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Pathophysiology of Protein Kinase C Isozymes in Chronic Lymphocytic Leukaemia

John C. Allen and Joseph R. Slupsky
Department of Molecular and Clinical Cancer Medicine, University of Liverpool, Liverpool, United Kingdom

1. Introduction

This chapter will review the roles of protein kinase C (PKC) isozymes in chronic lymphocytic leukaemia (CLL) cells. PKC family proteins are central to many signalling pathways within cells, and some have been implicated in the oncogenesis of numerous cancers (Benimetskaya, *et al.*, 2001;Keenan, *et al.*, 1999;O'Brian, 1998). In CLL, inhibitors of PKC signalling have been shown to have cytotoxic effects on the malignant cells, and the α, β and δ isoforms of PKC have been shown to have pathophysiological roles (Holler, *et al.*, 2009;Nakagawa, *et al.*, 2006;Ringshausen, *et al.*, 2002). The aim of this chapter is to discuss whether PKC can be considered a drug target in the treatment of this disease. We will examine how inhibitors of PKCs have been used in past preclinical studies of CLL, and will discuss the roles of various PKC isozymes (namely PKCβII, PKCα, PKCδ and PKCε) in the pathology of CLL. This chapter will end with the proposal that inhibition of PKC may be useful in combination therapy through a potential role in regulating Mcl-1 expression.

2. PKC in CLL

Survival and expansion of the malignant clone in CLL involves a myriad of intrinsic and extrinsic signals and most, if not all of these signals will involve the kinase function of PKC. For example, chronic antigen stimulation of the B-cell receptor (BCR) is thought to play a key role in CLL cell survival (Chiorazzi, *et al.*, 2005), and the β isoform of PKC (PKCβ) is known to play an important role in BCR signalling (Kang, *et al.*, 2001;Saijo, *et al.*, 2002). In this context, specific targeting of PKCβ in CLL cells may either enhance or inhibit the pro-survival signals that BCR engagement provides.

A role for PKC function in CLL cell survival was first suggested in experiments using PKC agonists such as the phorbol ester 12-*0* tetradecanoylphorbol 13-acetate (TPA) and bryostatin (al-Katib, *et al.*, 1993;Drexler, *et al.*, 1989;Forbes, *et al.*, 1992;Totterman, *et al.*, 1980). These compounds are natural product analogues of diacylglycerol, which is the ligand of PKC within cells, and act to stimulate kinase activity of PKC. Initial observations showed that treatment of CLL cells with either TPA or bryostatin-1 resulted in the induction of differentiation and inhibition of spontaneous apoptosis (al-Katib, *et*

al., 1993;Barragan, *et al.*, 2002;Drexler, *et al.*, 1989;Forbes, *et al.*, 1992;Totterman, *et al.*, 1980;Varterasian, *et al.*, 2000). Exploration of the mechanism through which TPA and bryostatin induced CLL cell differentiation showed that this was likely due to PKC-mediated activation of the ERK pathway (Figure 1A). These early experiments prompted a phase I (Varterasian, *et al.*, 1998) and phase II (Varterasian, *et al.*, 2000) clinical trial of bryostatin in CLL. The findings of these studies showed that bryostatin could induce *in vivo* differentiation of the malignant cells in CLL patients (Varterasian, *et al.*, 2000). Combination of bryostatin with 2-chlorodeoxyadenosine showed promise in treating CLL in both an animal model of CLL (Mohammad, *et al.*, 1998) as well as a case report of a single patient (Ahmad, *et al.*, 2000), however, the use of bryostatin as a therapeutic agent has not been followed up. This could be because other studies have shown that TPA and bryostatin provide protection against dexamethasone- and fludarabine-induced apoptosis of CLL cells (Bellosillo, *et al.*, 1997;Kitada, *et al.*, 1999). Investigation of the mechanism through which this protection is provided showed that these compounds stimulate upregulation of the anti-apoptotic proteins Mcl-1 and XIAP (Thomas, *et al.*, 2004) (Figure 1A).

A second approach to address the role of PKC in CLL cell survival has used inhibitors of this enzyme. Thus, compounds such as UCN01 (Byrd, *et al.*, 2001;Kitada, *et al.*, 2000), PKC412 (Ganeshaguru, *et al.*, 2002), LY379196 (Abrams, *et al.*, 2007) and Bisindolymaleimide (Barragan, *et al.*, 2002;Snowden, *et al.*, 2003) have all been shown to potently induce apoptosis of CLL cells *in vitro*. Interestingly, treatment of CLL cells with UCN01 or Bisindolylmaleimide reduces the expression of Mcl-1 and XIAP (Kitada, *et al.*, 2000;Snowden, *et al.*, 2003), thereby making treated cells more susceptible to apoptosis (Figure 1B). This observation, when taken together with others showing that activation of PKC results in an upregulation of Mcl-1 and XIAP, strongly suggest that PKC is an important mediator of CLL cell survival signals.

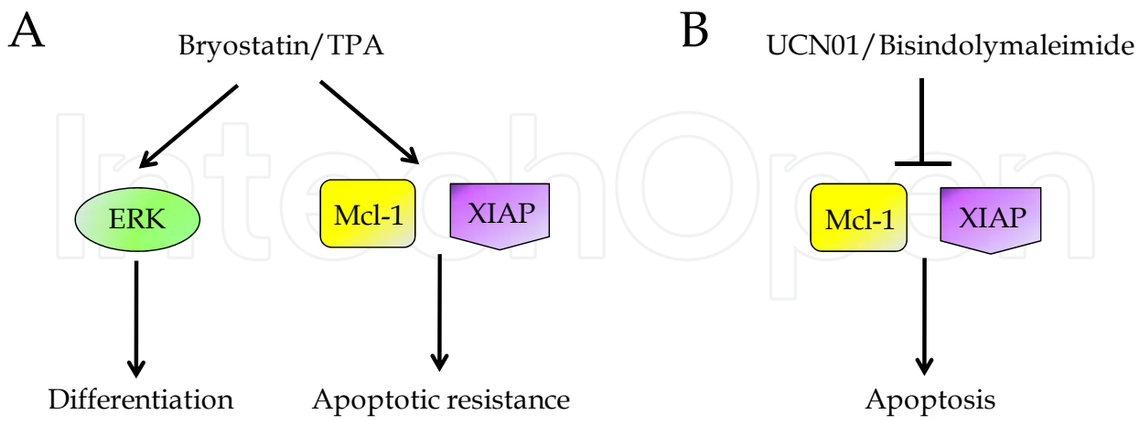

Fig. 1. Effects of PKC agonists and antagonists on CLL cells. (A) PKC agonists such as TPA and bryostatin induce ERK-mediated differentiation in CLL cells, and inhibit spontaneous apoptosis by stimulating the expression of Mcl-1 and XIAP. (B) PKC antagonists such as UCN01 or Bisindolymaleimide reduce the expression of Mcl-1 and XIAP in CLL cells thereby increasing the potential of CLL cells to undergo apoptosis.

