  $p=0.03$ ), but the more complete removal of dasatinib with the OPT wash lead to a significant increase in colony-forming cells (84.8% CFU,  $p=0.008$ ) compared to the STD wash (Figure 5.7B), suggesting that 30 min exposure to dasatinib followed by efficient dasatinib removal is not sufficient to impair colony forming ability.



**Figure 5.5: Extension of dasatinib exposure prior to OPT washout induces cell death in *BCR-ABL1+* cell lines.**

Dasatinib treatment of greater than 4 h prior to optimal (OPT) wash induces cell death. KU812 **(A)** cells were treated with 100 nM dasatinib for 0-24 h, followed by standard (STD) or OPT wash and culturing for 72 h in drug-free media. Meg01 cells **(B)** were incubated with 100 nM Das for 0-8 h, followed by STD or OPT wash and culturing for 72 h in drug-free media. Cells were then analysed by Annexin V/7-AAD staining (n=3 mean + SEM). \* p<0.05, \*\* p<0.01, \*\*\*p<0.001 compared to 30 min OPT wash.



**Figure 5.6: Extension of imatinib exposure prior to OPT washout induces cell death in *BCR-ABL1*+ cell lines.**

Imatinib treatment for >2 h prior to optimal (OPT) wash induces cell death. KU812 **(A)** and Meg01 **(B)** cells were incubated with 32.5  $\mu$ M imatinib for 0-8 h, followed by standard (STD) or OPT wash and then cultured for 72 h in drug-free media. Cells were analysed by Annexin V/7-AAD staining (n=1).

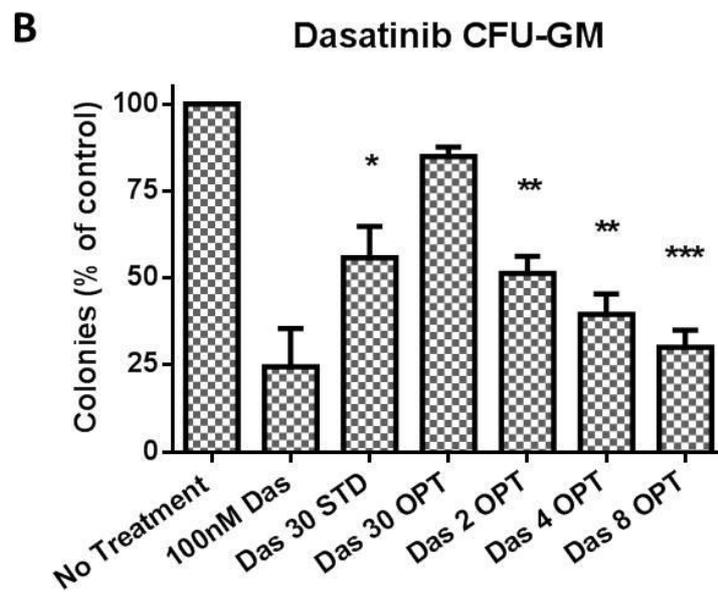
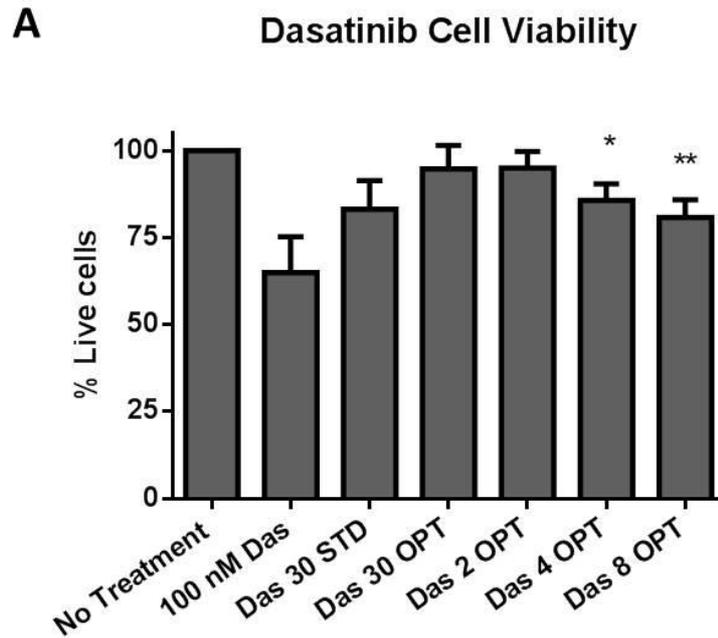
## **Chapter 5 | Critical factors involved in commitment of CML cells to apoptosis**

However, increasing the length of 100 nM dasatinib exposure to 4 h (85% viable,  $p=0.03$ ) and 8 h (80.6% viable,  $p=0.004$ ) significantly increased cell death compared to the 30 min treatment, despite complete removal of dasatinib with the OPT wash (Figure 5.7A) and resulted in significant reduction in CFUs compared to 30 min dasatinib treatment prior to the OPT wash, at 2h (51.3%,  $p=0.004$ ), 4h (39.6%,  $p=0.002$ ) and 8h (30.1%,  $p=0.002$ )(Figure 5.7B).

Similarly, 30 min treatment with 32.5  $\mu$ M imatinib followed by STD wash resulted in decreased viability of CP-CML CD34+ patients cells (76.6% viable cells) (Figure 5.8A). In this setting, cell viability was also maintained using the OPT wash following a 30 min treatment (85.5% viable), however not when the initial exposure to imatinib was extended to 2 h (69.5% viable), 4 h (61.6% viable) or 8 h (54.1% viable). Extension of imatinib treatment prior to the OPT wash resulted in significant reduction in CFUs compared to 30 min OPT imatinib (82.9% CFUs), at 2h (41.2%,  $p=0.01$ ), 4h (24.4%,  $p=0.0006$ ) and 8h (14.4%,  $p=0.0003$ ) (Figure 5.8B), indicating that the observation does not only apply in the dasatinib setting.

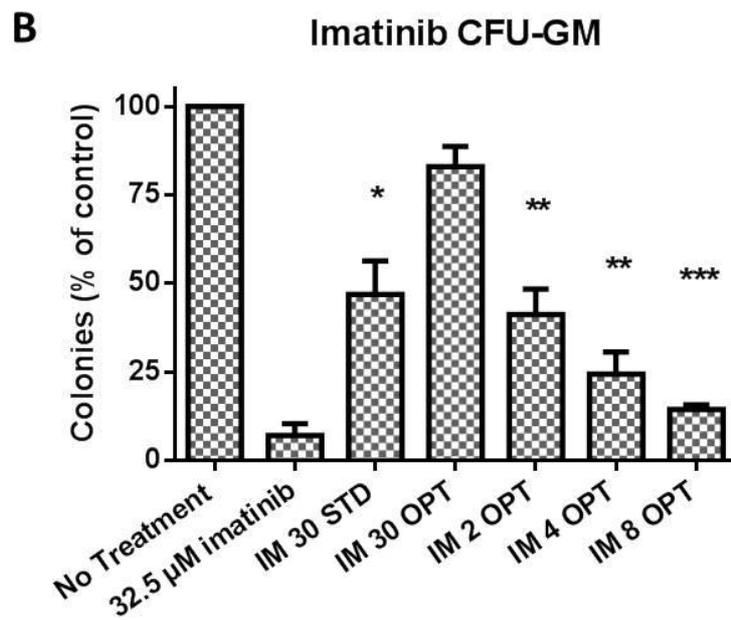
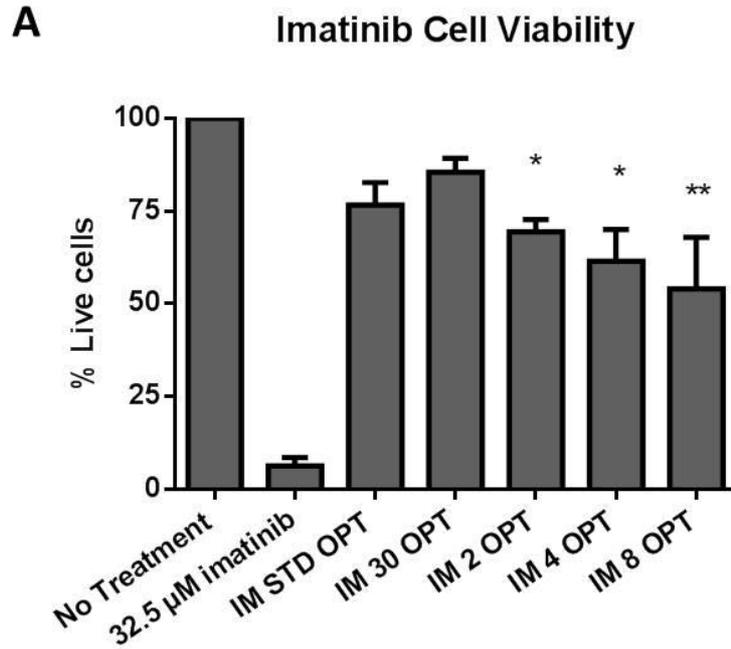
### **5.2.4 Induction of apoptosis markers following 8 h 100 nM dasatinib exposure followed by optimal washout, despite reactivation of STAT5 and Erk**

To determine the critical components which induce cell death following transient treatment with dasatinib, Bcr-Abl and related signalling (Erk and STAT5), along with apoptotic signalling was assessed following 8 h exposure to 100 nM dasatinib with OPT wash (which induces cell death, Figure 5.5) and compared to the 30 min 100 nM OPT wash (which does not induce cell death, Figure 5.2). The longer 8 h exposure to dasatinib prior to OPT wash, lead to a reduction in Bcr-Abl phosphorylation with



**Figure 5.7: Extension of 100 nM dasatinib exposure prior to OPT washout induces cell death and impairs clonogenic potential in CP-CML CD-34+ patient cells.**

*De novo* CP-CML CD34+ cells were incubated either continuously with 100 nM dasatinib (Das) for 72 h or transiently exposed to 100 nM dasatinib for 30 min-8 h followed either by standard washout (STD) or optimal washout (OPT) and culturing for 72 h in drug-free media. Cells were then analysed for **(A)** cell death by Annexin V/7-AAD staining at 72 h and **(B)** clonogenic potential by enumeration of CFUs following the CFU-GM assay at 2 weeks (n=3, mean + SEM). \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 compared to Das 30 OPT.



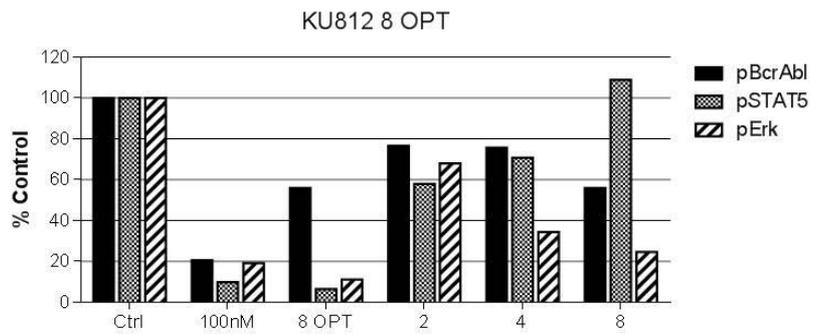
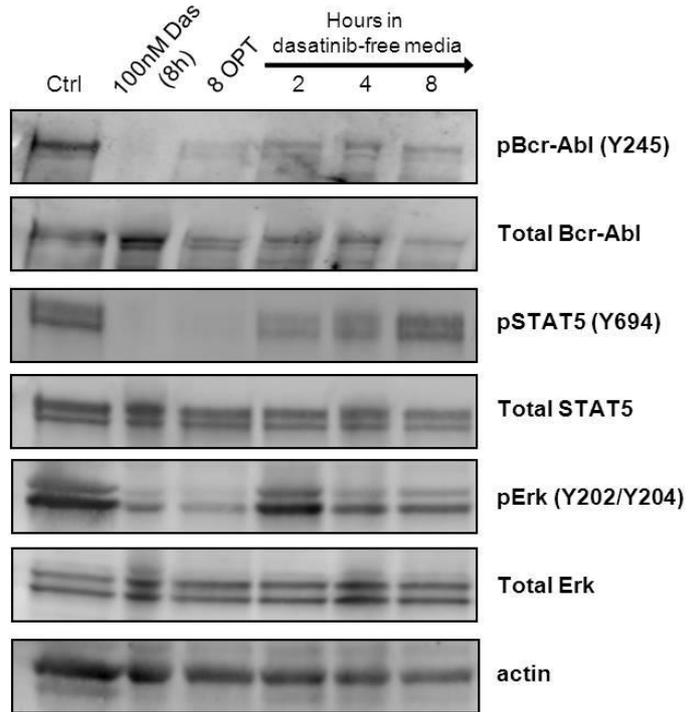
**Figure 5.8: Extension of imatinib exposure prior to OPT washout induces cell death and impairs clonogenic potential in CP-CML CD-34+ patient cells.**

*De novo* CP-CML CD34+ cells were treated with either continuously with 32.5  $\mu$ M imatinib (IM) for 72 h or transiently exposed to 32.5  $\mu$ M imatinib (IM) for 30 min-8 h followed either by standard washout (STD) or optimal washout (OPT) and culturing for 72 h in drug-free media. Cells were then analysed for **(A)** cell death by Annexin V/7-AAD staining at 72 h and **(B)** clonogenic potential by enumeration of CFUs following the CFU-GM assay at 2 weeks (n=3, mean + SEM). \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 compared to IM 30 OPT.

## **Chapter 5 | Critical factors involved in commitment of CML cells to apoptosis**

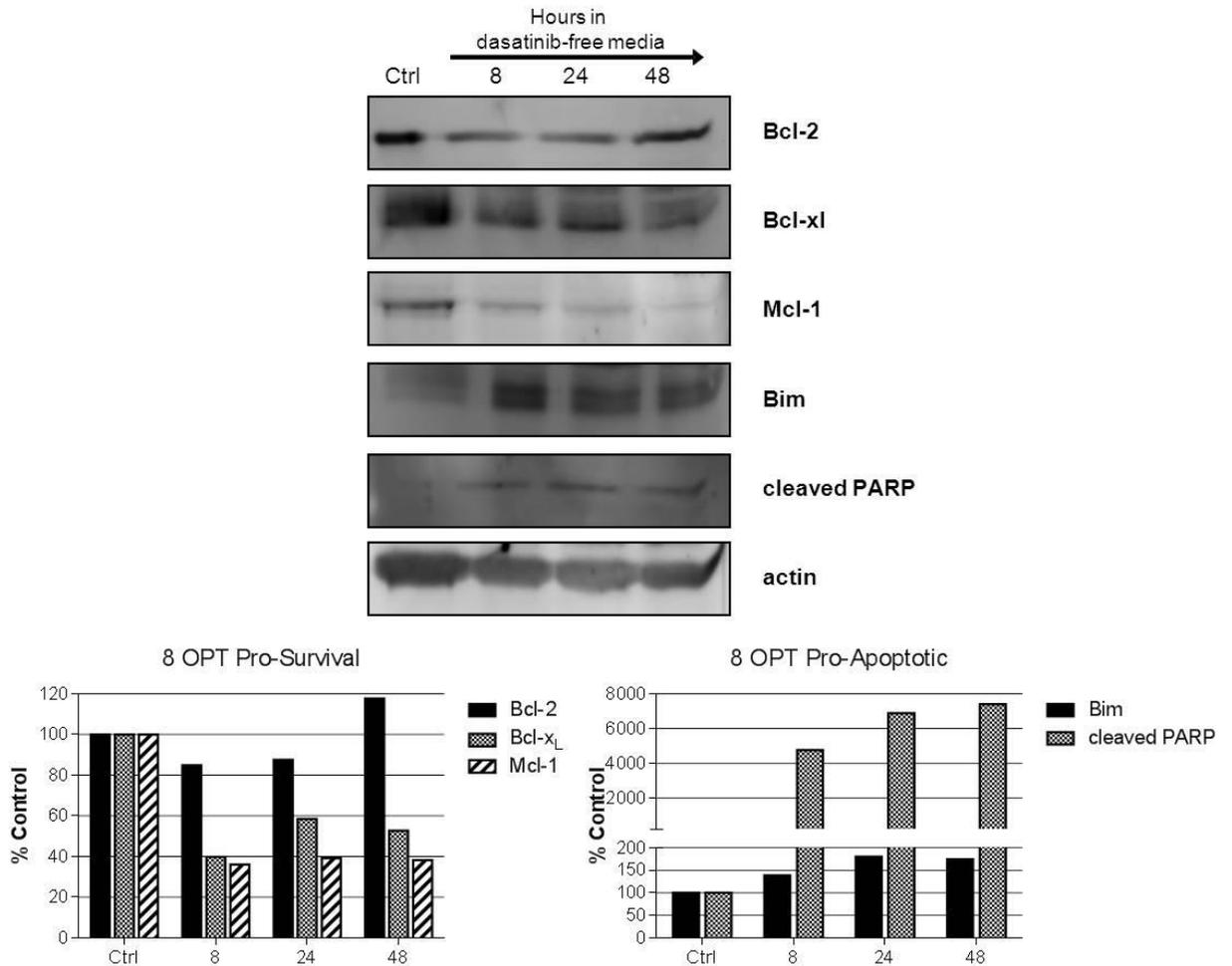
concomitant inhibition of STAT5 and Erk phosphorylation (Figure 5.9). These results are in contrast to the 30 min dasatinib exposure where Bcr-Abl activity was evident immediately after the OPT wash (Figure 5.3). This extended treatment induced a pro-apoptotic state as indicated by loss of the STAT5 targets Mcl-1 and Bcl-x<sub>L</sub>, but not Bcl-2 expression (associated with the Ras/Raf/MEK/Erk pathway) (Figure 5.10), indicating the loss of STAT5 phosphorylation as an important factor. Induction of apoptosis markers Bim and cleaved PARP were also observed (Figure 5.10).

To validate that the maintenance of STAT5 inhibition following exposure of greater than 30 min to dasatinib prior to the OPT wash occurs in primary cells, pSTAT5 was measured by flow cytometry in CD34<sup>+</sup> cells from *de novo* CP-CML patients (Figure 5.11). In these primary leukaemic cells, STAT5 is constitutively activated by Bcr-Abl. The percentage of pSTAT5 positive cells significantly decreased from 27.4% in untreated samples, to 6.6% in cells treated with 100 nM dasatinib for 30 min without washout ( $p=0.007$ ). In cells treated for 30 min with 100 nM dasatinib followed by the STD wash, pSTAT5 was significantly inhibited (9.7%,  $p=0.02$ ), however upon OPT wash pSTAT5 was restored (27.7%) (Figure 5.11). In contrast, increasing the initial dasatinib exposure to 4 h (18% pSTAT5,  $p=0.01$ ) or 8 h (3.5% pSTAT5,  $p=0.002$ ) demonstrated continued inhibition of STAT5 phosphorylation despite OPT wash (Figure 5.11).



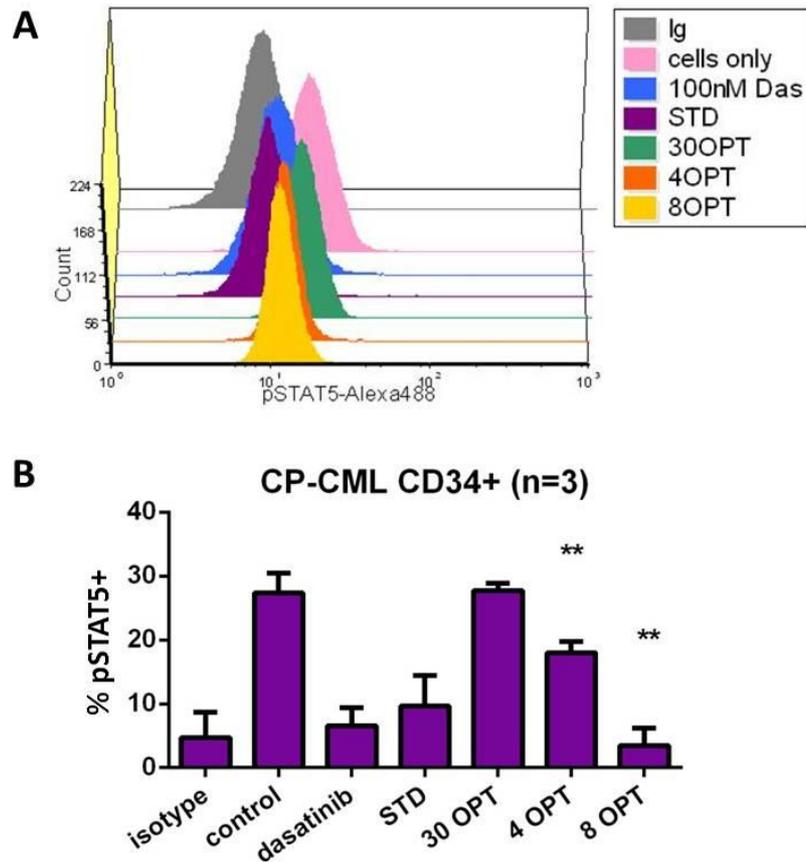
**Figure 5.9: Extension of Bcr-Abl inhibition results in the inhibition of pSTAT5 and pErk prior to OPT washout.**

KU812 cells were transiently exposed to 100 nM dasatinib (Das) for 8 h followed by optimal (OPT) wash and culturing for 0-8 h in dasatinib-free media. Lysates were analysed by western blot for Bcr-Abl-related signalling. Data is representative of three independent experiments. Quantitation of phosphorylated protein normalised to total protein and then actin (loading control). Percentage is relative to control (Ctrl). Graph is representative of western blots presented here.



**Figure 5.10: Extension of Bcr-Abl inhibition induces apoptosis markers and inhibits Mcl-1 and Bcl-xL.**

KU812 cells were transiently exposed to 100 nM dasatinib (Das) for 8 h followed by optimal (OPT) wash, then cultured for 0-48 h in dasatinib-free media. Lysates were analysed by western blot for apoptotic signalling proteins. Data is representative of three independent experiments. Quantitation of protein normalised to actin and percentage is relative to control (Ctrl). Graph is representative of western blots presented here.



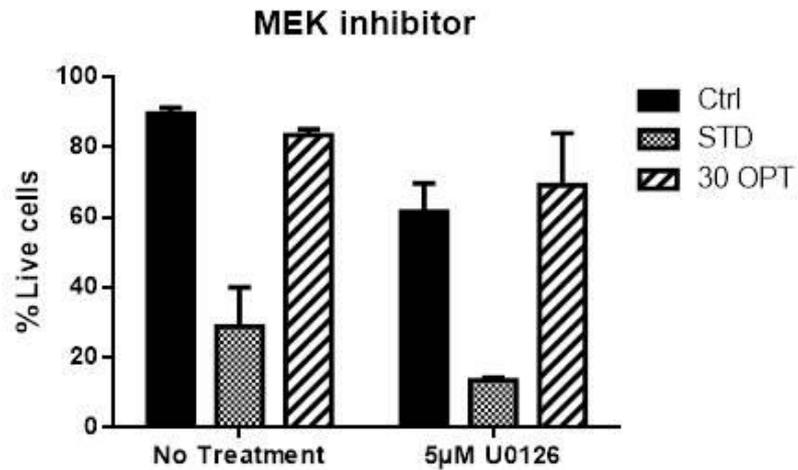
**Figure 5.11: Extension of Bcr-Abl inhibition prior to OPT washout inhibits pSTAT5.**

*De novo* CP-CML CD34+ patient cells were incubated either continuously with 100 nM dasatinib for 72 h or transiently exposed to 100 nM dasatinib for 30 min-8 h followed either by standard washout (STD) or optimal washout (OPT) and culturing for 72 h in drug-free media. Cells were then analysed for pSTAT5 (pY694) by intracellular phosphoflow. **(A)** Histogram of representative data. **(B)** Percentage of cells in pSTAT5 positive gate (n=3, mean + SEM). \*\* p<0.01 compared to 30 OPT. Ig denotes isotype control.

**5.2.5 Inhibition of JAK1/2, MEK/ERK or autophagy survival signally does not restore cell death following 30 min exposure to 100 nM dasatinib with OPT washout.**

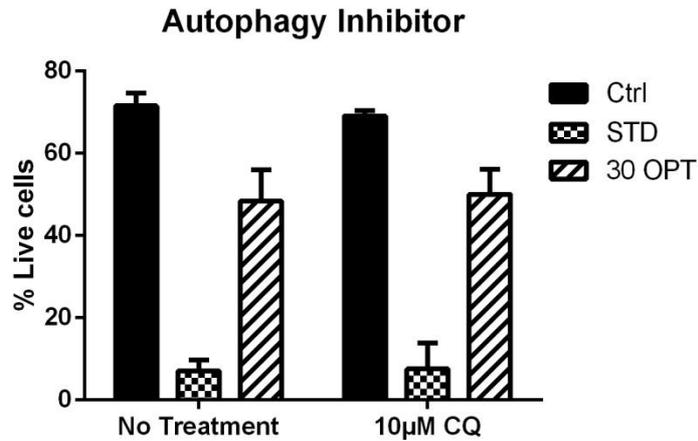
The observation that prolonged Bcr-Abl inhibition correlates with cell death lead us to hypothesise that application of a treatment which targets known CML resistance signalling, in combination with TKI treatment, may enhance cell death despite complete removal of TKI. As inhibition of Erk phosphorylation was observed following the STD wash, but recovered following the OPT wash, the ability of the MEK inhibitor U0126 to restore induction of cell death in the OPT wash setting was assessed. Surprisingly, no additional effect of MEK/Erk inhibition on commitment to cell death was observed following the 30 min 100 nM dasatinib OPT wash in combination with U0126 (69.2% viable cells) compared to U0126 alone (61.1% viable,  $p=0.73$ ) or dasatinib 30 min OPT wash (83.4% viable,  $p=0.39$ ) (Figure 5.11). Recent evidence suggests that autophagy protects *BCR-ABL1+* cells from TKI-induced cell death and induction of death can be restored by blocking autophagy with chloroquine.<sup>185</sup> Therefore the involvement of TKI-induced autophagy was assessed for a role in the protection of *BCR-ABL1+* cells from cell death. There was no significant effect on cell viability from the addition of chloroquine to the 30 min 100 nM dasatinib OPT treatment (69.1% viable) compared to treatment with chloroquine alone (48.4%,  $p=0.1$ ) or dasatinib 30 min OPT wash (50.1% viable,  $p=0.88$ ) (Figure 5.13).

The mechanism of STAT5 activation by Bcr-Abl is unclear, but it has been reported that JAK2 is involved in the activation of STAT5.<sup>148</sup> Intriguingly, combination of ruxolitinib (a pan JAK inhibitor) with 30 min 100 nM dasatinib OPT wash (77.6% viable cells) was



**Figure 5.12: MEK/Erk inhibition had no effect on cell viability when used in combination with 30 min 100 nM dasatinib exposure followed by OPT wash in KU812 cells.**

KU812 cells were transiently exposed to 100 nM dasatinib (Das) followed by either standard wash (STD) or optimal wash (OPT) in the presence or absence of 5µM U0126 and then cultured for 72 h in Das-free media with or without U0126. Cells were then analysed by Annexin V/7-AAD staining (n=3, mean + SEM).



**Figure 5.13: Inhibition of autophagy had no effect on cell viability when used in combination with 30 min 100 nM dasatinib exposure followed by OPT wash in KU812 cells.**

KU812 cells were transiently exposed to 100 nM dasatinib (Das) followed by either standard wash(STD) or optimal wash(OPT) in the presence or absence of 10 µM of the autophagy inhibitor chloroquine (CQ) and then cultured for 72h in dasatinib-free media with or without CQ. Cells were then analysed by Annexin V/7-AAD staining (n=3, mean + SEM).



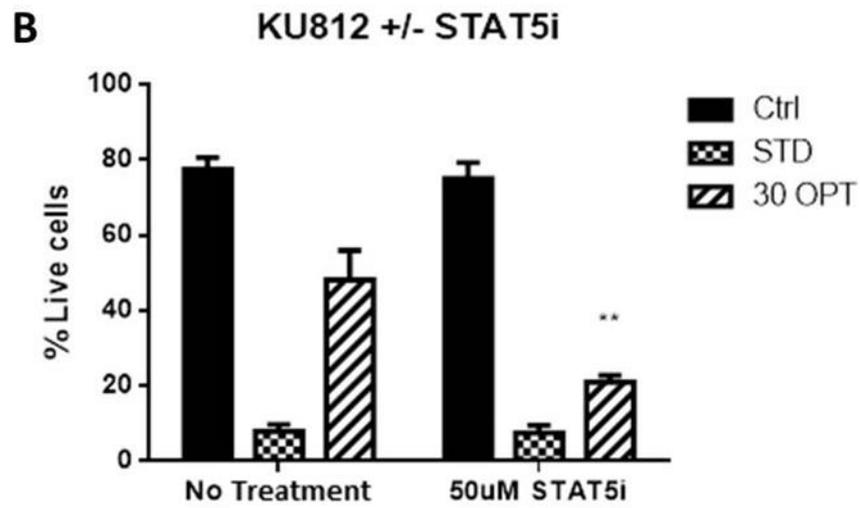
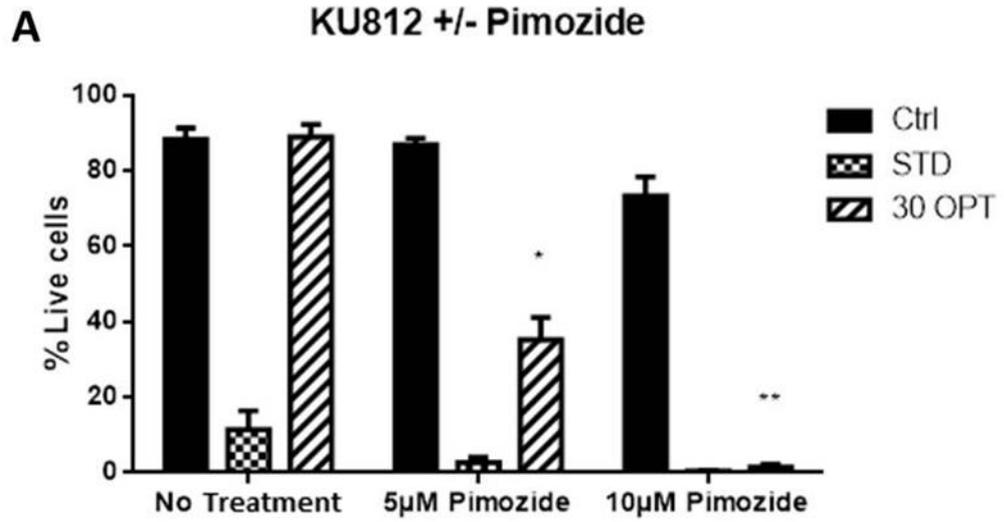
**Figure 5.14: JAK1/2 inhibition had no effect on cell viability when used in combination with 30 min 100 nM dasatinib exposure followed by OPT wash in KU812 cells.**

KU812 cells were transiently exposed to 100nM dasatinib (Das) followed by either standard wash (STD) or optimal wash(OPT) in the presence or absence of 1 µM of the JAK1/2 inhibitor ruxolitinib and then cultured for 72 h in dasatinib-free media with or without ruxolitinib (n=3, mean + SEM). Cells were then analysed by Annexin V/7-AAD staining (n=3, mean + SEM).

unable to induce a further reduction in live cells compared to treatment with the pan JAK inhibitor ruxolitinib alone in KU812 cells (71%,  $p=0.57$ ) or 30 min dasatinib OPT wash (72.3% viable,  $p=0.92$ ) (Figure 5.14).

