

ROLE OF AVEN IN APOPTOTIC RESPONSE FOLLOWING PACLITAXEL AND
DOCETAXEL TREATMENT IN MDA-MB231 BREAST CANCER CELLS

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ROLE OF AVEN IN APOPTOTIC RESPONSE FOLLOWING PACLITAXEL AND
DOCETAXEL TREATMENT IN MDA-MB231 BREAST CANCER CELLS

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ABSTRACT

Clinically useful, taxane group of drugs paclitaxel and docetaxel act by stabilizing microtubules and disrupting microtubular dynamics, which are important for cell cycle progression, leading to cell cycle arrest and apoptosis. Among numerous proteins involved in this response, Aven was shown have an anti apoptotic role, therefore, our study aimed at elucidating the role of Aven in apoptotic response to taxane treatment in p53 deficient and estrogen receptor (-) MDA-MB231 breast cancer cell line.

Our results demonstrated that Aven is regulated in response to 50 nM paclitaxel and docetaxel treatments in a time dependent manner according to western blot and Real-Time analysis. Aven was upregulated in early time points 2-4 hours, with a similar pattern of expression of Bcl-2, Apaf-1, caspase-9, Puma and Parp-1 but not with Bcl-xl or Bax. Aven expression was downregulated after 24 hours coinciding with a major decrease in cell viability measured by MTT assay in response to drug treatments. It was also shown that when Aven was silenced by siRNA treatment endogenous levels of Bcl-2 and Bax was upregulated. Furthermore in overexpression studies Bcl-2 was found to be downregulated. The effect of Aven silencing and overexpression on cell death was also investigated. Aven overexpression did not protect MDA-MB231 cells from taxane induced apoptosis; however Aven silencing increased vulnerability of these cells to docetaxel treatment. Finally, Aven was shown to be immunoprecipitated by phospho-tyrosine antibody indicating that it is phosphorylated on its tyrosine residue(s).

ÖZET

Bu çalışmada anti-apoptotik protein Aven'in mutant p53 fenotipli ve östrojen reseptör negatif MDA-MB231 göğüs kanseri hücrelerinde taksan ilaçlarının tetiklediği apoptoz üzerindeki etkisi araştırılmıştır. Taksan grubu ilaçları paklitaksel ve dosetaksel mikrotübülleri stabilize edip hücre döngüsü için önemli mikrotübüler dinamiği bozarak hücre döngüsü hapsine ve apoptoza sebep olmaktadır.

Western blot ve Real-Time PCR analizlerinden alınan sonuçlara göre Aven, 50 nM paklitaksel ve dosetaksel tarafından zamana bağlı olarak regüle edilmektedir. Bu çalışmalara göre Aven 2-4 saatten başlayan erken zaman aralıklarında artarak Bcl-2, Apaf-1, caspase-9, Puma ve Parp-1 ile benzer, Bcl-xl ve Bax ile ise farklı ekspresyon paterni göstermiştir. Aven'in ekspresyonu 24 saat sonra düşerek aynı zaman diliminde MTT deneylerinde saptanan ilaç kullanımına bağlı hücre ölümündeki artışla paralellik göstermiştir. Aynı zamanda Aven ekspresyonunun Bcl-2 ve Bax'ın endojen seviyelerini etkilediği, siRNA çalışmaları sonucunda iki proteinin de seviyelerini artırdığı ve overekspresyon çalışmaları sonucunda da Bcl-2 proteinini azalttığı gösterilmiştir. Aven'in susturulmasının ve overeksprese edilmesinin hücre ölümü üzerindeki etkisi de araştırılmıştır. Buna göre, Aven'in overeksprese edilmesi MDA-MB231 hücrelerini taksan ilaçlarının tetiklediği apoptozdan koruyamamıştır, ancak Aven'in susturulması bu hücrelerin dosetaksel uygulamasına karşı duyarlılığını artırmıştır. Son olarak, Aven'in tirozin aminoasitleri üzerinde fosforlandığı, Aven'in fosfo-tirozin antikoru tarafından çökeltilmesiyle gösterilmiştir.

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ABBREVIATIONS

AIF: Apoptosis inducing factor

Apaf-1: Apoptosis protease activating factor-1

BH: Bcl-2 homology

C-terminus: Carboxyl terminus

CAD : Caspase-activated DNAase

ICAD : Inhibitor of CAD

CARD : Caspase recruitment domain

CED: Cell death defective

DD: Death domain

DED : Death effector domain

DISC: Death inducing signaling complex

DIABLO: Direct inhibitor of apoptosis binding protein with low pI

DMP : Dimethylpimelimidate

DMSO: Dimethylsulfoxide

DR: Death receptor

ER: Endoplasmic reticulum

ERK: extracellular signal-regulated kinase

FADD: Fas-associated death domain

FBS: Foetal bovine serum

IAP : Inhibitor of apoptosis protein

JNK: c-Jun N-terminal kinase

MAPK: Mitogen-activated protein kinase

MOM : Mitochondrial outer membrane

MW: Molecular weight

NF- κ B: Nuclear factor-kappa B

N-terminus: Amino terminus

PARP: Poly-(ADP-ribose) polymerase

PI: Propidium iodide

SMAC: Second mitochondria-derived activator of caspases

STAT: signal transducer and activator of transcription

TNF: Tumor necrosis factor

TRADD: TNF-R-associated death domain

TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand

1 INTRODUCTION

Apoptosis is a conserved cell suicide mechanism important during development for proper organogenesis and organization of multicellular tissues whereas in adult tissues it is essential for maintenance of cellular homeostasis. Decreased apoptosis can lead to diseases such as cancer and autoimmunity, whereas increase in apoptosis can result in degenerative diseases, immunodeficiency and infertility. [3][6] Aven is an apoptosis inhibitor protein with intracellular membrane localization. Among Bcl-2 family members, Aven interacts with anti-apoptotic proteins like Bcl-2 and Bcl-xL, but not with proapoptotic proteins like Bax and Bak. Aven can inhibit apoptosis induced by Apaf-1 and caspase-9 in a number of cell lines functioning in apoptosome level. [9]

Docetaxel and Paclitaxel are taxane group of drugs with proven efficacy in breast, ovarian and lung cancers. They act via inhibition of microtubular depolymerization by binding to a distinct site in β -tubulin leading to cell cycle arrest and apoptosis. [22]

In this project we investigated how pro-survival protein Aven correlates with other anti- and pro-apoptotic proteins, its mechanism and role in apoptotic resistance following taxane (paclitaxel and docetaxel) treatment in MDA-MB 231 (estrogen receptor negative and p53 deficient) breast cancer cell line. For this purpose, we did western blot and Real-Time PCR analysis of Aven expression and also western blot analysis of other apoptosis proteins. Using siRNA and overexpression techniques, we checked the effect of Aven expression on endogenous levels of Bcl-2 family members

and cell death in response to taxane treatment. Finally, phosphorylation of Aven was analyzed by immunoprecipitation experiments.

2 BACKGROUND

2.1 Apoptosis

The first information on apoptosis was provided by Kerr *et al.* (1971) and it was initially called “shrinkage necrosis”. [1] Apoptosis consists of irreversible, genetically determined and complex biological pathways conserved through evolution and it is used to specifically remove unwanted cells to maintain homeostasis. Apoptosis is different from necrosis which results from ATP depletion, involves faster disintegration of organelles, rupture of plasma membrane and leakage of lysosomes and cell content leading to inflammation. It results from non-physiological inducers such as hypoxia, trauma and accumulation of toxic substances. Necrosis affects groups of cells around the necrotic cell area whereas apoptosis affects single cells. [2][8]

Apoptotic pathways can be grouped into four phases consisting of initiation by a stimulus that can either be intrinsic or extrinsic, signal transduction pathways inlaying the inducing signal to death machinery, activation of effector proteases, and engulfment of cell debris to prevent inflammation (Figure 2.2). [2] The morphological hallmarks of apoptosis include condensation of nuclear chromatin, cell shrinkage, formation of protuberances with buddings on the cell surface, cytoplasmic blebs and apoptotic bodies. [2]

Apoptosis is important during development for proper organogenesis and organization of multicellular tissues whereas in adult tissues it is essential for maintenance of cellular homeostasis. This function is especially important for long-lived animals which are exposed to various pathological death signals and infectious agents. Decreased apoptosis can lead to diseases such as cancer and autoimmunity, whereas increase in apoptosis can result in degenerative diseases, immunodeficiency and infertility. [3][6]

The first genes identified related to apoptotic cell death were *ced-3* and *ced-4* in *C. elegans*. Another gene, *ced-9*, was found to be protective and it was shown that it is the worm homolog of mammalian *Bcl-2* gene. *Bcl-2* was first identified as a protooncogene related to human follicular B cell lymphoma preventing cell death against various death stimuli rather than promoting cell proliferation. [3][6][7] (Figure 2.1)

The *Bcl-2* family includes both pro-survival and pro-apoptotic proteins. The first pro-apoptotic protein to be identified was *Bax* which was found through its interaction with *Bcl-2* protein. The *Bcl-2* family proteins are divided into three subclasses according to their shared *Bcl-2* homology (BH 1-4) domains which are roughly α -helices that are important for structure and function. The anti-apoptotic members include *Bcl-2*, *Bcl-xl*, *Mcl-1*, *Bcl-w* and *A1* which have all four BH domains. The structural studies on *Bcl-xl* revealed that the BH1-3 domains create a hydrophobic pocket which can interact with the BH3 domain of a pro-apoptotic member. The multi-domain *Bcl-2* family members are *Bax* and *Bak* which have the BH1-3 domains and they seem to require an activation process probably to expose the hydrophobic side of their BH3 domain so that they can interact with *Bcl-2* and *Bcl-xl*. The BH3-only proteins consist of only the minimal death domain BH3. [3][6]

Bcl-xl, Bcl-2, Bcl-W, MCL-1, Bax, Bak and Bid have similar structures despite having opposing functions. They all have a helix-turn-helix hairpin which is flanked by pairs of amphipathic helices on both sides, creating a helical bundle. They also have C-terminal membrane anchoring domains. In Bax, Bcl-W and Mcl-1 this C-terminal region is sequestered by a hydrophobic pocket formed by the BH1, BH2 and BH3 regions. This pocket can also bind to BH3 sequences of Bak, Bad and Bim creating dimers. [3][6] Among BH3 –domain proteins only Bid has a structure that is similar to core Bcl-2 family members sharing properties with multi-domain Bcl-2 family members such as oligomerization and membrane permeabilization. [4]

Bax and Bak have a central role in mediating apoptosis except for cell death in early development which is not disrupted in the absence of Bax and Bak. In other cases, their double knock-outs abolish cell death in response to various forms of stress signals. Bax and Bak function both at mitochondrion and the endoplasmic reticulum (ER). [3] These two proteins act by mediating cytochrome c release from mitochondria. Their action is under the regulation of pro-apoptotic BH3-only proteins and anti-apoptotic Bcl-2 like proteins which counter one another's function in order to control Bax and Bak. [5] [3]

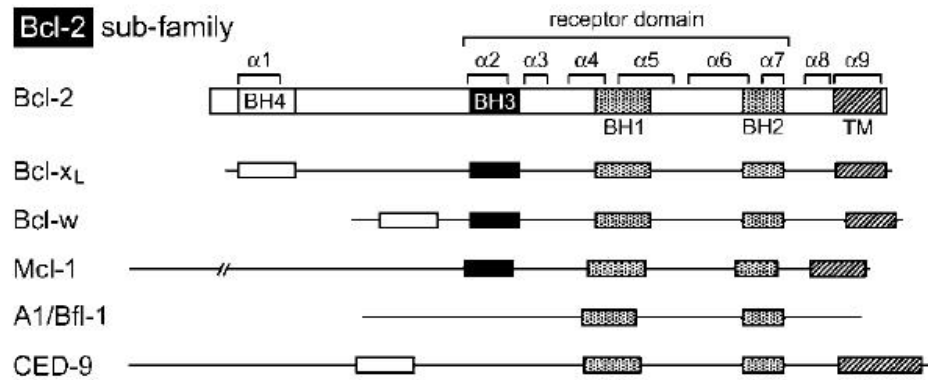
In the absence of death signals Bax resides in cytosol or is loosely attached to membranes as a monomer and its hydrophobic pocket is occupied by its C-terminal helix. As a result of an apoptotic inducer Bax is inserted to the outer mitochondrial membrane (MOM) and forms oligomers. [3] [5] Bak also undergoes oligomerization at the mitochondria following a conformational change and leads to the release of cytochrome c. This leakage may be due to pore formation by Bax and Bak as they have structural similarity to pore-forming toxins and Bax can form channels in artificial membranes. In addition to that, Bcl-2 proteins may work with intrinsic mitochondrial

proteins and lead to permeability transition or different MOM permeabilization mechanisms such as lipid pores and changed membrane curvature may be involved. [3][6]

There are several evidences indicating that Bax may be directly activated by BH3-only proteins. Some of the BH3-only proteins such as Bim, Bid and Puma bind Bax, the killing action of BH3-only proteins requires Bax and Bak, and the structure of Bax resembles Bcl-x1 which can bind BH3-only proteins through a hydrophobic groove which can explain how Bax bind these proteins. In addition to these, studies with purified components suggested that peptides spanning the BH3 regions in Bim and Bid work together with Bax to permeabilize mitochondria or liposomes leading to release of their contents. Based on these observations BH3-only proteins were divided into two subclasses consisting of ‘activators’ such as Bim and Bid and ‘sensitizers’ or ‘derepressors’ such as Bad and Noxa. Activator proteins bind Bax and presumably Bak. Pro-survival proteins try to prevent these activator proteins from binding Bax and Bak. Sensitizer proteins do not bind Bax or Bak, instead they bind the pro-survival proteins at their hydrophobic pockets and they activate Bax indirectly by counteracting the action of survival proteins. [3] The role of Puma is controversial, some think it is an activator protein; others classify it as a sensitizer. [5]

