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Protein kinase C (PKC) as a drug target in chronic lymphocytic leukemia

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Abstract

Protein kinase C (PKC) belongs to a family of ten serine/threonine protein kinases encoded by nine genes. This family of proteins plays critical roles in signal transduction which results in cell proliferation, survival, differentiation and apoptosis. Due to differential subcellular localization and tissue distribution, each member displays distinct signaling characteristics. In this review, we have summarized the roles of PKC family members in chronic lymphocytic leukemia (CLL). CLL is a heterogeneous hematological disorder with survival ranging from months to decades. PKC isoforms are differentially expressed in CLL and play critical roles in CLL pathogenesis. Thus, isoform specific PKC inhibitors may be an attractive option for CLL treatment.

1. Introduction

The mammalian genome encodes more than 500 protein kinases which mainly phosphorylate serine, threonine and tyrosine residues in substrate proteins (1,2). These kinases are important regulators of nearly all cellular processes. Apart from controlling normal cellular processes many kinases are also overexpressed or mutated in cancers. Thus, kinases are valuable drug targets for the treatment of cancers. The protein kinase C (PKC) family of protein serine/threonine kinases was initially described in 1977 as a cyclic nucleotide-independent kinase (3). Gradually this family became a multi-gene family of ten protein serine/threonine kinases encoded by nine genes (4), which are shown to be important intermediate regulators of many signaling cascades (5,6). Due to the varying tissue distribution as well as subcellular localization PKC family proteins regulate diverse cellular processes and have been proven to be

attractive drug targets in many cancers.

Chronic lymphocytic leukemia (CLL) accounts for 30% of all leukemia and is the most common type of adult hematological malignancy in the Western world. CLL is a neoplastic disease characterized by the accumulation of small B lymphocytes with a mature appearance in bone marrow, blood, lymph nodes or other lymphoid tissues. Although initially CLL was thought to be a homogeneous disease, based on clinical and biological characteristics, this disease shows a high degree of heterogeneity (for a review see (7)). Thus, treatment of CLL is apparently complicated and survival varies from month to decades. For example, expression of ZAP-70 is associated with poor prognosis, and mutation in the tumor suppressor genes p53 and ATM are markers of aggressive disease with a phenotype resistant to conventional chemotherapy (7). Therefore, invention of novel therapeutic agents will be highly beneficial for these groups of CLL patients.

2. The structure and classification of PKC isoforms

PKC isoforms are broadly subdivided into three subfamilies based on their domain structure and cofactor requirement (4). The general structure of PKC proteins includes an amino-terminal regulatory domain and a carboxy-terminal catalytic domain. These two domains are composed of several conserved and variable regions (Fig. 1). The classical or conventional PKC isoforms include PKCα, PKCβII and its splice variant PKCβI, and PKCγ. These four isoforms share equivalent domain structure including C1 and C2 domains in the regulatory part and a catalytic domain divided into two sub-domains (8). Two C1 domains are referred to as C1A and C1B which mediate association of phospholipids or diacylglycerol (DAG). The C2 domain is capable of interaction with two calcium ions. Thus, classical PKC isoforms are regulated by

lipids and calcium ions. The two sub-domains in the catalytic domain are so-called C3 and C4 domains (8). While the C3 domain creates a cavity for ATP binding, C4 mainly act as substrate binding site. Novel PKC isoforms include PKCδ, PKCε, PKCη and PKCθ. These PKC isoforms also have C1A and C1B domains while they lack a functional C2 domain. They are thus regulated by phospholipids and DAG but not by calcium ions. Instead of a functional C2 domain, the novel PKC isoforms contain a novel C2 domain that has important roles in cell signaling despite lacking affinity for calcium ions (9). Atypical PKC isoforms, PKCζ and PKCι, completely lack C2 domain and one C1 domain, and are therefore independent of DAG and calcium for their regulation, but can be regulated by phospholipids. Atypical PKC isoforms contain a PB1 domain that plays a role in protein dimerization. Binding of DAG and calcium to the regulatory domain helps specific PKC isoforms to localize to the specific subcellular compartments. A common feature of all PKC isoforms is the presence of a pseudo-substrate (PS) region in the regulatory domain that occupies the substrate binding site and thereby keeps the enzyme in an inactive state (4). Binding of factors to the regulatory domain causes necessary structural changes allowing PS to release the substrate binding site. These steps make PKC active and allows substrates to interact with the enzyme. Furthermore, association with factors can influence subcellular localization of PKC isoforms and thus PKC changes their subcellular localization upon activation.

