POSSIBLE ANTICANCER EFFECTS OF PMC-A THROUGH THE MECHANISMS OF MITOCHONDRIAL APOPTOSIS AND CELL CYCLE ARREST IN COLON CARCINOMA CELL LINES; HCT116 WT and Bax(-/-)

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Key words: PMC-A, Apoptosis, Bcl-2 protein family, Cell cycle arrest, HCT116 cell lines

Abstract

In previous studies reported from our lab, pramanicin (PMC) induced apoptosis in Jurkat leukemia cells in a dose- and time-dependent manner; and this effect has been shown to be caspase dependent. Therefore, in this study, possible anticancer effects of PMC and its 9 analogs were determined; and further insight into the mechanism of action is provided by focusing on Bcl-2 family proteins and cell cycle arrest in HCT-116 WT and Bax(-/-) colon cancer cell lines.

Among 9 analogs screened PMC-A was the most effective chemical in inducing apoptosis as shown by M30 and time-dependent Annexin V assays. Dose and time kinetics indicated that PMC-A was the most effective in inducing apoptosis (25 μM, ≈30% in 24 hours), therefore, it was chosen for further experiments. To understand the role of Bax in apoptotic pathway, parallel experiments were carried out in HCT116 WT and Bax(-/-) cells. Compared to the WT cell lines, the apoptotic response of Bax(-/-) cells after the treatment with PMC-A at 25 μM was detected at 24th hour indicating a late apoptotic response. Caspase-9 activation and apaf-1 up-regulation were observed consistently in a time-dependent manner. Although caspase-3 cleavage was

observed in WT cells, there was no caspase-3 cleavage in HCT116 Bax(-/-) cells indicating a caspase-3- independent apoptotic pathway.

To provide further insight into the apoptotic response, regulation of Bcl-2 protein family members (among pro-survivals, Bcl-2, Mcl-1 and Bcl-xL; among pro-apoptotics Bax, Bak, Bim, Puma, Bad and Bid) were monitored by immunoblotting in a time dependent manner. As Bcl-2 was down-regulated, Mcl-1 was up-regulated and Bcl-xL levels remained the same. On the other hand, among pro-apoptotic proteins, Bim and Bax was up-regulated, Bak was down-regulated, Bad and Puma levels remained constant. Since Bcl-2 and Mcl-1 were the regulated proteins among pro-survivals, their modulation in Bax(-/-) cells were determined. Their pattern did not change in comparison to WT cells, which can be explained by the upstream effect of these proteins. Among other pro apoptotic proteins, Bid was truncated in WT cells, whereas was not truncated in Bax(-/-) cells.

Since equilibrium between cell proliferation and death has been proposed to be a fundamental point in carcinogenesis, effect of PMC-A on cell cycle was identified. There was an increase in p21 level, a general inhibitor protein, in both cell types; WT and Bax(-/-). WT cells did not arrest at any phases of the cell cycle, however, Bax(-/-) cells arrested at G_2-M phase, indicating a sensitive profile of Bax(-/-) cells to cell cycle arrest.

Our data indicate that PMC-A induced intrinsic apoptosis pathway, triggering caspase-cleavage and Bcl-2 regulation in HCT116 WT cells, whereas Bax deficiency induced a different pathway in programmed cell death response. In summary, apoptosis is a dominant effect of PMC-A in HCT116 cells, on the other hand Bax deficiency made cells more sensitive to PMC-A induced cell cycle arrest indicating alternative response to apoptosis.

HCT116 WT VE BAX(-/-) KOLON KANSER HÜCRELERİNDE PMC-A'NIN MİTOKONDRİYEL APOPTOZ VE HÜCRE DÖNGÜSÜNDEN KAÇIŞ MEKANİZMLARI ÜZERİNDEN OLASI ANTİ-KANSER ETKİLERİ

Gizem Karslı

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Anahtar kelimeler: PMC-A, Apoptoz, Bcl-2 protein ailesi, Hücre döngüsünden kaçış, HCT116 hücre hattı

Özet

Laboratuvarımızın önceki çalışmalarında, Pramasin'in (PMC) Jurkat lösemi hücrelerini apoptoza teşvik ettiği doza ve zamana bağlı olarak gösterilmiştir. Buna dayalı olarak, bu çalışmada, HCT116 kolon kanser hücrelerinde PMC ve 9 analoğunun olası anti-kanser etkileri belirlenmiş, etki mekanizmaları Bcl-2 protein ailesine ve hücre döngüsünden kaçışa odaklanılarak araştırılmıştır.

M30 ve zamana bağlı Annexin V deneyleriyle gösterildiği üzere, taramaları yapılan 9 analog içerisinde PMC-A, HCT116 hücrelerini apoptotoza indükleyen en etkili kimyasaldır. Doz ve zaman kinetikleri, PMC-A'nın apoptoza indükleyen en etkili ajan olduğunu göstermiştir (25 μM, ≈30%, 24 saat), bu nedenle, PMC-A ileriki deneylerde çalışılmak üzere seçilmiştir. Bax'ın apoptotik yolaktaki rolünü anlamak için HCT116 Bax(-/-) hücreler üzerinde de paralel deneyler gerçekleştirilmiştir. Bax(-/-) hücreleri 25 μM PMC-A ile muamele edildiğinde, WT hücrelere kıyasla geç olarak apoptotik cevap 24. saatte gözlenmiştir. WT hücrelerde, kaspaz-9 aktivasyonu ve uyumlu olarak apaf-1 artışı zamana bağlı olarak gözlenmiştir. WT hücrelerde kaspaz-3 aktivasyonu gözlenmesine rağmen, HCT116 Bax(-/-) hücrelerde kaspaz-3 aktivasyonu

gözlenmemiştir, bu durum belirtilen hücrelerde kaspaz-3'ten bağımsız bir apoptotik yolağa işarettir.

Apoptotik cevabı daha ayrıntılı incelemek üzere, Bcl-2 protein ailesi üyelerinin düzenlenmesi (yaşam destekleyicilerden, Bcl-2, Mcl-1 ve Bcl-xL; apoptoz destekleyicilerden, Bax, Bak, Bim, Puma, Bad ve Bid) immünoblotlama tekniği ile zamana bağlı olarak incelenmiştir. Bcl-2 miktarı azalırken, Mcl-1 miktarı artmış ve Bcl-xL miktarı değişmemiştir. Diğer taraftan, apoptoz destekleyici proteinlerden, Bim, ve Bax miktarı artarken, Bak miktarı azalmış, Bad ve Puma miktarı sabit kalmıştır. WT hücrelerde miktarında değişim gözlenen yaşam destekleyici proteinlerden Bcl-2 ve Mcl-1 seviyeleri, Bax(-/-) hücrelerde kontrol edilmiştir, apoptotik yolakta Bax'ın öncesinde düzenlendiklerinden, beklendiği üzere anti-apoptotik proteinlerin patterninde bir değişim olmamıştır. Apoptoz destekleyici proteinlerden Bid, WT hücrelerde kesilirken Bax(-/-) hücrelerde kesilmemiştir.

Hücre çoğalması ve ölümü arasındaki denge kanser gelişiminde önemli bir nokta olduğundan, PMC-A'nın hücre döngüsü üzerindeki etkisi belirlenmiştir. Genel siklin-bağlı kinaz engelleyici proteinlerden p21 miktarı WT ve Bax(-/-) hücrelerde artmıştır. WT hücrelerde hücre döngüsünün hiçbir fazında kaçış gözlenmezken, Bax(-/-) hücrelerde hücre döngüsünün G_2-M fazında kaçış gözlenmiştir; bu durum Bax(-/-) hücrelerin hücre döngüsünden kaçma hassasiyetinin olduğuna işarettir.

Verilerimiz göstermektedir ki PMC-A etkisi ile HCT116 WT hücreleri kaspaz aktivasyonu ve Bcl-2 düzenlenmesi ile iç apoptoz yolağına indüklemiştir, fakat bunun yanında Bax eksikliği programlanmış hücre ölümünün farklı bir yolak üzerinden gerçekleşmesine neden olmaktadır. Özet olarak, apoptoz PMC-A'nın HCT116 üzerinde baskın bir etkisidir; diğer taraftan Bax eksikliği PMC-A uygulanmış hücrelerin, hücre döngüsünden kaçma hassasiyetini artırmaktadır; bu durum apoptoza alternatif bir cevaptır.

"To my family"

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TABLE OF ABBREVIATIONS

HCT116	Human colon carcinoma cell line
PMC	Pramanicin
TNF	Tumor necrosis factor
TRADD	TNFRSF1A-associated via death domain
FADD	Fas-Associated protein with Death Domain
FLIP	FLICE-inhibitory protein
DISC	Death-inducing signaling complex
IAP	Inhibitor of Apoptosis family of proteins.
BH	Bcl-2 Homology Domain
MMR	Mismatch Repair
CEBP	CCAAT-Enhancer Binding Protein
AIF	Apoptosis Inducing Factor
ROS	Reactive oxygen species
JNK	C-Jun N-terminal kinases
ASK	Apoptosis signal-regulating kinase
MAPK	Mitogen-activated protein kinases
PAK2	p21 (CDKN1A)-activated kinase 2
STAT	Signal Transducers and Activators of Transcription protein
DD	Death domain
MOMP	Mitochondrial outer membrane permeabilization
PARP	Poly (ADP-ribose) polymerase
CDK	Cyclin dependent kinase
CDKI	Cyclin dependent kinase inhibitor
ICAD	Inhibitor of caspase activated DNase

LIST OF FIGURES

1 INTRODUCTION

Colon cancer (also called as colorectal cancer or bowel cancer) is the third most common cancer type and the second leading cause of death related to cancers in the western world observed in both women and men. Cancerous growth is generally localized in colon or rectum, it has different stages from I to IV according to its spreading profile over the tissues. More than 50% of colorectal cancer patients can be cured by surgery alone, but afterwards some recurrent diseases can be observed, therefore there is a need to identify which of these patients would benefit from adjuvant therapy. The most important treatment available for stage IV patients is systemic chemotherapy. There are several kinds of agents/compounds identified which are effective against colorectal carcinoma through different mechanisms; as targeting special enzymes, growth factors, microtubules; and as a result impairing cell cycle especially for fast-dividing cells and/or inducing cells apoptosis. As an example; combretastatins and 2-methoxyestradiol affect by apoptosis, however angiostatin and cetuximab affect by cell cycle arrest [1, 2].

Mismatch repair (MMR) system is a DNA repair pathway important for genome integrity. Deficiency in this system is an advantage for tumor cell development, it acquires a competitive advantage, such as enhanced proliferation, and/or a survival advantage with apoptosis resistance, and subsequently an increased metastatic potential. Because of that, patients with colon cancer give variable responses to chemotherapy; cancer cells that have MMR deficiency are resistant to many cytotoxic drugs. HCT116 cells have defects in their mismatch repair system because of MLH1-deficiency, an important MMR protein. Therefore it is hard to find an effective chemical to beat this aggressive cell line [3].

There are several molecules discovered or designed for cancer therapy which eliminate the barriers to programmed cell death and restore the natural autodestruction pathways of cancer cells through affecting components of cell death machinery such as Bcl-2 protein family. In addition to targeting programmed cell death mechanism, elimination of cancerous cells can also be carried out by targeting cell cycle. In general, tumor cells are not capable to stop at the certain points of a cell cycle phase as a result of deficient checkpoint integrity. Some anti-cancer drugs can induce critical cell cycle inhibitor proteins as cyclin dependent kinase inhibitors to make cells arrest at a defined phase, and as a consequence destructing cell cycle can facilitate the induction of apoptosis [4, 5].

Pramanicin (PMC) is a novel anti-fungal agent, was recently isolated from a fungal fermentation, its general structure is composed of a polar head group and a simple aliphatic side chain [6]. Kutuk et.al. showed that PMC induces apoptosis in Jurkat leukemia cells in a dose- and time-dependent manner. They stated that PMC triggers cytochrome-c release, caspase activation and JNK/p38 kinase cascade activation as proapoptotic signaling pathways [7].

In order to eliminate tumor cells, apoptosis is an effective way to be induced by chemotherapeutic agents. Apoptosis has two different pathways; extrinsic and intrinsic pathways. Extrinsic pathway is activated via death receptor activation and intrinsic pathway is activated by stress-inducing stimuli. In extrinsic pathway, triggering of cell surface death receptors of the tumour necrosis factor (TNF) receptor superfamily causes rapid activation of the initiator caspases (2, 8, 9, 10) after forming a trimerized receptor-ligand complex (DISC) through the adaptor molecule Fas-associated death domain protein (FADD). However, in intrinsic pathway, stress-induced apoptosis causes perturbation of mitochondria and the release of several proteins, as cytochrome c, Smac, Diablo, AIF. The release of cytochrome c, from mitochondria is regulated in part by Bcl-2 family members, with anti-apoptotic (Bcl2, Bcl-xL and Mcl-1) and proapoptotic (Bax, Bak and tBid) members inhibiting or promoting the release, respectively. By the release of cytochrome c, it binds to apoptotic protease-activating factor 1 (apaf1), which then forms apoptosome complex; apaf1–caspase9 apoptosis. Then effector caspases (3, 6, 7) are activated; these are responsible for the cleavage of important cellular substrates

leading to classical biochemical and morphological changes associated with the apoptotic phenotype [8, 9].

Cell cycle is a regulated series of tightly integrated events that allow the cell to grow and proliferate, lots of important protein families regulates the cycle which are common targets for mutational inactivation or overexpression in human tumours. The main players of cell cycle are the cyclin dependent kinases (CDKs) which, when activated, makes the cell to move from one phase of the cell cycle to the next. (Figure.5) Cancer is the dysregulated result of the cell cycle such that cyclins are overexpressed or CDKIs (CDK inhibitors) are not expressed, then the cells undergo unregulated cell growth. As an example, cyclin D and E families and their cyclin-dependent kinase partners induces the phosphorylation of the retinoblastoma tumor suppressor protein and subsequent transition through the cell cycle. If cell cycle checkpoints abrogated before DNA repair is complete, it can activate the apoptotic cascade leading to cell death. Because of that mechanism, a series of targeted agents have been developed that directly inhibit the CDKs, unrestricted cell growth, and trigger a growth arrest. Also, there are some chemotherapeutics that abrogate the cell cycle checkpoints at critical time points and make the tumor cell susceptible to apoptosis. To sum up, in order to investigate a chemotherapeutics' effectiveness on an organism, its effect on cell cycle must be studied [10, 11].

2 OVERVIEW

2.1 Apoptosis

Multicellular organism is a highly organized cellular community. The cell number of this community is tightly regulated for homeostasis; in addition to controlling the rate of cell division, regulation is also performed by controlling the rate of cell death. If cells are no longer needed in dynamic balance of the living system, a suicide process is carried out by activating an intracellular programmed cell death mechanism; apoptosis; which can be triggered by intrinsic or extrinsic signals. Apoptosis is not an accidental passive mechanism as necrosis; it is an energy requiring and evolutionary conserved mechanism. Apoptosis is involved in several processes from embryogenesis to adult organ and tissue development, additionally in elimination of dangerous cells for organism as virally infected cells [12, 13, 14]. Therefore, any drawback involved in apoptosis signaling pathway can generate serious consequences as neurodegenerative diseases, developmental defects, cancer, or autoimmunity [15].

Apoptosis is a distinctive type of cell death with specific morphological and biochemical characteristics; including cell membrane blebbing without losing its integrity, chromatin condensation, genomic DNA fragmentation, and exposure of specific phagocytosis signaling molecules (i.e. phosphatidylserine) on the cell surface. After these morphological changes cells are fragmented into membrane surrounded apoptotic bodies and engulfed by macrophages or neighboring cells before any leakage of its contents (Figure 2.1 1) [16].

Molecular mechanism of apoptosis simply consists of initiation, mediation, execution and regulation. Mechanistic studies of apoptosis started with the genetic

studies in the nematode *C. elegans*. The first genes identified in the signaling pathway were *Ced-3*, *Ced-4, Ced-9, Egl-1*; homologs of the human genes for a defining member of *Caspase*, *apaf-1,* an anti-apoptotic member of *Bcl-2* gene family, a pro-apoptotic member of *Bcl-2* gene family, respectively. In a normal cell *Ced-4* is inactive associated with *Ced-9; Egl-1* binds to *Ced-9* and displace*s Ced-4*, finally Ced-3 is activated to induce apoptosis. It is a basic evolutionary model for apoptosis with their homologs in different organisms, the higher organism the more complexity [17].

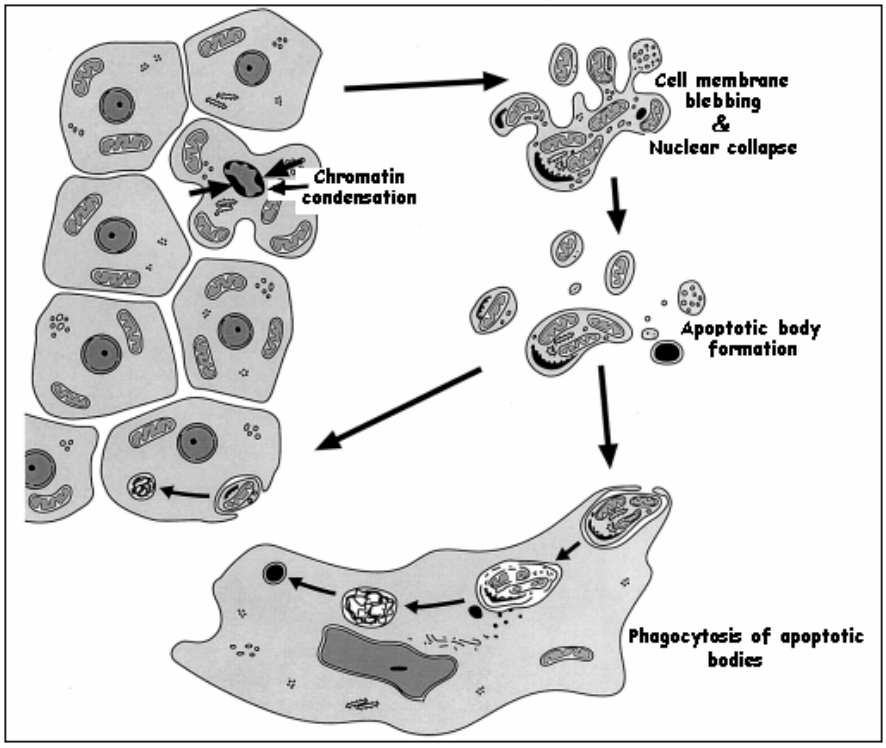

Figure 2.1 1: Morphological characteristics of apoptosis (modified from [16])

2.1.1 Caspases

The intracellular machinery of apoptosis is mediated by an intracellular proteolytic cascade through a family of proteases called caspases (cysteine-dependent aspartate-specific acid proteases). They have a cysteine at their active site and cleave their target proteins after specific aspartic acid residues. Caspases are expressed as inactive precursors called procaspases, activated by cleavage at aspartic acids by other caspases. The caspases at the upstream called initiators (caspase-2, -8, -9, and -10), the

downstream caspases activated by initiators are called executioners or effectors (caspase-3, -6 and -7). Caspases are the signaling mediators leading apoptotic execution pathways by cleaving and/or activating/inactivating several cellular proteins. Caspase targets can be classified to four subgroups according to their cellular function;

1. Mediators and regulators of apoptosis (apoptotic proteins); effector caspases, Bcl-2 protein family members, inhibitor caspase activated DNase (ICAD), some protein kinases (i.e. AKT, PAK2)
2. Structural proteins; lamins, intermediate filament proteins, adherence junction proteins (i.e. β-catenin)
3. Cellular DNA repair proteins; DNA dependent protein kinases, DNA repair enzymes
4. Cell cycle related proteins; cell cycle inhibitors (i.e. CDK inhibitors), cell cycle regulators (i.e. Cdc2) [14, 18, 19, 20].