BH3-only proteins act as stress sensors working under distinct upstream controls. For example DNA damage leads to the activation of p53 which in turn induces transcription of Noxa and Puma. [5] [3] In a similar way, Bim is induced by FOXO3A (class O forkhead box transcription factor-3A) in response to growth-factor deprivation and by CEBPa or CHOP in response to ER stress. Bad is activated via phosphorylation in response to growth factors creating a link between extracellular factors and cell survival. [3]

Pro-survival



Pro-apoptosis

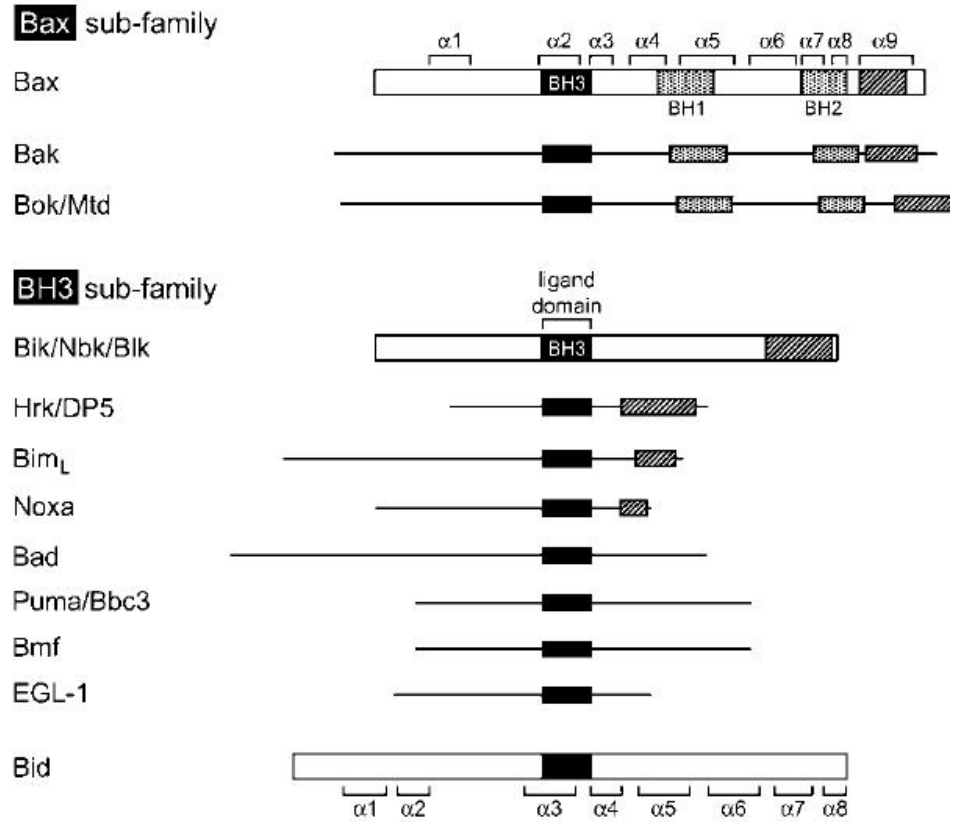


Figure 2.1 Bcl-2 family members [6]

Caspases are cysteine-dependent aspartate-specific acid proteases that are highly conserved through evolution and they are important for the regulation and execution of apoptosis. In mammalian cells there are 14 different caspases all of which are produced as inactive precursor zymogens. Caspases have three common domains: a large internal domain which contains the active site called the death effector domain (DED), a small C-terminal domain which is called the caspase recruitment domain (CARD) and a pro-domain at the N-terminal called the death domain (DD). The DD is a member of TNF receptor family and is important for early signaling events. DED and CARD are important for the recruitment of caspases to the plasma membrane before activation. [2]

The active site of caspases is formed from Arg and His residues on the large subunit and one Arg residue on the small subunit. Caspases are divided into three groups depending on their structure and function. The group-I caspases (caspase-1, -4 and -5) are important for cytokine maturation and inflammatory responses and they have large pro-domains. The group-II caspases either have DED (caspase-8 and -10) or CARD (caspase-9 and -2) and are also called the initiator caspases. The third group of caspases (caspase-3, -6 and -7) are the executioner caspases with short pro-domains. [3] [2] Caspase-12, -13 and -14 are not categorized yet but they are thought to be important for cytokine processing. [2][7]

One of the initiator caspases, Caspase-9, goes under self-processing after binding to Apaf-1 which makes sure that proper protein concentration and conformation are provided for the activation of caspase-9. Apaf-1 binds cytochrome c through its WD40 repeats, and then it can recruit caspase-9 in the presence of ATP/dATP. Apaf-1 and caspase-9 interact through their caspase recruitment domains (CARD). In the absence of apoptotic signals, the CARD domain on Apaf-1 is bound by two of the

WD40 repeats and it is released with the binding of cytochrome c to the WD40 domains. Following binding of ATP/dATP Apaf-1 undergoes a conformational change leading to the formation of a heptamer assembly creating a wheel-like shape which is called the apoptosome. [3][6][8][7]

However, in some cell types, the absence of caspase-9 or Apaf-1 do not prevent intrinsic pathway from proceeding. The loss of caspase-9 and Apaf-1 delays the onset of apoptotic morphology in myeloid progenitors and mast cells which are exposed to growth-factor withdrawal or DNA damage, but does not prevent cell death except for the cells that overexpress Bcl-2 or Bcl-XL. [4]

Inhibitors of apoptosis proteins (IAPs) inhibit caspases via their BIR (baculovirus IAP repeat) domains. IAPs inhibit caspase-3, -7 and -9 by binding to the substrate groove of these proteins via their BIR2 domains preventing the access of substrate to the enzymes. XIAP inhibits caspase-9 by directly interacting with the small subunit of caspase-9 with its BIR3 domain. In mammalian cells IAPs are controlled by several mechanisms including interaction with intermembrane space proteins (IMS) Smac/Diablo/Omi and Htra2 . These proteins prevent IAP inhibition of caspases via their IAP binding motifs (IBM). For example, XIAP is displaced from caspase-9 by the monomeric form of Smac through its IBM similar to that found in caspase-9. In order to make sure that caspase-9 remains active, an amplification loop is used in which the IAP binding domain of caspase-9 is cleaved by caspase-3 and this released domain binds to XIAP to keep it inert. As deletion studies of XIAP indicated that apoptosis can proceed in its absence, IAPs may function in a cooperative way. One example is that in sympathetic neurons for the deprivation of NGF to lead to caspase activation cytochrome c release is not sufficient, but the degradation of IAPs is also required. [3][6]

One alternative way of caspase activation is the inhibition of IAPs (inhibitor of apoptosis proteins) such as XIAP by the binding of Diablo which is released from the mitochondria following the activation of Bax and Bak. However, the lack of XIAP or Diablo in mice does not lead to a major increase in apoptosis. This suggests that new mechanisms need to be discovered. [4]

The extrinsic pathway is mediated by death receptors that depend on a set of signaling proteins containing death domains (DD) and death effector domains (DED) which are capable of homotypic interaction. The overall fold of DD and DED domains is very similar to the CARD domain structurally. Following the binding of a ligand Fas which is a trimer goes through a conformational change and assembles the DISC (Death-Inducing Signaling Complex) on its cytoplasmic tail. Then, pro-caspase-8 is recruited via its DED domain by the adaptor protein Fadd/Mort which has both DED and DD domains and binds DD domain of Fas. Caspase-8 is activated at the DISC complex due to its high local concentration through autoproteolytic cleavage and activation of caspase-3 and -7 follows. [3] [2][6] In type I cells like thymocytes, Fas activation leads to enough caspase-3 and -7 activation and is not subject to inhibition by Bcl-2. However, in type II cells, such as hepatocytes, a mitochondrial amplification loop is required to produce enough active effector caspase concentration to initiate apoptosis. This is provided when Bid is cleaved by caspase-8 and translocates to mitochondria leading to release of cytochrome c and initiation of the caspase cascade. As a result in these type II cells Fas mediated apoptosis can be inhibited by Bcl-2 protein. [3][8]

In order to prevent inflammation following apoptosis the cell debris is engulfed by phagocytes. The engulfment proceeds after the phagocytes recognize the signal on the apoptotic cell surface. One of the best characterized cell surface signals for

engulfment in mammalian cells is phosphatidylserine (PS) which normally faces cytosol but is displayed on cell surface of apoptotic cells. Many engulfment receptors may be involved including CD91, CD14, CD36 and phosphatidylserine receptor. When apoptotic cells are bound by engulfment receptors cytoskeletal pathways are triggered. [3]

One of the morphological hallmarks of apoptosis is the condensation and fragmentation of nuclei. DNA is degraded by a caspase activated DNAase (CAD) which is inhibited by ICAD in the absence of apoptotic inducer. During apoptosis ICAD is eliminated by caspase-3 and -7 and CAD is activated to cleave DNA into 200 kb fragments. [3][6]

As an alternative pathway, DNA degradation may occur in a caspase-independent manner using two mitochondrial proteins endonucleases G and apoptosis-inducing factor (AIF) which translocates to nucleus upon its release. This translocation depends on the activity of poly (ADP-ribose) polymerase-1 (PARP-1) which in turn requires AIF for its pro-apoptotic function. [3][6][8]

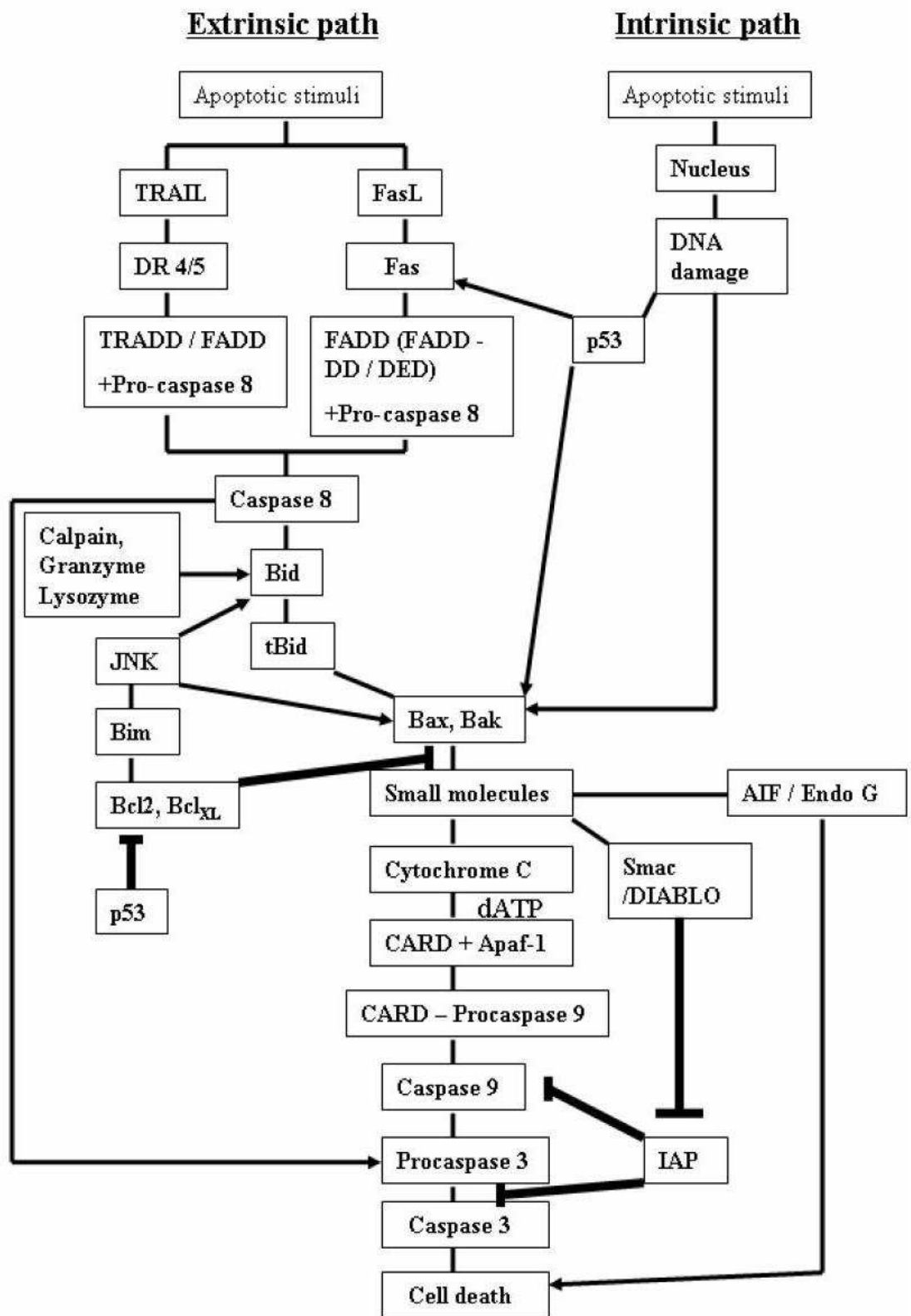


Figure 2. 2 Extrinsic and intrinsic pathways in apoptosis. [2]

2.2 Taxanes

Microtubules are filamentous protein polymers that are important for maintaining cell shape, cellular dynamics, cell signaling and mitosis. They are cylindrical cores composed of α - and β -tubulin monomers. First, these monomers form a heterodimer which then come together in a head-to-tail fashion to form a microtubule nucleus which elongates into protofilaments. These protofilaments associate laterally to form microtubules which have a kinetically more dynamic (+) end with exposed β -tubulin and a (-) end with exposed α -tubulin. The ability of microtubules to polymerize and depolymerize is important for cell division and chromosomal segregation during mitosis and this makes them a suitable target for anticancer drugs. [22][23]

Paclitaxel is isolated from the Pacific yew tree, *Taxus brevifolia*. Since its natural source is limited, eventually its semi-synthetic version Docetaxel was produced by the esterification of a side chain of the inactive taxane precursor 10-deacetylbaccatin III from the European yew tree *Taxus baccata*. [22]

