LEF-1 is a potential therapeutic target in the treatment of Chronic lymphocytic leukemia

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To my Parents,

Brother, Sister

& Gowri

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1. Summary

B-Chronic lymphocytic leukemia (B-CLL) is characterized by accumulation of apoptotic resistant $CD5^+$ B lymphocytes. There is an increased secretion of Wnt ligands indicating an autocrine loop leading to the extended survival of B-CLL cells. Lymphoid enhancer factor 1 (LEF-1) is a potent transcription factor regulating the expression of several Wnt induced target genes. A comprehensive gene expression profiling from two independent studies revealed that LEF-1 mRNA was ~3000 fold overexpressed in B-CLL when compared to its healthy counterpart.

The objective of this present study is to demonstrate the therapeutic benefit of inhibiting LEF-1 expression in B-CLL cells using novel small molecule inhibitors CGP049090 and PKF115-584 *in vivo* and *in vitro*. In order to explore the anti-leukemic potential of CGP049090 and PKF115-584 we tested its effects on freshly isolated B-CLL cells, prolymphocytic cell line (JVM-3 & MEC-1) and in a subcutaneous mouse xenograft model.

The present study shows that, in freshly isolated B-CLL cells there was high protein expression and nuclear localization of LEF-1 and β-catenin indicating active LEF-1 mediated transcription whereas LEF-1 remained undetectable in healthy B cells. Preliminary experiments of LEF-1 inhibition using siRNAs resulted in increased apoptosis indicating LEF-1 plays an important role in the survival of B-CLL cells. This observation was extended using CGP049090 and PKF-115584 as they induce dose dependent cytotoxicity in B-CLL, whereas the healthy B cells are not significantly affected. The half maximal inhibitory concentration (IC50) was less than 1 μM in primary B-CLL cells and cell lines whereas it was more than 5 μM in healthy B cells. CGP049090 and PKF-115584 induced apoptotic cell death in primary B-CLL cells and cell lines by cleavage of caspases 8, 9, 3 and 7 and subsequent cleavage of Poly (adenosine diphospate-ribose) polymerase (PARP). Both inhibitors also altered the expression of several anti-apoptotic proteins like X-linked Inhibitor of Apoptosis

Protein (XIAP), Mantle cell lymphoma-1 (Mcl-1) and B cell lymphoma-2 (Bcl-2). Co-Immunoprecipitation experiments revealed that both the inhibitors effectively disrupt the β-catenin/LEF-1 interaction, resulting in the down regulation of LEF-1 target genes such c-myc, cyclin D1 and LEF-1. Furthermore, when the inhibitors were tested in an *in vivo* JVM-3 subcutaneous xenograft nude mouse model, more than 70% inhibition of tumor growth and an increase in the median survival of the treated group without leading to systemic toxicity was observed. Immunohistochemistry analysis of the tumor sections revealed LEF-1 down regulation and subsequent inhibition of proliferation by down regulation of Proliferating Cell Nuclear Antigen (PCNA) and increase in apoptosis (cleaved PARP).

In summary, the data showed that LEF-1 is a potential therapeutic target in the treatment of B-CLL. Both CGP049090 and PKF115-584 showed potent inhibitory effects on the survival of CLL cells *in vitro* and *in vivo* without affecting the healthy cells. Both CGP049090 and PKF115-584 are hence, potential anti-cancer agents in B-CLL and other neoplastic malignancies with aberrant LEF-1/ T cell factor (TCF) transcriptional activity. Further investigations are warranted to determine the feasibility of these small molecules for therapeutic approach in humans.

2. Zusammenfassung

Die chronische lymphatische B-Zell Leukämie (B-CLL) ist durch eine Akkumulation von apoptose-resistenten CD5-positive B-Lymphozyten charakerisiert. In letzter Zeit wurden verschiedene Faktoren beschrieben, welche zu einer aberranten Aktivierung der Wnt-Signalkaskade in B-CLL Zellen beitragen. Eine vermehrte Sekretion von Wnt-Liganden weist auf einen autokrinen Rückkopplungsmechanismus hin, welcher eine Rolle im gesteigerten Überleben der B-CLL Zellen spielt.

LEF-1 ist ein potenter Transkriptionsfaktor, welcher die Expression verschiedener Wnt-induzierter Zielgene reguliert. LEF-1 wurde in zwei unabhängigen Genexpressionsanalysestudien als Gen mit etwa 3000-facher Überexpression in der B-CLL, relativ zum Expressionslevel in gesunden B-Zellen, beschrieben. Der Transkriptionsfaktor LEF-1 ist damit exklusiv auf B-CLL Zellen exprimiert.

Das Ziel der vorliegenden Studie ist es, den therapeutischen Nutzen einer gezielten Inhibierung von LEF-1 in B-CLL Zellen, durch die Verwendung zweier sogenannter „small molecule“ Inihibitoren CGP049090 and PKF115-584, sowohl in vivo als auch *in vitro* zu zeigen.

Um den anti-leukämischen Effekt dieser Substanzen zu zeigen, wurden deren Effekte auf frisch isolierte B-CLL Zellen und prolymphozytischen Zelllinien ((JVM-3 & MEC-1) untersucht. Zudem wurden beide Substanzen auf Ihre Wirksamkeit in einem JVM3-Xenograft Mausmodel getestet.

In frischen B-CLL Zellen konnten wir große Proteinmengen von LEF-1 detektieren, welche größtenteils im Zellkern zu finden waren. Auch β-Catenin war vorrangig im Zellkern vorhanden, was darauf schließen lässt, dass LEF-1 in B-CLL Zellen transkriptionell aktiv ist. LEF-1-Protein war in gesunden B-Zellen nicht detektierbar. Eine Runterregulierung von LEF1 mittels siRNA hat zu einem vermehrten Zellsterben durch Apoptoseinduktion geführt, welches die wichtige Funktion von LEF-1 für das Überleben der B-CLL Zelle zeigt. Diese

Beobachtung wurde dadurch erweitert, dass auch CGO049090 und PKF-115584 sowohl zeit- als auch konzentrationsabhängig zytotoxisch auf B-CLL Zellen wirken, während gesunde B-Zellen in signifikant geringerem Maße betroffen sind. Die IC50 in primären B-CLL Zellen war <1 µM und >5 µM in gesunden B-Zellen. CGO049090 und PKF-115584 führten zu apoptotischem Zelltod durch eine Spaltung der Caspasen 8, 9, 3 und 7 und eine darauffolgende Spaltung von poly (adenosine diphospate-ribose) polymerase (PARP). Beide Inhibitoren führten zudem zu einer veränderten Expression verschiedener antiapoptotischer Proteine wie X-linked Inhibitor of Apoptosis Protein (XIAP), Mantle cell lymphoma-1 (Mcl-1) und B cell lymphoma-2 (Bcl-2). Co-Immunoprezipitationsexperimente zeigten, dass beide Inhibitoren effektiv den β-Catenin/LEF-1 Komplex trennen, was zur Herunterregulation der LEF-1 Zielgene c-myc, cyclin D1 und LEF-1 führt. Darüber hinaus wurden beide Inhibitoren in einem subkutanen JVM-3-Xenograft Nacktmausmodel getestet und erzielten eine Tumorinhibitionsrate von >70%. Zudem erhöhte sich die mittlere Überlebensrate der Mäuse durch die Behandlung ohne erkennbare systemisch toxische Effekte. Eine Gewebeuntersuchung der Tumore ergab eine Herunterregulation von LEF-1 und eine darausfolgende Herunterregulation des Proliferating Cell Nuclear Antigens (PCNA), welches auf eine erniedrigte Proliferationsrate hinweist. Zudem wurde eine verstärkte Aktivität von PARP detektiert, wodurch auf eine erhöhte Apoptoseinduktion im Tumorgewebe behandelter Mäuse geschlossen werden kann.

Zusammenfassend lässt sich sagen, dass LEF-1 einen potentiellen therapeutischen Ansatzpunkt in der B-CLL darstellt. Die beiden Substanzen CGP049090 and PKF115-584 weisen eine hohe Wirksamkeit bei der Zelltodinduktion B-CLL Zellen gegenüber auf, während sie gesunde B-Zellen kaum beeinträchtigen. CGP049090 and PKF115-584 eignen sich deshalb als potentielle Substanzen in der B-CLL Therapie und auch in anderen neoplastischen Erkrankungen, die eine aberrierende transkriptionelle Aktivität von LEF-

1/TCF aufweisen. Weitere Untersuchungen sind wünschenswert um die Anwendbarkeit dieser zwei Substanzen für therapeutische Zwecke im Menschen genauer zu bestimmen.

3. Introduction

During normal lymphocyte differentiation B cells jeopardize their genomic integrity through the formation and revision of their antigen receptors. A second potentially dangerous event is the response to antigens, which under normal circumstances, is a tight homeostatic regulation of clonal expansion of B cells. A compromise in either of the two events would result in oncogenic genomic hits that block differentiation, prevent apoptosis and/ or promote proliferation leading to several types of lymphoma and leukemia. B-Chronic lymphocytic leukemia (B-CLL) is one such disease which results due to a prolonged and unregulated antigenic stimulation contributing to clonal expansion of leukemic B cells.

3.1. Hematopoiesis

To comprehend the origin of B-CLL we have to understand the mechanism of generation of different cell types of the lymphoid and myeloid systems (Figure 1). All the mature blood cells in the body are generated from a relatively small number of hematopoietic stem cells (HSCs) and progenitors (Weissman 2000). In the mouse, a single HSC can reconstitute the entire hematopoietic system for the natural lifespan of the animal (Osawa 1996). HSCs generate the multiple hematopoietic lineages through a series of intermediate progenitors. Those are the common lymphoid progenitors (CLPs) that give rise to natural killer cells (NK), T cells, and B cells, and the common myeloid progenitors (CMPs), which can generate monocytes, granulocytes, megakaryocytes, and erythrocytes (Kondo 1997; Akashi 2000). Out of the CMPs develop more specialized progenitors, that are further restricted to a number and type of cell lineages that they can generate. These are the granulocyte/monocyte progenitors (GMP), which give raise to the granulocytes and monocytes, and the megacarythrocyte/erythrocyte progenitors (MEP), which can develop to megakaryocytes and erythrocytes (Akashi 2000). The terminally differentiated cells produced cannot divide any longer and undergo apoptosis after days to decades depending on their cell type.

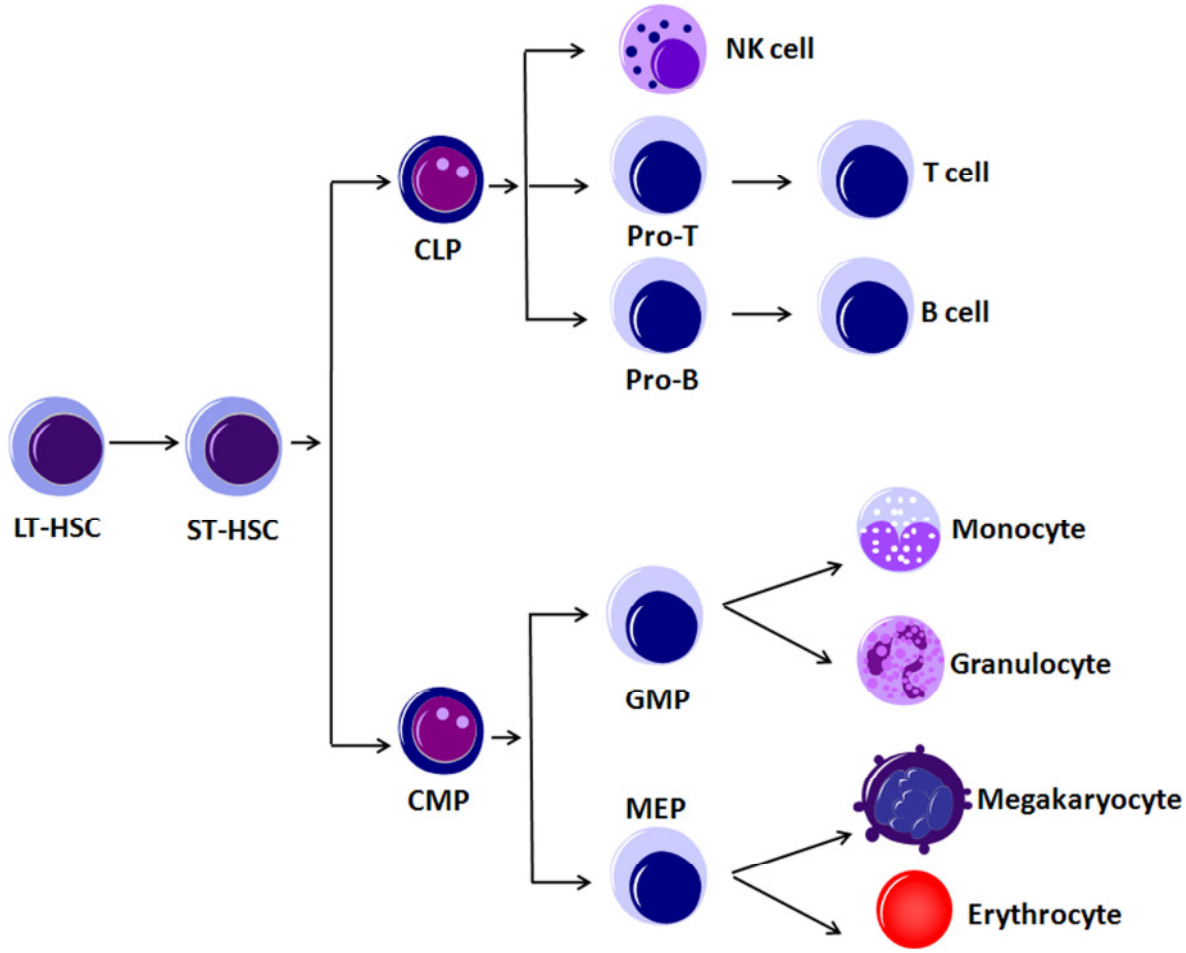


Figure 1: Hematopoiesis

Long term hematopoietic stem cells (LT-HSC) give rise to short term (ST) HSCs. Due to different stimuli they either become common lymphoid progenitors (CLP) or common myeloid progenitors (CMP). Downstream of CLPs the cells either develop to natural killer cells (NK), to B or T cells. The CMPs give rise to more specialized progenitors, granulocyte/monocyte progenitors (GMP) and megakaryocyte/erythrocyte progenitors (MEP), which finally differentiate to granulocytes, monocytes, megakaryocytes and erythrocytes.

During the process of B cell differentiation the B cells generate the B cell receptors (BCRs) by V(D)J recombination involving double strand DNA breaks initiated by recombination activated genes (RAG1 and RAG2) that are resolved by the non-homologus end joining repair apparatus (Fugmann et al. 2000). In this process, the cells are often susceptible to chromosomal translocations replacing the usual regulatory sequences of a gene with heterologous regulatory elements which lead to inappropriate gene expression at the breakpoints, leading to lymphomas (Pelicci et al. 1986).

On the other hand when a differentiated but naïve B cell encounters an antigen, the naïve B cell gets activated and interacts with follicular dendritic cells, T cells and the antigen within the microenvironment of the Germinal Centre (GC). Here the B cell undergoes Class Switch Recombination (CSR) via DNA breaks and extensive remodeling of the DNA and somatic hypermutation (SHM) using point mutations of the immunoglobulin genes. This again makes the B cell genome prone to genetic alterations leading to gross chromosomal mutations (Boehm et al. 1989; Honjo et al. 2002).

Interestingly, B-CLL does not represent any of these typical gross chromosomal translocations generated by the above mechanisms (Montserrat & Rozman 1995), but represent in two distinct subtypes i.e. B-CLL cells which have undergone SHM (IgV_H mutated) in the germinal centre and those cells which did not undergo SHM (IgV_H unmutated) (Shaffer *et al.* 2002). Hence the origin of this disease is still a mystery. The current hypothesis favors the role of an antigen in the pathogenesis of B-CLL.

3.2. The Role of Antigens in B-CLL

The role of antigens have been implicated in some lymphomas such as Marginal Zone Lymphoma (MALT) where the 70% of the patients present an infection with helicobacter pylori leading to gastric ulcers (Wotherspoon et al. 1993; Wotherspoon et al. 1994). Helicobacter pylori specific T cells stimulate the proliferation of MALT lymphoma cells in culture and these patients can be cured of their MALT simply by antibiotics (Hussell et al. 1993).

Several gene expression studies have been carried out in determining the difference between the IgVH unmutated and IgVH mutated forms of B-CLL (Rosenwald *et al.* 2001; Klein *et al.* 2001). These evidences indicate a potential role of an antigen in the clonal expansion of B-CLL cells. CLL B cells use a biased VH repertoire and have non-random combinations of V, D and J segments that are not characteristic of normal blood B cells. Furthermore, certain VH genes are used differentially by immunoglobulin-unmutated and -

mutated forms of CLL. For example, the VH1-69 gene is associated almost exclusively with immunoglobulin-unmutated CLL, whereas other VH genes, such as VH4-34, VH1-07 and VH3-21, are over-represented in immunoglobulin-mutated CLL, indicating role of antigen in the expansion of these cells (Damle *et al.* 1999; Fais *et al.* 1998; Tobin *et al.* 2002;). However the nature of this antigen is still unknown, it is predicted that it is presumably auto antigens such as single- and double-stranded DNA, and IgG. (Sthoeger et al. 1989; Borche et al. 1990).

3.3. Biology of chronic lymphocytic leukemia

The ability of one cell to influence the behavior of another cell is achieved through cell communication, termed cell signaling. Over the time several different signaling pathways have been developed, as for example the Notch-, the Phosphatidylinositol-3 kinase (PI3K), the BMP-, and the Wnt signaling pathway which are crucial in early development and maintenance of the cells. At the end of each intracellular signaling pathway are target proteins, which when altered changes the behavior of the cell. Moreover, a crosstalk between the different pathways occurs, which leads to a big network of signals and a tight regulation within a cell. Disturbance of the balanced systems often leads to diseases, as for example cancer. B-CLL is such a condition wherein there is a deregulation of several signaling pathways, which leads to extended survival. We will discuss some of the important signaling defects in the pathogenesis of CLL but will mainly focus on the role of Wnt signaling pathway in B cell development and pathogenesis of CLL. Figure 2 shows an overview of the pathogenesis of B-CLL during early stages of differentiation and junctures where additional oncogenic hits/antigenic stimulation might occur resulting in its activation of variety of pathways involved in prolonged survival (Shaffer *et al.* 2002).

Signaling defects in CLL is considered to be one of the factors prolonging the survival of the B cells than the normal lymphocytes (Hamblin & Oscier 1997). The BCR signaling is important in determining the B-cell fate, and the level of BCR engagement is modulated by the antigenic valency, epitope density and epitope organization. Upon BCR engagement,

receptor aggregation induces phosphorylation of the immunoreceptor tyrosine-based

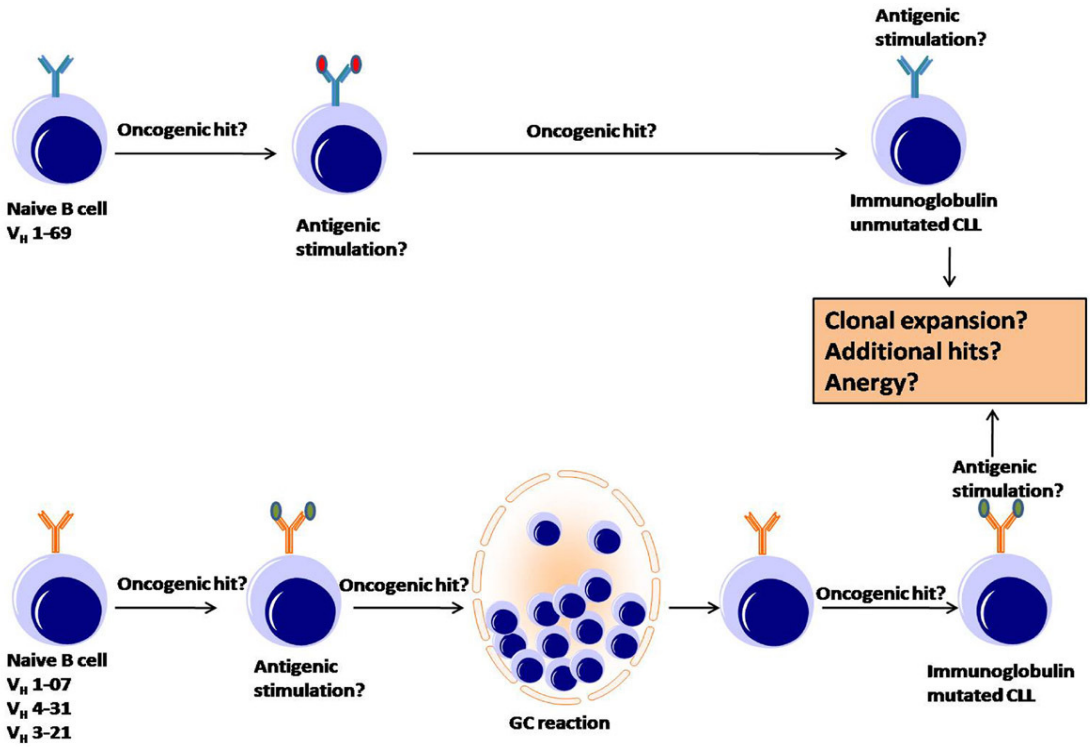


Figure 2: An overview of the pathogenesis of B-CLL *(Adapted from A. L. Shaffer 2002) The two subtypes of chronic lymphocytic leukemia (CLL) — immunoglobulin-mutated and immunoglobulin-unmutated CLL — are distinguished by the presence or absence of immunoglobulin variable (V)-region mutations, by differences in gene expression and by their clinical courses. This model emphasizes the potential role of antigenic stimulation in the progression of this disease. Immunoglobulin-unmutated and -mutated forms have a different repertoire of heavy-chain V-region (VH) gene rearrangements, which indicates that the type of CLL that results is dictated by the specificity of the B-cell receptor (BCR). Many studies have implicated an antigen or autoantigen indirectly in the pathogenesis of CLL. Antigenic stimulation might occur before and/or after the B cell acquires a genetic change (oncogenic hit) and becomes a CLL cell. It is also possible that early stages of CLL occur without oncogenic hits to the B cell. Immunoglobulin-unmutated CLL most probably originates from a pre-germinal centre (pre-GC) B cell. Immunoglobulin-mutated CLL might originate from a post-GC B cell. Alternatively, immunoglobulin-mutated CLL might originate from a pre-GC B cell that is nevertheless driven by antigen through a GC reaction. The clinical manifestations of CLL might be related to whether antigen drives continued clonal expansion or induces an anergic state. Disease progression might be influenced by the accumulation of additional oncogenic hits.*

activation motifs (ITAMs) by Src-family tyrosine kinases (Pierce 2002). This leads to recruitment of other kinases triggering activation of intracellular signaling cascades. Low expression of BCR is the hallmark of CLL (Payelle-Brogard et al. 2002; Vuillier et al. 2005) and its stimulation is considered to be important for survival and proliferation of CLL (Stevenson & Caligaris-Cappio 2004).

PI3K and mitogen activated protein kinases (MAPK) are activated through membrane receptor tyrosine kinases and are involved in several signal transduction pathways in B cells such as CD40 signaling, BCR signaling and signaling of variety of cytokines (Ringshausen et al. 2002). Studies have shown the constitutive activation of these pathways in CLL cells and their requirement in the maintenance of CLL viability (Ringshausen et al. 2004; Longo et al. 2007; Plate 2004).

Failed programmed cell death or apoptosis is a characteristic feature of CLL (Reed 1998). The Bcl-2 family members (bcl-2 bax, bclxL) are over-expressed in CLL (Hanada et al. 1993). The imbalance in the ratio of major pro- and anti- apoptotic family proteins is associated with the treatment response of CLL (Kitada et al. 1998; Pepper et al. 2001). Mcl-1, another anti-apoptotic protein is also overexpressed in CLL cells and contributes to failure of cytotoxic therapy (Kitada *et al.* 1998; Saxena *et al.* 2004). There is also evidence that indicate Mcl-1, Bcl-xL and XIAP are regulated by Akt and Erk pathway but only Mcl-1 is essential for CLL survival (Longo et al. 2008).

Most recently great emphasis is being placed on the role of aberrant Wnt signaling in malignant diseases and the different mechanisms, major players of Wnt signaling and its role in B cell development will now be elaborated further.

3.3.1. Overview of Wnt signaling mechanism

The Wnt signaling consists of three different pathways. The classical Wnt/β-catenin pathway, termed canonical Wnt pathway, the frizzled regulated planar cell polarity pathway

(PCP), and the Wnt/Ca2+ pathway (Kuhl et al. 2000; Wang & Malbon 2003). The PCP pathway involves the small GTPases - rho and cdc42 as well as the Jun N-terminal kinase (JNK) (Weber et al. 2000) and regulates Drosophila development independently of β-catenin (Tree *et al.* 2002). The mechanism is not completely understood, but it seems that it is not a linear signaling pathway from the receptor frizzled (Fzd) through a downstream cytoplasmic protein Dishevelled (Dsh), to tissue specific proteins, but that the signaling involves asymmetric distribution of Fzd and Dsh and this pathway is functioning through a feedback loop (Tree *et al.* 2002).

In the Wnt/Ca2+ pathway, Fzd appears to act through heterotrimeric guanine nucleotide-binding proteins (G proteins) (Slusarski et al. 1997) and seems to activate phospholipase C (PLC) and phosphodiesterase (PDE) (Ahumada et al. 2002), which lead to increased concentrations of free intracellular calcium and to decreased intracellular concentrations of cyclic guanosine monophosphate (cGMP).