3. Subcellular localization of PKC isoforms

The subcellular localization of PKC isoforms has been studied extensively (10-15). All PKC isoforms are expressed and retained in the cytosol before binding to their regulatory factors. Binding to DAG, or its analogs, and calcium ions increases membrane localization of the

classical PKC isoforms (10,11). Novel PKC isoforms also display similar membrane localization in response to DAG analogs, while some of them are also localized to the cytosolic organelles (11). Membrane translocation of PKC isoforms is required for PKC activation and further regulation of downstream signaling pathways. Although the DAG analog TPA is categorized as a potent tumor promoter, a macro-cyclic lactone bryostatin 1 (a mimic of TPA), can activate PKC by binding to the C1 domain, but does not induce differentiation of HL-60 cells and is also not a complete tumor promoter (12). Thus activation of PKC might have differential functions depending on associating molecules. Differential subcellular localization might also play a role in this process. The same factors can direct different PKC isoforms to the different cellular compartments. Bryostatin 1 treatment leads to translocation of PKCα from the cytosol to the plasma membrane while it leads to translocation of PKCβII to the plasma membrane as well as to the nuclear membrane in the HL60 cell line (12). In contrast, 4β-phorbol 12, 13-dibutyrate treatment induces complete translocation of PKCα to the plasma membrane, but only partial translocation of PKCβII to the same cellular compartment. Besides their translocation to the cell membrane, PKC isoforms have also been shown to be associated with other intracellular compartments. Many of them reside at the nuclear membrane or anchor with Golgi and mitochondrial membranes (16). Thus, subcellular localization of PKC isoforms is directed by factor binding to the specific PKC isoforms, and probably dependent on associating factors, different PKC isoforms either act as activators or suppressors of downstream signaling.

4. PKC isoforms and cancer

Although the tumor promoting properties of phorbol esters have been known for many years, the mechanism behind its activity remained a mystery before identification of PKC as a

receptor of phorbol esters. Later many investigators established the complex roles of this family proteins in cancers (17). PKC isoforms are mainly associated with activation of survival pathways leading to increased proliferation and survival. The classical PKCα and novel PKCε are the most potent activators of cell survival. While other PKC isoforms are also to some extent involved in oncogenicity, PKCδ was described as both an oncogene and a tumor suppressor dependent of its tissue distribution. Although a majority of the studies established PKCα as an oncogene, its role in cancer is complex. PKCα triggers apoptosis in glioma cells (18) and inhibits growth of pancreatic cancer cells (19). In addition, loss of PKCα expression potentiates cell proliferation in CaCo-2 cells (20). One recent report suggests that PKCα expression is downregulated in CLL, colon cancer and glioblastoma (21) which is in line with previous observations that PKCα acts as a tumor suppressor in certain cancers. The expression levels of other PKC isoforms also differ between cancer types (Fig. 2). Elevated PKCβ expression was observed in B-cell lymphoma, while expression was lost in a melanoma cell line (for review see (22)). PKCβII is also involved in enhancement of B-cell receptor (BCR) signaling in ZAP-70 expressing CLL cells (23). Even though PKCδ acts as a tumor suppressor in many cancers, it plays the opposite role in CLL. Inhibition of PKCδ induces apoptosis in B-CLL cells (24).

Although mutations in PKC isoforms are very rare, some earlier reports described certain types of mutations in different PKC isoforms. The D294G mutation in PKCα is the most studied mutation and was initially identified in a subpopulation of pituitary tumors (25). Later this mutation was also observed in thyroid and breast cancers (26,27). Patients carrying PKCα-D294G mutation is linked to poor prognosis. The D294G mutation is a loss of function mutation (28) and thus PKCα acts as a tumor suppressor in these tumors. The tumor suppressor role of PKC isoforms has also been described in cancer, where treatment with ingenol mebutate (also

called ingenol angelate or PEP005), an activator of a broad range of PKC isoforms is effective against skin cancer and leukemia (29-31). These observations suggest that different PKC isoforms play differential roles dependent on the cancer type.