2.1.2 Apoptosis Pathways

Procaspase activation can be triggered by a wide range of signals inducing two major apoptotic pathways in mammals; extrinsic (death receptor mediated) pathway and intrinsic (mitochondrial mediated) pathway. These pathways can intersect each other in some circumstances (Figure 2.1.2 1) [15].

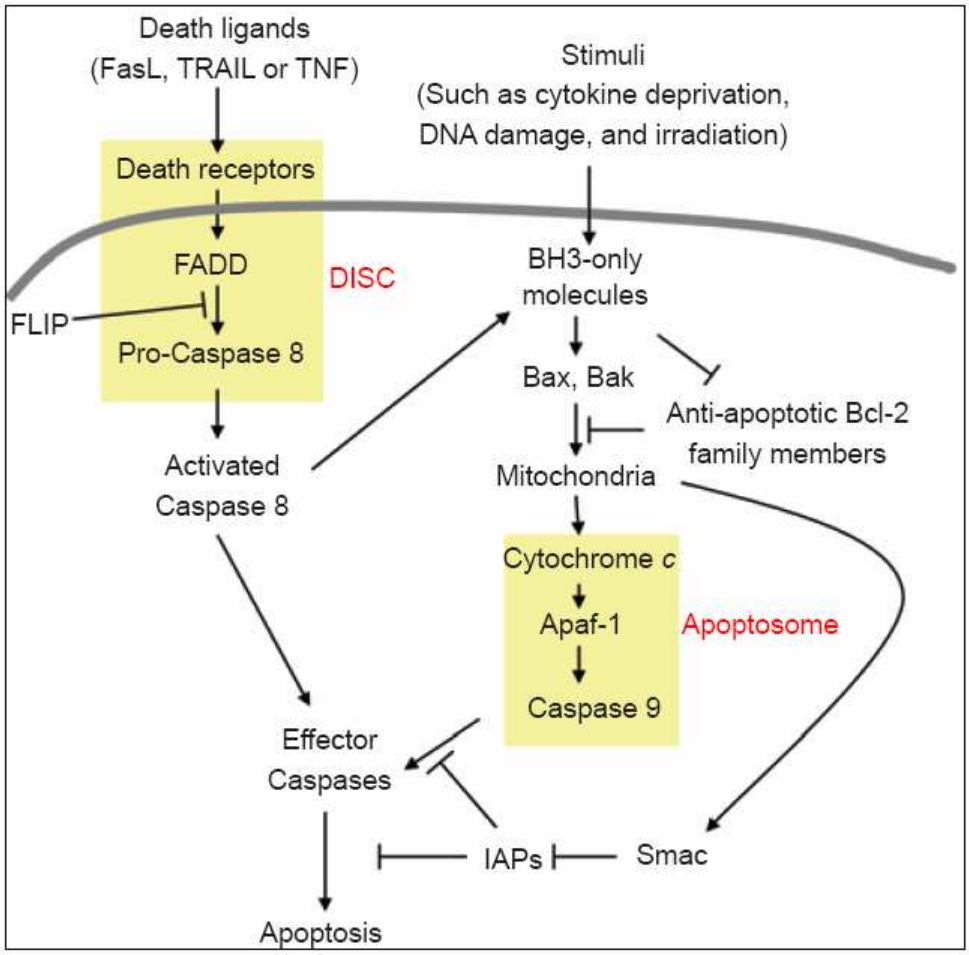

Figure 2.1.2 1: Simple illustration of extrinsic and intrinsic apoptosis pathways [15].

2.1.2.1 Extrinsic Pathway

Extrinsic pathway is activated by binding of death inducing signals to cell surface receptors such as Fas, tumor necrosis factor receptor or TRAIL receptors in order to maintain tissue homeostasis, especially for the immune system. When the death ligand (i.e. Fas ligand) binds to its receptor (i.e. Fas), receptors are oligomerized, recruit intracellular adaptor proteins bearing death domains (DD) (i.e. FADD) and procaspase-8; forming a death inducing signaling complex (DISC). As a consequence of DISC formation, Caspase-8 is activated. Activated caspase-8 activates one another to amplify the signal and then activates the downstream effector caspases, including caspase-3, 6 and 7 [13, 21, 22].

The direct activation mechanism of caspase dependent extrinsic pathway is believed to occur in certain cell types (type I cells, Fas responsive cells), type I cells directly activates sufficient amounts of caspase-8 in the DISC, consequently caspase-3 activation occurs. This simplified extrinsic pathway is important for immune system involved in the deletion of transformed cells. Whereas in some cell types the process is mitochondrial dependent, such as hepatocytes (type II cells), type II cells require a mitochondrial amplification loop to activate sufficient effector caspase, this process is mediated by proapoptotic Bid truncation and translocation to mitochondria, then cytochrome c is released and effector caspases are activated. When caspase-8 is insufficient, the action of Bid on mitchondria can determine whether a cell will be induced mitochondrial apoptosis, where the intersection of the two pathways (intrinsic and extrinsic pathways) occurs. The cleavage of the C terminal of Bid makes it active and as a result it is translocated from cytosol to mitochondria, where it oligomerizes with Bax and Bak. After the oligomerization, cytochrome c is released from the mitochondria. In other words, extrinsic apoptosis of type II cells is sensitive to Bcl-2 protein family [19, 23, 24, 27].

2.1.2.2 Intrinsic Pathway

Intrinsic apoptosis is induced through intrinsic signals when cells are damaged or stressed by irradiation, oxidative stress, chemicals, protein misfolding or growth factor deprivation. Mitochondrion is induced to release cytochrome c, electron carrier protein, to the cytosol in response to membrane permeabilization; cytosolic cytochrome c binds to and activates an adaptor protein apoptosis protease-activating factor (apaf-1). Aggregated apaf-1 binds to procaspase-9, forming an intracellular DISC-like complex called as “apoptosome”. Within this complex, caspase-9 is activated and activated caspase-9 leads the process of caspase-3 activation. Although it is not clearly identified, there are two major pathways for the release of caspase activating protein from mitochondria to the cytosol; the first is the opening of mitochondrial permeability transition (MPT) pore; the second is generation of specific release channels in the outer mitochondrial membrane, regulated by pro-apoptotic members of Bcl-2 [25, 26].

Mitochondrial outer membrane permeabilization (MOMP) is a critical event in intrinsic apoptosis, it is considered to be the point of no-return in apoptosis induction. MOMP induces release of some apoptotic proteins from mitochondria to cytoplasm leading a caspase cascade. In a non-apoptotic cell, it is prevented by anti-apoptotic Bcl-2 protein family, which is a determinant family of proteins containing anti- and pro-apoptotic members and rather than Bcl-2 control induction through mitochondrial permeability transition in response to the Ca^{2+} from endoplasmic reticulum stores is a second release mechanism [15, 34]. As the outer mitochondrial membrane disruption occurs, a group of mitochondrial membrane proteins in the intermembrane space of mitochondria is released, these include cytochrome c, Smac/DIABLO, Omi/HtrA2, AIF and endonuclease G; Smac/DIABLO and Omi/HtrA2 inhibit IAP. Since these apoptotic proteins released to cytosol, execution of apoptosis by proteolytic caspase cascade (Smac/DIABLO and Omi/HtrA2) or even by caspase independent death effectors (such as AIF and endonuclease G) is triggered [28, 29, 30, 31].

Additionally, there are other apoptotic pathways described in mammalian cells, perforin/granzyme-dependent pathway is an example; cytotoxic T cells (CTLs) induce apoptosis of their targets through a pore forming protein (perforin) and a serine protease (granzyme), which is released into cytoplasm of the target cell upon degranulation of the activated CTLs. Granzyme can either cleave the effector caspase, caspase-3, or cleave other substrates leading a caspase-independent pathway [32, 33].

2.1.3 Bcl-2 Protein Family

BCL-2 is an oncogene discovered at the translocation breakpoint of chromosome 14, in B-cell follicular lymphomas. The protein product of this oncogene does not promote cell proliferation as other oncogenes, rather its overexpression inhibits cell death. Several Bcl-2 family members were discovered after the identification of Bcl-2 protein itself. These members are similar to each other at the structural level; they have either tertiary or secondary structural similarity with Bcl-2 protein, they share one or more Bcl-2 homology (BH) domains; BH1, BH2, BH3 and BH4. Bcl-2 protein family has two different groups in a functional manner;

1. Pro-apoptotic members (i.e. Bax, Bak, Bok, Bid, Bim, Bik, Bmf, Noxa and Puma) promoting apoptosis

2. Anti-apoptotic members (i.e. Bcl-2, Bcl-xL, Bcl-w and Mcl-1) inhibiting apoptosis (Figure 2.1.3 1).

Anti-apoptotic proteins contain all BH domains (BH1-4) except Mcl-1. Among the pro-apoptotic proteins, there are groups according to their homolog structures; multidomain pro-apoptotic members and BH3-only pro-apoptotic members. Additionally, anti-apoptotic proteins and multidomain pro-apoptotic proteins contain a mitochondrial transmembrane (TM) domain [27, 34, 35].

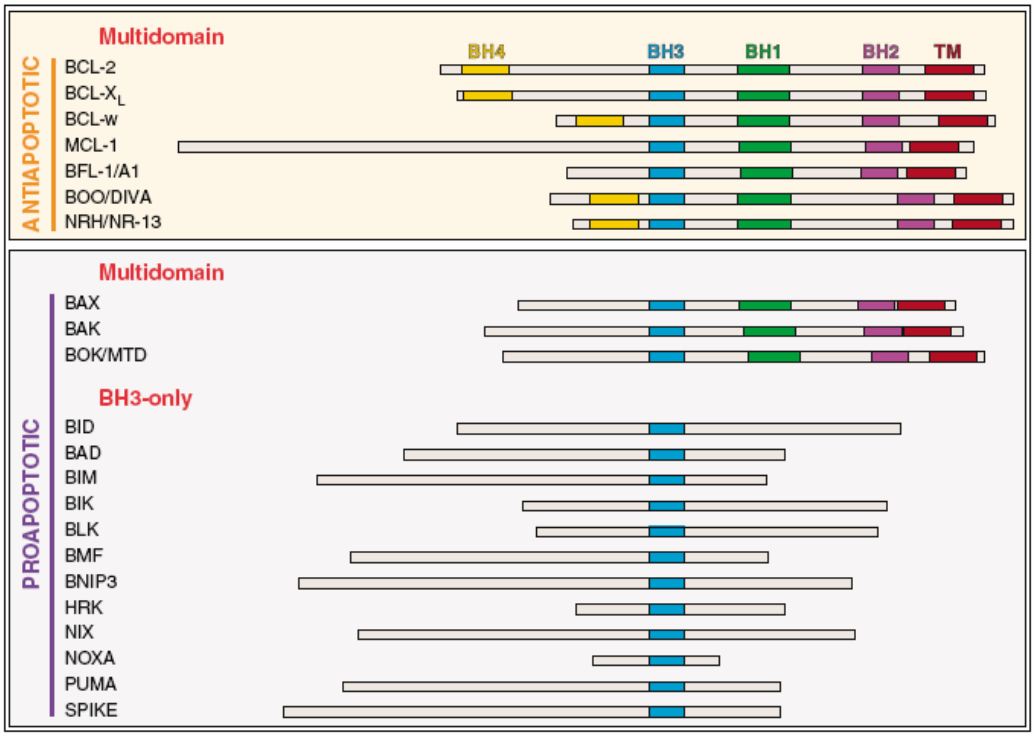

Figure 2.1.3 1: Bcl-2 protein family members [35].

2.1.3.1 Bcl-2 Protein Family Members

Anti-apoptotic Bcl-2 family of proteins, in other words pro-survivals, are the proteins protecting the cells against apoptosis (Figure 3). All of them are structurally similar with four BH domains (BH1-4). Bcl-2 is the first discovered member of this family, identified with its role for the survival of mature lymphocytes, melanocyte stem cells and cells of the developing kidney. Besides, Mcl-1 and Bcl-xL lacking embryos can not survive development [36, 37]. The survival induction mechanism of the proteins is not clearly identified, whereas their structures are generally established. Each has a similar helical fold placed on a core hydrophobic helix with BH1-3 domains generating a hydrophobic groove on the molecule required for their activity and binding to their target pro-apoptotic proteins. All anti-apoptotic proteins have a BH4 domain except Mcl-1, for this reason this domain is thought to be anti-death domain [38, 39, 40]. Additionally, Mcl-1 is a highly-regulated protein and has a short half life; it is not only an anti-apoptotic protein, but also can be cleaved by caspases during apoptosis to produce an apoptosis promoting protein through a positive feedback mechanism [41]. Their importance in tumorigenesis is extremely important, overexpression studies of them lead to several malignancies as acute promyelocytic leukemia, breast cancer, and pancreatic β-cell cancer [42].

Pro-apoptotic Bcl-2 family proteins are the apoptosis promoting proteins, consist of two structurally defined group; multidomain pro-apoptotics containing BH1-3 domains and BH3 only apoptotics. Among multi-domain pro-apoptotic proteins, Bax and Bak are the critical mediator proteins for MOMP, they form size-indeterminate openings in the outer mitochondrial membrane [43]. Bax and Bak deficient or mutant cells show resistance to several apoptotic stimuli [44, 45]. It is still not clear yet which structural composition determine proteins' anti- or pro- characteristics; interestingly, although each belongs to a different group, Bcl-xL, Bcl-w, Bax and Bid have similar seven or eight helices containing folds, two of these helices are hydrophobic and can be inserted within a membrane [46].

According to genetic and biochemical studies, BH3 only proteins are critical proteins for intrinsic apoptosis, whereas Bax/Bak like proteins are at the downstream of

the signaling, BH3 only proteins except Bid have different tertiary structures, there are four conserved hydrophobic residues which can insert themselves separate pocket in the hydrophobic groove of the interacting anti-apoptotic protein [39, 47]. Additionally, Bcl2 family members have apoptosis independent functions as Bcl-2, Bcl-xL, Bad and Bid; Bad opposes both cell cycle and anti-apoptotic functions of Bcl-2 and Bcl-xL through BH3 binding, besides Bid has a function on S-phase checkpoint after DNA damage [48]. BH3 only members' activation is not clearly identified, but thought be controlled by transcriptional and post-translational mechanisms; as an example p53 induces Puma and Noxa in response to DNA damage in transcription level, but other BH3 members are considered to be regulated at posttranslational level [49].

Binding pattern of Bcl-2 proteins is relatively a clear issue; BH3 only members share a short α-helix and this domain fits to pro-survival Bcl-2 protein's docking site in a stable manner, whereas Bax binding with pro-survival Bcl-2 proteins is not clear; detected by crystal structural studies, immunoprecipitation and yeast two hybrid experiments [50, 51]. Beside these, it was discovered that Bak can form complex with either Bcl-xL or Mcl-1, but not with Bcl-2 [52]. Additionally, BH3 only proteins do not bind to Bax or Bak, except Bim and tBid; whereas Bim's affinity was proposed to be higher [53].

Bcl-2 protein family members can be inserted mitochondrial and/or endoplasmic reticulum (ER) membranes or they can be localized free in cytosol; as an example Bax is cytosolic or loosely associated (not integrated) with mitochondria in a healthy cell, on the other hand Bak is a tail-anchored integral membrane protein which is located on the cytosolic face of mitochondrion and ER. In response to an apoptotic stimulus, Bax translocates to the membranes with an unknown mechanism, Bak changes conformation; and they form membrane-associated oligomers [54, 55]. Among pro-survival Bcl-2 proteins, Bcl-2 is located to mitochondrial and ER membranes, whereas Bcl-xL and Bcl-w are localized between the cytosol and mitochondrial membranes [56]. Among BH3 only pro-apoptotic proteins, Bim, Bik and Hrk has found to localise to intracellular membranes in response to overexpression and tBid similarly can insert into mitochondrial membrane [57, 58].

There are two models proposed to identify BH3 only proteins interaction and activation; direct binding and displacement model (Figure 2.1.3.1 1) [59]. According to direct activation model, among BH3 only family members Bim and tBid directly bind and activate Bax; they are normally inactive by pro-survival Bcl-2 proteins; other BH3 only proteins bind and occupy pro-survival Bcl-2 proteins to prevent them sequestering Bim and tBid. Bim and tBid are the activators, whereas the other BH3 only proteins (i.e. Bad and Noxa) are identified as sensitizers (derepressors) [60, 61, 62]. Puma is also suggested as an activator [63]. tBid and Puma bind to Bax at its N-terminus [64]. The displacement model also called as indirect activation model depends on the BH3 only proteins' binding affinity differences to pro-survival Bcl-2 proteins [60, 61]. According to this model; Bim, tBid and Puma are the most effective killers since they bind all pro-survival Bcl-2 proteins, whereas Bad only binds to the group Bcl-2, Bcl-xL and Bcl-w; Noxa binds Mcl-1 and A1 (Figure 2.1.3.1 2) [65, 66].

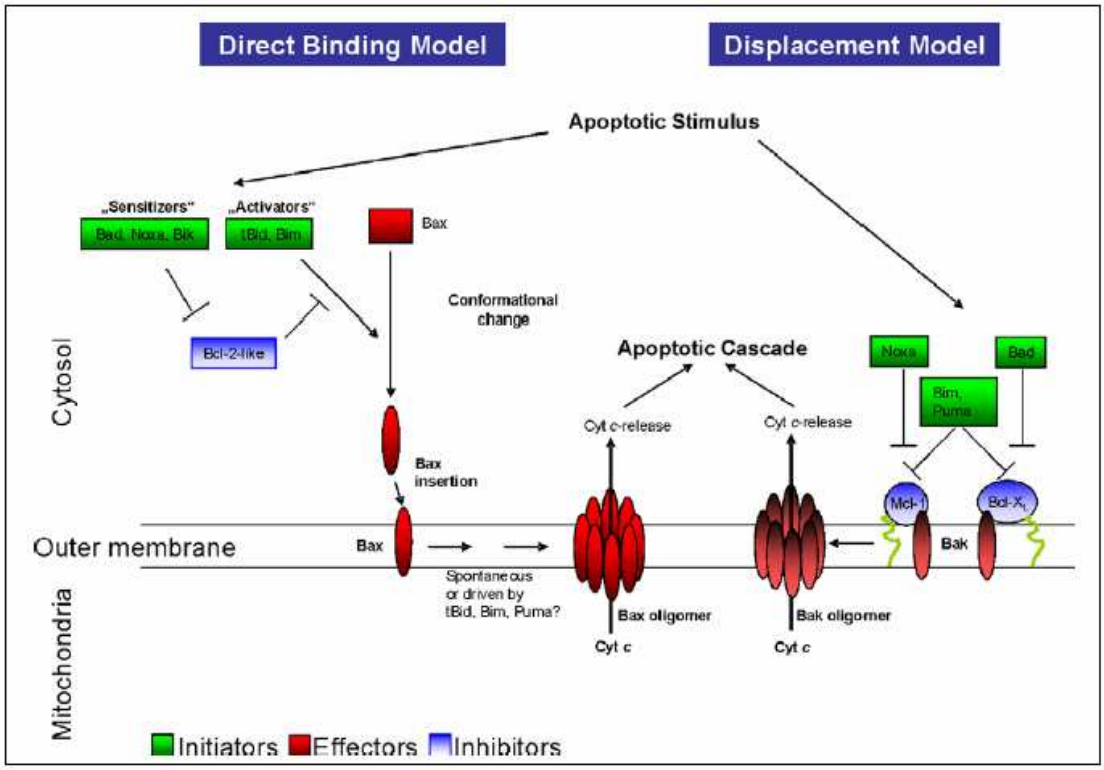

Figure 2.1.3.1 1 : Models proposed for the action of BH3 only proteins. In the direct binding model, activators directly bind and activate Bax or Bak, sensitizers bind to pro-survival Bcl-2 proteins to make release activators. In the displacement model, according to their binding affinity, BH3 only proteins bind to Bcl-2 pro-survivals to make release Bax or Bak to auto-activate themselves by oligomerization [59].

