Apoptin and its derivatives as molecular clues towards the development of novel tyrosine kinase inhibitors

by

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Abstract

The non-receptor tyrosine kinase activity of fusion gene *BCR-ABL* derived oncoproteins is the key factor responsible for development and progress of Philadelphia positive (Ph+) chronic myeloid leukemia (CML). In the search for a superior and novel peptide-based inhibitor of Bcr-Abl, here I investigated a naturally occurring molecule, called apoptin. Apoptin is a 13.6 kDa protein derived from chicken anemia virus (CAV) and known to induce apoptosis in a wide range of transformed but not in primary cells. Apoptin is a protein without any reported structural and/or functional homolog and is an interesting candidate to initiate protein-protein interactions and subsequent downstream effects.

Initially by an array-based analysis I found that apoptin interacts with the SH3 domain of Abl. By high stringency pull-down and co-immunoprecipitation assays the apoptin and Bcr-Abl interaction was further confirmed. Subsequently, a set of apoptin and Bcr-Abl deletion mutants were used to map this interaction precisely that mainly occurred between a proline rich domain of apoptin and the SH3 domain of Bcr-Abl. I further investigated the role of apoptin on Bcr-Abl. Apoptin was able to modify the phosphorylation of a series of targets (e.g. CrkL, STAT5, c-Myc) downstream of Bcr-Abl kinase. In addition, I used computational algorhythms for protein modeling to study the 3D structure of apoptin and it's docking with Bcr-Abl at the molecular level. In controlled studies using the 2-pheny-laminopyrimidine derived specific tyrosine kinase inhibitor Imatinib® I found that apoptin has comparable effects on CML cells, suggesting that the interacting segment of the apoptin molecule acts as an adaptor and negatively regulates the Bcr-Abl kinase by deactivating many cell proliferation and anti-apoptotic pathways in CML cells. Briefly, this work provides important insights towards the development of peptide based tyrosine kinase inhibitors as new anti-cancer agents.

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I dedicate this work

To my mother Amita Panigrahi

To my wife Tatiana

&

To my little daughter DASHA

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aa	Amino acid
Abl	Abelson murine leukemia virus encoded protein
Abi	Abl interactor protein
AGC kinases	cAMP-dependent protein kinase A/protein kinase G/protein kinase C
Ala, A	Alanine
AIF	Apoptosis Inducing Factor
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
Apaf-1	Apoptotic protease-activating factor-1
APC	Anaphase Promoting Complex
Arg, R	Arginine
ATCC	American Type Culture Collection
Atm	Ataxia-telangiectasia mutated
ATP	Adenosine triphosphate
bcr	Breakpoint cluster region
BH	Bcl-2 homology
BMT	Bone marrow transplantation
BSA	Bovine serum albumin
CaLB	Calcium-dependent lipid binding
CaCl2	Calcium chloride
CARD	Caspase recruitment domain
CAK	CDK activating kinase

CAV	Chicken anemia virus
Cbl	Casitas B-lineage lymphoma
CDK	Cyclin dependent kinase
CML	Chronic Myeloid Leukemia
CO-IP	Co-Immunoprecipitation
CREB	cAMP response element-binding
CrkL	V-crk sarcoma virus CT10 oncogene homolog-like
Cyt c	Cytochrome c
DAPI	4',6-diamidino-2-phenylindole
Dbl	A family of cell growth regulatory proteins and oncogene products
DD	Death domain
DED	Death effector domain
DH	Dbl homology
DISC	Death inducing signaling complex
DLI	Donor lymphocyte infusion
DMEM	Dulbecco’s modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA protein kinase
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
DTT	Dithiothreitol
Glu, E	Glutamic acid (Glutamate)
ECL	Enhanced chemical luminescence

EDTA	Ethylenediaminotetraacetic acid
eIF4E	Eukaryotic translation initiation factor 4E
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence activated cell sorter
FADD	Fas-associating protein with death domain
FBS	Fetal bovine serum
FFFs	Fuzzy functional forms
FITC	Fluorescein isothiocyanate
FGFR	Fibroblast growth factor receptor
FKHRL1	A fork head transcription factor
GEF	Guanidine nucleotide exchange factors
GFP	Green-fluorescent protein
Grb	Growth factor binding protein
GST	Glutathione-S-transferase
HA	Hemagglutinin
HDI	Histone deacetylase inhibitor
HPC	Hematopoietic progenitor cells
HRP	Horseradish peroxidase
HSC	Hematopoetic stem cell
IAP	Inhibitor of apoptosis proteins
IFN	Interferon
IgG	Immunoglobulin G
IgM	Immunoglobulin M

IKK	I kappa B kinase
IL	Interleukin
Im	Imatinib/Gleevec® (2-phenyl-amino-pyrimidine)
IR	Ionizing radiation
iSH2	Inter-SH2 domain
Jak	Janus kinase
JNK	c-Jun-N-terminal kinase
kDa	Kilo-Dalton
Leu, L	Leucine
LRS	Leucine rich sequence
µg	Micro-gram
µl	Micro-liter
µM	Micro-molar
MAPK	Mitogen activated protein kinase
MDS	Myelodysplastic Syndromes
ml	Milliliter
NEMO	NF-κB essential modulator
NES	Nuclear export signal
NF-κB	Nuclear factor-κB
NLS	Nuclear localization signal
nM	Nano-molar
NMR	Nuclear magnetic resonance
NP-40	Nonidet P-40

NRTK	Non-receptor tyrosine kinase
NST	Non-myeloablative allogeneic stem cell transplant
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Nur77	Nuclear receptor 77
OD	Optical density
Omi/HtrA2	A mitochondrial pro-apoptotic serine protease
Pro, P	Proline
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly (ADP-ribose) polymerase
p-Akt	Phosphorylated Akt
PBLs	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PDB	Protein data bank
PDGFR	Platelet-derived growth factor receptor
PI3-K	Phophatidylinositol 3-kinase
PI	Propidium iodide
PH	Pleckstrin homology
Ph	Philadelphia chromosome
PML	promyelocytic leukemia
PMSF	Phenylmethylsulfonyl fluoride
PKB	Protein kinase B
PKC	Protein kinase C
PRS	Proline rich sequence

PTD	Protein transduction domain
PTEN/MMAC1	Phosphatase and tensin homolog
PTP	Permeability transition pore
PTP1B	Protein tyrosine phosphatase-1B
RAIDD	RIP-associated ICH-1/CED-3 homologous protein with death domain
RIP	Receptor interacting protein
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute (medium)
RT	Room temperature
RTK	Receptor tyrosine kinase
SAPK	Stress-activated protein kinase
Saos	Sarcoma osteogenic
SDF	Stromal cell-derived factor
SDS	Sodium dodecyl sulfate
STAT	Signal Transduction and Activators of Transcription
SH	Src homology
Shc	Src homology 2 domain containing
Shp	Small heterodimer partner
STI	Signal transduction inhibitor
Syp	Synaptophysin
TAT	Trans-acting activator of transcription
Th1	T helper cell 1

Thr, T	Threonine
TK	Tyrosine kinase
TNF	Tumor necrosis factor
TRADD	TNF receptor associated death domain containing adaptor protein
TRAIL	TNF-related apoptosis-inducing ligand
TRAF	TNF receptor associated factor
Tyr, Y	Tyrosine
UV	Ultraviolet
v/v	Volume/volume
w/v	Weight/volume
wt	Wild type

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1. Introduction: Leukemias and oncogenic kinase activation

Like other cancers, leukemias arise through a series of physical alterations in DNA that ultimately results in unrestricted clonal proliferation of the affected cell. Many of these changes involve mutational alterations of the DNA sequence that may arise either as random replication error or as a consequence of germline mutation of a cancer gene. Among others, the most important genetic events crucial for leukemic transformation are: modifications of the transcription factors that govern hematopoietic differentiation and activation of signal transduction cascades secondary to specific mutations. The highly co-orchestrated molecular events that alter the transcriptional regulations in hematopoietic progenitor cells also modify the functionality of signal transduction molecules responsible for the clonal expansion of leukemias. To be precise, the molecular and genetic analyses of chronic myeloid leukemia (CML) and some of the myeloproliferative disorders have revealed acquired abnormalities in the tyrosine kinase (TK) genes (Lugo et al, 1990). Chromosomal translocations resulting in the creation of a fusion kinase gene, examples of which include 'Abelson murine leukemia viral oncogene homolog' (*ABL*), 'fibroblast growth factor receptor' (*FGFR*), and 'platelet-derived growth factor receptor' (*PDGFR*) are seen in disorders like CML, 8p11 myeloproliferative syndrome, atypical CML and chronic eosinophilic leukemia (Macdonald & Cross, 2007; Melo & Barnes, 2007). These fusion protein TKs are in general non-responsive to the tight inhibitory regulation seen in normal cells and are constitutively active (Macdonald & Cross, 2007). This mechanism is best understood in CML that can be considered as a paradigm for leukemias evolving through multi-step processes. CML is perhaps the most prominent

example where this fundamental knowledge leads the development of highly precise molecular therapy.

1.1 Chronic myeloid leukemia (CML)

Chronic myelogenous leukemia (CML) is a disorder characterized by clonal proliferation of the myeloid hematopoetic stem cell (HSC). CML is diagnosed by identifying a reciprocal translocation between chromosome 9 and chromosome 22. This translocation results in the reciprocal fusion of the breakpoint cluster region (*BCR*) gene on chromosome 22q11 with the *ABL* gene located on chromosome 9q34. In the initial chronic phase, myeloid progenitors and mature cells accumulate in the blood and extramedullary tissues. If left untreated, CML inevitably undergoes transition from chronic to an accelerated phase and then to the aggressive blast crisis phase (Giles et al, 2004).

1.1.1 Chronic myeloid leukemia: disease profile

Studies on animal models of radiation exposure suggested that only large doses of ionizing radiation (IR) can induce CML-like chromosomal changes; otherwise, no clear correlation with exposure to cytotoxic drugs or evidence of a viral etiology has been found (Deininger et al, 1998). Some CML patients are diagnosed while still asymptomatic, during health screening tests; other patients present with fatigue, malaise, and weight loss, or have symptoms secondary to an enlarged spleen. In more advanced situations, some of the patients present with high white blood cells and platelets count. Progression of the disease is generally associated with worsening symptoms characterized by fever, weight loss, bone and joint pain, bleeding, and infections.

Elevated white blood cell counts, with various degrees of immaturity of the granulocytic series, are present at diagnosis. Leukocyte alkaline phosphatase is characteristically low in CML cells. Serum levels of vitamin B_{12} and vitamin B_{12}-binding proteins are generally elevated. Histamine production secondary to high basophil count is increased in later stages, causing pruritus, diarrhea and flushing (Wetzler et al, 2006).

In 90 to 95% of patients the consistently observed chromosomal anomaly is t(9;22)(q34;q11.2). In the 1960's this was initially recognized by the presence of a shortened chromosome 22 (22q-) that was designated as the *Philadelphia chromosome* (Ph) (Lozzio & Lozzio, 1975; Nowell & Hungerford, 1960; Rowley, 1973). The Philadelphia chromosome first identified by Nowell and Hungerford is the cytogenetic hallmark of chronic myeloid leukemia CML. Interestingly, some patients may have complex or variant of these translocations involving more than two chromosomes but including chromosomes 9 and 22 with similar molecular consequences (Bartram et al, 1983).

The chimeric gene resulting from t(9;22) is transcribed into a hybrid *BCR-ABL* mRNA in which exon 1 of *ABL* is replaced by variable numbers of 5'*BCR* exons and the end product is fusion protein Bcr-Abl, that contains NH_2-terminal domains of Bcr and the COOH-terminal domains of Abl (Groffen et al, 1984; Walker et al, 1987). This results in critical functional changes: the Abl-TK becomes constitutively active and subsequently activates downstream kinases that prevent apoptosis, promote cell proliferation and decrease stromal adhesion of the transformed cells (Lugo et al, 1990; Melo, 1996). However, the events associated with transition to the acute phase are poorly understood. Acquisition of additional genetic and/or molecular abnormalities is critical to the

phenotypic transformation. Unlike normal Abl, Bcr-Abl can undergo uncontrolled autophosphorylation activating downstream cell growth, proliferation and anti-apoptotic pathways (Pendergast et al, 1991). Among numerous downstream targets, Crk and CrkL are the most prominent tyrosine-phosphorylated proteins in *BCR-ABL* transformed cells, and they are involved in the regulation of cellular motility and integrin-mediated cell adhesion (ten Hoeve et al, 1994). CrkL acts as an adaptor molecule and also activates Ras. Ras is one of the important mediators of cell proliferation via the downstream Mitogen Activated Protein Kinase (MAPK) pathway. Similarly, Bcr-Abl autophosphorylation at tyrosine residue 177 (Tyr-177) provides a docking site for other adapter molecules like Grb-2 (& Grb-1) that stabilizes Ras in its active GTP-bound form (Cortez et al, 1995; Meng S, 2005; Million & Van Etten, 2000). This implies that the Ras pathway is constitutively active during the pathogenesis of CML (Kiyokawa E, 1997). Moreover, activation of the stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) pathway by Bcr-Abl has been demonstrated and is also required for malignant transformation (Kang et al, 2000). Constitutive phosphorylation of STATs (Signal Transduction and Activators of Transcription, STAT1 and STAT5) has also been reported in several Bcr-Abl expressing transformed cell lines and in primary CML cells. STAT5 activation, in general, contributes to the clonal malignant transformations; however the role of STAT5 in *BCR-ABL* transformed cells is primarily anti-apoptotic (Danial NN, 2000). Similarly, Phosphoinositide 3-kinase (PI3-K) activity is required for the proliferation of Bcr-Abl positive cells (Danial NN, 2000). Interestingly, Bcr-Abl forms complexes with PI3-K where Crk and CrkL act as adaptor molecules. PI3-K is phosphorylated in this protein complex leading to trans-activation of the serine-threonine

kinase ‘Akt’ (protein kinase B) that is the main downstream substrate of PI3-K. Akt has a proven role in anti-apoptotic signaling in malignant cells (Arslan et al, 2006; Burchert et al, 2005). The critical role of some of these signal transductions proteins and pathways in the initiation and progress of CML will be discussed in the following sections (1.2, 1.3).

1.2 Major signal transduction proteins associated with the pathogenesis of CML

1.2.1 Abl and its functions

The evolutionarily conserved cellular oncogenes are a group of transforming genes homologous to that of oncogenic retroviruses. For example, in human the cellular homologue of v-Abl is similar to the transforming sequence of Abelson murine leukemia virus; the gene that encodes a tyrosine kinase (TK) is located on chromosome 9 (Abelson & Rabstein, 1970; Cohen et al, 1995). Abl is a large 145 kDa protein with 2 isoforms arising from alternative splicing of the first exon, one that is N-terminally myristoylated (c-Abl 1b) and another that is 19 residues shorter at the N terminus and not known to be myristoylated (c-Abl 1a) (**Figure 1**). In normal cell Abl is ubiquitously expressed in many cell types, and is found mostly in the nucleus (Baltimore et al, 1995; Laneuville, 1995). Abl has several structural domains recruited for specific functions.

Structure of Abl and the Src homology domains: The proto-oncogene *Src* is a family of TKs similar to the *v-Src* gene of Rous sarcoma virus. Towards the NH2 terminus of Abl molecule, three Src homology domains (SH1-3) are located (**Figure 1**). The SH1 domain of Abl molecule has the tyrosine kinase function, whereas the SH2 and SH3 domains allow Abl to interact with other proteins (Cohen et al, 1995). The proline-rich sequences in the center of Abl molecule act as the interaction motif that is able to interact with SH3 domains of other proteins (Alexandropoulos et al, 1995; Feller et al, 1994). This SH3

domain mediated recognition of proline-rich sequences takes place through a conserved mode of interaction. The hydrophobic surface in the binding site of the SH3 domains consists of three shallow pockets defined by the side chains of preserved aromatic residues (Sicheri & Kuriyan, 1997). The polyproline II helical conformation of the interacting peptide favors the recognition by SH3 domains. The SH3-SH2 unit docks onto the back of the kinase domain and restricts its conformational flexibility (Hou et al, 2006; Moarefi et al, 1997). The lack of a phosphorylated ligand for the SH2 domain in c-Abl is compensated for by an interaction that depends on the N-terminal myristoyl modification. The myristoyl group of c-Abl is inserted into the base of the kinase domain, and is phosphorylated on a serine residue that interacts with the connector between the SH3 and SH2 domains maintaining the inactive state of the protein (Nagar et al, 2006).

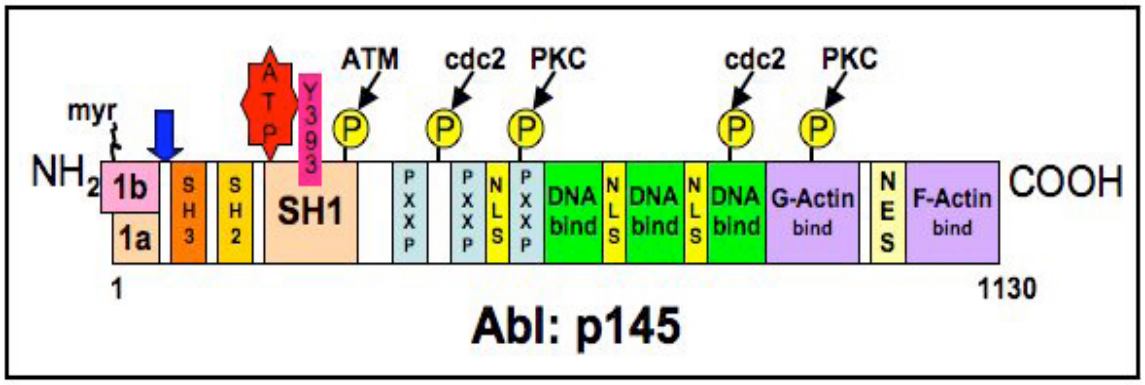


Figure 1: *Structure of the Abl protein*: The 145 kDa Abl protein has two isoforms. The type 1a isoform is shorter than type 1b. Type 1b Abl has a myristoylation (myr) site for its attachment to the structural proteins of cell membrane. The Src-homology (SH) domains situated toward the NH2 terminus. In Abl the Y393 is a major site for autophosphorylation within the kinase domain. The proline-rich regions (PxxP) are capable of binding to SH3 domains of other proteins. Abl also has nuclear localization signals (NLS) in its structure. The COOH terminus contains DNA binding domains (DNA bind) and also G-Actin and F-Actin binding domains. Phosphorylation sites by proteins like ATM, cdc2, and PKC are indicated. The position of the breakpoint in the Bcr-Abl fusion protein is indicated by arrow (blue) [*Based on:* (Cohen et al, 1995; Deininger et al, 2000; Feller et al, 1994; Kipreos & Wang, 1992; McWhirter & Wang, 1993; Van Etten et al, 1989)].

The nuclear localization and the DNA-binding as well as actin-binding motifs are found toward the 3' end (Kipreos & Wang, 1992; McWhirter & Wang, 1993; Van Etten et al, 1989). So, Abl can physically associate with a broad range of targets, and appears to function in a diverse array of signaling pathways. Thus, a range of complex and interrelated functions has been attributed to Abl.

Functions of normal Abl: The functions of normal Abl are very important to humans as well. The normal Abl protein is involved in the regulation of the cell cycle, in the response of cells to oxidative stress, and also mediating integrin signalling that is responsible for the transmission of information about the cellular environment (Afar et al, 1994; Kipreos & Wang, 1990; Sawyers et al, 1994; Van Etten, 1999). During oxidative stress like IR exposure, the serine/threonine kinase and DNA repair protein Atm (Ataxia-telangiectasia mutated) constitutively interacts with c-Abl and enhances its TK activity by phosphorylating the Ser-465 residue, and protein kinase C (PKC) plays an important intermediary role (Baskaran et al, 1997; Li et al, 2004). *In vitro* evidence indicates that DNA-dependent protein kinase (DNA-PK) can also potentially activate Abl kinase in response to IR exposure (Shangary et al, 2000). Eventually the activated normal c-Abl may promote apoptosis by up-regulating p73 that is a pro-apoptotic protein and p53 homologue (Gong et al, 1999). Another area of emerging interest is the role of a novel protein, termed Cables, that links Abl to cyclin-dependent kinase 5 (Cdk5) (Zukerberg et al, 2000). In addition to the C-terminal Cdk5 binding domain, Cables has six potential SH3 binding motifs (PxxP) clustered around its N terminus, two of which are similar to motifs known to bind the Abl SH3 domain. Biochemical tests determined that Cables does bind to Abl and that a trimolecular complex of Cdk5, Abl, and Cables exists in vivo.

These results provide a critical link between Abl and Cdk5 (Zukerberg et al, 2000). The merging of Cdk5 and Abl pathways opens a new area for experimentation and the interrogation of putative therapeutic targets. Overall, the Abl protein plays several complex and critical roles as a cellular module that integrates signals from various extracellular and intracellular environments, thus it has significant impact on the cellular decision making in regard to progression towards cell cycle or apoptosis.

Regulation of Abl kinase: Under physiological conditions the endogenous Abl is highly regulated. The SH3 domain plays an important role in the inhibitory process of Abl-TK because its positional alteration or deletion activates the kinase (Mayer & Baltimore, 1994; Van Etten et al, 1989). A conformational change that inhibits interaction of Abl-SH3 domain with its substrates that could bind internally to the proline-rich region in the center of the Abl protein is one of the main inhibitory mechanisms of Abl (Goga et al, 1993). Also, as mentioned, several proteins have been identified that bind to the SH3 domain (Cicchetti et al, 1992; Dai & Pendergast, 1995; Shi et al, 1995). Presence of an endogenous inhibitor of Abl was first presumed from the fact that the rates of dephosphorylation of Abl and Bcr-Abl fusion protein by phosphotyrosine-specific phosphatases are approximately the same. This suggested the existence of a cellular component that interacts noncovalently with Abl to inhibit its autophosphorylation (Pendergast et al, 1991). Later, two proteins, Abi-1 and Abi-2 were identified with inhibitory functions on Abl. Both, Abi-1 and Abi-2 (Abl interactor proteins 1 and 2) activate the inhibitory function of the SH3 domain. Interestingly, the activated Abl protein promotes proteasome-mediated degradation of Abi-1 and Abi-2 (Dai et al, 1998). It has also been reported that the degradation of Abi-1 and Abi-2 is predominant in Ph+ve

acute leukemias but not seen in the Ph- disease phenotype. Another possible inhibitor of Abl is Pag/Msp23 that dissociates from Abl on exposure of cells to oxidative stress such as ionizing radiation (Wen & Van Etten, 1997). These observations explain why the highly purified Abl protein possesses an active kinase and the less purified Abl, associated with these inhibitor proteins does not (Mayer & Baltimore, 1994).

1.2.2 Bcr and its functions

Bcr is a ubiquitously expressed protein. In the 160 kDa molecular structure of Bcr several functional motifs have been identified (Wetzler et al, 1993).

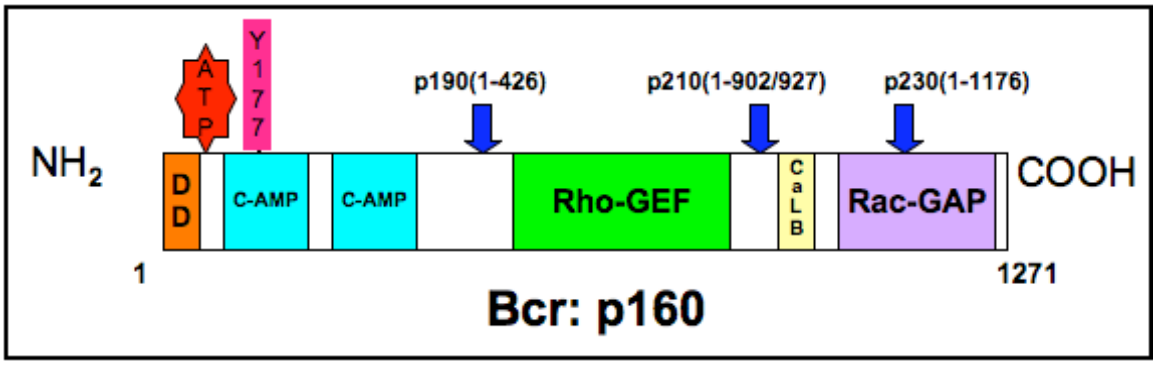


Figure 2: *Structure of the Bcr protein*; Bcr is a 1271 amino acid (160 kDa) protein. At the N terminus of Bcr the dimerization domain and two cyclic AMP kinase homologous domains are located. The Y177 is autophosphorylation site for Bcr and crucial for its Grb-2 binding. At the center of the molecule there are domains homologous to Rho guanidine nucleotide exchange factors (Rho-GEF), dbl-like and pleckstrin homology (PH) domains. At the C terminus of Bcr, a site for calcium-dependent lipid binding (CaLB) and a domain with activating function for Rac-GTPase (Rac-GAP) are located. Block arrows (blue) indicate different level of cleavage during different Bcr-Abl fusion protein formation. [Based on: (Deininger et al, 2000; Denhardt, 1996; Diekmann et al, 1995; Laneuville, 1995; Ma et al, 1997; Wetzler et al, 1993; Wu et al, 1998)].

The N-terminus of Bcr has a coiled-coil domain that allows *in-vivo* dimerization of Bcr (**Figure 2**) (McWhirter et al, 1993). The N-terminal 'coiled-coil' dimerization interface of Bcr, its serine/threonine kinase activity, and presence of the tyrosine phosphorylation site at position 177 (Y177) are crucial for its physiological functions (Deininger et al, 2000; Maru & Witte, 1991; Radziwill et al, 2003). The identified substrates of this serine/threonine kinase are Bap-1 which is a member of the 14-3-3 family of proteins (Reuther et al, 1994). Possibly Bcr itself is another substrate for its own kinase (Hawk et al, 2002; Lu et al, 1993). Bcr is also a Rho-GEF due to the presence of a Dbl homology (DH) domain and a pleckstrin homology (PH) domain (Maru & Witte, 1991).

Functions of Bcr: Both the Rho-GEF function and the oncogenic potential of Bcr depends on its DH-domains (Van Aelst & C, 1997). These GEFs activate members of the Ras superfamily by increasing the proportion of their GTP-bound form with respect to the GDP-bound form; also this Rho guanidine exchange factor possibly activates transcription factors like NF-κB (Boguski & McCormick, 1993; Denhardt, 1996; Montaner et al, 1998). In addition, Bcr can be phosphorylated on several of its tyrosine residues (**Figure 2**) (Wu et al, 1998). The predominantly phosphorylated Tyr-177 residues binds Grb-2 adaptor molecule and further activates the downstream Ras pathway (Ma et al, 1997). Bcr also contains a C-terminal Rac-GAP domain. In fact, Bcr is a negative regulator of Rac, as demonstrated by the fact that it reduces the Rac1-dependent activation of the protein kinase Pak1, an activator of the JNK pathway, via its GAP function (Lu & Mayer, 1999). The Rho-GEF and Rac-GAP functions strongly suggest an important role of Bcr in cytoskeleton modeling by regulation of Rho-like GTPases, such as Rac, cdc42, and Rho (Radziwill et al, 2003; Van Aelst & C, 1997; van Buul &

Hordijk, 2004). Bcr is also a recognized negative regulator of cell proliferation and oncogenic transformation. In the reciprocal t(9;22) Bcr-Abl fusion proteins both Bcr and Abl lose their normal physiological functions and Bcr gains novel functions.

1.2.3 Molecular biology of the fusion protein Bcr-Abl

The first exon of *ABL* is replaced by the 5′ end of *BCR* in the *BCR-ABL* fusion gene. The breakpoints within the *ABL* gene at 9q43 are observed in a region spanning the 5′ end of *ABL* either downstream or upstream of the first alternative exon 1b (Melo, 1996). However, irrespective of the precise location of the breakpoint, splicing of the primary hybrid transcript yields an mRNA molecule in which *BCR* sequences are fused to *ABL* exon a2 (**Figure 3**) (Melo, 1996). Interestingly, in sharp contrast to *ABL* splicing the breakpoints within *BCR* localize to any of the three so-called breakpoint cluster regions (bcr) spanning a 5.8 kb area in the *Bcr* exons 12-16 (exons b1-b5). This area in the *Bcr* exons is defined as the major breakpoint cluster region (M-bcr). Because of alternative splicing, the fusion transcripts with either b2a2 or b3a2 junctions can be formed (**Figure 3**). This fused mRNA is subsequently translated to a 210 kDa chimeric protein (Bcr-Abl^{p210}) in patients with Ph+ CML and about one third of the patients with Ph+ ALL (Hochhaus et al, 1996). In the remaining Ph+ ALL patients (and a tiny number of patients with CML) the breakpoints are in the alternative BCR exons e2′ and e2 (minor breakpoint cluster region or m-bcr) and the e1a2 fusion mRNA is translated into a kinase active 190 kDa protein (p190$^{Bcr-Abl}$) responsible for the characteristic disease phenotype (Melo et al, 1994; Ravandi et al, 1999; Spencer et al, 1996; van Rhee et al, 1996).

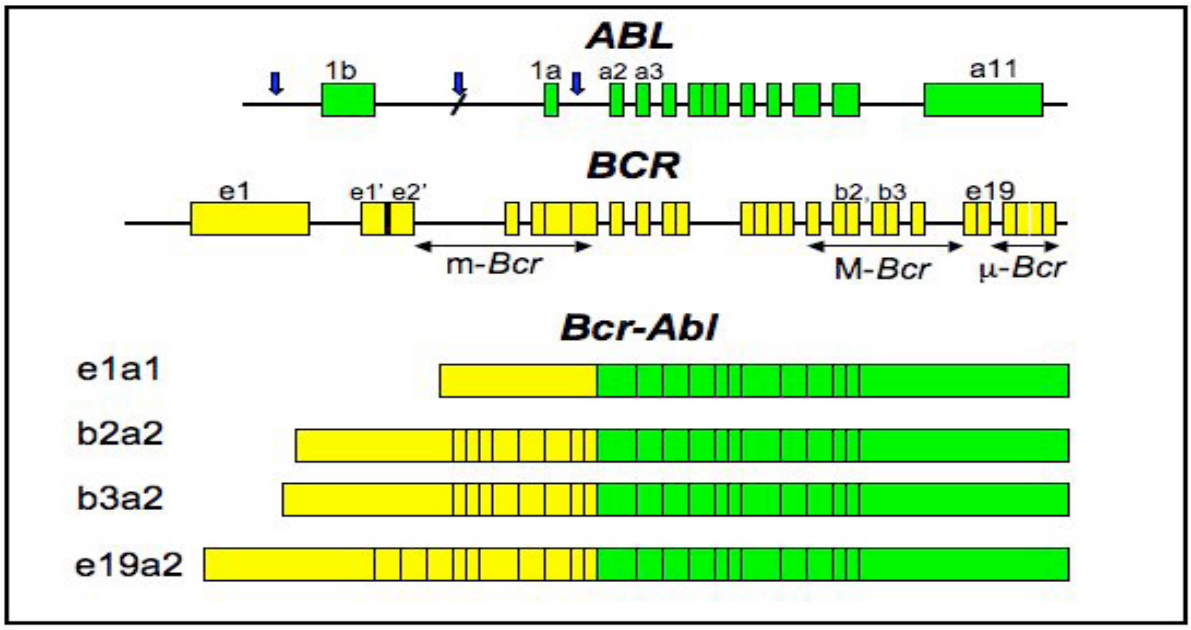


Figure 3: *BCR-ABL*; Positions of the breakpoints in the *ABL* and *BCR* genes and structure of the chimeric mRNAs derived from the various breaks. [Based on: (Deininger et al, 2000; Melo, 1996; Melo, 1997; van Rhee et al, 1996)]

Deregulation of Abl tyrosine kinase in Bcr-Abl: Presumably, the fusion of Bcr sequences to the Abl SH3 domain counteracts the physiologic suppression of Abl-TK (Afar et al, 1994). This event in the pathogenesis of CML is possibly a consequence of the formation of homodimers because the N-terminal dimerization domain is an essential feature of the Bcr-Abl protein (Golub et al, 1996). However, the same molecular event is observed when Bcr is functionally replaced by other sequences that allow dimer formation, for example the N-terminus of the *Tel* (*ETV-6*) transcription factor in the *Tel-Abl* fusion associated with t(9;12) (Janssen et al, 1995). The Bcr-Abl fusion protein is a potent kinase and capable of inducing self-phosphorylation. As a consequence of this auto-phosphorylation there is a significant increase of phosphotyrosine on *Bcr-Abl* itself. This phosphorylated Bcr-Abl-TK creates binding sites for the SH2 domains of other proteins.

Table 1

Downstream Bcr-Abl substrates

Substrate	Function	Sites	Reference
Bap-1	14-3-3 protein	-	(Reuther et al, 1994)
Cbl	Multifunctional adapter protein	-	(Schmidt & Dikic, 2005)
Caspase 9	Apoptosis	Tyr153	(Raina et al, 2005)
CD19	BCR co-receptor	Tyr508	(Zipfel et al, 2000)
CrkL	Adapter protein	-	(Oda et al, 1994)
Crk	Adapter protein	Tyr 221	(Feller et al, 1994)
DNA-PK	Protein kinase	Not mapped	(Kharbanda et al, 1997)
Dok1	Docking protein	Tyr361	(Woodring et al, 2004)
Fak	Cytoskeleton/cell membrane	-	(Gotoh et al, 1995)
Fes	Myeloid differentiation	-	(Ernst et al, 1994)
Fe65	Adapter protein	Tyr547	(Perkinton et al, 2004)
FOXO3a	Tumor Suppressor	-	(Jagani et al, 2008)
GAP-associated proteins	Ras activation	-	(Druker et al, 1992)
Hdm2, Mdm2	Cell cycle regulation	Tyr394	(Goldberg et al, 2002)
HPK1 / p62DOK	Hematopoietic progenitor kinase	Not mapped	(Ito et al, 2001)
MEKK1, MAP3K1	Serine/threonine kinase	Not mapped	(Kharbanda et al, 2000b)
Paxillin	Cytoskeleton/cell membrane	-	(Salgia et al, 1995b)
PLCγ	Phospholipase	Tyr69/Tyr74	(Gotoh et al, 1994)
PI3 kinase (p85 subunit)	Serine kinase	-	(Skorski et al, 1995)
PKD (protein kinase D)	Protein kinase	Tyr463	(Storz et al, 2003)
p73	Transcription activation	-	(Agami et al, 1999)
Rad9	DNA damage repair	Tyr28	(Yoshida et al, 2002)
Rad 51	DNA damage repair	Tyr54	(Yuan et al, 1998)
Ras-GAP	Ras-GTPase	-	(Gotoh et al, 1994)
RNA-Pol II	RNA polymerase	C-terminus	(Baskaran et al, 1993)
RAFT1, FRAP1	Rapamycin associated protein	Not mapped	(Kumar et al, 2000)
Shc	Adapter	-	(Matsuguchi et al, 1994)
Syp	Cytoplasmic phosphatase	-	(Tauchi et al, 1994)
Talin	Cytoskeleton/cell membrane	-	(Salgia et al, 1995a)
TERT	Telomerase reverse transcriptase	Not mapped	(Kharbanda et al, 2000a)
Vav	Hematopoietic differentiation	-	(Matsuguchi et al, 1995)

Substrates for Bcr-Abl: Autophosphorylation of Bcr-Abl^{p210} causes an overwhelming increase of phosphotyrosine on Bcr-Abl itself. The protein tyrosine kinase activity of Bcr-Abl^{p210} is essential for its leukemogenic potential *in vivo*, where a number of substrates can be tyrosine phosphorylated. Identification of the substrates of Bcr-Abl^{p210} and investigating the mechanisms by which Bcr-Abl targets specific substrates are therefore essential for understanding the malignant transformation process in CML.

The Bcr-Abl-TK has selective behaviour towards its specific substrates and the Bcr-Abl binding proteins are either regulators or substrates of the Abl kinase. Moreover, the choice of substrate by Bcr-Abl-TK is mostly dependent on the cellular context. For example, CrkL is the major tyrosine-phosphorylated protein in neutrophils of CML patients whereas the phosphorylated p62DOK is predominantly found in early hematopoetic progenitor cells (Carpino et al, 1997; Oda et al, 1994). In general, these substrates for Bcr-Abl are grouped according to their specific physiological functions. Although the list is growing, an overview on the Bcr-Abl substrates with their physiological functions in brief and know phosphorylation sites by Bcr-Abl kinase are presented above in **Table 1.**

Role of Tyrosine phosphatase in Abl kinase deregulation: Tyrosine phosphatases are responsible for regulating and counterbalancing the effects of tyrosine kinases in normal physiologic conditions. Tyrosine phosphatases like PTP1B, Syp83, and Shp1 form complexes with Bcr-Abl and downregulate its kinase activity. The PTP1B levels increase in a kinase-dependent manner and in fibroblasts the cellular transformation by Bcr-Abl is impaired by the overexpression of PTP1B. PTP1B recognizes Bcr-Abl^{p210} as a substrate, disrupts the formation of a Bcr-Abl^{p210}/Grb2 complex, and inhibits signaling events

initiated by the oncoprotein Bcr-Abl^{p210} (LaMontagne et al, 1998). The SH2 domain-containing protein tyrosine phosphatase Shp1 is a major inhibitory mediator of Bcr-Abl kinase. Shp1 levels are markedly decreased, due to post-transcriptional modifications, in blast crisis or advanced phase of CML compared to the level of Shp1 during the chronic phase of CML. This is a process shown to be independent of DNA methylation. Overall, the decreased level of Shp1 expression is implicated in the progression of CML (Amin et al, 2007).

