Identification and Characterization of a Novel Palladacycle, AJ-5, to treat advanced melanoma and breast cancer

By

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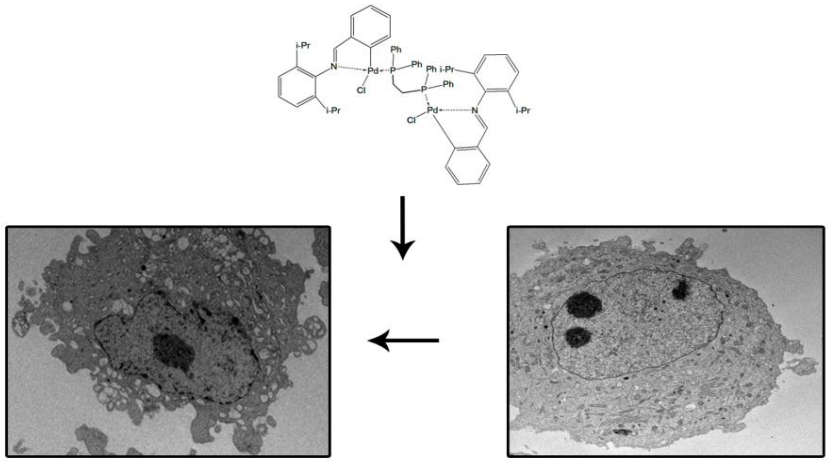

Thesis presented for the Degree of DOCTOR OF PHILOSOPHY in the department of Human Biology in the Faculty of Health Science

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Dedicated to my wife

Mervat

Declaration

I, Saeb Aliwaini, hereby declare that the work on which this thesis is based is my original work and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other University. I empower the University to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

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Saeb Aliwaini

February, 2014

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Abstract

Cancer is the second leading cause of death among both men and women and accounts for 13% of total deaths worldwide. Enormous efforts have therefore been invested to cope with this problem, but unfortunately limited success has been achieved with most of the current therapeutic strategies. Compared to organic chemotherapeutic molecules, metal complexes offer a much more diverse chemistry and have been shown to have important chemotherapeutic applications. Recently, palladium based compounds have been investigated as potential anti-tumour agents against several cancers including melanoma and breast cancer. However, very little is known about their mechanism of action and whether they have any side effects in vivo is not known. The aim of this study was therefore to identify novel chemotherapeutic palladium based drugs and to understand how these compounds exert their anti-tumour activity in vitro and in vivo. To achieve this, several novel palladium based compounds were initially screened for possible anti-tumour activity in advanced melanoma and breast cancer. From this initial screening one candidate, AJ-5 was identified as a potential metallodrug. This study describes the anti-tumour activity of AJ-5, a novel binuclear palladacycle complex in vertical growth phase (ME1402), metastatic (WM1158) melanoma and MCF7 and MDA-MB-231 metastatic breast cancer cell lines. Compared to normal control cell lines, AJ-5 was shown to be more effective in inhibiting the proliferation of melanoma and breast cancer cells with IC_{50} values of less than or equal to 0.20 µM. Flow cytometry analyses showed that AJ-5 induced apoptosis which was confirmed by Annexin V-FITC/propidium iodide double-staining, nuclear fragmentation and an increase in the levels of PARP cleavage.

Furthermore, AJ-5 was shown to induce both intrinsic and extrinsic apoptotic pathways as measured by PUMA, Bax, cytochrome c release and active caspases. Interestingly, AJ-5 treatment also simultaneously induced the formation of autophagosomes and led to an increase in the autophagy markers LC3II and Beclin1. Inhibition of autophagy reduced AJ-5 cytotoxicity suggesting that AJ-5 induced autophagy was a cell death and not cell survival mechanism. Moreover, it was shown that AJ-5 induces the ATM-CHK2 DNA damage pathway and that its anti-tumour function is mediated by the p38 and ERK1/2 signalling pathways. Importantly, AJ-5 treatment efficiently reduced tumour growth in melanoma bearing mice and induced high levels of autophagy and apoptosis markers. Finally this study also provides novel evidence to show that TBX3, an oncogenic transcription factor, is a repressor of the pro-apoptotic target gene, *PUMA* and that knocking down TBX3 sensitized MCF7 breast cancer cells to AJ-5. Together these findings suggest that AJ-5 may be an effective chemotherapeutic drug in the treatment of advanced melanoma and breast cancers.

Chapter 1: Literature Review

1.1 Introduction

Cancer is one of the most serious health problems worldwide, affecting individuals of all ages, sexes and races. It accounted for 13% of total deaths worldwide in 2013 and it is estimated that by 2030, deaths from cancer will exceed 13.0 million worldwide [1, 2]. Cancer is multifactorial in nature and it is unlikely to be cured by a single therapeutic approach. The primary method of treating most cancers is surgery and few patients elect to bypass this option as it is considered necessary to stop cancer progression. Other forms of cancer treatment include radiation therapy, which uses x-rays and other radioactive materials to damage DNA and cell membranes [3], and chemotherapy, which uses chemical agents that destroy dividing cells or stop their division [4]. In addition, cancer treatments may also involve hormone therapy such as tamoxifen and aromatase inhibitors for breast cancer treatment and localized cancers may be treated by administering immunotherapy or targeted therapies [3]. Unfortunately, in spite of the enormous efforts invested to treat cancers there has been limited success because most cancers are still diagnosed too late and several types of tumours develop resistance to the current chemotherapies [5, 6]. There is therefore a need to develop more effective therapies to treat this devastating disease.

This review will provide a general overview of the key areas of research pertaining to this thesis.

1.2 Chemotherapy

Chemotherapy, anti-neoplastic therapy and cytotoxic therapy are three medical terms used to describe chemical agents used in cancer therapy but the most commonly used is chemotherapy. Unlike surgery and radiation, chemotherapy is used as a systemic approach to treat cancer and is especially important for patients with advanced stages of cancer. Currently, more than 100 chemotherapeutic agents are used either as single treatments or in combination with other treatments. These chemotherapeutic agents have distinct chemical compositions, mechanisms of action, side effects and their efficacies differ for different cancers [7]. The most common types of chemotherapeutics are: (1) Alkylating agents, which are compounds that react with electron-rich atoms in biological molecules to form covalent bonds which results in DNA damage leading to cell cycle arrest and cell death. The discovery of metal based DNA alkylators, especially platinum Pt(II) analogues, was considered a major discovery in anti-cancer drug research. Pt(II) compounds such as cisplatin (from now on referred to as CDDP) and carboplatin, as well as other alkylating agents are still frequently used chemotherapies for several cancers [2]; (2) Anti-metabolites which are a class of chemotherapeutic agents that interfere with DNA replication by generally targeting metabolic pathways for nucleotide synthesis and this prevent cancer cells from proliferating [8]. The pyrimidine analog 5-fluorouracil is one of the best known and widely used anti-metabolites. During DNA synthesis, 5-fluorouracil can be incorporated into the DNA preventing chain elongation, which induces cell cycle arrest and apoptosis; (3) Anti-tumour antibiotics which function by inhibiting DNA and RNA synthesis. The most commonly used for the treatment of a variety of cancers is anthracyclines which include doxorubicin, daunorubicin, epirubicin and idarubicin. These drugs