2.1 PKC structure and function

PKCs are a family of serine/threonine kinases that share extensive structural homologies between different isotypes. Despite this homology, PKCs regulate different cellular functions in a variety of cell types, including proliferation, differentiation, apoptosis and cell survival (Tan & Parker, 2003). PKCs are divided into three subfamilies based on their regulatory domain composition, which determines what co-factors help induce their activation. Classical PKCs (PKC α, βI, βII, and γ) require the presence of DAG and calcium for activation, while novel PKCs (PKC δ, ε, η, θ) require only the presence of DAG. In contrast, atypical PKCs (PKC ζ, λ/ι) are both calcium and diacylglycerol-independent (Mellor & Parker, 1998).

The structure of all PKC family members is comprised of a C-terminal kinase domain linked by a flexible hinge segment to an N-terminal regulatory domain (Parker & Murray-Rust, 2004) (Figure 2). The kinase domain of PKC is highly conserved among isoforms and shows homology to the AGC superfamily of serine/threonine protein kinases. This domain contains the ATP- and substrate-binding sites, and also serves as a phosphorylation-dependent docking site for the regulatory molecules that interact with PKC (Newton, 2010).

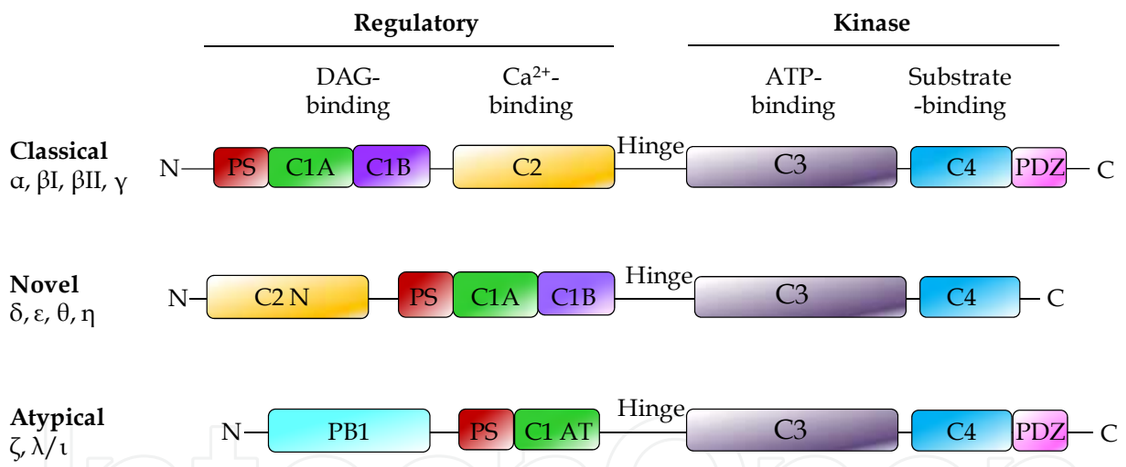

Fig. 2. Schematic representation of PKC isoform structure. The regulatory domain of PKC isoforms contain the regions necessary for membrane association and activation of the kinase. The C1 domain binds DAG/phorbol esters, and also contains a pseudosubstrate (PS) sequence at its N-terminus. The PS binds to the substrate-binding site within the catalytic domain to hold PKC in an inactive state. Atypical PKCs have a unique C1 region (C1 AT) as well as a Phox and Bem1 (PB1) region which are likely responsible for protein interaction resulting in kinase activation. The C2 domain regulates Ca^{2+}-mediated phospholipid binding in classical PKCs. Novel PKCs have a C2-like domain that does not bind Ca^{2+} (C2 N). The catalytic domain of all PKCs is conserved and contains the regions necessary for ATP-binding (the C3 domain) and for binding to substrate (the C4 domain). A PDZ region is also present in some PKC isoforms, and is responsible for protein-protein interactions following kinase activation.

The regulatory domain of PKC is divided into two regions. At the N-terminus there is a pseudosubstrate (PS) sequence that is responsible for binding the catalytic domain and maintaining the enzyme in an inactive conformation when it is in the cytoplasm (House & Kemp, 1987). This domain of PKC also contains the regions responsible for membrane targeting. Thus, classical PKCs contain motifs, termed C1 domains, that are able to bind DAG as well as phorbol esters (Newton, 1995a). Classical PKCs also have motifs, termed C2 domains, that are responsible for binding membrane phospholipids such as phosphatidylserine (PtS) and phosphatidylinositol-4-5-biphosphate (PIP_2) in a Ca^{2+}-dependent manner (Newton, 1995a). Novel and atypical PKC isoforms have a different regulatory domain structure. Novel PKCs contain tandem C1 domains that bind DAG with an affinity that is high enough to induce translocation to the membrane (Giorgione, *et al.*, 2006), and use a C2-like domain to bind phospholipids in a Ca^{2+}-independent manner (Newton, 1995a). In contrast, atypical PKCs lack a C2 domain in any format, and contain an impaired C1 domain that does not bind diacylglycerol (Newton, 1995a). Instead, atypical PKC isoforms depend largely upon protein-protein interactions for activation. For this purpose, these isoforms contain an N-terminal PB1 domain and a C-terminal PDZ ligand binding domain.

The flexible hinge region of PKCs is important in as much as it allows the close apposition of the regulatory and catalytic domains when PKC is in an inactive state. When PKC becomes activated, the hinge region allows the protein to unfold to the extent needed for the catalytic domain to interact with substrates and regulatory proteins.

2.2 PKC regulation

PKC is regulated by four key mechanisms: phosphorylation, co-factor binding, protein-protein interactions and regulated degradation. All help regulate the subcellular localisation, structure, and function of the enzyme.

