PRAMANICIN-A INDUCES APOPTOSIS IN HCT116 COLON CARCINOMA CELLS: ACTIVATION OF JNK, P38, ERK1/2 AND INDUCTION OF OXIDATIVE STRESS

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Abstract

Pramanicin (PMC) is a novel anti-fungal agent. In this study, among eight analogues screened by MTT cell proliferation assay for their potential cytotoxic effect on HCT116 colon cancer cells, Pramanicin-A and Pramanicin-F were found to be the most effective candidates at the concentration of 25uM and 75uM respectively. Flow-cytometric analyses with Annexin-V staining and M30 apoptosense eliza assay confirmed that PMC-A is a more effective apoptotic agent compared to PMC-F, thus PMC-A was selected for further studies. Moreover, no difference in cytotoxicity was observerved in puma and bax defficient cell lines, therefore further studies were conducted with HCT116 wild type cells. In order to get insight into the mechanism of apoptotic response by PMC-A, we followed MAP kinase pathways with using specific MAPK antibodies and inhibitors. Our immunoblotting data reveals that PMC-A induced the activation/phosphorilation of c-jun terminal kinase (JNK), p38 and extracellular signal-regulated kinases (ERK1/2) in different time kinetics. Inhibition of caspase-3, and caspase-9 with their specific inhibitors prevent apoptosis. Interestingly inhibition of JNK and p38 activations/phosphorilations potentiated the apoptotic response. These data indicate that PMC-A induced apoptosis is mediated by caspase dependent pathways, activation of JNK and p38 but not ERK 1/2 may have a pro-survival role.Finally our data from flow-cytometric and flourometric analyses with D2CDF-DA staining revealed induction of reactive oxygen species (ROS) acting as second messengers which may activate MAPK signaling pathways as well as other signaling pathways in apoptotic response of cells to PMC-A at early (1h, 2h) and late (24h) time points.

ÖZET

Pramanicin yeni bulunan bir anti-fungal ajandır. Bu çalışmada, HCT116 kolon kanseri hücrelerinde, sekiz analoğun MTT proliferasyon yöntemi kullanılarak sitotoksik potansiyelleri taranmıştır. PMC-A (25uM) ve PMC-F (75uM), bu analoglar içerisinde en güçlü potansiyele sahip ajanlar olanlar olarak bulunmuştur. Flowsitometrik ve M30 apoptosense eliza yöntemine dayanarak elde edilen sonuçlar PMC-A' nın PMC-F' e gore daha güçlü bir apoptotic ajan olduğunu ortaya koymaktadır. Bu sebeple çalışmanın tümünde PMC-A kullanılmıştır. Bununla birlikte bax ve puma knock-out hücreler ile yapılan çalışmalarda, sitotoksisite bakımından anlamlı bir fark bulunamamış ve çalışmanın tamamında normal tip HCT116 hücreleri kullanılmıştır. Daha ileriki aşamada PMC-A' nın apoptotic etkisinin moleküler düzeyde araştırılması için, MAPK yolakları spesifik antikor ve inhibitorler kullanılarak araştırılmıştır. Yapılan immunoblot çalışmalarında PMC-A' nın JNK, p38 ve ERK1/2 üzerinde farklı zaman kinetiklerine uygun olarak aktive edici etkisinin olduğu ortaya çıkarılmıştır. Kaspaz-3 ve Kaspaz-9' un spesifik inhibitorler ile baskılanması apoptozu başarılı bir biçimde durdururken, tam ters bir etki ile JNK ve p38 aktivasyonlarının inhibisyonları apoptozu potansiyelize ederek daha fazla hücrenin ölmesine yol açmıştır. Bu sonuçlar göstermektedir ki, PMC-A kaynaklı apoptoz Kaspaz yolaklarına bağlı iken, JNK ve p38' in içerisinde bulundukları yolaklar hücrenin yaşaması ile ilişkili yolaklar ile ilişkili olabilir. Bununla birlikte ERK1/2' nin inhibisyonu ne apoptoz nede sağ kalım üzerinde herhangi bir etki oluşturmamaktadır. Son olarak, çalışmamızda florometrik ve flowsitometrik D2CDF-DA boyama yöntemi kullanılarak yapılan çalışmalarda, PMC-A'nın MAPK yolakları gibi birçok yolağı aktive edebilen veya kendi başına bir ikincil haberci gibi davranarak apoptoz oluşumunda rol alabilen reaktif oksijen ürünlerinin oluşumuna aracılık ettiği hem erken (1saat, 2saat) hemde geç zaman (24saat) dilimlerinde ortaya konulmaktadır.

"To my family and my soul"

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TABLE OF CONTENTS

TABLE OF ABBREVIATIONS

AP-1: Activator protein-1

Apaf-1: Apoptosis protease activating factor-1

ASK: Apoptosis stimulating kinase

Bak: Bcl-2 antagonist/killer

C-terminus: Carboxyl terminus

DCHF-DA: Dichlorodihydrofluorescein diacetate

ER: Endoplasmic reticulum

ERK: extracellular signal-regulated kinase

FADD: Fas-associated death domain

GST: Glutathione S-transferase

HOCl: Hypochlorous acid

HSP: Heat shock proteins

JNK: c-Jun N-terminal kinase

MAPK: Mitogen-activated protein kinase

MW: Molecular weight

NAC: N-acetyl-cystein

N-terminus: Amino terminus

OH: Hydroxyl radicals

PARP: Poly-(ADP-ribose) polymerase

PDGF: Platelet derived growth factor

PMC: Pramanicin

PKC: Protein kinase C

SOD: Superoroxide dismutase

ROS: Reactive oxygen species

TNF: Tumor necrosis factor

TRAIL: TNF-related apoptosis-inducing ligand

LIST OF FIGURES

LIST OF TABLES

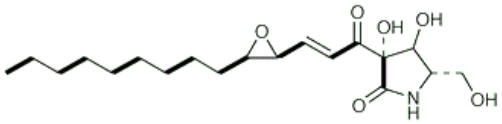
1 INTRODUCTION

Pramanicin (PMC) is a recently discovered antifungal molecule with an aliphatic side chain and a polar head group. It was synthesized in Stagonospora Sp. cultured in liquid medium. After seven days of culturing, the cultures were centrifuged and the supernatant extracted with methyl ethyl ketone. After concentration, the organic extracts were purified by column chromatography (SiO2, 10% MeOH/EtOAc). Final purification was done with MPLC on a Merck LOBAR RP-8 column in MeOH–H2O (70:30), finally giving approx. 75 mg of pramanicin, as previously described in [1]. PMC has ten different analogues with different molecular structures as shown in Figure 1.1. Minimal inhibitory concentrations of 20-100uM is found effective in growth-inhibition on fungal organisms. It has been previously shown that PMC increases cytosolic calcium concentration and induces cell death in endothelial cells [2]. Also Pramanicin was recently found as an apoptotic agent which acts through MAPK pathways and activates specific caspases in jurkat leukemia cells [16]. Targeting apoptotic machinery components and restoring apoptotic regulators that are impaired is nowadays seem the best approach for treating cancer. Day by day knowlegde of mechanisms of the apoptosis and how its regulated is being increased, many of the novel approaches to target the apoptotic cascades are being tried on many cancer therapies in vivo and in vitro. Many of these are still in preclinical stage, some of them are showing a good potential and are progressing into the clinical stage, [3], In cancer therapy we are using very powerful weapons such as chemotherapy and radiotheraphy, many drugs are investigated and produced for killing cancer cells by targeting the apoptotic machinery, although many patients could take advantage of these chemotherapautics and their cancer could be regressed by using those drugs, but it must not be forgotten that all those drugs have many side-effects including forming new cancer cells within body. Maybe the worst part of the chemotherapy is effected normal proliferative cells such as intestine and epidermal cells in body just because of unspecificity of the cancer drugs. Now it seems that we are quite far away from finding a drug that is only specific to cancer cells. But in future, in the light of the scientific advances, of course we will have better drugs to fight against the cancer without disturbing the normal and functional cells within body. Apoptosis is mediated by the signaling cascades, there are two well-characterized apopotic pathways, one of them is initiated through the interactions of the surface death receptors

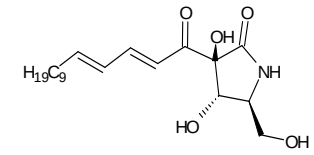
and their ligands and other one is related to changes in internal cellular integrity. These two distinct pathways generally activate the caspases and cause apoptosis, briefly cleavage of initiator caspases and the effector caspases has been defined the typical features of apoptosis in response to death receptor activation or intracellular insult. The apoptotic machinery highly depends on the finaly tuned protein-protein interactions and protein modifications after intrinsic and extrinsic pathways activation. Most of the chemotherapeutics target the regulator proteins of the apoptotic machinery and give us an opportunity to eliminate the cancer cells from the body. Intrinsic and extrinsic pathways and the regultor proteins have been broadly defined [4]. One of the apoptosis mediating signaling pathway is defined mitogen-activated protein kinases (MAPK) pathway. In mammals there are three different groups of mitogen-activated protein kinases (MAPKs), many types of stress can activate c-Jun N-terminal kinase and p38 MAPKs like UV radiation, growth factor withdrawal and pro-inflammatory cytokines [5,6]. After activation through a dual tyrosine/threonine phosphorilation mechanism by their upstream kinases, p38 and JNK phosphorilate many transcription factors like c-Jun, p53 and ATF-2 [7]. JNK and p38 kinases have been shown to function pro-apoptotic or anti-apoptotic in many different studies [8, 9, 10, 11]. Even if apoptosis is not seen in response to many stimuli, JNK and p38 MAP kinases activations were shown [12, 13]. The other MAP kinase called extracellular signal regulated kinases (ERK1/2, p42/p44 kinases) is activated by growth factors and mitogenic stimuli [14, 15]. There have been intensive work on MAP kinases and they were reported that they can function in response to many stimuli and they can be involved pro-apoptotic and anti-apoptotic pathways that decide cells to go apoptosis or to survive. Their function is determined by cell type, stimuli origin, duration, initial magnitude, further amplification of the activated signal trunsduction pathway, co-activation of other signaling pathways. Also they have reported that they can be also activated without apoptosis in response to many stimuli. Thus the activation patterns of MAP kinases must be carefuly evaluated in their pro-/anti-apoptotic properties in the light of the above parameters [12, 13, 16]. Oxidative stress is also one of the activator of the molecular signaling cascades and apoptotic machinery in the cells. Oxidative stress can be described as an increase in the reduction potential or a large decrease in reducing capacity of the cellular redox couples. Production of reactive oxygen species (ROS) which include free radicals and peroxides can be so harmful for the cells. However some of the ROS species are less reactive then others, they can be easily converted to more reactive and dangerous species by oxidoreduction reactions with

transition metals. These highly active ROS species can cause extensive cellular demage. In animals, ROS may influence cell proliferation, cell death, and expressions of some ROS related genes, activation of several signling pathways, acivation of cellular signalig cascades such as those involving mitogen activated protein kinases. Most of the ROS species can be produced by aerobic metabolism at a low level from mitochondria and can be easily repaired by some of the spesific mechanism in the cells. In cells, there are enzymes and antioxidants which preserve the cellular redox environment and maintain the reduced state through a constant input of metabolic energy [17]. There has been a broad research on oxidative stress and its functions to activate the molecular signaling cascades. Oxidative stress is also one of the major causes of apoptosis which is involved many diseases like diabetes and neurodegenerative diseases. It is so important to understand the mechanism of oxidative stress to eliminate the problems which threat the human health.

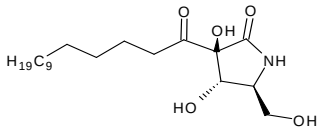
In this study, we investigated three major topics, Potential apoptotic effect of PMC-A in HCT116 cells, activation of MAPK pathway by PMC-A and finally induction of ROS production in response to PMC-A treatment. For this purpose, here we will focus on the apoptosis to understand the signaling mechanisims mediating this event. Then one of the most important apoptosis mediating pathway, MAPK pathway will be reviewed, finally reactive oxygen species production mechanisms and its relationship with MAPK pathway and apoptosis which could be mediated by MAPK will be discussed.



PMC



PMC-A



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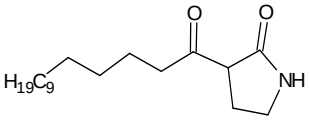

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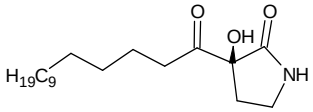
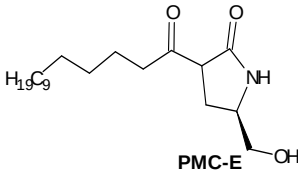
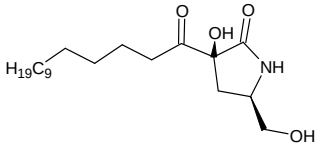


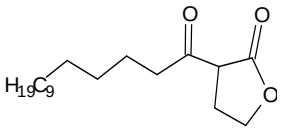
PMC-D



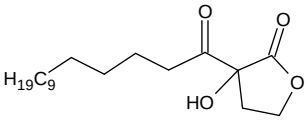
PMC-E



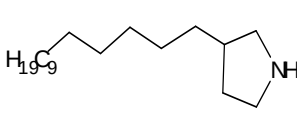
PMC-F



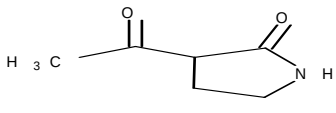
PMC-G



PMC-H



PMC-I



PMC-J

Figure 1.1 PMC and its analogues (PMC, PMC-A, PMC-B, PMC-C, PMC-D, PMC-F, PMC-G, PMC-H, PMC-I, PMC-J)

2 BACKGROUND

2.1 Apoptosis

Apoptosis is the Greek word for “falling off” or “dropping off” and describes the specific and unique changes in cells [18] . Apoptosis, first described by Kerr et al in 1972 [19], It is now seen as a potential target for cancer therapy during the past 30 years. When tumor cells are failured to undergo apoptosis, they progress into cells which have malignant potential and chemotherapeutic resistance. The process of apoptosis is a dynamic interplay of several molecules with upregulatory and downregulatory properties that is largely dependent on the cell type and the form of insult. No single factor in the machinery of apoptosis operates in isolation. Activation or inactivation of a single component alters the fate of the cell and lead them to programmed cell death named apoptosis. The typical executioners of apoptosis are the proteolytic enzymes called cysteinyl aspartate-specific proteases. Caspases are divided into two groups, the first one is initiator caspases (8 and 10), characterized by a long N-terminal, and the other is execution caspases (3, 6, and 7), characterized by a short N-terminal. Caspases 3, 6, and 7 are activated by way of two classical pathways: the extrinsic (death receptor) and the intrinsic (mitochondrial) (Fig. 2.1.1). In the extrinsic (death-receptor) pathway, binding of tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand treatment (TRAIL), or Fas ligands to their receptors, in association with adaptor molecules such as Fas-associated death domain (FADD) or TNF receptor–associated death domain, leads to cleavage and activation of initiator caspase 8 and 10, after that cleavage and activation of executioner caspases 3, 6, and 7, mediate apoptosis [20,21] . In the intrinsic (mitochondrial) pathway, proapoptotic proteins results in a net increase of free cytosolic cytochrome c. Once released, cytochrome c interacts with apoptosis-activating factor-1 (Apaf-1), adenosine triphosphate, and procaspase 9 to form the apoptosome. The apoptosome cleaves caspase 9 and activate it, which in turn caspases 3, 6, and 7 are activated, thus apoptosis is stimulated [22]. The relative permeability of the mitochondrial membrane to cytochrome c is determined by the ratio of proapoptotic and antiapoptotic mediators. When

proapoptotic molecules BAX and Bcl-2 antagonist/killer (Bak) (see Table 2.1.1 summary of molecules in the same family with similar functions) are translocated from the mitochondrial intermembrane, they cause a net increase of cytochrome c which in turn interacts with the apoptosome. The effect of these proapoptotic molecules is mediated by either altering mitochondrial membrane permeability; by coupling of proapoptotic molecules with antiapoptotic factors (ie, Bcl-2, Bcl-xL, and Mcl-1), thereby neutralizing their antiapoptotic actions; or a combination of these [23]. Therefore the relative ratio of proapoptotic and antiapoptotic mediators determines the relative amount of cytochrome c available to interact with the apoptosome. Release of second mitochondrial-derived activator (Smac/DIABLO) and Omi/HTRA-2 from the mitochondrial intermembrane neutralizes the actions of inhibitors of apoptosis (IAPs) such as cIAP1, cIAP2, and X chromosome–linked inhibitor of apoptosis (XIAP), thus causing a net stimulus of downstream caspases [24] . Synthesis and activation of IAPs are modulated by the transcription factor nuclear factor kappa beta (NF kB), NfkB is found in an inactive form in the cytoplasm bound to Ik B. Stimulation of apoptosis causes phosphorylation and subsequent degradation of I kB which in turn NF kB is freed. After that NFkB translocates into the nucleus and it mediates transcriptinal activation of IAPs [25] . For induction of apoptosis by chemotherapeutic agents, mitochondrial pathway is relatively more important than the death-receptor pathway. in particular, caspase 9–eficient cells and Apaf-1–negative thymocytes are resistant to chemotherapeutic agents, but induction of apoptosis can be mediated by Fas, TRAIL, or TNF (the death- receptor pathway) [26,27] . In contrast, embryonic fibroblast cells are FADD negative and caspase 8 negative which are resistant to apoptosis in response to death receptor pathway activation, yet they are still sensitive to cytotoxic drugs [28,29] . The death-receptor pathway, conversily, appears to be more important than the mitochondrial pathway in rendering cancer cells survival advantage by providing immune privilege. The mitochondrial and death-receptor pathways have crosstalks at various levels. Activation of caspase 8 causes stimulation of BH3-interacting domain death agonist, which in turn it leads to release of cytochrome c and apoptosome formation (in type II cells) [30] . Similarly, downstream stimulation of caspase 6 may in turn activate caspase 8 [31]. Both pathways can be regulated by heat shock proteins (HSP), which can have proapoptotic and antiapoptotic features [32]. Antiapoptotic HSP include HSP27, which inhibits release of cytochrome c, and HSP70 and HSP90, which bind to Apaf-1, thereby inhibiting the apoptosome.

Proapoptotic HSP60 and HSP10 directly stimulate caspase3 [33]. Other mechanisms of apoptosis which are independent of the caspase cascade have been described. The mediators involved are still not known well and have not been characterized, but they may involve proteases, apoptosis-inducing factors (AIF), endonuclease G, calpains, and cathepsins [34].

BAX	Bak, BAD
BID	BIM, PUMA, NOXA
Bcl-2	Bcl-xL, Mcl-1
cIAP-1	cIAP0, cIAP-2, ML-IAP, BRUCE, Survivin

Figure 2.1.1 Apoptotic molecules with similar functions [35].

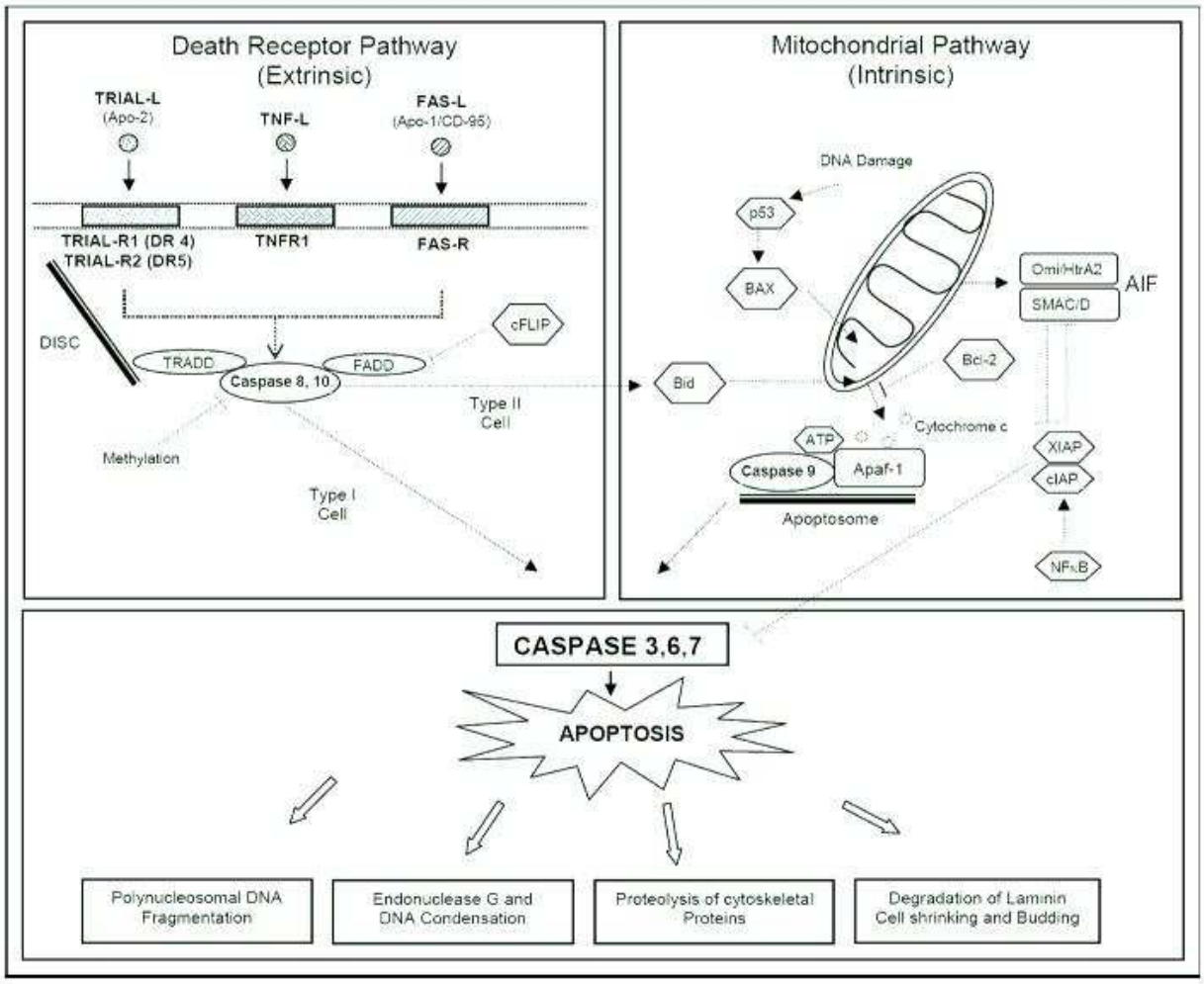


Figure 2.1.2 Mechanisms of apoptosis. The death receptor (extrinsic) and mitochondrial (intrinsic) pathway of apoptosis. (Arrow) Stimulatory effects. (Dashed line) Inhibitory effects[35].