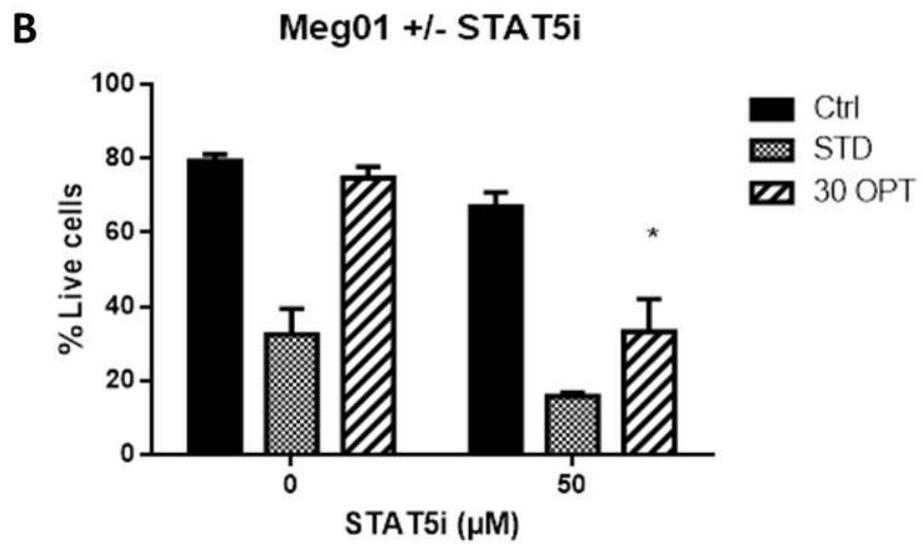
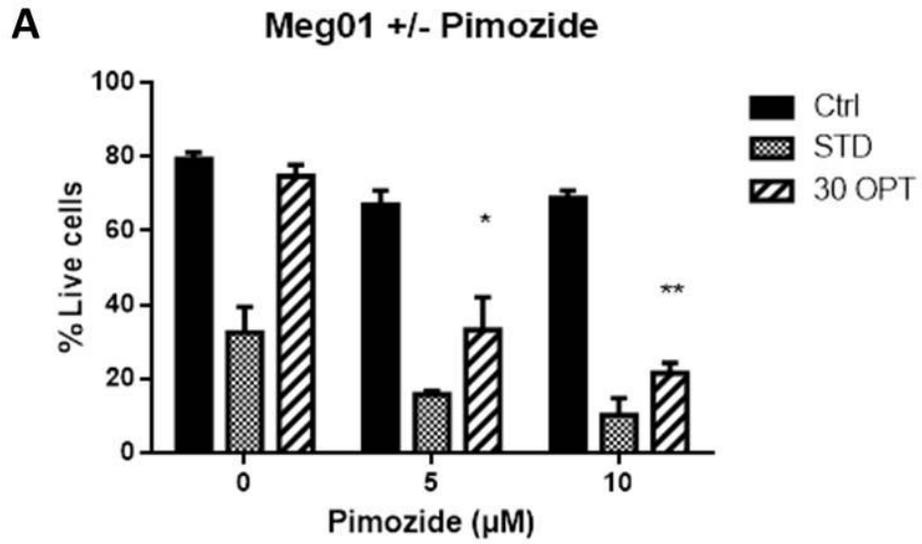
### **5.2.6 Inhibition of STAT5 activation with pimozide and STAT5i maintains cell death following 100 nM dasatinib with 30 min optimal washout in *BCR-ABL1+* cell lines**

To assess whether the loss of pSTAT5 was simply a surrogate for subtle inhibition of Bcr-Abl activity, or if it was itself a critical element of Bcr-Abl survival signalling, inhibitors which directly target STAT5 were utilised. STAT5 inhibition with pimozide (Figure 5.15A), and the specific STAT5 inhibitor N'-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide (from now on referred to as STAT5i) (Figure 5.15B) had little effect on viability. However continuous inhibition of STAT5 with either pimozide or STAT5i in combination with 30 min exposure to 100 nM dasatinib (with dasatinib completely removed by the OPT wash) effectively induced cell death in KU812 cells (2.3% and 21% viable cells respectively) (Figure 5.15). This finding was further evidenced by similar observation in Meg01 cells (24.4% and 29.8% viable cells) (Figure 5.16) and K562 cells (16.6% and 26.1% viable cells) (Figure 5.17) when 30 min 100 nM dasatinib OPT wash was combined with either pimozide or STAT5i.



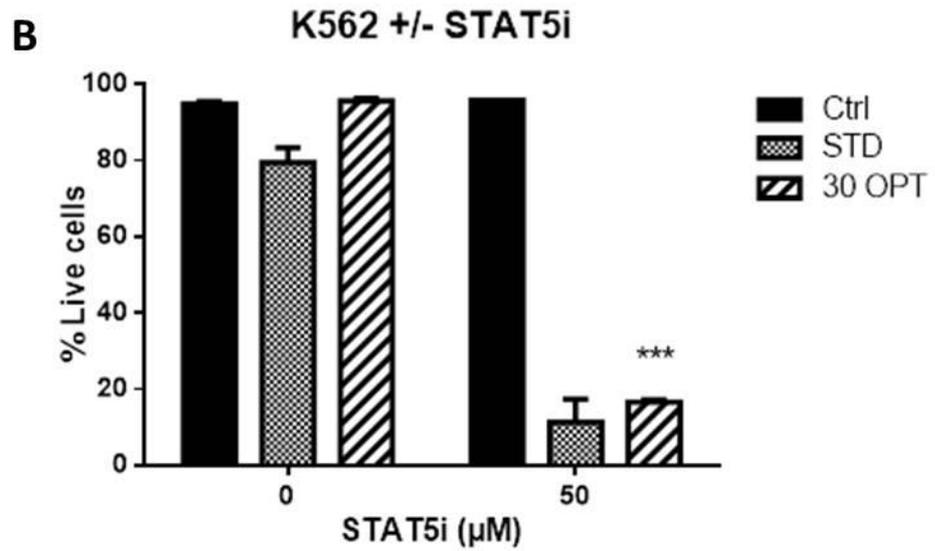
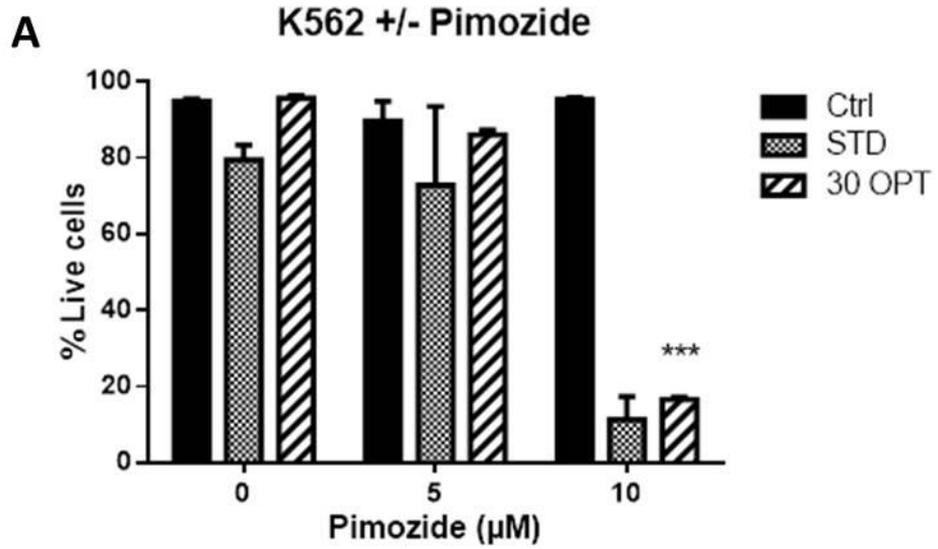
**Figure 5.15: Combination of STAT5 inhibition, with 30 min 100 nM dasatinib OPT wash, induces cell death in KU812 cells.**

Pimozide and STAT5i induce cell death following 100 nM dasatinib OPT wash. KU812 cells were transiently exposed to 100 nM Das followed by either STD wash or OPT wash in the presence or absence of STAT5 inhibitors and then cultured for 72 h in Das-free media with or without 5 $\mu$ M or 10 $\mu$ M of the STAT5 inhibitor pimozide **(A)** or 50 $\mu$ M of the STAT5 inhibitor N'-((4-Oxo-4H-chromen-3-yl)methylene) nicotinothiazide (STAT5i) **(B)**. Cells were analysed by Annexin V/7-AAD staining (n=3, mean + SEM). \* p<0.05 and \*\* p<0.01 compared to 30 OPT wash alone.



**Figure 5.16: Combination of STAT5 inhibition, with 30 min 100 nM dasatinib OPT wash induces cell death in Meg01 cells.**

Pimozide and STAT5i induce cell death following the OPT wash. Meg01 cells were incubated transiently exposed to 100nM Das followed by either STD wash or OPT wash in the presence or absence of inhibitors and then cultured for 72h in Das-free media with or without 5 $\mu$ M or 10 $\mu$ M of pimozide **(A)** and 50 $\mu$ M of STAT5i **(B)**. Cells were then analysed by Annexin V/7-AAD staining (n=3, mean + SEM). \* p<0.05 and \*\* p<0.01 compared to 30 OPT wash alone.



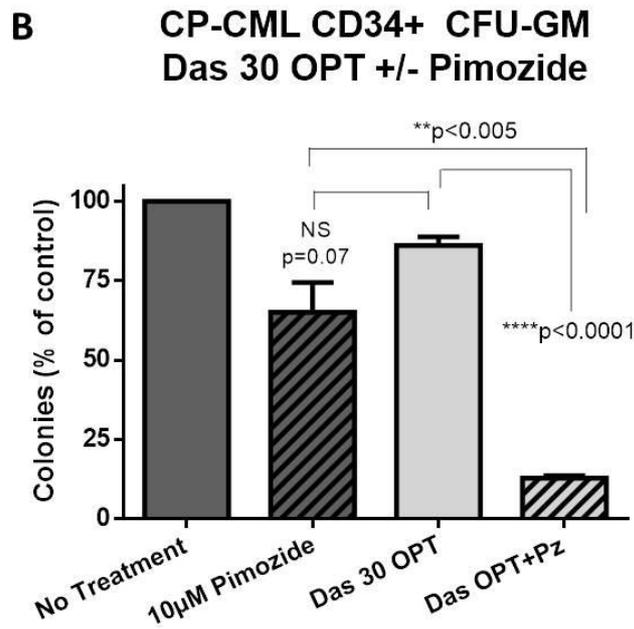
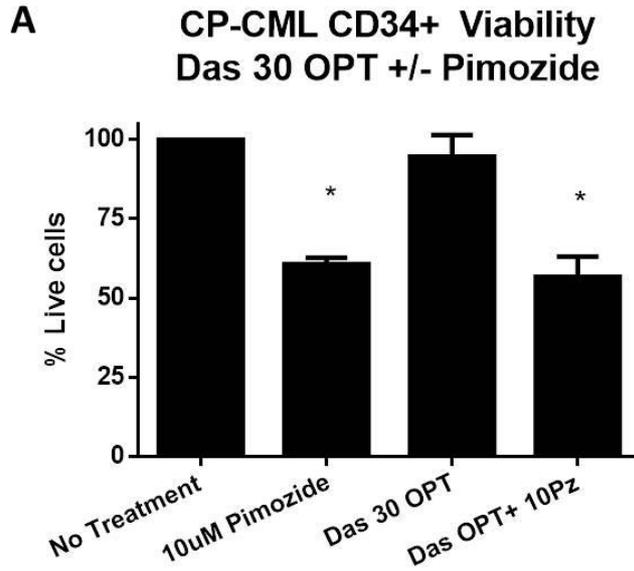
**Figure 5.17: Combination of STAT5 inhibition, with 30 min 100 nM dasatinib OPT wash induces cell death in K562 cells.**

Pimozide and STAT5i induce cell death following the OPT wash. K562 cells were incubated transiently exposed to 100nM Das followed by either STD wash or OPT wash in the presence or absence of inhibitors and then cultured for 72h in Das-free media with or without 5 $\mu$ M or 10 $\mu$ M of pimozide (A) and 50 $\mu$ M of STAT5i(B). Cells were then analysed by Annexin V/7-AAD staining (n=3, mean + SEM). \*\*\* p<0.0001 compared to 30 OPT wash alone.

**5.2.7 Inhibition of STAT5 activation with pimozone and STAT5i maintains cell death and impair colony formation following 30 min 100 nM dasatinib OPT wash in CP-CML CD34+ patient cells**

To determine the clinical relevance of these observations, the effect of STAT5 inhibition on the viability of *de novo* CP-CML CD34+ patient cells was determined. Interestingly, although addition of 10  $\mu$ M pimozone to 100 nM dasatinib 30 OPT wash (56.8% viable,  $p=0.018$ ) significantly reduced cell viability compared to the untreated control (89.6% viable), contrary to observations in cell lines, pimozone alone also reduced cell viability (61.6% viable) (Figure 5.18A). Furthermore, assessment of the ability to form colony forming units (CFUs) demonstrated that the addition of pimozone (12.8% CFUs,  $p<0.0001$ ) (Figure 5.18B) significantly reduced colony formation in comparison to the 100 nM dasatinib 30 OPT treatment alone (86.1% CFUs compared to untreated control).

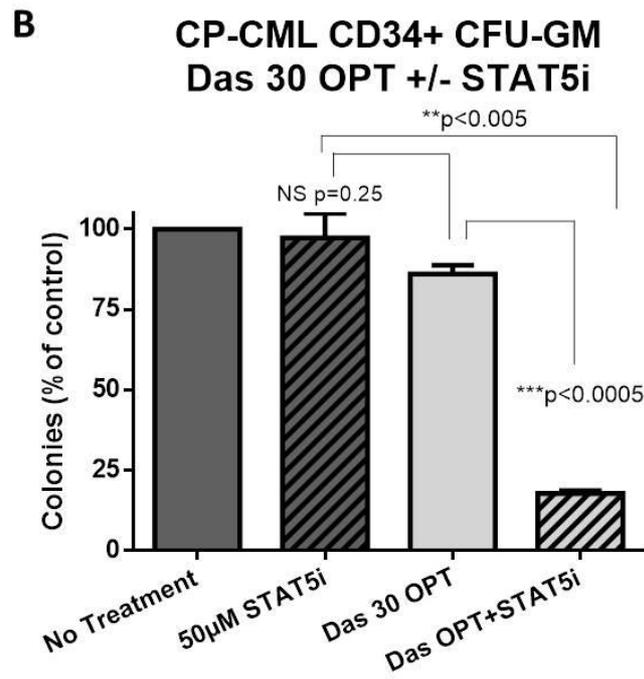
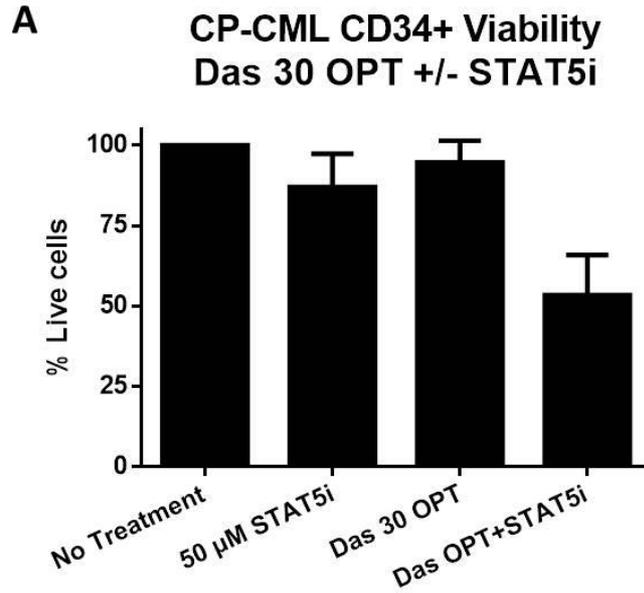
Similarly, combination of STAT5i with 100 nM dasatinib 30 OPT wash resulted in a significant reduction in live cells (53.5%,  $p=0.009$ ) compared to cells treated with 100 nM dasatinib 30 OPT wash in the absence of STAT5i (89.6% viable) (Figure 5.19A). However, unlike treatment with pimozone, the use of STAT5i as a single agent had little effect on viability (87.1% viable). Sequential assessment of clonogenic potential demonstrated that the addition of STAT5i to 100 nM dasatinib 30 OPT wash (29.5% CFUs,  $p<0.01$ ) significantly reduced colony formation in comparison to the 100 nM dasatinib 30 OPT wash treatment alone (86.1% CFUs compared to untreated control) whilst demonstrating minimal impairment alone (97.2% CFUs) (Figure 5.19B).



**Figure 5.18: STAT5 inhibition with pimozone and 30 min dasatinib 30 min OPT wash exposure induces cell death in CP-CML CD34+ patient cells.**

Pimozone induced cell death following the OPT wash in primary CP-CML CD34+ patient cells. *De novo* CP-CML CD34+ patient cells were treated with 100 nM dasatinib for 30 min, followed by OPT wash in the presence or absence of the STAT5 inhibitor pimozone (Pz) and then cultured for 72 h in dasatinib-free media containing STAT5 inhibitors.

Cells were then analysed for **(A)** viability by Annexin V/7-AAD staining and for **(B)** clonogenic potential by the CFU-GM assay at 2 weeks (right, n=3, mean + SEM). \* p<0.05, \*\* p<0.01 and \*\*\*\* p<0.0001 compared to 30 OPT wash alone.



**Figure 5.19: STAT5i in combination with 30 min 100 nM dasatinib OPT wash induces cell death in CP CD34+ CML cells.**

STAT5i induced cell death following the OPT wash in primary CML cells. *De novo* CP-CML CD34+ patient cells were treated with 100 nM dasatinib (Das) for 30 min, followed by optimal (OPT) wash in the presence or absence of the STAT5 inhibitor STAT5i and then cultured for 72 h in dasatinib-free media containing that STAT5 inhibitor. Cells were then analysed for **(A)** viability by Annexin V/7-AAD staining and for **(B)** clonogenic potential by the CFU-GM assay at 2 weeks (n=3, mean + SEM). \* p<0.05, \*\* p<0.01 and \*\*\* p<0.001 compared to 30 OPT wash alone.\*

## **Chapter 5 | Critical factors involved in commitment of CML cells to apoptosis**

This reduction in cell viability and colony forming ability was not dasatinib specific, as 30 min 32.5  $\mu$ M imatinib OPT wash alone (93.9% viable and 85.7% CFUs) was significantly reduced when treated in combination with pimozone (38.1% viable,  $p=0.01$  and 8.9% CFUs,  $p<0.001$ ) (Figure 5.20) or STAT5i (47.2% viable,  $p=0.008$  and 16.9% CFUs,  $p<0.005$ ) (Figure 5.21). Pimozone alone also induced a reduction in colonies (56.4% CFUs compared to untreated control) (Figure 5.18), however this may be attributed to off-target effects of pimozone, as STAT5i alone had little effect on the CFU-GM (97.2% CFUs compared to untreated control,  $p=0.24$ ) (Figure 5.19).

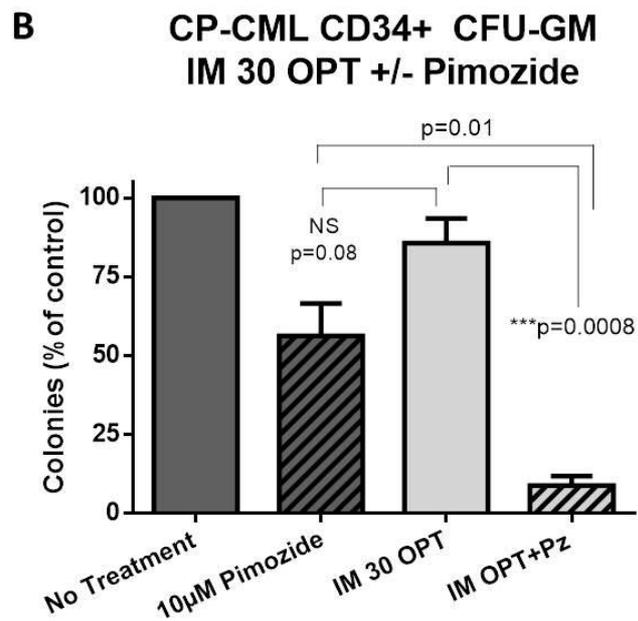
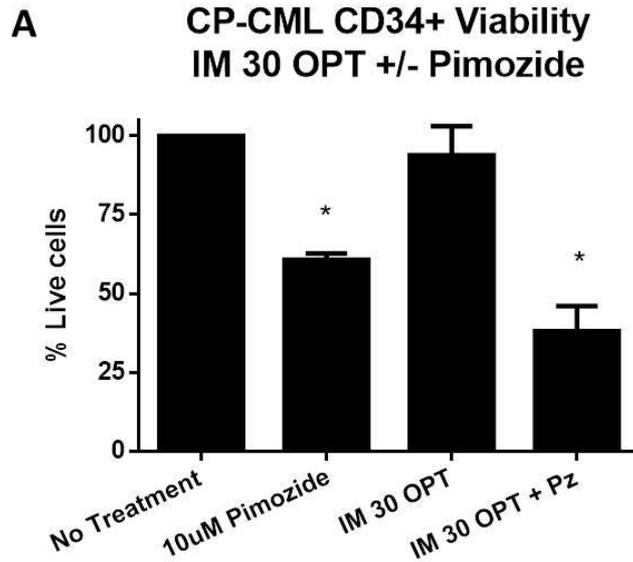
### **5.2.8 Following 30 min exposure to 100 nM dasatinib and pimozone, STAT5 remains inhibited despite complete removal of dasatinib**

Analysis of Bcr-Abl-related and apoptotic signalling was assessed following a 30 min exposure to 100 nM dasatinib plus OPT wash in combination with STAT5 inhibition by pimozone. Initial inhibition of Bcr-Abl and Erk phosphorylation, was completely restored 2 h post the removal of dasatinib (Figure 5.22). However, sustained inhibition of STAT5 phosphorylation was observed following the OPT wash which confirmed efficient blockade of STAT5 activation by pimozone. Furthermore, increased expression of Bim and cleaved PARP were observed indicating the induction of apoptosis (Figure 5.22). This was accompanied by a decrease in the STAT5 partners Bcl-x<sub>L</sub> and Mcl-1, but not Bcl-2 signalling, revealing STAT5 as an important player in commitment to cell death in this setting (Figure 5.23).

In CP-CML CD34<sup>+</sup> patient cells, treatment with 10  $\mu$ M pimozone alone caused a decrease in pSTAT5 positive cells (10.3%) compared with untreated cells (27.4%) (Figure 5.24).

## **Chapter 5 | Critical factors involved in commitment of CML cells to apoptosis**

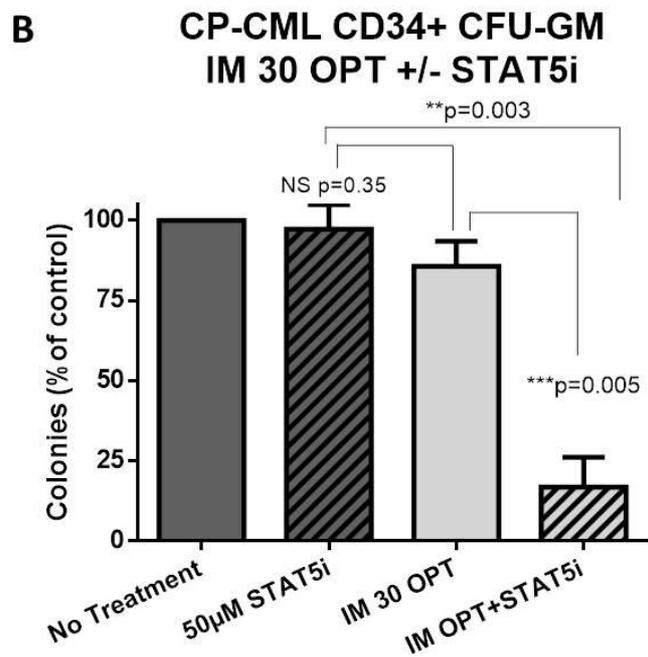
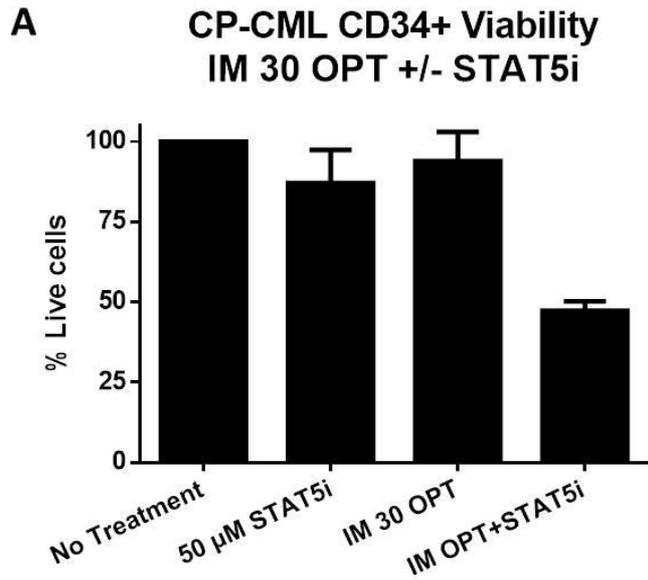
In cells treated with a combination of 10  $\mu$ M pimozide and 100 nM dasatinib for 30 min followed by the OPT wash, pSTAT5 remained inhibited (7.1%) compared to the dasatinib alone OPT wash treatment (21%). The combination of STAT5i with the 30 min 100 nM dasatinib OPT in CP-CML CD34+ patient cells, also resulted in sustained inhibition of pSTAT5 (Figure 5.24).



**Figure 5.20: Pimozide in combination with 30 min imatinib induces cell death following the OPT wash.**

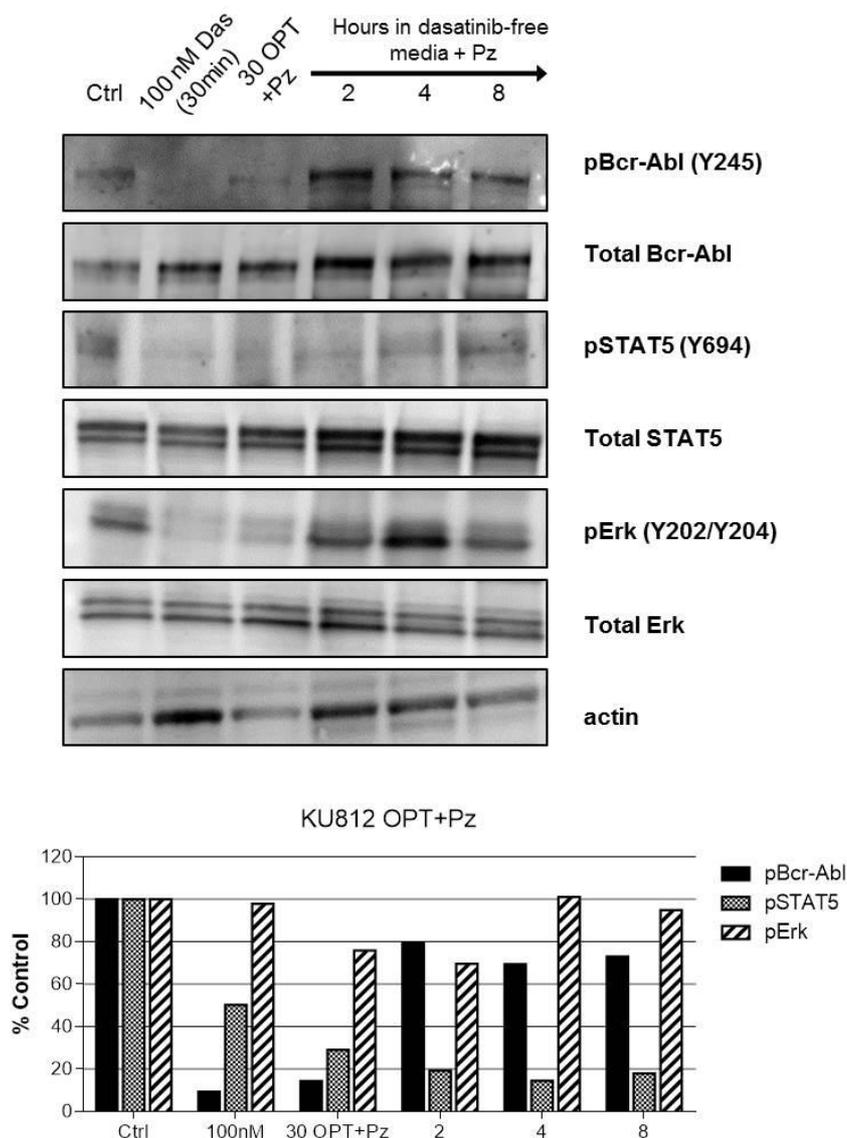
Pimozide and STAT5i induced cell death following the OPT wash in primary CML cells.

*De novo* CP-CML CD34+ cells were treated with 32.5  $\mu$ M imatinib (IM) for 30 min, followed by optimal (OPT) wash in the presence or absence of the STAT5 inhibitor pimozide and then cultured for 72 h in dasatinib-free media containing that STAT5 inhibitor. Cells were then analysed for (A) viability by Annexin V/7-AAD staining and for (B) clonogenic potential by the CFU-GM assay at 2 weeks (n=3, mean + SEM). \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  compared to 30 OPT wash alone.



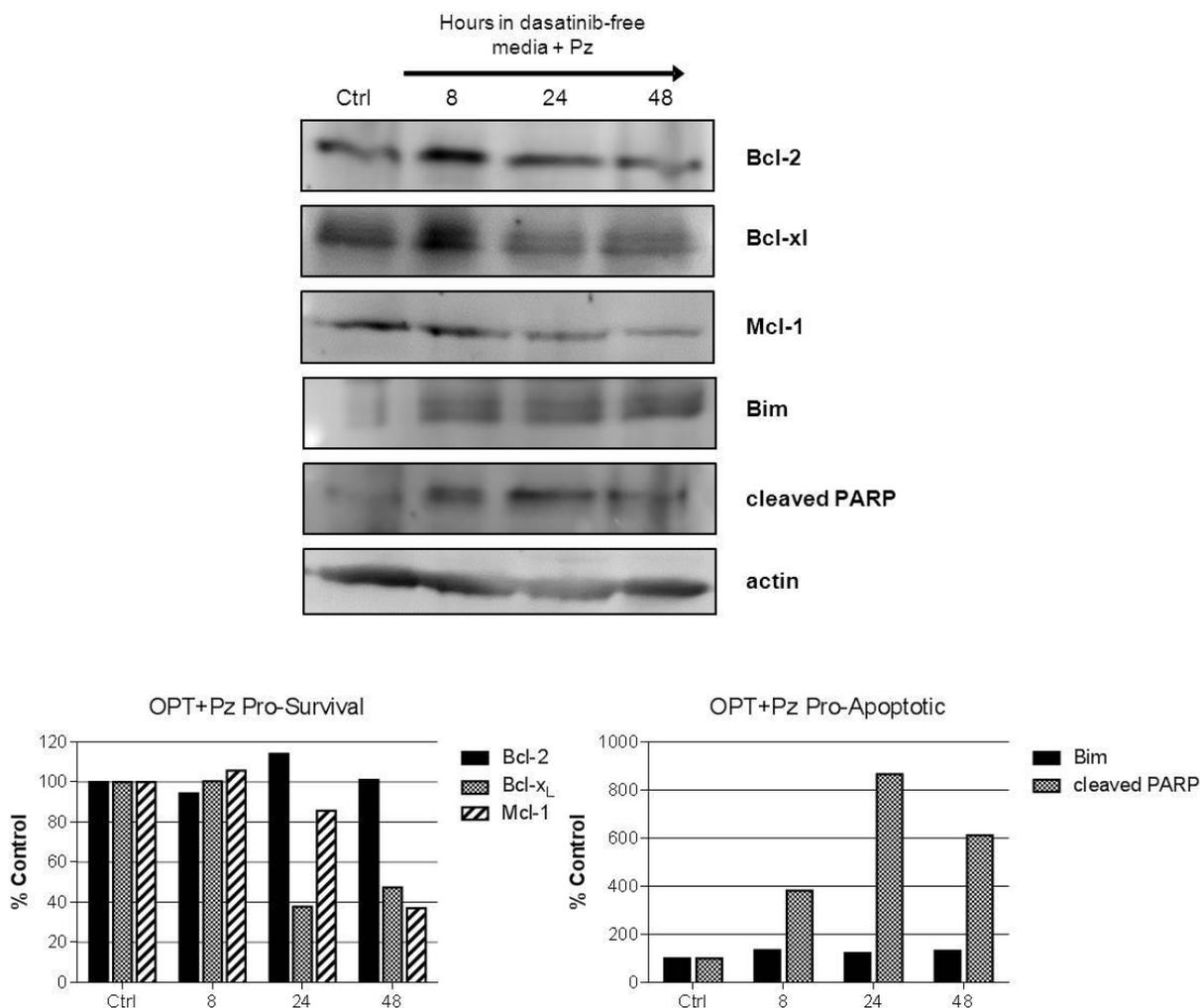
**Figure 5.21: STAT5i in combination with 30 min imatinib induces cell death following the OPT wash.**

STAT5i induced cell death following the OPT wash in primary CML cells. *De novo* CP-CML CD34+ cells were treated with 32.5  $\mu$ M imatinib (IM) for 30 min, followed by optimal (OPT) wash in the presence or absence of the STAT5 inhibitor STAT5i and then cultured for 72 h in dasatinib-free media containing that STAT5 inhibitor. Cells were then analysed for (A) viability by Annexin V/7-AAD staining and for (B) clonogenic potential by the CFU-GM assay at 2 weeks (right, n=3, mean + SEM). \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*\*  $p < 0.0001$  compared to 30 OPT wash alone.\*



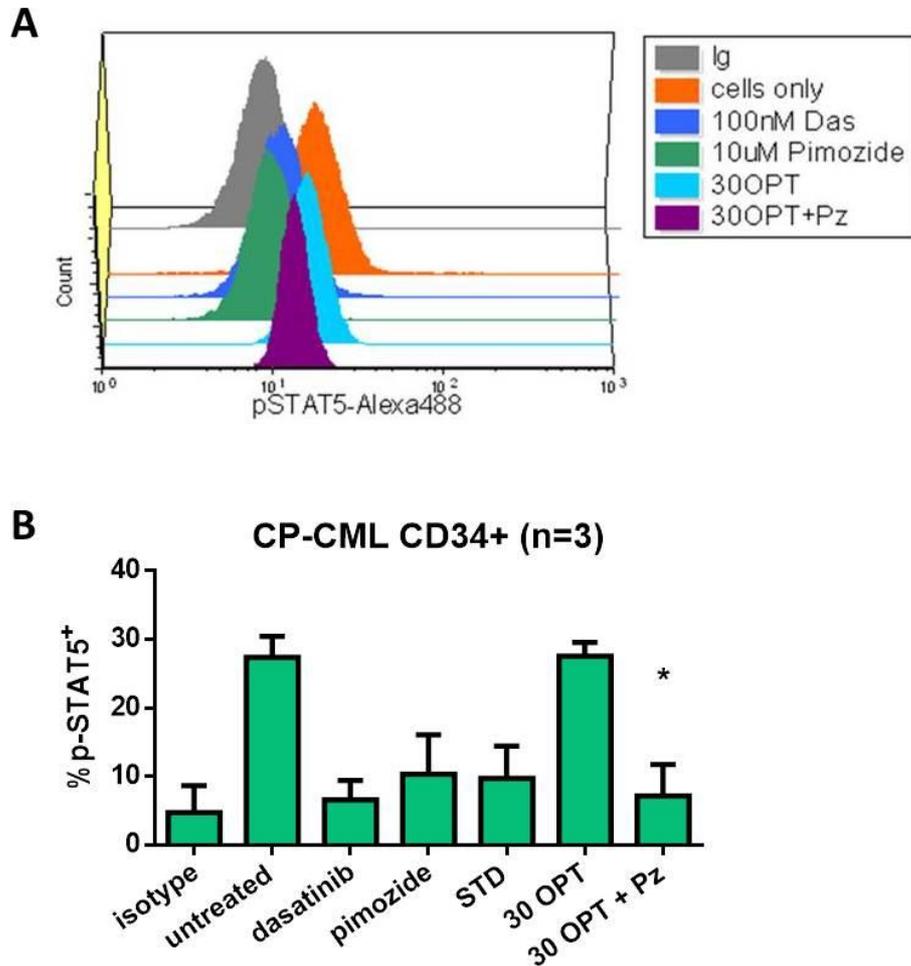
**Figure 5.22: Pimozide in combination with 30 min 100 nM dasatinib exposure inhibits pSTAT5.**

KU812 cells were transiently exposed to 100 nM dasatinib (Das) together with 10  $\mu$ M of the pimozide (Pz), followed by optimal (OPT) wash and then cultured for 0-8 h in dasatinib-free media containing pimozide. Lysates were analysed by western blotting for Bcr-Abl-related signalling. Data is representative of three independent experiments. Quantitation of protein phosphorylation normalised to total protein, then actin (loading control) and percentage is relative to control (Ctrl). Graph is representative of western blots presented here.