work in all phases of the cell cycle via several mechanisms such as inactivating enzymes involved in DNA replication, DNA intercalation, generating free radicals, binding and alkylating DNA and crosslinking DNA which make them very efficient but extremely toxic [9]; (4) Topoisomerase inhibitors which inhibit a class of enzymes responsible for separating the strands of DNA during DNA replication. The topoisomerase inhibitors work by maintaining the DNA-enzyme intermediate as a complex, preventing re-ligation of the break between DNA strands and thus inhibiting the replication process. Several studies carried out on the early topoisomerase inhibitors, etoposide and teniposide, showed that these compounds are unable to bind purified DNA. Further investigations revealed that these inhibitors bind to the topoisomerase-DNA complexes causing double strand breaks [10, 11]; (5) Mitotic inhibitors which can stop mitosis by inhibiting different enzymes important for cellular proliferation. Although these compounds target cells mainly in the M-phase, they are able to damage cells in all phases of the cell cycle, making them highly toxic drugs. The common limitations of using these drugs lie in their potential ability to cause peripheral nerve damage and to induce chemoresistance in various cancer types [12]. However, members of this chemotherapeutic class such as paclitaxel and ixabepilone are still used to treat patients with breast cancer, lung cancer and leukaemia [7].

Most of the current drugs have limited efficiency against cancers, partly due to, tumour cells acquiring resistance to chemotherapy [13]. In many cases, cancer cells modify the expression of cell surface receptors to reduce the drug uptake and to increase the drug efflux. In the case of enzyme-inhibiting chemotherapies, drug resistance can arise from genetic mutation or by gene

amplification of the targeted enzymes [14]. Tumour cell resistance to DNA damaging agents are usually associated with modifications in the DNA damage response to increase the DNA repair capacity [15]. Specific combination treatments can be used to improve the effect of a certain chemotherapy and to overcome drug resistance [2]. However this approach is usually associated with more adverse toxicity to normal tissues and can bring about extremely serious health problems. For example, use of doxorubicin and other anthracyclines have been linked to cardio-toxicity, including cardiomyopathy and congestive heart failure [9].

1.3 Metal based alkylating agents

The five major structural classes of alkylating agents are the nitrogen mustards, the aziridines, alkyl sulphonates, the nitrosoureas and the mechanistically distinct metal-containing drugs [16]. Compared to the organic alkylating agents, metal complexes offer a much more diverse chemistry for chemotherapy. Although precious metals, such as gold and silver, have long been used in the treatment of different types of cancers, the significance of metal-based anti-cancer drugs has only been appreciated and explored since the discovery of Pt(II)-based compounds such as CDDP [17, 18].

1.3.1 Platinum based compounds

To date, CDDP remains one of the most important chemotherapeutic agents and has been used extensively to treat several cancers including breast, ovarian, cervical, head and neck, and non-small-cell lung cancer [19]. It is particularly successful for the treatment of testicular cancer with approximately 100% cure rate if tumours are detected early [18]. It is widely accepted that

CDDP cytotoxicity is initiated by forming DNA adducts and subsequently blocking replication and/or preventing transcription [15]. Considerable evidence indicates that CDDP may induce several signalling pathways leading to cell cycle arrest and cell death depending on the cell type and the treatment conditions [20]. The most common types of cell death induced by CDDP are apoptosis and necrosis [15]. More recently, autophagy, a self-degradative process in which a cell degrades misfolded proteins and damaged organelles [21], has also been associated with CDDP treatment [22, 23]. The effectiveness of CDDP against most tumours, however, declines severely due to tumour-acquired resistance and the high dose of CDDP used has been associated with severe side-effects such as neuro-, hepato- and nephron-toxicity [24]. Huge efforts to overcome the limitations of CDDP treatments have been made and as a result thousands of other Pt(II) analogues have been synthesized and screened for anti-cancer activity. The second generation of Pt(II) analogues, called carboplatin has been used in combination with taxane as a first line therapy for ovarian cancer [25] and with other therapies for lung cancer [26]. Oxaliplatin represents the third-generation of Pt(II) drugs and is now considered as standard therapy, together with 5-fluorouracil/leucovorin, for colon and rectal cancers [27]. Although carboplatin and oxaliplatin undergo renal elimination successfully, both cause neurological and other side effects and many cancers also acquire mechanisms of resistance to these drugs [28].

1.3.1.1 Mechanism of anti-tumour activity of Platinum based compounds

Of the Pt(II) agents, the mechanism of action of CDDP has been the most studied and therefore this section will focus on it. The cytotoxic effects exerted by CDDP result mainly from its ability

to induce DNA double-strand breaks which triggers a canonical DNA damage signalling pathway through activating ataxia telangiectasia mutated (ATM) [29]. Active ATM together with ataxia telangiectasia mutated and Rad3 related (ATR) drives the accumulation of the phosphorylated form of the histone variant H2AX (γH2AX) to the area surrounding the DNA break [29, 30]. γH2AX facilitates the activation of the checkpoint kinase CHK2 and the multifunctional transcription factor p53 that mediate phenotypic responses to DNA damage including cell cycle arrest, DNA repair and/or the induction of programmed cell death [31]. p53 plays an important role in deciding cell fate in response to DNA damage through trans-activating the cyclin-dependent kinase inhibitor p21 as well as pro-apoptotic proteins [32]. Early studies showed that p53 binds to CDDP-modified DNA [33, 34] and several studies have demonstrated that the sensitivity of cancer cells to CDDP correlates positively with the presence of wild-type p53 [35, 36]. In agreement with these observations, studies on the triple negative breast cancer cell line MDA-MB-231 which has mutant p53, have shown that CDDP induces DNA damage but not cell death [37, 38]. In addition, a study on 8 melanoma cell lines showed that CDDP efficiently induced cell death in only cell lines with wild-type p53 and not p53 mutant cell lines [39]. While these studies show that p53 plays an important role in the cytotoxic effect of CDDP and defects in this pathway seem to reduce the efficacy of CDDP, other reports showed that p53 may be associated with CDDP resistance. For example, a very recent study showed that compared to metastatic melanoma cells with wild-type p53, those with mutant p53 underwent higher levels of apoptosis in response to CDDP [40]. The study suggests that functional p53 up-regulates DNA-binding protein 2 (DDB2) and xeroderma pigmentosum complementation group C (XPC) which subsequently enhance the DNA damage repair leading to drug resistance. In addition,

MCF7 breast cancer cells have wild-type p53 and are resistant to CDDP and disruption of p53 sensitized these cells to CDDP [41]. Similarly, ovarian cancer cells shown to be resistant to CDDP have higher levels of wild-type p53 compared to their CDDP-sensitive counterparts [42]. Transfection of a mutant p53 into the CDDP resistant cells significantly increased their sensitivity to the drug, suggesting that p53 is a direct determinant of CDDP resistance in these cells. It is unclear at this stage why in response to CDDP wild-type p53 may induce cell death in some cancer cells and confer drug resistance to other cancer cells.