2.2 Functions of MAP Kinases

Mitogen-activated protein kinases (MAPKs) are a family that control various cellular physiological functions of cells in number of organisms ranging from yeast to mammals, they highly conserved serine/threonine sites to be phosphorilated, these phosphorilations regulate their activities in response to various stimuli. As shown in Table 2.2.1 Gene-targeting studies have revealed their functions in vivo. In particular, embryos deficient in extracellular signal-regulated kinase (ERK2) 2 lack mesoderm differentiation and placental angiogenesis. Knockout mice of c-Jun amino-terminal kinases have revealed roles for these kinases in neural apoptosis and activation/differentiation of T cells. Deletion of p38α MAPK results in angiogenic defects in the placenta and peripheral vessels. ERK5-deficient embryos are embryonic lethal due to defects in angiogenesis and cardiovascular development. Also disease pathogenesis MAPKs are having a significant role, especially in cancer, thus working with MAPKs and reveal their functions in both cancer and healthy cases are so important to understand the mechanisms related to cancer progression and some diseases that MAPKs involved. Mitogen-activated protein kinases (MAPKs) are a family of highly conserved kinases. Serine and threonine residues of target protein substrates are phosphorylated by specific MAPKs and number of cellular activities including gene expression, mitosis, cell movement, metabolism, cell survival and apoptosis are regulated by them. MAPKs are one part of a three tiered cascade composed of a MAPK kinase (MAPKK, MKK, or MEK) and a MAPKK kinase (MAPKKK or MEKK). In mammals, at least four distinct groups of MAPKs have been recognized. Two extracellular signal regulated kinases (ERK1/2) are phosphorylated by specific MAPKKs, MKK1 and MKK2. MKK1/2 are known to be activated by upstream MAPKKKs such as RAF proteins whose functions are regulated by many growth factors and the proto-oncogene named RAS. In response to various stress stimuli, three JUN-amino-terminal kinases (JNK1/2/3) and four p38 protein kinases (p38α/β/γ/δ) are phosphorylated by MKK4/7 and MKK3/6, respectively. A number of MEKKs for JNKs and p38 protein kinases have been identified, some of which activate both JNK and p38 cascades [6,36]. Detailed signalling cascades for MAPK are reviewed in [37].

2.2.1 Extracellular-Regulated Kinase 1,2(ERK) Pathway

Mitogens and growth factors are activating mainly the ERK pathway (A-Raf, B-Raf, Raf-1→MEK1, 2→ERK1,2). This pathway has been related to cell growth, cell proliferation, and survival. Receptor-mediated activation of the small G-protein, Ras, is activating most of the ERK pathway signals. Ras is a membrane-bound protein activated by the exchange of GDP that is bound to Ras to GTP. The activation of Ras, thus requires the relations with proteins responsible for initiating GDP/GTP exchange to the membrane. Activated Ras, then recruits cytoplasmic Raf (MAPKKK) to the cell membrane for its activation. There are three mammalian serine/threonine Raf kinases: A-Raf, B-Raf, and Raf-1 (named as C-Raf). Gene knock-out studies in mice have showed that, these proteins have distinct biological functions. All three Raf proteins share the same downstream MAPKK substrate mitogen activated protein kinase kinases 1,2 (MEK1,2). MEK1,2 is activated by dual phosphorylation on two serine residues by Raf proteins. In addition, recent studies have shown evidence for Ras/Raf-independent activation of MEK1,2 by both p21 kinase (PAK) and MEKK1–3 kinases. MEK1 and MEK2 are named dual-specificity kinases and they share 80% amino acid sequence identity. ERK1,2 is activated by MEK1,2, specifically by phosphorylating a tyrosine and a threonine residue, separated by a glutamate residue (TEY) within the activation loop of the ERK protein (38,39). ERK1 and ERK2 share 85% amino acid identity and they are ubiquitously expressed. Active ERK1,2 can translocate into the nucleus, immediately after it activates various transcription factors, such as c-Fos, ATF-2, Elk-1, c-Jun, c-Myc, and Ets-1 shown in Figure 2.2.1.1 Activated ERK1,2 can also phosphorylate cytoplasmic and nuclear kinases, for example MNK1, MNK2, MAPKAP-2, RSK, and MSK1,2 (38,39). In mouse fibroblasts, serum-elicited ERK1,2 activation was originally shown to be required for proliferation and transformation. Moreover, it was shown that in human fibroblasts and mammary epithelial cells, Ras-mediated activation of Raf was identified as one of the requirements for transformation [40,41]. It was shown that, mutations of B-Raf, that increase the activity of the MEK1,2- ERK1,2 pathway, were revealed in several malignancies. and expression of such mutants in NIH3T3 cells lead to transformation. In particular, the B-Raf mutation V600E was detected in 70% of malignant melanomas, strongly supporting a positive role for ERK pathway activation in melanoma progression[42]. In addition to proliferation, ERK1,2-mediated

signaling also has a pivotal role mediating cell survival. For example, activated alleles of MEK1 and MEK2 mediate cell survival without survival factors. Dominant interfering mutants of MEK1 and MEK2 alleles disrupt cell survival signaling(reviewed in 43). Bonni A. Et al.(1999) proposed that ERK1,2-dependent survival signaling has been found to be mediated mainly through activation of RSK kinase. Active RSK phosphorylates, and thereby inactivates, the proapoptotic protein BAD. RSK can also activate the transcription factor CREB, which promotes cell survival through transcriptional up-regulation of antiapoptotic Bcl-2, Bcl-xL, and Bcl-1 proteins[44]. Moreover, Fas mediated apoptosis can be suppressed by ERK1,2 activity through inhibiting the formation of the death-inducing signaling complex (DISC) [45]. The utility of MEK-ERK pathway inhibition in cancer therapy was originally demonstrated by suppression of colon tumor growth in a mouse model by chemical inhibition of MEK1,2 [46]. In many other studies such as work by Rosen and collaborators using chemical inhibition of the B-Raf V600E chaperone Hsp90, have further proposed that MEK-ERK pathway inhibition as an attractive opportunity for cancer therapy.

2.2.2 C-jun N-Terminal Kinase (JNK) Pathway

C-Jun N-terminal kinase (JNK) pathway is mainly activated by cytokines and cellular stress. JNKs are acrivated by these stimuli through several upstream kinases (MAPKKKs), such as ASK1, HPK1, MLK-3, MKKK1–4, TAK-1, and TPL-2 [47,48]. MAPKKs, MKK4 and MKK7 are needed for JNKs fully activation. Both MKK4-/- and MKK7-/- mice are embryonic lethal [49,50], but in fibroblasts derived cell culture experiments, MKK4 and MKK7 knockout mice revealed that MKK7 mediates JNK inflammatory responses, and both MKK4 and MKK7 are crucial for stress-induced JNK activation [49]. Interestingly, a recent study provided evidence of an alternative pathway for JNK activation, through reactive oxygen mediated suppression of JNK phosphatase activity [51]. Three JNK genes—JNK-1, JNK-2, and JNK-3—are susceptible to alternative splicing, resulting in more than 10 JNK isoforms (47,48). As well as all other MAPKs,

JNKs activation needs phosphorylation of a tyrosine and a threonine residue, although specificity from the other MAPKs is ensured by the separating proline (TPY) within the activation loop of the kinase. JNKs has 85% sequence identity and are expressed ubiquitously. JNK pathway activity can function in indusing apoptosis, proliferation, or survival, depending on the stimuli and cellular conditions. Interestingly, sustained JNK activity is necessary for cellular homeostasis, whereas strong stress stimuli in non-transformed cells primarily leads to JNK mediated apoptosis. In JNK-knockout mice, the removal of any JNK isoform alone resulted in healthy and viable offspring, although some T cell abnormalities were observed in JNK1 and JNK2 knock-out mice [47,48]. Double knockout mice, lacking both JNK1 and JNK2, were embryonic lethal because of altered apoptosis during brain development (48,52,53). JNK3-/- mice showed differences in neuronal apoptosis compared to normal mice [54]. According to these findings demonstrate that JNKs isoforms has specific functional differences. The most classical JNK substrate is the transcription factor c-Jun, from which JNK derived its name. JNK can activate other transcription factors, such as ATF-2, Elk-1, MEF-2c, p53, and c-Myc. JNK also has other nontranscriptional substrates, for example the antiapoptotic proteins, Bcl-2 and Bcl-xL [47, 48].

2.2.3 P38 Pathway

The p38 MAPK pathway (MAPKKKs/MKK3,4,6/p38) can be activated by inflammatory cytokines, as well as pathogens and by environmental stress, including osmotic stress, ultraviolet light, hypoxia and heat shock. p38 MAPK pathway can also be activated by some mitogens, such as erythropoietin, colony stimulating growth factor 1, and granulocyte macrophage colony stimulating factor [reviewed in 55]. Considering the broad range of signals that can activate the p38 MAPK pathway, it is not unexpecting that several MAPKKKs can activate the p38 MAPK signaling module and that the specificity of activation may be determined by the stimuli. For instance, MTK1 cannot mediate cytokine signaling but can only stress signaling [55]. The p38 MAPK protein is represented by four isoforms: p38α, p38β, p38γ, and p38δ. Activation of all the p38 isoforms is needed dual phosphorylation of at hreonine and a tyrosine within the threonine-

glycine-tyrosine (TGY) sequence in the activation domain of the kinase [55, 56]. Activated p38 proteins can activate several transcription factors, such as ATF-2, CHOP-1, MEF-2, p53, and Elk-1. Importantly, p38 can also activate other kinases, including MNK1 and MNK2, MSK1, PRAK, MAPKAPK-2, and MAPKAPK-3. p38 MAPK pathway activation has a pivotal role for apoptosis induction in various cellular models [57-63]. In addition, stress-induced activation of p38 was shown that it causes G2/M cell cycle arrest and regulates the cell cycle through modulation of p53 and p73 tumor suppressor proteins [64,65]. Conversely,p38 MAPK pathway activity has been reported to promote cancer cell growth and survival. For instance, high p38 MAPK activation has been observed in some cancer types, as compared to their matched controls (66-68). p38 MAPK activity also correlated with the invasiveness of several cancer cell lines and inhibition of p38 activity reduced their proliferation, survival, and invasion [68]. The molecular mechanisms determining whether p38 signaling either promotes or inhibits cell proliferation and survival have not been elucidated but it could potentially be depend on the transformation state of the cell or could be related to the nature of p38-activating signal. Moreover, the p38 pathway has a pivotal role to regulate the expression of many inflammatory molecules, differentiation of epidermal keratinocytes, myoblasts, and immune cells, as well as mediates innate immune responses [69].

Genes	Summary of phenotypes
ERK1	Decreased T cell responses in the thymus
ERK2	Lack of mesoderm differentiation
	Defects in the placenta
JNK1	Defects in inhibiting Th2 differentiation
	Defects in T cell activation and apoptosis of thymocytes
	Less susceptible to insulin resistance in diabetes models
JNK2	Defects in Th1 differentiation
	Defects in T cell activation and apoptosis of thymocytes
JNK1+JNK2	Defects in neural tube closure
	Increased proliferation and IL-2 production in T cells
	Resistance to UV-induced apoptosis in embryonic fibroblasts
JNK3	Resistance to kainate-induced neural damage
p38α	Defects in placental angiogenesis
	Defects in Epo production
p38β	No obvious phenotype
p38γ	No obvious phenotype
ERK5	Defects in angiogenesis and cardiovascular development

Table 2.2.1 Summary of MAPK knockout phenotypes [6]

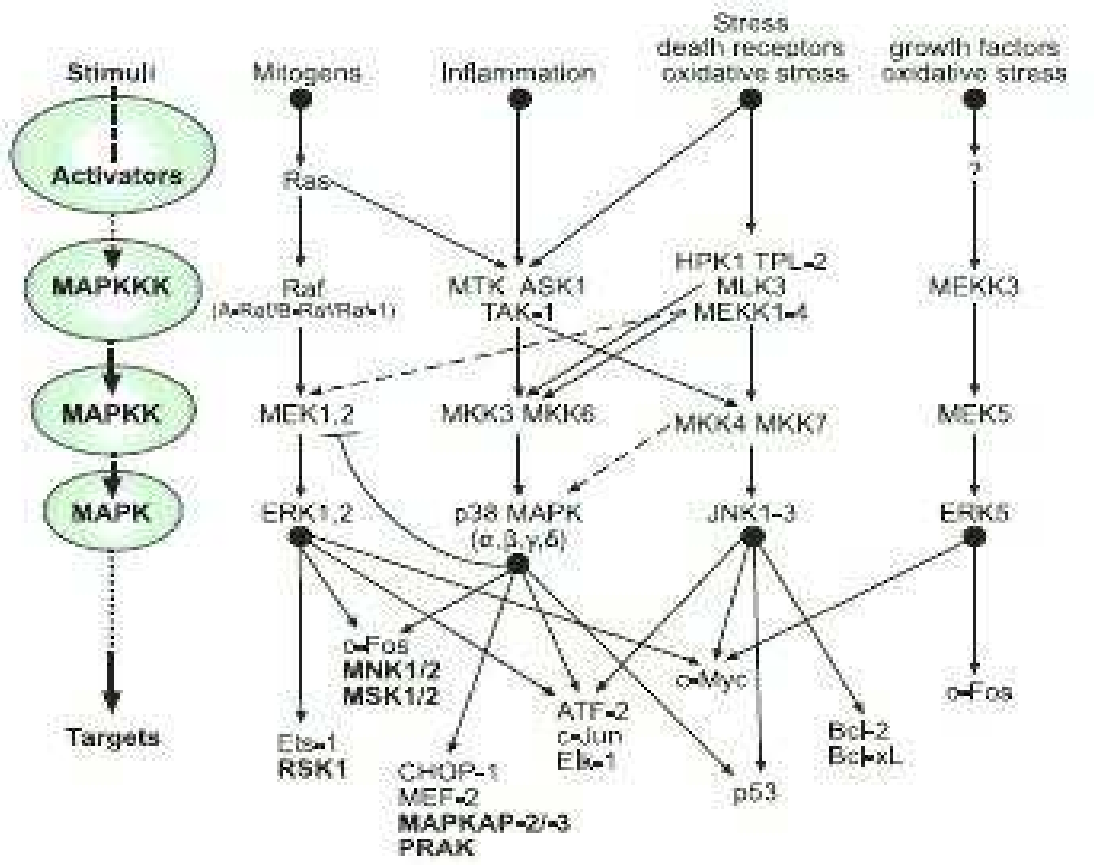


Figure 2.2.1.1. MAPK signaling pathways. MAPK signaling pathways are organized in modular cascades in which activation of upstream kinases by cell surface receptors lead to sequential activation of a MAPK module (MAPKKK - MAPKK - MAPK). Shown are the major MAPK pathway components and examples of the MAPK pathway target proteins. Target kinases are in bold. Dotted lines indicate context-dependent signaling connections between MAPK modules [37].

2.3 Oxidative Stress

Cells have to constantly deal with highly reactive and dangerous oxygen-derived free radicals. Defence mechanism against reactive oxygen species (ROS) involves antioxidant molecules and the antioxidant enzymes. ROS are generated by aerobic methabolism in cells and they are believed that they play a pivotal role in aging and several degenerative diseases. Finding of new specific genes and pathways related by oxidants showed us ROS function as a subcellular messenger in gene regulatory and signal trunsduction pathways. There are so many examples suggest that ROS effects on cellular compartments and regulatory levels shown in Table 2.3.1[70,71]. Growth factors, cytokines and several ligands can trigger ROS production in nonphagocytic cells upon their specific membrane receptors. Thus ROS production can be occured in a positive feedback mechanism on signal trunsduction from these receptors since intracellular signalling is often induced by ROS or pro-oxidative shift of the intracellular thiol/disulfide redox state. The reductive process is depend on the presence of transition metals, such as copper and iron, or specific enzymes (certain ozidases, monooxygenase) . This activation occurs in several cellular compartments such as mitochondria, microsomes, peroxysomes, and cytoplasmic membrane[72-76]. There are specific defense mechanisms called antioxidant defense system against the potential of the oxygen toxicity in cells. One of them works up the radical chain , inhibiting activation mechanisms. The other one neutralizes the free radicals already formed and stops the chain prrogression which involves some of the specific detoxifying enzymes, such as superoxide dismutase (SOD) and catalase. Some of the molecules act like a suicidal molecules or an anti-oxidant shield against ROS. Some of these molecules are in lipidic phase such like tocopherols, carotenoids, ubiquinones. Other molecules such as ascorbic acid, uric acid which are lipophobic are active in hydrated environment. When the defense mechanisms of the cells are weakened or excess production of ROS, a state of oxidative stress occurs. Thus many targets such as lipids, DNA,proteins are harmed by the radicals[72,77,78].

Source of ROS	*Cellular changes*	*Cell system*	*Biol. implication*	*Reference*
H_2O_2	↑adenyl cyclase	A10, murine vascular smooth muscle cells	Vasodilation	Tan *et al.*, 1995
H_2O_2	↑MAPK	Neonatal rat ventricular myeocytes	↑Na^+, H^+ exchange	Taher *et al.*, 1998; Sabri *et al.*, 1998
H_2O_2	↑BMK1/ERKs ↑c-*src*	Mouse fibroblasts		Abe *et al.*, 1997
H_2O_2	↑MAPK	Vascular smooth muscle cells		Hayashi *et al.*, 1998
Hypoxia/re-Oxygenation	↑JNK	Cardiac myeocytes		Laderoute and Webster, 1997
H_2O_2	↑ERK1/2, ↑BMK1	Rat vascular smooth muscle cells		Abe *et al.*, 1996 Warner *et al.*, 1996
H_2O_2 Doxorubicin	α actin, treponin I creatine kinase	Cardiocytes	Cardiotoxicity	Torti *et al.*, 1998
TNFα/H_2O_2	↑MnSOD, ↑NF-κB	Human pulmonary adenocarcinoma cells H441		
Aziridinylebenzoquinones or H_2O_2	↑$p21^{waf}$	HCT 116 colon carcinomas cells and K562 human chronic myelogenous leukemia cells	Apoptosis	Qui *et al.*, 1996
H_2O_2	↑MAPK, ↑p21ras	Jurkat T cells		Lander *et al.*, 1995
H_2O_2	↑ERK, ↑JNK, ↑p38	HeLa cells	Apoptosis	Wang *et al.*, 1998b
HIV-1		Jurkat and H9 Human T cells (Cd4)	Apoptosis	Bank *et al.*, 1998
ROS	↓SP1	Rat hepatoma	Regulating glycolytic enzymes in resting vs proliferating cells	Hamm-Kunzelmann *et al.*, 1997
ROS	↓Phospho-tyrosine, P105; p81	Human sperm	Sperm capacitation	Leclerc *et al.*, 1997
Sulfhydryl	↑c-Src		Mouse NIH3T3 fibroblasts	Pu *et al.*, 1996
Oxygen	Dimerized S100B	Glial cells	DNA synthesis	Scotto *et al.*, 1998
H_2O_2, NO	↑MAPK p42/p44	Rat hippocampal		Kanterewicz *et al.*, 1998
Oxidative stress	↑ras/MAPK/ERK2, ND	*In vitro*		Muller *et al.*, 1997
Oxygen	↑cAMP-dependent kinase I, II, ND	T lymphocytes	Lymphocyte activation	Dimon-Gadal *et al.*, 1997
Mitogenic stimulation	↑MAPK/ERK2, NF-κB/AP1	T lymphocytes		Goldstone and Hunt, 1997
N-acetylcysteine	↑JNK, ↑Jun ↑fos	Jurkat T lymphocytes		Gomez del Arco *et al.*, 1996
NO	↑MAPK ↑$p21^{ras}$ ↑NF-κB ↑ERK, ↑p38, ↑JNK (×100)	Jurkat T cells		Lander *et al.*, 1996
NO	PI3K (p110β/110γ) recruitment to $p21^{ras}$	Jurkat T cells		Deora *et al.*, 1998
NO	↓Angiotensin II activation of MAPK	Cardiac fibroblasts	Hormonal activation of mitogenic steps are ROS dependent	Wang *et al.*, 1998
UV	↓GST-p (24 h) ↑c-Jun (1–4 h) ↑c-*fos*	Rat keratinocytes		Nakano *et al.*, 1997
Paraquat	↑PKC ↑ODC	WI-38 human lung cells		Kuo *et al.*, 1995
Arsenite	MAPK, JNK, p38 AP1	CHO-k1	Apoptosis	Cavigelli *et al.*, 1996;
BPV-E5	↑NF-κB			Ludwig *et al.*, 1998
Asbestos	↑PKC, PLC PTK	Alveolar macrophages	Transformation	Kilk *et al.*, 1996
NO, thiol redox	↓JAK2/3	Ba/F3 cells		Lim *et al.*, 1997
Sodium orthovanadate	↑Syk, ERK2, ↑NF-κB		Proliferation	Duhe *et al.*, 1998
Oxidized dopamine	↑JNK ↓Bcl_2	PC12	Apoptosis	Krejsa *et al.*, 1997 Kang *et al.*, 1998
Thiuramdisulfides	↓cdk2, ↓Bcl2, ↑p53 ↓NF-κB ↓AP1	Hep G2 human hepatoma	Apoptosis	Lui *et al.*, 1998
Dopamine	↑JNK, Jun	293 primary neonatal cells	Apoptosis	Luo *et al.*, 1998
Oxidized LDL	↓PKC, PTK, ↑Fas ↓Bcl_2	Human coronary endothelial cells	Apoptosis	Li *et al.*, 1998
Cytoxic xenobiotics	↑MKK4, JNK, p38, ↑c-Jun, ↑c-*fos*			Wilhelm *et al.*, 1997
Angiotensin II	↑p38 ↑p42/44	Vascular smooth muscle cells	Vascular hypertrophy	Ushio-Fakai *et al.*, 1998
Diethyl-maleate	↑MAPK, ↑$p21^{waf}$	Neural cell lines PC12 GT10–7		Esposito *et al.*, 1997
BCL2	↑glutathione reduced ratio of oxidized glutathione, ↑superoxide dismutase ↑catalase			Ellerby *et al.*, 1996
TGFβ	↓Reduced glutathione	Primary fetal rat hepatocytes	↑Apoptosis blocked by cyclohexamide	Sanchez *et al.*, 1997
$p21^{rasV12}$	↓MAPK ↑Rac1 (no effect on JNK)	Mouse 3T3 fibroblasts	Transformation	Irani *et al.*, 1997
Hypoxia	↓MAPK, ↓c-*fos* ↓Elk1	HeLa		Muller *et al.*, 1997
Hypoxia	↓NF-κB not MAPK dependent	A549 human alveolar epithelial cells	Protection from apoptosis	Li *et al.*, 1997

Table 2.3.1 Protein kinases and transcription factors effected by ROS [70].