**Figure 5.23: Downstream targets of STAT5, Bcl-xL and Mcl-1, are inhibited in the presence of pimoziide.**

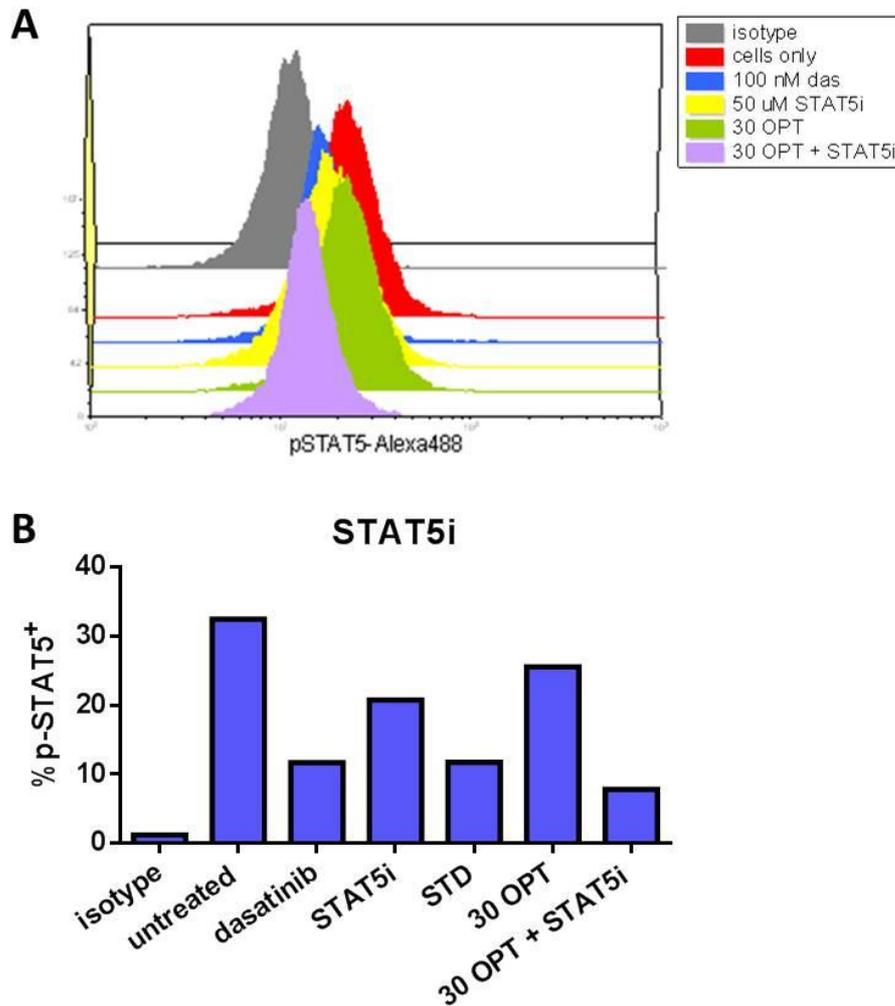
KU812 cells were transiently exposed to 100 nM dasatinib (Das) together with 10  $\mu$ M pimoziide (Pz), followed by optimal (OPT) wash and then cultured for 0-48 h in dasatinib-free media containing pimoziide. Lysates were analysed by western blotting for apoptosis signalling. Data is representative of three independent experiments. Quantitation of protein normalised to actin (loading control) and percentage is relative to control (Ctrl). Graph is representative of western blots presented here.



**Figure 5.24: Pimozide inhibits pSTAT5 in primary CP-CML CD34+ patient cells following complete removal of dasatinib.**

*De novo* CP-CML CD34+ patient cells were transiently exposed to 100 nM dasatinib (Das) together with 10  $\mu$ M pimozide (Pz), followed by either the standard (STD) or the optimal (OPT) wash and culturing in dasatinib-free media with or without pimozide. Cells were then analysed for pSTAT5 (pY694) by intracellular phosphoflow (n=3 mean + SEM).

\*p<0.01 compared to 30 OPT. Ig denotes isotype control.



**Figure 5.25: STAT5i inhibits pSTAT5 in primary CP-CML CD34+ patient cells following complete removal of dasatinib.**

*De novo* CP-CML CD34+ patient cells were transiently exposed to 100 nM dasatinib (Das) together with 50  $\mu$ M STAT5i, followed by either the standard (STD) or the optimal (OPT) wash and culturing in dasatinib-free media with or without STAT5i. Cells were then analysed for pSTAT5 (pY694) by intracellular phosphoflow (n=1).

### 5.3 Discussion

Bcr-Abl confers protection against apoptosis by harnessing cell survival pathways through proteins including STAT5<sup>238</sup> and Erk<sup>52</sup>, inducing expression of anti-apoptotic proteins to prevent apoptosis and hence promote survival.<sup>195,239,240</sup> *BCR-ABL1+* cells appear to be addicted to Bcr-Abl kinase activation for survival and therefore the targeting of *BCR-ABL1+* cells with Abl tyrosine kinase inhibitors (TKIs) commits these cells to death. Imatinib established the treatment paradigm that continuous TKI availability, and therefore continuous Bcr-Abl kinase inhibition, is required for induction of apoptosis in *BCR-ABL1+* cells.<sup>104,241</sup> However, studies of dasatinib therapy have challenged this as the accepted paradigm of continuous Bcr-Abl inhibition fails to account for the clinical efficacy of once-daily dasatinib therapy, which only transiently inhibits Bcr-Abl *in vivo* due to its short serum half-life.<sup>135,137</sup>

In the previous chapter, *in vitro* treatments with 100 nM dasatinib for 30 min followed by a standard drug washout demonstrated induction of cell death despite the reactivation of Bcr-Abl, which supports the concept that continuous Bcr-Abl kinase inhibition, is not required for optimal therapeutic response. However, recent reports demonstrate that low concentrations of TKI remain in cells following the standard washout (three consecutive washes) protocol following a 30 min exposure to high dose TKI; 100 nM dasatinib, 32.5  $\mu$ M imatinib or 1  $\mu$ M nilotinib.<sup>227,232,234</sup> The observations of Simara *et al.*<sup>227</sup> indicated that following the standard washout protocol for removal of 100 nM dasatinib, a residual amount of dasatinib remains that is similar to 1 nM continuous dasatinib for 72 h. As established in the previous chapter, sensitive inhibition of STAT5 and Erk signalling occurs during continuous 1 nM dasatinib treatment (Figure 4.11) which was comparable to the signalling observed following 30

## **Chapter 5 | Critical factors involved in commitment of CML cells to apoptosis**

min treatment with 100 nM dasatinib and the standard (STD) wash treatment (Figure 4.9). These results support the findings of others that low levels of dasatinib remain in cells following the standard washout procedure and that the low level inhibition of Bcr-Abl that this affords, is sufficient to induce apoptosis, despite substantial reactivation of Bcr-Abl signalling.

Lipka and colleagues<sup>232,234</sup> demonstrated that the removal of residual dasatinib using an optimal drug washout procedure (OPT wash) prevents induction of apoptosis. Using a similar washout procedure (in which cells are allowed to equilibrate in TKI-free media between washes), this investigation demonstrates that Bcr-Abl, STAT5 and Erk signalling are restored following efficient removal of 100 nM dasatinib (Figure 5.3), suggesting that no residual dasatinib remained in the cells to induce apoptotic signals (Figure 5.4). Pharmacokinetic studies suggest that a significant amount of dasatinib still remains in patient serum 4 h and 8 h after taking dasatinib, doses equating to approximately 100 ng/ml and 50 ng/ml respectively,<sup>242</sup> but that Bcr-Abl activation is inhibited for at least 7 h post treatment.<sup>230</sup>

Consequently, cellular signalling following longer exposures to dasatinib were interrogated. For the first time, this study demonstrates that commitment of *BCR-ABL1+* cells to death requires at least 4 h exposure to 100 nM dasatinib in cultured cell lines (Figure 5.5) and 2 h exposure in CP-CML CD34+ patient cells (Figure 5.7). This is despite the complete removal of dasatinib and the reactivation of Bcr-Abl and Erk, but interestingly not STAT5. Induction of cell death following transient exposure to high dose (32.5  $\mu$ M) imatinib for greater than 2 h (Figure 5.6 and Figure 5.8) was also observed, indicating that this phenomenon is not TKI specific.

Importantly, this investigation has clearly established distinct phospho-signalling dynamics upon 30 min or 8 h transient high dose dasatinib exposure, correlating with the commitment to cell death, offering a rationale for the therapeutic success of once daily dasatinib in the clinical setting. Comparison of 30 min and 8 h phospho-signalling profiles highlights STAT5 and its downstream signalling partners, Bcl-x<sub>L</sub> and Mcl-1 as key determinants of whether cells ultimately survive or die (Figure 5.9 and Figure 5.10). We therefore sought to target signalling pathways associated with Bcr-Abl resistance to overcome the OPT wash-induced prevention of cell death.

Recent data highlights STAT5 as a critical participant in TKI resistance<sup>153,154</sup> and the clinically available dopamine reuptake inhibitor pimozide, has been demonstrated to specifically inhibit STAT5 activation in *BCR-ABL1*<sup>+</sup> cells resulting in increased sensitivity to TKIs.<sup>157,243</sup> Addition of the STAT5 inhibitors pimozide or STAT5i induced cell death in both *BCR-ABL1*<sup>+</sup> cultured cell lines as well as primitive CP-CML CD34<sup>+</sup> patient cells despite complete removal of dasatinib or imatinib after 30 min high dose exposures. STAT5i, which targets the SH2 domain of STAT5,<sup>12</sup> had little effect as a sole agent in cell lines or primary samples, however pimozide alone induced cell death and decreased colony formation in primary CD34<sup>+</sup> CP-CML cells (Figure 5.16). The difference in effects as sole agents may be attributed to non-specific inhibition of other targets, as pimozide is also thought to inhibit proliferation in breast cancer cells by blocking T-type calcium channels.<sup>12,155</sup> Importantly, both STAT5 inhibitors increased the sensitivity of *BCR-ABL1*<sup>+</sup> cells to dasatinib and imatinib, whilst combination with inhibitors of JAK1/2, MEK/Erk or autophagy had no additional effects on cell viability when compared to TKI alone treatments (Figure 5.11-14). Consequently, the Bcr-Abl-related and apoptotic

## **Chapter 5 | Critical factors involved in commitment of CML cells to apoptosis**

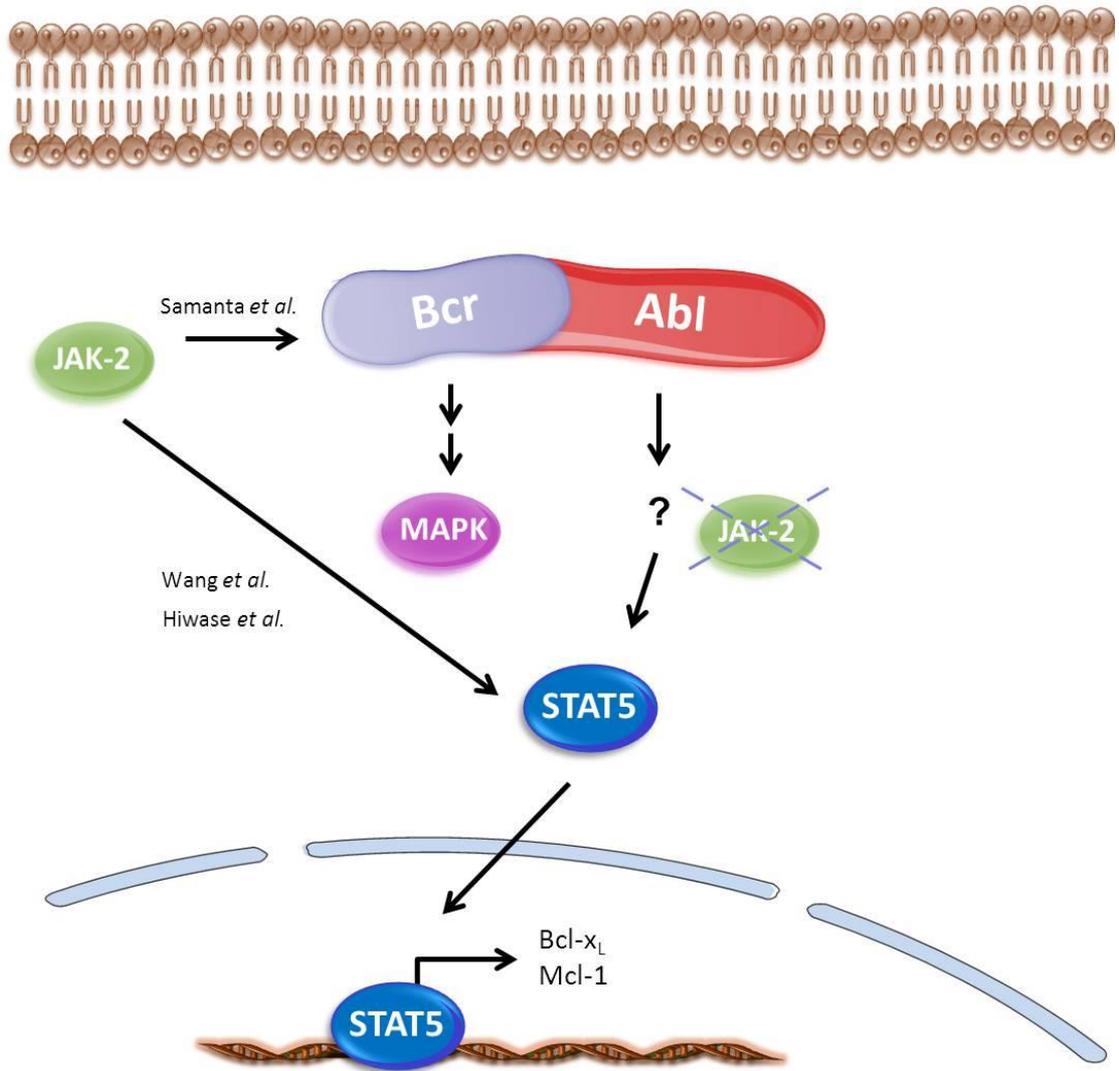
signalling profiles following the addition of pimozone to a 30 min transient treatment with 100 nM dasatinib were assessed and confirmed inhibition of STAT5, and subsequently Bcl-xL and Mcl-1 (Figure 5.22 and Figure 5.22), indicating the specific targeting of STAT5 to induce cell death. Importantly, induction of cell death was achieved despite reactivation of Bcr-Abl signalling, indicating that STAT5 inhibition is an important factor in commitment to cell death in addition to being a sensitive measure of residual TKI.

In the setting of a transient exposure to dasatinib (30 min OPT wash), which did not commit cells to cell death, surprisingly no benefit from the addition of the pan JAK inhibitor, ruxolitinib, in the induction of cell death was observed (Figure 5.14). Although there are reports that JAK2 may phosphorylate or complex with Bcr-Abl<sup>148,149,244</sup>, JAK2 inhibition alone had little effect on induction of cell death and appears to target extrinsic survival signalling (growth factor related) as opposed to Bcr-Abl-dependent signalling through STAT5.<sup>150</sup> Therefore, we propose that STAT5 activation as a result of active Bcr-Abl does not occur through JAK2, but instead is in close correlation to phosphorylation at the kinase site of Abl (see Figure 5.26).

This study demonstrates that continuous inhibition of Bcr-Abl activity is not required to induce cell death in *BCR-ABL1*<sup>+</sup> cells, rather the continuous inhibition of its downstream partner STAT5 is essential. Critically, this study convincingly establishes that additional inhibition of STAT5, and not JAK or MAPK inhibition, together with transient high dose dasatinib treatment, results in marked cell death (additional to the cell death noted from dasatinib treatment alone), in both cultured *BCR-ABL1*<sup>+</sup> cell lines as well as in primary

## **Chapter 5 | Critical factors involved in commitment of CML cells to apoptosis**

CP-CML CD34+ patient cells. These findings highlight STAT5 as a potential target for combination therapeutic approaches.



**Figure 5.26: Proposed activation of STAT5**

We propose that Bcr-Abl activation of STAT5 occurs independently of JAK2, despite previous observations that JAK2 may phosphorylate STAT5 and that cytokines activate STAT5 via JAK2.

## **CHAPTER 6.**

# **TARGETING CYTOKINE PROTECTION FROM TKI-INDUCED CELL DEATH WITH THE PAN JAK INHIBITOR RUXOLITINIB**

## 6.1 Introduction

Long term follow up of chronic phase CML (CP-CML) patients has demonstrated that imatinib does not eradicate leukaemic progenitor cells (LPCs) and that most patients experience persistent disease. Despite the complete cytogenetic response (CCyR) achieved in 87% of newly diagnosed CP-CML patients following treatment with imatinib for 60 months, only a minority of patients achieve the deeper response indicated by complete molecular response (CMR).<sup>103</sup> Moreover, discontinuation of imatinib in patients who had achieved a CMR for at least 2 years resulted in the relapse of 50% of patients within 6 months,<sup>158</sup> suggesting that the majority of the CP-CML patients are not cured by imatinib treatment and thus they are required to remain on imatinib therapy.

Recent studies have demonstrated the persistence of LPCs in patients who have achieved CCyR with imatinib treatment.<sup>245</sup> *In vitro* data has demonstrated that although imatinib and the more potent second generation tyrosine kinase inhibitors, nilotinib and dasatinib, have antiproliferative effects on CD34+ cells<sup>125,246</sup> and the more primitive CD34+38- LPCs,<sup>162</sup> the treatment did not result in the induction of apoptosis.<sup>247</sup> It had been postulated that LPCs are refractory to imatinib due to inadequate Bcr-Abl kinase inhibition within progenitors.<sup>162</sup> However, superior inhibition of Bcr-Abl kinase activity with the more potent second generation TKIs nilotinib and dasatinib was insufficient to increase the levels of apoptosis in quiescent CML progenitors.<sup>162,163</sup> Thus, clinical studies as well as *in vitro* studies demonstrate that early LPCs are refractory to TKI and may be responsible for long-term disease persistence and resistance to imatinib treatment. Therefore, it is likely that the resistance of these progenitor cells to TKIs is independent of Bcr-Abl kinase function.

Haematopoietic stem cells (HSCs), including LPCs, reside in the bone marrow niche, which provides a cytokine rich microenvironment for the maintenance of both normal and CML HSCs.<sup>9</sup> In a non-pathological setting, the cytokine dependent control of proliferation, differentiation and cell death is tightly regulated by negative feedback mechanisms (Figure 6.1). Of particular interest are the interleukin-3 (IL-3)/granulocyte-macrophage colony stimulating factor (GM-CSF)/IL-5 receptor family pathways, which activate downstream survival signalling through Janus kinase (Jak)/signal transducers and activators of transcription (STATs), the Ras-mitogen-activated protein kinase (MAPK) pathway and the phosphatidyl-inositol-3-kinase (PI3K) pathway.<sup>248</sup> It is therefore possible that the presence of cytokines in the bone marrow niche may provide protection of CML progenitor cells from the cytotoxic effects of TKI therapy.

Several studies have now demonstrated the capability of cytokines in providing protection from TKI-induced cell death. Initial studies with imatinib found that cell lines expressing Bcr-Abl (M07p210<sup>Bcr-Abl</sup> and 32Dp210<sup>Bcr-Abl</sup>), which are IL-3 responsive, are partially rescued from imatinib-induced cell death in the presence of IL-3.<sup>93,166</sup> Abnormal activation of the autocrine production of IL-3<sup>167,168,172</sup> and GM-CSF<sup>170,171</sup> appears to be associated with autonomous growth in cell lines and CD34+ CML progenitors. Dorsey *et al.*<sup>173</sup> also found that in Bcr-Abl-transformed cell lines (BaF3p210<sup>Bcr-Abl</sup> and 32Dp210<sup>Bcr-Abl</sup>) IL-3 protected cells from apoptosis, suggesting that in conjunction with autocrine secretion of IL-3, CD34+ progenitor cells may escape complete eradication by this mechanism.

Liu *et al.*<sup>249</sup> recently demonstrated that conditioned media from resistant DA1-3bp210<sup>Bcr-Abl</sup> cells, which over-express IL-3, induced TKI resistance in previously

sensitive (non-mutated) DA1-3bp210<sup>Bcr-Abl</sup> cells through the activation of STAT5 and MEK/ERK pathways. Inhibition of JAK2 or MEK1/2 abrogated the protective effect of the conditioned media from TKI-induced apoptosis. Jiang *et al.*<sup>168</sup> suggested that primitive CML cells become detectably more sensitive to TKI as they differentiate. This study demonstrated that autocrine secretion of IL-3 and G-CSF induced TKI resistance in CML CD34<sup>+</sup>/CD38<sup>-</sup> cells, even in the absence of exogenous growth factors. Whereas in the absence of exogenous growth factors, more mature CML cells (CD34<sup>+</sup>/CD38<sup>+</sup>) are more sensitive to imatinib. This variation in TKI sensitivity can be explained on the basis of IL-3 and G-CSF expression. The levels of G-CSF and IL-3 transcripts are both highest in the CML stem cells and then appear to be switched off when the cells begin to differentiate, resulting in increased sensitivity to TKI as the cells mature.<sup>168</sup>

Wang *et al.*<sup>171</sup> recently demonstrated that adaptive autocrine secretion of GM-CSF can initiate Bcr-Abl-independent survival signalling in the presence of imatinib and nilotinib. This resistance was mediated by the activation of the anti-apoptotic JAK2/STAT5 pathway. c-KIT has also been shown to circumvent Bcr-Abl inhibition and apoptosis by activation of survival signals.<sup>176,250</sup> Such data proposes that CML cells may not completely rely on Bcr-Abl for survival signalling.

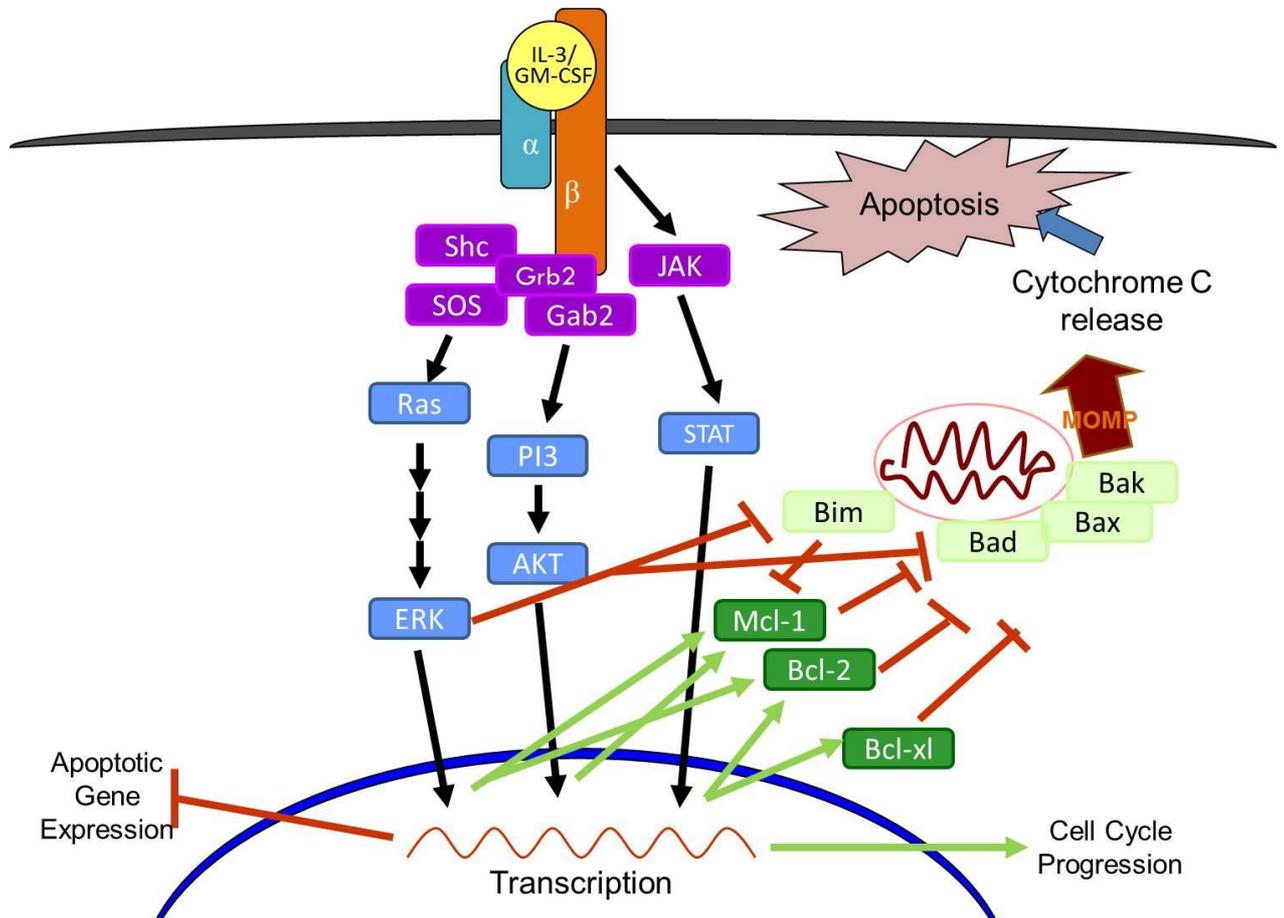
The IL-3 receptor  $\alpha$ -chain (IL-3R $\alpha$ ) has been described as a unique marker in the identification of acute myeloid leukaemia (AML) stem cells.<sup>177,178</sup> A recent murine study demonstrated that selectively targeting IL-3R $\alpha$  in AML with a specific monoclonal antibody results in reduced AML burden in BM and improved mouse survival.<sup>179</sup> The expression of IL-3R $\alpha$  on CML CD34<sup>+</sup>CD38<sup>-</sup> progenitor cells has subsequently been

observed in CML patients.<sup>180,251</sup> Collectively, this data suggests that IL-3 could be implicated in the protection of CML progenitors from cell death.

Inhibition of JAK2 with TG101209 and CYT387 was recently observed to induce little apoptosis in CML cells and appears to inhibit extrinsic cytokine-mediated survival signalling rather than Bcr-Abl-dependent signalling,<sup>150</sup> however there is some evidence to suggest that JAK2 interacts directly with Bcr-Abl<sup>148,149</sup>. Moreover, the absence of JAK2 did not affect leukaemia maintenance in a CML-like murine model<sup>151</sup>. The mechanism of STAT5 activation by Bcr-Abl is unclear, and it has been reported that JAK2 is involved<sup>148</sup>. Additionally, *in vivo* evidence suggests that JAK kinase signalling is only relevant in Bcr-Abl-independent, extrinsic activation of STAT5, as JAK2 inhibition had no effect on Bcr-Abl driven STAT5 signalling or cell death as a sole agent in the absence of cytokines<sup>150,151</sup>.

Ruxolitinib (formerly INCB018424), is a selective inhibitor of JAK kinases demonstrating potent inhibition of JAK1 (IC<sub>50</sub> ~3.3 nM) and JAK2 (IC<sub>50</sub> ~2.8 nM), with additional action against JAK3 (IC<sub>50</sub> ~428 nM) and Tyk2 (IC<sub>50</sub> ~19 nM).<sup>252</sup> Ruxolitinib has now been approved by the FDA for the treatment of myelofibrosis. Preclinical trials determined the ability of ruxolitinib to inhibit mutant JAK2<sup>V617F</sup> signalling through STAT3, STAT5 and Erk1/2, resulting in the inhibition of the proliferation and survival of BaF3<sup>EpoR-V617F</sup> and HEL cell lines, as well as primary MF patient cells.<sup>253</sup> Recent interest in targeting CML leukaemic progenitor cells has focused on inhibition of cytokine survival signalling, therefore this study evaluates for the first time the use of ruxolitinib in combination with TKI, in the induction of cell death in *BCR-ABL1*+ cells.

Here STAT5 activation is proposed to occur in close association to the kinase site of Abl as a result of active Bcr-Abl and not through JAK2. This project evaluates cytokine mediated persistence of LPCs during TKI treatment and the effectiveness of combining TKI treatment with JAK1 and JAK2 inhibition to eliminate the leukaemic progenitor population.



**Figure 6.1 Normal Haematopoietic Cytokine Signalling Pathways**

In a non-pathological setting, cytokine activation of IL-3/GM-CSF/IL-5 receptors results in activation of the Ras/MEK/Erk, PI3K/Akt and JAK/STAT pathways. This results in the inhibition of apoptosis and the activation of survival and proliferation signalling. Such signalling is tightly regulated by negative feedback mechanisms.

### 6.1.1 Approach

*BCR-ABL1*<sup>+</sup> cell lines and CP-CML CD34<sup>+</sup> patient cells were screened for expression of the cytokine receptors IL-3R $\alpha$  and GM-CSFR $\alpha$ . Cytokine receptor surface expression was assessed by flow cytometry. Examination of p-STAT5 by flow cytometry was used to measure both the loss of Bcr-Abl signalling due to inhibition by dasatinib (as STAT5 is constitutively activated by Bcr-Abl in the absence of cytokines) and additionally the subsequent restoration of survival signalling upon the addition of exogenous cytokines. The effect of cytokines on the protection of cells from death was assessed using Annexin V and 7AAD staining.

### 6.1.2 Summary and Research Contribution

It appears that the majority of CP-CML patients are not cured by imatinib treatment. LPCs may be refractory to TKI treatment as a result of inadequate Bcr-Abl kinase inhibition or survival signalling independent of Bcr-Abl. LPCs exist in a cytokine rich microenvironment in the bone marrow and survival pathways activated by cytokines can protect these cells from the cytotoxic effects of TKI treatment. Therefore, the maintenance of survival signals in leukaemic progenitors by cytokines is likely to be a significant contributor to their persistence during TKI therapy. An understanding of this cytokine mediated TKI resistance is essential for developing new therapeutic strategies to target and eliminate these leukaemic progenitors and potentially provide a cure for CML patients.

The research presented in this chapter contributes to the current understanding of cytokine mediated protection from TKI-induced cell death and demonstrates that

blocking of cytokine signalling via JAK inhibition along with Bcr-Abl kinase inhibition has the potential to eradicate primitive CML cells.

### **6.1.3 Publications**

**Schafranek L**, Nievergall E, Powell JA, Hiwase DK, Leclercq TL, White DL and Hughes TP. Sustained inhibition of STAT5, but not JAK2, is essential for TKI-induced cell death in chronic myeloid leukemia. *Leukemia advance online publication*, June 27, 2014; doi:10.1038/leu.2014.156; accepted article preview online May 12, 2014.

