THE ROLE OF BAX IN PMC-F INDUCED CELL DEATH MECHANISM IN HCT116 COLON CARCINOMA CELL LINES

by

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ABSTRACT

In a previous study reported from our lab, 2 analogous of Pramanicin (PMC); PMC-A and PMC-F were found to be the most toxic drugs in HCT116 colon carcinoma cell line, among 9 analogues.

In this study, cytotoxicity of PMC-A and PMC-F has been compared and death signaling pathways have been identified in HCT116 WT and Bax-/- cells. Bax-/- cells exhibited resistance in the early times of drug treatment, followed similar death response with WT cells. PMC-F was more effective than PMC-A inducing the initial cleavage of caspase-3, -9 and -8. Therefore, PMC-F was used in the further experiments.

To understand the role of MAP kinases in PMC-F induced apoptosis, their phosphorylation levels were investigated. The results showed that Bax-/- cells exhibited higher level of ERK 1/2 and JNK phosphorylations. Also, WT cells presented an increasing phosphorylation level of p38 sustained longer than Bax-/- cells. We also demonstrated that PMC-F induced ROS production in both cell lines, but less and with a delayed manner in Bax-/- cells.

These data indicate that PMC-A and PMC-F may stand for new potential anti-cancer drugs for the treatment of colon cancer. Moreover, ROS might be the key

signaling mechanism which determines the level of MAPK phosphorylation and the earlier resistance of HCT116 Bax-/- cells to death in PMC-F induced apoptosis.

HCT116 KOLON KANSERİ HÜCRE HATLARINDA PMC-F TARAFINDAN İNDÜKLENMİŞ HÜCRE ÖLÜM MEKANİZMASINDA BAX'IN ROLÜ

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Anahtar Kelimeler: PMC-F, Apoptoz, MAPK sinyalizasyonu, ROS üretimi, HCT116 hücre hatları

Özet

Laboratuarımızdan rapor edilen bir önceki çalışmada PMC'nin 2 analoğu; PMC-A ve PMC-F 9 analog içerisinden HCT116 kolon karsinoma hücreleri üzerinde en toksik ilaçlar olarak bulunmuştur.

Bu çalışmada, PMC-A ve PMC-F'in sitotoksisteleri karşılaştırılmış ve HCT116 WT ve Bax -/- hücrelerindeki hücre ölüm sinyal yolları belirlenmiştir. Bax-/- hücreleri ilaç muamelesinin erken zaman dilimlerinde dirençlilik göstermiş, WT hücreleriyle benzer ölüm tepkisini takip etmiştir. PMC-F, caspase-3, -9 ve -8'in ilk kesimlerini tetikleyerek PMC-A'ya göre daha etkili bulumuştur. Bu nedenle, ilerleyen deneylerde PMC-F kullanılmıştır.

PMC-F ile indüklenmiş apoptozizde MAP kinazların rolünü anlayabilmek için fosforilasyon seviyeleri incelenmiştir. Sonuçlar göstermiştir ki Bax-/- hücreleri daha fazla ERK 1/2 ve JNK fosforulasyonu sergilemiştir. Ayrıca, WT hücreleri Bax-/- hücrelerine göre daha uzun süre artan bir p38 fosforulasyonu göstermiştir. Diğer taraftan PMC-F'in her iki hücre hattında da ROS üretimini tetiklediğini; ancak Bax-/-

hücrelerinin bu üretimi daha az ve daha geç bir tepkiyle gerçekleştirmiş olduğunu gösterdik.

Bu sonuçlar işaret etmiştir ki PMC-A ve PMC-F kolon kanseri'nin tedavi edilmesinde yeni potansiyel anti-kanser ilaçları olarak değerlendirilebilir. Ayrıca, ROS MAP kinaz fosforilasyon seviyesini ve Bax-/- hücrelerinin PMC-F ile indüklenmiş apoptozdaki ölüme karşı olan erken dirençliğini belirleyen muhtemel temel sinyal mekanizması olarak durmaktadır.

To my family

and

my grandfather,

Mehmet Orhan Özol..

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LIST OF ABBREVIATIONS

HCT116	Human colon carcinoma cell line
PMC	Pramanicin
NK	Natural Killer
Cdk	Cyclin dependent kinase
tBID	Truncated BID
MAPK	Mitogen activated protein kinase
ERK	Extra cellular signal regulated kinase
JNK	c-Jun N-terminal kinase
MDR	Multi drug resistance
APC	Adenomatous polyposis coli
5-FU	Fluorouracil
PARP	Poly ADP ribose polymerase
FAP	Familial adenomatous polyposis
HNPCC	Hereditary nonpolyposis colorectal cancer
mCRC	Metastatic colorectal cancer
MMR	Mismatch repair
MIN	Microsatellite instability
CIN	Chromosome instability
EGFR	Epidermal growth factor receptor
PI3K	Phosphatidylinositol-3 kinase
DCHF-DA	Dichlorodihydrofluorescein diacetate
VEGF	Vascular endothelial growth factor
LV	Leucovorin
IAP	Inhibitor of apoptosis
PCD	Programmed cell death
TRAIL	TNF-related apoptosis-inducing ligand

TNF	Tumor necrosis factor
DR	Death receptor
FADD	Fas-associated via death domain
XIAP	X-linked inhibitor of apoptosis
SMAC	second mitochondria-derived activator of caspase
TNFR	Tumor necrosis factor receptor
DISC	Death-inducing signaling complex
AIF	Apoptosis inducing factor
PDGF	Platelet derived growth factor
FGF	Fibroblast growth factor
AP-1	Activator protein 1
ROS	Reactive oxygen species
FasL	Fas ligand
Ψm	Mitochondrial membrane potential
MnSOD	Manganese Superoxide Dismutase
NO	Nitric oxide
HLA	Human leukocyte antigen
COX	cylooxygenase
HSP-90	Heat shock protein-90
mTOR	Mammalian target of rapamycin

1. INTRODUCTION

Cancer is one of the leading causes of death in the world. Lung, stomach, colorectal, liver and breast cancers stands at the top of the list as being the most prevalent cancers in the world [1].

Colorectal cancer is the fourth most prevalent cancer in the world [2]. There are also some factors affecting the occurrence of it such as age, sex and ethnicity. On the other hand, there are risk factors for colorectal cancer listed as modifiable and non-modifiable. While family history and personal history of colorectal cancer, colorectal polyps or chronic inflammatory bowel diseases are classified as non-modifiable factors, physical activity, overweight and obesity, diabetes and diet are among the modifiable factors [3]. Bacterial carcinogenesis is another aspect contributing to colon cancer formation. For example, a person with *Helicobacter pylori* infection has a predisposition to have colon cancer by two ways; the initiation of chronic inflammation and the assembly of carcinogenic bacterial metabolites [4].

Cancer cells can be eliminated by radiotherapy, surgery and chemotherapy applications; however, these strategies may also cause many side effects in the patients. In terms of chemotherapeutic approaches, anti-cancer vaccines can be used to either enhance the activation of tumor-specific T-cells to kill the cancer cell lines in the body or directly inhibit the proliferation as wells as induce death in cancer cells. In the latter strategy, chemotherapeutic drugs are aimed to induce apoptotic cell death mechanism in cancer cells in order to reduce the toxic effects of the drug for the surrounding normal cells.

The execution of apoptosis involves the binding death stimulating factors to death receptors for the activation of extrinsic pathway and cellular stress to induce intrinsic apoptotic pathway [5]. The direct activation of extrinsic pathway may also use the

intrinsic pathway inhibiting anti-apoptotic Bcl-2 proteins with tBid [6]. In the presence of DNA damage, p53 enhances the activation intrinsic mitochondrial pathway by preventing the anti-apoptotic Bcl-2 proteins and activating the pro-apoptotic Bcl-2 proteins [6]. MAP kinases are also one of the regulatory signaling molecules which determine the function of Bcl-2 family proteins and affect the appearance of apoptosis. ERK signaling cascade which was mostly defined to contribute cell survival, can inhibit the pro-apoptotic Bcl-2 proteins by phosphorylation [7-9]. JNK may positively regulate apoptotic pathway phosphorylating anti-apoptotic Bcl-2 proteins which results with their inhibition [10]. p38 protein, which is mostly known as death inducing protein in the case of stress, can target the Bcl-xL and the activity of p53 [11].

The drug should also overcome the multidrug (mdr) resistance which colon cancer cells can exhibit. Upon the activation of *MDR1* gene, colon cancer cells enable to efflux the drug into extracellular field [12]. In addition, there may not be a unique population of colon cancer cell line, since genetic changes in the pathway of APC-Ras-p53 leads to microsatellite and chromosomal instability resulting with mutation accumulation in DNA mismatch repair genes. The genetic instability causes various gene expression profile between the individual cells that the response of each cancer cell becomes different against an anti-cancer drug which make the treatment of colon cancer more difficult. So, we need to find new and effective chemotherapeutic drugs for the treatment of patients with colon cancer.

1.1. The Aim of the Study

There are two aims of the study;

1. identification of the chemotherapeutic potential of PMC-A and PMC-F drugs for the treatment of colon cancer.

2. elucidation of the key signaling molecules activated in PMC-F-induced apoptosis in HCT116 cell lines.

Bax is a significant pro-apoptotic protein for the activation of mitochondrial apoptotic pathway. The aim of using Bax-/- cells is to obtain apoptotic deficient cells. In the Bax-/- cells, the mitochondrial apoptotic pathway is inhibited partially, due to Bax deficiency. So, we aimed to see the involvement of mitochondrial apoptotic pathway in

PMC-F induced cell death mechanism and the potential of the drug to induce apoptosis in the Bax-/- cells as well as to be a candidate of chemotherapeutic drug for the treatment of colon cancer. Additionally, the different responses of the WT and Bax-/- cells to the drug would serve for the determination of the key signaling molecules in PMC-F induced death in HCT116 colon carcinoma cell lines.

2. BACKGROUND

2.1. Cancer

2.1.1. Colon Cancer

Colorectal cancer which is also called as colon cancer covers the area of both colon and rectum in the body which are the units of digestive system or gastrointestinal. Colon is the part of large intestine in which water and minerals are absorbed and rectum is the place where wastes are transferred to be expelled thorough anus [3].

2.1.1.1. Common Mutations in Colon Cancer

Colorectal cancer has three types of form; sporadic, familial and inherited. In sporadic form, there is no genetically inherited mutation which makes the person to be susceptible to develop cancer. It results from the accumulation of somatic mutations in the cell [13]. Familial colon cancer is not an inherited form; however it appears due to polymorphisms. On the other hand, less than 10% patients with colon cancer have an inherited tendency for this disease. There are 2 types of inherited colon cancer including polyposis syndrome and non-polyposis syndrome. The polyposis syndrome includes familial adenomatous polyposis (FAP) and hamartomatous polyposis. The non-polyposis syndrome is also subdivided into hereditary nonpolyposis colorectal cancer (HNPCC) & the cancer family syndrome (Lynch syndrome II) [14].

Mutations on gatekeeper and caretaker genes are required for the progressing of colon neoplasia. Gate keeper genes are related with cell proliferation and death mechanisms [15]. The most known gate keeper gene in colorectal cancer is Adenomatosis polyposis coli (APC) which affects constant cell proliferation. APC mutation and less common β-catenin mutation initiate neoplastic formation [16, 17].

Caretaker genes are mismatch DNA-repair genes (MMR) covering *MSH2* and *MLH1* which provide the genome stability. They are also required for chromosome metabolism, spindle assembly and dynamics, cell-cycle regulation and mitotic check point regulation. Dysfunction of these genes increases the probability of mutation on other genes and genomic instability [17, 18]. Additional mutations on proto-oncogenes and tumor suppressor genes constitute the inductive pathways resulting in colon cancer. The estimated order and the involvement of mutated genes for colon cell progression from adenoma to carcinoma are depicted in Figure 2.1 [15].

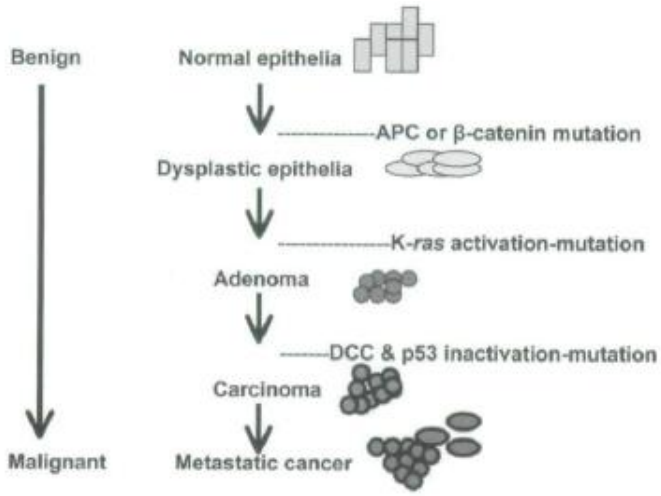


Figure 2. 1: The genetic model for the progress of colon cancer. Most of the early development of colorectal cancer is based on the mutations related with APC pathway [15].

Large adenomas located in the colon comprise the mutations in the RAS/RAF pathway. Along with the next step mutations in p53 pathway and many other pathways cause the tumor progression through malignancy and metastasis [19]. During the progress of cancer depending on APC-Ras-p53 pathway, microsatellite instability (MIN) and chromosome instability (CIN) which exists in nearly 60-80% of colon cancer are somehow triggered because of the deficiency in MMR genes [15].

Mutation Type	Genes Involved	Types of disease caused
Germline	APC MMR Oncogenes (*myc, ras, src, erbB2*)	FAP HNPCC
Somatic	Tumor suppressor genes (*p53, DCC, APC*) MMR genes (*hMSH2, hMLH1, hPMS1, hPMS2, hMSH6, hMSH3*)	Sporadic disease
Genetic polymorphism	APC	Familial disease

Figure 2. 2: Gene mutations involved for the occurrence of different types of colon cancer [14].

2.1.1.2. Therapeutic Approaches for Colon Cancer

During the treatment of colon cancer, mostly the molecules related with cell surface growth factor receptors, proliferation signaling, cell cycling, apoptosis, angiogenesis and matrix metalloproteinases are targeted.

2.1.1.2.1. Chemotherapy Strategies

The cancer vaccines aim to trigger the activation of tumor specific T-cells against providing an antitumor immune environment [20]. The way for cancer cells to avoid from immune recognition is the down-regulation of antigen expression [21]. However, there are some tumor-specific antigens or tumor associated antigens expressed in tumor cells that can be targeted by $CD8^+$ T-cells. In order to prevent the metastasis of cancer cells, the molecules which are expressed in cancer cells to enable them epithelial-to-mesenchymal transition (EMT) are also targeted with anti-cancer vaccines. For example, the stable silencing of Brachury transcription factor which is highly expressed druing EMT process has been shown to eliminate invasion of cancer cells [22]. Another strategy of cancer vaccines is to enhance the activation of tumor specific T-cells. In

order to achieve this, tumor antigen and costimulatory factors are given together in the vaccine [20].

Epidermal growth factor receptor (EGFR), which is one of HER-family tyrosine kinase, is related with proliferation. This signaling pathway affects on Ras/Raf/MEK/ERK, PI3K/AKT survival pathways, production of vascular endothelial growth factor (VEGF) involved for metastasis of cancer cells (Figure 2.3) [23, 24]. VEGF is one of the cytokines which has an inhibitory effect for anti-tumor T cell responses as well as contributing to angiogenesis. Thus, the vaccines including anti-VEGF antibodies can be used to prevent metastasis of cancer cells as well as inducing the efficiency of dendritic cells (DCs) pulsed with peptide [25]. Bevacizumab, cetuximab and panitumumab can be listed as the monoclonal antibodies targeting proliferation of cancer cells. While bevacizumab inhibits the VEGF derived angiogenesis pathway, cetuximab and panitumumab target the EGF pathway. Addition of bevacizumab into the combination of irinotecan, 5-FU and LV (IFL) led to higher survival in both Phase II and III of cancer [26]. 5-FU has been also shown that the drug initiates the expression of ICAM-1 and Fas in cancer cells which subsequently enhances the activation of tumor specific T-cells to kill the cells [27].

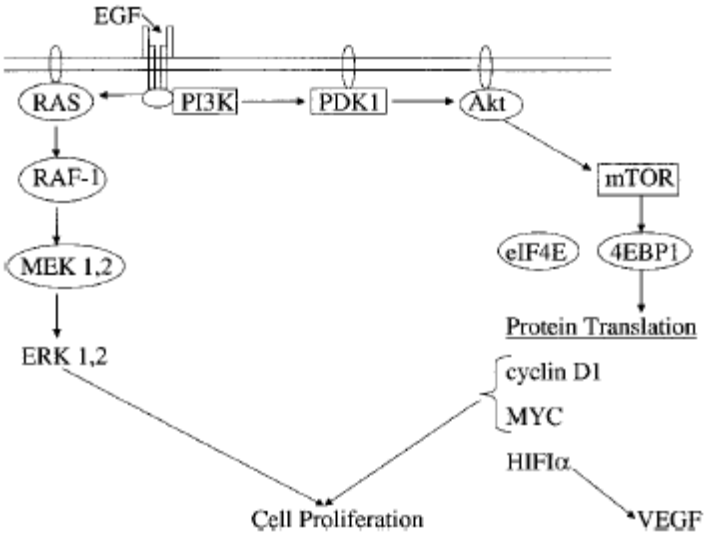


Figure 2. 3: EGFR signaling pathways [24].

K-ras gene encoding Ras protein includes missense mutation in 50% of colorectal cancers rendering the gene constitutive active [28]. Farnesyl transferase inhibitors (FTIs) such as R1157777 (tipifarnib) and SCH66336 (lonafarnib) obstructing the post translational farnesylation of Ras are needed to be investigated for its benefit when applied with chemotherapy in Phase II/III [29].

Another approach as a therapeutic way in colon cancer is apoptosis. There is an increased level of survivin (one of IAPs) and Bcl-2 in colon cancer cells. Antisense Bcl-2 constructs and drugs targeting Bcl-2 family members are being improved to be applied with the aim of facilitating apoptosis in colon cancers [30].

Additionally, cell cycle regulation is mostly defective in cancer cells which enable them to proliferate continuously. As a defective mechanism, cyclin D1 is required in the progress through G1 stage is overexpressed in nearly one-third of colorectal cancers [31]. There is also increased activity of cdk2 which is the kinase of cyclin E in nearly all colorectal carcinoma. There are some agents such as flavopiridol, UCN-01 (7-hydroxystaurosporine) targeting cdks [32]. Other potential targets can be classified as cylooxygenase (COX)-2, mammalian target of rapamycin (mTOR), proteasome and heat shock protein 90 (HSP-90) [24].

2.1.1.2.2. Gene Therapy Strategies

Another approach to prevent cancer progression is gene therapy. In spite of the presence of various genetic mutations and clonal heterogeneity in colon cancer, some mutations such as p53 and K-ras which are significant for malignant transformation can be targeted [33]. For example, injection of adenovirus encoding wild-type p53 into subcutaneous tumor concluded with tumor regression in mice. Due to its high activity of K-ras protein, K-ras oncogene is silenced with anti-sense mRNA in colon cancer cells. The inhibition of K-ras restricted cell growth, colony formation in Phase I trial lung cancer patients without affecting normal cells and colon cancer cells with K-ras mutation [34].