5. Signaling from hematopoietic growth factor receptors

Growth factors mediate their biological effects through binding to the cell surface receptors. Binding of specific growth factor to the specific cell surface receptor activates downstream signaling resulting cell proliferation and differentiation. Signal from growth factor is transduced by a number of intermediate signaling proteins including adaptors, scaffold proteins, non-receptor kinases and phosphatases (32-38). Activation of PKC isoforms occurs in response to growth factors under physiological conditions. This process is partially mediated through growth factor-mediated phospholipase C (PLC) activation which by generating DAG and inositol trisphosphate (IP_3) from membrane phospholipids in turn activates classical and novel PKC isoforms. A variety of hematopoietic receptor tyrosine kinases signal through PKC isoforms as well. Binding of growth factors to these receptors leads to dimerization and auto-phosphorylation of the receptors on tyrosine residues which further creates docking sites for a number of signaling molecules (32-37). PKC isoforms amplify signals from these receptor by phosphorylating downstream substrates, while phosphatases counteract these processes (39).

The role of type III receptor tyrosine kinases in hematopoietic malignancies has been extensively investigated. This group includes PDGFRα, PDGFRβ, FLT3, KIT and M-CSFR (CSF1R). The ligand for platelet-derived growth factor receptor (PDGFR), PDGF was originally purified from platelet extracts. Following injury PDGF is released by monocytes and platelets. Although PDGFR plays a role in wound healing, its main role is in embryonal development.

PDGF induces PKC activation through activation of phospholipase C (PLC), in particular PLCγ1 (40). Activation of PKC by PDGF results in induction of c-Fos promoter which subsequently enhances gene expression (41). Furthermore, PKCα is involved in PDGF induced DNA-synthesis (42). PDGF-stimulation induced nuclear translocation of PKCα (43) and nuclear localization of PKCδ (44). Thus, PDGF is able to regulate the subcellular localization of PKC isoforms and PDGF-mediated biological effects are partially mediated through PKC isoforms.

The stem cell factor (SCF) serves as a ligand for the c-Kit receptor. Both SCF and c-Kit are crucial regulators of early hematopoiesis. SCF stimulation activates PKC isoforms independent of PLC activity. Activated PKC then act as negative feedback loop in c-Kit signaling. PKC directly phosphorylates c-Kit receptor on multiple serine residues resulting in partial inhibition of SCF signaling (45). Although, SCF is practically unable to activate PLC, it can activate phospholipase D (PLD), which leads to release of phosphatidic acid that can further be dephosphorylated to generate DAG (46). M-CSF induced NF-κB activation can be blocked by specific PKC inhibitors or siRNA mediated depletion of PKCα in monocytes (47). M-CSF-mediated Erk activation and cell proliferation was also found to be dependent on PKC activation (48). Another hematopoietic growth factor receptor, FLT3 is a frequently mutated gene in hematopoietic malignancies (49). FLT3 is capable of activating PLCγ (50,51) suggesting that FLT3 can activate PKC isoforms. Furthermore, the PKC activator TPA activates Akt independent of PI3K in B-CLL and using a specific PKCβ inhibitor TPA-induced Akt phosphorylation could be inhibited (52). These findings suggests that hematopoietic growth factors can activate PKC isoforms through either PLC or PLD (Fig. 3).