2.2.1 Processing of PKC

Newly synthesised PKC is associated with membrane fractions where it is processed by a series of tightly coupled phosphorylations on serine and/or threonine residues in the catalytic domain (Newton, 2010) (Figure 3). These phosphorylations are essential before PKC can become activated, and the series in which they take place is analogous to other AGC protein kinases such as Akt. The binding of the chaperone protein heat shock protein 90 (HSP90) was identified as an initial step in the maturation of both classical and novel PKC isoforms (Gould, *et al.*, 2009). It binds to the catalytic domain of PKC and primes the enzyme for phosphorylation within the activation loop of the catalytic domain (Figure 3A). Failure of PKC to bind HSP90 results in inhibited phosphorylation at this site, misfolding of the entire protein and its consequent degradation (Balendran, *et al.*, 2000;Gould, *et al.*, 2009). Phosphorylation of the activation loop of PKC is catalyzed by 3-phosphoinositide-dependent kinase (PDK)-1, which binds to the exposed C-terminus of newly synthesised PKC that is in complex with HSP90 (Chou, *et al.*, 1998;Dutil, *et al.*, 1998;Dutil & Newton, 2000) (Figure 3A). This is followed by phosphorylation of the turn motif by the mTORC2 complex (Ikenoue, *et al.*, 2008) (Figure 3B). Phosphorylation of the turn motif stabilises the active conformation of PKC prior to autophosphorylation of the hydrophobic motif and

generation of catalytically competent PKC (Behn-Krappa & Newton, 1999). Whether this latter step results from autophosphorylation is controversial because phosphorylation of the hydrophobic motif does not take place in mTORC2 deficient cells (Newton, 2010). However, because phosphorylation of the turn motif must take place before phosphorylation of the hydrophobic motif, it is likely to be very difficult to fully define the kinase(s) responsible. It is important to note here that phosphorylation of the activation loop, turn and hydrophobic motifs within PKC only results in an enzyme that is fully matured and catalytically competent, it should not be mistaken for active PKC as these sites will be phosphorylated on inactive PKC located within the cytoplasm of cells.

2.2.2 Mechanism(s) of activation

Fully matured PKC is predominantly localised to the cytosol, where it is likely maintained in specific microenvironments by interacting with regulatory proteins (Schechtman & Mochly-Rosen, 2001). Here, the enzyme is held in an inactive conformation by the N-terminal PS binding to the substrate-binding site of the catalytic domain (House & Kemp, 1987). Processes that result in a structural change in the protein so that the N-terminus of PKC is no longer in close proximity to the C-terminus result in activation of the enzyme. Typically, activation of classical isoforms of PKC occurs following the induction of PIP_2 hydrolysis within certain pathways of intracellular signalling. This generates Ca^{2+} and DAG, two second messengers crucial for the activation of classical PKCs (Beaven, 1996;Nishizuka, 1988). Ca^{2+} binds to the C2 domain of classical PKCs causing their translocation to the plasma membrane where they bind phospholipids such as PtS and PIP_2 (Cho, 2001;Newton, 1995b) (Figure 3C). Once at the membrane the C1 domain of PKC binds to membrane-bound DAG, an interaction aided by the binding of PtS (Bolsover, *et al.*, 2003;Cho, 2001). The engagement of both the C1 and C2 domains then causes a structural change in PKC that induces the release of the PS from the substrate-binding site of the catalytic domain, freeing PKC to catalyze the phosphorylation of downstream substrates (Newton, 1995a). The greater affinity of the C1 domain of novel PKC isoforms for DAG (Giorgione, *et al.*, 2006) allows the recruitment of these isoforms to membranes without the need for Ca^{2+}. Once at the membrane, novel PKC isozymes, like classical ones, unfold the regulatory domains from the catalytic domains and kinase activities ensue.

However, there are additional mechanisms of activation involving post-translational modification. Tyrosine kinases such as $pp60^{src}$ are able to bind some PKC isoforms, such as PKCδ, and catalyze their tyrosine phosphorylation (Joseloff, *et al.*, 2002;Kronfeld, *et al.*, 2000;Yuan, *et al.*, 1998). Phosphorylated tyrosine residues within PKCδ then act as docking sites for SH2 domain-containing proteins, which can further regulate the function of this PKC isoform (Leitges, *et al.*, 2002). The specific tyrosine residue where phosphorylation occurs dictates the response induced by PKCδ. The location of this phosphorylation and resultant cellular response is largely dependent upon the inciting stimulus and cell type. For example, the use of a mutant form of PKCδ containing several tyrosine residue mutations found that phosphorylation of Y^{64} and Y^{187} were important sites for regulating etoposide-induced apoptosis in C6 glioma cells (Blass, *et al.*, 2002). In contrast, viral infection of PC12 cells induced the phosphorylation of Y^{52}, Y^{64} and Y^{155} in PKCδ and these sites proved essential in mediating the antiapoptotic effects of this PKC isoform (Wert & Palfrey, 2000).