2.3.1 ROS Generation

Researchers have been interested in ROS for many years in all areas of biology. Originally ROS were found as being instrumental for mammalian host defense, and early work led to the characterization of the respiratory burst of neutrophils[79] and originally the NADPH oxidase complex[79,80], it is now recognized as a primary source of ROS. However, recent works have revealed a more widespread and exciting role of ROS: that of key signalling molecules. ROS are species of oxygen which are in a more reactive state than molecular oxygen, and by which therefore, oxygen is reduced to varying degrees. A primary ROS is superoxide (O2−), which is formed by the one electron reduction of molecular oxygen. This is the reaction catalysed by NADPH oxidase, with electrons supplied by NADPH[81]. Oxygen's further reduction produces hydrogen peroxide (H2O2). This can come from the dismutation of O2−, which can occur spontaneously, especially at low pH. However, this reaction can also be catalyzed by SOD enzyme family. Therefore, under physiological conditions, once O2− is formed the presence of H2O2 becomes almost inevitable. Further reactions may lead to the formation of hydroxyl radicals (OH), especially in the presence of metal ions through the Fenton or Haber±Weiss reactions[81,82]. Hydroxyl radicals are highly reactive and dangerous, and their halflife is very short, they generaly react with the first molecule nearby. In neutrophils, myeloperoxidase catalyses the formation of hypochlorous acid (HOCl), while O2− may also react with nitric oxide (NO) to form another relatively reactive molecule, peroxynitrite : $NO + O^{-}_{2} \rightarrow ONOO^{-}$ (peroxynitrite). It thus appears that, following the formation of superoxide anions, a cascade of ROS production is likely. Some of these ROS, especially H2O2 , are key signalling molecules, while others appear to be extremely detrimental to biological systems, effects that are dependent on the concentrations that are perceived by the cells. However, to be considered as a potential signalling molecule, ROS must: (a) be produced by a cell when stimulated to do; (b) have an action in a cell, either the cell which produces it or a nearby cell; and (c) be removed in order to turn off, or reverse, the signal[83].

2.3.2 ROS and Signalling Pathways

In many ways, ROS are ideal signalling molecules because of the small size, and they can diffuse short distances; there are several mechanisms of their production, some of them are rapid and controllable; and cells have several mechanisms for their quick removal. Many studies have showed a role for ROS in the induction or inhibition of cell proliferation, in both activation and inhibition of apoptosis, and, at higher concentrations, in the induction of necrosis[84-88]. Some of the biochemical effects of ROS on cells will be showed in Figure 2.1. It has been shown that several enzymes involved in cellular signalling mechanisms are potential targets of ROS. Some examples are; Several enzymes which are involved in cell signalling mechanisms are also potential targets of ROS. These include guanylyl cyclase [105], phospholipase C [106,107], phospholipase A2 [108–109] and phospholipase D [110], the latter again from direct attack of cysteine. Ion channels too may be targets [111,112], including calcium channels [113]. Signalling mechanisms that respond to changes in the thiol/disulfide redox state includes AP-1 transcription factor in human T cells, nuclearfactor κB (NF-κB) transcription factor in human T cells [114], control of K+ channel activity in the carotid body [115], human insulin receptor kinase activity [116], Src family kinases, JNK and p38 mitogen-activated protein kinase (MAPK) signalling pathways [117] and signalling in replicative senescence [118].

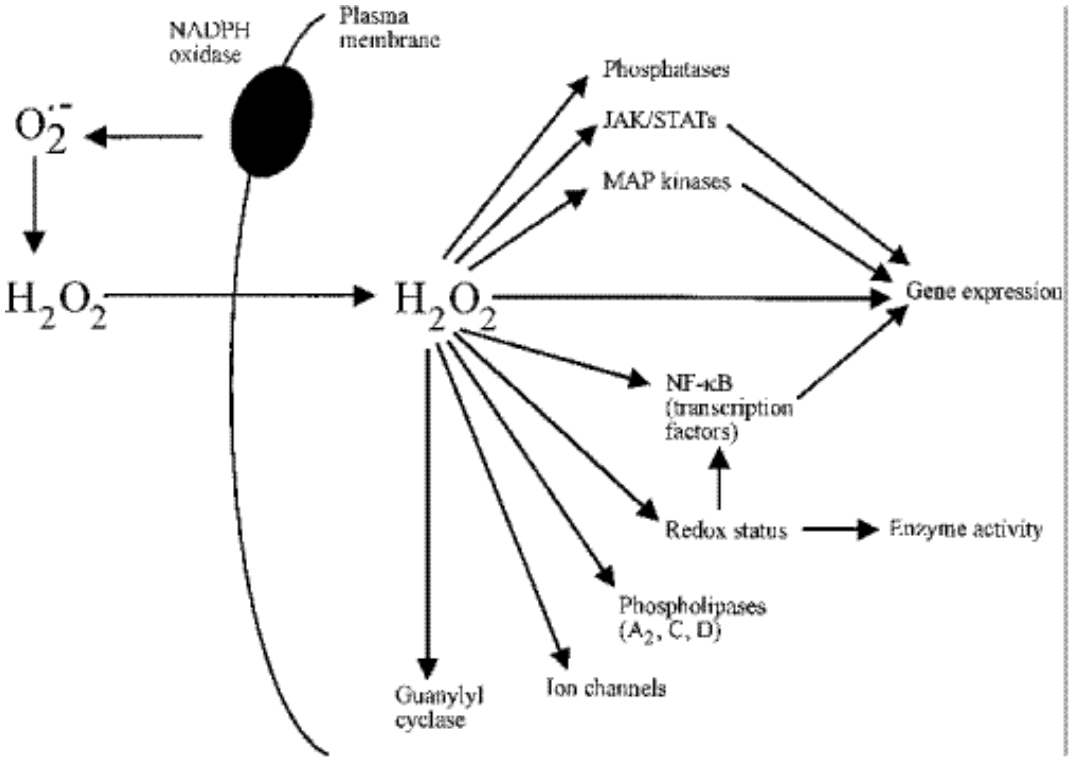


Figure 2.3.2.1 Potential intracellular signaling pathways mediated by NADPH oxidase

2.3.3 ROS, Gene Expression and Protein Phosphorilation

Reactive oxygen speices are involved in many problems that we face such as aging, degenerative diseases, diabetes mellitus. They are produced by all aerobic cells by many ways. Thanks to the usage of numerous molecular genetic techniques, expression of a wide range of genes is regulated by hydrogen peroxide have now revealed. [89]. It has been reported that the addition of H2O2 (or xanthine oxidase/xanthine) stimulated the expression of c-fos and c-myc[90], and increased expression of c-jun, egr-1 and JE has also been reported [91,92]. Other examples include increased expression of clones identified as fibronectin and p105 co-activator in rat aorta smooth muscle cells, as shown by subtractive hybridization [93]. If H2O2 is altering gene expression patterns in cells, how is this being achieved? Transcription factors have been shown to be activated by H2O2. Schreck et al. [94] showed that H2O2 activates the transcription factor nuclear factor-kB (NF-kB). NF-kB usually is in the cytoplasm of the cell in association with an inhibitor protein, Ik-B, but addition of H2O2 to cells results in the dissociation of NF-kB from Ik-B, and translocation of NF-kB to the nucleus. Other transcription factors affected by exogenous H2O2 include AP-1, Myb and Ets [95-97]. Reversible protein phospholiration is the one of key modulators in cellular signalling, and plays a critical role in many cellular metabolic processes in eukaryotes. In particular protein phosphorilation mediates numerous signal trunsduction pathways. For the balance of the intracellular signalling environment, protein phosphorilation must be reversible, thus phosphatases are involved in the phosphorilation process as well as protein kinases are involed in. Cellular target proteins are phosphorilated at specific cellular transduction sites(usually serine/threonine or tyrosine residues) by one of the protein kinases and the phosphates are removed by a specific phosphatase. ROS production is also an event which protein phosphorilations occur. Mitogen activated protein kinases are one of the key elements that can be regulated by phosphoilation in response to ROS production both in animals and plants, this phosphorilations of MAP kinases leads to gene expressions which regulates the cell response[98-101]. H2O2 is one of the rective oxygen species and H2O2 has been shown to inhibit phosphatases, probably by the direct oxidation of cysteine in the active site of these enzymes [102-103]. The JAK/STAT (Janus kinase/signal transducers and activators of transcription) pathways in animal cells are also activated by H2O2 [104], these findings suggest that H2O2 may transduce its message into the nucleus by at least two signal transduction

pathways. H2O2 may be sythesized endogenously in certain cell types as a response to activation by specific cytokines or growth factors. this endogenous H2O2 then plays as a second messenger role to stimulate protein kinase cascades related to inflammatoryvgene expression, or in control of the cell cycle[119]. In vascular smooth cells it was shown that after stimulation of platelet derived growth factor(PDGF), PDGF receptor binding caused peroxide formation which could be inhibited by the catalase. catalase expression inhibited PDGF signal trusduction by supressing protein tyrosine phosphorilation. Anti-oxidants, especially thiol-reducing agents such as N-acetyl- cystein, could mimic the inhibitory effects of catalase and prevent redox activation of ligand-coupled protein kinase cascades. Exposure to high concentrations of H2O2 or strong pro-oxidative changes in the intracellular thiol/disulfide redox state will generally lead to increased tyrosine phosphorilation in numerous proteins[120-123]. DNA demage is also seen in oxidative stress and it is one of the most published area. One of the most dangerous radical is hydroxyl radicals which arise either from the radiolysis of water by ionizing radiation, or from purely chemical source. Also metal-bound oxyl radicals are active intermediates in DNA demaging reactions and may be formed from syntetic compounds or from natural products such as bleomycin which is also used for cancer therapy[124,125]. Free radicals can cause DNA demage which can result in deletirous biological consequences such as the initiation and promotion of cancer. Thus characterization and quantitation of free radical induced DNA demage is really important for understanding of its biological consequences and cellular repair. Methodologies incorporating the technique of gas chromatography/mass spectrometry (GC/MS) have been developed in recent years for measurement of free radical induced DNA damage. The use of GC/MS with selected-ion monitoring (SIM) facilitates unequivocal identification and quantitation of a large number of products of all four DNA bases produced in DNA by reactions with hydroxyl radical, hydrated electron, and H atom. Hydroxyl radical induced DNA-protein cross-links in mammalian chromatin, and products of the sugar moiety in DNA are also unequivocally identified. The sensitivity and selectivity of the GC/MS-SIM technique enables the measurement of DNA base products even in isolated mammalian chromatin without the necessity of first isolating DNA, and despite the presence of histones[126,127]. Redox status inside the cell is crucial for the correct enzymatic activity, this redox status can alter the enzymatic activity and its thought that alterations of the redox status could acct as a signalling mechanism. One of the most impotant redox-sensitive molecule is glutathione(GSH), GSH forms the GSH-GSSG couple.

Changes in GSH-GSSG status have been measured after cell stimulation. GSH content can be lowered by H2O2 , then redox status is altered and so propogation of a signal is induced by H2O2 through this route. It is suggested that some of the enzymes such as ribonucleotide reductase and thioredoxin reductase and some of the transcription factors might be among the targets for altered redox status. GSH does not only act as an anti-oxidant, also it can modulate the activity of the various proteins via S-glutathionylation of cysteine sulfhydril groups. Also the thioredoxin system works with GSH system via reducing inter- and intrachain protein disulfide bonds as well as maintaining the activity of mportant antioxidant enzymes such as peroxiredoxins and methionine sulfoxide reductases[128-130]. NfkB/rel family also was shown to be activated not only by receptor-targeted ligands but also by direct application of oxidasing agents, particularly H2O2, or ionizing radiation[131,132].

2.3.4 ROS and MAPK Cascade

MAP kinase sinalling cascades are regulated by phosphoriltion and dephosphorilation on serine and or/threonine residues and they respond to stimulation of receptor tyrosine kinases, protein tyrosine kinases, receptors of cytokines and gowth factor, and heterotrimeric G protein-coupled receptors[133-136]. There are currently four known MAPKs: the extracellular regulated kinase (ERK1/2), the c-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK), the p38, and the big mitogen-activated protein kinase 1 (BMK1). These pathways can be defined by a dual-phosphorylation motif that is required for activation: Thr– Glu– Tyr, Thr– Pro– Tyr, Thr–Gly–Tyr, and Thr– Glu– Tyr, for ERK1/2, JNK, p38, and BMK1,respectively[137,138]. In MAP kinase cascade, Each MAP kinase pathway has reltively distinct upstream mediators and specific, although multiple, substrates[139]. In many studies, it was shown that JNK and p38 are strongly activated by ROS or by a mild oxidative shift of the intracellular thiol/disulfide redox state[140-146]. The extracellular signal-regulated kinase 1 (ERK-1) and ERK-2 were found to be activated in vascular smooth muscle cells by O2− but not by H2O2[147]. Angiotensin II induces the

production of O2− and H2O2 and it activates ERK-1, ERK-2, and p38 MAPK [148]. Platelet-Derived Growth Factor Receptor (PDGF) was found to induce the activation of ERK-1 and ERK-2. JNK and p38 MAPK were shown to be activated by H2O2 in perfused rat hearts. The redox sensitivity of JNK and p38 MAPK is depend on the oxidative activation of upstream tyrosine kinases called the Src family [148-151]. Numerous of cellular stimuli like inflammatory cytokines and environmental stresses can activate the c-Jun NH(2)-terminal kinase (JNK), ROS also cause activation of JNK [152,153]; however, the signalling cascade that leads to JNK activation remains to be elucidated. Because recently it was reported that expression of Cas, a putative Src substrate, stimulates JNK activation, it was thought that [152] the Src kinase family and Cas would be related to JNK activation by ROS. An essential role for both Src and Cas was demonstrated. First, the specific Src family tyrosine kinase inhibitor, PP2, inhibited JNK activation by H2O2 in a concentration-dependent manner but it had no effect on extracellular signal-regulated kinases 1 and 2 and p38 activation. Second, JNK activation in response to H2O2 was completely inhibited in cells harvested from transgenic mice deficient in Src but not Fyn. Third, expression of a dominant negative mutant of Cas stops H2O2-mediated JNK activation but there is no effect on extracellular signal-regulated kinases 1 and 2 and p38 activation. The importance of Src was supported by the inhibition of both H2O2-mediated Cas tyrosine phosphorylation and Cas. Crk complex formation in Src−/− but not Fyn−/− cells [151,152]. The JNK/glutathione S-transferase Pi (GSTp) complex has also been characterized as a redox-responsive signalling element. In normal growing cells of the mouse fibroblast cell line 3T3-4A, JNK is related with and catalytically inhibited by glutathione-S-transferase (GSTp). Complex formation between GSTp and JNK limits the degree of Jun phosphorylation under normal conditions. Exposure to low micromolar H2O2 concentrations causes the oligomerization of GSTp and the dissociation of the GSTp-JNK complex, supporting that JNK inhibition requires monomeric GSTp [153]. There is another kinase called apoptosis signalling kinase-1 (ASK1) plays a role in the activation of MKK3/6, MKK4/MKK7 and the MAP kinase species including p38, JNK. This leads to the phosphorilation of some transcription factors such as ATF-2, c-Jun and p53[154,155]. p38 MAP kinase pathway is a potential target of antioxidant antagonism in inflammatory diseses. p38 MAP kinase plays a role in expression of some of the cytokines such as IL1B, iNOS and COX-2[156-158].

2.3.5 ROS and Apoptosis

Apoptosis is a mechanism that can be described as a cellular suicidal process in response to various stimuli, ROS formation is one of the stimuli that can trigger apoptosis. Apoptosis also can be seen to regulate the body homeostasis in normal circumstances especially in developmental stage. An increase of ROS is generally seen in the apoptotic process triggered by such stimuli as APO-1/Fas/CD95 ligands. however some authors described that triggering of APO-1/Fas/CD95 ligands does not induce ROS production[159,160]. Also some authors observed membrane changes typical for apoptosis in the absence of ROS, these findings suggest that pro-oxidative conditions are not a general prerequisite for apoptotic cell death.[161].

3 PURPOSE OF THE STUDY

Pramanicin is a newly synthesized anti-fungal agent which has been shown to cause cell death and Calcium release in vascular endothelial cells by Kwan et al. (2003). Another study has recently shown its apoptotic effect in jurkat leukemia cell line in our laboratory by Kutuk et al. (2005) In the light of previous studies, Pramanicin analogues (PMC-A, C, E, F, G, I, H, J) have been screened by MTT assay and the most effective analog, PMC-A, has been studied mechanistically for a possible apoptotic effect in HCT 116 cells. In this study we tried to reveal;

- The efficiency of pramanicin analogues and their time and dose kinetics in inducing apoptosis in HCT 116 cells.
- The role of MAPK in this event by using of specific antibodies and inhibitors by immunoblotting and flowcytometric analyses with annexin-V staining.
- The effect of caspase 3, 9 and general caspase inhibitors in apoptotic signaling.
- To identify the role of ROS as second messenger in apoptotic signaling of PMC-A.

4 MATERIALS AND METHODS

4.1 Materials

4.1.1 Chemicals

(in alphabetical order)

Name of Chemical	Supplier Company	Catalog Number
Acetic acid	Sigma, Germany	A9967
Acrylamide/Bis-acrylamide	Sigma, Germany	A3699
Ammonium persulfate	Sigma, Germany	A3678
Antibiotic solution	Sigma, Germany	P3539
Bradford solution	Biorad Inc.,USA	500-001
Chloroform	Merck, Germany	102431
CM-H2DCF-DA	Molecular Probes, USA	C-6827
Coomassie Brilliant Blue	Merck, Germany	115444
DMSO	Sigma, Germany	D2650
EDTA	Riedel-de Haén, Germany	27248
Ethanol	Riedel-de Haén, Germany	32221
Foetal Bovine Serum	Sigma, Germany	F2442
Glycerol	Riedel-de Haén, Germany	15523

Glycine	Amnesa, USA	0167
HCl	Merck, Germany	100314
Hepes	Sigma, Germany	H7006
Hyperfilm ECL	Amersham Bio., UK	RPN2114K
Isopropanol	Riedel-de Haén, Germany	24137
KCl	Fluka, Switzerland	60129
KH2PO4	Riedel-de Haén, Germany	04243
KOH	Riedel-de Haén, Germany	06005
Liquid nitrogen	Karbogaz, Turkey	
2-Mercaptoethanol	Sigma, Germany	M370-1
Methanol	Riedel-de Haén, Germany	24229
MgCl2	Sigma, Germany	M9272
Milk Diluent concentrate	KPL, USA	50-82-00
NaCl	Riedel-de Haén, Germany	13423
NaO2C2H3.3H2O	Riedel-de Haén, Germany	25022
NaOH	Merck, Germany	106462
NaPO4H2	Riedel-de Haén, Germany	04269
NP-40	Sigma, Germany	I3021
PD98059	Calbiochem, USA	513000
Phenol	Applichem, Germany	A1153
Phenol/chloroform /isoamylalcohol	Applichem, Germany	A0889
Phosphate buffered saline	Sigma, Germany	P4417
PMSF	Sigma, Germany	P7626

Propidium iodide	Sigma, Germany	P4170
SB203580	Calbiochem, USA	559389
SP600125	Calbiochem, USA	420123
Sodium Dodecyl Sulphate	Sigma, Germany	L4390
TEMED	Sigma, Germany	T7029
Triton X-100	Applichem, Germany	A1388
Tris	Fluka, Switzerland	93349
Tween® 20	Merck, Germany	822184

All chemicals used in this study were purchased from, Amresco, Applichem, Fluka(Switzerland), Calbiochem(USA), Merck(Germany), Riedel de Haen(Germany), Sigma-Aldrich(Germany) otherwise indicated. Pramanicin Analogues (PMC-A and others) were synthesized and sent from McMaster University, Canada.