Colon cancer may escape from elimination by CD8+ because of the deficiency of human leukocyte antigen (HLA) presentation and also gut is the place where cytokine

response is reduced inhibiting Th1 cell activation. So, one of the aspects of gene therapy is the transduction of colon cancers with adenovirus encoding a cytokine which helps the activation of CD8+ or NK cells against these cancer cells. Another approach in gene therapy of colon cancer is virus-directed enzyme prodrug therapy (VDEPT). The aim of this method is enabling cancer cells to express a viral or bacterial enzyme which converts a pro-drug into an active metabolite [33].

2.1.1.2.3. Neoadjuvant Therapy

Neoadjuvant therapy is the application of chemotherapy or/and radiotherapy before surgery. The survival rate of patients exposed to neoadjuvant radiotherapy is more than the ones operated only [35]. In another study, in which German Rectal Cancer Study combined radiotherapy and chemotherapy with 5-FU in phase II and III rectal cancer before surgery, the recurrence rate of the cancer was lower than the non-treated patients. There are still current tests considering neoadjuvant therapy depending on various dose, frequency and duration of the therapy in the way of improving the survival rate and impeding the recurrence rate of local cancer [36].

2.2. Cell Death

Cell death is an essential cellular response which plays a crucial role during embryogenesis, shaping our bodies, morphogenesis, in regulating the homeostasis, deletion of damaged and dangerous cells [37-40]. There are various cell death types up to 12 different modalities which are defined by the Nomenclature Committee on Cell Death (NCDD). These types are classified depending on morphological appearance, enzymological criteria, functional aspects (programmed or accidental, physiological or pathological) or immunological characteristics [41].

2.2.1. Apoptosis

After the introduction of programmed cell death (PCD) in 1964, PCD was identified as apoptosis firstly in *Caenorhabitis elegans* (*C. Elegans*) in 1990s. However, apoptosis is known as one of the programmed cell death mechanisms in recent literature. Apoptotic cell death is characterized by cell shrinking following the cleavage

of lamins and actin filaments in the cytoskeleton, breakdown of chromatin leading to nuclear condensation, membrane blebbing and formation of apoptotic bodies containing organelles, cytosol and nuclear fragments to be phagocytosised by macrophages [42]. In addition to these morphological characteristics, apoptosis can be defined with biochemical analysis such as DNA fragmentation, phosphatidylserine exposure, loss of mitochondrial permeability and caspase activation.

The factors contributing to PCD were elucidated in *C.elegans* and studies done for the analysis of apoptosis in mammalian cells led to the identification of proteins which have homologues in this organism [43]. The apoptotic machinery of *C.elegans* covers the proteins as egl-1 (pro-apoptotic BH3-only member of Bcl-2 protein family), CED-9, CED-4 and CED-3. Normally, anti-apoptotic CED-9 inhibits the adaptor protein CED-4 which activates the Cys protease CED-3 causing the cell death [44]. So, activation of apoptotic pathway in *C.elegans* starts with the transcriptional up-regulation of egl-1 which inhibits CED-9 [45].

In mammalians, the mechanism of apoptosis becomes more complex by the addition of multiple family members in each class of the apoptotic regulators. These regulators can be exemplified as caspases, Bcl-2 family and Apaf-1, NLR and PIDD adaptors (Figure 2.4) [46].

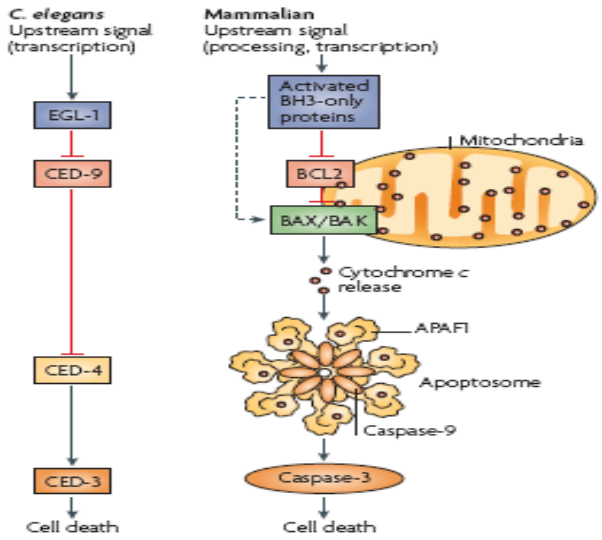


Figure 2. 4: Evolutionary comparison of apoptotic machinery between *C.elegans* and mammalian cells [46].

2.2.1.1. Two Main Apoptotic Pathways: Extrinsic and Intrinsic Mitochondrial Apoptotic Pathways

There are two pathways overlapping in animal cells during apoptosis; extrinsic and intrinsic mitochondrial apoptotic pathways. Extrinsic apoptosis is mediated by death receptors found on the cell surface and involves the direct activation of initiator caspases followed by the activation of effector caspases [42]. Death receptors are derived from tumor necrosis factor receptor (TNFR) superfamily comprising TNFR-1, Fas/CD95 and TNF-related apoptosis-inducing ligand (TRAIL) receptors including DR4 and DR5. Upon the binding of ligands such as TNF-α, Fas ligand (FasL), TRAIL to their own receptors, adaptor molecules such as Fas-associated via death domain (FADD), TNFR1-associated DEATH domain protein (TRADD) bind to the death domain (DD) of the receptors by their DDs. FADD adaptor protein also includes death effector domain (DED) which recruits pro-caspase 8 with DED-DED homotypic interaction. This complex is called death-inducing signaling complex (DISC). Autocatalytic cleavage of recruited pro-caspase 8 leads to the formation of active caspase 8 which subsequently processes the activation of effector caspases-3, -6 and -7 [47, 48]. The activation of caspase-8 is enough for cell death and the extrinsic pathway is not prevented by over-expression of Bcl-2 family or by using inhibitor of caspase-9 [49]. In the case of weak signal, extrinsic pathway also uses the intrinsic mitochondria-dependent pathway amplifying the apoptotic signal [5].

Intrinsic pathway which can be activated by receptor-independent stimuli including DNA damaging agents, UV, γ-irradiation, hypoxia, lack of growth factors [50-52]. The connection of this pathway with extrinsic pathway is provided by the cleavage of Bid into truncated Bid (tBid) by caspase-8. Subsequently, tBid translocates to the mitochondria and induce the oligomerization of Bax/Bak causing permeability transition (PT) pores. After the opening of PT pores, solutes with molecular mass of up to 1500Da nonselectively pass through the mitochondrial inner membrane leading to mitochondrial depolarization, swelling, ATP depletion and cell death. These solutes include cytochrome c, apoptosis inducing factor (AIF), endonuclease endo G,

Smac/DIABLO (direct IAP [inhibitor of apoptosis protein]-binding protein with low pI) and Omi/HtrA2. These released proteins may also mediate caspase-independent cell death. Released cytochrome c makes a complex with Apaf-1 forming apoptosome complex with heptameric form. Next, monomeric caspase-9 proteins bind to this complex to make dimmers followed by autoproteolytic cleavage. The activation of caspase-9 leads to the activation of caspase-3, -6 and -7 through their cleavage subsequently.

Poly (ADP-ribose) polymerase (PARP-1) functions in DNA recombination and repair in the presence of DNA strand breaks. This protein is cleaved by caspase-3 and -7 *in vivo* during apoptosis. This cleavage results with 89 kDa C-terminal fragment and 24 kDa N-terminal fragment. This cleavage makes the protein inactive in response to DNA damage. In addition to apoptosis, PARP-1 cleavage can also be observed in non-apoptotic cells [53].

2.2.1.2. Regulators of Apoptosis: Bcl-2 Family Proteins

Bcl-2 family member proteins which are involved in the apoptotic pathway are the main regulators of apoptotic mechanism in mammalian cells. They can be classified as multidomain anti-apoptotic (Bcl-2, Bcl-xL, Mcl-1, Bcl-w, A1/Bfl-1, Boo/Diva and NR-13), multi-domain pro-apoptotic (Bax, Bak and Bok/Mtd) and BH3-only members of Bcl-2 family (Bid, Bim, Bad, Noxa, Puma, Bik, Blk, Hrk, BNIP3, Nix and BMF) proteins according to their structural and functional properties (Figure 2.5) [5].

Anti-apoptotic Bcl-2 proteins are essential for cell survival and protecting the cell from death against apoptotic stimuli. They reside on the cytoplasmic side of nuclear membrane, endoplasmic reticulum and the outer mitochondrial membrane [42]. The function of Bcl-2 proteins to protect cell death is assumed by interacting with pro-apoptotic Bcl-2 proteins and preventing the translocation of Bax and Bak to mitochondria and oligomerization of them in the outer membrane of mitochondria. The structure of Bcl-xL and Bak suggests that there is a functional interaction between them [6, 54-56].

The activity of Bcl-2 may be regulated with post-translational modifications and cleavage. As the quantity and specificity of phosphorylation on Bcl-2 affects the function of the protein, the N-terminal (BH4 domain) cleavage of Bcl-2/Bcl-xL in a caspase dependent manner converts them into pro-apoptotic ones [57].

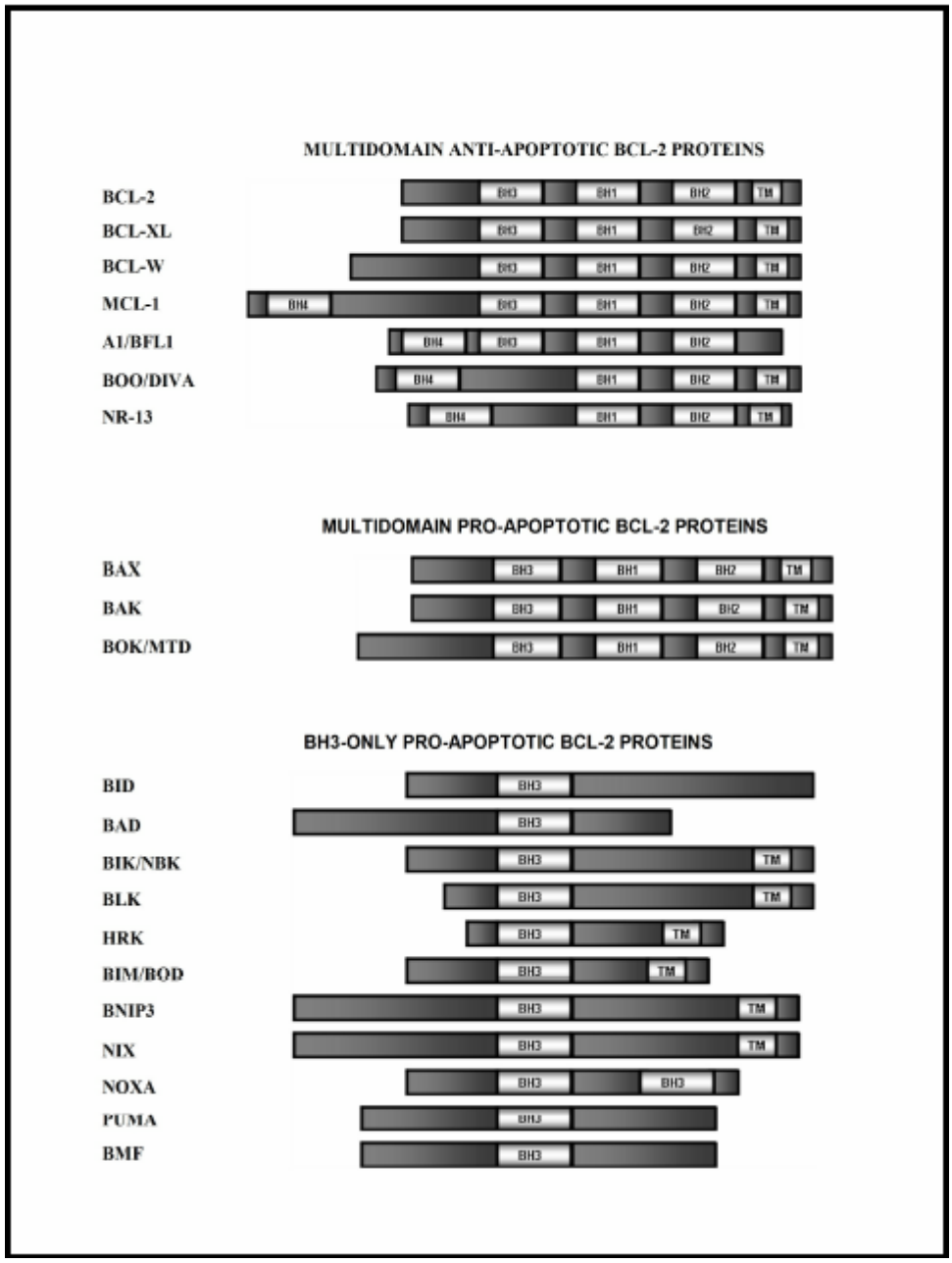


Figure 2. 5: Bcl-2 protein family. The domains of Bcl-2 family family proteins are shown. Most of them include the transmembrane protein (TM) that provides them to anchor intracellular membranes of mitochondria, nucleus or endoplasmic reticulum (ER) [58].

Pro-apoptotic Bcl-2 proteins are the adaptor molecules such as Bax and Bak being the mediators of mitochondrial apoptotic pathway [59]. Bax resides mainly in the cytosol or localized loosely on the outer membrane of mitochondria [60]. On the other hand, Bak is found as an integral protein of the outer membrane of mitochondria [61]. In the case of apoptotic stimuli, both Bax and Bak go into conformational change and they form dimers, oligomers or high-order multimers in the outer membrane of mitochondria leading to permeability of the membrane (Figure 2.6) [62].

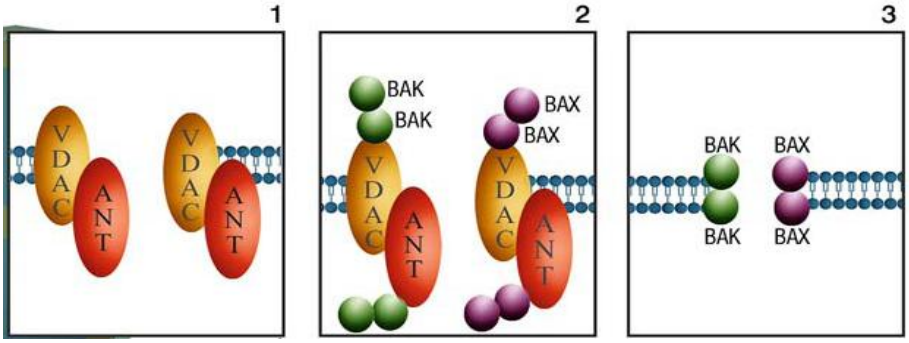


Figure 2. 6: Possible ways for Bax and Bak oligomerization in the membrane of mitıchondria. They may interact with voltage dependent anion channels inside the inner membrane of mitochondria or they may generate oligomers between each other [5].

BH3-only members of Bcl-2 family trigger the initiation of mitochondria-dependent apoptotic pathway by sensitizers and direct activators either inhibiting the activity of Bcl-2/Bcl-xL interacting with them or activating Bax and Bak respectively. Noxa as a sensitizer interacts with Bcl-2, Bcl-xL and Mcl-1 and Puma as an activator protein affects the conformational change and oligomerization of Bax and Bak. In fact, there are 2 models about how BH3-only proteins act on oligomerization of Bax and Bak; direct activation model and indirect activation model (Figure 2.7) [58].

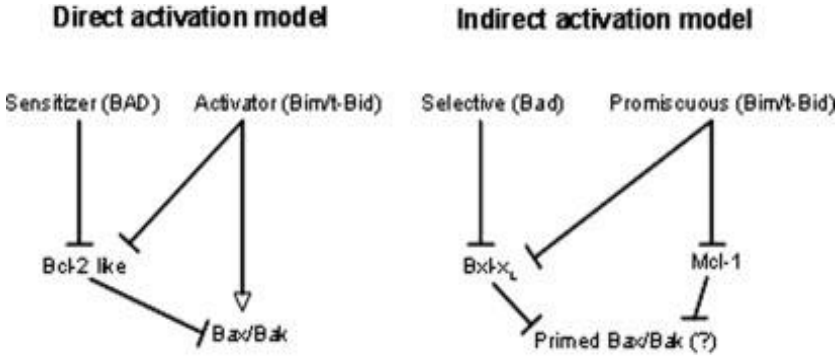


Figure 2. 7: Activation models for Bax and Bak proteins. In direct activation model, BH3-only proteins activate Bax/Bak directly, as the sensitizer neutralizes Bcl-2 like proteins. In indirect activation model, sensitizer prevents the activity of Bcl-xL, as the other BH3-only proteins act on the other Bcl-2 like proteins to induce the activation of Bax/Bak **[58].**

The activity of BH3-only proteins is regulated by phosphorylation, transcriptional control and cleavage. Depending on the specificity of Bad phosphorylation its activity can be regulated. While survival signals sequesters Bad by phosphorylating it, JNK-mediated phosphorylation of Bad and Bim results with their activity [63-66]. In the case of DNA damage, p53 transcription factor is usually expressed. p53 promotes the transcription of several pro-apoptotic proteins such as Bax, Puma, Noxa, Apaf-1, Fas and DR5 in addition to p21. Additionally, it contributes to the suppression of anti-apoptotic Bcl-2 and Bcl-xL proteins.

2.2.1.2.1. Bax: A Pro-apoptotic Bcl-2 Family Protein

Induction of apoptosis as a programmed cell death is the fundamental approach for the treatment of cancer. Bcl-2 family member proteins play significant roles in the regulation of mitochondrial apoptotic pathway. Among the pro-apoptotic Bcl-2 family proteins, Bax and Bak positively affect the increase of the mitochondrial membrane permeability and the release of cytochrome c to the cytosol from the mitochondria [67]. When Bax protein is impaired, the activation of mitochondrial apoptotic pathway is either partially or completely inhibited. The level of the inhibition changes depending on the type of the cell and death inducing agents.

For example, a study in which deoxycholic acid (DCA) was used as a drug for the treatment of HCT116 cell lines showed that (DCA) could trigger the releases of cytochrome c from the mitochondria into the cytoplasm and activation of caspase-3, subsequently. This situation indicated that DCA –induced mitochondrial pathway was Bax-independent. But, the drug triggered apoptosis faster in HCT116 Bax-/- cells than Bax+/- cells, surprisingly [68]. In another study, hepatocyte cells with Bax deficiency treated with an agent, TNF- α also presented similar result that the cytochrome c release and the following caspase activities were initiated by the drug [69].

Although, Bax protein is known as a pro-apoptotic protein, its function needs to be clarified more in drug-induced apoptosis in various cell lines [65]. In addition, the possible inhibition of mitochondrial apoptotic pathway in Bax-/- cells can help to identify the role of this signaling pathway in drug treatments. Thus, new approaches can be developed for cancer treatments by targeting the key molecules which may sensitize the cancer cells to death. The activity patterns of signaling pathways can also give knowledge about the potential of the chemotherapeutic drugs.

2.2.1.3. Caspase Family Proteins

Caspases are cysteine aspartases and the essential activators of apoptotic process [70]. They function as transmitting and amplifying the death signals, causing drastic morphological changes by proteolyzing many key substrates such as structural proteins, gelsolin, p21-activated kinase, PAK2 and focal adhesion kinase [71-74].