The canonical Wnt cascade plays a critical role in many developmental processes. It has been implicated in the development of B and T cells (Okamura *et al.* 1998; Reya *et al.* 2000) and in the self-renewal of hematopoietic stem cells (HSC) (Reya et al. 2003). The transcription factors LEF/TCF mediate a nuclear response to Wnt signals by interacting with β-catenin. Following a Wnt signal, β-catenin is stabilized and transported to the nucleus, and is binding to the LEF/TCF proteins to turn on target genes.

3.3.2. Molecular Mechanism of Canonical Wnt signaling

A schematic overview of the canonical Wnt signaling pathway in the presence and absence of stimulation by Wnt ligand is depicted in Figure 3. In unstimulated cells, β-catenin is present in the cytoplasm together with the tumor suppressor adenomatous polyposis coli (APC), the constitutively active Glycogen synthase kinase 3β (GSK-3β), Casein kinase α-1 (CSKA-1) and Axin (Kimelman & Xu 2006). In this complex, any free β-catenin is captured

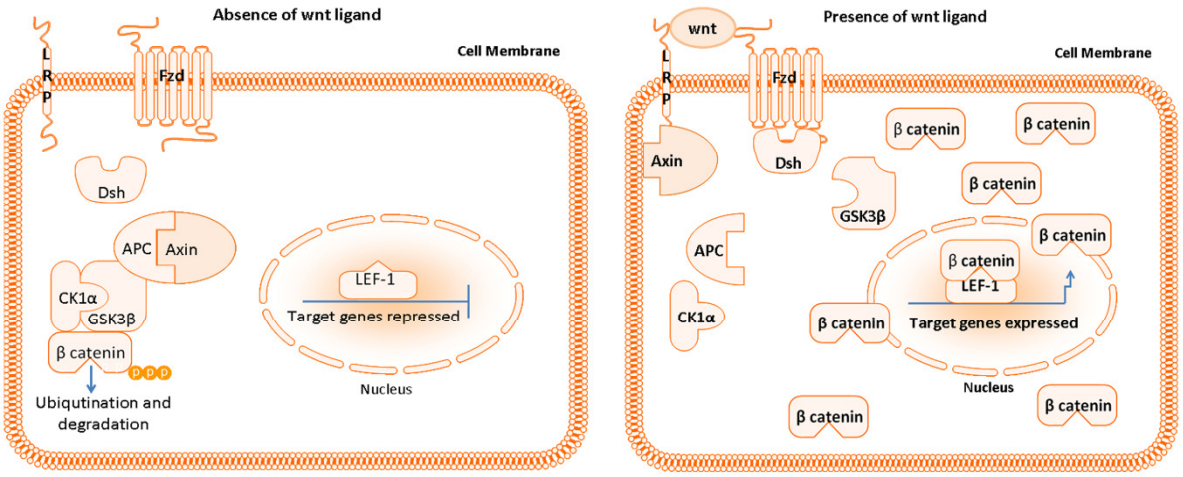


Figure 3: Schematic overview of the canonical Wnt signaling pathway. In the absence of wnt ligand β-catenin is phosphorylated in the destruction complex and tagged for ubiquitination. But in the presence of wnt ligand the destruction complex of β-catenin is inactivated and hence β-catenin is translocated in to the nucleus where it binds with LEF-1 and plays a role of transcriptional co-activator expressing β-catenin/LEF-1 target genes.

and subjected to phosphorylation by GSK-3β at four N-terminal serine and threonine residues (Ikeda et al. 1998). The phosphorylation of β-catenin is recognized by different proteins like (Slimb/TrcP) and get conveyed to ubiquitin conjugating enzymes, which mark β-catenin for degradation (Jiang & Struhl 1998; Marikawa & Elinson 1998). The β-catenin is then rapidly degraded via the ubiquitin-proteasome pathway (Aberle et al. 1997).

The proteins of the Wnt family stimulate the cells via binding of the Wnt ligands to the Fzd family of serpentine receptors (Bhanot et al. 1996). Wnt proteins comprise a large family of 19 identified family members till date, that have been found in round worms, insects, and vertebrates (Sidow 1992). Wnt proteins are secreted glycoproteins that have been shown to be associated with the cell surface receptors. (Mcmahon & Bradley 1990; Papkoff & Schryver 1990). They are involved in a number of developmental and physiologic processes. The Low-density lipoprotein receptor-related proteins (LRP) can bind together with Fzd to the Wnt proteins, thus activating the Wnt cascade (Pinson et al. 2000; Tamai et al. 2000). As a

consequence of this Wnt signal, GSK-3β is inhibited by the cytoplasmic protein Dishevelled (Dsh) (Noordermeer et al. 1994; Kishida et al. 1999; Smalley et al. 1999; Itoh et al. 2000), preventing the phosphorylation of β-catenin and its degradation. The cytoplasmic pool of free β-catenin, thus stabilized, translocates into the nucleus where it can interact with the nuclear mediators of Wnt signaling, the LEF/TCF proteins. This interaction leads to activation of the Wnt target genes by interaction with the mediators (Hsu et al. 1998). In the absence of a Wnt signal, LEF/TCF proteins cannot activate target genes.

3.3.3. The LEF/TCF protein family

The first members of the LEF/TCF family to be identified were T cell factor 1 (TCF-1) (Oosterwegel et al. 1991; Vandewetering et al. 1991) and Lymphoid enhancer factor 1 (LEF-1) (Travis *et al.* 1991; Carlsson *et al.* 1993). Schematic representations of the structure of LEF-1 isoforms have been illustrated in figure 4. Proteins of the LEF/TCF family share an 80-amino-acid high mobility group (HMG) box. It was shown that the HMG box can bind to DNA as a monomer in a sequence specific manner (Travis *et al.* 1991; Giese *et al.* 1991). Other features of LEF-1 are the β-catenin binding domain (βBD), through which the interaction with β-catenin is achieved, and the context dependent activation domain (CAD) that can interact for example with the Ally of AML-1 and LEF-1 (ALY), an ubiquitously expressed nuclear protein that was shown to be necessary for the T cell receptor α (TCRα) enhancer function (Bruhn et al. 1997) The LEF/TCF family members are expressed in a great variety of tissues such as immature T and B cells of adult mice and in the neural crest, mesencephalon, tooth germs, whisker follicles, and other sites during embryogenesis. It was shown that LEF-1 has an architectural function and can interact with different proteins that result in either activation or repression of target genes. For the activating effect, the LEF/TCF

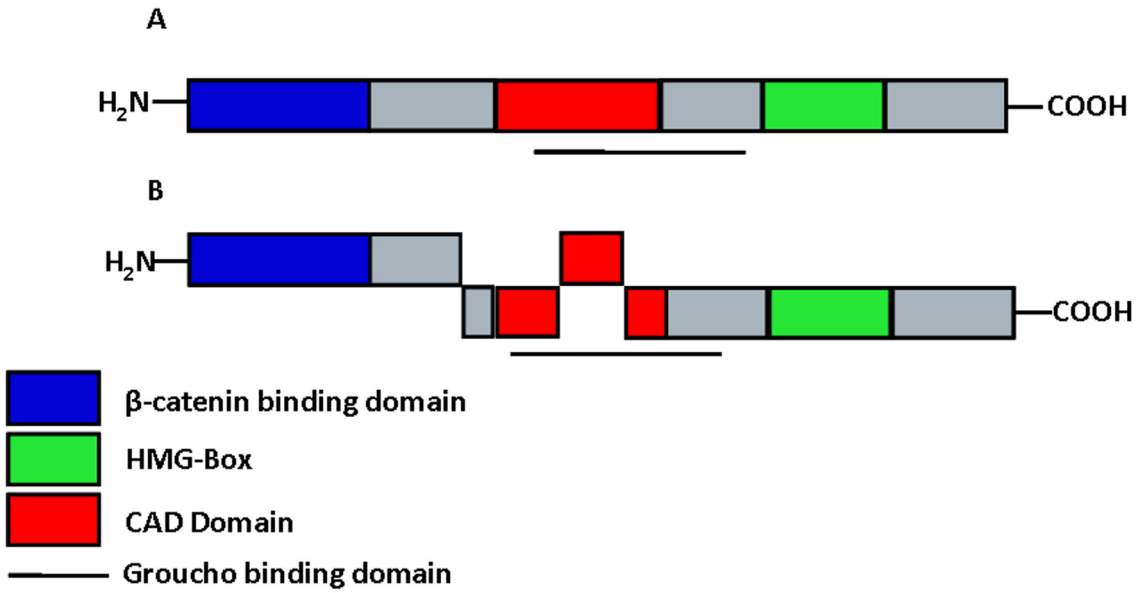


Figure 4: LEF-1 Isoforms (A) Schematic representation of LEF-1 splice variants and their most conserved domains. (B) Short forms of Lef-1 lack the N-terminal domain, which interacts with β-catenin. The CAD domain in Lef-1 is required for context-dependent activation of the TCR enhancer. The HMG box mediates sequence-specific DNA binding.

family members mostly interact with β-catenin to turn on Wnt target genes what makes them a member of the Wnt signaling pathway for this regulation. In a distinct number of cases, LEF-1 can also positively regulate target genes without the help of β-catenin, thus acting independently of the Wnt pathway, as shown for example in the regulation of TCRα by LEF-1 (Travis *et al.* 1991).

On the other hand LEF/TCF proteins can also actively repress transcription. This was first observed with experiments in Drosophila and Xenopus, showing that in the absence of a Wnt/Wg signal the repression of Ultrabithorax (Ubx) and Siamois is released by mutating the LEF/TCF consensus sites in their transcriptional control elements (Brannon et al. 1997; Riese et al. 1997; Bienz 1998). There are some co-repressors known to directly interact with LEF/TCF proteins that help to repress target genes. Groucho is once such protein that binds to a part of the CAD domain of LEF-1, thereby allowing its binding to β-catenin at the same time leading to a repressive effect in the context of a Wnt signal. Repression of E-cadherin

through LEF-1 and β-catenin interaction without the help of any co-repressors has also been shown (Jamora et al. 2003). Nevertheless it seems to be more likely, that the main mechanism for repression is mediated without the help of β-catenin. However the reports about the mechanism of repression through LEF-1 are contradictory and no main pathway was discovered yet. Thus, for the repressive effect of LEF/TCF proteins there are still a lot of questions to be answered.

3.3.4. The Role of Wnt signaling in B-cell development

LEF-1 is known to be expressed in transformed pre-B cell lines but rapidly down regulated in mature B cells (Travis *et al.* 1991). The other family members of the LEF/TCF family are not found to be expressed in any stage of the B cell development. Only little is known about the influence of Wnt signaling on B cell development. The first evidence that Wnt might play a critical role came from the finding, that some leukemic B cell lines overexpress a novel Wnt protein, Wnt 16 (McWhirter et al. 1999). The exact expression pattern of the Wnt proteins and LEF-1 in B cells and the role of Wnt signaling and LEF-1 in the development remained unclear. Recently the effects of LEF-1 on B cell development were subject to intensive analysis (Reya *et al.* 2000). First the precise pattern of LEF-1 in developing B cells was studied. It could be shown by lacZ reporter assay that LEF-1 is expressed during early B cell development in the fetal liver and adult bone marrow (Galceran et al. 2000). The upregulation occurs in fraction B pro-B cells and LEF-1 can also be detected in fraction C cells. There is no LEF-1 expression found in IgM-positive B cells from the adult spleen or adult bone marrow. To test for a correlation between the expression pattern and the function, fetal liver of $Lef1^{-/-}$ embryos and perinatal bone marrow was analyzed, as an analysis of older mice is not possible due to the early death of $Lef1^{-/-}$ mice. The number of $B220^{+}$ cells was reduced by more than two fold and was even more obvious after excluding the dying and dead cells. To specify the stage of the cells, $B220^{+}$ positive cells were tested for

other surface markers and it could be shown, that the majority of the $B220^+$ cells were also $CD43^+$, placing them in the pro-B cell compartment. To test if LEF-1 deficiency results in a differentiation defect, they tested bone marrow of mice at postnatal day 13 (P13) for the ratio of IgM^- to IgM^+ B lymphocytes. Although the total number of the cells was reduced as shown before, the ratio remained still the same and there were no defects in rearrangement of the immunoglobulin heavy and light chains occurring. These findings were also confirmed with adoptive transfer experiments where the mutant B cells behaved like wildtype cells in a wildtype environment. On further analysis the reduced number of $B220^+$ cells was found to be due to reduced cell survival. With TUNNEL assay and Annexin V staining it could be shown that indeed the $B220^+$ cells of Lef-$1^{-/-}$ mice die up to a 20-fold higher frequency. As a cause for the reduced survival, Reya and coworkers analyzed the expression level of several genes known to be involved in apoptosis. The levels of Bcl-2, Bcl-x, and p53 remained unchanged in sorted pro-B cells (fraction B) of Lef$1^{-/-}$ mice compared to wildtype, whereas the expression of Fas and c-myc was elevated. A second defect that can contribute to the reduced size of the B cell compartment is the diminished proliferation of the B cells. With a thymidine incorporation assay it could be shown, that in addition to the increased apoptosis, the decreased proliferation of B cells signifies that LEF-1 has an important function for the proliferation of B cells. As LEF-1 and β-catenin together are members of the Wnt signaling pathway, they went on testing the responsiveness of B cells to Wnt stimuli. While Wnt10B, Wnt3A, and Wnt5A were found to be expressed in bone marrow, only Wnt5A was expressed in the stromal cells of the bone marrow, indicating that the other family members are produced by the hematopoietic cells themselves. It could be shown that proliferation of wildtype pro-B cells is increased after LiCl stimulation and that the soluble Wnt3A could stabilize β-catenin in the cells. Furthermore comparing the responsiveness of wildtype and Lef$1^{-/-}$ cells to Wnt3A stimulation revealed the LEF-1 dependence. Only a small proportion of cells deficient for LEF-1 started to proliferate after Wnt3A addition whereas the majority of

the wildtype cells were found to start dividing. These findings taken together strongly indicate an essential role of Wnt signaling and LEF-1 expression for B cell development. Table 1 shows the list of few validated target genes of TCF/LEF-1 based on the consensus binding sequence (5'-CCTTGAA-3') in the promoter region of the gene.

Table 1 List of target genes of Wnt/β-catenin/TCF/LEF-1 signaling

Gene	Organism/system	References
c-myc	Human/colon cancer	(He *et al.* 1998)
n-myc	Mesenchyme limbs	(ten Berge *et al.* 2008)
Cyclin D1	Human/colon cancer	(Shtutman *et al.* 1999; Tetsu & McCormick 1999)
LEF-1	Human/colon cancer	(Hovanes *et al.* 2001; Filali *et al.* 2002)
c-jun	Human/colon cancer	(Mann *et al.* 1999)
Fra-1	Human/colon cancer	(Mann *et al.* 1999)
Axin-2	Human/colon cancer	(Jho *et al.* 2002)
CD44	Human/colon cancer	(Wielenga *et al.* 1999)
Dickkopf-1	Various cells/tumors	(Chamorro *et al.* 2005; Gonzalex-Sancho *et al.* 2005)
MMP-7	Human/colon cancer	(Crawford *et al.* 1999; Gonzalex-Sancho *et al.* 2005)

3.4. Wnt Signaling and Diseases

The Wnt signaling being an important pathway in the development of B cells and in the delicate balance between apoptosis, survival and proliferation of B cells clearly implies that deregulation of this pathway would lead to manifestation of pathological conditions. Besides considerable advances in investigating the mechanisms of Wnt signaling and their role in development, recent studies implicate Wnt signaling in cancer and other clinical conditions (Moon et al. 2004; Polakis 2000; Giles et al. 2003; Lustig & Behrens 2003).

3.4.1. Role of Wnt signaling in human cancers and its therapy

There have been numerous reports describing overexpression or under-expression of Wnt genes in cancer. A transgenic mouse model has established that tumor growth is dependent on Wnt-1 expression (Gunther et al. 2003). Studies of Wnt expression in human breast tumors have tended evidence to support Wnt signaling in development of breast cancer (Lane & Philip 1997; Huguet et al. 1994; Smalley & Dale 2001; Howe & Brown 2004). Cells expressing Wnt1 were resistant to cancer therapy mediated apoptosis. Wnt1 signaling inhibited cytochrome c release and the subsequent caspase-9 activation that was induced by chemotherapeutic drugs, including both vincristine and vinblastine. Further research showed that Wnt1 signaling inhibited apoptosis by activating β-catenin/Tcf mediated transcription (Chen et al. 2001). It was recently demonstrated that inhibition of Wnt2 mediated signaling induced apoptosis in both malignant melanoma cells and non-small-cell lung cancer cells (You et al. 2005; You et al. 2004). These studies show that activation of the β-catenin/TCF/LEF-1 signaling pathway by Wnt ligands not only provides a growth advantage to cancer cells, but also significantly affects the clinical outcome by inhibiting chemotherapy-induced apoptosis. Blocking Wnt signaling, for instance with a monoclonal antibody or development of small molecules can be useful to inhibit the Wnt/β-catenin signaling pathway for treatment of cancer patients and may improve the efficacy of chemotherapy by enhancing apoptosis in cancer cells.

3.4.2. Role of Wnt signaling in B Chronic lymphocytic leukemia (B-CLL)

As previously mentioned Wnt signaling is active during early B cell development in a LEF-1 dependent mechanism (Reya *et al.* 2000). However some studies exist that indicate factors governing aberrant activation of Wnt signaling in mature B-CLL cells. A comprehensive gene expression profiling from two independent studies revealed that LEF-1 mRNA was ~3000 fold overexpressed in B-CLL when compared to its healthy counterpart

(Gutierrez et al. 2007; Howe & Bromidge 2006). Later series of studies demonstrated autocrine activation of Wnt signaling in different clinical subsets of CLL. Based on a cDNA microarray study, Wnt-3 was found to be uniformly upregulated in B-CLL (Rosenwald *et al.* 2001). Lu and co workers demonstrated that B-CLL cells are in an autocrine Wnt action where several Wnts and frizzled (Wnt-3, Wnt-5b, Wnt-6, Wnt-14, Wnt-16, Fzd-3) mRNA were found to be elevated in CLL when compared to healthy B cells. The induction of β-catenin by inhibiting GSK3-β also demonstrated an increased survival of B-CLL cells *in vitro*, whereas inhibiting Wnt signaling by R-etodolac led to apoptosis in CLL cells indicating that this pathway plays a crucial role in survival of CLL cells. (Lu et al. 2004).

There are also evidences for epigenetic alterations of modulators of Wnt signaling at the genomic level. Secreted frizzled-related proteins (SFRP-1, SFRP-2 SFRP-4 and SFRP-5) was found to be frequently methylated in CLL (Liu *et al.* 2006; Chim *et al.* 2008). SFRPs are physiological inhibitors of Wnt pathway, which compete with the Fzd receptors for Wnt binding through the CRD domain or by binding directly to Fzd, resulting in the formation of inactive complexes with the receptor. The Wnt inhibitory factor-1 (Wif-1) which can bind to different Wnts and render them inactive is infrequently methylated in CLL (Chim et al. 2006).

Taken together, there is an autocrine Wnt secretion in CLL cells, with overexpression of LEF-1 and concordant methylation of physiological inhibitors of Wnt pathway. As a result of such activation, LEF-1 target genes such as c-myc cyclin D1 are overexpressed in CLL cells providing extended survival of CLL cells (Faderl *et al.* 2002; Nagy *et al.* 2003; Plate *et al.* 2000).

3.5. Clinical perspective of B-Chronic lymphocytic leukemia (B-CLL)

Given the potential therapeutic value of the Wnt signaling pathway in the treatment of B-CLL, it is critical to understand the clinical perspective of the disease with regard to the epidemiology, symptoms, diagnosis, disease staging, and prognosis in order to develop the most effective therapeutic strategies.

3.5.1. Epidemiology

CLL is the most common type of leukemia in the western countries representing 22-30% of all leukemia's with an incidence rate of between 1 and 5.5 per 100,000 people and is considered incurable with currently available therapy (Redaelli et al. 2004; Zent et al. 2001). Geographically it is most prevalent in Australia, USA, Italy, Switzerland and Ireland, while low incidence is seen among Asian countries and US asian origin and is slightly more common in whites than in blacks, which emphasizes the racial factors in the occurrence of this disease (Dores et al. 2007; Groves et al. 1995; Morton et al. 2006). It mostly affects the elderly (>50yrs of age) however an increase in the incidence among the younger individuals has been reported (de Lima *et al.* 1998; Derossi *et al.* 1989; Mauro *et al.* 1999). CLL is more common in men than women with a sex ratio of about 2:1 (Cartwright et al. 2002).

3.5.2. Clinical manifestations

CLL is initially asymptomatic and often diagnosed during routine examination. Despite its lack of visible manifestation in terms of symptoms it affects the immune system leading to the complications like infections and secondary malignancies (Anaissie et al. 1998). Apart from infection patients can present features like painless lymphoadenopathy (enlargement of lymph nodes), bone marrow infiltration with leukemia cells causing anemia (low RBC levels) and thrombocytopenia (low platelets levels) (Mauro *et al.* 1999). Autoimmune hematological conditions are frequently associated with CLL, of which autoimmune haemolytic anemia (AHA), idiopathic thrombocytopenic purpura and red cell aplasia are more common (Diehl & Ketchum 1998; Mauro et al. 2000). Hypogammaglobulinemia (low gamma globulin levels) is found in approximately 50% of CLL patients (Dighiero G. 1988).

3.5.3. Diagnostic criteria

The current diagnosis of CLL is based on the revised guidelines from the national cancer institute working group (NCI-WG) (Cheson *et al.* 1996; Hallek *et al.* 2008). Accordingly CLL can be diagnosed when the following conditions exist:

1) A persistent (>3 month) peripheral blood lymphocyte count > 5 x 10^9 cells/L of mature lymphocytes in the absence of other causes
2) Distinct immunophenotype of CLL
    a) Predominant expression of B cell markets like CD19, CD20, CD23 along with T cell marker CD5 antigen, in the absence of other T cell markers
    b) Light chain restriction and FMC7 negative
    c) Surface immunoglobulin (sIg) of low density and absence or low expression of CD79b

Several hematological disorders like mantel cell lymphoma (MCL), splenic marginal zone lymphoma (SMZL), B cell prolymphocytic leukemia (PLL), Hairy cell leukemia (HCL) and Waldenstrom's macroglobulinemia resemble CLL in their clinical presentation and microscopic appearance (Jaffe 2001). Therefore a differential diagnosis is necessary to distinguish CLL from other disorders. MCL differs from CLL by CD23 negativity and presence of t (11; 14) translocation (Sanchez *et al.* 2002). In addition, PLL and HCL are morphologically and immunophentoypically distinct from CLL. Clinically PLL and HCL have prominent splenomegaly without lymphoadenopathy. The immunophenotypic features of PLL and HCL differ from CLL, since they are negative to CD5 and CD23, while positive for FMC7 (Melo 1986; Sanchez *et al.* 2002). SMZL cells are morphologically distinct from CLL lymphocytes, with a plasmacytoid basophilic cytoplasm (Catovsky & Matutes 1999). Waldenstrom's macroglobulinemia is distinct from CLL, with exception of hyper viscosity syndrome associated with as a result of elevated immunoglobulin M (Chng et al. 2006).

3.5.4. Disease staging

Clinical staging is important in CLL to predict prognosis, which aids in comparison of clinical findings and establishment of therapeutic guidelines. The Rai and Binet staging systems (Table 2) are two traditional clinical staging systems which are currently in practice (Binet et al. 2006; Rai et al. 1975).

Table 2: **Staging Systems for Chronic Lymphocytic Leukemia**

Stage		**Clinical features**	**Median survival (y)**
Rai staging system			
Original	**Modified**		
0	Low	Lymphocytosis only	>10
I	Intermediate	Lymphocytosis and lymphoadenopathy	9
II		Lymphocytosis and splenomegaly with or without lymphoadenopathy	7
III	High	Lymphocytosis and anemia (H<11g/dL)	5
IV		Lymphocytosis and lymphoadenopathy (platelets <100 x 10^9/L)	5
Binet staging system			
A		Lymphocytosis and lymphoadenopathy in <2 LN regions, Hb≥10g/dL and platelets ≥100 x 10^9/L	>7-10
B		Lymphoadenopathy at >3 sites in the absence of anemia or thrombocytopenia, Hb≥10g/dL and platelets≥ 100 x10^9/L	5-7
C		Anemia (Hgb<10g/dL) and /or thrombocytopenia (platelets <100x10^6/dL) with or without lymphoadenopathy	<2-5

3.5.5. Prognostic factors

With the development of technology and growing insight into the biology of the disease, new prognostic markers became available such as cytogenetics, the mutational status of the immunoglobulin heavy chain variable genes (IgVH), zeta associated protein kinase 70 kDa (ZAP-70) and CD38. These new prognostic markers allow stratifying patients into risk categories at the moment of the initial diagnosis. However further prospective trials are necessary to verify and establish their usefulness in the management of the patients with CLL (Binet et al. 1981).