The extracellular signal-regulated kinase (ERK), p38 and c-Jun NH_2-terminal kinase (JNK) are members of the Mitogen activated protein kinases (MAPKs), a class of serine/threonine kinases that have also been implicated in the DNA damage response and regulating CDDP-induced cell death [43]. The activation of the p38 pathway by CDDP has been reported in different experimental model systems and have been shown to result in a CDDP-sensitive phenotype [44]. Furthermore, inhibition of p38 using two pharmacological inhibitors decreased the cytotoxic activity induced by CDDP. Similarly, inhibition of p38 rendered ovarian cancer cells resistant to CDDP and re-stimulation of the p38 pathway re-sensitized these cells to CDDP by increasing the expression of Fas Ligand [45]. Further studies have shown that p38 mediates its apoptotic effect via p18 (Hamlet) which interacts with p53 to stimulate the transcription of pro-apoptotic genes PUMA and NOXA [46].

While ERK signalling plays a critical role in the survival of a number of cell types [47], a number of studies have revealed that ERK also plays a pro-apoptotic role in cancer cells treated with

chemotherapeutic agents such as CDDP [49, 50]. For example, the level of CDDP induced apoptosis in cervical adenocarcinoma cells was significantly decreased when ERK1/2 was inhibited [51]. Importantly, ERK has been shown to induce extrinsic apoptotic pathways through increased expression of death receptor ligands and activation of caspase 8 [52]. The transduction of DNA-damage signals by JNK has also been reported and defective JNK signalling has been linked to CDDP resistance in vitro [53–56]. An understanding of how JNK contributes to CDDP induced cytotoxicity was supported by the observation that p73, a pro-apoptotic member of the p53 family, forms a complex with JNK leading to CDDP-induced apoptosis [57].

The phospho-inositide 3-inase (PI3K)/ AKT signalling pathway is constitutively active in many types of cancers and has also been implicated in rendering cancer cells resistant to CDDP [58]. AKT belongs to a family of serine/threonine kinases which act downstream of PI3K and plays a critical role in cell survival and proliferation [59]. Different mechanisms have been proposed for how AKT contributes to acquired CDDP resistance including the downregulation of p53 levels and the inhibition of the pro-apoptotic PUMA and BAD proteins [60–64].

1.3.2 Palladium based compounds

Recently palladium complexes, especially Pd(II) species, have been reported to exert a significant cytotoxic effect against cancer cells [65]. Importantly, they have been shown to exert anti-tumour activity in CDDP resistant cells and to have less side effects than CDDP [66]. While Pd(II) complexes are 10^5 times chemically more active than their Pt(II) analogues, many Pd(II) compounds were previously shown to have low anti-tumour activity [67]. However, this low

anti-cancer activity has now been attributed to the rapid hydrolysis of the ligands which in many cases dissociates readily in solution. This results in very reactive species which are assumed to undergo further chemical transformations preventing them from reaching their pharmacological targets. The development of effective anti-tumour Pd(II) drugs therefore requires strong stabilization, such as co-ordination by nitrogen ligands and incorporation of suitable chelating groups, to prevent such dissociation. In this regard, many studies have focused on the preparation of Pd (II) complexes bearing one (mononuclear), two (dinuclear) or more (multinuclear) Pd(II) centres with different types of ligands to stabilize these compounds [68]. The most common types of ligands used in stabilizing Pd(II) compounds are described below.

(1) Bidentate nitrogen ligands

Spermidine and its derivative spermine are natural polyamines known to be essential regulators of various cellular processes including DNA stability, cellular growth, differentiation and apoptosis [69]. Because of their relevant biological activity, several research groups used them as chelating ligands to stabilize anti-cancer novel Pd(II) compounds. An early study by Navarro-Ranninger and colleagues reported the synthesis of dichloro Pd(II) complexes with spermidine and spermine ligands [70]. The compounds were assayed for in vitro anti-proliferative activity against breast cancer cells (MDA-MB-468) and human leukaemia cells (HL-60) and showed potential cytotoxic activity. Another study also investigated the cytotoxicity of a dinuclear Pd(II) compound chelated with a spermine ligand, Pd_2-Spm [$(PdCl_2)_2$(spm), (spm = spermine, $H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$)] in breast cancer cell lines as well as towards an

untransformed cell line called BJ fibroblasts [71]. This compound exhibited strong anti-proliferative effects towards both Estrogen Receptor (ER)-positive (MCF7) and ER-negative (MDA-MB-231) breast cancer cell lines, compared to the non-tumourigenic BJ cell line model. Importantly, the results show that this compound was more effective against the MDA-MB-231 which is notoriously unresponsive to current treatments.

Other examples of bidentate nitrogen containing ligands are the aromatic N-heterocyclic ligands like pyridine, quinoline, 1,10-phenanthroline and their derivatives which have also been used to stabilize Pd(II) complexes [72, 73]. Some of these ligands are able to intercalate DNA and have been shown, in combination with Pd(II) salts, to be effective against experimental tumours in animals, as well as human cancers. For example, the ligand 2,6-dimethyl-4-nitro-pyridine (dmnp) displayed anti-cancer activity in different cancer cell lines including SW707 (adenocarcinoma of the rectum), T47D (breast cancer), HCV (bladder cancer) and A549 (non-small cell lung carcinoma) and coordinated to Pd(II) in the complex $[Pd(dmnp)_2Cl_2]$, it was found to be much more efficient [74]. Indeed, $Pd(dmnp)_2Cl_2$ was more effective than CDDP in lung, liver and breast cancer cell lines tested. Moreover, Opolski and colleagues showed that $Pd(dmnp)_2Cl_2$ had the greatest activity against the T47D breast cancer cell line which is known to be poorly responsive to Pt(II)-based drugs [75]. Another study, investigated the anti-tumour activity of three Pd(II) complexes of the form: trans-$PdCl_2L_2$ (where L = 3-hydroxypyridine, 2-hydroxypyridine and 4-hydroxypyridine) code named TH5, TH6 and TH7 respectively [76]. While all three complexes were generally less active than CDDP against ovarian cancer cells, TH6 was found to be effective against the CDDP resistant ovarian cancer cell line, A2780cisR (IC_{50} ± SD =

5.6 ± 0.1 μM compared to CDDP IC_{50} ± SD = 12.9 ± 0.4 μM) [76]. In more recent studies, the bioorganic and medicinal chemistry of 2,2':6',2"-terpyridine (terpy) was further used in the synthesis of a group of novel Pd(II) and Pt(II) complexes [77]. The Pd(II) complex [Pd(sac)(terpy)](sac).4H_2O] where (sac= saccharinate) was found to exhibit significant cytotoxic effects against MCF7 and MDA-MB-231 breast cancer cells [65]. Very recently, two novel compounds [$PtCl_2$(L)] and [$PdCl_2$(L)] (L = 2-deoxy-2-[(2-pyridinylmethylene)amino]-α-D-glucopyranose):Dichloro (2-deoxy-2-[(2- pyridinylmethylene)amino]-α-D-glucopyranose) Pt. were synthesized and tested against CDDP resistant gastric cancer cells [78]. The study showed that [$PdCl_2$(L)] was able to induce a high level of apoptosis and to overcome cross-resistance to CDDP both in vitro and in vivo.