4.1.2 Antibodies

p44/42 MAP Kinase (137F5) Rabbit mAb # 4695 (Cell Signaling)

SAPK/JNK (56G8) Rabbit mAb # 9258 (Cell Signaling)

p38 MAP Kinase Antibody # 9212 (Cell Signaling)

Phospho-p44/42 MAPK(Thr202/Tyr204)(D13.14.4E)Rabbit mAb#4370(Cell Signaling)

Phospho-p38 MAPK (Thr180/Tyr182) Antibody # 9211 (Cell Signaling)

Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) Rabbit mAb # 4668 (Cell Signaling)

Anti-rabbit IgG, HRP-linked Antibody # 7074 (Cell Signaling)

4.1.3 Commercial Kits

M30-Apoptosense™ ELISA Kit Peviva AB

ECL Advance Chemiluminescence Amersham Biosciences , Detection Kit

Cell Proliferation Kit I (MTT) Roche, Germany

ECL Kit (Amersham Pharmacia)

4.1.4 Cells

HCT 116 Wild Type Colon Cancer Cells

4.1.5 Buffers and Solutions

4.1.5.1 Cell Culture Media, Additions and Other Solutions for Cell Culture

MACCOYs 5A medium with %5 L-glutamine

Sterile FBS (Sigma)

Penicillin-Streptomycin (Sigma)

Sterile 1X PBS (Biological Industries)

Sterile Tyripsin (Biological Industries)

Sterile DMSO (Sigma)

L-Glutamine (Sigma)

4.1.5.2 Western Blotting Buffers

1X Tris-Glycine-SDS (sodium dodecyl sulfate) buffer was used for polyacrylamide gel electrophoresis. Gels were run at constant voltage, 100 mV, for about 1 hour. Transfer buffer (Tris base, Glycine and methanol) was used for blotting the proteins into PVDF membrane. The membranes were blocked with blocking solution, 5% milk powder in PBS-Tween 20 (0,25%) and washed with washing buffer, PBSTween 20 (0,25%). The antibodies were diluted in 5% milk diluent, 10% PBS-Tween 20 (0,25%) and 80% sterile distilled water.

1X Running Buffer Tris-Glycin-SDS (10X Stock)

1X Transfer Buffer Tris-Glycin (10X Stock)

2X Laemli Buffer (Fermentas)

10X PBS

1X PBS-Tween (%0,2 Tween 20)

4.1.6 Equipment

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

4.2 Methods

All methods used in this study are described below;

4.2.1 Cell Culture

HCT116 colon cancer cells were cultured in MACCOYs 5A medium with %5 L-glutamine supplemented with 10% heatinactivated FBS, 100 IU/ml penicillin and streptomycin. Cultures were maintained in 37°C in a humidified 5% CO2 atmosphere. Cells were seeded in 6-well culture plates (1 x 106 cells/well), 60 mm culture flasks (1x 107 cells/well) or 96-well plates (1 x 104 cells/well) and treated as indicated in the experimental protocols. Ethanol was added to all control wells in each experiment. For cryopreservation, cells were trypsinized and resuspended in complete medium containing 10% heat-inactivated FBS and 10% DMSO (freezing medium). The cell suspension in freezing medium transferred into cryovials, frozen at -70 º C for 24 hours, and then stored in liquid nitrogen to remain until thawing.

4.2.2 Cryopreservation

For cryopreservation, cells were trypsinized and resuspended in complete medium containing 10% heat-inactivated FBS and 10% DMSO (freezing medium). The cell suspension in freezing medium transferred into cryovials, frozen at -70 º C for 24 hours, and then stored in liquid nitrogen to remain until thawing.

4.2.3 Pramanicin-A(PMC-A) and PMC-F Treatment

HCT116-wt colon cancer cells were seeded 200.000cells/well to 6-well plates, then cells were treated with PMC-A(25uM from 20mM stock) and PMC-F(75uM from 20mM stock) at %80 confluency for 30min,1h,2h,4h,8h. PMC-A and PMC-F were delivered in lyophilized powder and they were dissolved in ethanol for stock solutions, 2,5ul of each pramanicin analogue were applied to wells, 2,5ul ethanol was added to controls. At each time points mediums were discarded and proteins were isolated as described below.

4.2.4 Total protein isolation

Treated and control HCT116 wild-type(wt) cells were harvested, washed with ice-cold phosphate buffered saline and lysed on ice in a solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, Nonidet P-40 0.5%, (v/v), 1 mM EDTA, 0.5 mM PMSF, 1mM DTT, protease inhibitor cocktail (Complete from Roche, Mannheim, Germany) and phosphatase inhibitors

(Phosphatase inhibitor cocktail 1 and 2, Sigma, Darmstadt,Germany). After cell lysis, cell debris was removed by centrifugation 15 min at 13000*g* and protein concentrations were determined with Bradford protein assay.

4.2.5 Protein Content Assay

Protein concentrations were determined with DC protein assay (Biorad). 3 - 5 dilutions of a protein standards containing from 0.2 mg/ml to about 1.5 mg/ml protein were prepared. (Note: A standard curve should be prepared each time the assay is performed). 5 µl of standards and samples were pipeted into a clean, dry microtiter plate.Then 25 µl of reagent A' or reagent A was added into each well. After adding reagent A, 200 µl reagent B was added into each well and mixed by pipeting up and down. After 15 minutes in room temperature, absorbances was read at 695 nm.

4.2.6 SDS-PAGE

Biorad mini potein gel systems were used for protein electrophoresis. %12 SDS-PAGE was prepared by mixing the proper amount of distilled water, pH 8.8 1.5M Tris-HCl-SDS, %30/%0.8 acrylamide/bisacrylaide, %10 APS and TEMED for seperating gel. Then gels were overlaid with isopropanol to initiate polymerization. Once gels were formed then isopropanol was removed and gels were washed with distilled water. Then stacking gels were prepared by mixing the proper amount of distilled water, pH 6.8 0.5M Tris-HCl-SDS, %30/%0.8 acrylamide/bisacrylaide, %10 APS and TEMED and poured onto the seperating gels. Well combs were placed for the formation of wells. Polymerized gels were put into tanks and tanks were fulled with 1X running buffer. Equal amount of protein samples were prepared and mixed with 2X loading dye (125mM Tris

pH6.8, %4 SDS, %10 glycero, %0.5 bromophenol blue and %1.8-2 mercaptoethanol). Samples were denaturated at 95°C for 3 minutes, then centrifuged shortly at high speed for 10 seconds. After that, each of the samples was loaded to the wells along with a 4ul protein marker. Gels were run at 100V for 1-1,5 hours. After running step, proteins were transfered to PVDF membranes at 60V for 1 hour, and immunoblottings were done described as below.

4.2.7 Immunoblots

Proteins (50 μg) were separated on a 10-15% SDS-PAGE and blotted onto PVDF membranes. The membranes were then blocked with 5% dried milk in PBS-Tween20 and incubated with appropriate primary(1:2000) and horseradish peroxidase (HRP)-conjugated secondary antibodies(1:10000) (Amersham Pharmacia Biotech, Freiburg, Germany) in antibody buffer containing 5% (v/v) Milk Diluent/Blocking concentrate. After required washes with PBS-Tween 20, proteins were finally analyzed using an enhanced chemiluminescence detection system (ECL-advanced, Amersham Pharmacia Biotech, Freiburg, Germany) and exposed to Hyperfilm-ECL (Amersham Pharmacia Biotech, Freiburg, Germany).

4.2.8 Apoptosis and cell death

4.2.8.1 MTT assay

In order to determine cell viability, Cell Proliferation Kit I (MTT Roche, Mannheim, Germany) has been used. MTT Assay is designed for the spectrophotometric quantification of

cell growth and viability without the use of radioactive isotopes. The assay is based on the cleavage of yellow tetrazolium salt, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromid) to purple formazan crystals by viable cells. The formazan crystals formed are solublized and the resulting solution, which is colored, can be quantified by spectrophotometer. According to the protocol, HCT116-wt cells(1X106) were grown on 96-well plates, starved with MACCoy's Modified medium supplemented with 0.2% Fetal calf serum overnight. After treatment, the medium was changed and 10 mL of the MTT labeling reagent was added to each well and incubated under humidified atmosphere containing % 5 CO2 at 37°C for 4 hours. Then 100 mL of the solubilization solution was added into each well and incubated under humidified atmosphere containing % 5 CO2 at 37°C overnight. Spectrophotometrical absorbance of the samples was measured by Model 680 Microplate Reader (Bio-Rad, CA, USA) at 550 nm. Percent viability was calculated as (OD of drug-treated sample/control OD) X 100.

4.2.8.2 M30 Apoptosense®Elisa Assay

First day HCT116-wt colon cancer cells were seeded the density of 10,000 cells per well in a 96-well plate. Second day cells were washed once with PBS and add fresh medium (200 µl/well).Then cells were exposed to PMC-A(25uM from 20mM stock) and PMC-F(75uM from 20mM stock). After 24h treatment, 10 µl 10% NP-40 added per well and allowed lysis for 5 minutes at room temperature. 2x25 µl of the medium/lysate were transfered to wells of an M30-Apoptosense plate. And absorbance was read at 450nm with microplate elisa reader.

4.2.9 ROS Production

4.2.9.1 Flourometric Analysis for ROS Production

In order to quantify the ROS production in HCT116-wt colon cancer cell line. Cells were grown in 12-well plate, after treatment of PMC-A(25uM from 20mM Stock) cells were washed with PBS twice, and then incubated with DC-FHDA (1 mM) for 30 minutes. Cells were washed with PBS to remove the excess dye and read on Microplate Reader (Spectra Max Gemini XS) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The increase in absorbance indicates ROS production.

4.2.9.2 Flow-cytometric Analysis of ROS Production

In order to show the ROS production in HCT116-wt colon cancer cells with flow-cytomety. Cells were grown in 12-well plates, after treatment of PMC-A (25uM from 20mM Stock) for 24 hours, cells were washed with PBS twice, and then incubated with DC-FHDA (1 mM) for 30 minutes. Cells were washed with PBS to remove the excess dye, again 500ul PBS were added to wells, then 200ul PBS with cells were taken to 96-well plate by pipet up and down, and read on BD-FACS CANTO with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

4.2.9. Statistical Analysis

The results are expressed as mean ± SEM and the mean values were compared using Students *t*-tail test or one-way ANOVA analysis. Values of $P<0.05$ and $P<0.01$ were considered statistically significant.

5. RESULTS

5.1 Determination of Pramanicin Analogs-Induced Cytotoxicity in HCT116-wt Colon Carcinoma Cells

In order to investigate the potential effect of pramanicin analogs (A,C,E,F,G,H,I,J) on HTC-wt cells, cell viability was checked by using MTT assay. As shown in Figure 5.1, treatment of HCT-wt cells with pramanicin's analogs, some of the analogs significantly affects viability of the HCT116 cells. Treatment of HCT116-wt cells with 100uM each of the pramanicin analogs for 24 hour, some of the analogs (esp. PMC-A and PMC-F) showed significant potential in decreasing cell viability compared with untreated control and ethanol control. Because of the significant potential of PMC-A and PMC-F, they were selected for further investigations. Ethanol control was used because of all PMC analogues were dissolved in pure ethanol.

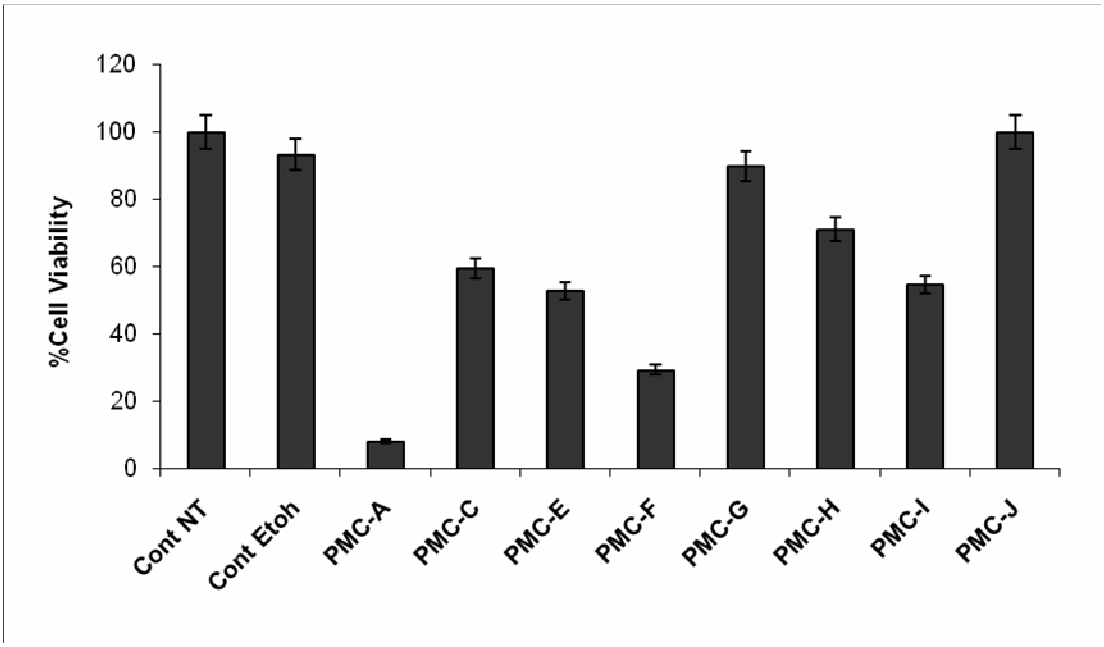


Figure 5.1 Pramanicin's analogs induce cell death. HCT-wt cells were treated with 100uM each of the pramanicin's analogs for 24 h and after incubation, cell viability was assessed using MTT assay. Results are expressed as means ±SEM from the experiment performed in triplicate.

5.2 Determination of the PMC-A and PMC-F Concentration

For determining the minimum potential dose of PMC-A and PMC-F, HCT-wt cells were treated with differing dose (from 25uM to 75uM, 100uM were not screened since the previous experiments showed the effect of 100uM concentrations of PMC-A, F) of PMC-A and PMC-F for 24 h. After incubation, cell viability was checked by using MTT assay. As demonstrated in Figure 5.2 25uM for PMC-A and 75uM for PMC-F were selected for further experiments as minimum effective dose.

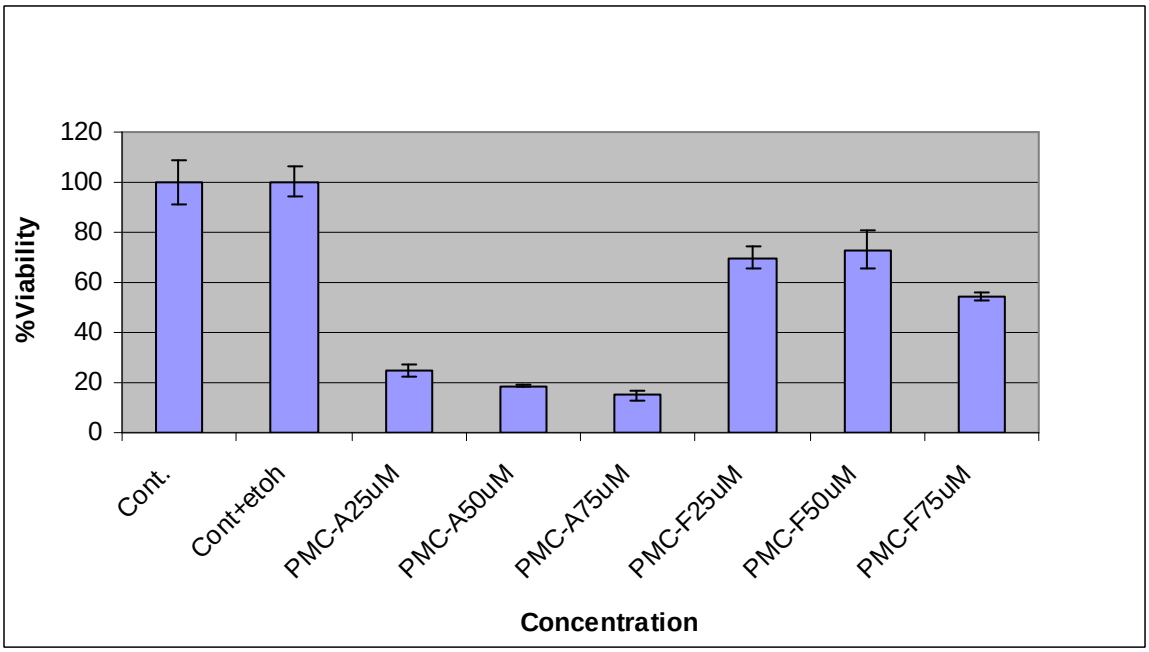


Figure 5.2 Different dose of Pramanicin-A and Pramanicin-F induce cell death in HCT-wt cells. HCT cell lines were treated with differing dose of the pramanicin-A and pramanicin-F analogs for 24 h and after incubation, cell viability was assessed using MTT assay. Results are expressed as means ±SEM from the experiment performed in triplicate.

5.3 Determination of PMC-A Cytotoxic Potential on Different HCT Cell Lines

In order to show the effect of the most potent PMC analog PMC-A on different HCT cell lines (wt, Bax-/-, Puma-/-), MTT assay was used at the concentration of 100uM PMC-A. Treatment of different HCT cell lines with 100uM of the pramanicin-A analog for 24 hour. As shown in Figure 5.3. There was no significant change in cell viability among the different HCT cell lines. This result suggests that PMC-A induced cytotoxicity is independent from BAX and PUMA mediated pathways.Thus HCT-wt was selected for the further investigations

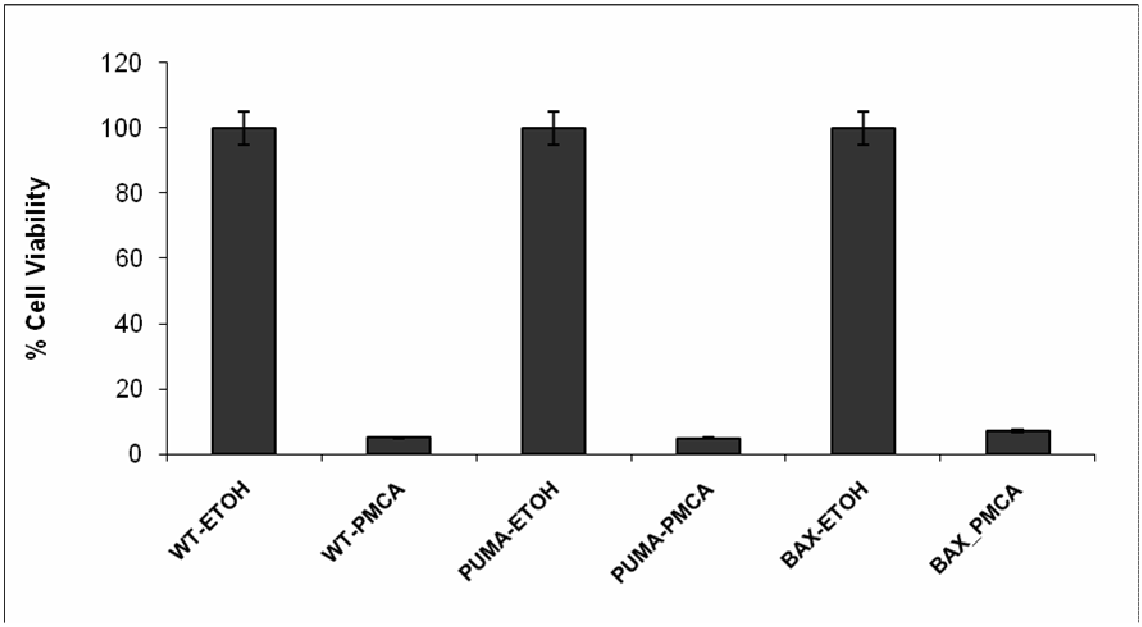


Figure 5.3 Pramanicin-A induce cell death in different HCT cell lines (wt, puma-/-, bax-/-). HCT cell lines were treated with 100uM of the pramanicin-A analog for 24 h and after incubation, cell viability was assessed using MTT assay. Results are expressed as means ±SEM from the experiment performed in triplicate.

5.4 Comparison of PMC-F and the First Synthesized Pramanicin Analog PMC Potentials

PMC was the first synthesized drug among the other analogs and was demonstrated its potential of apoptotic function on Jurcat cells (Kutuk et. al, Apoptosis 2005 ; 10:597-609) we compared the PMC-F and PMC potentials on HCT-wt cells in a dose- dependent manner. As shown in Figure 5.4. HCT-wt cells were treated with differing dose of PMC and PMC-F for 24 h. After incubation, cell viability was checked by using MTT assay. As shown in Figure 5.4, PMC is not potent as PMC-F, Kutuk et al. (2005) showed PMC very effective on jurkat cells, but in our case probably because of the cell type difference PMC is not as effective as PMC-F. Also we could conclude that PMC-F is only as potent as PMC-A at the concentration of 200-250uM compared to previous MTT assay results in Figure 5.4, Figure 5.1.