Mechanism of Bcr-Abl mediated malignant transformation: One of the essential features of Bcr-Abl onco-protein is its own high level of tyrosine phosphorylation. As mentioned, this Bcr-Abl-TK plays the role of a key-signalling molecule to initiate CML pathogenesis (Skorski et al, 1998a). In CML the normal Abl functions are deregulated (see above) and the fusion protein Bcr-Abl kinase is highly active. The active Abl kinase in Bcr-Abl transactivates a number of downstream signal transduction pathways. This switch in the signalling cascades lead to a series of changes in cellular behaviors, which include *altered adhesion properties, degradation of inhibitory proteins, activation of mitogenic signaling* and *inhibition of apoptosis* (**Figure 4**) (Bedi et al, 1994; Gordon et al, 1987a; Puil et al, 1994). CML progenitor cells exhibit decreased adhesion to bone marrow stroma cells and extracellular matrix (Gordon et al, 1987a). In this scenario, adhesion to stroma negatively regulates cell proliferation, and CML cells escape this regulation by virtue of their perturbed adhesion properties (Gordon et al, 1987b). The β-integrins mediate the interaction between bone marrow stromal cells and CML progenitor cells (Verfaillie et al, 1997a). In CML an adhesion-inhibitory variant of this protein is expressed, which is not found in normal progenitors (Verfaillie et al, 1997b).

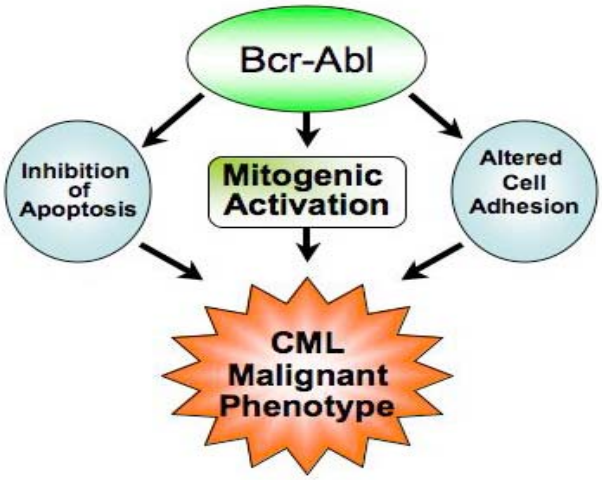


Figure 4: *Pathogenesis of Chronic Myeloid Leukemia*; Mechanisms implicated in the malignant transformation by *Bcr-Abl:* i). Altered adhesion to bone marrow stromal cells; ii). Inhibition of apoptotic response and iii) Constitutively active mitogenic signaling [Based on: (Bedi et al, 1994; Gordon et al, 1987a; Puil et al, 1994)].

Integrins are responsible for initiating normal signal transduction from the extracellular environment to the cell (Lewis et al, 1996). Thus expressing an abnormal variant of integrin, the transfer of signals that normally inhibits proliferation, facilitates the proliferation of CML cells. In Ph+ CML cells the c-Abl kinase inhibitory endogenous proteins Abi-1 and Abi-2 show accelerated proteasome-mediated degradation in the presence of active Bcr-Abl kinase (Dai et al, 1998). This is possibly another way by which the uncontrolled Bcr-Abl induces malignant transformation of the cell. It can be speculated that other Bcr-Abl regulatory proteins, whose cytoplasmic level is controlled through the proteasome pathway, may also undergo similar degradation. The activation of mitogenic signaling pathway is discussed in the following section (1.3). Detailed introduction to different apoptotic pathways and the role of kinase active Bcr-Abl as an anti-apoptotic factor in Bcr-Abl positive CML cells are also discussed in the subsequent section (1.4).

1.3 Major downstream signal transduction pathways involved in CML

Bcr-Abl can undergo uncontrolled autophosphorylation activating downstream cell growth, proliferation and anti-apoptotic pathways (**Figure 5**). Crk and CrkL are the most prominent tyrosine-phosphorylated proteins in *Bcr-Abl* transformed cells (ten Hoeve et al, 1994). The stromal cell-derived factor-1α (SDF-1α) is a potent chemoattractant for hematopoietic progenitor cells (HPC). Modulation of VLA-4 mediated CD34+ bone marrow cell adhesion by SDF-1α plays a key role in their migration within and to the bone marrow (BM) and therefore influence their proliferation and differentiation (Hidalgo et al, 2001). Moreover, activation of the SAPK/JNK pathway by Bcr-Abl is also required for malignant transformation (Kang et al, 2000).

Constitutive phosphorylation of the STATs, particularly STAT5 activation, contributes to clonal malignant transformation (Danial NN, 2000). Similarly, the proliferation of Bcr-Abl positive cells was linked to their PI3-K activity (Danial NN, 2000; Skorski et al, 1995). PI3-K is phosphorylated in this protein complex leading to trans-activation of the serine-threonine kinase Akt, which has a proven role in anti-apoptotic signaling (Arslan et al, 2006; Burchert et al, 2005). (Major pathways outlined in **Figure 6**).

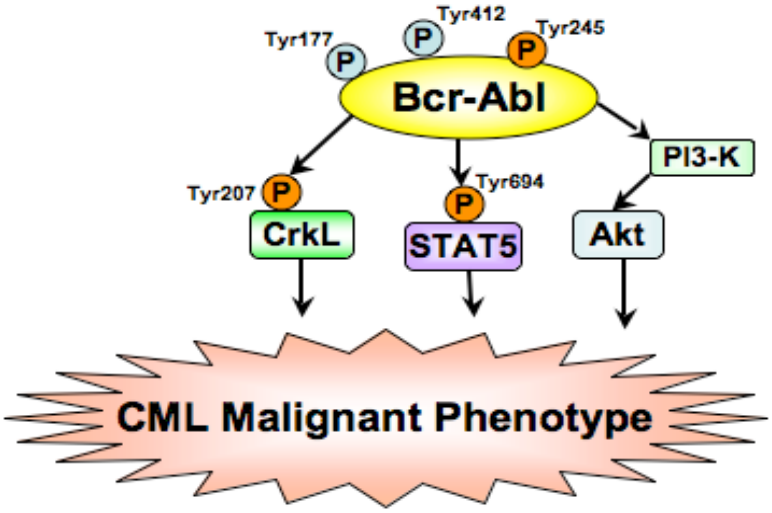


Figure 5: *Major signaling pathways activated in BCR-ABL-transformed cells*; Multiplefactors including the cell types determine the activation of each of the signaling pathways. Crk and CrkL are the most prominent Bcr-Abl-TK phosphorylated adhesion proteins acting as cell motility regulator. Activation of Jak/STAT pathway leads to the cellular, transformation and the constitutively active PI3-K/Akt pathway acts as brake to the apoptotic machinery. In addition the MAPK system plays the crucial role towards cell proliferation by modulating the transcriptional machinery [Based on: (Danial NN, 2000; Ilaria & Van Etten, 1996; Jagani et al, 2008; Nieborowska-Skorska et al, 1999; Oda et al, 1994; Skorski et al, 1997)]

1.3.1 CrkL, the integrin pathway and altered adhesion properties of CML cells

CML cells escape the negative regulation of cell proliferation by the diminished cellular adhesion to the BM stromal cells and extracellular structural proteins in the matrix microenvironment (Gordon et al, 1987a; Verfaillie et al, 1997a). This adhesion defect in CML is at least partially reversed by interferon α (IFNα) (Bhatia et al, 1994). The β-integrins play important role in this interaction between BM stromal cells and CML progenitor cells. CML cells express an adhesion-inhibitory variant of β1-integrin, which is not found in the normal hematopoetic progenitors (Bhatia et al, 1999). Integrins can initiate normal signal transduction from outside to inside of the cell after binding to their receptors (Lewis et al, 1996). Therefore, it can be assumed that the signals conveyed by

integrins normally inhibit cellular proliferation and this effect is impaired in Bcr-Abl expressing CML cells. As mentioned earlier, the Abl kinase has an important role in the intracellular transduction of such signals and this process is subsequently jeopardized by the presence of a large pool of highly kinase active Bcr-Abl protein in the cytoplasm. Besides, the cell motility regulator integrin mediated adhesion proteins like Crk and CrkL are found most prominently tyrosine-phosphorylated by Bcr-Abl-TK in CML cells (Oda et al, 1994; Sattler et al, 1996). CrkL binds to Bcr-Abl through its N-terminal SH3 domain and is known to interact with several signaling proteins that have been implicated in integrin signaling, like Paxillin; the focal adhesion kinase Fak, p130Cas and Hef1 are also de-regulated in presence of an active pool of Bcr-Abl tyrosine kinase (Salgia et al, 1995b; Salgia et al, 1996; Sattler et al, 1997; Turner, 2000). Furthermore, a CrkL-C3G complex activates VLA-4 and VLA-5 in hematopoetic cells, by activating the small GTP binding proteins R-Ras, through the guanine exchange activity of C3G (Arai et al, 1999). The erythropoietin or IL3 induced and Ras-dependent activation of Raf/ERK pathway that further activates Elk-1 and *c-fos* gene transcription is modulated by CrkL-C3G complex in hematopoetic progenitor cells as well (Nosaka et al, 1999). Reportedly, a similar Cbl-CrkL-C3G complex is involved in migration signaling suggesting the involvement of CrkL in signaling pathways that regulate migration, possibly through a complex with Cbl and C3G (Uemura & Griffin, 1999; Uemura et al, 1999). Therefore, we can conclude that Bcr-Abl causes direct or indirect interference to integrin function. In CML cells this altered integrin function leads to diminished stromal adhesion; although reportedly in other cellular systems, integrin functions are enhanced rather than reduced by Bcr-Abl (Bazzoni et al, 1996).

1.3.2 Jak-STAT pathway in CML

Constitutive tyrosine phosphorylation of Jak1 and Jak3 and induction of STAT DNA binding has been demonstrated in v-Abl transformed pre-B cell lines. The oncoprotein v-Abl (p120) was also found to co-immunoprecipitate with Jak1 and Jak3. These results suggest that activation of the Jak-STAT pathway may play a role in transformation by oncogenic forms of Abl (Danial NN, 2000). Although the human oncogenic form of Abl, Bcr-Abl and the murine v-Abl share many common characteristics, there are many structural and functional differences. Presumably, sequences within the Abl SH3 domain or in the Bcr region of Bcr-Abl fusion protein contribute to these differences (Danial NN, 2000).

The physiological function of STAT5 is primarily in the regulation of cell proliferation (Mui et al, 1996). It has important structural domains that include a DNA-binding sequence, SH2 domain, and a transactivation domain. Phosphorylation of tyrosine 694 of STAT5A and tyrosine 699 of STAT5B creates a binding site for the SH2 domain of another STAT5 molecule. Interestingly, deletions of various domains within the STAT5 molecule also modify STAT5 function. In particular, expression of a STAT5B mutant truncated at position 683 exert a dominant negative effect on endogenous STAT5 (Mui et al, 1996). Moreover, the hyperactivity of the STAT transcription factors (STAT1 and STAT5) have been reported in several Bcr-Abl positive CML cells (Ilaria & Van Etten, 1996). Bcr-Abl may directly activate STAT1 and STAT5 whereas the activation of the Jak-STAT pathway by physiologic stimuli requires prior phosphorylation of Jak proteins (Ilaria & Van Etten, 1996). The malignant transformation of CML takes place partially as a consequence of this STAT5 activation (de Groot et al, 1999).

1.3.3 Ras and MAPK pathway in CML

Mitogen activated protein kinases (MAPK) are a family of Ser/Thr protein kinases widely conserved among eukaryotes. They are responsible for the transduction of a large variety of external signals that lead to a wide range of cellular responses including growth, differentiation, inflammation and apoptosis The external signals are delivered to the downstream cascades by small GTP binding proteins (Ras, Rap1) or by small GTPases of the Rho family (Rac, Rho, cdc42). Three major MAPK signaling cascades are organized hierarchically into three-tiered modules: (i) MAPK/ERK, (ii) SAPK/JNK, and (iii) p38 MAPK (**Figure 6**) [review (Platanias, 2003)].

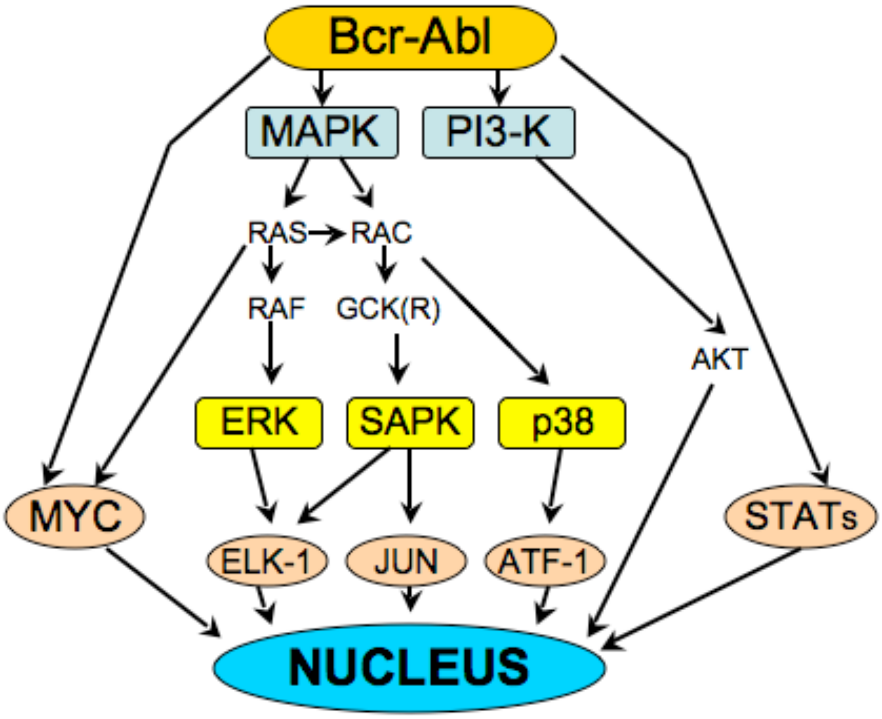


Figure 6: *Signaling pathways responsible for the transformation and proliferation of Bcr-Abl expressing cells*; the level of activation of the individual pathways are mostly cell type dependent. However, the MAP-kinase system plays a pivotal role in cell proliferation. [Based on: (Danial NN, 2000; Galan-Moya et al, 2008; Notari et al, 2006; Platanias, 2003; Skorski et al, 1995; Skorski et al, 1998b)]

The MAPK/ERK Cascade: A diverse range of receptors involved in cellular growth and differentiation that includes non-receptor/receptor tyrosine kinases (NRTKs/RTKs), integrins, and ion channels can activate the MAPK/ERK signaling cascade. The specific components of the cascade usually includes a set of adaptors (e.g. Shc, Grb-2, Crk) that link the receptor to a guanine nucleotide exchange factor (SOS, C3G) transducing the signal to small GTP binding proteins (Ras, Rap1), which in turn activate the core unit of the cascade composed of Raf, Mek1/Mek2 and ERK. Briefly, Raf initiates the signaling cascade involving the serine-threonine kinases Mek1/Mek2 and ERK, which ultimately lead to the activation of gene transcription (Cahill et al, 1996). Bcr-Abl and Ras are linked in several ways. The autophosphorylated Bcr-Abl tyrosine residue Tyr-177 provides a docking site for the adaptor molecule Grb-2 (Growth factor-binding protein 2) that has important role in the malignant transformation (Gishizky et al, 1995; Pendergast et al, 1993). Next, Grb-2 binds to the SOS protein and stabilizes Ras in its active GTP-bound form. Two other adaptor molecules, Shc and CrkL, can also activate Ras. Both Shc and CrkL are substrates of Bcr-Abl and bind Bcr-Abl through their SH2 (Shc) or SH3 (CrkL) domains (Oda et al, 1994; Pelicci et al, 1995). Circumstantial evidence that Ras activation is important for the pathogenesis of Ph+ leukemias comes from the observation that activating mutations are uncommon, even in the blastic phase of the disease unlike in most other tumors (Radich, 2007).

The SAPK/JNK: The stress-activated protein kinases (SAPK)/Jun amino-terminal kinases (JNK) are members of the MAPK family. The SAPK/JNK pathway is also activated by Bcr-Abl and is demonstrated to be essential for malignant transformation in CML (Raitano et al, 1995).

This signaling from Ras is then relayed through the GTP-GDP exchange factor Rac down to SAPK/JNK (Skorski et al, 1998b). The small GTPases belonging to the Rho family (Rac, Rho, cdc42) transmit cellular stress signals to this cascade. The phosphorylated SAPK/JNK translocates to the nucleus where it can regulate the activity of multiple transcription factors (Burgess et al, 1998).

The p38 MAPK: The p38 MAPKs (α, β, γ and δ) are members of the MAPK family and are activated by a variety of environmental stresses and inflammatory cytokines (Ono & Han, 2000). A growing body of evidence supports the role of c-Abl as a major determinant of genotoxic stress induced p38 MAPK activation. As an essential component of the MAP kinase pathway, p38 is known to be activated in Bcr-Abl transformed cells by a non Bcr-Abl-TK dependent manner and plays a critical role in the induction of the antileukemic effects of IFN-α (**Figure 6**) (Galan-Moya et al, 2008; Jansson et al, 2003). Also, p38 is known to be involved in regulation of HSP27 and several transcription factors including ATF-2, STAT1, the Max/Myc complex, MEF-2, Elk-1 and indirectly CREB (Zarubin & Han, 2005). The signal is eventually transduced to the transcriptional machinery of the cell. It is also possible that Bcr-Abl expressing cells use more direct growth factor pathways. The c subunit of the IL-3 receptor and the Kit receptor are observed to be associated with cell proliferation in CML (Hallek et al, 1996). Interestingly, the pattern of tyrosine-phosphorylated proteins seen in normal progenitor cells after stimulation with the Kit ligand is similar to an activated Myc pathway. The Myc pathway has been demonstrated to be overexpressed in CML progenitor cells (Notari et al, 2006).

1.3.4 MYC pathway in CML and other cancers: the dual role

MYC was discovered as the cellular homologue of the transduced oncogene of several avian retroviruses (Sheiness, 1978; Vennstrom, 1982). The gene encodes a transcription factor of the HLH/leucine zipper family of proteins that activates transcription as part of a heteromeric complex with a protein termed Max. Altogether, the Myc family members function as regulators of gene transcription by heterodimerization with Max at the molecular level through network of Myc/Mad/Max proteins at the E-box element (Blackwell et al, 1990). Some of the biological functions of Myc family proteins are accomplished by sequence-specific DNA binding that is mediated by the carboxyl-terminal region of the protein (Blackwell et al, 1990); a negative feedback mechanism can act as a homeostatic regulator of c-Myc expression *in vivo* (Penn, 1990). *MYC* genes are among the most frequently affected genes in human cancers (Henriksson & Luscher, 1996). In fact, the expression of Myc proteins is deregulated in approximately one third of human cancers through a number of different mechanisms (Spencer, 1991). Likewise, overexpression of Myc is common in certain advanced cancers with poor prognosis such as colorectal cancers, hormone dependent breast and prostate cancers (Borg, 1992; Jenkins, 1997; Watson, 2006). Overexpression of c-Myc is also documented in CML. Activation of c-Myc in CML cells is dependent on the SH2 domain of Bcr-Abl. An overexpression of *c-Myc* partly rescues the SH2 deletion mutants with defective transformation (Park et al, 2007; Sawyers et al, 1992). Results obtained from v-Abl transformed cells and Bcr-Abl transformed murine myeloid cells suggest that the signal is transduced through Ras/Raf, cyclin-dependent kinases (CDKs), and E2F transcription factors finally activating the *MYC* promoter (Birchenall-Roberts et al, 1997; Stewart et al,

1995; Zou et al, 1997b; Zou et al, 1997c) . Presumably the effects of Myc in Ph+ cells are not different from those in other tumors.

In mammalian fibroblasts, Myc acts as an upstream regulator of CDKs and functionally antagonizes the action of at least one CDK inhibitor, p27 (Steiner, 1996). Myc is one of the few proteins that can solely drive resting cells into cell cycle and promote DNA synthesis. Interestingly, overexpression of Myc in these cells also blocks their differentiation.

It is now well documented that translocations of c-Myc locus into the heavy or light chain immunoglobulin loci frequently occurs in Burkitt's lymphoma and the amplification of N-*MYC* and L-*MYC* genes is a feature of neuroblastoma and small cell lung cancer respectively (Heerema, 2005).

As mentioned, along with its transforming effect, Myc can also sensitize the cells towards apoptosis, indicating Myc acting as a part of the intracellular proliferative or an apoptotic switching system (Desbarats, 1996). This dual role of Myc is outlined below in **Figure 7**.

The effects of human c-Myc on cells' susceptibility to apoptosis were investigated by introducing them into immortalized rat fibroblasts, or by introduction of Myc expression systems that could be on demand activated by the addition of estrogen (Alexander et al, 2007; Stroh et al, 2002). The transfected cells proliferated to a similar extent upon Myc activation or over expression, but differed by up to fifteen-fold in the level of apoptosis, that correlated inversely with the expression of Myc. A necessary role for c-Myc was observed in apoptosis induced by T-cell receptor activation and further studies also established that c-Myc is a critical determinant of apoptosis induced by TNF-α (Dong J, 1997; Shi, 1992).

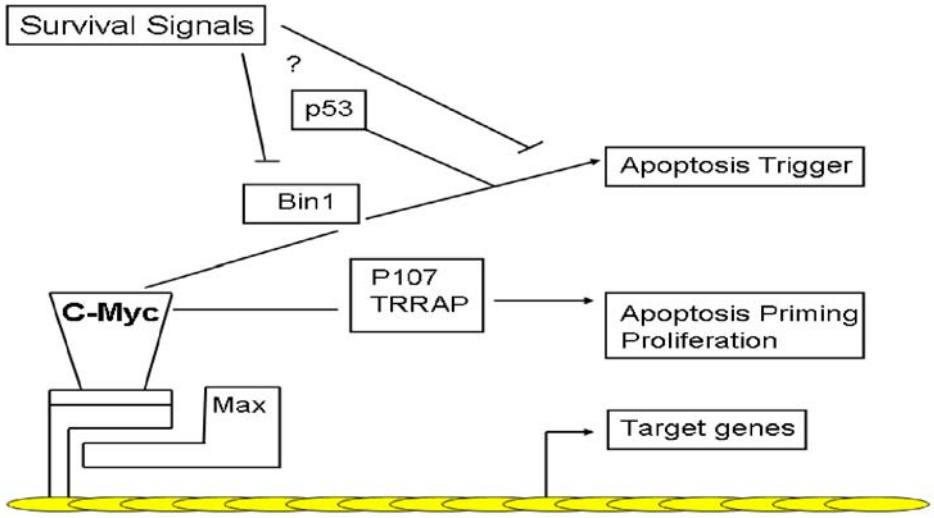


Figure 7: *A dual signal model for c-Myc function; the model proposes that c-Myc activates proliferation and primes apoptosis through one pathway and triggers apoptosis through a mechanistically distinct second pathway*; In this model, death priming and proliferation involves regulation of specific target genes by c-Myc/Max complexes. The links between TRRAP and the transcription complex SAGA suggest possible involvements with the proliferation pathway. The interaction of p107 is proposed to co-ordinate gene regulation with the cell cycle. Max and gene regulation is required for proliferation and death priming but may be dispensable for death triggering. Conversely, Bin1 is dispensable for proliferation and death priming but necessary to trigger death once it is primed. p53 may contribute to sensitizing cells to the trigger, but is mechanistically distinct from Bin1 since the latter can mediate p53-independent death. Survival signals from cytokine and adhesion receptor pathways are proposed to target the inactivation of triggering pathway [Adapted with permission from (Maddika et al, 2007a)].

However, it now appears that c-Myc is required for the efficient response to a variety of apoptotic stimuli, including transcription and translation inhibitors, heat shock, hypoxia, glucose deprivation, chemotherapeutic agents and DNA damage (Alarcon, 1996; Dong J, 1997; Rupnow, 1998; Shim, 1998). Interestingly, the anti-apoptotic Bcl2-family members counteract the Myc-induced apoptotic program in cancer cells (Bissonnette et al, 1992; Maddika et al, 2007a). Therefore, in CML the apoptotic arm of c-Myc's dual function is possibly counterbalanced by both constitutively active proteins of Bcl-2-family and by Bcr-Abl.

1.3.5 PI3-Kinase/Akt pathway

1.3.5.1 The Phosphoinositide 3- kinases (PI3-Ks)

The Phosphotidyl inositols (PI) are a family of phospholipids that represent a small fraction of total cellular lipids but play vital roles in signal transduction acting as the precursors of many second messengers. The free hydroxyl groups of PI have the potential to be phosphorylated (phosphoinositides) by PI3-Kinases. PI3-Ks are defined by their ability to phosphorylate the 3-OH group of the inositol ring in phosphotidyl inositols. Until recently, three families of phosphoinositides kinases have been characterized: phosphoinositide 3- kinases (PI3-Ks), phosphoinositide 4- kinases (PI4-Ks) and PtdIns-P (PIP) kinases (PIP5-Ks). PI3-Ks are further classified into 3 classes according to their specific protein domain structures and adaptors as: Class I, Class II and Class III PI-3Ks. (Fruman et al, 1998).

PI3-Ks are heterodimers composed of a catalytic subunit (p110) and an adaptor/regulatory subunit (p85). The p110 proteins consist of an N-terminal p85 interaction domain, a Ras binding domain, a PIK domain and a C-terminal catalytic domain (Hiles et al, 1992; Hu et al, 1993; Vanhaesebroeck et al, 1997). The p85 subunit contain an N-terminal src-homology 3 (SH3) domain, two or three proline rich sequences, a Bcr-homology domain, two src homology2 (SH2) domains and an inter-SH2 (iSH2) domain that interacts with the p110 subunit (Vanhaesebroeck & Alessi, 2000).

Many important cellular stimuli like hormones and growth factors that regulate key cellular functions like growth, proliferation, survival, transcription, translation, cell cycle and apoptosis are responsible for the activation of phosphatidylinositol 3`-kinase PI3-K/Akt signaling pathway (Cantley, 2002; Vanhaesebroeck & Alessi, 2000). Precisely, in

many cancers the frequent deregulation of the PI3-K/Akt signaling pathway plays a major role in tumor growth and in the potential response to anti-cancer treatment (Vivanco & Sawyers, 2002). The proven role of PI3-K in certain disease conditions has created new possibilities of drug design (Divecha & Irvine, 1995; Shi et al, 2006).

Activation and Regulation of PI3-Kinases: Activated PI3-Ks increase the levels of PtdIns-3,4,5-P3 (PIP3) by selectively phosphorylating PtdIns 3,4-P2 (PIP2) *in vivo* (Stephens et al, 1991). This activation occurs primarily by a classical pathway involving the receptor tyrosine kinase dimerization and autophosphorylation as well as by involving the non-receptor tyrosine kinases. The regulatory p85 subunit directly associates with the active tyrosine kinases via the physical interaction of its SH2 domains selectively with the phosphotyrosine residues of the receptor protein tyrosine kinases (Cantley, 2002). The conformational switch within the p85-p110 holoenzyme also occurs via the interactions of SH3 domain/proline rich sequences, *Bcr-homology domain*/GTP loaded adaptor proteins and others. The p85 proline rich sequence interaction with the SH3 domains of src family kinases including src itself, lck, lyn and Fyn or SH3 domain of cytoplasmic tyrosine kinase Abl are all known to activate PI3-K. The members of the Ras super family like Rac and cdc42 also bind to the BH domain and this interaction is shown to stabilize and activate the p85/p110 complex (Liu et al, 1993; Pleiman et al, 1994; Prasad et al, 1993; Zheng et al, 1994).

1.3.5.2 Akt (Protein kinase B): Akt/PKB is a serine/threonine protein kinase and a cellular homologue of the viral onco-protein v-Akt. It belongs to the family of AGC kinases (cAMP-dependent protein kinase A/protein kinase G/protein kinase C) with a high homology to protein kinases A and C. Akt is phosphorylated at two specific sites (kinase domain Thr-308 and C-terminal regulatory motif Ser-473) during its full activation.

Regulation of PKB/Akt activity: PKB/Akt exists in an inactive state and is localized in the cytosol in resting cells. Akt activity is augmented by the stimulation of cells with growth factors, insulin, G-protein coupled signaling or integrin signaling (Welch et al, 1998). Upon activation of the upstream PI3-K, PIP3 is synthesized at the plasma membrane and Akt interacts with these lipids via its PH domain. This anchors Akt to the plasma membrane, where conformational change occurs in Akt as a result of site-specific phosphorylation. Next, the phosphorylated and activated Akt is translocated either to cytosol or the nucleus to interact on its downstream targets (Crowell et al, 2007; Fruman et al, 1998; Maddika et al, 2007b).

Physiological functions of Akt - Role in cell survival and apoptosis: Maintenance of cell survival via inhibition of apoptosis is an important function of activated PI3-K/Akt in the cell. Akt regulates this process by phosphorylating different downstream substrates responsible for direct or indirect regulation of the apoptosis. Bad (a pro-apoptotic Bcl-2 family member), caspase-9 (a pro-cell death protease), FKHRL1 (a fork head transcription factor), IKK, Mdm2 and cAMP response element-binding (CREB) are some of the important targets for activated Akt (Manning & Cantley, 2007). Akt mediated phosphorylation of Bad promotes cell survival by inhibiting its interaction with the

antiapoptotic Bcl-2 family members like Bcl-2 itself and Bcl-xL and further preventing cytochrome c (Cyt c) release (Datta et al, 1997). Akt also phosphorylates Caspase-9 causing a conformational change that leads to the inhibition of its proteolytic activity (Cardone et al, 1998; Hu et al, 2005). Also, Akt promotes cell survival by activation of NF-κB via phosphorylating IKKα, which in turn mediates the degradation of IκB, an NF-κB inhibitor (Kane et al, 1999; Madrid et al, 2001; Romashkova & Makarov, 1999). Akt mediated phosphorylation of CREB also enhances cell survival by increasing transcription of pro-survival genes like Bcl-2, Mcl-1 and Akt itself (Pugazhenthi et al, 2000; Zheng & Quirion, 2006). Further, Akt also negatively regulates apoptosis by enhancing the degradation of p53 by enhanced nuclear localization and enhancing its binding to the negative regulator Mdm2 (Mayo & Donner, 2001).

1.3.5.3 PI3-K/Akt pathway in CML and other cancers

The proliferation of Bcr-Abl positive cells requires consistent PI3-kinase activity. Bcr-Abl induced activation of PI3-K is secondary to the formation of multimeric complexes where Cbl, and the adaptor molecules Crk and CrkL are also associated (Sattler et al, 1996). Studies with isolated mutant domains revealed that association of p85 subunit of PI3-K with Bcr-Abl was dependent on the SH2 and SH3-mediated interactions and the p85 mutant inhibits the growth factor-independent *in vitro* proliferation of Bcr-Abl positive cells. This observation further verifies a selective and critical role of PI3-K in Bcr-Abl dependent signaling (Ren et al, 2005). Akt is the next relevant substrate. An interesting report placed Akt in the downstream cascade of the IL-3 receptor and identified the pro-apoptotic protein Bad as a key substrate (Skorski et al, 1997). Phosphorylated Bad is inactive because it is no longer able to bind anti-apoptotic proteins

such as Bcl-2 or Bcl-xL but trapped by cytoplasmic 14-3-3 proteins. Thus, Bcr-Abl imposes a profound effect on phosphoinositol metabolism, which might again shift the balance to a pattern similar to physiologic growth factor stimulation.

The gene that encodes p110α catalytic subunit of PI3-K is also overexpressed in some ovarian and cervical cancers (Shayesteh et al, 1999). Overexpression of the downstream kinase Akt2 has been reported in some breast, ovarian and pancreatic cancers as well (Bellacosa et al, 1995; Ruggeri et al, 1998). In addition, some primary human colon and ovarian cancers have mutations in the p85α regulatory subunit, which leads to PI3-K activation (Philp et al, 2001). These structural alterations presumably release the p85-p110 complex from negative regulation, bypassing the normal role of RTK signaling in PI3-K activation.

The role of PTEN mutations: Most compelling evidence for the role of PI3-K/Akt pathway comes from the fact that the second most mutated protein in different cancers, MMAC1/PTEN (phosphatase and tensin homolog deleted on chromosome 10) is frequently mutated in various types of cancers particularly in glioblastomas, prostate and endometrial cancers. PTEN is a dual specificity phosphatase that has both lipid and protein substrates and functions as a negative regulator of PI3-K/Akt pathway (Ali et al, 1999; Steck et al, 1997). The identification of frequent PTEN mutantations revealed the importance of this protein in regulating the different aspects of many cancers. However, in malignant myeloid disorders like acute myeloid leukemia (AML), myelodysplastic syndromes (MDS) and CML the mutations in the *MMAC1/PTEN* gene on chrosome 10q23.3 was reportedly less frequent although *MMAC1/PTEN* displays many essential features of a classical tumor suppressor gene (Aggerholm et al, 2000).

1.4 Apoptosis: Introduction

Programmed cell death as an important cellular event was reported earlier but only in 1972 Kerr and colleagues first introduced the term *Apoptosis* to describe the ATP dependent programmed cell death (Kerr et al, 1972; Lockshin & Williams, 1965). The fundamental understandings on apoptosis and the related cellular pathways came from genetic studies in the nematode *C. elegans.* The breakthrough report in apoptosis research on the genetic basis of programmed cell death was a predictable death pattern of about 13% of newly formed somatic cells in the developing embryo of *C. elegans.* This study provided a simple animal model to investigate the genetic basis of apoptosis (Sulston & Horvitz, 1977). By the year 1982, the existence of genes, such as *ced-3*, that controlled essentially all the somatic cell deaths was established (Horvitz et al, 1983). In another report, Vaux and colleagues identified the B-cell lymphoma gene *Bcl-2* as an anti-apoptosis gene (Vaux et al, 1988). Then, p53 and c-Myc were identified as pro-apoptosis regulators (Evan et al, 1992; Lowe et al, 1993b; Vaux et al, 1988). A third discovery in this series was the identification and characterization of Fas/Apo-1 as a death-transducing cell-surface receptor and the involvement of caspase activity in this process (Los et al, 1995; Trauth et al, 1989). Since 1990, when Bcl-2 was found in the mitochondrial membrane, it has been known that mitochondria were implicated in the regulation of mammalian cell apoptosis (Chen-Levy & Cleary, 1990; Zamzami et al, 1996).

The characteristic features of apoptosis include cell membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation, finally ending with engulfment by macrophages or neighboring cells, thereby avoiding an inflammatory response in the surrounding tissues (Darzynkiewicz et al, 1997; Khosravi-Far & Esposti, 2004; Schultz &

Harrington, 2003). Apoptosis is distinct from necrosis. Necrosis as a cell death process is initiated after the cells suffer a major insult that leads to swelling, loss of membrane integrity and disruption of the cells, resulting in a strong inflammatory response in the surrounding tissue. On the contrary, the apoptotic cell death program does not affect the extra-cellular environment. Genetic screening revealed an association of four important genes that control the programmed cell death in *C. elegans Ced-3, Ced-4, Ced-9* and *Egl-1* (Hengartner et al, 1992; Liu & Hengartner, 1999a; Liu & Hengartner, 1999b). Homologues of these apoptosis regulatory genes of *C. elegans* have been identified in mammals. *Ced-3* is functionally homologous to the mammalian caspase family, *Ced-9* represents the anti-apoptotic Bcl-2 members, *Ced-4* is homologous to Apaf1 and *Egl-1* is homologous to pro-apoptotic BH-3 (Liu & Hengartner, 1999a; Yuan et al, 1993; Zou et al, 1997a). However, compared to the programmed cell death in *C. elegans*, apoptosis in mammals is tightly regulated through more complex interactions between positive and negative apoptosis regulating machineries. In mammalian cells induction of apoptosis or programmed cell death occurs via two major pathways where caspases act as the central effector molecules. The sequential activation of these caspases is triggered by either the death receptor-dependent (extrinsic) pathway or via the mitochondrial (intrinsic) pathway induced by factors like UV radiation, chemotherapeutics, free radicals or DNA damage (Brouckaert et al, 2005; Los et al, 1995; Maddika et al, 2006).

1.4.1 Caspases – The key executioner proteolytic enzymes of apoptosis

The caspase family: The caspases are a family of calcium dependent-cysteine proteases that cleave cellular substrates at specific aspartate residues (Alnemri et al, 1996). Currently, from an initial discovery of 14 caspases, 11 human caspases have been verified and recognized: caspase-1-10 and caspase-14 (Alnemri et al, 1996; Pistritto et al, 2002). Caspase-11 is absent in human, caspase 12 is often inactive, caspases -4 & -5 correspond to murine caspase-11 and caspase-13 does not exist. Interestingly, mice lack caspases -4, -5 and -10 and there is an evolutionary tendency of increasing the number of caspases over phylogenetic time, from four in *C. elegans* to seven in Drosophila and eleven in mice and humans (Lamkanfi et al, 2002; Los et al, 1999).

Caspases serve as signaling mediators and once activated some of them can propagate the activation response to other family members initiating a proteolytic cascade. Caspases can be classified into three major groups according to their specific functions: (1) *Apoptotic initiator caspases*: with either a death effector domain (DED) or with a caspase activation and recruitment domain (CARD). They are initiator caspases responsible for activating the downstream effector caspases; (2) *Apoptotic effector caspases:* which are activated by initiator caspases and perform the final steps in the apoptotic program; and (3) *Inflammatory caspases* are involved mainly in the inflammatory response (Boyce et al, 2004).

Caspase activation: In general, caspases are synthesized as inactive zymogens and during the induction of apoptosis these zymogens can be cleaved to form active enzymes (Mikolajczyk et al, 2003). The apoptotic signaling pathways (extrinsic and intrinsic) lead to this caspase zymogens processing. The homodimerization of caspase-9 and caspase-8

in the apoptosome and death inducing signaling complex (DISC) complexes respectively, leads to their activation (Boatright et al, 2003). Similarly the homophilic interactions of the CARD domain in caspase-9 and caspase-2 and the DED motifs of caspase-8 and caspase-10 with CARD or DED motif containing proteins like Apaf-1 or Fas-associating protein with death domain (FADD), respectively, may mediate the adaptor driven caspase activation (Boatright & Salvesen, 2003; Donepudi et al, 2003). Studies on transgenic knock-out mice indicate that caspase-8 is required for all known death receptor mediated apoptotic pathways, whereas caspase-9 is mostly involved in the mitochondrial pathway (Hakem et al, 1998; Kuida et al, 1998; Varfolomeev et al, 1998).