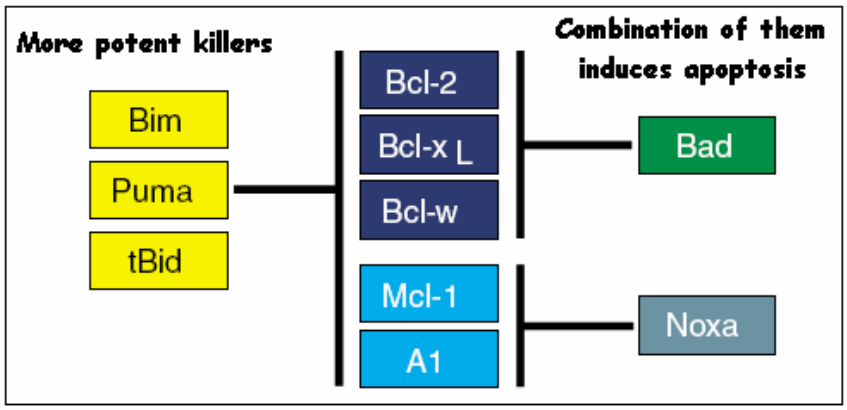

Figure 2.1.3.1 2: Apoptotic characteristics of BH3 only proteins. Bim, Puma and tBid are more potent killers with binding pattern to all pro-survivals, whereas Bad and Noxa selectively bind pro-survivals. [66]

According to Leber et. al., both of the activation models neglect the importance of the membrane. For this reason, they proposed another model called embedding model stating that both pro- and anti-apoptotic Bcl-2 family proteins can involve in similar dynamic interactions which are leaded by membrane dependent conformational changes and culminate in either aborted or productive membrane permeabilization depending on the final oligomeric state of pro-apoptotic Bax and/or Bak [67].

2.2 Cell Cycle

Cell cycle is a tightly regulated series of events that processes cells to proliferate, composed of duplication of the cell contents and division in eukaryotic cells. It has four phases that are controlled by a number of important protein families at certain checkpoints, failure in these checkpoints is hallmark of cancer. The main phases are S phase (synthesis or synthetic phase) and M phase (mitosis phase) involving DNA synthesis and mitosis, respectively; between these phases there are gaps called G1 (Gap 1) and G2 (Gap 2). G1 phase is the phase that cell grows with synthesis of RNAs and proteins; as it gets a certain size it enters S phase where the DNA synthesis occurs from

the diploid value of 2n to 4n and chromosomes are duplicated. After DNA synthesis is completed, the cell enters G2 phase to prepare itself for the division, in this phase the cell has two diploid sets of chromosomes. Then the cell enters M phase where mitosis takes place, during this phase diploid sets of chromosomes are separated and segregated to daughter cells; after completion of cell cycle, cell again enters back in G1 phase. (Figure 2.2 1) [10, 68, 69].

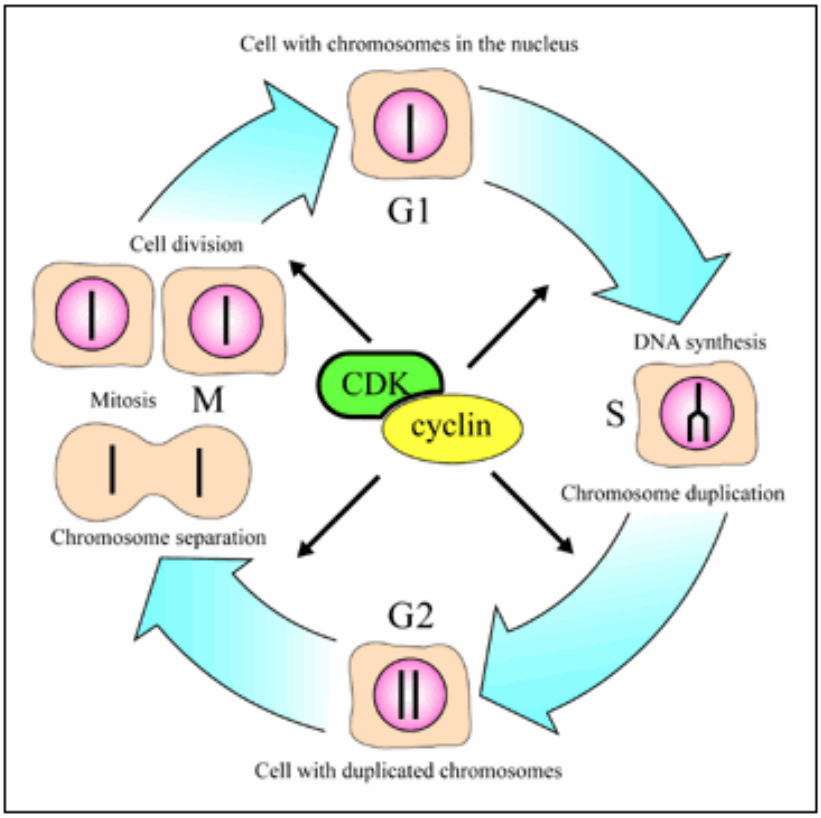

Figure 2.2 1 : Phases of cell cycle [69].

Cell cycle is tightly controlled by a family of protein kinases, cyclin-dependent kinases (CDKs, serine threonine kinases with catalytic properties); these kinases' activity increases or decreases depending on the progress through the cell cycle. CDKs are controlled by several enzymes and other proteins; the most specific regulatory proteins are cyclins. If CDKs are bound to cyclins, they have protein kinase activity. Cyclin level increases and decreases in each cell cycle, whereas CDK level is constant. Beside these, there are cell cycle inhibitory proteins negatively regulating cell cycle and prevents cell to enter to the next cell cycle phase (Figure 2.2 2) [10, 70, 71]. Restriction

point is one of the important checkpoints in cell cycle occurs in mid G1; after restriction point cell cycle becomes independent of growth factors and follow cell division [72].

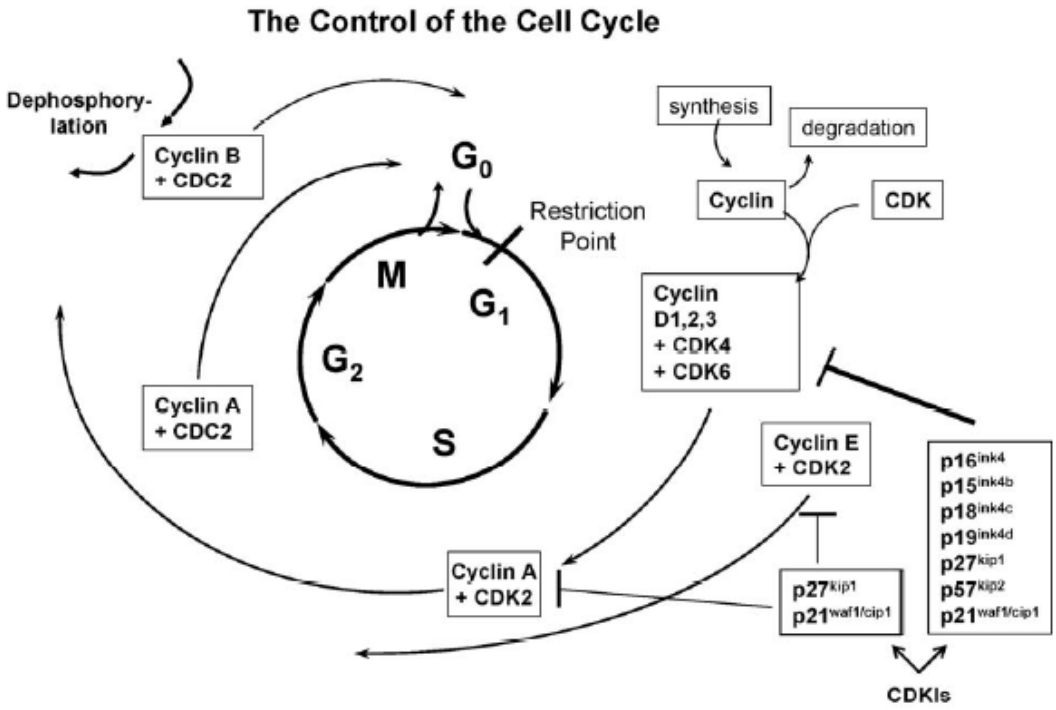

Figure 2.2 2: Network of the cell cycle regulator proteins [10]

2.2.1 Regulation of Cell Cycle

Deficiency in restriction point is a common hallmark of human cancer types. Restriction point passage is managed by two cyclin families; cyclin D (D1, D2 and D3) and cyclin E families. Cyclin D family binds CDK4 and CDK6 to activate them and cyclin E family interacts with CDK2 to activate it. Cyclin A and cyclin B are responsible for the coordination of other checkpoints where they bind and activate CDK1 (cdc2). Since CDKs protein concentration remains same through the cell cycle, regulation of CDKs is thought to occur at the post-translational level. Inactive form of CDKs is the phosphorylated form on N-terminal threonine and tyrosine residues; for the activation of CDKs, initially threonine and tyrosine residues are dephosphorylated by cdc25 phosphatase family, after that a CDK activating kinase (CAK, cyclin H-CDK7)

phosphorylates a central threonine residue. Moreover, further regulation of cyclin/CDK complex is followed by CDK inhibitors (CDKIs) [10, 73, 74].

CDKIs are grouped as below;

1. INK4 (inhibitor of CDK4, p16 family): p15, p16, p18 and p19 are the members of this group; they inhibit CDK2, 4 and 6 to prevent complex formation with cyclin D, and as a result to stop progression of G1.

2. KIP (kinase inhibitor proteins, p21 family): p21, p27 and p57 are the members of this group; they inhibit cyclin E and A/CDK2 complexes, to stop entry into S phase and progression of S phase respectively. Beside these, these inhibitor proteins are important at cyclin A/CDK1 (Cdc2) complex in G2 phase and cyclin B/CDK1 complex for mitosis phase (Figure 2.2 2) [10, 75].

Among p21 protein family, p21 can interact both with the cyclins and CDKs, whereas other CDKIs generally interact only with CDKs; because of that p21 is thought to be important in cell cycle arrest induced by genotoxic stress. Gamma or ultraviolet irradiation induces G1 arrest in a p53-dependent manner, with increased p21 protein and inhibition of cyclin-E/CDK2-kinase activity [76, 77]. Although p21 is not required for p53 induced apoptosis, it is a direct transcriptional target of p53 and it can also be activated by different mechanisms as E2Fs, AP2, STATs, C/EBPα and C/EBPβ at transcriptional level. p21 overexpression leads to cell cycle arrest through G1, G2, S phases and the transitions between these phases. Additionally, p21 can also interact with other cellular proteins important in growth, DNA synthesis and DNA repair; to exemplify, GADD45 is a DNA damage inducible and growth arrest inducer protein that interacts with p21 [78, 79].

In addition to cell cycle regulation characteristics of p21, it was also stated as an important regulator protein in apoptosis, previous studies on p21 generally specified p21 as an inhibitor of p53-dependent and -independent apoptosis in cancer cells. The anti-apoptotic mechanism of p21 is not clarified, but it was proposed that either cell cycle arrest caused by p21 may make cells resistant to apoptosis or it may block apoptosis through a direct mechanism. In human HepG2 cells, p21 was discovered to

bind to the procaspase-3 to block its cleavage and activation, and as a result blocking Fas-induced apoptosis. Besides, p21 can interact with ASK1 to inhibit its activity [80, 81, 82]. Therefore, it was proposed that small molecules targeting p21 can be used in cancer therapy to make tumor cells sensitive to anticancer drugs [83].

However, the interaction pattern of p21 is not that simple, it has a dual role in apoptosis; in addition to its anti-apoptotic effect it is a pro-apoptotic protein in some circumstances. As an example, overexpression of p21 elevates apoptotic response to cisplatin treated glioma and ovarian carcinoma cell lines; bile acid-induced MAPK-dependent apoptosis in hepatocytes [84, 85, 86].

p21 also shows pro-apoptotic characteristics under certain conditions in specific biological systems lacking functional p53 when it is overexpressed either upon p21 induction or upon TNF family receptors activation. p21's dual role was observed in the same cells (HEp-2 cancer cells lacking p53 expression) treated with different chemotherapeutic agents; overexpressed levels of the protein increases apoptotic response induced by cisplatin, whereas inhibits apoptotic response caused by methotrexate probably by effecting GADD45a gene expression (Figure 2.2.1 1). They proposed that, enhanced apoptotic effect caused by cisplatin was a consequence of JNK, caspase-3 and caspase-7 activity through GADD45a expression. On the other hand they stated that the lack of caspase-3 activation in HEp-2 cells probably make p21 effect apoptosis-protective [78, 87, 88, 89, 107].

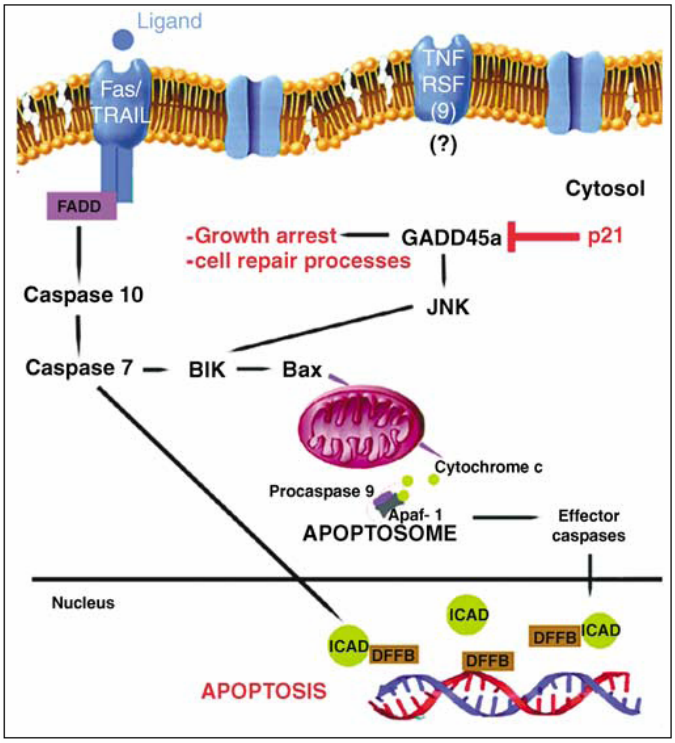

Figure 2.2.1 1 : The model proposed for the effects of cisplatin and methotrexate in Hep-2 cells in response to p21 protein overexpression [107]

The tumor suppressor p53 has a central role oncogenesis by inhibiting the development of abnormal cells; it is not a required protein for growth and development of a cell but loss of p53 leads to carcinogenesis. Since it is an important regulator for cell cycle regulation addition to its characteristics related to apoptosis induction of an undesired cell, the mutations on that gene increases the cell's sensitivity to development of cancer [90]. If there is a problem ongoing in cell cycle, p53 is activates and afterwards p53 activates p21 to manage initial cell cycle arrest. As cell cycle arrest occurs, p21 levels decrease and another CDK inhibitor, p16, becomes unregulated to maintain cell cycle arrest. This cascade was stated by gene knockout and targeted inhibition studies [91, 92, 93].

Apoptosis dysregulation and destruction in cell cycle mechanism are the two important hallmarks of cancer development. For this reason, targeting apoptosis and cell cycle pathways for the treatment of human malignancies is an outstanding approach.

Targeting these two pathways is followed by targeting the critical components as kinases, activators, inhibitors and regulator proteins [94].

For apoptosis, the aim is to reconstruct the integrity of natural apoptotic pathways by modulating pro-apoptotic and pro-survival proteins' pattern. This could be carried out either by small molecules to eliminate apoptosis blockers or by replacing/reactivating endogenous death inducers/activators. There are several families targeted for this purpose, such as Bcl-2 protein family, cytokines and inhibitors of apoptosis. In tumor cells, pro-apoptotic proteins can be inhibited or anti-apoptotic proteins can be up-regulated in response to several mutations or deficiencies in control mechanisms. The drugs identified can upregulate pro-apoptotic proteins as apaf-1 (5-aza-cytosine, Vidaza®, Pharmion Corp., Boulder, CO), compounds such as nutlins that activate pro-apoptotic transcription factors as p53 by binding Mdm2, chemical inhibitors of c-AKT and compounds that prevent activation of nuclear factor kappa B (NF-κB) family transcription factors to reduce levels of c-FLIP, c-IAP2, and other antiapoptotic proteins [4, 95, 96, 97].

Cell cycle can be modulated by targeting CDKs either by small molecules binding to the ATP binding pocket of CDKs or by affecting the composition of the CDK and its inhibitor complexes by different mechanisms. According to the CDK functions, inhibition of them can lead to different outcomes as cell cycle arrest, induction of differentiation, inhibition of transcription and apoptosis [98, 99]. In such a complex network, cell cycle arrests by CDKIs finally induce cells to apoptosis [5]. Cell cycle and apoptosis are overlapping pathways; different cell cycle regulators as p21, p27, pRb, CDK1, cdc27 and Weel1 are cleaved by caspases stating that cell cycle is modulated during apoptosis [100].

Tumor cells lose ability to stop at the certain points of a cell cycle phase as a consequence of deficient checkpoint integrity caused either by inactivation of CDKIs or by overexpression of cyclins. p21 and p16 are the general targeted CDKIs in cell cycle targeted cancer therapies; loss of these inhibitors is associated with several malignancies including melanoma, breast, lung and colorectal tumors. In addition to specific CDKs and their inhibitor proteins, growth state of the cell, the presence of specific cell cycle

components and the tissue type are the important parameters for cell cycle targeted therapies [5, 99, 101].

2.3 Pramanicin and its analogs

Pramanicin (PMC) is a potent anti-fungal agent which was recently isolated from a fungal fermentation; it has a highly functionalized polar head group and a simple aliphatic side chain. PMC and its analogs have been shown to exert growth-inhibitory effect with minimal inhibitory concentration [6] There are 11 analogs of PMC synthesized and/or obtained as by-products, which have slight differences on their aliphatic side chains. (Figure 2.3 1) [102].

Its effects on mammalian cells have came forward very recently in pharmacological studies. In previous studies, it has been demonstrated that PMC triggers endothelium-dependent and NO-mediated vascular relaxation, besides it increases cytosolic calcium levels and induces cell death in vascular endothelial cells [103, 104]. Therefore it was thought to be a candidate to induce cancer cells to undergo apoptosis. In the previous studies, Kutuk et.al. stated that PMC induces apoptosis in Jurkat leukemia cells in a dose- and time-dependent manner through triggering cytochrome-c release, caspase-3,-9 activation and JNK/p38 kinase cascade activation as proapoptotic signaling pathways (Figure 2.3 2) [7].