6. PKC expression in CLL

PKC isoforms display differential expression in various cancers. Since overexpression of PKC isoforms play a role in cancer and since PKC is rarely mutated in cancers, it is important to know PKC expression profile in CLL before attempting to use this family of proteins as a target of intervention. Although an early study demonstrated lower total PKC activity in CLL as well as in three other hematopoietic disorders, a higher PKCβ expression was found in CLL patients compared to PKCα and PKCγ (53). A more recent study investigated a more complete profile of PKC isoform expression. While all CLL patient samples analyzed displayed a significant level of PKCβ, PKCγ, PKCδ and PKCζ protein expression, the protein expression of PKCα, PKCε, PKCθ and PKCι was found to vary from 0% to 67% (54). Western blotting analysis of different PKC isoform from hairy cell leukemia (HCL), normal B-cell and CLL cells demonstrated that PKCβII is the dominant isoform in CLL but the expression of PKCζ is also elevated (Fig. 4). Moreover, PKCβII expression is 7 fold higher in CLL compared to that in HCL and B-cells (55). A recent study using microarray data from patient samples showed an elevated expression of PKCβII and PKCζ mRNA in CLL (21). Differential expression of PKC isoforms in CLL patients opens for a possibility of targeting specific PKC isoforms in this disease. Although multiple PKC isoforms are found to be overexpressed in CLL, several CLL studies have pointed towards PKCβII. These studies suggests that elevated expression of PKCβII correlates with aggressive disease phenotype in CLL (56-58). Furthermore, elevated PKCβII expression is sufficient to promote carcinogenesis (59,60). Therefore targeting PKCβII might provide a novel approach in treatment of CLL.

7. The role of PKC isoforms in CLL

It is now widely accepted that BCR signaling is of importance for CLL pathogenesis.

Mutations of BCR sequences contributes to the survival and proliferation of B-cells. The expression of the cell surface molecules CD38 further supports this process. Survival signals from the cell surface B-cell receptor are propagated through a number of signaling proteins including the non-receptor tyrosine kinases Syk, Lyn and ZAP70 (61). Patients with clones carrying few IgV_H mutations or with many CD38+ or ZAP70+ B-cells suffer from aggressive disease, while patients with IgV_H mutated clones or few CD38+ and ZAP70+ B-cells have a favorable prognosis (61). Thus, targeting BCR signaling is a potential avenue in developing CLL therapies.

Upon antigen binding BCR creates a complex with multiple proteins including SFKs and SYK. This complex activates PLCγ2 through membrane-recruited Bruton tyrosine kinase (BTK) (62). PLCγ2 catalyzes the hydrolysis of PIP_2 into DAG and IP_3. Generation of IP_3 results in increased intracellular calcium ion levels. Thus, enrichment of DAG and calcium ion triggers activation of classical PKC isoforms which further activates survival pathways through NF-κB (Fig. 5) (63). Activation of PKCβII can phosphorylate BTK (64). This phosphorylation inhibits membrane translocation of BTK resulting in negative regulation of BCR signaling (65). Thus activation of PKC not only activates pro-survival pathways, it also activates a negative feedback loop.

ZAP70 is an important regulator of T-cell receptor signaling playing equivalent role like Syk in BCR signaling. ZAP70 has also been described as a key component of BCR signaling. In BCR signaling ZAP70 acts as an adaptor protein enhancing BCR signaling (66-68). The exact mechanism behind this regulation was unknown until investigators observed that PKCβII is constitutively associated with lipid rafts in ZAP70 positive CLL cells (23). Since, ZAP70 is constitutively associated with lipid rafts, it probably recruits PKCβII to the rafts. This

recruitment enhances PKCβII activation and activated PKCβII translocates to the mitochondria where it associates with and phosphorylates the anti-apoptotic protein BCL2 and the pro-apoptotic protein BIM_{EL} (23). While phosphorylation of BIM_{EL} directs this protein for proteosomal degradation, BCL2 phosphorylation enhances this process. Therefore, PKCβII-mediated anti-apoptotic effects in CLL (69) is mediated through early activation of NF-κB pathway as well as down regulation of BIM_{EL}.

8. PKC inhibitors in CLL

Treatment of CLL generally involves combination chemotherapies which generate severe side-effects. Targeted therapy allows more specific treatment option. This approach appears to be more popular but still suffers from moderate response and secondary resistance to the drugs. Recent studies suggest that mutations in the inhibitor binding pocket confers secondary resistance (70). Thus, robust drug targets are required for better treatment outcome. Specific signaling molecules are important players of malignant transformation and promising targets for cancer treatment. Multiple small molecule PKC inhibitors have been used in clinical trials including Staurosporine, Enzastaurin, Aprinocarsen, Midostaurin, UCN-01 and Bryostatin 1 with disappointing results (For review see (71)). Besides these synthetic molecules short polypeptide sequence for different PKC isoforms exhibited promising results in animal models (71). Although most small molecule inhibitors lack specificity to the different isoforms of PKC and inhibits a wide range of kinases, short polypeptides are highly specific inhibitors.