### **6.1.4 Conference Presentations**

**Schafranek L**, Nievergall E, Hiwase H, Powell J, Leclercq T, White D, Hughes T. Direct inhibition of STAT5 in combination with transient Bcr-Abl inhibition commits cells to apoptosis despite reactivation of Bcr-Abl. ASH Dec 2013, New Orleans, USA (poster presentation) **Abstract Achievement Award**

**Schafranek L**, Hiwase H, Powell J, Melo J, White D, Hughes T. Blocking Cytokine Signalling Along with Intense BCR-ABL Kinase Inhibition may be necessary to Eradicate CML cells. Health Sciences Postgraduate Research Conference Aug. 2011, Adelaide, Australia (poster presentation).

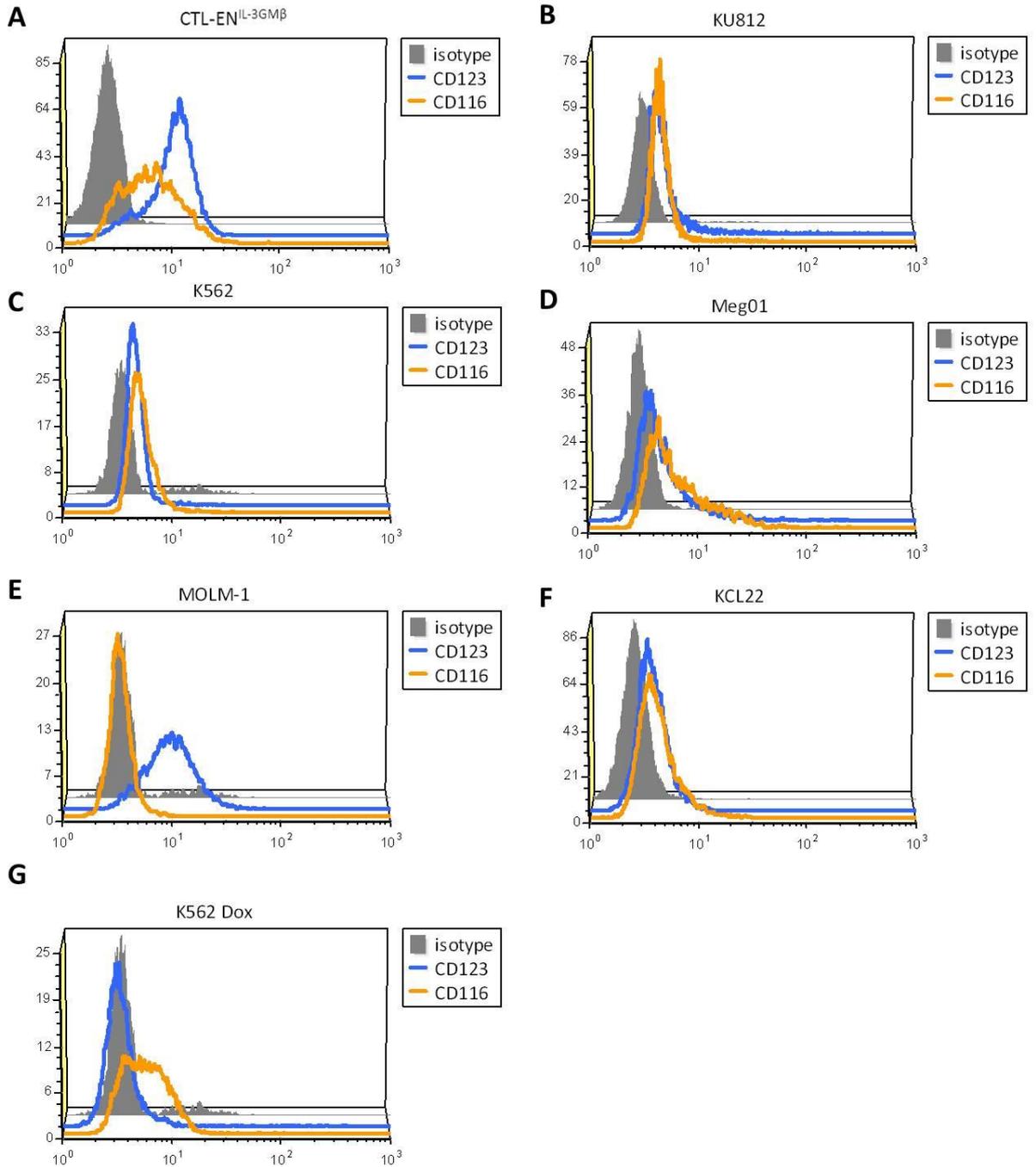
## 6.2 Results

### 6.2.1 *BCR-ABL1*+ cell lines express IL-3 and GM-CSF receptors

Previous studies have indicated that CML progenitor cells may escape TKI-induced cell death by harnessing IL-3 and GM-CSF survival signalling.<sup>171,173</sup> To assess the requirement of cytokine signalling to enable CML cells to escape from apoptosis, *BCR-ABL1*+ cell lines were first screened for the surface expression of IL-3 receptor alpha (IL-3R $\alpha$ ) and GM-CSF receptor alpha (GM-CSFR $\alpha$ ). The mouse CTL-EN<sup>IL-3GM $\beta$</sup>  cell line, which overexpresses the alpha receptors for both IL-3 and GM-CSF together with the  $\beta$ -common receptor, was used as a positive control (Figure 6.2A). The *BCR-ABL1*+ cell lines KU812, K562, Meg01 and KCL-22 demonstrated some expression of both IL-3R $\alpha$  and GM-CSFR $\alpha$  (Figure 6.2B-E), MOLM-1 cells only expressed IL-3R $\alpha$  (Figure 6.2F) and K562 Dox only expressed GM-CSFR $\alpha$  (Figure 6.2G).

### 6.2.2 Cytokines stimulate STAT5 activation in the presence of dasatinib

Having determined the expression of the IL-3R $\alpha$  and GM-CSFR $\alpha$  in *BCR-ABL1*+ cell lines, the ability of cytokines to induce survival signalling in the absence of active Bcr-Abl was assessed. Bcr-Abl constitutively activates STAT5<sup>152,254</sup> and IL-3 and GM-CSF activates STAT5 via the JAK-STAT pathway.<sup>248</sup> As both cytokine- and Bcr-Abl-dependent signalling converge on STAT5, phosphorylation of STAT5 (pSTAT5) was assessed by flow cytometry to determine the reactivation of survival signals in the presence of cytokines following dasatinib treatment.



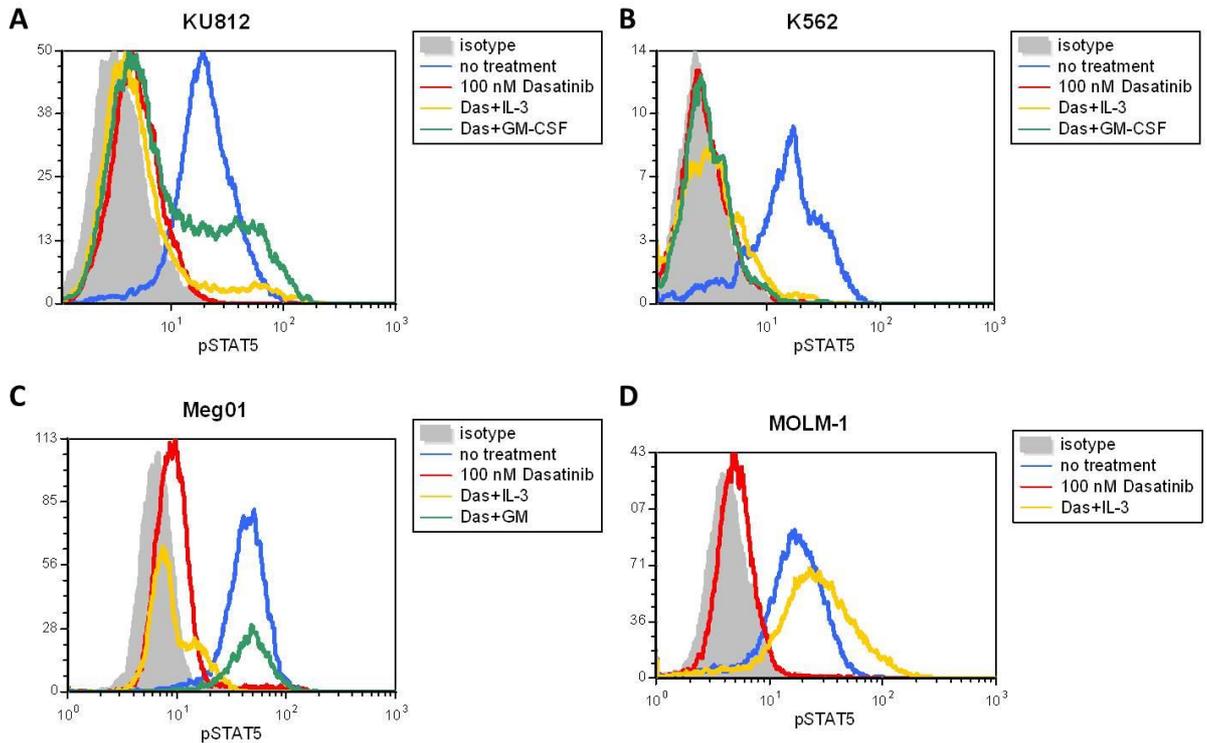
**Figure 6.2: Expression of IL-3R $\alpha$  and GM-CSFR $\alpha$  in *BCR-ABL1*+ cell lines.**

Cells were stained with antibodies against IL-3R $\alpha$  (CD123, blue) and GM-CSFR $\alpha$  (CD116, orange) and expression was determined by flow cytometry. **(A)** CTL-EN mouse cell line overexpressing IL-3R $\alpha$  and GM-CSFR $\alpha$  was used as a positive control, and the *BCR-ABL1*+ cell lines **(B)** KU812, **(C)** K562, **(D)** KCL-22, **(E)** Meg01 **(F)** MOLM-1 and **(G)** K562 Dox. Grey peaks demonstrate isotype negative control staining. Histograms are representative of at least 2 independent experiments

*BCR-ABL1+* cell lines were exposed to 100 nM dasatinib for 30 min in the presence or absence of either IL-3 or GM-CSF. In the absence of cytokines, exposure to dasatinib blocked pSTAT5 in KU812, K562, Meg01 and MOLM-1 cells (Figure 6.3). In KU812 cells, stimulation with GM-CSF resulted in recovery of pSTAT5 in the presence of dasatinib, whilst IL-3 showed only a slight reactivation of STAT5 (Figure 6.3A). K562 cells express both IL-3R $\alpha$  and GM-CSFR $\alpha$ , however stimulation with IL-3 or GM-CSF did not result in recovery of pSTAT5 in the presence of dasatinib (Figure 6.3B). Meg01 cells stimulated with cytokines demonstrated reactivation of STAT5 with GM-CSF in the presence of dasatinib, but not IL-3 despite expressing receptors for both (Figure 6.3C). MOLM-1 cells only express IL-3R $\alpha$ , and stimulation with IL-3 resulted in the reactivation of STAT5 in the presence of dasatinib (Figure 6.3D). These results indicate that cytokines can activate Bcr-Abl-independent survival signalling and the dominant survival signalling pathway/s are specific to each cell line.

### 6.2.3 Newly diagnosed primary CP-CML CD34+ cells express IL-3 and GM-CSF receptors

To determine whether the expression of cytokines receptors were relevant to primary CML cells, the receptor expression of IL-3R $\alpha$ , GM-CSFR $\alpha$  and the  $\beta$ -common chain (required for signalling) was assessed in primary CML CD34+ cells. Surface expression of both the IL-3R $\alpha$  and GM-CSFR $\alpha$  were evident in CP-CML-CD34+ cells (Figure 6.4A). Importantly, expression of the  $\alpha$ -receptors are found in conjunction with the  $\beta$ -common chain which is essential for downstream signal transduction (Figure 6.4B).



**Figure 6.3: Cytokine receptor activation of STAT5 signalling in *BCR-ABL1*+ cell lines**

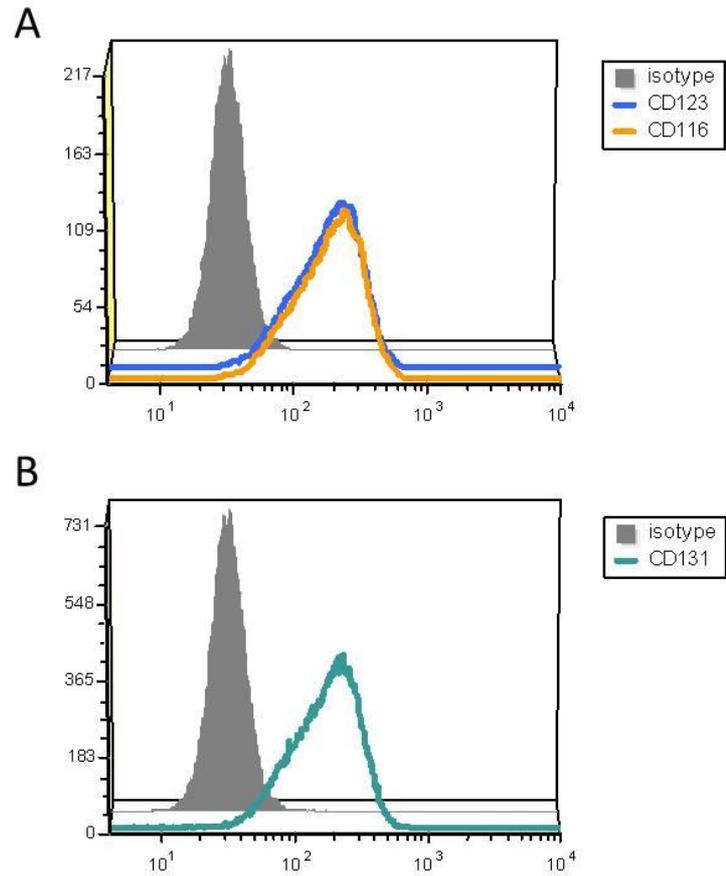
*BCR-ABL1*+ cells were treated with 100 nM dasatinib (Das, red) in the presence or absence of 20 ng/ml GM-CSF (green), or 20ng/ml IL-3 (yellow). **(A)** KU812, **(B)** K562, **(C)** Meg01 and **(D)** MOLM-1. Phosphorylation of STAT5 was measured by flow cytometry. Grey peaks demonstrate isotype negative control staining. Data are representative of 3 independent experiments.

### 6.2.4 Cytokines stimulate STAT5 activation in CP-CML CD34+ cells

As primitive CP-CML CD34+ cells possess the required components for IL-3 and GM-CSF signalling, accordingly the ability of IL-3 and GM-CSF to stimulate STAT5 in the presence of dasatinib was assessed. CP-CML CD34+ cells were exposed to 100 nM dasatinib in the presence or absence of either IL-3 or GM-CSF (Figure 6.5). The presence of dasatinib inhibited activation of STAT5 (as measured by pSTAT5). Addition of IL-3 or GM-CSF, resulted in activation of STAT5 even in the presence of dasatinib. These results indicate that cytokines have the potential to protect CP-CML CD34+ cells from TKI-induced cell death.

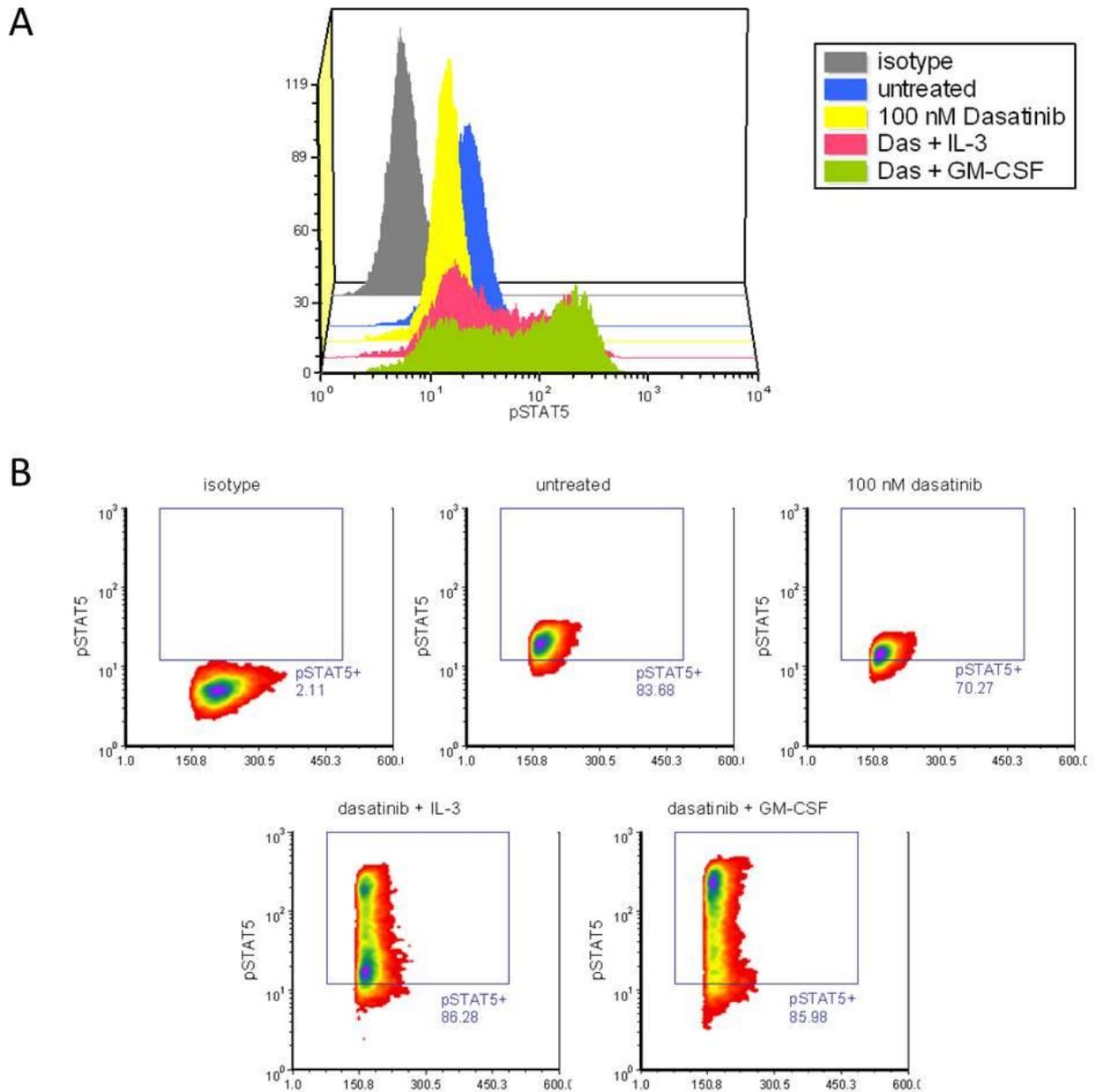
### 6.2.5 Cytokines protect cells from cell death

Activation of STAT5 signalling was established as an important component for cell survival in the previous chapter. Here, the effect of cytokines on TKI-induced cell death was determined in the *BCR-ABL1*+ KU812 cell line. Cells were cultured either with 1 nM dasatinib for 72 h or transiently with 100 nM dasatinib for 30 min followed by the standard washout procedure (STD wash) and recultured for 72 h in dasatinib free media. Cell death was induced by 72 h exposure to both 1 nM dasatinib (11.4% viable,  $p < 0.0001$ ) and 100 nM dasatinib 30 min STD wash (17.9% viable,  $p < 0.0001$ ) (Figure 6.6A). When exposed to 1 nM dasatinib for 72 h or 100 nM dasatinib for 30 min followed by STD wash, respectively, cells were protected from TKI-induced death by a mix of six growth factors (6GF) (69.3% and 66.9% viable), with only partial protection by IL-3 (48.3% and 29% viable), GM-CSF (20% and 44.3% viable), and erythropoietin (EPO) (15.9% and 22.3% viable), but not thrombopoietin (TPO) (10.8% and 15.4% viable).



**Figure 6.4: Expression of IL-3R $\alpha$ , GM-CSFR $\alpha$  and the  $\beta$  common chain in CP-CML CD34+ cells.**

Cells were stained with antibodies against **(A)** IL-3R $\alpha$  (CD123, blue) and GM-CSFR $\alpha$  (CD116 orange), or **(B)** the  $\beta$ -common chain (CD131, green), with expression determined by flow cytometry in CP-CML CD34+ progenitor cells. Grey peaks demonstrate isotype negative control staining. Histograms are representative of at least 2 independent experiments. Results were obtained with the assistance of Dr. Eva Nievergall.



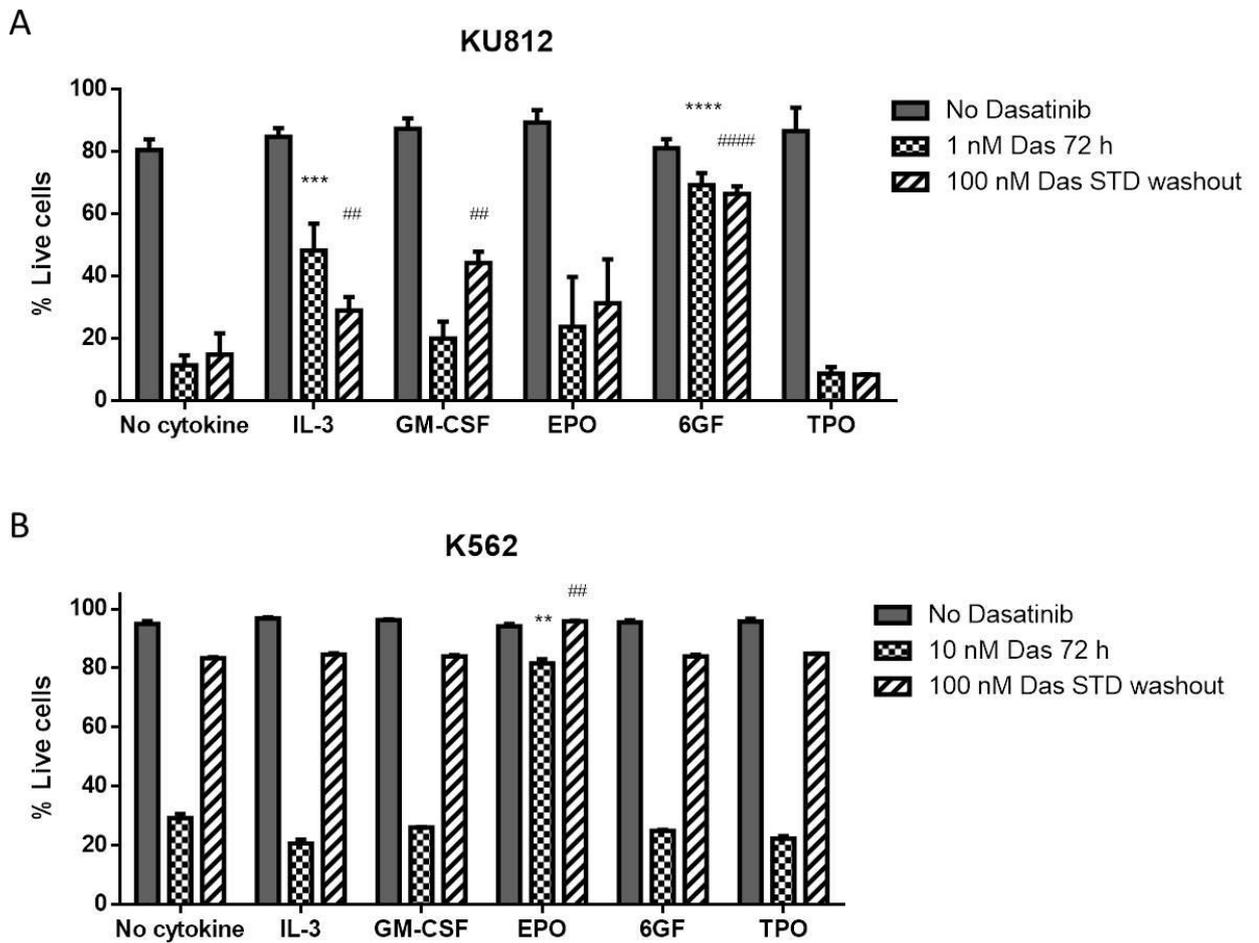
**Figure 6.5: Cytokine receptor activation of STAT5 signalling in CP-CML CD34+ cells**  
CP-CML CD34+ cells were treated with 100 nM dasatinib (Das) in the presence of absence of 20ng/ml IL-3 or 20 ng/ml GM-CSF. **(A)** Representative histogram. **(B)** Representative density plots. Phosphorylation of STAT5 was measured by flow cytometry. Grey peaks demonstrate isotype negative control staining. Data are representative of 3 independent experiments. Results were obtained with the assistance of Dr. Eva Nievergall.

Comparative to KU812 cells, continuous exposure to 10 nM dasatinib induced death in K562 cells (29.6% viable,  $p=0.0007$ ), however transient exposure to 100 nM dasatinib 30 min followed by STD wash had little effect on cell viability (83.3% viable,  $p=0.008$ ) compared to untreated cells (94.9% viable) (Figure 6.6B). Unlike KU812s, no protection from TKI-induced cell death was observed with IL-3 (20.7% and 84.5% viable), GM-CSF (26.1% and 83.9% viable) or 6GF (25% and 83.2% viable). Interestingly, EPO (81.6% and 95.9% viable), but not TPO (22.4% and 84.8% viable) protected cells from dasatinib induced cell death.

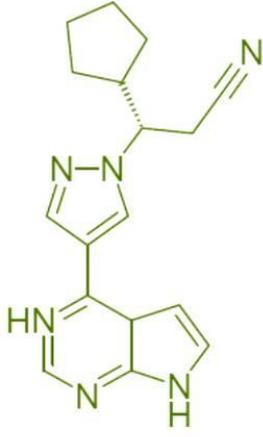
### 6.2.6 Ruxolitinib inhibits JAK1 and JAK2 activation in *BCR-ABL1*+ cell lines

Ruxolitinib is a pan JAK inhibitor approved for use in myeloproliferative neoplasms (myelofibrosis, polycythemia vera and essential thrombocythemia).<sup>252,255</sup> It potently inhibits JAK1 and JAK2 over JAK3, opposed to other more specific JAK2 inhibitors (Figure 6.7).<sup>252</sup> To determine the effect of ruxolitinib in *BCR-ABL1*+ cell lines, KU812 cells were assessed for activation of JAK1, JAK2 and their downstream signalling partners STAT3 and STAT5, following exposure to increasing concentrations of ruxolitinib for 3 h.

Ruxolitinib exposure demonstrated sensitive inhibition of JAK1 with an  $IC_{50}$  of  $\sim 10$  nM and also resulted in inhibition of JAK2 ( $IC_{50} \sim 100$  nM) and STAT3 ( $IC_{50} \sim 100$  nM) (Figure 6.8). However, ruxolitinib had no inhibitory effect on the phosphorylation of STAT5. As it has previously been reported that inhibition of JAK2 may result in inhibition of Bcr on Bcr-Abl itself,<sup>148</sup> phosphorylation of Bcr (Y177) and Abl (Y245) were also investigated. Surprisingly, neither phosphorylation of Bcr-Abl at Y177 nor Y245 was inhibited in the presence of ruxolitinib (Figure 6.8).



**Figure 6.6: Cytokine protection from TKI-induced death in *BCR-ABL1*+ cell lines**  
 (A) KU812 or (B) K562 cells were treated with 1 nM dasatinib for 72 h or 30 min with 100 nM dasatinib (Das) followed by standard wash and culturing for 72 h in drug-free media, in the presence or absence of IL-3, GM-CSF, erythropoietin (EPO), a 6 growth factor mix (6GF) or thrombopoietin (TPO). Cell viability was analysed by Annexin V/7-AAD staining (n=3 mean + SEM). \*\* p<0.005, \*\*\* p<0.0005, \*\*\*\*p<0.0001 compared to Das 72 h and ## p<0.005, #### p<0.0001 compared to Das STD washout.

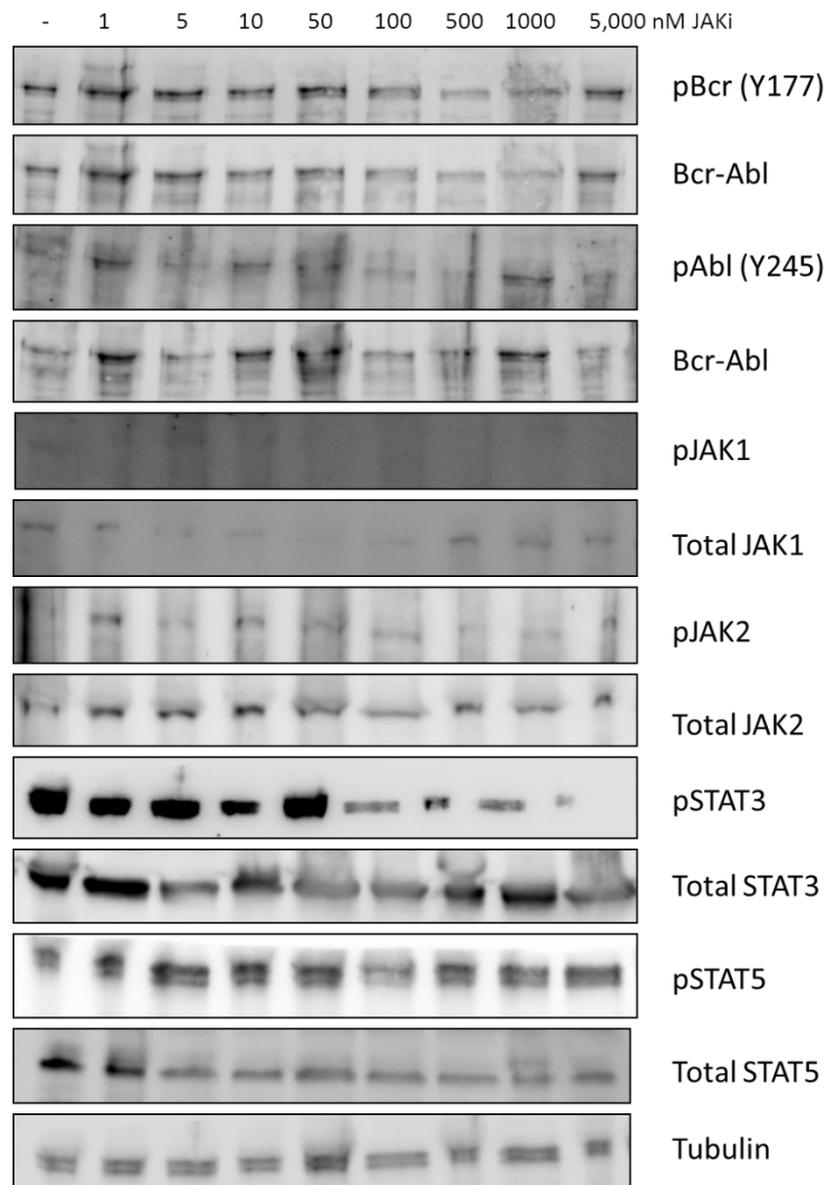


Potency of JAK2 inhibitors	Targets	IC50 (nM)
<b>Ruxolitinib</b>	JAK1	3.3
	JAK2	2.8
	JAK3	428
	Tyk2	19
	CHK2	<1000
	cMET	<10 000
<b>TG101348</b>	JAK1	105
	JAK2	3
	JAK3	996
<b>XL019</b>	JAK1	132
	JAK2	2
	JAK3	250

INCB-018424 (Ruxolitinib)

**Figure 6.7: The structure and activity of JAK inhibitor ruxolitinib in comparison to other JAK2 inhibitors.**

Ruxolitinib (formerly, INCB018424) is a specific JAK kinase inhibitor with preferentially selectivity for JAK1 and JAK2. Adapted from Weinberg *et al.*<sup>43</sup> and Quintàs-Cardama *et al.*<sup>256</sup>



**Figure 6.8: Dose-dependent inhibition of JAK1, JAK2 and STAT3, but not Bcr-Abl or STAT5, with ruxolitinib in KU812 cells.**

KU812 cells were exposed to 0-5000 nM ruxolitinib for 3 h. Lysates were analysed by western blotting for Bcr-Abl-related survival signalling. Data is representative of three independent experiments. Tubulin western blot included as a loading control.

### 6.2.7 Ruxolitinib blocks cytokine-dependent activation of STAT5, but not Bcr-Abl-dependent activation of STAT5 in *BCR-ABL1*+ cell lines

In the setting of transient exposure to dasatinib, no additional benefit of the pan JAK inhibitor ruxolitinib was observed, however chemical inhibition of STAT5 in combination with 30 min TKI treatment induced cell death (refer Results Chapter 3 Figure 2.13-18), suggesting JAK signalling is not significantly involved in this setting. The effect of JAK inhibition on pSTAT5 was assessed by flow cytometry in the presence and absence of GM-CSF in KU812 cells. Following a 3 h incubation, a minor reduction in pSTAT5 was observed using ruxolitinib at concentrations of 100 nM (6.8 MFI), and 500 nM (6.8 MFI) compared to untreated control (7.8 MFI), with maximum effect at 1000 nM (6.3 MFI) (Figure 6.9A). Stimulation with GM-CSF slightly increased pSTAT5 (8.3 MFI), which could be reduce back to unstimulated levels with ruxolitinib at 100 nM (7.7 MFI), 500 nM (7.6 MFI) and 1000 nM (6.5 MFI) (Figure 6.9B).