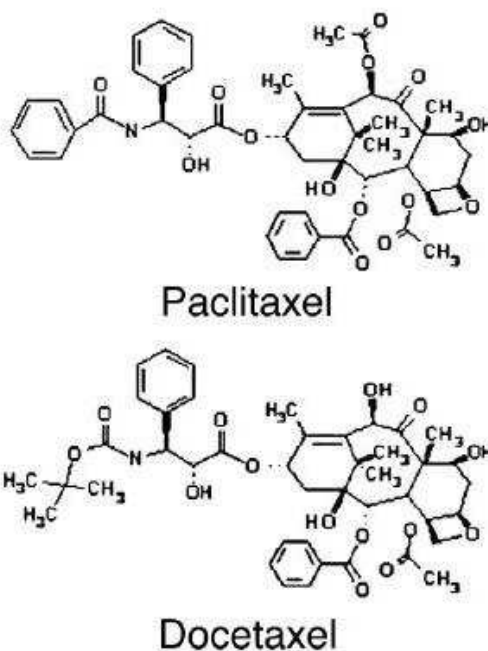


Figure 2. 3 Chemical structures of paclitaxel and docetaxel [22]

Paclitaxel is a highly hydrophobic compound and it is administered in solution with alcohol and purified Cremophor EL (polyoxyethylated castor oil), which can cause severe hypersensitivity reactions. Docetaxel is administered in solution with ethanol and polysorbate 80 which is a vector less toxic than Cremohor EL. [22] Both paclitaxel and docetaxel have increased disease-free survival and overall survival in breast cancer patients, especially when used together with anthracycline-based regimens. [22]

Both paclitaxel and docetaxel bind to the taxane site which is only present in the assembled microtubules. Docetaxel binds with greater affinity. The exact binding site of paclitaxel on the intermediate domain on B-tubulin has been determined by electron crystallography. It is near a hydrophobic cleft close to the surface of β -tubulin where paclitaxel can make hydrogen bonds and have hydrophobic contact with surrounding proteins. The binding of paclitaxel results in lateral polymerization and increases microtubule stability. [22][23]

Both microtubule-stabilizing agents like paclitaxel and docetaxel and microtubule-destabilizing agents such as colchicines suppress spindle microtubule dynamics and lead to the inhibition of the metaphase anaphase transition which results in cell cycle arrest and apoptosis. [22][23]

Following paclitaxel treatment spindle microtubule dynamics is disrupted due to the change in tension across the kinetochore during mitosis, although microtubule attachment is allowed. Docetaxel disrupts centrosome organization affecting S, G2 and M phases leading to incomplete mitosis, accumulation of cells in G2/M phase resulting in cell death. [2]

In vitro, it was demonstrated that the cytotoxic effects of paclitaxel and docetaxel are time and concentration dependent. They also suppressed proliferation, docetaxel being more effective. In paclitaxel treatments, using concentrations above 50 nM did not lead to higher cytotoxicity after 24 hours. However, exposing cells to 50 nM paclitaxel from 24 to 72 hours was more effective. [22]

In HeLa cells, 10 nM paclitaxel does not affect microtubule mass, but suppresses microtubule dynamics resulting in mitotic arrest and apoptosis, also aberrant mitosis containing multipolar spindles which cause aneuploidy. With higher concentration of paclitaxel such as 100 nM stabilized microtubule bundles are formed, tubulin is recruited to microtubule which increases the mass of microtubule polymers. Mitosis is blocked resulting in G2/M cell cycle arrest and apoptosis. [22][23]

Paclitaxel treatment leads to downregulation of Bcl-xl, and upregulation of Bax and Bak. Its effect can be inhibited by overexpressing pro-survival proteins Bcl-2 and Bcl-xl and enhanced with expression of Bak, Bad and Bax. Also, Bcl-2 is phosphorylated in response to paclitaxel treatment and more effectively following docetaxel treatment. This Bcl-2 phosphorylation occurs independently of JNK/SAPK

pathway which is required for the early phase of taxane-induced apoptosis, up to 16 hours. Low concentrations of paclitaxel (less than 200 nM) mainly inhibit mitotic spindle formation and leads to cell cycle arrest and the overall architecture of microtubule cytoskeleton is not disrupted. However, with high concentrations (higher than 200 nM) paclitaxel leads to massive microtubular damage and cells may die with mechanisms other than cell cycle arrest. [24][25]

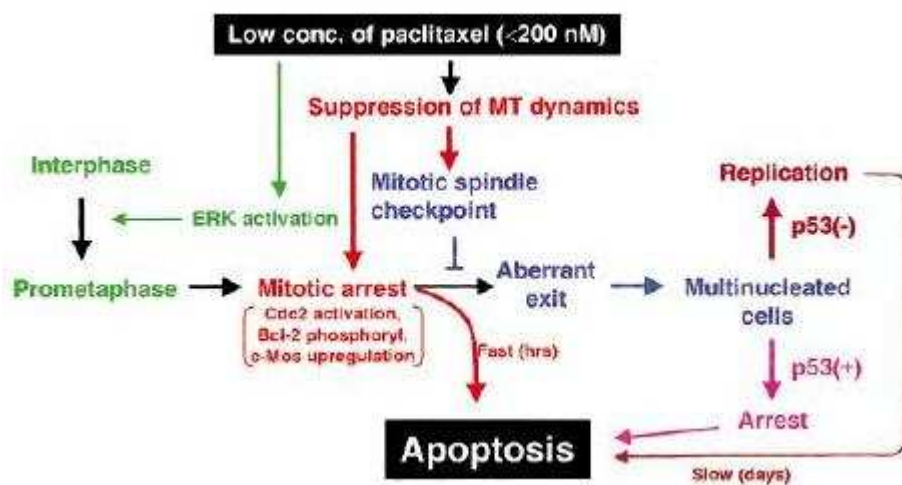


Figure 2. 4 Apoptotic mechanism following low concentrations of paclitaxel treatment. [24]

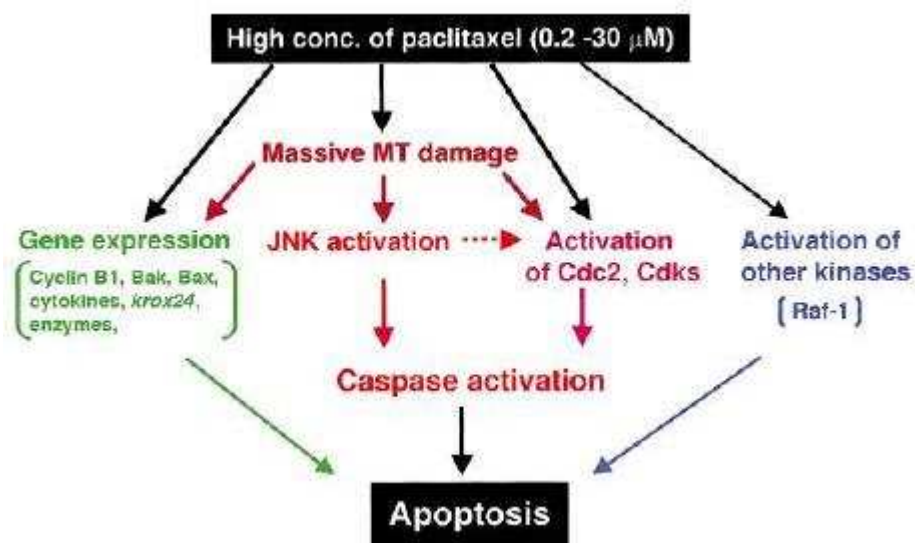


Figure 2. 5 Apoptotic mechanism following high concentrations of paclitaxel treatment. [24]

2.3 Aven

Aven was discovered by Chau *et al.* (2000) during a yeast two hybrid screen performed to identify factors other than Bcl-2 family members that interact with Bcl-xl. For this, a mutant form of Bcl-xl which could not bind to Bax or Bak, but maintained its anti-apoptotic function, was used as bait for screening a human B cell cDNA library. Among interacting cDNA clones, five encoded different lengths of the Aven protein. [9]

Aven is expressed in all adult tissues prominently in heart, skeletal muscle, kidney, liver, pancreas and testis. It is transcribed from a 1.7 kb mRNA, and although it is predicted to have a molecular weight of 38.6 kDa, it migrates with apparent weight of 55 kDa. [9]

In order to determine Subcellular localization of Aven, cell fractionation experiments were performed on HeLa cell lysates. Aven was primarily found in the light membrane portion, and also in heavy membrane and nuclear fractions. It was absent in the S-100 fraction which would mean that Aven is a membrane protein mainly associated with endoplasmic reticulum, and also mitochondria and nucleus. In cells co-transfected with Aven and Bcl-xl, some of Aven co-localized with Bcl-xl. [9]

In the co-immunoprecipitation assays, Aven interacted with Bcl-2 but not proapoptotic Bcl-2 family members Bax and Bak. Aven also did not interact with Bcl-xl with its N-terminus removed. This version of Bcl-xl is produced upon caspase cleavage and acts in a pro-apoptotic way. These results showed that Aven was linked to anti-apoptotic functions of Bcl-xl. [9]

It was shown that Aven could cause protection against apoptosis in a manner comparable to Bcl-2 in CHO (Chinese hamster ovary) cells infected by Sindbis virus which effects central nervous system and induces apoptosis. In addition to that, Aven overexpression increased cell survival in FL5.12 cells in response to IL-3 withdrawal and γ -irradiation demonstrating that Aven acts in an anti-apoptotic against different death stimuli. Aven also enhanced anti-apoptotic activity of Bcl-xl in BHK (baby hamster kidney) cells from caspase-1 induced apoptosis. [9]

Besides having interactions with Bcl-2 and Bcl-xl it was demonstrated that Aven can also interact with Apaf-1 independently of Bcl-xl since Aven interacted with these two proteins through different domains. However, the interaction between Aven and Apaf-1 was not detected with in vitro translated proteins indicating that other factors are involved. Aven overexpression reduced apoptosis induced by co-transfection of Apaf-1 and caspase-9 in 293, MCF-7 and BHK cells by inhibiting oligomerization of Apaf-1. Aven also prevented caspase-9 cleavage in response to addition of cytochrome c and dATP in COS-1 cell extracts. [9]

The role of Aven in cell survival was investigated by Figuerosa *et al.* (2004) in CHO cells exposed to various model insults such as serum deprivation, spent medium, Sindbis virus infection and staurosporine, a chemical inducer of apoptosis. Stable Aven overexpression increased cell survival in these cells in response to staurosporine treatment, serum deprivation and spent medium. Aven also enhanced Bcl-xl's antiapoptotic activity. Cells overexpressing both Aven and Bcl-xl had higher effect than the total effect of separate overexpression of these genes. However, Aven had a mildly pro-apoptotic effect in response to Sindbis virus infection in CHO cells when expressed on its own. When it was expressed together with Bcl-xl, it again enhanced Bcl-xl's pro-survival function. [10]

In those studies Aven provided initial protection against the insults when majority of the cells were viable, but failed to have a significant effect with lower viabilities. According to these results, Aven may be functioning in the early steps of apoptosis during caspase-9 activation. The mechanism of Aven's pro-apoptotic effect against Sindbis virus infection is not known. It may be due to a modification of Aven to turn it into a pro-apoptotic protein or Aven may have a dual role in the regulation of apoptosis. [10]

Aven may enhance Bcl-xl's anti-apoptotic function by stabilizing it against degradation by caspases or other proteases, or it may act by inhibiting apoptosome and caspase-9 activation. Both mechanisms would be consistent with Aven having a role in the early steps of apoptosis. [10]

The protection provided by overexpression of Aven and Bcl-xl at the same time was improved even further with the addition of an inhibitor of caspase such as XIAP in CHO cells [11] Like in Bcl-xl, Aven also had an enhancing effect on the anti-apoptotic function of E1B-19K in CHO [12] and in baby hamster kidney (BHK) cell lines [13].

Choi *et al.* (2006) demonstrated that increase in Aven expression correlates with the occurrence of childhood leukemia and poor treatment outcome. Aven overexpression also strongly correlated with unfavorable numerical cytogenic abnormalities that lead to childhood ALL whereas its expression was significantly lower in translocations with good prognosis [14].

Paydas *et al.* (2003) checked mRNA levels of aven and IAP protein survivin patients with acute leukemia and acute lymphoblastic leukemia and found that both genes were expressed more in the study groups compared to healthy control groups. This result indicates that Aven and survivin can be novel prognostic factors in acute leukemias [15].

The role of Aven in mutant superoxide dismutase-1 (mSOD1) toxicity in mouse spinal cords was investigated in amyotrophic lateral sclerosis (ALS) mSOD1 transgenic mice [16]. Aven had perinuclear localization in the cell body of motor neurons in 8 week-old mSOD-1 mice and was overexpressed compared to wild-type spinal cords. Aven's overexpression in these motor neurons was higher than the overexpression of caspase inhibitor XIAP. The expression level of both proteins was decreased after 14 weeks. The overexpression of Aven was linked to non-apoptotic nature of cell deaths in these ALS spinal cords [16].

Aven was also investigated in the damaged heart tissue of rat following traumatic brain injury. When the rats were injected with erythropoietin and methylprednisolone Aven was upregulated together with IAP protein survivin. These results showed that erythropoietin and methylprednisolone may play an important role in the regulation of Aven in heart tissue following brain injury [17].

Aven was also associated with dermal tumors, histone deacetylase inhibitors, glutamine and RNA helicases. Aven was found to be upregulated in the keloid disease which results from fibroproliferative dermal tumors [18]. It was downregulated in response to histone deacetylase inhibitor LBH589 leading to increased apoptosis in multiple myeloma cells [19]. Glutamine, which is important for cell growth, was shown to upregulate Aven in human epithelial intestinal HCT-8 and prevents apoptosis [20]. And finally, the expression of Aven was suppressed in mouse testes when mouse Vasa-homolog protein (Mvh) which is a germ cell specific RNA helicase was knocked out. It was shown that Aven is important for spermatogenesis in mouse [21].