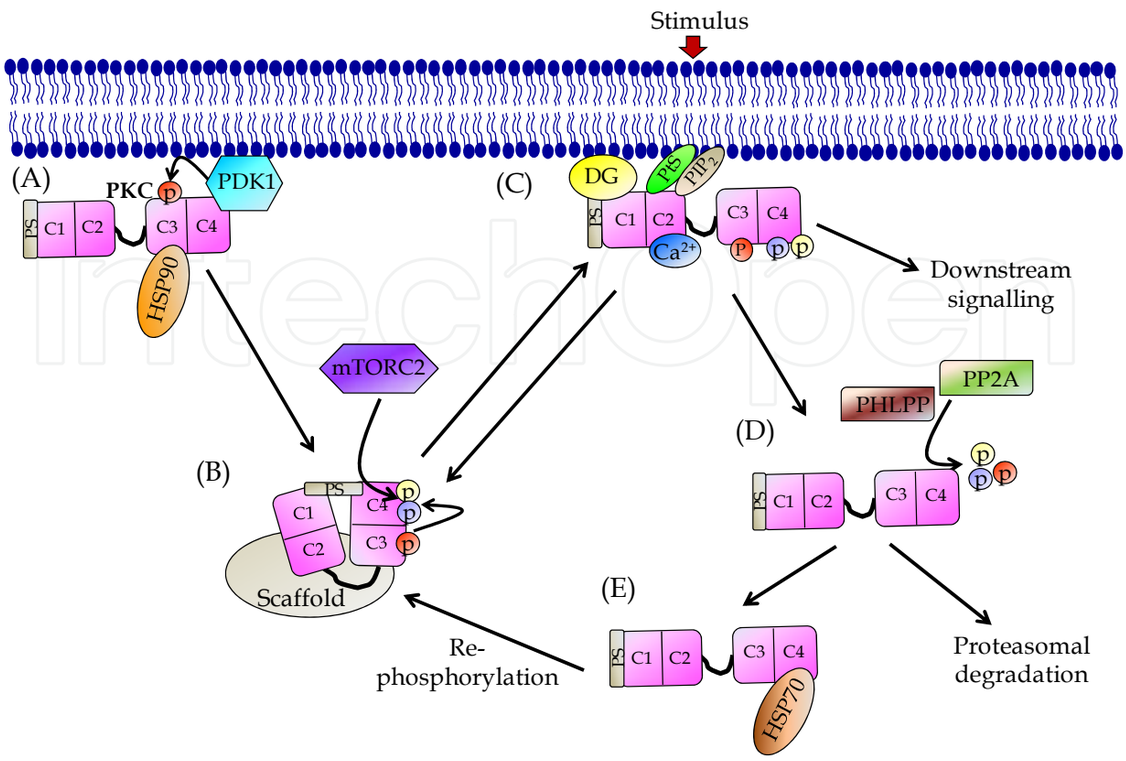

Fig. 3. PKC regulation (adapted from Newton, *et al.*, 2010). (A) HSP90 binds the kinase domain of newly synthesised PKC within the C3 region and primes it for phosphorylation of the activation motif by PDK-1. (B) mTORC2 and/or autophosphorylation is then responsible for phosphorylating the turn and then hydrophobic motifs within the C4 region of the catalytic domain. This results in fully-matured PKC which is then maintained in different cytosolic locations by interacting with scaffold proteins, and in an inactive state through interaction of the pseudosubstrate (PS) in the C1 region of the regulatory domain with the substrate binding site (C4) in the catalytic domain. (**C**) Specific stimuli induce the production of PIP_2, DAG and Ca^{2+}. This causes the recruitment of PKC to the membrane where the C1 domain binds DAG and the C2 domain binds phospholipids such as PIP_2 and phosphotidylserine (PtS) in a Ca^{2+}-dependant way. Membrane association of PKC releases the PS from the substrate-binding site to allow the protein to assume an active conformation and induce downstream signalling. (**D**) Active PKC is prone to dephosphorylation by phosphatases. PHLPP (PH domain leucine-rich repeat protein phosphatase) dephosphorylates the hydrophobic motif, while the activation and turn motifs are likely dephosphorylated by PP2A. (**E**) Dephosphorylated PKC is then either degraded, or HSP70 can bind the unphosphorylated turn motif and allow rephosphorylation of PKC to occur.

Other examples of post-translational modification include the oxidation of cysteines within the C1 domain. This causes a conformational change similar to that induced by lipid binding to result in increased PKC activity (Knapp & Klann, 2000). This phenomenon has been observed for PKCα, -βII, -γ and ε isoforms following exposure to superoxide anions (Knapp & Klann, 2000). Finally, nitrosylation of tyrosine residues can also activate certain PKC

isozymes. Tyrosine nitrosylation occurs in the presence of peroxynitrite and can affect PKCε activation (Balafanova, *et al.*, 2002). Pathologically, this is important for constitutive activation of ERK pathway signalling in hairy cell leukaemia cells (Slupsky, *et al.*, 2007).

Another mechanism of PKC activation is through the generation of an autonomous kinase by caspase-cleavage of the hinge domain. This form of the enzyme lacks the regulatory PS and is therefore maintained in an active conformation. This mechanism is best exemplified by PKCδ, which can be cleaved by caspases following the onset of apoptosis. Such cleavage causes the now autonomous catalytic domain of PKCδ to translocate to the nucleus where it catalyzes histone phosphorylation and chromosomal decondensation to aid in the production of DNA ladders that are characteristic of apoptotic cell death (Brodie & Blumberg, 2003;Kikkawa, *et al.*, 2002).

2.2.3 Co-factor binding

The activity of all PKC isoforms is tightly coordinated by interacting with different scaffold proteins. These interactions help localise the enzyme to different microenvironments where they are in proximity to particular lipid regulators, key proteins and substrates. The C2 (Brandman, *et al.*, 2007) , PB1 (Hirano, *et al.*, 2004;Moscat & Diaz-Meco, 2000) and PDZ (Staudinger, *et al.*, 1997) binding domains of PKCs are specifically engineered for this purpose, and define the individual functions of each isozyme. Examples of such proteins include receptors for activated C kinase (RACKs). RACK1 and RACK2 can competitively bind PKC isozymes, trapping them in active conformations by relieving autoinhibition by the N-terminus (Ron & Mochly-Rosen, 1995). Such interactions have the potential of localising active PKC in areas where sustained ligand activation is not possible. PKC can also interact with the cytoskeleton, either directly through protein-protein interaction or by binding to cytoskeleton-associated proteins (Larsson, 2006). Like PKCs interaction with RACK proteins, these interactions can replace the need for lipid second messengers and induce an active PKC conformation.

Co-factor binding to PKC can also prevent activation of this enzyme. For example, the overexpression of 14-3-3 in jurkat cells inhibits phorbol ester-induced PKCθ translocation from the cytosol to the membrane (Meller, *et al.*, 1996). Taking this into consideration it is important to note that there are different scaffold proteins for all conformations of PKC, all helping to regulate PKC from the moment it is synthesised and activated, until when it is deactivated and degraded.

2.2.4 Downregulation and degradation

Despite having a long half-life in the absence of stimulation, sustained activation of PKC, such as that achieved when cells are treated with phorbol esters, results in its rapid degradation (Hansra, *et al.*, 1999;Huang, *et al.*, 1989;Szallasi, *et al.*, 1994). Active PKC adopts a membrane-bound open conformation that is vulnerable to dephosphorylation by phosphatases (Dutil, *et al.*, 1994). PH domain leucine-rich repeat protein phosphatases (PHLPP) are able to dephosphorylate the hydrophobic motif of novel and classical PKC isoforms when they are in this open membrane-bound conformation (Figure 3D). This dephosphorylation causes these PKC isozymes to shunt to a detergent-insoluble cell fraction where they are then further dephosphorylated on the turn motif, possibly by PP2A, before

being degraded (Brognard & Newton, 2008;Gao, *et al.*, 2008). However, in some instances HSP70 can rescue PKC from this mechanism of degradation. Like HSP90, HSP70 can bind to the dephosphorylated turn motif of PKC and stabilise its conformation, and, in turn, promote its rephosphorylation and catalytic competence (Gao & Newton, 2002) (Figure 3E). This may be important because HSP70 is upregulated in cells undergoing stress, such as in response to chemotherapeutic agents (Jensen, *et al.*, 2009).

2.3 The role of different PKC isoforms in CLL

To gain a greater insight into the function of PKC in CLL pathobiology it is first important to determine the expression profile of this enzyme in CLL cells and to define the specific role each isoform plays in CLL signalling. Together, these findings may help design more customised clinical therapies targeting specific PKC isoforms.

2.3.1 Expression profile

Work in our lab discovered that CLL cells express PKCβI, -βII, -α, -δ, –ε, -ζ and PKC λ/ι (Abrams, *et al.*, 2007;Alkan, *et al.*, 2005). Furthermore, upon comparing the expression levels of these isoforms to the levels expressed in normal B cells, we discovered that CLL cells express less PKCβI and PKCα, and more PKCδ. However, what clearly distinguished CLL cells from normal B cells and other B-lymphoid malignancies was an overexpression of PKCβII equating to 0.53% ± 0.25% of total cellular protein (Abrams, *et al.*, 2007).