PMC-A is one of PMC analogs, synthesized as a by-product; epoxy group in PMC is replaced by a CC bond. As its effect is compared with PMC's effect, it causes little endothelial-dependent relaxation, but it can cause endothelial cell dysfunction. This suggests that epoxy group of PMC is required for its vasorelaxant effect [104].

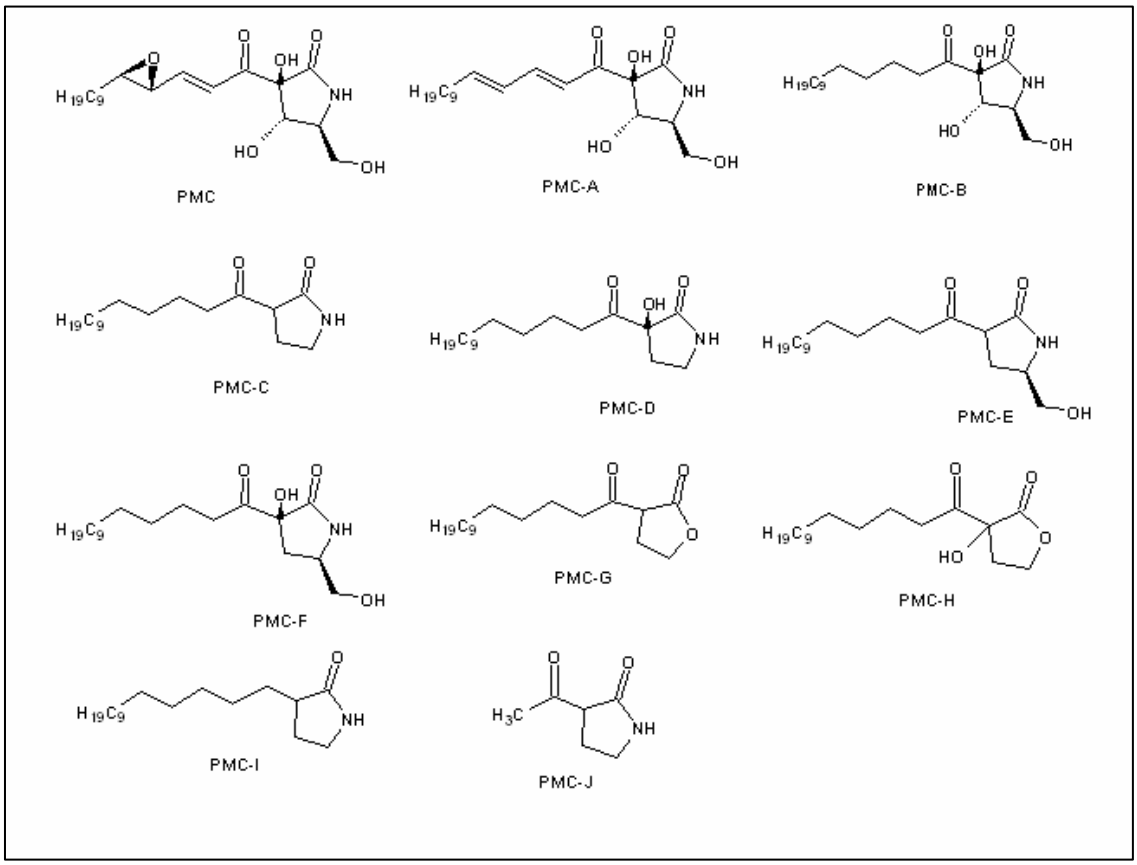

Figure 2.3 1 : PMC and its analogs (PMC-A, B, C, D, E, F, G, H, I and J).

2.4 HCT116 and Deficient Mismatch Repair System

Mismatch repair (MMR) system is a DNA repair mechanism important for genome integrity. Elimination of the errors occurred during DNA replication, DNA damage surveillance, and the prevention of recombination between non-identical sequences is under the control of MMR. Not surprisingly, deficiencies in this system may directly lead to cancer; generating mutations at a rate that is 1000-fold higher than the rate observed in normal cells. This is an advantage for tumor cell development with a competitive advantage, such as enhanced proliferation, and/or a survival advantage with apoptosis resistance, and subsequently an increased metastatic potential indicating a direct role for MMR proteins in recognizing and signaling DNA damage responses. The dedicated role of MMR proteins is not related to MMR catalytic response. Loss of

MMR in mammalian cells generates resistance to certain DNA damaging agents including some of the chemotherapeutics; because of that, patients with colon cancer give variable responses to chemotherapy. HCT116 cells have defects in their mismatch repair system because of a deficiency of an important MMR protein (MLH1). Therefore it is hard to discover/identify an effective chemical to beat this aggressive cell line [3, 111, 112, 113].

Additionally, MMR deficiency may lead to defects in G_2/M cell cycle arrest at low doses of some agents as Cisplatin, whereas it can induce apoptosis at high doses. It is not clarified that how G_2 arrest relates to cytotoxicity. The complex correlation between the scope and time of G2 arrest and cell killing by methylating agents proposes that cell cycle arrest reflects the processing by MMR of both lethal and non-lethal DNA damage [114, 115, 116].

The aim of this study was to investigate possible anticancer effects of PMC analogs in HCT116 WT and Bax (-/-) cell lines, for this purpose the effective PMC analog, PMC-A, was studied in detail through the molecular mechanisms of apoptosis and cell cycle arrest. Subsequent to determining dose and time kinetics of PMC-A by cytotoxicity and apoptosis assays, apoptosis was verified at the protein level through cleaved caspase-3, 9 and apaf-1 immunoblottings. Since Bcl-2 protein family has an established role in apoptosis, levels of pro- and anti-apoptotic Bcl-2 protein members were examined by immunoblotting. In order to investigate the effect of PMC-A on cell cycle, the level of a general cyclin-dependent kinase inhibitor protein, p21, was identified. Furthermore, cell cycle analysis was performed in order to understand if PMC-A induced cell cycle arrest in colon carcinoma cell lines.

3 MATERIAL AND METHODS

3.1 Materials

3.1.1 Chemicals and Antibodies

Chemicals and antibodies that are used are listed in Appendix A

3.1.2 Molecular Biology Kits

Molecular biology kits that are used for apoptosis screening and protein analysis are listed in Appendix B.

3.1.3 Equipments

Equipment that is used for general laboratory procedures are listed in Appendix C.

3.1.4 Buffers and Solutions

Standard buffers and solutions used in cloning and molecular manipulations were prepared according to the protocols in *Molecular Cloning: A Laboratory Manual*, Sambrook *et al*., 2001

3.1.4.1 Buffers and solutions for cell viability and apoptosis assays

MTT Solution:

5 mg/ml MTT was dissolved in PBS and filtered for removing insoluble particles.

Glycine Buffer:

605 mg Tris and 3750 mg Glycine were dissolved in 50 ml ddH_2O and pH was adjusted to 10.5.

Annexin V-FITC incubation buffer:

10 mM Hepes, 140 mM NaCl and 2.5 mM $CaCl_2$ were dissolved in 500 ml of ddH_2O.

3.1.4.2 Buffers and solutions for protein analysis

Cell Lysis Buffer:

150 mM NaCl, 1% NP-40, 50 mM Tris-HCl were dissolved in dd H_2O. pH was adjusted at 8, buffer was stored at -20^{o}C till usage. 0.1 mM PMSF and protease inhibitor cocktail were added prior to use.

10X PBS (Phosphate Buffered Saline):

80 g NaCl, 2.25 g KCl, 23.27 g $Na_2HPO_4.12H_2O$ and 2.05 KH_2PO_4 were dissolved in 1L ddH_2O of and pH was adjusted to 7.4.

1X PBS-T:

10X PBS was diluted, 0.2% Tween20 was added and pH was adjusted to 7.4

10X Running Buffer:

250 mM Tris base, 1.92 M Glycine and 1% (w/v) SDS were dissolved in 500 ml ddH_2O; pH was adjusted to 8.5.

10X Transfer Buffer:

15.41 gr Tris, 72.1 gr Glycine (for 500 ml)

1X Transfer Buffer:

10X transfer buffer was diluted, 20% methanol was added prior to use.

Stripping Buffer:

62.5 mM Tris-HCl and 2% SDS (w/v) were dissolved in 500 ml ddH_2O and pH was adjusted to 6.7. 352.1 µl of β-mercaptoethanol was added for 50 ml of solution prior to use.

3.1.4.3 Solutions for cell cycle analysis

PI solution:

2 ml PBS and 12 µl TritonX100 were mixed, 10 µl RNase and 20 µl PI were added to 1ml of mixture for the samples, and 2 µl RNase was added to 200 µl of mixture for no dye sample.

3.2 Methods

3.2.1 Cell Culture

HCT116 WT and Bax(-/-) cells are kindly provided by Bert Vogelstein (Johns Hopkins Oncology Center, Baltimore). The cells were cultured in McCoy's 5A medium modified containing L-glutamine; 10 % fetal bovine serum (FBS), penicillin/ streptomycin (100 U/ml, respectively; 1% of the medium). Cells were maintained in a humidified incubator at 37^{o}C and 5% CO_2. When cells were grown to confluency, cells were washed with 1X cell culture grade phosphate buffer saline (PBS) before trypsinization, then they were detached by 1X sterile trypsin at 37^{o}C incubator, then trypsinization was inhibited by complete medium and cells were seeded in 96-well cell culture plates (for MTT $1x10^4$ cells/well and for M30 $5x10^3$ cells/well), 12-well cell culture plates ($2x10^5$ cells/well) and 60 mm culture dishes ($6x10^5$ cells/well) in complete medium before experiments. Cells were counted with a hematocytometer and cells are seeded in flasks or dishes in the appropriate amounts.

For cryopreservation, HCT 116 cells were first trypsinized, resuspended and frozen in freezing mix that is composed of heat inactivated FBS with 10% DMSO. The mixture transferred into cryovials and were kept at -80^{o}C, after 24 hours they were put in liquid nitrogen until next usage.

3.2.2 PMC Treatment

PMC and its analogs were dissolved in ethanol to 20 mM and stored at +4^{o}C. HCT116 cells were seeded in complete medium 36 hours before the treatment. After that, cells were treated with the chemicals at the indicated concentrations. Additional to untreated cells, ethanol treated cells were used as controls.

3.2.3 Cell Viability and Apoptosis Assays

3.2.3.1 MTT Assay

Cytotoxicity induced by the chemicals and their effective concentration were determined with MTT assay. HCT116 cells were seeded in 96-well plate ($1x10^4$ cells/well) and treated with the chemicals as indicated above. After the incubation with PMC and its analogs for 24 hours, medium was removed; afterwards 100 µl fresh medium and 10 µl MTT solution was added. Then the plate was kept in 37^{o}C at dark. After 4 hours incubation MTT solution-medium mixture was removed, 200 µl DMSO and 10 µl Glycine buffer were added. In the last step, the absorbance was recorded with a microtiter plate reader at a wavelength of 570 nm. Percentage of relative cell viability was calculated as (OD of drug treated sample x 100)/ (OD of control sample).

3.2.3.2 M30 Apoptosense Assay

Apoptotic cell death induced by PMC-A and PMC-F in HCT116 WT cells were determined by M30 Apoptosense Assay. The cells were seeded in 96 well plates ($5x10^3$ cells/well) in 100 µl complete medium, after 24 hours cells were treated 25 µM PMC-A and 75 µM PMC-F. After 24 hours incubation at 37^{o}C incubator, 10 µl of 10% NP40

was added to each well and mixed with a shaker for 5 minutes at room temperatue. 25 µl of medium-cell lysate mixture was transferred to M30 apoptosense plate's wells. After that, the assay was performed according to the manufacturer's protocol; 75 µl of diluted HRP-conjugate solution was added to each well containing cell lysates-medium mixture; subsequently it was agitated on a shaker for 4 hours at 200 rpm at room temperature. Then fluid was aspirated from the wells and washed for five times with 250 µl wash solution. After that, 200 µl TMB substrate solution was added and plate was incubated for 20 minutes in dark at room temperature. After incubation, 50 µl of stop solution was added and mixed. In the last step, after 5 minutes incubation with stop solution, absorbance was measured with microtiter plate reader at a wavelength of 450 nm.

3.2.3.3 Annexin V Apoptosis Assay

To further verify the apoptotic effect of PMC-A and PMC-F in HCT116 cells, AnnexinV-FITC staining assay was performed. The cells were seeded in 12 well-plates ($2x10^5$ cells/well); after 36 hours cells were treated with PMC-A and PMC-F. After 24 hours of incubation, cells were detached by trypsinization, transferred to flow-cytometer tubes and centrifuged at 300g for 5 minutes. Supernatant was removed in order to discard medium. Cells were washed with 1ml of 1X PBS then centrifuged again. PBS was discarded and cell pellets were resuspended in 102 µl of staining solution consisting of 100 µl of incubation buffer and 2µl of AnnexinV-FITC. After that, cells were incubated in staining solution for 15 minutes in dark at room temperature. In the last step, in order to stop the reaction 500 µl of incubation buffer was added. Then the cells were analyzed by a flow cytometer using lasers and filters required for FITC detection. Additionally, HCT116 Bax(-/-) cells was tested in comparison with WT cells in a time dependent manner; subsequent to treatment cell were collected after 2, 4, 8, 12 and 24 hours, analyzed by flow cytometer.

3.2.3.4 Total Protein Isolation

HCT116 WT and HCT116 Bax(-/-) cells were seeded in 60 mm culture dishes ($6x10^5$ cells/well) and after treatment with PMC-A, cell lysates were collected in a time

dependent manner at 2, 4, 8, 12 and 24 hours as explained below. After treatment dishes were washed with ice cold 1XPBS at the indicated time points. After centrifugation, 1ml of ice cold 1X PBS was added again and cells were scraped from the plates by a scrapper. The scraped cells were put in eppendorf tubes and centrifuged at 13200 rpm for 30 seconds at +4^{o}C. After discarding supernatant, cell lysis buffer was added on the pellet and left on ice for 30 minutes. Then, cell debris was removed from the lysate by centrifugation at 13200 rpm for 25 minutes at +4^{o}C. Supernatant was transferred to another eppendorf tube; these protein isolates were stored at -80^{o}C.

3.2.4 Protein Concentration Determination

Concentrations of the protein isolates were determined by DC Assay for equal loading to SDS gel. The bovine serum albumin was used as standard; different concentrations (0.1, 0.25, 0.5, 0.75, 1.0, 1.5 μg/μl) of BSA standards were obtained by serial dilutions. 5 μl from BSA standards were put in 96-well plates in triplicates; 4μl of ddH_2O and 1μl of protein isolate was put in 96-well plates in triplicates. 25 μl of solution A and 200 μl of solution B of DC Assay Kit were added to each well. The absorbance of each sample and standards were measured in a microtiter plate reader at a wavelength 655nm. A standard curve was obtained using BSA standards and unknown samples' concentrations were calculated using its graph's equation.

3.2.5 SDS-PAGE Gel Electrophoresis

Isolated proteins were seperated in 12% SDS-PAGE, 30 μg of proteins were loaded to gels. Gels were prepared according to Bio-Rad Mini Protean SDS-PAGE gel apparatus -for 1mm gel- manufacturer's instructions; first separating gel was prepared, a mixture containing 6 ml of 30% acrylamide / 0.8% bisacrylamide, 3.75 ml of 4X Tris-Cl/SDS at pH 8.8, 5.25 ml of ddH_2O, 50 μl of 10% ammonium per sulphate (APS) (dissolved in ddH_2O) freshly prepared and immediately 10 μl of TEMED in the given order for the running gel. The gels were overlaid with isopropanol to initiate polymerization and to block the association of the gel with the air. After the separating gel polymerized, isopropanol was discarded. The stacking gel was prepared with mixing sequentially 0.65 ml of 30% acrylamide / 0.8% bisacrylamide, 1.25 ml of 4X Tris-

Cl/SDS at pH 6.8, 3.05 ml of ddH_2O, 25 µl of 10% APS freshly prepared and immediately 5 µl of TEMED. After that, combs were inserted to the gel for the formation of the wells to load the samples. Then the protein samples to be loaded were prepared; 30 µg protein mixture and same volume of 2X Laemmli loading dye were mixed in eppendorf tubes. Proteins were denatured in 95^oC for 3 minutes. Equal amounts of proteins were loaded to SDS-PAGE gel in reference to DC Assay. The proteins were seperated on the gels at constant voltage 70V for 2 hours at room temperature.

3.2.6 Immunoblotting

After separating proteins with SDS-PAGE, they were blotted on a PVDF membrane. As an initial step, PVDF membrane was washed in methanol for 30 seconds, besides sponges and Whatman papers were washed with 1X transfer buffer before immunoblotting. After that gels and membranes were placed in the casettes, proteins were blotted onto PVDF membrane via the application of 60V constant voltage for 70 minutes at 4°C. After transfer, the blots containing protein bands were blocked in 5% dried milk in 1X PBS-Tween20 (0.2%) for 2 hours at room temperature. The primary antibody was diluted in 5% dried milk in 1X PBS-Tween20 (0.2%) in 1:2000 proportion. The primary antibody incubation was performed for overnight at +4^oC. After the primary antibody incubation, the blot was washed with 1X PBS-Tween20 (0.2%) for 2 times during 15 minutes. Prior to secondary antibody incubation, blot was further blocked in 5% dried milk in 1X PBS-Tween20 (0.2%) for 15 minutes. The secondary antibody was diluted in 5% dried milk in 1X PBS-Tween20 (0.2%) in 1:10000 proportion and incubation was performed at rom temperature for 2 hours. The blot was washed with 1X PBS-Tween20 (0.2%) for 3 times, 15 minutes for each. Finally the last washing was done with 1X PBS for 15 minutes. After washing steps, detection of blots were performed by using an enhanced chemiluminescence detection system and the developed results were exposed to Hyperfilm-ECL. Antibodies used in immunoblotting are listed in Appendix A.

3.2.7 Cell Cycle Analysis

In order to determine cell cycle arrest at cellular level, cell cycle analysis was performed by PI staining using flow cytometer in a time dependent manner parallel with protein isolation time points. HCT116 WT and Bax(-/-) cells were seeded in 12 well-plates ($2x10^5$ cells/well); after 36 hours cells were treated with PMC-A. After 24 hours incubation with PMC-A, cells were detached by trypsinization, then were washed with 1Xcold PBS. Supernatant was discarded and pellet was vortexed gently. 5 ml 70% ethanol was added to the vortexed tube and kept at $+4^oC$ while collecting the samples of other time points. After collecting all the samples, they were left at room temperature for 15 minutes, and then the cells were spin down for 5 minutes at 300 g. After centrifuge, cells were washed with 1Xcold PBS, supernatant was removed and 100 µl PI solution was added to each sample, except for no dye. Then the plate was left in dark for 45 minutes at room temperature. In order to stop reaction 500 µl PBS was added to make analysis by flow cytometer.

3.2.8 Statistical Analysis

Data are given as means ± SEM. For statistical comparison t-test was assesed. P values smaller than 0.05 was considered to be * statistical significant, smaller than 0.01 was considered to be ** statistical significant.