2.4 Aim of the work

- To investigate regulation of Aven in response to paclitaxel and docetaxel treatment and how it correlates with Bcl-2, Bcl-xl, Bax, Apaf-1, Caspase-9, Caspase-3, Puma and Parp-1 protein levels in MDA-MB231 breast cancer cells (p53 deficient, ER -).

- To assess the effect of Aven's overexpression and silencing on Bcl-2 family proteins. Aven was shown to interact with Bcl-xl, Bcl-2 and Apaf-1, but not with Bax. Therefore, any regulatory effect Aven can have on these proteins was studied.

- To study the effect of its overexpression and silencing on cell death following taxane treatments. Downregulating key pro-survival proteins can lead to increased apoptosis or in contrary upregulation of these proteins can increase resistance of the cells against apoptotic stimuli. For this reason, the role of Aven in apoptotic resistance was investigated.

- To check its possible post-translational modifications, mainly phosphorylation. The predicted molecular weight of Aven is 38.6 kDa. However, it migrates with an apparent MW of 55 kDa which may indicate post-translational modifications. Phosphorylation of proteins plays an important role in apoptotic pathways affecting the proteins' activity level or function. For example, the pro-survival effect of Bcl-2 is prevented as a result of phosphorylation. In a similar manner, phosphorylation of Aven may provide useful information about its mechanism of action.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and antibodies

Chemicals and antibodies that are used are listed in Appendix A.

3.1.2 Molecular biology kits

Molecular biology kits that are used for DNA isolation, plasmid isolation and protein analysis are listed in Appendix B. Other specialty materials including DNA and protein markers are indicated in Appendix C.

3.1.3 Equipment

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

3.1.4 Buffers and solutions

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

10X TBE (Tris-Borate-EDTA) pH: 8.0

890 mM Tris, 890 mM boric acid, 20 mM EDTA

50X TAE Buffer pH: 8.5

242 g of Tris-Base, 57.1 g of acetic acid, 100 ml of 0.5 M EDTA, add H₂O up to 1 liter.

10X TBS

1 M Tris-HCl, 9% NaCl, NaOH to make pH: 7.4

1X TBS-T

1X TBS, 0.5% Tween-20

10X Running Buffer

30.3 gr Tris, 144,1 gr Glycine, 10 gr SDS (for 1 L)

10X Transfer Buffer

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

Stripping Buffer

62.5 mM Tris-HCl pH:6.7, 2% SDS, 100 mM B-mercaptoethanol (added before usage)

1X Chaps Buffer

1% Chaps, 1mM NaF, 1 mM Na₃VO₄, 1mM PMSF, 1mM EDTA in 20mM Tris-HCl pH:7.4

Separating gel

For 12% polyacrylamide gel: 6 ml of 30%acrylamide / 0.8% bisacrylamide, 3.75 ml of 4X Tris-Cl/SDS pH 8.8, 5.25 ml dH₂O, 0.05 ml 10% ammonium persulfate, 0.01 ml TEMED.

Stacking gel

0.65 ml of 30%acrylamide / 0.8% bisacrylamide, 1.25 ml of 4X Tris-Cl/SDS pH 6.8, and 3.05 ml H₂O.

3.1.5 Buffer for agarose gel electrophoresis

1 X TAE (Tris-EDTA-Acetate) buffer was used for preparation of 1% agarose gel for DNA samples and 1X TBE buffer was used for RNA samples. Gels were run at 100mV for 30 minutes. DNA was visualized by including 0.005% ethidium bromide in the gel during its preparation.

3.1.6 Aven Primers, vectors and siRNA

Aven primers were AF 5'-GATTTTCAGTGTCTCCTTAG-3' AND AR 5'-TTGCCATCATCAGTTCTC-3' (obtained from Operon Tech.).

pSG5-HA-Aven plasmid was kindly provided from Johns Hopkins University, USA (Figure 3.1).

Hs_Aven_3_Hp siRNA Sense r(CGUAUCAAGAUUUGAAA)dTdT

Antisense r(UUCAAUAUCUUGAUUUCG)dAdT

Non-silencing siRNA is designed so that it cannot bind to any mRNA expressed in human tissues. Both Aven siRNA and control siRNA were obtained from Qiagen.

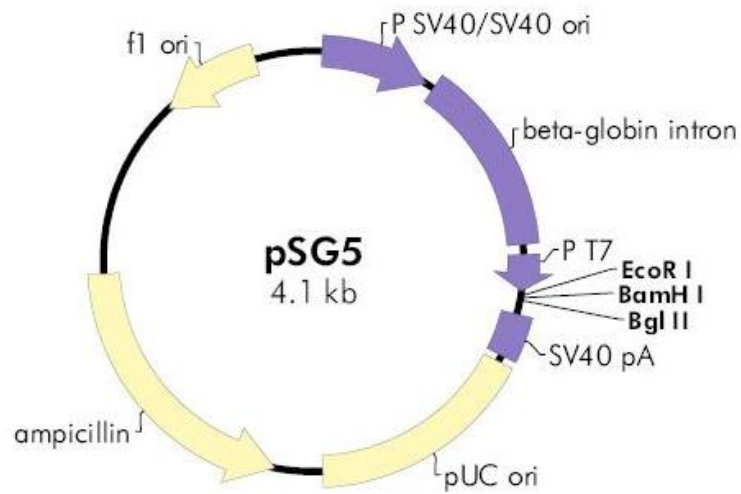


Figure 3.1 Circular map of pSG5 vector

3.1.7 Other primers

Bcl-2 upstream: 5'-TGTGGCCTTCTTTGAGTTCG-3'

Bcl-2 downstream: 5'-TCACTTGTGGCTCAGATAGG-3'

Actin Reverse: 5'-TGGCATAGAGGTCTTTACG-3'

Actin Forward: 3'-CATCACTATTGGCAACGAG-3'

All primers were obtained from Operon Technologies.

3.2 Methods

3.2.1 Cell culture

MDA-MB231 cells were grown in RPMI 1640 medium supplemented with 10 % FBS and 100 IU/ml penicillin and streptomycin in incubator (37 °C and 5% CO₂) and they were split twice a week. For splitting cells, first medium is removed, and then cells are washed once with sterile 1 X PBS. Trypsin is added and the cells are put back in incubator for 7 minutes for detachment of cells. After that, cells are centrifuged at 300 g for 5 minutes, supernatant is removed and the pellet is resuspended in medium. Cells are counted using a hemacytometer and appropriate amount of cells are seeded in flasks or dishes.

For cryopreservation, cells were trypsinized and resuspended in freezing mix containing 10% DMSO in FBS. The cells were transferred into cryovials, frozen at -80 °C for 24 hours, and then stored in liquid nitrogen.

3.2.2 Drug treatments

For drug treatments fresh 1:1000 dilutions of paclitaxel, docetaxel and the same amount of pure ethanol are prepared in serum free medium and vortexed. 50 nM from both drugs are added to the cells and corresponding amount of diluted ethanol is added to control cells.

3.2.3 MTT Assay

MTT assay is used to determine general cell viability. It is a colorimetric assay which measures the activity of the enzymes in mitochondria that reduce yellow MTT to purple colored formazan. When the cells are dead yellow color is obtained instead of purple as mitochondria is destroyed and the enzymatic activity cannot be measured.

10.000 cells are seeded in 96-well plates with 100 μ l complete medium 24 hours before experiment. For the MTT assay 10 μ l of MTT reagent is added to each cell and incubated for 4 hours at 37 °C. Following this incubation 100 μ l solubilization buffer is added and cells are kept in the incubator at 37 °C overnight to dissolve insoluble formazan and obtain purple color. The resulting absorbance is measured in spectrophotometer at 550 nm and at 655 nm as reference point.

3.2.4 Protein isolations

400 000 cells were seeded in 6-well plates 30 hours before treatments. Proteins were isolated by scraping cells in 1 % Chaps buffer following removal of medium and washing with 1 X PBS. The lysed cells are put in eppendorf tubes and left on ice for 30 minutes for complete lysis. Then, the tubes are centrifuged at 13200 rpm for 10 minutes to remove cell debris and the supernatant is kept at -80 °C.

3.2.5 Protein Assay

In order to determine the protein concentrations Bio-Rad's protein assay kit was used. 1 μ l protein lysate is spotted in replicates to wells of 96-well plate together with 4 μ l of ddH₂O. Then, 25 μ l of reagent A is added to wells followed by addition of 200 μ l

of reagent B. The proteins are incubated at room temperature for 15 minutes in the dark and the absorbance is measured at 655 nm in spectrophotometer. In order to determine the protein concentration the measured absorbance corresponds to, BSA standards were used in concentrations of 1.5 $\mu\text{g}/\mu\text{l}$, 1.0 $\mu\text{g}/\mu\text{g}$, 0.75 $\mu\text{g}/\mu\text{g}$, 0.50 $\mu\text{g}/\mu\text{g}$, 0.25 $\mu\text{g}/\mu\text{g}$ and 0.10 $\mu\text{g}/\mu\text{l}$. An equation was obtained from the linear graph of absorbance vs. concentrations of BSA standards and the concentrations of the unknown samples were determined according to this equation.

3.2.6 Western Blots

30-50 μg of protein samples were separated with SDS-PAGE in 12% polyacrylamide gels and transferred onto PVDF membranes. The membranes were blocked with 5% dried milk in TBS-T and incubated with primary antibodies (Aven (BD Biosciences), all the other antibodies are from Cell Signaling) and horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-mouse for Aven Ab, anti-rabbit for the other antibodies) with concentrations of 1:2000 and 1:10 000 respectively in antibody buffer containing 5% dried milk in TBS-T. Following washes with TBS-T proteins were analyzed using an enhanced chemiluminescence detection system (ECL-advanced, Amersham Pharmacia Biotech, Freiburg, Germany) and exposed to Hyperfilm-ECL (Amersham Pharmacia Biotech, Freiburg, Germany). For β -actin controls, blots were stripped of the bound antibodies in the stripping buffer at 65 °C for 30 minutes. Following washes in 1X TBS-T normal blotting procedure was followed.

3.2.7 RNA isolation

RNA was isolated according to Trizol method. MDA-MB231 cells were seeded in 6-well plates. First, medium is removed and cells are washed once with 1 X PBS. 500 μ l of Trizol is added under hood; cells are scrapped with blue tip and put in eppendorf tubes on ice. Then 100 μ l (1/5 of Trizol volume) of ice-cold chloroform is added and mixed by inverting the tubes several times. The tubes are kept on ice for 5 minutes and centrifuged at 1200 g for 15 minutes at 0 °C. Following centrifuge, the clear supernatant is taken and put into new tubes again on ice. 500 μ l of ice-cold isopropanol is added, mixed thoroughly by inverting the tubes and left at room temperature for 10 minutes. Then, they are centrifuged for 15 minutes at 16100 g and 0 °C. The supernatant is removed, the pellet is washed with 500 μ l of ice-cold 70% ethanol and the tubes are centrifuged again for 15 minutes at 16100 g and 0 °C. The supernatant is removed, the pellet is air-dried in refrigerated vacuum dryer, resuspended in 50 μ l DEPC H₂O and the samples are kept in -80 °C. RNA concentrations are measured using Amersham Ultrospec 21000 pro spectrophotometer.

3.2.8 Reverse Transcriptase - PCR

For RT-PCR 2 μ l of 10X RT buffer, 2 μ l of 5mM dNTP mix, 1 μ l of 10mM Oligo dT, 1 μ l of RTS, 1ul of RNA sample and 13 μ l of DEPC H₂O were mixed and incubated at 37 °C for 1.5 hours. The cDNA concentrations were measured using Amersham Ultrospec 21000 pro spectrophotometer and the samples were kept at -20 °C.

3.2.9 Real-Time PCR

Quantitative Real-Time PCR was used to determine mRNA levels of Aven and Bcl-2. Reaction mixtures were prepared from 500 ng of cDNA samples, 1X SybrGreen buffer from Quantifast Real-Time PCR kit (Qiagen) and 1µl of forward and reverse primers. DEPC H₂O was added to a final volume of 25 µl. Real-Time cycler conditions were 5 minutes at 95 °C for PCR initial activation step, 10 seconds at 95 °C for denaturation step and 40 seconds at 60 °C for combined annealing and extension steps. These reaction steps were repeated for 40 cycles. Bio-Rad iCycler was used for these Real-Time experiments.

Standards were prepared using control cDNA and either 18sRNA or β-actin primers. Amounts of cDNA were calculated by the iCycler program according to the equation of the graph obtained from standards and their corresponding threshold cycle values (Ct) at which they cross the baseline which is calculated according to the data collected from cycle 2 to 10. Aven and Bcl-2 cDNA levels were normalized to either 18sRNA or β-actin levels.

The efficiency of the PCR was calculated by iCycler program using the equation $\text{PCR efficiency} = 10^{1/\text{slope} - 1}$.

3.2.10 Aven siRNA Transfection

250 000 cells were seeded to 6-well plates, or 125 000 cells were seeded to 12-well plates 30 hours before transfection. Transfection complexes were prepared by mixing 20 nM of Aven siRNA Hs_Aven_3 (from 20µM stock) with 24 µl of Hiperfect transfection reagent for 6-well plates or 12 µl transfection reagent for 12-well plates in 100 µl serum free medium. The complex was incubated at room temperature for 10

minutes and added to cells dropwise. Suppression of Aven expression was confirmed after 48 hours by western blotting.

3.2.11 Aven overexpression

250 000 cells were seeded to 6-well plates, or 125 000 cells were seeded to 12-well plates 30 hours before transfection. Transfection complexes were prepared with 2 µg of psG5-HA-Aven vector DNA and 3 µl or 4 µl of FuGene 6 transfection reagent in 100 µl serum free medium for 6-well plates and half of these amounts for 12-well plates. The complexes were incubated in room temperature for 45 minutes and added to cells dropwise. The overexpression of Aven was checked after 24 hours.