(2) Phosphine ligands

It has been suggested that the organometallic biphosphine-based cyclopalladated complexes are more stable and less toxic and that they could have a more specific anti-tumour activity in vivo. In this regard four novel Pd(II) compounds derived from 2-oxo-1,2-dihydroquinoline-3-carbaldehyde thiosemicarbazones with triphenylphosphine as co-ligands have been investigated for possible anti-tumour activity [79]. The compounds exhibited significant reduction in cell viability accompanied with a cell cycle arrest in a group of cancer cell lines including skin, liver and cervical cancer cells. Other studies showed that cyclopalladated complexes derived from dppf [1,1'-bis(diphenyl-phosphino)ferrocene] were able to induce apoptosis in K562, HL60 and Jurkat leukaemia cell lines [80, 81].

A group of dinuclear Pd(II) compounds, biphosphinic cyclopalladated, were produced and tested in vitro and in vivo against melanoma B16F10-Nex2 cells [82]. Out of the seven compounds tested in this study, three compounds showed a robust cytotoxic effect in vitro with an IC_{50} lower than 1.25 μM. The compound C7a significantly inhibited the growth of melanoma tumours and the combined treatment of C7a with immunotherapy further enhanced the anti-tumour activity of C7a [83]. More recent reports described the anti-cancer properties of another compound 7b in human leukaemia cells which involve the induction of cell death by mitochondrial apoptosis [84].

(3) Other ligands

Recently other ligands have been used to stabilize Pd(II) compounds. For example, Matović et al. used amide-containing ligands, especially derivatives of malonic and oxalic acid to synthesize Pd(II) complexes and described the anti-tumour activity of four of these compounds [85]. The authors compared the activity of these compounds to CDDP and carboplatin in the human cancer cell lines: Hs294T (melanoma), K562 (chronic myelogenous leukemia) and HeLa (human cervix carcinoma). The HeLa cells were the most sensitive to all compounds and one of the Pd(II) complexes had an IC_{50} value 3×10^3 less than the Pt(II) compounds. However, the other cancer cell lines were also found to be slightly more sensitive to the Pd(II) compounds than to the Pt(II) compounds with small variations between each cell line. Recently, a water-soluble iminophosphorane ligand, TPA=N-C(O)-2BrC_6H_4 (C,N-IM; TPA = 1,3,5-triaza-7-phosphaadamantane) was used in the synthesis of a group of Pd(II) based compounds. All

compounds showed potential anti-cancer activity against Jurkat-T, human prostate cancer (DU-145) as well as normal T-lymphocytes (PBMC) [86].

1.3.2.1 Mechanisms of anti-tumour activity of Palladium based compounds

While there is substantial evidence to suggest that Pd(II) based complexes exert their anti-cancer effects by inducing cell cycle arrests and apoptosis, very little is known about the molecular basis of their action. Indeed, to the best of my knowledge, there are only a few reports that contribute to a detailed understanding of how they initiate their anti-cancer activity and two mechanisms have been proposed. The one mechanism involves the induction of DNA damage and the other suggests that Pd(II) based compounds target specific organelles such as the mitochondria, Endoplasmic Reticulum (ER) and lysosomes [83, 87, 88].

Induction of DNA damage

Based on the structural similarity between Pd(II) and Pt(II) ions it was suggested that Pd(II) based compounds might also induce their cytotoxic effects via a mechanism involving the induction of DNA damage. In agreement with this proposal, some studies showed that Pd(II) based compounds increase the level of γH2AX, a marker of double-strand DNA breaks, in different types of cancer cells [71, 78, 88]. In a very recent study, a novel Pd(II)-based compound [PdCl(terpy)](-sac).4H_2O where (sac=saccharinate, and terpy=2,2':6'2"-terpyridine) showed strong anti-tumour activity against different prostate cancer cell lines [88]. Following the treatment with this Pd(II) complex, the cells showed a high level of γH2AX accompanied by an increase in cells with sub-G1 DNA content and the induction of apoptosis and autophagy.

However, the high cytotoxic activity of many Pd(II) compounds in CDDP-resistant cells [86], led to suggestions that they may induce different forms of DNA damage to that of CDDP. Furthermore, studies showed that while novel Pt(II) and Pd(II) dichloride compounds induce DNA damage markers, the Pd(II) compounds were more cytotoxic against cancer cells [89, 90]. Indeed, nuclear magnetic resonance (^{1}H NMR) spectroscopy showed this to be the case [72, 91, 92]. For example, studies on the interactions of CDDP and its isoform (trans-DDP) with DNA have demonstrated that these compounds result in intra-DNA strand binding to guanine residues with 50-GG(N7-N7) and 50-GNG(N7-N7) being reported [66]. However, research on different interactions of Pd(II) salts with DNA revealed that they showed sequence selective binding with the oligonucleotide $[d(CGCGAATTCGCG)]_2$ at T8 imino and G4, N7 atoms [93]. However, the nature of Pd(II) covalent interaction with DNA appears to be influenced also by the type of ligand associated with it as mentioned earlier.

Targeting organelles

As indicated earlier, there are also reports suggesting that the anti-tumour activity of Pd(II) complexes results mainly from organelle-specific actions, such as lysosomal and mitochondrial membrane permeabilisation, rather than through DNA interactions. For example, cyclopalladated complexes derived from dppf [1,1'-bis(diphenyl-phosphino)ferrocene] were able to induce lysosomal permeabilization in the K562, HL60 and Jurkat leukaemia cell lines which resulted in the release of cathepsin B and apoptotic cell death [80, 81].