4 RESULTS

4.1 Determination of effective drugs and their effective concentrations

The effective PMC analogs and their effective concentration against HCT116 cell line were determined by MTT cell viability assay. In order to determine effective praminicin analogs, HCT116 cells were treated with PMC analogs (PMC-A, PMC-C, PMC-E, PMC-F, PMC-G, PMC-H, PMC-I and PMC-J) at a concentration of 100 μM for 24 hours. EtOH treated cells were used as an additional control because PMC analogs were dissolved in EtOH. At this concentration, all of the analogs, except for PMC-J, caused cell death in HCT116 cells (Figure 4.1 1). PMC-A and PMC-F were determined as most effective analogs with 8% and 29% cell viability ratios, respectively (column 3 and 6, respectively). According to these results PMC-A and PMC-F were used for further experiments.

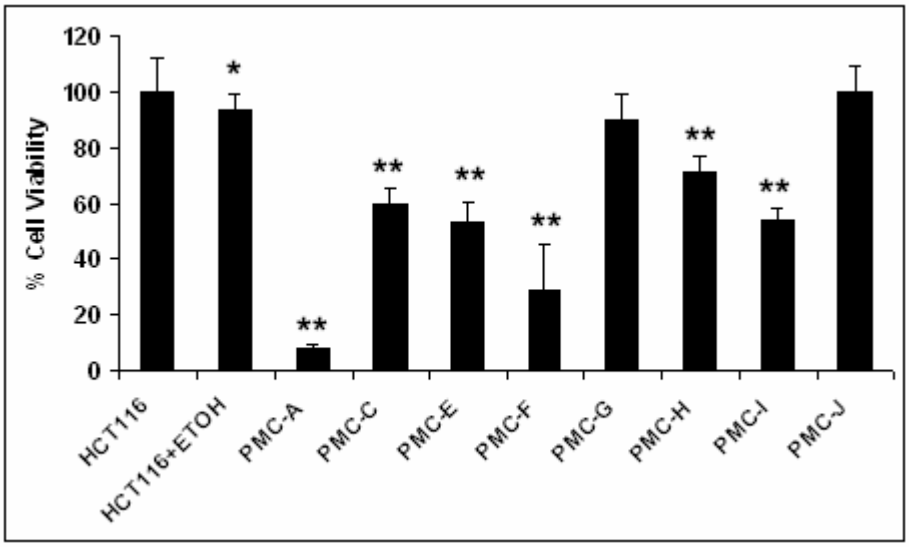

Figure 4.1 1 : Cytotoxic PMC analogs for HCT116 cells. HCT116 cells were seeded in 96 well-plate ($1x10^4$ cells/well) and treated with 100 μM PMC analogs for 24 hours. Cell viability was assessed using MTT assay. Data are shown as mean ±SEM representative of eight experiments. * $P < 0.05$, ** $P < 0.01$

To determine the effective concentrations of PMC-A and PMC-F, HCT116 cells were treated with 25, 50 and 75 μM PMC-A and PMC-F for 24 hours (Figure 4.1 2). 25 μM PMC-A and 100 μM PMC-F were chosen for further experiments. Beside these, PMC was checked whether it induced cytotoxicity in HCT116 cells as in Jurkat leukemia cells [7]. HCT116 cells were treated with 50, 100 and 150 μM PMC for 24 hours. Based on the results, HCT116 cells were not sensitive to PMC (Figure 4.3).

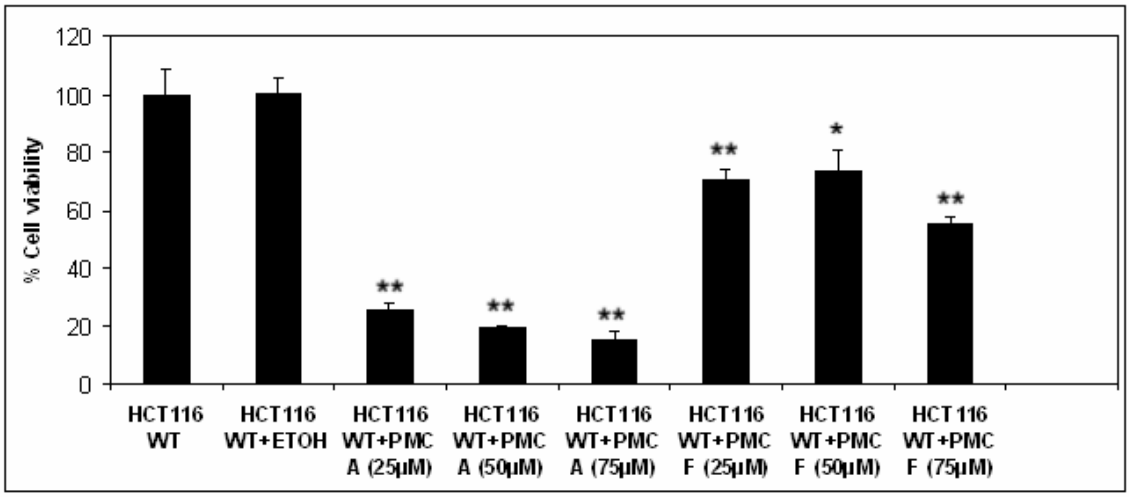

Figure 4.1 2 : Dose response analysis of PMC-A and PMC-F on HCT116 cells. HCT116 WT cells were seeded in 96 well-plate ($1x10^4$ cells/well) and treated with 25, 50 and 75 μM PMC-A and PMC-F for 24 hours. Cell viability was assessed using MTT assay. Data are shown as mean ±SEM representative of four experiments. * $P < 0.05$, ** $P < 0.01$

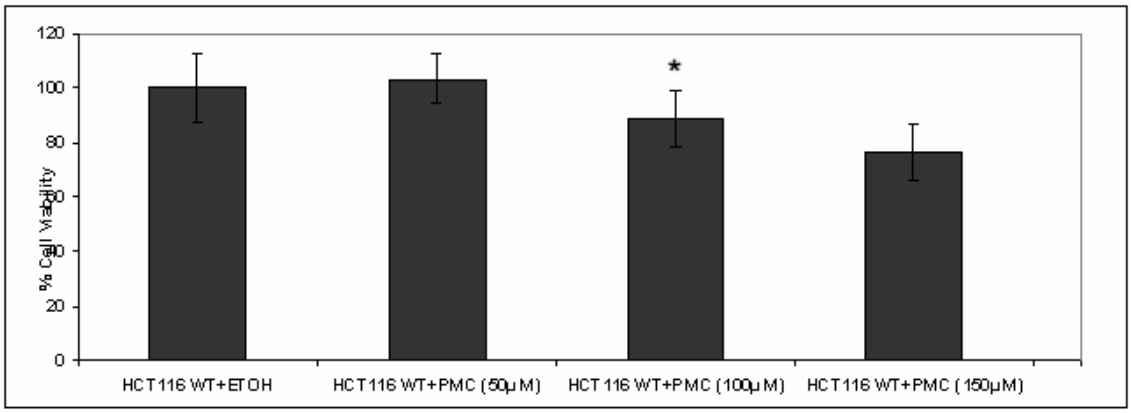

Figure 4.1 3 : PMC cytotoxicity for HCT116 cells. HCT116 cells were seeded in 96 well-plate ($1x10^4$ cells/well) and treated with 50, 100 and 150 μM PMC for 24 hours. Cell viability was assessed using MTT assay. Data are shown as mean ±SEM representative of three experiments. * $P < 0.05$, ** $P < 0.01$

4.2 Apoptotic effect of PMC-A and PMC-F on HCT116 cells

In order to understand if PMC-A and PMC-F's cytotoxicity was due to apoptosis, M30 Apoptosense and Annexin V apoptosis assays were performed. Initially, M30 Apoptosense ELISA Test was performed, cells were treated with 25 µM PMC-A, 75 and 100 µM PMC-F for 24 hours; according to the M30 Apoptosense Value varying from 0 to 3000 U/L representing M30 antigen levels, apoptosis induction was evaluated (Figure 4.2 1) (Figure 4.2 2).

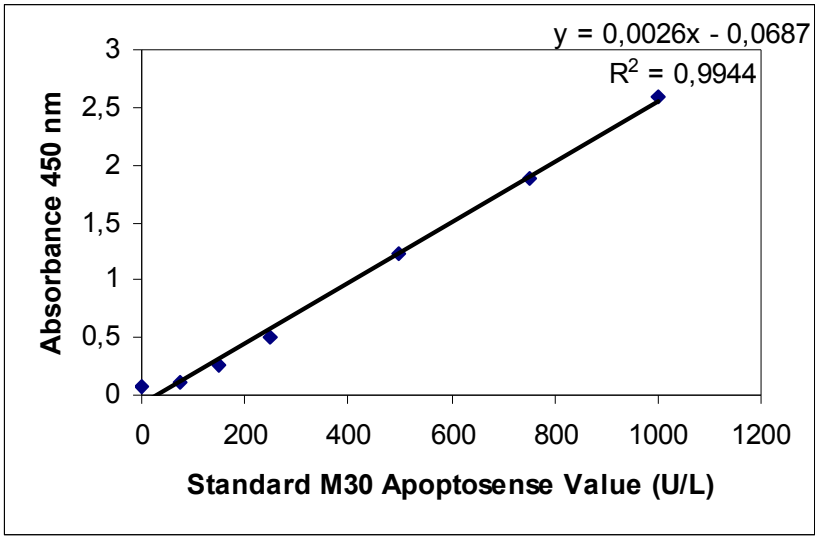

Figure 4.2 1 : Standard curve for M30-Apoptosense ELISA Test indicating absorbance values proportional to M30 antigen quantities, lot PE0024.

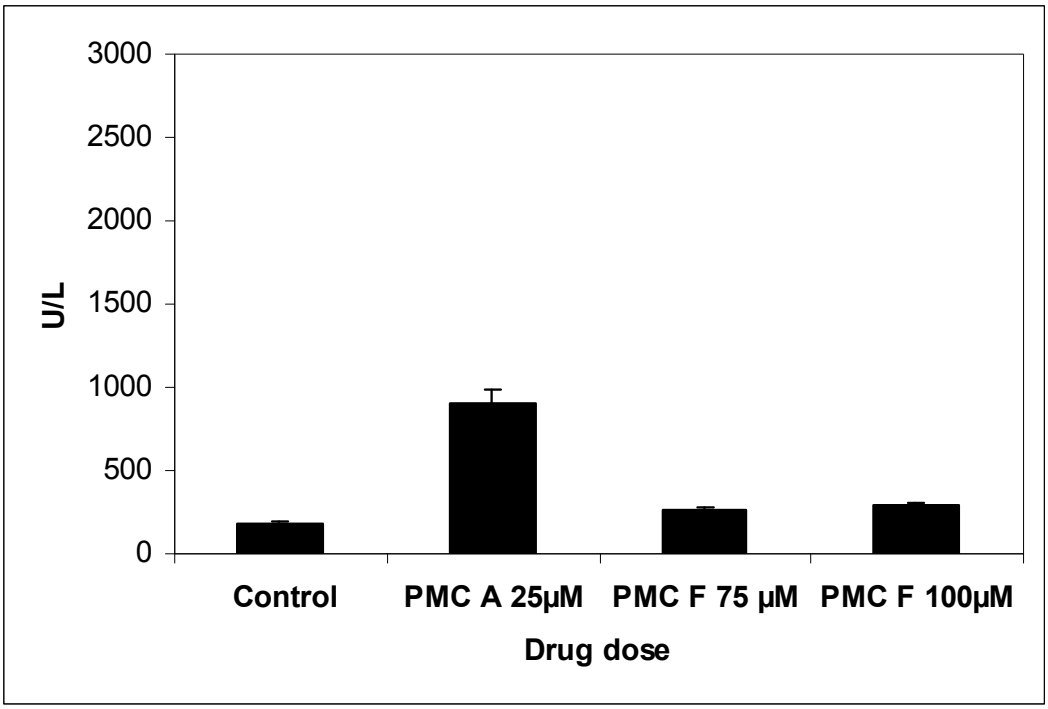

Figure 4.2 2 : PMC-A and PMC-F induced apoptosis in HCT116 cells. HCT116 cells were seeded in 96 well-plate ($5x10^3$ cells/well) and treated with 25 µM PMC-A, 75 and 100 µM PMC-F for 24 hours. Apoptosis was assessed using M30 Apoptosense ELISA assay. U/L represents 1,24 pmol M30 antigen levels per liter. Data are shown as mean ±SEM representative of two experiments.

Based on M30 results, PMC-A and PMC-F's effects might have been related to apoptosis; to further verify the apoptotic effect, Annexin V apoptosis assay was performed. HCT116 cells were treated with 25 µM PMC-A and 75 µM PMC-F for 24 hours, after that Annexin V staining and FACS analysis was performed (Figure 4.2 3). Since, apoptosis was observed in control (untreated and EtOH treated) cells, by ignoring its 11% apoptosis induction, 25 µM PMC-A induced 36% apoptosis and 75 µM PMC-F 9% apoptosis. Both apoptosis assays indicate that 25 µM PMC-A induced ≈30%, whereas 75 µM PMC-F induced only ≈8% apoptosis; according to these results, effects of 25 µM PMC-A at protein level were followed to be identified.

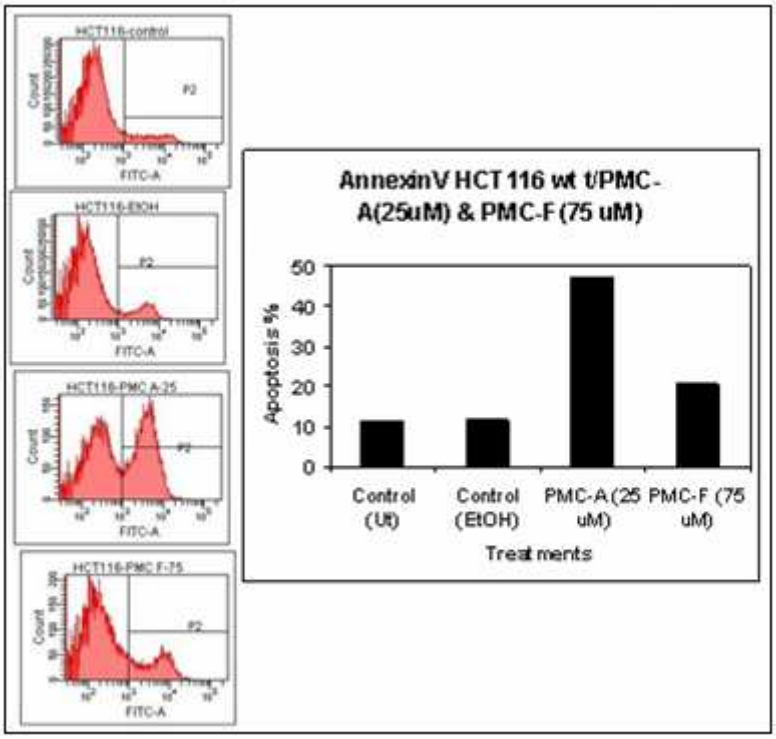

Figure 4.2 3: PMC-A and PMC-F induced apoptosis in HCT116 cells. HCT116 cells were seeded in 12 well-plate ($2x10^5$ cells/well) and treated with 25 μM PMC-A and 75 μM PMC-F for 24 hours. Apoptosis was assessed using Annexin V assay.

Since PMC-A induced apoptosis in HCT116 cells, a derivative cell line that lacks pro-apoptotic Bax gene (HCT116 Bax(-/-)) was additionally tested in order to understand the requirement of Bax in this apoptotic machinery. For this purpose, both cell lines were treated with 25 μM PMC-A, after that Annexin V Assay was performed in a time dependent manner (for t=2h., 4h., 8h., 12h., 24h.). EtOH treated cells were used as control and collected at the indicated time points (Figure 4.2 4) (Figure 4.8). According to the results, there was a ≈15% difference in apoptosis up to 12th hour in HCT116 Bax(-/-) cells as seen in Figure 4.2 5.

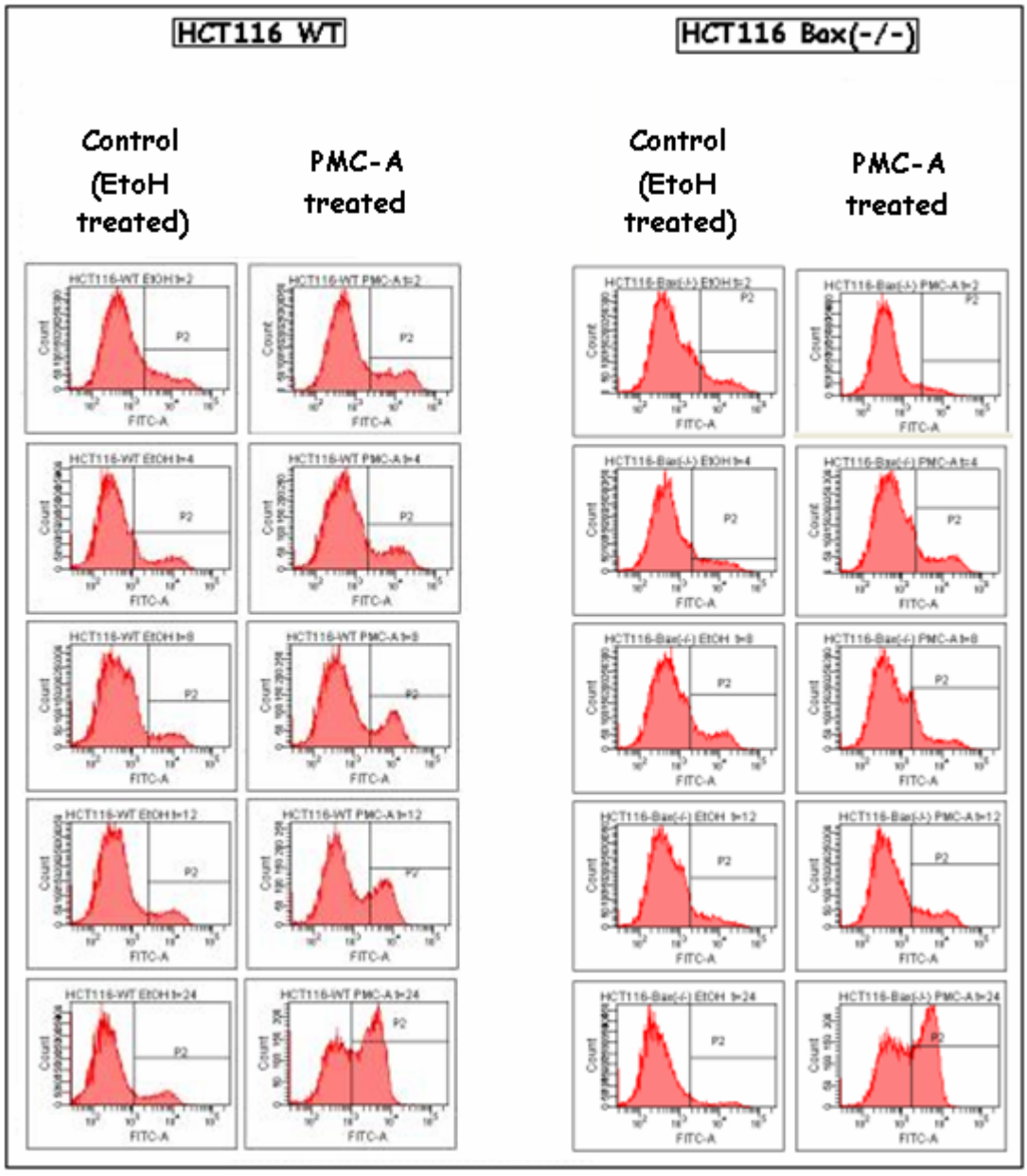

Figure 4.2 4: Apoptotic effect of PMC-A on HCT116 WT and HCT116 Bax(-/-) cells in a time dependent manner. HCT116 cells were seeded in 12 well-plate ($2x10^5$ cells/well) and treated with 25 µM PMC-A and collected at the indicated time points (t=2h., 4h., 8h., 12h., 24h.). Apoptosis was assessed using Annexin V assay.