There is a growing list of identified caspase substrates. Some of the important caspase substrates are apoptotic proteins (such as Bcl-2, Bcl-xL, Akt, PKC, IAP, FLIPL, MEKK1, Bid, ICAD, RIP, PAK2, and NF-κB), cellular DNA repair proteins (such as ATM, DNAPK, Rad51 and PARP-1), cell cycle proteins (such as Cdc27, Wee1 $p27^{kip1}$, $p21^{cip1}$ and cyclin A), cytoskeletal and structural proteins (actin, lamins, β-catenin, Fodrin and Gas-2), and few other cellular proteins (Ras, STATs, Gap, SP1, Nedd4, ataxin-3, atrophin-1, presenilins and caspases themselves) (Fischer et al, 2003).

1.4.2 Extrinsic and intrinsic apoptotic pathways

1.4.2.1 Death receptor pathway (Extrinsic): The ligand bound death receptors like CD95L-CD95, TNF-TNFR-1 and TRAILDR4 or DR-5 acts as the key mediators of the extrinsic apoptotic pathway.

The CD95 pathway: The cell surface molecule CD95 (Fas/Apo-1) was identified as a receptor responsible for mediating apoptotic cell death of transformed cells and

regression of experimental tumors developed in nude mice (Trauth et al, 1989). Intracellular clustering of death domains (DD) follows CD95 receptor trimerization as a result of CD95 ligand binding. This allows the adaptor protein FADD (Fas-associated death domain containing protein) to associate with the receptor. Subsequently FADD recruits pro-caspase-8 that results in the formation of DISC (Chinnaiyan et al, 1995; Kischkel et al, 1995). The CD95 ligand induced clustering of CD95, FADD and pro-caspase-8 within the DISC leads to autoproteolytic processing of caspase-8 (Medema et al, 1997b). Following this cleavage, caspase-8 is released from the DISC as an active heterotetramer (Medema et al, 1997a; Scaffidi et al, 1997).

The TNF pathway: As a multifunctional pro-inflammatory cytokine TNF (tumor necrosis factor) elicits a number of biological responses that includes neutrophil activation, cytokine production, chronic inflammation, and is involved in the pathogenesis of autoimmune diseases, cancer and neurodegenerative disorders (Wajant et al, 2003). Clustering and trimerization of TNF receptors, recruits the death domain containing adaptor protein TRADD to the death domain of TNF-R mediating the binding of TRAF2 (TNF receptor associated factor) and RIP, a serine/threonine kinase containing a death domain (Hsu et al, 1996). The TRAF-2/TRADD/RIP complex (complex-I) subsequently recruits a stable IKK (IκB kinase) complex containing IKKα, IKKβ and a regulatory protein NEMO (NF-κB essential modulator), where activated IKK complex phosphorylates and targets IκB for proteosome-mediated degradation (Devin et al, 2000; Hsu et al, 1995). This finally results in the release of NF-κB that allows it to translocate to the nucleus where genes involved in different cellular functions are activated. Complex-I also mediates the TNF induced p38 MAPK and JNK activation, which again

results in the activation of gene transcription via the c-Jun-Fos transcription factor complex (Devin et al, 2003; Lee et al, 1997; Tournier et al, 2001).

The TRAIL pathway: TRAIL receptors have five distinct identified subtypes with high sequence homology in their extracellular domains and with significant differences in their intracellular domains, namely: Death Receptor 4 (DR4/TRAILR1), DR5 (TRAIL-R2), DcR1 (TRAIL-R3), DcR2 (TRAIL-R4) and Osteoprotegrin (Emery et al, 1998; Kharbanda et al, 1997; Pan et al, 1997; Sheridan et al, 1997; Walczak et al, 1997). TRAIL induced apoptotic signaling is similar to that of CD95. Binding of TRAIL to its receptors DR4 or DR5 triggers the formation of the DISC by recruiting FADD, caspase-8 and caspase-10, followed by activation of caspase-8 and caspase-3 (Ashkenazi, 2002). TRAIL induced apoptosis also involves the activation of the mitochondrial death pathway, similar to CD95 induced cell death in type II cells (Deng et al, 2002). TRAIL also activates NF-κB and utilizes this activation for the expression of pro-apoptotic genes during apoptotic cell death by recruiting the adaptors RIP and TRAF2 (Lin et al, 2000).

1.4.2.2 Mitochondrial death pathway (Intrinsic): A range of diverse cell death stimuli such as chemotherapy drugs, DNA damage, and growth factor withdrawal can activate the intrinsic pathway of apoptosis (Reed, 2000). Subsequently, there is convergence of intracellular signals at the level of mitochondria causing loss of mitochondrial membrane potential (Ψm) and the release of pro-apoptotic molecules such as Cyt c, apoptosis inducing factor (AIF), Endonuclease G, Omi/HtrA2 and SMAC/Diablo (Green & Reed, 1998). The released Cyt c forms a high molecular weight pro-apoptotic protein complex in the cytosol called the *apoptosome* which contains oligomerized Apaf-1, pro-caspase-9, Cyt c and dATP. Apoptosome triggers the activation of pro-caspase-9 to caspase-9 (Jin &

El-Deiry, 2005). The activated caspase-9 further activates the downstream effector caspases like caspase-3, caspase-6 and caspase-7 by proteolytic cleavage (Los & Walczak, 2002; Slee et al, 1999). However, tight regulation of this caspase activation is essential to prevent inadvertently committing a cell to apoptosis. Once activated, a family of specific inhibitory proteins imposes further regulations on caspase function. The 'inhibitor of apoptosis proteins' (IAPs) directly binds and inhibits active caspases (Deveraux & Reed, 1999). Furthermore, the mitochondrial IAP antagonists that include SMAC/Diablo and Omi/HtrA2, selectively bind to IAPs via their IAP-binding motifs that promote their auto-ubiquitination and proteosome mediated degradation (van Loo et al, 2002a). Also, Omi/HtrA2, a serine protease can directly cleave and inactivate the IAP proteins (van Loo et al, 2002b; Verhagen & Vaux, 2002). Mitochondria also release pro-apoptotic proteins that are unrelated to activation of caspase such as AIF and Endonuclease G, in addition to proteins that trigger or mediate caspase activation. The mitochondrial protein AIF is a flavoprotein that, once released, is translocated to the nucleus and induces peripheral chromatin condensation and large-scale DNA fragmentation (Joza et al, 2001). Endonuclease G, an apoptotic DNase, released from the mitochondria, is also translocated to the nucleus causing DNA fragmentation (Li et al, 2001). In addition, AIF also has other functions including vital regulatory roles during oxidative phosphorylation (Vahsen et al, 2004). Studies indicate AIF is important in maintenance of glutathione levels during cellular stress and during oxidative phosphorylation in mitochondria (Cande et al, 2004).

1.4.3 Bcl-2 family proteins - Regulators of cell survival and cell death

Classification of Bcl-2 family: *Bcl-2* was identified as an oncogene that is involved in human B-cell follicular lymphoma (Bakhshi et al, 1985; Tsujimoto et al, 1984). *Bcl-2* is pro-survival to various apoptotic stimuli such as IL-3 or serum deprivation, heat shock and cytotoxic chemotherapy (Strasser et al, 1991; Tsujimoto, 2003). The proteins of Bcl-2 family regulate mitochondrial outer membrane permeability and constitute a critical checkpoint in the intrinsic pathway of apoptosis. About 20 mammalian Bcl-2 family members have been identified, which contain at least one of the four conserved motifs known as Bcl-2 homology domains (BH1-BH4) (Adams & Cory, 1998; Cory & Adams, 1998). The Bcl-2 family members are mainly grouped according to their structure and function into three sub-families: (1) *The anti-apoptotic Bcl-2 sub family* (Bcl-2, Bcl-xL, Mcl-1 and Bcl-w) members contain at least BH1 and BH2 domains. (2) *The Bax subfamily* (Bax, Bak and Bok) members contain BH1, BH2 and BH3 domains and are very similar in structure to the Bcl-2 subfamily. Functionally, however, they antagonise the anti-apoptotic Bcl-2 subfamily members. (3) *The BH3-only sub family* (Bik, BNIP3, Bim, Bad, Bid, Blk and Crk) members are pro-apoptotic molecules with a central BH3-domain (Reed, 1997).

Mechanisms of apoptosis regulation by Bcl-2 family members: The Bcl-2 proteins prevent or activate the apoptotic process respectively by regulating the release of mitochondrial Cyt c and other pro-apoptotic mediators. The BH3 region of Bax and Bak are able to induce apoptotic cell death. Interestingly, the replacement of the BH3 region of Bcl-2 by the BH3 region of Bax converts Bcl-2 from an anti-apoptotic molecule to a pro-apoptotic molecule (Hunter et al, 1996; Hunter & Parslow, 1996). Bcl-2 and Bcl-xL

prevent the apoptosis-associated release of Cyt c, AIF and other pro-apoptotic factors by binding either directly to pro-apoptotic Bcl-2 members like Bax and Bad or even Cyt c and Apaf-1 (Adachi et al, 1997; Hu et al, 1998). The ratio of anti- to pro-apoptotic Bcl-2 family members constitutes a regulator system that sets the threshold of cellular susceptibility to the induction of apoptosis (Korsmeyer et al, 1993). Both Bax and Bak undergo conformational changes, polymerization and are re-located to the outer mitochondrial membrane during apoptosis (Antonsson & Martinou, 2000; Gottlieb, 2000). Death signals activate some BH-3 members which in turn either induce direct oligomerization of pro-apoptotic Bax and Bak to insert into the outer mitochondrial membrane or indirectly release the pro-apoptotic proteins from the anti-apoptotic Bcl-2 proteins (Desagher et al, 1999; Zha et al, 1997). The pro-apoptotic Bcl-2 proteins engage either permeability transition pore (PTP) dependent or independent mechanisms for mitochondrial outer membrane permeabilization to release the apoptogenic factors (Kim et al, 2000; Marzo et al, 1998; Shimizu et al, 1999). Opening the PTP causes ion flux in and out of the mitochondria from the cytosol and thus results in the loss of mitochondrial membrane potential, and rupture of the outer mitochondrial membrane releasing the mitochondrial proteins. In the contrary, the pro-apoptotic Bcl-2 proteins like Bax and Bak can directly generate pores in the outer mitochondrial membranes in a permeability transition independent process and causes the release of mitochondrial pro-apoptotic proteins (Antonsson & Martinou, 2000; Gross et al, 1999).

Regulation of Bcl-2 family members: Bcl-2 expression is regulated both at the transcriptional and posttranslational levels. The p53 protein transcriptionally up-regulates Bax and NF-κB upregulates Bad that further facilitates the pro-cell death process (Baetz

et al, 2005; Chipuk & Green, 2004; Chipuk & Green, 2006; Chipuk et al, 2004; Lowe et al, 1993a). On the other hand, NF-κB also upregulates the anti-apoptotic Bcl-2 family members like Bcl-2, Bcl-xL, Bfl1/A1 and Nrl3 assisting cell survival (Perkins & Gilmore, 2006). Phosphorylation as a form of post-translational regulation plays a crucial role in regulating Bcl-2 members (Deng et al, 2001; Ruvolo et al, 1998). Akt phosphorylates Bad and sequesters it away from the mitochondria via 14-3-3 proteins leading to cell survival (Datta et al, 1997). However, other studies support a transcriptional and post-translational independent regulation of Bcl-2 family members. For example, p53 translocates from nucleus to mitochondria, binds and inhibits Bcl-2/Bcl-xL or activates Bax (Chipuk & Green, 2004; Chipuk et al, 2004).

1.4.4 Antiapoptotic signals induced by Bcr-Abl in CML

Several investigators observed that Bcr-Abl expressing cells show resistance to apoptosis when DNA damaging agents are applied (Bedi et al, 1994; Sirard et al, 1994). The cultures of murine and human hematopoetic progenitor cell lines are growth factor dependent and withdrawal of these factors (e.g. rIL3) from the media induces apoptosis. When the kinase active Bcr-Abl is expressed these cells continue proliferating in absence of external rIL3 (Daley et al, 1992; Sirard et al, 1994). This effect is critically dependent on the tyrosine kinase activity of Bcr-Abl and correlates with Ras activation (Cortez et al, 1995; Puil et al, 1994). Other key downstream signal transduction pathways activated by Bcr-Abl also, reportedly prevent apoptosis in hematopoietic cells. The PI3-K/Akt pathway, for example, is an important bridge between the survival signal triggered by Bcr-Abl and modulators of apoptosis, like Bad (Gelfanov et al, 2001; Neshat et al, 2000). As a proapoptotic member of the Bcl-2 family de-phosphorylated Bad binds to the pro-

survival Bcl-2 family member Bcl-xL, inhibiting its function (Yang et al, 1995). Thus, the phosphorylation of Bad is correlated with the inhibition of apoptosis in CML. The survival signal from Bcr-Abl is partially mediated by Bad and requires targeting of Raf-1 to the mitochondria (Neshat et al, 2000). However this phosphorylation of Bad does not account for the complete protection of CML cells from apoptosis, suggesting the existence of alternative Bad-independent survival pathways. Bcr-Abl blocks the release of mitochondrial cytochrome c and prevents activation of caspases (Amarante-Mendes et al, 1998; Dubrez et al, 1998). The Bcl-2 family of proteins mediates this specific antiapoptotic effect that is upstream of caspase activation. Bcr-Abl was previously reported as a factor up-regulating Bcl-2 in a PI3-K depended manner (Skorski et al, 1997). Moreover in Bcr-Abl expressing cells Bcl-xL is also transcriptionally activated by STAT5 (Horita et al, 2000; Sillaber et al, 2000). Possibly the relative importance of the different survival, proliferative and antiapoptotic pathways depends on the cellular context, and the cross talk between these pathways is essential for the complete protection against apoptosis. Thus, Bcr-Abl can be considered as a factor that shifts the balance toward the inhibition of apoptosis while simultaneously providing a proliferative stimulus (Fernandez-Luna, 2000)

1.5 Apoptin: A cancer selective killer molecule

1.5.1 Chicken anemia virus (CAV)

CAV is a small (23-35 nm) encapsulated avian-specific icosahedral virus of the *Circoviridae* family with a single stranded 2.3 kb DNA and it has recently been classified as the only member of the genus *Gyrovirus* (Pringle, 1999). CAV is a known infectious agent for young chicks with highest infectivity rate on the hatch day (Noteborn et al,

1991). However, older chickens acquire rapid resistance against the effects of the virus. Infection with CAV in young chickens is characterized by generalized lymphoid atrophy, development of subcutaneous hemorrhages with necrotic dermatitis, small bursas of Fabricius (BFs) and small thymuses (histology: atrophy or hypoplasia of thymuses and BFs), pale bone marrow and watery blood, increased incidence of bacterial secondary infections like septic necrotizing clostridial dermatitis and hepatitis and ultimately death (Adair, 2000; Noteborn, 2004). According to the previous studies hematopoetic cells of bone marrow origin and the T cell precursors in thymus are the targets of CAV infection. The early infection of the hematopoetic precursor cells in the bone marrow results in the reduction of the precursor erythroid cells and megakaryocytes that leads to anemia and thrombocytopenia causing intramuscular hemorrhages. In the thymus, cortical lymphocytes like the CD3+/TCR+ T-cell precursor cells were among the first cells to be destroyed, whereas generally the non-lymphoid leukocytes and stromal cells are spared. In addition the CD8+/Cd4+ mature T cells are reportedly reduced by CAV infection (Taniguchi et al, 1983). Studies have shown that induced apoptosis is the mechanism of thymocyte and hematopoetic precursor depletion following CAV infection.

Sequences in the 5′- nontranscribed region of the CAV genome are believed to be the sole promoter enhancer for CAV (Noteborn et al, 1994b). The CAV genome contains three partially overlapping open reading frames; together they produce a single strand of unspliced RNA which encodes three different viral proteins (Miller et al, 2005). VP1 is a 51.6kDa structural protein; VP2 is a 24kDa non-structural protein and the third protein VP3, also known as ‘apoptin’, is a 13.6kDa protein. All three proteins are found in CAV infected cells (Noteborn et al, 1994a; Phenix et al, 1994). The triggering of apoptosis by

CAV infection has been attributed to the non-structural protein VP3 or apoptin (Adair, 2000; Maddika et al, 2006; Noteborn et al, 1994a; Noteborn, 2004).

1.5.2 Apoptin (VP3)

Apoptin is a 13.6 kDa viral protein encoded by the *VP3* gene of Chicken Anemia Virus (Adair, 2000). It is well documented that apoptin induces apoptosis in a broad range of transformed and cancer cells but not in non-transformed or primary cells (Heilman et al, 2006; Poon et al, 2005b). Apoptin induced apoptosis is independent of death receptor pathways (Maddika et al, 2005; Maddika et al, 2006). When applied, apoptin remains in the cytoplasm of primary cells. Interestingly, apoptin is accumulated into the nucleus of transformed cells which induces apoptosis by the activation of mitochondrial death pathway following phosphorylation and cytoplasmic transfer of orphan nuclear steroid receptor Nur77 (Maddika et al, 2005). The cellular localization of apoptin plays a crucial role for its selective toxicity (Danen-van Oorschot et al, 2003; Maddika et al, 2007c; Poon et al, 2005b). Apoptin associates itself with the anaphase-promoting complex resulting in subsequent G2/M arrest and apoptosis (Teodoro et al, 2004); also it was reported that SAPK/JNK signaling pathway is possibly activated during apoptin induced cell death (Ben et al, 2005). However, targeted translocation of apoptin into the nucleus is not sufficient for induction of apoptosis of primary cells. An acceptable explanation towards apoptin's tumor specific toxicity, therefore, requires identification of additional interaction partners or specific activation of other signalling pathways in the cancer cells preceding the nuclear accumulation of apoptin. Apoptin was also found to be very effective in inducing tumor specific apoptosis in animal models of tumor formation. Single intra-tumor injection of recombinant apoptin into nude mice with human

hepatoma cells resulted in reduction of tumor growth and symptoms of regression within a week of apoptin administration (Pietersen et al, 1999). In another study, tumor immunization with adjuvant recombinant apoptin along with IL-18 was proved to induce a higher Th1 (T helper cell 1) response against Lewis lung carcinomas demonstrating significant inhibition of the tumor growth compared to apoptin alone (Lian et al, 2007). No effect of apoptin was seen in normal cells suggesting the tumor specific toxicity of apoptin *in vivo* in these studies. Similarly, a combined treatment approach with recombinant adenovirus expressing apoptin and etoposide showed an additive cytotoxic effect on human osteosarcoma U2OS cells. When paclitaxel treatment was combined with apoptin, the survival of p53-positive human osteosarcoma U2OS and non-small lung carcinoma A549 cells, p53-negative human osteosarcoma Saos-2 cells and p53-mutant human prostate cancer Du145 cells responded at a significantly lower dose of cytotoxic chemotherapy. This observation indicates that application of apoptin in combination with other conventional chemotherapeutic agents might offer a more efficient form of cancer therapy (Olijslagers et al, 2007).

1.5.3 Apoptin: its structure and the molecular basis of functions: One of the key properties of apoptin, which was also confirmed by others, and us is its ability to induce tumor specific cell death by apoptosis. This event does not dependent on the p53 status of the cell (Burek et al, 2005; Maddika et al, 2005; Zhuang et al, 1995). Apoptin does not require p53 to induce apoptosis as the expression of apoptin in cells either having functional p53, mutant p53 or no p53 shows equal frequencies of apoptosis induction (Zhuang et al, 1995). Apoptin induces of endogenous TAp73 and the p53/p73 BH3-only pro-apoptotic target, PUMA, independently of the p53 function. Interestingly, P73, a

member of the p53 family, plays its critical role here. In the absence of the p53 function, apoptin utilizes the p73 pathway to induce efficient tumor cell death (Klanrit et al, 2008). It has also been reported that apoptin mediated cell death is independent of the Bcl-2 status of the cell, in fact the co-expression of apoptin and anti-apoptotic Bcl-2 in Saos-2 cells was shown to accelerate the cell death compared to apoptin alone (Danen-Van Oorschot et al, 1999a; Danen-Van Oorschot et al, 1999b). However, the role of Bcl-2 family members during apoptin induced cell death is still not clear and requires further studies. The selective nuclear localization of apoptin has a critical role in the induction of apoptosis and it was previously proposed that apoptin's nuclear translocation depends on the cellular concentration of apoptin (Wadia et al, 2004). Apoptin has also been reported to bind to the nuclear DNA, but the functional significance of this association is not yet understood (Leliveld et al, 2004). According to reports, apoptin modulates the sphingolipid-ceramide pathway that leads to an increased concentration of the lipid ceramide in human prostate cancer cells independent of the expression of p53, Bax, survivin, LIP, XIAP and CIAP (Liu et al, 2006a; Liu et al, 2006b). Ceramide is a second messenger molecule and a central molecule in sphingolipid metabolism; it plays a crucial role as a tumor suppressor lipid that mediates an intracellular response to diverse therapeutic agents such as Fas ligand, TNF-α, ionizing radiation and chemotherapeutic drugs. Inhibition of ceramide synthesis is associated with drug resistance (Ogretmen & Hannun, 2004). Interestingly, apoptin inhibits acid ceramidase and increases the levels of ceramide by preventing enzymatic deacylation of ceramide to sphingosine that can be explained as a part of its mechanism of action (Liu et al, 2006b). In addition it is reported that apoptin activates c-Jun N-terminal kinase (JNK) signalling during the cell death

process in U937 human lymphoma cells (Ben et al, 2005), but again the mechanism of JNK signalling activation is not yet well understood. The selective toxicity of apoptin is mainly attributed to its differential localization in tumor and normal cells. Apoptin mainly localizes to the nucleus of cancer cells, but its transport to the nucleus is severely impaired in normal cells (Danen-van Oorschot et al, 2003). Recent data indicate that apoptin is a nucleo-cytoplasmic shuttling protein whose localization is regulated by nuclear localization signals (NLS) and nuclear export signals (NES). Studies using various apoptin deletion and point mutant derivatives revealed a C-terminal bipartite basic nuclear localization signal (spanning the amino acids 82-88 and 111-121) which mediates the nuclear localization of apoptin, transporting it via the nuclear pore complex (Poon et al, 2005b). The NLS of apoptin is active both in normal and cancer cells as apoptin is shown to translocate to the nucleus in both types of cells though the amount of nuclear apoptin is significantly lower in normal cells compared to the cancer cells (Poon et al, 2005a; Rohn & Noteborn, 2004; Wadia et al, 2004). Recently, however, a CRM1 nuclear export sequence (NES residues 97-105) has been identified. This NES is functionally active in normal cells. But, in tumor cells the apoptin NES is inactive which determines the tumor cell specific nuclear localization of apoptin (Poon et al, 2005b). In addition to the tumor specific nuclear targeting sequences, apoptin has a phosphorylation site at threonine residue 108 (Thr-108) adjacent to the NES (Rohn et al, 2002). We have recently identified the Ser-Thr protein kinase CDK2 is essential for apoptin-induced cell death and acts as the principal kinase that phosphorylates apoptin at Thr-108 residue (Maddika et al, 2008b). The phosphorylation at Thr-108 residue is reported to be responsible for tumor specific apoptin's nuclear accumulation via inactivating the NES in

tumor cells thus inhibiting the nuclear export specifically in tumor cells. However, another opposing report indicated that this Thr-108 phosphorylation is not essential for the tumor-specific nuclear localization of apoptin and abolishing it only partially affect apoptin's apoptotic activity in tumor cells (Lee et al, 2007). Apoptin also contains a leucine rich sequence (LRS; aa: 33-46) at the N-terminus, which is important for apoptin's interaction with promyelocytic leukemia (PML) proteins (Heilman et al, 2006). The PML nuclear bodies formed via the PML proteins are the structured protein complexes associated with the nuclear matrix and they play a role in regulating apoptosis, DNA replication, repair, transcription and RNA transport (Salomoni & Pandolfi, 2002). Interestingly the inactivating mutations in the LRS sequence not only reduce the ability of apoptin to associate with PML bodies, but also reduce apoptin's nuclear accumulation in tumor cells (Outlined in **Figure 8**). It has been recently reported that apoptin interacts with the SH3 domain of the p85 regulatory subunit of PI3-K, through its proline rich sequence (PRS; aa: 81-86). Surprisingly, when p85 is downregulated, apoptin is moved out of the nucleus and this impairs cell death induction by apoptin. This observation indicates that interaction of apoptin with the p85 subunit of PI3-K is essential for the cytotoxic activity of apoptin (Maddika et al, 2007c).

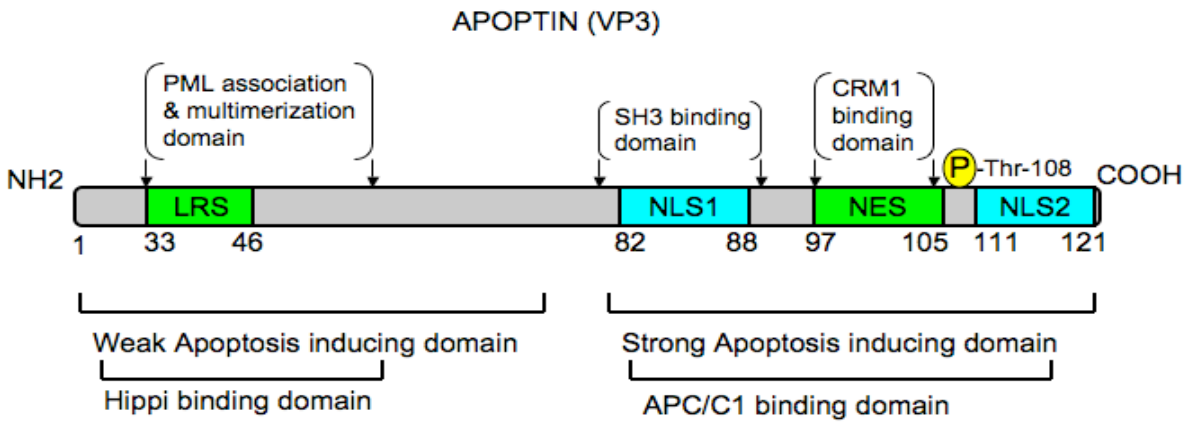


Figure 8: *Diagrammatic representation of structural and protein binding domains of apoptin;* the numbers indicate amino acids at each structural motif. The proline rich sequence (PRS; aa: 81-86) is merged within the NLS1 domain (aa: 82-88). [Based on: (Heilman et al, 2006; Noteborn et al, 1994a; Poon et al, 2005a; Poon et al, 2005b; Rohn et al, 2002; Rohn et al, 2005; Wadia et al, 2004)].

1.5.4 Derivatives of apoptin and their applications

Purified apoptin is a 13.6 kDa viral protein with 121 amino acid residues. Like many other viral proteins, apoptin is also prone to multimerization (Leliveld et al, 2003b). For biological experiments that involve the use of cell extracts, intact or live cells and anima l models, different modified forms of apoptin are used to fit within the limitations of that specific experiment. Most of the experiments in the present study make use of apoptin derivatives including a complete recombinant apoptin molecule conjugated with: (i) Glutathione S-transferase (GST-Apoptin), (ii) Green Fluorescence Protein (GFP-Apoptin), (iii) TAT-conjugated peptide (TAT-Apoptin) and (iv) Synthetic apoptin derivatives.

GST-Apoptin: The GST-Apoptin conjugate (~ 40 kDa) is typically used for pull-down assays as an *in vitro* method to determine physical interaction between apoptin and other proteins. Such pull-down assays are useful for both confirming the existence of a

predicted protein-protein interaction and as an initial screening assay for novel interactions. Availability of purified GST tagged apoptin (the bait) is the minimal requirement for an apoptin pull-down assay that captures and 'pulls-down' an apoptin-binding partner (the prey).

GFP-Apoptin: The GFP conjugate of apoptin (~ 40 kDa) is typically used to identify specific intracellular localization of apoptin in eukaryotic cells upon induced expression by exogenous expression vectors. Applying modern epi-fluorescent or laser confocal microscopy it is also possible to identify specific protein interaction in the cellular environment. Additionally, using large amount of extract of cells expressing GFP-Apoptin, co-immunoprecipitation experiments are conducted to identify intracellular interactions of apoptin with specific other proteins.

TAT-Apoptin: The TAT (trans-acting activator of transcription) protein is a HIV1 virus derived polypeptide with 72 (p14) amino acid residues. The TAT protein from human immunodeficiency virus 1 (HIV-1) was shown to enter cells when added exogenously (Fawell et al, 1994). This provides the potential for intracellular therapeutic use of oligonucleotides, peptides and larger proteins. The mechanism of cellular entry of TAT-conjugated proteins is not yet completely understood. The large charge of the peptide at physiological pH excludes the possibility of passive diffusion across the lipid bilayer. It is known that the exclusive involvement of cationic charges within the TAT sequence plays a central role in its cellular uptake (Wender et al, 2000). The rate of cellular uptake also depends on the number of basic residues present, specifically the number of arginine residues (Wadia & Dowdy, 2002). The TAT-conjugated apoptin has a small sequence of 11 amino acid residues from the protein transduction domain (PTD) of TAT. The TAT

PTD has six arginine and two lysine residues, making the peptide highly cationic. The TAT PTD allows apoptin to traverse the cell membrane like many other large cargo molecules tested previously. The peptide sequence is able to pass through the cells membranes from almost all tissues including brain cells (Schwarze et al, 1999). This opens new possibilities for direct delivery of proteins into animal models and patients in the context of protein or peptide based therapy. However, the potential immunological consequences related to such technology involving the use of cell-penetrating peptides to overcome the lipophilic barrier of the cellular membranes and delivering larger molecules, require careful investigations in the future.

Synthetic apoptin derivatives Purified and biologically active peptide sequences of apoptin can be custom prepared by solid phase peptide synthesis technique. Such production methods of biologically active synthetic apoptin derivatives are recently reported (Heckl et al, 2008). Since the use of apoptin derivatives in diagnostics and therapy require large amount of purified product, such artificially synthesized apoptin conjugates are a significant alternative to the recombinant products. However, the first objective of the production of biologically active apoptin is to test the comparative potency and efficacy of such custom prepared peptides for both *in vitro* and *in vivo* experiments to evaluate their uptake by the nuclei of tumor cells and healthy cells. Although the specificity of tumor cell killing was found to be modified, the synthetic dephosphorylated apoptin with relatively shorter peptide sequences showed apoptin's predicted tumor cell specific nuclear accumulation and retention (Heckl et al, 2008; Poon et al, 2005b). This interesting observation indicates the importance of in-depth structure and functional analyses of apoptin and its derivatives.

1.6 Computational modeling of protein structure and molecular interaction studies:

In many cellular processes, proteins recognize and bind their specific targets in a highly organized manner. The specificity of such interactions is, however, determined by the physical, structural and chemical properties of two interacting proteins. Knowledge about 3D structures of these proteins is necessary to study *in silico* their interactions. However, construction of the 3D structure of a specific protein either by X-ray crystallography or by NMR methods may not be always possible because of a number of reasons. When these conventional methods appear inadquate to build reasonable 3D structures of the proteins of interest, the alternative technique to study the protein structure and to predict its interactions with other proteins is homology modeling and virtual protein docking experiments. This novel method to identify the function of proteins, based directly on the sequence-to-structure-to-function paradigm is broadly known as ‘computational protein modeling’. In this system, it is possible to build the best-predicted structure of the protein from its amino acid sequences (target) on the basis of known 3D structure of related family members (templates). The 'low resolution' models obtained by homology modeling provide essential information of the spatial arrangement of important groups of residues.

Today, computerized digital modeling tools can predict the structure and functions of new proteins and can shed light on their putative functions and underlying mechanisms (Rychlewski et al, 1999). Such software can also provide insight into design experiments and suggest possible ways towards custom drug design (Mandal et al, 2007). The structures generated through these computational methods are used as homology modeling templates that enable genomic-scale levels of structural and functional

annotations. The protein active sites, termed fuzzy functional forms (FFFs), can be simulated based on the geometry and conformation of the active site (Cammer et al, 2003). The FFFs are applied to identify their corresponding active sites in a library of known protein models produced by crystallography or NMR spectroscopy. Next, to screen for active sites, these FFFs are used in threading prediction algorithms (Fetrow & Skolnick, 1998). Eventually, the functional domains of these proteins from their predicted structures are specifically identified. Thus, large-scale functional screening of genomic sequence databases based on the prediction of structure from sequence and identification of functional active sites in the predicted structure is possible (Marsden et al, 2006). Moreover, the deleterious functional effects of amino acid mutations can also be predicted using a library of structure-based function descriptors (Herrgard et al, 2003).

The Pairwise Sequence Alignment is an approach to find possible homology for a protein in the sequence databases (**Table 2**). It also acts as a foundation for more complex sequence comparison methods. A pairwise sequence alignment method is applied to compare two protein sequences according to a predetermined matrix match criterion with preference (score) to replace the amino acid types. Several types of algorithms have been developed to obtain an optimal alignment. Needleman and Wunsch developed the first algorithm where a dynamic programming technique was applied to serve this purpose (Needleman & Wunsch, 1970). Currently, *BLAST* is the most frequently used and fastest local alignment tool. Another reason for its wide acceptance is that *BLAST* gives a predicted value for an alignment, which estimates how many times one expects to see occurrence of such an alignment by chance alone. This allows a user to quantitatively assess the significance of the alignment.

In contrast, the Multiple Sequence Alignment strategy aligns several sequences to obtain the best similarity among them. 'Multiple sequence alignment' is considered as the foundation for identification of functionally important protein regions, for building a sequence profile and further sequence search as well as for protein family classification and phylogenetic reconstruction. The conserved motifs in multiple sequence alignment frequently have biological significance. A profile derived from multiple sequence alignment is sensitive with less noise and can be categorized into global alignment and local alignment. Short similar regions across the different sequences are focused in the local multiple sequence alignment (Krissinel & Henrick).

In global multiple sequence alignment clusters of previously aligned sequences are treated as a linearly weighted profile when they are subsequently aligned with another sequence or cluster. The 'progressive method' is a widely used algorithm for global alignment (Gotoh, 1999). It initially aligns all possible pairs of sequences and then uses the 'pairwise similarity scores' to construct the model. Several variants of the progressive algorithm have also been developed (**Table 2**). The related multiple sequence alignment based software *CLUSTAL* allows a user to choose between the dynamic programming algorithm and an algorithm that is less sensitive but much faster (Wilbur & Lipman, 1983).

The multiple sequence alignment method is useful in classifying protein sequences into families that subsequently indicates transitional structural, functional, and evolutionary relationships.

Table 2

Selected Sequence Comparison, Modeling and Docking Tools

Pairwise Sequence Alignment		
BLAST	www.ncbi.nlm.nih.gov/BLAST/	server/executable
SSEARCH	www.vega.igh.cnrs.fr/bin/ssearch-guess.cgi	server
FASTA	www.embl-heidelberg.de/cgi/fasta-wrapper-free/	server
GCG/BESTFIT	www.gcg.com	executable
KESTREL	www.cse.ucsc.edu/research/kestrel/	server
Multiple Sequence Alignment		
CLUSTAL	www.ubik.microbiol.washington.edu/ClustalW/	executable
Tcoffee@igs	http://igs-server.cnrs-mrs.fr/Tcoffee/	server
BlockMaker	www.blocks.fhcrc.org/blocks/blockmkr/	server
GCG/PILEUP	www.gcg.com	executable
MEME	www.sdsc.edu/MEME/meme/website/	server
Multalin	www.toulouse.inra.fr/multalin.html	server
BCM Search Launcher	www.dot.imgen.bcm.tmc.edu:9331/multi-align/	server
PAUP	www.lms.si.edu/PAUP/	executable
Search Based on Multiple Sequence Alignment		
HMMER	www.hmmer.wustl.edu	executable
PSI-BLAST	www.ncbi.nlm.nih.gov/BLAST/server/	executable
SAM-T98	www.cse.ucsc.edu/research/compbio/HMM-apps/	server
Three Dimensional Modeling		
EBI-SSM	http://www.ebi.ac.uk/msd-srv/ssm/	server
SWISS-MODEL	www.swissmodel.expasy.org	server
DeepViewer	www.swissmodel.expasy.org/spdbv/	server
Docking Tools		
AutoDock	http://autodock.scripps.edu/	server
CAPRI	http://www.ebi.ac.uk/msd-srv/capri/	server
ClusPro	http://nrc.bu.edu/cluster	server
PPI Server	http://www.biochem.ucl.ac.uk/bsm/PP/server/	server
PIC	http://crick.mbu.iisc.ernet.in/~PIC	server

The Ramachandran Plot: The 'Ramachandran plot' of an unknown protein is essential for testing the level of accuracy in its computed 3D model. Briefly: the N-Cα and Cα-C bonds in a polypeptide chain are relatively free to rotate. These rotations are represented by the torsion angles phi (Φ) and psi (Ψ), respectively. In 1963 'GN Ramachandran' first used computer models of small polypeptides to find their stable conformations by systematically varying these torsion angles (Ramachandran et al, 1963). The structure was examined for close contacts between atoms for each of these conformations. Atoms were treated as hard spheres with dimensions corresponding to their van der Waals radii. Therefore, angles, which cause spheres to collide, correspond to sterically disallowed conformations of the polypeptide backbone. Disallowed regions involve steric hindrance between the side chain methylene group and main chain atoms. Interestingly, glycine has no side chain and therefore can adopt phi and psi angles in all four quadrants of the 'Ramachandran plot'.

Protein Docking: Protein docking is defined as a bound structure complex formed from two proteins or a protein and a substrate, starting with two separate unbound proteins. A widely used docking program is *DOCK* (Kuntz et al, 1982). Predicted rigid binding by this method is often proved in wet-lab experiment.

AutoDock (http://autodock.scripps.edu/) is another software that is designed to predict the bound conformations between flexible ligands and rigid proteins (Rosin et al, 1998). A number of such docking tools are listed in **Table 2**.

The computational tools for protein modeling are a rapidly developing area of science and play an important role in protein and peptide sciences, from the genome scale to the atomic level. However, one cannot use modeling tools blindly. Experimental evidences

are often needed for some predictions, which could be otherwise inaccurate. There are still many challenging problems in protein modeling and the related research is very active.