3.2.12 Annexin V / PI staining

Annexin V staining is used to detect exposed phosphatidylserine on cell surface which is a marker for early apoptosis and PI staining is used to detect necrotic cells. First, cells were trypsinized and centrifuged at 300 g for 5 minutes. Then, the cell pellets were resuspended in 1X PBS, transferred to FACS tubes and centrifuged again at 300 g for 5 minutes. The pellets were resuspended in 100 µl of incubation buffer including 2 µl of Annexin V fluorescein and 2 µl of PI stain. The tubes were incubated at room temperature in the dark for 15 minutes and the Annexin V and PI stained cells were analyzed using BD FACS Diva.

3.2.13 Immunoprecipitation

600 µg of total protein lysate was used and 1% Chaps buffer is added to a final volume of 500 µl. 1 µg of phospho-tyrosine antibody was added and the mixture was

incubated at 4 °C for 1 hour on shaker. 50 µl of 50% slurry protein A beads were added and incubated for 2 hours at 4 °C rotating. Following these incubations beads were washed 6 times with 1 ml of 1% Chaps buffer and each time they were centrifuged at maximum speed for 30 seconds and the supernatant was removed. Finally the pellet was resuspended in sample buffer and analyzed by western blotting using Aven antibody.

For cross-linking phospho-tyrosine antibodies to protein A beads DMP (dimethylpimelimidate) was used. First, phospho-tyrosine antibodies were coupled to 50% slurry protein A beads by incubating at room temperature for 1 hour on a shaker. The beads were washed with 10 X volume of 0.2M sodium borate (pH9.0) and centrifuged 10,000 g for 30 seconds. The beads were resuspended in 10 X volume of 0.2M sodium borate (pH9.0) and DMP was added to a final concentration of 20 mM. The mixture was incubated at room temperature for 30 minutes. The reaction was stopped by washing the beads once with 0.2M ethanolamine (pH8.0) and the beads were incubated for 2hr at room temperature in 0.2M ethanolamine with gentle mixing. Following this final wash the beads were resuspended in PBS. 50 µl of cross-linked beads were incubated with 600 µg of total lysate proteins at room temperature for 1 hour. The beads were washed 6 times with 1% Chaps buffer, the resulting pellet was resuspended in sample buffer and analyzed with western blot using Aven antibody.

3.2.14 Image J

An image processing and analysis program Image J (<http://rsbweb.nih.gov/ij/>) was used for densitometric analysis of western blots. Densitometric measurements were made for each band and normalized according to their corresponding β-actin controls.

3.2.15 Phosida

In order to determine putative phosphorylation sites and their accessibility on Aven Phosida database was used (<http://www.phosida.com/>).

3.2.16 miRNA binding sites

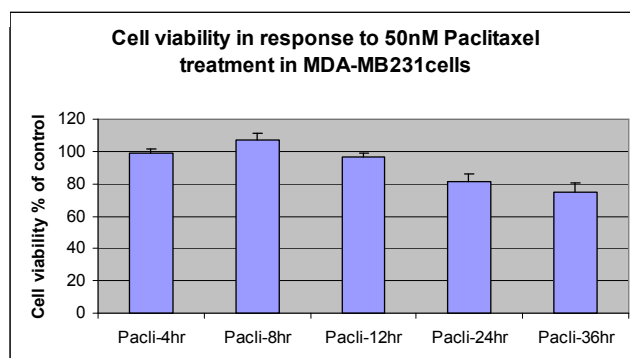
Possible miRNA binding sites on Aven was searched through <http://microrna.sanger.ac.uk/> of Sanger Institute.

4 RESULTS

4.1 Cell viability in MDA-MB231 cells in response to 50 nM paclitaxel and docetaxel treatment

Before analyzing how taxane treatment affects expression of Aven and other apoptosis proteins, we wanted to check the general effect of paclitaxel and docetaxel on cell viability using MTT assay in a time-dependent manner. For these experiments, 50 nM of taxane drugs was used as it was reported before that exposing cells to 50 nM of these drugs for 24-72 hours is more effective than using higher concentrations for 24 hours showing that after 50 nM concentration the effect of taxanes become more dependent on exposure time than concentration. [22] In both paclitaxel (Figure 4.1a) and docetaxel (Figure 4.1b) treatments cell viability was not affected until 12 hours and it became pronounced at 24 hours leading to 32% cell death following docetaxel treatment and 26 % cell death following paclitaxel treatment after 36 hours.

a)



b)

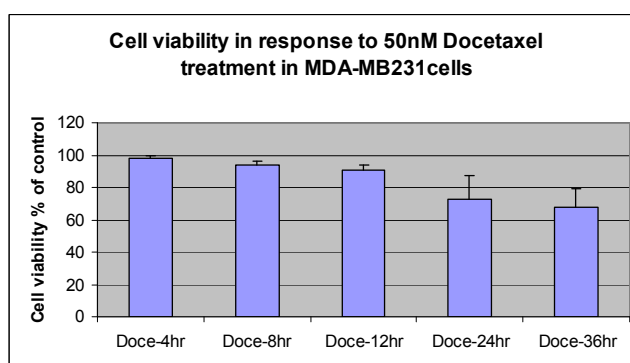


Figure 4. 1 MTT assay to measure cell viability in MDA-MB231 cells in response to 50nM Paclitaxel (a) and 50 nM Docetaxel (b) treatment in a time-dependent manner. Error bars represent standard deviations of replicates.

4.2 Regulation of Aven in response to 50 nM taxane treatment

Docetaxel and paclitaxel treatment lead to cell cycle arrest and eventually apoptosis through the intrinsic pathway with concentrations as low as 10 nM. [22][23] In order to increase apoptosis without using high concentrations of these drugs, which cause a lot of toxicity, it is important to find key pro-survival proteins that lead to apoptotic resistance so that by suppressing their expression levels induction of apoptosis can be augmented. We wanted to check whether Aven, a novel pro-survival protein, can be such a candidate to play a role in apoptotic resistance and investigated how it is regulated and how it correlates with other apoptosis proteins in response to taxane

treatment. Therefore, the expression profile of Aven in response to taxane treatment was compared to those of Bcl-2, Bcl-x1, Bax, Apaf-1, caspase-3, PARP-1 and Puma in a time-dependent manner using western blot analysis.

Aven expression was increased in protein levels (Figure 4.2a) following 50 nM paclitaxel treatment starting from 2 hours, peaking at 4 and 8 hours and lasting until 12 hour time point. However, after 24 hours the protein levels of Aven was dropped. According to the Real-time analysis (Figure 4.2b), the mRNA levels of Aven decreased after 12hours being downregulated further after 24 hours.

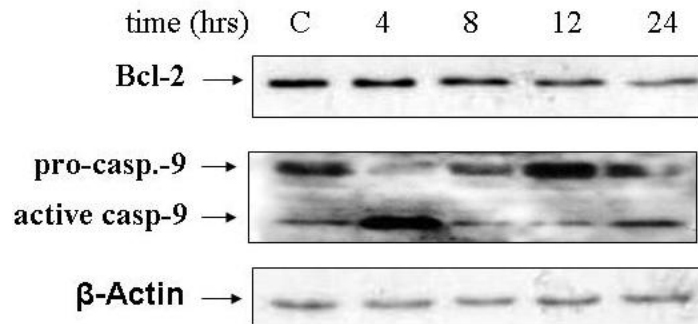
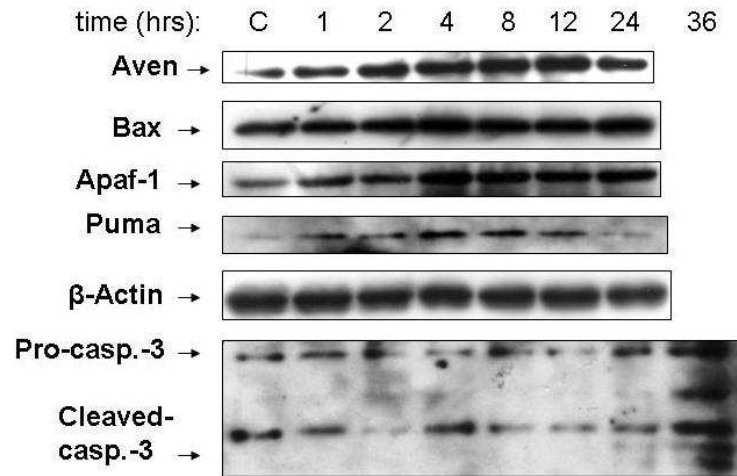
As can be seen from Figure 4.2a, following 50 nM paclitaxel treatment Apaf-1 was upregulated in protein levels starting from 4 hours and lasting through 24 hours, Puma was upregulated at 4, 8 and 12 hour time points and was decreased after 24 hours, the protein levels of Bcl-2 was downregulated after 12 hours and was decreased further after 24 hours. In addition to this caspase-9 was activated at 4 hour time point, but the cleaved caspase-9 levels dropped after that until 24 hour time point. In parallel, the protein levels of caspase-9 first decreased at 4 hours due to cleavage then increased starting from 8 hours, peaking at 12 hours again becoming similar to control levels and then started decreasing again after 24 hours due to the re-initiation of caspase-9 cleavage. Active caspase-3 was increased starting at 24 hours through 36 hour time point. Besides this, Bax remained unchanged except for a slight increase at 4 hours.

When MDA-MB231 cells were treated with 50 nM docetaxel Aven was upregulated in early time points at 4 and 8 hours, then decreased at 12 hour time point and then it was slightly upregulated again at 24 hours in protein levels (Figure 4.3a). The mRNA level of Aven was decreased after 12 hours, increasing slightly after 24 hours (Figure 4.3b) correlating with the western blot results. Early regulation of Aven coincided with upregulation of Bcl-2 which peaked at 4 and 8 hours and decreased after

12 hours being downregulated even more at 24 hour time point. Similar to paclitaxel treatments, following docetaxel treatment Apaf-1 was upregulated at 4 hours until 12 hour time point and it decreased at 24 hours and caspase-3 activation increased starting from 24 hours through 36 hours. Furthermore, Bax was upregulated and 1 and 2 hours and then decreased to its control levels, and Bcl-xl remained relatively unchanged having a slight increase starting from 8 hours through 24 hours. We also analyzed Parp-1 cleavage which is a downstream effector of caspase-3. Parp-1 cleavage started at 1 hour peaking at 2 hours and then decreased after 4 hours. This pattern was also true for the uncleaved version of Parp-1. The early response of Parp-1 was interesting as its known upstream effector caspase-3 is activated after 24 hours (Figure 4.3a).

50 nM Paclitaxel Treatment

a)



b)

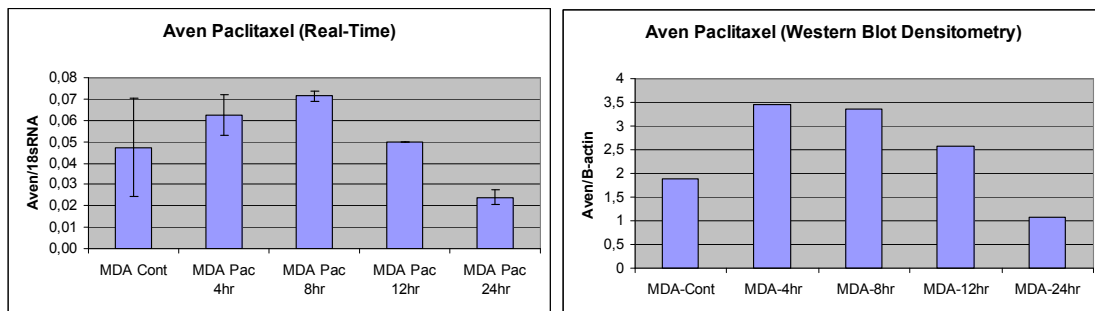
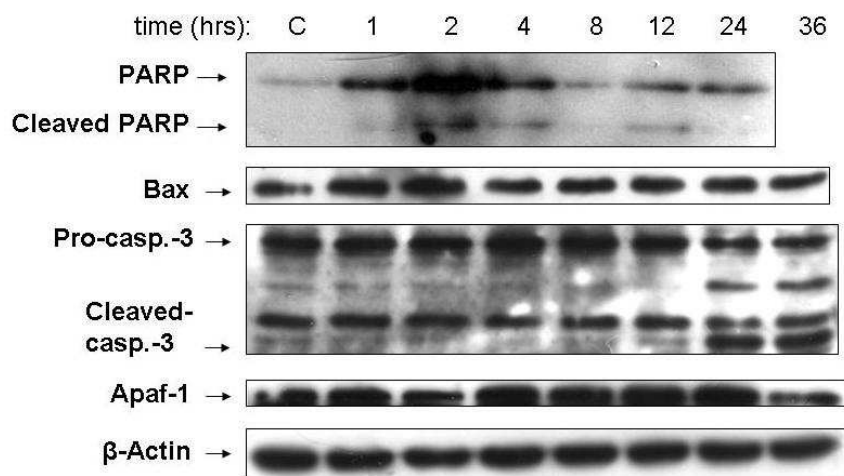


Figure 4. 2 Aven is regulated in response to 50 nM paclitaxel treatment in MDA-MB231 cells. **a)** Western blot analysis of Aven, Bcl-2, Bax, Apaf-1, Puma, caspase-3 and caspase-9 following 50 nM paclitaxel treatment in a time-dependent manner. **b)** Comparison of Aven mRNA (Real-time analysis) and protein levels (western blot densitometry) in response to 50 nM paclitaxel treatment. Error bars represent standard deviation of replicates.

a)

50 nM Docetaxel Treatment



b)

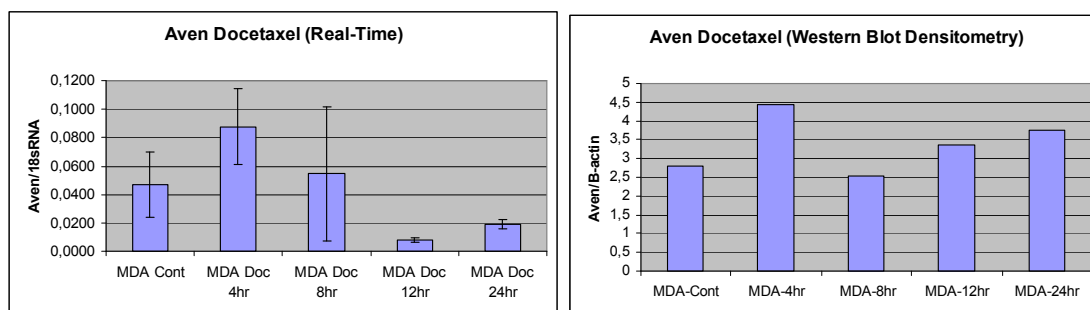
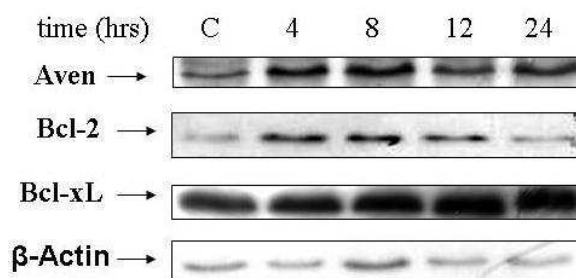


Figure 4. 3 Aven is regulated in response to 50 nM docetaxel treatment in MDA-MB231 cells. **a)** Western blot analysis of Aven, Bcl-2, Bcl-xl, Bax, Apaf-1, PARP and caspase-3 following 50 nM docetaxel treatment in a time-dependent manner. **b)** Comparison of Aven mRNA (Real-time analysis) and protein levels (western blot densitometry) in response to 50 nM docetaxel treatment. Error bars represent standard deviation of replicates.