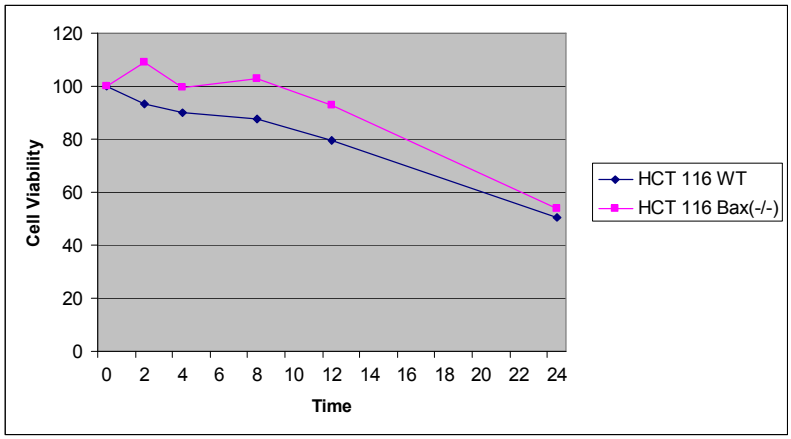

Figure 4.2 5: Comparison of PMC-A induced apoptotic effect on HCT116 WT and HCT116 Bax(-/-) cells.

Furthermore, in order to understand the effect of PMC-A at the morphological level, after treatment the cells were observed under the light microscope for t=12h. and 24h.. As in Annexin V assays, in apoptotic manner induced by PMC-A, WT and Bax(-/-) were morphologically distinct as seen in Figure 4.2 6.

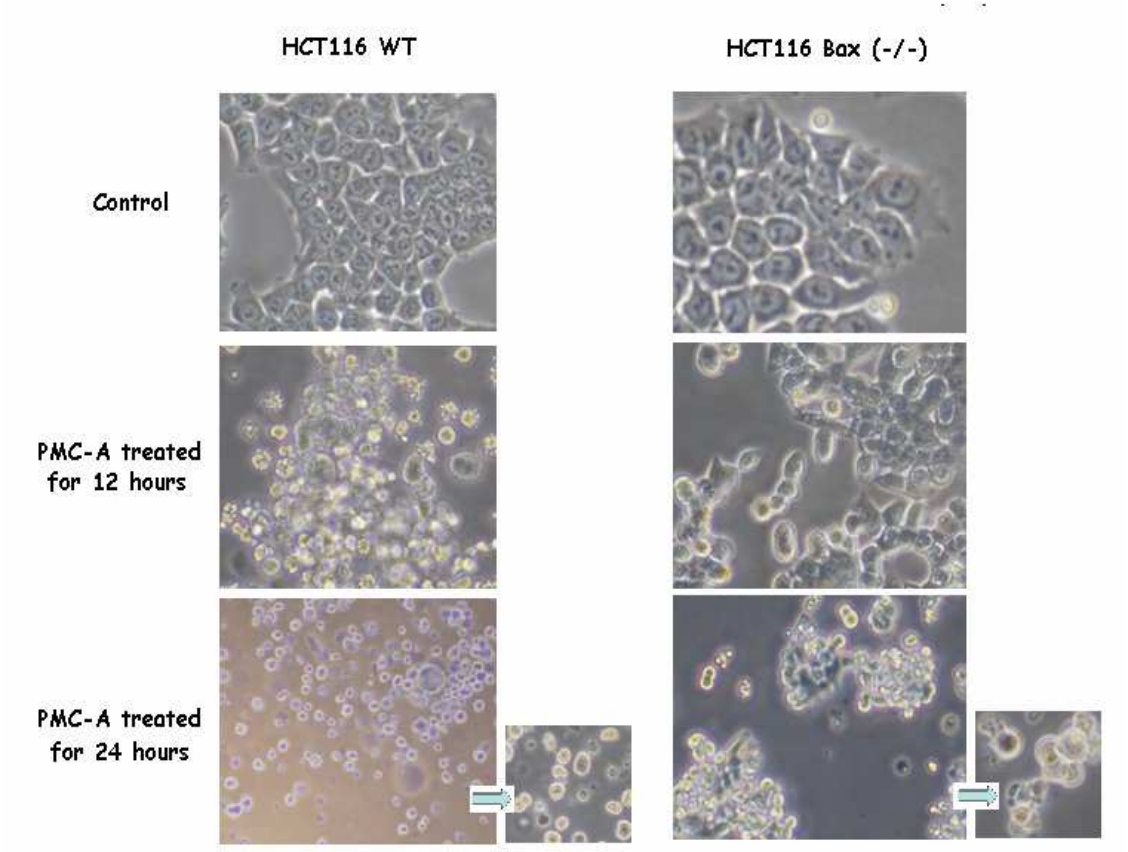

Figure 4.2 6: Morphological effect of PMC-A on HCT116 WT and HCT116 Bax(-/-) cells. Cells were treated with 25 μM PMC-A and observed at the indicated time points. The cells were observed under the light microscope at 400X magnification.

4.3 Determination of apoptosis at protein level: caspase activation and apaf-1 up-regulation

In order to determine apoptosis at the protein level, caspase cleavage and apaf-1 protein levels were examined by immunoblotting. As an initial setup, the pathway's caspase dependence was identified. Since caspase-3 is an effector caspase, its protein level was studied first. HCT116 cells were treated with 25 μM PMC-A and then cell lysates were collected in a time dependent manner, from 2 hours to 24 hours corresponding with time dependent Annexin V assay. Cell lysates were immunoblotted with anti-cleaved caspase-3 antibody which detects both pro- and cleaved caspase-3 proteins. Furthermore, equal protein loading was confirmed by immunoblotting the

same membrane with anti-β-actin antibody (Figure 4.3 1). The results indicate that cleavage of caspase-3 started at 8th hour after treatment with 25μM PMC-A (lane 5) with an increasing profile.

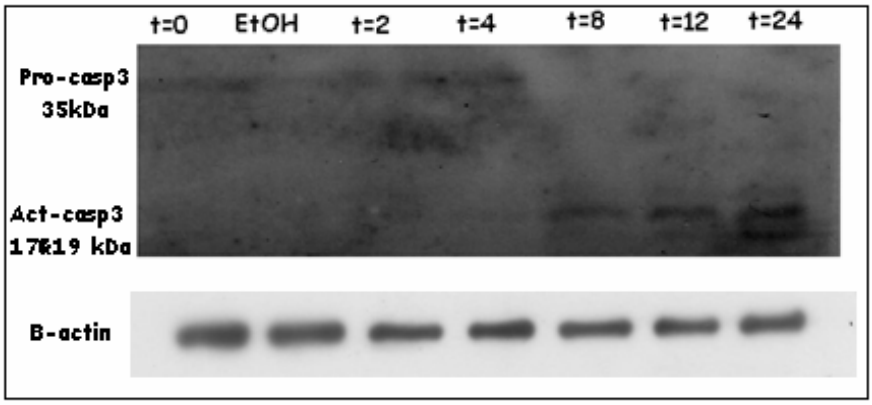

Figure 4.3 1: PMC-A induced caspase-3 activation in HCT116 cells. Cells were seeded in 60 mm dishes ($6x10^5$ cells/dish) and treated with 25 μM PMC-A from 2 hours to 24 hours. Proteins were isolated at the indicated time points, the activation of caspase-3 were detected by immunoblotting using specific antibodies. B-actin was used as loading control for immunoblots.

According to the Annexin V results PMC-A induced apoptosis in HCT116 Bax (-/-) cells, caspase-3 cleavage was additionally tested in these cells. For this purpose, HCT116 Bax (-/-) cells were treated with 25 μM PMC-A and then cell lysates were collected in a time dependent manner, same as caspase-3 immunoblotting in HCT 116 WT cells. Moreover, an initial control was used to be sure to observe caspase-3 clevage; samples of 24 hours PMC-A treated HCT116 WT cells were loaded as an additional lane (lane 8). The immunoblots were treated with anti-cleaved caspase-3 antibody. Equal protein loading was confirmed by immunoblotting the same membrane with anti-β-actin antibody (Figure 4.3 2). According to the initial control confirmation (lane 8, HCT116 WT treated with PMC-A), caspase-3 activation was not observed in PMC-A treated HCT116 Bax (-/-) cells (lane 1-7).

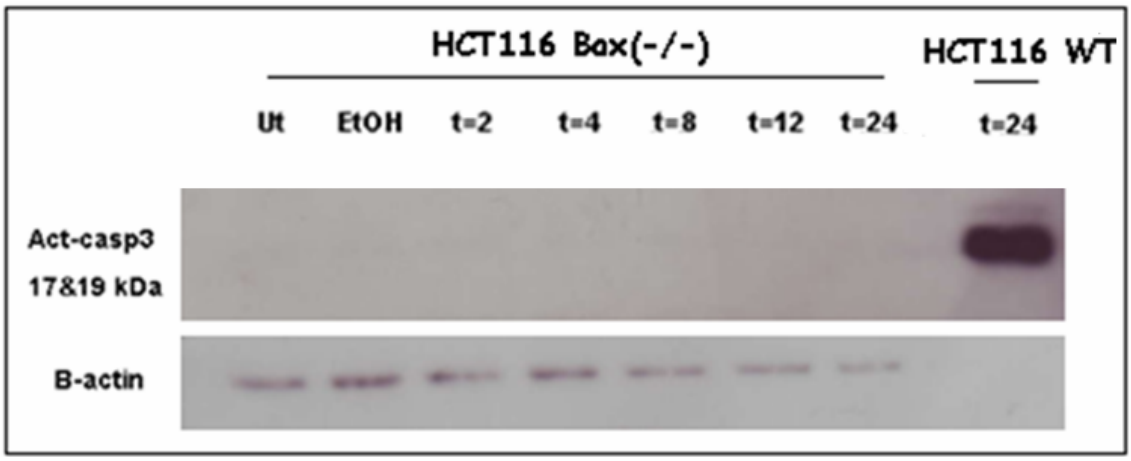

Figure 4.3 2: PMC-A induced caspase-3 activation in HCT116 Bax (-/-) cells. Cells were seeded in 60 mm dishes ($6x10^5$ cells/dish) and treated with 25 μM PMC-A from 2 hours to 24 hours. Protein isolations were performed at the indicated time points, the activation of caspase-3 were detected by immunoblotting using specific antibodies. The last lane (HCT116 WT 24h. PMC-A treated) was used as an initial control to show caspase-3 cleavage. B-actin was used as loading control for immunoblots. Ut represents untreated cells.

To identify if the apoptotic effect of PMC-A works through the intrinsic pathway, caspase-9 cleavage was also tested in order to understand whether it was the initiator caspase for caspase-3 activation. HCT116 cells were treated with 25 μM PMC-A and then cell lysates were collected in a time dependent manner, similar to caspase-3 immunoblotting. The immunoblots were treated with anti-cleaved caspase-9 and anti-apaf-1 antibodies. Anti-cleaved caspase-9 antibody recognized both non-claved (pro) and cleaved forms of caspase-9 protein. The results achieved by immunoblotting demonstrate that caspase-9 activation and apaf-1 up-regulation correlates with each other. Equal protein loading was confirmed by immunoblotting the same membranes with anti-β-actin antibody (Figure 4.3 3). Based on the results, caspase-9 cleavage started at early time points (t=4h.; lane 3 of caspase-9 blot), preceeding apaf-1 up-regulation was additionally observed (lane 5 of apaf-1 blot).

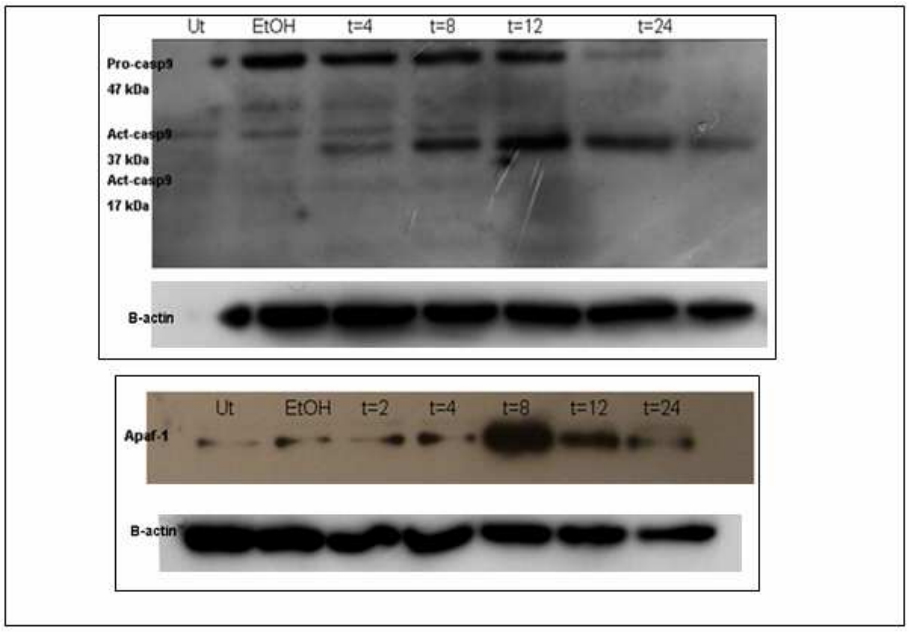

Figure 4.3 3: PMC-A induced caspase-9 and apaf-1 regulation in HCT116 cells. Cells were seeded in 60 mm dishes ($6x10^5$ cells/dish) and treated with 25 μM PMC-A from 2 hours to 24 hours. Protein isolations were performed at the indicated time points, the activation of caspase-9 and regulation of apaf-1 were detected by immunoblotting using specific antibodies. B-actin was used as loading control for immunoblots. Ut represents untreated cells.

4.4 Modulation of Bcl-2 Pro-survival proteins; Bcl-2, Bcl-xL & Mcl-1

Bcl-2 family of proteins is the key modulators of apoptosis. To investigate the role of Bcl-2 protein family, whole cell lysates were probed with Bcl-2 antibodies. First of all, regulation of Bcl-2 pro-survivals was identified. Bcl-2, Bcl-xL and Mcl-1 protein levels were checked by immunoblotting. HCT116 cells were treated with 25 μM PMC-A and then cell lysates were collected in a time dependent manner. The immunoblots were treated with anti-Bcl-2, anti-Bcl-xL and anti-Mcl-1 antibodies. Equal protein loading was confirmed by immunoblotting the same membranes with anti-β-actin antibody (Figure 4.4 1A). At the early time points (t=2h.; lane 3 of PMC-A treated HCT116WT Bcl-2 blot) decrease in Bcl-2 protein amount with a slight increase starting

at 12th hour (lane 3-7 PMC-A treated HCT116WT Bcl-2 blot), and increase in Mcl-1 protein levels (lane 1-5 of PMC-A treated HCT116WT Mcl-1 blot) were determined whereas there was no change in the protein level of Bcl-xL.

Beside these, there was an additional band at the lower part of Mcl-1 blot, which may indicate the cleaved product of Mcl-1, had an increasing pattern in a time dependent manner with a decrease in 24th hour (lane 3-5 of PMC-A treated HCT116WT Mcl-1 blot). Since Bcl-2 and Mcl-1 were shown to be regulated as anti-apoptotic members of Bcl-2 family in PMC-A induced apoptosis in HCT116 WT cells, their role were also studied in HCT116 Bax (-/-) cells. For this purpose, HCT116 Bax (-/-) cells were treated with 25 μM PMC-A and then cell lysates were collected in a time dependent manner. The immunoblots were treated with anti-Bcl-2 and anti-Mcl-1 antibodies. Equal protein loading was confirmed by immunoblotting the same membranes with anti-β-actin antibody (Figure 4.4 1B). The decrease in Bcl-2 protein levels were similar with the HCT116 WT cells' Bcl-2 protein levels, but increase in Mcl-1 protein levels was not much as HCT116 WT cells' Mcl-1 protein levels, however at the late time point (t=24h.) Mcl-1 protein level was much more similar (lane 7 of PMC-A treated HCT116 Bax(-/-) blot).

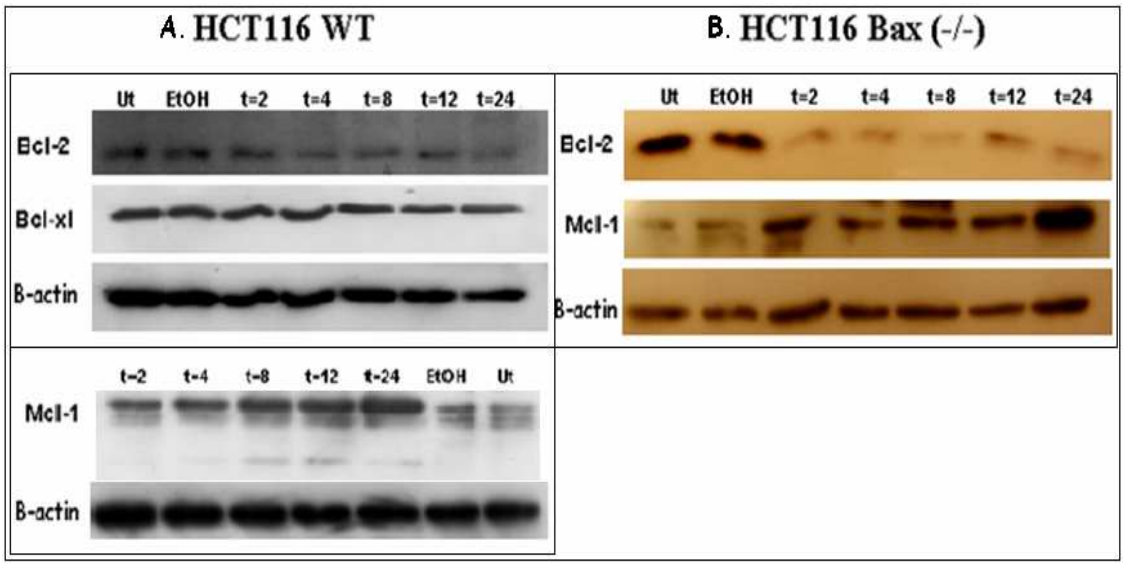

Figure 4.4 1: Involvement of Bcl-2 pro-survivals; Bcl-2, Bcl-xL and Mcl-1 in PMC-A induced apoptosis in HCT116 cells. **A.** HCT116 WT cells were seeded in 60 mm dishes ($6x10^5$ cells/dish) and treated with 25 μM PMC-A from 2 hours to 24 hours. Protein isolations were performed at the indicated time points, the regulation of Bcl-2, Bcl-xL and Mcl-1 were detected by immunoblotting using specific antibodies. **B.** HCT116 Bax(-/-) cells were seeded in 60 mm dishes ($6x10^5$ cells/dish) and treated with 25 μM PMC-A from 2 hours to 24 hours. Protein isolations were performed at the indicated time points, the regulation of Bcl-2 and Mcl-1 were detected by immunoblotting using specific antibodies. B-actin was used as loading control for each immunoblots. Ut represents untreated cells.