In addition, palladacyclic compounds obtained from the reaction of dmpa (N,N-dimethyl-1-phenethylamine) with the dppe [1,2-ethanebis(diphenyl phosphine)], were able to induce a Ca^{2+}-independent mitochondrial swelling as well as mitochondrial permeabilization in isolated rat liver [94]. In addition, Rodrigues and his colleagues synthesized a group of palladacyclic compounds from the reactions of dmpa (N,N-dimethyl-1-phenethylamine) with diphenyl phosphine derivatives and studied their anti-cancer effects in several cancer cell lines [25, 82, 86, 95, 96]. One of the compounds, C7a, was further tested in vivo and the promising results obtained motivated this group to investigate the mechanism behind its anti-tumour activity [83, 97]. The data showed that C7a interacts with thiol groups on the mitochondrial membrane proteins, inducing Bax translocation from the cytosol to the mitochondria and in this way disrupting the mitochondrial membrane potential. Importantly, C7a treatment significantly decreased the cytoplasmic ATP levels and activated the apoptotic effector caspases in mouse melanoma cells. Similar results were obtained in human tumour cells which suggest that C7a induces a mitochondria-dependent cell death [96]. More recently the same group reported on the anti-cancer activity of another palladacycle compound named compound 7b against the K562 human leukemia cells [84]. The compound 7b oxidized protein thiol residues in the membrane causing dissipation of the mitochondrial transmembrane potential and leading to an induction of the intrinsic apoptotic pathway.

A very recent study reported that the ER may also be a target for the Pd(II) based compound, Pd(II) bisacetylacetonate [$Pd(acac)_2$] [98]. The authors showed that the cytotoxic effect in the human non-small-cell lung cancer H460 cells involves an increase in the levels of the CHOP

protein, an ER stress marker. Furthermore, the treated cells exhibited swollen ER and decreasing levels of the ER calcium content. Together these results suggest that $Pd(acac)_2$ treatment results in the accumulation of misfolded protein and ER stress. Importantly, this Pd(II) compound inhibited the tumour growth of H460 cells in mice by more than 80% while CDDP treatment showed approximately 56% inhibition.

Taken together while both Pd(II) and Pt(II) based compounds are able to induce DNA damage and/or interact with other organelles to induce cell death, the exact molecular mechanism(s) of cell death induced by Pd(II) compounds has yet to be elucidated.

1.4 Cell death

The main objective of chemotherapeutic approaches is to kill cancer cells and for many years it was thought that they do so primarily by inducing apoptosis [99, 100]. However, accumulating evidence suggests that they also induce other forms of cell death including necrosis, mitotic catastrophe and autophagy [99]. For the aim of this study apoptosis and autophagy will be further elucidated.

1.4.1 Apoptosis

"Apoptosis" is an ancient Greek word which means "leaves falling from a tree" and biologically it was the first programmed cell death to be identified [101]. Apoptosis can be classified into two main pathways: caspase dependent and caspase independent apoptosis. The best known

mechanism is the caspase dependent apoptosis which includes extrinsic (receptor-mediated) and intrinsic (mitochondria-mediated) pathways **(Fig. 1.1)** [102, 103].

1.4.1.1 Intrinsic apoptosis

The intrinsic or mitochondrial mediated apoptosis is activated by intracellular signals such as DNA damage, hypoxia and ER stress [104]. The main mediators of intrinsic apoptosis are the BCL-2 family proteins which include the anti-apoptotic subfamily (BCL-2 and Bcl-xl) and the pro-apoptotic subfamily (Bax, Bok, Bak and the BH3 domain-only proteins including Bid, Bad, Bim and PUMA) [105]. Bax and Bak are considered the main pro-apoptotic proteins and their activation results from an extremely controlled process where they are trans-located from the cytoplasm to the outer mitochondrial membrane (OMM) [106]. The main role of Bax/Bak in the intrinsic apoptosis pathway is to permeabilize the mitochondrial membranes and to allow efflux of apoptotic factors such as cytochrome c through a poorly understood mechanism [105]. Upon permeabilization of the mitochondria, cytochrome c transports to the cytoplasm and interacts with apoptosis protease activating factor 1 (Apaf-1) to trigger apoptosome assembly. The apoptosome, a heptameric platform, then activates caspase 9 which in turn activates downstream effector caspases such as caspase 3 and caspase 7. This starts a proteolytic cascade which results in the degradation of different cellular structures and leads to cell death [107, 108]. In healthy cells there are low levels of Bax/Bak in the OMM but it has been observed that Bax moves back and forth continuously from the cytoplasm to the mitochondria [109]. The anti-apoptotic BCL-2/Bcl-xL proteins are found to retro-translocate Bax to the cytoplasm by an unknown mechanism. Several factors such as p53 and BH3-only have been shown to be

