Evaluation of Anti-proliferative and Pro-apoptotic Effects of Tyrosine Kinase Inhibitors on CML-CD34+ Cells

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ABSTRACT

Although imatinib (IM) has revolutionalised CML management, 30 to 40% patients fail IM therapy. Many of these patients can be rescued with second generation tyrosine kinase inhibitors (TKI), dasatinib, nilotinib and bosutinib. This research elucidates the dasatinib cellular transport pathways and its role in mediating dasatinib resistance. It also assesses dynamics of Bcr-Abl kinase inhibition and apoptosis in CML lines and CML-$CD34^+$ progenitors. Lastly it addresses the role of cytokines in mediating TKI resistance and possible combination therapy to circumvent cytokine mediated TKI resistance.

The organic cation transporter (OCT-1) mediates IM influx and low OCT-1 activity is a major contributor to suboptimal response in CML patients treated with IM. In the current study the relevance of OCT-1 activity and efflux pumps in determining intracellular concentration (IUR) of dasatinib were assessed. In contrast to IM, dasatinib cellular uptake is not significantly affected by OCT-1 activity, so that expression and function of OCT-1 is unlikely to affect response to dasatinib. Dasatinib is a substrate of efflux proteins, ABCB1 and ABCG2. Overexpression of these proteins can mediate dasatinib resistance. There is increasing evidence that nilotinib is an ABCB1 inhibitor. These different interactions of dasatinib and nilotinib with ABCB1 were exploited for combination therapy. Nilotinib increased ^{14}C-Dasatinib IUR and had synergistic effect in inducing cell death in ABCB1 overexpressing cells. These data suggest that combinations of these two TKI can overcome ABCB1 mediated dasatinib resistance and may allow the use of lower concentrations of each drug.

In contrast to IM, dasatinib cellular influx is predominantly passive and maximum intracellular concentration is achieved within a few minutes. This was further confirmed by the observation of maximum Bcr-Abl kinase inhibition within 30 minutes of culture with dasatinib. Despite reactivation of Bcr-Abl kinase within 30 minutes of drug washout, short-term (30 minutes) intense (>90%) Bcr-Abl kinase inhibition with dasatinib triggers apoptosis in CML cell lines. This is in contrast to the previously established paradigm that continuous kinase inhibition is required for optimal response to IM. These results were further supported by a recently published dasatinib dose optimisation study. Further work in this thesis demonstrated that although Bcr-Abl kinase reactivates within 30 minutes of drug washout, the prosurvival proteins Erk, AKT and STAT5 dephosphorylated rapidly while the apoptotic proteins remained phosphorylated. This differential degradation of prosurvival and apoptotic proteins might be responsible for a state of "oncogenic-shock", as described by Sharma *et al.*

Subsequent studies demonstrated that in the absence of cytokines, short-term intense Bcr-Abl kinase inhibition with therapeutically achievable concentration of dasatinib (100 nM dasatinib) eliminated 70 to

80% of CML-CD34$^+$ progenitors. However, in the presence of cytokines despite >90% Bcr-Abl kinase inhibition it did not trigger cell death in CML progenitors. These results suggest that intense Bcr-Abl kinase inhibition alone may not be adequate to trigger cell death in CML progenitors. Further studies demonstrated that cytokines mediate TKI resistance by activating JAK2-STAT5 pathway and that the combination of JAK2 inhibitor and TKI can circumvent cytokine mediated TKI resistance.

DECLARATION

Name: Devendra Hiwase Program: Doctorate of Philosophy

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Retrospectively reflecting on what really spurred me to enter the dynamic world of cancer research in particular and medicine in general, I realized that while my PhD began only in late 2005/early 2006, it has its beginnings on the precise day of 5th October 1984, this is a day seared in my memory as it was on this day that my father passed away. He had been diagnosed with cancer when I was still in year 10. Treatment in those days was vastly different in kind and expectation as to the outcome compared to what it is today, and my father declared that he would accept treatment only at the hands of his son when he became a doctor. Unfortunately that was not to be as he passed away soon after, but I promised him that I would one day join the fight against cancer. That is the day the seed was sown and led me to pursuing a career path in medicine with a PhD in cancer research being the apotheosis of my formal learning. Because it was my father who was my first inspiration, I dedicate this thesis to him. I feel in reaching the culmination of my PhD- and in keeping my word given to my father- I have contributed to some extent to cancer research, but going through this journey has made me realize that my progress is a mere blip on the wide horizon of cancer research, the seeming apotheosis just the beginning of what will likely be a lifetime of never-ending learning.

My PhD itself- like the ones who have trod this path before and doubtless will follow after me- would not have been possible without my mentor and principal supervisor Professor Timothy Hughes. He was the driving force behind my progress to an immeasurable degree, making me realize my potential to the extent I would not have thought possible. There were times when my progress seemed to be rather a weary trudge, and it would be Tim who would encourage me, and if necessary, guide me in the right direction. Sunny days appeared to be few and far between, but when experiments worked, as they ought to, there used to be a spring in my stride. During those times he encouraged me to go further, and tended to ask after a meeting "But, what about…" and a mental image of the prospect of another month of experiments would loom before me initially, though the challenge would edge me on. Apart being a researcher at the pinnacle of his chosen field Tim is a clinician, and a supervisor of several other students. Despite this he always found time to provide his input on the chapters of my thesis within a remarkable two to three days. He fostered in me the idea of forging national and international collaborative research, and I look forward to taking this further in the future.