2.3.2 PKCβ

The PRKCB gene is transcribed as a single mRNA that is then alternatively spliced to produce PKCβI and PKCβII (Ono, *et al.*, 1986). In CLL cells, PKCβII is the predominant isoform and its elevated expression is thought to be due to increased transcription of the PKC gene by autocrine VEGF stimulation (Abrams, *et al.*, 2010). Furthermore, PKCβII is constitutively active in CLL cells and contributes to cell survival by protecting the cells from pro-apoptotic BCR signalling (Abrams, *et al.*, 2007). The importance of PKCβ in CLL development and propagation was more recently shown in a study using a CLL mouse model where the T-cell leukaemia (TCL1) protein is specifically overexpressed in B cells (Holler, *et al.*, 2009). This particular mouse model of CLL develops an aggressive disease that is similar to the aggressive form of CLL in humans (Yan, *et al.*, 2006). Thus, when this TCL1 transgenic mouse model of CLL was crossed with mice in which PKCβ was disrupted it was found that the CLL-like disease did not develop (Holler, *et al.*, 2009) (Figure 4A). Interestingly, in this same study the TCL1 transgenic PKCβ(+/-) heterozygous mice developed the CLL like disease with a slower kinetic than did TCL1 transgenic PKCβ wild type mice. Taken together, these data indicate that not only is PKCβ expression important for the development of CLL, but the level of expression plays a key role too. This same study also showed that the specific PKCβ inhibitor enzastaurin induced apoptosis of human CLL cells *in vitro*, suggesting that PKCβ was important in maintaining CLL cell survival.

Signals through the BCR are important for CLL cell survival and PKCβII activity inversely correlates with CLL cell response to BCR engagement (Abrams, *et al.*, 2007). An important substrate of PKCβ in B cells is Bruton's tyrosine kinase (Btk). Phosphorylation of Btk on

serine 180 results in its removal from the cell membrane and downregulation of its contribution to BCR signal transduction (Kang, *et al.*, 2001) (Figure 4B). During BCR signalling PKCβ is downstream of Btk activation, therefore, PKCβ acts in a feedback fashion to provide inhibition of this signalling. In CLL cells, PKCβII activity provides inhibition of BCR-induced intracellular calcium release and other downstream signals. We believe that this effect is largely pro-survival because strong, pro-apoptotic BCR signals would be largely suppressed in these cells. However, in CLL cells with high levels of PKCβII activity the pro-survival effects of BCR signalling are lost. Experiments comparing cell survival and Mcl-1 protein levels have shown that both these parameters are increased in response to BCR signalling in CLL cells with low levels of PKCβII activity, whereas there was little effect on these parameters when CLL cells with high levels of PKCβII activity were stimulated by BCR engagement. The regulation of PKCβ activity in CLL cells is likely to involve factors such as VEGF and bFGF, which have been shown to increase PKCβ activity and downregulate BCR signalling (Abrams, *et al.*, 2010).

In addition to its role in downregulating BCR signalling in CLL cells, PKCβII has also been shown to augment anti-apoptotic BCR signalling pathways in CLL cells (Barragan, *et al.*, 2006;zum Buschenfelde, *et al.*, 2010). The expression of ZAP70 in CLL cells is associated with poor disease prognosis and it is thought to enhance BCR signal transduction by acting as a platform to recruit downstream signalling proteins (Chen, *et al.*, 2005). In CLL cells, ZAP70 was recently demonstrated to enhance the BCR signal by recruiting PKCβII into lipid raft domains (zum Buschenfelde, *et al.*, 2010). Here, PKCβII becomes active and is shuttled to the mitochondrial membrane where it is able to phosphorylate anti-apoptotic Bcl-2 and pro-apoptotic Bim_{EL} (Figure 4B). This process provides important pro-survival signals because phosphorylation of Bcl-2 increases its ability to sequester Bim_{EL} and promote cell survival, whilst the phosphorylation of Bim_{EL} results in its proteasomal degradation and protection from its pro-apoptotic effects (zum Buschenfelde, *et al.*, 2010). Another example of how PKCβII mediates BCR-induced survival signals in CLL cells is by activating Akt, a kinase that provides an important source of survival signals to CLL cells (Barragan, *et al.*, 2006;Longo, *et al.*, 2008) (Figure 4B).

Finally, one study has shown that PKCβII may provide pro-survival signals in B cells by inducing the activation of Akt following stimulation by B cell-activating factor (BAFF) (Patke, *et al.*, 2006). This may be important for the pathophysiology of CLL cells because both BAFF and Akt are important sources of pro-survival signals for CLL cells (Barragan, *et al.*, 2006;Nishio, *et al.*, 2005). In B cells, PKCβII also transmits BCR signals to the NFκB pathway by phosphorylating CARMA1, which, together with MALT1, Bcl10 and TAK1 acts to stimulate I-κB kinase activity and NFκB pathway activation (Shinohara, *et al.*, 2005, 2007). Again, this may be pathophysiologically important in CLL because constitutive activation of the NFκB pathway is a feature of the malignant cells of this disease (Hewamana, *et al.*, 2008). Support for this idea comes from studies of diffuse large B cell lymphoma. PKCβ has been shown to be a therapeutic target in the malignant cells of this disease that bear the activated B cell phenotype because of the role it plays in activating the NFκB pathway through the CARMA1/MALT1/Bcl10 complex (Naylor, *et al.*, 2011). Taken together, these studies provide strong support for PKCβII in maintaining CLL cell survival by decreasing pro-apoptotic signals and increasing anti-apoptotic signals.