There are 3 major classes of caspases; initiator caspases such as caspase-2, -4, -8, -9, -10 and -12, effector caspases including caspase-3, -6, and -7 and inflammatory caspases such as caspase-1, -5 and -11 [46]. In healthy cells, caspases are found inactively in the form of zymogens including a prodomain, two catalytically active sites that are separated by a linker domain [75, 76]. For caspases to be activated the pro-domain of the protein is removed and the linker domain is cleaved resulting with the active form of caspase with both a large subunit and a small subunit [76].

The zymogens of initiator caspases like caspase-2, -8, -9 and -10 exist as monomeric and they need to dimerize for activation. In fact, it is not known exactly, whether proteolytic cleavage is necessary for their activation since dimerization may be enough to provide a conformational change [77, 78]. Caspase-9 is activated binding to apoptosome followed by the dimerization and autocleavage of caspase-9 subsequently. After the cleavage of caspase-9 via apoptosome, 2 subunits exist; p35 and p12. P12 is assumed to be inhibited by XIAP (X-linked inhibitor of apoptosis) due to its structural similarity with SMAC (second mitochondria-derived activator of caspase) which binds to XIAP and inactivates it and allowing the cleavage of caspase-3 via the activated caspase-9. XIAP inactivates caspase-9 by binding to it. Unlike initiator caspases, effector caspases are found as inactive dimers in the cytosol and they are cleaved to become activated [79].

2.3. MAPK Signaling Pathways

Mitogen activated protein kinase (MAPK) signaling pathway is involved to handle divergent stimulus inside the cell. This cascade can be activated by various receptors such as receptor tyrosine kinases, G-protein-linked receptors and cytokine receptors. The stimulus taking role for this activity can be classified as extracellular signal proteins such as hormones and growth factors [platelet derived growth factor (PDGF), epidermal growth factor (EGF) and fibroblast growth factor (FGF), e.g.], inflammatory cytokines of tumor necrosis factor (TNF) and environmental stresses such as radiation, osmotic shock and ischemic injury [80].

The main MAP kinases are listed as ERK (extracellular signal regulated protein kinases), JNK (c-Jun N-terminal kinase) and p38. In the first step of cascade, Serine/Threonine MAP3Ks are activated. In the following, tyrosine (Tyr) and threonine (Thr) MAPKKs and Ser/Thr MAPKs are phosphorylated in turn. The proteins contributing to this pathway, the transcription factors which are activated by MAP kinases and the possible outcomes of each cascade are shown in Figure 2.8 [80].

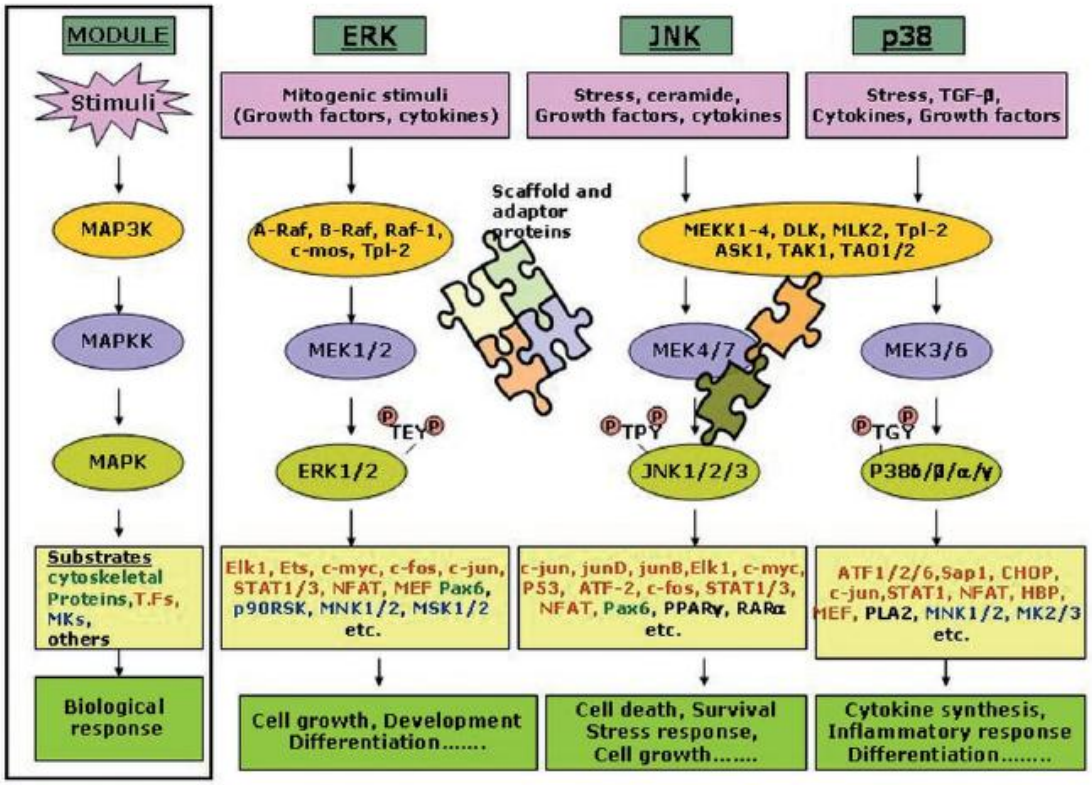


Figure 2. 8: MAP kinase signaling pathways. There are at least 5 parallel MAPK modules directed by 12 MAPKs, 7 MAPKKs and 7 MAPKKKs in mammals. The precise combination of these kinases is provided by scaffold proteins [80].

It is almost difficult to attribute certain roles for MAP kinases. ERK pathway is generally activated by mitogens and utilized in cancer cell proliferation, while JNK and p38 MAP kinases participate in stress signaling pathways, although their functions are still ambiguous [80-83]. Since, the functional outcomes of these MAP kinases change depending on the type of the cells, strength, duration and type of the stimulus.

There are two isoforms of ERK including ERK 1 and ERK 2. ERK 1/2 pathway is generally activated by receptor tyrosine kinases and G-protein coupled receptors (GPCRs). Dual phosphorylated ERK 1/2 by MAPKKs translocates into the nucleus resulting with the activation of transcription factors. Also, it can remain in the cytosol or pass to multiple cellular compartments and phosphorylates many different cytosolic and membrane proteins and cytoskeletal proteins [84]. The phosphorylated transcription factors covering c-myc, c-fos, SRF, CREB and AP-1 play significant role in cell proliferation [80]. ERK activation is also required in angiogenesis, cell migration,

invasion and metastasis of cancer cells [85, 86]. Colon carcinoma cells were shown to have less apoptosis rate in the presence of high ERK activity and initiate cell cycle arrest with CDK inhibitors p21 and p27. It prevents the caspase activation and also set Bad protein apart from mitochondria by phosphorylating it [7-9].

JNK has three isoforms; JNK 1, JNK 2 and JNK 3. JNKs are one of stress activated protein kinases and are mainly activated by cytokines, UV irradiation, growth factor deprivation, anticancer drugs and DNA damaging agents. On the other hand, GPCRs, serum and growth factors may participate in less JNK activation [87-89]. Bcl-2 family proteins (Bcl-2, Bcl-xL, Bad, Bim and Bax) are also among the targets of JNK. For instance, Bcl-2 and Bcl-xL can be obstructed once they are phosphorylated by JNK [10]. In contrary, JNK may contribute to cell survival in a positive way. In a study, a crosstalk between ERK and JNK in which ERK phosphorylates JNK resulting with enhancement of cell proliferation in response to VEGF was reported [90].

p38 includes p38α, β, γ and δ. Upon the activation of p38, it may remain in the cytosol or localize in the nucleus. In addition to the phosphorylation of several cellular targets in the cytosol, it also phosphorylates transcription factors including p53, NFAT, Elk1, e.g [91]. p38 may take part in various cellular responses such as apoptosis, differentiation, survival, proliferation, development, inflammation and other stress responses [92]. There are so many studies suggesting a tumor suppressor function of p38α mediating the cell cycle arrest and inducing apoptosis. Depending on the type of the stress stimuli, apoptosis may involve p38α activation [93]. Reactive oxygen species (ROS) as an apoptotic stimulus can sometimes induce activity of p38α. The pro-apoptotic mechanism of p38 was shown in a study that it triggered apoptosis in endothelial cells by down-regulating Bcl-xL and up-regulating p53 in endothelial cells [11].

2.4. ROS Signaling

Reactive oxygen species (ROS) are derivatives of oxygen which are formed by the reduction of oxygen molecules into various degrees and it covers the molecules of superoxide (O_2^-), H_2O_2 and hydroxyl radical ($OH^\cdot$) [94, 95]. Mitochondria play an important role for the production of ROS in most mammalian cells [96]. The source of ROS comes from the superoxide (O_2^-) produced in mitochondrial matrix. The reduction of O_2 to O_2^- is indeed thermodynamically favorable in mitochondria [97, 98].

2.4.1. ROS Production

There are some extracellular signals contributing to ROS production. These factors can be exemplified as hormones, inflammatory cytokines, interferons and growth factors [99]. Increase in Ca^{+2} level can activate Ca^{+2} dependent proteases which cleaves xanthine dehydrogenase enzyme to be able to produce O_2^- [100]. Cytokines may induce mitochondria dependent ROS production via ceramide signaling by the activation of ceramide producing sphingomyelinase and other membrane-associated enzymes which take place in phospholipid metabolism.

2.4.2. The Effect of ROS in Protein Levels

In addition to the role of mitochondria as being the central organ mediator of intrinsic apoptotic pathway, it has also critical role in cellular metabolism and redox balance. The recent studies have shown that kinases can transfer to mitochondria [101, 102]. Thus, they can affect the phosphorylation of respiratory chain proteins and release of mitochondrial components into cytosol. In this sense, there is a bilateral and sensitive relationship between mitochondria and kinases including MAP kinases, Akt/protein kinase B, protein kinase C and protein kinase A [103, 104].

ROS level or H_2O_2 concentration plays an important role in the regulation of MAP kinase activation. Different concentration levels of H_2O_2 produced in mitochondria may determine the activity level and subcellular compartmentalization of

each MAP kinase individually. The impact mechanism of H_2O_2 on kinase is that catalytic cysteine residue found in the active side of the protein is oxidized to sulfenic acid (Cys-SOH) intermediate which regulates the interaction of MAP kinase with their upstream regulators, MAPKKs. For instance, low H_2O_2 concentration stimulates the activity of ERK, whereas JNK and p38 MAP kinases become activated at high H_2O_2 concentrations (Figure 2.9) [81].

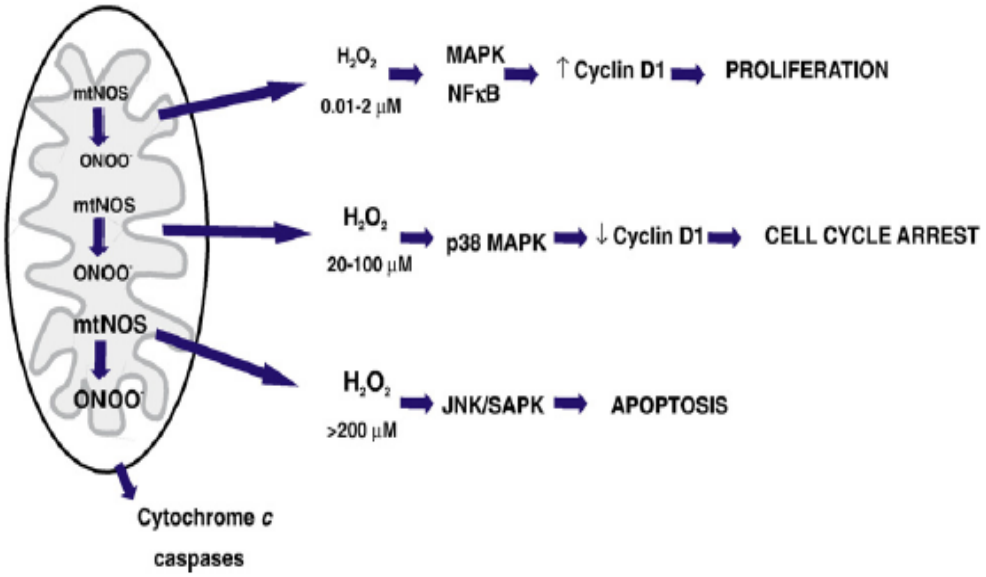


Figure 2. 9: Different H_2O_2 concentration levels produced by mitochondria affect the activation of MAP kinases and the cellular response.

In addition to the phosphorylation of MAP kinases, redox can also switch the localization of them. In a study done with GFP-hERK2 showed that low amount of H_2O_2 leaded the kinase into the nucleus and triggered proliferation. In contrast, GFP-hJNK1 translocated into nucleus at high level of hydrogen peroxide resulting with cell cycle arrest and apoptosis [102]. However, high level of hydrogen peroxide changed cysteine residue of ERK into $–SO_2H$ and $–SO_3H$ resulting with the inhibition of ERK phosphorylation. Thus, unphosphorylated ERK can't translocate into nucleus and induce cellular proliferation (Figure 2.10) [81].

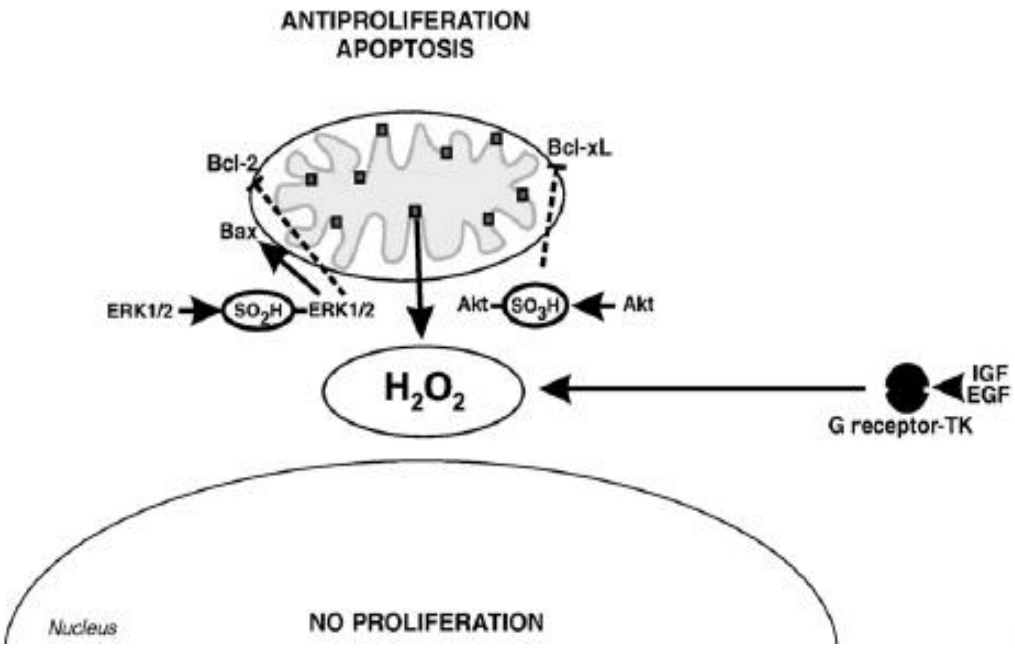


Figure 2. 10: High production of H_2O_2 hinders the activation of both ERK1/2 and Akt kinases[78].

There are also some studies demonstrating the presence of ERK, p38 and JNK in mitochondria. ERK localization in mitochondria contributes to sustain mitochondrial membrane potential, sequester cytochrome c release and deactivate Bad protein [105, 106]. On the other hand, Bax is initiated to be translocated from cytosol to mitochondria by p38 in order to start apoptosis. JNK initiates apoptosis by preventing the activity of Bcl-2 and Bcl-xL and enhancing the release of cytochrome c from mitochondria [107].

2.5. p53 Signaling

p53 is a tumor suppressor protein being responsible for genomic integrity upon DNA damage which is activated mainly in response to different types of stress contributing to apoptosis, autophagy, cell cycle arrest, senescence, DNA repair and cell metabolism. The activity of such an important protein requires different regulatory levels including p53 stabilization, DNA binding and transcriptional activation. Although there are several post-translational modifications acted on p53 such as phopshorylation of p53 by ATM/ ATR/ DNA-PK and Chk1/Chk2 which is the classical regulatory mode for p53 stabilization preventing the degradation of it by mdm2 dependent ubiquitination

and enhancing the DNA binding of p53 [108]. According to the type of post-translational modification on p53, the transcription of target genes are manipulated. For instance, partial acetylation of p53 induces the transcription of p21, while full acetylation of p53 is involved for transcription of pro-apoptotic genes such as Bax, Puma, Noxa and Fas [108, 109].

Indeed, the translocation of p53 to mitochondria is another aspect of p53 activation except of nuclear translocation. In the case of all stress stimuli, p53 translocates to mitochondria and trigger the ROS production by inhibiting MnSOD (manganase superoxide dismutase) resulting with apoptosis [110]. The place of oxidative stress is also an important factor for the determination of p53 activity. For instance, as some chemotherapeutic drugs cause excessive ROS generation in mitochondria leading to apoptosis without transactivation of p53, p53 initiates the transcription of genes related with DNA-repair in the presence of oxidative stress in the nucleus. ROS may also affect the DNA binding ability of p53 by attacking the cysteine residues found in DNA-binding domain of p53.

2.6. Pramanicin and Its Analogous

Pramanicin is a strong antifungal agent which is obtained from fungal fermentation and biosynthesized. It includes a highly functionalized polar head group and a simple, long aliphatic side chain [111]. Analogous of pramanicin are also available having some little differences in their structure (Figure 2.11).

The effect of pramanicin which has an anti-growth impact on fungal organisms with minimal inhibitory concentrations was examined on dog carotid artery. Another study showed that the same concentration of pramanicin as in the previous study caused progressive elevation of cytosolic Ca^{+2} mostly seen in the peripheral regions of endothelium cells. Additionally, in the medium lack of Ca^{+2}, there is a transient elevation cytosolic Ca^{+2} which may indicate that pramanicin might be inhibiting sarcoplasmic reticulum (SR) Ca^{+2}-ATPase pump, thus triggering the increase of cytosolic Ca^{+2} [111, 112].

Since pramanicin has a hydrophobic fatty acid side chain, it is possible that it may integrate into the lipid bilayer of plasma membrane leading to increase of membrane permeability to ions such as Ca^{+2} which accounts for nitric oxide (NO) synthase activation [111]. In addition to the possible importance of hydrophobic fatty acid side chain, epoxy group in the aliphatic side chain of pramanicin might also be significant for the function of the molecule. Pramanicin A which is an analogue of pramancin which is lack of epoxyl group, induced relaxation effect and cell death in dog vascular endothelial cells following prolonged incubation. However, the relaxation effect of PMC-A was lower than PMC. This study showed that the epoxyl group is not essential for the function of pramanicin [113].