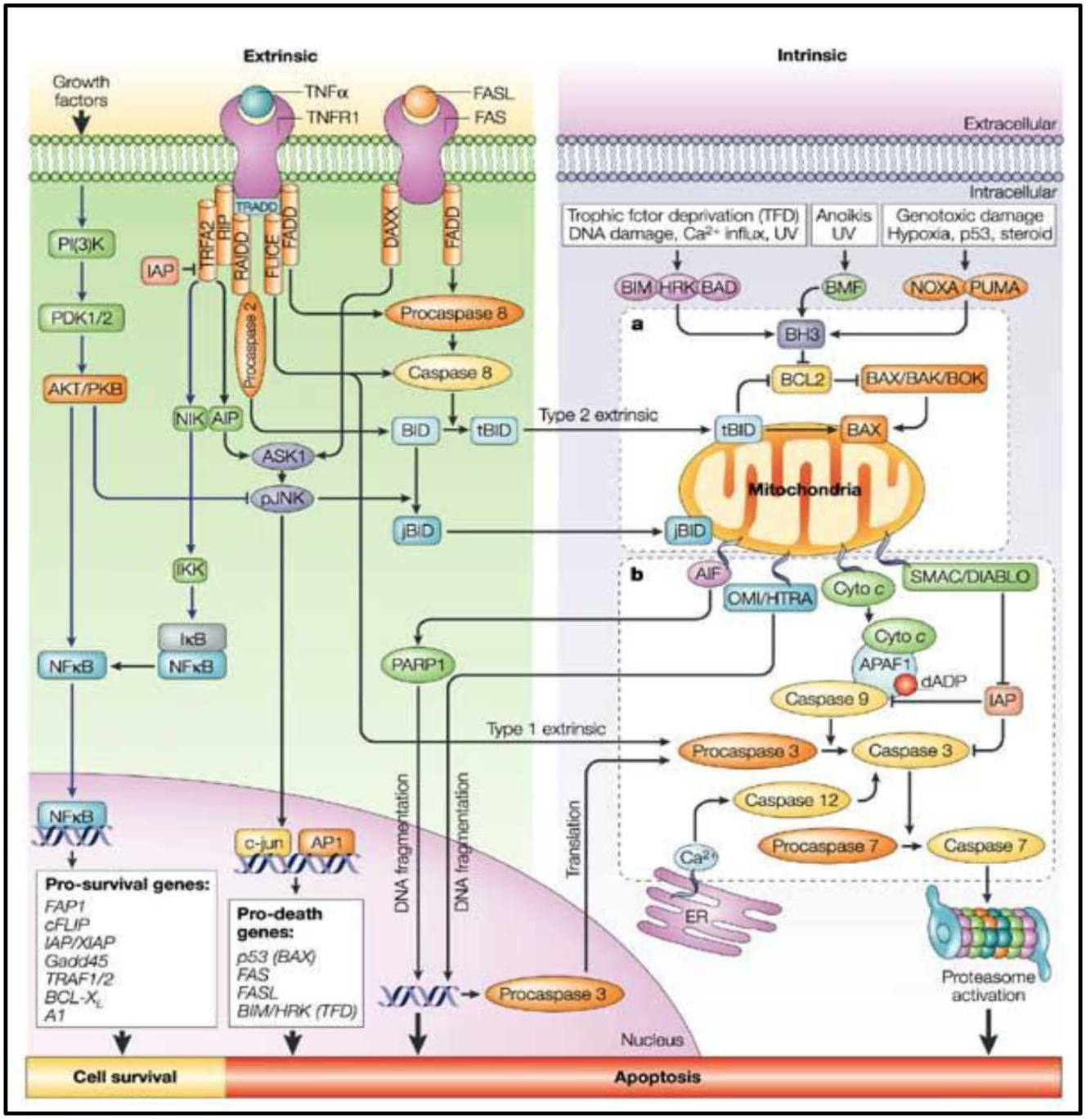


Figure 1.1 Regulation of caspase dependent apoptotic pathways. Pro-apoptotic pathways are in black and anti-apoptotic (survival) pathways are in blue. Upon binding of a ligand to a death receptor, the extrinsic apoptosis pathway is triggered. Adapter proteins FADD and TRADD facilitate recruitment and activation of caspase 8 or caspase 2. Active caspase 8 then either initiates apoptosis directly (extrinsic apoptosis type 1) via activating executioner caspase 3 and caspase 7 or trigger extrinsic apoptosis type 2 where it activates the intrinsic apoptotic pathway through cleavage of Bid to induce cytochrome c release. The intrinsic (mitochondrial) apoptotic pathway can be triggered through different cellular stresses that result in cytochrome c release from the mitochondria, formation of the apoptosome and activation of caspase 9. Active caspase 9 then activates the executioner caspases [110].

responsible for sending Bax/Bak to the mitochondria and for triggering changes in its conformation [111].

1.4.1.2 Extrinsic apoptosis

The extrinsic apoptotic pathway is initiated when cell death ligands bind to death receptors such as TNF-1, CD95 (Fas/Apo-1), DR3, DR4, DR5 and DR6. This induces conformational changes in the intracellular domains of the death receptors resulting in their activation [102]. The now active death receptors recruit apoptotic proteins to form a death inducing signalling complex (DISC) which activates a caspase cascade and initiate extrinsic apoptosis. The initiator procaspases 2 or 8 must be recruited to this complex to trigger the extrinsic apoptotic pathway irreversibly [104]. In response to CDDP treatment of a number of cancer cell lines such as ovarian carcinoma, osteosarcoma, melanoma and lung cancers it would appear that caspase 8, and not caspase 2, is involved [112–114].

Interestingly, CDDP treatment of non-small-cell lung cancer (NSCLC) cells resulted in increasing levels of both intrinsic and extrinsic apoptotic markers [114]. Furthermore, blocking either the intrinsic apoptotic pathway or caspase 8 was unable to block CDDP induced apoptosis in these cells. However, blocking both pathways strongly decreased the CDDP induced cell death. In addition, human melanoma cell lines treated with different concentrations of CDDP showed a significant decrease in cell survival and both the intrinsic and extrinsic apoptotic pathways were responsible for the cytotoxicity [113].

1.4.2 Autophagy

Initially, autophagy was considered only a pro-survival mechanism which enables cells to adapt to stress conditions and it has been associated with anti-cancer drug resistance [21]. However, more recently, the significant activation of autophagy in response to stress, such as exposure to certain chemotherapeutic agents, has also been shown to lead to cell death which was classified by Clarke in 1990 as a non-apoptotic programmed cell death pathway also referred to as type II programmed cell death. To appreciate how different chemotherapeutic agents may elicit autophagy as a cell survival mechanism or a cell death mechanism requires a general understanding of the morphological and molecular basis of autophagy which will therefore be described first.

Autophagy is a multistep process **(Fig. 1.2)** characterized by morphological changes starting with the appearance of intracellular double membrane autophagic vacuoles called autophagosomes. Maturation of these autophagosomes involves the fusion with lysosomes to form the more advanced autophagic vacuoles referred to as autolysosomes [115]. Autolysosomes then degrade all the sequestered cellular organelles and misfolded/long-lived proteins providing the cell with more nutrients and energy. To maintain cellular homeostasis, autophagy is kept at a low basal level in healthy cells. Disruption of the autophagic machinery results in different pathological processes such as neurodegenerative diseases [116], inflammatory diseases [117], aging [118] and cancer [119]. In response to stress, different cells can activate various levels of autophagy, and the cellular outcome of this activation is complex and depends on the cellular context, type and magnitude of stress. In minor stress conditions a