4.5 Modulation of Bcl-2 Pro-apoptotic proteins; Bim, Puma, Bad, Bak, Bax and Bid

Regulation of Bcl-2 pro-apoptotic proteins is known to be important in apoptosis, especially for the intrinsic pathway. In order to identify their regulation and modulation, Bim, Puma, Bad, Bak and Bax protein levels were checked by immunoblotting. HCT116 cells were treated with 25 μM PMC-A and then cell lysates were collected in a time dependent manner. The immunoblots were treated with anti-Bim, anti-Puma, anti-Bad, anti-Bak and anti-Bax antibodies. Equal protein loading was confirmed by immunoblotting the same membranes with anti-β-actin antibody (Figure 4.5 1). Based on the results; there was a clear increase in the protein level of Bim in a time dependent

manner (lane 3-7 of PMC-A treated HCT116WT Bim blot) and slight increase in the levels of Bax (lane 1-3 of PMC-A treated HCT116WT Bax blot) with a sharp decrease at 24th hour (lane 5 of PMC-A treated HCT116WT Bax blot). Additionally, Puma and Bad protein levels were constant, but surprisingly there was a sharp decrease in both of the protein levels at 24th hour as Bax protein. (lane 7 of PMC-A treated HCT116WT Puma blot and lane 5 of PMC-A treated HCT116WT Bad blot). Beside these, there was a sharp decrease in Bak level (lane 3-5 of PMC-A treated HCT116WT Bak blot).

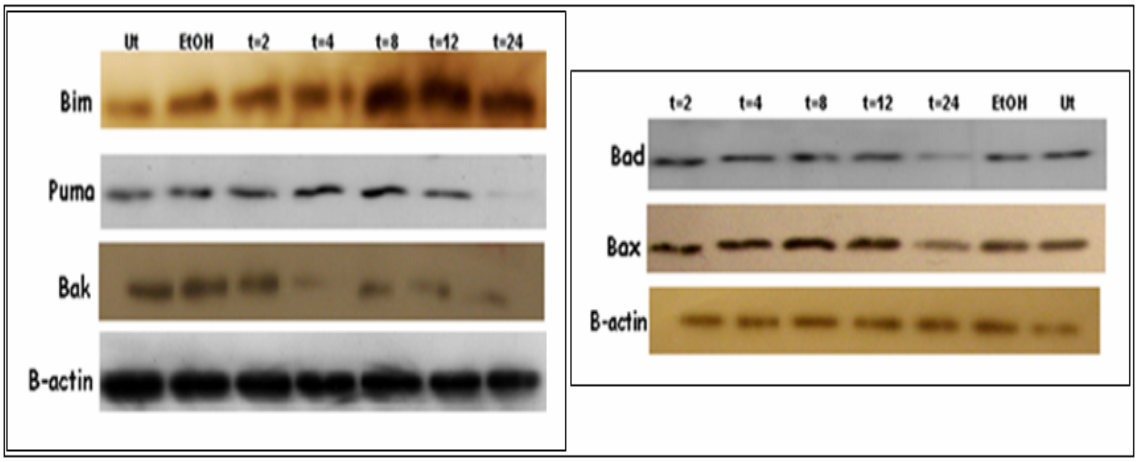

Figure 4.5 1: Involvement of Bcl-2 pro-apoptotic proteins; Bim, Puma, Bak, Bad and Bax in PMC-A induced apoptosis in HCT116 cells. HCT116 WT cells were seeded in 60 mm dishes ($6x10^5$ cells/dish) and treated with 25 μM PMC-A from 2 hours to 24 hours. Protein isolations were performed at the indicated time points, the regulation of Bim, Puma, Bak, Bad and Bax were detected by immunoblotting using specific antibodies. B-actin was used as loading control for both immunoblots.

Additionally, Bid truncation was determined in HCT116 WT cells; since Bid is a player in different pathways its truncation was also determined in HCT116 Bax(-/-) cells. Both cell lines were treated with 25 μM PMC-A and then cell lysates were collected in a time dependent manner. The immunoblots were treated with anti-Bid antibody. (Figure 4.5 2). There were slight bands that might be identified as tBid in HCT116 WT cells (represented by red circles in the figure; lane 4-6 of PMC-A treated HCT116WT Bid blot), whereas there were no bands in HCT116 Bax(-/-) cells.

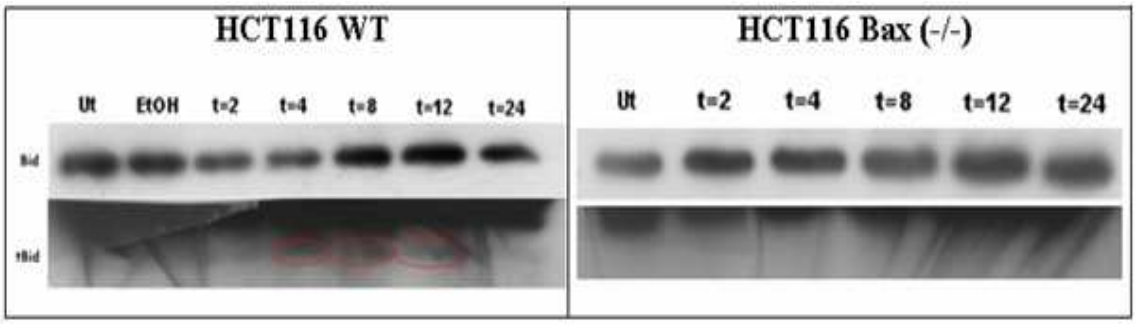

Figure 4.5 2: PMC-A induced Bid truncation in HCT116 cells. HCT116 WT and HCT116 Bax(-/-) cells were seeded in 60 mm dishes ($6x10^5$ cells/dish) and treated with 25 μM PMC-A from 2 hours to 24 hours. Protein isolations were performed at the indicated time points, the truncation of Bid was detected by immunoblotting using specific antibodies. B-actin was used as loading control for both immunoblots.

4.6 Effect of PMC-A on Cell Cycle

In order to determine PMC-A's effect on cell cycle, HCT116 cells were analyzed in cell cycle manner to understand alternative pathways. Initially, a general cyclin-dependent kinase inhibitor protein, p21, level was checked in both cell types. HCT116 cells were treated with 25 μM PMC-A and then cell lysates were collected in a time dependent manner. The immunoblots were treated with anti-p21 antibody. Equal protein loading was confirmed by immunoblotting the same membranes with anti-β-actin antibody (Figure 4.6 1). Since Bax deficiency made a delay and/or inhibition on PMC-A induced apoptosis, the cells could be more sensitive to cell cycle arrest, for this purpose p21 protein levels are additionally checked in HCT116 Bax(-/-) cell lines. For both of the cells, the results indicate that p21 protein levels increased in a time dependent manner (lane 3-7 of PMC-A treated HCT116WT and HCT116 Bax(-/-) p21 blots).

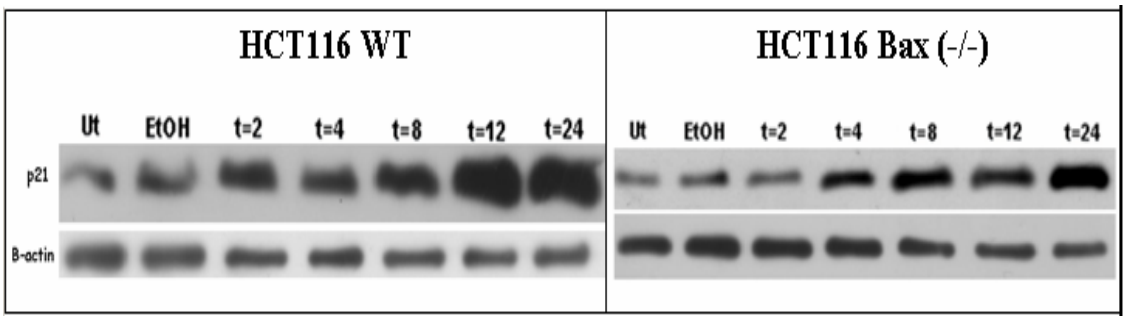

Figure 4.6 1: PMC-A induced p21 regulation in HCT116 cells. HCT116 WT and HCT116 Bax(-/-) cells were seeded in 60 mm dishes ($6x10^5$ cells/dish) and treated with 25 μM PMC-A from 2 hours to 24 hours. Protein isolations were performed at the indicated time points, the regulation of p21 was detected by immunoblotting using specific antibodies. B-actin was used as loading control for both immunoblots**.**

Furthermore, in order to determine cell cycle arrest at cellular level, cell cycle analysis was performed by FACS analysis. For this purpose, both of the cell lines were treated with 25 μM PMC-A, after that cell cycle analysis was performed in a time dependent manner (for t=2h., 4h., 8h., 12h., 24h.) (Figure 4.6 2). According to the FACS analysis after PI staining, there were not significant difference between the phases of different time points of HCT116 WT cells; but in HCT116 Bax(-/-) cell lines, G_2-M phase column showed an increasing pattern corresponding increasing time points, remarking an arrest at that phase. On the other hand, G_0-G_1 phase column showed a decreasing pattern and S phase column remained same; except for t=12h. These data also specified the an arrest at G_2-M phase in HCT116 Bax(-/-) cell lines. For t=0 cells, the untreated cells were collected with 24 hours PMC-A treated cells in order to make the cell-based results consistent with protein data.

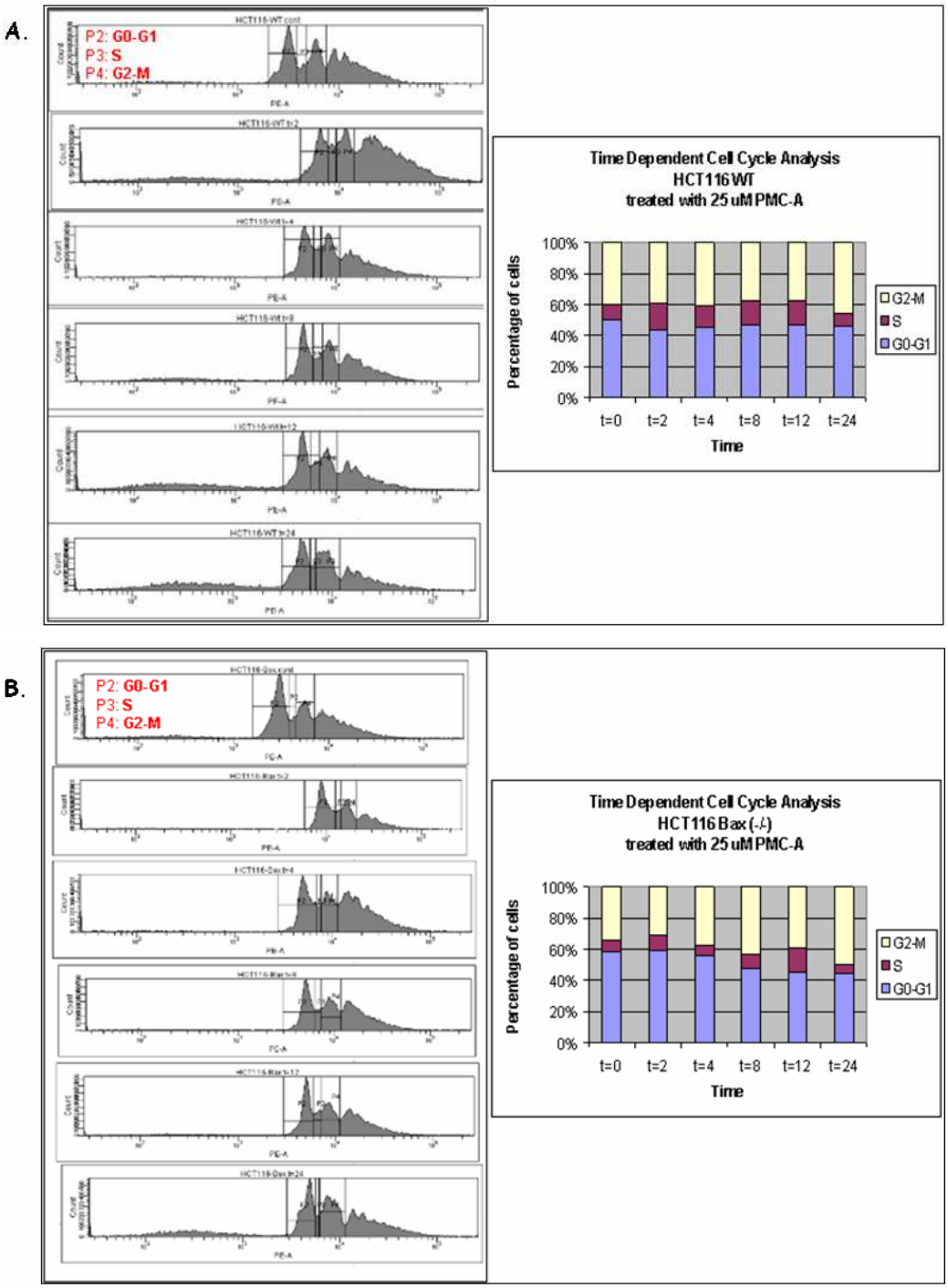

Figure 4.6 2: Time dependent cell cycle analysis in PMC-A treated HCT116 WT and HCT116 Bax(-/-) cells. **A.** HCT116 cells and **B.** HCT116 Bax(-/-) cells were seeded in 12 well-plate ($2x10^5$ cells/well) and treated with 25 μM PMC-A and collected at the indicated time points (t=2h., 4h., 8h., 12h., 24h.). Cell cycle analysis was assessed by PI staining.

5 DISCUSSION

In the present study, initially it was shown that among 24 hours treated PMC analogs, 25 μM PMC-A and 75 μM PMC-F were the most cytotoxic chemicals for HCT116 cells according to MTT results (Figure 4.1 1 and Figure 4.1 2). In the previous studies of our laboratory, PMC was found to be cytotoxic to Jurkat leukemia cells, whereas it was not cytotoxic to HCT116 cells as shown in Figure 4.1 3 [7]. PMC-A contains CC bond instead of epoxy group of PMC, according to Kwan et.al PMC-A had induced little endothelial dependent relaxation than PMC and they suggested epoxy group could be a determinant for vasorelaxant effect which was related to Ca^{2+} release and as a result may be related to apoptosis [104]. However, for HCT116 cells, PMC-A was more cytotoxic and PMC was not; the drugs' effects can be dissimilar for different cell types. HCT116 cells are aggressive cell lines because of deficient mismatch repair system; lots of effective drugs can be ineffective to HCT116 cells [3]. For that reason PMC could be ineffective for HCT116 cell lines, on the other hand since PMC-A is effective on such an aggressive cell line it could be more effective other cancer cell lines.

In our studies M30 Apoptosense and Annexin V assay results were confirmative; 25 μM PMC-A induced ≈30%, whereas 75 μM PMC-F induced only ≈8% apoptosis (Figure 4.2 2 and Figure 4.2 3). Following these initial results further experiments were done with PMC-A to understand the apoptotic response the cellular and molecular level.

Bax is one of the key regulators in apoptosis, Vogelstein et.al. generated HCT116 cells lacking functional Bax genes by deletion to determine its role in drug-induced apoptosis. The deletion of Bax made HCT116 cells partially resistant to apoptosis in response to 5-fluorouracil, on the contrary some of the chemopreventive agents as sulindac and other nonsteridal anti-inflammatory drugs completely eliminated

apoptosis [109]. Since it was determined that PMC-A induced apoptosis in HCT116 cell lines, HCT116 Bax(-/-) was additionally tested in a time dependent manner to understand the role of pro-apoptotic Bax, Interestingly, apoptosis induction was observed after 12th hour in HCT116 Bax(-/-) cell lines, whereas HCT116 WT cells' apoptotic response was monitored starting from 8th hour, this may indicate Bax involvement in PMC-A induced apoptosis. On the other hand, at 24th hour the apoptotic effect of the drug on both cell lines were similar to each other, according to this result a different signaling pathway may be activated (Figure 4.2 4 and Figure 4.2 5) In other words, Bax deficiency partially abolish apoptosis, cells can follow either a delayed intrinsic pathway or an alternative apoptotic pathway different from intrinsic pathway.

Additionally, in order to determine apoptotic effect of PMC-A at the cellular level, HCT116WT and Bax(-/-) cells were observed under light microscope. As HCT116 WT cells were detached at 12th hour, similar morphology was observed in Bax(-/-) cells within 24th hour as shown in Figure 4.2 6. These results further indicated PMC-A induces Bax dependent apoptosis in HCT116 cell lines.

Determination of apoptosis at molecular level indicated that caspase-9 cleavage and apaf-1 up-regulation started at 4th hour (Figure 4.3 3) indicating apoptosome formation as a consequence of intrinsic pathway, after these modulations effector caspase-3 was activated at 8th hour (Figure 4.3 1). The apoptosome formation occurred at 4th hour before caspase-3 cleavage at 8th hour consistent with apoptosis kinetics [25, 26].

Surprisingly, caspase-3 was not activated in HCT116 Bax(-/-) cells indicating an alternative Bax- and caspase-3-independent apoptotic pathway (Figure 4.3 2). which may indicate granzyme-dependent pathway, that can follow caspase-3 independent progression. As reviewed by Elmore S., there are different apoptotic pathways rather than intrinsic and extrinsic pathways; granzyme pathway is one of these alternative pathways. In this pathway, there is a serine protease called granzyme released to cytoplasm in response to degranulation of the activated CTLs. Granzyme pathway can follow cleavage of substrates different than caspase-3 to induce apoptosis [110]. In order to understand its granzyme dependency, granzyme levels could be determined in response to PMC-A treatment. This experiment must be achieved in both cell lines to

recognize whether it is a part of normal apoptotic response or followed only in Bax deficient cells

Bcl-2 family of proteins is the key modulator proteins of apoptosis. Among Bcl-2 pro-survivals such as Bcl-2, Mcl-1 and Bcl-xL; Bcl-2 was down-regulated starting at very early time points (2 hours) with a slight inrease starting at 12th hour in HCT116 cells as shown in Figure 4.4 1A, additionally in Bax(-/-) cells, there was a decrease in Bcl-2 protein levels also and its pattern was similar to HCT116 WT cells' this could be explained by the upstream effect of Bcl-2 (Figure 4.4 1B) [15].

Mcl-1 was up-regulated starting at very early time points (2 hours) in contrast to Bcl-2 down regulation in HCT116 WT cell lines (Figure 4.4 1B), suggesting a possible compensating role of Mcl-1. When similar experiments were conducted in Bax(-/-) cells, Mcl-1 up-regulation was also observed; however the increase of Mcl-1 levels of WT cells was more pronounced than the increase observed in Bax(-/-) cells up to 24 hours. Though, at 24th hour the Mcl-1 profile of both cells became similar to each other, which might be related with the converged apoptotic response at 24 th hour shown by time dependent Annexin V assay (Figure 4.2 5).

Lower band observed in Figure 4.4 1A lane 2-5 in Mcl-1 blot, may indicate the cleaved product of Mcl-1, had an increasing pattern in a time dependent manner with a decrease in 24th hour. Mcl-1 is a highly-regulated protein and has a short half life; in addition to its anti-apoptotic characteristics, it can also be cleaved by effector caspases during apoptosis to produce an apoptosis promoting protein through a positive feedback mechanism [41]. This additional band was only observed in HCT116 WT cells which may be a consequence of caspase-3 activation, since there was not caspase cleavage in HCT116Bax (-/-) cells, there was not expected any cleaved products. Clearly, Mcl-1 data indicate that in PMC-A induced apoptosis Mcl-1 might take a role trying to compensate Bcl-2's down-regulation, and its possible cleaved product may involve in promoting apoptosis.