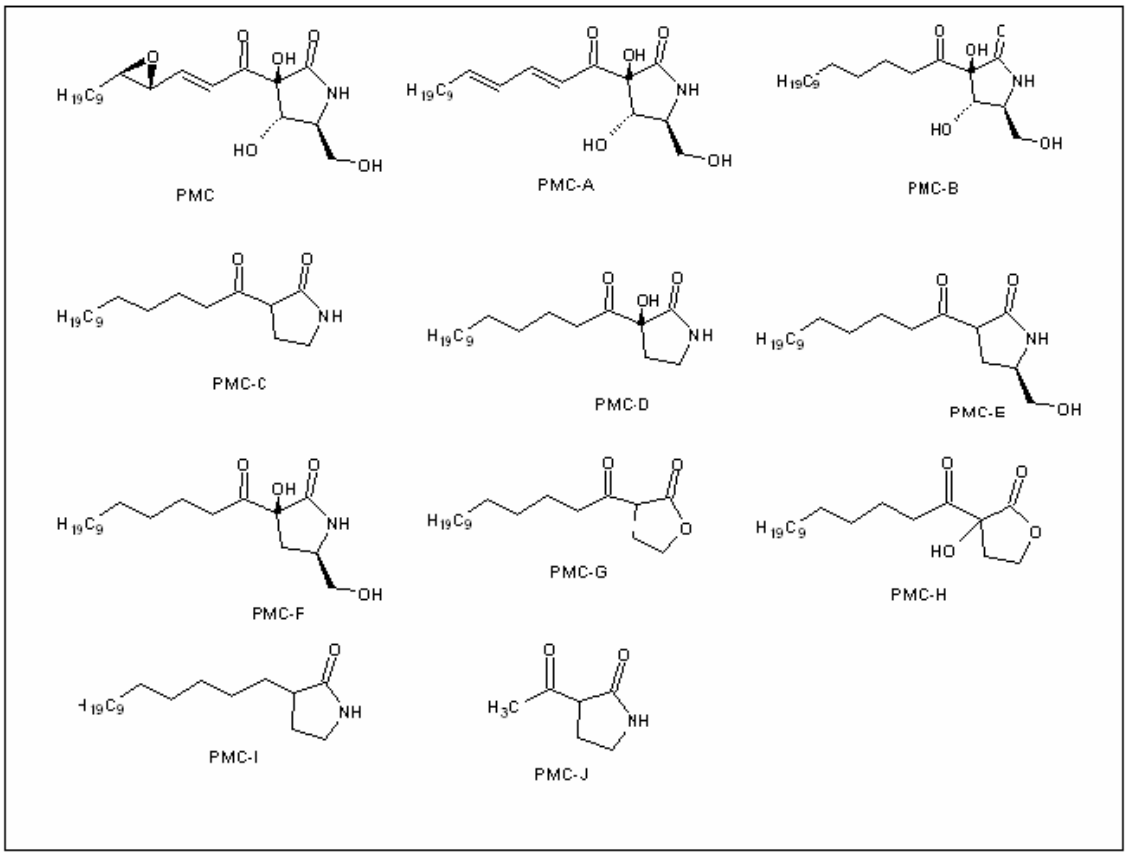


Figure 2. 11: The chemical structure of pramanicin and its analogues. Pramanicin has 11 analogues listed form –A to –J. These derivatives show differences in their aliphatic and polar head group structure [114].

The cell death effect of pramanicin was observed by plasmalemmal blebs formation as a morphological change on endothelial cells. The prolonged exposure of

the cells to PMC-A showed the similar effect with some floating cells, representing numerous blebs, heavily stained nuclei and large intracellular vacuoles [112].

The cell death mechanism which pramanicin induces was elucidated by the study done on Jurkat leukemia cells. It was shown that pramanicin activated the apoptotic pathway by the processing of caspase-9 cleavage and cytochrome c release from mitochondria. Moreover, MAP kinases were demonstrated to have been activated. JNK and p38 phosphorylation were determined after pramanicin treatment. Additionally, pramanicin induced the phosphorylation of ERK1/2 in dose dependent manner. The apoptotic effect of JNK and p38 were confirmed using inhibitors against them that resulted with the decrease in cell death and the inhibition of these MAP kinases also abrogated the cytochrome c release, caspase-9 and -3 activations which may indicate that both JNK and p38 have a regulatory role upstream of mitochondrial pathway [115].

3. MATERIALS 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. Equipment

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

3.1.3. Buffers and Solutions

3.1.3.1. Buffers and Solutions for Cell death assays

AnnexinV-FITC incubation buffer:

10 mM Hepes, 140 mM NaCl and 2.5 mM $CaCl_2$ were dissolved in 500 ml of ddH_2O. The buffer is stored at 4^0C.

3.1.3.2. Buffers and Solutions for Protein Isolation

3.1.3.2.1. Total Protein Isolation

Cell Lysis Buffer:

150 mM NaCl, 1% NP40 and 50 mM Tris were dissolved in ddH_2O and the pH was adjusted at 8 with HCl. The buffer was stored at 4^0C. Prior to protein isolation, complete cell lysis buffer was prepared by adding 1X protease and phosphatase inhibitors and 0.5 M PMSF freshly.

10X PBS (Phosphate Buffered Saline):

80 g NaCl, 2.0 g KCl, 14.4 g Na_2HPO_4 and 2.4 g KH_2PO_4 were dissolved in 1L of ddH_2O.

3.1.3.2.2. Cytoplasmic and Nuclear Protein Fractionation

T1 buffer: 10mM HEPES-KOH (pH:7.9), 2mM MgCl2.6H2O, 0.1mM EDTA, 10mM KCl, 1% NP-40 and freshly added DTT, 0.5mM PMSF, complete protease and phosphatase inhibitors.

T2 buffer: 20 mM HEPES-KOH (pH:7.9), 1.5 mM MgCl2, 0,2 mM EDTA, 650 mM NaCl, glycerol (25%,v/v) with freshly added 1 mM of DTT, 0,5 mM of PMSF, 1X protease and phosphatase inhibitors.

3.1.3.3. Buffers and Solutions for SDS-Polyacrylamide Gel Electrophoresis

1.5 M Tris-HCl pH 8.8:

1.5 M Tris was dissolved in ddH_2O and pH was adjusted at 8.8 with HCl.

0.5M Tris-HCl pH 6.8:

0.5M Tris was dissolved in ddH_2O and pH was adjusted at 6.8 with HCl.

3.1.3.4. Buffers and Solutions of Western Blotting

1X PBS-Tween20 (PBS-T):

0.2% Tween 20 was prepared in 1X PBS.

10X Running Buffer:

30.3 g Tris, 144.1 gr Glycine, 10 gr SDS were dissolved in 1L of ddH_2O and pH was adjusted at 8.3.

10X Transfer Buffer:

30.3 gr Tris and 144 gr Glycine were dissolved in 1L of ddH_2O.

1X Transfer Buffer:

Prior to use for transfer of proteins to the membrane, 20% methanol was added into 1X transfer buffer and the remaining volume was completed with ddH_2O.

Blocking Solution:

0.05% (w/v) dried milk powder was dissolved in PBS-T.

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.

3.2. Methods

3.2.1. Cell Culture

HCT116 WT and Bax -/- cells were grown in McCoy's 5A Modified Medium (Modified) with L-glutamine added with %10 fetal bovine serum (FBS) and 1% penicillin/streptomycin (100 U/ml). Cultures were sustained in the incubator at 37 ^{0}C and %5 CO_2. T-75 and T-25 flasks were used for the seeding of the cells depending on the need of cell amount. Cells were splitted by trypsin before they had a high confluency on the flasks. As a first step, the old medium was withdrawn to wash the cells with 1X PBS. After PBS was removed, cells were incubated with trypsin for 5 min in the incubator until they detached from the surface of the flasks. Cells were suspended in the mixture of trypsin and complete medium in order to be centrifuged at 300 g for 5 min in the following. The pellet including the cells was dissolved within the complete medium for further culturing.

Cells were grown on 60 mm dishes with a seeding density of $6x10^5$ cells/dish for 36 h for protein isolation experiments. Flow Cytometry analysis including AnnexinV and DCFH-DA labeling involved the seeding of cells on 12 well plates ($1x10^5$ cells/well) for 36h and treated with the drugs for various time points. As a control of immunoblot analysis and FACS analysis, cells were always treated with EtOH.

For cryopreservation, cells were trypsinized and resuspended in the medium containing 10% DMSO (freezing medium) and 90% FBS. These resuspended cells were transferred into cryovials in order to be stored at -80 ^{0}C for 1 day and placed into liquid nitrogen tank.

3.2.2. Protein Isolation

3.2.2.1. Total Protein Isolation

HCT116 cell lines were grown on 60 mm dishes with a seeding density of $6x10^6$cells/dish for 36 h. Drug treated cells for the given time points in the results were washed with 1X cold PBS after the removal of old complete medium from the dishes. Cells found in 1X cold PBS were harvested with scrapping and put into 1.5 ml eppendorf tubes in order to be centrifuged at 13.200 rpm at 4 ^{0}C for 30 seconds. The pellets containing the whole cells were dissolved in complete lysis buffer which includes incomplete cell lysis buffer, 1X protease inhibitor, 1X phosphatase inhibitor and 0.5 mM PMSF. These dissolved cells were vortexed briefly and left on ice for 30 min and stored at -80 ^{0}C subsequently.

3.2.2.2. Cytoplasmic and Nuclear Protein Isolation

The cells were grown on 6-well plates with a seeding density of $3x10^5$ cells/well for 36 h. Drug treated cells for the defined time points were washed with 1X cold PBS. Cells were detached from the wells by scrapping them in 1X cold PBS and collected into 1.5 ml of eppendorf tubes. Next, they were centrifuged at 13.200 rpm for 30 seconds at 4^0C. The supernatant was sucked out and the pellet containing the cells was resuspended with 120 µl of T1 buffer [10 mM Hepes/KOH, (pH 7.9), 10 mM KCl, 2 mM MgCl2, 0.1 mM EDTA, with freshly added of 1 mM dithiothreitol (DTT) 0.5 mM of PMSF, 1X proteases and phosphatase inhibitors] . Resuspeneded cells were incubated on ice for min. 20 min. Subsequently, they were vortexed briefly and centrifuged at 13.200 rpm for 1 min at 4 ^{0}C. The supernatant containing the cytoplasmic proteins were transferred into new eppendorf tubes to be kept at -80 ^{0}C immediately. The remaining pellet was dissolved in 20 µl of T2 buffer [20 mM Hepes/KOH (pH 7.9), 1.5 mM MgCl2, 0,2 mM EDTA, 650 mM NaCl, glycerol (25%,v/v) with freshly added 1 mM of DTT, 0,5 mM of PMSF, 1X protease and phosphatase inhibitors] and incubated on ice for min. 20 min. Later, they were vortexed briefly and centrifuged at 13.200 rpm for 20 min at 4 ^{0}C. The supernatant including the nuclear proteins were kept at -80 ^{0}C.

3.2.3. Determination of Protein Concentration

Bradford assay was utilized to identify the protein concentrations. Bovine Serum Albumin (BSA) with 1ug/µl was used as a reference protein. The concentration of BSA was diluted into the following concentrations; 5, 2.5, 1.25, 0.625, 0.3125, 0.15625 µg/µl. This was achieved with serial dilution of BSA mixing 5µl of the previous sample with the next 5 µl of mQH_2O in 96-well plate. One well containing only 5 µl of mQ H_2O functioned as a blank well. The sample wells included 4 µl of mQ H_2O and 1 µl of protein sample. After the addition of 95 µl of Bradford dye on the wells containing BSA and protein samples, ELISA plate was put into the spectrophotometer in order to determine the density of the colors in terms of absorbance values measured at 595 nm of wavelength. A standard curve was generated firstly depending on the concentrations of BSA and their corresponding absorbance values. The concentrations of the samples were calculated on the basis of this standard curve as long as R^2 is smaller than 1.0 and larger than 0.9.

3.2.4. SDS-Polyacrylamide Gel Electrophoresis

Proteins were separated on a 9, 12 and 15% SDS-PAGE according to their sizes. The separating gel of a typical 12% SDS-PAGE was prepared as shown in the list below.

Table 3.1. The concentrations of components for 12% SDS-PAGE.

12% Seperating Gel	
dH2O	3,4 ml
1,5 M Tris-HCl pH 8,8	2,5 ml
20% (w/v) SDS	50 µl
30% Acrylamide/0.8% Bis-Acrylamide	4 ml
10% Ammonium persulfate (APS)	50 µl
TEMED	5 µl

As soon as the separating gel was prepared, it was poured in between the two glass panes immediately. Next, the empty part remained up-side of the gel was completed with isopropanol to let the gel to be frozen quickly. Once the gel was polymerized and the top of the gel was washed with dH_2O to remove isopropanol, stacking gel was prepared with the following concentrations of ingredients.

Table 3.2. The concentrations of the components for stacking gel of SDS-PAGE.

Stacking Gel	
dH2O	3,075 ml
0,5 M Tris-HCl pH 6,8	1,25 ml
20% (w/v) SDS	25 µl
30% Acrylamide/0.8% Bis-Acrylamide	670 µl
10% Ammonium persulfate (APS)	25 µl
TEMED	5 µl

The prepared stacking gel was poured on to the frozen separating gel and a comb with defined number of wells was placed quickly into the stacking gel and left to be polymerization. Once the stacking gel was ready, the glass panes including the SDS polyacrylamide gel were inserted into the running tank containing 1X running buffer. Before loading the sample proteins into the wells, they were mixed with 2X laemmli loading dye in eppendorf tubes and exposed to 95 ^{0}C for 3 min. After the comb was removed from the gel allowing the formation of empty wells, proteins were loaded into the gel. SDS-PAGE was run for about 2 hours at room temperature with a constant voltage of 100V.

3.2.5. Immunoblot Assay

The separated proteins in SDS-PAGE were transferred onto PVDF membrane as a next step. The components of the transfer system were placed inside the cassettes with the electrical direction of negative side to positive side in the following order; sponge, Whatman paper, SDS-polyacrylamide gel, PVDF membrane, Whatman paper and sponge. Prior to alignment, PVDF membrane was activated for 30 min with methanol and sponges and Whatman papers were pre-wet with 1X Transfer Buffer (TB). The cassettes were placed into the transfer tank filled with 1X TB and containing an ice-block to prevent heating and a constant voltage of 100V was applied for about 1.5 hours at 4 ^{0}C.

The membranes were blocked with 1X Blocking solution [5% dried milk powder in PBS-Tween20 (PBS-T)] for 1h at room temperature. After a quick washing of the blocked membrane with PBS-T, it was incubated with primary antibodies 1/2000 (v/v) diluted in 1X Blocking solution overnight at 4 ^{0}C. Subsequently, the membranes were washed with 1X PBS-T three times for 15 min. Afterwards, they were treated with horseradish peroxidase (HRP)-conjugated secondary antibodies with 1/5000-1/10.000 (v/v) diluted in 1X Blocking solution overnight at 4 ^{0}C. Once the membranes were rinsed with PBS-T three times for 15 min in order to remove the excess secondary Immunoblots were determined with addition of enhanced chemiluminescence solution on the membrane and the expanded light was captured with Hyperfilm-ECL.

3.2.6. Cell Death Analysis with AnnexinV-labeling

Death analysis of HCT116 cell lines were achieved with Flow Cytometry by AnnexinV labeling. Since AnnexinV binds to the phosphoditylserine which is exposed to outside of plasma membrane during apoptosis, it was also utilized to estimate the probable apoptosis induced by the drugs in the cells. Cells were grown on 12-well plates with a seeding density of $1x10^{5}$ cells/well for 36 h. The cells were treated with the drugs for pre-set durations. After the removal of old medium for the wells, cells were rinsed with 1X cold PBS and 0.5 ml of trypsin was added to make the cells detach from the wells. The detached cells were harvested into FACS tubes to be centrifuged at 300 g for

5 min. After the removal of supernatant, 1 ml of 1X cold PBS was added onto the pellet and they were centrifuged at 300 g for 5 min. The supernatant was discarded again and cells were incubated with 100ul of AnnexinV buffer containing 2 µl of AnnexinV tagged with Fluorescein isothiocyanate (FITC) for 15 min in dark at room temperature. Upon the addition of 500 µl of AnnexinV buffer to the mixture lastly, cells became ready to be analyzed with Flow Cytometry.

3.2.7. ROS Detection with DCFH-DA Labeling

HCT116 cell lines were seeded on 12-well plates at a density of $1x10^5$ cells/well for 36 h. The durations for the treatment of the cells with PMC-F were given in the results. The pre-determined concentrations of DCFH-DA dye was added into the medium of the cells 1 hour before the end of the time. Thus, the cells were incubated with the dye for 1 h in the medium. Subsequently, cells were prepared for FACS analysis with the same protocol applied in AnnexinV assay. As a difference from the previous protocol, after the last centrifuge done for the washing of the cells, cells were resuspended in 500 ml of 1X cold PBS and examined by Flow Cytometry.

3.2.8. Statistical Analysis

The graphic results were expressed as means ± SEM and the statistically significance of the mean values were measured using Students t-tail test. The values of $P< 0.01$ were determined as significant.

4. RESULTS

4.1 Cytotoxicity of PMC-A and PMC-F on HCT116 cell lines

Pramanicin (PMC) which is an anti-fungal agent has 11 types of analogous whose structures are slightly different from each other. The probable death effects of all these analogous of PMC were tested on HCT116 colon carcinoma cell lines in a previous study done in our laboratory. Cell death analysis results showed that two of the analogues, PMC-A and PMC-F were the most effective agents inducing death response on HCT116 cancer cells. In the present study, one drug had to be selected from these 2 agents to analyze the possible chemotherapeutic effect of the drug on HCT116 colon carcinoma cells. The potential of PMC-A and PMC-F drugs were compared with the experiments including time dependent cell death analysis and detection of apoptotic signaling molecules.

The concentration of the drugs was adjusted by Flow Cytometry - AnnexinV labeling. The concentration inducing 50% of cell death was determined as the amount of drugs for the treatment of HCT116 cell lines. Results of AnnexinV assay indicated that 25 μM of PMC-A and 50 μM of PMC-F was able to induce nearly 50% death in HCT116 WT and Bax-/- cells at 24 h (Figure 4.1. and Figure 4.2.). Thus, these concentrations of the drugs were used for further experiments.