3.5.5.1. Cytogenetic abnormalities

There is no single characteristic cytogenetic abnormality in CLL (Montserrat & Rozman 1995). With the development of interphase fluorescence in situ hybridization (FISH) techniques, it has become possible to detect numerical and structural chromosomal abnormalities in non dividing cells. Using FISH probes cytogenetic lesions were found in more than 80% of examined CLL cases, which is more than those detected in conventional metaphase cytogenetics and indicates prognostic applicability (Dohner *et al.* 2000; Krober *et al.* 2002). The most common cytogenetic alterations include deletion of 13q14.1, deletion of 11q22.3-23.1, trisomy 12 and deletion of 17p13 (Anastasi & Le Beau 1992; Stilgenbauer et al. 1998). Table 3 shows the correlation of specific chromosome aberration with clinical characteristics and outcome in CLL patients (Montillo et al. 2005). A recent study analysed survival of CLL patients in relation to chromosomal abnormalities using FISH. There is increasing evidence that chromosomal abnormalities were associated with prognosis in retrospective and prospective clinical trials (Catovsky 1989; Shanafelt *et al.* 2006). In a prospective study 13q deletion was correlated with a favourable prognosis and 17p deletion is associated with most unfavourable prognosis among the occurring abnormalities (Grever *et al.* 2007; Shanafelt *et al.* 2006). Chromosome 17 abnormalities have been associated with p53 mutation and with poor response rates and resistant to standard chemotherapeutic regiments

employing alkylating drugs and purine analogues (Byrd et al. 2006). For all these reasons during clinical trials, it is recommended to perform cytogenetics prior to treating a patient (Hallek *et al.* 2008).

Table 3: Cytogenetics in CLL (Modified from Montillo et al., 2005)

Chromosomal Aberrations	Clinical Outcome	Frequency %	Median Survival (months)
13q	Favourable outcome if isolated aberration	55	133
Trisomy 12	Atypical morphology and intermediate outcome	16	114
11q	Extensive lymphoadenopathy, shorter treatment and free survival	7	32
17p	Resistance to chemotherapy	7	32
Normal karyotype	Favourable outcome	18	≥ 133

3.5.5.2. IgVH mutational status

During recent years, the somatic hypermutation status of the immunoglobulin variable heavy chain (IgVH) genes has been vividly investigated and found to be an important prognostic marker in CLL (Damle *et al.* 1999; Hamblin *et al.* 1999). A rearranged clonal IgVH sequences that differ from their germline counter parts by 2% are defined as 'mutated' (Dighiero G. 1988; Hashimoto et al. 1995; Schroeder & Dighiero 1994). Based on this subjective threshold value, two subgroups of CLL with clear difference in survival have been

identified as 'mutated' CLL, where leukemic cells have rearranged VH genes with 2% or more mutations and 'unmutated' CLL with few or no mutations (Fais *et al.* 1998; Stevenson & Caligaris-Cappio 2004). Mutational status has been strongly correlated with prognosis of CLL. Patients with unmutated gene sequences had aggressive disease, shorter survival and poorer prognosis than those with mutated genes (Krober *et al.* 2002; Oscier *et al.* 2002). Also there are reports indicating that patients with unmutated IgVH genes had a higher risk of relapse after stem cell transplantation (Ritgen et al. 2003). However, there is an exception, where the usage of IgVH gene V3-21 expression has an inferior outcome independent of the mutational status (Thorselius *et al.* 2006; Tobin *et al.* 2002).

3.5.5.3. Zeta-associated protein (ZAP-70)

Zeta-associated protein (ZAP-70) is an intracellular tyrosine kinase that is predominantly expressed in T and natural killer cells and functions in transmitting activation signals involved in T-cell receptor signaling and T cell activation (Chan et al. 1992). Microarray studies reveal that CLL cells share a characteristic gene-expression profile and found that a small number of genes (ZAP-70 and C-type lectin) correlated with the mutational status of IgVH genes (Klein *et al.* 2001; Rosenwald *et al.* 2001). Several studies using western blotting, RT-PCR and flow cytometry have confirmed the ability to distinguish between IgVH mutated (ZAP-70-) and unmutated (ZAP-70+) (Crespo *et al.* 2003; Orchard *et al.* 2004; Rassenti *et al.* 2004). Many groups have validated a threshold of 20% by flow cytometry to separate ZAP-70- from ZAP-70+ cases (Crespo *et al.* 2003). As IgVH sequencing is laborious and costly, ZAP-70 expression is recommended as surrogate marker for IgVH gene mutational status in CLL (Del Principe et al. 2006; Wiestner et al. 2003).

3.5.5.4. CD38 expression

CD38 is a cell surface molecule that regulates cell activation, proliferation and adhesion and present in various lineages of hematopoietic cells, including B-cells (Cruse et al.

2007). Recently it has been shown to promote proliferation and to prolong survival of CLL cells (Deaglio et al. 2006). CD38 was initially suggested as a surrogate marker for IgVH mutation status in CLL (Damle *et al.* 1999; Ibrahim *et al.* 2001). High CD38 expression correlates with unmutated IgVH and is associated with significantly shorter overall survival, progression-free survival times and poor response to chemotherapy (Del Poeta et al. 2001; Ghia et al. 2003; Jelinek et al. 2001). However other studies found CD38 expression gives discordant results with respect to IgVH mutation status (Hamblin *et al.* 2000; Krober *et al.* 2002). This discrepancy is due to the lack of a defined cut-off value for CD38 expression (Boonstra *et al.* 2006; Krober *et al.* 2002) and also change in the expression of CD38 with time, especially after initiation of therapy (Hamblin et al. 2002).

3.6. Current Therapeutic strategies

Remarkable progress in elucidating the biology of CLL has been made over the last two decades. Improved understanding of CLL has lead to new prognostic tools and therapeutic options, and holds promise for eventually finding a cure for this disease. Challenges lie in incorporating the various treatment modalities, including chemotherapy, monoclonal antibodies, immunotherapeutic strategies and novel small molecules, into a comprehensive treatment strategy guided by the biological complexity of CLL.

3.6.1. Criteria for patient treatment

The disease course for CLL is highly variable and the criterion for initiating the therapy is an important consideration in the clinical management of CLL. The decision to treat is guided by the stage of the disease, the presence of symptoms and disease progression (Eichhorst & Hallek 2007). According to the guidelines for diagnosis and treatment (Hallek *et al.* 2008) set at the recent International Workshop on Chronic Lymphocytic Leukemia (IWCLL) indicate that in routine clinical practice, newly diagnosed patients with asymptomatic early stage disease should be kept under ‘wait and watch’ unless there is evidence of disease progression. Several research groups confirm that the treatment of early-

stage disease with alkylating agents does not prolong the survival (Dighiero *et al.* 1998). However symptomatic and progressive disease patients (Rai III and IV or Binet B or C) should be immediately treated to benefit initiation of treatment (Molica et al. 1991; Robak et al. 2006a). Improvement in therapeutic regiments and advances in the understanding the biology of the disease have made it possible to achieve higher percentage of remissions, but never a complete remission of the disease. A complete cure of this disease is still a far-to-reach goal of modern medicine (Ghia et al. 2007).

3.6.2. Alkylating agents as single agents and in combination therapy

For many years alkylating agents like chlorambucil have been the gold standard for the first line therapy in CLL, given daily or intermittently, alone or in association with corticosteroids. Chlorambucil produced overall remission rate of 40-80% in previously untreated patients and all the patients showed a relapse (Binet 1990; Rai *et al.* 2000; Keller *et al.* 1986). Despite higher response rates chlorambucil does not prolong survival and use of corticosteroids does not provide any benefit in the treatment (Catovsky *et al.* 1991; Dighiero *et al.* 1998; Jaksic *et al.* 1997). Another alkylating agent, cyclophosphamide is used when chlorambucil is poorly tolerated. A higher response rate was achieved when this drug used in combination therapies with anthracyclines. The best combination therapies were CHOP (cyclophosphamide, adriamycin, vincristine and prednisone), CAP (cyclophosphamide, doxorubicin and prednisone) and COP (cyclophosphamide, vincristine and prednisone) (Keating *et al.* 1988; Jaksic *et al.* 1997; Binet 1990). In most randomized studies and meta analyses of randomized trials of these combination chemotherapy regiments showed a higher response rates, but none showed a benefit in terms of survival (Binet 1990; Raphael *et al.* 1991).

3.6.3. Purine analogs as single agents and in combination therapy

Purine analogs have become the gold standard for first line treatment in CLL replacing monotherapy with alkylating agents (Keating *et al.* 1991). Three purine analogues are currently used in CLL: fludarabine, pentostatin and cladribine. Fludarabine is most extensively studied and used for therapy in the west and in Europe. Fludarabine monotherapy induces more complete remissions when compared with other treatment regiments containing alkylating agents or corticosteroids (Keating *et al.* 1991; Rai *et al.* 2000; Johnson *et al.* 1996; Keating *et al.* 1998; Leporrier *et al.* 2001). Single agent fludarabine has superior response rates in previously treated patients (Hallek et al. 2001) and also in elderly patients with progressive disease (Eichhorst & Hallek 2007). Randomized studies of cladribine show similar overall and complete remission as with fludarabine as single agent or in combination in CLL (Robak 2002; Montillo et al. 2003).

3.6.4. Chemoimmunotherapy

Monoclonal antibodies and immunotoxins are emerging as attractive agents and have been investigated in clinical trials in patients with CLL (Del Poeta et al. 2008; Robak et al. 2006b; Tam & Keating 2007). Rituximab (Rituxan, Mabthera) is a chimeric human mouse monoclonal antibody that targets CD20 antigen. CD20 antigen is expressed in all B-cell phases except stem cells and plasma cells. Rituximab's anti-leukemia action includes elimination of B cells includes complement dependent lysis, antibody dependent cell mediated cytotoxicity (ADCC) and direct induction of apoptosis (Voso *et al.* 2002). Single agent rituximab has limited efficacy at standard FDA approved doses and most studies suggested a higher dose of rituximab in CLL when compared to other lymphomas (Voso *et al.* 2002; Byrd *et al.* 2001; Itala *et al.* 2002; O'Brien *et al.* 2001). The combination of rituximab with fludarabine (Byrd et al. 2003; Schulz et al. 2002) or fludarabine and cyclophosphamide (Keating et al. 2004) increased the overall and complete remission rates in previously

untreated patients and in the relapsed CLL patients (Wierda et al. 2005). Alemtuzumab (Campath) is a recombinant, fully humanized, monoclonal antibody against CD52 antigen. Several reports have confirmed significant overall response rate and survival activity alemtuzumab in relapsed or refractory CLL (Keating et al. 2002; Osterborg et al. 2002; Rai et al. 2002). In addition, alemtuzumab showed clinical response in patients with poor prognostic factors, including high-risk genetic markers such as deletions of chromosome 11 or 17 and p53 mutations (Lozanski et al. 2004; Stilgenbauer et al. 2001).

3.6.5. Allogeneic transplant

A recent consensus paper by EMBT (European Group for Bone Marrow Transplantation) provided standard indications for allogeneic transplant in CLL. The authors contend that allogeneic HCT (Hematopoietic cell transplantation) has proven efficacy in poor-risk CLL, for younger patients with 1) non response or early relapse with 12 months after purine analog treatment. 2) Relapse within 24 months after achieving CR (Complete Remission) with a purine analog based regimen. Or 3) patients with 17p- abnormalities with dismal responses with conventional chemotherapy regimens (Dreger et al. 2007). Several nonrandomized trials have evaluated the efficacy of allogeneic stem cell transplantation in CLL. However, most trials were limited by size, absence of long term follow up and high treatment related mortality (Jabbour et al. 2004). A recent Spanish study reported 30 patients with poor-prognosis CLL and or high-risk molecular/cytogenetic characteristics, treated with reduced intensity conditioning (RIC) allogeneic transplant indicate that this therapy may overcome adverse prognostic factors even in patients with 17p-, and provide patients with long term remission suggesting potential cure.

3.6.7. Other Targeted therapies

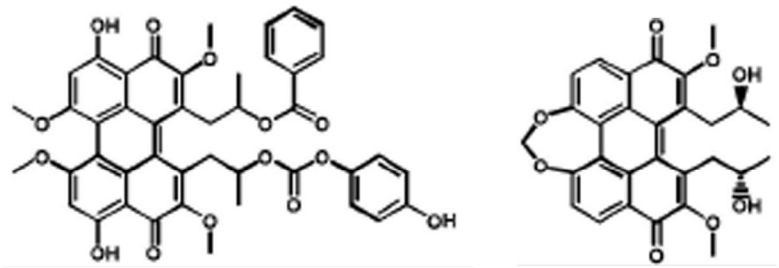
Apart from immunotherapy, few targeted therapies are being tested in clinical trials for CLL. Oblimersen, an antisense phosphothioate oligonucleotide targets the messenger RNA of

the anti-apoptotic molecule BCL-2. As a single agent oblimersen confirmed only modest activity in patients with relapsed/refractory CLL (O'Brien et al. 2005) but demonstrated favourable activity in combination with FC (Fludarabine-Cytoxan), compared to FC alone (O'Brien et al. 2007). ABT-263 is a BH3 mimetic that binds and inhibits multiple anti-apoptotic BCL-2 family proteins and potent against cell lines derived from lymphoid and small cell lung cancers as single agent. Currently combination strategies using ABOUT-263 with rituximab or chemotherapy are underway or in planning phase (O'Connor et al. 2008).

As CLL is still an incurable disease with high relapse rates with current treatment regimens, there is still a great need to investigate novel drugs and to understand in detail the underlying molecular mechanism in the pathogenesis of the disease

3.6.8. Novel TCF/LEF-1/β-catenin Inhibitors CGP049090 and PKF115-584

The interaction between TCF family transcription factors and β-catenin requires a minimal N-terminal C fragment and central domain of 12 armadillo repeats in β-catenin. A recent high throughput screening explored the compound libraries in search of small molecules which inhibit the TCF/LEF-1/β-catenin interaction. Out of approximately 7,000 natural compounds, very few displayed reproducible and dose-dependent inhibition of the protein-protein interaction between β-catenin and Tcf4 in an immunoenzymatic assay with IC50 values lower than 10μM. Six compounds were evaluated in a range of *in vitro* secondary assays such as reporter gene activation, expression analysis, cell proliferation, and xenopus duplication assay. Three compounds (PKF115- 584, CGP049090, and PKF222-815), with the same chemical core structure, scored consistently in the different assays (reporter gene assays, GST-pulldown assay, electrophoretic mobility shift assay) used to confirm the antagonistic properties of the compounds. We obtained two of these compounds CGP409090 and PKF115-584 (Synonym: Calphostin C) from Novartis Pharma AG (Basel) (Figure 5). Both CGP049090 and PKF115-584 are extracts from fungal organisms and are aromatic



PKF115-584 **CGP049090**

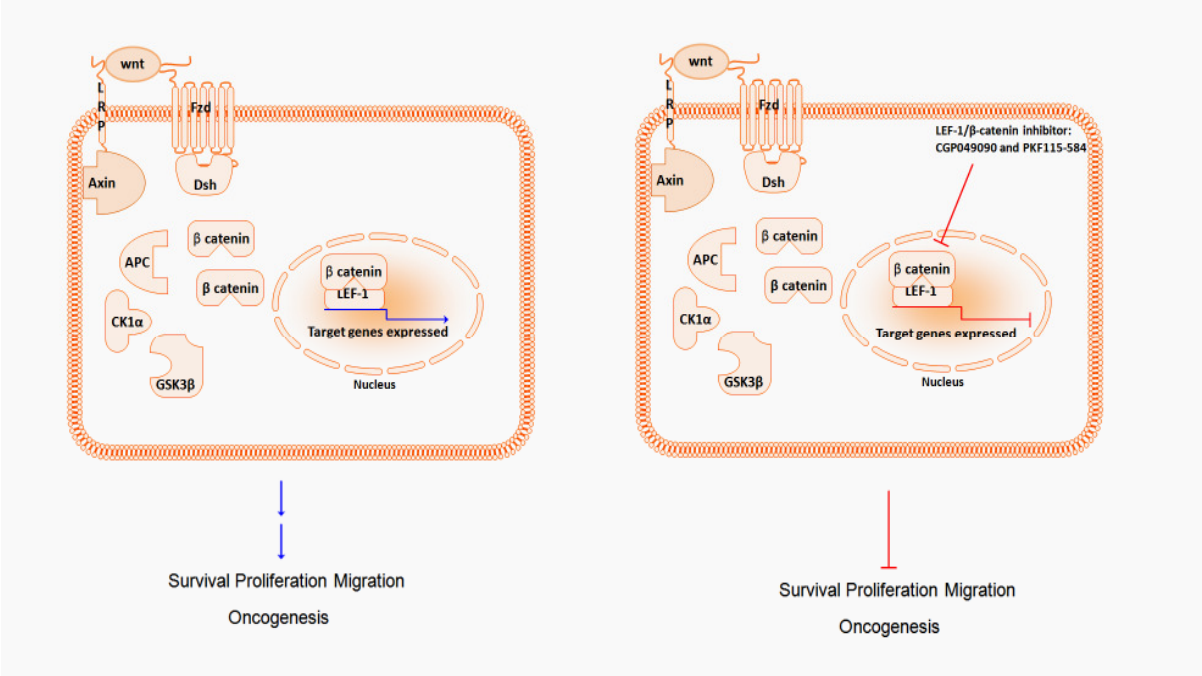


Figure 5: Molecular structure of PKF115-584 and CGP049090 and the potential mechanism of the disruption of Wnt signaling pathway

heterocyclic compounds. Due to their heterocyclic nature they possess auto-fluorescence properties. The Molecular weight of PKF115-584 is 790.70g/mol and CGP049090 is 534.52g/mol. Figure 5 also depicts the molecular mechanism of the action of these compounds in the disruption of the Wnt signaling pathway

3.7. Project Objectives

B-Cell Chronic lymphocytic leukemia (B-CLL) is the result of progressive accumulation of dysfunctional monoclonal B lymphocytes. Several aberrant pathways have been implicated in this disease leading to its extended survival. Current treatment options include the use of traditional chemotherapy with or without in combination of antibody therapy and allogeneic transplantation. Despite availability of current treatment options, B-CLL still remains an incurable disease.

Over the last few years there has been considerable focus on role of Wnt signaling and malignant diseases. Classically Wnt signaling is active in early developmental tissues and is turned off in adult differentiated tissues. However, positive Wnt signaling has been fairly established in most of the cancer types. Little is known about the Wnt signaling in B-CLL. A micro-array study revealed that an important transcription factor of Wnt signaling pathway LEF-1, is 3000 fold over-expressed in CLL cells when compared to healthy B cells.

Therefore, the central aim of this thesis is to determine whether LEF-1 is an attractive therapeutic target for B-CLL therapy.

This would be systematically accomplished by

- Confirming the expression of Wnt signaling components in B-CLL cells, cell lines and healthy cells at the protein level
- Investigating the role of LEF-1 in extended survival of B-CLL cells and cell lines by siRNA knockdown
- To test novel small molecule inhibitors (CGP049090 & PKF115-584) of the LEF-1/ β-catenin complex in B-CLL *in vitro* and in a suitable mouse model *in vivo*.

4. Results

The current study was undertaken to investigate if LEF-1 could be a potential therapeutic target for B-CLL. In order to study this, the activation of Wnt signaling pathway was first characterized in the various cell lines used and the need for LEF-1 for the survival of B-CLL was ascertained. Following this, the two small molecule inhibitors, CGP049090 & PKF115-584 were tested and analysed for inhibition of LEF-1/ β-catenin interaction in the *in vitro* and *in vivo* context.

4.1. Activation of Wnt signaling in B-CLL

The extracellular Wnt signal is transduced by transmembrane and cytoplasmic mediators which ultimately lead to dephosphorylation of β-catenin. β-catenin can then translocate into the nucleus where it can interact with LEF-1 and initiate target gene expression (Morin & Weeraratna 2003). This LEF-1/ β-catenin complex is considered to be a trademark canonical Wnt pathway. Hence, monitoring the increase of dephosphorylated β-catenin and LEF-1 served as a surrogate marker for activation of Wnt pathway.

4.1.1. Expression of downstream signaling components of Wnt signaling in B-CLL and healthy B cells.

The levels of LEF-1/ dephospho β-catenin were compared in primary CLL samples and CLL cell lines to that of the healthy B cells and PBMCs. Briefly total protein extracts were made from primary B-CLL cells (n=7) healthy B cells (n=2) healthy PBMCs (n=2) and JVM-3 and MEC-1 cell lines. 10µg of the cell lysate were subjected to western blot analysis to detect LEF-1, total β-catenin, dephospho β-catenin (active beta catenin). Equal protein loading was assured by measuring ubiquitously expressed β-actin (Figure 5). There was a significant over-expression of LEF-1 in B-CLL samples when compared to the healthy B cells and PBMCs. LEF-1 expression is completely turned off in healthy B cells. The total β-catenin and dephospho β-catenin pool was also higher in B-CLL samples when compared to healthy

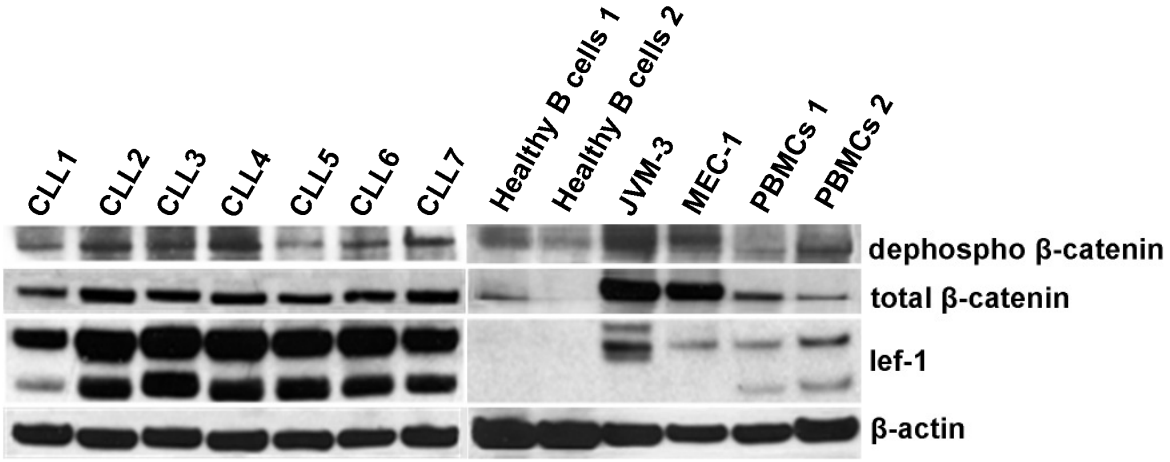


Figure 6: LEF-1 and β-catenin expression in CLL, cell lines and healthy B cells*. Total protein extracts from primary B-CLL (n=7), healthy CD 19+ B cells (n=2), healthy PBMCs (n=2), and B-CLL cell lines were probed for LEF-1, total β-catenin, and dephospho β-catenin and β-actin as loading control.*

B cells and PBMCs. The presence of LEF-1 and dephospho β-catenin in B-CLL confirms the LEF-1/ dephospho β-catenin complex formation thereby the potency to transcribe target genes.

4.1.2. Nuclear localization of LEF-1 and β-catenin in primary B-cells

LEF-1 which exists as a repressor in the nucleus is transformed to a transcriptional co-activator by complexing with dephospho β-catenin in the nucleus upon stimulation with Wnt ligands. Hence the presence of this complex in the nucleus regarded as the functional significance of this pathway as it can direct transcription of various LEF-1 target genes. To demonstrate that the leukemic B-CLL cells have an aberrant and constitutive activation of Wnt signaling nuclear and cytoplasmic extracts from three B-CLL patient samples were made and probed for the localization of LEF-1, total β-catenin and β-actin as loading/purity control (Figure 7). It was evident that LEF-1 is primarily localized in the nucleus along with β-catenin indicating both proteins interact and can transcribe LEF-1 target genes in B-CLL. However residual amount LEF-1and β-catenin was also seen in the cytoplasmic extract. Interestingly

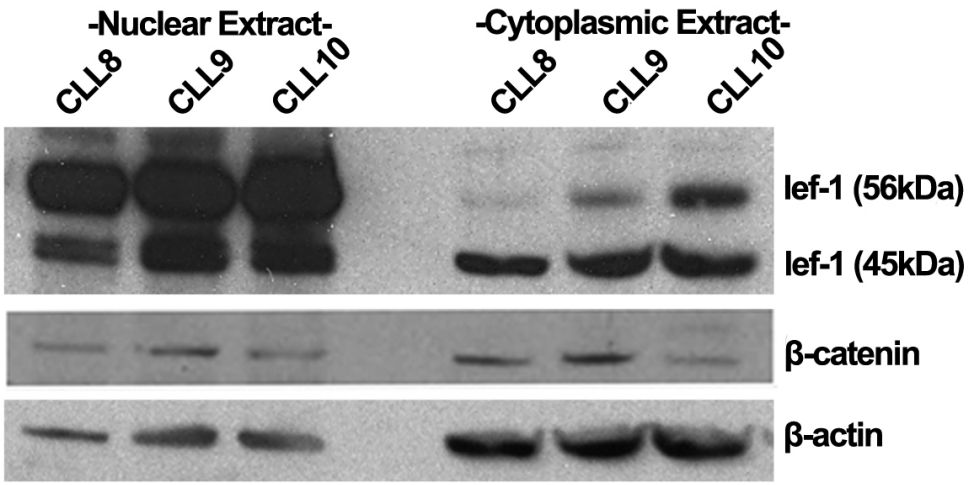


Figure 7: Nuclear localization of LEF-1 and β-catenin in primary B-CLL cells. *Nuclear and cytoplasmic extracts from 3 primary B-CLL samples indicate both LEF-1 isoforms are predominantly localized in the nucleus along with β-catenin. β–actin served as the loading control and to determine purity of the nuclear extract.*

the B-CLL cells also express a short isoform (~45kDa) and a longer isoform (~56kDa) of LEF-1. The antibody against LEF-1 used in this study only detects the dominant from of LEF-1 which has the β-catenin binding domain. Hence both isoforms of LEF-1 can interact with β-catenin and then act as a transcriptional co-activator in the nucleus. As seen from the figure 7 the longer isoform of LEF-1(56kDa) is predominantly localized in the nucleus whereas the shorter isoform (45kDa) is located in both cytoplasm and the nucleus.