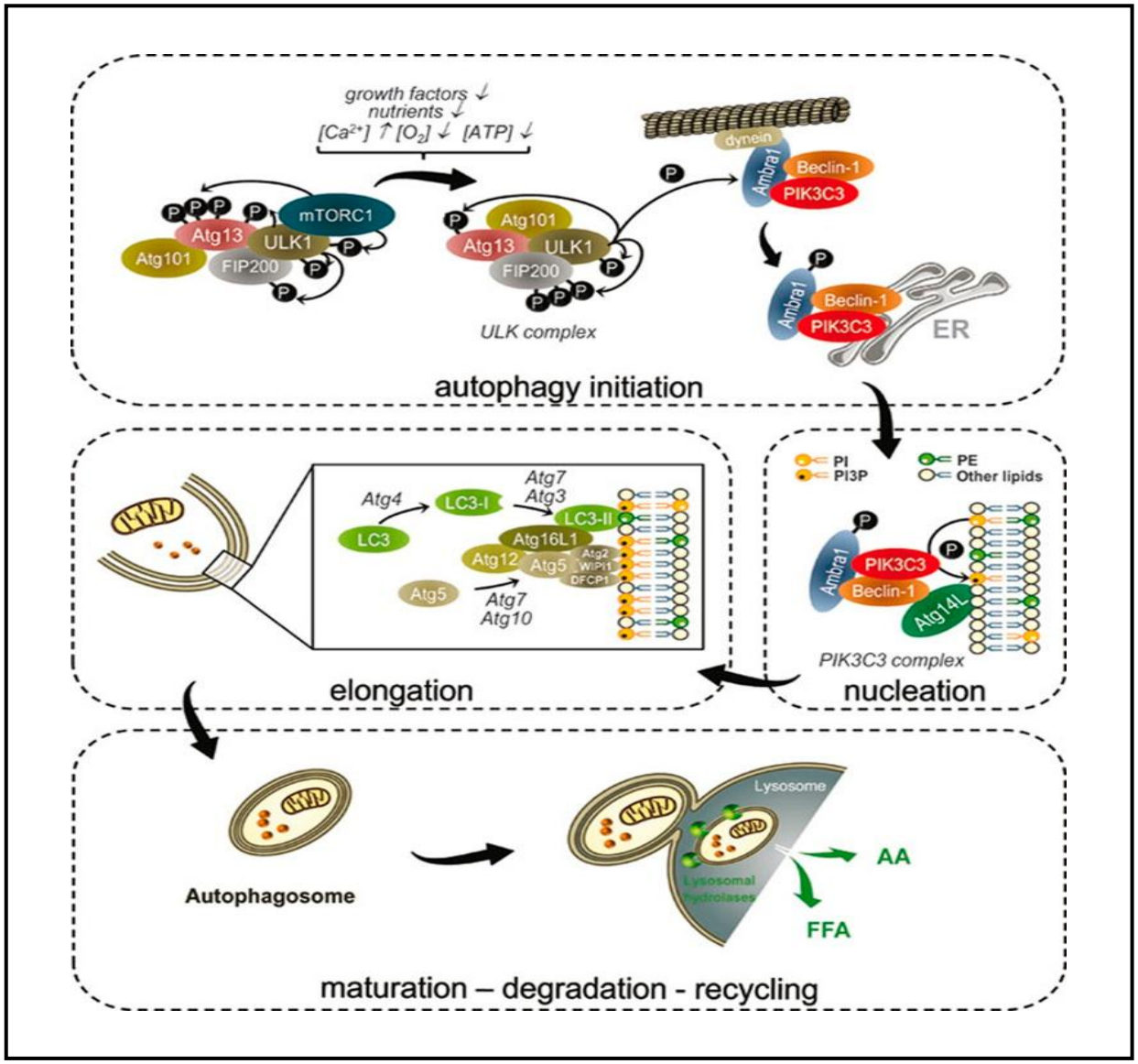


Figure 1.2 Molecular mechanism of autophagy. In response to stress, mTORC1 is inhibited which therefore allows activation of ULK complex and subsequently phosphorylation of Ambra1 which is necessary to activate and translocate PIK3 complex to the ER where it starts the nucleation stage. PI3P generated by PIK3 complex then recruits other Atg proteins to form the Atg5-Atg12 complex to carry on to the elongation stage. Mature autophagosome then fuses with the lysosome, releasing the autophagic cargo into the lysosomal lumen to be degraded [120].

low level of autophagy is induced to enhance cell survival by allowing the cell to activate checkpoints and repair the damage. On the other hand, higher levels of stress causing irreparable damage over a prolonged period of time induces autophagic cell death which has been observed for several chemotherapeutic agents [121].

The understanding of the molecular mechanism underpinning autophagy started recently with the discovery of autophagy-related genes (Atg). Three main complexes consisting of Atgs (Atg1, Vps34-Atg8 and Atg5-Atg12) were identified at the different stages of autophagy **(Fig. 1.2)** [120, 122]. The initiation or the first stage of autophagy involves the formation and activation of the Atg1 complex (ULK complex). This stage is regulated by the mammalian protein target of rapamycin (mTOR) which forms two different complexes (mTORC1 and mTORC2). Under normal conditions, mTORC1 binds and phosphorylates the ULK1 and Atg13 proteins which prevents the activation of the ULK complex [119, 122]. Upon stress conditions, mTORC1 is inhibited and subsequently it dissociates from the ULK complex which leads to its activation [123]. Activation of the ULK complex is followed by the nucleation or the second stage of autophagy which depends mainly on the activation of the phosphati- dylinositol-3-kinase class-III (PIK3C3) complex, also called Vps34 complex, which includes the Ambra1 and BCL-2-interacting (Beclin1)/Atg6 proteins [122]. In mammals there are two types of the lipid kinase Vps34 (PIK3) called class I and class III which have completely different roles [124]. While PIK3 class I activates mTOR and inhibits autophagy, PIK3 class III (PIK3C3) forms the second important autophagic complex with the Beclin1 protein, the homolog of Vps30/Atg6 in yeast [125]. The PIK3C3 complex is held inactive by Ambra1 and once the ULK complex is active it will

phosphorylate and release Ambra1 from the PIK3C3 complex leading to its activation. Activation of Vps34 lipid kinase activity is essential for generating phosphati-dylinositol (3)-phosphate (PI3P) to allow the recruitment of other important Atg proteins. The generated PI3P interacts with Atg2 and WIPI1 to recruit Atg7 and Atg10 which facilitate forming the third complex, Atg5-Atg12. This complex is responsible for the third stage of autophagy called "membrane elongation" which requires the ubiquitin-like protein LC3 (Atg8 in yeast). LC3 has to be recruited to the Atg5-Atg12 complex where it plays a critical role in the expansion of the phagophore (also called the isolated membrane). Upon the initiation of autophagy the C-terminal arginine-117 residue of the full length inactive LC3 will be cleaved by cysteine Atg4. This process leads to the exposure of a glycine region in the LC3 protein which binds to active Atg7 [126]. Active LC3 is then linked to Atg3 which facilitates the joining of LC3 to phos-phatidylethanolamine (PE) on the C-terminal glycine. Electron microscopy has shown that LC3-PE (also called LC3II) localizes to the phagophore and organizes its expansion [127]. LC3II shows an equal distribution on both sides of the phagophore but when the autophagosomes mature the LC3II portion in the outer membrane will be released by a second Atg4-dependent cleavage. The population of LC3II inside the autophagosome however remains and is delivered into the autolysosomes where it is degraded [128]. Upon autophagosome maturation, its outer membrane fuses with lysosomes forming autolysosomes. The last step requires the presence of the lysosomal membrane protein, LAMP-2, the small GTPase, RAB7 and the UVRAG and LC3 proteins. The loss of any of these proteins may stop autophagy at this stage and prevent autolysosome formation. Finally, under acidic conditions the cargo inside the autolysosome can be degraded by the lysosomal enzymes [129, 130].

The AMP-activated protein kinase (AMPK) and PI3K- AKT signalling pathways play an important role in regulating autophagy by controlling the activity of the mTORC1 complex [131]. Low cellular energy status is one of the most common conditions leading to autophagy. Under such conditions, ATP levels drop and AMP levels increase which activate AMPK, a central sensor of cellular energy status. Active AMPK triggers tuberous sclerosis protein 2 (TSC2) to inhibit mTORC1 and activate autophagy [21]. Initiation of autophagy through inhibition of mTORC1 is usually accompanied by inhibition of p70S6 kinase, a downstream target of mTORC1 [132]. While this has led to the theory that p70S6 kinase is a negative regulator of autophagy, other studies have suggested that it may be necessary for the initiation of autophagy and that it plays an important role in the synthesis of proteins that participate in autophagosome formation and maturation [133]. Based on more recent reports it would however appear that p70S6 kinase plays dual roles in the regulation of autophagy [134, 135]. The PI3K- AKT pathway is constitutively active in many types of cancer where it functions as a pro-survival pathway. Nutrients and growth factors activate PI3K Class I which catalyse the production of second messengers to activate AKT [136]. Activated AKT phosphorylates and inhibits TSC2 leading to mTORC1 activation and autophagy inhibition [136–138].