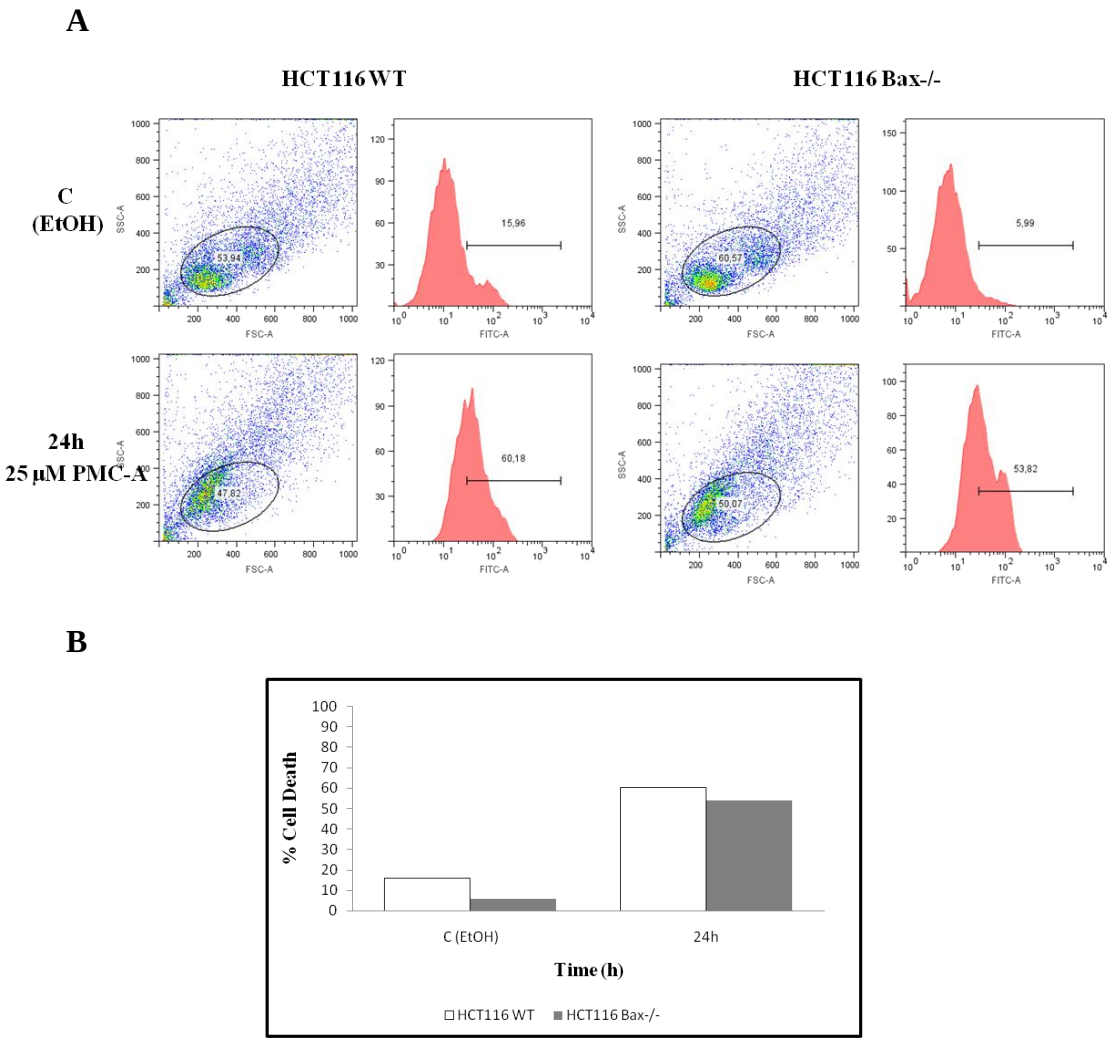


Figure 4. 1: Cell death analysis of HCT116 WT and Bax-/- cells treated with 25 µM of PMC-A. Cells were treated with the drug and EtOH for 24 h. Cell death analysis was performed by Flow Cytometry – AnnexinV labeling. A) The populations selected for the analysis are represented and the histograms show the shift in the intensity of fluorescence dye between control and drug treated cells. B) The graphic represents the mean percentage of cell death in control cells treated with EtOH and the cells treated with the drug.

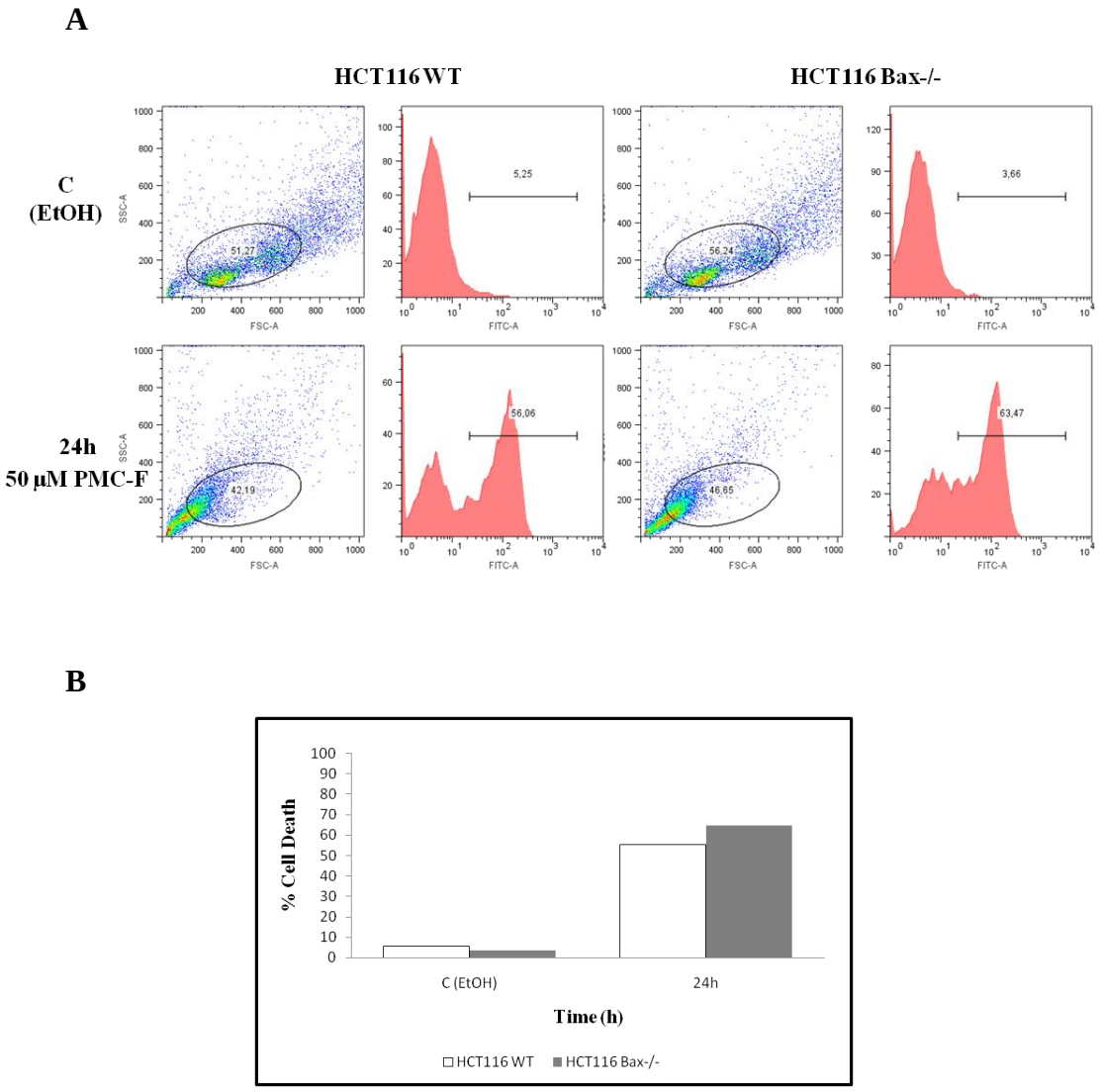


Figure 4. 2: Cell death analysis of HCT116 WT and Bax-/- cells treated with 50 µM of PMC-F. Cells were treated with the drug and EtOH for 24 h. Cell death analysis was performed by Flow Cytometry – AnnexinV labeling. A) The populations selected for analysis were presented and the histograms show the shift in the intensity of fluorescence dye between control and drug treated cells. B) The graphic represents the mean percentage of cell death in control cells treated with EtOH and the cells treated with the drug.

In order to identify the death profile in HCT116 WT and Bax-/- cells induced by 25 μM of PMC-A and 50 μM of PMC-F, the cell lines were analyzed for various time periods. As shown in Figure 4.3 and Figure 4.4, both of the cell lines presented an increasing death response until 48 h after treatment with 25 μM of PMC-A and 50 μM of PMC-F. However, when the percentage of the death responses exhibited by the cell lines was compared for the specified time points, apparent differences were observed between HCT116 WT and Bax-/- cells.

While HCT116 WT cells displayed nearly 50% of death in response to 25 μM of PMC-A incubation for 12 h, the amount of death exhibited by HCT116 Bax-/- cells was only around 10% until that time. But, the resistance of the Bax-/- cells to death disappeared after 12 h. After that point, HCT116 Bax-/- cells exhibited a rapidly increasing death response and presented the same ratio of death with the WT cells at 24 h. The death response exhibited by the WT and the Bax-/- cells continued in the same manner during the next 48 h (Figure 4.3.). PMC-F-induced time dependent cell death analysis gave the similar results as in PMC-A treatment. 50 μM of PMC-F treatment of HCT116 cell lines induced an increasing death response in both of the cell lines in a time dependent manner. However, the Bax-/- cells exhibited resistance to death early in the treatment of PMC-F at 8 h, significantly. The percentage of death observed at 8 h in the Bax-/- cells was nearly half the death of noticed in the WT cells. As in the PMC-A treatment, the resistance of the Bax-/- cells to PMC-F induced death did not continue and started to increase in the following hours. At 12 h, the Bax-/- cells were able to show the same amount of death exhibited in the WT cells (Figure 4.4). These findings suggest that HCT116 Bax-/- cells are significantly resistant to death early in PMC-A and PMC-F treatments. On the other hand, the deficiency of Bax could not inhibit the death of Bax-/- cells completely. This fact suggests that Bax is important but not indispensible for PMC-A and PMC-F-induced death in HCT116 colon carcinoma cell lines.

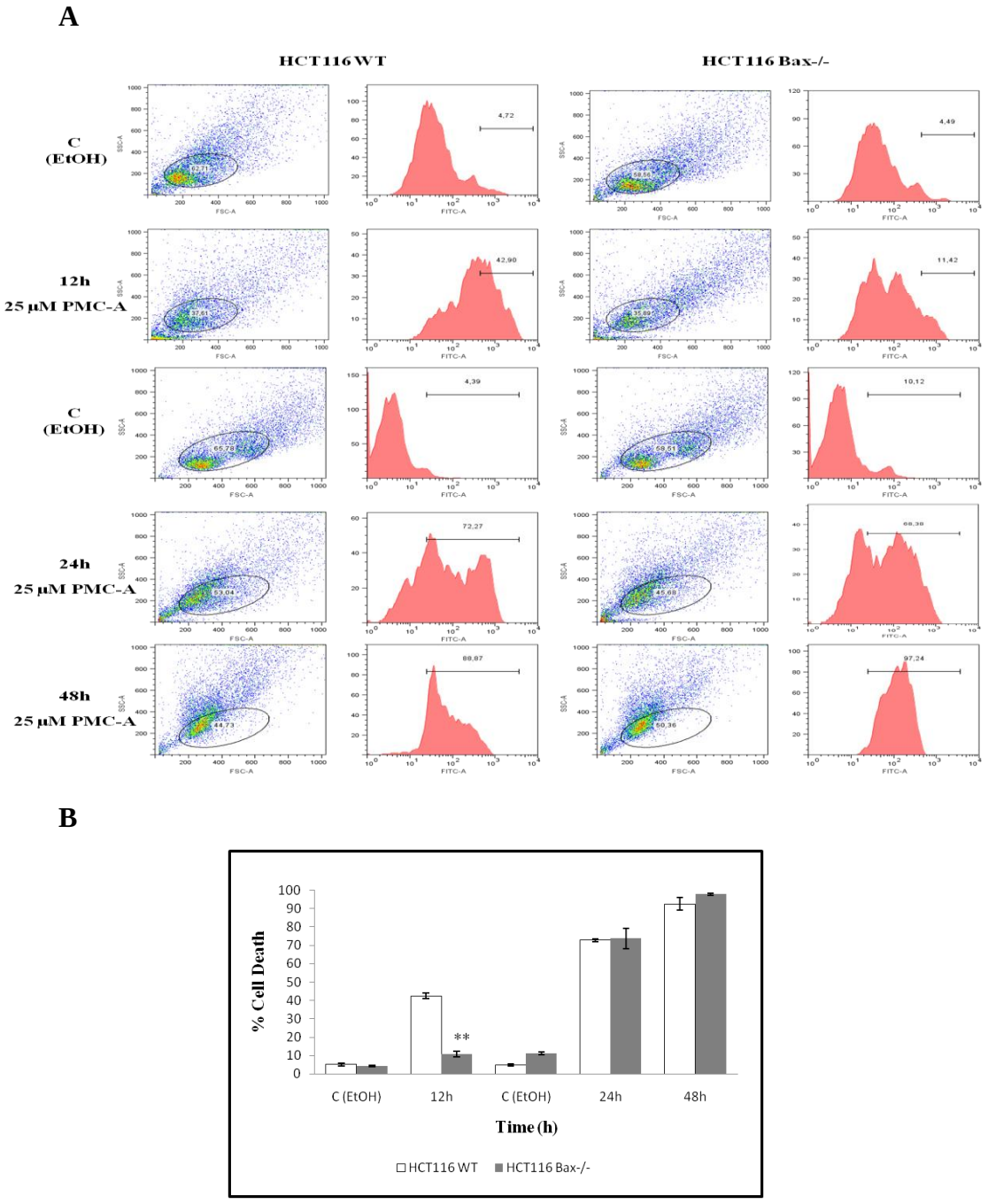


Figure 4. 3: Death analysis of HCT116 WT and Bax-/- cells treated with 25 µM of PMC-A in a time dependent manner. The cell lines were treated with the drug for the indicated time periods. Cell death analysis was done with Flow Cytometry – AnnexinV labeling. A) The percentage of cell death after the drug treatment was obtained according to the gate defined in the histogram of the control cells for the indicated time periods. Since the voltage was different during the analysis at 12 h drug treatment when compared to 24 h and 48 h, its control gate was defined independently. B) The graphic shows the mean percentage of cell death in the control cells and the drug treated cells at 12 h, 24 h and 48 h. Data are shown as mean ± SEM representative of the three experiments. ** $P < 0.01$.

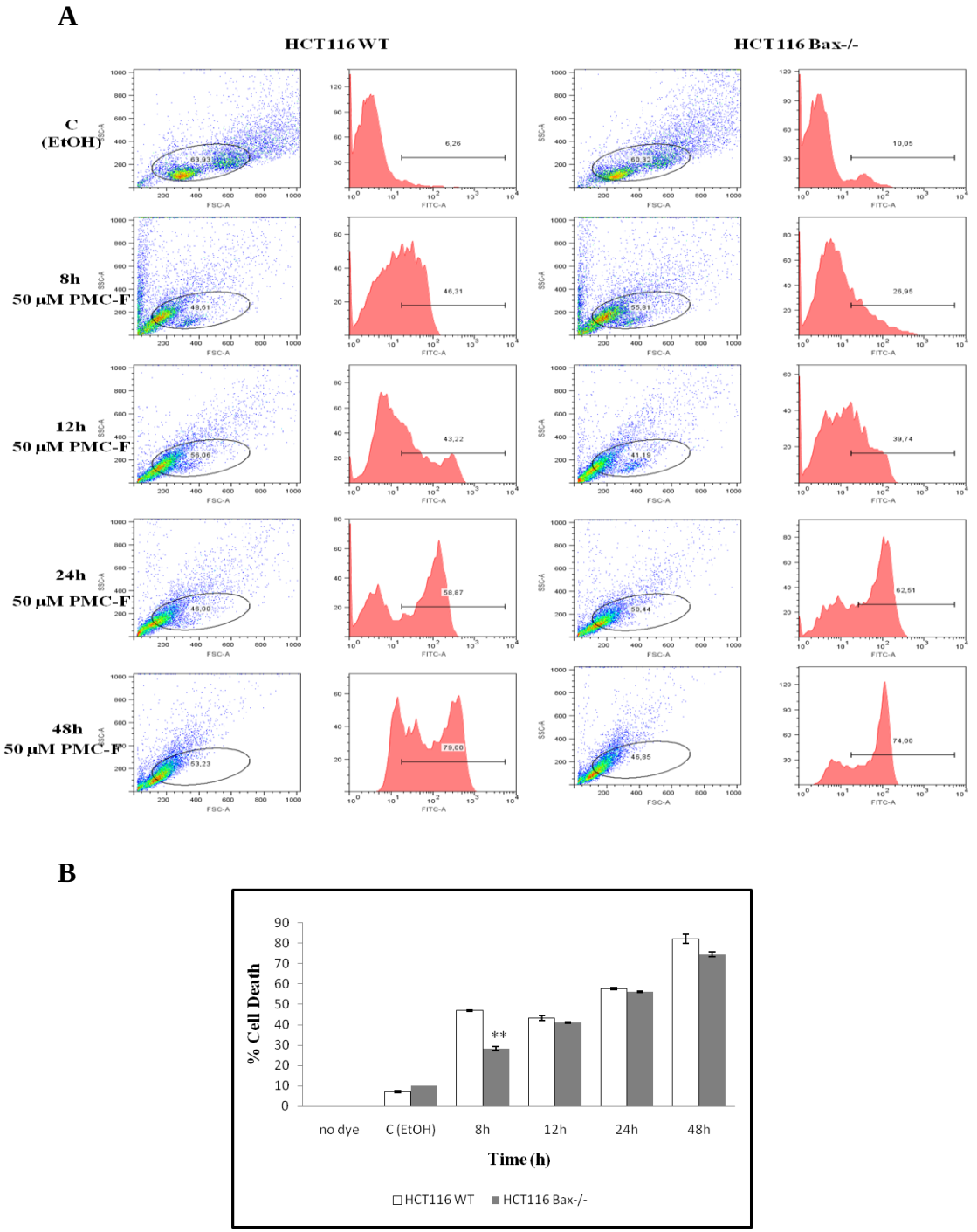


Figure 4. 4: Death analysis of HCT116 WT and Bax-/- cells treated with 50 µM of PMC-F in a time dependent manner. The cell lines were treated with the drug for the indicated time periods. Cell death analysis was done with Flow Cytometry – AnnexinV labeling. A) The percentage of cell death after the drug treatment was identified in the basis of the gates defined in the histogram of the control cells. B) The graphic shows the mean percentage of cell death in the control cells and the drug treated cells at 8 h, 12 h, 24 h and 48 h as well as the no dye as a negative control. Data are shown as mean ± SEM representative of the three experiments. ** $P < 0.01$.

When the data obtained from PMC-A and PMC-F-induced time dependent cell death analysis were compared, it was found out that the drugs had similar effects. Both of the drugs induced an increasing death response in both of the cell lines. Bax-/- cells also showed a resistance to death until a time point in both of the drug treatments. However, these time points were different that the resistance of Bax-/- cells lasted for 12 h after PMC-A treatment, but 8 h after PMC-F treatment which probably suggest that PMC-F might be more effective drug than PMC-A starting an earlier death response in the Bax-/- cells.

4.2. Apoptotic effect of PMC-A and PMC-F on HCT116 WT and Bax-/- cells

A chemotherapeutic drug is selected for clinical use according to some criterions. Firstly, the drug should target only the cancer cells. Secondly, the drug is expected to induce a programmed cell death in cancer cells. In this sense, such a chemotherapeutic drug initiating apoptosis would be preferred for the treatment of patients with cancer, because, only the cells exhibiting apoptotic cell death are eliminated specifically in the body not affecting or damaging the surrounding cells. As a result, the type of the cell death mechanism which anti-cancer drugs induce is a crucial factor for a successful treatment of the patients.

In order to elucidate if PMC-A and PMC-F can activate apoptosis in HCT116 cell lines, some of the signaling molecules related with apoptotic signaling pathways were investigated in a time dependent manner. Immunoblot analysis of caspase cleavage showed that PMC-A induced the activation of caspase-3, -9 and -8 in WT and Bax-/- cells. The cleavage of caspase-9 and the up-regulation of Apaf-1 protein provided evidence for the activation of mitochondrial apoptotic pathway in HCT116 WT and Bax-/- cells. Additionally, the cleavage of caspase-8 was an indicator of active extrinsic apoptotic pathway. Caspase-3 is one of the effector caspases targeted by extrinsic and mitochondrial apoptotic pathways inducing irreversible apoptosis. Thus, the cleavage of caspase-3 observed in the cell lines also confirmed the activation of apoptosis induced by PMC-A (Figure 4.5).