4.2. LEF-1 plays a central role in for survival of B-CLL cells

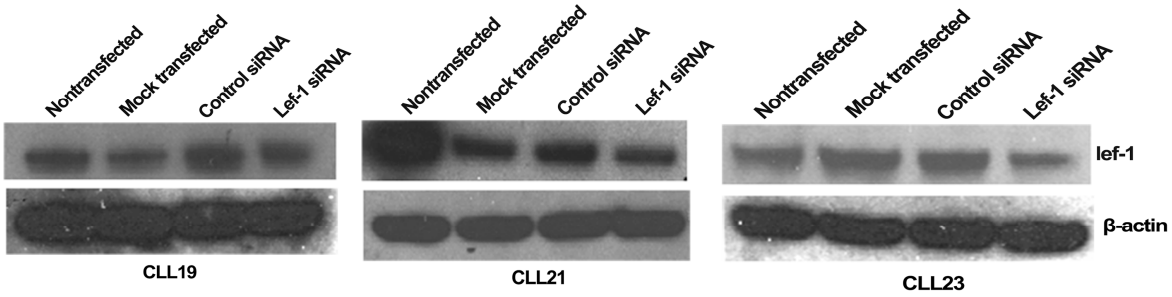
Following characterisation of the Wnt signaling pathway in the various cell lines, it was ascertained that LEF-1 is indeed required for the survival of B-CLL before investigating Lef-1 as a target for inducing apoptosis of B-CLL.

4.2.1. Knockdown of LEF-1 in primary CLL cells by siRNA

Having established that the Wnt signaling is constitutively activated in B-CLL, it was essential to determine the role of LEF-1/β-catenin transcription in the survival of B-CLL cells. Most of the LEF-1 targets are known oncogenes and play a vital role in tumorigenesis.

SiRNA mediated LEF-1 knockdown experiments was carried out in primary B-CLL cells by nucleofection. LEF-1 knock down was determined at the protein level in three representative samples by immunoblotting using anti LEF-1 antibody and anti β-actin antibody as loading control (Figure 8A). Figure 8B indicates the % inhibition of in LEF-1 in the sample transfected with LEF-1 siRNAs when compared to nontargeting control siRNA by densitometry analysis using β-actin as the reference band. This validates that LEF-1 knockdown promotes apoptosis of B-CLL cells.

A



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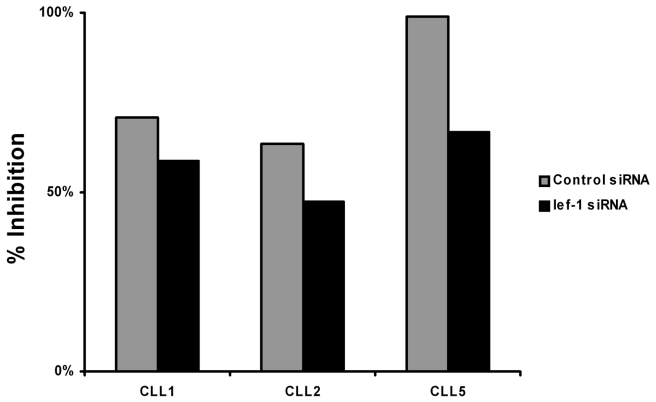

Figure 8: (A) Knockdown of LEF-1 in primary CLL cells by siRNA

*LEF-1 expression was analyzed by western blot in total cell extracts from of indicated patient samples with anti-β-actin as the loading control.**(B)Densitometry analysis of % Inhibition of Lef-1***

For each of the 3 samples, blot lanes corresponding to control nontargeting and LEF-1 siRNA were subjected to densitometric analysis and normalized to β-actin levels. Columns represent the percentage inhibition of LEF-1 induced by LEF-1 siRNA compared to control siRNA.

4.2.2. siRNA mediated LEF-1 knockdown induces apoptosis in primary CLL cells

The cell viability following the siRNA knockdown of Lef-1 was determined 16 hours post nucleofection by flow cytometry by annexinV/PI staining. The cells nucleofected with LEF-1 siRNAs had a significantly increased rate of apoptosis when compared to the cells nucleofected with nontargeting siRNAs. The percent survival (mean ± s.e of six samples) was 45.02% ± 3.7 in the control siRNA transfected cells and 32.46% ± 3.6 in the LEF-1 transfected cells (p value= 0.031) as shown in the Figure 9.

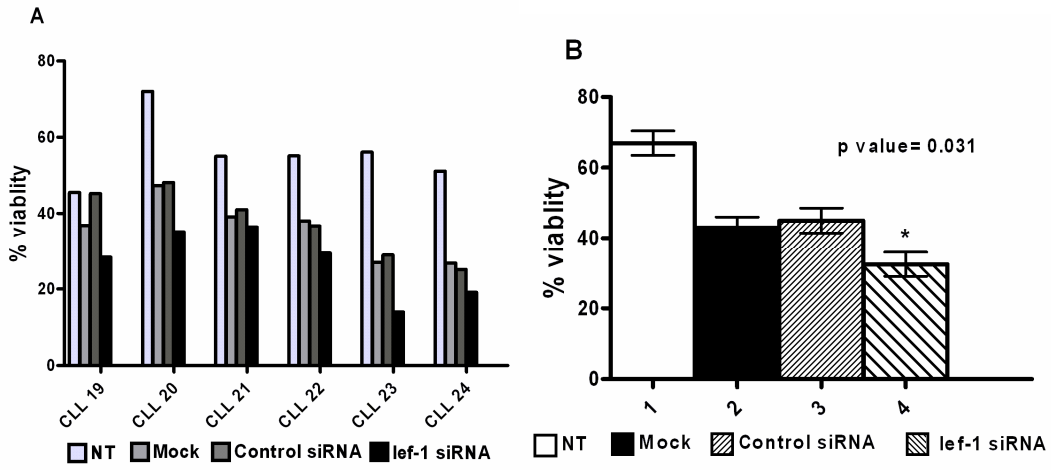

Figure 9: siRNA mediated LEF-1 knockdown induces apoptosis in primary CLL cells. *Freshly isolated B-CLL cells were non transfected (NT), mock transfected (no siRNA), transfected with 0.5μM control non targeting or LEF-1 siRNA (n=6) and cultured for 16h in complete medium. (A) Cell viability was evaluated by annexin-V/PI staining and the results from each patient are presented as the percentage of annexin-V-/PI- cells (%viable cells). (B) The same result represented as M±SE indicates the significant difference between the control siRNA Vs LEF-1 siRNA (p value =0.031)*

4.2.3. Knockdown of LEF-1 in JVM-3 cell lines by siRNA leads to down regulation of target genes and reduced proliferation

Till date, JVM-3 and MEC-1 is among the few cell lines that can be considered CLL like cells. They were originally derived from a patient with B-prolymphocytic leukemia (PLL) at diagnosis and EBV-transformed during treatment with phorbol ester TPA. JVM-3 cell lines express κ light chains, surface CD19, and CD23. To evaluate the effects of LEF-1 knock down in JVM-3 cell lines siRNA mediated LEF-1 knock down experiments were carried out.

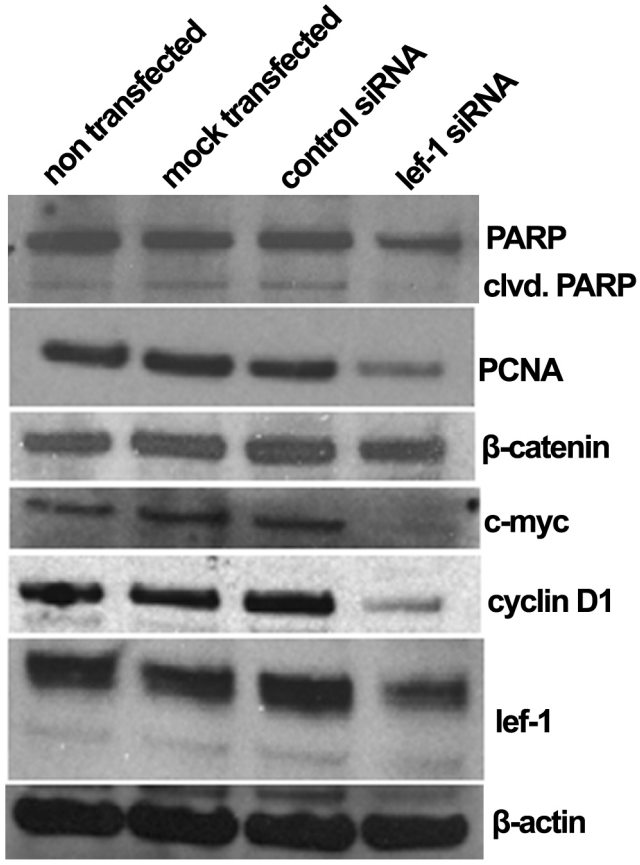

Figure 10: Knockdown of LEF-1 in JVM-3 cell line by siRNA leads to reduced proliferation.

LEF-1 knock down experiments in JVM-3 cell lines shows down regulation of target gene expression of c-myc, cyclin D1, PARP and PCNA when compared to the control. Levels of β-catenin remained unchanged. This observation was confirmed in two independent experiments.

48 hours post siRNA nucleofection cells were harvested and protein extracts were immunoblotted for lef-1, β-catenin, c-myc, cyclin D1, PCNA, cleaved PARP and β-actin as the loading control. Figure 10 shows that LEF-1 knock down, leads to expected inhibition of LEF-1 target genes like c-myc and cyclin D1. Surprisingly, LEF-1 knockdown lead to decreased rate of proliferation rather than apoptosis, as seen by decreased expression of PCNA. PCNA is expressed in the nuclei of cells during the DNA synthesis phase of the cell cycle and hence considered as a marker for proliferation (Leonardi *et al.* 1992). Significant inhibition of PARP was also seen but cleaved PARP was not detectable indicating lack of apoptotic induction. However, the levels of β-catenin remained stable; indicating β-catenin expression is independent of LEF-1 expression in B-CLL.

4.3. *In vitro* cytotoxicity of small molecules (CGP049090 and PKF115-584)

The cytotoxic potential of the small molecule inhibitors, CGP049090 and PKF115-584 by the disruption of β- catenin/LEF-1 signaling in CLL was ascertained in vitro in B-CLL and healthy cells.

4.3.1. Cell lines

MEC-1 and JVM-3 cell lines were treated with different concentrations of CGP049090 and PKF115-584 (10, 1, 0.1, 0.01 μM) along with the DMSO vehicle control for 24h. The cell viability was determined using an ATP dependent cell viability assay Dose response curves and LC50 were calculated using GraphPad Prism™. Figure 10 shows both small molecule inhibitors inhibited the survival of these cells in a dose-dependent manner. The LC50 value was found to be 0.12 μM for CGP049090 and 0.49 μM for PKF115-584 in MEC-1 cells (Figure 11B) and 0.42 μM for CGP049090 and 0.93 μM for PKF115-584 in JVM-3 cells (Figure 11A).

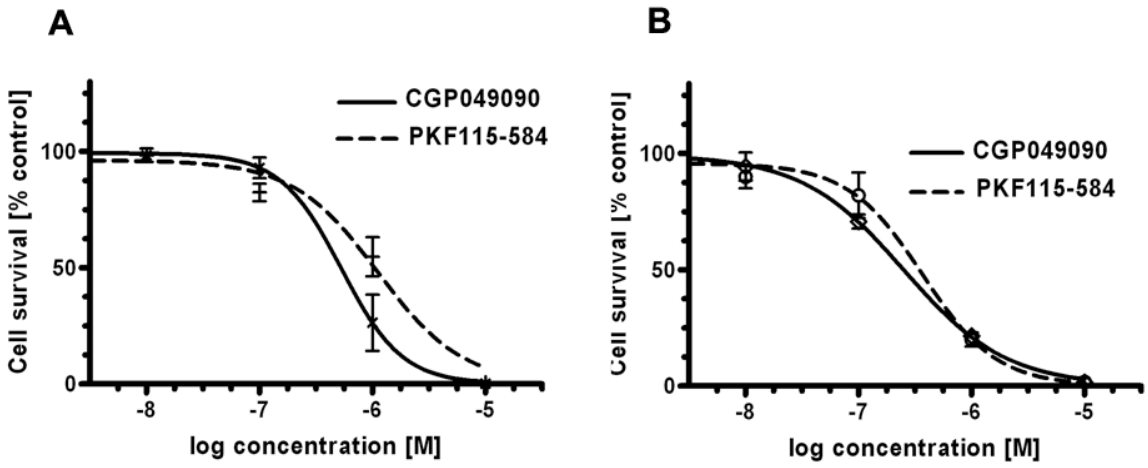

Figure 11: Dose dependent cytotoxicity of GP049090 and PKF115-584 in CLL cell lines. *JVM-3 cell line (A) and MEC-1 Cell line (B) were incubated with different concentrations of the two small molecule inhibitors for 24h and survival was measured using a luminescent ATP cell survival assay. The % cell survival is shown as the ratio of the DMSO vehicle control. The experiment was repeated three independent times*

4.3.2. Healthy B cells and Primary CLL cells

As B-CLL is a highly complex and heterogeneous disease we performed ATP dependent cell survival assays in 20 different B-CLL samples, in order to illustrate the distinct in vitro cytotoxic response that these compounds have with respect to different subsets of CLL. CLL cells from patients and purified healthy B cells from healthy volunteers were similarly treated as the cell lines. Figure 12 shows the dose response curves in 24 B-CLL patients and 3 healthy B cell volunteers. Interestingly The LC50 value was found to be e 0.89µM for CGP049090 and 0.77 µM for PKF115-584 in primary CLL cells and 8.5µM for CGP049090 and 5.7µM for PKF115-584 in healthy B cells. Both small molecules have their effect at the sub nanomolar range in leukemic CLL cells but significantly do not affect the healthy B cells, Complete cell kill was not achieved in healthy B cells at the highest concentration (10µM) indicating specificity of the small molecules to CLL cells. Table 4 tabulates the LC50 values from different cell types.

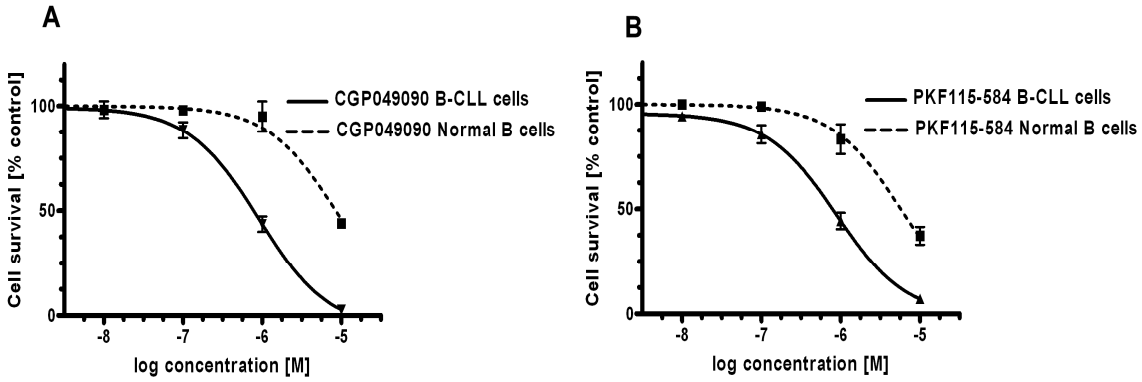

Figure 12: In vitro cytotoxicity of CGP049090 and PKF115-584 in primary CLL cells and Healthy B cells. *Primary B-CLL cells (n=24) and Normal B cells from healthy volunteers (n=3) were incubated at different concentrations (10, 1, 0.1, 0.01μM) for 24h. The % cell survival is shown as the ratio of the DMSO vehicle control. The experiment was repeated three independent times and the the results are presented as the Mean ± SE.*

Table 4: LC_{50} of PKF115-584 and CGP049090 in different cell types in B-CLL

Cell Type (n)	LC_{50} PKF115584 (μM)	LC_{50} CGP049090 (μM)
JVM-3 cell line (3)	0.93±0.12	0.42±0.2
MEC-1 cell line (3)	0.49±0.03	0.12±0.05
Primary CLL cells (24)	0.77 ±0.13	0.89±0.07
Healthy B cells (3)	5.7±0.04(estimated)	8.5±0.05(estimated)

4.3.3. Patient Sample Characteristics

Peripheral blood samples were obtained from patients who were previously diagnosed for CLL according to standard criteria (Cheson *et al.* 1996) Table 5 shows the patient's clinical characteristics in terms of their prognostic factors including ZAP-70 and CD38 expression and % apoptotic induction by the two inhibitors derived from the in vitro cytotoxicity assay. The results indicate that both CGP049090 and PKF115-584 invoke heterogeneous cytotoxic responses which are not correlative to any of the prognostic markers

Table 5: *In vitro* cytotoxicity of B-CLL samples to CGP049090 and PKF115-584

Patient ID	ZAP70 / CD38 Status*	Binet Stage†	% Cell Kill at 1 µM	
			CGP049090	PKF115-584
17	NA	NA	54	51
18	$ZAP70^-$/ $CD38^-$	A	45	10
19	$ZAP70^-$/ NA	NA	64	67
20	$ZAP70^+$/ NA	NA	53	75
21	$ZAP70^-$/ $CD38^+$	B	55	60
22	$ZAP70^-$/ $CD38^-$	NA	32	74
23	NA / NA	NA	73	62
24	$ZAP70^-$/ $CD38^+$	B	60	50
25	NA / NA	NA	54	44
26	NA / NA	NA	50	29
27	$ZAP70^-$/ $CD38^-$	C	42	38
28	$ZAP70^+$/ $CD38^-$	A	71	71
29	$ZAP70^-$/ $CD38^-$	A	31	14
30	$ZAP70^-$/ $CD38^-$	C	53	58
31	NA / NA	B	41	31
32	$ZAP70^-$/ $CD38^-$	B	40	56
33	$ZAP70^-$/ $CD38^-$	B	91	88
34	$ZAP70^-$/ $CD38^-$	A	45	52
35	$ZAP70^-$/ $CD38^+$	B	88	88
36	$ZAP70^-$/ $CD3^-$	A	47	42
37	$ZAP70^-$/ $CD38^+$	B	55	56
38	$ZAP70^-$/ $CD38^+$	B	70	60
39	$ZAP70^-$/ $CD3^-$	B	95	98
40	NA / NA	A	56	45
		Mean ± s.e.	**56.8 ± 4.0**	**54.9 ± 3.9**

**ZAP70 and CD38 expression was determined by flow cytometry. Cells expressing >20% ZAP70 and >30% CD38 were considered positive. †Patients classified according to Binet staging system indicating progressiveness of the disease. NA represents data not available at the time of completion of the study. The % cell kill at 1 µM calculated from the normalized (vehicle control) raw data.*

4.3.4. CGP049090 & PKF115-584 induces apoptotic cell death in B-CLL

To verify that both inhibitors CGP049090 and PKF115-584 induce apoptotic cell death, primary CLL and JVM-3 cell lines were incubated with 5 µM of the inhibitors for 16 hours. Protein extracts were made and the levels of cleaved poly (ADP-ribose) polymerase (PARP) were detected by immunoblotting (Figure 13). PARP is DNA repair enzyme involved in repairing single strand DNA nicks. However upon induction of apoptosis PARP is cleaved in by active caspase-3. This cleavage is intracellular marker of activation of apoptotic

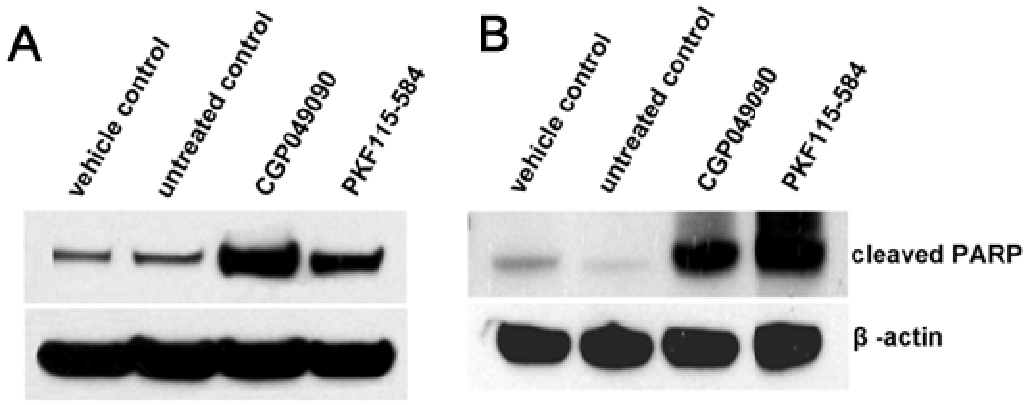

Figure 13: Analysis of PARP cleavage upon incubation with CGP049090 and PKF115-584. *Primary CLL cells (A) and JVM-3 cell line (B) were treated with 5μM of the inhibitors for 16 hours and the cell lysates were immunoblotted for anti cleaved PARP antibody. β-actin was used as the loading control. This observation was confirmed in three independent experiments*

machinery. Figure 13 shows increase in accumulation of 89 kDa cleaved PARP in the CLL and JVM-3 cell lines following 16 hour incubation. Altogether the cytotoxicity induced by these inhibitors is mediated by apoptotic mechanisms

4.3.5. CGP049090 & PKF115-584 induced apoptotic cell death is dependent on activation of caspase pathway

Apoptosis is a tightly regulated death pathway which includes activation of cysteine proteases of the caspase family. Caspases exist as inactive pro-caspases but upon apoptotic induction is cleaved in to its active form whereby it then cleaves several intracellular targets. These are characterized by the cleavage of specific substrates after an aspartic residue, and considered essential in the execution stage of the apoptotic process (Fadeel et al. 2000). To distinguish if the inhibitors induced apoptosis was driven through the intrinsic or extrinsic pathway activation status of two apical proteases caspase-8 and caspase-9 were examined along with downstream effectors proteases caspase-3 and caspase-7 (Figure 14).

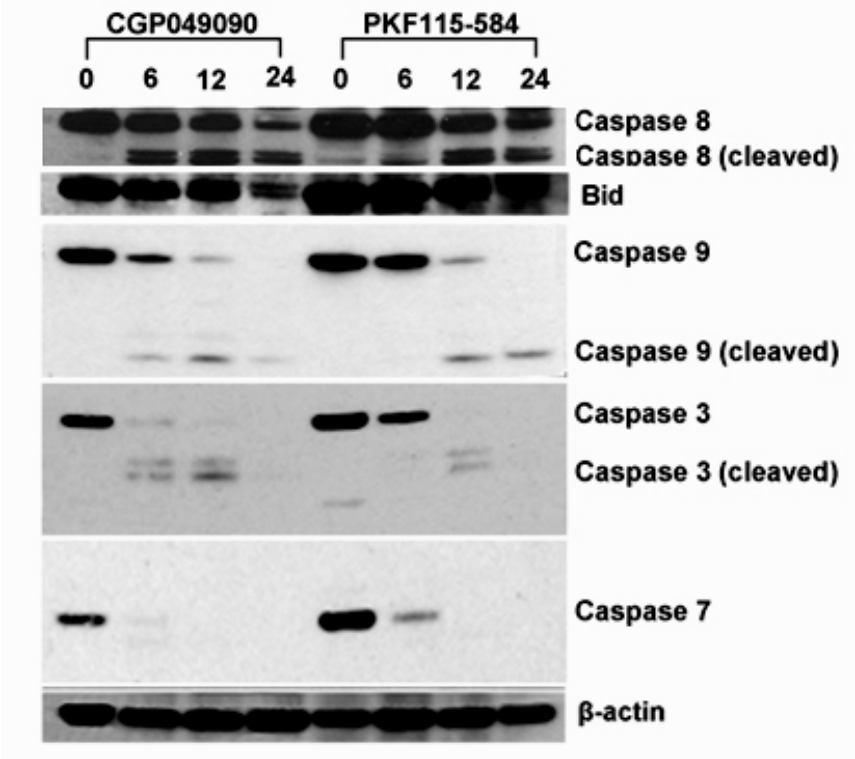

Figure 14: Caspase activation upon incubation with CGP049090 and PKF115584. *JVM-3 cells were cultured with 10μM of the inhibitors for the indicated time points. Lysates were subjected to immunoblot analysis by using specific antibodies for caspase 8, 9, 3 and 7. β actin was used the loading control. This observation was confirmed in three independent experiments.*

Caspase activation was assessed on the basis of reduction or disappearance of pro-caspases on western blot analysis. To avoid the heterogeneity seen in primary CLL cells derived from different donors, the JVM-3 cell line was used as a model system to enumerate the apoptotic mechanism involved. Time course experiments on JVM-3 cells treated with 10μM of the inhibitors for 6, 12, and 24 hours. Subsequent immunoblotting of whole cell extracts revealed cleavage of apical caspases 9 leading to consequent activation of effector caspases 3 and 7, which cleave several cellular targets promoting apoptosis (Figure 14). However there was no bid cleavage indicating activation of intrinsic mechanism of apoptosis. These data suggest that both PKF115-584 and CGP049090 induce apoptotic cell death in CLL cells.