2. Rationale

2.1 CML: Current treatment options

The currently available treatment options in CML have different risks and benefits and the appropriate choice for an individual patient depends upon age as well as phase and duration of the disease. One of the most commonly used oral anti-CML chemotherapy is hydroxyurea (Hydrea). Oral hydroxyurea treatment is with relatively few side effects but capable of controlling peripheral white blood cell (WBC) counts in CML (Deininger et al, 2000). Similarly, IFNα also induce complete cytogenetic response in approximately 13% of Ph+ CML patients but IFNα therapy has many potential side effects (Bonifazi et al, 2001). These include fever, sweating, weight loss, fatigue, bone and muscle pain, which may interfere with quality of life. The currently practiced curative therapy option for CML is allogeneic bone marrow transplantation (BMT). High-dose chemotherapy, with or without radiation, may wipe out CML in the patients. To avoid life-threatening bone marrow failure and reconstitute the bone marrow with immuno-competent donor bone marrow, a bone marrow stem cell graft is introduced to the patient after the myeloablative conditioning treatment. The other more advanced and relatively easier BMT option is non-myeloablative allogeneic stem cell transplant (NST) with post BMT donor lymphocyte infusion (DLI), where a milder but specific conditioning protocol is applied to induce ‘transient immuno-ablation’. This conditioning spares the vital bone

marrow cells creating a window of opportunity for the donor origin stem cells and T-lymphocytes to fight against the CML cells (Kapelushnik et al, 1996; Slavin et al, 1998). If no donor is available, it is possible to use the patient's own cultured bone marrow cells - an experimental procedure for purging or cleansing the marrow of leukemia cells. However, autologous bone marrow transplant in CML patients is rarely being used as a therapy option for CML after the introduction of imatinib (STI571/Gleevec®). Effective inhibitors of Bcr-Abl kinase which is the key offending oncogenic fusion protein found in CML, were searched for, identified and some of them are currently in routine clinical application owing to the fact that Bcr-Abl is an attractive drug target for CML. Drugs like Desatinib® and Nilotinib® belong to this new generation of signal transduction inhibitors (STI) that are in clinical use or just passing through clinical trials (Inokuchi, 2006; Noble et al, 2004). However, the major logistical problem associated with these specific molecules is their failure as rapid and durable oncolytic agents during advanced disease conditions like blast crisis in comparison to radiation or cytotoxic chemotherapy (Piccaluga et al, 2007). While the ‘domino effect’ of multiple signal transduction pathway activation is at its peak, the efficacy of imatinib decreases (Jacquel et al, 2003; Krystal, 2001). Because imatinib is a selective and potent inhibitor of Bcr-Abl kinase activity, the inability of imatinib to cure CML blastic phase in mice suggests that only the specific inactivation of Bcr-Abl kinase is not sufficient to cure CML (Hu et al, 2004). Therefore, one of the tempting options is developing newer drugs that can target multiple activated kinase pathways in CML for effective elimination of malignant cells (Hu et al, 2006).

2.2 Bcr-Abl tyrosine kinase inhibitors and the need to identify such novel molecules

Imatinib is one of the first small molecules, recently introduced to CML therapy that can specifically inhibit the Bcr-Abl kinase activity in leukemic cells without adversely affecting the normal cell population. Imatinib mesylate (also called Gleevec or STI571) is chemically a benzamide methanesulfonate (2-phenyl-amino-pyrimidine) that binds to the kinase domain of Bcr-Abl through an induced-fit mechanism and stabilizes the protein in its closed, inactive conformation (Cohen et al, 2002). Imatinib inhibits *in vivo* activity of Bcr-Abl kinase and currently it is considered as the first line of adjuvant therapy for CML (Cohen et al, 2002; Lugo et al, 1990; Soverini et al, 2008). However, in many patients the emergence of resistance to imatinib therapy is of major concern and the mechanisms underlying this resistance have been urgently investigated. The most prominent finding from clinical samples is that 50–90% of cases in which resistance develops after imatinib therapy involve point mutations in the Bcr-Abl kinase domain. Until recently more than 17 different clinically relevant point mutations within this domain have been identified. Apparently some mutations specifically cause amino acid substitutions that interfere with the ability of imatinib to interact directly with the Bcr-Abl kinase domain while in other cases the ability of the Bcr-Abl kinase domain to adopt a conformation required for imatinib binding is destroyed or modified (Sawyers, 2004; Shah & Sawyers, 2003). Another mechanism of resistance may be due to the amplification of the *BCR-ABL* gene and increased expression of Bcr-Abl fusion protein (Shah & Sawyers, 2003). These kinase domain mutations ultimately lead to a relapse of CML under treatment. Interestingly, relapse may occur despite receiving imatinib as a first line of therapy with good initial hematological and cytogenetic response (Weisberg & Griffin, 2001). The

high frequency of mutations within the kinase domain causing imatinib resistance necessitates renewed efforts on the identification of unique inhibitors that are active against imatinib-resistant mutants of Bcr-Abl. Several other compounds, such as PD180970 and CGP76030 are able to inhibit Bcr-Abl by binding to the ATP-binding site and were shown to induce apoptosis in selected cases of imatinib-resistant leukemias (Hoover et al, 2002; Huang et al, 2002; La Rosee et al, 2002; Nimmanapalli et al, 2002). The T315I mutation is one of the most frequently occurring point mutation in *BCR-ABL* gene that leads to the generation of clones highly resistant to imatinib. These imatinib resistant clones are also less responsive to the newer generation ATP-competitive Src kinase inhibitors like BMS-354825 (Dasatinib®) and AMN107 (Nilotinib®) (Schindler et al, 2000; Shah et al, 2004). The T315I *BCR-ABL* mutation was found to be resistant to many other known kinase inhibitors. Several different approaches have been described to overcome this resistance. In some work, farnesyltransferase inhibitors like SCH66336 and the proteasome inhibitor Bortezomib were demonstrated to have growth inhibitory effects on some imatinib-resistant CML cells (Yu et al, 2003a). In another novel approach, induction of apoptosis in Bcr-Abl expressing cells by histone deacetylase inhibitors (HDIs) involved coordinate inactivation of the cytoprotective Raf/Mek/ERK pathway in conjunction with the ROS-dependent activation of JNK (Yu et al, 2003b).

Briefly, none of the currently established CML therapy is without drawbacks and effective equally against CML in any phase of the disease.

2.3 Apoptin as a novel Bcr-Abl tyrosine kinase inhibitor

In a search for signal transduction inhibitors that could modulate multiple proliferation pathways, activate apoptosis and also possibly bypass some of the above described inherent problems with currently known Bcr-Abl tyrosine kinase inhibitors, apoptin is considered as a promising candidate molecule (Maddika et al, 2006; Poon et al, 2005a). Apoptin induced apoptosis is independent of death receptor pathways (Maddika et al, 2005; Maddika et al, 2006). When applied, apoptin remains in the cytoplasm of primary cells, but it migrates into the nucleus and induces apoptosis in transformed cells by activating the mitochondrial death pathway mediated by the orphan steroid receptor Nur77 (nuclear receptor 77) (Maddika et al, 2005). The cellular localization of apoptin plays a crucial role for its selective toxicity (Danen-van Oorschot et al, 2003; Maddika et al, 2007b; Poon et al, 2005b).

On the other hand, the Src homology domains (SH3 ~ 60 kDa) are the most commonly found protein-protein interaction domain in c-Abl and tends to bind the proline rich sequences forming a poly proline type II helix (Hou et al, 2006; Moarefi et al, 1997; Sicheri & Kuriyan, 1997). Interestingly, apoptin has a proline rich region (aa:81-86) and it has documented regulatory interaction with the SH3 domain of other kinases (Maddika et al, 2005; Maddika et al, 2007c). Besides, Bcr-Abl is a known inhibtor of caspases activation by blocking the release of the mitochondrial Cyt c whereas apoptin triggers the release of Cyt c and apoptosis inducing factor (AIF) from mitochondria (Dubrez et al, 1998; Maddika et al, 2005). Altogether, these evidences and correlations place apoptin as a highly potential candidate protein that deserves its evaluation as a Bcr-Abl tryrosine kinase inhibitor in the *in vitro* and *in vivo* experimental environments.

3. Hypothesis: The interaction of apoptin with Bcr-Abl-kinase constitutively inhibits the phosphorylation of Bcr-Abl and redirects the Bcr-Abl-kinase and related downstream survival and proliferation pathways towards the cell death pathways during apoptin induced apoptosis in *Bcr-Abl* expressing chronic myeloid leukemia cells.

4. Objectives:

Aim 1. To test the possible interactions of apoptin with Bcr-Abl and to study the effects of these interactions on the phosphorylation of Bcr-Abl and other down stream kinases.

Aim 2. To elucidate the effects of apoptin on Bcr-Abl-kinase downregulation during apoptin mediated cell death in Bcr-Abl expressing murine and human cell lines.

Aim 3. To establish a three-dimensional model of the apoptin molecule and to identify the possible interaction sites precisely at the molecular level.

5. Materials and Methods

5.1 Reagents: All the chemicals were purchased from either Sigma-Aldrich® Inc. (Oakville, ON. Canada), unless otherwise indicated. All antibodies were purchased from Sigma-Aldrich. Inc (Oakville, ON. Canada), Abcam® Inc. (Cambridge, MA. USA) or Cell Signaling Technology®, Inc. (Danvers, MA. USA).

5.2 Bcr-Abl kinase Inhibitor: Imatinib; Imatinib mesylate or Imatinib® (also know as Gleevec®/STI-571; Novartis, Dorval QC. Canada), is a synthetic (2-phenyl-amino-pyrimidine) and site-specific tyrosine kinase inhibitor (Cohen et al, 2002). One 400 mg clinical grade tablet of imatinib was dissolved into sterile phosphate buffer solution (PBS) at a concentration of 1 mg/ml and filtered by 0.2mm syringe filter (Fisherbrand®). The final concentration in cell culture applications was optimized to 1mM according to our earlier dose reponse studies and as described by others (Olijslagers et al, 2007).

5.3 Antibodies: The following antibodies were used: murine/rabbit anti-Bcr-Abl/anti-Bcr (monoclonal: Abcam® Inc.), murine Anti-Akt (monoclonal), rabbit anti-c-Myc-phospho & murine anti-c-Myc (monoclonal: Abcam® Inc.), rabbit anti-cleaved PARP-1 (polyclonal: Cell Signaling Technology®, Inc.), rabbit anti-CrkL (polyclonal: Sigma-Aldrich. Inc), (rabbit-anti-phospho-Bcr-Abl, anti-phospho-STAT5 and anti-phospho-CrkL were purchased as a polyclonal antibody cocktail; Multiplex Western detection kit from Cell Signaling Technology®, Inc), anti-rabbit Cy3, anti-mouse Cy3 (all from Sigma, Oakville, ON) and murine monoclonal anti-apoptin antibody (kind gift from Dr. D. Jans, Victoria, Australia).

5.4 Expression plasmids: The following plasmids were used: GFP, GFP-Apoptin (Apoptin cloned into pEGFP-C1 vector, Clontech), GST, GST-Apoptin (apoptin cloned into PGEX-2T vector, Amersham Biosciences), apoptin mutant plasmids as previously described (Poon et al., 2005a), pTAT-GFP, pTAT-Apoptin (obtained from Dr. M. Tavassoli, London, UK). The p210Bcr-Abl mutants, ΔSH2 (deletion of the SH2 domain), ΔSH3+ΔSH2 (deletion of both the SH3 and the SH2 domains), ΔSH3+R1053L (deletion of the SH3 domain and single amino acid substitution in the SH2 domain) and P1013L+R1053L (single amino acid substitutions in the SH3 and SH2 domains, respectively), cloned in pSRαMSVtkneo vector (kind gift of Prof. Tomasz Skorski, Temple University, Philadelphia, USA) (Nieborowska-Skorska et al, 1999). The plasmids were propagated either in BL21(DE3)pLysS or DH5α *E. coli* bacterial strains, after transforming the respective competent bacterial cells using standard $CaCl_2$ mediated chemical transformation. The plasmid isolations from the positive clones were done with Sigma GenElute^{TM} Plasmid Maxi-prep kits using their standard protocols.

5.5 Cells and cell culture: The mIL3 dependent murine primary hematopoetic stem cells $32D_{DSMZ}$ (ACC 411: DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstraße 7 B, 38124 Braunschweig, Germany) and external growth factor independent $32D^{p210}$wt:b3:a2/e13:a (will be referred as $32D^{p210}$) Bcr-Abl variant were kind gifts from Dr. Heiko van der Kulip, Dr. Margarete Fischer-Bosch-Institut für Klinische Pharmakologie, Stuttgart, Germany. Both of the cell lines were grown in DSMZ recommended RPMI-1640 medium (Gibco BRL) 500 ml supplemented with 20% FBS (Hyclone), 0.05g penicillin/streptomycin (Gibco BRL), 10mM HEPES (2-(4-hydroxyethyle)-1-piperazinyl)ethanolsulfonsäure), 2mM L-Glutamine (Gibco BRL),

0.13mM L-Asparagin, 0.05 nM β-Mercaptoethanol, 1mM Na-Pyruvate (Gibco BRL), and 3 ml 100x non-essential amino acid (Gibco BRL). $32D_{DSMZ}$ cells are strictly dependent on murine interleukin 3 (mIL3) so the media was supplemented with 10% supernatant from WEHI-3B cells as described elsewhere (McCubrey et al, 1991). The model human CML cell line K562 (ATCC® Number: CCL-243™) was cultured in ATCC recommended Iscove's modified Dulbecco's medium (Gibco BRL) with 4mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 10% FCS (v/v) and antibiotics (Klein et al, 1976; Lozzio & Lozzio, 1975). All cells were grown at 37°C with 5% CO_2 in a humidified incubator and maintained in a logarithmic growth.

5.6 Transfection of mammalian cells: Different mammalian primary and cancer cell lines were transfected with the desired plasmids by Lipofectamine^{TM}2000 (Invirogen Canada Inc. Burlington, Ontario, L7P 1A1)) reagent. The cells were plated in an antibiotics free medium 24 hours prior to transfection and plasmid DNA was added to 100µl of medium in a tube at the recommended concentrations (typically 2µg for a 12 well plate and 5µg for a 6 well plate) at the time of transfection. The Lipofectamine reagent (5µl for 12 well and 10µl for 6-well plate) was diluted in 100µl of medium in a second tube. Next, the DNA and Lipofectamine reagents were mixed after 5 minutes and then incubated for 20 minutes at room temperature to allow the formation of DNA-liposome complexes. Following the incubation, the DNA-lipid mixture was gently added directly to the cells that had previously been rinsed with PBS and replaced with fresh medium.

5.7 Protein purifications

5.7.1 GST-fusion protein purification: The GST and the recombinant GST-Apoptin were purified according to the manufacturer's protocol by high performance glutathione sepharose beads (Amersham Biosciences®). In brief, the GST and GST-Apoptin expressing bacterial clones grown overnight in 50 ml of LB were transferred in 500 ml of LB medium with 50μg/ml ampicillin to amplify further. The bacteria were allowed to grow at 37°C until they reached an OD600 of 0.6- 0.8 and then induced with Isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM for 3 hours. The cells were then harvested by centrifugation at 5000 RPM (Beckman JA-10 rotor) for 10 min and the cell pellets were washed once in PBS, resuspended in 10 ml of lysis buffer (1X PBS, 100 mM EDTA, 0.5 mM DTT and protease inhibitors) and sonicated three times for 45 seconds. The cell lysate was centrifuged for 15 minutes at 13000 RPM; the supernatant containing the GST fusion proteins were transferred to new tubes and 1 ml of 50% glutathione sepharose beads were added. The beads were then washed three times with 50 volumes of 1X PBS/1% Triton X-100 and once with 50 mM Tris (pH 7.5)/150 mM NaCl following an incubation of the fusion proteins for 4 hours at 4°C. Finally, the fusion proteins were eluted in 3 ml of 10mM reduced glutathione in 50mM Tris (pH 8.0) and the excess salt was removed by PD-10 desalting columns. The recombinant proteins were analysed by SDS-PAGE followed by Coomassie blue staining (**Figure 11**).

5.7.2 TAT-fusion protein purification: The recombinant TAT-GFP and TAT-Apoptin were expressed in BL21 bacterial cells and purified as previously described (Guelen et al, 2004). In brief, the recombinant histidine tagged TAT-Apoptin expressing bacterial clone was grown overnight in 50 ml of LB and used to inoculate 1 liter of LB medium added

with 50μg/ml ampicillin. The bacteria were harvested by centrifugation at 5000g (Beckman JA-10 rotor) for 10 minutes after a 4-5 hours of growth at 37°C. The collected cell pellets were then washed once in PBS and were resuspended in 10 ml of buffer (8M urea, 100mM NaCl and 20mM HEPES, pH 7.0) and sonicated by three pulses of 45 seconds each in ice. Cell debris was removed by centrifugation at 10000g for 10 minutes at 4°C. Imidazole was added to the TAT-fusion proteins in the buffer to a final concentration of 10mM. TAT-fusion proteins from the supernatant were isolated by Ni-ion affinity chromatography (Amersham Pharmacia Biotech) with stepwise elution in 1 ml fractions with increasing imidazole concentrations (100, 250 and 500 mM). Excess urea was removed by elution through PD 10 desalting columns (Amersham Pharmacia Biotech) using sterile PBS with 10% glycerol and protease inhibitors (complete mini, Roche). The concentration of TAT fusion proteins was assessed by mixing with Bradford reagent (BioRad) and the absorbance measured at 595nm using a 96 well plate reader. The recombinant TAT conjugated proteins were analysed by SDS-PAGE followed by Coomassie Blue staining (**Figure 30**).

5.7.3 Apoptin derived synthetic peptides:

To evaluate the biological effects of apoptin derived peptide sequences were custom synthesized (GenScript Corporation, PO Box 855, NJ, 07076-0855, USA) as TAT-conjugates that ensured its entrance to the live cells in active form (Hellgren et al, 2004). In the actual pilot experiments the TAT-conjugated apoptin derived peptide sequence (aa:81-90/rkkrrqrrr-**PKPPSK**KRSC) was used as test and TAT-conjugated scrambled sequence (aa:81-90 scrambled sequence: rkkrrqrrr-PRKPSKSPKC) was used as the control in a concentration of 1 μM. The TAT-conjugated deca-peptide (aa:81-90/

rkkrrqrrr-**PKPPSK**KRSC) was used for the array based SH3 domain interactions study as well.

5.8 GST-pull down assay and protein identification: GST pull-down assay is a method to test interactions between a tagged protein or the bait (here GST tagged apoptin) and another protein (test protein, or prey: here Bcr-Abl^{p210}). The bait protein GST-Apoptin was purified from the E. coli expression system and it served as the secondary affinity support to confirm Bcr-Abl^{p210} as a protein interaction partner to the bait. Briefly, either purified GST or GST-Apoptin along with total 32D^{p210} or K562 cell lysate (by sonication) was immobilized on glutathione sepharose beads overnight at 4°C in IP-Buffer containing protease and phosphatase inhibitors (50mM Tris-Hcl pH 8.0, NaCl 150mM, NP-40 0.5%, EDTA 1mM, PMSF 1mM, NaF 10mM, Na_3VO_4 1mM, β-glycerophosphate 25mM & H_2O). The beads were washed six times with ice-cold lysis buffer and the bound proteins were isolated on SDS-PAGE to be identified by immunoblotting. Supernatants of these pull-down products were used as positive controls for the test protein (Bcr-Abl^{p210}) and identical experiments performend in parallel where of bait protein (GST-Apoptin) were omitted served as negative controls (Beads control).

5.9 TAT mediated protein transduction Cells were seeded in either 6 well or 12 well culture plates (Corning Incorporated COSTER®). His-tagged TAT-fusion proteins were diluted in culture medium to the desired concentrations (1μM) and added directly to the cells. Cells were incubated with TAT-fusion proteins at 37°C in a 5% CO2 humidified incubator for 16 hours.

5.10 TransSignal SH3™ Domain Array: The SH3 domain array was performed according to the manufacturer's protocol (Panomics, Inc. Redwood City, CA, USA). In brief, purified TAT-Apoptin was incubated with TransSignal SH3™ Domain Array1 membrane at 4ºC, and after necessary washing steps the image was acquired on high performance chemiluminescence film (Hyperfilm™ECL, Amersham Biosciences). The proteins in the array are spotted in duplicates. Histidine tagged ligands were spotted along the bottom and in duplicate along the right side of the membrane for alignment purpose.

5.11 Immunoblotting: The protein concentration in the cell lysate was estimated by Bradford assay. 30-50 μg of the protein lysate was mixed with 5X SDS loading dye and heated to denature for 5 minutes at 99°C. The protein samples were resolved by SDS PAGE and transferred to PVDF-membrane (Amersham Biosciences®) using a Wet transfer apparatus (BioRad®) for 1 hour at constant current of 85mA. Membranes were blocked for 1 hour with 5% non-fat dry milk powder in Tris-buffered saline with 0.25% v/v Tween-20 (TBS-T) and incubated overnight with the appropriate primary antibody diluted in TBS-T containing 1% non-fat dry milk powder or 5% bovine serum albumin (BSA). Membranes were washed three times for 5 minutes in TBS-T and later incubated with an appropriate secondary antibody conjugated with horseradish peroxidase (HRP) for 45 minutes at room temperature. Following the repeated washing steps (15 minutes, x3) the specific proteins on the membrane were detected by using enhanced chemiluminescent (ECL) staining (Amersham Biosciences®).

5.12 Co-Immunoprecipitation (CO-IP):

Co-immunoprecipitation (CO-IP) is a technique for protein interaction study. In a CO-IP experiment the target antigen is co-precipitated as a protein-antibody complex from a lysate by an antibody that is attached to a binding partner. It is generally assumed that the interaction partners of the co-precipitated protein are functionally related at the cellular level. In the current study, depending on the protein, 100-500 μg of cell lysate (by sonication) was added with 2-5 μg of appropriate antibody in IP buffer and incubated for 4 hours at 4°C agitating on a rotary shaker. Subsequently, 100μl of 50% protein-G Sepharose beads (Amersham Pharmacia Biotech®) washed three times with 1X PBS were added to the protein-antibody immune complexes and further incubated for 1 hour at 4°C. After the incubation, the beads were washed five to six times with the lysis buffer, each time centrifuging at 4°C and removing the supernatant. After final wash, the beads were suspended in 50 μl of 5X SDS loading dye and heated for 5 minutes at 99°C to denature and release the adhered proteins to the loading dye. After brief centrifugation of these samples at 13000g to separate the beads, the proteins were resolved on SDS-PAGE (8%) and detected by immunoblotting with appropriate antibodies. In these experiments either purified recombinant target proteins or supernatants containing the residual target proteins are used as positive controls and the protein precipitate from identical parallel experiments without the first precipitating antibody were used as negative controls.

5.13 Immunocytochemistry and fluorescent imaging: Cells were grown overnight in 10 ml Petri dishes and then transfected with appropriate plasmids according to the lipofectamine protocol (Invirogen®). After 16-18 hours of incubation the cells were collected and washed with PBS and then fixed in 4% w/v paraformaldehyde in PBS. Thin

smears were made on pre-washed standard microscope glass slides and then air-dried. Next, the cells were permeabilized with permeablization buffer (0.1% triton X-100 in PBS), blocked with 5% BSA/PBS for 1 hour, and subsequently incubated overnight at 4°C with an appropriate primary antibody diluted in the blocking buffer. An appropriate secondary antibody conjugated with Cy3 or FITC depending on the experiment was used following adequate washing steps. To visualize nuclei and to prevent photo bleaching of the fluorescent dye, slides were mounted with Vectashild® with DAPI (4',6-diamidino-2-phenylindole). The slides were then imaged for fluorescent signals and analyzed using Zeiss fluorescent microscope and Zeiss Axiovision 3.1 software.

5.14 Bcr-Abl multiplex kinase assays: The *in vitro* kinase assays were performed using a non-radioactive method. Briefly, the phosphorylation status of Bcr-Abl, STAT5, CrkL and Akt were measured by scanning the signal strength of phosphorylated proteins on Western blot membranes. The kinase reaction was performed in K562 and $32D^{p210}$ cells by overnight (16 hours) incubation with TAT-Apoptin (test), Imatinib® (positive control) TAT-GFP, or no treatment (negative controls). The cell extracts were resolved by SDS-PAGE (10%) and detected by immunoblotting using their respective phospho-specific antibodies or antibodies detecting both phosphorylated and non-phosphorylated kinase. A high-resolution scanner (STORM 860: Molecular Dynamics®: Amersham Pharmacia Biotech) was used to scan the immunoblot membranes after ECL treatment and individual band strengths were measured by Image Quant 5.2 software (Molecular Dynamics®) after background corrections. The final results were normalized according to respective loading controls and expressed as a ratio of measured values for phosphorylated proteins versus total protein bands.

5.15 Apoptosis and proliferation assays

5.15.1 Morphological detection of cell death: Suspended cells were grown in liquid media and treated with appropriate apoptosis inducing agents or apoptotic inhibitors. Cells were then harvested and spread over microscope slides, fixed for 15 minutes in 3.7% v/v paraformaldehyde in PBS and stained with the DNA dye DAPI (10μg/ml, Molecular probes, Eugene, OR) and mounted with Vectashild®. The morphology of the cells was examined by using phase contrast microscopy and the hallmarks of apoptosis such as nuclear condensations and fragmentations were visualized by Zeiss fluorescent microscope using DAPI specific filter. The image data was analyzed and stored by Zeiss Axiovision 3.1 software.

5.15.2 Identification of apoptotic nuclei by detecting cleaved PARP-1: PARP-1 is one of the earliest targets of activated caspase-3 and presence of cleaved PARP-1 is a specific indicator of initiation of apoptosis (Fischer et al, 2003). So we wanted to see if cleaved PARP-1 can be detected in apoptin treated CML cells. As described above, Bcr-Abl kinase active suspended cells and Bcr-Abl negative cells as control were treated for 16 hours with appropriate apoptosis inducing agents like imatinib, TAT-Apoptin as test and positive control and TAT-GFP as negative control. Microscope slides were prepared according standard immunocytochemistry protocol using FITC conjugated cleaved PARP-1 specific antibody and counter stained with the DNA dye DAPI (10μg/ml) and mounted with Vectashild®. The presence of cleaved PARP-1 and nuclear morphology were studied for 100 cells in each of the different control and test groups by epi-fluorescent microscopy as described above.

5.15.3 MTT assay: The MTT assays were carried out as previously described (Mosmann, 1983). Briefly, 10,000 cells, grown in 96 well plates, were added with 10µl of 5mg/ml tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)] dissolved in phenol red free RPMI medium (Roswell Park Memorial Institute medium, Gibco). The cells were then incubated for 4 hours at 37°C. Following the incubation, the cells were centrifuged at 90g for 10 minutes and the supernatant was discarded. Next, the formazan crystals were dissolved in 200 µl 1:1 solution of ethanol & DMSO. Finally, the absorbance was measured at λ=570 nm using a spectrophotometer and the percentage of living cells in experimental wells was calculated by comparing to the control cells. The MTT assay is a rapid and relatively simple method of studying cell survival following any drug treatment but this method does not differentiate cell death by necrosis or by apoptosis.

5.15.4 Measurement of cell death by propidium iodide uptake: The number of apoptotic cell nuclei was assessed by the Nicoletti method (Nicoletti et al, 1991). Cells subjected to appropriate experimental conditions of apoptosis were washed once with PBS and collected after centrifugation at 800g for 10 minutes at room temperature. The cells were suspended in 1X PBS and propidium iodide was added (1µg/ml) and the propidium iodide (PI) fluorescence was measured directly by flow cytometer using FL-2 channel. Living cells with intact cell membrane were PI-negative, whereas dead cells with a permeable membrane were PI positive with a stronger fluorescence signal. The PI-positive cells were gated using 'BD CellquestPro' software and represented in terms of percentage of cell death.

5.16 Computational studies of apoptin structure and interactions:

The modeling procedure included the following steps: (i). Sequence analysis: - for the determination of globular domains and low complexity, transmembrane or coiled-coil regions that is based on the primary structure of the protein, (ii). Homology search: - for proteins/domains of known 3D structure that will provide the basis of the structural alignment and (iii). Structural alignment: - to determine the average position of each atom in the target sequence based on the position of the corresponding atom in the template sequences, depending on the degree of sequence similarity. This step provides the determination of the backbone of the protein is followed by the completion of the side chains and the refinement of the generated model, (iv). Model presentation: to display the structure in a convenient manner. Next, to examine the binding interactions of apoptin and Bcr-Abl, I performed the molecular modeling of apoptin where I used the following softwares: (view detailed in **Table 2**)

(i) ClustalW2 (http://www.ebi.ac.uk/) for multiple sequence alignment

(ii) ModellerSoft9V1 for 3D modeling of apoptin

(iii) DeepViewer for viewing the 3D models

(iv) AutoDock4.0.1 for virtual docking experiments

THE SEQUENCE OF APOPTIN:

MNALQEDTPP GPSTVFRPPT SSRPLETPHC REIRIGIAGI TITLSLCGCA NARAPTLRSA TADNSESTGF KNVPDLRTDQ **PKPPSK**KRSC DPSEYRVSEL KESLITTTPS RPRTARRCIR L (121)
(Bold: SH3 interacting domain)

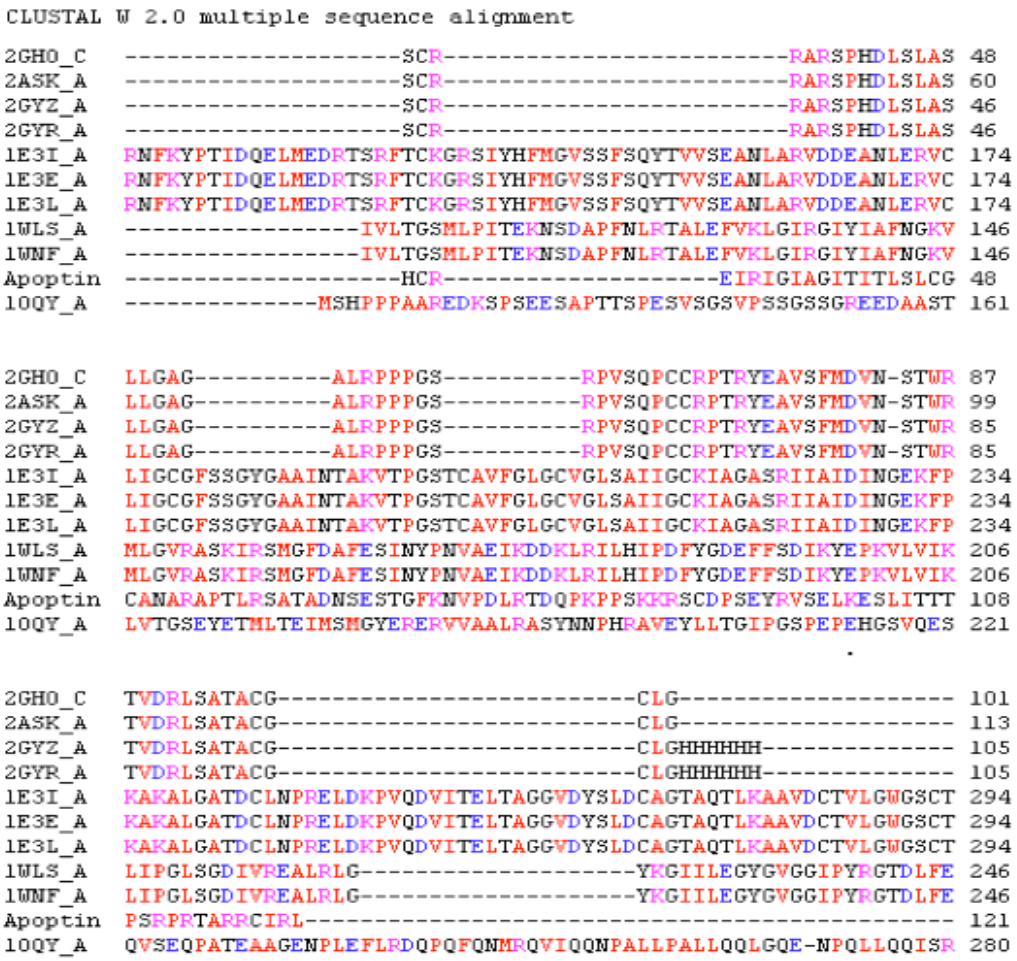


Figure 9. *Multiple sequence alignment table for Apoptin (aa:33-90):* Ten proteins which serve as templates were identified (PDB code: 2GHO_C, 2ASK_A, 2GYZ_A, 2GYR_A, 1E3I_A, 1E3E_A, 1E3L_A, 1WLS_A, 1WNF_A, 1OQY_A: Detailed in **Table 3**) and the 11 sequences including apoptin were aligned by using ClustalW2 (European Bioinformatics Institute, http://www.ebi.ac.uk/).

To build the 3D model of apoptin, I identified ten proteins which served as templates (**Table 3**). The 3D structures of these templates were obtained from The Protein Data Bank (PDB). Next, using ClustalW2, the 11 sequences including apoptin were aligned (**Figure 9**). In the following step, a model of apoptin from amino acid 33 to 90 was built in which about 80% of the sequence was in the allowed region predicted by the corresponding *Ramachandran plot*. The ModellerSoft9V1 software was used to build the 3D model for apoptin that was viewed and further verified by DeepViewer.

Table 3 **Apoptin template proteins**

PDB codes*	PROTEINS
2GHO_C	DNA-directed RNA polymerase β chain
2ASK_A	Human artemin
2GYZ_A	Neurotrophic factor artemin, isoform 3
2GYR_A	Neurotrophic factor artemin, isoform 3
1E3I_A	Murine alcohol dehydrogenase, class II
1E3E_A	Murine alcohol dehydrogenase, class II
1E3L_A	P47H mutant murine alcohol dehydrogenase, class II
1WLS_A	L-asparaginase I homologue from Archacea (*pyrococcus horikoshii*)
1WNF_A	PH0066 (L-asparaginase) from Archacea
1OQY_A	Human UV excision repair protein RAD23 homolog A

* Data source: Protein Data Bank (RCSB-PDB; http://www.rcsb.org/pdb/explore.do)

After building the 3D model for apoptin, required hydrogen atoms were added to the backbone structure of the apoptin to rectify the 3D conformation further. Then, all atoms of the molecule were locked except hydrogen atoms and molecular dynamic simulations were performed unlocking one turn from each end of the sequence at a time.

Finally, the simulated molecular docking experiments with apoptin and the SH3 domain of Abl were preformed by AutoDock4.0.1 software. The identification of this specific protein-protein interactions required prior determination of accessible surface in a 3D model. An accessible surface is the surface of a protein that could be touched by a 1.4Å sphere.

5.17 Statistical Analysis: Unless stated otherwise, all normalized band intensity data were statistically analyzed by *Student t-test* assuming equal variance by Microsoft® Excel software. The variance patterns in each set of data were previously checked by ANOVA from Excel data analysis tool package.

6. Results

6.1 Bcr-Abl and apoptin interactions

6.1.1 The Src homology domain 3 (SH3) of Bcr-Abl interacts with the proline rich domain of apoptin: Several well-characterized SH3 domains were previously identified as potential sites critical to ligand binding on the basis of alignment with their structures (Lim & Richards, 1994). In this study, I first performed an array-based screening to identify the interaction of apoptin with the SH3 domain of a known set of proteins. A high stringency SH3 domain interaction array study indicated that apoptin strongly interacts with the SH3 domain of Abl (**Figure 10**, D3-4) and Plc-ɣ (**Figure 10**, D5–6). Here, I focused primarily on studying the consequences of this apoptin and Bcr-Abl interaction while understanding the significance of apoptin and Plc-γ interaction was not the main interest of this part of the study although Plc-γ is a known downstream target of activated Bcr-Abl kinase (Gotoh et al, 1994).

This observation was further confirmed by 'pull-down assay' and co-immunoprecipitation studies (CO-IP) using $32D^{p210}$ - a mouse myeloid cell line stably transfected to express $Bcr\text{-}Abl^{p210}$, as the model cell line. The data was further compared and comfirmed with Bcr-Abl non-expressing $32D_{DSMZ}$ cell lines (**Figure 12 - 14**).

For the GST-pull down assay recombinant GST and GST-conjugated apoptin were purified from IPTG stimulated transformed bacterial clones harbouring the respective plasmids (**Figure 11**). The immunoblot PVDF membrane from the GST *pull-down assays* were treated sequentially with anti-BCR-Abl, and Anti-Akt antibodies. In **Figure 12** representative image from such an experiment shows presence of Bcr-Abl and Akt (Lane 5) in the GST-Apoptin pull-down product.