To assess the contribution of the JAK-STAT pathway to Bcr-Abl survival signalling, the effects of 1000 nM ruxolitinib on the cytokine activation of STAT5 were examined in the presence of 100 nM dasatinib. Ruxolitinib had little effect on STAT5 phosphorylation as a sole agent (7 MFI) however when STAT5 was activated by 20 ng/ml GM-CSF (8.5 MFI), ruxolitinib reduced pSTAT5 comparable to untreated control (7.7 MFI) (Figure 6.10A). However, this only inhibited the GM-CSF stimulated portion of STAT5 and did not reduce Bcr-Abl activation of STAT5. Contrariwise, dasatinib inhibited STAT5 in the absence of GM-CSF (4.1 MFI), but was unable to completely inhibit STAT5 in the presence of GM-CSF (8.8 MFI). The combination of dasatinib with ruxolitinib was required to completely inhibited STAT5 in the presence of GM-CSF (3.8 MFI) (Figure

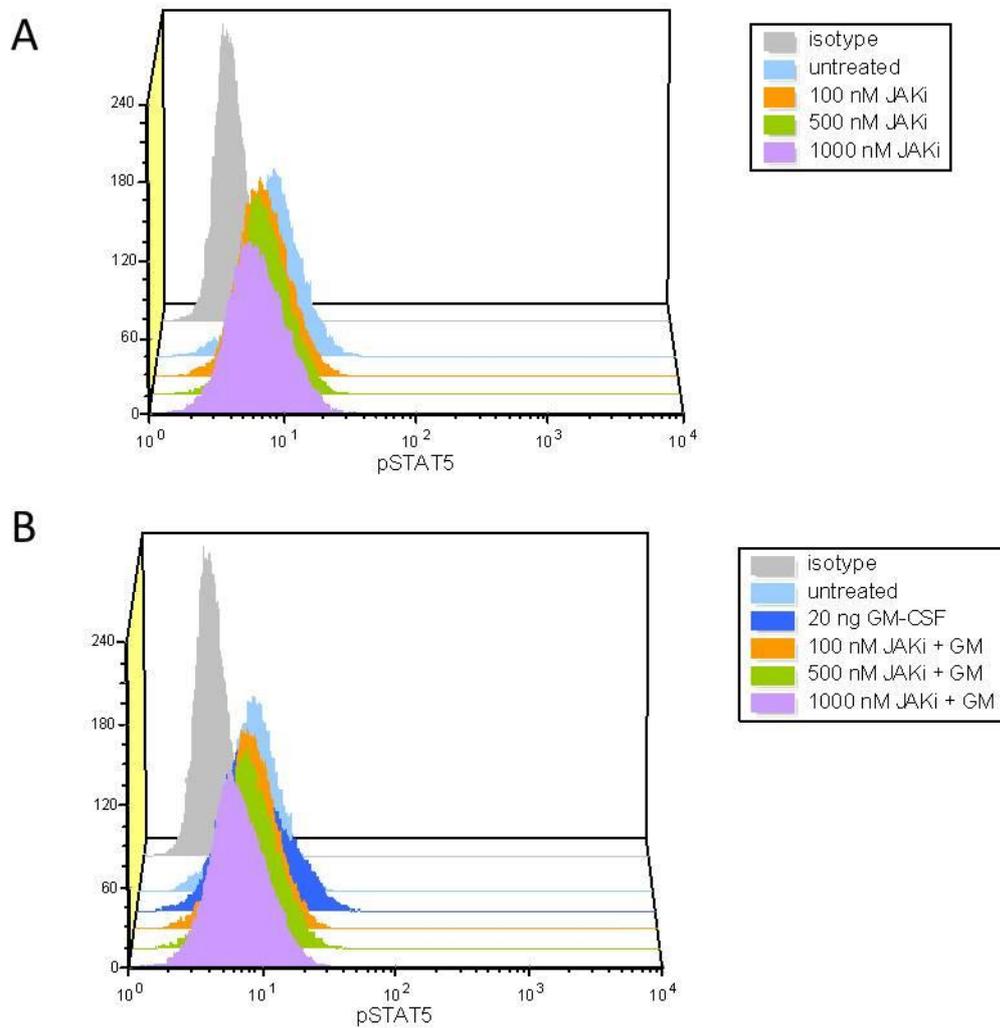
6.10B). JAK inhibition appears to only target cytokine-dependent activation of STAT5 and therefore acts independently of Bcr-Abl.

### **6.2.8 Ruxolitinib blocks cytokine-dependent activation of STAT5, but not Bcr-Abl-dependent activation of STAT5 in CP-CML CD34+ cells**

Subsequently, the activation of STAT5 was assessed in the presence and absence of a mix of growth factors (5GF) in CP-CML CD34+ cells. In the absence of 5GF, ruxolitinib alone had little effect on STAT5 phosphorylation, however 100 nM dasatinib reduced pSTAT5 whether alone or in combination with ruxolitinib (Figure 6.11A). Addition of 5GF stimulated further activation of STAT5 (Figure 6.11B). Although dasatinib completely inhibited pSTAT5 in the absence of 5GF, this could be partially restored by 5GF stimulation. Ruxolitinib removed 5GF stimulated activation of STAT5 both in the presence and absence of dasatinib, suggesting that the role of JAK2 activation of STAT5 in CP-CML CD34+ cells may preferentially occur through cytokine signalling and not via Bcr-Abl.

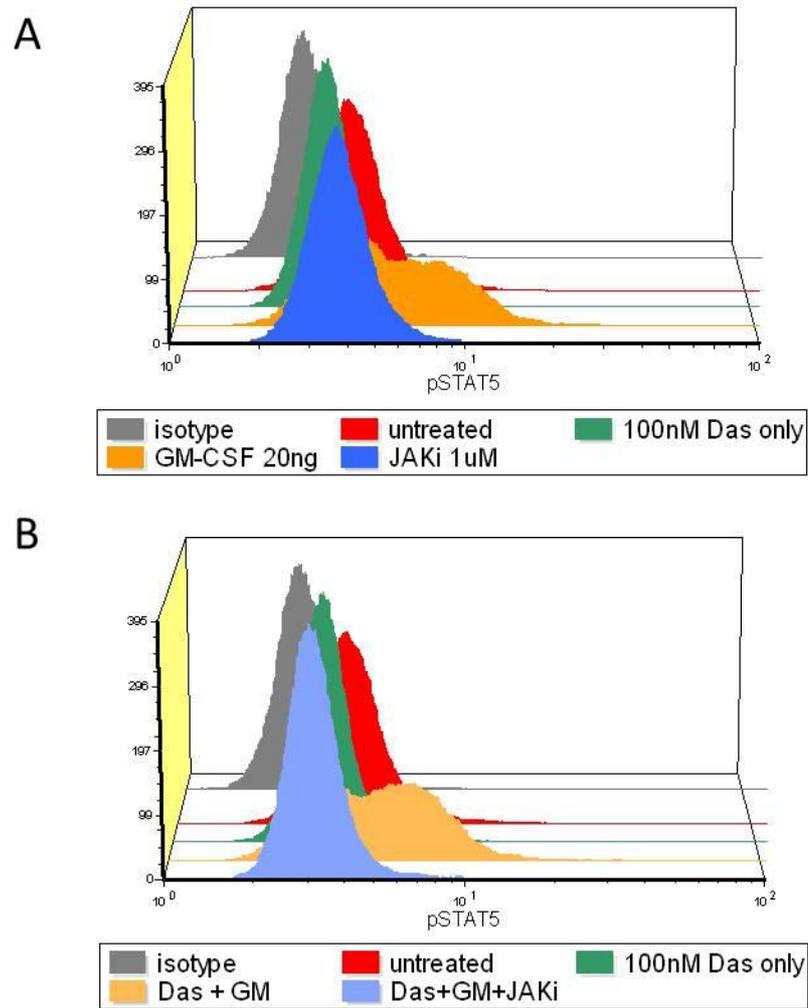
### **6.2.9 Ruxolitinib has little effect as a sole agent or in combination with dasatinib in the absence of cytokines, but abrogates cytokine protection of dasatinib-induced cell death.**

As ruxolitinib successfully inhibited cytokine-dependent activation of STAT5, the ability of JAK inhibition to induce cell death in the presence and absence of cytokines was assessed. Ruxolitinib specifically inhibits JAK kinases producing IC50s of JAK1 at 2.7 nM, JAK2 at 4.5 nM and JAK-3 at 322 nM and is commonly used in *in vitro* assays at 300-1000 nM.<sup>253,257</sup> To determine the optimal dose in this setting, a titration in the presence and



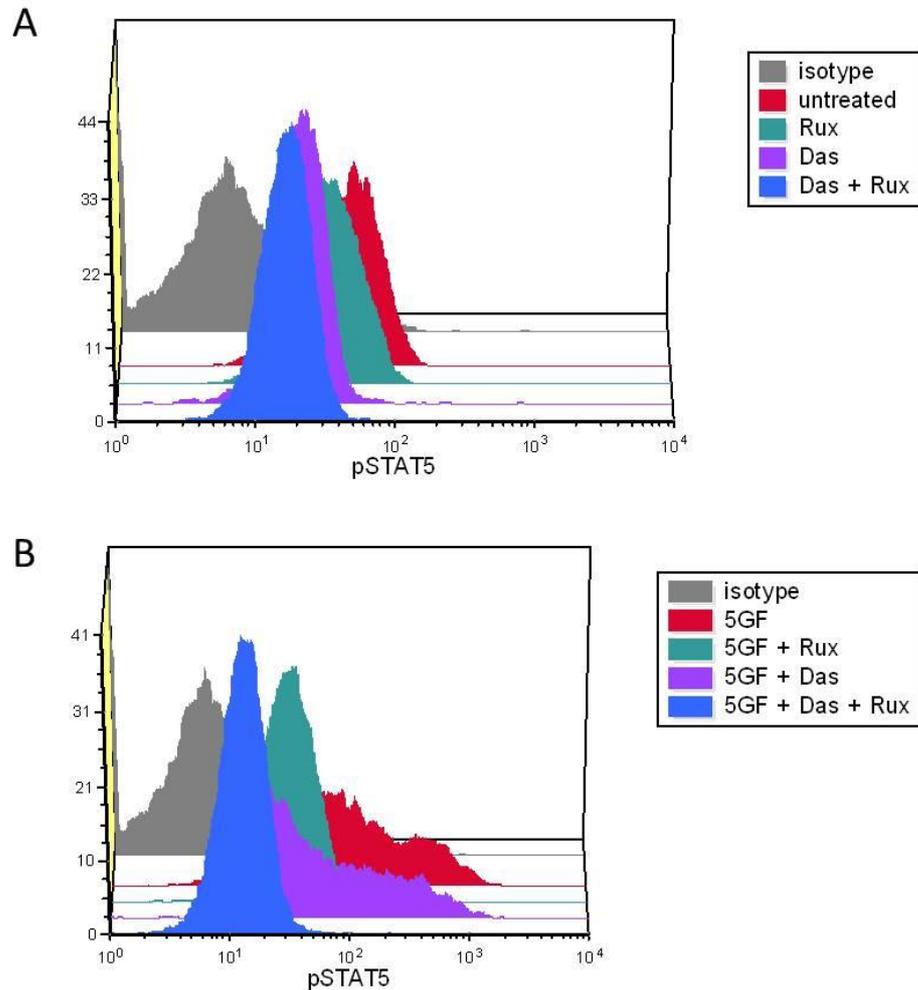
**Figure 6.9: Ruxolitinib demonstrates minimal reduction of Bcr-Abl dependent pSTAT5 in KU812 cells.**

KU812 cells were treated with 100 nM, 500 nM and 1000 nM ruxolitinib (JAKi) in the **(A)** absence or **(B)** presence of 20 ng/ml GM-CSF (GM). Phosphorylation of STAT5 (pSTAT5) was measured by flow cytometry. Histograms are representative of at least 2 independent experiments.



**Figure 6.10: Pan JAK inhibition removes cytokine stimulated pSTAT5 but not Bcr-Abl-dependent pSTAT5 in KU812 cells**

KU812 cells were treated with or without 20ng GM-CSF (GM) in the presence or absence of 1000 nM ruxolitinib (JAKi) **(A)** alone or **(B)** in combination with 100 nM dasatinib (Das). Phosphorylation of STAT5 was measured by flow cytometry. Histograms are representative of at least 2 independent experiments.



**Figure 6.11: Ruxolitinib reduces cytokine stimulated pSTAT5 but not Bcr-Abl-dependent pSTAT5 in CP-CML CD34+ cells.**

Newly diagnosed CP-CML CD34+ cells were treated **(A)** with or **(B)** without a 5 growth factor mix (5GF) in the presence or absence of ruxolitinib (Rux) with or without 100 nM dasatinib (Das). Phosphorylation of STAT5 was measured by flow cytometry.

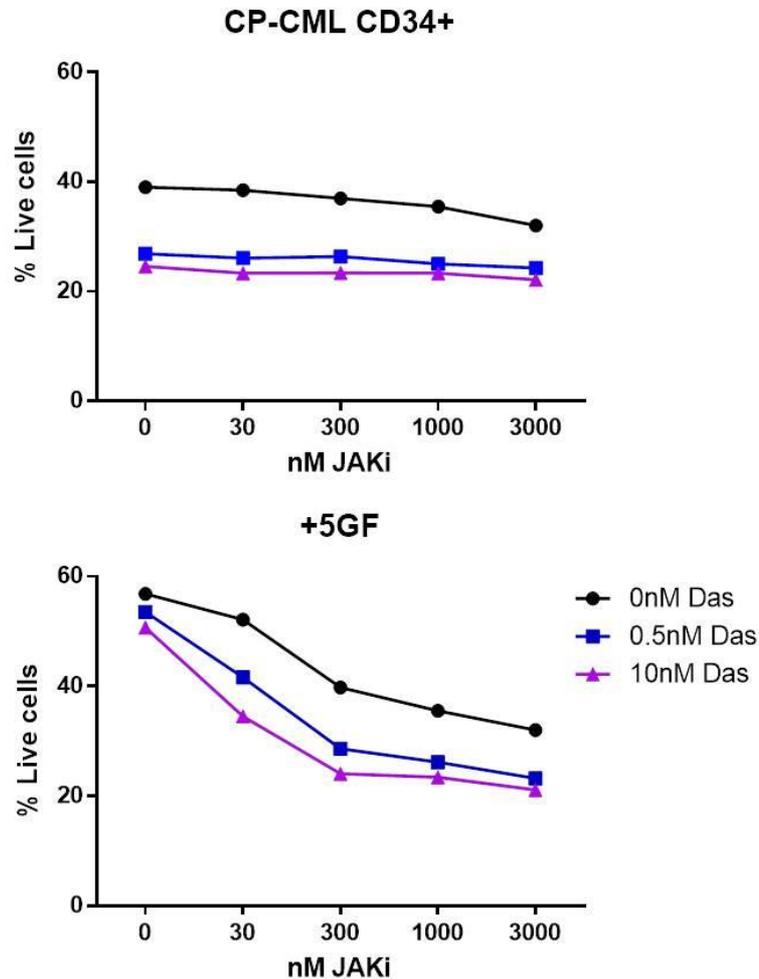
Histograms are representative of at least 2 independent experiments.

absence of 5GF in CP-CML CD34+ cells was performed. In the presence of 5GF, ruxolitinib resulted in a dose dependent reduction in live cells, however in the absence of cytokines ruxolitinib had little effect on cell viability after 72 h culture (Figure 6.12).

### **6.2.10 In primary CP-CML CD34+ cells ruxolitinib removes cytokines protection from dasatinib-induced cell death, having no additional effect to dasatinib treatment in the absence of cytokines**

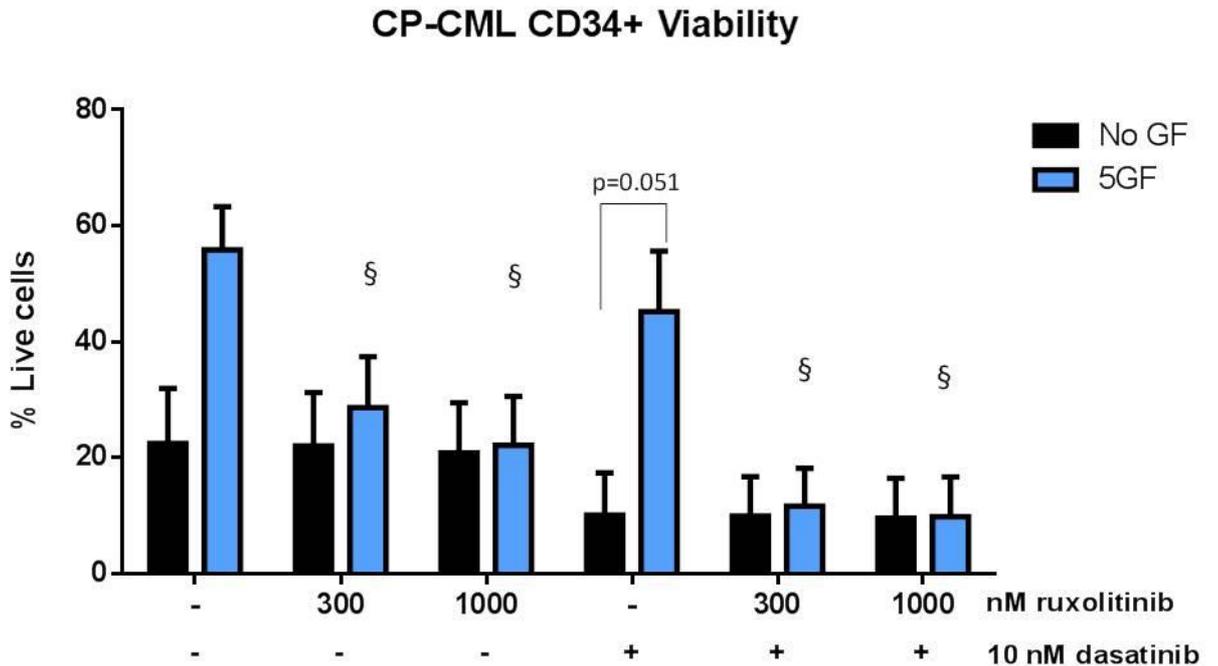
As there was no additional effect of 3000 nM ruxolitinib (Figure 6.12), concentrations of 300 nM and 1000 nM were chosen for use in subsequent experiments. In the absence of cytokines, cell viability was not significantly reduced from control cells (22.4% viable) by JAK inhibition alone with ruxolitinib at either 300 nM (22% viable,  $p=0.73$ ) or 1000 nM (20.8% viable,  $p=0.24$ ) (Figure 6.13). Dasatinib alone (10 nM) induced significant cell death compared to control (10.1% viable,  $p=0.04$ ), but this cell death was not significantly enhanced by addition of either 300 nM (9.9% viable,  $p=0.75$ ) or 1000 nM ruxolitinib (9.5% viable,  $p=0.3$ ). Cell viability was significantly increased by the presence of 5GF compared to control (55.8% viable,  $p=0.04$ ), and could then be significantly reduced by the addition of 300 nM (28.6% viable,  $p=0.03$ ) and 1000 nM (22.1% viable,  $p=0.04$ ) ruxolitinib. The presence of growth factors protected cells from dasatinib induced cell death, where dasatinib alone was unable to significantly decrease viability compared to 5GF alone (45.2% viable,  $p=0.45$ ).

The combination of dasatinib with 300 nM (11.6% viable,  $p=0.03$ ) or 1000 nM (9.8% viable,  $p=0.04$ ) ruxolitinib significantly enhanced the cell death achieved by dasatinib treatment alone in comparison to 5GF alone. However ruxolitinib was unable to reduce viability more than dasatinib treatment alone, suggesting that ruxolitinib abrogates



**Figure 6.12: Cytokine protection of CP-CML CD34+ cells from dasatinib-induced cell death is blocked by ruxolitinib.**

CP-CML CD34+ cells were treated with increasing concentrations of ruxolitinib in the presence or absence of 0.5 nM or 10 nM dasatinib (Das), **(A)** without cytokines or **(B)** with a 5 growth factor (5GF) mix. Cells were then analysed by flow cytometry for Annexin V/7-AAD staining following 72 h culture.



**Figure 6.13: Ruxolitinib abrogates cytokine-dependent protection from dasatinib-induced cell death, but does not further increase cell death compared to dasatinib alone.**

CP-CML CD34+ cells were treated with either 300 nM or 1000 nM ruxolitinib +/- 5 growth factor (5GF) mix, +/- 10 nM dasatinib. Cells were then analysed for Annexin V/7-AAD staining at 72 h by flow cytometry (n=3 mean + SEM). § p<0.05, compared to 0 nM dasatinib with 5GF.

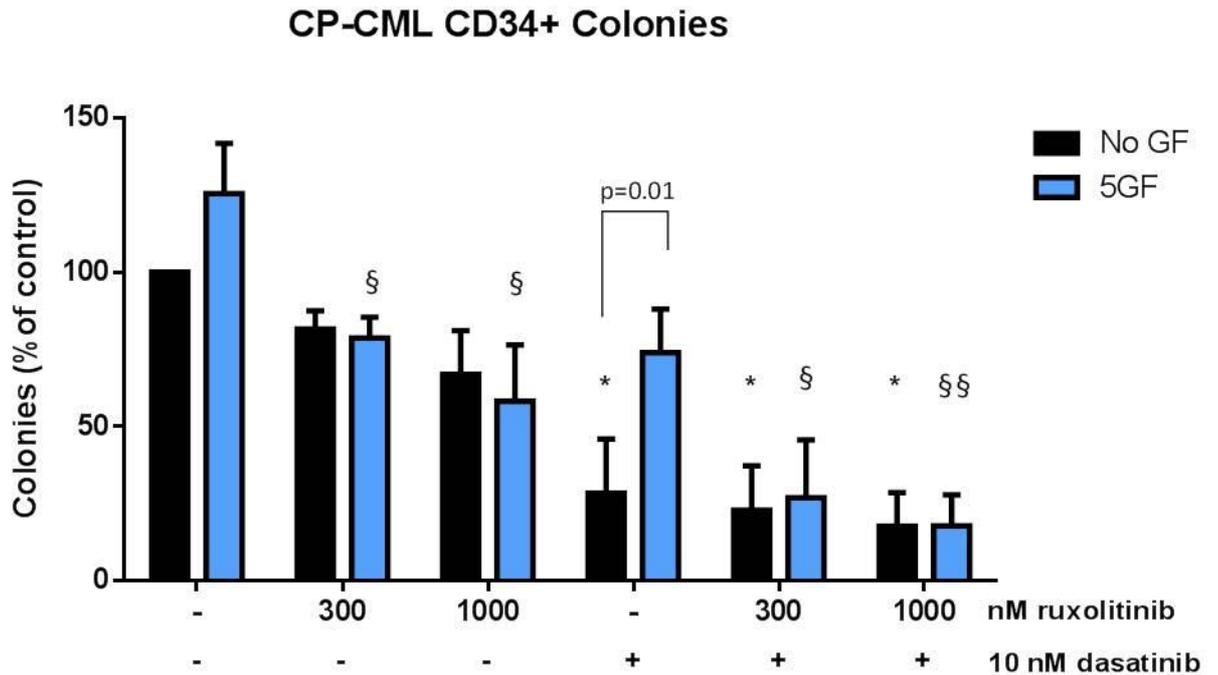
cytokine-dependent protection from dasatinib induced death. These results indicate that JAK inhibition may only be effective in the context of cytokine stimulation, irrespective of Bcr-Abl activity.

### **6.2.11 Ruxolitinib impairs the ability of CP-CML CD34+ cells to form colonies in the presence of 5GF**

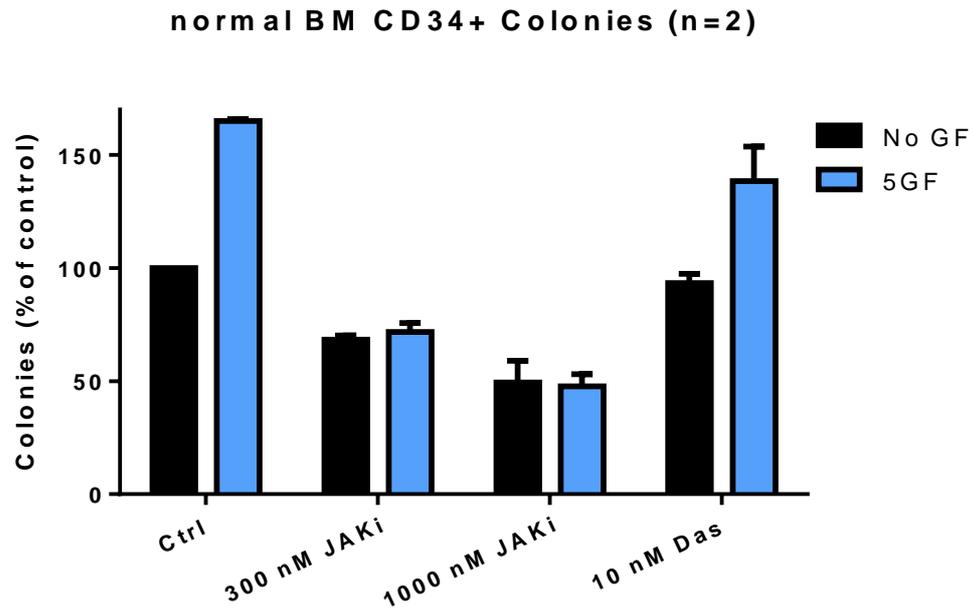
The ability of CP-CML CD34+ cells to form colonies was assessed following the 72 h cell death assay (previously described in Section 5.2.7). In the absence of cytokines, 10 nM dasatinib alone significantly reduces CP-CML CD34+ colony formation (24.1% CFUs of untreated control (arbitrarily set at 100%),  $p=0.01$ ), however 300 nM ruxolitinib alone was unable to significantly reduce CFUs (87.8% CFUs compared to untreated control,  $p=0.27$ ) and the combination of dasatinib with 300 nM ruxolitinib (24.7% CFUs compared to untreated control,  $p=0.98$ ) did not further reduce CFUs compared to dasatinib alone (Figure 6.14). The addition of 5GF significantly increased colony formation (156.5% CFUs compared to no 5GF control) which was significantly reduced by the addition of 10 nM dasatinib (71.9%  $p=0.03$ ) or 300 nM ruxolitinib (58.1%,  $p=0.01$ ). The combination of dasatinib with 300 nM ruxolitinib in the presence of 5GF, resulted in a further decrease in colonies (31.3% CFUs,  $p=0.01$  compared to 5GF alone). Again, indicating that the effect of JAK inhibition is to specifically block survival signalling induced by cytokine stimulation. Interestingly, 1000 nM ruxolitinib not only significantly reduced colony formation in the presence of cytokines (46.5%,  $p=0.04$ ), but also reduced CFUs in the absence of cytokines (53.3%,  $p=0.07$ ) (Figure 6.14).

However, this may occur due to loss of specificity of ruxolitinib for JAK1 and JAK2 at this high dose, <sup>237,252</sup> therefore the effect of ruxolitinib on CD34+ cells from normal bone marrow donors was assessed to confirm that ruxolitinib-induced cell death is not due to

Bcr-Abl. Exposure to dasatinib alone had little effect on the reduction of colony formation (93.3% CFUs compared to untreated control) (Figure 6.15). Alternatively, ruxolitinib resulted in a decrease in colonies at both 300 nM (68.4% CFUs) and 1000 nM (49.4% CFUs). Therefore, it's likely that dasatinib results in effective inhibition of Bcr-Abl signalling and that JAK inhibition offers no further benefit except in the presence of cytokines, with STAT5 common to both pathways.



**Figure 6.14: JAK inhibition prevents colony formation in the presence of 5GF**  
 CP-CML CD34+ cells were treated with 300 nM or 1000 nM ruxolitinib, +/- 10 nM dasatinib, in the absence or presence of a 5 growth factor (5GF). Clonogenic potential was assessed by enumeration of colonies at 2 weeks following CFU-GM assay (n=3, mean+SEM). \* p<0.05 compared to 0 nM dasatinib without 5GF, § p<0.05, §§ p<0.01 compared to 0 nM dasatinib with 5GF.



**Figure 6.15: Non-specific effect of ruxolitinib on clonogenic potential of normal CD34+ cells.**

CD34+ cells were treated with increasing concentrations of ruxolitinib (JAKi) +/- 5 growth factor (5GF) mix. Clonogenic potential was assessed by enumeration of CFUs at 2 weeks following CFU-GM assay (n=2, mean+SEM).

## 6.3 Discussion

STAT5 is constitutively activated due to the presence of the Bcr-Abl oncoprotein in CML cells.<sup>75</sup> Bcr-Abl confers protection against apoptosis through the activation of cell survival signals, like STAT5, as well as the expression of anti-apoptotic proteins, to promote cell survival. In the previous chapter, STAT5 was found to have a critical role in the balance between life and death in *BCR-ABL1*+ cells exposed to TKIs. Furthermore, the activation of STAT5 by Bcr-Abl has recently been demonstrated to play an important role in protection of CML cells from oxidative stress.<sup>154</sup>

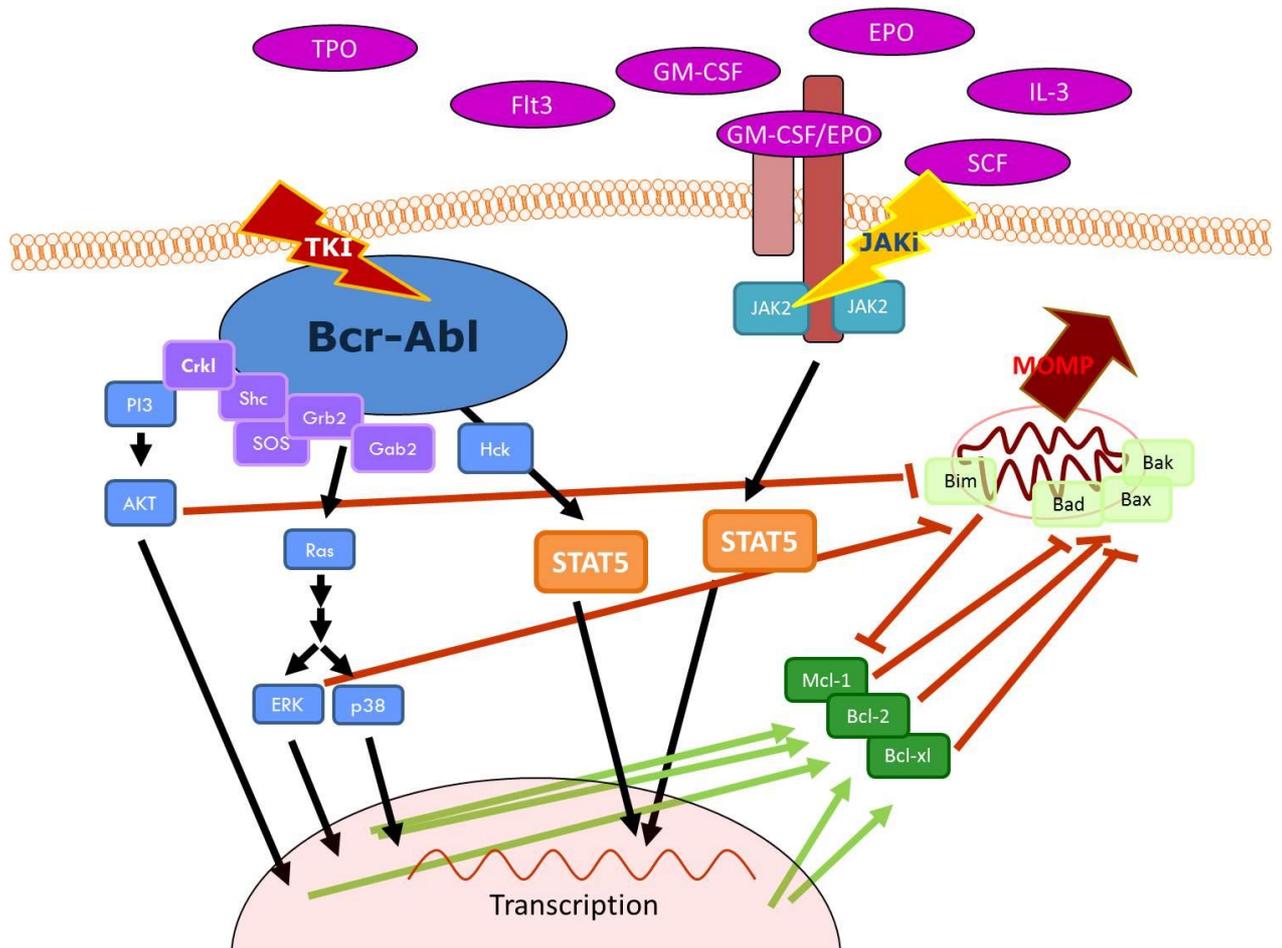
In a non-pathological setting, activation of STAT5 occurs through cytokine and growth factor signalling by way of JAK2 kinases.<sup>146,165</sup> Although there are reports that JAK2 may phosphorylate or complex with Bcr-Abl,<sup>148,149,244</sup> inhibition of JAK2 alone has little effect on induction of apoptosis and appears to specifically target extrinsic survival signalling (cytokine dependent) as opposed to Bcr-Abl-dependent signalling through STAT5.<sup>150</sup> As such, the focus of this chapter was to interrogate the function of cytokine signalling as a mechanism of TKI resistance. Expression of the cytokine receptors IL-3R $\alpha$  and GM-CSFR $\alpha$  were observed in *BCR-ABL1*+ cell lines and CP-CML CD34+ cells (Figure 6.2 and Figure 6.4). Stimulation of the *BCR-ABL1*+ KU812 cell line and CP-CML CD34+ cells with IL-3 and GM-CSF was found to enhance pSTAT5 in the presence of Bcr-Abl inhibition with dasatinib (Figure 6.3 and Figure 6.5) and consequently, the presence of these cytokines was able to prevent the induction of dasatinib-induced cell death (Figure 6.6 and Figure 6.12).