4.3.6. CGP049090 & PKF115-584 suppress the expression of anti-apoptotic proteins

B-CLL is a disease which is linked to aberrant expression of anti-apoptotic proteins such as Bcl-2, Mcl-1 and XIAP (Kitada et al. 1998). To determine whether CGP049090 and PKF115-584 induced apoptosis is modulated by anti apoptotic proteins, JVM-3 cells were cultured in the presence of the inhibitor for 6 12 and 24 hours and the lysates were analyzed by western blot. Western blotting analysis (Figure 15) reveals that the exposure of the cells with the inhibitors steadily decreases XIAP and Bcl-2 proteins. However there was a dramatic decrease in the levels of Mcl-1 in the time course experiments. These observations indicate that the inhibitors hasten the apoptotic mechanism by down regulating Inhibitors of Apoptosis Proteins (IAPs) proteins.

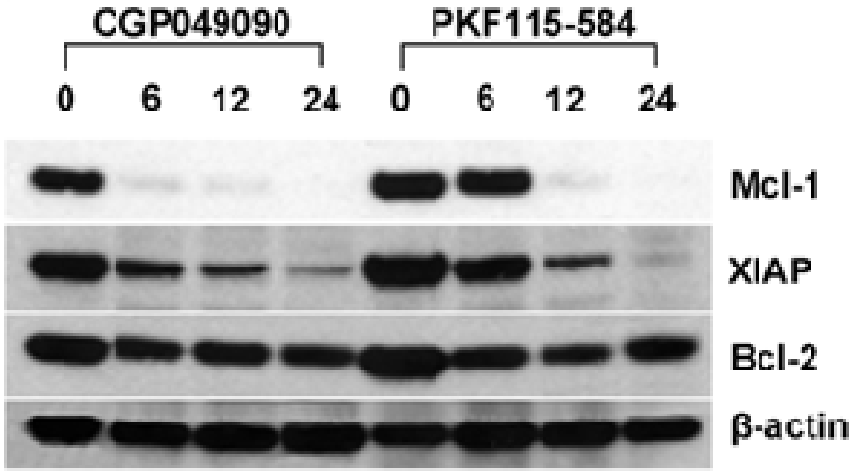

Figure 15: CGP049090 and PKF115584 down regulates anti-apoptotic proteins.
JVM-3 cells were cultured with 10μM of the inhibitors for the indicated time points. Lysates were subjected to immunoblot analysis by using specific antibodies for Mcl-2, XIAP and Bcl-2. β -actin was used the the loading control. This observation was confirmed in three independent experiments.

4.3.7. β-catenin levels are down regulated upon initiation of apoptosis by CGP049090 and PKF115-584

β-catenin has been shown to perform two apparently unrelated functions: it has a crucial role in cell–cell adhesion in addition to a signaling role as a component of the Wnt pathway. Furthermore it is evident that β-catenin is an important protein which have several putative regulators involving in cell proliferation and adhesion (Morin & Weeraratna 2003). It was interesting to know the fate of β-catenin upon CGP049090 and PKF115-584 incubation. So we treated JVM-3 cell line with the inhibitors for 6 hours, and the total cell extracts were immunoblotted for β-catenin (Figure 16). There was a complete reduction of β-catenin upon incubation with both the inhibitors when compared to the vehicle control. This is attributed to the fact that β-catenin is an important intracellular target of caspase-3 which leads to its cleavage and reduces its transactivation potential upon apoptotic induction (Steinhusen *et al.* 2000) .

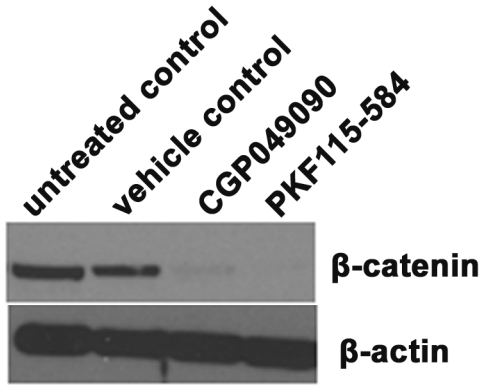

Figure 16: Reduction of β-catenin levels upon apoptotic induction. *JVM-3 cells were cultured with 10μM of the inhibitors for 6 hours. Lysates were subjected to immunoblot analysis by using specific antibody for β-catenin. β -actin was used the the loading control. This observation was confirmed in three independent experiments.*

4.4. CGP049090 and PKF115-584 inhibit the interaction between LEF-1/β-catenin

A Previous study on PKF115-584 and CGP049090 pin point that both lead compounds specifically inhibit the LEF-1/β –catenin interaction in several in vitro and in vivo assays (Lepourcelet et al. 2004). We further tested the potency of these substances to inhibit this interaction in CLL cells. Since there was rapid induction of apoptosis on in vitro culture, we incubated the inhibitors ex-vivo with the CLL lysates. 10 μM of the inhibitors was incubated with total protein extracts from CLL patients for 16 hours along with the vehicle control. Co-immunoprecipitation was carried out using β–catenin as the precipitating antibody. The immuneprecipitate was later subjected to western blot analysis to detect LEF-1(Figure 17). The results reveal reduced precipitation of LEF-1 in the lysate incubated with CGP049090 or PKF115-584. Vehicle control (0.02% DMSO) also served as the positive control for the

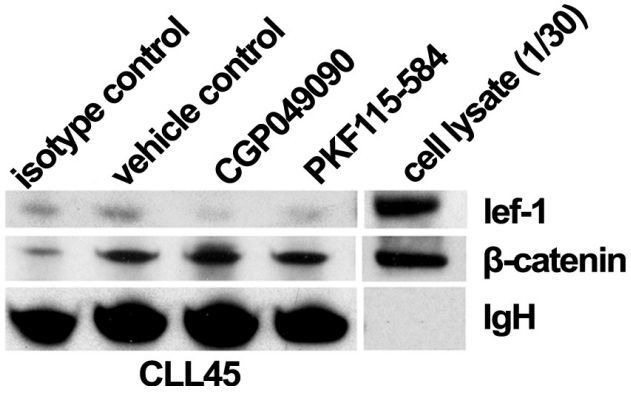

Figure 17: CGP049090 and PKF115-584 specifically inhibit co-immunoprecipitation of β-catenin and LEF-1. *Total cell protein extracts from primary CLL cells were incubated with 10 μM of CGP049090 or PKF11584 for 16h at 4°C and later immunoprecipitated with β-catenin antibody. The immunoprecipitation was probed for lef-1 and β-catenin by immunoblotting. The results reveal reduced precipitation of lef-1 in the lysate incubated with CGP049090 or PKF115-584. This observation was confirmed in three independent experiments.*

interaction. Starting extract is denoted as cell lysate (1/30). IgH is the immunoglobulin heavy chain of the precipitating antibody. This observation was confirmed in three independent experiments

4.4.1. CGP049090 and PKF115-584 down regulate LEF-1/β-catenin target genes in Primary CLL cells

Since both the inhibitors specifically inhibit the LEF-1/β-catenin interaction, it is likely that there should be an inhibition of LEF-1 transcribed target genes. To investigate whether the resulting apoptosis is mediated by paralleled down regulation of LEF-1 target genes we treated primary CLL cells (n=3) with 5 µM of the inhibitors for 48 hours. The resulting total cell lysate was immunoblotted for detecting c-myc, cyclin D1 and LEF-1 (Figure 18).

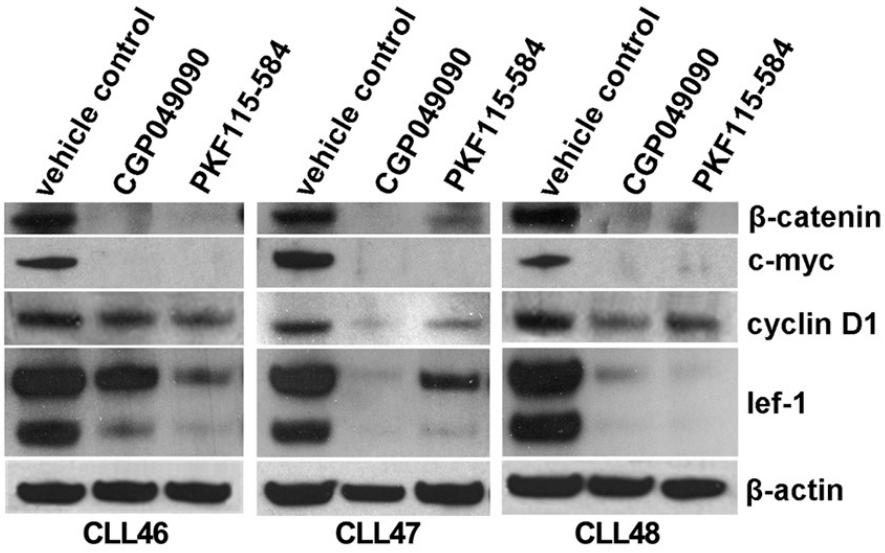

Figure 18: CGP049090 and PKF115-584 specifically inhibit LEF-1 target genes expression. *Total cell protein extracts from primary CLL cells were incubated with 5µM of CGP049090 or PKF11584 or the vehicle control (0.01% DMSO) for 48 hours. The cell lysates were immunoblotted and probed for lef-1, β-catenin, c-myc and cyclin D1. The results reveal there is a concordant downregulation of lef-1 target genes in the lysate incubated with CGP049090 or PKF115-584. This observation was confirmed in three different primary CLL samples.*

As expected, there was a reduction of LEF-1 target genes in all the three CLL samples confirming the fact that CGP049090 and PKF115-584 down regulate LEF-1 target genes and are fairly specific in their activity. Furthermore there was also a down regulation of β-catenin due to apoptotic induction as described earlier in JVM-3 cell lines.

4.4.2. CGP049090 and PKF115-584 down regulate LEF-1/β-catenin target genes in JVM-3 cell lines

The down-regulation of LEF-1 target genes was also confirmed in the JVM-3 cell lines. The cells were treated with 5 μM of the inhibitors for 24 hours. The protein extract was immunoblotted for c-myc, cyclin D1, LEF-1 and β-catenin. There was down-regulation of LEF-1 target genes and reduction of β-catenin (Figure 19). This observation was confirmed in 3 independent experiments.

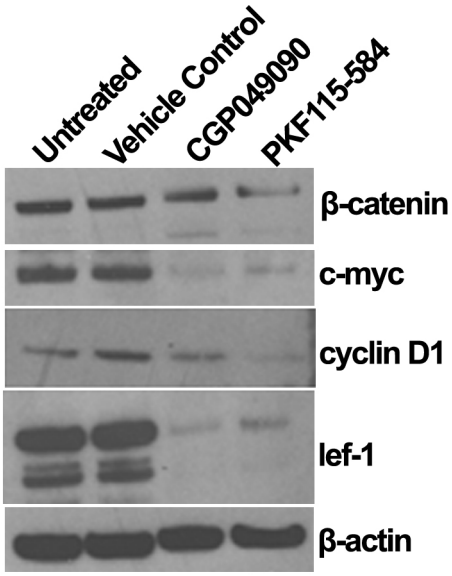

Figure 19: CGP049090 and PKF115-584 specifically inhibit LEF-1 target genes expression in JVM-3 cells. *Total cell protein extracts from JVM-3 cell lines were incubated with 5 μM of CGP049090 or PKF11584 or the vehicle control (0.02% DMSO) for 24 hours. The cell lysates were immunoblotted and probed for lef-1, β-catenin, c-myc and cyclin D1. The results reveal there is a concordant downregulation of lef-1 target genes in the lysate incubated with CGP049090 or PKF115-584. This observation was confirmed in three independent experiments.*

4.4.3 Apoptosis induced by CGP049090 and PKF115-584 is inhibited by Pan Caspase inhibitors ZVAD.FMK

Caspases exhibit highly conserved catalytic and substrate recognition motifs. This allows amino acid sequences (ZVAD) to interact with both positive and negative regulators of their activity. The feature has been exploited in the generation of peptides that compete for caspase binding (Cryns & Yuan 1998). Fluoromethyl ketone (FMK)-derivative peptides act as effective irreversible inhibitors with no added cytotoxic effects. These pan caspase inhibitors successfully inhibit their activity in vivo and in vitro (Guo & Kyprianou 1999). We used pan caspase inhibitors to determine a) whether the apoptosis induced by CGP049090 and PKF115-584 is inhibited by pre-treatment with ZVAD-fmk b) to determine whether the IAPs and lef-1 target proteins are down regulated preceding or succeeding caspase activation. We treated primary CLL cells with 3 μM of inhibitors for 16h in the presence or absence of 50 μM pan caspase inhibitor (ZVAD-fmk) with respective vehicle control for 16h. Pre treatment of CLL cells with pan caspase inhibitors lead to inhibition of caspase 3 and PARP cleavage confirming the cell death induced by the small molecule inhibitors is arrested in the presence of pan caspase inhibitors (Figure 20).

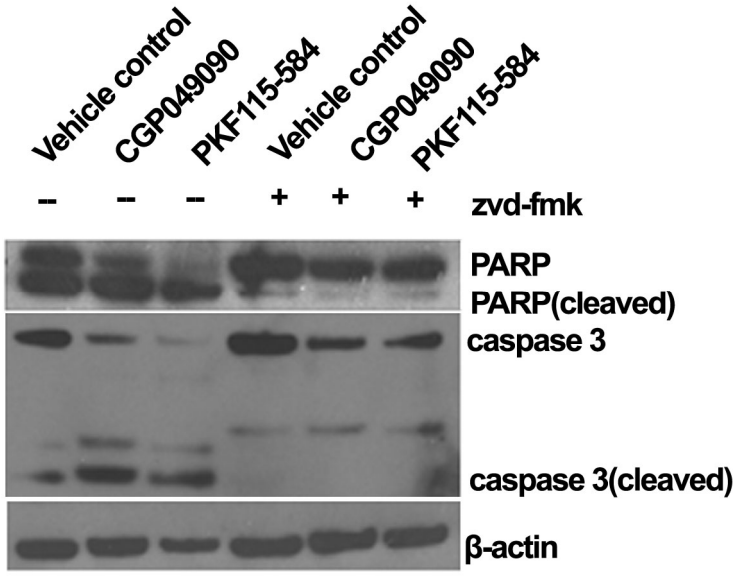

Figure 20: CGP049090 and PKF115-584 induced apoptosis in inhibited by pan caspase inhibitors. *Primary CLL cells were incubated with 50μM of pancaspase inhibitoirs for 1 hour followed by 5 μMCGP049090 or PKF11584 or the vehicle control (0.4% DMSO) for 16 hours. The cell lysates were immunoblotted and probed for cleaved PARP and caspase 3 with actin as loading control. The results reveal there is inhibition of PARP cleavage and caspase 3 cleavage in the lanes treated with pancaspase inhibitors (+) when compared to lanes without pancaspase inhibitors (-). This observation was confirmed in two independent experiments.*

4.4.4 Apoptosis induced by CGP049090 and PKF115-584 leads to cleavage of LEF-1 target proteins

The rapid onset of apoptosis also raises the question whether the downregulation of LEF-1 target genes precedes apoptosis as previously observed. However the results from the caspase inhibition experiments reveals that there was no complete down regulation of anti apoptotic proteins (XIAP, Mcl-1) and LEF-1 target genes (LEF-1, c-myc, and cyclin D1) in the presence of pan caspase inhibitors. (Figure 21). This leads to the conclusion that the CGP049090 and PKF115-584 induced caspase mediated proteolytic cleavage is responsible in

general degradation of cellular proteins rather solely on LEF-1 mediated downregulation *in vitro*.

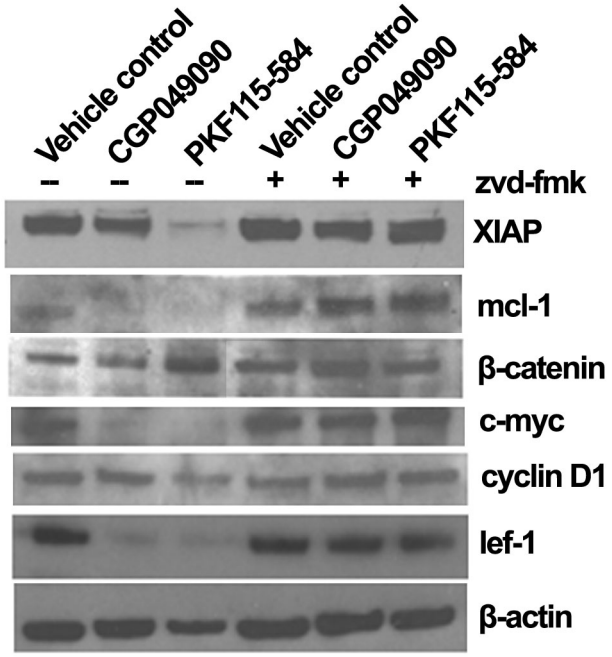

Figure 21: CGP049090 and PKF115-584 induced apoptosis cleaves several intracellular target proteins. *Primary CLL cells were incubated with 50μM of pancaspase inhibitoirs for 1 hour followed by 3 μMCGP049090 or PKF11584 or the vehicle control (0.4% DMSO) for 16 hours. The cell lysates were immunoblotted and probed for LEF-1target genes (lef-, cmyc, cyclin D1) IAPs (XIAP, mcl-1) and β catenin. The results show cleavage of all of the indicated proteine in the lanes without pancaspase inhibitors (-) when compared to lanes treated with pancaspase inhibitors (+). This observation was confirmed in two independent experiments..*

4.5. Enhanced auto-fluorescence exhibited by PKF115-584

Both the inhibitors had considerable auto-fluorescence properties due to their heterocyclic carbon rings in their molecular structure. But PKF115-584 displayed enhanced auto-fluorescent properties which could be used in localization studies. Briefly $5x10^6$ primary B-CLL cells were treated with 5μM of PKF115-584 for 6 hours. Cell were then washed in twice in PBS and resuspended in a final volume of 200μl of PBS. Cells were later fixed on to

the slides immersed in ice cold ethanol and mounted with DAPI mounting medium. Fluorescence microscopy was used to capture nuclear DAPI at excitation wavelength of 350nm and PKF115-584 at excitation wavelength of 536nm. Figure 22A shows the merged images indicating the localization of PKF115-584 in the CLL cells at 200X magnification. PKF115-584 is mostly predominantly localized in the cytosol and not in the nucleus. Figure 22B shows an inset of figure 22A revealing formation of condensed nuclei (dotted arrow) and apoptotic bodies (continuous arrow) in cells undergoing apoptosis at 1000X magnification.

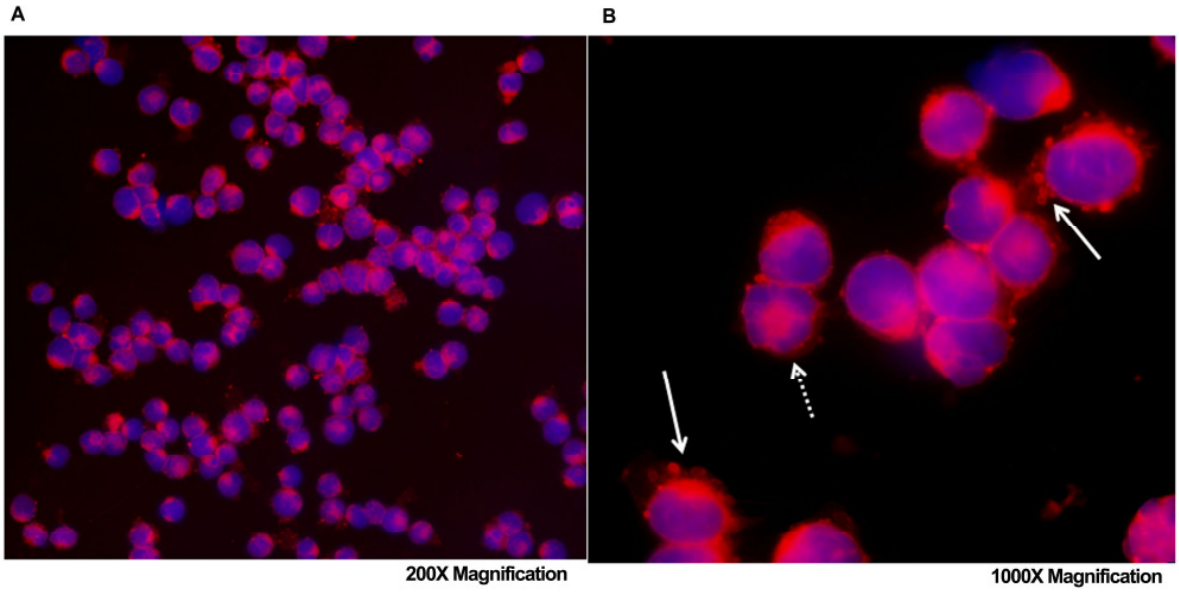

Figure 22: Intracellular localization of PKF115-584 expression. *Primary CLL cells were incubated with 5μM of PKF115-584 for 6 hours. The cells were fixed and counterstained with DAPI and later subjected to fluorescence analysis. A) Shows DAPI stained nuclei (blue) and localization of PKF115-584 (red) B) an inset of (A) showing apoptotic blebs (arrow) and condensed nuclei (dotted arrow)*

4.5.1. LEF-1 Overexpression and Intracellular Co-localization with PKF115-584 in CLL Cells.

Of the two inhibitors tested PKF115-584 had substantial auto fluorescence properties. Next we decided to treat the cells with PKF115-584 for 3 hours and lable LEF-1 by indirect immunofluorscence. Results of indirect immunofluorescence studies revealed a high

expression of LEF-1 in both, the cytoplasm and nucleus in primary CLL cells. PKF115-584 had a strong cytoplasmic localization during apoptosis, implying that the inhibition occurs in the cytoplasmic level at LEF-1 or β-catenin. Figure 23 shows the independent and merged channels of a CLL cell treated with PKF115-584 for 3h and labeled with FITC lef-1 antibody and nucleus stained in DAPI. The yellow region in the cytoplasm of the merged picture represents co-localization of lef-1and PKF115-584, suggesting possible inhibition of lef-1 transport into the nucleus by the inhibitors.

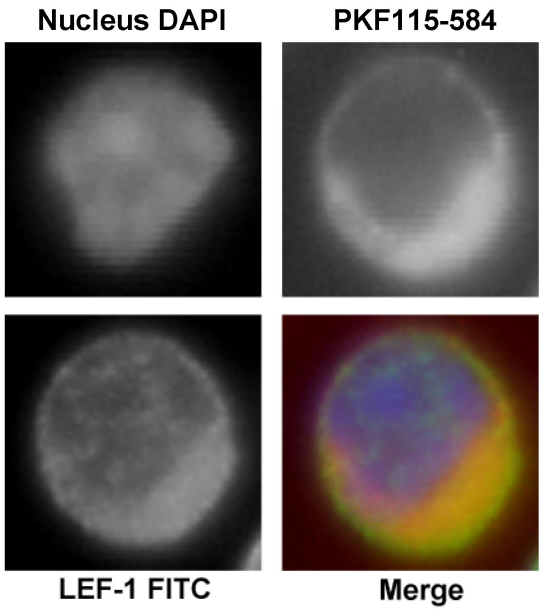

Figure 23: Co-localization of PKF115-584 and LEF-1. *$5x10^6$ primary CLL cells were incubated with 5μM PKF115-584 for 3h. The cells fixed and incubated with primary antibody (LEF-1 or isotype control) for 60 min. Cells were later incubated with secondary FITC labeled antibody for 30min. Cells were applied to ethanol rinsed microscopic glass slides, air dried counterstained by DAPI. Images were acquired by fluorescence microscopy.*

4.6. In Vivo efficacy of CGP049090 and PKF115-584 in pre-clinical mouse model.

Many of the lead substances involved in drug discovery fail to make in to the pre-clinical and clinical trial phase mostly due to lack of convincing pharmacokinetic and toxicological properties. Hence it is essential to test these inhibitors in a suitable in vivo

mouse model to elucidate the response in an in vivo setting. The in vivo efficacy studies of CGP049090 and PKF115-584 were carried in a JVM-3 subcutaneous xenograft nude mouse tumor model.

4.6.1. CGP049090 and PKF115-584 inhibit tumor growth in vivo

We first investigated the in vivo efficacy and systemic tolerance to the inhibitors in a small cohort of mice (n=4). JVM-3 cell lines were injected subcutaneously in the athymic nude mice and the tumor growth was monitored for a week. When the tumor volume reached a minimum of 80mm3, the mice were divided into 3 groups (4 mice per group) and treated intraperitoneally with 25mg/kg of CGP049090, PKF115-584, or the vehicle control every other day for 5 doses. The tumor volume was measured prior to therapy and every third day after initiation of therapy. Figure 24 shows the tumor growth response following administration of 5 doses at 25mg/kg over a period of 12 days after initiation of therapy. There was a significant cessation of tumor growth in the mice treated with CGP049090 and PKF115-584.

Table 6 shows this observation in terms of the tumor inhibitory rate (IRmax) and the rate of body weight reduction (ΔBWmax). The ΔBWmax in the vehicle treated group is was slightly elevated due to the tumor load in the animals. All of the mice in the treated group survived the course of the therapy and the observation period. Both small molecule inhibitors exert their activity specifically on tumor cells without affecting the normal tissues.