Since PKC isoforms are specifically overexpressed in many cancers including CLL, targeted therapy against specific isoforms could be beneficial. The non-specific PKC inhibitor Midostaurin (PKC 412) inhibits growth of B-cell chronic lymphocytic leukemia (B-CLL) in

vitro (72). Treatment of CLL cells with the PKC inhibitor UCN-01 efficiently abrogates cell growth (73). Both UNC-01 and Midostaurin induce cell death independent of p53 or IgV_H mutational status (72,73). Since these drugs are non-specific PKC inhibitors effects might not be exclusive mediated by PKC inhibition. Furthermore, PKC-induced B-CLL cell survival can be inhibited by BisI (74). BisI induces apoptosis by blocking PKC activation and accelerates dexamethasone- and fludarabine-induced apoptosis in the presence of survival factors. The PKC inhibitor safingol controls growth of CLL by inducing apoptosis (54). Although a PKCα antisense oligonucleotide, aprinocarsen, has been used in preclinical and clinical studies (75), patients with CLL would not benefit from this therapy since PKCα expression has been found to be down regulated in this disease (21). These studies suggest that PKC inhibitors are capable of CLL growth control by inducing apoptosis.

The PKCβII inhibitor Enzastaurin effectively kills CLL cells and enhances toxicity of chemotherapy (23), and the specific inhibitor against PKCδ induces apoptosis of CLL cells even in presence of survival factors (24,76). These findings further suggest that targeted therapy against PKC isoforms might be a valuable approach for CLL treatment. Thus, involvement of PKC isoforms in CLL pathogenesis is proven and the simultaneous administration of PKC inhibitor with other drugs has the potential to be beneficial for CLL patients.

9. Conclusion

Recent studies have established that several PKC isoforms are overexpressed in CLL and are essential for CLL cell survival (21,52,54,55,77). Clinical trials using the PKC inhibitors PKC412 and enzastaurin have provided promising results with low side effects in CLL treatment (78-80). Therefore, inhibition of distinct PKC isoforms may offer an important contribution to

the targeted therapy of CLL.

Conflict of Interest: The authors declare no conflict of interest.

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Figure Legends

Figure 1. Domain structure and brief classification of PKC isoforms.

Figure 2. PKC expression in different cancers (generated from figure 1 of reference (21)).

Figure 3. Type III receptor kinase signaling in CLL.

Figure 4. PKC expression in B-cell, HCL and CLL (generated from figure 1B of reference (55)).

Figure 5. BCR signaling in CLL.