Cytokine activation of JAK2 has previously been described to have a protective effect on CML cells.<sup>144,171</sup> Thus, the pan JAK inhibitor ruxolitinib was utilised to determine whether the involvement of JAK kinases was specific to cytokine-dependent signalling, or also played a role in Bcr-Abl dependent signalling, as recently suggested.<sup>148</sup> Dose-dependent inhibition of JAK1, JAK2, and STAT3 was observed following ruxolitinib treatment (Figure 6.8). Interestingly however, no inhibition of STAT5 was observed following treatment with ruxolitinib in KU812 cells, suggesting a role for STAT3 but not STAT5 in JAK signalling in the *BCR-ABL1*+ setting (Figure 6.8). Interestingly, JAK inhibition had no effect on pBcr-Abl at either the Y245 site, or the Y177 site, which has been previously reported to be inhibited by JAK2 inhibition.<sup>258</sup> However, in the observations made by Samanta *et al.*<sup>258</sup>, there was also a decrease in total Bcr-Abl. This disparity may be due to use of different techniques. The approach for these analyses used only the soluble fraction of the lysates which may have resulted in loss of proteins associated with the cytoskeleton,<sup>259</sup> whereas our study utilised the whole cell lysate and we noted no reduction in total Bcr-Abl (Figure 6.8).

In the absence of growth factors, it was observed that increasing concentrations of ruxolitinib alone was unable to cause a significant reduction in CD34+ CP-CML cell viability (Figure 6.12). In the presence of a growth factor cocktail, JAK inhibition was only able to reduce the live cell population stimulated by cytokines, and didn't further enhance dasatinib mediated cell killing (Figure 6.12 and Figure 6.13). Although a significant reduction in CD34+ colonies was observed following treatment with 1000 nM ruxolitinib alone, there was no significant additive effect when ruxolitinib was used in combination with dasatinib (Figure 6.14). Furthermore, we demonstrated that pan JAK inhibition alone was unable to inhibit Bcr-Abl-dependent phosphorylation of STAT5,

only inhibiting the cytokine stimulated portion of pSTAT5 (Figure 6.9-11). These findings support previous reports that cytokines protect cells from TKI induced apoptosis by Bcr-Abl independent activation of the JAK2/STAT5 pathway.<sup>171</sup>

The data presented here is also supported by *in vivo* evidence which suggests that JAK kinase signalling is only relevant in Bcr-Abl-independent, extrinsic activation of STAT5 as JAK2 inhibition had no effect on Bcr-Abl driven STAT5 signalling or cell death as a sole agent in the absence of cytokines<sup>150,151</sup>. Therefore, we propose that STAT5 activation as a result of active Bcr-Abl does not occur through JAK2, but rather that JAK2 activation of STAT5 occurs through cytokine signalling (Figure 6.16).



**Figure 6.16: Proposed cytokine- and Bcr-Abl-dependent signalling in CML**

Bcr-Abl activates survival signalling through components such as STAT5. When Bcr-Abl is inhibited by tyrosine kinase inhibitors (TKI), pSTAT5 is subsequently reduced. Cytokine receptor activation results in the dimerization of JAK2 which can activate STAT5 resulting in cell survival in the presence of Bcr-Abl inhibition which can be targeted by JAK inhibition (JAKi).

## **CHAPTER 7.**

### **GENERAL DISCUSSION**

Chronic myeloid leukaemia is reliant on the constitutive activation of Bcr-Abl for survival and maintenance.<sup>26</sup> Bcr-Abl signalling potentiates survival of cells by harnessing growth factor survival pathways, like STAT5 signalling,<sup>53,260</sup> and consequently results in uncontrolled factor-independent proliferation,<sup>47</sup> reduced apoptosis (ie via the overexpression of Bcl-2)<sup>67</sup> and aberrant adhesion.<sup>261</sup> TKI therapy, which targets Bcr-Abl, removes this survival signalling promoting cell death.

The introduction of imatinib therapy has revolutionised the treatment of CML and become a model for targeted therapy in other malignancies.<sup>262</sup> Nevertheless, not all CML patients achieve optimal responses when treated with imatinib therapy. The inability to achieve a landmark response to imatinib treatment may be due to intrinsic factors (primary resistance), a loss of response after a successful treatment phase (secondary resistance); and/or progression to a more advanced disease stage.<sup>122</sup> As such, deeper investigations into the conditions required for optimal patient outcomes are essential. The aim of such investigations is to optimize treatment for CML patients and avert both TKI resistance and persistent disease. The studies presented in this thesis examine the critical factors involved in cell death following treatment with TKIs; specifically, assessing the signalling mechanisms CML cells employ in order to avoid death. Aspects of Bcr-Abl dependent and independent signalling which govern leukaemic cell survival in response to TKI treatment explored in these studies include:

- Examination of the extent of Bcr-Abl signalling inhibition required to commit cells to death and subsequently, the important Bcr-Abl dependent factors involved in these signalling pathways that allow the cells to survive TKI treatment

- 
- Investigation into the mechanism of TKI-induced autophagy and assessment of autophagy inhibition as a strategy for combination therapy
  - Evaluation of cytokine signalling as a Bcr-Abl independent mechanism of TKI-resistance

## 7.1 Major Findings

### 7.1.1 Deactivation of STAT5 is critical for the induction of cell death in situations of low level or transient Bcr-Abl kinase inhibition.

Utilizing *in vitro* models of transient TKI inhibition of Bcr-Abl, Chapter 4 and Chapter 5 challenge the validity of the current clinical dogma of continuous Bcr-Abl kinase inhibition. Understanding the duration and degree of kinase inhibition needed for optimal efficacy in CML patient is critically important.

Long term partial kinase inhibition is effective at killing *BCR-ABL1*+ cells in CP-CML patients receiving once daily dasatinib treatment.<sup>263</sup> Chapter 4 examines the disparity between the dose of dasatinib required to inhibit Bcr-Abl kinase activity and the dose required to induce cell death. *BCR-ABL1*+ cells are believed to be “addicted” to Bcr-Abl survival signalling, resulting in dysregulated proliferation and survival of malignant cells in the absence of exogenous growth factors. Additionally, oncogene addiction has been established in other malignancies, for example *MYC*<sup>229</sup> and *EGFR*<sup>230</sup> are drivers of cancer cell maintenance, thus understanding oncogene addiction reaches beyond the scope of CML.

Preliminary studies of imatinib demonstrated *that* continuous and potent Bcr-Abl kinase inhibition was required to trigger cell death and inhibit cell proliferation, whereas a short exposure of 6-7 h was not.<sup>132</sup> Clinical observations using dasatinib challenged the paradigm that a continuous high dose of TKI was required for optimal clinical responses, demonstrating that transient dasatinib exposure was capable of inducing cell death in *BCR-ABL1+* cells *in vitro*<sup>137</sup> and that reduced frequency of dasatinib intake from 50-70mg twice daily to 100-140mg once daily did not appear to affect clinical outcome in patients with CML.<sup>135,264,265</sup> Initial studies suggested that very brief exposure to kinase inhibitors (30 min) was sufficient to induce apoptosis in CML cells,<sup>136-138</sup> however, flaws in this experimental model have subsequently been identified.<sup>227,232,234,266</sup> Several groups have now demonstrated that the removal of residual dasatinib using an optimal washout procedure (OPT wash) reverses apoptosis induction and that 30 min exposure to TKI while sufficient to result in inhibition of Bcr-Abl is insufficient to induce apoptosis.<sup>227,232,234,266</sup> They hypothesised that it is the residual TKI present in cells, due to an inferior washout procedure, which induced cell death and not the initial short exposure to high dose TKI.<sup>227,232,234,266</sup> The results presented in Chapter 4 demonstrate that low doses of dasatinib committed *BCR-ABL1+* cells to death (Figure 4.2). Sensitive inhibition of pSTAT5 was observed despite <10% inhibition of Bcr-Abl (Figure 4.10). This suggests that low levels of dasatinib may cause the sustained inhibition of pSTAT5 following the standard washout (STD wash) and that cell death may arise from technical considerations associated with the original washout protocol.

Chapter 5 assesses recent reports that low concentrations of TKI remain following the STD wash.<sup>227,232,234</sup> Here we confirm that an equilibration between washes (i.e. the OPT wash) restores viability to *BCR-ABL1+* cells treated for 30 min with 100 nM dasatinib,

suggesting that a low level of dasatinib remained in the cells following the STD wash. At the time of these experiments, sensitive detection of dasatinib by liquid chromatography and/or mass spectrometry was unavailable to us, and observations presented here using radiolabelled dasatinib were inconclusive due to lack of sensitive detection (Figure 4.13). However, during the establishment of these procedures in our laboratory, several other groups reported sensitive detection of dasatinib using chromatography and mass spectrometry procedures,<sup>227,232,234,266</sup> although they have expressed difficulty in accurately quantifying low nanomolar concentrations. Along with the OPT wash, our analysis of Bcr-Abl signalling, in particular the reactivation of STAT5 (Figure 5.3), indicated the complete removal of dasatinib. This data indicated that sufficient dasatinib remained following the 30 min 100 nM dasatinib STD wash to maintain continuous pSTAT5 inhibition, despite reactivation of Bcr-Abl (Figure 4.8), and subsequently achieved induction of cell death (Figure 4.6).

These results raised two questions;

- 1) Is there a minimum length of exposure to TKI which commits *BCR-ABL1+* cells to death (thus supporting the theory of “oncogenic shock”<sup>226</sup>)?
- 2) is STAT5 simply a sensitive measure of low intensity Bcr-Abl inhibition, or is it important for the decision of *BCR-ABL1+* cells to survival following challenge with TKI?

### **7.1.2 STAT5 is a critical component of the time-dependent sensitivity of *BCR-ABL1+* cells to TKI treatment**

The clinical efficacy of dasatinib therapy suggests that continuous potent exposure, and consequent complete and continuous inhibition of Bcr-Abl, is not required for optimal

clinical response in CML patients. Although recent reports have established that 30 min exposure to TKI is not sufficient to induce apoptosis,<sup>227,232,234</sup> the *in vitro* length of Bcr-Abl inhibition required to induce cell death has not been assessed. For the first time, the data presented in this thesis establishes a requirement of at least 2-8 h exposure to potent TKI (100 nM dasatinib, or 32.5 µM imatinib) to induce death and decrease colony forming potential in CP-CML CD34+ cells (Figure 5.7 and Figure 5.8). These results are in line with serum availability of dasatinib<sup>130</sup> in patients treated on a once daily regime and thus provides mechanistic support for the clinical efficacy of once daily dasatinib therapy.

Recent data highlights STAT5 as a critical factor in TKI resistance<sup>153,154</sup> with chemical inhibition of STAT5 phosphorylation in CML cells increasing sensitivity to TKI induced apoptosis.<sup>157,243</sup> Investigations of Bcr-Abl signalling comparing low dose dasatinib, 30 min 100 nM dasatinib STD wash and 30 min 100 nM dasatinib optimal wash highlighted STAT5 as a potentially critical target in the survival of *BCR-ABL1*+ cells following challenge with TKI. Bcr-Abl activation of STAT5 has previously been implicated in the protection of cells from DNA-damage-induced apoptosis<sup>267</sup> and Warsch *et al.*<sup>153</sup> recently emphasised the role of STAT5 expression as an important mechanism of resistance to TKI treatment. Interestingly, high expression of STAT5 was found to facilitate imatinib resistance, independent of JAK2, significantly reducing sensitivity to TKI induced apoptosis.<sup>153</sup>

Recent data has revealed chemical inhibition of STAT5 activation in CML cells increasing sensitivity to TKI induced apoptosis.<sup>157,243</sup> Here the addition of the STAT5 inhibitors pimozone or N'-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide (STAT5i) in

combination with only a 30 min exposure to TKI, was demonstrated to induce cell death in both *BCR-ABL1*+ cell lines (**Figure 5.15**) and CP-CML CD34+ progenitor cells (**Figure 5.19**) despite complete removal (optimal washout) of dasatinib or imatinib after a 30 min exposure, a novel observation. If JAK2 is involved in Bcr-Abl activation of STAT5, it would be expected that inhibition of JAK2 would produce the same result as direct inhibition of STAT5, however treatment with ruxolitinib (a JAK2 inhibitor in clinical use in other malignancies) did not enhance cell death following 30 min dasatinib OPT wash (Figure 5.14). Treatment with STAT5i, which targets the SH2 domain of STAT5<sup>12</sup>, had little effect as a sole agent in *BCR-ABL1*+ cell lines or primary CP-CML CD34+ progenitor cells, thus supporting the recent findings mentioned above and suggesting that direct inhibition of STAT5 may be a promising therapeutic target in combination with TKI therapy.

To further define the role of STAT5 in TKI induced cell death, further genetic studies investigating the knockdown and rescue of STAT5 are required to confirm its role in the commitment to cell death following transient treatment with TKIs. Casetti *et al.*<sup>154</sup> recently described a role for STAT5 in protection of CML cells from stress (i.e. from the loss of Bcr-Abl dependent survival signals). This work demonstrated that attenuation of STAT5A with RNAi was unable to induce apoptosis, but instead resulted in increased DNA damaging reactive oxygen species (ROS) and subsequent activation of the p53/Chk2 pathway. This is supported by the results presented in Chapter 3, where an increase in Chk2 was observed following dasatinib treatment (Figure 3.11). As such, DNA damage as a result of STAT5 inhibition may contribute to the induction of autophagy in response to TKIs. Collectively, the results presented in Chapters 4 and 5

provide new insight into the biology of TKI therapy, challenging the premise that continuous Bcr-Abl kinase inhibition is required for sustained disease control.

### **7.1.3 TKI-induced autophagy occurs by inhibiting Bcr-Abl driven survival signalling and may involve energy sensing pathways**

The inactivation of Bcr-Abl oncogenic activity by TKI therapy results in a reduction of pro-survival and the induction of pro-apoptotic signalling consequently inducing cell death.<sup>93,142,164</sup> The loss of Bcr-Abl dependent survival signals creates a highly stressful environment for *BCR-ABL1*+ cells and recent observations have highlighted a role for STAT5 in protection of CML cells from TKI-induced stress.<sup>154</sup> Although knockdown of STAT5 was unable to induce apoptosis directly, *BCR-ABL1*+ cells were sensitised to TKI-induced apoptosis due to lack of protection from stress.<sup>154</sup> Intriguingly, current research suggests that chemotherapy-induced stress responses induce an autophagic response to protect cells from apoptosis,<sup>206,268-270</sup> rendering these studies applicable to a range of malignant diseases.

### **7.1.4 Tyrosine kinase inhibition of Bcr-Abl induces autophagy by activating stress response pathways**

Previous studies have established a role for TKI-induced autophagy as a mechanism of resistance to TKIs in CML,<sup>185,186</sup> however there is only speculation in regard to the processes by which inhibition of Bcr-Abl induces autophagy.<sup>271</sup> Thus, the underlying mechanisms of autophagy induction were investigated here following TKI inhibition. In Chapter 3, induction of autophagy by inhibition of Bcr-Abl with TKIs is confirmed in *BCR-ABL1*+ cell lines as demonstrated by increased LC3-II, and additionally, increased levels of p62 demonstrated induction of autophagy.

Bcr-Abl induces the overexpression of anti-apoptotic Bcl-2 to prevent apoptosis.<sup>47,67</sup> Beclin-1 is a signalling protein required for the induction of autophagy, which has previously been demonstrated to interact with both Bcl-2 and Bcl-x<sub>L</sub>, thereby preventing its availability for the induction of autophagy.<sup>210-212</sup> Chapter 3 provides novel insight into the availability of Beclin1 in the presence or absence of TKIs. Although Beclin-1 expression is unchanged following TKI treatment, the reduction in Bcl-2 and Bcl-x<sub>L</sub> observed would liberate Beclin-1 for initiation of autophagy (Figure 3.6). This interesting finding could be applicable when therapy-induced autophagy is observed in other diseases. It is hypothesised that autophagy could be induced by most cancers in response to chemotherapy,<sup>268</sup> especially targeted therapy. In our experience we did not observe induction of autophagy following targeted therapy in JAK2 driven MPN (D Ross, L Schafranek unpublished data). Thus understanding what drives autophagy following TKI treatment in CML may provide additional information about critical targets encompassing other malignancies.

A phosphoproteomic approach was undertaken to assess changes in kinase signalling following dasatinib treatment and revealed additional Bcr-Abl driven signals involved energy sensing and regulation of mTOR. The energy sensing LKB1/AMPK pathway, in conjunction with Pim3 kinases, is presented as a possible mechanism for TKI-induced autophagy (Figure 2.9 and Figure 2.11). Bcr-Abl constitutively activates several survival pathways, including but not limited to, those involving Pim kinases.<sup>272</sup> Pim kinases inhibit the activation of AMPK,<sup>200</sup> thereby preventing LKB1/AMPK inhibition of mTOR which consequently inhibits autophagy. Preliminary evidence presented here demonstrates that TKI inhibition of Bcr-Abl reduces Pim3 and increases LKB1 protein

expression (Figure 3.10 and Figure 3.8). Thus, this data implicates LKB1/AMPK inhibition of mTOR as a possible mechanism for TKI induced autophagy in *BCR-ABL1+* cells.

Additionally, preliminary observations in our laboratory demonstrate TKI-induced reductions in glucose transport genes (e.g. Glut3), indicating a TKI-induced decrease in glycolysis that could also stimulate an increase in LKB1/AMPK activity. Interestingly, change STAT5 phosphorylation is associated with autophagy in breast cancer<sup>273</sup> and STAT5 is known to activate Pim kinases<sup>274</sup> suggesting that inactivation of STAT5 may be involved in the induction of an autophagic response (Figure 7.1).

This preliminary research is also relevant to other chemotherapeutic resistant malignancies which utilise these metabolic pathways and highlights the requirement for investigations into the metabolic processes in CML cells following TKI therapy as a means of identifying important targets to counteract resistance to therapy.

#### **7.1.5 Combination treatment of TKI with clarithromycin targets autophagy in *BCR-ABL1+* cells**

Inhibition of autophagy with chloroquine (CQ) is currently being investigated in the CHOICES trial to determine its effectiveness as a combination treatment in conjunction with TKI therapy.<sup>275</sup> Traditionally a treatment for malaria, CQ is not a pleasant treatment long-term. Therefore it is desirable to find other therapies, in particular those already available with thoroughly evaluated pharmacokinetic profiles, to substitute for CQ that might be more amenable to a good quality of life for CML patients.

Clarithromycin (CAM) has previously been reported to be involved in autophagy in other cancers.<sup>189,276</sup> When CAM is used in combination with TKI a rapid reduction in BCR-ABL transcripts was observed in TKI-resistant CML patients.<sup>188</sup>

For the first time, CAM is demonstrated here to effectively increase the sensitivity of *BCR-ABL1*+ cells to TKI-induced cell death *in vitro*, whilst having little or no effect on cell death as a sole agent (Figure 3.13). The mechanism of induction of cell death by CAM combined with dasatinib appears to be via inhibition of late stage autophagy. Results presented here suggest that CAM inhibits autophagy in a similar way to the late stage autophagy inhibitor CQ, providing strong evidence to support CAM as a candidate for combination therapy, in particular in patients with resistant disease. These findings were published in the peer reviewed journal *Leukemia and Lymphoma*.<sup>277</sup>

#### **7.1.6 JAK2 activation of STAT5 occurs in a cytokine-dependent, but Bcr-Abl-independent manner**

Persistent disease in the majority of CML patients means that continuation of TKI therapy is required for life in most patients. Cytokine pathways including IL-3 and GM-CSF are utilised by Bcr-Abl in mature cells to maintain survival and proliferation.<sup>54</sup> It is therefore possible that the presence of cytokines in the bone marrow (BM) niche may provide protection to CML progenitor cells from the cytotoxic effect of TKI therapy.

The JAK/STAT pathway has been proposed to play a role in Abl oncogenic transformations.<sup>278,279</sup> Bcr-Abl constitutively activates STAT5,<sup>53,260</sup> however the mechanism by which it is activated is currently controversial. Ilaria *et al.*<sup>280</sup> observed no

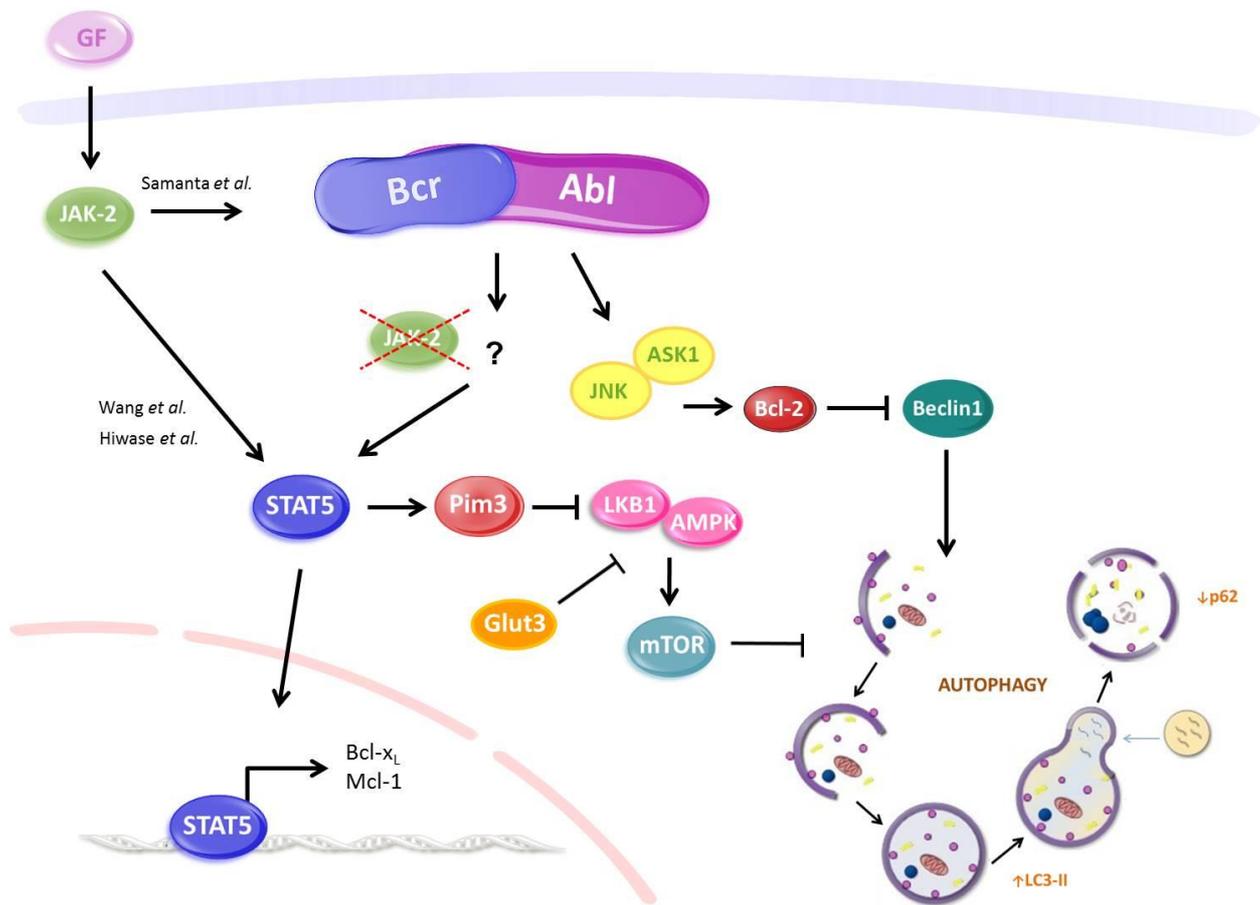


Figure 7.1: Proposed STAT5 and autophagy signalling

detectable complex between JAK2 and Bcr-Abl, however, Arlinghaus and colleagues have suggested that a direct interaction occurs between JAK2 and Bcr-Abl<sup>73</sup> and that JAK2 subsequently regulates Bcr-Abl signalling at the Y177 phosphorylation site on Bcr.<sup>148,258</sup> Recent findings by Jiang and colleagues postulate that the Abelson helper integration site 1 (AHI-1) interacts with Bcr-Abl and JAK2 and thus destabilisation of this complex by inhibition of JAK2 sensitizes cells to TKIs.<sup>281,282</sup> Our observations of JAK/STAT signalling demonstrated that inhibition of STAT5 increased the sensitivity of *BCR-ABL1*+ cells to TKIs, whilst combination with ruxolitinib (JAK2 inhibitor) had no additional effect on cell viability when compared to TKI alone. These results suggest that JAK2 may not be an important Bcr-Abl-dependent target, rather the specific targeting of STAT5 is required to induce cell death. Thus, we sought to clarify the role of JAK2 beyond Bcr-Abl-dependent signalling by assessing extrinsic/Bcr-Abl-independent, cytokine signalling.

As reported in Chapter 6, an investigation was undertaken into cytokine signalling as a mechanism of resistance to TKI. These studies determined the presence of cytokine receptors surface expression in *BCR-ABL1*+ cell lines and CP-CML CD34+ cells (Figure 6.4), which could be stimulated with appropriate cytokines to activate STAT5 (Figure 6.5). Inhibition of JAK2 with ruxolitinib demonstrated little effect alone and in combination with dasatinib demonstrated no increase in dasatinib sensitivity in the absence of cytokines (Figure 6.12). However, in cells cultured in the presence of cytokines, ruxolitinib effectively inhibited pSTAT5 (Figure 6.11) and thus abrogated cytokine protection of cells from dasatinib induced cell death (Figure 6.13), confirming previous observations that cytokine activation of JAK2 has a protective effect on CML cells.<sup>144,171</sup> These results are supported by *in vivo* evidence which suggests that JAK

kinase signalling is only relevant in Bcr-Abl-independent<sup>150</sup> extrinsic activation of STAT5; JAK2 inhibition had no effect on Bcr-Abl driven STAT5 signalling or cell death as a sole agent in the absence of cytokines.<sup>150,151</sup> Moreover, the absence of JAK2 did not affect disease maintenance following initial myeloid transformation in a CML-like murine model.<sup>151</sup> Conversely, STAT5 deletion resulted in failure to maintain leukemic hematopoiesis,<sup>152</sup> reinforcing the pivotal role for the STAT5 protein in leukemic cell survival.

In non-malignant cells, activation of STAT5 occurs by cytokine and growth factor signalling through JAK kinases. Thus, several lines of evidence suggest the critical involvement of STAT5<sup>153,157</sup> but not JAK2<sup>150</sup> in the sensitivity of Bcr-Abl to TKI therapy. Further investigations to determine the requirement for STAT5 over JAK2 would benefit from inducible knockouts of STAT5 and JAK2, and will further define the importance of STAT5 in resistance to TKI therapy.

The results presented here establish that STAT5 activation, as a result of active Bcr-Abl, does not occur through JAK2, however, further investigation is required to precisely determine the mechanism by which Bcr-Abl activates STAT5.

## 7.2 Future Directions

To further define the role of STAT5 and the kinetics of Bcr-Abl inhibition resulting from exposure to TKIs from that presented in this thesis, the following lines of investigation have been proposed:

1. An important next step would be to investigate the sensitive detection of dasatinib by LC or MS to conclusively define intracellular levels of dasatinib
  - a. following 1 nM dasatinib, 100 nM STD wash or 100 nM OPT wash over 24 h
  - b. *in vivo* snapshots of intracellular dasatinib at day 0 (pre-dose), day 1 (pre- and post-dose), day 7 (pre- and post-dose) and day 28 (pre- and post-dose)
2. To rule out off target effects of chemical inhibitors used in this study, genetic knockdown of STAT5 and JAK2 and rescue experiments utilising inducible STAT5 would demonstrate whether STAT5 aids in the protection cells of from TKI-induced stress and subsequently cell death.
3. To eliminate non-specific toxicity effects of STAT5 inhibitors experiments involving non-malignant cells *in vitro* in *BCR-ABL1*-ve cell lines and additionally in an *in vivo* murine model are required.

In this thesis, autophagy was determined by assessment of proteins known to be involved in autophagy (LC3 and p62) and by morphological changes of *BCR-ABL1*+ cells following TKI exposures in the presence or absence of autophagy inhibitors. Further assessment of autophagy should also assess;

1. Localisation of LC3 to vacuoles using fluorescence microscopy, electron microscopy of cells and genetic impairment/deletion of molecules required for the induction of autophagy (i.e. Beclin-1)

2. Further studies which define the underlying mechanisms of TKI induction of autophagy, will assess knockdown and overexpression experiments of top kinases from the kinase protein array and thus determine the value of these kinases to TKI-induced autophagy

### **7.3 Conclusion**

Continuous inhibition of Bcr-Abl activity is not required to induce cell death in *BCR-ABL1*<sup>+</sup> cells, rather the continuous inhibition of its downstream partner STAT5 is essential. This offers a plausible explanation for why transient exposure to once daily dasatinib therapy in a clinical setting is successful. The findings detailed here will help establish critical Bcr-Abl signaling components which may be targeted in combination therapeutic approaches, and may enable a better understanding of the potential clinical effectiveness of dose reductions during dasatinib therapy.

This thesis defines critical CML signalling components that may be targeted in combination therapeutic approaches. These studies will have broad implications for ongoing therapeutic strategies in TKI-resistant cases of CML and ultimately in making progress towards the elusive curative treatment.