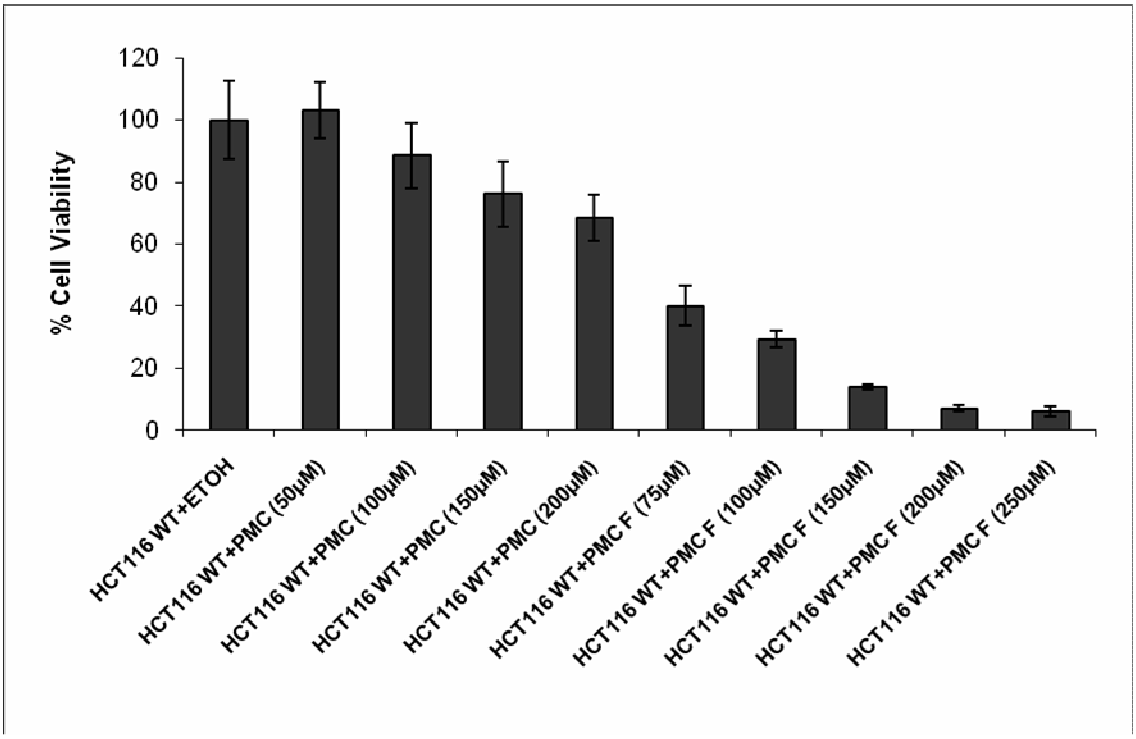
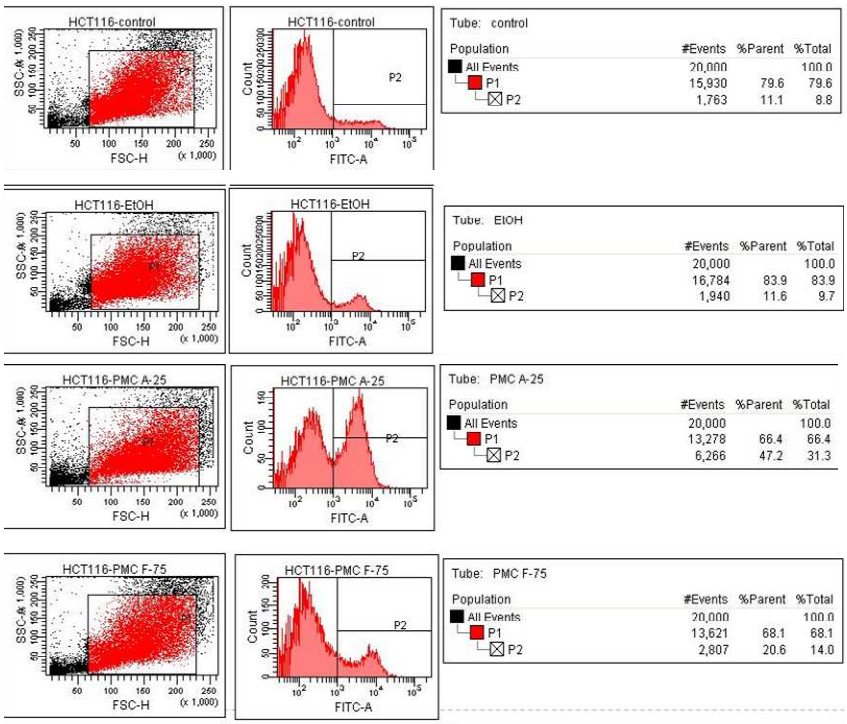


Figure 5.4 Different dose of Pramanicin (PMC) and Pramanicin-F(PMC-F) induce cell death in HCT-wt cells. HCT cells were treated with differing dose of the pramanicin and pramanicin-F for 24 h and after incubation, cell viability was assessed using MTT assay. Results are expressed as means ±SEM from the experiment performed in triplicate.

5.5 PMC-A and PMC-F Induce Apoptosis in HCT-wt Cells

In order to investigate if PMC-A and PMC-F induce apoptosis, Flow Cytometric analysis Figure 5.5A and M30-Apoptosense® ELISA Figure 5.5B were performed. HCT-wt cells were treated with 25uM PMC-A and 75uM PMC-F for 24 h. As demonstrated in Figure 5.5A, Figure 5.5B. Compared to PMC-A, PMC-F was found less potent for its apoptotic efficiency, thus PMC-A were selected for further experiments.



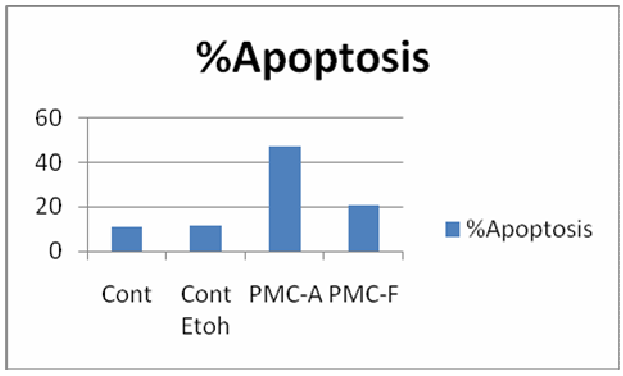


Figure 5.5A PMC-A and PMC-F induce apoptosis, HCT-wt cells were treated with 25uM PMC-A and 75uM PMC-F for 24 h, after incubation flowcytometry was performed with FITC(Fluorescein isothiocyanate).

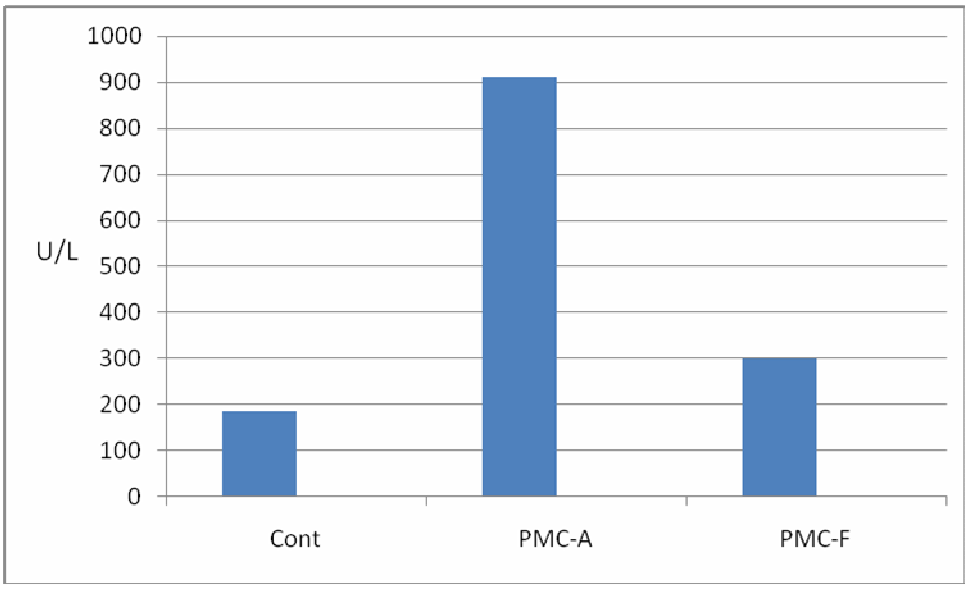


Figure 5.5B PMC-A and PMC-F induce apoptosis, HCT-wt cells were treated with 25uM PMC-A and 75uM PMC-F for 24 h, after incubation M30-Apoptosense® ELISA was performed.

5.6 PMC-A Activates P38, JNK, ERK-1, 2

In order to understand the expression profiles of MAPKs to PMC-A treatment in HCT116-wt cells, immunoblot analysis was performed. HCT116-wt cells were treated with 25uM PMC-A in a time dependent-manner, after incubation, proteins were isolated at 30min-8h time period. From each sample tube 50ug protein were loaded to the SDS-PAGE, after blotting, membranes were probed suitable antibodies at 1/2000 for primary and 1/10000 for secondary antibody dilution from the stocks. As shown in Figure 5.6A, Pramanicin-A induces P38 activation at early time point at 1h without any changes of P38 protein level. Maximum level of phosphorilation of P38 was observed at 1h and P38 returns its basal level at 8h compared to nontreated control. As shown in Figure 5.6B, Pramanicin-A also induces ERK-1, 2 phosphorilation at 30 min and maximum level of phosphorilation was observed at 4h, then there is a slight decrease of its activation at 8h. Finally as demonstrated in figure 5.6C there is a slight activation of JNK at 30min and maximum level of JNK activation is seen at 8h compared to control.

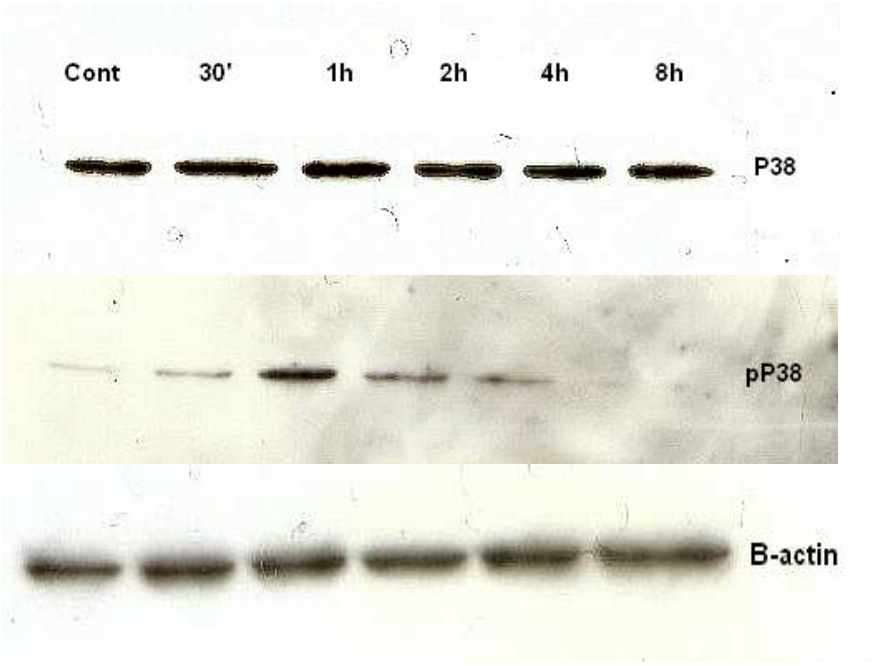


Figure 5.6A PMC-A induces P38 activation, HCT116-wt cells were grown on 60mm cell culture flasks and treated with 25uM PMC-A for 0-8 h, activities of P38 was shown in immunoblot analysis. Specific antibodies were used against total P38, phospho-P38, B-actin was used as a loading control for immunoblots.

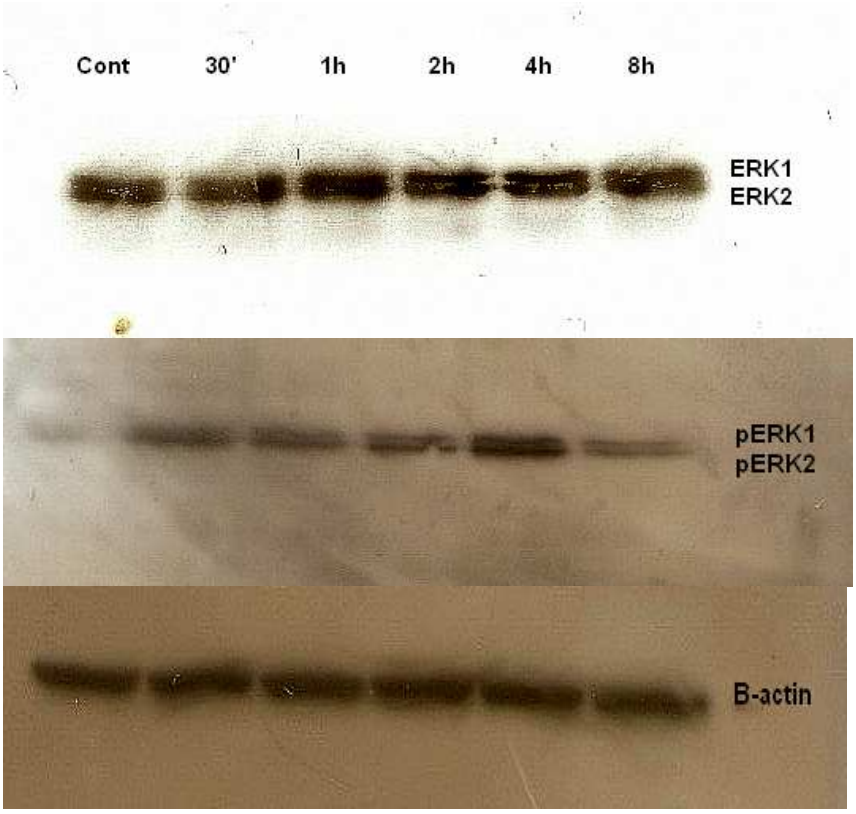


Figure 5.6B PMC-A induces ERK-1,2 activation, HCT116-wt cells were grown on 60mm cell culture flasks and treated with 25uM PMC-A for 0-8 h, activities of ERK-1,2 were shown in immunoblot analysis. Specific antibodies were used against total ERK-1,2, phospho-ERK-1,2, B-actin was used as a loading control for immunoblots.

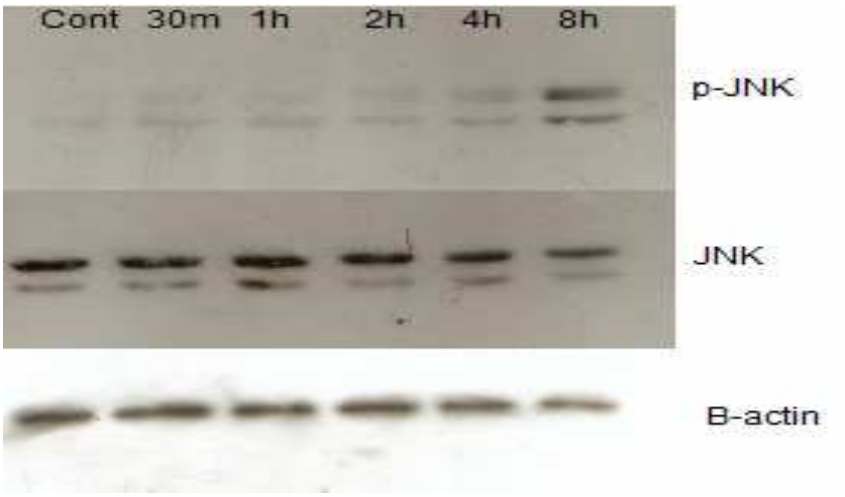
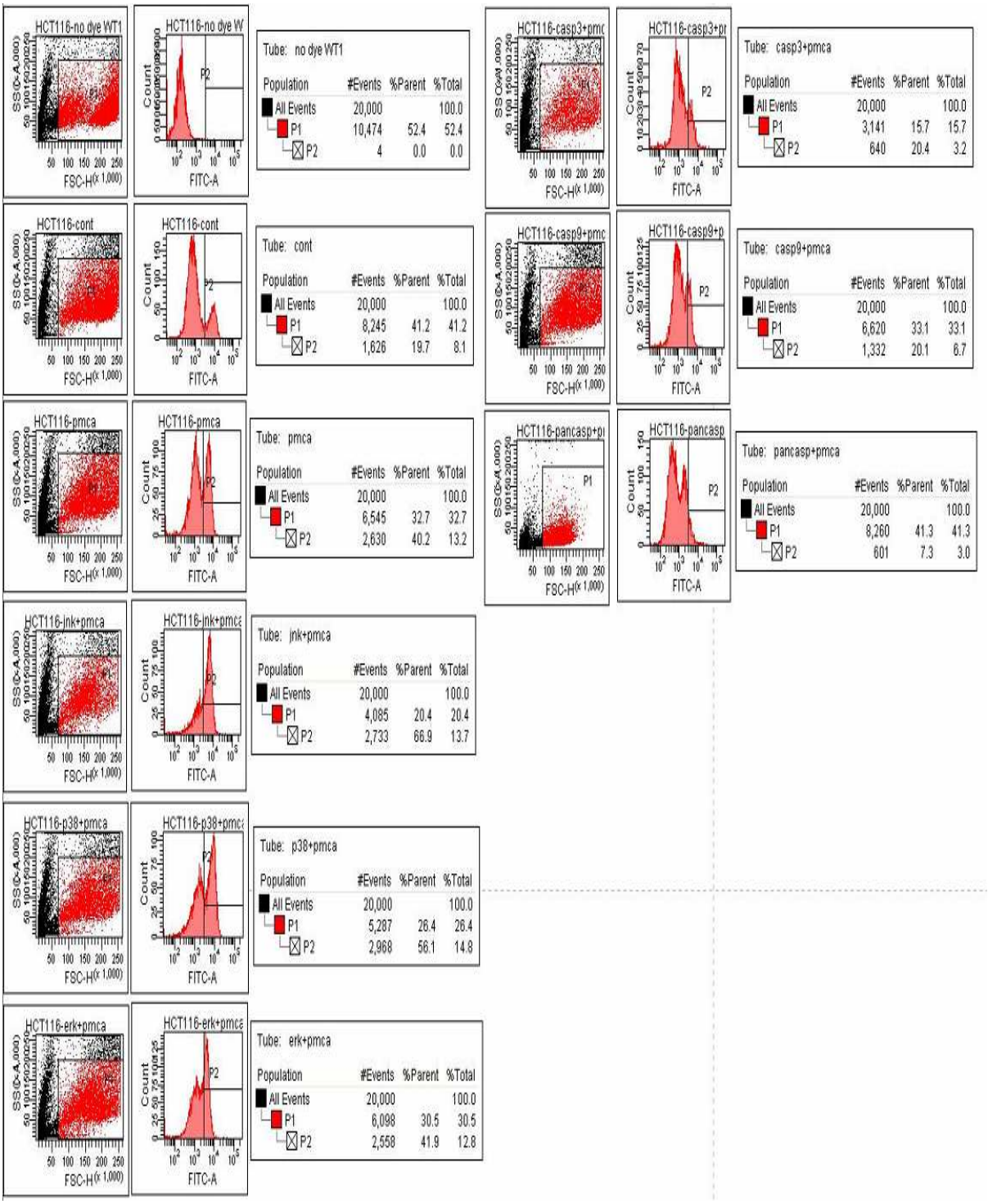


Figure 5.6C PMC-A induces JNK activation, HCT116-wt cells were grown on 60mm cell culture flasks and treated with 25uM PMC-A for 0-8 h, activities of JNK were shown in immunoblot analysis. Specific antibodies were used against, phospho-JNK, B-actin was used as a loading control for immunoblot.

5.7 Effect of JNK, p38, ERK1/2 and Caspase Inhibition on PMC-A Induced Apoptosis

In order to investigate the role of MAPKs, JNK, p38, ERK1/2 and Caspases, Caspase-3, Caspase-9 in apoptotic response of HCT116-wt cells to PMC-A, we applied specific inhibitors of MAPKs and Caspases. As shown in Figure 5.7, Caspase inhibitors effectively prevent PMC-A induced apoptosis, in contrast MAPK inhibitors did not show any preventive effect, but JNK and p38 inhibition did potentiate the apoptotic response of HCT116-wt cells to PMC-A treatment. For the experiment HCT-wt cells were treated with 25uM PMC-A for 24 h, 10uM of each specific MAPKs inhibitors were applied 1 hour before the PMC-A treatment (JNK inhibitor, p38 inhibitor, ERK1/2 inhibitor; SP600125, SB203580, PD98059). Also 20uM of each specific caspase inhibitors (Caspase3,9 and pancaspase inhibitor) were applied 30 minutes before the treatment. After 24 hours treatment, samples were stained with Annexin-V dye for 15 minutes and they were read with BD-Facs Canto flow-cytometry machine. These results indicate that, PMC-A induced apoptosis in HCT116-wt cells depends on caspase cascades, in contrary inhibition of JNK and p38 did not show any preventive effect on apoptosis. Interestingly, JNK and p38 inhibition potentiated the apoptotic response of HCT116-wt cells to PMC-A treatment. Even if ERK1/2 signaling cascades play a survival

role in many cells lines, in our case ERK1/2 inhibition also did not effect the survival response of HCT116 cells.