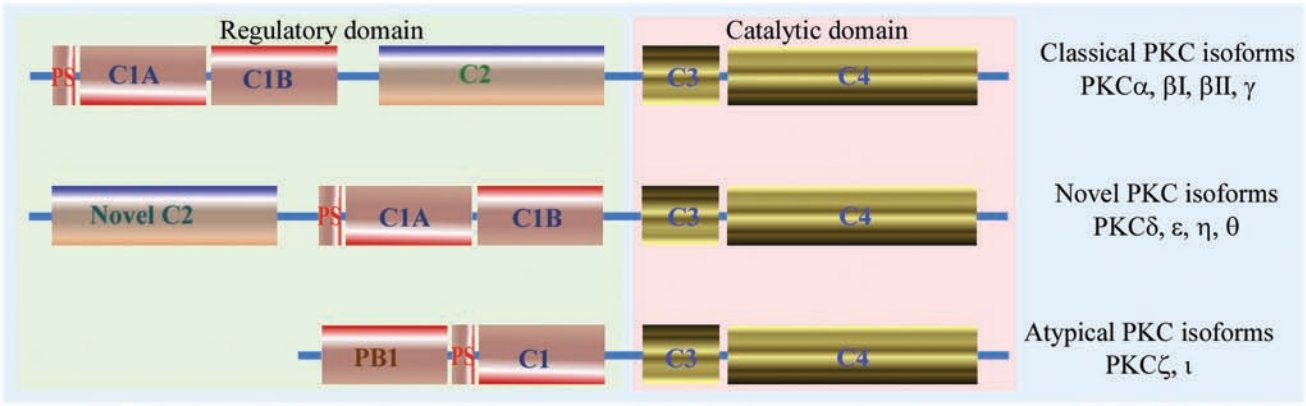
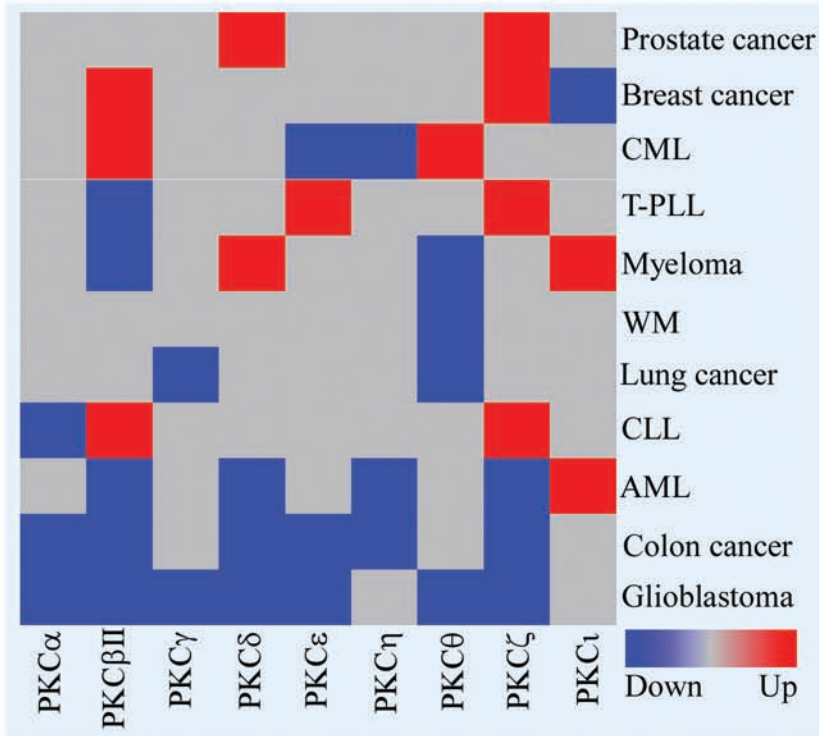

Regulatory domain
Catalytic domain
PS
C1A
C1B
C2
C3
C4
Classical PKC isoforms
PKCα, βI, βII, γ
Novel C2
PS
C1A
C1B
C3
C4
Novel PKC isoforms
PKCδ, ε, η, θ
PB1
PS
C1
C3
C4
Atypical PKC isoforms
PKCζ, ι

Figure 1


Prostate cancer
Breast cancer
CML
T-PLL
Myeloma
WM
Lung cancer
CLL
AML
Colon cancer
Glioblastoma
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PKCγ
PKCδ
PKCε
PKCη
PKCθ
PKCζ
PKCι
Down
Up