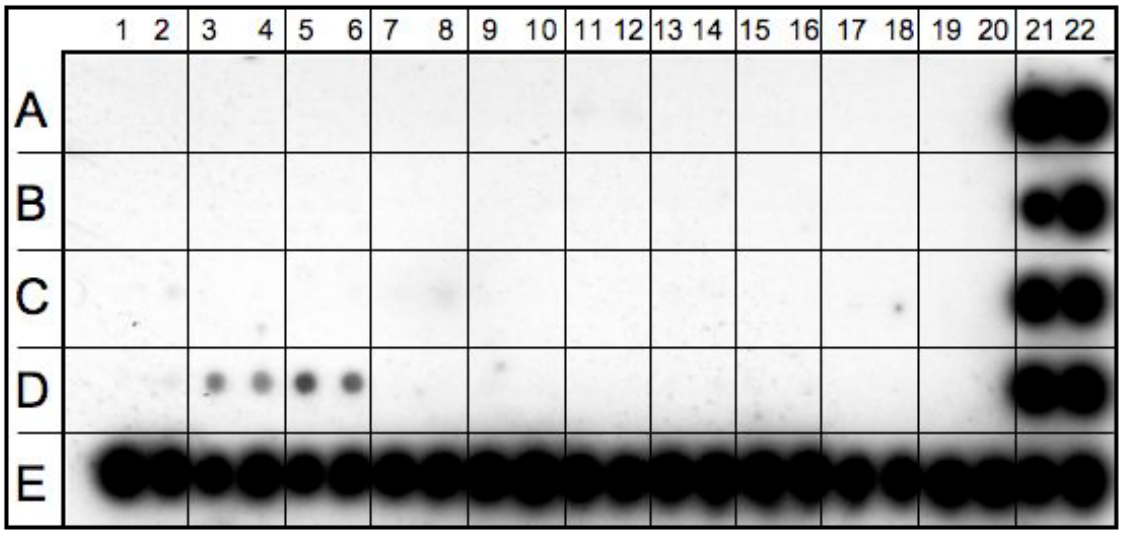


Figure 10. *TransSignal SH3™ Domain Array1: interaction of apoptin and SH3 domains of* Abl *(D3, 4) and* [*Plc-γ (D5, 6)*]. The TransSignal SH3™ Domain Array1 was incubated in purified recombinant TAT-Apoptin in buffer and the resulting interactions were imaged on high performance chemiluminescence film (Hyperfilm™ECL, Amersham Biosciences). Spots with stronger intensity indicate higher binding affinity with ligand of interest to SH3 domain(s). The proteins in the array are spotted in duplicates. Histidine tagged ligands have been spotted along the bottom (row E) and in duplicate along the right side of the membrane (column 21, 22) for alignment purpose. These results were confirmed by pull down assay, co-immunoprecipitation assay and imunofluorescence study as shown below in **Fig. 12-14**.

Figure 11: *Production and purification of recombinant GST conjugated apoptin (SDS-PAGE gel image)*: Lane 2, shows purified GST which is a ~ 26kDa protein and in Lane 3, the affinity purified GST-Apoptin (~40kDa) which was used for pull-down assays is seen.

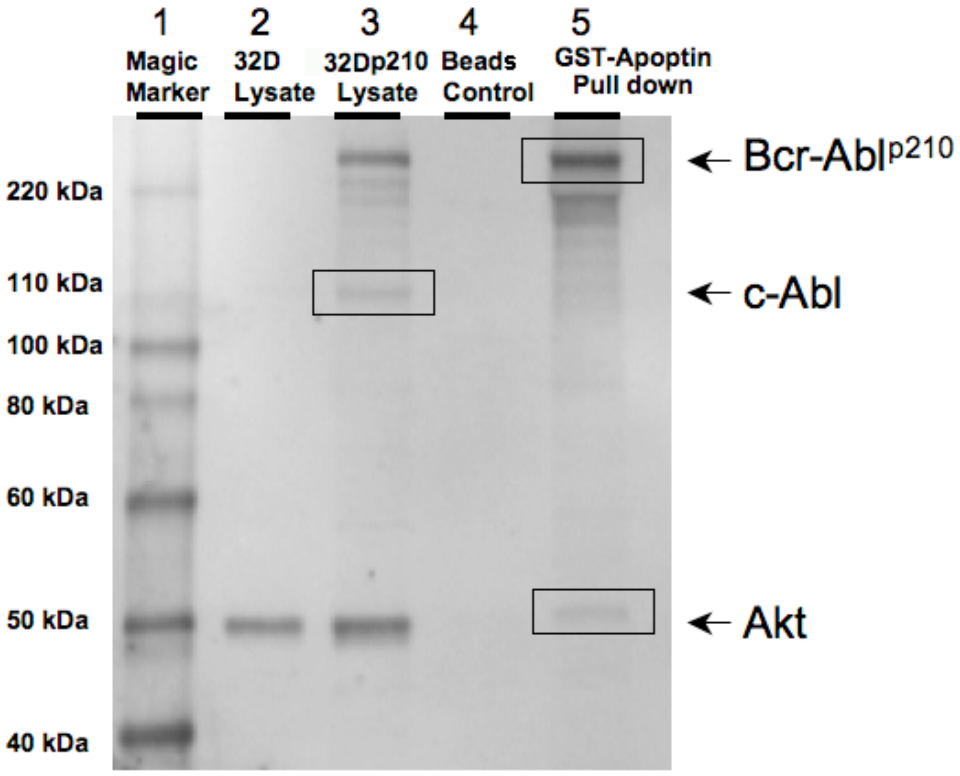


Figure 12: *Apoptin, Bcr-Abl and Akt interaction: Class lb SH3 ligand specifically 'pulls down' its corresponding protein-protein interaction domains.* GST conjugate of apoptin was used in the 'pull down assay' along with the total cell lysates from Bcr-Abl expressing $32D^{p210}$ cells as test and Bcr-Abl non-expressing $32D_{DMSZ}$ as control. Lane 1: Molecular weight marker. Lane 2: pull-down assay on $32D_{DMSZ}$ extract (-Ve control). Lane 3: $32D^{p210}$ extract (+Ve control), Lane 4: $32D^{p210}$ extract and pulled with glutathione sepharose bead (Bead control). Lane 5: $32D^{p210}$ extract incubated with GST-apoptin and pulled with glutathione sepharose bead. The PVDF membrane was sequentially treated with three primary antibodies (mouse Bcr-Abl, mouse Abl and rabbit Akt).

This implies apoptin interacted with Bcr-Abl and Akt in this *in vitro* reaction. Notably, the 'Beads control' (Lane 4) where GST-Apoptin was absent, does not show any non-specific interaction. It was reported previously that apoptin interacts with the SH3 doamin of PI3-K and co-transports activated Akt to the nucleus of cancer cells (Maddika et al, 2008a; Maddika et al, 2007b). In this study, I observed the Bcr-Abl and apoptin interacting complex possibly contains Akt as well (**Figure 12**). The possible biological significance of this observation is highlighted later in the discussion section.

Similar Bcr-Abl and apoptin interactions were noted in the intracellular environment by co-immunoprecipitation (CO-IP) and imunofluorescence studies. GFP-conjugated apoptin was transiently expressed in $32D^{p210}$ cells and GFP-Apoptin was immunoprecipitated by Bcr-Abl antibody tagged protein G beads (**Figure 13**). Notably, the immunoprecipitated GFP-Apoptin (40 kDa) band is prominantly seen in lane 3. Absence of such a band at 40 kDa in the lanes 5, 6, 7 and 8 indicates absence of any non-specific interactions. Other bands seens in all the lanes belong to immunoglobulin heavy and light chains. These results were reproducible in subsequent 5 experiments.

In the imunofluorescence studies, the GFP conjugated apoptin that was expressed in $32D^{p210}$ cells showed nuclear transport and co-localization of Bcr-Abl and apoptin. Unlike c-Abl, Bcr-Abl is predominantly a cytoplasmic protein. Hence, in Bcr-Abl expressing $32D^{p210}$ cells which was not transfected with GFP-Apoptin expressing plasmids, the Bcr-Abl is mostly seen in the cytoplasm. This experiment further supported the apoptin and Bcr-Abl interaction and also demonstrated that the apoptin and Bcr-Abl protein complex is possibly transported to the nucleus (**Figure 14**).

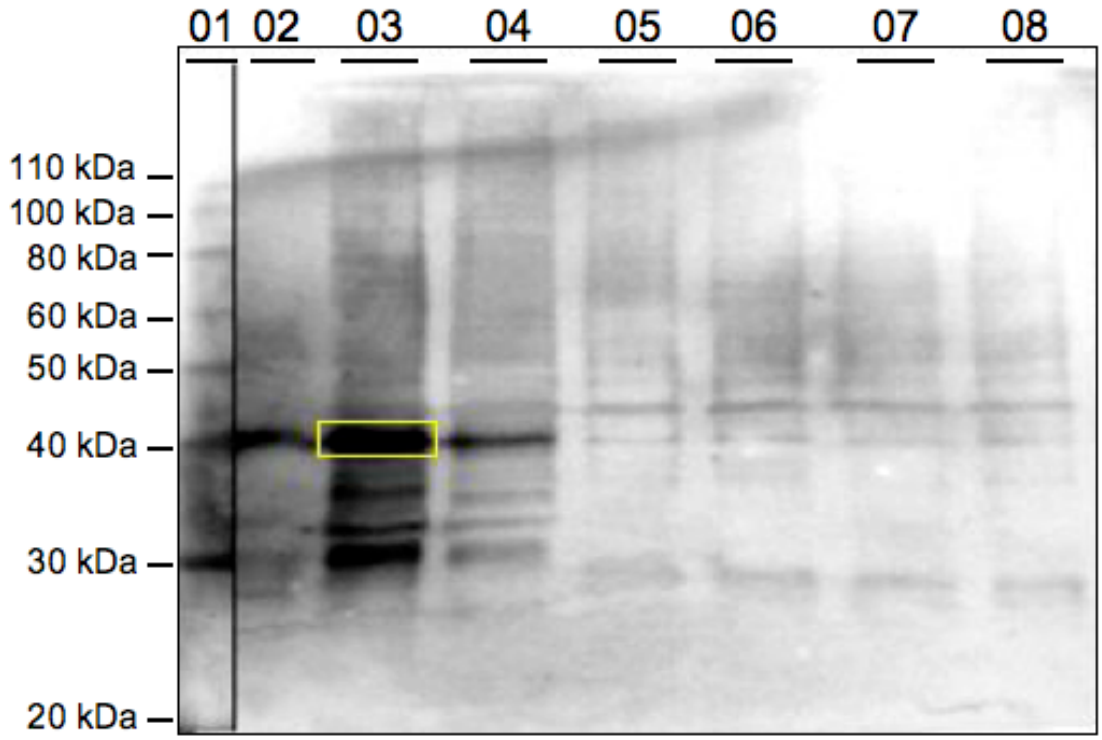


Figure 13: *Apoptin and Bcr-Abl interaction as demonstrated by co-immunoprecipitation (CO-IP) assay:* Co-immunoprecipitation was done with Bcr-Abl antibody on transiently transfected $32D^{p210}$ (wt) cells. The IP product was positive for apoptin (GFP-Apoptin: ~ 40 kDa) by immunoblot with anti-apoptin antibody. LANE 1: Molecular weight marker, LANE 2: GST-Apoptin (+Ve control), LANE 3: $32D^{p210}$ cells transfected with GFP-Apoptin showing co-immuno-precipitated GFP-Apoptin in yellow box, LANE 4: $32D^{p210}$ transfected with GFP-Apoptin (CO-IP Supernatant), LANE 5: $32D^{p210}$ transfected with GFP (CO-IP), LANE 6: $32D^{p210}$ No transfection (CO-IP, -Ve control), LANE 7: $32D_{DMSZ}$ transfected with GFP-Apoptin (CO-IP), LANE 8. $32D_{DMSZ}$ transfected with GFP (CO-IP)

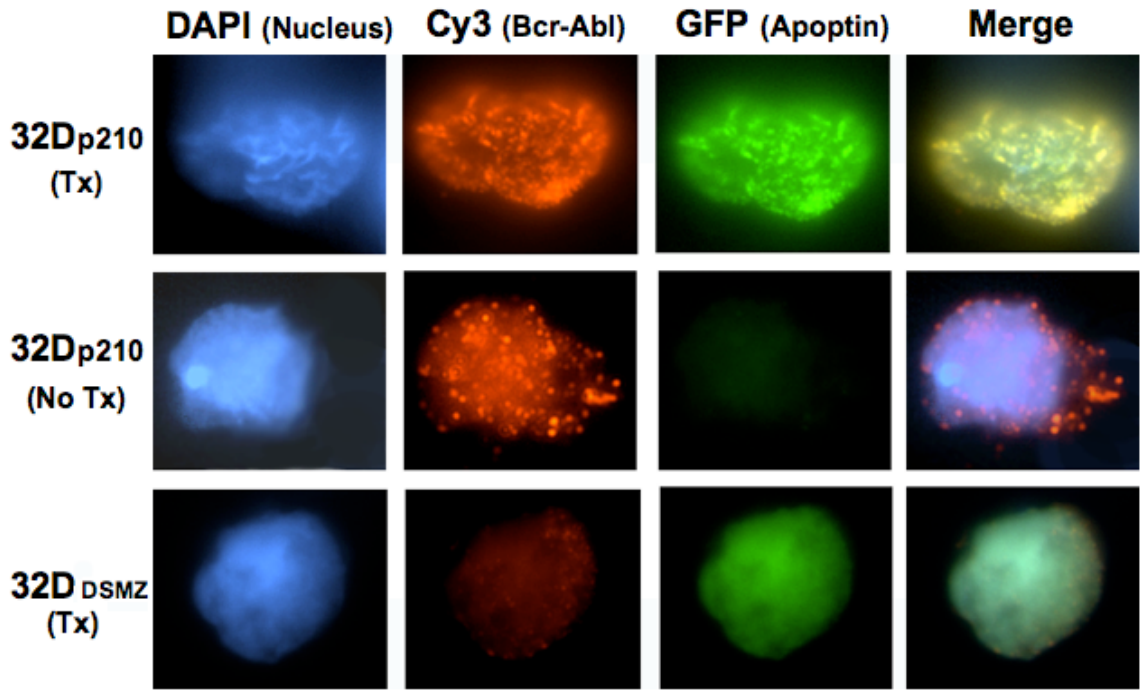


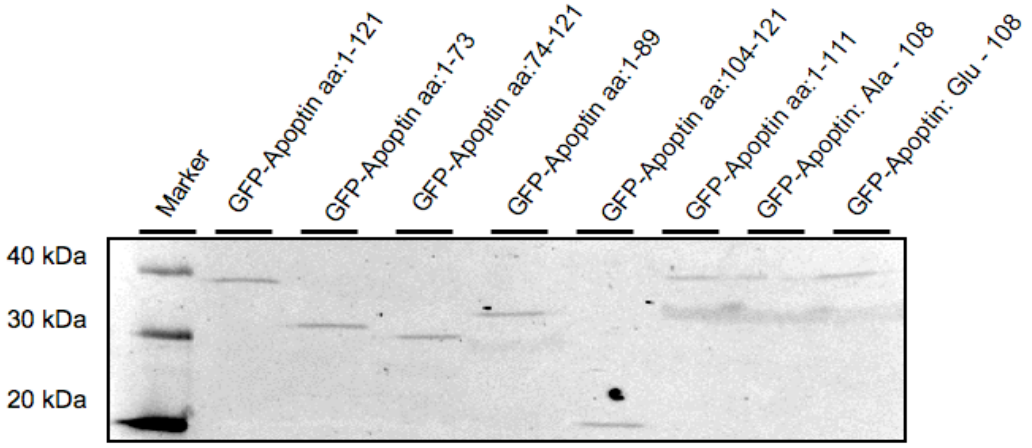
Figure 14: *Immunocytochemistry studies showing nuclear co-localization of Bcr-Abl after interacting with apoptin. 32D^{P210} and 32D*DMSZ *cells were transiently transfected (Tx) for GFP-Apoptin expression.* Nuclei: blue (DAPI); Bcr-Abl^{p210} with Cy3 tagged secondary antibody: Red and GFP-Apoptin: Green. Representative images in Lane 1: transfected 32D^{p210} cells (Tx) expressing GFP-Apoptin that is co-localized with Bcr-Abl^{p210} in the nucleus (column 4); Lane 2: non-transfected (No-Tx) 32D^{p210} cells where Bcr-Abl protein is mostly extra-nuclear (column 4) and Lane 3: transfected (Tx) Bcr-Abl non-expressing 32DDMSZ cells.

Specific interacting motif of apoptin is responsible for its interaction with Bcr-Abl: To identify the precise nature of apoptin and Bcr-Abl interaction in CML cells I mapped the sites on apoptin responsible for interaction with specific region of Bcr-Abl^{p210}. The murine bone marrow derived 32D$_{DMSZ}$, 32D^{p210} cells and human CML cell line K562 were grown in appropriate media and transfected with different apoptin mutant constructs by lipofectamine protocol.

The expression of these mutant derivatives of apoptin tagged with an N-terminal GFP was verified by SDS-PAGE and immunoblotting with mouse monoclonal anti-GFP antibody (representative image from transfected 32D$_{DMSZ}$ cells; **Figure 15a**).

In the experimental groups apoptin was immuno-precipitated by murine anti-GFP antibody from the lysates of transfected cells expressing various forms of mutant apoptin. The protein complexes were analyzed to detect the presence of Bcr-Abl^{p210} by immunoblotting using rabbit monoclonal anti-Bcr antibody. Bcr-Abl^{p210} was found in the immuno-precipitates of full-length apoptin (aa: 1-121) and apoptin derivatives that harbored amino acids from 74-100 (including the PRS), implying that this region of apoptin is important for interaction with Bcr-Abl^{p210} wt (**Figure 15b**). Interestingly, in this study the mutant proteins Ala-108 and Glu-108 where the Thr-108 residue of apoptin was replaced by alanine or glutamine respectively, converting them to be non-phosphorylatable and non-toxic to the cells (Rohn et al, 2002), also interacted with Bcr-Abl^{p210}wt.

a.



b.

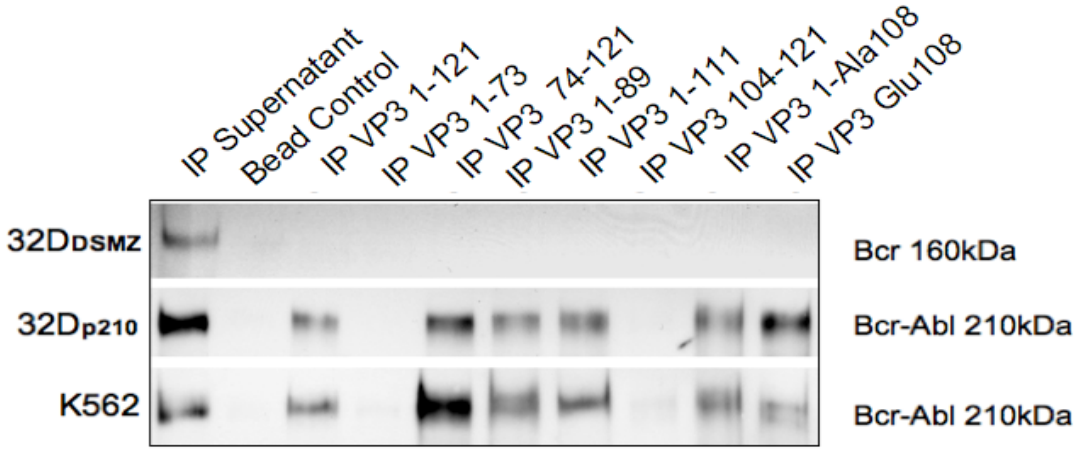


Figure 15. *Apoptin interacts with Bcr-Abl^{p210} via its proline-rich sequence.* ***a.*** *(Upper Panel): Various N-terminal GFP-tagged apoptin deletion mutants expressed in 32D$_{DSMZ}$ cells and detected by murine anti-GFP antibody.* Last two columns indicate full-length GFP-conjugated apoptin molecules with single amino acid replacement at the phosphorylation site (Thr-108) by Ala and Glu respectively. ***b.*** *(Lower Panel): Apoptin CO-IP experiment from transfected 32D$_{DMSZ}$, 32D^{p210} and K562 cells with various mutant forms of apoptin;* The GFP conjugated apoptin mutants (identified as VP3) were co-immunoprecipitated with protein G sepharose coated with murine anti-GFP antibody and the antibody bound protein complexes were analyzed by immunoblotting for the presence of Bcr-Abl^{p210} with rabbit monoclonal anti-Bcr primary antibody. Bcr-Abl was identified in the immuno-precipitates of full-length apoptin and apoptin derivatives that harbored amino acids from 74-100 (includes the proline rich region, aa: 81-86) indicating a part of this region of apoptin is important for apoptin's interaction with Bcr-Abl^{p210}.

Subsequently, the specific interaction between full-length GST-conjugated apoptin and Bcr-Abl^{p210}wt or various Src homology domain mutant-constructs of Bcr-Abl expressed in 32D$_{DMSZ}$ cells were studied by GST-*pull-down assay*. The Bcr-Abl mutant constructs, namely: the Bcr-Abl^{p210}wt with intact SH3, SH2 & SH1 (Kinase); Bcr-Abl^{p210}ΔSH2 with intact SH3, deleted SH2 and intact SH1; Bcr-Abl^{p210}ΔSH2 ΔSH3 with deleted SH2, deleted SH3 and intact SH1; Bcr-Abl^{p210}ΔSH3-R1053L with deleted SH3, single aa substitution at SH2 and intact SH1; Bcr-Abl^{p210}P1013L-R1053L single aa substitution at SH2 & SH2 domains respectively and intact SH1 domain, were selected accoding to their specific nature of src homology domain mutations to detect if apoptin interacts with the SH3 domain of Bcr-Abl. Expression of these deletion and single amino acid substitution mutant derivatives of Bcr-Abl^{p210} were confirmed from the lysates of 32D$_{DMSZ}$ cells transfected with specific Bcr-Abl mutant constructs by SDS-PAGE and immunoblotting (**Figure 16** and **Figure 17** upper panel). Bcr-Abl expressing cell lysates and GST-Apoptin were incubated overnight for the interaction to occur. Next, GST-Apoptin was pulled down after 4 hours of incubation using glutathione-sepharose beads. The Bcr-Abl in the protein complexes was verified by mouse monoclonal anti-Bcr-Abl antibody. Both full length and Src homology domain mutant of Bcr-Abl^{p210} with intact SH3 domain interacted with GST-Apoptin and were 'pulled-down' by glutathione sepharose beads, while other mutants lacking the SH3 domain failed to show such interaction. However apoptin and Bcr-Abl interaction was also observed when a single aminoacid was substituted (P1013L) in the SH3 domain (**Figure 17**, lower panel). Taken together, this high stringency assay indicated apoptin's SH3 domain specific interaction with Bcr-Abl (**Figure 17**).

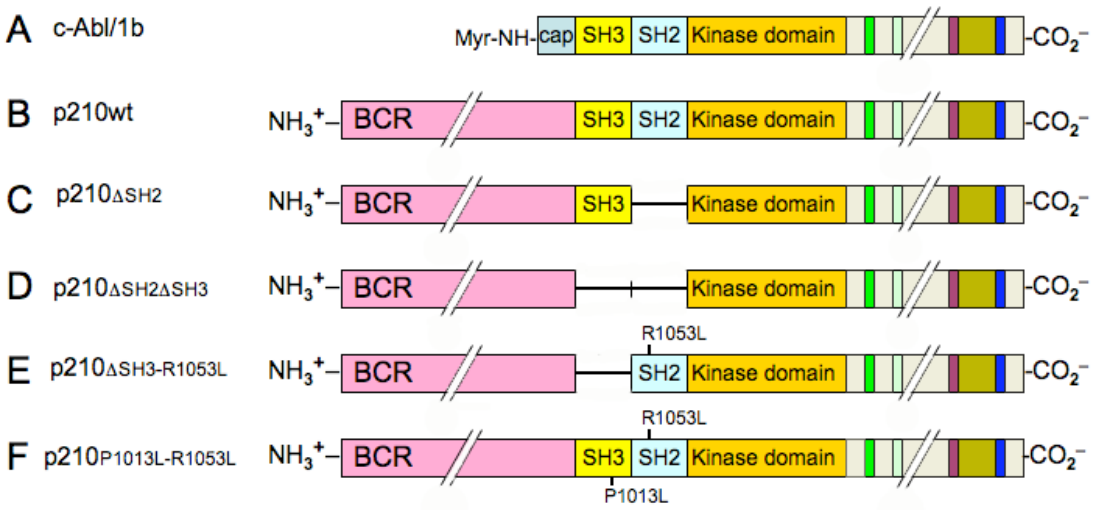


Figure 16: *Schematic representation of Bcr-Abl Src homology domain mutants;* (A) c-Abl/1b with intact N-terminal 'cap', intact SH3, SH2 and SH1 (kinase) domain; (B) Bcr-Abl^{p210}wt with intact SH3, SH2 and SH1 (kinase) domain; (C) Bcr-Abl^{p210}ΔSH2 with intact SH3, deleted SH2 and intact SH1 (kinase) domain; (D) Bcr-Abl^{p210}ΔSH2 ΔSH3 with deleted SH2, deleted SH3 and intact SH1 (kinase) domain; (E) Bcr-Abl^{p210}ΔSH3-R1053L with deleted SH3, single aa substitution at SH2 and intact SH1 (kinase) domain; (F) Bcr-Abl^{p210}P1013L-R1053L single aa substitution at SH2 and SH2 domains respectively and intact SH1 (kinase) domain.

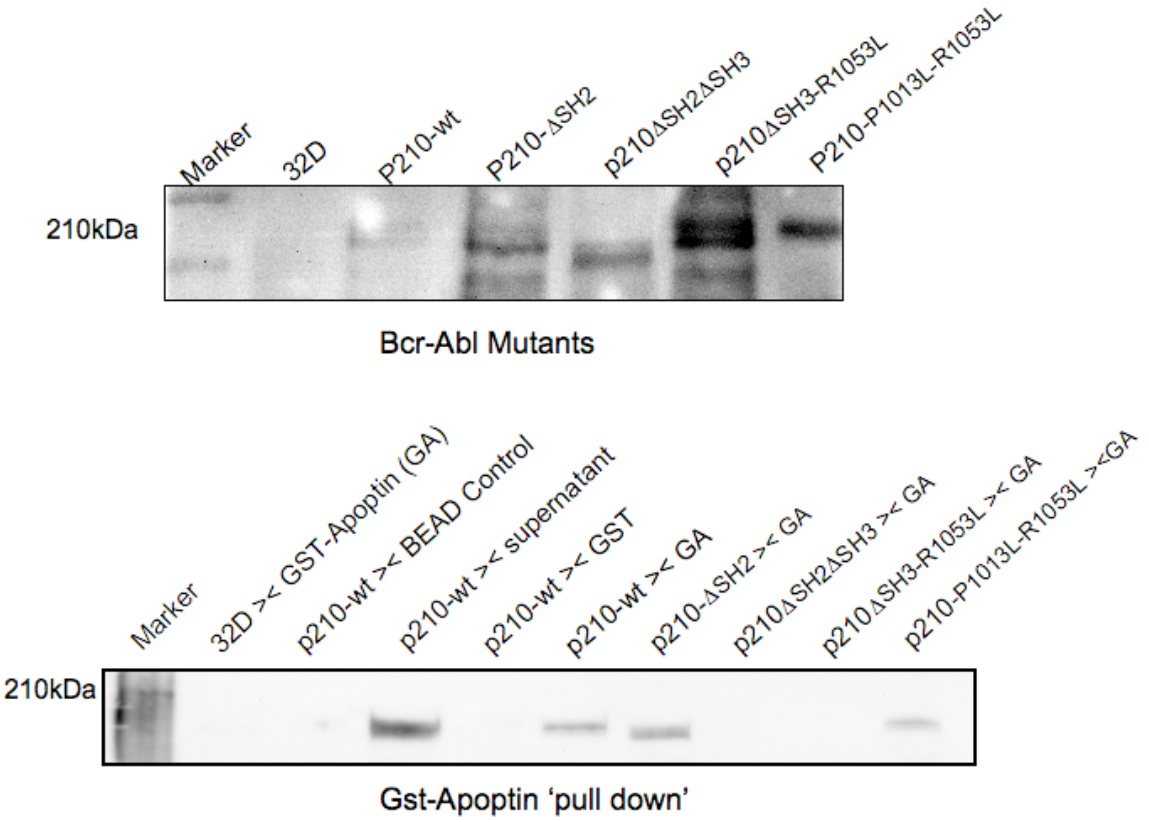


Figure 17: *'Pull-down assay' showing differential interactions of GST-Apoptin with different Bcr-Abl mutants. UPPER PANEL:* Representative immunoblot image of the mutant Bcr-Abl. *LOWER PANEL:* Src Homology domain mutant Bcr-Abl^{p210} and GST-Apoptin (GA) 'pull-down' (><) experiment using the lysates of transfected 32D$_{DSMZ}$ cells expressing different mutant forms of Bcr-Abl. The protein complexes were 'pulled down' by glutathione sepharose beads and analyzed for possible interaction with apoptin by immunoblotting. Presence of an intact SH3 domain in the Bcr-Abl molecule was found to be essential for its interaction with apoptin (p210-wt><GA, p210-ΔSH2><GA). Some Bcr-Abl><apoptin interaction was also noted where the Abl-SH3 domain in Bcr-Abl was partially modified by single amino-acid (aa) substitution (p210-P1013L-R1053L).

6.1.2 Apoptin down-regulates Bcr-Abl kinase auto-phosphorylation and alters the activation of down-stream signaling pathways: To study the down-stream effects of apoptin and Bcr-Abl interaction I checked the expression and phosphorylation status of Bcr-Abl^{p210} and other major down-stream Bcr-Abl target molecules, STAT5, CrkL, c-Myc and Akt in both murine and human cell lines. Experiments were performed in triplicates to measure phosphorylated and total proteins.

Apoptin induced inhibition of Bcr-Abl kinase: To study the inhibition of activated Bcr-Abl by apoptin, I measured the relative auto-phosphorylation level of Bcr-Abl^{p210} detected by immunoblotting with Bcr-Abl specific phospho-antibodies. Individual band intensity of total and phosphorylated Bcr-Abl was measured by dedicated software as described, and these values were normalized against the loading control. Using K562 and 32D^{p210} as model CML cell lines at least five independent experiments were performed. Average values expressed as ratio of phosphorylated and total Bcr-Abl from three of such experiments are presented in **Figure 18-19** (from K562 and 32D^{p210} cells respectively). In both apoptin and imatinib treated cell lines the phosphorylation of Bcr-Abl was downregulated significantly ($p < 0.01 - 0.04$). However, imatinib showed a more pronounced inhibitory effect in comparison to apoptin in these experiments where a fixed concentration of 1μM of imatinib and apoptin was applied in the culture media.

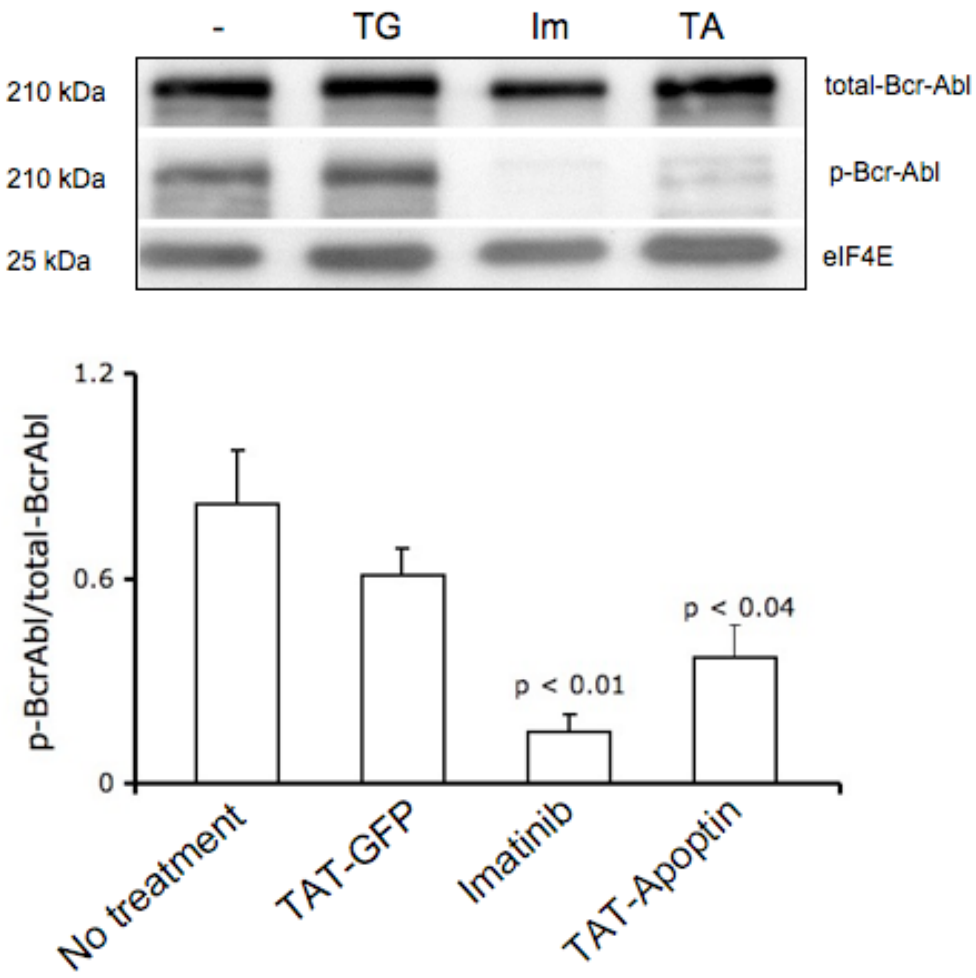


Figure 18: *Apoptin inhibits Bcr-Abl kinase (K562 cells)*; TAT-Apoptin, TAT-GFP (-Ve control) and imatinib (+Ve control) were applied (all at concentration: 1 μM) to K562 cells 24 h after splitting. Cells were harvested at 16 hours and cell lysates were checked for phosphorylated and total Bcr-Abl by SDS-PAGE and immunoblotting. The final quantitative data was normalized according to the loading control and expressed as a ratio of phosphorylated & total Bcr-Abl. COLUMN 1: *Untreated cells*; Lane 1: Total Bcr-Abl, Lane 2: phospho-Bcr-Abl and Lane 3: loading control; COLUMN 2: *TAT-GFP treated cells*; Lane 1: Total Bcr-Abl, Lane 2: phospho-Bcr-Abl and Lane 3: loading control, COLUMN 3: *Imatinib treated cells*; Lane 1: Total Bcr-Abl, Lane 2: phospho-Bcr-Abl and Lane 3: loading control, COLUMN 4: *TAT-Apoptin treated cells*; Lane 1: Total Bcr-Abl, Lane 2: phospho-Bcr-Abl and Lane 3: loading control. The quantitative estimation of Bcr-Abl phosphorylation is significantly less ($p<0.04$) according to the estimated values (histogram), indicating apoptin-induced inhibition of Bcr-Abl phosphorylation is comparable to that of imatinib. (Student's t-test assuming equal variance; $p<0.05$ indicates statistical significance; n=5).

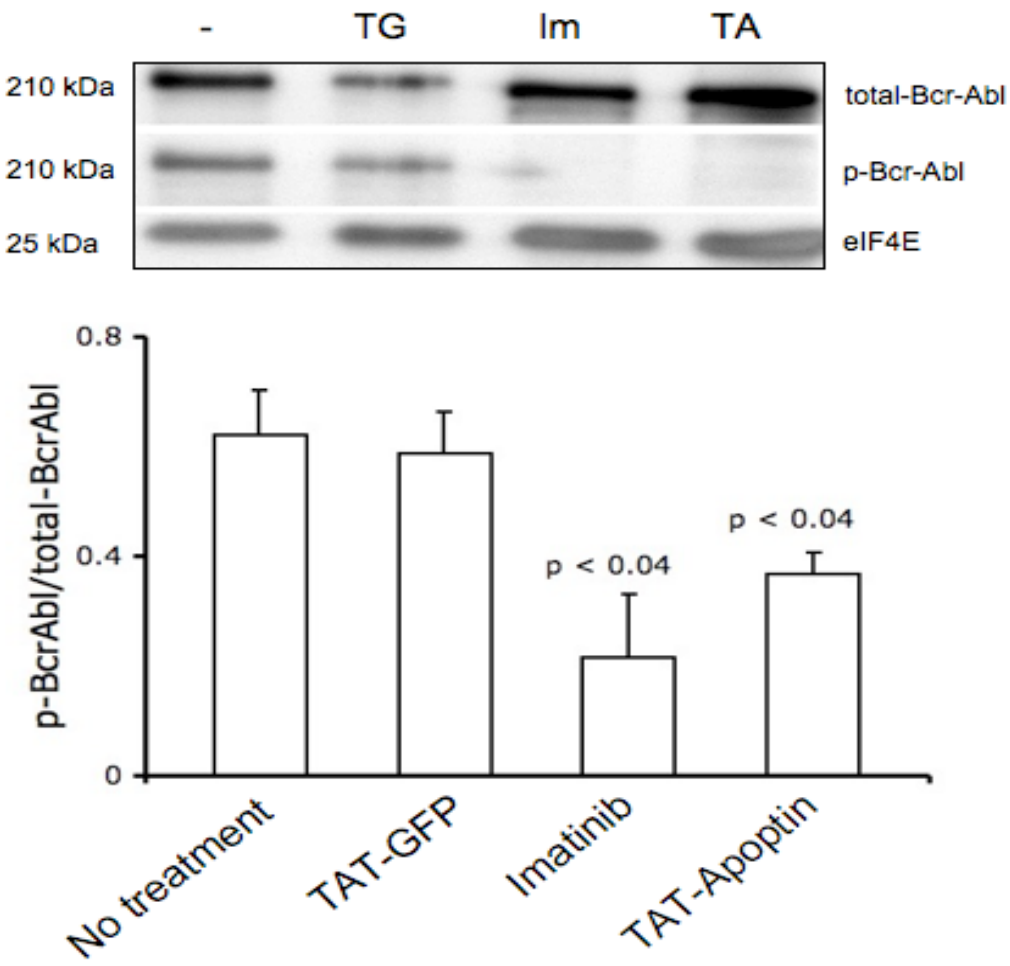


Figure 19: *Apoptin inhibits Bcr-Abl kinase (32D^{p210} cells)*; TAT-Apoptin, TAT-GFP (-Ve control) and imatinib (+Ve control) were applied (all at concentration: 1 μM) 32D^{p210} cells 24 h after splitting. Cells were harvested at 16 hours and cell lysates were checked for phosphorylated and total Bcr-Abl by SDS-PAGE and immunoblotting. The final quantitative data was normalized according to the loading control and expressed as a ratio of phosphorylated & total Bcr-Abl. COLUMN 1: *Untreated cells*; Lane 1: Total Bcr-Abl, Lane 2: phospho-Bcr-Abl and Lane 3: loading control; COLUMN 2: *TAT-GFP treated cells*; Lane 1: Total Bcr-Abl, Lane 2: phospho-Bcr-Abl and Lane 3: loading control, COLUMN 3: *Imatinib treated cells*; Lane 1: Total Bcr-Abl, Lane 2: phospho-Bcr-Abl and Lane 3: loading control, COLUMN 4: *TAT-Apoptin treated cells*; Lane 1: Total Bcr-Abl, Lane 2: phospho-Bcr-Abl and Lane 3: loading control. The quantitative estimation of Bcr-Abl phosphorylation is significantly less ($p<0.04$) according to the estimated values (histogram), indicating apoptin-induced inhibition of Bcr-Abl phosphorylation is comparable to that of imatinib. (Student's t-test assuming equal variance; $p<0.05$ indicates statistical significance; n=5).