In the following, PMC-A-induced caspase activation kinetic was examined in the HCT116 cell lines. The patterns of the caspase cleavage detected for each time periods suggested that incubation of the cell lines with PMC-A for 8 h was enough to see the initial activation of caspase-3 in the WT and Bax-/- cell lines. Increase in the level of caspase-9 cleavage could be detected at 12 h in the WT cells, but 8 h in the Bax-/- cells after PMC-A treatment. Being parallel to the preceding results, caspase-8 activation could be detected starting at 8 h and continued through 24 h in both of the cell lines which means that extrinsic and intrinsic mitochondrial apoptotic pathways were activated in a parallel manner. The increase in the protein level of Apaf-1 could also be detected in the WT and Bax-/- cell lines in the indicated time points (Figure 4.5).

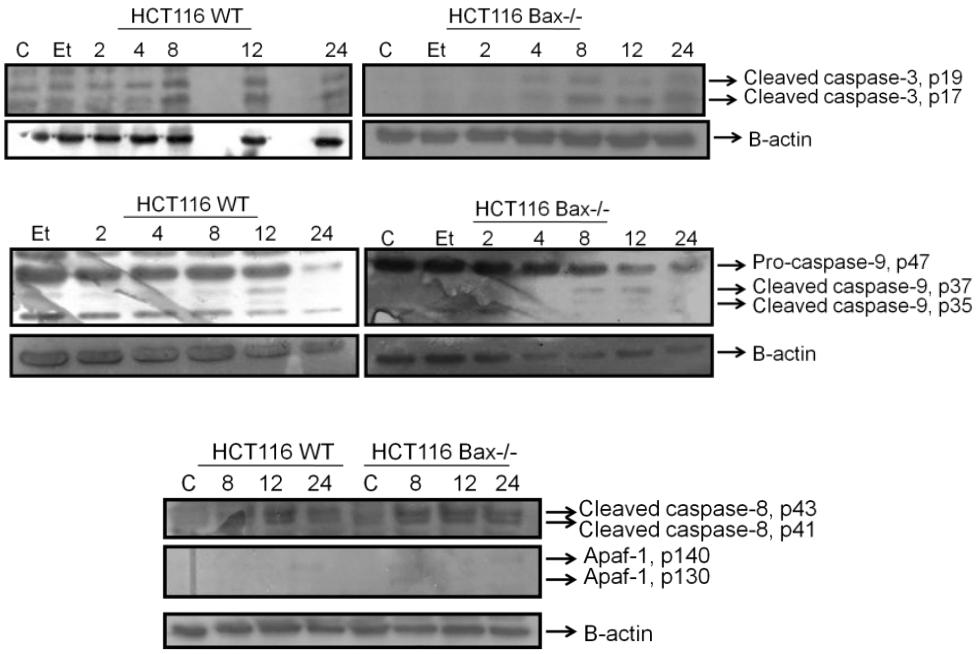


Figure 4. 5: Immunoblot analysis of apoptosis in HCT116 WT and Bax-/- cells treated with 25 μM of PMC-A in a time dependent manner. The cell lines were treated with 25 μM of PMC-A for 2 h, 4 h, 8 h, 12 h and 24 h. Total cytosolic proteins were isolated and loaded into SDS-PAGE. The cleavage of caspase-3, -9 and -8 and Apaf-1 protein level were detected with specific antibodies. β-actin was used as a loading control.

PMC-F also induced a similar apoptotic response in HCT116 WT and Bax-/- cells. The drug activated both extrinsic and mitochondrial apoptotic pathways by triggering the cleavage of caspase-8 and caspase-9, respectively. In addition to detection of caspase-3 cleavage, PARP cleavage was also detected to confirm the caspase-3

activation, since PARP is one of the target proteins of caspase-3 during apoptosis (Figure 4.6). The analysis of PMC-F-induced caspase activation kinetic revealed that 4 hour was the time when the drug stimulated the initial cleavage of caspase-3 in the WT cells. Even as early as at 2 h, it is possible to see a slight increase in the cleavage pattern of caspase-9 and caspase-8 in the WT and Bax-/- cells. The kinetic analysis data of the caspase activities obtained from PMC-A and PMC-F treatments suggested that PMC-F induced apoptotic response earlier than PMC-A in HCT116 cell lines.

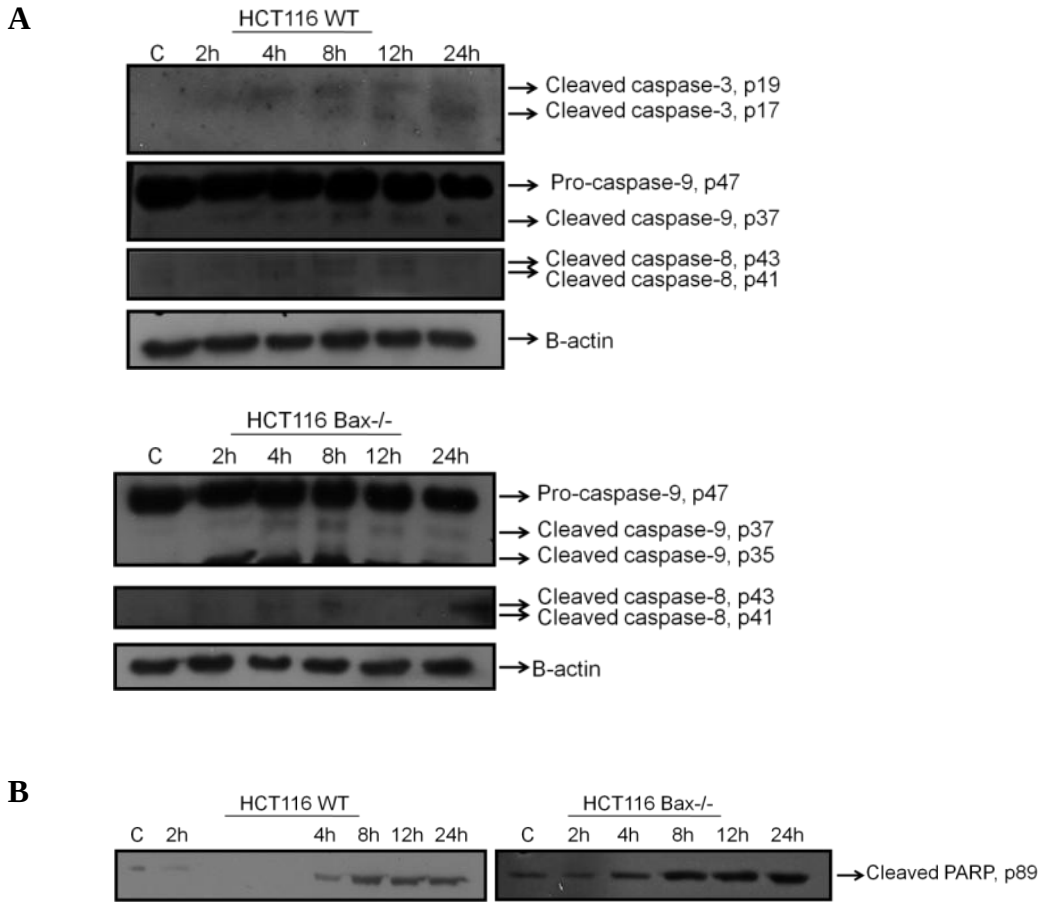


Figure 4. 6: Immunoblot analysis of apoptosis in HCT116 WT and Bax-/- cells treated with 50 μM of PMC-F in a time dependent manner. The cell lines were treated with 50 μM of PMC-F for 2 h, 4 h, 8 h, 12 h and 24 h. Total cytosolic proteins were isolated and loaded into SDS-PAGE. A) Cleavage of caspase-3, -9 and -8 were determined in the cell lines. B) PAPR cleavage provided evidence for the caspase-3 activity in PMC-F induced apoptosis. β-actin was used as a loading control.

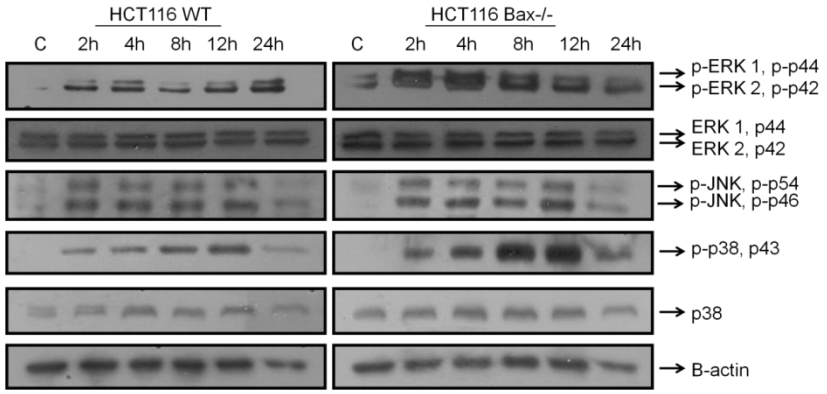
In the light of the results derived from time dependent cell death analysis and the kinetic analysis of caspase activations after drug treatments, PMC-F was likely to be more active than PMC-A. Because it induced earlier death response after 8 h in the Bax-/- cells when compared to PMC-A treatment in which Bax-/- cells could only exhibit a significant death after 12 h. In addition, PMC-F triggered the initial activation of caspase proteins starting from 4 h and even 2 h for some caspases which are earlier time points when compared to 8 h observed in PMC-A treatment. Thus, PMC-F was selected for the treatment of HCT116 cell lines in the further experiments.

4.3. Role of MAP kinase pathway in PMC-F induced apoptosis in HCT116 WT and Bax-/- cells

MAP kinases which are composed of the isoforms of ERK, JNK and p38 proteins have a significant role in the determination of cell survival and cell death regulating cellular responses to various stimuli. They can modulate the balance between cell survival and apoptosis by affecting the activation of Bcl-2 family proteins and p53. In order to establish the involvement of MAP kinases in PMC-F-induced apoptosis, their phosphorylation levels were examined in the cell lines in a time dependent manner.

As shown in Figure 4.3.1.A, PMC-F-induced an increase in the phospho-level of ERK1/2 protein without any distinct change in its total protein level in the cell lines. The sudden increase of ERK 1/2 phosphorylation at 2 h and decrease after 4 h may indicate an early involvement of ERK 1/2 activation in the cell lines after treatment with PMC-F. The immunoblot analysis of phospho-JNK revealed a strong and stable band for a longer period extending up to 12 h. The activity level of JNK started to diminish between 12 h and 24 h. These results suggested that PMC-F treatment might involve the robust activation of JNK in HCT116 cell lines. On the other hand, phospho-level of p38 protein exhibited an activity pattern similar to ERK 1/2 and JNK phosphorylations that p38 protein also started to be active after 2 h incubation with PMC-F. It displayed an increasing phosphorylation level until 12 hour without any change in its total protein level in the cell lines (Figure 4.7.A).

A



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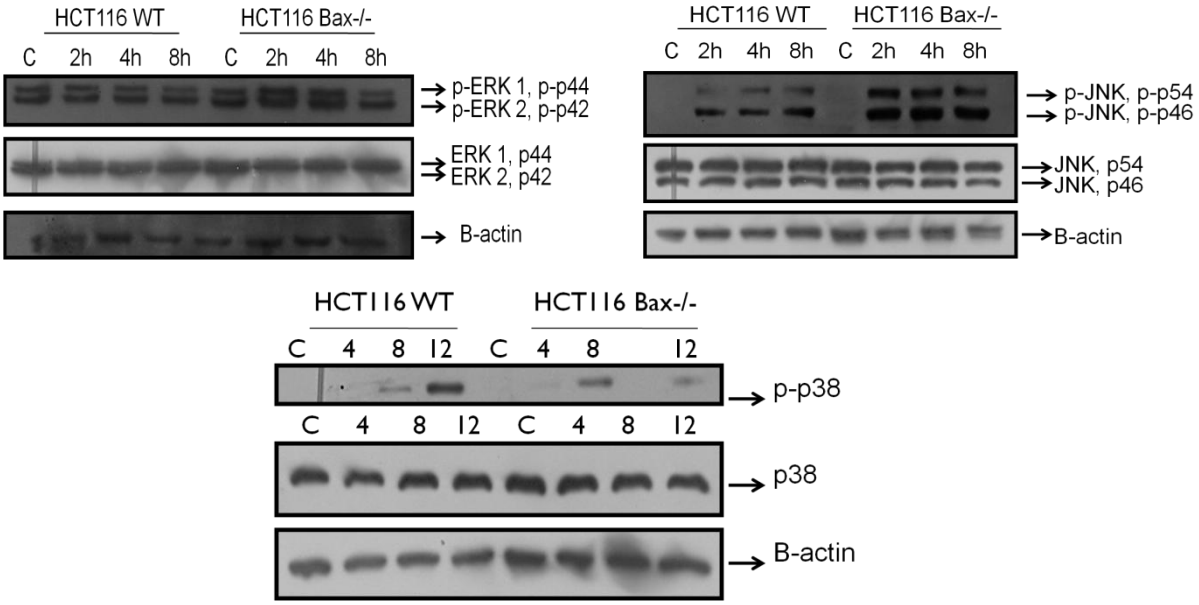


Figure 4. 7: PMC-F-induced MAPK phosphorylation in HCT116 cell lines. Cells were treated with 50 μM of PMC-F. Total cytosolic proteins isolated at 2 h, 4 h, 8 h, 12 h and 24 h were loaded into SDS-PAGE. Specific antibodies were used targeting the specific phosphorylation sites in MAP kinases. A) The activity level of ERK 1/2, JNK and p38 were analyzed in a time dependent manner. B) The phosphorylation levels of MAPKs were compared between WT and Bax-/- cells. β-actin was probed as a loading control.

To summarize, 2 hours treatment of HCT116 WT and Bax-/- cells with 50 μM of PMC-F was enough for the appearance of initial phosphorylation of MAP kinases. However, the duration of activity consistency was different for each MAP kinase proteins. For instance, the phophorylation of ERK 1/2 lasted for 4 hour and disappeared after that point in the cell lines. On the other hand, JNK protein followed a longer consistency in its phospho-level. The bands on the film, which represent the phospho-

level of JNK, were available during 12 h of PMC-F treatment. In addition, the protein level of phospho-p38 showed a gradually increasing pattern up to 12 h (Figure 4.7.A).

In order to identify the possible differences in the activity level of MAP kinases between the WT and Bax-/- cells, the phosphorylation levels of the proteins were compared for various time periods. The time periods indicated in the relevant immunoblot assays were selected depending on the hours when phospho-MAP kinases showed the strongest band pattern in the first western blot detections (Figure 4.7.B).

To investigate the possible difference in the level of active ERK 1/2, the total lysate proteins isolated from HCT116 WT and Bax-/- cells at 2 h, 4 h and 8 h were loaded into the same SDS-PAGE. The results showed that the level of phospho-ERK 1/2 protein was significantly higher particularly at 2 h and 4 h in the Bax-/- cells than in the WT cells. JNK phosphorylation was also examined for the same time periods. The immunoblot analysis indicated that the phosphorylation level of JNK isoforms was obviously more in the Bax-/- cells than in the WT cells during the 8 h incubation with 50 μM of PMC-F. In addition to the phospho-level detection of ERK and JNK proteins, the total levels of the proteins were also analyzed with another immunoblot assay. This detection showed that there was no distinct change in the total level of ERK 1/2 and JNK proteins. The result confirmed that the increase observed in the phospho level of the proteins indeed raised from the increased phosphorylation of the proteins, not due to any increase in the total level of the proteins. The level of phospho-p38 was compared between the cell lines at 4 h, 8 h and 12 h. While HCT116 WT cells exhibited an increasing phospho-level of p38 during 12 h, the phospho-level of p38 could increase until only 8 h in the Bax-/- cells. Its level followed a decrease during the next 12 h. As an additional difference, p38 protein was significantly more phosphorylated at 8 h, but less phosphorylated at 12 h in the Bax-/- cells than in the WT cells (Figure 4.7.B).

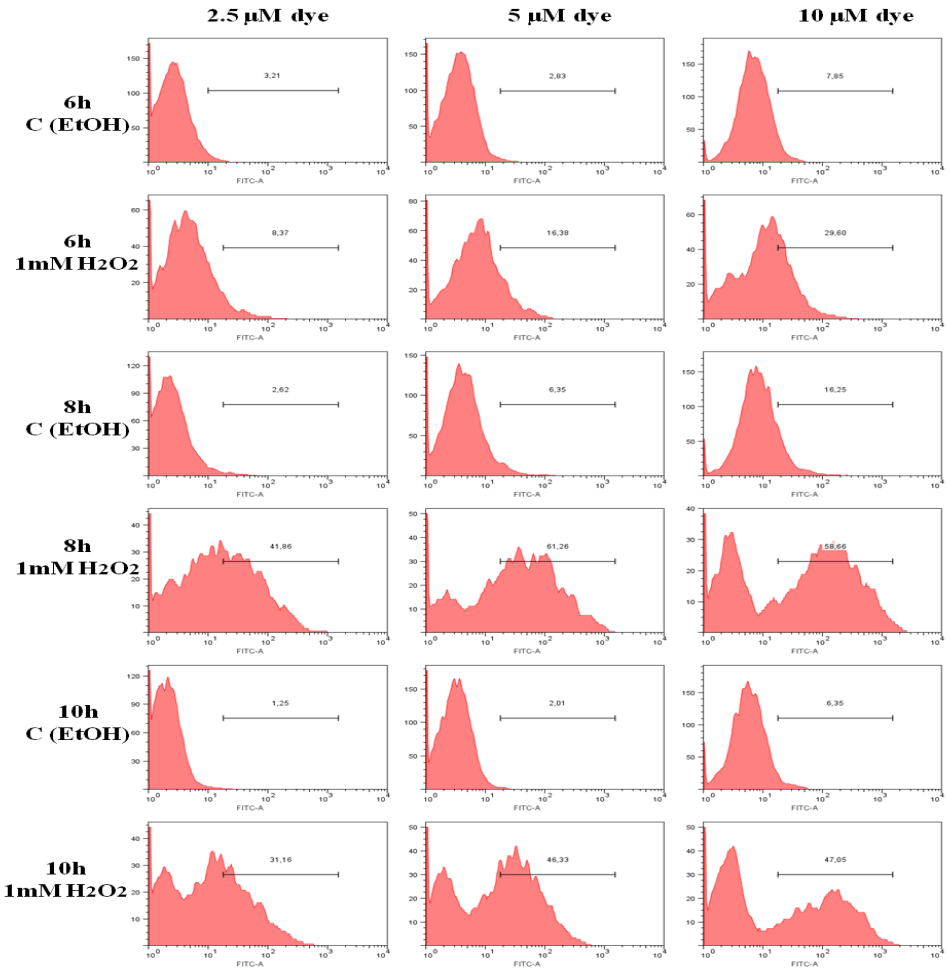
4.4. Detection of ROS production induced by PMC-F in HCT116 WT and Bax-/- cells

Mitochondria is the main organelle responsible for ROS generation in the cells. The electron transport chain favors the formation of O_2^- which is the source of ROS in normal physiological conditions. In the case of mitochondrial damage and the dysfunction of electron transport chain, ROS can be produced in a high concentration in the cells. Based on the finding that PMC-F could induce the cleavage of caspapse-9 and, thereby, the intrinsic mitochondrial apoptotic pathway in the cell lines, possible ROS production that would be caused by mitochondrial damage was investigated in HCT116 WT and Bax-/- cells treated with PMC-F.