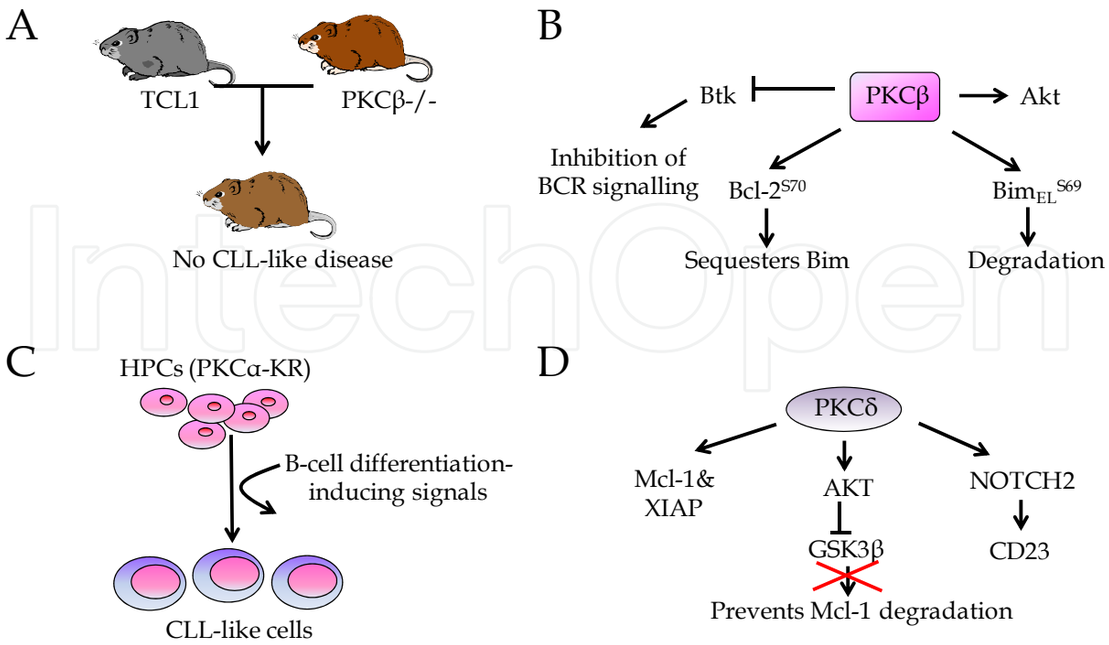

Fig. 4. PKC isoforms in CLL. (A) The T-cell leukaemia-1 (TCL1) mouse overexpresses TCL1 protein and develops an aggressive disease similar to aggressive CLL. TCL1 mice that do not express PKCβ (PKCβ-/-) do not develop the CLL-like disease. (B) PKCβII signalling. CLL cells express elevated levels of PKCβII, likely due to VEGF stimulation. High PKCβII-expressing CLL cases inhibit BCR-signalling by phosphorylating Btk on S^{180} which prevents its activation. Additionally, PKCβII augments antiapoptotic signalling by inducing S^{69} phosphorylation of Bim_{EL} and S^{70} phosphorylation of Bcl-2. Phosphorylation on these residues results in Bim_{EL} proteasomal degradation, and sequestration of Bim_{EL} by Bcl-2, respectively. PKCβII can also activate Akt which is an important mediator of CLL-cell survival. (**C**) Tumour suppressive effects of PKCα in CLL. When fetal-derived hematopoietic progenitor cells (HPCs) overexpressing a dominant negative form of PKCα (PKCα-KR) are induced to differentiate into B lineage cells, a population of CLL-like malignant cells is generated. (**D**) PKCδ signalling. PKCδ is constitutively active in CLL cells via a PI3Kδ-sensitive mechanism. Active PKCδ induces Akt phosphorylation which can then phosphorylate GSK3β. Hyperphosphorylated GSK3β is inactive, preventing it from phosphorylating Mcl-1 and inducing its proteasomal degradation. PKCδ may also induce the transcription of Mcl-1 and XIAP. More recent work has shown that PKCδ can induce the expression of CD23 by activating NOTCH2.

2.3.3 PKCα

Expression and function of PKCα is associated with both tumour promoting and tumour suppressing effects. For example, high levels of PKCα expression are associated with breast, prostrate, gastric and brain cancers (Griner & Kazanietz, 2007;Michie & Nakagawa, 2005) whilst low levels of PKCα expression are associated with cancers of epithelium, pancreas, colon and CLL (Abrams, *et al.*, 2007;Alvaro, *et al.*, 1997;Detjen, *et al.*, 2000;Neill, *et al.*, 2003). In its tumour promoting role PKCα is typically associated with anti-apoptotic signalling

achieved through the ability of this kinase to phosphorylate Bcl-2 at the mitochondrial membrane and increase its ability to sequester Bim (Jiffar, *et al.*, 2004;Ruvolo, *et al.*, 1998). The tumour suppressive functions of PKCα are unclear. It has been shown that PKCα knockout mice spontaneously develop intestinal lesions with greater frequency than wild type littermate controls, and that the mitotic index of the malignant cells derived from the PKCα knockout mice is greater than that of malignant cells derived from wild type mice (Oster & Leitges, 2006). However, the mechanism through which this happens remains undefined.

With respect to CLL, a very interesting study by Nakagawa *et al* (Nakagawa, *et al.*, 2006) has suggested that PKCα may have important tumour suppressive effects in this disease (Figure 4C). Using a system whereby fetal liver-derived hematopoietic progenitor cells (HPCs) are induced to differentiate into B lineage cells, this group show that stable overexpression of a dominant negative PKCα (PKCα-KR) leads to the generation of a population of malignant cells bearing a CLL phenotype ($CD19^{hi}$, $CD23^{+}$, $CD5^{+}$, $sIgM^{lo}$) (Figure 4C). This population of malignant cells, like human CLL cells, are arrested in G_0/G_1 phase of the cell cycle and have enhanced expression of Bcl-2 (Hanada, *et al.*, 1993;Kitada, *et al.*, 1998;Mariano, *et al.*, 1992). However, when these cells are injected into SCID mice they have an enhanced proliferative capacity over mock-transfected and non-transfected control populations. This effect was specific for PKCα-KR because expression of other kinase dead PKC isoforms within the same system did not produce cells bearing the same phenotype (Michie & Nakagawa, 2006).

This system as a model for CLL is interesting for the reason that the malignant cells it generates have a high resemblance to the human CLL phenotype, as well as to CLL-like cells in mouse models of the disease (Holler, *et al.*, 2009;Nakagawa, *et al.*, 2006). This is important because in virtually all mouse models of CLL there is expansion of the B1 population of cells prior to development of disease (Hamano, *et al.*, 1998), and B1 cells in the mouse mainly derive from progenitor cells within the fetal liver (Dorshkind & Montecino-Rodriguez, 2007). Importantly, where the B1 population is absent, such as in PKCβ knockout mice, CLL does not develop in mouse models of this disease (Holler, *et al.*, 2009). Thus, by subverting the function of PKCα, this group has created a system whereby malignant expansion of B1 cells is promoted at the haematopoietic stage. Although this system may not represent the actual mechanism of CLL pathogenesis, it does reveal some important aspects within this mechanism. Since this system is easily manipulated at a genetic level, further study will provide important information on the tumour suppressive function of PKCα not only in CLL cells, but in other cancers as well.

