TKI resistance in CML cell lines: Investigating resistance pathways

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Declaration

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

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Carine Tang

1st September 2011
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Romans 13:7  *Render therefore to all their dues: tribute to whom tribute is due; custom to whom custom; fear to whom fear; honour to whom honour.*

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Psalms 18:30 As for God, His way is perfect: the word of the LORD is tried: He is a buckler to all those that trust in Him.
### Glossary

<table>
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<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>ABCB1/ABCG2</td>
<td>ATP binding cassette (ABC) transporter proteins B1 and G2</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute lymphoblastic leukaemia</td>
</tr>
<tr>
<td>AP</td>
<td>Accelerated phase</td>
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<td>BCR-ABL</td>
<td>Breakpoint cluster region-Ableson kinase fusion transcript/protein</td>
</tr>
<tr>
<td>Bcr-Abl</td>
<td>Breakpoint cluster region-Ableson kinase fusion gene</td>
</tr>
<tr>
<td>C</td>
<td>Celcius</td>
</tr>
<tr>
<td>CCR</td>
<td>Complete cytogenetic response</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CHR</td>
<td>Complete haematologic response</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myeloid leukaemia</td>
</tr>
<tr>
<td>CP</td>
<td>Chronic phase</td>
</tr>
<tr>
<td>Crkl</td>
<td>CT10 regulator of kinase-like</td>
</tr>
<tr>
<td>DABCO</td>
<td>1,4-diazabicyclo[2.2.2]octane</td>
</tr>
<tr>
<td>DAS</td>
<td>Dasatinib</td>
</tr>
<tr>
<td>ddNTP</td>
<td>Dideoxynucleotide triphosphate</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>dmin</td>
<td>Double minutes</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphates</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescent in situ hybridisation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GUSB</td>
<td>Beta-glucuronidase</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks Balanced Salt Solution</td>
</tr>
<tr>
<td>Hck</td>
<td>Haemopoietic cell kinase</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HSR</td>
<td>Homogenously staining region</td>
</tr>
<tr>
<td>IC50</td>
<td>Inhibitory concentration 50%</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IM</td>
<td>Imatinib mesylate</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>KD</td>
<td>Kinase domain</td>
</tr>
<tr>
<td>kD</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>KDR</td>
<td>Kinase insert domain protein receptor</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>Lyn</td>
<td>V-yes-1 Yamaguchi sarcoma viral related oncogene homolog</td>
</tr>
<tr>
<td>M</td>
<td>Molar (Moles per litre)</td>
</tr>
<tr>
<td>Mbq</td>
<td>Mega Becquerel ($10^6$ Becquerel)</td>
</tr>
<tr>
<td>MCR</td>
<td>Major cytogenetic response</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
</tr>
<tr>
<td>µCi</td>
<td>Micro Curie ($10^6$ Curie)</td>
</tr>
<tr>
<td>µg</td>
<td>Micro gram ($10^{-6}$ gram)</td>
</tr>
<tr>
<td>µM</td>
<td>Micro molar ($10^{-6}$ Molar)</td>
</tr>
<tr>
<td>MMR</td>
<td>Major molecular response</td>
</tr>
<tr>
<td>mM</td>
<td>Milli molar ($10^{-3}$ Molar)</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end-joining</td>
</tr>
<tr>
<td>NIL</td>
<td>Nilotinib</td>
</tr>
<tr>
<td>nM</td>
<td>Nano molar ($10^{-9}$ Molar)</td>
</tr>
<tr>
<td>OCT-1</td>
<td>Organic cation transporter 1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Ph</td>
<td>Philadelphia chromosome</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PI3-K</td>
<td>Phosphatidylinositol – 3-kinase</td>
</tr>
<tr>
<td>P-loop</td>
<td>Phosphate binding loop</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene Fluoride</td>
</tr>
<tr>
<td>P-value</td>
<td>Probability value</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear factor kappa-b ligand</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver operating characteristic</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute (-1640 medium)</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RQ-PCR</td>
<td>Real-time quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SH</td>
<td>Src Homology domain</td>
</tr>
<tr>
<td>SN</td>
<td>Supernatant</td>
</tr>
<tr>
<td>Src</td>
<td>Sarcoma</td>
</tr>
<tr>
<td>SSC</td>
<td>Saline-Sodium Citrate buffer</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline with 0.1% Tween20</td>
</tr>
<tr>
<td>TKI</td>
<td>Tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
</tbody>
</table>
Abstract