4.3 Effect of Aven expression on Bcl-2 family proteins, Apaf-1 and Caspase-9

Because Aven was shown to interact with Bcl-2, Bcl-xl and Apaf-1 but not with Bax and it was shown that Aven inhibited caspase-9 activation [9], the endogenous levels of these proteins were investigated in response to Aven siRNA and overexpression to check whether Aven has any regulatory effects on the proteins it interacts with.

4.3.1 Effect of Aven overexpression on Bcl-2, Bcl-xl, Bax, Apaf-1 and Caspase-9

MDA-MB231 cells were transfected with psG5 empty vector as control and psG5-HA-Aven vector for Aven overexpression. Following this treatment the endogenous protein levels of Bcl-2, Bcl-xl, Bax, Apaf-1 and caspase-9 were analyzed by western blot (Figure 4.4a).

In the absence of apoptotic inducers, overexpression of Aven leads to a decrease in Bcl-2 and slightly in Bcl-xl and Bax protein levels. However, no detectable change was observed in the protein levels of Apaf-1 and caspase-9. This may show that Aven can be a substitute for the pro-survival proteins at the same time slightly suppressing pro-apoptotic protein Bax. The decrease in Bcl-2 expression is also confirmed with Real-Time PCR analysis of Aven and Bcl-2 mRNA levels (Figure 4.4b).

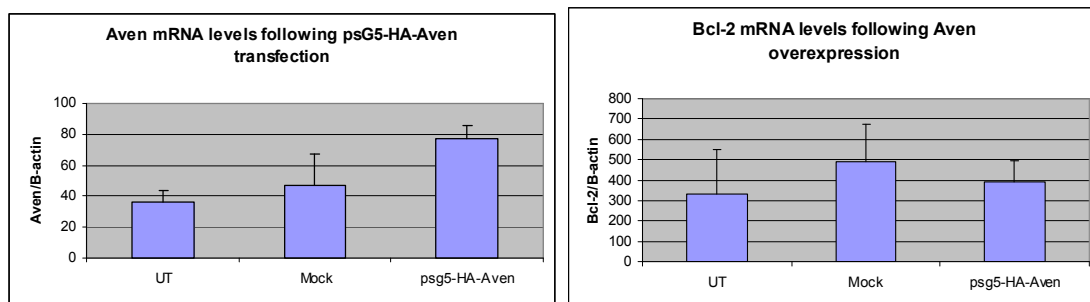
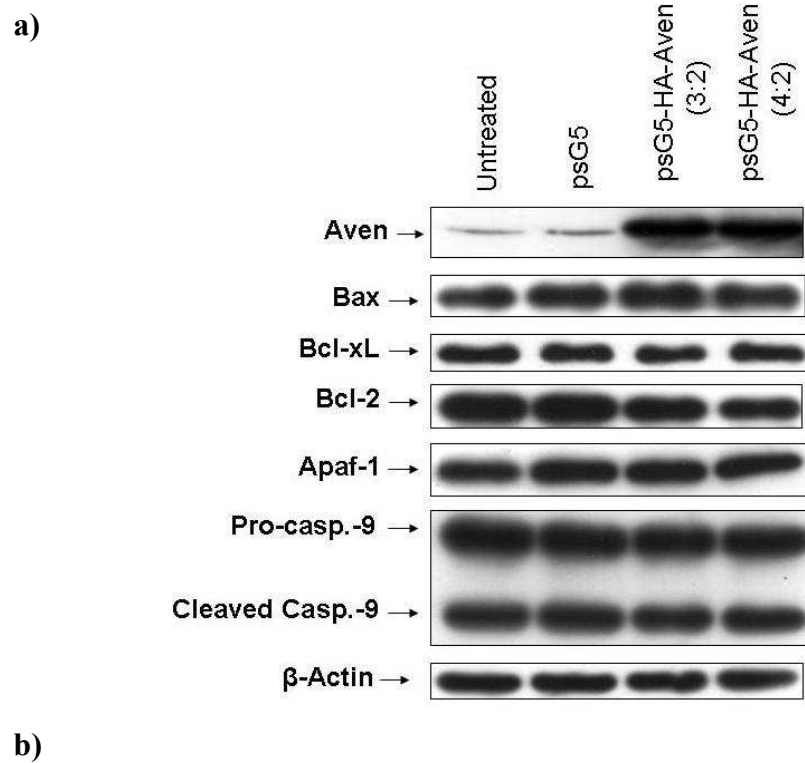


Figure 4. 4 Aven overexpression decreases Bcl-2 levels. Effect of Aven overexpression on Bcl-2 proteins in MDA-MB231 cells **a)** Cells are transfected with mock and pSG5-HA-Aven vectors using 2 μ g of DNA and either 3 μ l (3:2) or 4 μ l (4:2) of FuGene 6 transfection reagent and Bcl-2 protein levels are analyzed with western blot. **b)** Real-Time PCR analysis of Aven and Bcl-2 mRNA levels following vector transfections. (Real-Time PCR efficiency: 91.7 %) Error bars represent standard deviations of replicates.

4.3.2 Effect of Aven silencing on Bcl-2, Bcl-xL and Bax

MDA-MB231 cells were transfected with non-silencing siRNA as control and Aven siRNA Hs_Aven_3 from Qiagen for silencing Aven gene expression. Following this treatment the endogenous protein levels of Bcl-2, Bcl-xL and Bax were analyzed with western blot (Figure 4.5a).

The endogenous levels of Bcl-2 and Bax were regulated in response to Aven silencing. Both pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 were upregulated, the increase in Bax expression being more prominent. However, Bcl-xL expression levels did not have a detectable change. The increase in Bcl-2 expression is also confirmed with Real-Time PCR analysis of Aven and Bcl-2 mRNA levels (Figure 4.5b).

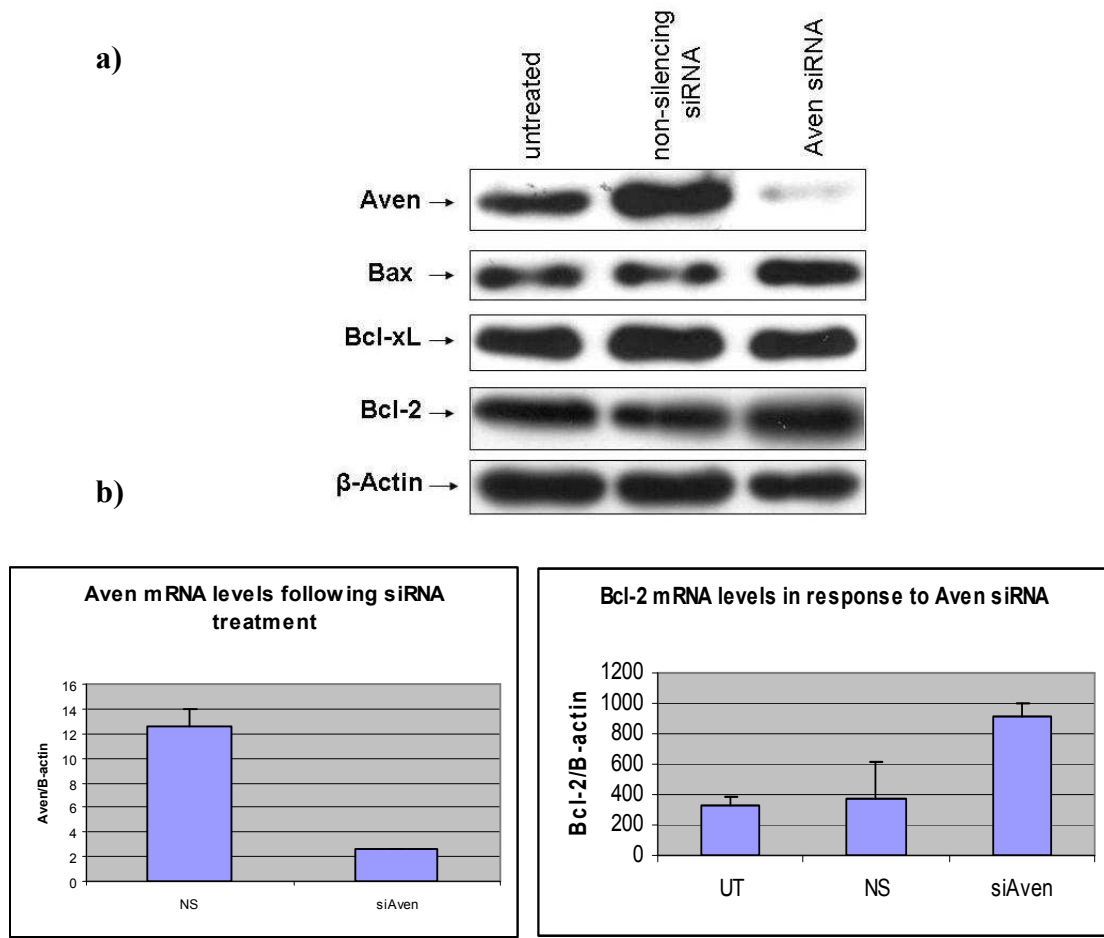


Figure 4. 5 Aven suppression increases Bcl-2 and Bax levels. Effect of Aven siRNA on Bcl-2 proteins in MDA-MB231 cells **a)** Cells are transfected with non-silencing control and Aven siRNA. Bcl-2 members are analyzed with western blots. **b)** Real-Time analysis of Aven and Bcl-2 mRNA levels following siRNA treatment. (Real-Time PCR efficiency: 150% original, 99% modified)

4.4 Effect of Aven expression on apoptosis induced by docetaxel and paclitaxel

The role of Aven in protection against apoptosis in response to taxane group of drugs was investigated. MDA-MB231 cells were exposed to 50 nM paclitaxel and docetaxel following either silencing or overexpression of Aven. The apoptotic cells were detected using Annexin V/PI staining method (Figure 4.6).

Transfection of cells with either pSG5 vector or pSG5-HA-Aven before drug treatment resulted in increased cell death compared to cells that were treated with 50 nM taxanes only. There was no detectable difference in cell death between the mock vector and Aven vector treated cells. The increase in cell death in transfected cells compared to untreated ones was probably due to the mechanism of transfection which made cells more vulnerable to drug treatment and was not related to Aven overexpression (Figure 4.6a).

When MDA-MB231 cells were transfected with non-silencing siRNA and Aven siRNA prior to 50 nM taxane treatment, cells with Aven siRNA became more vulnerable to docetaxel treatment. However, Aven silencing did not result in increased cell death in paclitaxel treated cells (Figure 4.6b).

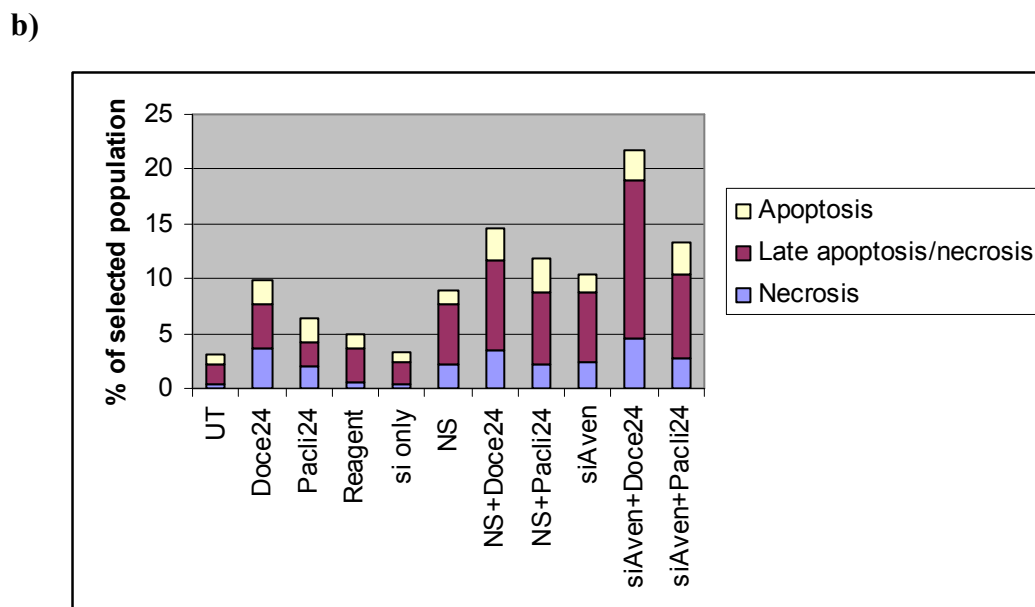
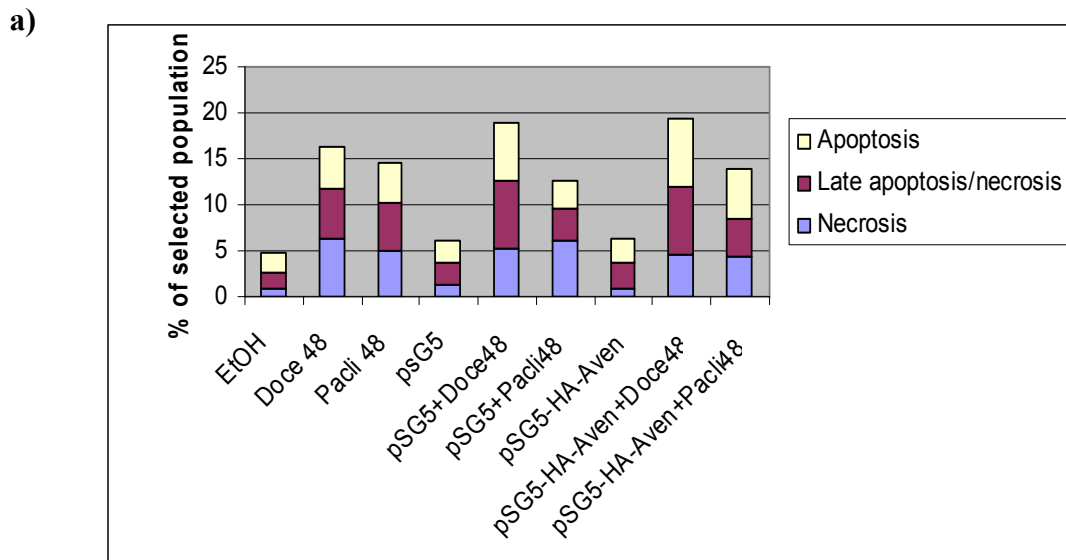