Figure 2

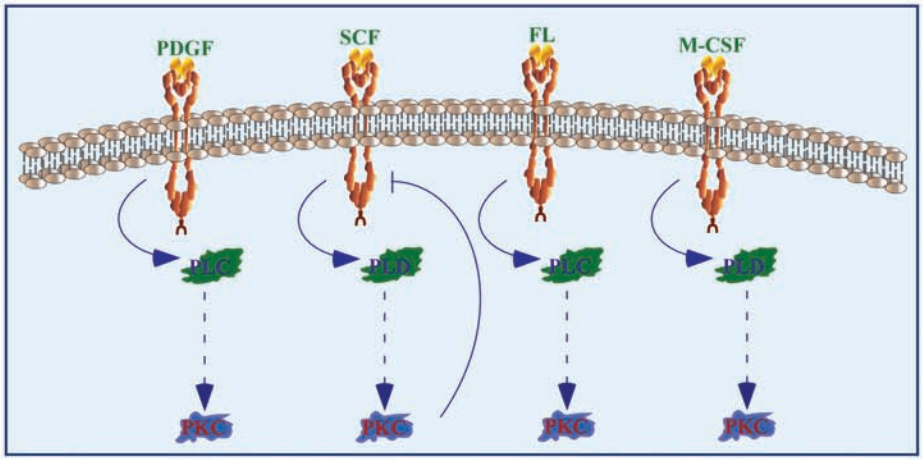

Figure 3

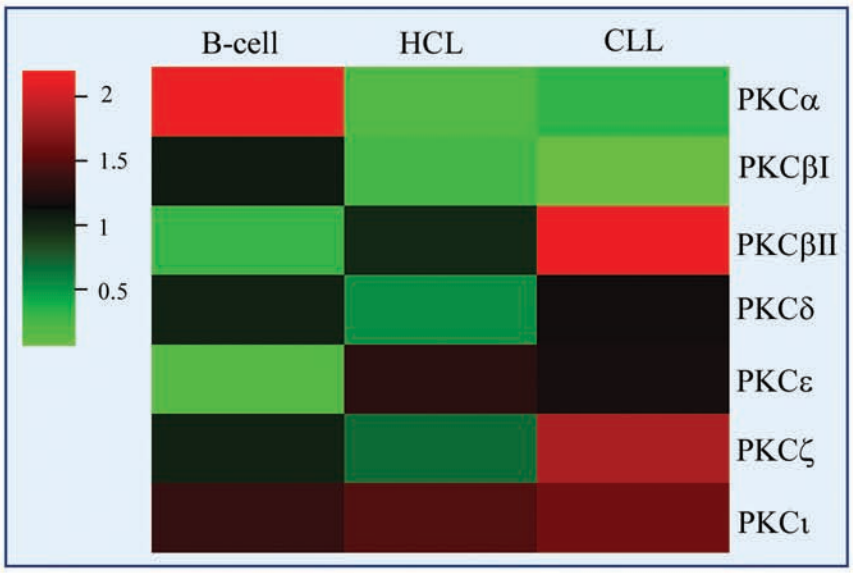

Figure 4

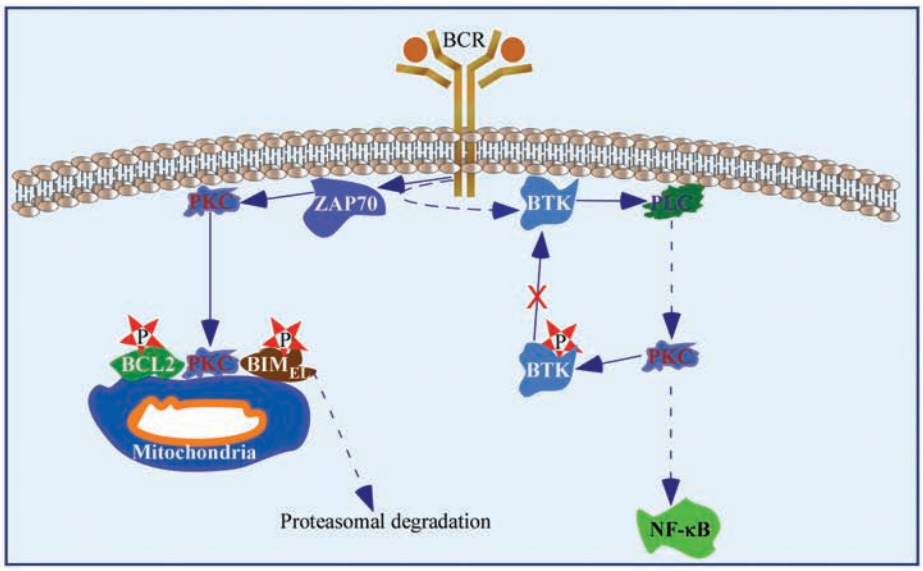
BCR
PKC
ZAP70
BTK
PLC
P
BCL2
PKC
P
BIM
EL
Mitochondria
Proteasomal degradation
P
BTK
PKC
NF-κB

Figure 5