2.3.4 PKCδ

In normal B cells PKCδ plays a key role in mediating signals for cell survival in response to BAFF (Mecklenbrauker, *et al.*, 2004). BAFF signalling is important for maintaining B cell survival in the periphery, particularly with respect to B cells that have become tolerant to autoantigens (Ota, *et al.*, 2010). Thus, mice in which BAFF is overexpressed develop autoimmune diseases such as systemic lupus erythematosis because autoreactive B cells are able to escape tolerance (Stohl, *et al.*, 2005). A similar situation is observed in mice where PKCδ expression is disrupted (Mecklenbrauker, *et al.*, 2004), indicating a pro-apoptotic role

for this PKC isozyme within a process that maintains survival of tolerant B cells. The relationship of PKCδ to BAFF is illustrated by experiments showing that this cytokine prevents nuclear localization of this PKC isoform. The absence of BAFF-stimulated signals results in nuclear localisation of PKCδ where it contributes to phosphorylation of histone H2B at serine 14 and initiation of apoptosis (Mecklenbrauker, *et al.*, 2004). Whether BAFF-mediated signalling stimulates pro-survival functions of PKCδ or merely prevents nuclear localisation is not clear at the present time, however, the pro-survival signalling capabilities of PKCδ, particularly those potentially induced by BAFF, may be highly relevant for CLL cells.

A potential role of PKCδ in maintaining CLL cell survival was first proposed in a paper by Ringshausen *et al*. (Ringshausen, *et al.*, 2002, 2006). This paper demonstrated that PKCδ was constitutively active in a phosphatidylinositol 3 kinase (PI3K)-dependent manner in CLL cells, and that inhibition of this isozyme with rottlerin induced apoptosis of treated cells by reducing the expression of Mcl-1 and XIAP (Ringshausen, *et al.*, 2002, 2006) (Figure 4D). However, with respect to this latter aspect there are some controversies regarding the use of rottlerin. Rottlerin is described as a specific inhibitor of PKCδ, but its pro-apoptotic activity is associated with PKC-independent effects (Villalba, *et al.*, 1999), particularly with respect to the uncoupling and depolarization of mitochondrial membranes (Soltoff, 2007). This uncoupling in cells leads to a reduction in ATP levels and consequent activation of 5'-AMP-activated protein kinase (AMPK), with the end result resembling that produced by direct inhibition of PKCδ. Therefore, results using this inhibitor lack specificity and must be approached with caution. Nevertheless, there is evidence linking PI3K activity to PKCδ in B cells. BAFF signalling in B cells is impaired by the absence of the p110δ isoform of PI3K (Henley, *et al.*, 2008), and inhibition of PI3Kδ with a compound known as CAL101 has shown clinical efficacy both *in vitro* and *in vivo* in CLL cells (Herman, *et al.*, 2010, 2011;Hoellenriegel, *et al.*, 2011). Moreover, other recent findings have shown that PKCδ regulates CLL cell survival by activating the Akt pathway and stabilising the expression of Mcl-1 (Baudot, *et al.*, 2009) (Figure 4D). These findings provide confirmation that PKCδ may play an anti-apoptotic role in CLL cells.

Our own work has discovered that PKC may be important for the survival of CLL cells through its ability to phosphorylate STAT3 on serine 727, and cause increased transcription of the gene for Mcl-1 (Allen, *et al.*, 2011) (Figure 4D). We found that treatment of CLL cells with Bis-1, a specific inhibitor of the novel isoforms PKCδ and PKCε, inhibited the phosphorylation of $STAT3^{S727}$ and decreased the expression of Mcl-1. Conversely, treatment with bryostatin, to activate classical and novel PKC isoforms, induced $STAT3^{S727}$ phosphorylation and increased Mcl-1 expression in CLL cells. Of course, the identity of the PKC isoform phosphorylating STAT3 in CLL cells remains to be characterised by more specific investigations, such as siRNA-mediated knock down of specific PKC isoform expression. Indeed, investigation of the mechanism of Syk-mediated CLL cell survival used siRNA to knock down PKCδ expression and showed a concomitant downregulation of Mcl-1 expression (Baudot, *et al.*, 2009). This study does not address whether PKCδ can phosphorylate STAT3 in CLL cells, but studies using other cell types have shown that PKCδ and PKCε can perform this function (Aziz, *et al.*, 2010;Gartsbein, *et al.*, 2006). Thus, there is ample support for our findings that these PKC isoforms may promote CLL cell survival by activating STAT3-mediated Mcl-1 transcription. Such a mechanism may be useful

therapeutically. High expression levels of Mcl-1 correlate with more aggressive and poor prognosis disease in CLL by providing the malignant cells with protection against chemotherapy (Pepper, *et al.*, 2008). Inhibiting PKC may reduce Mcl-1 expression in CLL cells, thereby lowering the apoptosis threshold and making them more susceptible to other chemotherapeutic agents.

Another way in which PKCδ may contribute to CLL cell pathophysiology is through NOTCH2. One study has used RNAi to knock down PKCδ expression in CLL cells and found that this procedure antagonises PMA-induced NOTCH2 activation (Hubmann, *et al.*, 2010). This result is important because one characteristic of CLL cells is high expression of CD23, and NOTCH2 is known to regulate CD23 expression in these cells (Hubmann, *et al.*, 2002). Taken together, these results suggest that PKCδ and NOTCH2 are critically involved in maintaining the malignant phenotype of CLL cells.

2.3.5 Other PKC isoforms

The function of the remaining PKC isoforms in CLL cells remains poorly defined. However, their role in other cell types is well documented and may provide clues as to what function they have in CLL cells. Intriguingly, PKCε is known to phosphorylate and activate signalling proteins such as Akt (Matsumoto, *et al.*, 2001), PKD (Waldron & Rozengurt, 2003) and STAT3 (Aziz, *et al.*, 2010), pathways which all provide an important source of anti-apoptotic signals to CLL cells. Furthermore, in hairy cell leukaemia, PKCε is activated by nitration of a tyrosine residue causing it to co-localise with ERK1/2 at the mitochondrial membrane and induce activation of the MAPK pathway (Slupsky, *et al.*, 2007). Given that ERK1/2 has been shown to phosphorylate and stabilise the expression of Mcl-1 (Domina, *et al.*, 2004), its activation in CLL cells may provide additional anti-apoptotic signals to CLL cells. Moreover, knockout mouse models have highlighted the importance of the atypical PKCζ in B-cell survival and proliferation by regulating the activation of ERK and NFκB signalling pathways (Martin, *et al.*, 2002).