## **CHAPTER 8.**

## **APPENDICES**

## Fullmoon kinase array antibody list

ID	Antibody Name	ID	Antibody Name	ID	Antibody Name	ID	Antibody Name
1	LRRK2	36	GRAP2	71	CAMK1D	106	EPHA7
2	BRAF	37	ILK	72	CAMK4	107	STK11
3	AHSA1	38	IRAK1	73	CAMKK1	108	SGK3
4	NTRK2	39	LATS1	74	CAMKK2	109	ANP32A
5	IRAK2	40	MAPK11	75	CDG2L2	110	FGFR2
6	SLPI	41	MAPK12	76	MAPKAPK3	111	EPHA4
7	STAT6	42	MAPK3	77	MILL4	112	CSF1
8	AXL	43	MAPK4	78	EFNA3	113	ZAP70
9	LINK1	44	MAPK6	79	FES	114	PDGFRA
10	EPB41L3	45	MAP2K1IP1	80	LOK	115	ROCK1
11	PTK7	46	MAP2K3	81	PRKACB	116	RPS6KA3
12	HIPK1	47	MAP3K5	82	ERBB2IP	117	INSRR
13	SGK2	48	MAPKAPK5	83	MAPK14	118	DMPK
14	STAT5A	49	MLKL	84	PRKAR1B	119	EIF2AK2
15	GRK7	50	PAK4	85	EPHA5	120	TGFB1
16	YARS	51	PA2G4	86	EPHB2	121	PRKRIR
17	RPS6KC1	52	PRKGBP1	87	EPHB4	122	MYLK
18	GRK4	53	PRKCZ	88	MAPK8	123	EPBA1
19	MYO3A	54	PRKD2	89	CSNK1G1	124	IKBKE
20	ACVR1B	55	PRKD3	90	BUB1B	125	MYO7A
21	BCR	56	PRKAA1	91	CSNK1G2	126	BMPR1A
22	CDKL2	57	PRKAG2	92	HIPK2	127	STAT3
23	CDKL3	58	PRACA	93	RPS6KA6	128	ATM
24	CDKL4	59	PRKDC	94	GRK6	129	PRKAB2
25	CDKL5	60	PRKRA	95	IKBKG	130	EPHB1
26	DYRK1A	61	PTK2	96	GRK1	131	MELK
27	DYRK4	62	PTK2B	97	MUSK	132	EPHA2
28	EFNA1	63	PTK6	98	FGR	133	RPS6KB2
29	EFNA5	64	RET	99	GRK5	134	PDGFRB
30	FGFR1OP	65	SAB	100	MST1R	135	EGFR
31	FGFR1OP2	66	TEK	101	TIE1	136	RAFI
32	FRAP1	67	TYK2	102	ABL2	137	MAPK1
33	FLT1	68	YES1	103	SYK	138	STAT1
34	FLT3LG	69	ROR2	104	CDK5	139	CSF2RA
35	FLT4	70	CAMK2A	105	FLT3	140	CSNK1D

## Appendix I

# Appendix I continued

ID	Antibody Name
141	CSNK1E
142	ROCK2
143	FGFR1
144	EPB41L1
145	ARG2
146	NTRK3
147	RPS8KA5
148	MYO9A
149	FGFR4
150	BMPR2
151	CSNK2A2
152	HIPK4
153	FGFR1
154	PTK9L
155	EPB42
156	MAP2K1
157	RPS8KA2
158	PRIN2A
159	PRKCA
160	GSK3B
161	FUSIP1
162	WNK2
163	MYLIP
164	RPS8KA1
165	AKT2
166	CDC2
167	CDKGR1
168	CDK8
169	CHEK2
170	STAT2
171	STAT5B
172	MYO9B
173	RNASEL
174	CDC25A

ID	Antibody Name
175	MYLK2
176	HERC4
177	EPHX1
178	ACTR1B
179	CDK2
180	EPDR1
181	CDK6
182	CDK4
183	TYRO3
184	INS
185	TGFB11
186	CDK7
187	CSF2
188	IGFBP1
189	CDC25B
190	SRC
191	NPR2
192	EPN2A
193	MAP2K2
194	RNMT
195	CDKGRAP3
196	CDK3
197	CDKL1
198	DYRK1B
199	DYRK2
200	ILKAP
201	IRAK3
202	IRAK4
203	JAK3
204	MAST2
205	MASTL
206	MAPK10
207	MAPK13
208	MAP3K13

ID	Antibody Name
209	MAP3K15
210	MAP3K4
211	MAP4K5
212	MAPKAPK2
213	PAK3
214	PIM1
215	PIM3
216	PRKCABP
217	PRKAA2
218	PRKAB1
219	ERBB3
220	KDR
221	CAMK2D
222	CAMK1G
223	CAMK2B
224	ROR1
225	CDK9
226	MAP2K6
227	MAP3K1
228	MAP3K11
229	MAP3K3
230	PRKAR2A
231	PRKX
232	MAP4K4
233	ERBB2
234	PTK9
235	EPHB3
236	EPHB6
237	PRKCD
238	MAPK9
239	CHEK1
240	IKBK1
241	PAK2
242	SGK

ID	Antibody Name
243	RNASEH1
244	RPS6KB1
245	BUB1
246	AKT1
247	RNASEH2A
248	MAP2K5
249	PAK1
250	PRKG1
251	NEK2
252	CAMK1
253	BMPRI1B
254	CSNK2A1
255	MAP3K7
256	CAMKV
257	CDC25C
258	PRKCSH
259	HERC3
260	PRKACG
261	RPS6
262	LNK2
263	WNK3
264	MAP3K7IP1
265	IGFBP6
266	PLK1
267	IKBKAP
268	ANK1
269	STAT4
270	PRKCI
271	DYRK3
272	PLK4
273	FYN
274	CSF1R
275	MERTK
276	EPHA6

Fullmoon Kinase array plate layout

**Block 1**

r1	P	E	E	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
r2	P	E	E	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
r3	P	E	E	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
r4	P	E	E	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
r5	P	E	E	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
r6	P	E	E	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29

**Block 2**

r1	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61
r2	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61
r3	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61
r4	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61
r5	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61
r6	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61

**Block 3**

r1	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93
r2	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93
r3	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93
r4	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93
r5	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93
r6	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93

**Block 4**

r1	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125
r2	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125
r3	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125
r4	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125
r5	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125
r6	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125

Appendix II

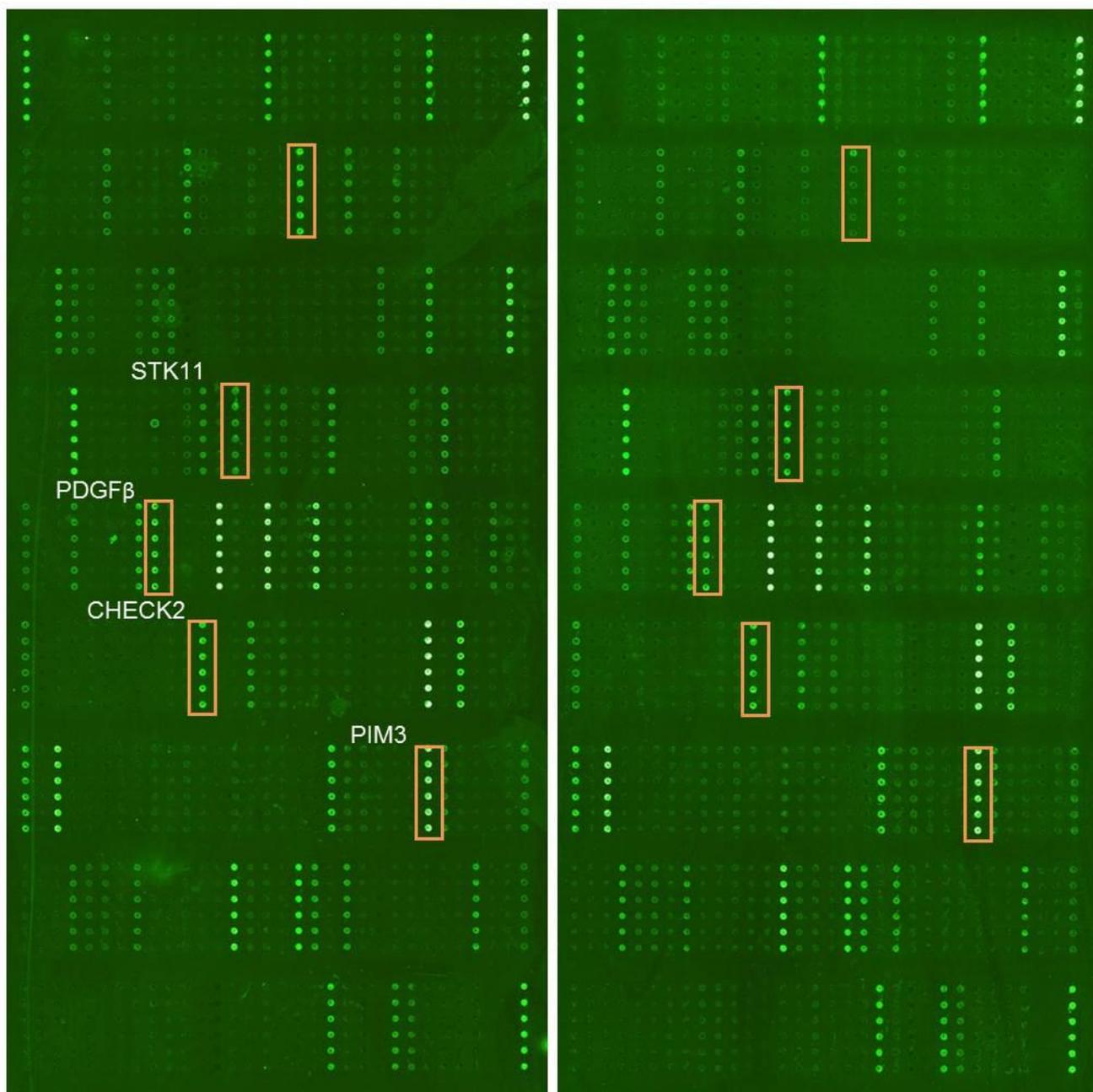


# Appendix III

KU812

0nM Dasatinib

100nM Dasatinib



## **CHAPTER 9.**

## **REFERENCES**

1. Galloway JL, Zon LI. Ontogeny of hematopoiesis: examining the emergence of hematopoietic cells in the vertebrate embryo. *Curr Top Dev Biol* 2003;53:139-58.
2. Cumano A, Godin I. Ontogeny of the hematopoietic system. *Annual review of immunology* 2007;25:745-85.
3. Tenen DG, Hromas R, Licht JD, Zhang DE. Transcription factors, normal myeloid development, and leukemia. *Blood* 1997;90:489-519.
4. Li CL, Johnson GR. Stem cell factor enhances the survival but not the self-renewal of murine hematopoietic long-term repopulating cells. *Blood* 1994;84:408-14.
5. Yang L, Dybedal I, Bryder D, et al. IFN-gamma negatively modulates self-renewal of repopulating human hemopoietic stem cells. *J Immunol* 2005;174:752-7.
6. Borge OJ, Ramsfjell V, Veiby OP, Murphy MJ, Jr., Lok S, Jacobsen SE. Thrombopoietin, but not erythropoietin promotes viability and inhibits apoptosis of multipotent murine hematopoietic progenitor cells in vitro. *Blood* 1996;88:2859-70.
7. Nilsson SK, Debatis ME, Dooner MS, Madri JA, Quesenberry PJ, Becker PS. Immunofluorescence characterization of key extracellular matrix proteins in murine bone marrow in situ. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* 1998;46:371-7.
8. Clark BR, Gallagher JT, Dexter TM. Cell adhesion in the stromal regulation of haemopoiesis. *Baillieres Clin Haematol* 1992;5:619-52.
9. Benveniste P, Cantin C, Hyam D, Iscove NN. Hematopoietic stem cells engraft in mice with absolute efficiency. *Nature immunology* 2003;4:708-13.
10. Orkin SH. Diversification of haematopoietic stem cells to specific lineages. *Nat Rev Genet* 2000;1:57-64.
11. Whetton AD, Graham GJ. Homing and mobilization in the stem cell niche. *Trends Cell Biol* 1999;9:233-8.
12. Velpeau A. Sur la résorption du pus et sur l'altération du sang dans les maladies. *Revue Médicale Française et étrangère* 1827:216-8.
13. Bennett JH. Case of hypertrophy of the spleen and liver in which death took place from suppuration of the blood. *Edinburgh Medical and Surgical Journal* 1845;64:413-23.
14. Donne A. *Cours de microscopie complémentaire des études médicales*. Balliere, Paris 1844;135:196.
15. Virchow R. Weisses Blut. *Froriep's Neue Notizen aus dem Gebiet der Natur.-und Heilkunde*. *Frorieps Notizen* 1845;36:151-6.
16. Nowell P, Hungerford D. A minute chromosome in human chronic granulocytic leukemia. *Science* 1960;132:1497.
17. Fialkow PJ, Denman AM, Jacobson RJ, Lowenthal MN. Chronic myelocytic leukemia. Origin of some lymphocytes from leukemic stem cells. *J Clin Invest* 1978;62:815-23.
18. Takahashi N, Miura I, Saitoh K, Miura AB. Lineage involvement of stem cells bearing the philadelphia chromosome in chronic myeloid leukemia in the chronic phase as shown by a combination of fluorescence-activated cell sorting and fluorescence in situ hybridization. *Blood* 1998;92:4758-63.
19. Rieger MA, Schroeder T. Hematopoiesis. *Cold Spring Harbor Perspectives in Biology* 2012;4.
20. Rowley JD. Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. *Nature* 1973;243:290-3.
21. Abelson HT, Rabstein LS. Lymphosarcoma: virus-induced thymic-independent disease in mice. *Cancer Res* 1970;30:2213-22.
22. de Klein A, van Kessel AG, Grosveld G, et al. A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukaemia. *Nature* 1982;300:765-7.

23. Groffen J, Stephenson JR, Heisterkamp N, de Klein A, Bartram CR, Grosveld G. Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. *Cell* 1984;36:93-9.
24. Canaani E, Gale RP, Steiner-Saltz D, Berrebi A, Aghai E, Januszewicz E. Altered transcription of an oncogene in chronic myeloid leukaemia. *Lancet* 1984;1:593-5.
25. Grosveld G, Verwoerd T, van Agthoven T, et al. The chronic myelocytic cell line K562 contains a breakpoint in bcr and produces a chimeric bcr/c-abl transcript. *Mol Cell Biol* 1986;6:607-16.
26. Shtivelman E, Lifshitz B, Gale RP, Canaani E. Fused transcript of abl and bcr genes in chronic myelogenous leukaemia. *Nature* 1985;315:550-4.
27. Konopka JB, Witte ON. Activation of the abl oncogene in murine and human leukemias. *Biochim Biophys Acta* 1985;823:1-17.
28. Ben-Neriah Y, Daley GQ, Mes-Masson AM, Witte ON, Baltimore D. The chronic myelogenous leukemia-specific P210 protein is the product of the bcr/abl hybrid gene. *Science* 1986;233:212-4.
29. Faderl S, Talpaz M, Estrov Z, Kantarjian HM. Chronic myelogenous leukemia: biology and therapy. *Ann Intern Med* 1999;131:207-19.
30. Sandberg AA. Cytogenetic staging of chronic myeloid leukemia (CML). *Bollettino dell'Istituto Sieroterapico Milanese* 1978;57:247-56.
31. Wong S, Witte ON. The BCR-ABL story: bench to bedside and back. *Annual review of immunology* 2004;22:247-306.
32. Faderl S, Talpaz M, Estrov Z, O'Brien S, Kurzrock R, Kantarjian HM. The biology of chronic myeloid leukemia. *N Engl J Med* 1999;341:164-72.
33. Altekruse SF, Krapcho M, Neyman N, Aminou R, Waldron W, Ruhl J, Howlander N, Tatalovich Z, Cho H, Mariotto A, Eisner MP, Lewis DR, Cronin K, Chen HS, Feuer EJ, Stinchcomb DG, Edwards BK. SEER Cancer Statistics Review, 1975-2007: National Cancer Institute; 2010.
34. Cortes J. Natural history and staging of chronic myelogenous leukemia. *Hematology-Oncology Clinics of North America* 2004;18:569-+.
35. Daley GQ, Van Etten RA, Baltimore D. Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. *Science* 1990;247:824-30.
36. Stam K. Dynamical-mass generation in large-N three-dimensional QED. *Physical review D: Particles and fields* 1986;34:2517-8.
37. Dhut S, Dorey EL, Horton MA, Ganesan TS, Young BD. Identification of two normal bcr gene products in the cytoplasm. *Oncogene* 1988;3:561-6.
38. Collins S, Coleman H, Groudine M. Expression of bcr and bcr-abl fusion transcripts in normal and leukemic cells. *Mol Cell Biol* 1987;7:2870-6.
39. Arlinghaus RB. Multiple BCR-related gene products and their proposed involvement in ligand-induced signal transduction pathways. *Mol Carcinog* 1992;5:171-3.
40. Wetzler M, Talpaz M, Yee G, et al. Cell cycle-related shifts in subcellular localization of BCR: association with mitotic chromosomes and with heterochromatin. *Proc Natl Acad Sci U S A* 1995;92:3488-92.
41. Wetzler M, Talpaz M, Van Etten RA, Hirsh-Ginsberg C, Beran M, Kurzrock R. Subcellular localization of Bcr, Abl, and Bcr-Abl proteins in normal and leukemic cells and correlation of expression with myeloid differentiation. *J Clin Invest* 1993;92:1925-39.
42. Kipreos ET, Wang JY. Cell cycle-regulated binding of c-Abl tyrosine kinase to DNA. *Science* 1992;256:382-5.
43. Van Etten RA. c-Abl regulation: a tail of two lipids. *Current biology : CB* 2003;13:R608-10.

44. Konopka JB, Witte ON. Detection of c-abl tyrosine kinase activity in vitro permits direct comparison of normal and altered abl gene products. *Mol Cell Biol* 1985;5:3116-23.
45. McWhirter JR, Wang JY. An actin-binding function contributes to transformation by the Bcr-Abl oncoprotein of Philadelphia chromosome-positive human leukemias. *EMBO J* 1993;12:1533-46.
46. Wetzler M, Talpaz M, Van Etten RA, Hirsh-Ginsberg C, Beran M, Kurzrock R. Subcellular localization of Bcr, Abl, and Bcr-Abl proteins in normal and leukemic cells and correlation of expression with myeloid differentiation. *Journal of Clinical Investigation* 1993;92:1925-39.
47. Cambier N, Chopra R, Strasser A, Metcalf D, Elefanty AG. BCR-ABL activates pathways mediating cytokine independence and protection against apoptosis in murine hematopoietic cells in a dose-dependent manner. *Oncogene* 1998;16:335-48.
48. Goldman JM, Melo JV. Mechanisms of disease: Chronic myeloid leukemia - Advances in biology and new approaches to treatment. *New England Journal of Medicine* 2003;349:1451-64.
49. Hehlmann R, Hochhaus A, Baccarani M. Chronic myeloid leukaemia. *Lancet* 2007;370:342-50.
50. Sawyers CL. Chronic myeloid leukemia. *New England Journal of Medicine* 1999;340:1330-40.
51. Puil L, Liu J, Gish G, et al. Bcr-Abl oncoproteins bind directly to activators of the Ras signalling pathway. *EMBO Journal* 1994;13:764-73.
52. Skorski T, Kanakaraj P, Nieborowska-Skorska M, et al. Phosphatidylinositol-3 kinase activity is regulated by BCR/ABL and is required for the growth of Philadelphia chromosome-positive cells. *Blood* 1995;86:726-36.
53. Danial NN, Rothman P. JAK-STAT signaling activated by Abl oncogenes. *Oncogene* 2000;19:2523-31.
54. Steelman LS, Pohnert SC, Shelton JG, Franklin RA, Bertrand FE, McCubrey JA. JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in cell cycle progression and leukemogenesis. *Leukemia* 2004;18:189-218.
55. Pendergast AM, Quilliam LA, Cripe LD, et al. BCR-ABL-induced oncogenesis is mediated by direct interaction with the SH2 domain of the GRB-2 adaptor protein. *Cell* 1993;75:175-85.
56. Sattler M, Mohi MG, Pride YB, et al. Critical role for Gab2 in transformation by BCR/ABL. *Cancer Cell* 2002;1:479-92.
57. Roskoski Jr R. Signaling by Kit protein-tyrosine kinase--The stem cell factor receptor. *Biochemical and Biophysical Research Communications* 2005;337:1-13.
58. Goga A, McLaughlin J, Afar DE, Saffran DC, Witte ON. Alternative signals to RAS for hematopoietic transformation by the BCR-ABL oncogene. *Cell* 1995;82:981-8.
59. ten Hoeve J, Kaartinen V, Fioretos T, et al. Cellular interactions of CRKL, and SH2-SH3 adaptor protein. *Cancer Res* 1994;54:2563-7.
60. ten Hoeve J, Arlinghaus RB, Guo JQ, Heisterkamp N, Groffen J. Tyrosine phosphorylation of CRKL in Philadelphia+ leukemia. *Blood* 1994;84:1731-6.
61. Neshat MS, Raitano AB, Wang HG, Reed JC, Sawyers CL. The survival function of the Bcr-Abl oncogene is mediated by Bad-dependent and -independent pathways: Roles for phosphatidylinositol 3-kinase and Raf. *Molecular and Cellular Biology* 2000;20:1179-86.
62. Tauchi T, Boswell HS, Leibowitz D, Broxmeyer HE. Coupling between p210bcr-abl and Shc and Grb2 adaptor proteins in hematopoietic cells permits growth factor receptor-independent link to ras activation pathway. *J Exp Med* 1994;179:167-75.
63. Plataniias LC. Map kinase signaling pathways and hematologic malignancies. *Blood* 2003;101:4667-79.

64. Nguyen TK, Rahmani M, Harada H, Dent P, Grant S. MEK1/2 inhibitors sensitize Bcr/Abl<sup>+</sup> human leukemia cells to the dual Abl/Src inhibitor BMS-354/825. *Blood* 2007;109:4006-15.
65. Raitano AB, Halpern JR, Hambuch TM, Sawyers CL. The Bcr-Abl leukemia oncogene activates Jun kinase and requires Jun for transformation. *Proc Natl Acad Sci U S A* 1995;92:11746-50.
66. Yu C, Krystal G, Varticovski L, et al. Pharmacologic mitogen-activated protein/extracellular signal-regulated kinase kinase/mitogen-activated protein kinase inhibitors interact synergistically with STI571 to induce apoptosis in Bcr/Abl-expressing human leukemia cells. *Cancer Research* 2002;62:188-99.
67. Sánchez-García I, Martín-Zanca D. Regulation of Bcl-2 gene expression by BCR-ABL is mediated by Ras. *Journal of Molecular Biology* 1997;267:225-8.
68. Li QF, Huang WR, Duan HF, Wang H, Wu CT, Wang LS. Sphingosine kinase-1 mediates BCR/ABL-induced upregulation of Mcl-1 in chronic myeloid leukemia cells. *Oncogene* 2007;26:7904-8.
69. Aichberger KJ, Mayerhofer M, Krauth MT, et al. Identification of mcl-1 as a BCR/ABL-dependent target in chronic myeloid leukemia (CML): Evidence for cooperative antileukemic effects of imatinib and mcl-1 antisense oligonucleotides. *Blood* 2005;105:3303-11.
70. Aichberger KJ, Mayerhofer M, Krauth MT, et al. Low-level expression of proapoptotic Bcl-2-interacting mediator in leukemic cells in patients with chronic myeloid leukemia: Role of BCR/ABL, characterization of underlying signaling pathways, and reexpression by novel pharmacologic compounds. *Cancer Research* 2005;65:9436-44.
71. Kuroda J, Puthalakath H, Cragg MS, et al. Bim and Bad mediated imatinib-induced killing of Bcr/Abl<sup>+</sup> leukemic cells, and resistance due to their loss is overcome by a BH3 mimetic. *Proceedings of the National Academy of Sciences of the United States of America* 2006;103:14907-12.
72. Klejman A, Schreiner SJ, Nieborowska-Skorska M, et al. The Src family kinase Hck couples BCR/ABL to STAT5 activation in myeloid leukemia cells. *EMBO Journal* 2002;21:5766-74.
73. Xie S, Wang Y, Liu J, et al. Involvement of Jak2 tyrosine phosphorylation in Bcr-Abl transformation. *Oncogene* 2001;20:6188-95.
74. Samanta AK, Lin H, Sun T, Kantarjian H, Arlinghaus RB. Janus kinase 2: a critical target in chronic myelogenous leukemia. *Cancer Res* 2006;66:6468-72.
75. Frank DA, Varticovski L. BCR/abl leads to the constitutive activation of Stat proteins, and shares an epitope with tyrosine phosphorylated Stats. *Leukemia* 1996;10:1724-30.
76. Gesbert F, Griffin JD. Bcr/Abl activates transcription of the Bcl-X gene through STAT5. *Blood* 2000;96:2269-76.
77. Horita M, Andreu EJ, Benito A, et al. Blockade of the Bcr-Abl kinase activity induces apoptosis of chronic myelogenous leukemia cells by suppressing signal transducer and activator of transcription 5-dependent expression of Bcl-x(L). *Journal of Experimental Medicine* 2000;191:977-84.
78. Goldman JM, Apperley JF, Jones L, et al. Bone-marrow transplantation for patients with chronic myeloid leukemia. *New England Journal of Medicine* 1986;314:202-7.
79. Clift RA, Storb R. Marrow transplantation for CML: The Seattle experience. 1996. p. S1-S3.
80. van Rhee F, Savage D, Blackwell J, et al. Adoptive immunotherapy for relapse of chronic myeloid leukemia after allogeneic bone marrow transplant: equal efficacy of lymphocytes from sibling and matched unrelated donors. *Bone Marrow Transplantation* 1998;21:1055-61.

81. Pusey WA. Report of cases treated with roentgen rays. *J Am Med Assoc* 1902;XXXVIII:911-9.
82. Senn N. Therapeutical value of Roentgen ray in treatment of pseudoleukemia. *New York Med J* 1903;77:665.
83. Osgood EE, Seaman AJ. Treatment of chronic leukemias; results of therapy by titrated, regularly spaced total body radioactive phosphorus, or roentgen irradiation. *J Am Med Assoc* 1952;150:1372-9.
84. Gollerkeri MP, Shah GB. Management of chronic myeloid leukemia: a five-year survey with a comparison of oral busulfan and splenic irradiation. *Cancer* 1971;27:596-601.
85. Dowling MD, Haghbin, M., Gee, T., Wakonig-Vaartaja, T., Clarkson, B. Attempt to induce true remissions in chronic myelocytic leukemia (CML) *Cancer Chemother Rep* 1973;57:102.
86. Clarkson B, Strife A, Wisniewski D, Lambek CL, Liu C. Chronic myelogenous leukemia as a paradigm of early cancer and possible curative strategies. *Leukemia* 2003;17:1211-62.
87. Goto T, Nishikori M, Arlin Z, et al. Growth characteristics of leukemic and normal hematopoietic-cells in Ph+ chronic myelogenous leukemia and effects of intensive treatment. *Blood* 1982;59:793-808.
88. Hehlmann R, Heimpel H, Hasford J, et al. Randomized comparison of interferon-alpha with busulfan and hydroxyurea in chronic myelogenous leukemia. The German CML Study Group. *Blood* 1994;84:4064-77.
89. Allan NC, Richards SM, Shepherd PC. UK Medical Research Council randomised, multicentre trial of interferon-alpha n1 for chronic myeloid leukaemia: improved survival irrespective of cytogenetic response. The UK Medical Research Council's Working Parties for Therapeutic Trials in Adult Leukaemia. *Lancet* 1995;345:1392-7.
90. Kloke O, Niederle N, Opalka B, Hawig I, Seeber S, Becher R. Prognostic impact of interferon alpha-induced cytogenetic remission in chronic myelogenous leukaemia: long-term follow-up. *Eur J Haematol* 1996;56:78-81.
91. Hochhaus A, Lin F, Reiter A, et al. Variable numbers of BCR-ABL transcripts persist in CML patients who achieve complete cytogenetic remission with interferon-alpha. *Br J Haematol* 1995;91:126-31.
92. Lee MS, Kantarjian H, Talpaz M, et al. Detection of minimal residual disease by polymerase chain reaction in Philadelphia chromosome-positive chronic myelogenous leukemia following interferon therapy. *Blood* 1992;79:1920-3.
93. Druker BJ, Tamura S, Buchdunger E, et al. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. *Nat Med* 1996;2:561-6.
94. Buchdunger E, Zimmermann J, Mett H, et al. Inhibition of the abl protein-tyrosine kinase in vitro and in vivo by a 2-phenylaminopyrimidine derivative. *Cancer Research* 1996;56:100-4.
95. McGary EC, Onn A, Mills L, et al. Imatinib mesylate inhibits platelet-derived growth factor receptor phosphorylation of melanoma cells but does not affect tumorigenicity in vivo. *J Invest Dermatol* 2004;122:400-5.
96. McGary EC, Weber K, Mills L, et al. Inhibition of platelet-derived growth factor-mediated proliferation of osteosarcoma cells by the novel tyrosine kinase inhibitor STI571. *Clin Cancer Res* 2002;8:3584-91.
97. Dewar AL, Cambareri AC, Zannettino ACW, et al. Macrophage colony-stimulating factor receptor c-fms is a novel target of imatinib. *Blood* 2005;105:3127-32.
98. Okuda K, Weisberg E, Gilliland DG, Griffin JD. ARG tyrosine kinase activity is inhibited by STI571. *Blood* 2001;97:2440-8.

99. Cohen MH, Williams G, Johnson JR, et al. Approval summary for imatinib mesylate capsules in the treatment of chronic myelogenous leukemia. *Clinical Cancer Research* 2002;8:935-42.
100. Buchdunger E, Cioffi CL, Law N, et al. Abl protein-tyrosine kinase inhibitor STI571 inhibits in vitro signal transduction mediated by c-kit and platelet-derived growth factor receptors. *J Pharmacol Exp Ther* 2000;295:139-45.
101. Druker BJ. Perspectives on the development of a molecularly targeted agent. *Cancer Cell* 2002;1:31-6.
102. Hughes T, Kaeda J, Branford S, et al. Molecular responses to imatinib (STI571) or interferon plus Ara-C as initial therapy for CML; Results in the IRIS study. *Blood* 2002;100:345.
103. Druker BJ, Guilhot F, O'Brien SG, et al. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *New England Journal of Medicine* 2006;355:2408-17.
104. Larson RA, Druker BJ, Guilhot F, et al. Imatinib pharmacokinetics and its correlation with response and safety in chronic-phase chronic myeloid leukemia: a subanalysis of the IRIS study. *Blood* 2008;111:4022-8.
105. Branford S, Lawrence R, Grigg A, et al. Long Term Follow up of Patients with CML in Chronic Phase Treated with First-Line Imatinib Suggests That Earlier Achievement of a Major Molecular Response Leads to Greater Stability of Response. 2008: *Blood*. p. 735-6.
106. Hochhaus A, O'Brien SG, Guilhot F, et al. Six-year follow-up of patients receiving imatinib for the first-line treatment of chronic myeloid leukemia. *Leukemia* 2009.
107. Manley PW, Breitenstein W, Bruggen J, et al. Urea derivatives of STI571 as inhibitors of Bcr-Abl and PDGFR kinases. *Bioorg Med Chem Lett* 2004;14:5793-7.
108. Weisberg E, Manley PW, Cowan-Jacob SW, Hochhaus A, Griffin JD. Second generation inhibitors of BCR-ABL for the treatment of imatinib-resistant chronic myeloid leukaemia. *Nat Rev Cancer* 2007;7:345-56.
109. Kantarjian H, Giles F, Wunderle L, et al. Nilotinib in imatinib-resistant CML and Philadelphia chromosome-positive ALL. *N Engl J Med* 2006;354:2542-51.
110. Saglio G, Kim DW, Issaragrisil S, et al. Nilotinib versus imatinib for newly diagnosed chronic myeloid leukemia. *N Engl J Med* 2010;362:2251-9.
111. Talpaz M, Shah NP, Kantarjian H, et al. Dasatinib in imatinib-resistant Philadelphia chromosome-positive leukemias. *N Engl J Med* 2006;354:2531-41.
112. Jabbour E, Kantarjian H, Jones D, et al. Characteristics and outcomes of patients with chronic myeloid leukemia and T315I mutation following failure of imatinib mesylate therapy. *Blood* 2008;112:53-5.
113. Jabbour E, Kantarjian HM, Jones D, et al. Characteristics and outcome of chronic myeloid leukemia patients with F317L BCR-ABL kinase domain mutation after therapy with tyrosine kinase inhibitors. *Blood* 2008;112:4839-42.
114. Shah NP, Tran C, Lee FY, Chen P, Norris D, Sawyers CL. Overriding imatinib resistance with a novel ABL kinase inhibitor. *Science* 2004;305:399-401.
115. Mauro MJ, Baccarani M, Cervantes F, et al. Dasatinib 2-year efficacy in patients with chronic-phase chronic myelogenous leukemia (CML-CP) with resistance or intolerance to imatinib (START-C). *J Clin Oncol (Meeting Abstracts)* 2008;26:7009-.
116. Hochhaus A, Baccarani M, Deininger M, et al. Dasatinib induces durable cytogenetic responses in patients with chronic myelogenous leukemia in chronic phase with resistance or intolerance to imatinib. *Leukemia* 2008;22:1200-6.
117. Hochhaus A, Muller MC, Radich J, et al. Dasatinib-associated major molecular responses in patients with chronic myeloid leukemia in chronic phase following imatinib failure: response dynamics and predictive value. *Leukemia* 2009;23:1628-33.

118. O'Brien SG, Guilhot F, Goldman JM, et al. International Randomized Study of Interferon Versus STI571 (IRIS) 7-Year Follow-up: Sustained Survival, Low Rate of Transformation and Increased Rate of Major Molecular Response (MMR) in Patients (pts) with Newly Diagnosed Chronic Myeloid Leukemia in Chronic Phase (CMLCP) Treated with Imatinib (IM). *ASH Annual Meeting Abstracts* 2008;112:186-.
119. Deininger MWN. Optimizing therapy of chronic myeloid leukemia. *Experimental Hematology* 2007;35:144-54.
120. Hochhaus A. Prognostic factors in chronic myeloid leukemia (CML). *Onkologie* 2008;31:576-8.
121. Jabbour E, Cortes JE, Kantarjian HM. Suboptimal Response to or Failure of Imatinib Treatment for Chronic Myeloid Leukemia: What Is the Optimal Strategy? *Mayo Clinic Proceedings* 2009;84:161-9.
122. Baccarani M, Saglio G, Goldman J, et al. Evolving concepts in the management of chronic myeloid leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet. *Blood* 2006;108:1809-20.
123. Barnes DJ, Melo JV. Primitive, quiescent and difficult to kill: The role of non-proliferating stem cells in chronic myeloid leukemia. *Cell Cycle* 2006;5:2862-6.
124. Bhatia R, Holtz M, Niu N, et al. Persistence of malignant hematopoietic progenitors in chronic myelogenous leukemia patients in complete cytogenetic remission following imatinib mesylate treatment. *Blood* 2003;101:4701-7.
125. Graham SM, Jørgensen HG, Allan E, et al. Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. *Blood* 2002;99:319-25.
126. Barnes DJ, Palaiologou D, Panousopoulou E, et al. Bcr-Abl expression levels determine the rate of development of resistance to imatinib mesylate in chronic myeloid leukemia. *Cancer Res* 2005;65:8912-9.
127. Branford S, Rudzki Z, Walsh S, et al. Detection of BCR-ABL mutations in patients with CML treated with imatinib is virtually always accompanied by clinical resistance, and mutations in the ATP phosphate-binding loop (P-loop) are associated with a poor prognosis. *Blood* 2003;102:276-83.
128. Delbaldo C, Chatelut E, Re M, et al. Pharmacokinetic-pharmacodynamic relationships of imatinib and its main metabolite in patients with advanced gastrointestinal stromal tumors. *Clinical Cancer Research* 2006;12:6073-8.
129. Peng B, Lloyd P, Schran H. Clinical pharmacokinetics of imatinib. *Clinical Pharmacokinetics* 2005;44:879-94.
130. Picard S, Titier K, Etienne G, et al. Trough imatinib plasma levels are associated with both cytogenetic and molecular responses to standard-dose imatinib in chronic myeloid leukemia. *Blood* 2007;109:3496-9.
131. White DL, Saunders VA, Dang PO, et al. OCT-1-mediated influx is a key determinant of the intracellular uptake of imatinib but not nilotinib, (AMN107): reduced OCT-1 activity is the cause of low in vitro sensitivity to imatinib. *Blood* 2006;108:697-704.
132. le Coutre P, Mologni L, Cleris L, et al. In vivo eradication of human BCR/ABL-positive leukemia cells with an ABL kinase inhibitor. *J Natl Cancer Inst* 1999;91:163-8.
133. White D, Saunders V, Lyons AB, et al. In vitro sensitivity to imatinib-induced inhibition of ABL kinase activity is predictive of molecular response in patients with de novo CML. *Blood* 2005;106:2520-6.
134. Brave M, Goodman V, Kaminskas E, et al. Sprycel for chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia resistant to or intolerant of imatinib mesylate. *Clin Cancer Res* 2008;14:352-9.