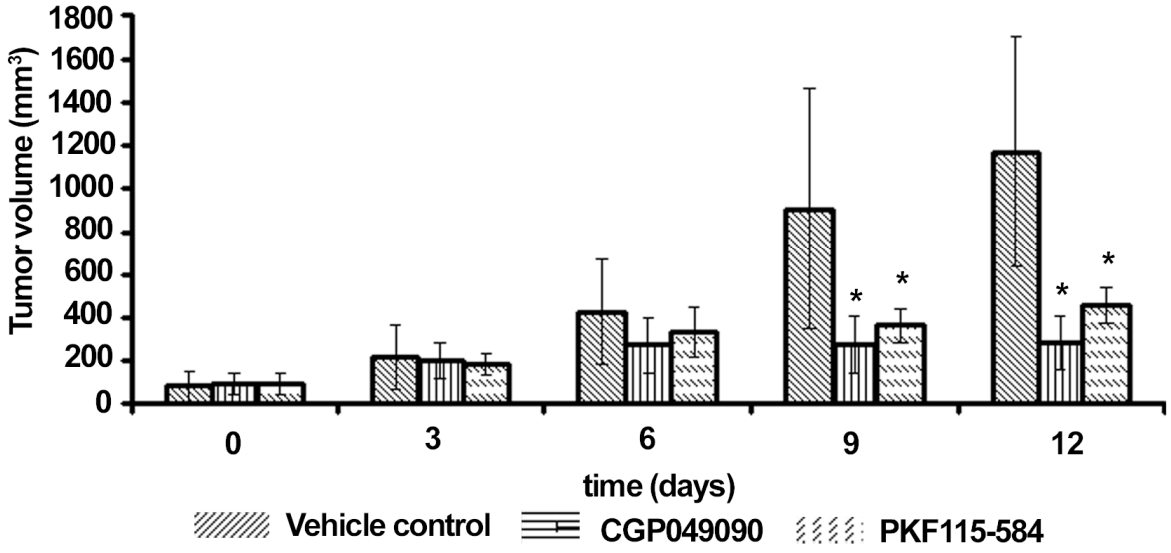

Figure 24: CGP049090 and PKF115-584 inhibit tumor growth in vivo. *Tumor volume in mice (n=four/group) treated with CGP049090 or PKF115-584 following administration of 5 doses at 25mg/kg over a period of 12 days after initiation of therapy. There was a significant cessation of tumor growth in the mice treated with both CGP049090 and PKF115-584 inhibitors.*

Group	Body weight [g]		Tumor volume [mm³]		IR_{max} [%]	ΔBW_{max} [%]
	Initial	*Final*	*Initial*	*Final*		
Control	21.5 ± 3.1	20.1 ± 3.4	84.5 ± 68.6	1166.8 ± 532		5.12
CGP049090	21.6 ± 2.4	18.7 ± 3.3	97.8 ± 47.4	†284.5 ± 12.0	78.9	7.45
PKF115-584	22.6 ± 0.8	22.0 ± 1.3	91.3 ± 50.3	†455 ± 86.4	63.8	1.46

Mean ± s.e. †$p<0.05$

Table 6. Therapeutic effect and systemic toxicity of CGP049090 and PKF115-584 (25mg/kg) in JVM-3 xenografts in nude mice*. IRmax indicates the greatest effect of each test inhibitor. ΔBW is the rate of body weight reduction. The maximal value for ΔBW was designated as ΔBWmax, from which each test compound's toxicity to mice was evaluated. *Mean±s.e. † $p<0.05$*

4.6.2. CGP049090 and PKF115-584 increases the median survival of the treated mice

The previous experiment was repeated in a larger cohort of mice (n=8 per group) and treated with a higher dose of 25mg/kg bodyweight for 12 days and tumor volume and survival was observed for 30 days. The tumor volume was measured every third day and mice which reached a maximum tumor volume of $1000mm^3$ were removed from the study and euthanized due to ethical reasons. The day of euthanization due to the enlarged tumor volume was considered as a death event for Kaplan-Meier survival analysis. Figure 25 shows the Kaplan-Meir analysis of CGP049090 and PKF115-584 treated mice compared to the control.

Kaplan-Meier survival curves reveal that treatment significantly improves median survival by 12.5 days (p= 0.003) with CGP049090 and 15.5 days (p=0.0023) with PKF115-584. These data suggest that both the both the inhibitors have a respectable tendency to inhibit tumor growth and extend the survival in the control group.

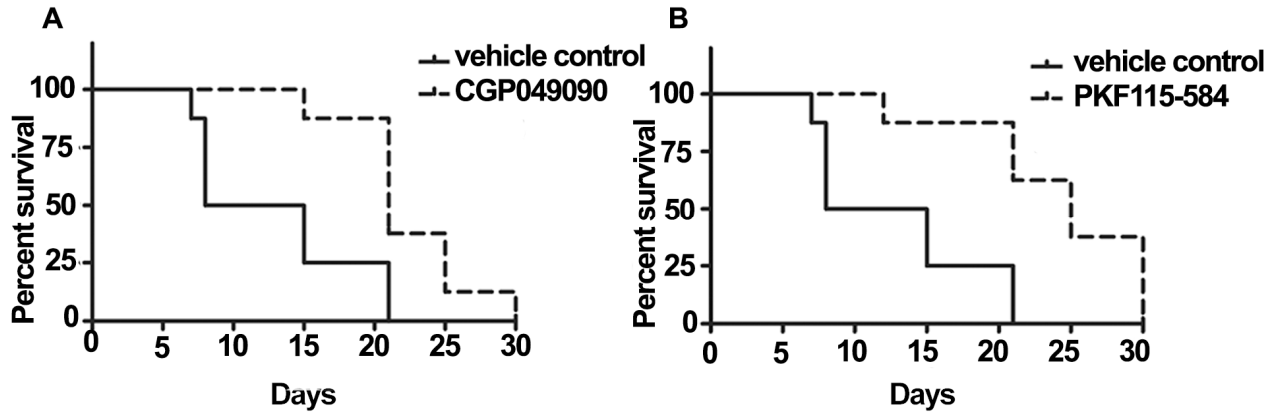

Figure 25: CGP049090 and PKF115-584 increases the median survival of the treated mice. *The survival benefit conferred to the group treated with CGP049090 was 12.5 days (p=0.003) and 15.5 days (p=0.0023) with the group treated with PKF11-584 when compared to the group treated with vehicle only.*

4.6.3. Tumor growth cessation by CGP049090 and PKF115-584 in vivo is mediated by inhibition of cellular proliferation and apoptosis

We could demonstrate that both small molecule inhibitors inhibit tumor cell proliferation in the pre-clinical JVM-3 xenograft mouse model. Figure 25 shows the visual tumor growth in a representative mouse of each group. The mice treated with the vehicle shows an enlarged subcutaneous tumor (A) whereas the mice treated with CGP049090 and PKF115-584 show a smaller tumor with a central red necrotic patch indicative of cellular termination (B & C).

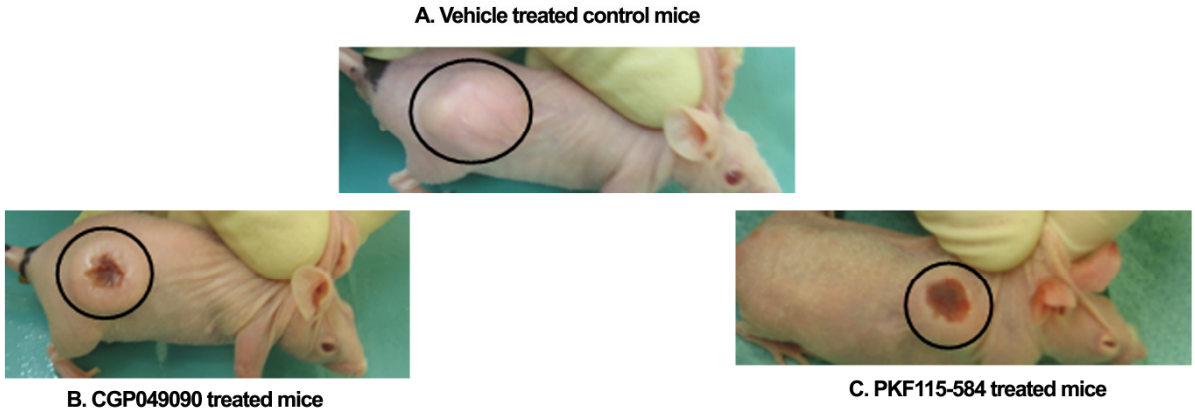

Figure 26: CGP049090 and PKF115-584 inhibit in vivo tumor growth. *After 6 doses of therapy (25mg/kg) a representative mouse from each group was inspected for tumor growth. The vehicle treated mice (A) shows an enlarged tumor without central area of necrosis, whereas mice treated with the respective inhibitors (B & C) show a reduced tumor with a central necrosis indicative of cell death at the central regions of the tumor due to the therapy*

4.6.4. LEF-1 is downregulated upon treatment with CGP049090 and PKF115-584 *in vivo*

To understand the mechanism of inhibition of CGP049090 and PKF115-584 *in vivo* paraffin embedded tumor sections were made from the tumors derived from the vehicle control, CGP049090 and PKF115584 for immunohistochemistry analysis. Immunohistochemical staining using LEF-1 antibody revealed that there was a distinct downregulation of LEF-1 in the treated samples when compared to the vehicle control. Figure 26 shows uniform LEF-1 expression in the vehicle treated control, whereas sporadic expression in the treated samples. This indicates that both small molecule inhibitors function in an expected manner by targeting LEF-1 expression *in vivo*.

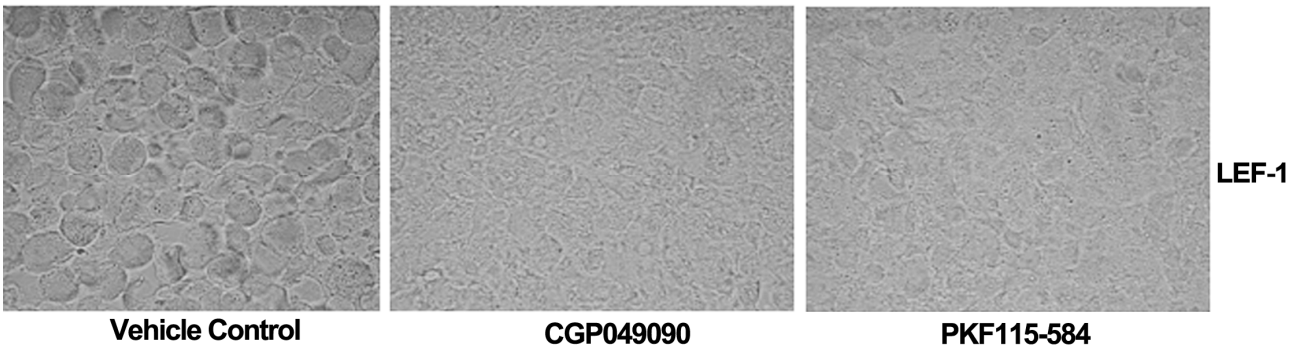

Figure 27: CGP049090 and PKF115-584 inhibit LEF-1 expression in vivo. *After 12 doses of therapy (25mg/kg) a representative mouse from each group was sacrificed and the tumor was used to generate paraffin embedded sections and subsequently labeled for LEF-1 by immunohistochemistry. The vehicle treated mice (Control) shows uniform expression of LEF-1 when compared to the sporadic expression seen in mice treated with CGP049090 and PKF115-584.*

4.6.5. *In Vivo* Inhibition of Proliferation (PCNA) and Increase of Apoptosis (cleaved PARP) after CGP049090 and PKF115-584 treatment.

In order to distinguish the mode of cell death from apoptosis and necrosis due to possible acute toxicity of the inhibitors in vivo, paraffin embedded tumor sections were made from the tumors derived from the vehicle control, CGP049090 and PKF115584 for immunohistochemistry analysis. Proliferating Cell Nuclear Antigen (PCNA) is a member of the DNA sliding clamp family of proteins that assist in DNA replication and acts as a co-factor for DNA polymerase delta (Kelman & O'Donnell 1995). PCNA is expressed in the nuclei of cells during the DNA synthesis phase of the cell cycle and hence considered as a marker for proliferation (Leonardi *et al.* 1992). The immunohistochemical analysis revealed that PCNA expression was completely inhibited in the mice treated with CGP049090 and PKF115-584 when compared to the vehicle control. This indicates that the cells in the treated group are not in an active state of proliferation due to the treatment with the small molecule inhibitors (Figure 27A).

On the other hand to reveal the inhibition of cell proliferation resulted in apoptotic cell death the tumor sections were probed for the presence of cleaved PARP by immunohistochemistry. As mentioned earlier PARP, a 116kDa nuclear poly (ADP-ribose) polymerase, appears to be involved in DNA repair in response to environmental stress (Satoh & Lindahl 1992). This protein is one of the main cleavage targets of caspase-3 *in vivo*. In human PARP, the cleavage occurs between Asp214 and Gly215, which separates the PARP amino-terminal DNA binding domain (24kDa) from the carboxy-terminal catalytic domain (89kDa) (Nicholson et al. 1995) hence cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis. The immunohistochemical analysis reveal that there was no cleaved PARP detectable in the control tumor sample but increased incidence of cleaved PARP in the mice treated with CGP049090 and PKF115-584. This indicates that cells in the treated group are undergoing apoptotic cell death (Figure 27B).

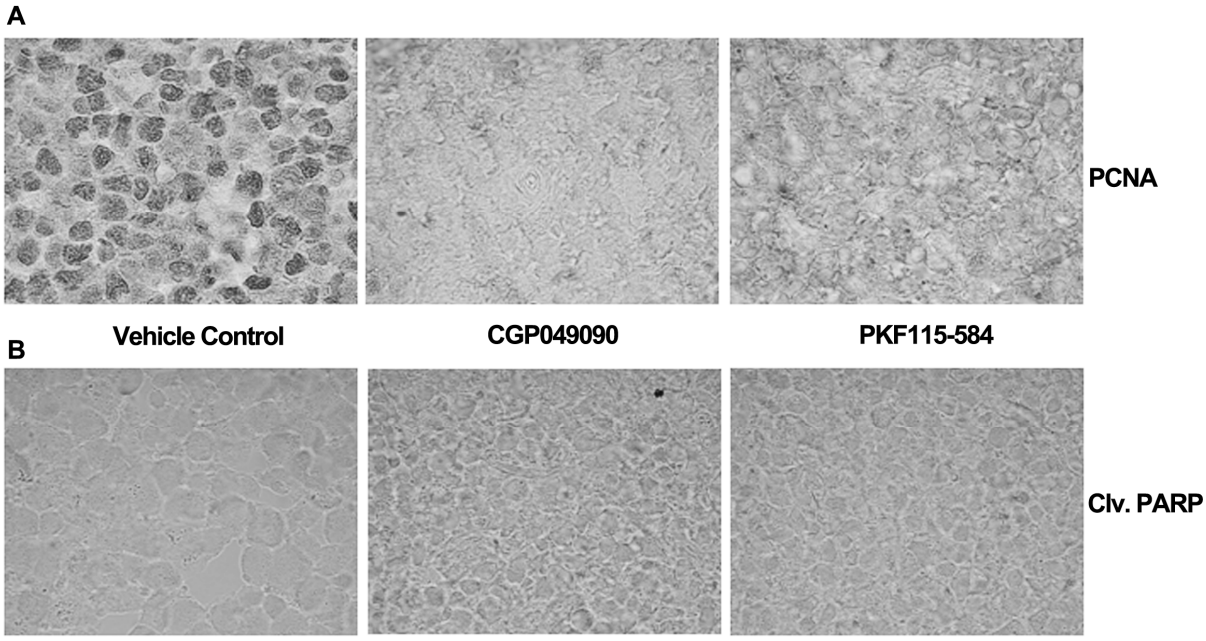

Figure 28: CGP049090 and PKF115-584 inhibit proliferation and increase apoptosis in vivo. *After 12 doses of therapy (25mg/kg) a representative mouse from each group was sacrificed and the tumor was used to generate paraffin embedded sections and subsequently labeled for PCNA (A) as marker of proliferation and Cleaved PARP as marker for apoptosis (B) by immunohistochemistry. There was a decrease in PCNA expression and increased cleaved PARP in mice treated with CGP049090 and PKF115-584.*

5. Discussion

The concept of translational research has received very strong focus in the biomedical community over the last few years, as a new way of thinking and conducting life science research to accelerate healthcare outcomes. According to NCI definition (National Cancer Institute, USA), 'Translational research transforms scientific discoveries arising from laboratory, clinical, or population studies into clinical applications to reduce cancer incidence, morbidity, and mortality.(2009). Hence there is a constant need for rendering the findings of basic research to into clinical application. This study is one such attempt to translate findings from basic research into clinical relevance.

Chronic lymphocytic leukemia (CLL) is a disease characterized by accumulation of dysfunctional B-cells with significant resistance to apoptosis, thereby enhancing their survival *in vivo* (Dighiero & Binet 2000). As CLL is not driven by a single genetic variation, targeting intracellular proteins would be an interesting therapeutic approach. Understanding the pathogenesis of this disease would unravel critical therapeutic targets. Among the signaling pathways involved in the manifestation of the B CLL, the Wnt pathway is a major signaling system that is constitutively turned on in B CLL cells leading to expression of several genes involved in survival.

Much of the understanding about Wnt signaling has been primarily derived from lower organisms such as drosophila, C.elegans and xenopus embryos. The Wnt pathway is a critical determinant of cell proliferation during early development and regenerative processes, whereas its completely switched off in mature cellular phenotypes except stem cells in the adults (Clevers 2006). Aberrant activation of the Wnt pathway has been linked to oncogenesis in multiple systems usually involving gain/loss of function mutations of different proteins of the pathway (Janssens et al. 2006). In CLL aberrant expression of Wnt ligands and epigenetic alterations of physiological Wnt inhibitors has been implicated in the extended survival of CLL cells in vivo (Lu *et al.* 2004; Chim *et al.* 2008). Moreover LEF-1 the transcription factor

which is highly over-expressed exclusively in CLL cells has been overlooked regarding its role in transcriptional significance in the overall survival of CLL cells. There are currently no reports on the role of LEF-1 in survival of CLL cells. The purpose of this study is to determine the role LEF-1 in the survival of CLL cells and validate it as a therapeutic target using the novel LEF-1/β-catenin small molecule inhibitors CGP049090 and PKF115-584.

5.1. Expression of LEF-1 and β-catenin in CLL

Previous reports have indicated the indicated the high LEF-1 expression in mRNA levels but there are no reports on actual expression levels at the protein level (Klein *et al.* 2001; Howe & Bromidge 2006). Hence we first set to examine the endogenous basal expression of LEF-1 in primary B-CLL cells when compared to healthy B cells at the protein level. LEF-1 was consistently and highly expressed in CLL cells but completely undetectable in healthy B cells. B-CLL cells also express short isoform of LEF-1 with the β-catenin binding domain derived from an intronic promoter. However the shorter isoform without the β-catenin binding domain cannot function in transcription activity in the nucleus (Hovanes *et al.* 2001). The peripheral blood mononuclear cells (PBMCs) express restrained amount of LEF-1 as they are expressed in T cell compartments (Travis *et al.* 1991). However the JVM-3 and MEC-1 cell lines cells do not express similar levels of LEF-1 as the primary CLL cells since these cell lines are prolymphocytic cell lines and do not represent the true CLL characteristics (Figure 6). These findings are coherent with previous studies undertaken at the mRNA levels.

β-catenin is essential for LEF-1 transactivation however, we could observe a steady detectable pool of both phosphorylated and de-phosphorylated forms of β-catenin. The maintenance of de-phosphorylated β-catenin is attributed to autocrine (Wnt ligands) and paracrine action (microenvironment) (Caligaris-Cappio 2003; Ghia et al. 2002). Upon BCR ligation, activation of PI3K/Akt pathway which inhibits gsk3-β in the CLL cells, also leads to stabilization of de-phosphorylated β-catenin (Christian et al. 2002).

On the other hand, the grounds for the overexpression of LEF-1 in CLL have not yet been identified. Wnt signaling can promote the expression of several Wnt pathway components, indicating that feedback control is a key feature of Wnt signaling regulation. One could speculate that any loss in one or several of these feedback loops could contribute to the overexpression of LEF-1 in leukemic B cells.

Moreover, the functional significance of LEF-1 and β-catenin is only complete when both of the proteins are localized in the nucleus involving in transactivation of target genes. Figure 7 shows that LEF-1 (short and long forms) is predominantly localized in the nucleus along with β-catenin indicating active LEF-1/ β-catenin transcriptional co-activation.

Hence the aberrant activation of Wnt signaling is in cooperation with massive expression of LEF-1 and a steady pool of de-phosphorylated β-catenin in primary CLL cells.

5.2. LEF-1 and B-CLL survival

Since LEF-1 is over-expressed in CLL and has several target genes to be involved in survival and proliferation (Table 1) it is expected that they have a vital role to in survival of CLL cells. siRNA mediated LEF-1 knockdown experiments in primary CLL cells resulted in increased apoptosis in CLL cell which directly implies the important role of LEF-1 in CLL survival. One major drawback in this approach is the that primary CLL cells undergo spontaneous apoptosis upon in vitro culture (Collins et al. 1989) and is a major hurdle in performing functional experiments in vitro in CLL cells (Seiffert et al. 2007). This has led to poor transfection efficiencies (max 25%) and poor viability post transfection as seen from figure 9. Nevertheless there was a statistically significant change in cell survival upon siRNA mediated LEF-1 inhibition. To further understand role of LEF-1 in B-CLL cells we transfected JVM-3 cell lines with LEF-1 siRNAs. Interestingly, the there was a decrease of LEF-1 target genes (c-myc, cyclin D1) but no PARP or β-catenin cleavage at 72 hours. This indicates that there was no initiation of apoptosis upon LEF-1 inhibition in JVM-3 cell line. But there was decrease in PCNA expression which is widely considered as a proliferation

marker indicating growth arrest in the cell line. The difference in the outcome (apoptosis Vs growth arrest) of siRNA mediated LEF-1 inhibition in primary CLL cells and cell lines is that the primary CLL cells are already in G0 cell cycle arrest and are mostly non proliferative (Hamblin & Oscier 1997), whereas JVM-3 cell lines are EBV transformed and are in high proliferative rate (Melo et al. 1988). But in either of cases the LEF-1 knockdown leads to apoptosis in resting CLL cells or leads to growth arrest in JVM-3 cell lines indicating the clinical importance of targeting LEF-1 signaling in CLL cells. Thus LEF-1 might represent a promising therapeutic target in CLL belonging to both the subgroups of CLL.

5.3. In vitro Efficacy of CGP049090 and PFK115-584 CLL

Both CGP049090 and PKF115-584 are known to inhibit TCF/LEF-1/β-catenin interaction (Lepourcelet et al. 2004). It was most interesting to test the efficiency of these inhibitors in B-CLL model due to the over-expression of LEF-1. The dose response curves generated showed similar results in CLL cell lines (MEC-1 and JVM-3) and primary CLL cells. The LC50 was less than 1µM in the cell lines tested leading to a complete cell kill at the highest tested concentration (10µM). Similarly treating 24 heterogeneous primary CLL samples we determined the LC50 to be 0.89µM for CGP049090 and 0.77µM for PKF115-584. LC50 values fall in the same range as in the previous studies, which tested these inhibitors in colon carcinoma, prostate cancer and multiple myeloma cell lines (Lepourcelet *et al.* 2004; Lu *et al.* 2004; Sukhdeo *et al.* 2007). It has to be noted that another study attempted the same in CLL using the β-catenin-inhibitor R-etodolac which was unsuccessful due to its high LC50 for more than 250µM (Lu et al. 2004). Similarly, another novel etodolac SDX-308, tested in multiple myeloma cells was found to target the TCF/LEF-1/ β-catenin pathway but the LC50 values were more than 30µM and was not tested *in vivo*.

The % cell kill at 1µM (Table 5) showed variable cytotoxcity and no correlation to the prognostic factors which indicates that the inhibitors have their cytotoxic effects in both subsets of CLL.

The foremost importance of any targeted therapy is that the healthy counterparts are spared from the cytotoxic activity. Hence we tested these compounds in purified B cells from healthy volunteers. Interestingly healthy B cells were not affected as 10µM was not enough to achieve the same complete cell kill as observed in leukemic cells. The estimated LC50 was 5.7µM for PKF115-584 and 8.5µM for CGP049090 respectively. Furthermore these inhibitors do not affect the healthy PBMCs as seen from the estimated LC50 values of 37.83µM and 113.31µM for PKF115-584 and CGP049090, shown previously by our group (Minke et al. 2009).

Since both CGP049090 and PKF115-584 are heterocyclic compounds they exhibited pronounced auto-fluorescence properties. We utilized this property in PKF115-584 to localize the drug in the cell with respect to LEF-1. As observed earlier in western blots (Figure 7) we could see LEF-1 expression in both cytoplasm and in the nucleus which was confirmed by immunofluorescence studies (Figure 23). This concludes that the inhibition of LEF-1/β-catenin occurs at the cytoplasmic level as PKF115-584 was not localized in the nucleus.