Bcl-xL protein levels remain constant in a time dependent manner in HCT116 cells (Figure 4.4 1A). Since it is hard to observe modulations of each pro-survivals, the apoptotic pathway in response to PMC-A treatment could be Bcl-xL independent.

Although the pro-survivals are classified in the same group, their modulation has been shown to have distinct patterns in different dose and time kinetics.

Among Bcl-2 pro-apoptotic proteins, there was a clear increase in the level of Bim in a time dependent manner which is shown to be up-regulated by Ca^{2+} flux [105]. Also Bim directly inhibits Bcl-2, which was found to be down-regulated indicating apoptotic response induced by PMC-A in HCT116 cells is Bim dependent. Bim has the higher affinity to directly bind and activate Bax [53], this could be one of the reasons for the possible delayed or modulated apoptosis in HCT116 Bax(-/-) cells. Although the level of Bim was up-regulated, Bax and Bim association could not occur according to Bax deficiency. Modulated apoptotic response in HCT116 Bax(-/-) cells could also be related to this reason.

Beside these, there was a sharp decrease in Bak level, this was an expected result either, because Bak can form complex with either Bcl-xL or Mcl-1, but not with Bcl-2 [52]. Since Bcl-xL level remained constant and Mcl-1 level was upregulated then Bak could be trapped by these inhibitors with an increasing pattern in a time dependent manner. In addition to this, Bak and Bax have the same importance in Bcl-2 activation models, but in HCT-116 cell lines it was discovered that apoptotic sensitivities existed in the order of $Bax^{+}/Bak^{+} > Bax^{+}/Bak^{-} >> Bax^{-}/Bak^{+} >> Bax^{-}/Bak^{-}$, then Bax could be qualified as a more critical modulator for HCT116 cell lines [106].

Since Bid is a player in different pathways, it can make the connection with intrinsic and extrinsic pathways its truncation was determined in HCT116 WT and HCT116 Bax(-/-) cells. There were slight bands that might be identified as tBid in HCT116 WT cells, whereas there were no bands in HCT116 Bax(-/-) cells. It is not for sure that the bands in HCT116 WT blots represented tBid, but the important point was that there was not Bid truncation in HCT116 Bax(-/-) cells thus preventing an alternative pathway in between intrinsic and extrinsic pathways.

Studying anti-cancer therapies to eliminate tumor cells means investigating the fate of a cell by both apoptosis and cell cycle. For this purpose, PMC-A treated HCT116 cells were analyzed in cell cycle manner to understand alternative pathways. p21 is one of the general targeted CDKIs in cell cycle targeted cancer therapies; loss of p21 is

associated with several malignancies [99, 101]. Also, calcium is a critical modulator of epithelial cell differentiation and growth, HCT116 cells are epithelial cells and PMC-A induces Ca^{2+} flux, taking these into consideration cell cycle analyzing is an outstanding issue for this study [108].

Since Bax deficiency made a delay and/or modulation on PMC-A induced apoptosis, the cells could be more sensitive to cell cycle arrest, for this purpose p21 protein levels were additionally checked in HCT116 Bax(-/-) cell lines. As shown in, Figure 4.6 1; p21 levels were increased in PMC-A treated HCT116 WT and HCT116 Bax(-/-) cells in a time dependent manner. In WT cells increase in p21 levels was more than increase in Bax (-/-), which might be a result of state that "cell cycle is modulated during apoptosis" [100]. These distinct networks, cell cycle and apoptosis, are interconnected at some specific points as dedicated.

Additionally p21 plays a dual role in apoptosis, for some cells in response to some specific drugs it can behave either as a pro-apoptotic protein or as an pro-survival protein [107]. p21 pattern in this study might also serve as cell cycle arrest inducer and/or apoptosis promoter.

In order to understand p21 increase association with cell cycle arrest, PMC-A treated HCT116 WT and HCT116 Bax(-/-) cells were analyzed by flow cytometer. Untreated cells were collected with 24 hours PMC-A treated HCT116 cells to be consistent with p21 protein data. As the phases were compared in a time dependent manner there was not a major difference between the phases of HCT116 WT cells, whereas HCT116 Bax(-/-) cells were arrested in G_2-M phase indicating a sensitive profile of Bax(-/-) cells to cell cycle arrest (Figure 4.6 2).

HCT116 cells are aggressive cancerous cell lines with deficient mismatch repair system; it is hard to find out an agent to beat this kind of cells. They demonstrate unique characteristics involving enhanced proliferation, and/or a survival advantage with apoptosis resistance and problems in cell cycle arrest especially at G_2/M phase [3,116]. Our results specify a prominent agent eliminating the problems associated with MMR deficiency.

6 CONCLUSION AND FUTURE STUDIES

In conclusion, here we report that PMC-A induced intrinsic apoptotic pathway, triggering caspase-cleavage and Bcl-2 regulation in HCT116 WT cells (Figure 6 1), on the other hand Bax deficiency lead cells to a modulated and/or delayed response in programmed cell death (Figure 6 2).

Apoptosis is a dominant effect of PMC-A in HCT116 cells, whereas Bax deficiency made cells more sensitive to PMC-A induced cell cycle arrest as shown in p21 levels indicating an alternative response to apoptosis.

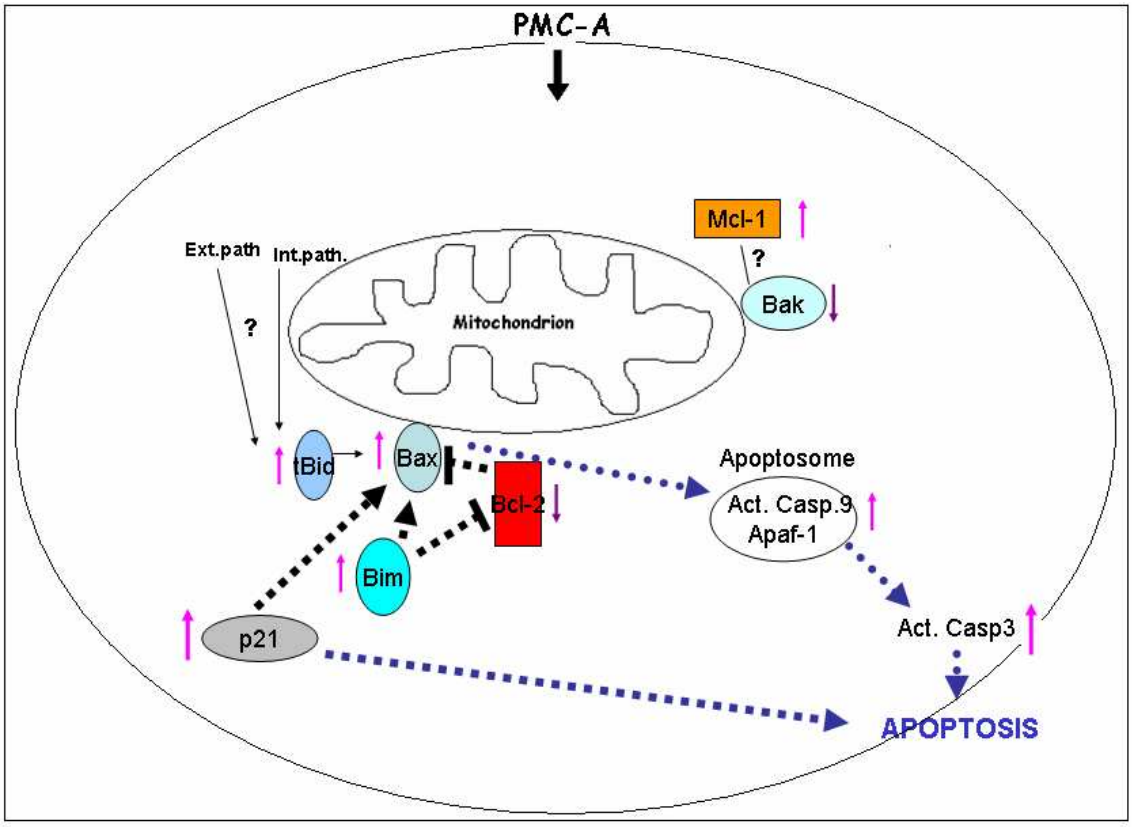

Figure 6 1 The proposed apoptotic mechanism of PMC-A in HCT116 cell lines, through the regulation of Bcl-2 protein family.

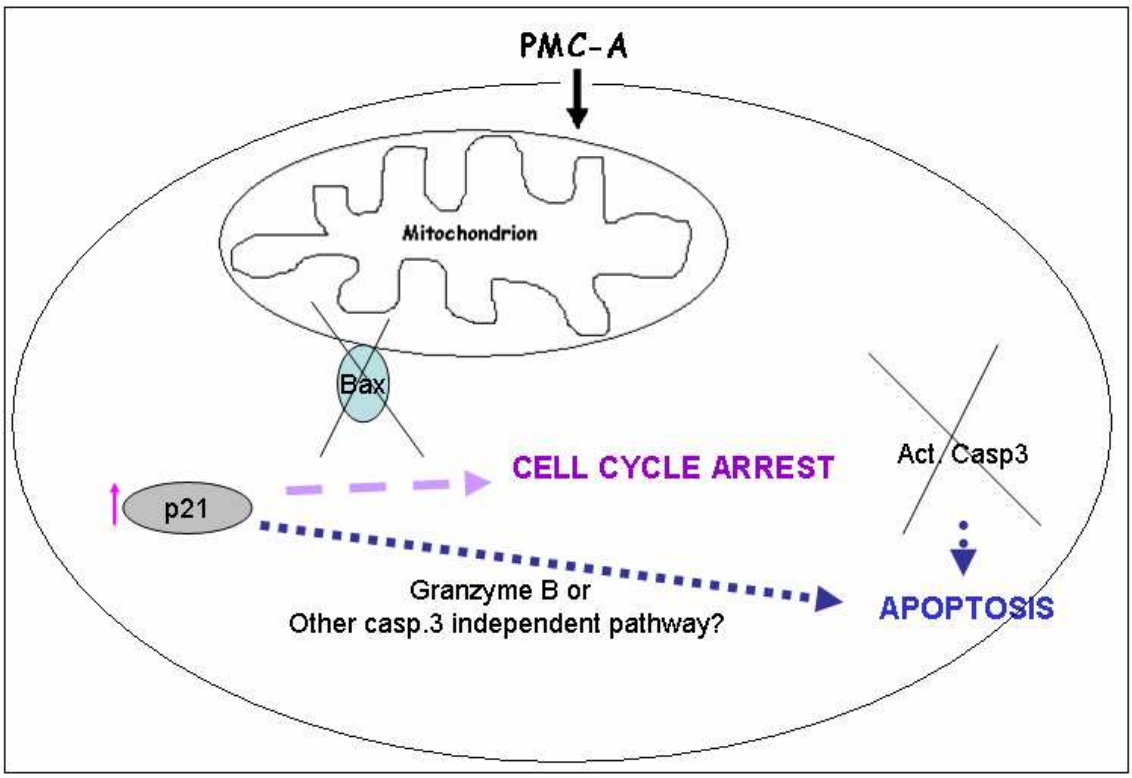

Figure 6 2 The proposed apoptotic mechanism of PMC-A in HCT116 Bax(-/-) cell lines.

In view of the results obtained future studies can be suggested as:

- In order to determine apoptotic pathway in more detail, Bcl-2 overexpression or Bim silencing can be achieved. If PMC-A's apoptotic effect persists on cells indicating alternative apoptotic pathways, other possible pathways have to be identified.

- To clarify apoptotic pathway in response to PMC-A, HCT116 cells can be treated with different caspase inhibitors to observe whether apoptotic response changes. Additionally, caspase-8 cleavage can be examined to investigate if PMC-A induces extrinsic pathway, this result will also identify Bid truncation.

- To investigate molecular effects of PMC-A, it must be studied on other cancer cell types, as an example on less aggressive cells to understand its

different effects, and DNA binding studies can be performed to understand its mode of action.

- To understand the role of Bax in PMC-A treated HCT116 cells, other possible pathways can be identified, as a consequence it would be obvious that if the response observed in HCT116 Bax(-/-) cells is a delayed and/or an alternative response.

- Mcl-1 up-regulation and Bak down-regulation have to be studied in more detail in order to detect if their unexpected modulations are related to each other. Immunoprecipitation and overexpression of Bak, silencing of Mcl-1 can be performed to explore their possible relation. Besides, possible Bak involvement in Bax deficiency in response to PMC-A have to be discovered.

- Since p21 is a target for p53, the major tumor suppressor, with the purpose of to enlighten interconnection between cell cycle arrest and apoptosis; p53 modulation, responses to its inhibition have to be recognized.

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APPENDIX A

CHEMICALS

(in alphabetical order)

Name of Chemical	Supplier Company	Catalog Number
Acrylamide/Bis-acrylamide	Sigma, Germany	A3699
Ammonium persulfate	Sigma, Germany	A3678
Annexin V-FITC	Alexis Biochemicals	ALX-209-250-T100
Anti-Beta-actin Ab	Cell Signal Tech., USA	4967
Anti-rabbit IgG HRP linked	Cell Signal Tech., USA	7074
Anti-mouse IgG HRP linked	Amersham Biosciences, UK	RPN4201
Anti-Bad Ab	Cell Signal Tech., USA	9292
Anti-Bcl-2 Ab	Cell Signal Tech., USA	2872
Anti-Bcl-xL Ab	Cell Signal Tech., USA	2762
Anti-Mcl 1 Ab	Cell Signal Tech., USA	4572
Anti-Bak Ab	Cell Signal Tech., USA	3814
Anti-Bax Ab	Cell Signal Tech., USA	2772
Anti-Bim Ab	Cell Signal Tech., USA	2819

Anti-Bid Ab	Cell Signal Tech., USA	2002
Anti-cleaved Casp 3 Ab	Cell Signal Tech., USA	9661
Anti-cleaved Casp 9 Ab	Cell Signal Tech., USA	9501
Anti-Mcl 1 Ab	Cell Signal Tech., USA	4572
Anti-p21 Ab	Cell Signal Tech., USA	2946
Anti-Puma Ab	Cell Signal Tech., USA	4976
Protease inhibitor cocktail tablet	Roche, Germany	11697498001
EDTA	Riedel-de Haén, Germany	27248
Ethanol	Riedel-de Haén, Germany	32221
Foetal Bovine Serum	Sigma, Germany	F2442
Glycerol	Riedel-de Haén, Germany	15523
Glycine	Amnesa, USA	0167
Heparin	BiochromAG, Germany	L6510
HCl	Merck, Germany	100314
Hyperfilm ECL	Amersham Biosciences, UK	RPN2103K
Hybond P-membrane (PVDF)	Amersham Biosciences, UK	RPN2020F
Isopropanol	Riedel-de Haén, Germany	24137
KCl	Fluka, Switzerland	60129
KH_2PO_4		
KOH		
Liquid nitrogen	Karbogaz, Turkey	
Mccoy's 5A Medium	Biological Industries	01-075-1

2-Mercaptoethanol	Sigma, Germany	M370-1
Methanol	Riedel-de Haén, Germany	24229
MgCl2	Sigma, Germany	M9272
Milk Diluent concentrate	KPL, USA	50-82-00
NaCl	Riedel-de Haén, Germany	13423
NaOH	Merck, Germany	106462
$NaPO_4H_2$		
NP-40	Sigma, Germany	I3021
Pen/Strep. Solution	Biological Industries	
Phosphate buffered saline	Sigma, Germany	P4417
PMSF	Sigma, Germany	P7626
Prestained Protein MW Marker	Fermentas,Germany	#SM0441
RPMI 1640	PAN Biotech, Germany	P04-22100
Sodium Dodecyl Sulphate	Sigma, Germany	L4390
TEMED	Sigma, Germany	T7029
Triton X-100	Applichem, Germany	A1388
Tris	Fluka, Switzerland	93349
Trypsin/EDTA 1X	Biological Industries, Israel	03-050-1
Tween® 20	Merck, Germany	822184

APPENDIX B

MOLECULAR BIOLOGY KITS

(in alphabetical order)

Name of Kit	**Supplier Company**	**Catalog Number**
ECL Advance Chemiluminescence Detection Kit	Amersham Biosciences , UK	RPN2135
Cell Proliferation Kit I (MTT)	Roche, Germany	1465007-001
DC Assay		
M30-Apoptosense ELISA	Peviva, Sweden	PE0024

APPENDIX C

Autoclave: Hirayama, Hiclave HV-110, JAPAN

Certoclav, Table Top Autoclave CV-EL-12L, AUSTRIA

Balance: Sartorius, BP211D, GERMANY

Sartorius, BP221S, GERMANY

Sartorius, BP610, GERMANY

Schimadzu, Libror EB-3200 HU, JAPAN

Blot Module X Cell II ™ Blot Module, Novex, USA

Centrifuge: Eppendorf, 5415C, GERMANY

Eppendorf, 5415D, GERMANY

Eppendorf, 5415R, GERMANY

Kendro Lab. Prod., Heraeus Multifuge 3L, GERMANY

Hitachi, Sorvall RC5C Plus, USA

Hitachi, Sorvall Discovery 100 SE, USA

Deepfreeze: -70^{o} C, Kendro Lab. Prod., Heraeus Hfu486 Basic, GERMANY

-20^{o} C, Bosch, TURKIYE

Distilled Water: Millipore, Elix-S, FRANCE

Millipore, MilliQ Academic, FRANCE

Electrophoresis: Biogen Inc., USA

X Cell SureLock ™ Electrophoresis Cell, Novex USA

Gel Documentation: UVITEC, UVIdoc Gel Documentation System, UK

Biorad, UV-Transilluminator 2000, USA

Ice Machine: Scotsman Inc., AF20, USA

Incubator: Memmert, Modell 300, GERMANY

Memmert, Modell 600, GERMANY

Laminar Flow: Kendro Lab. Prod., Heraeus, HeraSafe HS12, GERMANY

Magnetic Stirrer: VELP Scientifica, ARE Heating Magnetic Stirrer, ITALY

VELP Scientifica, Microstirrer, ITALY

Microliter Pipette: Gilson, Pipetman, FRANCE

Mettler Toledo, Volumate, USA

Microwave Oven: Bosch, TÜRKIYE

pH meter: WTW, pH540 GLP MultiCal®, GERMANY

Power Supply: Biorad, PowerPac 300, USA

Wealtec, Elite 300, USA

Refrigerator: +4o C, Bosch, TÜRKiYE

Shaker: Forma Scientific, Orbital Shaker 4520, USA

GFL, Shaker 3011, USA

New Brunswick Sci., Innova™ 4330, USA

C25HC Incubator shaker New Brunswick Scientific, USA

Spectrophotometer: Schimadzu, UV-1208, JAPAN

Schimadzu, UV-3150, JAPAN

Speed Vacuum: Savant, Speed Vac® Plus Sc100A, USA

Savant, Refrigerated Vapor Trap RVT 400, USA

Thermocycler: Eppendorf, Mastercycler Gradient, GERMANY

Vacuum: Heto, MasterJet Sue 300Q, DENMARK

Water bath: Huber, Polystat cc1, GERMANY