Apoptin induced inhibition of the STAT5 phosphorylation: Signal transducers and activators of transcription (STATs) are proteins serving the dual role of signal transducers and activators of transcription in cells exposed to polypeptide signaling. Among the large family of over 30 STAT proteins, STAT5 has been identified as a key factor involved in anti-apoptotic signaling and malignant transformation in CML. As evidenced by our five independent experiments, STAT5 phosphorylation is markedly reduced in K562 cells, 16 hours after addition of 1μM of apoptin in the culture system and this is comparable to that of imatinib treated groups (**Figure 20**). Similar results with statistical significance ($p < 0.03$) were also observed when experiments were repeated on Bcr-Abl^{p210} expressing murine cell line 32D^{p210} **(Figure 21).**

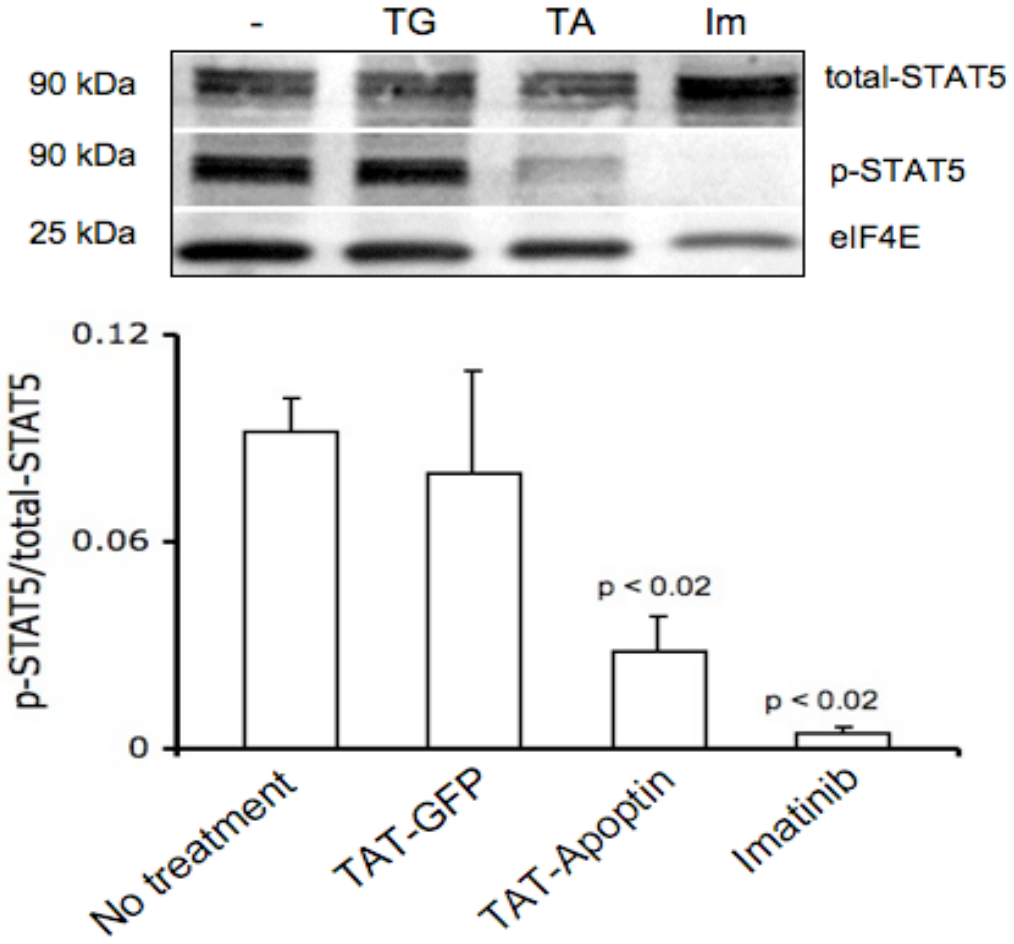


Figure 20: *Apoptin induced inhibition of Bcr-Abl phosphorylation leads to the down-regulation of* STAT5 *activity in K562 cells*; TAT-Apoptin, TAT-GFP (-Ve control) and imatinib (+ Ve control) were applied (1 μM) to K562 cells 24 hours after splitting. Cells were harvested at 16 hours and cell lysates were checked for phosphorylated and total STAT5 by SDS-PAGE and immunoblotting. The final quantitative data were normalized according to the loading control and expressed as a ratio of phosphorylated & total STAT5. COLUMN 1: *Untreated cells*; Lane 1: Total STAT5, Lane 2: phospho-STAT5 and Lane 3: loading control; COLUMN 2: *TAT-GFP treated cells*; Lane 1: Total STAT5, Lane 2: phospho-STAT5 and Lane 3: loading control, COLUMN 3: *Imatinib treated cells*; Lane 1: Total STAT5, Lane 2: phospho- STAT5 and Lane 3: loading control, COLUMN 4: TAT-*Apoptin treated cells*; Lane 1: Total STAT5, Lane 2: phospho-STAT5 and Lane 3: loading control. The quantitative estimation of STAT5 phosphorylation was significantly less ($p<0.02$) according to the estimated values (histogram) in most of the experiments, indicating apoptin-induced inhibition of Bcr-Abl phosphorylation decreases activation of STAT5, a down-stream substrate for Bcr-Abl. This data is a summary of three independent experiments. (Student's t-test assuming equal variance; $p<0.05$ indicates statistical significance; $n=3$).

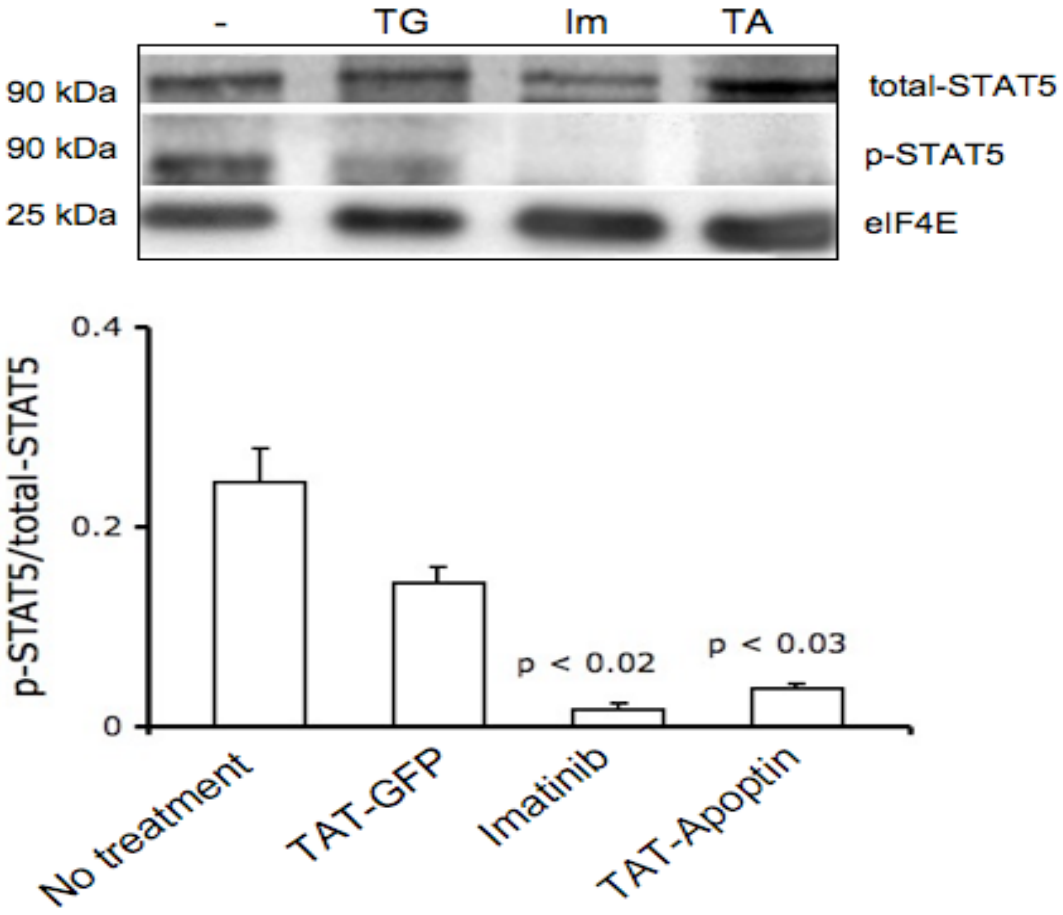


Figure 21: *Apoptin induced inhibition of Bcr-Abl phosphorylation leads to the down-regulation of* STAT5 *activity in 32D^{p210} cells*; TAT-Apoptin, TAT-GFP (-Ve control) and imatinib (+ Ve control) were applied (1 μM) to 32D^{p210} cells 24 hours after splitting. Cells were harvested at 16 hours and cell lysates were checked for phosphorylated and total STAT5 by SDS-PAGE and immunoblotting. The final quantitative data were normalized according to the loading control and expressed as a ratio of phosphorylated & total STAT5. COLUMN 1: *Untreated cells*; Lane 1: Total STAT5, Lane 2: phospho-STAT5 and Lane 3: loading control; COLUMN 2: *TAT-GFP treated cells*; Lane 1: Total STAT5, Lane 2: phospho-STAT5 and Lane 3: loading control, COLUMN 3: *Imatinib treated cells*; Lane 1: Total STAT5, Lane 2: phospho- STAT5 and Lane 3: loading control, COLUMN 4: TAT-*Apoptin treated cells*; Lane 1: Total STAT5, Lane 2: phospho- STAT5 and Lane 3: loading control. The quantitative estimation of STAT5 phosphorylation was significantly less ($p<0.03$) according to the estimated values (histogram) in most of the experiments, indicating apoptin-induced inhibition of Bcr-Abl phosphorylation decreases activation of STAT5, a down-stream substrate for Bcr-Abl. This data is a summary of three independent experiments. (Student's t-test assuming equal variance; $p<0.05$ indicates statistical significance; n=3).

Apoptin induced inhibition of the CrkL phosphorylation: To study other downstream consequences of apoptin induced reduction of Bcr-Abl phosphorylation we checked the phosphorylation status of CrkL (39 kDa) that is involved in β-integrin signaling and is a prominent substrate for activated Bcr-Abl kinase. Interestingly, Bcr-Abl is the most vividly characterized stimulus known to activate CrkL (Feller, 2001). I documented for the first time here from three independent experiments that CrkL phosphorylation is significantly inhibited ($p < 0.04$) in K562 cells, 14~16 hours after addition of 1μM apoptin in the culture system and this result is comparable to that of imatinib treated cells ($p<0.02$) (**Fig. 22**). Similar marked inhibition of CrkL phosphorylation was observed when these experiments were repeated on Bcr-Abl^{p210} expressing 32D^{p210} cells (**Fig. 23**).

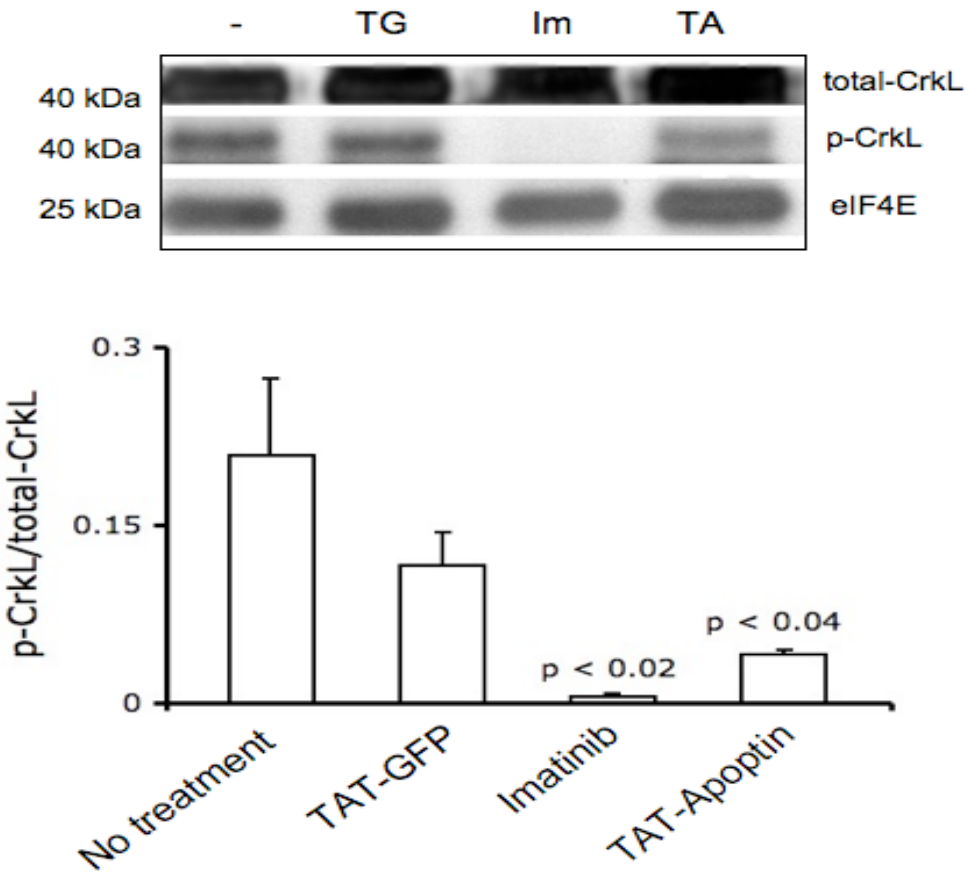


Figure 22: *Apoptin induced inhibition of Bcr-Abl phosphorylation leads to the down-regulation of CrkL phosphorylation in K562 cells*; TAT-Apoptin, TAT-GFP (-Ve control) and imatinib (+ Ve control) were applied (1 μM) to K562 cells 24 hours after splitting. Cells were harvested at 16 hours and cell lysates were checked for phosphorylated and total CrkL by SDS-PAGE and immunoblotting. The final quantitative data was normalized according to the loading control and expressed as a ratio of phosphorylated & total CrkL. COLUMN 1: *Untreated cells*; Lane 1: Total CrkL, Lane 2: phospho- CrkL and Lane 3: loading control; COLUMN 2: *TAT-GFP treated cells*; Lane 1: Total CrkL, Lane 2: phospho- CrkL and Lane 3: loading control, COLUMN 3: *Imatinib treated cells*; Lane 1: Total CrkL, Lane 2: phospho- CrkL and Lane 3: loading control, COLUMN 4: TAT-*Apoptin treated cells*; Lane 1: Total CrkL, Lane 2: phospho-CrkL and Lane 3: loading control. The quantitative estimation of CrkL phosphorylation was significantly less ($p<0.04$) according to the estimated values (histogram) in most of the experiments, indicating apoptin induced inhibition of Bcr-Abl phosphorylation decreases activation of CrkL, a down-stream substrate for Bcr-Abl. (Student's t-test assuming equal variance; $p<0.05$ indicates statistical significance; n=3).

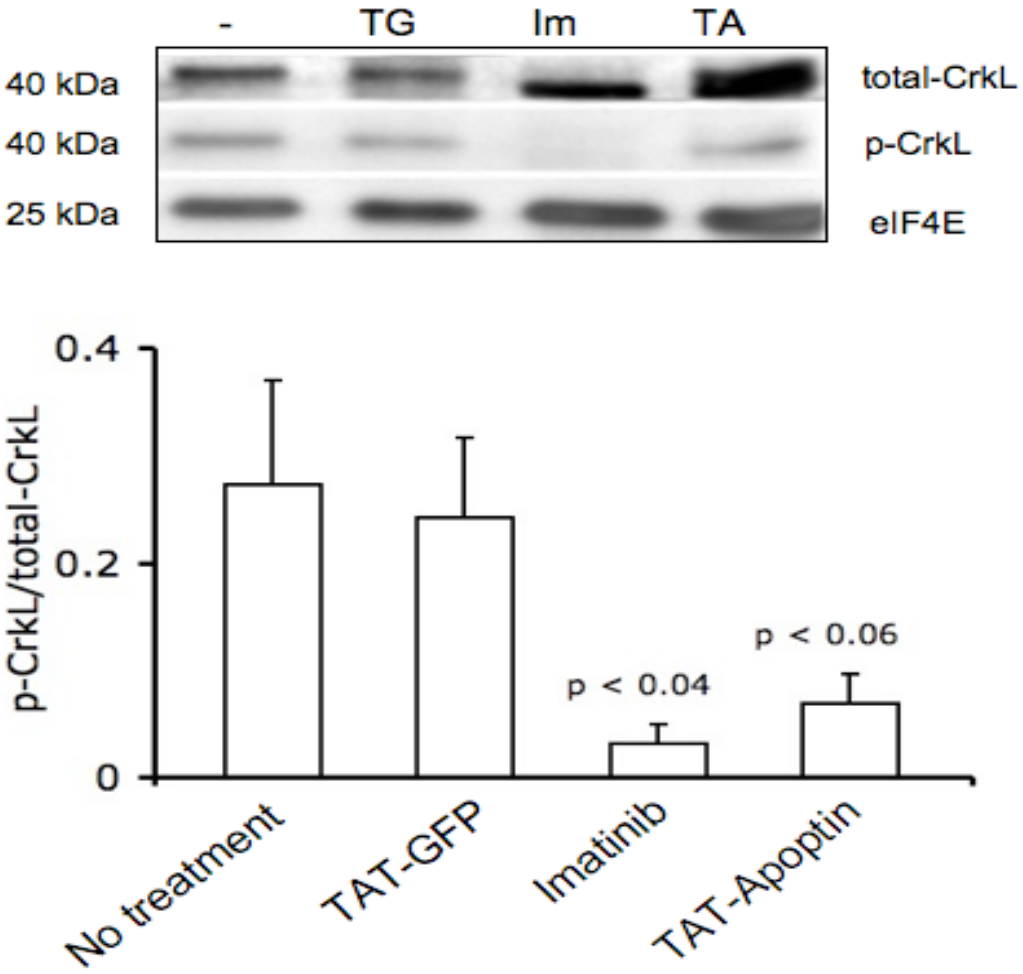


Figure 23: *Apoptin induced inhibition of Bcr-Abl phosphorylation leads to the down-regulation of CrkL phosphorylation in 32D^{p210} cells*; TAT-Apoptin, TAT-GFP (-Ve control) and imatinib (+ Ve control) were applied (1 μM) to K562 and 32D^{p210} cells 24 hours after splitting. Cells were harvested at 16 hours and cell lysates were checked for phosphorylated and total CrkL by SDS-PAGE and immunoblotting. The final quantitative data was normalized according to the loading control and expressed as a ratio of phosphorylated & total CrkL. COLUMN 1: *Untreated cells*; Lane 1: Total CrkL, Lane 2: phospho- CrkL and Lane 3: loading control; COLUMN 2: *TAT-GFP treated cells*; Lane 1: Total CrkL, Lane 2: phospho- CrkL and Lane 3: loading control, COLUMN 3: *Imatinib treated cells*; Lane 1: Total CrkL, Lane 2: phospho- CrkL and Lane 3: loading control, COLUMN 4: TAT-*Apoptin treated cells*; Lane 1: Total CrkL, Lane 2: phospho-CrkL and Lane 3: loading control. The quantitative estimation of CrkL phosphorylation was remarkably less according to the estimated values (histogram) in most of the experiments, indicating apoptin induced inhibition of Bcr-Abl phosphorylation decreases activation of CrkL, a down-stream substrate for Bcr-Abl. (Student's t-test assuming equal variance; $p<0.05$ indicates statistical significance; n=3).

Apoptin modifies Akt phosphorylation: To further characterize the pro-apoptotic effect of apoptin on Bcr-Abl expressing cells we analyzed its effect on the signaling protein Akt and its downstream target Bad. As a positive control, I used imatinib, a known inhibitor of Bcr-Abl kinase. Interestingly, although Akt is known as a mediator of cell survival pathways, I repeatedly recorded marked augmentation of Akt phosphorylation 14~16 hours after both apoptin and imatinib treatment of K562 and $32D^{p210}$ cells (results are summarized in **Fig. 24** and **Fig. 25** respectively). These observations lead us to re-think about role of p-Akt in Bcr-Abl expressing cells after apoptin or imatinib treatment. Akt activation by apoptin and cytotoxic agents was previously reported (Maddika et al, 2007c). A possible correlation to this finding do exists with the nuclear transport of p-Akt, CDK2 phosphorylation, phosphorylation of apoptin and activation of suppressor network in the apoptin treated cancer cells (Maddika et al, 2008a; Trotman et al, 2006). [Discussed in section 7.2]

The overall results indicate that the apoptin induced inhibition of Bcr-Abl phosphorylation downregulates STAT5, CrkL phosphorylation but paradoxically activates Akt. Presumably, these modified signal transduction cascades act as the key initiator of antiproliferative and apoptotic reponses in CML.

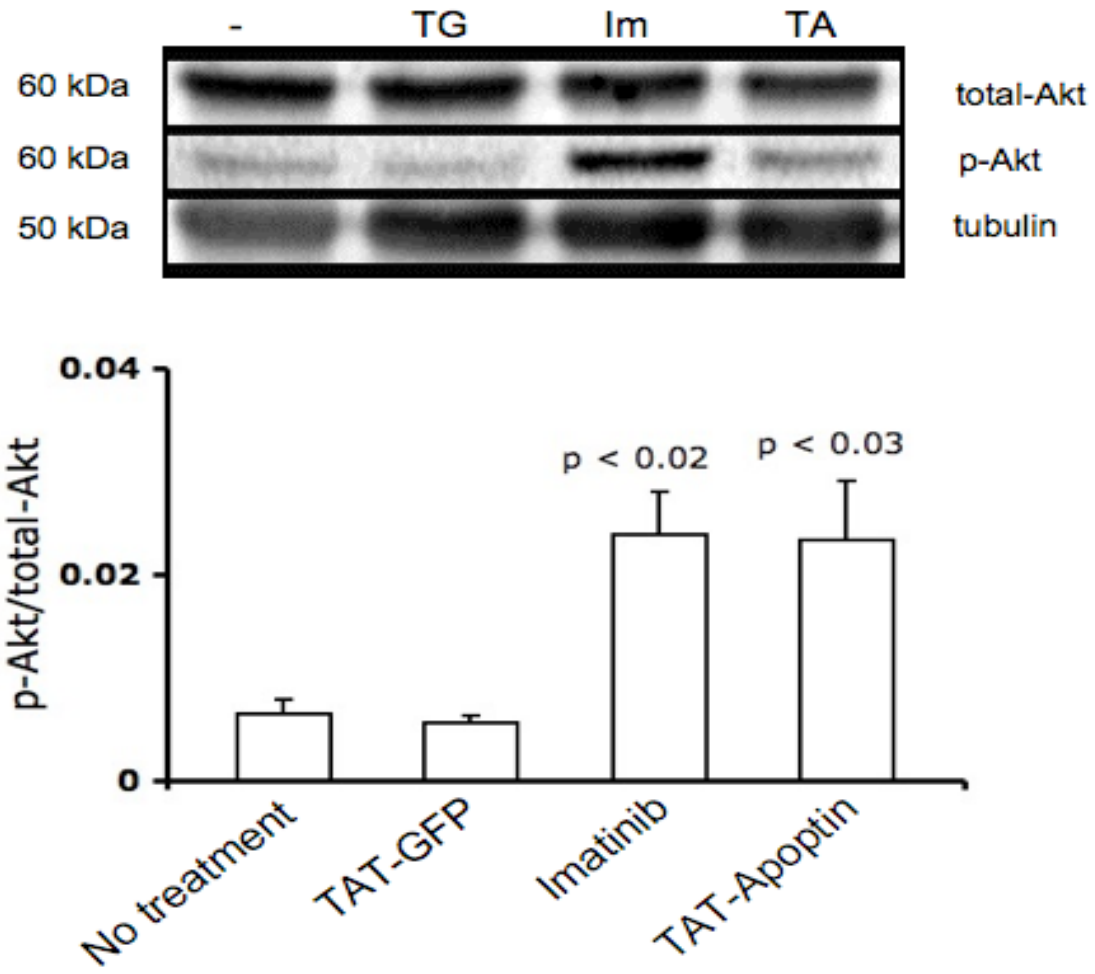


Figure 24: *Apoptin and imatinib activate Akt in K562 cells*; TAT-Apoptin, TAT-GFP (-Ve control) and imatinib (+ Ve control) were applied (1 μM) to K562 cells 24 hours after splitting. Cells were harvested at 16 hours and cell lysates were checked for phosphorylated and total Akt by SDS-PAGE and immunoblotting. The final quantitative data was normalized according to the loading control and expressed as a ratio of phosphorylated & total Akt. COLUMN 1: *Untreated cells*; Lane 1: Total Akt, Lane 2: phospho-Akt and Lane 3: Tubulin; COLUMN 2: *TAT-GFP treated cells*; Lane 1: Total Akt, Lane 2: phospho-Akt and Lane 3: Tubulin; COLUMN 3: *Imatinib treated cells*; Lane 1: Total Akt, Lane 2: phospho-Akt and Lane 3: Tubulin; COLUMN 4: TAT-*Apoptin treated cells*; Lane 1: Total Akt, Lane 2: phospho-Akt and Lane 3: Tubulin. The quantitative estimation of Akt phosphorylation were higher according to the estimated values (histogram), indicating apoptin induced inhibition of Bcr-Abl phosphorylation induce significant Akt activation ($p<0.03$) that is comparable to that of imatinib (see discussion section 7.2). (Student's t-test assuming equal variance; $p<0.05$ indicates statistical significance; n=3).

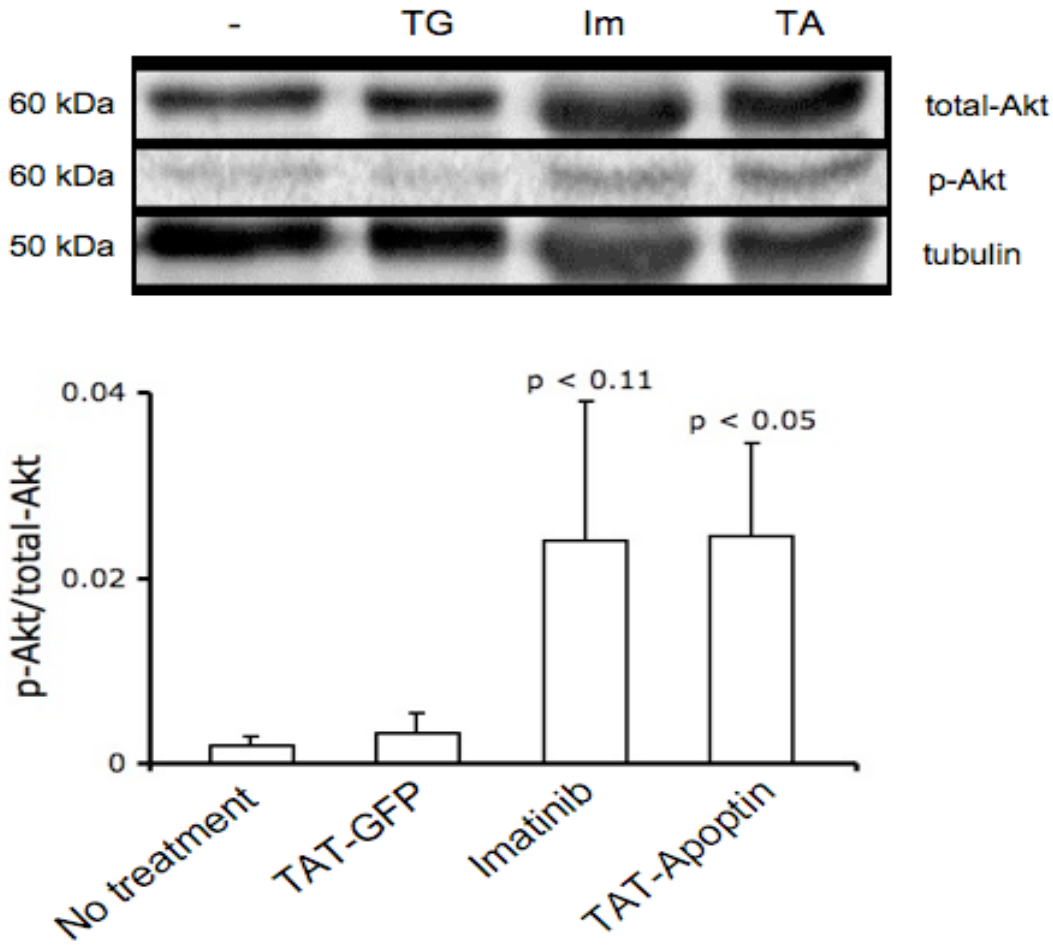


Figure 25: *Apoptin and imatinib activate Akt in 32D^{p210} cells*; TAT-Apoptin, TAT-GFP (-Ve control) and imatinib (+ Ve control) were applied (1 μM) to 32D^{p210} cells 24 hours after splitting. Cells were harvested at 16 hours and cell lysates were checked for phosphorylated and total Akt by SDS-PAGE and immunoblotting. The final quantitative data was normalized according to the loading control and expressed as a ratio of phosphorylated & total Akt. COLUMN 1: *Untreated cells*; Lane 1: Total Akt, Lane 2: phospho-Akt and Lane 3: Tubulin; COLUMN 2: *TAT-GFP treated cells*; Lane 1: Total Akt, Lane 2: phospho-Akt and Lane 3: Tubulin; COLUMN 3: *Imatinib treated cells*; Lane 1: Total Akt, Lane 2: phospho-Akt and Lane 3: Tubulin; COLUMN 4: TAT-*Apoptin treated cells*; Lane 1: Total Akt, Lane 2: phospho-Akt and Lane 3: Tubulin. The quantitative estimation of Akt phosphorylation were higher according to the estimated values (histogram), indicating apoptin induced inhibition of Bcr-Abl phosphorylation induce significant Akt activation ($p<0.05$) that is comparable to that of imatinib (see discussion section 7.2). (Student's t-test assuming equal variance; $p<0.05$ indicates statistical significance; n=3).

Apoptin inhibits c-Myc phosphorylation: Like many other human malignancies c-Myc is also overexpressed in Bcr-Abl expressing CML cells. As mentioned earlier, acting in dual-mode c-Myc may constitute a proliferative or an apoptotic signal. In my experiments, it was detected that TAT-Apoptin can successively diminish the phosphorylation of c-Myc in Bcr-Abl^{p210} expressing K562 cells and this effect is comparable to imatinib, a well know inhibitor of Bcr-Abl tyrosine phosphorylation (**Figure 26)**.

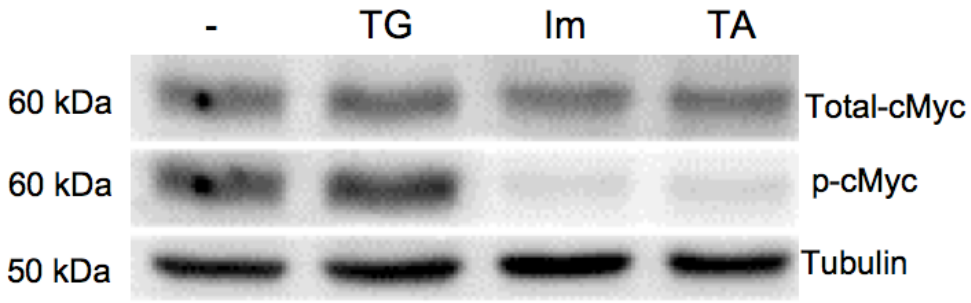


Figure 26: *Apoptin inhibits c-Myc phosphorylation*; Western blot analysis of extracts from K562 cells, untreated (-) or treated with TAT-GFP (TG; –ve control), imatinib (Im; +ve control) and TAT-Apoptin (TA) using *rabbit monoclonal antibody* to c-Myc (phospho T58 + S62). (Representative image from one of three independent experiments; tubulin serves as loading control).

6.1.3 Apoptin induces apoptosis in Bcr-Abl expressing cells:

Apoptin induces apoptosis in Bcr-Abl expressing cells: It is already documented that apoptin triggers the activation of caspases via the intrinsic/mitochondrial death pathway, and not the death receptor/extrinsic pathway in cancer cells (Maddika et al, 2005). To further verify the nature of apoptin induced cell death in Bcr-Abl expressing leukemia cells I compared nuclear morphology of the apoptin/imatinib untreated and treated K562 and $32D^{p210}$ cells to study the features of apoptotic nuclei (**Figure 27**). Furthermore, I estimated the presence of cleaved PARP-1, which is a key target of 'activated caspase-3' in pro-apoptotic cells by Western blot analysis and immunocytochemistry (**Figure 28** and **Figure 29**). In these experiments, the charateristic apoptotic nuclear morphology and presence of cleaved PARP-1 in the cytoplasm of apoptin treated $32D^{p210}$ cells clearly indicate the induction of apoptosis following the application of apoptin.

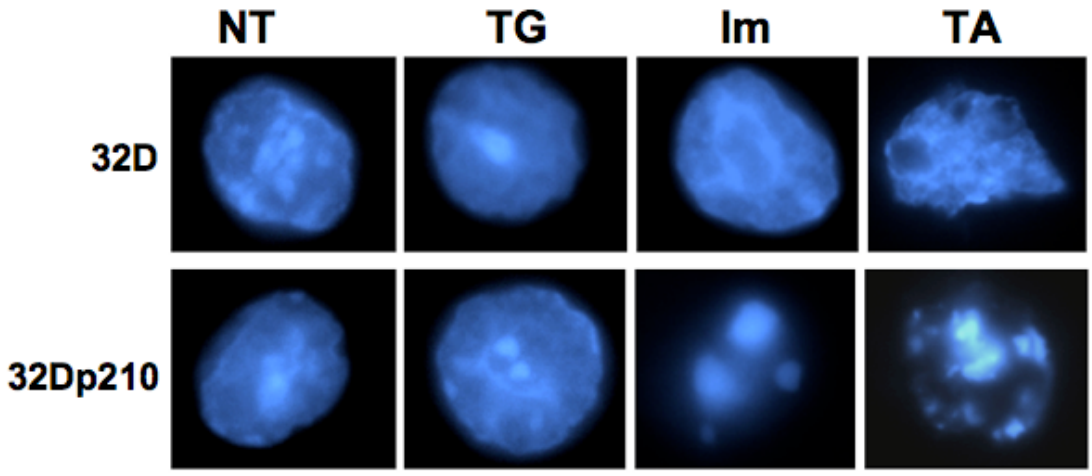


Figure 27: Apoptin *induces apoptosis in Bcr- 32D^{p210} expressing cells;* 16 hours following the treatment of primary murine cells, 32D$_{DSMZ}$ and the Bcr-Abl^{p210}wt expressing murine 32D^{p210} cells, with blank (NT; -Ve control), TAT-GFP (TG; -Ve control), imatinib (Im; +Ve control) and TAT-Apoptin (TA; Test): DAPI stained nuclear morphology of 100 nuclei in each group were studied by epi-fluorescent microscopy and significant number of apoptotic nuclei, characterized by nuclear fragmentations was observed in the Im and TG treated groups as shown in the representative images.

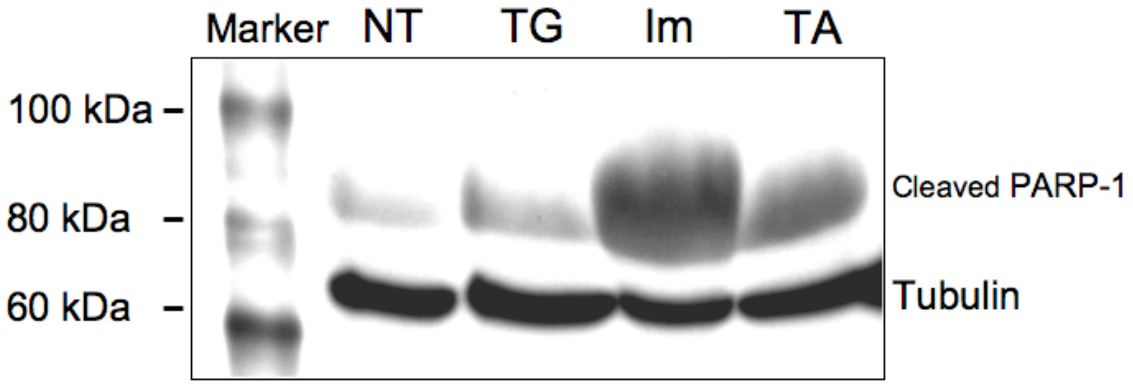


Figure 28: *Elevated level of cleaved PARP-1 in 32D^{p210} cells treated with apoptin.*16 hours following the treatment of primary murine cells, 32D$_{DSMZ}$ and the Bcr-Abl^{p210}wt expressing murine 32D^{p210} cells, with blank (NT; -Ve control), TAT-GFP (TG; -Ve control), imatinib (Im; +Ve control) and TAT-Apoptin (TA; Test): high level of cleaved PARP-1 (89 kDa) in the Im and TA treated groups was also detected by anti-cleaved PARP-1 (Asp214) specific mouse monoclonal antibody that does not recognize full length PARP-1.