5.4. Mechanism of apoptotic Induction by CGP049090 and PKF115-584

CLL is the disease of failed apoptosis. In the present work we show that both small molecule inhibitors show pro-apoptotic activity in CLL patient cells and cell lines. Upon treatment the CLL cells exhibited all the typical hallmarks of apoptosis, such as activation of caspases, cleavage of PARP and membrane blebing. Bcl-2 family of proteins are known to be critical regulators of apoptosis and have been implicated in CLL (Packham & Stevenson 2005). We found moderate down regulation of Bcl-2 upon incubation with CGP049090 and PKF115-584. However we observed complete down-regulation of Mcl-1 and XIAP upon treatment. Recent study shows that Mcl-1 expression in CLL is correlated with survival and chemoresistance in CLL cells (Saxena *et al.* 2004), hence downregulation of Mcl-1 plays an important role in apoptotic induction in CLL cells. XIAP is physiological inhibitor of caspase 3, 7 and 9 and efficiently inhibits apoptosis (Deveraux & Reed 1999). However upon

treatment with CGP049090 and PKF115-584 there is a downregulation of XIAP which accelerates the apoptotic process in CLL cells.

Apoptosis can be initiated by the death-receptor (extrinsic) pathway that acts through caspase 8 or mitochondrial (intrinsic) pathway that acts through caspase 9, but both pathways converge to activate the effector caspases (caspase 3), which act on the death substrates (Reed 2000). In order to differentiate the mode of apoptotic induction from intrinsic or extrinsic pathway we checked for bid cleavage. If the apoptotic signal is from the extrinsic pathway, caspase 8 is activated which cleaves bid to tbid (Li et al. 1998). The tbid translocates from the cytoplasm into the mitochondria thereby initiating the release of cytochrome C which in turn forms a complex with APAF-1 (apoptosis-activating factor 1) which in turns activates caspase 9. However the time course experiments on JVM-3 cells indicate that there was no significant bid cleavage between 6-12 hours despite caspase 8 cleavage. But during the same 6-12 hour time frame we could see a significant cleavage of caspase 9 (Figure 14). The active caspase 9 cleaves pro-caspase 3 into it active form which in turn cleaves several intracellular targets like PARP and β-catenin.

However upon cellular stress, by radiation or cytotoxic drugs the cytochrome C is released by the intrinsic pathway from the mitochondria which then forms complex with APAF-1 leading to subsequent activation of caspase 9 and caspase 3 (Reed 2000). Since there was no bid cleavage upon incubation with CGP049090 and PKF115-584, it is obvious that these small molecules induce apoptosis through the intrinsic pathway.

Recently dasatinib, a src kinase inhibitor was found to be only effective *in vitro* with preference for a subgroup of patients with unmutated IgVH genes (Johnson *et al.* 2006; Veldurthy *et al.* 2008). Since CLL is a heterogeneous disease with different subsets determining the prognosis of the disease, our results show that both CGP049090 and PKF115-584 have similar effects independent of both subsets of CLL tested (ZAP70/CD38) which could be advantageous in a clinical perspective.

Both inhibitors induce apoptosis in non-proliferative primary CLL cells and an EBV-transformed CLL cell line, in a dose dependent mechanism indicating that these inhibitors are excellent choices for targeting LEF-1 in clinical treatment of the disease.

5.5 Specificity of the CGP049090 and PKF115-584

Both substances are known to have higher affinity to inhibit the TCF/LEF-1/ β-catenin complexation (Lepourcelet et al. 2004). Co-immunoprecipitation (co-IP) experiments were done verify this claim in CLL model. Our study was limited to perform co-IP experiments ex vivo on crude total cell protein extracts as there was a rapid induction of apoptosis and a steady loss of β-catenin due to caspase activation (Steinhusen *et al.* 2000). As expected both small molecule inhibitors effectively antagonize LEF-1/ β-catenin complex formation *in vitro* confirming previous studies. There are considerable evidences from previous studies that these small molecule inhibitors inhibit LEF-1/catenin interaction from TOPFLASH/FOPFLASH reporter assays (Sukhdeo *et al.* 2007; Doghman *et al.* 2008). However our *in vitro* studies employing pan caspase inhibitors revealed that these small molecules inhibitors induced apoptosis preceding down regulation of LEF-1 target proteins in CLL cells (Figure 21). This suggests that silencing LEF-1 activity and induction of apoptosis are dual properties/events exhibited by both CGP049090 and PKF115-584. This can be supported from comparing the results of the siRNA mediated LEF-1 knockdown (Figure 10) Versus CGP049090 and PKF115-584(Figure 19) experiments in JVM-3 cell line. siRNA mediated LEF-1 knockdown in JVM-3 cell line lead to growth inhibition (reduction of PCNA, PARP) but no apoptosis in JVM-3 cells, whereas CGP049090 and PKF115-584 induced apoptosis(cleaved PARP). However the underlying mechanism explaining the induction of apoptosis by these inhibitors remains to be elucidated. Current evidences suggest that both CGP049090 and PKF115-584 induce cell cycle arrest at G1/S phase (Sukhdeo *et al.* 2007).

This was also reflected in the downregulation of TCF/LEF-1 target genes in CLL. In the present work we have show downregulation of LEF-1 target genes c-myc, cyclin D1 and LEF-1 in primary CLL cells as upon incubation with the small molecules. This downregulation is due to apoptotic induction rather than complete LEF-1 knockdown as seen from figure 21. Nevertheless LEF-1 knockdown experiments in using siRNA in JVM-3 cell line indicate downregulation of the target genes (Figure 10) c-Myc is one of the most important targets of LEF-1 signaling with respect to malignant diseases. c-Myc is a transcription factor which governs the expression of 15% genes in the human genome involved in cell division, cell growth, and apoptosis (Dang et al. 2006; Zeller et al. 2003). Hence indirect c-myc knockdown deprives the survival advantages of the CLL cells. Another study which targeted c-myc in MCF-7 breast tumor cells by siRNAs lead to significant inhibition growth of tumor cells *in vitro* and *in vivo* (Wang *et al.* 2005b).

Cyclin D1 has been disregarded in B-CLL proliferation and its apoptotic block. Very little is known about its role in the pathogenesis in CLL, although in some cases, it has been used as a prognostic marker in CLL (Faderl *et al.* 2002). Hence, cyclin DI downregulation must be regarded as a surrogate for LEF-1 inhibition in the context of our work, but no statement can be made about its functional relevance. Moreover, further studies are needed to examine the complete set of genes which are modulated upon incubation with the inhibitors by microarray analysis.

Although one would expect that the selective leukemic cell killing is directly related to inhibition of LEF-1 expression, one has to admit that most of the small molecule inhibitors *do* show off-target effects on cells. However to what extent CGP049090 and PKF115-584 show off target effects remains to be elucidated.

5.6. In vivo efficacy of CGP049090 and PKF115-584

Despite the promising results *in vitro*, there are many factors that influence the activity of a compound during *in vivo* applications. Factors such as solubility, bioavailability and

toxicity of the drug, play a vital role in the pre-clinical drug development. On the other hand, the drug has to overcome the cellular interactions and reactions in the *in vivo* microenvironment which can rescue leukemic cells from cytotoxic agents. For example flavopiridol, a cyclin-dependent kinase inhibitor, showed promising results *in vitro* but failed to have any activity in the clinical setting (Flinn et al. 2005).

To address these issues we first treated a small cohort of non-transplanted mice with 50mg/kg bodyweight daily of CGP049090 or PKF115-584 for 14 days and ascertained that the mice did not develop any gross systemic toxicity (data not shown). This is also consistent to a previous study which investigated pharmacokinetics and metabolism of another compound, calphostin C, which structurally resembles PKF115-584 (Chen et al. 1999). The authors reported that i.p. administration of 40mg/kg bodyweight in CD1 mice resulted in therapeutically relevant plasma levels (Cmax = 2.9 μM). Moreover, no toxic signs or fatalities could be observed in the animals during a 30 day observation period after administration.

Although it is always appropriate to test the small molecules in a suitable B-CLL mouse model, due to the nature of the disease, there is no single animal model representing true CLL characteristics in a mouse model. Although several transgenic mouse models are being currently studied to understand the pathogenesis of CLL (Pekarsky et al. 2007), none of these models were suitable for testing pre-clinical drugs. Nevertheless we attempted to test these inhibitors in a transgenic Eμ-TCL1 model (Johnson *et al.* 2006) and were highly unsuccessful due to unpredictable deaths in both treated and control groups and the extended period of time for the Eμ-TCL-1 transgenic mice to develop leukemia. Therefore, owing to the lack of a convincing *in vivo* tumor model for CLL we carried out *in vivo* studies in JVM-3 subcutaneous xenografts. The results of the experiments indicated that all of the mice in the treated groups survived the entire regimen with a significant tumor inhibition (Figure 24, 26; Table 6) and showed extended survival (Figure 25) when compared to the controls regardless of high proliferative rate exhibited by the EBV transformed JVM-3 cells.

Both substances were well tolerated *in vivo,* as observed from the ΔBWmax values. Interestingly there were no toxicity induced deaths encountered in the animal experiments indicating both CGP049090 and PKF115-584 do not show lethal systemic toxicity. Although PKF11-584 was tested *in vivo* in multiple myeloma xenograft model with convincing results, but revealed bone marrow hypoplasia due to inhibition of Wnt signaling in hematopoietic stem cells which is vital for their self-renewal and proliferative capacity, thereby leading to depleted stem cell populations (Sukhdeo *et al.* 2007). In this present study for the first time we showed that CGP049090 also possesses anti-leukemic properties *in vivo*.

We set to determine the mechanism of tumor inhibition in vivo. Immunohistochemistry analysis of the tumor tissue from the treated and control groups clearly indicate reduced of LEF-1 expression in the treated group (Figure 27). This is then reflected in reduced proliferation as seen PCNA expression and increase in cleaved PARP (Figure 28A & B) indicating ongoing apoptosis. PKF115-584 has been previously demonstrated to block cells entering into the S phase of the cell cycle and inducing apoptotic genes (Sukhdeo *et al.* 2007). Our result correlated with this finding, evidenced by the reduced PCNA expression and increased apoptosis (PARP cleavage) seen *in vivo* seen upon treatment with CGP049090 and PKF115-584.

5.7 Off-target effects of CGP049090 and PKF115-584

Although one would expect that the selective leukemic cell killing is directly related to inhibition of LEF-1 expression, the small molecule inhibitors also *do* show off-target effects in the cells. There are recent debates in the scientific community on the need of 'dirty drugs' that can target multiple proteins in the tumor cell rather than one single target to cure cancer, since there are several deregulated pathways in cancer promoting oncogenesis and the cancer cells are able to adapt and generate drug resistance (Fojo 2008). For instance, in Glioblastoma Multiforme (GBM) a brain tumor involving glial cells have activation of several receptor tyrosine kinases (RTKs) and hence targeting any one of these RTKs with available tyrosine

kinase inhibitors did not inhibit the tumor growth (Stommel et al. 2007). Imatinib (Gleevec™), a small molecule tyrosine kinase inhibitor, that specifically targets a kinase in the bcr-abl fusion protein, had a tremendous impact in the treatment of Chronic Myeloid Leukemia (CML) (Druker & Lydon 2000). However after promising initial response, the patients started to relapse dramatically and further investigations revealed 17 different point mutations in the binding domain of imatinib rendering it ineffective (Shah et al. 2004).

Since CLL is a disease of multiple deregulated pathways there is need for novel drugs which can bind to several signaling pathways to cure the disease. Whether CGP049090 and PKF115-584 exhibit this property is worth to be investigated.

5.8 Future Perspectives:

LEF-1 is a potent transcription factor regulating the expression of several important genes. Oncogenic forms of LEF-1 are known to be expressed in both solid and hematological malignancies. Colorectal and epithelial cancers express the highest LEF-1 transcripts followed by brain and testicular cancers in solid tumors. In hematological malignancies, overall LEF-1 level was higher in lymphocytic leukemias compared with myeloid leukemias. However, acute myeloid leukemia and acute lymphocytic leukemia showed a significantly increased fraction of the oncogenic LEF-1 compared with chronic lymphocytic leukemia and chronic myeloid leukemia (Wang *et al.* 2005a). The present study resolved LEF-1 to be an effective therapeutic target in B-CLL. Hence inhibiting LEF-1 could be an effective therapeutic strategy to counter other malignancies with oncogenic LEF-1.

There is also a need to understand the underlying mechanism governing LEF-1 overexpression in CLL. The trigger which drives LEF-1 expression in CLL cells but not in healthy B cells is yet to be identified. Since LEF-1 is controlled by a negative feedback loop, it can be speculated that any mutations in genes involved in the feedback loop (Dickkopf-1, LRP) may result in loss of regulation of LEF-1 in CLL cells.

Despite acceptable efficacy of CGP049090 and PKF115-584 *in vitro* and *in vivo* in the present study further pharmacokinetic studies are necessary on the small molecules to determine the bioavailability in higher primates. Both CGP049090 and PKF115-584 are potential anti-cancer agents in other neoplastic malignancies with aberrant LEF-1/TCF transcriptional activity

6. *Materials & Methods*

6.1. Materials

6.1.1. Instruments

Equipment	Supplier
Automatic pipettes	Eppendorf, Germany
Amaxa Nucleofector device I	Amaxa, Germany
Balance	Sartorius, Germany
Cell counting chambers	Neunauer, Germany
Cell freezing containers	Nalgene, Belgium
Centrifuge (bench top/4°C)	Eppendorf, Germany
Centrifuge (cell culture)	Heraeus, Germany
Electrophoresis system (protein)	Invitrogen, USA
Electrophoresis System (DNA)	Biorad, USA
Fluorescent microscope	Olympus, Japan
Incubators	Thermo Scientific, USA
Ice machine	Scotsman, USA
FACS Canto Flow cytometer	BD Bioscience, Germany
Fridge 4°C & -20°C	Bosch, Germany
Freezer -80°C	Bosch, Germany
Heating blocks	Biometra, Germany
Laminar flow hoods	Heraeus, Germany
Light Microscopes	Leitz, Germany
Microtome	Leica, Germany
pH-meter	InoLab, Germany
Shaker	IKA, Germany

Ultrasonicator	Bandelin Sonoplus, Germany
Spectrophotometer	Eppendorf, Germany
Tissue processor	Leica Histokinette, Germany
Vortex	Bender & Hobein, Germany
Water bath	GFL, Germany
Western Blot system	X cell II Blot Module, Invitrogen, USA

6.1.2. Consumables

Consumables	**Supplier**
8-12% gradient pre cast gels	Invitrogen, Carlsbad .USA
Hyperfilm ECL	Kodak, United Kingdom
Nitrocellulose membranes	Invitrogen, Carlsbad/USA .USA
Parafilm	American national Can, IL, USA
Needles	BD Biosciences, NJ, USA
Tissue culture material	
Cryotube vials	Nunc, Roskilde, Denmark
Pipette tips, tubes, dishes flasks	Sarstedt, Germany
Transwell 96 well plates	Corning Life sciences, MA, USA

6.1.3. Chemical and Reagents

Substance	Supplier
4x sample buffer	Invitrogen, Carlsbad .USA
BSA (albumin)	Carl Roth GmbH, Karlsruhe, Germany
Cremophor-EL	Sigma, Steinheim, Germany

Dithiothreitol (DTT)	Sigma, Steinheim, Germany
DMEM	Gibco Karlsruhe, Germany
DMSO	Carl Roth GmbH, Karlsruhe, Germany
Ethanol	Carl Roth GmbH, Karlsruhe, Germany
Fetal Calf Serum (FCS)	Gibco Karlsruhe, Germany
Formaldehyde 4%	Carl Roth GmbH, Karlsruhe, Germany
Ficoll-hypaque	Amersham Bioscience, Buckinghamshire, UK
Hydrogen peroxide 3%	Carl Roth GmbH, Karlsruhe, Germany
Isopropanol	Carl Roth GmbH, Karlsruhe, Germany
L-15 media	Gibco Karlsruhe, Germany
Methanol	Carl Roth GmbH, Karlsruhe, Germany
Non fat dry milk powder	Carl Roth GmbH, Karlsruhe, Germany
PBS (D-PBS)	Gibco Karlsruhe, Germany
PBS (ca & mg free)	Gibco Karlsruhe, Germany
Penicillin/streptomycin	Gibco Karlsruhe, Germany
Ponceau S	Sigma, Steinheim Germany
Protein G beads	Miltenyi Biotec, Bergisch Gladbach, Germany
RPMI-1640	Gibco Karlsruhe, Germany
SDS PAGE molecular weight standard	Invitrogen, Carlsbad/USA
Trypsin EDTA	Gibco Karlsruhe, Germany
Xylene	Carl Roth GmbH, Karlsruhe, Germany

6.1.4. Reagent/ Kits

Annexin V-FITC Apoptosis Detection Kit	BD Biosciences, New Jersy, USA
Amaxa cell line nucleofection kit V	Amaxa, Cologne, Germany
CellTiter-GLo® Luminiscent Cell viablity Assay	Promega, Madison, USA
ECL Plus immunoblot detection reagents	Amersham Bioscience, New Jersy,USA
M-PER Mammalian Protein Extraction	Reagent Thermo Fisher Scientific, Leicestershire UK.
Protease Inhibitor cocktail tablets	Roche Applied Science, Mannheim Germany
Phosphatase Inhibitor cocktail tablets	Roche Applied Science, Mannheim Germany
Rossettesep® B cell enrichment kit	Stemcell Technologies, Vancouver, Canada
Vectastain ABC KIT	Vectorlabs, Burlingame, USA
Vectastain DAB reagent	Vectorlabs, Burlingame ,USA
Vectashield mounting medium with DAPI	Vectorlabs, Burlingame ,USA

6.1.5. Antibodies

Antibody specificity	Species	Supplier
dephospho β-catenin	Mouse monoclonal	Enzo Life Sciences, NY, USA
β-catenin	Mouse monoclonal	BD Biosciences, NJ, USA
β-actin	Mouse monoclonal	BD Biosciences, NJ, USA
Bcl-2	Mouse monoclonal	BD Biosciences, NJ, USA
Bid	Rabbit monoclonal	Cell Signaling, MA, USA
Caspase 3	Rabbit polyclonal	Cell Signaling, MA, USA

Caspase 7	Rabbit polyclonal	Cell Signaling, MA, USA
Caspase 8	Mouse monoclonal	BD Biosciences, NJ, USA
Caspase 9	Rabbit polyclonal	Cell Signaling, MA, USA
c-myc	Rabbit monoclonal	Cell Signaling, MA, USA
Cyclin D1	Mouse monoclonal	BD Biosciences, NJ, USA
IgG1 κ isotype control	Mouse monoclonal	Ebiosciences, CA, USA
LEF-1 (C12A5)	Rabbit monoclonal	Cell Signaling, MA, USA
LEF-1(REMB1)	Mouse monoclonal	Abcam, MA, USA
Mcl-1	Rabbit Polyclonal	Santa cruz Biotech, CA, USA
PARP	Rabbit polyclonal	Cell Signaling, MA, USA
XIAP	Mouse monoclonal	BD Biosciences, NJ, USA

Secondary Antibodies

Anti-mouse IgG-HRP	Dako cytomation, Glostrup, Denmark
Anti-rabbit IgG-HRP	Dako cytomation, Glostrup, Denmark
Anti-mouse IgG-FITC	Dako cytomation, Glostrup, Denmark
Anti-rabbit IgG-FITC	Dako cytomation, Glostrup, Denmark

6.1.6. Inhibitor stock Solutions

3.3 mM CGP049090 stock solution: The substance was dissolved in 70% DMSO and aliquoted in sterile vials and stored in -20 °C in dark.

3.3 mM PKF115-584 stock solution: The substance was dissolved in 70% DMSO and aliquoted in sterile vials and stored in -20 °C in dark.

6.1.7. Cell lines

Cell Line	Description	Source
JVM-3	Human CLL cells	DMSZ, Germany
MEC-1	Human CLL cells	DMSZ, Germany
HS-5	Human bone marrow stromal cells	DMSZ, Germany

6.2. Methods

6.2.1. Culture Conditions

All the indicated cell lines were cultured at 37°C, relative humidity of 90% and 5% CO_2. Cell culture media was supplemented with 20% (v/v) heat inactivated Fetal Calf Serum (FCS) and 1% penicillin/streptomycin antibiotics.

6.2.2. Culture of primary CLL samples

6.2.2.1. Isolation of PBMCs by Ficoll gradient

Ficoll is a neutral, highly branched, high mass, hydrophilic polysaccharide, which is used to separate blood to its components. Peripheral blood mononuclear cells were isolated in heparinized blood samples by Ficoll- hypaque plus sedimentation. The samples were diluted with PBS in 1:1 ratio and layered on to 20ml of Ficoll and centrifuged at 2300 g for 20 min without breaks applied. Mononuclear cells were retrieved from the interface and washed twice with PBS and centrifuged at 1200 g for 5 mins. The pellet is thoroughly resuspended and cultured in Rosewell's park memorial institute (RPMI)-1640 medium supplemented with 20% (v/v) heat inactivated Fetal Calf Serum (FCS) and 1% antibiotics at 37°C in a humidified atmosphere containing 5% CO_2.

6.2.2.2. Isolation of B-cells by Rosette Sep

To obtain pure CLL cells, the Ficoll gradient was preceded by incubation of whole blood with Rosette Sep B cell purification antibody cocktail (2 ml of whole blood + 100µl of cocktail) at room temperature to aggregate unwanted cells with erythrocytes.

6.2.3. Maintenance of mammalian cells

6.2.3.1. Culture of suspension cells

JVM-3 cells and MEC-1 cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) heat inactivated Fetal Calf Serum (FCS) and 1% antibiotics at 37°C in a humidified atmosphere containing 5% CO_2. Cells were subcultured at confluence every 2-3 days by dilution of the cells to a concentration of 0.5 x 10^6 cells/ml.

6.2.3.2. Culture of adherent cells

Adherent cell line HS-5 cells were maintained at confluence in Dulbecco's Modified Eagles's Medium (DMEM) supplemented with 10% (v/v) heat inactivated Fetal Calf Serum (FCS) and 1% antibiotics at 37°C in a humidified atmosphere containing 5% CO_2. Confluent cells were washed two times with PBS and incubated with 2 ml of trypsin/EDTA for 2-3 min at 37°C. The cells were later detached and washed with DMEM with 10% FCS and centrifuged at 1200 g for 5 min. The pelleted cells are thoroughly resuspended in DMEM with 10% FCS and diluted to a concentration of 1 x 10^6 cells/ml.

6.2.3.3. Freezing and thawing cells

Cells were grown to confluence and centrifuged at 1200 g for 5 min. They were resuspended in freezing medium at a density of 1 x 10^7 cells/ml. 1 ml aliquots were pipetted into cryovials and frozen at -80°C in cell freezing containers. For long time storage the cryotubes were transferred to liquid nitrogen tank.

Freezing medium

90% RPMI-1640 with 20% FCS

10% DMSO

Frozen cells were thawed at 37°C in water bath, and immediately washed in 10 ml fresh RPMI-1640 media and centrifuged at 1200 g for 5 min. The cell pellet is resuspended in appropriate fresh culture medium

6.2.3.4. Co-culturing cells

Co-culturing experiments were performed with HS-5 cell line and with CLL primary cells. To obtain a lawn of confluent HS-5 cells, the cells were trypsinized and plated at a density of 1 x 10^5 in 6 well plates with 2 ml DMEM with 10% FCS one day before the experiment. On the day of the experiment the cells were washed twice in RPMI with 20% FCS and incubated with fresh RPMI with 20% FCS containing primary CLL cells.

6.2.4. Cytotoxcity Assay

We carried out cytotoxicity assays in an ATP dependent cell viability assay, CellTiter-GLo® (Promega) according to manufacturer's instructions. The CellTiter-Glo® Luminescent Cell Viability Assay is a homogeneous method to determine the number of viable cells in culture based on quantitation of the ATP present, which signals the presence of metabolically active cells (Crouch *et al.* 1993). This assay relies on the standard luciferase reaction as described below. The Luciferase catalyses luciferin in the presence of Adenosinetriphosphate (ATP) and Magnesium ions (Mg^{2+}) into luciferyl adenylate and pyrophosphate. This luciferyl adenylate in the presence of Oxygen (O_2) is converted into oxyluciferin with the release of Adenosine monophosphate and light.

$$\text{Luciferin} + \text{ATP} \rightarrow \text{Luciferyl adenylate} + PP_i$$

$$\text{Luciferyl adenylate} + O2 \rightarrow \text{Oxyluciferin} + \text{AMP} + \text{Light}$$

The assay reagent is a lysis buffer with all the necessary ingredients for luciferase reaction except ATP. Once this reagent is added to the culture, the cells are lysed releasing intracellular ATP to participate in the luciferase reaction. The amount of luminescent light generated is directly proportional to the ATP levels which directly correlate to the number of viable cells. The luminescent signal is read by MircoLumatPlus LB96V (Berthhold) Luminometer.

To determine the LC_{50} values from dose response curves, cells were seeded in 96-well plates at $2x10^5$ cells per well (triplicates) and treated with various concentrations (0.01, 0.1, 1, and 10 µM) of the inhibitors, DMSO vehicle control (0.0002, 0.002, 0.02, 0.2 % v/v) and untreated control in a final volume of 100 µl for 24 hours. After incubation 100 µl of the CellTiter-GLo® reagent was added to each well and incubated for 10 min at room temperature and read on a 96 well plate luminometer at exposure of 1 sec/well. All the experiments were done in triplicates.

The data was then normalized with the respective vehicle control for each concentration and analyzed using non-linear regression using Graphpad Prism 5 (statistical software suite) and the LC_{50} was determined.

6.2.5. Quantification of apoptosis flow cytometry

Fluorescence activated cell sorting (FACS) provides a method to study cells based upon specific light scattering and fluorescent characteristics of each cell. Expression of proteins on the surface or in the cytoplasm can be detected by staining the cells with specific fluorescently-labeled antibodies, whereas cells expressing endogenous fluorescent proteins can be directly detected.