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ABBREVIATIONS

7-AAD	7-Aminoactinomycin D
Ab	Antibody
ABL1	Abelson murine leukaemia virus human homologue 1 gene
ACD-A	Anticoagulant citrate dextrose solution formula A
Akt	a serine threonine kinase also known as protein kinase B
ALL	Acute lymphoblastic leukaemia
AMN107	Second generation kinase inhibitor – nilotinib
AP	Accelerated phase
ATP	Adenosine triphosphate
BAD	Bcl-Xl/Bcl-2 associated death promoter
BAX	Bcl-2 associated X protein
Bcl-XL	A 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
BFU-E	Burst forming unit erythroid
Bim	Bcl-2 interacting mediator of cell death
BM	Bone Marrow
bp	Nucleotide base pairs
BSA	Bovine serum albumin
CML	Chronic myeloid leukaemia
C	Degrees Celsius
^{14}C	Carbon ^{14} labelled drugs
CCR	Complete cytogenetic response
cDNA	Complementary deoxyribonucleic acid
CE	Clonal evolution
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CFU	Colony forming unit
CFU-GM	Colony forming unit granulocytes and macrophage
CFU-GEMM	Colony forming unit granulocyte, erythroid, macrophages and megakaryocyte
CFC	Colony forming cells

CHR	Complete haematologic response
CP	Chronic phase
Crkl	Crkl oncogene like protein
CV	Coefficient of variation
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulphoxide
DMEM	Dulbecco's minimum essential media
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide triphosphates
DSB	Double strand break
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
Erk	Extracellular signal related kinase
e.g	*exempli gratia*
et al	*et alia*
FACS	Fluorescence activated cells sorting
FBS	Foetal bovine serum
FISH	Fluorescent *in situ* hybridisation
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
FLT-3 ligand	FMS-like tyrosine kinase 3 ligand
FOXO	Member of the forkhead family of transcriptional regulators
gDNA	Genomic DNA
Gab2	GRB2-associated-binding protein 2
G-CSF	granulocyte-colony stimulating factor
GFP	green fluorescent protein
6-GF	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
GVHD	graft versus host disease
h	Hours
HBSS	Hanks Balanced Salt Solution
HLA	Human leucocyte antigen
HSC	Haemopoietic stem cells
HSCT	Haemopoietic stem cell transplantation

IC50	Concentartion of drug required to inhibit Bcr-Abl kinase activity by 50%
$IC50^{dasatinib}$	Dasatinib concentration required to inhibit Bcr-Abl kinase activity by 50%
i.e.	*id est*
IFN-AraC	interferon-_ plus low dose cytarabine
IL-3	Interleukin-3
IL-6	Interleukin-6
Ig	Immunoglobulin
IM	Imatinib mesylate
IMDM	Iscove's modification of Dulbecco's medium.
IMVS	Institute of Medical & Veterinary Science
IRIS	International randomised study of interferon versus STI571
IS	International Standard (*BCR-ABL* mRNA level)
JAK	Janus Kinase
Kb	Kilo base pairs
kD	kilo Dalton
L	Litres
LBC	Lymphoid blast crisis
LB	Luria broth
log	$Logarithm_{10}$
LT	Long-term
M	Molar
mA	mili Amp (10^{-3} Amps)
MACS	Magnetic activated cell sorting
MBC	Myeloid blast crisis
MAPK	Mitogen activated protein kinase
M-bcr	Major breakpoint cluster region
m-bcr	Minor breakpoint cluster region
MCR	Major cytogenetic response
mIL3	Murine IL3
MMR	Major molecular response
mM	Milli Molar (10^{-3} Molar)
MNC	Mononuclear cells
mRNA	Messenger ribonucleic acid
m	Milli (10^{-3})
min	Minutes

MoAB	monoclonal antibody
μ	Micro (10^{-6})
μM	Micro Molar (10^{-6} Molar)
μg	Micro gram (10^{-6} gram)
n	Nano (10^{-9})
ND	not detected
Ng	nano gram (10^{-9} gram)
nt	Nucleotide
O_2	oxygen
p	Pico (10^{-12})
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PCyR	Partial cytogenetic response
PE	Phycoerythrin
PFS	Progression free survival
Pg	Pico gram (10^{-12} gram)
Ph	Philadelphia chromosome
Phe	Phenylalanine
PHR	Partial haematologic response
PI3-K	Phosphotidylinositol – 3-kinase
P-loop	Phosphate binding loop
PB	Peripheral Blood
%	*per centum*
Q-PCR	Quantitative Polymerase chain reaction
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute (media)
RT-PCR	Reverse transcription polymerase chain reaction
rpm	Revolutions per minute
s	Seconds
SA Pathology	South Australia Pathology Services
SCF	Stem cell factor
SC	side scatter
SCT	Stem cell transplantation
SD	standard deviation
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Elctrophoresis

SH	Src homology
ST	Short-term
STI571	signal transduction inhibitor 571 (imatinib)
TKI	Tyrosine kinase inhibitor
TRM	transplantation related morbidity
TNC	total nucleated cells
TPO	Thrombopoietin
Tyr	Tyrosine
U	Units
UV	Ultraviolet
v/v	Volume per volume
V	Volts
vs	*versus*
WCC	white cell count
w/v	Weight per unit volume
y	year