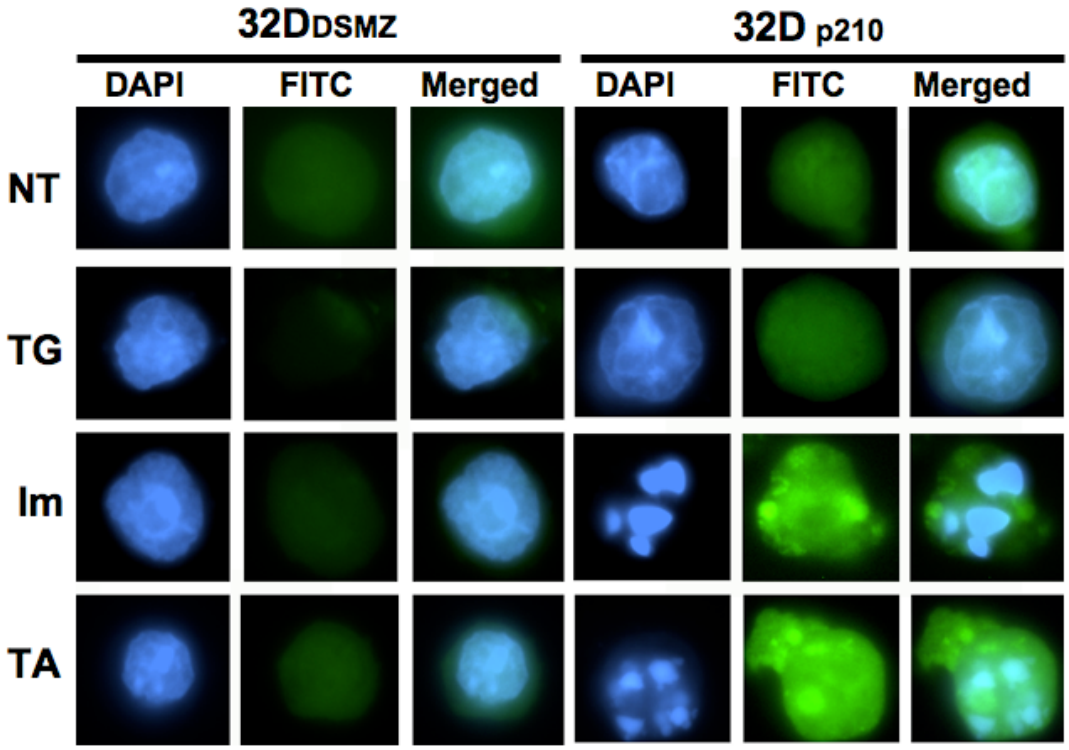


Figure 29: *Appearance of cleaved PARP-1 and induction of apoptosis in Bcr-Abl expressing 32D ^{p210}cells when treated with apoptin or imatinib;* 16 hours following the treatment of primary murine cells, $32D_{DSMZ}$ and the Bcr-Abl^{p210}wt expressing murine 32D^{p210} cells, with blank (NT; -Ve control), TAT-GFP (TG; -Ve control), imatinib (Im; +Ve control) and TAT-Apoptin (TA; Test)**:** These immuno-cytochemistry experiments were performed on the cells from similar experiments as described to show the intracellular presence of cleaved PARP-1 in TA and Im treated cells. Accumulated cleaved PARP-1 was identified by FITC-tagged anti-cleaved PARP-1 specific mouse monoclonal antibody that does not recognize full length PARP-1. Representative images show the presence of cleaved PARP-1 (green) and apoptotic nuclei (blue) in the Im and TA treated Bcr-Abl^{p210}wt expressing 32D^{p210} cells (column 6 and row 4, 5 respectively).

Apoptin induces kills both Bcr-Abl positive and negative transformed cells: To study the biological activity of the cell-penetrating TAT-Apoptin on $32D^{p210}$ cell lines expressing Bcr-Abl^{p210}, I added affinity purified recombinant TAT-Apoptin (1μM) (**Figure 30**) to the growing cells in culture. The specific 1μM dose of TAT-Apoptin was selected for these experiments according to previous dose response studies by us and others (Olijslagers et al, 2007). The cell survival was estimated by MTT cell proliferation assay at different time points. In some other experiments cells were stained according to the Nicoletti method 24 hours after treatment with TAT-Apoptin and analyzed by FACS. The results are summarized in **Figure 31.** Cells grown without any treatment were set as 100% proliferation.

Below, **Figure 30** shows purified TAT-GFP (control) and TAT-Apoptin. The N-terminal leucine rich PML binding domain of apoptin (LRS) also facilitates apoptin polymerization. Multiple bands in this gel image possibly indicates the presence of different stable polymeric forms of apoptin, which are reportedly with equal biological activity (Leliveld et al, 2003a).

In **Figure 31,** the results show that treatment of 32^{p210} cell lines with either TAT-Apoptin or the positive control imatinib caused significant reduction ($p < 0.03$) in cell survival compared to the negative control group receiving TAT-GFP treatment. A marked inhibition of $32D^{p210}$ growth was observed 24 hours after the application of TAT-Apoptin, imatinib in comparison to the control groups treated with TAT-conjugated GFP.

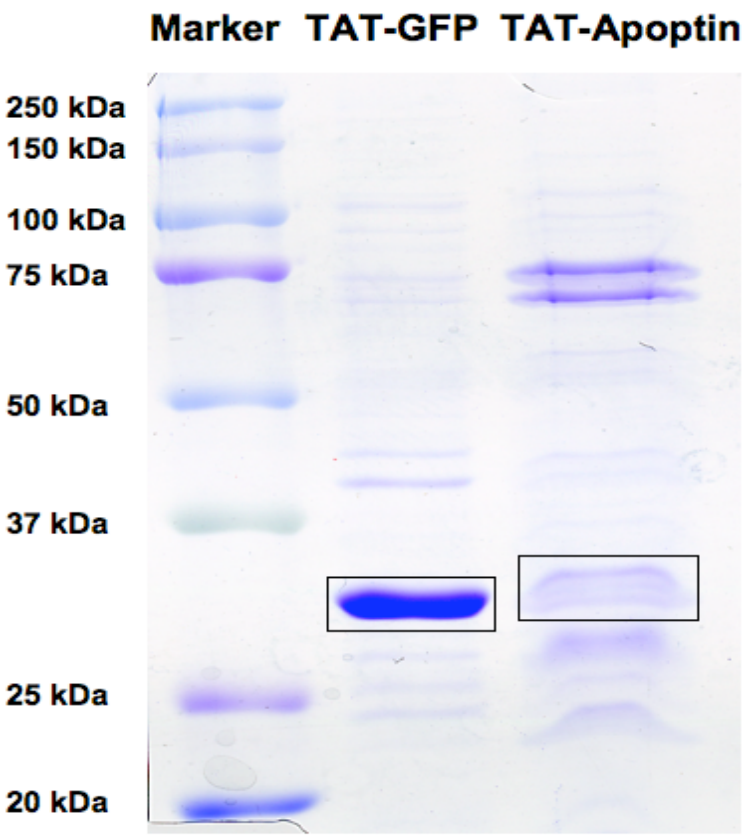


Figure 30: *Production and purification of recombinant His-tagged TAT-conjugated apoptin;* Recombinant histidine tagged TAT-conjugated apoptin was produced from transformed BL21 clones following IPTG stimulation. The His-tagged TAT-Apoptin was purified from bacterial lysate by Ni-affinity column. As reported, this His-tagged apoptin can spontaneously form non-covalent globular aggregates comprising 30 to 40 subunits *in vitro*. This multimerization is virtually irreversible, and the globular aggregates are functionally active and stable in cell extracts (Leliveld et al, 2003b). Presence of such apoptin multimers is evidenced above by multiple apoptin bands of different molecular weights.

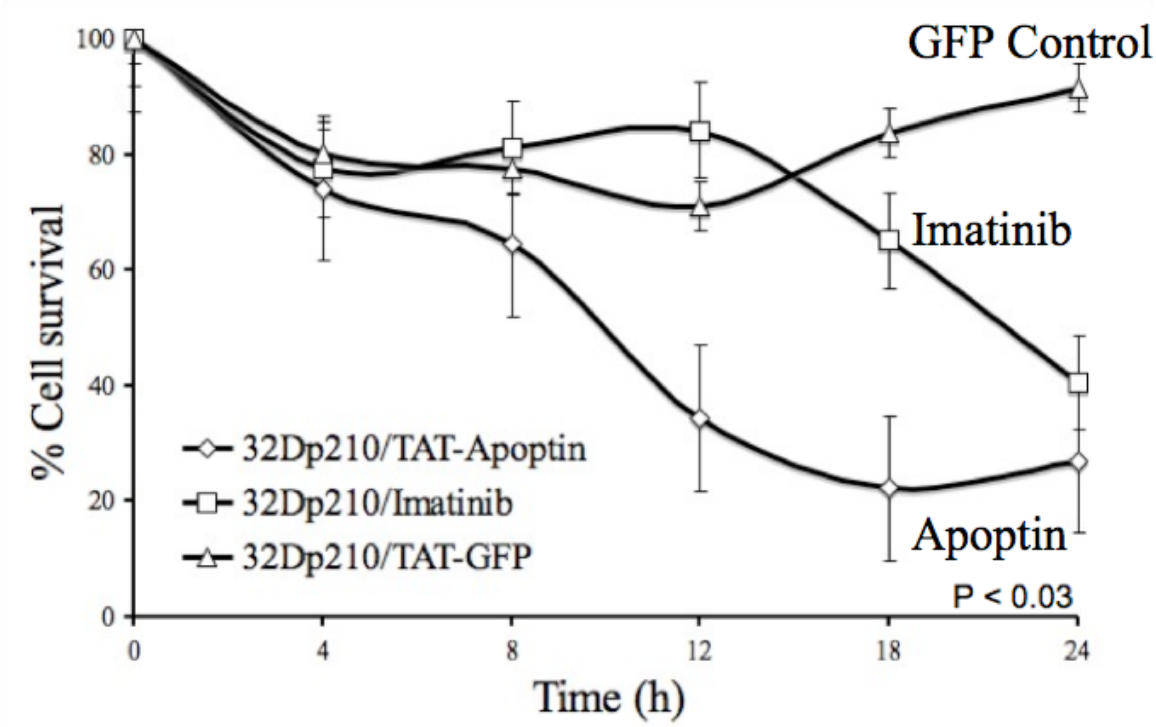


Figure 31: *The effects of apoptin on Bcr-Abl expressing cell as estimated by MTT method;* $32D^{p210}$ cells were grown in a 96 well plate (10,000 cells/well). His-tagged TAT-Apoptin, imatinib (+Ve control) and TAT-GFP (-Ve control) were applied (in triplicate, 1μM) at an interval of 0, 4, 8, 12, 18 and 24 hours. The % of live cells was estimated my MTT method and average and normalized (with untreated cells) values of cell survival data as plotted here shows significant ($p < 0.03$) killing effect of apoptin. (Student's t-test assuming equal variance, $p<0.05$ indicates statistical significance; n=3).

Similar interesting results results were obtained from other experiments where cells were stained according to the Nicoletti method and analyzed by flow cytometry 24 hours after the treatment with apoptin or imatinib (**Figure 32a-b**). Here, I analyzed the Bcr-Abl^{p210} expressing 32D^{p210} cells and the parental 32D$_{DSMZ}$ cells that normally require murine IL-3 for survival and proliferation. The treatment with imatinib only reduced about 50% of the 32D^{p210} growth whereas a combination treatment of imatinib and apoptin on both cell lines showed significant cell killing potentials. Notably, imatinib alone did not show any effect on 32D$_{DSMZ}$ cells that do not express Bcr-Abl^{p210}. As it is with the 32D^{p210} cells expressing Bcr-Abl^{p210} (grows in the absence of IL-3), when the 32D$_{DSMZ}$ cells are exposed to apoptin, the resultant 24 hours survivals were similar. This similarity in cell death pattern in both Bcr-Abl expressing and non-expressing cells following apoptin treatment is possibly due to its unique transformed cell specific apoptosis induction property. We have to remember that 32D$_{DSMZ}$ is an immortalized cell line. Interestingly, when the same cell lines were treated with imatinib, there was visibly no effect on the survival of 32D$_{DSMZ}$ cells and only about half of the 32D^{p210} cells were killed (**Figure 32b**) confirming the potent anti-proliferative effect of apoptin on transformed and Bcr-Abl expressing cells.

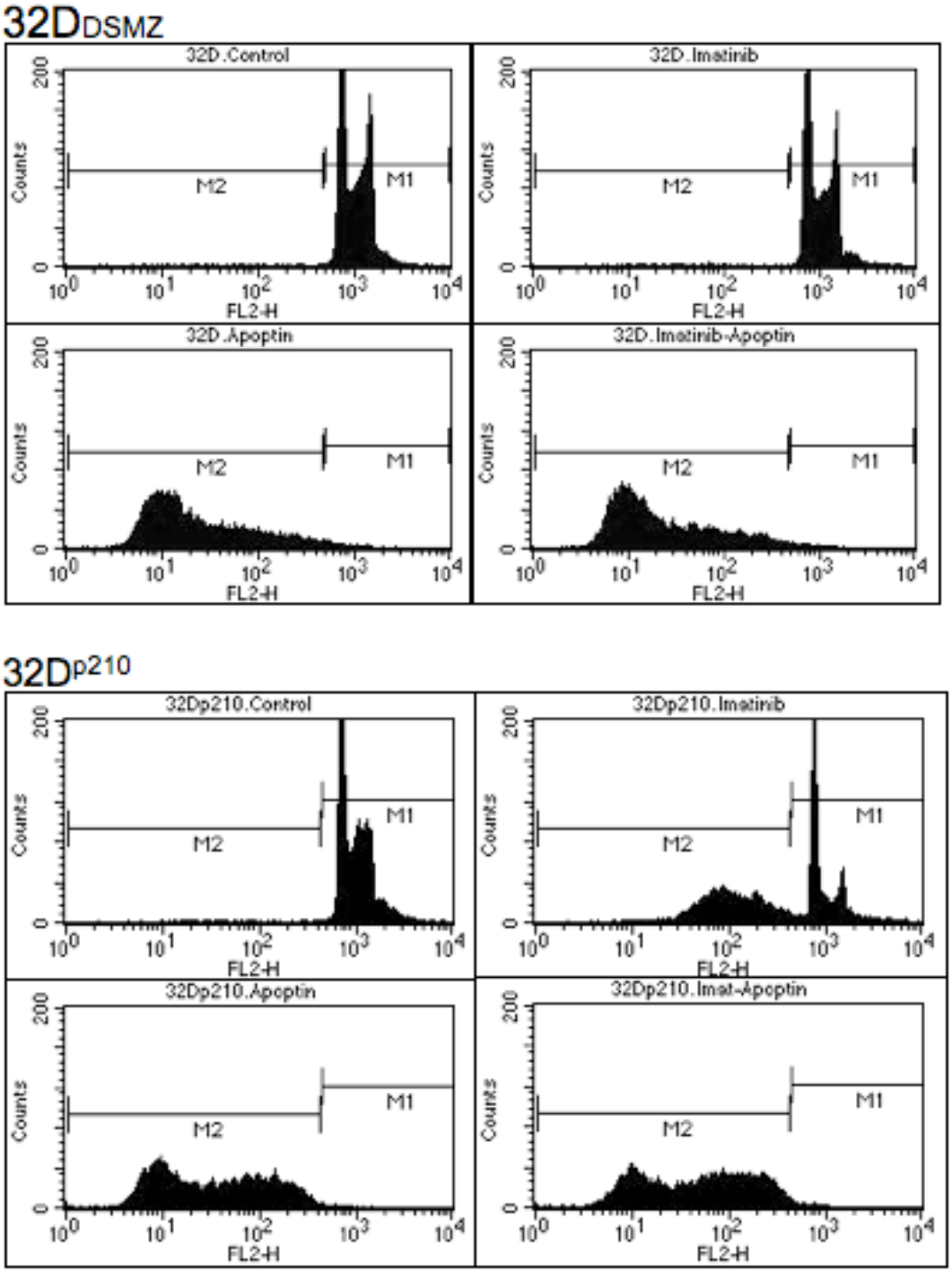


Figure 32a: *The effects of apoptin on Bcr-Abl expressing cells as determined by Nicoletti method:* (i-iv) *32D$_{DSMZ}$ cells and* (v-viii) *32D^{p210} cells*; were treated with apoptin, imatinib or in combination. Each of the treatment groups were analyzed for survival after 24 hours by FACS (Nicoletti method) with FL2 gating for 10,000 cells where M2 indicates dead cells and M1 indicates live cells.

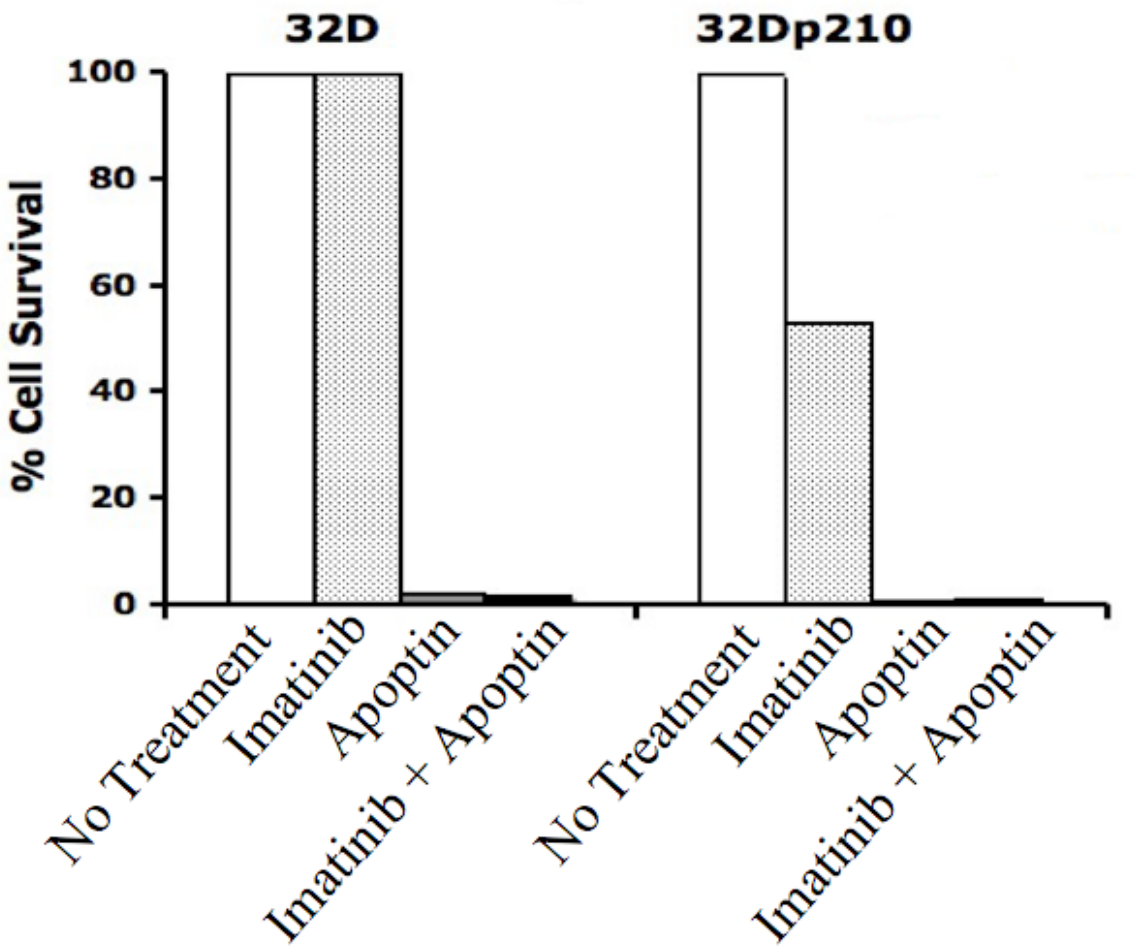


Figure 32b: *The effects of apoptin on Bcr-Abl expressing cells as determined by Nicoletti method* (*Compiled data from* ***Fig. 32a***): *(i-viii)*: Cell growth without any treatment was considered as 100% proliferation (control). Normalized values, expressed as percent of cell survival, indicates imatinib has minimum effect on the survival of Bcr-Abl non-expressing *32D$_{DSMZ}$* cells that is comparable to the untreated control group whereas when applied to 32D^{p210} cells, imatinib alone killed about 50% of the cells. Apoptin alone or in combination with imatinib significantly killed both 32D$_{DSMZ}$ and 32D^{p210} cells.

6.2 A simulated three-dimensional model of apoptin, built for the first time, shows apoptin and Bcr-Abl SH3 domain interactions

In this part of study, the unknown 3D structure of apoptin was approximated by a comparative or homology protein structure modeling system. For this purpose, the protein sequence (target) based on the known 3D structure of related family members was used (**Figure 9**). The 3D structures of ten such known templates with identified partial homology to apoptin were used to build a 3D structure. This 'low resolution' model obtained by homology modeling provided essential information of the spatial arrangement of biologically important groups of residues. Using ClustalW2, a multiple sequence alignment tool (Krissinel & Henrick), we aligned the 11 sequences including apoptin. A set of 3D models of apoptin (Ball and cylinder; Ribbon view, Space-filled) from amino acid 33 to 90 was built (**Figure 33a-b**). After building the model based on the ten sequences, all atoms of the molecule were locked and molecular dynamic simulations were performed unlocking one turn from each end of the sequence at a time. Subsequently, as an essential component for testing and verification of the level of accuracy in this 3D model, a *Ramachandran plot* was used (**Figure 34**). The N-Cα and Cα-C bonds in a polypeptide chain are relatively free to rotate. These rotations are represented in the plot by the torsion angles phi (Φ) and psi (Ψ), respectively. The structure was examined for close contacts between atoms for each of these conformations. Atoms were considered as hard spheres with dimensions corresponding to their van der Waals radii. Therefore angles, which cause spheres to collide, correspond to sterically disallowed conformations of the polypeptide backbone. Disallowed regions involve steric hindrance between the side chain methylene group and main chain atoms.

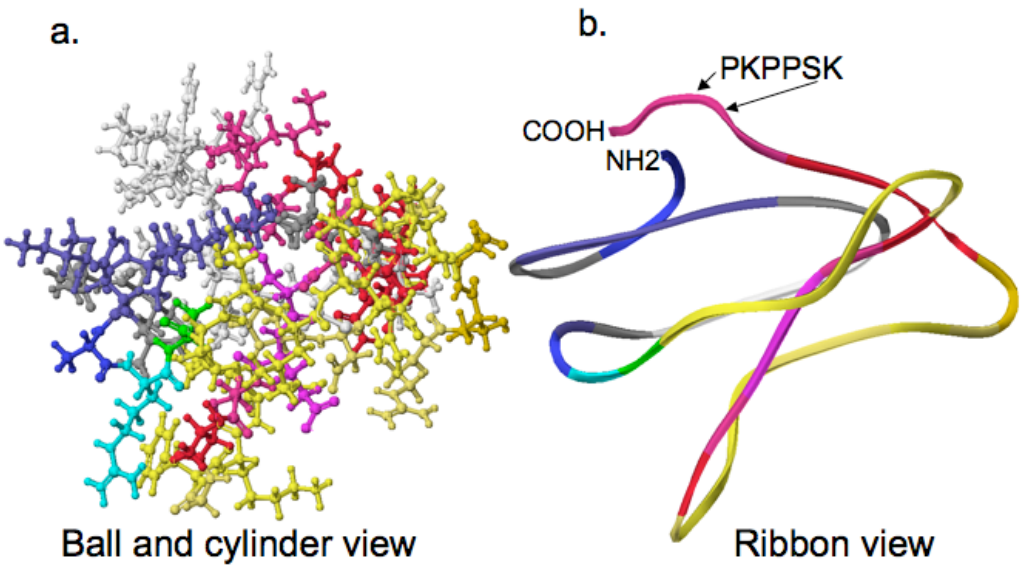


Figure 33: *Computer generated three-dimensional model of apoptin (aa:33-90) demonstrating potential hydrophobic proline rich interacting area* (PKPPSK). **a**. *3D apoptin – Ball and cylinder view and* **b**. *3D apoptin – Ribbon view*; the multiple sequence alignment tool (Krissinel & Henrick), ClustalW2 was used to align the homology sequences. Subsequently the model of apoptin from amino acid 33 to 90 was built and verified. This model of apoptin was about 80% in the allowed 3D configuration obtained from the corresponding 'Ramachandran plot' (see below).

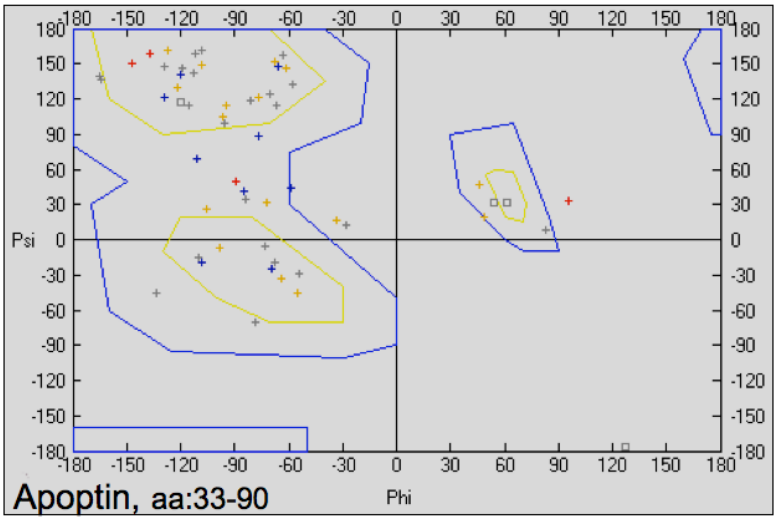


Figure 34: *Ramachandran plot:* Showing the relatively free to rotate N-Cα and Cα-C bonds respectively represented by the torsion angles phi (Φ) and psi (Ψ) in the apoptin (aa:33-90*)* polypeptide chain. The structure was examined for close contacts between atoms for each of these conformations.

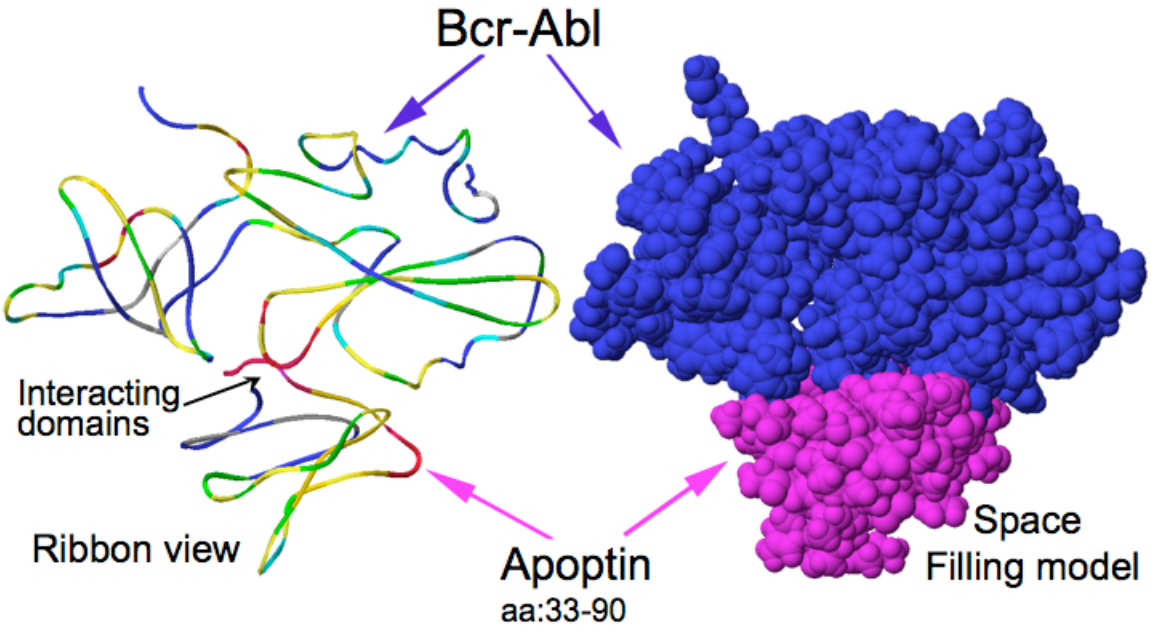


Figure 35: *Computational 3D model of apoptin sequence (aa:33-90) demonstrating molecular docking of apoptin with the known 3D model of* Bcr-Abl-SH3*;* In this computational virtual docking experiment 16 amino acids of Bcr-Abl SH3 domain came within 3Å of 16 amino acids of apoptin including its proline rich PxxP sequence and at least three pairs of direct hydrogen bonding are possible in between them (**Table 4**).

Table 4 Interacting amino acid residues at the docking site of apoptin and Bcr-Abl

Apoptin (aa:1-121)			Bcr-Abl (p210)	
Amino acid residue	**Position***		**Amino acid residue**	**Position**
Isoleucine	33 (1)		Histidine	40
Arginine	34 (2)		Asparagine	41
Alanine	38 (6)		Glycine	42
Glycine	39 (7)		Glutamic acid	43
Isoleucine	40 (8)		Serine	58
Threonine	43 (11)		Asparagine	59
Leucine	46 (14)		Glutamine	68
Leucine	76 (44)		Glycine	75
Aspartic acid	78 (47)		Proline	76
Proline	81 (49)		Serine	78
Proline	83 (51)		Asparagine	80
Proline	84 (52)		Alanine	81
Serine	85 (53)		Tyrosine	84
Lysine	86 (54)		Glutamic acid	100
Lysine	87 (55)		Serine	101
Arginine	88 (56)		Proline	103
Amino acid residues forming direct hydrogen bond				
Isoleucine	33 (1)	>	< Glutamic acid	100
Glycine	39 (7)	>	< Asparagine	41
Threonine	43 (11)	>	< Serine	101

- Numbers in (-) indicate the relative positions of the same amino acid residues in apoptin 33-90 peptide.
- The SH3 interacting amino acids in the proline rich PxxP region of apoptin are marked as bold.
- The pairs of amino acid residues (> <) in apoptin and Bcr-Abl, forming direct hydrogen bonding are presented at the bottom part of **Table 4**.

Apoptin modeling is challenging because it is difficult to find templates spanning the full length of its amino acid sequence. However, using 10 to 11 sequences, we were able to build the full length (aa:1-121) 3D structure of apoptin with 78.8% accuracy and another partial model (aa:7-110) with about 86% accuracy. Here, the most critically verified model of apoptin (aa:33-90) is presentated as an example of these predicted 3D models (summerized in **Figure 33** and **Figure 34**). Furthermore, this model was used to virtually examine various binding interactions with Bcr-Abl. For example, to study the interaction of apoptin with the SH3 domain of Bcr-Abl, we first noted the possible hydrophobic surfaces on the 3D models of SH3-SH2 domains in Bcr-Abl and apoptin and in this

model system 16 amino acids of Bcr-Abl SH3 domain were approximated within 3Å of 16 amino acids of apoptin including its proline rich PxxP sequence (**Figure 33** and **Figure 35**) and at least three pairs of direct hydrogen bonding are possible in between them (**Table 4**). Thus, these results further confirm the previous findings and provide a platform for designing novel peptide based Bcr-Abl TK inhibitors.

6.3 Specific apoptin-derived peptides also interact with the Abl-SH3 and induce apoptosis in CML cell: a pilot study:

6.3.1 Three-dimensional modeling of the proline rich peptide sequence of apoptin

In this part of the study, the 3D structure of apoptin's proline rich interacting region (NLS1) spanning amino acid residues 81-90 (**PKPPSK**KRSC) was built by the computational protein modeling system as described earlier. All atoms of this small peptide sequence were locked and molecular dynamic simulations were performed in the final model. This model of an apoptin derivative shows the spatial arrangement of the SH3 domain interacting proline residues (colored red) in its 3D structure (**Figure 36**).

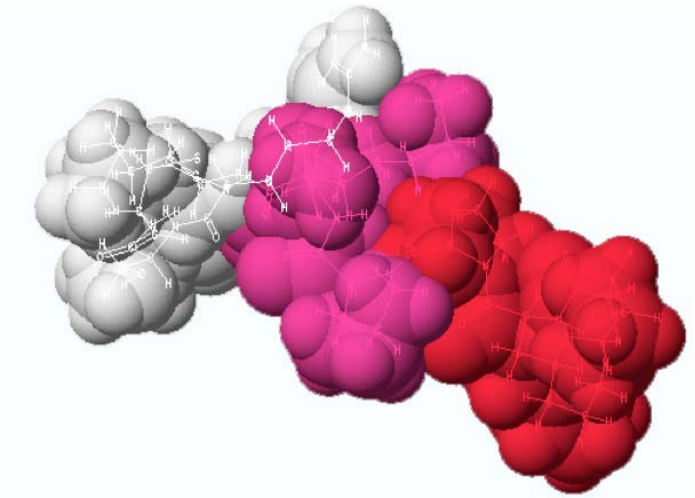


Figure 36: *The three-dimensional model of proline rich apoptin sequence* (aa:81-90/ **PKPPSK**KRSC), *space-filled model*: the sequence alignment tool (European Bioinformatics Institute, http://www.ebi.ac.uk/), ClustalW2 was used to build this model of apoptin derivative from amino acid 81 to 90 and verified by *Ramachandran plot*.

6.3.2 The Src homology domain 3 (SH3) of Abl interacts with synthetic proline rich peptide sequence (aa: 81-86) of apoptin:

In this part of the study, a high stringent array-based SH3 domain interaction experiment was performed to identify if the apoptin derived proline rich peptide domain interacts with the SH3 domain of a known set of proteins as described earlier (5.10). The previous observation with such interaction array experiment showed a precise binding selection of the recombinant TAT-conjugated full-length apoptin sequence towards the SH3 domain of c-Abl (**Fig. 10**). Interestingly, in this assay, the short apoptin derived synthetic peptide also showed similar interaction with the Abl SH3 domain but with a remarkably reduced specificity. As seen in **Figure 37,** the synthetic TAT-conjugated peptide sequence (rkkrrqrrr-**PKPPSK**KRSC) also had interactions with the SH3 domains of a number of proteins namely, Amphiphysin, Cortactin, Yes1, SJHUA, CDK-D2, EMP55, c-Src, FYB-D1, Y124, PEXD and PSD95. Presumably, other areas of full-length apoptin and its intact phosphorylation site (Thr-108) play important roles for its interaction specificity with proteins like Bcr-Abl. Evaluation of the significance of this observation is beyond the limit of the current work.

In the following section (6.3.3) results from a set of pilot studies indicate the biological cell-killing efficacy of this apoptin derived TAT-conjugated short peptide and the TAT-conjugated scrambled sequence as control.

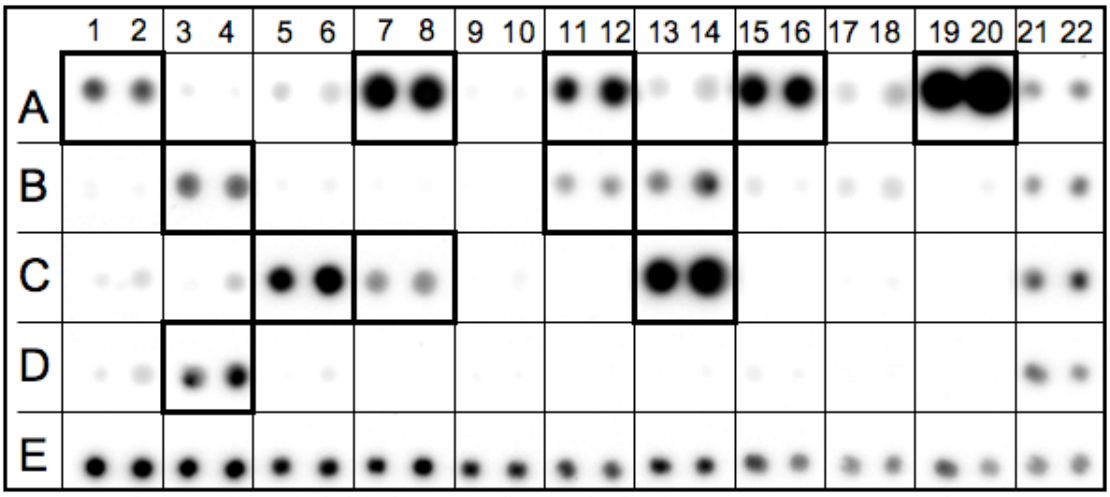


Figure 37: *TransSignal SH3™ Domain Array1: interaction of synthetic proline rich sequence of apoptin and SH3 domain of* Abl *(D3, 4).* The TransSignal SH3™ Domain Array1 was incubated in synthetic TAT-conjugated proline rich sequence of apoptin (aa: 81-90) in buffer and the resulting interactions were imaged on high performance chemiluminescence film (Hyperfilm™ECL, Amersham Biosciences). Spots with stronger intensity (boxed) indicate higher binding affinity with ligand of interest to SH3 domain(s). The proteins in the array are spotted in duplicates. In this study, other than the interaction with Abl SH3 (D3, 4), the proline-rich peptide sequence of apoptin (aa: 81-90) also shows interactions with the SH3 domains of Amphiphysin (A1, 2), Cortactin (A7, 8), Yes1 (A11-12), SJHUA (A15, 16), CDK-D2 (A19, 20), EMP55 (B3, 4), c-Src (B11, 12), FYB-D1 (B13, 14), Y124 (C5, 6), PEXD (C7, 8) and PSD95 (C13, 14). Histidine tagged ligands have been spotted along the bottom (row E) and in duplicate along the right side of the membrane (column 21, 22) for alignment purpose.

6.3.3 The TAT conjugated synthetic proline rich sequence of apoptin (aa: 81-90) kills Bcr-Abl expressing cells:

The proline-rich apoptin derived peptide induced differential cell death in Bcr-Abl expressing cells: To study the biological activity of the apoptin derived cell-penetrating synthetic peptide on murine $32D^{p210}$ cell lines and human K562 cell lines expressing Bcr-Abl^{p210}, TAT-conjugated peptide (rkkrrqrrr-**PKPPSK**KRSC) was added in a concentration of 1μM to the growing cells in culture and cell survival was estimated by MTT cell survival assay at different time points over a period of 48 hours. The murine IL3 dependent primary hematopoetic murine cell line $32D_{DSMZ}$ was used as the control cell line. In another set of parallel experiments a scrambled TAT-conjugated peptide sequence (rkkrrqrrr-PRKPSKSPKC) was used as treatment control. The results obtained from these three cell lines treated with both test and control peptides are summarized in (**Figure 38a, Figure 38b, Figure 38c).** Cells grown without any treatment were set to 100% proliferation and the cell survival was expressed as normalized average. This result further confirms the anti-proliferative effect of apoptin and apoptin derived peptides that are mediated through their SH3 domain interacting proline rich regions. Here, I compared the Bcr-Abl^{p210} expressing $32D^{p210}$ cells and the parental $32D_{DSMZ}$ cells that normally require murine IL-3 for survival and proliferation. The differential killing properties are notable from **Figure 38a** and **Figure 38b** respectively. Interestingly, similar peptide treatment of the Bcr-Abl^{p210} expressing K562 cells also yielded comparable results (**Figure 38c**).