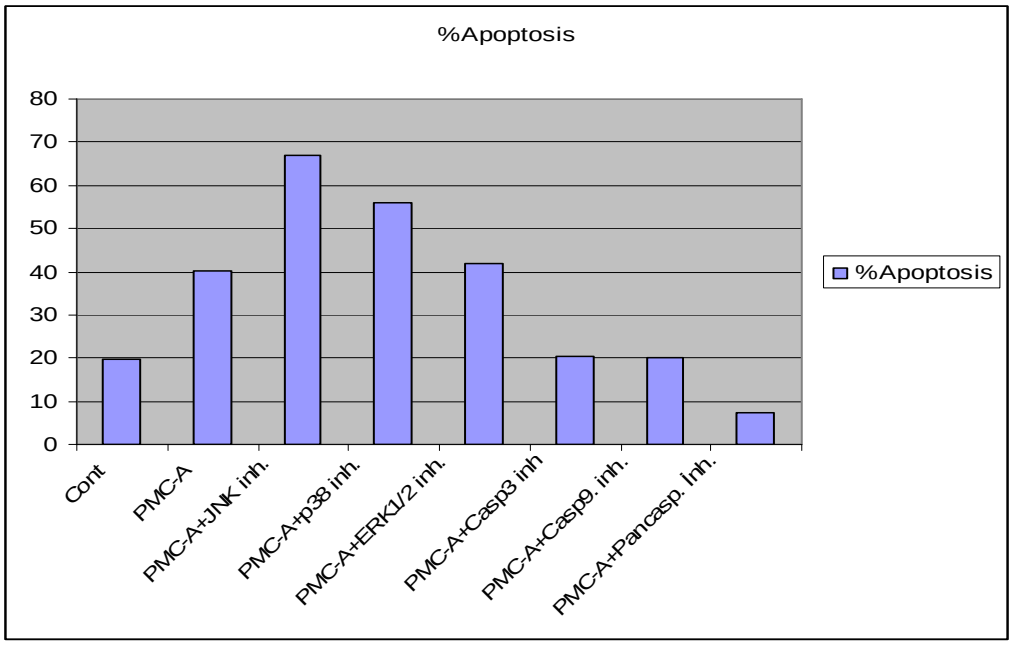
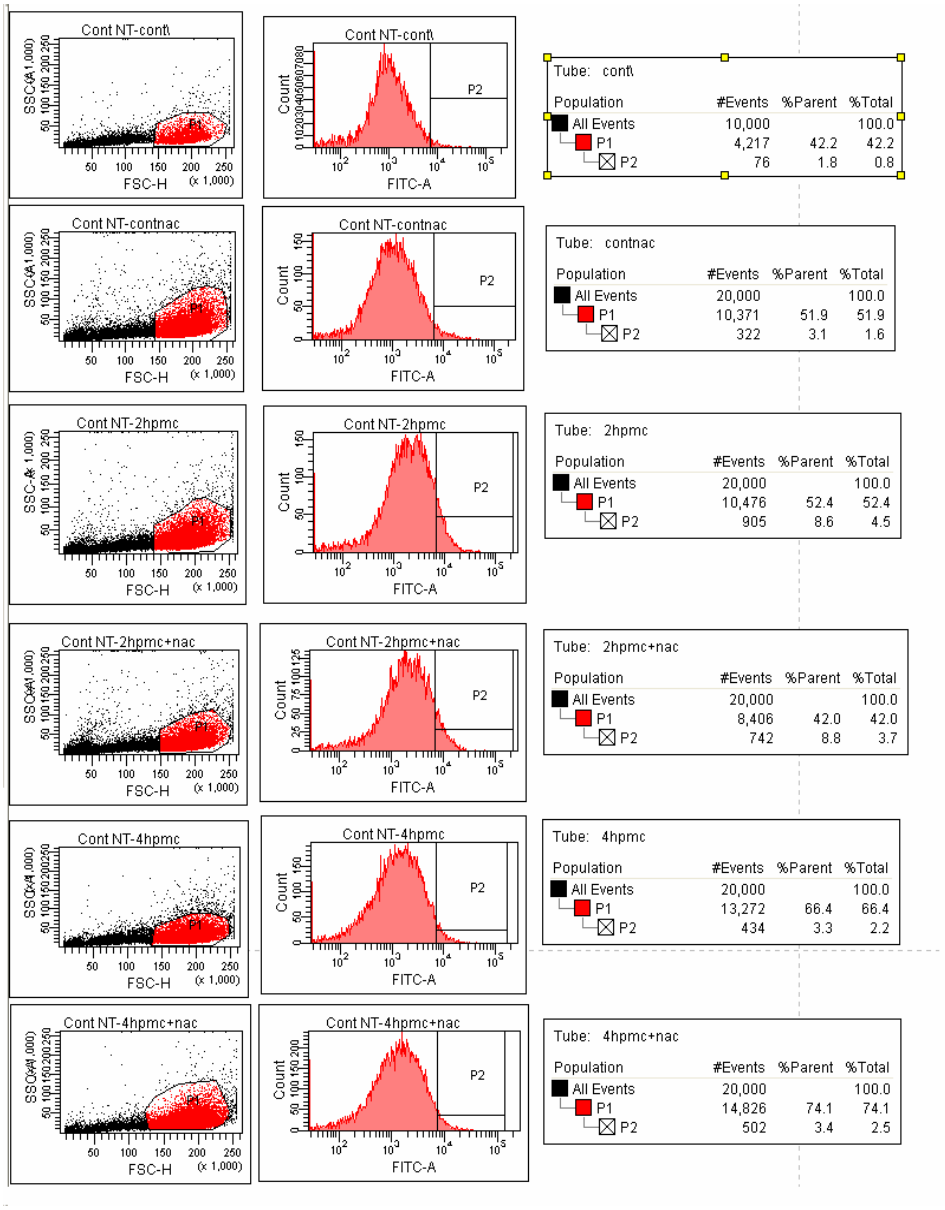


Figure 5.7 Effect of specific Caspase and MAPK inhibitors on apoptosis induced by PMC-A HCT116-wt cells were treated with 25uM PMC-A for 24h with or without 10uM specific MAPKs inhibitors(JNK inhibitor, p38 inhibitor, ERK1/2 inhibitor; SP600125, SB203580, PD98059 1h prior to PMC-A treatment) and specific caspase inhibitors(Caspase-3, caspase-9 and general caspase inhibitor 30 minutes prior toPMC-A treatment), after 24 hours incubation, cells were stained with annexin-v dye for 15 min at room temperature and immediately flowcytometric analysis was performed.

5.8 PMC-A Mediates ROS Production in HCT116-wt Cells

To test the possible role of PMC-A in ROS production, H2DCF-DA was used with flowcytometric method. As shown in Figure 5.8A(early points) and Figure 5.8B(late point), HCT116-wt cells were treated with 25uM PMC-A for early points for 2h, 4h and late point for 24h with or without NAC(n-acetyl cysteine) as an antioxidant, after incubation, cells were stained with H2DCF-DA for 30 min at 37º and immediately flowcytometric analysis was performed with 200.000cells/tube. There is a significant increase of ROS production upon 25uM PMC-A treatment for 24h Figure 5.8B, whereas there is a slight increase after 2h of the treatment observed, a small decrease was observed at 4h Figure 5.8A. At 24h some of the sample's mediums were changed before staining with H2DCF-DA for 30 min at 37 centigrade degree. (24pmcM and 24pmcnacM samples shown in figure 5.8B), there is a significant recover of the cells from ROS production was observed after PMC-A was taken away. Samples with NAC pretreated prior to PMC-A treatment did not show any change of the ROS production, NAC is generally involved in H_2O_2 mediated oxidative stress, this result might

indicate that in our case the oxidative stress is independent from H_2O_2 mediated oxidative process, or apoptotic formation of the cells might have generated the ROS.



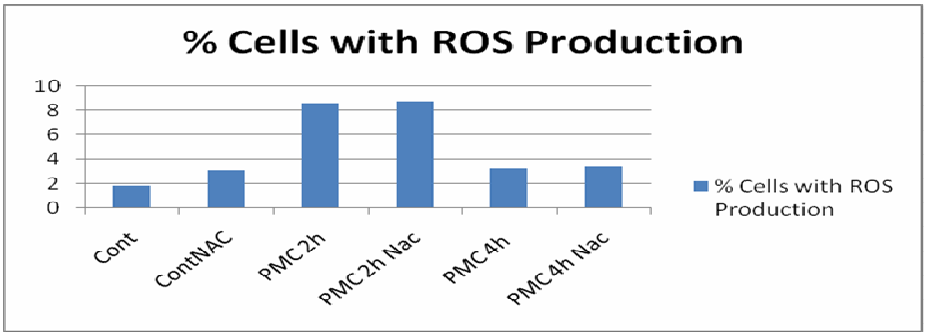


Figure 5.8A PMC-A induces ROS production, HCT116-wt cells were treated with 25uM PMC-A for early points as 2h, 4h with or without 50mM NAC(n-acetyl cysteine) as an antioxidant(2h prior to PMC-A treatment), after incubation, cells were stained with H2DCF-DA for 30 min at 37º and immediately flowcytometric analysis was performed.

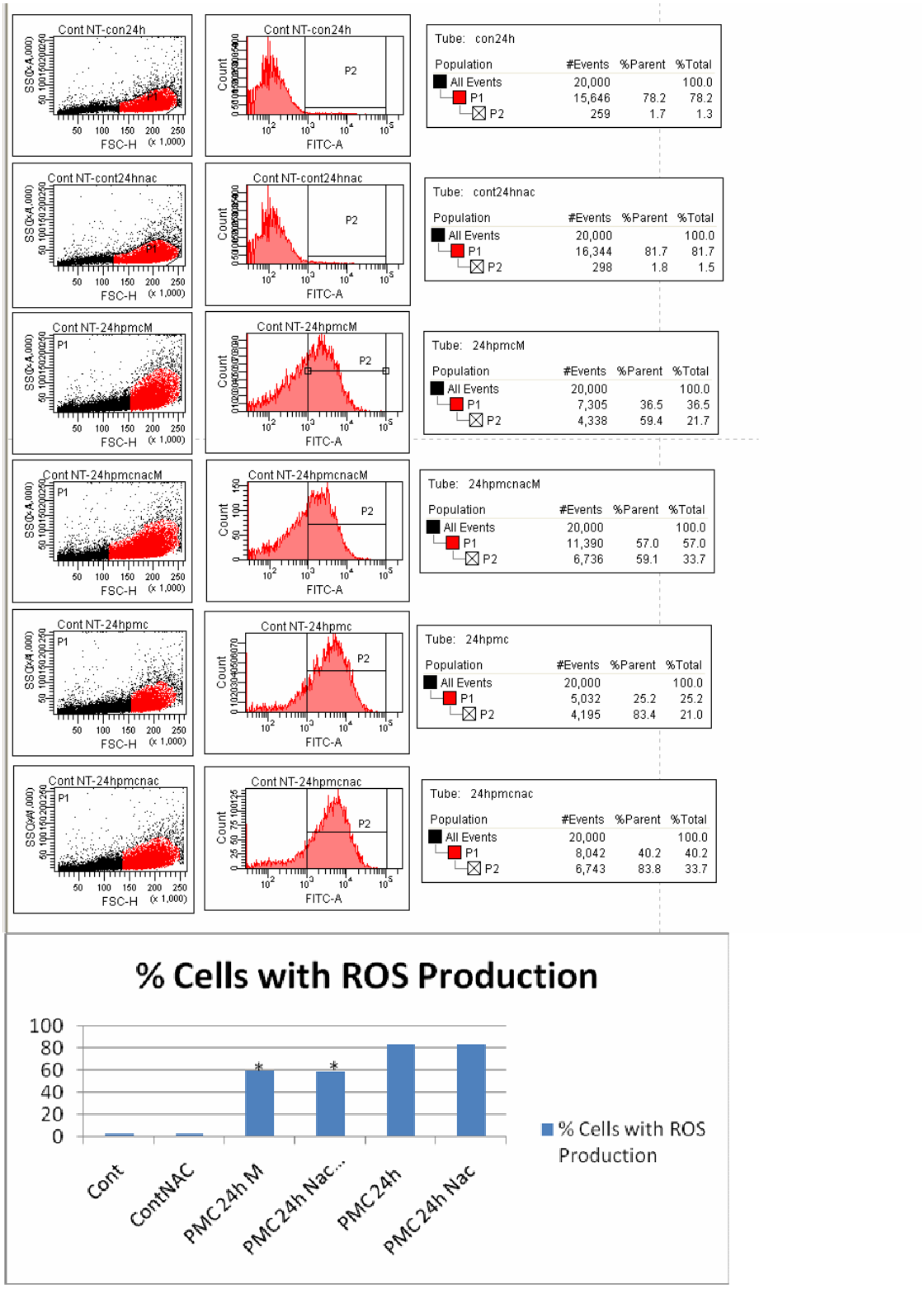


Figure 5.8B %Cells with ROS production induced by PMC-A. HCT116-wt cells were treated with 25uM PMC-A for late point as 24h with or without 50mM NAC(n-acetyl cysteine) as an antioxidant(2h prior to PMC-A treatment), after incubation, cells were stained with H2DCF-DA for 30 min at 37º and immediately flowcytometric analysis was performed. * Medium changed before staining for 30 min.

5.9 Flourometric Analysis of ROS Production in HCT116-wt Cells Treated with PMC-A in a Time-Dependent Manner

To investigate the ROS production in a time-dependent manner,and to understand when the ROS production starts after treatment of PMC-A, H2DCF-DA were used with flourometric method. HCT116-wt cells were treated with 25uM PMC-A for early points 0-8h with or without 50mM NAC(n-acetyl cysteine) as an antioxidant(adding 2h before the treatment of PMC-A), after incubation, cells were stained with H2DCF-DA for 30 min at 37º and immediately flourometric analysis was performed at 532 wave length. As demonstrated in Figure 5.9, ROS production slightly increases at early time points at 1h and a slight decrase was observed at 4h, this might have an experimental error or a defence response. As shown in figure 5.8B after 24h treatment ROS production is significantly increases. These results indicate that early ROS production might mediate MAPKs early signaling pathways in other word initiates MAPKs signaling but further experiments with antioxidants must be done for verifying. On the other hand late excessive ROS accumulation might be a cause for apoptosis as well as other activated pathways upon the PMC-A treatment including MAPK's signaling pathways in HCT116-wt cells.

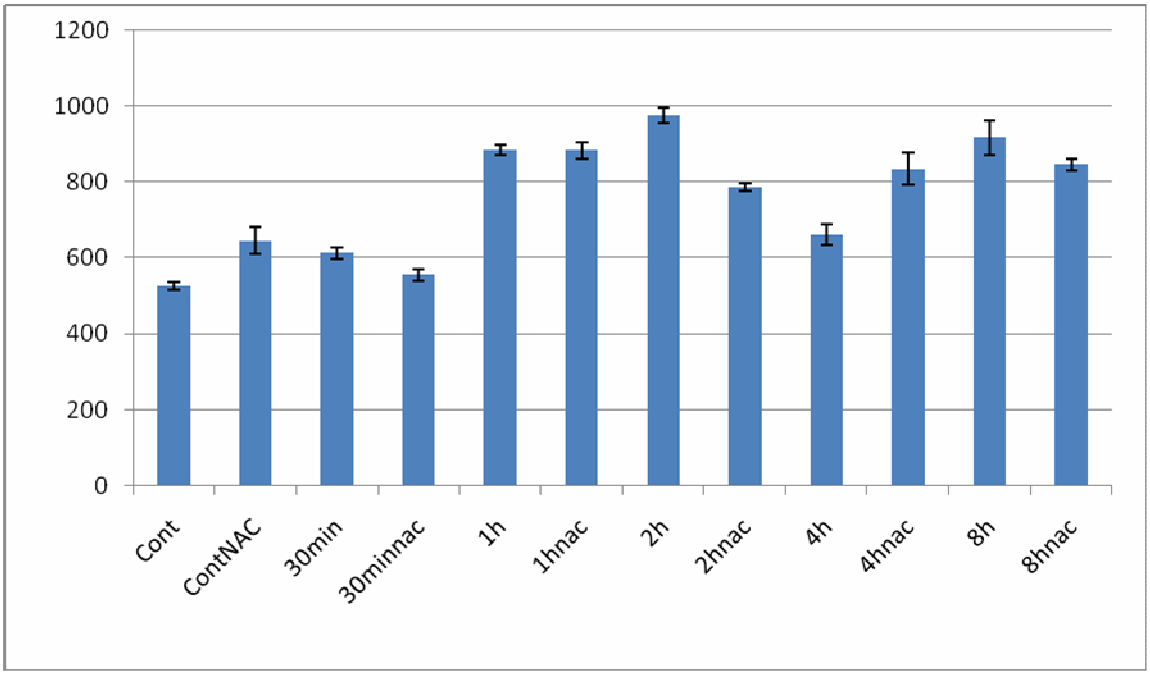


Figure 5.9 Flourometric analysis of ROS production induced by PMC-A, HCT116-wt cells were treated with 25uM PMC-A for early points 0-8h with or without 50mM NAC(n-acetyl cysteine) as an antioxidant(adding 2h before the treatment of PMC-A), after incubation, cells were stained with H2DCF-DA for 30 min at 37º and immediately flourometric analysis was performed at 532 wave length.

6. DISCUSSIONS AND CONCLUSIONS

6.1 PMC-A Induces Apoptosis in HCT116-wt Colon Cancer Cells

Cancer is now the most important health issue around the world. Milions are dying from various kinds of cancers every year. Among all types of cancer, Colon cancer is the 3. most seen and dangerous cancer type in both male and female. Finding novel strategies in cancer prevention, diagnosis and treatment has been the most important step for humankind in medical field, many researchers are now trying to reveal the mechanistic pathways of the cancer formation and its proliferation, methastasis mechanisms to find the new treatments for people suffering from cancer. Every day many agents are being discovered and their apoptotic functions are being shown in various kind of cancer cells. Pramanicin has been recently found as a potential apoptotic agent which can be an anti-cancer drug among many others. Pramanicin (PMC) is an anti-fungal agent which was isolated from a sterile fungal fermantation of Stagonospora. It has 10 analogues as shown in Figure 1.1 It has been recently shown that PMC increases cytosolic calcium concentration and induces cell death in endothelial cells[2]. Also Kutuk et al. have been recently shown the pramanicin's apoptotic effects on jurkat cells[16]. Thus the recent studies have been shown us, PMC and its analogues could be the potential chemotherapeutic agents which are important to be clarified their apoptotic machinery. Determination of cytotoxic effects of pramanicin analogues by MTT assays revealed that, PMC-A and PMC-F are the most effective analogues at 100uM concentration among others in HCT116-wt colon cancer cells Figure 4.1. Then apoptotic effects of PMC-A and PMC-F was determined with flowcytometric analyses and M30 apoptosense elisa assay, Our results demonstrated that PMC-A and PMC-F treatments induce apoptosis in HCT116-wt colon cancer cells in a dose and time dependent manner. PMC-A's apoptotic effect was found efficient at the concentration of 25uM, whereas PMC-F was efficient at the concentration of 75uM, Also in our results, we have found PMC-A more potential then PMC-F Figure 4.5A, Figure 4.5B. For further studies, PMC-A has been selected. Importantly, Screening of the pramanicin analogues for their cytotoxic potentials in HCT116-wt colon cancer cells showed us, their different molecular formations effect their

fuctionality, when PMC's molecular structure is changed, its functions can be totally impaired or potentiated. According to Kutuk et al., PMC is at 100uM concentration is a very potential apoptotic agent in jurkat cells, in our study, it was seen that PMC is at 100uM concentration not as potent as PMC-A and PMC-F in HCT116-wt colon cancer cells compared to jurkat cells. Thus, it is so clear that different cell lines effected apoptotic potentials of PMC and its analogues. Also shown in Figure 5.3 the most effective analogue,PMC-A, was applied to different HCT knock-out cell lines (bax-/- and puma-/-), our data indicate that, neither bax nor puma mediated pathways are involved in apoptotic response of HCT116 cells, thus HCT116 wild type cells were selected for further experiments. In conclusion, Our results demonstrated that PMC-A and PMC-F are very potential apoptotic agents for HCT116-wt colon cancer cells, and their apoptotic potentials should be clarified based on their different molecular structures in different cancer cell lines. Also animal experiments are needed to see their effects in vivo models.

6.2 PMC-A Activates JNK, ERK1/2 and P38

In mammals, three different groups of mitogen-activated protein kinases(MAPKs) are defined, many types of stress like UV radiation, growth factor withdrawal and pro-inflammatory cytokines can activate c-Jun N-terminal kinase and p38 MAPKs [5,6]. After phosphorilation by their upstream kinases on dual tyrosine/threonine, they are activated. P38 and JNK phosphorilate many transcription factors like c-Jun, p53 and ATF-2, and regulate essential functions in cells including proliferation, cell cycle progression, gene expression and apoptosis[7]. JNK and p38 kinases have been described to function pro-apoptotic or anti-apoptotic in many different studies[8,9,10,11]. Even if apoptosis is not seen in response to many stimuli, JNK and p38 MAP kinases activations were shown[12,13]. The other MAP kinase called extracellular signal regulated kinases (ERK1/2, p42/p44 kinases) can be activated by growth factors and mitogenic stimuli[14,15]. There have been many studies on MAP kinases. Their function is determined by cell type, stimuli origin, duration, initial magnitude, further amplification of the activated signal trunsduction pathway, co-activation of

other signaling pathways. Also they have reported that they can be also activated without apoptosis in response to many stimuli. Thus the activation patterns of MAP kinases must be carefuly evaluated in their pro-/anti-apoptotic properties in the light of the above parameters[12,13,16]. As described above, MAPKs pathways play an important role for cells to go apoptosis or to survive in response to many stimuli. After showing the apoptotic function of PMC-A, we investigated activation of MAPKs with their specific anti-bodies by immunoblotting and their roles in PMC-A induced apoptosis with their specific inhibitors. Our data demonstrated that, all three MAPKs including JNK, p38 and ERK1/2 are activated in different time kinetics and amplitudes. PMC-A at 25uM concentration led to an early activation of p38 at 1h, also increased p-JNK level was seen at early time point at 30min but it reached maximum level at 8h compared to non-treated control. ERK1/2 activation was seen at very early time point at 30min, the maximum level of ERK1/2 was seen at 4h in response to PMC-A treatment in HCT116-wt colon cancer cells. Involvement of MAPKs in PMC-A induced apoptosis was evaluated by using their specific inhibitors. Caspase inhibitors successfully inhibited the PMC-A induced apoptotic response compared to the nontreated control and revealed the functional involvement of the caspase pathway, also caspase-3 and caspase-9 activations were shown by Gizem Karslı with western blotting, thus this finding has been verified by her results as PMC-A induced apoptosis through caspase dependent pathway. However Pretreatment of caspase inhibitors (specific casp-3, casp-9 and general caspase inhibitor) effectively prevented the apoptosis induced by 25uM PMC-A. But pretreatment of specific JNK (SP6001259 and p38 (SB203580) inhibitors did not have any preventive effect on apoptosis induced by 25uM PMC-A, but they potentiated the apoptotic response of HCT116-wt cells. Also involvement of ERK1/2 was investigated by its specific inhibitor(PD98059) but results showed that, ERK1/2 inhibition did not effect apoptosis in response to PMC-A treatment, also did not potentiate apoptosis in HCT116-wt cells. All these data (inc. gizems data) indicate the functional role of caspases on apotosis induced by PMC-A. On the other hand, inhibition of JNK and p38 did not effect apoptosis, but potentiate the apoptotic response of HCT116-wt cells. As discussed in Lim SJ et. al. 2006 and Sung-Ho Kook et. al. 2008, inhibition of JNK and p38 in some cases sensitize tumor cells to apoptosis induced by various anti-cancer molecules. In the light of the previous studies, our data show that JNK and p38 might play a pivotal role of surviving response of HCT116-wt cells in response to PMC-A insult. Even if ERK1/2 play a survival role in many stimuli in many cell lines, our datas showed that in HCT116-wt cells, ERK1/2 was not involved in apoptosis induced by PMC-A. MAP kinases are a protein kinase family which can phosphorilate many

transcription factors including c-jun, atf-2 and p53, and regulate various kinds of gene expressions which can play a role in cell survival and death. In our study activation of MAPKs including JNK, p38, and ERK1/2 were shown, we further will investigate the downstream elements of MAPKs such as atf-2, p53 and especially c-jun which forms AP-1 with c-fos. Also specific inhibitors of MAPKs will be used and downstream elements will be checked for revealing the mechanistic pathway.