The phospholipid phosphatidylserine (PS) is translocated from the inner cytosolic leaflet of the plasma membrane bilayer to the outer cell surface, indicating the early events of apoptotic machinery. Annexin V is a 35 kDa phospholipid binding protein with high affinity for PS. PS

inversion is also a phenomenon during necrosis hence in order to differentiate apoptosis and necrosis; Annexin V staining is accompanied with a vita dye Propidium iodide (PI) which stains nucleic acid only when the membrane integrity is lost.

After culture conditions, 5 x 10^5 cells are washed in PBS and pipetted into FACS tubes. The cells are resuspended in 100 µl of 1 X Annexin binding buffer containing 3 µl of annexin-FITC and 2 µl of PI. The samples were incubated for 20 min at room temperature at dark. After 20 min samples were analysed using FACS-Cantor flow cytometer. The percentage of Annexin V^-/PI^- cells (% viable) were compared with untreated cells.

6.2.6. Immunofluorescence

Expression of proteins on the surface or in the cytoplasm can be detected by staining the cells with specific fluorescently-labeled antibodies this can be detected using standard fluorescence microscopy and high resolution images are obtained. Advantage of this particular method is that localization studies of proteins of interest can be carried out.

Immunofluorescence studies were undertaken in primary CLL cells and JVM-3 cell lines to visualise the expression and localisation of intracellular proteins. Briefly cells were washed in cold RPMI-160 media. Cells were then fixed in 4 % paraformaldehyde for 20 min on ice after which washed in twice in PBS supplemented with 1 % BSA. Cells were then permeablized with 0.2% saponin in PBS/1%BSA for 5 min followed by a wash in PBS/BSA. They were then blocked for 15 min in 5 % normal goat serum (NGS) in PBS/BSA. The cells were washed again in PBS/BSA. To this cell suspension primary antibody diluted in PBS/BSA was added and incubated at room temperature for 1 hour. This was followed by 3 washes in PBS/BSA. After staining with the primary antibody the cells were again blocked in 5% NGS, and followed by incubation with the secondary fluorescently labelled antibody diluted in PBS/BSA. After washing the cells thrice in PBS/BSA the cells were suspended in 100 µl of

VectaShield mounting medium. 15 µl of this cell suspension is spotted on to a glass microscopic slide and mounted with a coverslip. The slides are then subjected to fluorescence microscopic analysis.

PBS (Phosphate Buffer):

1 tablet dissolved in 500 ml of double distilled water (ddH_2O). pH adjusted to 7.4 with NaOH or HCl

PBS/BSA Buffer:

To 100 ml of PBS added 1 gm of Bovine Serum Albumin (BSA) and dissolved.

0.2% Saponin

To 10 ml of ddH2O added 200 mg of saponin and vortexed until dissolved.

6.2.7. siRNA mediated gene knockdown

Small interfering RNA (siRNA), or silencing RNA, is a class of double-stranded RNA molecules, 20-25 nucleotides in length that is involved in the RNA interference (RNAi) pathway, where it interferes with the expression of a specific gene. It was first identified as a part of post transcriptional gene silencing in plants, but later was shown that synthetic siRNAs were to be able to induce RNAi in mammalian cells (Elbashir *et al.* 2001).

Synthetic pool of siRNAs targeting different parts of LEF-1 mRNA (ON-TARGET plus SMARTpool *lef-1* siRNA) or the non-targeting negative control pools (ON-TARGETplus siCONTROL) were obtained from Dharmacon, Lafayette, USA. Transfection of siRNAs in CLL cells were carried out by Amaxa Nucleofection systems.

8 x 10^6 primary CLL cells were resuspended in 100 µl of cell line solution kit V (Amaxa) with 0.5 µM of LEF-1 siRNA or non-targeting control siRNAs or Mock transfected without siRNAs or Non-transfected with program U-013 using Amaxa nucleofector device I.

The cells were immediately transferred in to 6 well plates and co-cultured with HS-5 feeder layer in RPMI-1640 supplemented with 20% FCS for 16 hours at 37°C in a CO_2 incubator. The cell viability was monitored using Annexin V/PI staining by flow cytometry. The efficiency of the transfection was enumerated using pmax-GFP plasmid.

6.2.8. Methods in Protein chemistry

6.2.8.1. Preparation of cell lysates.

Cell lysis is the first step in cell fractionation and protein purification by distruption of cells either by physical or detergent methods or in combination of both. Detergents and high salt concentrations in the buffer break the lipid barrier surrounding the cells.

Cells were washed twice in ice cold PBS and the cell pellet was slowly resuspended in 200µl of M-PER protein extraction reagent supplemented with protease and phosphatase inhibitors on ice. The suspension was sonicated for 3 cycles (30 sec/cycle) at 50% power. Then the cell extracts were centrifuged at 13000 g at 4°C for 15 min. The clear supernatant was transferred into new tubes and used for subsequent western blots or frozen at -80°C for further biochemical analysis.

6.2.8.2. Preparation of nuclear and cytoplasmic fractions

Nuclear and cytoplasmic fractionation was carried using Active motif nuclear extract kit according to manufacturer's instructions. The principle involved is when cells are incubated in a hypotonic solution the water diffuses into the cell by osmosis, causing the cells to swell and possibly explode. Upon subsequent centrifugation the supernatant contains proteins from the cytoplasm alone leaving the nuclear pellet. Briefly the cells were washed twice in ice cold PBS and the cell pellet was resuspended in hypotonic buffer supplemented with protease and phosphatase inhibitors for 20 min on ice. 25 µl of detergent is added to the mix and vortexed briefly. The cell suspension was later centrifuged at 13000 g for 30 seconds in a

microcentrifuge at 4ºC. The supernatant (cytoplasmic fraction) was then transferred into a new tube and stored on ice or frozen at -80°C until further analysis. The nuclear pellet was washed twice in the hypotonic buffer to rinse of remaining cytoplasmic proteins. The resulting nuclear pellet is resuspended in 100-200 µl of MPER protein extraction reagent and sonicated for 3 cycles (30 sec/cycle) at 50% power. Then the nuclear extracts were centrifuged at 13000 g at 4°C for 15 min. The clear supernatant (nuclear fraction) was transferred into new tubes and used for western blots or frozen at -80°C for further analysis.

6.2.8.3. Quantification of proteins

For the quantification of proteins, the BCA (Bicinchoninic acid) protein assay reagent kit from Pierce, USA was employed. The principle involved in BCA assay is the formation of a Cu^{2+} protein complex in alkaline conditions, followed by reduction of the Cu^{2+} to Cu^{1+}. The amount of reduction is proportional to the protein present in the lysates. Two molecules of BCA chelate with one molecule of Cu^{1+} to form a purple-blue complex which can be monitored at its absorbance maximum of 562 nm.

For this assay 2 µl of protein lysates were used and diluted in 98 µl of distilled water. To prepare protein standards for calibration, 0, 5, 10, 15, 20 µg of bovine serum albumin (BSA) from 2 mg/ml stock solution was diluted in water to a final volume of 100 µl. working BCA reagents were freshly prepared by mixing reagent A and reagent B from the assay kit in 1:50 (v/v) ratio. The protein samples were diluted with 900 µl of working reagent mixture and the components were mixed with inversion. The reaction tubes were incubated for 10 min and samples were measured at 562 nm absorbance using a spectrophotometer. A standard curve of absorbance versus micrograms protein was plotted and the protein concentration in each sample was determined by linear regression.

6.2.8.4. Co-immunoprecipitation

Co-immunoprecipitation (Co-IP) is best preceded by a discussion of immunoprecipitation (IP) to help understanding of the principles involved. Immunoprecipitation (IP) involves the enrichment or separation of specific proteins from the cellular lysates by using specific antibodies. The immune complexes are then precipitated using immobilized protein A or protein G, bacterial proteins which have high affinity to the Fc part of the immunoglobulin which are covalently linked to agarose or magnetic beads. The process of capturing this complex from the solution is referred to as precipitation. Finally, components of the bound immune complex (both antigen and antibody) are eluted from the support and analyzed by SDS-PAGE often followed by western blot detection to verify the identity of the antigen. Co-immunoprecipitation (Co-IP) is an extension of IP except the fact that the precipitated protein has some interaction partners complexed to it *de novo* and will be useful to study protein interactions. This is only an assumption, however, that is subject to further verification.

For Co-IP experiments we used µMACS™ Protein A/G Kits from Miltenyi Biotec, Bergisch Gladbach Germany. Immunoprecipitation with µMACS Protein A/G MicroBeads involves a short incubation of cleared lysate with the MicroBeads coated with the specific antibody the magnetizable immune complex is passed over a separation column placed in the magnetic field of a MACS Separator. The labeled complex is retained within the column while other proteins are efficiently washed away. For SDS-PAGE analysis, the immunoprecipitated protein is eluted from the column with SDS gel loading buffer.

For Co-IP 500 µg of total protein extracts were incubated 10 µM of CGP049090 or PKF115-584 or 0.02% DMSO (vehicle control) for 16 hours. Later this extract 2 µg of precipitating antibody and 50 µl of µMACS Protein G beads were added and incubated overnight to magnetically label the immune complexes. The immune complexes were then passed through a µ separation column in a magnetic field. The column was rinsed 3 times with 200 µl of high

salt buffer (M-PER lysis buffer) which washes off unspecific and un-complexed proteins but retains the magnetically labeled immune complexes. 20 µl of pre-heated hot SDS sample buffer was added to the column and incubated for 5 min in room temperature. Later 40 µl of pre-heated SDS sample buffer was added to elute the immune complexes. The eluate was stored and later analyzed by western blotting.

1X SDS Sample Buffer:

4X NuPAGE SDS sample buffer:	25 µl
NuPAGE reducing agent (DTT):	10 µl
DdH2O:	65 µl
Total:	100µl

6.2.8.5. SDS PAGE ELECTROPHORESIS

In SDS polyacrylamide gel electrophoresis (SDS-PAGE), proteins are separated on the basis of molecular weight of the proteins. Two sequential gels are cast: the stacking gel which is slightly acidic with low polyacrylamide concentration and the separating gel which is more basic and higher polyacrylamide content which allows proteins to be separated according to their molecular sizes, where smaller proteins travel faster than larger proteins.

SDS is an anionic detergent which denatures the protein and imparts negative charge, and in an electric field the protein is attracted towards the anode and they are resolved solely on the basis of their sizes by the pores of the polyacrylamide gel.

In this study we used NuPAGE® 4-12% Bis-Tris gradient gels, NuPAGE® MES Running buffer, NuPAGE® LDS sample Buffer and NuPAGE® Sample reducing agent and NuPAGE® Antioxidant from Invitrogen. The SDS PAGE was carried out in a vertical Xcell surelock apparatus according to manufacturer's instructions.

6.2.8.6. Protein transfer (Western Blot)

For the detection of protein of interest, the resolved bands on the gel are transferred blotting membranes by the process referred to as blotting. This blotting process was achieved using Xcell blot module (Invitrogen) according to manufacturer's instructions. The proteins are transferred onto nitrocellulose membranes in the NuPAGE® Transfer buffer at 35V for 60 min. Membranes, filter paper, fiber pads, were all pre-soaked in the transfer buffer.

The following is the order of assembly in the blot module:

Anode (Red)

Fiber pad

Filter paper

Membrane

Gel

Filter paper

Fiber pad

Cathode (Black)

Care should be taken to remove all bubbles in the assembly. The transfer the efficiency of the western blot is monitored by staining the nitrocellulose membrane in Ponceu-S solution for 5 min. The membrane is destained using 0.1 M NaOH sodium hydroxide solution in ddH2O.

<u>0.1M NaOH</u>

4 grams of NaOH dissolved in 1 L of ddH2O.

<u>Ponceau-S stock Solution (100 ml)</u>

2% m/v	Ponceau S
30% m/v	Trichloroacetic acid
30% m/v	Sulphuric acid

The stain is dissolved in 100 ml of ddH2O. The working solution is made by diluting the stock solution 3 times with ddH2O.

6.2.8.7. Immunoblot

Immunoblotting involves the detection of specific proteins on membranes using antibodies. The membranes are first blocked with blocking agents to prevent unspecific

binding of antibodies. Then, primary antibodies that recognize specific proteins or tags on proteins are allowed to bind to their targets. After washing to remove non-specific binding and excess antibody, the secondary antibodies coupled with Horse Radish Peroxidase (HRP) recognize the primary antibodies. After washes the enzymes are detected using chemiluminiscence

After the proteins are transferred to the nitrocellulose membranes, the blots are blocked in Block I solution at room temperature for 1 hour. Subsequently the membranes are incubated overnight with respective primary antibody in a horizontal roller at 4°C. The membranes are then washed 3 x 10 min cycles in 50 ml PBS in a horizontal shaker. This is followed by incubation of the membranes in Block II solution with HRP conjugated secondary antibody for 1 hour at room temperature on a roller. Thereafter the membranes are washed 3 x 10 min cycles in 50 ml PBS in a horizontal shaker before the detection of proteins using the ECL detection reagents.

For detection of proteins by chemiluminiscence, 1 ml of fresh solution mixture was made by mixing Solution A & Solution B in a ration 1:1 (v/v). This mixture is immediately spread on the membrane and incubated for 1 min. The excess solution is drained off and the membrane is exposed to hyperfilm in dark for appropriated time and developed in X-ray film processor.

Block I solution (PBS)

5% milk powder

10% Rotiblock®

Block II solution (PBS)

5% milk powder

PBS (Phosphate Buffer)

1 tablet dissolved in 500 ml of ddH2O. pH adjusted to 7.4 with NaOH or HCl

6.2.9. Generation of JVM-3 Xenograft Subcutaneous Tumor Mouse Model

During molecular target therapies for cancer, some relevant and novel aspects from the clinical setting should be considered for the *in vivo* experimental studies aimed at the development of new therapeutic strategies. The preclinical development of new drugs or combination of drugs with molecular targets should be planned with a modern approach based firstly on the use of advanced animal models.

We carried out *in vivo* studies in JVM-3 subcutaneous xenografts in athymic nude mice as described previously(Loisel *et al.* 2005) . The nude mice were developed from the transfer of the nude gene (FOXN1) from Crl:NU-Foxn1nu to a CD-1 mouse through a series of crosses and backcrosses at Charles River Laboratories™. The animal lacks a thymus, is unable to produce T-cells, and is therefore immunodeficient. Hence the animal can graft any human/mouse transplanted tissue. This mouse is usually employed in pre-clinical drug development

CGP049090 and PKF115-584 Stock Solution for *in vivo* studies:

Both small molecules are extremely lipophilic and hence required stable yet non-toxic excipient for *in vivo* application. The drug was dissolved first dissolved in 1 part 100% ethanol followed by 1 part of cremophor EL and 9 parts of ddH2O. The final suspension (6.25mg/ml) is vortexed vigorously before intraperitoneal injection.

Mice were allowed 3 days acclimatization period in the local animal facility upon arrival. The mice were then pre treated with 3Gy of whole body irradiation 1 day prior to inoculation as this enhances tumor engraftment dramatically by depleting residual B or T cells. JVM-3 cells are grown in complete RPMI-1640 with 10% FCS and harvested at 75-80 % confluency. Cells are washed in PBS resuspend them in L-15 media without FCS and antibiotics at $1x10^7$ in 100µl. Cells are then injected subcutaneously (SC) in the nude mice under aseptic

conditions using 1 cm^3 syringe and a 19 gauge needle. The mice are observed for 2 weeks for tumour progression. Tumor volume is measured once it's palpable using the formula. The mice are then randomized into treated and vehicle control groups provided that both groups have the same tumor volume before initiation of therapy. The therapy is initiated once the tumor volume of the groups is around 90-100 mm^3. The mice are divided into three groups; Vehicle control, CGP049090 group and PKF115-584 group (n= 8 mice/group) and drug was administered at 25mg/kg body weight for 12 days intraperitoneally. The tumor volume and body weight is measured every third day after initiation of therapy in both control and treated group. Due to ethical reasons the maximal tumor volume in the mice will not be allowed to exceed 1000 mm^3. If it exceeds, the mouse was sacrificed and declared dead on that particular day.

6.2.9.1. Evaluation of in vivo antitumor activity

Tumor volume (TV) was measured prior to therapy and every third day after initiation of therapy as described previously (Kawato *et al.* 1991).

TV was calculated according to the formula

TV (mm^3) = L (mm) x W (mm) x H (mm) /2,

L is the tumor length

W is the tumor width

H is tumor height.

The inhibition rate (IR) of tumor growth on the basis of TV was calculated according to the formula

IR = (1 - RVt/RVc) x 100%,

RVt represents the mean ratio of TV on day n to TV on day 0 of a treated group

RVc indicates the ratio of the control group.

Largest value for IR was designated as IRmax which indicates the greatest effect of each tested inhibitor

The rate of body weight reduction (ΔBW) was calculated according to the following formula:

$$\mathbf{BW = (1 - BW_n / BW_o) \times 100\%}$$

$\boldsymbol{BW_n}$ represents the mean body weight of mice on day n

$\boldsymbol{BW_0}$ represented the mean body weight on day 0.

The maximum value for ΔBW was designated as ΔBW_{max}; from which each test compound's toxicity to mice was evaluated.

6.2.10. Histochemistry

Histochemistry involves the process in which a tissue is sliced into very thin sections, which can be either stained by certain dyes to study the cell morphology or using specific antibodies (immunohistochemistry) to localise proteins of interest. This procedure involves fixation, dehydration, paraffin embedment and subsequent sectioning with a microtome.

Fixation:

After euthanization of the mouse the tumor is dissected and sliced into sizes of 1 cm^3. These slices are immediately fixed in 4 % formaldehyde for 24 hours. This step leads to cross-linking of proteins in the tumor specimens thereby preserving the structural integrity of the cells. This step is done in an automatic tissue processor (Leica™ Histokinette)

Dehydration:

Formaldehyde and water are known to interfere with several downstream staining process, hence formaldehyde and remaining water is completely removed from the tissue by

incubating the tumor specimen in successive series of increasing concentrations of ethanol (30%, 50%, 70%, 80%, 90%, 95%, and 100%) for two hours each by the tissue processor.

Paraffin embedment:

The specimens are incubated in a clearing solution usually xylol which is totally miscible with the dehydrating alcohol and wax embedding agent for 2 hours. Later the tissue is impregnated with paraffin at 65°C for 4 hours for the final embedding stage which sets specimens in blocks of paraffin wax from which sections may be cut.

Sectioning:

The embedded tissue blocks are cooled on a cold plate and mounted onto a standard microtome and sections of 10 µm are made. These sections are then floated onto a warm water bath to smooth out the creases and then lifted out onto a glass microscope slide and allowed to dry on a hot plate set just below the melting point of the wax. Once the sections are dried onto the slide they are ready for immunohistochemistry analysis.

6.2.11. Immunohistochemistry

Immunohistochemistry is a useful tool to identify key changes in cellular behavior after a therapeutic intervention *in vivo.* This technique involves treating a part of a tumor section with antibodies that recognize cell proteins of interest. The method involves establishing a biotin link, through avidin, between the horseradish peroxidase enzyme and a secondary antibody reagent, enzyme localization can be achieved at the site of primary antibody interaction with the specimen.

The tumor sections embedded in paraffin are incubated in three washes of xylene for 5 min each to deparaffinze the section. Later the sections are rehydrated with decreasing concentration of ethanol (100% 95% 75%) 10 min each. After rinsing the slides in ddH2O, the slides are placed in antigen unmasking buffer (TE buffer) and boiled for 20 min. After cooling them down for 5 min the slides are washed in ddH2O and incubated in 3% hydrogen

peroxide for 10 min. The sections are then washed in ddH2O and blocked in 500 µl of blocking solution for 1 hour. After removing the blocking solution 500µl of primary antibody diluted in the antibody diluent to each is added to the sections and incubated overnight at 4°C in a humidified chamber.

The next day the sections are rinsed in wash buffer three times for 5 min, followed by incubation with 500 µl of biotinylated secondary antibody, diluted in TBST and incubated in room temperature for 1 hour. The slides are washed again three times for 5 min and 500 µl of (The Avidin-Biotin Complex) ABC reagent is added to the sections and incubated for 1 hour. The sections are washed in wash buffer thrice and 200 µl of 3,3'-Diaminobenzidine (DAB) substrate is added to each section and incubated for 20 min until the appropriate color reaction develops. The sections are washed in ddH2O and mounted with coverslips and observed under documented with the light microscope.

Wash Buffer

10X Tris Buffered Saline (TBS): 24.2 g Tris base ($C_4H_{11}NO_3$) and 80 g sodium chloride (NaCl) to 1 L ddH_2O. pH adjusted to 7.6 with concentrated HCl.

1X TBS/0.1% Tween-20 (1X TBST)**:** 100 ml 10X TBS to 900 ml dH_2O and 1 ml Tween-20 and mix.

Antibody diluent /Blocking solution

TBST/5% normal goat serum

Antigen unmasking buffer

TE Buffer**:** 10 mM Tris/1 mM EDTA/0.05% Tween–20, pH 9.0: To prepare 1litre add 1.21 g Tris base ($C_4H_{11}NO_3$) and 0.372 g EDTA ($C_{10}H_{14}N_2O_8Na_2•2H_2O$) to 950 ml dd$H_2O$. Adjust pH to 9.0, add 0.5 ml Tween–20, then adjust final volume to 1000 ml with ddH_2O.

3% Hydrogen peroxide

10 ml of 30% H_2O_2 to 90 ml ddH_2O.

ABC Reagent and DAB Substrate

(Vectastain ABC Kit, vector laboratories, Burlingame, CA)

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Abbreviations

ALY	Ally of AML-1 and LEF-1
APAF-1	apoptosis-activating factor 1
APC	adenomatous polyposes coli
B-CLL	B-Chronic Lymphocytic Leukemia
BCR	B cell receptor
Bcl-2	B cell lymphoma 2
BclxL	B-cell lymphoma-extra large
Bid	BH3 interacting domain death agonist
BMP	Bone Morphogenetic Proteins
βBD	β-catenin binding domain
CAD	context dependent activation domain
CD5+	Cluster of Differentiation 5
cGMP	cyclic guanosine monophosphate
CLL	Chronic Lymphocytic Leukemia
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CSKA-1	Casein kinase α-1
CSR	class switch recombination
Co-IP	Co-immunoprecipitation
DAB	3,3'-Diaminobenzidine
Dsh	Dishevelled
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSB	double strand break
DSBR	double strand break repair
dsDNA	double stranded DNA
DTT	Dithiothreitol
ECL	enhanced chemiluminescence
EBV	Epstein-barr virus
et al.	et alii
FCS	fetal calf serum
FISH	flourescence in situ hybridisation
Fzd	Frizzled
g	gram
G proteins	Guanine nucleotide binding protein
GC	germinal centre
GMP	granulocyte/monocyte progenitor
GSK3β	Glycogen synthase kinase 3β
GTPases	guanosine triphosphate hydrolases
h	hour
HMG	high mobility group
HSC	Hematopoetic Stem Cell
IC50	half maximal inhibitory concentration
Ig	immunglobulin
IgV_H	immunglobulin heavy chain variable region
IP	immunoprecipitation
ITAM	Immunoreceptor tyrosine-based activation motif
JNK	Jun N-terminal Kinase
kDa	kilo Dalton
l	Litre

LEF-1	Lymphoid enhancer factor 1
LRP	lipoprotein receptor-related protein
M	molar (Mol/Litre)
MAPK	mitogen activated protein kinase
Mcl-1	Mantle cell lymphoma-1
MEP	megakarythrocyte/ erythrocyte progenitor
mg	milligram
MgCl2	Magnesium chloride
min	min
ml	milliliter
mM	millimolar
mRNA	messenger ribonucleic acid
µg	microgram
µl	microlitre
µM	micromolar
NK	Natural Killer
NLS	nuclear loacalisation signal
PAGE	polyacrylamide gel electrophoresis
PARP	Poly (ADP-ribose) polymerase
PBS	phosphate buffered saline
PCP	planar cell polarity
PCR	polymerase chain reaction
PCNA	Proliferating cell nuclear antigen
PDE	phosphodiesterase
pH	negative logarithm of the hydrogen ion concentration
PI3K	Phosphatidylinositol-3 kinase
PLC	phospholipase C
RAG	recombination activated genes
RNA	Ribonucleic acid
RT	room temperature
RTKs	receptor tyrosine kinases
SDS	Sodium dodecyl sulphate
SHM	somatic hypermutation
siRNA	Small interfering RNA
TCF	T cell factor
TCR	T cell receptor
Tris	Tris hydroxyl methyl aminomethane
V(D)J	variable(diversity)joining segments of immunoglobulin genes
Vs	versus
Wif	Wnt inhibitory factor
Wnt	combination of Wingless (Drosophila) and Int (vertebrate) homologue genes
wt	wildtype
w/v	weight per volume
XIAP	X-linked Inhibitor of Apoptosis Protein
ZAP70	Zeta associated protein kinase 70

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Ehrenwörtliche Erklärung

Ich versichere hiermit ehrenwörtlich, dass die Dissertation von mir selbstständig, ohne unerlaubte Beihilfe angefertigt worden ist. Diese Arbeit hat in gleicher oder ähnlicher Form noch keiner anderen Prüfungsbehörde vorgelegen.

Köln, den 15. August 2009

Rajesh Kumar Gandhirajan

Declaration

I hereby declare that this thesis is the result of independent work done by me without any unauthorised source of assistance. This work, in the same or a similar form, has not been submitted to any other board of examination.

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