Figure 4. 6 Effect of Aven overexpression and silencing on cell death. Annexin V/PI staining of MDA-MB231 cells following siRNA and overexpression studies and drug treatments were analyzed. Annexin V positive cells indicate early apoptotic cells, PI positive cells are necrotic and late apoptotic cells are positive for both Annexin V and PI. **a)** Cells were exposed to 50 nM docetaxel and paclitaxel for 48 hours following transfection of cells with pSG5 vector as control and pSG5-HA-Aven vector for overexpression. **b)** Cells were exposed to 50 nM docetaxel and paclitaxel for 24 hours following transfection of cells with non-silencing siRNA (NS) as control and Aven siRNA for silencing.

4.5 Aven is phosphorylated at tyrosine residues

According to phosphorylation site databases (e.g. Phosida), Aven has three putative phosphorylation sites: Serine94, Threonine98 and Tyrosine99. In order to confirm Aven's phosphorylation on tyrosine residue(s), immunoprecipitation experiments were performed with phosphotyrosine antibody.

Cell lysates were precipitated with phosphotyrosine antibody (rabbit) and blotted with Aven antibody (mouse). Both Aven and heavy-chain IgG migrate at 55 kDa. Although antibodies of different sources were used for immunoprecipitation and blotting, due to cross-reaction between antibodies, the heavy-chain IgG band was not completely omitted (Figure 4.7a). In order to be sure that Aven protein was immunoprecipitated, the phospho-tyrosine antibody was crosslinked to protein A beads with DMP (Figure 4.7b). Following cross-linking heavy chain IgG band was not detected in immunoprecipitated cell lysate when blotted with anti-rabbit IgG antibody (Figure 4.7b). This shows that the 55 kDa band seen in the protein lysate lane in Figure 4.7b is Aven protein and not heavy-chain IgG and that Aven has tyrosine phosphorylation.

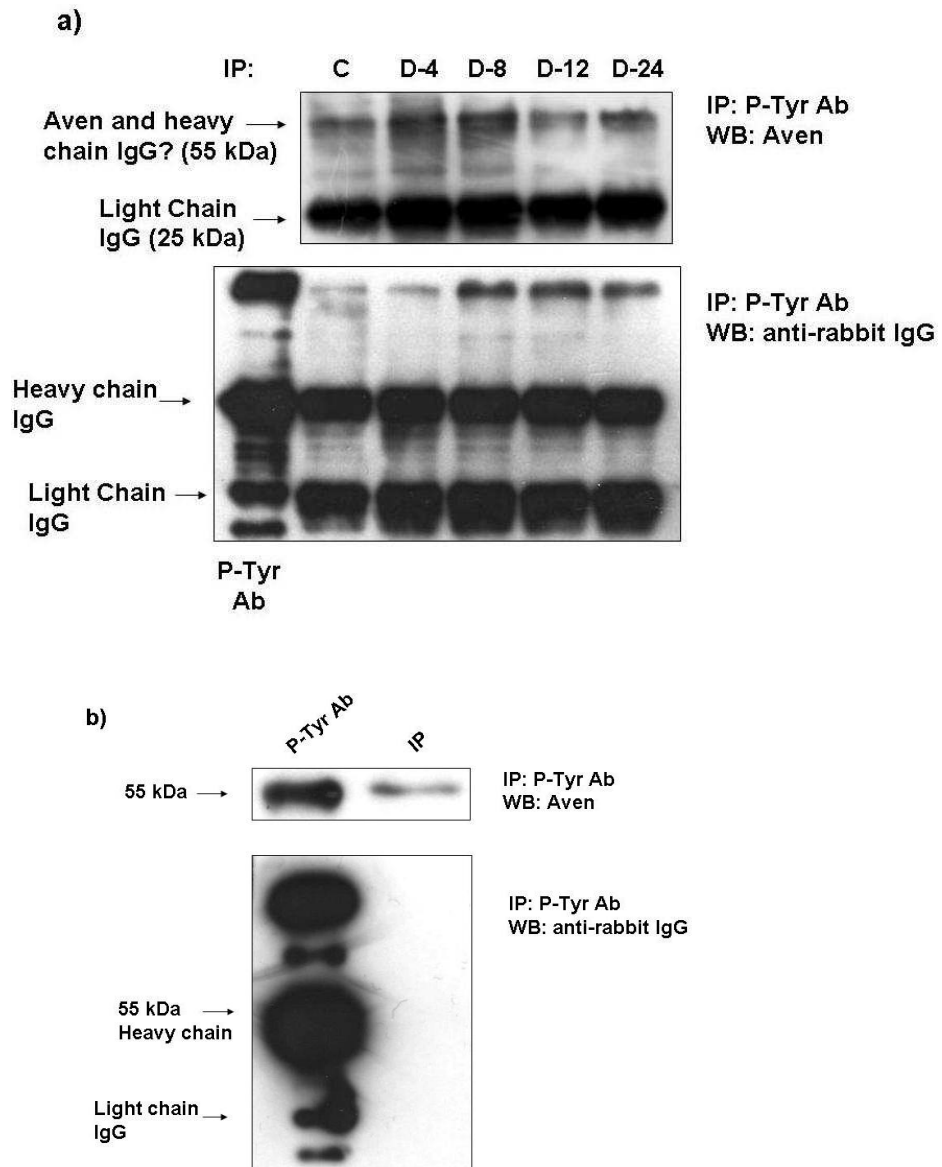


Figure 4. 7 Aven is immunoprecipitated by phospho-tyrosine antibodies **a)** Immunoprecipitation of MDA-MB231 cell lysates treated with 50 nM docetaxel with phosphotyrosine antibody (rabbit) and western blot analysis with Aven (mouse) and anti-rabbit IgG antibodies prior to crosslinking of IP antibodies to protein A beads. **b)** Immunoprecipitation of untreated cell lysate with phosphotyrosine antibody cross-linked to protein A beads and western blot analysis with Aven antibody and then with anti-rabbit IgG antibody after stripping.

5 DISCUSSION

Taxane group of drugs paclitaxel and docetaxel are clinically used for the treatment of breast, ovary and lung cancer patients. These chemotherapeutic agents bind to β -tubulin in microtubules and increase their stabilization disrupting microtubular dynamics which are very important for cell cycle progression. Low concentrations of taxanes (less than 200 nM) mainly affect mitotic spindles and not microtubule cytoskeleton leading to aberrant chromosomal segregations and cell cycle arrest. Higher doses of these drugs (more than 200 nM) lead to massive microtubular damage inside the cell and mechanisms other than cell cycle arrest may be employed.[23] Since cancerous cells divide more rapidly than healthy cells, they are more prone to cell cycle arrest induced by taxane treatment and so they are the main target for these drugs. The level of cell death is more time-dependent than dosage-dependent after 50 nM of paclitaxel and docetaxel meaning increasing exposure time to these drugs is more effective than increasing the concentration over 50 nM. [22] In MDA-MB231 cells 50 nM taxane treatment affected cell viability starting from 12 hours and resulted in approximately 30 % cell death after 36 hours (Figure 4.1). Silencing of key pro-survival proteins or overexpression of pro-apoptotic ones can lead to increased vulnerability in cancerous cells. As a result investigating novel inhibitor of apoptosis proteins such as Aven can prove useful in breaking the resistance of tumor cells in response to death stimuli such as drug treatments.

Aven is a pro-survival protein with intracellular membrane localization. It is expressed in all tissues. It interacts with pro-survival proteins Bcl-xl and Bcl-2, but not with pro-apoptotic Bcl-2 family members Bax or Bak. Aven also has interaction with Apaf-1 preventing its oligomerization and formation of apoptosome. [9] Aven was

found to be upregulated in acute leukemia, acute lymphoblastic leukemia and fibroproliferative dermal tumors [18], and was found to be upregulated to inhibit apoptosis in response to stimuli that lead to damage such as mutant superoxide dismutase-1 (mSOD1) toxicity in mouse spinal cord [16], traumatic brain injury in rats [17] or in response to growth inducers such as glutamine [20] to inhibit apoptosis. Downregulation of Aven lead to increased apoptosis following histone deacetylase inhibitor LBH589 treatment of multiple myeloma cells [19].

In this project, Aven's possible role in apoptotic resistance in MDA-MB231 cells following taxane treatment was investigated by checking how it is regulated and how it correlates with other apoptosis proteins. More insight into Aven's mechanism of function was obtained using siRNA and overexpression studies and its phosphorylation was analyzed using immunoprecipitation.

As can be seen from Figure 4.2a following 50 nM paclitaxel treatment Aven was upregulated between 2 and 12 hours peaking at 4 hours and decreasing after 24 hours. This pattern correlated with protein levels of Apaf-1, Caspase-9, Bcl-2 and Puma. Both Apaf-1 and Puma were upregulated starting from 4 hours. Puma levels started decreasing after 8 hours whereas upregulation of Apaf-1 was maintained at the same level. Interestingly, Caspase-9 was activated at 4 hours, but then the active caspase-9 levels dropped at 8 hours and pro-caspase-9 levels increased indicating new protein synthesis. The pro-caspase-9 and active caspase-9 levels became comparable to control levels at 12 hours and the cleavage of pro-caspase-9 started again at 24 hours. The downregulation of active caspase-9 at 8 hours may be due to degradation following sustained upregulation of Aven leading to replenishment of pro-caspase-9.

The upregulation of pro-survival Aven and pro-apoptotic proteins Apaf-1 and Puma, and activation of caspase-9 at the same time points following paclitaxel treatment

may indicate a competition between them trying to break the balance in favor of either survival or death. At early time points Aven is more dominant leading to degradation of active caspase-9 preventing it from relaying the death signal downstream. However, downregulation of pro-survival proteins Aven and Bcl-2 at 24 hours show that at this time point pro-apoptotic proteins win the competition leading to cell death. This is consistent with the MTT data presented in Figure 4.1 showing that cell death becomes prominent at 24 hours and until 12 hours cell viability does not change. This is further supported by the increase in activation of caspase-3 at 24 hours.

In response to 50 nM docetaxel treatment, Aven was upregulated at 4 and 8 hours, decreased at 12 hours and then increased again at 24 hour time point (Figure 4.3a). Accordingly, Apaf-1 was upregulated at 4 hours, but decreased at 24 hour time point. This decrease may be due to the upregulation of Aven at 24 hours. However, this increase in Aven levels did not prevent caspase-3 activation at 24 hours which lasted through 36 hour time point. The protein levels of Parp-1, cleaved Parp-1 and Bax were increased at 1 and 2 hours, but downregulated after 4 hours indicating that Aven upregulation at 4 hours had a dominant effect over these pro-apoptotic proteins probably leading to their degradation. Interestingly, activation of Parp-1 which is known to be a downstream effector of caspase-3 occurred hours before caspase-3 activation. A similar result exists in literature. [27] This result suggests that other proteases or caspases may be involved in Parp-1 activation. Bcl-2 also correlated with Aven expression being upregulated between 4 and 12 hours and decreasing at 24 hours. Caspase-9 was not analyzed in response to docetaxel treatment, but considering the similarities between docetaxel and paclitaxel regulated Apaf-1 protein levels as well as activation of caspase-3 it is expected that the expression pattern of caspase-9 would be similar to that of paclitaxel treated cells. Despite the upregulation of Aven at 24 hours,

due to the downregulation of Bcl-2 and upregulation of active caspase-3 cell viability dropped at 24 hours decreasing further after 36 hours.

Aven's early upregulation at protein level in these experiments is consistent with previous data in the literature showing that it is mainly effective at early stages of apoptotic pathway during caspase-9 activation. [10] The Real-Time PCR data showing the mRNA levels of Aven (Figure 4.2b and Figure 4.3b) indicates a decrease in mRNA levels following taxane treatment. This shows that the increase in protein levels may be a result of protein stabilization rather than an increase in gene expression suggesting that there may be ubiquitinylation mechanisms downregulating protein levels of Aven and these mechanisms may be inhibited in response to drug treatments leading to stabilization of Aven. This can be verified in future experiments by immunoprecipitating Aven and checking for ubiquitin using anti-ubiquitin antibodies in western blot analysis. Another way of Aven regulation can be through microRNAs that bind to Aven mRNA and downregulate its protein levels. According to the microRNA database of Sanger Institute there are 56 putative miRNA binding sites on Aven. These miRNAs may be removed in response to drug treatments leading to upregulation in Aven protein levels. However, this result showing that mRNA levels of Aven is downregulated without an initial upregulation may also be due to the relatively low (70%) efficiency of the Real-Time PCR data which probably occurred because of fluctuations in SybrGreen signals.