The relative level of ROS produced in the cell lines was detected with DCFH-DA fluorescent labeling. Firstly, the concentration of the dye had to be adjusted. HCT116 WT cells were treated with 1 mM of H_2O_2 to trigger ROS generation. The relative ROS level was detected in the cell lines incubated with 1 mM of H_2O_2 for 6 h, 8 h and 10 h to identify the time point when ROS was highly produced. ROS was measured with 2.5 μM, 5 μM and 10 μM of DCFH-DA for each time period.

The analysis of ROS production by the cell lines was done with two ways. The first analysis shown in Figure 4.8., was done through the percentage of the cells who found in the defined gates and so, expressed higher amounts of ROS when compared to control cells. The second analysis shown in Figure 4.9., was performed according to the calculated mean values of the signals derived from the fluorescence dye which correspond to relative ROS level that exhibited by the whole selected population and the number of these mean values were compared between the control and the drug treated cells to understand the change in relative ROS level.

A



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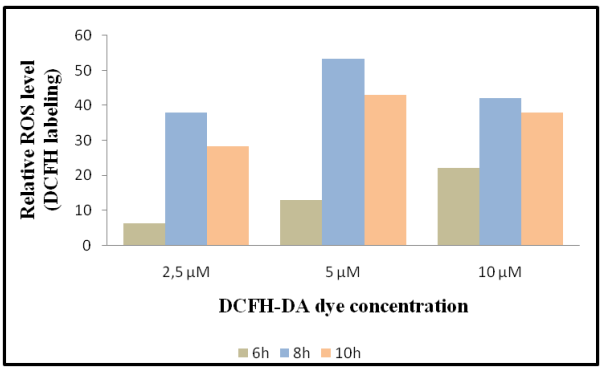

Figure 4. 8: Concentration adjustment of DCFH-DA dye for ROS measurement in the basis of percentage amount. HCT116 WT cells were treated with 1 mM of H_2O_2 for 6 h, 8 h, and 10 h. The cells were incubated with the dye for 1 h before FACS analysis. A) Histograms show the shift in the populations expressing high level of ROS when compared to control cells at the indicated treatment time points. B) The graphic shows the net percentage amount of cells producing ROS identified according to the gates defined in the histograms.

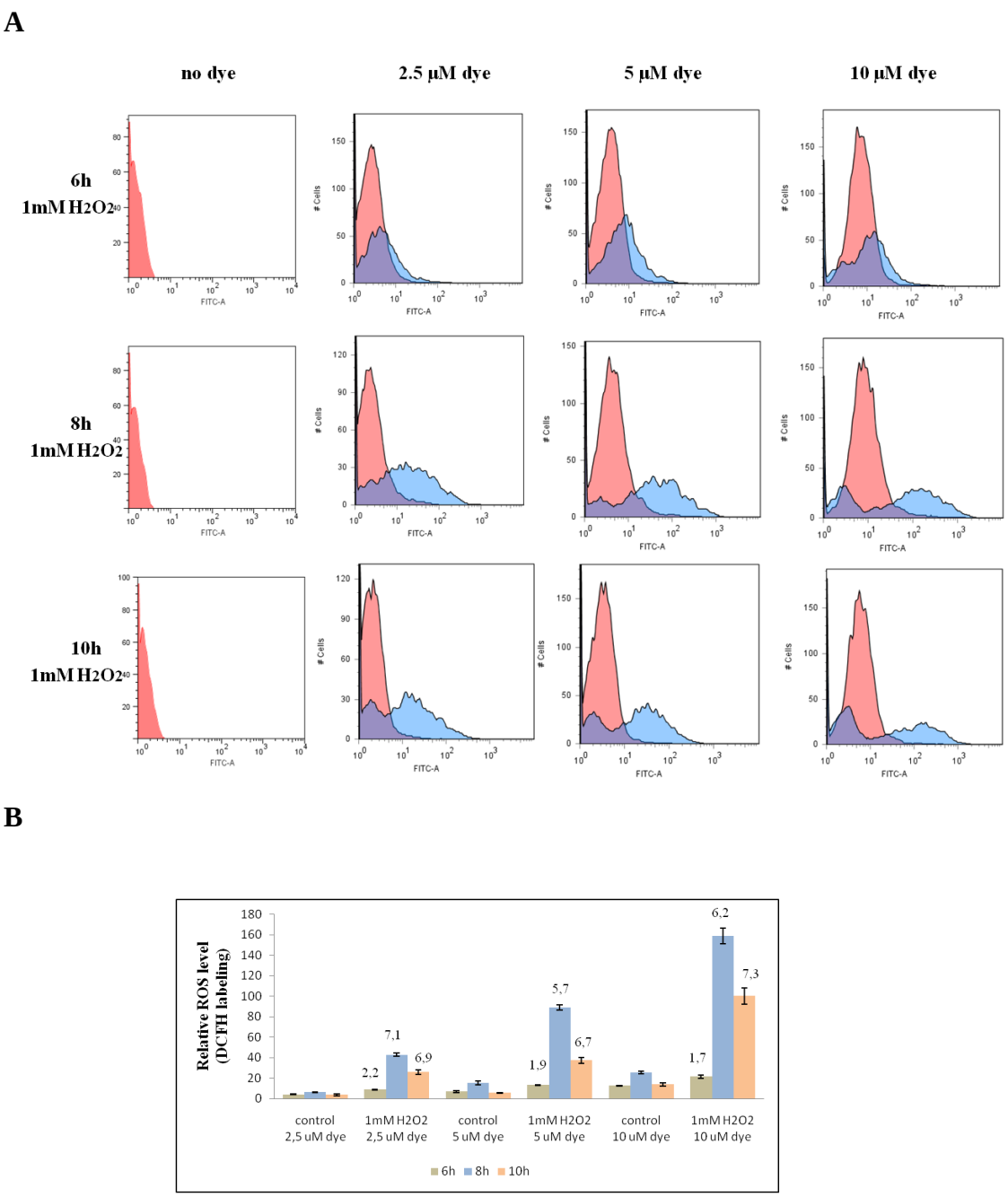

Figure 4. 9: Concentration adjustment of DCFH-DA dye for ROS measurement in the basis of mean value calculation. In this method, the mean values of relative ROS level detected in the control and drug treated cells were calculated. A) The overlapped histograms of control and treated WT cells show the shift in the pick of the population. B) The bars represent the mean values of the populations. The numbers shown on the bars indicate the number of fold in the increase of relative ROS level after 50 µM of PMC-F treatment.

Both of the analysis showed that HCT116 WT cells exhibited ROS production in the following of 1 mM of H_2O_2 treatment and the level of ROS produced in the cells followed an increase from 6 h to 8 h and a decrease during the next 10 h for each dye concentration. So, the results of the two analyses suggested that treatment of the cells for 8 h provided the most production of ROS. The overlapped layout histograms as shown in Figure 4.9 also indicated that the shift in the pick of 1 mM H_2O_2 treated cells was obviously the most at 8 h for each dye concentrations. However, when we compare the relative ROS level detected by 2.5 μM, 5 μM and 10 μM of DCFH-DA dye, we see that 5 μM of the dye presented the highest level of ROS in the first analysis at 8 h, whereas 2.5 μM of the dye was the most effective concentration showing 7.1 fold increase of ROS level at 8 h according to its control level in the second analysis. 5 μM and 10 μM dye concentrations had less impact than 2.5 μM dye concentration by inducing 5.7 and 6.2 fold increases of their control levels. In the basis of the first analysis, we decided to use 5 μM of DFCH-DA dye in the following detection of ROS produced in the cell lines treated with 50 μM of PMC-F. The second analysis also suggested us that we could also choose to use 2.5 μM of DFCH-DA dye concentration for further analysis.

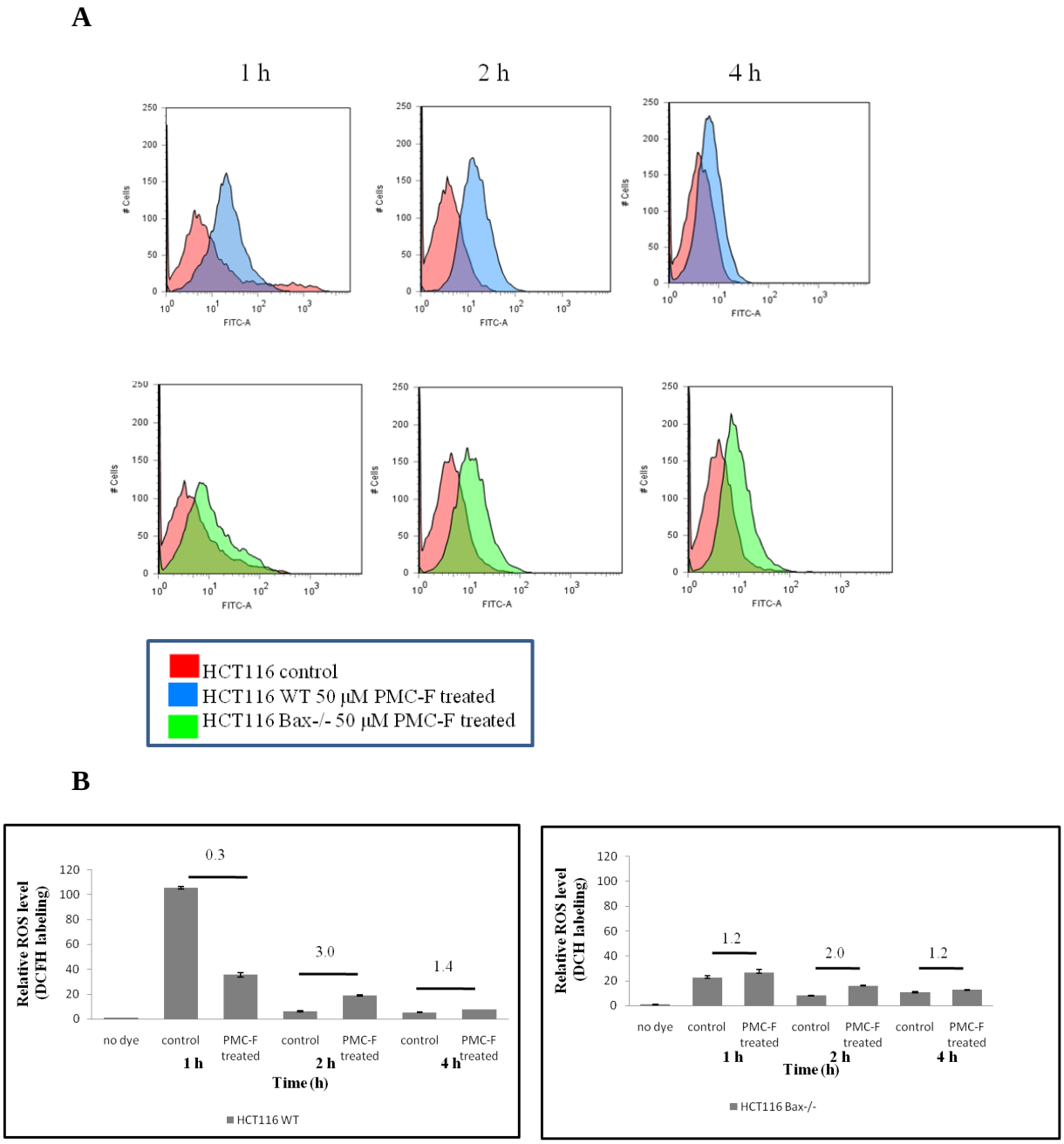

Figure 4. 10: Detection of ROS level produced in the cells induced by PMC-F at 1 h, 2 h and 4 h. HCT116 WT and Bax-/- cells were treated with 50 µM of PMC-F for the indicated time periods. The cells were incubated with 5 µM of DCFH-DA dye for 1 h before FACS analysis. A) The histograms indicate how much WT and Bax-/- cell lines show shift from their control populations which suggests about the level of ROS production after drug treatment. B) The graphics represent the mean values of ROS and the number of fold in the increase of relative ROS level after drug treatment for the indicated hours in the WT and Bax-/- cells. The mean values are shown as mean ± SEM representative of three experiments.

The detection of relative ROS level was analyzed in HCT116 WT and Bax-/- cells after incubation with 50 μM of PMC-F for 1 h, 2 h and 4 h. As it is seen from the shift of the drug treated populations in Figure 4.10.A, ROS production started in the WT and the Bax-/- cell lines at as early as 1 hour after PMC-F treatment. The drug increased the production of ROS level in the cell lines during 2 h drug incubation; however, the effect of the drug in ROS production started to diminish during the next 4 h. In order to observe the possible different response of WT and Bax-/- cells for ROS production, the mean values of ROS signal exhibited by the control and drug treated cell lines were calculated for each time points as it is seen in Figure 4.4.3B and the difference between these mean values gave us a number of fold to define the magnitude of change. Depending on the comparison of these values between the WT and the Bax-/- cell lines for each time point, we could detect the differences in their response to ROS production.

When the folds at 1 h were compared, it is seen as if there is a decrease in the ROS level in HCT116 WT cells after drug treatment. On the other hand, HCT116 Bax-/- cells had 1.2 fold increases (Figure 4.10.B). However, this apparently high ROS level seen in the WT control cells is probably because of the shift of a little population through the right side in the histogram which corresponds to high intensity of fluorescence. So, seen 0.3 fold incerase in the WT cells at 1 h should have been at least a number of fold > 1.0 which corresponds to an increase in ROS level. On the other hand, when the overlapped histograms of control and drug treated WT cells at 1 h is analyzed, it is easily seen that there is a distinct shift in the population giving higher intensity of fluorescence which supports the fact that WT cell started to produce ROS.
Even, the shift seen in the WT cell is more than the shift observed in the Bax-/- cells at 1 h which supports the fact that there should be an increase in ROS production higher in drug treated WT cells than the Bax-/- cells (Figure 4.10.A). So, we could also expect to see a number of fold increase in ROS level in the WT cells bigger than 1.2 fold increase seen in the Bax-/- cells at 1 h. At least, in the light of the overlapped histograms, it can be suggested that there is a late response of Bax-/- cells to ROS production in the early drug treatment when compared to the WT cells.

During the next 2 h, the Bax-/- cells and the WT cells depicted an increasing ROS generation with 3.0 fold increase in the WT cells and 2.0 fold increase in the Bax-/-

cells at 2 h. This result indicated that the WT cells had higher relative ROS level than the Bax-/- cells at this time point. Thus, the total level of ROS produced was less in the Bax-/- cells than the WT cells, also. In the following 4 h treatment, the decrease in the number of the folds when compared to the previous hours means that the relative level of ROS in the cells began to decrease. 1.2 fold increase depicted by the Bax-/- cells at 4 h may suggest that the cells exhibited faster decrease in the ROS level than the WT cells with 1.4 fold increase (Figure 4.10.B).

4.5. Determination of p53 Activation in PMC-F-induced Apoptosis

In the presence of cellular stress and genotoxic agents, mitochondrial apoptotic pathway is activated. As a regulatory protein of this signaling pathway, p53 can enhance the activation of mitochondrial apoptotic pathway by inducing the over expression of some pro-apoptotic genes in the nucleus, targeting the activation of Bax/Bak or inhibiting anti-apoptotic proteins in the cytoplasm.

In order to understand if PMC-F induced apoptosis is p53-dependent or –independent, the activation of p53 was investigated in the HCT116 cell lines in a time dependent manner. In the first immunoblot anlaysis, the possible change in the total level of p53 protein was observed at indicated hours; however, there was not a distinct change in the level of the protein in HCT116 WT and Bax-/- cells during PMC-F treatment. In order to estimate the activity of p53, the total level of p21 protein was also examined. Since p21 is one of the target genes of p53, active p53 might have up-regulated the expression level of p21 gene. Thus, the possible change in p21 level might give us information about the probable activity of p53. Indeed, the results of p21 immunoblot detection showed slight differences between the specified time periods in the cell lines upon PMC-F treatment (Figure 4.11.A). However, since the changes in the band levels of p21 did not follow a gradual increase or decrease through the following hours, the results were unreliable to assess the activity of p53.

In the next step, the possible translocation of p53 from the nucleus to the cytoplasm was investigated to analyze its activity. The nuclear and cytoplasmic proteins of HCT116 WT and Bax-/- cells were isolated at 1 h, 2 h and 4 h in the following of

PMC-F treatment. The times selected for protein isolation were determined according to the hours when the analysis of ROS production was performed after the drug treatment. The cytoplasmic and nuclear proteins derived from the same cell lines were loaded into the same SDS-PAGE.

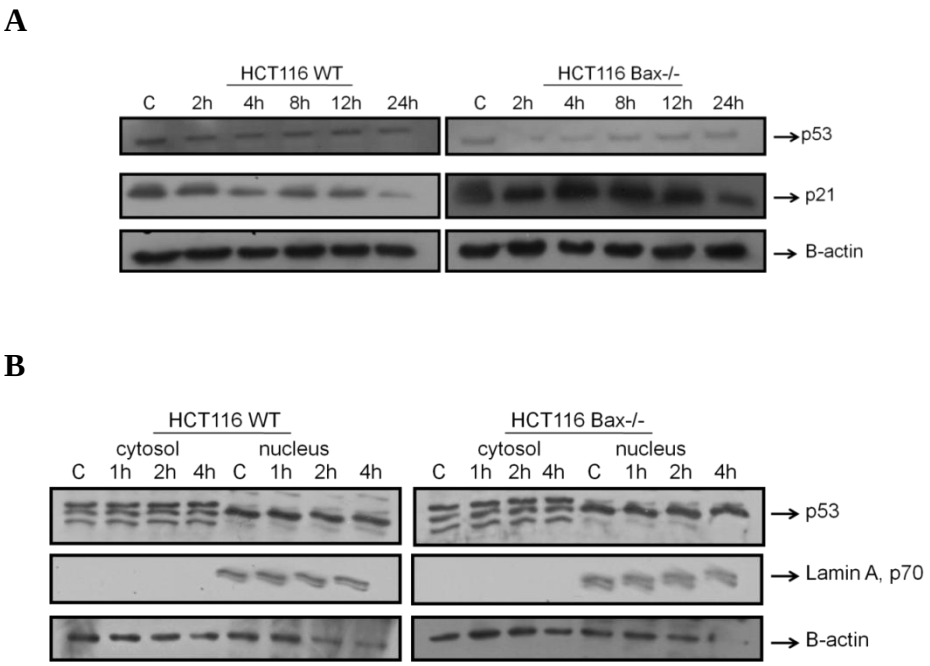

Figure 4. 11: p53 activity in HCT116 cell lines upon treatment with 50 μM of PMC-F. Total cytosolic proteins isolated from the cell lines treated with the drug for 2 h, 4 h, 8 h, 12 h and 24 h were isolated and loaded into SDS-PAGE. p53 and p21 proteins were detected with specific targeting antibodies. B) Cells were treated with the drug for 1 h, 2 h and 4 h. Cytosolic and nuclear proteins isolated from the cells loaded into the same SDS-PAGE. Laminin served as a control for the fractionation of cytosolic and nuclear compartments. β –actin was used as a loading control.

The latter immunoblot analysis of p53 revealed that there was no observable change in the level of the bands regarding the level of cytoplasmic p53 protein in both of the cell lines after 1 h, 2 h and 4 h incubation with PMC-F. As expected, no change was noticed in the level of nuclear p53 protein in the WT and Bax-/- cell lines (Figure 4.11.B). These findings suggest that PMC-F treatment did not induce a change in the localization of p53.