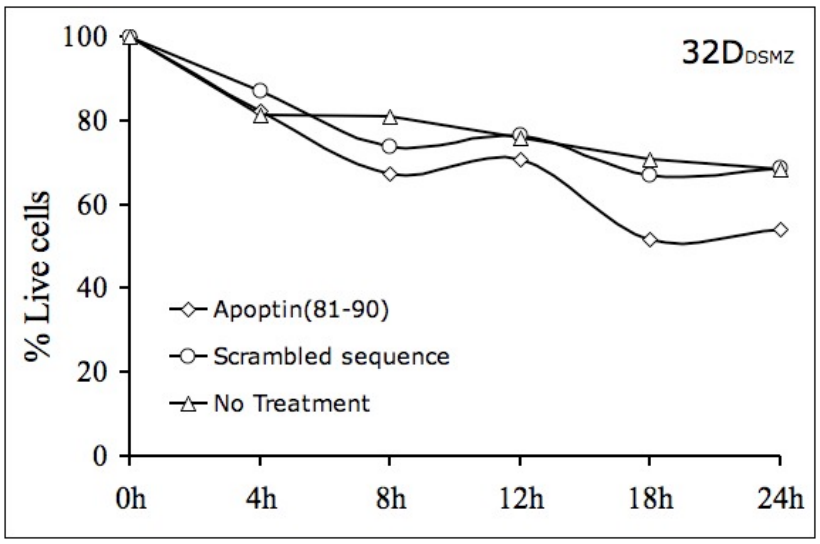


Figure 38a: *The effects of TAT-conjugated apoptin derived peptide on the survival of Bcr-Abl non-expressing 32DDSMZ cells (MTT assay)*: murine 32DDSMZ, cells were grown in 96 well plates (10,000cells/well). TAT-Apoptin (aa: 81-90), and TAT-Scrambled peptide sequence (-Ve control) were applied (in triplicate, 1μM) at an interval of 0, 4, 8, 12, 18, 24, 36 and 48 hours (H). The relative ratio of live cells in treated and un-treated groups was estimated by MTT method and the average normalized (with untreated cells) values of cell survival data as plotted here show promising killing effect of this apoptin derived peptide on Bcr-Abl expressing murine and human CML cells.

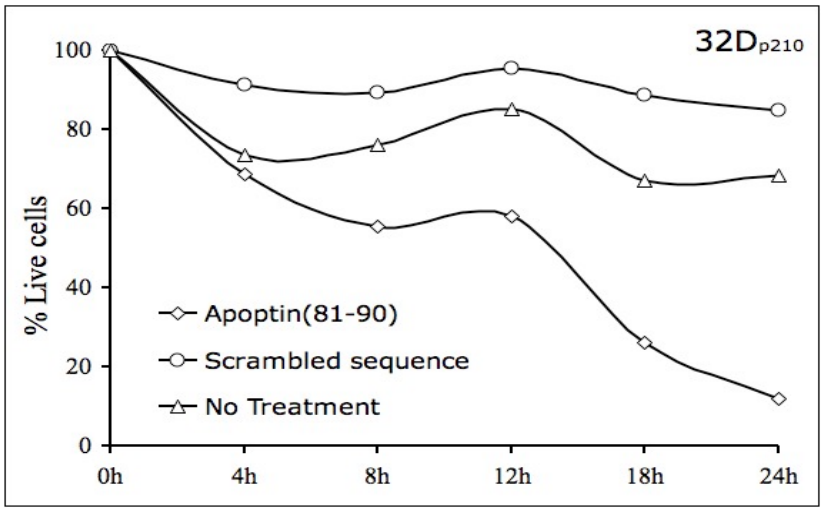


Figure 38b: *The effects of TAT-conjugated apoptin derived peptide on the survival of Bcr-Abl expressing* $32D^{p210}$ *cells (MTT assay)*: transformed murine $32D^{p210}$, cells were grown in 96 well plates (10,000cells/well). TAT-Apoptin (aa: 81-90), and TAT-Scrambled peptide sequence (-Ve control) were applied (in triplicate, 1μM) at an interval of 0, 4, 8, 12, 18, 24, 36 and 48 hours (H). The relative ratio of live cells was estimated by MTT method as described.

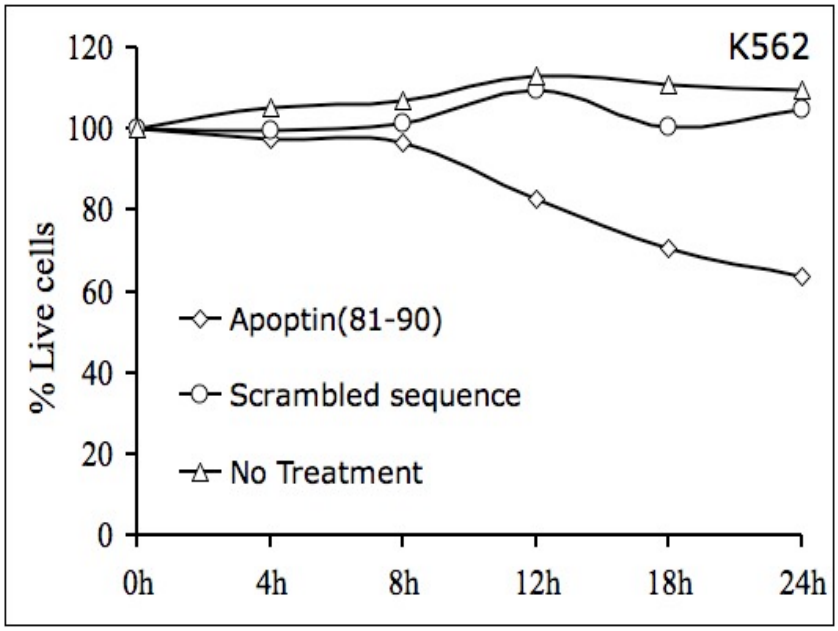


Figure 38c: *The effects of TAT-conjugated apoptin derived peptide on the survival of Bcr-Abl expressing* K562 *cells (MTT assay)*: K562 cells (human CML cells) were grown in 96 well plates (10,000cells/well). TAT-Apoptin (aa: 81-90), and TAT-Scrambled peptide sequence (-Ve control) were applied (in triplicate, 1μM) at an interval of 0, 4, 8, 12, 18, 24, 36 and 48 hours (h). The relative ratio of live cells was estimated by MTT method as described.

7. Discussion

Protein kinase pathways, active in malignant cells, are among the most promising strategic targets for new anti-cancer drug development. In CML the uniquely expressed onco-protein Bcr-Abl acts as an anti-apoptotic protein and contributes to the development and proliferation of CML. Therefore, Bcr-Abl is considered as an attractive molecular target for CML therapy. The Bcr-Abl (p210) protein is constitutively active in CML and specific synthetic small molecules that inhibit Bcr-Abl kinase activity in leukemic cells are already in clinical use (Soverini et al, 2008). Unfortunately, relapse of the disease occurs in a large percentage of these patients after receiving some of the newly introduced Bcr-Abl kinase inhibitors like imatinib mesylate (Gleevec), as a first line of therapy, although there are encouraging initial hematological and cytogenetic responses. Until recently, more than 17 different clinically relevant point mutations within the *BCR-ABL* gene have been identified and considered as the key reason behind the emergence of imatinib-resistance. Some of these mutations in the *BCR-ABL* gene cause amino acid substitutions that interfere with the ability of imatinib to interact with the Bcr-Abl kinase domain. These frequent mutations of the Bcr-Abl kinase domain that initiate imatinib resistance, renewed the efforts towards identification of unique inhibitors that are effective against imatinib-resistant mutants of Bcr-Abl. Several different approaches to overcome this resistance were previously reported, although many of the new molecules were found to be ineffective against the T315I Bcr-Abl mutation that is most frequently observed in imatinib-resistant CML patients (Schindler et al, 2000; Shah & Sawyers, 2003; Shah et al, 2004).

Apoptin is a protein derived from chicken anemia virus that can induce death-receptor and p53-independent apoptosis in a number of tumor and transformed cells, but not in normal primary cells as shown by both *in vitro* and *in vivo* studies (Maddika et al, 2006). This special feature is believed to be due to apoptin's ability of targeting multiple signal transduction proteins and relocating them into specific cellular compartments. Although there are some discrepancies in observation regarding the selective toxicity of apoptin, further studies in detail using broader normal cell systems and varied cancer cell lines will complete our understanding of this specificity.

Most of the cancer therapies available today, like cytotoxic chemotherapy or radiation do not selectively kill cancer cells. So normal cells are also affected. Apoptin shows cytoplasmic localization in primary cells, whereas it accumulates in the nuclei of transformed cells and it can be speculated that the selective nuclear localization in combination with the mitotic interference largely explains the cancer selective effects of apoptin. When apoptin is directed artificially to the nuclei of primary cells it still remains non-toxic for these cells. This observation indicates that apoptin's cancer cell specific toxicity requires interactions with other molecules or additional modifications in cancer cells. In several independent studies the molecular basis of apoptin's tumor specific nuclear localization was investigated that include the role of c-terminal nuclear localization signals, N-terminal nuclear export signals and post-translational modifications like Thr-108 tumor specific phosphorylation in the apoptin molecule. Another proposed mechanism of apoptin's specific killing is its ability to interfere with the function of the anaphase-promoting complex in cancer cells. This interference with the final stages of mitosis is a factor that causes cellular stress and most likely activates

the intrinsic/mitochondrial apoptotic-signaling cascade. At the mitochondrial level, the apoptin-triggered signal(s) cause(s) the release of various mitochondrial proapoptotic molecules that either directly propagate(s) the apoptotic process or activate downstream molecules in the apoptotic pathway.

In the current study, using apoptin as a model molecule, the direct and indirect roles on different activated intracellular signaling pathways in Bcr-Abl expressing cells were investigated. As postulated, the interaction of apoptin with Bcr-Abl-kinase inhibited the phosphorylation of Bcr-Abl constitutively and redirected Bcr-Abl-kinase and related down stream survival and proliferation pathways towards the cell death pathways. The Bcr-Abl expressing malignant or transformed cells were highly sensitive to apoptin induced cell death. The activated signaling molecule Bcr-Abl in CML was found to be a major target for apoptin. In addition, in an attempt to understand these protein-protein interactions at the molecular level and to have further insight towards designing novel peptide-based drug that targets the oncoprotein Bcr-Abl with higher efficiency, a three-dimensional model of apoptin was built and its molecular docking with the SH3 domain of Bcr-Abl has also been characterized for the first time in this work. Finally, a synthetic TAT-conjugated small apoptin derived proline rich peptide sequence (**PKPPSK**KRSC) was tested in pilot experiments to study its effects on Bcr-Abl expressing mouse and human cells.

This work on apoptin and apoptin based synthetic peptides targeted against CML will possibly be helpful in designing new generations of peptide-based tyrosine kinase inhibitors in the future without having the drawbacks of currently available Bcr-Abl kinase inhibitors in the market for treating CML.

7.1 Apoptin induced apoptosis in Bcr-Abl expressing cells

Apoptin's ability to induce tumor specific cell death by apoptosis is a key feature of this molecule. But, as mentioned earlier, apoptin does not require p53 to induce apoptosis. The selective toxicity of apoptin is mainly due to its differential localization in transformed and normal cells. Apoptin localizes to the nucleus of cancer cells and induce apoptosis. On the other hand, in normal cells apoptin mainly stays in the cytoplasm (Danen-van Oorschot et al, 2003). Apoptin is a nucleo-cytoplasmic shuttling protein and its specific localization is regulated by the NLS (nuclear localization signals) and NES (nuclear export signals). In normal cells NES remains functionally active, whereas in tumor cells the apoptin NES is inactive. This difference determines the tumor cell specific nuclear localization of apoptin (Poon et al, 2005b).

The Ser-Thr protein kinase CDK2 is essential for apoptin-induced cell death and acts as the principal kinase that phosphorylates apoptin at Thr-108 residue (Maddika et al, 2008b). The phosphorylation at Thr-108 residue is reported to be responsible for tumor specific apoptin's nuclear accumulation via inactivating the NES in tumor cells thus inhibiting the nuclear export specifically in tumor cells. In addition, activated PI3-Kinase indirectly mediates the phosphorylation of Nur77 and the phosphorylated Nur77 is translocated to the cytoplasm and transmit the nuclear apoptin induced signal to mitochondrial activation and shifts the balance between pro-and anti-apoptotic molecules at the mitochondria towards apoptosis (Maddika et al, 2005).

In the current study, we showed for the first time, that TAT-Apoptin, a cell-penetrating derivative of apoptin strongly binds to the SH3 domain of Bcr-Abl, modifies the phosphorylation and thus the activity of Bcr-Abl and several its downstream targets.

These changes lead to antiproliferative effect and induction of intrinsic apoptotic pathways in the rapidly growing CML cells. Using human CML cells K562 and Bcr-Abl (p210) expressing murine cell line $32D^{p210}$ as a model, we observed that these cells are highly responsive to apoptin, and that apoptin's anticancer activity favorably compares to imatinib. These human and murine cell lines are rapidly growing with a high cytoplasmic Bcr-Abl (p210) pool, thus the cell culture condition mimic blast crisis stage of CML. Furthermore, as it is in CML, the central mitogenic Ras-MAPK cascade is also activated in these cell lines. Our findings corroborate well with previous studies involving a similar approach directed towards the Grb2-SoS-Ras-MAP kinase (Erk) pathway (Kardinal et al, 2001). In those experiments Kardinal and colleagues applied small, high affinity peptides blocking the N-terminal SH3 domain of Grb2. Their results indicate that peptide based inhibitor of Bcr-Abl kinase or its down-stream targets could be valuable anti-CML tool if combined with conventional cytotoxic therapy (Kardinal et al, 2001). We have also observed in pilot studies that apoptin-derived peptides capable of interaction with SH3 domain are toxic against Bcr-Abl expressing cells (**Figure 37**).

In oncoprotein Bcr-Abl, the fusion of Bcr sequences to the Abl-SH3 domain abrogates the physiologic regulation of the tyrosine kinase of Abl. This fusion protein is capable of inducing uncontrolled auto-phosphorylation that activates the downstream cell growth, proliferation and anti-apoptotic pathways. These altered signaling cascades lead to a series of alterations in the behaviors of CML cells like diminished adhesion to the stromal environment, degradation of inhibitory proteins, activation of mitogenic signaling and inhibition of apoptosis. To test the possible interaction of apoptin with Bcr-Abl an initial attempt was made to identify the interaction partners for apoptin by using an SH3 specific

array that revealed that the SH3 regulatory subunit of c-Abl is a major candidate interacting with recombinant apoptin (**Figure 10**). The interaction of Bcr-Abl during the apoptin treatment was confirmed by GST-Apoptin pull down assays and apoptin/Bcr-Abl co-immunoprecipitation assays in Bcr-Abl expressing mouse ($32D^{p210}$) and human cell line (K562) transfected for the expression of GFP-Apoptin (**Figure 12-13**). The same set of cell lines was used for imunofluorescence studies to view protein-protein interaction directly in the cell (**Figure 14**). The cultured human CML cells K562 and transformed Bcr-Abl (p210) expressing mouse cell line $32D^{p210}$ are rapidly growing cells with a high cytoplasmic Bcr-Abl (p210) pool. As seen in the transfected mouse cell systems, the transiently expressed GFP conjugated apoptin co-transports the predominantly cytoplasmic Bcr-Abl protein pool to the nucleus and induces apoptotic response. We also observed that apoptin, unlike imatinib/Gleevec, was effective both against Bcr-Abl positive and Bcl-Abl negative transformed cells. Thus, hypothetically apoptin based therapeutics would not only be more effective, but they would be less prone to the development of a resistance, since, unlike imatinib, they have other multiple and diverse targets within the cancer or transformed cells.

We further verified the specific interaction domains of apoptin and Bcr-Abl fusion protein (p210) (**Figure 15 - 17**). As demonstrated, apoptin works by binding to the SH3 domain of Bcr-Abl and possibly acting as an adaptor molecule it prevents the activation of the kinase domain (SH1). Thus, the ATP binding and phosphorylation of Bcr-Abl is downregulated and this abrupt loss of signal leads to downregulation of cell proliferation and activation of intrinsic apoptotic pathways. The hypothesized nature of apotin induced downregulation of activated Bcr-Abl kinase in CML is summarized below in **Figure 39.**

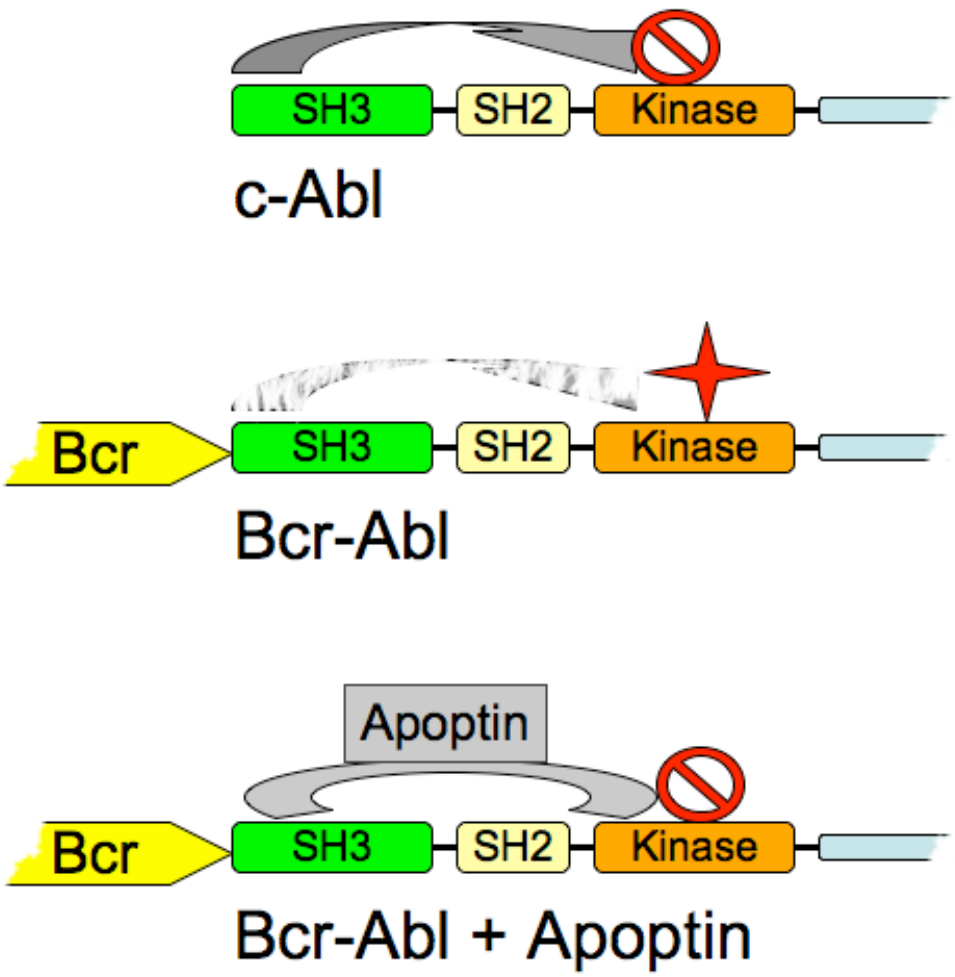


Figure 39: *Schematic diagram of apoptin induced Bcr-Abl kinase inhibition.* UPPER PANEL- In normal c-Abl the SH3 domain acts as an endogenous inhibitor of its own kinase (SH1 domain) due to its interaction with a proline rich sequence of the same molecule. MIDDLE PANEL: In the fusion protein Bcr-Abl, the attachment of Bcr to the SH3 domain of Abl disrupts the attachment to this internal proline rich sequence and abrogates the kinase inhibition leading to autophosphorylation and transactivation of other oncogenic kinase pathways. LOWER PANEL: Strong interaction between the proline rich sequence of apoptin and the SH3 domain of oncoprotein Bcr-Abl leads to re-institution of this inhibition on Bcr-Abl kinase.

7.2 Role of Bcr-Abl and downstream pathways on apoptin's selective toxicity

The apoptin-induced inhibition of Bcr-Abl phosphorylation also carried significant modifications of the functions of downstream kinases involved in proliferation, cellular stress response and apoptotic pathways. In the immunoblot assays the antibodies were targeted against phosphorylated and total STAT5, CrkL, Akt, c-Myc and against cleaved PARP-1 on the extracts of Bcr-Abl expressing mouse and human cell lines treated with TAT-Apoptin (with imatinib as positive control and TAT-GFP as negative control).

STATs act as regulators of cell proliferation (Meyer et al, 1998). The N-terminal regulatory region of Abl protein contains SH2 and SH3 domains which are important for the regulation of its activity *in vivo* (Nam et al, 1996). In the current work we reported for the first time that apoptin inhibits STAT5 activation in Bcr-Abl expressing cell lines (**Figure 20 - 21**). This observation strongly supports apoptin's role as a proliferation inhibitor of CML cells. STAT5 also regulates the Bfl-1 family gene A1 that reportedly collaborates with *c-myc* and is required for Bcr-Abl transformation (Sawyers et al, 1992; van Lohuizen et al, 1989). We also noticed that Bcr-Abl induced activation of c-Myc is downregulated in the presence of apoptin (**Figure 26**).

The gene encoding CrkL is located near the *BCR* locus on chromosome 22 (band q11) and the protein itself forms strong complexes with Bcr-Abl (ten Hoeve et al, 1993). These interactions and trans-activation of CrkL and c-Crk II by activated Bcr-Abl kinase and their functional consequences are already well documented (Feller, 2001; Posern et al, 2000; Voss et al, 2000). The resulting abnormally high TK activity of CrkL is maintained by activated Bcr-Abl causing reduced cell adhesion, increased proliferation and resistance against apoptotic cell death. In the current study, we demonstrated for the first

time that apoptin inhibits phosphorylation of CrkL in Bcr-Abl expressing cells (**Figure 22 - 23**). This observation indicates that apoptin can indirectly affect the growth-supportive role of phosphorylated CrkL by inhibiting Bcr-Abl kinase.

Overall, these observations point apoptin as a negative-modulator of Bcr-Abl kinase activity, and indirectly down-regulate the multiple cell proliferation and anti-apoptotic pathways that are fuelled by Bcr-Abl. Surprisingly, we also repeatedly observed the activation of Akt upon apoptin and/or Imatinib treatment in Bcr-Abl expressing cells.

Role of Akt in apoptin mediated apoptosis of Bcr-Abl expressing cells: In this part of the study, it was observed that apoptin induces activation of Akt in CML cells. Akt is a known downstream target for Bcr-Abl kinase and a documented interacting partner of apoptin (Maddika et al, 2007b; Skorski et al, 1995). Although, the role of the PI3-K/Akt pathway in cell survival is well established, there are exceptions, in which PI3-K and Akt are also involved in promotion of cell death (Aki et al, 2003; Lu et al, 2006; Nimbalkar et al, 2003; Shack et al, 2003). Interestingly, in a recent publication it was reported that instead of a proliferation response the direct apoptin-Akt interaction promotes nuclear trafficking of Akt and initiates apoptosis (Maddika et al, 2007c). As previously documented by others, agents like etoposide and IFN-β activate Akt phosphorylation preceding the onset of PARP-1 cleavage and apoptosis (Lei et al, 2005; Tang et al, 2001). Interestingly, imatinib therapy also lead to the activation of (PI3-K/Akt/mTor)-pathway in Bcr-Abl positive leukemia cells *in vitro* and in a chronic phase CML patient *in vivo* although it is well known that the PI3-K/Akt-activation mediates survival during the early phase of imatinib resistance (Burchert et al, 2005). In clinical trials as well, where imatinib resistance occurred through Bcr-Abl-kinase mutations, an autonomous

activation of the downstream targets of Bcr-Abl, Akt and mTor, was found in some patients (Burchert et al, 2005). Similarly, the next generation Bcr-Abl-TK inhibitor Nilotinib also did not suppress Akt activity in CML cells (Konig et al, 2008). Also reported, transient activation of Akt supported cell survival, whereas its sustained activation leads to apoptosis (van Gorp et al, 2006). These observations clearly support the hypothesis of the dual role of Akt in cell survival and apoptosis. Moreover, in a recent report cancer cell specific cytotoxicity was linked to apoptin's interactions with the SH3 domain of p85, the regulatory subunit of PI3-K, through its proline-rich region leading to constitutive activation of PI3-K. PI3-K/Akt pathways have a dual role in both survival and cell death processes depending on the stimulus and apoptin mediates the nuclear transport of Akt (Maddika et al, 2007b; Maddika et al, 2007c). However, a transient, physiological Akt/CDK2 activation is also necessary for cell cycle progression. CDK2 is an S-phase cyclin-dependent kinase and acts as a novel target for Akt during cell cycle and apoptosis. There is growing evidence that certain cyclin/CDK complexes might not only control cell proliferation, but cell death as well (Golsteyn, 2005). Akt that is re-localized to the nucleus phosphorylates CDK2 at Thr-39 residue and causes the temporary cytoplasmic localization of the CDK2-cyclin-A complex. Anti-cancer agents like methotrexate or docetaxel constitutively activate the Akt/CDK2 pathway. This activated Akt/CDK2 pathway and their changed subcellular localization promotes apoptosis (Maddika et al, 2008a; Maddika et al, 2007b). The nuclear Akt reportedly, acts as an apoptosis stimulator rather than as a repressor as it possibly gains access to a new set of substrates in the nuclei of cancer cells. Apoptin is phosphorylated by an unknown kinase at Thr-108 specifically in transformed cells, which facilitates its nuclear

localization and tumor specific activity and probably is a key regulatory mechanism for the apoptin mediated cell death (Rohn et al, 2002). We recently reported, CDK2 regulates apoptin's nuclear retention by direct phosphorylation that is specifically mediated by cyclin A but not cyclin E associated CDK2 (Maddika et al, 2008a).

Apoptin induced apoptosis essentially depends on an abnormal PI3-kinase/Akt activation that results in the activation of the cyclin-dependent kinase CDK2. We have recently identified CDK2 as the principal kinase that phosphorylates apoptin at Thr-108 residue and is crucially required for apoptin-induced cell death (Maddika et al, 2008a; Maddika et al, 2008b). This CDK2 activation occurs up-stream of the mitochondrial death pathway. Akt activates CDK2 by direct phosphorylation as well as by the phosphorylation-induced degradation of its inhibitor $p27^{Kip1}$. $p27^{Kip1}$ is a negative regulator of the cell cycle at G1/S phase and has an Akt consensus motif for phosphorylation. In the presence of apoptin, active Akt may target alternative substrates or pathways that may lead to this aberrant activation of CDK2, disturbance of cell cycle progression and cell death. These observations not only decipher the pathway of apoptin-induced cell death, but also provide novel mechanistic insights for the selective killing of tumor cells (Maddika et al, 2008b).

However, these recent data certainly do not exclude the fact that nuclear Akt may have a pro-survival function under some conditions. For example, it was reported that the tumor suppressor PML prevents cell growth by dephosphorylating and inactivating Akt inside the nucleus (Trotman et al, 2006). Nevertheless, CDK2 activation may be not restricted to apoptin-induced cell death. In this context, elevated activity of CDK2 has been found in

certain forms of apoptosis, while overexpression of CDK2 accelerated thymocyte cell death (Gil-Gomez et al, 1998).

Various regulators of the PI3-K/Akt pathway are involved in tumorigenesis and are highly active in various types of cancers. Hyperactivation of these pathways is associated with a poor clinical prognosis and contributes to drug resistance. Thus, apoptin's targeting of these pathways might be responsible for its unique tumor-specific effects. According to our previous work, apoptin possibly 'hijacks' survival pathways and redirects them from their survival function towards induction of cell death, therefore establishing a novel link between cell survival and cell death that may be important for the development of strategies to selectively kill tumor cells.

In the current study, we also repeatedly detected the presence of Akt in the bcr-Abl/Apoptin pull-down product (**Figure 12**). Besides, both murine and human CML cell lines showed significantly elevated Akt phosphorylation following apoptin or imatinib treatment (**Figure 24 - 25**). Interestingly, apoptin was able to mediate the nuclear transport of Bcr-Abl as it was previously shown for Akt (Maddika et al, 2007b). The nuclear transport of the cytoplasmic pool of Bcr-Abl that mostly stays attached to other cytoskeleton proteins indicates that possibly a similar mechanism of apoptin induced cancer cell death is taking place in Bcr-Abl expressing cells.

Different components of the Bcr-Abl downstream pathways are involved in the pathogenesis of CML and are highly active compared to normal cells. Hyper-activation of the STATs, Ras-MAPK or CrkL-Integrin pathways lead to the development of characteristic CML pathology. Apoptin affects many of these signaling events, thus it is well suited for targeting the cellular signaling environment of Bcr-Abl expressing cancer

cells. Overall, these observations imply apoptin's role as a potential inhibitor for Bcr-Abl induced multiple co-activated cell proliferation and anti-apoptotic pathways. Thus apoptin is well suited to serve as a model/lead molecule for the development of smaller peptides or peptidomimetics that would target multiple cell proliferation and anti-apoptotic pathways. This may be of advantage also for CML-treatment as advanced, highly mutated, CML-cells may no longer solely rely on Bcr-Abl as the driver of cell proliferation. Since apoptin attacks multiple targets related to cell proliferation, either alone, or in combination with imatinib/Gleevec, it offers higher curative potential than imatinib/Gleevec alone.

7.3 Three-dimensional modeling of apoptin and molecular interaction studies

The major purpose of computational construction of apoptin structure was to translate the knowledge of apoptin interactions with Bcr-Abl into designing of new peptide based targeted CML therapy. It could be concluded from both my experiments and the computer modeling that a proline rich sequence of apoptin (aa: 81-86, PKPPSK) has the highest possibility to interact with the SH3 domain of Bcr-Abl, and this interaction is primarily responsible for apoptin's cytotoxic functions on CML cells. Further investigations of the precise nature of these protein-protein interactions at the molecular level could provide enough information for the development of small peptide based Bcr-Abl inhibitors that structurally resemble those specific interacting areas of apoptin. Unfortunately, the precise 3D atomic structure of apoptin revealing its precise structure-function relationship has not yet been resolved by crystalography studies.

The methods of determining 3D molecular structure like X-ray or NMR spectroscopy require the molecule to be in crystal form suitable for X-ray crystallographic study or to

be of less than 30 kDa for accurate NMR study. The possible reasons behind the failure to reveal a crystallographic 3D molecular structure of apoptin are due to the difficulties in obtaining large quantities of chemically competent apoptin in its native form, inability to crystallize recombinant apoptin and apoptin's nature to stay in solution as active globular multimers – a property of the apoptin molecule that is mediated by the N-terminal leucine rich multimerization domain (Leliveld et al, 2003a; Leliveld et al, 2003b). In this situation, a simulated three-dimensional model of apoptin generated by computer was an effective alternative technique and in reality it provided the molecular details of apoptin and additional proofs for Abl-SH3 domain interactions. The most important requirement for a successful protein modeling is to find the provisional 3D structures with similarities to the target sequences. The target sequence we obtained was therefore compared to the database of sequences derived from PDB (http://www.rcsb.org/pdb/home/home.do). Several suitable template sequences were found for one target sequence and the final 3D model was tested for accuracy by examining the 3D model by *Ramachandran plot* that determined the contacts between atoms for each of the possible conformations. The *Ramachandran plot* provided an estimate of the number of α-helices with right and left hand turns and the number of β-pleated sheets in the apoptin molecule. Each chemical bond angle in a peptide sequence is considered as individual spherical unit that corresponds to the sterically disallowed conformations of the polypeptide backbone and this essential feature in the 3D structure of apoptin was determined by *Ramachandran plot*. As shown in **Figure 34** (*Ramachandran plot)*, about 80% of the amino acid residues were in the allowed regions.

The protein-protein interactions required prior determination of accessible surface in a 3D model. In the partial 3D model of apoptin (**Figure 33**) all atoms of the molecule were digitally locked to restrict the in-between movements and subsequently molecular dynamic simulations were performed to identify the accessible surfaces. On Abl the large patches of hydrophobic areas indicated that the Abl-SH3 domain interacts with the proline rich motif of other proteins like apoptin. Apoptin has a known consensus site for the binding of a proline dependent serine/threonine kinase CDK2 at Thr-108 residue. Detection of large patchy hydrophobic areas on the surface of this protein suggests that the Abl-SH3 domain is involved in interactions with other proteins as well. Eventually, using such computational algorhythms, we verified the molecular interactions of Bcr-Abl SH3 domains with apoptin. The molecular docking of these two individual proteins was determined by computer algorhythm, *AutoDock*. Once again, the predicted strong binding of apoptin and the SH3 domain of Bcr-Abl were confirmed by this method as it was previously proved in wet-lab experiment.

Overall, the computational modeling strategies and molecular docking studies provided us with important insight towards designing new peptide sequences of desired 3D configurations and functions. Here, we used apoptin as a model molecule to find the best-fit Bcr-Abl TK inhibitor and in this respect the amino-acid sequences covering its interaction and phosphorylation motifs are some of the key regions of interest.

7.4 Apoptin derived peptides as potential inhibitor of Bcr-Abl kinase

The array-based SH3 domain interaction study once again confirmed that the proline rich peptide of apoptin interacts with the SH3 domain of Bcr-Abl. However, in the pilot experiment we found partial loss of SH3 binding specificity by the short apoptin derived synthetic peptide. Presumably, other areas of apoptin molecule are also important for the interaction specificity of apoptin with proteins like Bcr-Abl. This observation indicates that shortening and modification of the peptide may find additional substrate proteins that act as the key regulator of activated signal transduction cascades in the cancer cell. Thus, there is potential for more effective targated anti-CML drug development that will be based on these observations. However, only further extensive investigation can tell us more. Here, the results from a set of cell survival studies showed the anti-proliferative effect of these apoptin derived peptides that are mediated through their SH3 domains interacting with the proline rich regions. During this early phase of toxicity study, these differential cell-killing properties of apoptin-derived peptides could be considered as an important step towards the development of peptide based Bcr-Abl TK inhibitor in the future. Finally, structural and functional studies of apoptin based peptides and Bcr-Abl interaction fresh opens possibilities for finding peptides or small molecular drugs that will be equally beneficial like apoptin and will overcome drug resistance against currently available therapy.

8. Future Directions

Novel apoptin derivatives as Bcr-Abl tyrosine kinase inhibitors

Targeted therapy of human malignancies is still in its early stage of development. The unquestionable proof of principle with regards to targeted therapy in cancer came with

the discovery and remarkably successful therapeutic use of imatinib in CML. CML is frequently associated with the unique and activated tyrosine kinase Bcr-Abl that remains the major target for novel molecular therapy. It is also likely that other cancers being treated with kinase inhibitor therapy will benefit from a similar treatment strategy. This project provides new knowledge about the apoptin mediated cell death mechanism(s) in Bcr-Abl expressing leukemias, particularly CML and creates a solid foundation for the development of apoptin based therapies that could confer anti-cancer activity against CML and other malignancies.

Apoptin's innate tumor specificity makes it a highly interesting anti-cancer agent that can kill tumor cells specifically and efficiently. Furthermore, it also identifies new molecular targets in cancer cells for the development of anti-cancer therapies that are more effective while displaying fewer side effects. Different components of the Bcr-Abl downstream pathways are involved in the pathogenesis of CML and they are highly active as compared to normal cells. Hyper-activation of STATs, Ras-MAPK or CrkL-integrin pathways lead to the formation of final disease morphology in CML. Apoptin's target towards many of these signaling proteins indicates that the presence of apoptin in the cellular environment of Bcr-Abl producing cancer cells selectively abrogates these pathways and can redirect their actions from survival to cell death/apoptosis pathways.

Previous studies with smaller but high affinity peptides blocking the N-terminal SH3 domain of Grb2 peptide indicated that peptide based inhibitor of Bcr-Abl kinase can be a valuable anti-CML tool if combined with conventional cytotoxic therapy (Kardinal et al, 2001). Further experimentations on specific TAT-conjugated apoptin derived peptide sequences as anti-CML agents are of high interest. Apoptin may also serve as a model;

specific smaller peptides can be designed as experimental anti-CML/anti-cancer agents that can target multiple cell proliferation and anti-apoptotic pathways.

It would be interesting to test the effects of apoptin on the imatinib resistant CML cells expressing commonly found mutants of Bcr-Abl (T315I). Based on our experimental results (apoptin was efficiently killing both Bcr-Apl positive and negative transformed cells), it is likely that apoptin and its derivatives would be useful in the treatment of imatinib resistant CML secondary to the amplification and overexpression of the Bcr-Abl gene, in addition to the kinase mutations other than the T315I.

The data presented here are supportive of the hypothesis that specific molecular interactions of apoptin with the SH3 domain of Bcr-Abl can significantly decrease the proliferation and induce apoptosis of CML cells *in vitro* (**Figure 31**). At this point we can speculate that as multi-directional signal transduction inhibitors, apoptin or its specific synthetic derivatives may be effective in overcoming primary resistance of the CML cells to imatinib and/or other conventional cytotoxic agents. However, to prove apoptin-induced apoptosis in CML as a relatively safe and efficient mode of therapy, strategic animal experiments for studing its *in vivo* toxicity and possible immunological consequences are essential future steps. Adequate scientific attention will be the key to develop a potent apoptin derived drug in the future that will work as efficiently against a molecular target like Bcr-Abl.

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