When Aven was overexpressed in MDA-MB231 cells there was a decrease in Bcl-2 protein and mRNA levels (Figure 4.4) which may indicate that Aven and Bcl-2 have similar functions and when Aven is overexpressed Bcl-2 is downregulated as Aven can take over some of Bcl-2's activities. Besides this, the protein levels of Bax, Bcl-xl, Apaf-1 and caspase-9 were unaffected. In these experiments transfection efficiency was

not checked as Aven was shown to be overexpressed successfully in western blot analysis. However, if the transfection efficiency is low despite overexpression of Aven this may explain why Bax, Bcl-xl, Apaf-1 and caspase-9 levels did not change. In this case, since the majority of the cells would not be transfected they would mask the effect of transfected cells. Therefore this result can be further improved by checking the transfection efficiency and enhancing it.

When Aven was silenced with siRNA both Bcl-2 and Bax were upregulated, the increase in Bax levels being more prominent whereas Bcl-xl levels remained unchanged (Figure 4.5). This may again indicate that Bcl-2 and Aven have similar functions and that with the upregulation of Bax the cells may be more vulnerable to apoptotic inducers. Similar to overexpression studies, this result can be improved by checking the transfection efficiency and increasing it. In siRNA studies it would be also interesting to check what happens to Bcl-2 and Bax levels following Aven siRNA and taxane treatment. In this case since Aven cannot be upregulated Bcl-2 would be increased even further to protect the cells and Bax would also be upregulated to overcome pro-survival effect of Bcl-2. In addition to this the interaction between Aven and Bcl-2 can be effected from these siRNA and overexpression studies as the two proteins are regulated in opposite fashion. For Aven silencing siRNA technique was used which provides transient suppression of expression. For stable transfections and hence sustained suppression shRNA can be used. Also different vectors such as retroviruses or different transfection techniques such as electroporation can be employed.

Aven overexpression did not result in protection against taxane-induced cell death showing similar cell death levels to mock vector treated cells. However, the vector treated cells had higher amount of cell death compared to untransfected ones following

drug treatments. This may be a toxic effect of the transfection method making cells more vulnerable to drugs (Figure 4.6a). This result is consistent with previous data in the literature showing that Aven overexpression has an enhancing effect when it is co-expressed with other pro-survival proteins such as Bcl-xl, but is not protective when expressed on its own. [10][12][13] Also, this may be due to a mechanism that skips apoptosome for caspase activation by-passing Aven's protective role and rendering it ineffective. [26] Another possibility is that MDA-MB231 cells are deficient in p53 and cannot relay the cell cycle arrest information to downstream effectors so it takes a longer time for these drugs to be effective on these cells through alternative pathways. Since Aven is upregulated at early time-points and downregulated after 24 hours it cannot be effective on the delayed activity of these taxane drugs.

Following Aven siRNA treatment MDA-MB231 cells responded to docetaxel treatment with increased apoptosis whereas Aven silencing had no effect in response to paclitaxel treatment (Figure 4.6b). The inability of Aven siRNA to increase vulnerability of MDA-MB231 cells against paclitaxel treatment may be due to increased levels of Bcl-2 in response to Aven silencing (Figure 4.5). Or, apoptosome may not be on the main pathway of action for these drugs. When cells were treated with docetaxel Aven was upregulated a second time at 24 hour time point. Therefore, it is also possible that when Aven was silenced that upregulation is abolished making cells more vulnerable in response to docetaxel treatment.

Phosphorylation can play very important roles for the functioning of apoptotic proteins. For example, phosphorylation of pro-survival protein Bcl-2 prevents its anti-apoptotic function and helps progression of apoptosis. In addition to this, phosphorylation can be important for stabilization or degradation of proteins. As a result, phosphorylation of Aven was studied. Aven has three putative phosphorylation

sites at Serine94, Threonine98 and Tyrosine99. We investigated tyrosine phosphorylation of Aven with immunoprecipitation assay.

First, total cell lysates were used following 50 nM docetaxel treatment at 4, 8, 12, and 24 hour time points (Figure 4.7a). Immunoprecipitation was performed using phospho-tyrosine antibody, and immunoblotting was done with Aven antibody. Since both Aven and heavy chain IgG migrate at 55 kDa, we chose the immunoprecipitation and immunoblotting antibodies from different sources namely rabbit and mouse respectively. Although this eliminated majority of the heavy chain IgG still two bands were apparent at 55 kDa, presumably one of them being Aven and the other being rabbit heavy chain IgG cross-reacting with mouse antibody. The light-chain IgG was seen around 25 kDa.

In order to make sure that Aven was really precipitated by phospho-tyrosine antibody, phospho-tyrosine antibodies were cross-linked to protein A beads using DMP. As a result, one band was obtained at 55 kDa at the immunoprecipitated protein lysate lane (Figure 4.7b) when membrane was blotted with Aven antibody. In order to check that this was not heavy-chain IgG the same blot was stripped and reblotted with anti-rabbit IgG and the band disappeared meaning that the 55 kDa band in the Aven blot was actually Aven protein precipitated by phospho-tyrosine antibody. This indicates that it is phosphorylated on its tyrosine residue(s) (Figure 4.7). This result can further be confirmed by using Aven antibody for immunoprecipitation and then immunoblotting with phospho-tyrosine antibody. Phosphorylation may have importance for the activity of Aven or its post-translational regulation affecting its stabilization and function. Among the putative phosphorylation sites of Aven threonine and serine have high accessibility on the protein surface whereas tyrosine has medium accessibility.

Therefore, investigating Aven phosphorylation on serine and threonine residues and finding about upstream kinase pathways is important for future experiments.

Aven was mainly isolated from light membrane portion of cell fractions according to Chau *et al.* (2000) which indicates endoplasmic reticulum. It was also found in heavy membrane and nuclear portions which would indicate mitochondrial and nuclear localization. Considering that Aven interacts with Apaf-1 and prevents its oligomerization, it can be speculated that Aven can induce conformational changes in proteins or affect protein folding. Together with the data from Chau *et al.* (2000) which showed that it was isolated from endoplasmic reticulum portion of cell extracts it can be very interesting to check Aven's role in protein folding and ER stress and ER-induced cell death. This would also be consistent with the data that Aven interacts with Bcl-x1 and Bcl-2 since both of these proteins can be found in the ER.

The main role of Aven was found to be related to apoptosome formation and hence the intrinsic pathway of apoptosis. However, Aven may also function in response to stimuli that act through extrinsic pathway if the mitochondrial amplification loop is used through BH3-only protein Bid.

If these experiments were done with another breast cancer cell line MCF-7 which is estrogen receptor positive and caspase-3 deficient, the p53 pathway would proceed, but the apoptotic pathways would be delayed at caspase-3 level. It has been previously reported that caspase-3 and another executioner caspase caspase-7 have redundant functions and can compensate for one another. Therefore, it is expected that apoptosis induced by taxanes would proceed through caspase-7 in MCF-7 cells. [61]

6 CONCLUSION

In this study we have shown that Aven is regulated in response to low concentrations of microtubule-stabilizing agents, paclitaxel and docetaxel in MDA-MB231 cells and is upregulated at early time points together with Bcl-2, Apaf-1, Puma, Parp-1 and Caspase-9. Aven overexpression lead to decreased Bcl-2 protein levels, whereas Aven silencing resulted in increased levels of both Bcl-2 and Bax. It cannot protect MDA-MB231 cells from taxane treatments when overexpressed. However, it leads to a small degree of vulnerability following docetaxel treatment when silenced. And finally, Aven is phosphorylated on its tyrosine residues.

The role of Aven in taxane induced cell death can further be investigated by expressing or silencing it together with other pro-survival proteins like Bcl-xl. Also, phosphorylations other than tyrosine residues can be checked and using kinase inhibitors upstream mechanisms can be investigated. Checking Aven's localization following different death inducers can also help elucidate its mechanism.

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APPENDIX A

CHEMICALS

(in alphabetical order)

Name of Chemical	Supplier Company	Catalog Number
Acrylamide/Bis-acrylamide	Sigma, Germany	A3699
Agarose low EO	Applichem, Germany	A2114
Ammonium persulfate	Sigma, Germany	A3678
Ampicillin Sigma,	Germany	A9518
Antibiotic solution	Sigma, Germany	P3539
Anti-Apaf-1 Ab	Cell Signal Tech., USA	4452
Anti-Aven Ab	BD Biosciences	612520
Anti-Bax Ab	Cell Signal Tech., USA	2772
Anti-Beta-actin Ab	Cell Signal Tech., USA	4967
Anti-Bcl-2 Ab	Cell Signal Tech., USA	2872
Anti-Bcl-xl Ab	Cell Signal Tech., USA	2762
Anti-Cleaved caspase-3 Ab	Cell Signal Tech., USA	9661
Anti-Cleaved caspase-9 Ab	Cell Signal Tech., USA	9501
Anti-Cleaved Parp-1 Ab	Cell Signal Tech., USA	9542
Anti-Phosphotyrosine Ab	BD Biosciences	610009
Anti-Puma Ab	Cell Signal Tech., USA	4976
Chloroform	Merck, Germany	102431

Coomassie Brilliant Blue	Merck, Germany	115444
DMP	Sigma, Germany	D8388
DMSO	Sigma, Germany	D2650
EDTA	Riedel-de Haén, Germany	27248
Ethanol	Riedel-de Haén, Germany	32221
Ethidium Bromide	Merck, Germany	OCO28942
Foetal Bovine Serum	Sigma, Germany	F2442
FuGene 6	Roche, Switzerland	11814443001
Glycerol	Riedel-de Haén, Germany	15523
Glycine	Amnesa, USA	0167
HCl	Merck, Germany	100314
HiPerfect Trans. Reagent	Qiagen	301705
Hyperfilm ECL	Amersham Biosciences, UK	RPN2114K
Isopropanol	Riedel-de Haén, Germany	24137
KCl	Fluka, Switzerland	60129
Liquid nitrogen	Karbogaz, Turkey	
Luria Agar	Sigma, Germany	L-3147
Luria Broth	Sigma, Germany	L-3022
2-Mercaptoethanol	Sigma, Germany	M370-1
Methanol	Riedel-de Haén, Germany	24229
MgCl ₂	Sigma, Germany	M9272
Milk Diluent concentrate	KPL, USA	50-82-00
NaCl	Riedel-de Haén, Germany	13423
NaOH	Merck, Germany	106462
NP-40	Sigma, Germany	I3021

Phenol	Applichem, Germany	A1153
Phosphate buffered saline	Sigma, Germany	P4417
PMSF	Sigma, Germany	P7626
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

APPENDIX B

MOLECULAR BIOLOGY KITS

(in alphabetical order)

Name of Kit	Supplier Company	Catalog Number
ECL Advance Chemiluminescence Detection Kit	Amersham Biosciences , UK	RPN2135
Cell Proliferation Kit I (MTT)	Roche, Switzerland	1465007-001
QIAGEN® Plasmid Midi Kit (100)	Qiagen, Germany	12145
Quantifast Real-Time PCR Kit	Qiagen, Germany	204054
Annexin V Fluos Stain. Kit	Roche, Switzerland	1988549

APPENDIX C

OTHER MATERIALS

(in alphabetical order)

Name of material	Supplier Company	Catalog Number
Hybond-P membrane (PVDF)	Amersham Biosciences, UK	RPN2020F
Hyperfilm ECL	Amersham Biosciences, UK	RPN2103K
Prestained Protein MW Marker	Fermentas, Germany	#SM0441
RNase A	Roche, Germany	1119915
T4 kinase	Promega, Germany	M4103
T4 DNA kinase Buffer (10X)	Promega, Germany	M4103-B

APPENDIX D

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 TM Blot Module, Novex, USA

Centrifuge: Eppendorf, 5415C, GERMANY

Eppendorf, 5415D, GERMANY

Eppendorf, 5415R, GERMANY

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

Hitachi, Sorvall RC5C Plus, USA

Hitachi, Sorvall Discovery 100 SE, USA

Deepfreeze: -70°C, Kendro Lab. Prod., Heraeus Hfu486 Basic, GERMANY

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-20°C, Bosch, TURKIYE

Distilled Water: Millipore, Elix-S, FRANCE

Millipore, MilliQ Academic, FRANCE

Electrophoresis: Biogen Inc., USA

X Cell SureLock TM Electrophoresis Cell, Novex USA

Gel Documentation: UVITEC, UVIdoc Gel Documentation System, UK

Biorad, UV-Transilluminator 2000, USA

Ice Machine: Scotsman Inc., AF20, USA

Incubator: Memmert, Modell 300, GERMANY

Memmert, Modell 600, GERMANY

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

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

VELP Scientifica, Microstirrer, ITALY

Microliter Pipette: Gilson, Pipetman, FRANCE

Mettler Toledo, Volumate, USA

Microwave Oven: Bosch, TÜRKIYE

pH meter: WTW, pH540 GLP MultiCal®, GERMANY

Power Supply: Biorad, PowerPac 300, USA

Wealtec, Elite 300, USA

Refrigerator: +4°C, Bosch, TÜRKIYE

Shaker: Forma Scientific, Orbital Shaker 4520, USA

GFL, Shaker 3011, USA

New Brunswick Sci., Innova™ 4330, USA

C25HC Incubator shaker New Brunswick Scientific, USA

Spectrophotometer: Shimadzu, UV-1208, JAPAN

Schimadzu, UV-3150, JAPAN

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

Savant, Refrigerated Vapor Trap RVT 400, USA

Thermocycler: Eppendorf, Mastercycler Gradient, GERMANY

Vacuum: Heto, MasterJet Sue 300Q, DENMARK

Water bath: Huber, Polystat cc1, GERMANY

BD Biosciences FACS Canto Flow Cytometer

Bio-Rad, iCycler Real-Time PCR systems