5. DISCUSSION

The induction of apoptosis or programmed cell death is a preferred mechanism for a successful treatment of cancer [67]. Chemotherapy-induced apoptosis depends on the cell-type, the type and the concentration of drug and the duration of treatment [116]. There are recently published studies in which various agents have been tested in terms of their apoptotic effects on cancer cells including HCT116 colon carcinoma cell lines. Since Bax is a very significant pro-apoptotic protein for the activation of mitochondrial apoptotic pathway, HCT116 Bax-/- cells are used in these studies in order to have at least partially apoptosis deficient cells and so, identify the role of apoptotic cell death mechanism that might be induced by many anticancer agents [67].

In the present study, HCT116 WT and Bax-/- cell lines provided us a good model to elucidate the role of Bax in the apoptosis induced by PMC-F and PMC-A. This model also let us identify the key signaling mechanism which is likely essential in PMC-F induced cell death mechanism. The ability of PMC-A and PMC-F drugs to induce death through the induction of apoptotic cell death pathways including mitochondrial apoptotic pathway suggested that Bax was important, but not essential for these drugs to induce apoptosis in HCT116 colon carcinoma cell lines.

If mitochondrial apoptotic pathway was activated in the lack of Bax in the Bax-/- cells, then what could be the mechanism which let the disruption of mitochondria to enhance apoptosis? There is also another pro-apoptotic Bcl-2 family protein, called Bak which collaborates with Bax to generate channels inside the outer membrane of mitochondria leading the release of cytochrome c into the cytoplasm and trigger apoptosis. We assume that Bak might have substituted the role of Bax and induced mitochondrial permeability by generating channels in the membrane of the mitochondria by Bak/Bak oligomerizations in HCT116 Bax-/- cells rather than Bax/Bak oligomerizations as in the WT cells. However, these oligomerizations should have been performed at the same time, because the kinetic of caspase activations were the same in

the WT and the Bax-/- cells after the drug treatments in both of drug treatment, PMC-A and PMC-F.

The next question; what was the mechanism which rendered the Bax-/- cells resistant to death until a time period if there is no delay in the activation of Bak in the Bax-/- cells? To elucidate the mechanism, we firstly investigated the involvement of MAP kinases in PMC-F induced apoptosis. Since, MAP kinase signaling cascade, which regulate different cellular responses against to various stimuli, is one of the up-stream regulators of mitochondrial apoptotic pathway through phosphorylating and affecting the activity of anti- and pro- apoptotic Bcl-2 family proteins [117-120]. Thus, any change in the activation of MAP kinase pathway might have affected the response of the cells to apoptotic cell death. We showed that PMC-F induced the phosphorylation of all MAP kinases including ERK1/2, JNK and p38 in a time dependent manner in the WT and the Bax-/- cell lines with the same manner (Figure 4.7.A.). However, the phosphorylation level of the kinases was not the same for the indicated time periods between the WT and the Bax-/- cells. ERK 1/2 and JNK were activated more in the Bax-/- cells than in the WT cells after PMC-F treatment. The level of phopho-p38 also exhibited differences between the cell lines during the drug treatment. While the activity level of p38 increased significantly between 8 h and 12 h in the WT cells, there was a decrease in the phosphorylation level of p38 during this time in the Bax-/- cells (Figure 4.7.B.).

The difference that we observed in the phosphorylation level of MAP kinases directed us to think if this difference arose from the different concentration of ROS produced in the WT and the Bax-/- cells after PMC-F treatment. There are evidences that MAP kinases are among the redox regulated proteins depending on ROS concentration produced in the cell. This case can also alter their localization in the organelles such as mitochondria and nucleus as well as their activation [81]. The results of ROS level analysis confirmed our assumption that Bax-/- cells started to produce ROS less than the WT cells at 1 h and the total produced level of ROS was also less in the Bax-/- cells than in the WT cells at 2 h as shown in Figure 4.10. If ROS signaling is the mainstay factor which is responsible for the resistance of Bax-/- cells to drug induced death by affecting the activity of the proteins related with death signaling

pathways including MAP kinases, we may suggest that ROS might be the key signaling molecule which sensitizes colon cancer cells to chemotherapeutic drugs.

Analysis of relative ROS level for the determination of the concentration of DCFH-DA dye was done in this study with two methods as indicated in Figure 4.8 and 4.9. The analysis shown in Figure 4.8 was performed according to the percentage of the cells that shifted into the gate defined before. These cells were determined as the cells that started to produce ROS after PMC-F treatment. Depending on this analysis 5 μM of DCFH-DA dye was found to give the most signal for 1 mM H_2O_2 treated cells and located in the gate of histogram at 8 h, so we used this concentration for ROS analysis during PMC-F treatment of HCT116 cell lines. In addition to this analysis method, there was also another method to measure the relative ROS level which is based on the calculation of mean values of fluorescence signal derived from the dye that was exhibited by the whole population. We also wanted to apply this method just to compare with the first method that we used at the beginning. This time, the number of fold increase in relative ROS level was calculated after the cells were treated with PMC-F. What we found out was different that 2.5 μM of DCFH-DA dye was seen as the concentration which gave the highest fold increase at 8 h. So, this comparison suggested us that we could also measure relative ROS level in PMC-F treated HCT116 WT and Bax-/- cell lines by using 2.5 μM rather than 5 μM of DCFH-DA dye.

The induction of cell death response and mitochondrial apoptotic pathway even in the Bax-/- cells gave us a preliminary data to anticipate the potential of the drugs for killing HCT116 colon carcinoma cell lines. Firstly, we showed with the cell death analysis done in a time dependent manner that PMC-A and PMC-F were able to eliminate the early resistance of the Bax-/- cells to death (Figure 4.3 and 4.4.). Next, their cell death mechanism was elucidated that both of the drugs were found to activate apoptosis inducing both the extrinsic and intrinsic mitochondrial apoptotic pathways through the cleavage of caspase-8 and caspase-9 as well as Apaf-1 up-regulation, respectively in HCT116 cell lines treated with the drugs as shown in Figure 4.5 and 4.6. The activation of apoptosis was also confirmed with one of the main effectors caspases activation, caspase-3 cleavage. These first data suggested that PMC-A and PMC-F drugs might be strong candidates of chemotherapeutic drug for the elimination of colon cancer.

PMC-F was probably more active drug than PMC-A that it induced death response in the Bax-/- cells after 8 h resistance, but PMC-A managed this after 12 h resistance of the Bax-/- cells (Figure 4.3 and 4.4). In addition, PMC-F was likely to induce the initial cleavage of caspae-3, -9 and – 8 at 4 h, even as early as at 2 h that it was also possible to see very faint capsase cleavage patterns at this time point. These time points were earlier than when PMC-A induced the initial caspase cleavage starting at 8 h as shown in immunoblot results in Figure 4.5 and 4.6. This difference in the activity level of the drugs might be due to the different structure they have in their fatty acid chain. It is possible that this hydrophobic chain might have affected their integration into the plasma membrane of the cells and the level of disrupting of the integrity of the plasma membrane which probably trigger the activation of apoptotic pathways, subsequently. For example, there are some chemotherapeutic drugs such as cisplatin and doxorubicin which were found to increase membrane permeability and some studies showed that upon the change in the permeability of the cell membrane, FasL death receptor can become activated to initiate apoptosis [121, 122]. So, this type of apoptosis activation might also be a model for the initial effect of PMC-A and PMC-F drugs to induce apoptosis in HCT116 colon carcinoma cell lines.

p53 is a crucial protein whose activity may results with three outcomes; 1) transient cell cycle arrest and DNA repair; 2) apoptosis; 3) cellular senescence. Post translational modifications directed on nearly 40 amino acid residues of p53 defines the specificity of the protein [123]. Ubiquitination of p53 is the primary regulator of the protein activity. Phosphorylation is the classic model of post translational modification for p53 stabilization [108]. p53 is typically found in the nucleus bound to mdm2 protein, which initiates the ubiquitination of p53 and its degradation in the cytosol. However, in the presence of DNA damage or stress conditions, p53 is phosphorylated to become activated. It either stays in the nucleus to let the over expression of target apoptosis related genes or translocates into the cytoplasm or mitochondria to enhance apoptosis.

We also investigated if PMC-F induced cell death was p53-dependent or – independent. Total level of p53 and p21 proteins and possible translocation of p53 from nucleus to cytoplasm were analyzed in the cell lines. As shown in Figure 4.11., the

results suggested that PMC-F did not involve p53 activity for the induction of apoptosis that there was no increase in the level of p53 and p21 as well as no translocation of p53 from nucleus to cytosol (Figure 4.11). The likely inactivation of p53 may also suggest that PMC-F may not be a DNA-damaging agent. However, the probable activation of p53 should be confirmed with further experiments. For instance, different phosphorylated sites of p53 can be detected with immunoblot analysis. According to the possibility that the cytoplasmic p53 might have accumulated in the mitochondria during apoptosis induced by PMC-F, the level of p53 protein isolated from mitochondria and cytoplasm can also be compared.

As a result, PMC-A and PMC-F drugs stand for potential candidates for chemotherapy of colon cancer. Because they induced apoptotic cell death even in HCT116 Bax-/- cells in which apoptosis or mitochondrial apoptotic pathway was partially inhibited. Moreover, the rate of death in the Bax-/- cells was not reduced when compared to HCT116 WT cells after treatment with the drugs. Thus, the drugs were able to trigger the same death ratio in both of the cell lines at the end. Colon carcinoma cell lines have two types of genetic instabilities including microsatellite instability (MIN) and chromosome instability (CIN) due to the dysfunction of mismatch repair genes. This fact results with a heterogenic population with different genomic expression profile and thereby, giving different response to anti-cancer drugs such as Cetixumab [14, 115, 124, 125]. In addition, colon carcinoma cell lines can utilize multi-drug resistance (MDR) genes to efflux the drug from the cell. Thus, while all these factors render the treatment of colon cancer difficult, PMC-A and PMC-F drugs might be effective drugs for the elimination of the whole colon cancer cells.

ROS seems to play the central role for the occurrence of death response in the HCT116 cell lines induced by PMC-F. The delayed production of ROS in the Bax-/- cells may probably be responsible for the early resistance of the cells to death and the following increase of relative ROS level in the Bax-/- cells might contribute to let the Bax-/- cell reach the same ratio of cell death with the WT cells in the following hours. On the other hand, in addition to MAP kinases, there might be other proteins whose activities are affected from the level of ROS produced in the cells and, thereby, induce the rapid execution of apoptosis in the Bax-/- cell lines. If this is really the case,

artificial agents can be designed which specifically target high level of ROS production for the treatment and elimination of colon cancer in the future.

PMC-F or PMC-A drugs might be applied also in combination with anti-cancer vaccines in the future. For example, the proliferation and the metastasis of colon cancer cells can be inhibited with the first step of anti-cancer vaccine treatment. For example, the mutated Ras gene may be silenced with shRNA which targets specifically the gene or in order to let the gain function of APC gene which limits the proliferation of cells in normal conditions, the mutated APC gene can be replaced with its wild type form by recombination. By these ways, non-proliferating cancer cells can't accumulate any more mutation and contribute to the extent of heterogenic population. In the second step, when PMC-F and PMC-A drugs are applied, they can kill the cancer cells in a more effective way. Additionally, in order to facilitate the drugs to target only the cancer cells, not the normal cells, these drugs can be tagged with antibodies which specifically target the antigens found on the colon cancer cells and kill them. Thereby, the antibodies tagged with the drugs can also be used in the treatment of patients with the cancer. This application can also help to reduce the severe conditions of chemotherapy in the patients.

6. CONCLUSION and FUTURE ASPECTS

In conclusion, PMC-A and PMC-F drugs induces apoptotic signaling pathways in HCT116 WT and Bax-/- cells through the cleavage of caspase-3, -9 and -8. The activation of mitochondrial pathway with caspase-9 activation and apaf-1 up-regulation in the Bax-/- cells suggests that Bax is not indispensible in PMC-A and PMC-F induced apoptosis.

The resistance of the Bax-/- cells to death until a time point in PMC-A and PMC-F treatments is probably due to the partially inhibited of mitochondrial pathway. The results obtained from the cell death analysis and kinetic of caspase activation suggested that PMC-F was more active than PMC-A. Thus, PMC-F was used in the further studies. In order to identify the signaling pathways which contribute to that early resistance of Bax-/- cells to death, the phosphorylation of MAP kinases as a regulatory mechanism of mitochondrial apoptotic pathway was investigated. The results showed that ERK 1/2 and JNK were highly phosphorylated in the Bax-/- cells when compared to the WT cells after treatment with PMC-F. On the other hand, HCT116 WT cells exhibited the increasing phospho-level of p38 for a longer period than the Bax-/- cells. The subsequent analysis of ROS level produced in the cell lines indicated that Bax-/- cells represented slower and less production of ROS than the WT cells. In addition, PMC-F induced apoptosis of WT and Bax-/- cells was found as p53-independent.

In the light of the data we obtained, ROS may be the key signaling mechanism which affects the phosphorylation of MAP kinases differently and the resistance mechanism of the Bax-/- cells to death.

The study provides the first insight for the potential of PMC-A and PMC-F as a chemotherapeutic drug. The agents seem to be strong candidates for chemotherapy to be used for colon cancer treatment in the future, because they can induce the apoptotic response even in the Bax-/- cells.

Future studies can be listed as in the following;

- In order to confirm the mitochondrial damage, cytochrome c release can be examined.

- We should intensify our assumption on the role of ROS playing the central role in the occurrence of apoptotic response by affecting the signal transduction mechanisms. For this purpose, the produced ROS can be inhibited after PMC-F treatment and death responses of the cells are analyzed.

- To clarify if ceramide production is the initial signaling mechanism which PMC-F induces, the activity level of sphingomyelinase enzymes can be investigated or its activity can be inhibited to see its effect on the cell death response.

- Although MAP kinases were shown that they were phosphorylated in PMC-F induced apoptosis, their contribution for the cell death response needs to be examined. Respectively inhibition of the phosphorylation of ERK 1/2, JNK and p38 kinases and observation of the probable change in death responses may give a worth data about their role.

- The activity of p53 needs to be confirmed with further experiments. Different phosphorylation levels of p53 can be detected by western blotting. Secondly, the possible translocation of p53 into mitochondria can be examined by mitochondrial fractionation.

- PMC-F should also be applied on healthy cells *in vitro* or *ex vivo* to see their effects on healthy cells, also. Since, an ideal chemotherapeutic drug would be a drug which only kills cancer cells.

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APPENDIX A

Name of Chemicals	Supplier Company	Catalog Number
Anti-β-actin ab	Cell Signal Tech., USA	4967
Anti-rabbit IgG HRP ab	Cell Signal Tech., USA	7074
Anti-mouse IgG HRP ab	Cell Signal Tech., USA	7076
Anti-cleaved caspase-3 ab	Cell Signal Tech., USA	9661
Anti-caspase-9 ab	Cell Signal Tech., USA	9502
Anti-p21 ab	Cell Signal Tech., USA	2946
Anti-p53 ab	Cell Signal Tech., USA	9282
Anti-cleaved caspase-8 ab	Cell Signal Tech., USA	9746
Anti-Apaf-1 ab	Cell Signal Tech., USA	5088
Anti-cleaved PARP ab	Cell Signal Tech., USA	9541
Anti-ERK 1/2 ab	Cell Signal Tech., USA	4695
Anti-p-ERK 1/2 ab	Cell Signal Tech., USA	4376
Anti-JNK ab	Cell Signal Tech., USA	9258
Anti-p-JNK ab	Cell Signal Tech., USA	9251
Anti-p38 ab	Cell Signal Tech., USA	9212
Anti-p-p38 ab	Cell Signal Tech., USA	4631
Annexin V-FITC	Alexis Biochemicals	ALX-209-250-T100
Protease Inhibitor coctail tablet	Roche, Germany	4693124001
Phosphatase Inhibitor coctail tablet	Roche, Germany	4906837001
Acrylamide/Bis-Acrylamide	Sigma, Germany	A3699
Ammonium persulfate	Sigma, Germany	A3768-25G
TEMED	Sigma, Germany	T7024-100ml
Tris	Molekula, UK	M11946779
HCl	Merck, Germany	100314

Isopropanol	Merck, Germany	1009952500
Sodium Dodecyl Sulphate	Sigma, Germany	L4390
Glycine	Molekula, UK	M10795955
Methanol	Sigma-Aldrich	24229
PVDF membrane	Roche, Germany	3010
NaCl	Duchefa Biochemie	S05205000
KCl	Fluka, Switzerland	60129
KH_2PO_4	Riedel-de Haén, Germany	4243
Na_2HPO_4	Merck, Germany	1065791000
Tween 20	Molekula, UK	18945167
Blocking agent	ECL Advanced ^{TM} blocking agent	CPK1075
Hyperfilm ECL	Amersham Biosciences, UK	RPN2103K
2-Mercaptoethanol	Fluka, Switzerland	63700
Fetal Bovine Serum	PAN, Germany	
McCoy's 5A Medium	PAN, Germany	P04-05500
Trypsin/EDTA	PAN, Germany	P10-0231SP
Page Ruler Prestained Ladder	Fermentas, Germany	#SM1811
PMSF	Amresco®,USA	0754-25G
NP-40	Sigma, Germany	I3021
EDTA	Riedel-de Haén, Germany	27248
Liquid nitrogen	Karbogaz, Turkey	
Ethanol	Riedel-de Haén, Germany	32221
Penicilin/Streptomycin	PAN, Germany	P06-07100
ECL Advance Chemiluminescence	Amersham Biosciences, UK	RPN2135
CM-H_2DCF-DA	Fluka, Switzerland	WA12820
Hepes	Molekula, UK	M55704197
Bradford Solution	Biorad Inc., USA	500-0006

APPENDIX B

Autoclave:	Hirayama, Hi-Clave HV-110, JAPAN
Balance:	Sartorius, BP211D, GERMANY
Centrifuge:	Eppendorf, 5415D, GERMANY
	Eppendorf, 5415R, GERMANY
	Kendro Lab. Prod. Heraues Multifuge 3L, GERMANY
Deepfreeze:	-70^0C, Kendro Lab. Prod. Hearus Hfu486 Basic, GERMANY
	-20^0C, Bosch, TURKEY
Distilled Water:	Milipore, MiliQ Academic, FRANCE
Electrophoresis:	Biorad Inc., USA
Flow Cytometry:	BD FACS Canto™, USA
Ice Machine:	Scotsman Inc., AF20, USA
Incubator:	Memmert, Modell 300, GERMANY
Laminar Flow:	Kendro Lab. Prod. Heraues, HeraSafe, HS12, GERMANY
Magnetic Stirrer:	VELP Scientifica, ARE Heating Magnetic Stirrer, ITALY
Microliter Pipette:	Eppendorf, Research
Microwave Oven:	Bosch, TURKEY
pH meter:	WTW, pH540 GLP MultiCal®, GERMANY
Power Supply:	Biorad, PowerPac 300, USA
	Wealtec, Elite 300, USA
Refrigerator:	+4o C, Bosch, TURKEY
Shaker:	Stuart® Gyratory rocker, SSL3, USA
Thermocycler:	Eppendorf, Mastercycler Gradient, GERMANY
Water Bath:	Huber, Polystat cc1, GERMANY