Chronic myeloid leukaemia (CML) is characterised by the presence of the Philadelphia chromosome which harbours the Bcr-Abl oncogene. BCR-ABL is a constitutively active tyrosine kinase that can be inhibited by rationally designed tyrosine kinase inhibitors (TKIs) such as imatinib, nilotinib and dasatinib. Although TKI therapy is an effective treatment for many patients, resistance can arise. There are currently four identified resistance mechanisms. These are 1) overexpression of drug-efflux proteins (ABCB1 and ABCG2), 2) BCR-ABL kinase domain (KD) mutations, 3) increased BCR-ABL expression and 4) BCR-ABL independent mechanisms such as Lyn kinase expression. In this study the interplay between these four recognised modes of TKI resistance is investigated.

Imatinib- and dasatinib-resistant cell lines were established and used to investigate TKI resistance in vitro. Viability and IC50 assays were used to demonstrate TKI sensitivity/resistance. Flow cytometry was used to screen for ABCB1 and ABCG2 cell surface expression, while conventional sequencing and the MassARRAY method were used to determine the mutation status of the BCR-ABL KD. Fluorescence in situ hybridisation (FISH) and quantitative DNA PCR were used to investigate Bcr-Abl DNA copy number, and RQ-PCR was used to investigate expression levels of BCR-ABL and Lyn mRNA.

These studies revealed that IM-resistant K562 cell lines exhibited increased BCR-ABL expression at the onset of resistance. Interestingly, these cell lines had increased viability and IC50s for IM and NIL, while the DAS IC50s were variable. Further investigation revealed Lyn overexpression in the cell line which was more sensitive to DAS. The development of a DAS-resistant K562 culture resulted in the emergence of the T315I mutation. Studies of the intermediate stages of resistance of this DAS-resistant cell line revealed that increased BCR-ABL expression occurred gradually, preceding the emergence of the mutation, at which time the BCR-ABL expression decreased and plateaued. Thus, it appears that increased BCR-ABL expression may be the initial mechanism of resistance, followed by the emergence of a KD mutation which has a clear selective advantage. This phenomenon was observed a further four times (in a DAS-resistant K562 Dox culture, and in three IM-resistant KU812 cultures) each time with the emergence of different KD mutations. Different KD mutations resulted in differential resistance to the three TKIs used in this study.
In contrast, three IM-resistant K562 Dox cell lines were not found to have any KD mutations, nor BCR-ABL overexpression. Instead, the primary cause of resistance in these lines appeared to be an increase in ABCB1 expression. The addition of PSC833 (an ABCB1 inhibitor) decreased the IM, NIL, and DAS IC50s for all three resistant lines to the level of the naïve control. This indicated that ABCB1 expression, facilitating active efflux of the drugs, is the primary mechanism of resistance in these lines.

This study demonstrates that KD emergence is a stochastic event, as the same mutation did not always occur twice when exposed to the same TKI. However, increased ABCB1 expression was more likely to arise recurrently in the predisposed K562 Dox cell line. Notably, different TKIs elicited different resistant mechanisms, and all but one (the Lyn overexpressing K562 cell line) were BCR-ABL dependent. Furthermore, all resistant cell lines showed cross-resistance (at least to some extent) to the three TKIs tested, suggesting that currently available TKIs share the same susceptibilities to drug resistance.