In conclusion, we report on the activation of JNK, p38 and ERK1/2 in response to PMC-A treatment, inhibitory experiments showed that, JNK and p38 were not major elements in apoptotic response of HCT116-wt cells, otherwise they dont play a pro-apoptotic role, but their inhibitions potentiate the apoptotic response of HCT116-wt cells. In contrast, inhibitions of caspases prevented the apoptotic response, these data suggest that, the apoptotic response depend on the caspase cascades, but not MAPKs cascades. For further evaluations of MAPKs functions in apoptotic response of HCT116-wt cells to PMC-A, downstream and upstream elements such as p53, atf-2, chop, elk-1 and c-jun should be investigated in further studies. Also focusing on different cancer cell lines and/or animal models will further extend our knowledge of mechanisms involved in apoptotic response to pramanicin analogues, and let us evaluate the anti-cancer potential of these molecules.

6.3 PMC-A Induces ROS Production

Molecular signaling cascades in the cells are activated by various stimuli, one of the major activator is oxidative stress. Increase in the reduction potential or a large decrease in reducing capacity of the cellular redox couples can be described as oxidative stress. Reactive oxygen species (ROS) which include free radicals and peroxides can be produced by many mechanism in cells and can be so dangeous. Oxidoreduction reactions with transition metals can easily convert less reactive ROS species to more reactive and dangerous species. These highly active ROS species can cause extensive cellular demage. In animals, ROS may cause cell proliferation, cell death, and expressions of some ROS related genes, activation of cellular signaling cascades such as those involving mitogen activated protein kinases

(MAPK). Most of the ROS species can be produced by aerobic metabolism at a low level from mitochondria and can be easily repaired by some of the spesific mechanism in the cells. In cells, there are enzymes and antioxidants which preserve the cellular redox environment and maintain the reduced state through a constant input of metabolic energy. Oxidative stress is also one of the major causes of apoptosis which is involved many diseases like diabetes and neurodegenerative diseases. In our study, we showed production of reactive oxygen species at early (1h, 2h) and late (24h) time points by flowcytometric and flourometric H2DCF-DA staining with maximum ROS production at 24h. Intrestingly a fluctuation at 4h was seen also in flourometric and flowcytometric anaylsis. At the same time point ERK1/2 activation was seen at maximum level, these results can be speculated that there might be a relationship between ERK1/2 activation and decreased level of ROS production at 4h in response to PMC-A treatment or independently cells might try to recover from ROS by activating their antioxidant mechanisms. ROS production is one of the activator of MAPK signaling pathway as well as other signaling cascades and also ROS can play as a secondary messenger role in various kinds of stimuli. Our results indicated activation of MAP Kinases including all three MAPKs, JNK, p38, ERK1/2. Their activations might related to early ROS formation or the early activations of MAPK pathways can mediate ROS production, but verifications with anti-oxidants or overexpression studies with MAP kinases are needed to reveal the relationship between ROS production and MAPK activations. It is reported that excessive ROS production can cause apoptosis in many studies, in our case ROS production might be at least one of the reasons that mediate PMC-A induced apoptosis in HCT116-wt cells. After showing ROS production, we then tryed to reverse oxidative stress with a general antioxidant, N-acetyl-cystein (NAC). However we could not see any effect on ROS production in response to NAC pre-treatment. Even if NAC is a general antioxidant which can particularly prevent H_2O_2 mediated oxidative stress, we further will try other anti-oxidants which have different anti-oxidant mechanism such as Vitamin E, catalase in our study, then we will further investigate the activation paterns of MAPKs and see whether MAPKs activations depend on ROS production or not. And also using anti-oxidants could let us see if the PMC-A induced apoptotic response of HCT116-wt cells is related to ROS production in our further studies. Finally, using different anti-oxidants in our case will also let us to find the mechanism of the ROS formation in response to PMC-A treatment.

7 FUTURE WORK

İn the light of the results reported herei we would like to suggest the following studies;

- Identifying the relationship between MAPK pathway activation and ROS generation by using specific antioxidants for reversing the oxidative stress and screening paterns of the phosphorilated MAPK family members with their specific anti-bodies by immunoblotting in apoptotic response of HCT116 cells induced by PMC-A. Also identifying the role of ROS on the apoptotic response through the use of specific anti-oxidants such as vitamin E and catalase.
- Identifying the role of ROS production on the activation of caspase-3 and caspase-9 via specific caspase enzymatic assays and immunoblotting with or without specific anti-oxidant treatment in HCT116 cells treated with PMC-A.
- Investigating the down-stream and up-stream elements of activated MAPK pathway such ATF-2, p53, CHOP, Elk-1 etc. in response to PMC-A treatment in HCT116 cells.
- The role of JNK and p38 on apoptotic response of HCT116 cells induced by PMC-A through overexpressions of these proteins.
- Possible cross-talk between MAPK pathway and BCL-2 family members in apoptotic response of HCT116 cells induced by PMC-A by western blotting and co-IP.
- Effects of PMC analogues on different cancer cell lines.

8 REFERENCES

1. Duspara P, Jenkins SI, Hughes DW, *et al.* The biosynthesis of pramanicin: Intact incorporation of serine and absolute configuration of the antibiotic. *Chem Comm* 1998; 23: 2643–2644.

2. Kwan CY, Harrison PH, Kwan TK. Pramanicin, an antifungal agent, raises cytosolic Ca^{2+} and causes cell death in vascular endothelial cells. Vascul Pharm 2003; 40: 35–42.

3. Tan TT, White E. Therapeutic targeting of death pathways in cancer: mechanisms for activating cell death in cancer cells. Adv Exp Med Biol. 2008;615:81-104.

4. Vucic D, Fairbrother WJ. The inhibitor of apoptosis proteins as therapeutic targets in cancer. Clin Cancer Res. 2007 Oct 15;13(20):5995-6000.

5. Kuida K, Boucher DM. Functions of MAP kinases: Insights from gene-targeting studies. J Biochem (Tokyo) 2004; 135:653-656.

6. Wada T, Penninger JM. Mitogen-activated protein kinases in apoptosis regulation. Oncogene 2004; 23: 2838-2849.

7. Davis RJ. Signal transduction to the nucleus by MAP kinases in signaling networks and cell cycle control. In: Gutkind JS, ed. The Molecular Basis of Cancer and Other Diseases. Totowa, NJ: Humana Press 2000: 153-164.

8. Xia Z, Dickens M, Raingeaud J, et al. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 1995; 270: 1326–1331.

9. Franklin RA, McCubrey JA. Kinases: Positive and negative regulators of apoptosis. Leukemia 2000; 14: 2019–2034.

10. Sung-Ho K, Young-Ok S, Yong-Suk J, Kyung-Yeol L, Seung-Ah L, Beom-Soo K, Hyun-Jeong L, Jeong-Chae L. Inhibition of c-Jun N-terminal kinase sensitizes tumor cells to flavonoid-induced apoptosis through down-regulation of JunD. Toxicology and Applied Pharmacology 227 (2008) 468–476.

11. Soo-Jeong L, Young-Ju L, Eunmyong L. p38MAPK inhibitor SB203580 sensitizes human SNU-C4 colon cancer cells to exisulind-induced apoptosis. Oncology Reports 16: 1131-1135, 2006.

12. Liu ZG, Hsu H, Goeddel DV, et al. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappa B activation prevents cell death. Cell 1996; 87: 565–576.

13. Rodrigues GA, Park M, Schlessinger J. Activation of the JNK pathway is essential for transformation by the Met oncogene. EMBO J 1997; 16: 2634–2645.

14. Stofega MR, Yu CL, Wu J, et al. Activation of extracellular signal-regulated kinase (ERK) by mitogenic stimuli is repressed in v-Src-transformed cells. Cell Growth Differ 1997; 8: 113–119.

15. Ory S, Morrison DK. Signal transduction: Implications for Ras dependent ERK signaling. Curr Biol 2004; 14: R277–R278.

16. Kutuk O, Pedrech A, Harrison P and Basaga H. Pramanicin induces apoptosis in Jurkat leukemia cells: A role for JNK, p38 and caspase activation. Apoptosis 2005; 10: 597–609.

17. Genestra M. Oxyl radicals, redox-sensitive signalling cascades and antioxidants. Cell Signal. 2007 Sep;19(9):1807-19. Epub 2007 May 1.

18. Cummings MC, Winterford CM, Walker NI. Apoptosis. Am J Surg Pathol 1997;21:88 – 101.

19. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972;26:239 –57.

20. Krammer PH. CD95's deadly mission in the immune system. Nature 2000;407:789–95.

21. Walczak H, Krammer PH. The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. Exp Cell Res 2000;256:58–66.

22. Adrain C, Martin SJ. The mitochondrial apoptosome: a killer unleashed by the cytochrome seas. Trends Biochem Sci 2001;26:390–7.

23. Watanabe T, Wu TT, Catalano PJ, et al. Molecular predictors of survival after adjuvant chemotherapy for colon cancer. N Engl J Med 2001;344:1196 –1206

24. Du C, Fang M, Li Y, et al. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell 2000;102:33– 42

25. Mayo MW, Baldwin AS. The transcription factor NF-kappaB: control of oncogenesis and cancer therapy resistance. Biochim Biophys Acta 2000;1470:M55–M62.

26. Hakem R, Hakem A, Duncan GS, et al. Differential requirement for caspase 9 in apoptotic pathways in vivo. Cell 1998;94:339 –52.

27. Yoshida H, Kong YY, Yoshida R, et al. Apaf1 is required for mitochondrial pathways of apoptosis and brain development. Cell 1998;94:739 –50.

28. Varfolomeev EE, Schuchmann M, Luria V, et al. Targeted disruption of the mouse caspase 8 gene ablates cell death induction by the TNF receptors Fas/Apo1 and DR3 and is lethal prenatally. Immunity 1998;9:267–76.

29. Yeh WC, Pompa JL, McCurrach ME, et al. FADD: essential for embryo development and signaling from some, but not all, inducers of apoptosis. Science 1998;279:1954–8.

30. Roy S, Nicholson DW. Cross-talk in cell death signaling. J Exp Med 2000;192:21– 6.

31. Slee EA, Adrain C, Martin SJ. Serial killers: ordering caspase activation events in apoptosis. Cell Death Differ 1999;6:1067–74.

32. Jaattela M. Escaping cell death: survival proteins in cancer. Exp Cell Res 1999;248:30–43.

33. Zhivotovsky B, Orrenius S. Defects in the apoptotic machinery of cancer cells: role in drug resistance. Semin Cancer Biol 2003;13:125–34.

34. Broker LE, Kruyt FA, Giaccone G. Cell death independent of caspases: a review. Clin Cancer Res 2005;11:3155– 62.

35. Huerta S, Goulet EJ, Livingston EH. Colon cancer and apoptosis. Am J Surg. 2006 Apr;191(4):517-26.

36. Chang, L. and Karin, M. (2001) Mammalian MAP kinase signalling cascades. Nature 410, 37–40.

37. Junttila MR, Li SP, Westermarck J. Phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival. FASEB J. 2008 Apr;22(4):954-65. Epub 2007 Nov 26.

38. Roux, P. P. And Blenis, J. (2004) ERK and p38 MAPK-activated protein kinases: a family of protein kinases with divese biologicacal functions. Microbiol Mol. Biol. Rev. 68, 320-344.

39. Zebisch, A., Czernilofsky, A. P., Keri, G., Smigelskaite, J., Sill, H., and Troppmair, J. (2007) Signaling through RAS-RAFMEK-ERK: from basics to bedside. Curr. Med. Chem. 14, 601–623.

40. Troppmair, J., Bruder, J. T., Munoz, H., Lloyd, P. A., Kyriakis, J., Banerjee, P., Avruch, J., and Rapp, U. R. (1994) Mitogenactivated protein kinase/extracellular signal-regulated protein kinase activation by oncogenes, serum, and 12-O-tetradecanoylphorbol-13-acetate requires Raf and is necessary for transformation. J. Biol. Chem. 269, 7030–7035.

41. Rangarajan, A., Hong, S. J., Gifford, A., and Weinberg, R. A. (2004) Species- and cell type-specific requirements for cellular transformation. Cancer Cell 6, 171–183.

42. Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M. J., Bottomley,W., Davis, N., Dicks, E., Ewing, R., Floyd, Y., Gray, K., Hall, S., Hawes, R., Hughes, J., Kosmidou, V., Menzies, A., Mould, C., Parker, A., Stevens, C., Watt, S., Hooper, S., Wilson, R., Jayatilake, H., Gusterson, B. A., Cooper, C., Shipley, J., Hargrave, D., Pritchard-Jones, K., Maitland, N., Chenevix-Trench, G., Riggins, G. J., Bigner, D. D., Palmieri, G., Cossu, A., Flanagan, A., Nicholson, A., Ho, J. W., Leung, S. Y., Yuen, S. T., Weber, B. L., Seigler, H. F., Darrow, T. L., Paterson, H., Marais, R., Marshall, C. J., Wooster, R., Stratton, M. R., and Futreal, P. A. (2002) Mutations of the BRAF gene in human cancer. Nature 417, 949–954

43. Rubinfeld, H., and Seger, R. (2005) The ERK cascade: a prototype of MAPK signaling. Mol. Biotechnol. 31, 151–174

44. Bonni, A., Brunet, A., West, A. E., Datta, S. R., Takasu, M. A., and Greenberg, M. E. (1999) Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. Science 286, 1358–1362

45. Holmstro¨m, T. H., Schmitz, I., Soderstrom, T. S., Poukkula, M., Johnson, V. L., Chow, S. C., Krammer, P. H., and Eriksson, J. E. (2000) MAPK/ERK signaling in activated T cells inhibits CD95/Fas-mediated apoptosis downstream of DISC assembly. EMBO J. 19, 5418–5428.

46. Sebolt-Leopold, J. S., Dudley, D. T., Herrera, R., Van Becelaere, K., Wiland, A., Gowan, R. C., Tecle, H., Barrett, S. D., Bridges, A., Przybranowski, S., Leopold, W. R., and Saltiel, A. R. (1999) Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. Nat. Med. 5, 810–816

47. Davis, R. (2000) Signal transduction by the JNK group of MAP kinases. Cell 103, 239–252.

48. Weston, C. R., and Davis, R. J. (2007) The JNK signal transduction pathway. Curr. Opin. Cell Biol. 19, 142–149

49. Dong, C., Yang, D. D., Tournier, C., Whitmarsh, A. J., Xu, J., Davis, R. J., and Flavell, R. A. (2000) JNK is required for effector T-cell function but not for T-cell activation. Nature 405, 91–94

50. Tournier, C., Dong, C., Turner, T. K., Jones, S. N., Flavell, R. A., and Davis, R. J. (2001) MKK7 is an essential component of the JNK signal transduction pathway activated by proinflammatory cytokines. Genes Dev. 15, 1419–1426

51. Kamata, H., Honda, S., Maeda, S., Chang, L., Hirata, H., and Karin, M. (2005) Reactive oxygen species promote TNFα-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. Cell 120, 649–661

52. Sabapathy, K., Kallunki, T., David, J. P., Graef, I., Karin, M., and Wagner, E. F. (2001) c-Jun NH2-terminal kinase (JNK)1 and JNK2 have similar and stage-dependent roles in regulating T cell apoptosis and proliferation. J. Exp. Med. 193, 317–328

53. Tournier, C., Hess, P., Yang, D. D., Xu, J., Turner, T. K., Nimnual, A., Bar-Sagi, D., Jones, S. N., Flavell, R. A., and Davis, R. J. (2000) Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. Science 288, 870–874

54. Yang, D. D., Kuan, C. Y., Whitmarsh, A. J., Rincon, M., Zheng, T. S., Davis, R. J., Rakic, P., and Flavell, R. A. (1997) Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. Nature 389, 865–870

55. Ashwell, J. D. (2006) The many paths to p38 mitogen-activated protein kinase activation in the immune system. Nat. Rev. 6, 532–540

56. Raingeaud, J., Gupta, S., Rogers, J. S., Dickens, M., Han, J., Ulevitch, R. J., and Davis, R. J. (1995) Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J. Biol. Chem. 270, 7420–7426

57. Li, S.-P., Junttila, M. R., Han, J., Ka¨ha¨ri, V.-M., and Westermarck, J. (2003) p38 Mitogen-activated protein kinase pathway suppresses cell survival by inducing dephosphorylation of mitogen-activated protein/extracellular signal-regulated kinase kinase1,2. Cancer Res. 63, 3473–3477

58. Tanaka, N., Kamanaka, M., Enslen, H., Dong, C., Wysk, M., Davis, R. J., and Flavell, R. A. (2002) Differential involvement of p38 mitogen-activated protein kinase kinases MKK3 and MKK6 in T-cell apoptosis. EMBO Rep. 3, 785–791

59. Tobiume, K., Matsuzawa, A., Takahashi, T., Nishitoh, H., Morita, K., Takeda, K., Minowa, O., Miyazono, K., Noda, T., and Ichijo, H. (2001) ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. EMBO. Rep. 2, 222– 228

60. Porras, A., Zuluaga, S., Black, E., Valladares, A., Alvarez, A. M., Ambrosino, C., Benito, M., and Nebreda, A. R. (2004) P38 alpha mitogen-activated protein kinase sensitizes cells to apoptosis induced by different stimuli. Mol. Biol. Cell 15, 922–933

61. Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J., and Greenberg, M. E. (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270, 1326–1331

62. Grethe, S., and Porn-Ares, M. I. (2006) p38 MAPK regulates phosphorylation of Bad via PP2A-dependent suppression of the MEK1/2-ERK1/2 survival pathway in TNF-alpha induced endothelial apoptosis. Cell. Signal. 18, 531–540

63. Efimova, T., Broome, A. M., and Eckert, R. L. (2004) Protein kinase Cdelta regulates keratinocyte death and survival by regulating activity and subcellular localization of a p38deltaextracellular signal-regulated kinase 1/2 complex. Mol. Cell. Biol. 24, 8167–8183

64. Bulavin, D. V., Higashimoto, Y., Popoff, I. J., Gaarde, W. A., Basrur, V., Potapova, O., Appella, E., and Fornace, A. J., Jr. (2001) Initiation of a G2/M checkpoint after ultraviolet radiation requires p38 kinase. Nature 411, 102–107

65. Bulavin, D. V., and Fornace, A. J., Jr. (2004) p38 MAP kinase's emerging role as a tumor suppressor. Adv. Cancer Res. 92, 95–118

66. Gauthier, M. L., Pickering, C. R., Miller, C. J., Fordyce, C. A., Chew, K. L., Berman, H. K., and Tlsty, T. D. (2005) p38 regulates cyclooxygenase-2 in human mammary epithelial cells and is activated in premalignant tissue. Cancer Res. 65, 1792–1799

67. Greenberg, A. K., Basu, S., Hu, J., Yie, T. A., Tchou-Wong, K. M., Rom, W. N., and Lee, T. C. (2002) Selective p38 activation in human non-small cell lung cancer Am. J. Respir. Cell Mol. Biol. 26, 558–564

68. Junttila, M. R., Ala-Aho, R., Jokilehto, T., Peltonen, J., Kallajoki, M., Grenman, R., Jaakkola, P., Westermarck, J., and Ka¨ha¨ri, V. M. (2007) p38alpha and p38delta mitogen-activated protein kinase isoforms regulate invasion and growth of head and neck squamous carcinoma cells. Oncogene 26, 5267–5279

69. Efimova, T., Broome, A. M., and Eckert, R. L. (2003) A regulatory role for p38 delta MAPK in keratinocyte differentiation.Evidence for p38 delta-ERK1/2 complex formation. J. Biol. Chem. 278, 34277–34285

70. Adler V, Yin Z, Tew KD, Ronai Z. Role of redox potential and reactive oxygen species in stress signaling. Oncogene. 1999 Nov 1;18(45):6104-11.

71. Dröge W. Free radicals in the physiological control of cell function. Physiol Rev. 2002 Jan;82(1):47-95.

72. Boscoboinik D, Szewczyk A, Hensey C, Azzi A. Inhibition of cell proliferation by alpha-tocopherol. Role of protein kinase C. J Biol Chem. 1991 Apr 5;266(10):6188-94.

73. Allen R.G., Tressini M.. Oxidative stress and gene regulation. Free Radic. Biol. Med. 28 (2000) 463–499.

74. Aikawa R., Komuro I., Yamazaki T., Zou Y., Kudoh S., Tanaka M., Shiojima I., Hiroi Y., Yazaki Y. Oxidative stress activates extracellular signal-regulated kinases through Src and Ras in cultured cardiac myocytes of neonatal rats. J. Clin. Invest. 100 (1997) 1813–1821.

75. Sulciner DJ, Irani K, Yu ZX, Ferrans VJ, Goldschmidt-Clermont P, Finkel T. rac1 regulates a cytokine-stimulated, redox-dependent pathway necessary for NF-kappaB activation. Mol Cell Biol. 1996 Dec;16(12):7115-21.

76. Wilden PA, Pessin JE. Differential sensitivity of the insulin-receptor kinase to thiol and oxidizing agents in the absence and presence of insulin. Biochem J. 1987 Jul 15;245(2):325-31.

77. Liochev SI, Fridovich I. The effects of superoxide dismutase on H2O2 formation. Free Radic Biol Med. 2007 May 15;42(10):1465-9. Epub 2007 Feb 28.