The above findings are important because Akt, NFκB and STAT3 signalling pathways are known to be constitutively active in CLL cells, and because these pathways are essential in maintaining CLL cell viability (Allen, *et al.*, 2011;de Frias, *et al.*, 2009;Hazan-Halevy, *et al.*, 2010;Hewamana, *et al.*, 2008;Zhuang, *et al.*, 2010). The level of NFκB activation is regarded as an essential component of CLL survival because it correlates with *in vitro* survival of CLL cells as well as with clinical disease progression (Hewamana, *et al.*, 2008). Furthermore, our own work has demonstrated that STAT3 mediates CLL cell survival by inducing Mcl-1 transcription (Allen, *et al.*, 2011), and more recent studies have shown that both pathways can work in concert to induce the expression of anti-apoptotic proteins (Liu, *et al.*, 2011). Collectively, these findings highlight that PKC has the potential to activate numerous anti-apoptotic pathways and that further work is now critical to help understand the specific role these isoforms play in CLL cell signalling.

2.4 CLL cell microenvironment and PKC

It is clear that PKC-mediated signalling pathways seem to provide important survival signals to CLL cells *in vitro*, but how close do these conditions mimic those of *in vivo*? The CLL microenvironment is a milieu rich in signals generated by the interaction of the

malignant cells with IL-6 (Moreno, *et al.*, 2001), IL-4 (Dancescu, *et al.*, 1992) , SDF-1 (Burger, *et al.*, 2000), BAFF and April (Endo, *et al.*, 2007). These cytokines have all been shown to have anti-apoptotic effects on CLL cells. Moreover, the interaction of CLL cells with bone marrow stromal cells (Lagneaux, *et al.*, 1998;Panayiotidis, *et al.*, 1996), follicular dendritic cells (Pedersen, *et al.*, 2002), endothelial cells (Moreno, *et al.*, 2001), nurse-like cells (Burger, *et al.*, 2000) and CD40L-expressing cells (Hallaert, *et al.*, 2008) results in an increase apoptotic threshold. This may be due to the induction of anti-apoptotic genes by these interactions; a comparison of the apoptosis regulatory genes and proteins in neoplastic B cells derived from CLL lymph node proliferation centres and peripheral blood found that lymph node-derived cells had increased expression of anti-apoptotic Mcl-1, Bcl-XL and A1/Bfl-1 (Smit, *et al.*, 2007). Moreover, co-culture of CLL cells on CD40L-expressing fibroblasts strongly induces the expression of these anti-apoptotic proteins, and this culminates in drug resistance (Hallaert, *et al.*, 2008). PKC activation is likely to play a role in all of the microenvironmental interactions CLL cells are likely to encounter in an *in vivo* setting. However, whether inhibition of PKC lowers the threshold of apoptosis in CLL cells within their microenvironment is unknown and requires proper assessment before PKC inhibition becomes a therapeutic target in the treatment of this disease. Recent studies have begun to address this area and have demonstrated the importance of PKC in the survival of CLL cells that have been co-cultured with accessory cells (Martins, *et al.*, 2011).

3. Conclusion

There are convincing demonstrations that PKC-mediated signalling is an important contributor to the development and propagation of the malignant clone in CLL. Inhibition of PKC, therefore, poses an attractive therapeutic approach for the treatment of this debilitating disease, particularly when we consider the roles of the individual PKC isozymes in CLL pathobiology. Within this review we have addressed the potential functions of PKCβ, α, δ and, to a lesser extent, PKCε and PKCζ. There is clear contribution of PKCβ to the pathogenesis of CLL, because disruption of PKCβ expression blocks the development of the CLL-like disease in TCL1-transgenic mice. This same type of experiment now needs to be done for the other PKC isoforms. Thus, disruption of PKCα may accelerate disease progression in TCL1 mice because of the tumour suppressive action of this PKC isoform. The effect of PKCδ disruption is harder to predict. On one hand, disruption of PKCδ should accelerate disease development because the pro-apoptotic effects of this isoform would be lost. However, this prediction does not take into account the pro-survival functions of PKCδ in CLL cells, particularly its potential role in regulating STAT3 phosphorylation and Mcl-1 protein expression. Finally, targeted disruption of PKC isoforms would potentially yield useful information on the role these isoforms play in CLL cell–microenvironment interactions.

Within this review we have not addressed opposing functions of different PKC isoforms. For example, PKCα and PKCδ can act antagonistically to regulate cellular proliferation and apoptosis in glioma cells (Mandil, *et al.*, 2001). It is conceivable that more general inhibitors of PKC, through their ability to inhibit tumour suppressive or pro-apoptotic functions of PKC, may have an adverse effect by actually promoting CLL cell survival and proliferation. Nevertheless, PKC inhibitors such as N-benzoyl-staurosporine (PKC412) have already been tested in clinical trials, and were found to be effective at inducing CLL cell apoptosis in

patients that were refractory to fludarabine and chlorambucil (Ganeshaguru, *et al.*, 2002). Furthermore, a more recent drug called sotrastaurin (AEB071) has been introduced as an immunosuppressant following organ transplant, and for the treatment of psoriasis. Early clinical trials suggest sotrastaurin has no clinically relevant side effects and has the potential to become a long term treatment option (Skvara, *et al.*, 2008). Another study has suggested that AEB071 may even be useful in the treatment of diffuse large B-cell lymphoma (DLCBL) through inhibition of BCR-mediated NFκB pathway activation (Naylor, *et al.*, 2011). Thus, given the potential role of PKC in regulating CLL cell survival and disease pathogenesis and that side effects associated with the use of some inhibitors can be adequately managed within a clinical setting, PKC inhibitors may have therapeutic application in the treatment of CLL.

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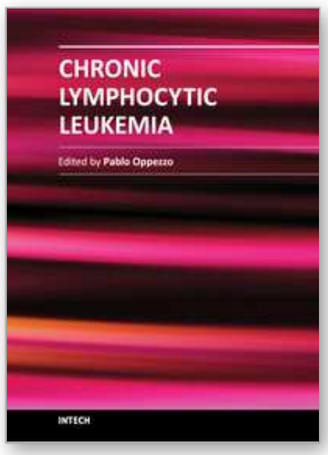

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B-cell chronic lymphocytic leukemia (CLL) is considered a single disease with extremely variable course, and survival rates ranging from months to decades. It is clear that clinical heterogeneity reflects biologic diversity with at least two major subtypes in terms of cellular proliferation, clinical aggressiveness and prognosis. As CLL progresses, abnormal hematopoiesis results in pancitopenia and decreased immunoglobulin production, followed by nonspecific symptoms such as fatigue or malaise. A cure is usually not possible, and delayed treatment (until symptoms develop) is aimed at lengthening life and decreasing symptoms. Researchers are playing a lead role in investigating CLL's cause and the role of genetics in the pathogenesis of this disorder. Research programs are dedicated towards understanding the basic mechanisms underlying CLL with the hope of improving treatment options.

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