135. Shah NP, Kantarjian HM, Kim DW, et al. Intermittent target inhibition with dasatinib 100 mg once daily preserves efficacy and improves tolerability in imatinib-resistant and -intolerant chronic-phase chronic myeloid leukemia. *J Clin Oncol* 2008;26:3204-12.
136. Hiwase DK, White DL, Saunders VA, Kumar S, Melo JV, Hughes TP. Short-term intense Bcr-Abl kinase inhibition with nilotinib is adequate to trigger cell death in BCR-ABL(+) cells. *Leukemia* 2009;23:1205-6.
137. Shah NP, Kasap C, Weier C, et al. Transient potent BCR-ABL inhibition is sufficient to commit chronic myeloid leukemia cells irreversibly to apoptosis. *Cancer Cell* 2008;14:485-93.
138. Snead JL, O'Hare T, Adrian LT, et al. Acute dasatinib exposure commits Bcr-Abl-dependent cells to apoptosis. *Blood* 2009;114:3459-63.
139. Weinstein IB. Cancer. Addiction to oncogenes--the Achilles heel of cancer. *Science* 2002;297:63-4.
140. Bagnyukova T, Serebriiskii IG, Zhou Y, Hopper-Borge EA, Golemis EA, Astsaturov I. Chemotherapy and signaling: How can targeted therapies supercharge cytotoxic agents? *Cancer Biology and Therapy* 2010;10:839-53.
141. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* 1995;270:1326-31.
142. Sharma SV, Gajowniczek P, Way IP, et al. A common signaling cascade may underlie "addiction" to the Src, BCR-ABL, and EGF receptor oncogenes. *Cancer Cell* 2006;10:425-35.
143. Sharma SV, Fischbach MA, Haber DA, Settleman J. "Oncogenic shock": explaining oncogene addiction through differential signal attenuation. *Clin Cancer Res* 2006;12:4392s-5s.
144. Hiwase DK, White DL, Powell JA, et al. Blocking cytokine signaling along with intense Bcr-Abl kinase inhibition induces apoptosis in primary CML progenitors. *Leukemia* 2010;24:771-8.
145. Orkin SH. Stem cell alchemy. *Nat Med* 2000;6:1212-3.
146. Watanabe S, Itoh T, Arai K. Roles of JAK kinase in human GM-CSF receptor signals. *Leukemia* 1997;11 Suppl 3:76-8.
147. Warsch W, Walz C, Sexl V. JAK of all trades: JAK2-STAT5 as novel therapeutic targets in BCR-ABL1+ chronic myeloid leukemia. *Blood* 2013.
148. Samanta A, Perazzona B, Chakraborty S, et al. Janus kinase 2 regulates Bcr-Abl signaling in chronic myeloid leukemia. *Leukemia* 2011;25:463-72.
149. Yamada O, Ozaki K, Furukawa T, et al. Activation of STAT5 confers imatinib resistance on leukemic cells through the transcription of TERT and MDR1. *Cell Signal* 2011;23:1119-27.
150. Traer E, MacKenzie R, Snead J, et al. Blockade of JAK2-mediated extrinsic survival signals restores sensitivity of CML cells to ABL inhibitors. *Leukemia* 2011.
151. Hantschel O, Warsch W, Eckelhart E, et al. BCR-ABL uncouples canonical JAK2-STAT5 signaling in chronic myeloid leukemia. *Nat Chem Biol* 2012;advance online publication.
152. Hoelbl A, Schuster C, Kovacic B, et al. Stat5 is indispensable for the maintenance of Bcr/Abl-positive leukaemia. *EMBO Molecular Medicine* 2010;2:98-110.
153. Warsch W, Kollmann K, Eckelhart E, et al. High STAT5 levels mediate imatinib resistance and indicate disease progression in chronic myeloid leukemia. *Blood* 2011;blood-2009-10-248211.
154. Casetti L, Martin-Lannerée S, Najjar I, et al. Differential contributions of STAT5A and STAT5B to stress protection and tyrosine kinase inhibitor resistance of chronic myeloid leukemia stem/progenitor cells. *Cancer Research* 2013.
155. Bertolesi GE, Shi C, Elbaum L, et al. The Ca(2+) channel antagonists mibefradil and pimozide inhibit cell growth via different cytotoxic mechanisms. *Mol Pharmacol* 2002;62:210-9.

156. Tecott LH, Kwong LL, Uhr S, Peroutka SJ. Differential modulation of dopamine D2 receptors by chronic haloperidol, nitrendipine, and pimozide. *Biological psychiatry* 1986;21:1114-22.
157. Nelson EA, Walker SR, Weisberg E, et al. The STAT5 inhibitor pimozide decreases survival of chronic myelogenous leukemia cells resistant to kinase inhibitors. *Blood* 2011;blood-2009-11-255232.
158. Rousselot P, Huguet F, Rea D, et al. Imatinib mesylate discontinuation in patients with chronic myelogenous leukemia in complete molecular remission for more than 2 years. *Blood* 2007;109:58-60.
159. Bocchia M, Ippoliti M, Gozzetti A, et al. CD34+/Ph+ cells are still detectable in chronic myeloid leukemia patients with sustained and prolonged complete cytogenetic remission during treatment with imatinib mesylate. *Leukemia* 2008;22:426-8.
160. Chu S, Lin A, McDonald T, Snyder DS, Forman SJ, Bhatia R. Persistence of Leukemia Stem Cells in Chronic Myelogenous Leukemia Patients in Complete Cytogenetic Remission on Imatinib Treatment for 5 Years. *ASH Annual Meeting Abstracts* 2008;112:194-.
161. Jorgensen HG, Allan EK, Jordanides NE, Mountford JC, Holyoake TL. Nilotinib exerts equipotent antiproliferative effects to imatinib and does not induce apoptosis in CD34+ CML cells. *Blood* 2007;109:4016-9.
162. Copland M, Hamilton A, Elrick LJ, et al. Dasatinib (BMS-354825) targets an earlier progenitor population than imatinib in primary CML but does not eliminate the quiescent fraction. *Blood* 2006;107:4532-9.
163. Konig H, Holtz M, Modi H, et al. Enhanced BCR-ABL kinase inhibition does not result in increased inhibition of downstream signaling pathways or increased growth suppression in CML progenitors. *Leukemia* 2008;22:748-55.
164. Gambacorti-Passerini C, le Coutre P, Mologni L, et al. Inhibition of the ABL kinase activity blocks the proliferation of BCR/ABL+ leukemic cells and induces apoptosis. *Blood Cells Mol Dis* 1997;23:380-94.
165. Okuda K, Foster R, Griffin JD. Signaling domains of the beta c chain of the GM-CSF/IL-3/IL-5 receptor. *Ann N Y Acad Sci* 1999;872:305-12; discussion 12-3.
166. Carroll M, Ohno-Jones S, Tamura S, et al. CGP 57148, a Tyrosine Kinase Inhibitor, Inhibits the Growth of Cells Expressing BCR-ABL, TEL-ABL, and TEL-PDGFR Fusion Proteins. *Blood* 1997;90:4947-52.
167. Hariharan IK, Adams JM, Cory S. bcr-abl oncogene renders myeloid cell line factor independent: potential autocrine mechanism in chronic myeloid leukemia. *Oncogene Res* 1988;3:387-99.
168. Jiang X, Lopez A, Holyoake T, Eaves A, Eaves C. Autocrine production and action of IL-3 and granulocyte colony-stimulating factor in chronic myeloid leukemia. *Proceedings of the National Academy of Sciences of the United States of America* 1999;96:12804-9.
169. Jiang X, Ng E, Yip C, et al. Primitive interleukin 3 null hematopoietic cells transduced with BCR-ABL show accelerated loss after culture of factor-independence in vitro and leukemogenic activity in vivo. *Blood* 2002;100:3731-40.
170. Sirard C, Laneuville P, Dick JE. Expression of bcr-abl abrogates factor-dependent growth of human hematopoietic M07E cells by an autocrine mechanism. *Blood* 1994;83:1575-85.
171. Wang Y, Cai D, Brendel C, et al. Adaptive secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF) mediates imatinib and nilotinib resistance in BCR/ABL+ progenitors via JAK-2/STAT-5 pathway activation. *Blood* 2007;109:2147-55.
172. Jiang X, Fujisaki T, Nicolini F, et al. Autonomous multi-lineage differentiation in vitro of primitive CD34<sup>+</sup> cells from patients with chronic myeloid leukemia. *Leukemia* 2000;14:1112-21.

173. Dorsey JF, Cunnick JM, Lanehart R, et al. Interleukin-3 protects Bcr-Abl-transformed hematopoietic progenitor cells from apoptosis induced by Bcr-Abl tyrosine kinase inhibitors. *Leukemia* 2002;16:1589-95.
174. Liu XH, Tang CS. [The paracrine and autocrine of stem cells: a new frontier of basic and clinical research]. *Sheng Li Ke Xue Jin Zhan* 2008;39:196-202.
175. Jiang X, Zhao Y, Smith C, et al. Chronic myeloid leukemia stem cells possess multiple unique features of resistance to BCR-ABL targeted therapies. *Leukemia* 2007;21:926-35.
176. Belloc F, Airiau K, Jeanneteau M, et al. The stem cell factor-c-KIT pathway must be inhibited to enable apoptosis induced by BCR-ABL inhibitors in chronic myelogenous leukemia cells. *Leukemia* 2009;23:679-85.
177. Jordan CT, Upchurch D, Szilvassy SJ, et al. The interleukin-3 receptor alpha chain is a unique marker for human acute myelogenous leukemia stem cells. *Leukemia* 2000;14:1777-84.
178. Munoz L, Nomdedeu JF, Lopez O, et al. Interleukin-3 receptor alpha chain (CD123) is widely expressed in hematologic malignancies. *Haematologica* 2001;86:1261-9.
179. Jin L, Lee EM, Ramshaw HS, et al. Monoclonal antibody-mediated targeting of CD123, IL-3 receptor alpha chain, eliminates human acute myeloid leukemic stem cells. *Cell Stem Cell* 2009;5:31-42.
180. Florian S, Sonneck K, Hauswirth AW, et al. Detection of molecular targets on the surface of CD34+/CD38-- stem cells in various myeloid malignancies. *Leuk Lymphoma* 2006;47:207-22.
181. Konig H, Copland M, Chu S, Jove R, Holyoake TL, Bhatia R. Effects of dasatinib on SRC kinase activity and downstream intracellular signaling in primitive chronic myelogenous leukemia hematopoietic cells. *Cancer Res* 2008;68:9624-33.
182. Rathmell JC, Vander Heiden MG, Harris MH, Frauwirth KA, Thompson CB. In the absence of extrinsic signals, nutrient utilization by lymphocytes is insufficient to maintain either cell size or viability. *Mol Cell* 2000;6:683-92.
183. Yang Z, Klionsky DJ. Eaten alive: a history of macroautophagy. *Nat Cell Biol* 2010;12:814-22.
184. Mishima Y, Terui Y, Taniyama A, et al. Autophagy and autophagic cell death are next targets for elimination of the resistance to tyrosine kinase inhibitors. *Cancer Sci* 2008;99:2200-8.
185. Bellodi C, Lidonnici MR, Hamilton A, et al. Targeting autophagy potentiates tyrosine kinase inhibitor-induced cell death in Philadelphia chromosome-positive cells, including primary CML stem cells. *Journal of Clinical Investigation* 2009;119:1109-23.
186. Crowley LC, Elzinga BM, O'Sullivan GC, McKenna SL. Autophagy induction by Bcr-Abl-expressing cells facilitates their recovery from a targeted or nontargeted treatment. *Am J Hematol* 2011;86:38-47.
187. Solomon VR, Lee H. Chloroquine and its analogs: a new promise of an old drug for effective and safe cancer therapies. *European journal of pharmacology* 2009;625:220-33.
188. Carella AM, Beltrami G, Pica G, Carella A, Catania G. Clarithromycin potentiates tyrosine kinase inhibitor treatment in patients with resistant chronic myeloid leukemia. *Leuk Lymphoma* 2012;13:13.
189. Nakamura M, Kikukawa Y, Takeya M, Mitsuya H, Hata H. Clarithromycin attenuates autophagy in myeloma cells. *Int J Oncol* 2010;37:815-20.
190. Kroemer G, Jaattela M. Lysosomes and autophagy in cell death control. *Nat Rev Cancer* 2005;5:886-97.
191. Carew JS, Nawrocki ST, Kahue CN, et al. Targeting autophagy augments the anticancer activity of the histone deacetylase inhibitor SAHA to overcome Bcr-Abl-mediated drug resistance. *Blood* 2007;110:313-22.

192. Choi KS. Autophagy and cancer. *Experimental and Molecular Medicine* 2012;44:109-20.
193. Kabeya Y, Mizushima N, Yamamoto A, Oshitani-Okamoto S, Ohsumi Y, Yoshimori T. LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. *J Cell Sci* 2004;117:2805-12.
194. Miyamura K, Takeo T, Kataoka T, Tahara T, Tanimoto M, Saito H. Detection of minimal residual disease in Philadelphia chromosome positive acute lymphoblastic leukemia: rationale for bone marrow transplantation from the polymerase chain reaction point of view. *Leuk Lymphoma* 1993;11:181-9.
195. Fernandez-Luna JL. Bcr-Abl and inhibition of apoptosis in chronic myelogenous leukemia cells. *Apoptosis* 2000;5:315-8.
196. Collins SJ, Groudine MT. Chronic myelogenous leukemia: amplification of a rearranged c-abl oncogene in both chronic phase and blast crisis. *Blood* 1987;69:893-8.
197. Green AS, Chapuis N, Lacombe C, Mayeux P, Bouscary D, Tamburini J. LKB1/AMPK/mTOR signaling pathway in hematological malignancies: From metabolism to cancer cell biology. *Cell Cycle* 2011;10:2115-20.
198. Lin F-R, Huang S-Y, Hung K-H, et al. ASK1 promotes apoptosis of normal and malignant plasma cells. *Blood* 2012;120:1039-47.
199. Webber LM, Garson OM. Fluorodeoxyuridine synchronization of bone marrow cultures. *Cancer Genet Cytogenet* 1983;8:123-32.
200. Beharry Z, Mahajan S, Zemsanova M, et al. The Pim protein kinases regulate energy metabolism and cell growth. *Proceedings of the National Academy of Sciences* 2011;108:528-33.
201. Tang C, Schafranek L, Watkins DB, et al. Tyrosine kinase inhibitor resistance in chronic myeloid leukemia cell lines: investigating resistance pathways. *Leuk Lymphoma* 2011.
202. Branford S, Melo JV, Hughes TP. Selecting optimal second-line tyrosine kinase inhibitor therapy for chronic myeloid leukemia patients after imatinib failure: does the BCR-ABL mutation status really matter? *Blood* 2009;114:5426-35.
203. Behrends C, Sowa ME, Gygi SP, Harper JW. Network organization of the human autophagy system. *Nature* 2010;466:68-76.
204. Lozy F, Karantza V. Autophagy and cancer cell metabolism. *Semin Cell Dev Biol* 2012;23:395-401.
205. Hammerman PS, Fox CJ, Birnbaum MJ, Thompson CB. Pim and Akt oncogenes are independent regulators of hematopoietic cell growth and survival. *Blood* 2005;105:4477-83.
206. Abedin MJ, Wang D, McDonnell MA, Lehmann U, Kelekar A. Autophagy delays apoptotic death in breast cancer cells following DNA damage. *Cell Death Differ* 2007;14:500-10.
207. Ren Y, Huang F, Liu Y, Yang Y, Jiang Q, Xu C. Autophagy inhibition through PI3K/Akt increases apoptosis by sodium selenite in NB4 cells. *BMB Rep* 2009;42:599-604.
208. Amaravadi RK, Yu D, Lum JJ, et al. Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. *J Clin Invest* 2007;117:326-36.
209. Klionsky DJ, Abdalla FC, Abeliovich H, et al. Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy* 2012;8:445-544.
210. Levine B, Sinha S, Kroemer G. Bcl-2 family members: dual regulators of apoptosis and autophagy. *Autophagy* 2008;4:600-6.
211. Marquez RT, Xu L. Bcl-2:Beclin 1 complex: multiple, mechanisms regulating autophagy/apoptosis toggle switch. *Am J Cancer Res* 2012;2:214-21.
212. Pattingre S, Tassa A, Qu X, et al. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* 2005;122:927-39.

213. He C, Levine B. The Beclin 1 interactome. *Current opinion in cell biology* 2010;22:140-9.
214. Kang R, Zeh HJ, Lotze MT, Tang D. The Beclin 1 network regulates autophagy and apoptosis. *Cell Death Differ* 2011;18:571-80.
215. Can G, Ekiz HA, Baran Y. Imatinib induces autophagy through BECLIN-1 and ATG5 genes in chronic myeloid leukemia cells. *Hematology* 2011;16:95-9.
216. Puissant A, Robert G, Fenouille N, et al. Resveratrol promotes autophagic cell death in chronic myelogenous leukemia cells via JNK-mediated p62/SQSTM1 expression and AMPK activation. *Cancer Res* 2010;70:1042-52.
217. Mukaida N, Wang YY, Li YY. Roles of Pim-3, a novel survival kinase, in tumorigenesis. *Cancer Sci* 2011;102:1437-42.
218. Degtyarev M, De Mazière A, Orr C, et al. Akt inhibition promotes autophagy and sensitizes PTEN-null tumors to lysosomotropic agents. *The Journal of Cell Biology* 2008;183:101-16.
219. Wu WKK, Cho CH, Lee CW, et al. Macroautophagy and ERK phosphorylation counteract the anti-proliferative effect of proteasome inhibitor in gastric cancer cells. *Autophagy* 2010;6:228-38.
220. Wu YT, Tan HL, Shui G, et al. Dual role of 3-methyladenine in modulation of autophagy via different temporal patterns of inhibition on class I and III phosphoinositide 3-kinase. *J Biol Chem* 2010;285:10850-61.
221. Tsujimoto Y, Shimizu S. Another way to die: autophagic programmed cell death. *Cell Death Differ* 2005;12 Suppl 2:1528-34.
222. Deininger MWN, Goldman JM, Melo JV. The molecular biology of chronic myeloid leukemia. *Blood* 2000;96:3343-56.
223. Skorski T, Bellacosa A, Nieborowska-Skorska M, et al. Transformation of hematopoietic cells by BCR/ABL requires activation of a PI-3k/Akt-dependent pathway. *EMBO J* 1997;16:6151-61.
224. Porkka K, Khoury HJ, Paquette RL, Matloub Y, Sinha R, Cortes JE. Dasatinib 100 mg once daily minimizes the occurrence of pleural effusion in patients with chronic myeloid leukemia in chronic phase and efficacy is unaffected in patients who develop pleural effusion. *Cancer* 2010;116:377-86.
225. Bandyopadhyay G, Biswas T, Roy KC, et al. Chlorogenic acid inhibits Bcr-Abl tyrosine kinase and triggers p38 mitogen-activated protein kinase-dependent apoptosis in chronic myelogenous leukemic cells. *Blood* 2004;104:2514-22.
226. Sharma SV, Settleman J. Exploiting the balance between life and death: Targeted cancer therapy and "oncogenic shock". *Biochemical Pharmacology* 2010;80:666-73.
227. Simara P, Stejskal S, Koutna I, et al. Apoptosis in chronic myeloid leukemia cells transiently treated with imatinib or dasatinib is caused by residual BCR-ABL kinase inhibition. *Am J Hematol* 2013.
228. Carlesso N, Frank DA, Griffin JD. Tyrosyl phosphorylation and DNA binding activity of signal transducers and activators of transcription (STAT) proteins in hematopoietic cell lines transformed by Bcr/Abl. *J Exp Med* 1996;183:811-20.
229. Jain M, Arvanitis C, Chu K, et al. Sustained loss of a neoplastic phenotype by brief inactivation of MYC. *Science* 2002;297:102-4.
230. Luo FR, Yang Z, Camuso A, et al. Dasatinib (BMS-354825) Pharmacokinetics and Pharmacodynamic Biomarkers in Animal Models Predict Optimal Clinical Exposure. *Clinical Cancer Research* 2006;12:7180-6.
231. Cogle CR. Overcoming chronic myeloid leukemia stem cell resistance to imatinib by also targeting JAK2. *J Natl Cancer Inst* 2013;105:378-9.

232. Lipka DB, Wagner MC, Dziadosz M, et al. Intracellular Retention of ABL Kinase Inhibitors Determines Commitment to Apoptosis in CML Cells. *PLoS One* 2012;7:e40853.
233. O'Hare T, Eide CA, Agarwal A, et al. Threshold levels of ABL tyrosine kinase inhibitors retained in chronic myeloid leukemia cells determine their commitment to apoptosis. *Cancer research* 2013;73:3356-70.
234. Wagner MC, Dziadosz M, Melo JV, Heidel F, Fischer T, Lipka DB. Nilotinib shows prolonged intracellular accumulation upon pulse-exposure: a novel mechanism for induction of apoptosis in CML cells. *Leukemia* 2012.
235. Simara P, Stejskal S, Koutna I, et al. Apoptosis in Chronic Myeloid Leukemia Cells Transiently Treated with Imatinib or Dasatinib Is Caused by Residual BCR-ABL Kinase Inhibition. *ASH Annual Meeting Abstracts* 2012;120:1668-.
236. White D, Saunders V, Grigg A, et al. Measurement of in vivo BCR-ABL kinase inhibition to monitor imatinib-induced target blockade and predict response in chronic myeloid leukemia. *Journal of Clinical Oncology* 2007;25:4445-51.
237. Sohn SJ, Barrett K, Van Abbema A, et al. A Restricted Role for TYK2 Catalytic Activity in Human Cytokine Responses Revealed by Novel TYK2-Selective Inhibitors. *J Immunol* 2013;191:2205-16.
238. Nieborowska-Skorska M, Wasik MA, Slupianek A, et al. Signal transducer and activator of transcription (STAT)5 activation by BCR/ABL is dependent on intact Src homology (SH)3 and SH2 domains of BCR/ABL and is required for leukemogenesis. *J Exp Med* 1999;189:1229-42.
239. Akgul C. Mcl-1 is a potential therapeutic target in multiple types of cancer. *Cellular and Molecular Life Sciences* 2009;66:1326-36.
240. Epling-Burnette PK, Bai F, Nimanapalli R, et al. Mcl-1 is a novel mediator of Bcr-Abl anti-apoptotic activity. *Blood* 2001;98.
241. le Coutre P, Tassi E, Varella-Garcia M, et al. Induction of resistance to the Abelson inhibitor STI571 in human leukemic cells through gene amplification. *Blood* 2000;95:1758-66.
242. Jiang J, Greulich H, Janne PA, Sellers WR, Meyerson M, Griffin JD. Epidermal growth factor-independent transformation of Ba/F3 cells with cancer-derived epidermal growth factor receptor mutants induces gefitinib-sensitive cell cycle progression. *Cancer Res* 2005;65:8968-74.
243. Soliera AR, Mariani SA, Audia A, et al. Gfi-1 inhibits proliferation and colony formation of p210BCR/ABL-expressing cells via transcriptional repression of STAT 5 and Mcl-1. *Leukemia* 2012;26:1555-63.
244. Jaffe ES, Harris NL, Diebold J, Muller-Hermelink HK. World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues. A progress report. *Am J Clin Pathol* 1999;111:S8-12.
245. Bhatia R, Holtz M, Niu N, et al. Persistence of malignant hematopoietic progenitors in chronic myelogenous leukemia patients in complete cytogenetic remission following imatinib mesylate treatment. *Blood* 2003;101:4701-7.
246. Jørgensen HG, Holyoake TL. Characterization of cancer stem cells in chronic myeloid leukaemia. *Biochemical Society Transactions* 2007;35:1347-51.
247. Corbin AS, Agarwal A, Loriaux M, Cortes J, Deininger MW, Druker BJ. Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. *Journal of Clinical Investigation* 2011;121:396-409.
248. Bagley CJ, Woodcock JM, Stomski FC, Lopez AF. The structural and functional basis of cytokine receptor activation: lessons from the common beta subunit of the granulocyte-macrophage colony-stimulating factor, interleukin-3 (IL-3), and IL-5 receptors. *Blood* 1997;89:1471-82.

249. Liu J, Joha S, Idziorek T, et al. BCR-ABL mutants spread resistance to non-mutated cells through a paracrine mechanism. *Leukemia* 2008;22:791-9.
250. Ohtomo T, Miyazawa K, Naito M, et al. Cytoprotective effect of imatinib mesylate in non-BCR-ABL-expressing cells along with autophagosome formation. *Biochem Biophys Res Commun* 2010;391:310-5.
251. Nievergall E, Ramshaw HS, Yong ASM, et al. Monoclonal antibody targeting of IL-3 receptor  $\alpha$  with CSL362 effectively depletes CML progenitor and stem cells. *Blood* 2013.
252. Quintás-Cardama A, Vaddi K, Liu P, et al. Preclinical characterization of the selective JAK1/2 inhibitor INCB018424: therapeutic implications for the treatment of myeloproliferative neoplasms. *Blood* 2010;115:3109-17.
253. Quintás-Cardama, K V, P L, et al. Preclinical characterization of the selective JAK1/2 inhibitor INCB018424: therapeutic implications for the treatment of myeloproliferative neoplasms. *Blood* 2010;115:3109-17.
254. Shuai K, Halpern J, Ten Hoeve J, Rao X, Sawyers CL. Constitutive activation of STAT5 by the BCR-ABL oncogene in chronic myelogenous leukemia. *Oncogene* 1996;13:247-54.
255. Verstovsek S, Kantarjian H, Mesa RA, et al. Safety and Efficacy of INCB018424, a JAK1 and JAK2 Inhibitor, in Myelofibrosis. *New England Journal of Medicine* 2010;363:1117-27.
256. Asmussen J, Lasater EA, Tajon C, et al. MEK-Dependent Negative Feedback Underlies BCR-ABL-Mediated Oncogene Addiction. *Cancer Discovery* 2013.
257. Shi JG, Chen X, McGee RF, et al. The Pharmacokinetics, Pharmacodynamics, and Safety of Orally Dosed INCB018424 Phosphate in Healthy Volunteers. *The Journal of Clinical Pharmacology* 2011.
258. Samanta AK, Chakraborty SN, Sun X, Schlette E, Priebe W, Arlinghaus RB. Jak2 Phosphorylates Tyr 177 of Bcr-Abl Activating the Ras and PI-3 Kinase Pathways and Maintains Functional Levels of Bcr-Abl in Chronic Myelogenous Leukemia. *ASH Annual Meeting Abstracts* 2009;114:39-.
259. Alvira D, Naughton R, Bhatt L, Tedesco S, Landry WD, Cotter TG. Inhibition of protein-tyrosine phosphatase 1B (PTP1B) mediates ubiquitination and degradation of Bcr-Abl protein. *J Biol Chem* 2011;286:32313-23.
260. Campàs-Moya C. Ruxolitinib. Tyrosine-protein kinase JAK1/2 inhibitor, treatment of myelofibrosis, treatment of myeloproliferative neoplasms, treatment of psoriasis. *Drugs of the Future* 2010;35:457-65.
261. Kuroda J, Taniwaki M. Life and death of leukemic cells under Bcr-Abl signaling control. *Current Cancer Therapy Reviews* 2009;5:303-9.
262. Baker SJ, Reddy EP. Targeted inhibition of kinases in cancer therapy. *Mount Sinai Journal of Medicine* 2010;77:573-86.
263. Shah NP, Kim DW, Kantarjian H, et al. Potent, transient inhibition of BCR-ABL with dasatinib 100 mg daily achieves rapid and durable cytogenetic responses and high transformation-free survival rates in chronic phase chronic myeloid leukemia patients with resistance, suboptimal response or intolerance to imatinib. *Haematologica* 2010;95:232-40.
264. Kantarjian H, Shah NP, Hochhaus A, et al. Dasatinib versus imatinib in newly diagnosed chronic-phase chronic myeloid leukemia. *New England Journal of Medicine* 2010;362:2260-70.
265. Visani G, Breccia M, Gozzini A, et al. Dasatinib, even at low doses, is an effective second-line therapy for chronic myeloid leukemia patients resistant or intolerant to imatinib. Results from a real life-based Italian multicenter retrospective study on 114 patients. *American Journal of Hematology* 2010;85:960-3.
266. O'Hare T, Eide CA, Adrian LT, et al. Cryptic Intracellular Retention of ABL Tyrosine Kinase Inhibitors within CML Cells Mediates Apoptosis Commitment Following Acute Drug Exposure. *ASH Annual Meeting Abstracts* 2011;118:3504-.

267. Hoover RR, Gerlach MJ, Koh EY, Daley GQ. Cooperative and redundant effects of STAT5 and Ras signaling in BCR/ABL transformed hematopoietic cells. *Oncogene* 2001;20:5826-35.
268. Amaravadi RK, Thompson CB. The Roles of Therapy-Induced Autophagy and Necrosis in Cancer Treatment. *Clinical Cancer Research* 2007;13:7271-9.
269. Gong K, Chen C, Zhan Y, Chen Y, Huang Z, Li W. Autophagy-related gene 7 (Atg7) and reactive oxygen species (ROS)/ extracellular-signal-regulated kinase (ERK) regulate tetradrine-induced autophagy in human hepatocellular carcinoma. *Journal of Biological Chemistry* 2012.
270. Kang R, Tang D, Loze MT, Zeh HJ. Apoptosis to autophagy switch triggered by the MHC class III-encoded receptor for advanced glycation endproducts (RAGE). *Autophagy* 2011;7:91-3.
271. Calabretta B, Salomoni P. Suppression of autophagy by BCR/ABL. *Frontiers in bioscience* 2012;4:453-60.
272. Van Etten RA. Cycling, stressed-out and nervous: cellular functions of c-Abl. *Trends Cell Biol* 1999;9:179-86.
273. Holmes FA, Espina V, Liotta LA, et al. Pathologic complete response after preoperative anti-HER2 therapy correlates with alterations in PTEN, FOXO, phosphorylated Stat5, and autophagy protein signaling. *BMC research notes* 2013;6:507.
274. Brault L, Gasser C, Bracher F, Huber K, Knapp S, Schwaller J. PIM serine/threonine kinases in the pathogenesis and therapy of hematologic malignancies and solid cancers. *Haematologica* 2010;95:1004-15.
275. ChOICES - CHlorOquine and Imatinib Combination to Eliminate Stem cells. (Accessed Jan, 2014, at <http://public.ukcrn.org.uk/search/StudyDetail.aspx?StudyID=8492>.)
276. Altman JK, Plataniias LC. A New Purpose for an Old Drug: Inhibiting Autophagy with Clarithromycin. *Leukemia & Lymphoma*;0:1-5.
277. Schafranek L, Leclercq TM, White DL, Hughes TP. Clarithromycin enhances dasatinib-induced cell death in chronic myeloid leukemia cells, by inhibition of late stage autophagy. *Leuk Lymphoma* 2013;54:198-201.
278. Danial NN, Pernis A, Rothman PB. Jak-STAT signaling induced by the v-abl oncogene. *Science* 1995;269:1875-7.
279. Sattler M, Griffin JD. Molecular mechanisms of transformation by the BCR-ABL oncogene. *Seminars in Hematology* 2003;40:4-10.
280. Ilaria Jr RL, Van Etten RA. P210 and P190(BCR/ABL) induce the tyrosine phosphorylation and DNA binding activity of multiple specific STAT family members. *Journal of Biological Chemistry* 1996;271:31704-10.
281. Zhou LL, Zhao Y, Ringrose A, et al. AHI-1 interacts with BCR-ABL and modulates BCR-ABL transforming activity and imatinib response of CML stem/progenitor cells. *Journal of Experimental Medicine* 2008;205:2657-71.
282. Chen M, Gallipoli P, DeGeer D, et al. Targeting primitive chronic myeloid leukemia cells by effective inhibition of a new AHI-1-BCR-ABL-JAK2 complex. *J Natl Cancer Inst* 2013;105:405-23.

# **PUBLICATIONS ARISING FROM THESIS**

# Statement of Authorship

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## Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

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Contribution to the Paper	Helped in data interpretation, evaluated and edited the manuscript.		
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Name of Co-Author	Deborah L White		
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