78. Calabrese V, Guagliano E, Sapienza M, Panebianco M, Calafato S, Puleo E, Pennisi G, Mancuso C, Butterfield DA, Stella AG. Redox regulation of cellular stress response in aging and neurodegenerative disorders: role of vitagenes. Neurochem Res. 2007 Apr-May;32(4-5):757-73. Epub 2006 Dec 27.

79. Babior BM. NADPH oxidase: an update. Blood. 1999 Jun 15;93(12):4449.

80. Jones O.T.G., Hancock J.T., in: Winyard P.G., Blake D.R., Evans C.H. (Eds.), Free Radicals in Inflammation, Birkha, Switzerland, 2000, pp.21–46.

81. Fridovich I.. The biology of oxygen radicals. Science 201 (1978) 875–880.

82. Deby C., Goutier R.. New perspectives on the biochemistry of superoxide anion and the efficiency of superoxide dismutases. Biochem. Pharmacol. 39 (1990) 399–405.

83. Hancock J.T., Desikan R., Neill S.J., Role of reactive oxygen species in cell signalling pathways, Biochemical Society Transactions, 2001, pp. 345–350.

84. Jones, O. T. G. and Hancock, J. T. (2000) Free Radicals in Inflammation (Winyard, P. G., Blake, D. R. and Evans C. H., eds), pp. 21–46, Birkhäuser, Switzerland

85. Henderson, L. M., Chappell, J. B. and Jones, O. T. G. (1988). Internal pH changes associated with the activity of NADPH oxidase of human neutrophils. Further evidence for the presence of an H+ conducting channel. Biochem. J. 251, 563–567.

86. Harrison, R. (2000) Free Radicals in Inflammation (Winyard, P. G., Blake, D. R. and Evans, C. H., eds), pp. 65–81, Birkhäuser, Switzerland

87. Bolwell, G. P., Butt, V. S., Davies, D. R. and Zimmerlin, A. The origin of the oxidative burst in plants. (1995) Free Radical Res. 23, 517–523

88. Gamaley, I. A. and Klyubin, I. V. Roles of reactive oxygen species: signaling and regulation of cellular functions. (1999) Int. Rev. Cytol. 188, 203–255.

89. Allen, R. G. and Tresini, M. Oxidative stress and gene regulation. (2000) Free Radical Biol. Med. 28, 463–499.

90. Crawford, D., Amstad, P., Zbinden, I. and Cerutti, P. A. (1988) Oncogene 3, 27–32.

91. Devary, Y., Gottlieb, R. A., Lau, L. F. and Karin, M. Rapid and preferential activation of the c-jun gene during the mammalian UV response. (1991) Mol. Cell. Biol. 11, 2804–2811.

92. Nose, K., Shibanuma, M., Kikuchi, K., Kageyama, H., Sakiyama, S. and Kuroki, T. Transcriptional activation of early-response genes by hydrogen peroxide in a mouse osteoblastic cell line. (1991) Eur. J. Biochem. 201, 99–106.

93. Sakamoto, K., Yamasaki, Y., Kaneto, H., Fujitani, Y., Matsuoka, T., Yoshioka, R., Tagawa, T., Matsuhisa, M., Kajimoto, Y. and Hori, M. Identification of oxidative stress-regulated genes in rat aortic smooth muscle cells by suppression subtractive hybridization.(1999) FEBS Lett. 461, 47–51.

94. Schreck, R., Rieber, P. and Baeuerle, P. A. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1.(1991) EMBO J. 10, 2247–2258.

95. Rao, G. N., Glasgow, W. C., Eling, T. E. and Runge, M. S. Role of hydroperoxyeicosatetraenoic acids in oxidative stress-induced activating protein 1 (AP-1) activity.(1996) J. Biol. Chem. 271, 27760–27764.

96. Wasylyk, C. and Wasylyk, B. Oncogenic conversion of Ets affects redox regulation in-vivo and in-vitro. (1993) Nucleic Acids Res. 21, 523–529.

97. Myrset, A. H., Bostad, A., Jamin, N., Lirsac, P. N., Toma, F. and Gabrielsen, O. S. DNA and redox state induced conformational changes in the DNA-binding domain of the Myb oncoprotein. (1993) EMBO J. 12, 4625–4633.

98. Fialkow, L., Chan, C. K., Rotin, D., Grinstein, S. and Downey, G. P. Activation of the mitogen-activated protein kinase signaling pathway in neutrophils. Role of oxidants.(1994) J. Biol. Chem. 269, 31234–31242.

99. Guyton, K. Z., Lui, Y., Gorospe, M., Xu, Q. and Holbrook, N. J. Activation of mitogen-activated protein kinase by H2O2. Role in cell survival following oxidant injury.(1996) J. Biol. Chem. 271, 4138–4142.

100. Desikan, R., Clarke, A., Hancock, J. T. and Neill, S. J. Harpin induces mitogen-activated protein kinase activity during defence responses in Arabidopsis thaliana suspension cultures. (1999) J. Exp. Bot. 50, 1863–1866.

101. Kovtun, Y., Wan-Ling, C., Tena, G. and Sheen, J. Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 2940–2945.

102. Sullivan, S. G., Chiu, D. T., Errasfa, M., Wang, J. M., Qi, J. S. and Stern, A. Effects of H2O2 on protein tyrosine phosphatase activity in HER14 cells. (1994) Free Radical Biol. Med. 16, 399–403

103. Wu, Y., Kwon, K.-S. and Rhee, S. G. Probing cellular protein targets of H2O2 with fluorescein-conjugated iodoacetamide and antibodies to fluorescein. (1998) FEBS Lett. 440, 111–115

104. Simon, A. R., Rai, U., Fanburg, B. L. and Cochran, B. H. Activation of the JAK-STAT pathway by reactive oxygen species. (1998) Am. J. Physiol. 275, C1640–C1652

105. Griendling K.K., Sorescu D., Lassegue B., Ushio-Fukai M.. Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. Arterioscler. Thromb. Vasc. Biol. 20 (2000) 2175–2183.

106. Cheng Y., Zhao Q., Liu X., Araki S., Zhang S., Miao J..Phosphatidylcholine-specific phospholipase C, p53 and ROS in the association of apoptosis and senescence in vascular endothelial cells. FEBS Lett. 580 (2006) 4911–4915.

107. Zhao J., Miao J., Zhao B., Zhang S. Upregulating of Fas, integrin beta4 and P53 and depressing of PC-PLC activity and ROS level in VEC apoptosis by safrole oxide. FEBS Lett. 579 (2005) 5809–5813.

108. Krjukov A.A., Semenkova G.N., Cherenkevich S.N., Gerein V.. Activation of redox-systems of monocytes by hydrogen peroxide. Biofactors 26 (2006) 283–292.

109. Muralikrishna Adibhatla R., Hatcher J.F.. Phospholipase A2, reactive oxygen species, and lipid peroxidation in cerebral ischemia. Free Radic. Biol. Med. 40 (2006) 376–387.

110. Tappia P.S., Dent M.R., Dhalla N.S. Oxidative stress and redox regulation of phospholipase D in myocardial disease. Free Radic. Biol. Med. 41 (2006)349–361.

111. Thomas M.P., Chartrand K., Reynolds A., Vitvitsky V., Banerjee R., Gendelman H.E. Ion channel blockade attenuates aggregated alpha synuclein induction of microglial reactive oxygen species: relevance for the pathogenesis of Parkinson's disease. J. Neurochem. 100 (2007) 503–519.

112. Moudgil R., Michelakis E.D., Archer S.L. The role of k+ channels in determining pulmonary vascular tone, oxygen sensing, cell proliferation, and apoptosis: implications in hypoxic pulmonary vasoconstriction and pulmonary arterial hypertension. Microcirculation 13 (2006) 615–632.

113. Hajnoczky G., Csordas G., Das S., Garcia-Perez C., Saotome M., Sinha Roy R., Yi M.. Mitochondrial calcium signalling and cell death: approaches for assessing the role of mitochondrial Ca2+ uptake in apoptosis. Cell Calcium 40 (2006) 553–560.

114. Galter D., Mihm S., Dröge W. Distinct effects of glutathione disulphide on the nuclear transcription factor kappa B and the activator protein-1. Eur. J. Biochem. 221 (1994) 639–648.

115. Acker H., Xue D. Nips 10 (1995) 211–216.

116. Schmid E., El Benna J., Galter D., Klein G., Dröge W. Redox priming of the insulin receptor beta-chain associated with altered tyrosine kinase activity and insulin responsiveness in the absence of tyrosine autophosphorylation. FASEB J. 12(1998) 863–870.

117. Hehner S.P., Breitkreutz R., Shubinsky G., Unsoeld H., Schulze-Osthoff K., Schmitz M.L., Dröge W. Enhancement of T cell receptor signaling by a mild oxidative shift in the intracellular thiol pool. J. Immunol. 165 (2000)4319–4328.

118. Smith J., Ladi E., Mayer-Pröschel M., Noble M. Redox state is a central modulator of the balance between self-renewal and differentiation in a dividing glial precursor cell. Proc. Natl. Acad. Sci. U.S. A. 97 (2000) 10032–10037.

119. Marciniak A., Borkowska E., Kedra A., Rychlik M., Beltowski J. Time-dependent transition from H(2)O(2)-extracellular signal-regulated kinase- to O(2)-nitric oxide-dependent mechanisms in the stimulatory effect of leptin on renal Na+/K+/-ATPase in the rat. Clin. Exp. Pharmacol. Physiol. 33 (2006) 1216–1224.

120. Staal F.J.T., Anderson M.T., Staal G.E.J., Herzenberg L.A., Gitler C., Herzenberg L.A. Redox regulation of signal transduction: tyrosine phosphorylation and calcium influx. Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 3619–3622.

121. Hardwick J.S., Sefton B.M. Activation of the Lck tyrosine protein kinase by hydrogen peroxide requires the phosphorylation of Tyr-394. Proc. Natl. Acad. Sci. U. S. A. 92 (1995)4527–4531.

122. Chiang G.G., Sefton B.M. Phosphorylation of a Src kinase at the autophosphorylation site in the absence of Src kinase activity. J. Biol. Chem. 275 (2000) 6055–6058.

123. Wang Y., Johnson P. Expression of CD45 lacking the catalytic protein tyrosine phosphatase domain modulates Lck phosphorylation and T cell activation. J. Biol. Chem. 280 (2005) 14318–14324.

124. McNeill D.R., Wilson D.M. dominant-negative form of the major human abasic endonuclease enhances cellular sensitivity to laboratory and clinical DNA-damaging agents.Mol. Cancer Res. 5 (2007) 61–70.

125. Rose J.L., Reeves K.C., Likhotvorik R.I., Hoyt D.G. Base excision repair proteins are required for integrin-mediated suppression of bleomycin-induced DNA breakage in murine lung endothelial cells. J Pharmacol Exp Ther. 321 (2007) 318–326.

126. Jaruga P., Rodriguez H., Dizdaroglu M. Measurement of 8-hydroxy-2'-deoxyadenosine in DNA by liquid chromatography/mass spectrometry. Free Radic. Biol. Med. 31 (2001) 336–344.

127. Dizdaroglu M., Jaruga P., Birincioglu M., Rodriguez H. Free radical-induced damage to DNA: mechanisms and measurement. Free Radic. Biol. Med. 32 (2002) 1102–1115

128. Galter D., Mihm S., Dröge W. Distinct effects of glutathione disulphide on the nuclear transcription factor kappa B and the activator protein-1.Eur. J. Biochem. 221 (1994) 639–648.

129. Arner E.S., Holmgren A. The thioredoxin system in cancer. Semin. Cancer Biol. 16 (2006) 420–426.

130. Gon S., Beckwith J. Ribonucleotide reductases: influence of environment on synthesis and activity. Antioxid. Redox Signal. 8 (2006) 773–780.

131. Baeuerle P.A. IkappaB-NF-kappaB structures: at the interface of inflammation control. Cell 95 (1998) 729–731.

132. Rogler G., Brand K., Vogl D., Page S., Hofmeister R., Andus T., Knuechel R., Baeuerle P.A., Scholmerich J., Gross V. Nuclear factor kappaB is activated in macrophages and epithelial cells of inflamed intestinal mucosa. Gastroenterology 115 (1998) 357–369.

133. Blenis J. Signal transduction via the MAP kinases: proceed at your own RSK. Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 5889–5892.

134. Kim Y.M., Song E.J., Seo J., Kim H.J., Lee K.J. Proteomic analysis of tyrosine phosphorylations in vascular endothelial growth factor- and reactive oxygen species-mediated signaling pathway. Proteome Res. 6 (2) (Feb 2007) 93–601.

135. Gerits N., Kostenko S., Moens U. In vivo functions of mitogen-activated protein kinases: conclusions from knock-in and knock-out mice. Transgenic Res. 16 (2007) 281–314.

136. Whitmarsh A.J. Regulation of gene transcription by mitogen-activated protein kinase signaling pathways. Biochim Biophys Acta. 2006 (in press).

137. Abe J., Kusuhara M., Ulevitch R.J., Berk B.C., Lee J.D. Big mitogen-activated protein kinase 1 (BMK1) is a redox-sensitive kinase. J. Biol. Chem 271 (1996) 16586–16590.

138. Yan C., Takahashi M., Okuda M., Lee J.D., Berk B.C. Fluid shear stress stimulates big mitogen-activated protein kinase 1 (BMK1) activity in endothelial cells. Dependence on tyrosine kinases and intracellular calcium. J Biol Chem. 274 (1999) 143–150.

139. Kusuhara M., Takahashi E., Peterson T.E., Abe J., Ishida M., Han J., Ulevitch R., Berk B.C. p38 Kinase is a negative regulator of angiotensin II signal transduction in vascular smooth muscle cells: effects on Na+/H+ exchange and ERK1/2. Circ. Res. 83 (1998) 824–831.

140. Abe J., Takahashi M., Ishida M., Lee J.D., Berk B.C. c-Src is required for oxidative stress-mediated activation of big mitogen-activated protein kinase 1. J. Biol. Chem. 272 (1997) 20389–20394.

141. Kamakura S., Moriguchi T., Nishida E. Activation of the protein kinase ERK5/BMK1 by receptor tyrosine kinases. Identification and characterization of a signaling pathway to the nucleus. J. Biol. Chem. 274 (1999) 26563–26571.

142. Marinissen M.J., Chiariello M, Gutkind J.S. Regulation of gene expression by the small GTPase Rho through the ERK6 (p38 gamma) MAP kinase pathway. GenesDev. 15 (2001) 535–553.

143. Suzaki Y., Yoshizumi M., Kagami S., Koyama A.H., Taketani Y., Houchi H., Tsuchiya K., Takeda E., Tamaki T. Hydrogen peroxide stimulates c-Src-mediated big mitogen-activated protein kinase 1 (BMK1) and the MEF2C signaling pathway in PC12 cells: potential role in cell survival following oxidative insults. J. Biol. Chem. 277 (2002) 9614–9621.

144. Ishizawa K., Izawa Y., Ito H., Miki C., Miyata K., Fujita Y., Kanematsu Y., Tsuchiya K., Tamaki T., Nishiyama A., Yoshizumi M. Aldosterone stimulates vascular smooth muscle cell proliferation via big mitogen-activated protein kinase 1 activation. Hypertension 46 (2005) 1046–1052.

145. Gomez-Lazaro M., Galindo M.F., Melero-Fernandez de Mera R.M., Fernandez-Gomez F.J., Concannon C., Segura M.F., Comella J.X., Prehn J.H., Jordan J., Jordan J. Reactive oxygen species and p38 mitogen-activated protein kinase activate Bax to induce mitochondrial cytochrome c release and apoptosis in response to malonate. Mol Pharmacol. 71 (2007) 736–743.

146. Nagai H., Noguchi T., Takeda K., Ichijo H. Pathophysiological roles of ASK1-MAP kinase signaling pathways. J. Biochem. Mol. Biol. 40 (2007) 1–6.

147. Ardanaz N., Pagano P.J. Hydrogen peroxide as a paracrine vascular mediator: regulation and signaling leading to dysfunction. Exp. Biol. Med. 231 (2006) 237–251.

148. Ushio-Fukai M., Alexander R.W., Akers M., Griendling K.K. p38 Mitogen-activated protein kinase is a critical component of the redox-sensitive signaling pathways activated by angiotensin II. Role in vascular smooth muscle cell hypertrophy. J. Biol. Chem. 273 (1998) 15022–15029.

149. Pu M., Akhand A.A., Kato M., Hamaguchi M., Koike T., Iwata H., Sabe H., Suzuki H., Nakashima I. Evidence of a novel redox-linked activation mechanism for the Src kinase which is independent of tyrosine 527-mediated regulation. Oncogene 13 (1996) 2615–2622.

150. Waldron R.T., Rozengurt E. Oxidative stress induces protein kinase D activation in intact cells. Involvement of Src and dependence on protein kinase C. J. Biol. Chem. 275 (2000) 17114–17121.

151. Sekimoto H., Boney C.M. C-terminal Src kinase (CSK) modulates insulin-like growth factor-I signaling through Src in 3T3-L1 differentiation. Endocrinology 144 (2003) 2546–2552.

152. Yoshizumi M., Abe J., Haendeler J., Huang Q., Berk B.C. Src and Cas mediate JNK activation but not ERK1/2 and p38 kinases by reactive oxygen species. J. Biol. Chem. 275 (2000) 11706–11712.

153. O'Hara K.A., Klei L.R., Barchowsky A. Selective activation of Src family kinases and JNK by low levels of chromium(VI). Toxicol. Appl. Pharmacol. 190 (2003) 214–223.

154. Adler V., Yin Z., Fuchs S.Y., Benezra M., Rosario L., Tew K.D., Pincus M.R., Sardana M., Henderson C.J., Wolf C.R., Davis R.J., Ronai Z. Regulation of JNK signaling by GSTp. EMBO J. 18 (1999) 1321–1334.

155. Ichijo H., Nishida E., Irie K., Dijke P., Saitoh M., Moriguchi T., Takagi M., Matsumoto K., Miyzaono K., Gotoh Y. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science 275 (1997) 90–94.

156. Da Silva J., Pierrat B., Mary J.L., Lesslauer W. Blockade of p38 mitogen-activated protein kinase pathway inhibits inducible nitric-oxide synthase expression in mouse astrocytes. J. Biol. Chem. 272 (1997) 28373–28380.

157. Bhat N.R., Zhang P., Lee J.C., Hogan E.L. J. p38 MAPK-mediated transcriptional activation of inducible nitric-oxide synthase in glial cells. Roles of nuclear factors, nuclear factor kappa B, cAMP response element-binding protein, CCAAT/enhancer-binding protein-beta, and activating transcription factor-2. Neurosci. 18 (1998) 1633–1641.

158. Steer S.A., Moran J.M., Christmann B.S., Maggi Jr. L.B., Corbett J.A. Role of MAPK in the regulation of double-stranded RNA- and encephalomyocarditis virus-induced cyclooxygenase-2 expression by macrophages. J. Immunol. 177 (2006) 3413–3420.

159. Hug H., Enari M., Nagata S. No requirement of reactive oxygen intermediates in Fas-mediated apoptosis. FEBS Lett. 351 (1994) 311–313.

160. Schulze-Osthoff K., Krammer P.H., Droge W. Divergent signalling via APO-1/Fas and the TNF receptor, two homologous molecules involved in physiological cell death. EMBO J. 13 (1994) 4587–4596.

161. Jacobson M.D., Burne J.F., Raff M.C. Programmed cell death and Bcl-2 protection in the absence of a nucleus. EMBO J. 13 (1994) 1899–1910.

APPENDIXES

APPENDIX A

Autoclave:

Hirayama, Hiclave HV-110, JAPAN

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

Balance:

Sartorius, BP211D, GERMANY

Sartorius, BP221S, GERMANY

Sartorius, BP610, GERMANY

Schimadzu, Libror EB-3200 HU, JAPAN

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

Centrifuge:

Eppendorf, 5415C, GERMANY

Eppendorf, 5415D, GERMANY

Eppendorf, 5415R, GERMANY

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

Hitachi, Sorvall RC5C Plus, USA

Hitachi, Sorvall Discovery 100 SE, USA

Deepfreeze:

-70o C, Kendro Lab. Prod., Heraeus Hfu486 Basic, GERMANY

-20o C, Bosch, TÜRKİYE

Distilled Water:

Millipore, Elix-S, FRANCE

Millipore, MilliQ Academic, FRANCE

Electrophoresis:

Biogen Inc., USA

Biorad Inc., USA

X Cell SureLock ™ Electrophoresis Cell, Novex USA

Gel Documentation:

UVITEC, UVIdoc Gel Documentation System, UK

Biorad, UV-Transilluminator 2000, USA

Ice Machine:

Scotsman Inc., AF20, USA

Incubator:

Memmert, Modell 300, GERMANY

Memmert, Modell 600, GERMANY

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

Magnetic Stirrer:

VELP Scientifica, ARE Heating Magnetic Stirrer, ITALY

VELP Scientifica, Microstirrer, ITALY

Microliter Pipette:

Gilson, Pipetman, FRANCE

Mettler Toledo, Volumate, USA

Microwave Oven:

Bosch, TÜRKİYE

pH meter:

WTW, pH540 GLP MultiCal®, GERMANY

Power Supply:

Biorad, PowerPac 300, USA

Wealtec, Elite 300, USA

Refrigerator:

+4o C, Bosch, TÜRKOYE

Shaker:

Forma Scientific, Orbital Shaker 4520, USA

GFL, Shaker 3011, USA

New Brunswick Sci., Innova™ 4330, USA

C25HC Incubator shaker New Brunswick Scientific, USA

Sonicator Vibracell 75043, Bioblock Scientific,FRANCE

Spectrophotometer:

Schimadzu, UV-1208, JAPAN

Schimadzu, UV-3150, JAPAN

Secoman, Anthelie Advanced, ITALY

Speed Vacuum:

Savant, Speed Vac® Plus Sc100A, USA

Savant, Refrigerated Vapor Trap RVT 400, USA

Thermocycler:

Eppendorf, Mastercycler Gradient, GERMANY

Vacuum:

Heto, MasterJet Sue 300Q, DENMARK

Water bath:

Huber, Polystat cc1, GERMANY