More recently the mitogen activated protein kinase (MAPK) subfamily members p38, ERK and JNK have also been implicated in the regulation of autophagy [139]. For example, p38 has been shown to mediate autophagy induced by different anti-tumour agents such as oridonin, resveratrol and polygonatum cyrtonema lectin [140–142]. Resveratrol-induced apoptosis and autophagy in leukaemia cells were also shown to result from the activation of p38 because the

suppression of p38 attenuated resveratrol induced apoptosis and autophagy [143]. Other studies revealed that the increasing levels of Atg1 and Atg5 in response to stress conditions were associated with the activation of p38. It is important to note that there are also studies that have suggested that autophagy is negatively regulated by p38 and that inhibition of p38 leads to the activation of Beclin1 and autophagy [144–146]. It has been suggested that the apparent conflicting roles for p38 in autophagy may relate to the different p38 inhibitors used in the various studies.

An early study demonstrated that ERK1/2 mediates starvation-induced autophagy in human colon cancer cells [147] and subsequently several studies confirmed that in response to anti-tumour agents ERK1/2 is activated and responsible for the initiation of autophagy [148, 149]. The overexpression of ERK2 was also found to be sufficient to induce autophagy in glioblastoma cells and recent studies suggest that ERK1/2 is specifically important for the maturation of autophagic vacuoles [150]. There is also evidence that stress conditions such as ultraviolet irradiation, osmotic shock and cytokines result in JNK1/2 activation [151, 152] and activation of autophagy in various types of cancers and normal cells [153–155]. The activation of JNK1/2 under these stress conditions was associated with the inhibitory phosphorylation of the BCL-2 protein which is known to inhibit both autophagy and apoptosis by binding to Beclin1 and Bax respectively [156, 157]. Activation of the JNK pathway by mild stress conditions has been shown to release Beclin1 from the BCL-2/Beclin1 complex whereas only sustained starvation and strong stress conditions were sufficient to release Bax from the BCL-2 complex [158]. These

results and similar data obtained from other studies suggest that the levels of BCL-2 phosphorylation determine whether the autophagic or apoptotic pathway is activated [159].

1.4.2.1 Therapeutic modulation of autophagy

There is accumulating evidence that autophagy can be induced by different types of cancer therapy and that it plays a critical role in determining how tumour cells respond to the treatment. In many cases, autophagy can be activated as a pro-survival response (see **Table 1.1**) to promote resistance to the cancer therapy. In these instances, the inhibition of autophagy enhances drug induced cell death. However, other studies have demonstrated that some anti-cancer agents exert their cytotoxicity by inducing autophagy as a cell death mechanism (see **Table 1.2**). In line with these observations, efforts have been made to focus on the design of novel drugs that can induce autophagy. The inhibition of the pro-survival pathway AKT/mTOR has the most potential for such therapies because it triggers cell cycle arrest and autophagy and apoptosis as cell death mechanisms [160]. Indeed, everolimus (RAD001) and temsirolimus specifically inhibit mTOR and have been used successfully for the treatment of renal cell carcinoma, mantle cell lymphoma and pancreatic tumours [99, 161–163]. It has been shown that everolimus reduces tumour mass very efficiently in vivo and mainly by autophagic cell death [161]. Moreover, everolimus showed strong anti-tumour activity in patients with advanced pancreatic tumours [162, 163] and daily treatment with this drug was well tolerated in patients with metastatic renal cell cancer [164]. In chemo-resistant cancers, synergetic use of everolimus with etoposide, CDDP or doxorubicin also resulted in co-operative anti-tumour effects [165–167].

Table 1.1 Examples of cancer therapies reported to induce autophagy which results in cell survival (drug resistance).

Anti-cancer drug	Cancer type	Treatment effect	Autophagy inhibitor	Results of autophagy inhibition	Reference
Tamoxifen	lymphoma	Apoptosis and autophagy	Chloroquine or Knocking down ATG5	apoptosis increased	[167]
Camptothecin	breast cancer	Apoptosis and autophagy	knocking down Beclin1	apoptosis increased	[168–170]
Cisplatin	lung adenocarcinoma	Apoptosis and autophagy	3-MA	apoptosis increased	[171]
5-fluorouracil	Colorectal , colon , lymphoma and oesophageal cancers	Apoptosis and autophagy	3-MA or Knocking down Atg7	apoptosis increased	[167, 172–174]

3-MA= 3-methyladenine

Table 1.2 Examples of cancer therapies reported to exert their cytotoxicity by inducing autophagy (cell death mechanism).

Anti-cancer drug	Cancer type	Treatment effect	Autophagy inhibitor	Results of autophagy inhibition	Reference
Tamoxifen	Breast cancer	Decreasing of cell viability and autophagy	3-MA	Increasing of cell viability	[175, 176]
Temozolomide	Glioma cancer	Cell cycle arrest and autophagy	3-MA	Increasing of cell viability	[177]
Doxorubicin	papillary thyroid cancer	Decreasing of cell viability and autophagy	3-MA	Increasing of cell viability	[178]
Gemcitabine	Pancreatic cancer	Decreasing of cell viability and autophagy	3-MA	Increasing of cell viability	[179]
imatinib	Glioma cancer	Apoptosis and autophagy	3-MA Baf	Increasing of cell viability	[180]
bozepinib	Breast cancer	Apoptosis and autophagy	chloroquine	Increasing of cell viability	[181]

It would appear that whether autophagy functions as a cell survival or cell death mechanism depends on the cancer cell type and/or the chemotherapy used.

1.4.3 Cross talk between apoptosis and autophagy

Several cancer therapies induce both apoptosis and autophagy at the same time and there is evidence to suggest that there is a cross talk between both processes and that they may impact on one another [168]. For example, while disruption of apoptosis by inhibiting different caspases increased autophagic cell death, blocking autophagy has been shown to potentiate both intrinsic and extrinsic apoptotic cell death pathways [169]. A study by Yu et al. showed that the inhibition of caspase 8 with a pharmacological inhibitor led to high levels of cell death in human U937 monocytoid and mouse L929 fibroblastic cells [170]. Using transmission electron microscopy these cells were confirmed to display no apoptotic features but displayed an increase in several large double membrane autophagic vacuoles. In the same study, the authors showed that inhibition of both apoptosis and autophagy was able to block cell death suggesting that both processes function as cell death mechanisms and that the one can possibly compensate for the other. Another interpretation of this data, however, is that apoptosis suppresses autophagy and there have been several recent reports to support this possibility.

The apoptotic caspase and calpain family members provide important points of cross talk between apoptosis and autophagy **(Fig. 1.3)**. Active members of these families cleave Beclin1, Atg4 and Atg5 proteins [171, 172] and once cleaved, Beclin1 (N and C-terminal) can't interact with Vps34 to facilitate autophagy.

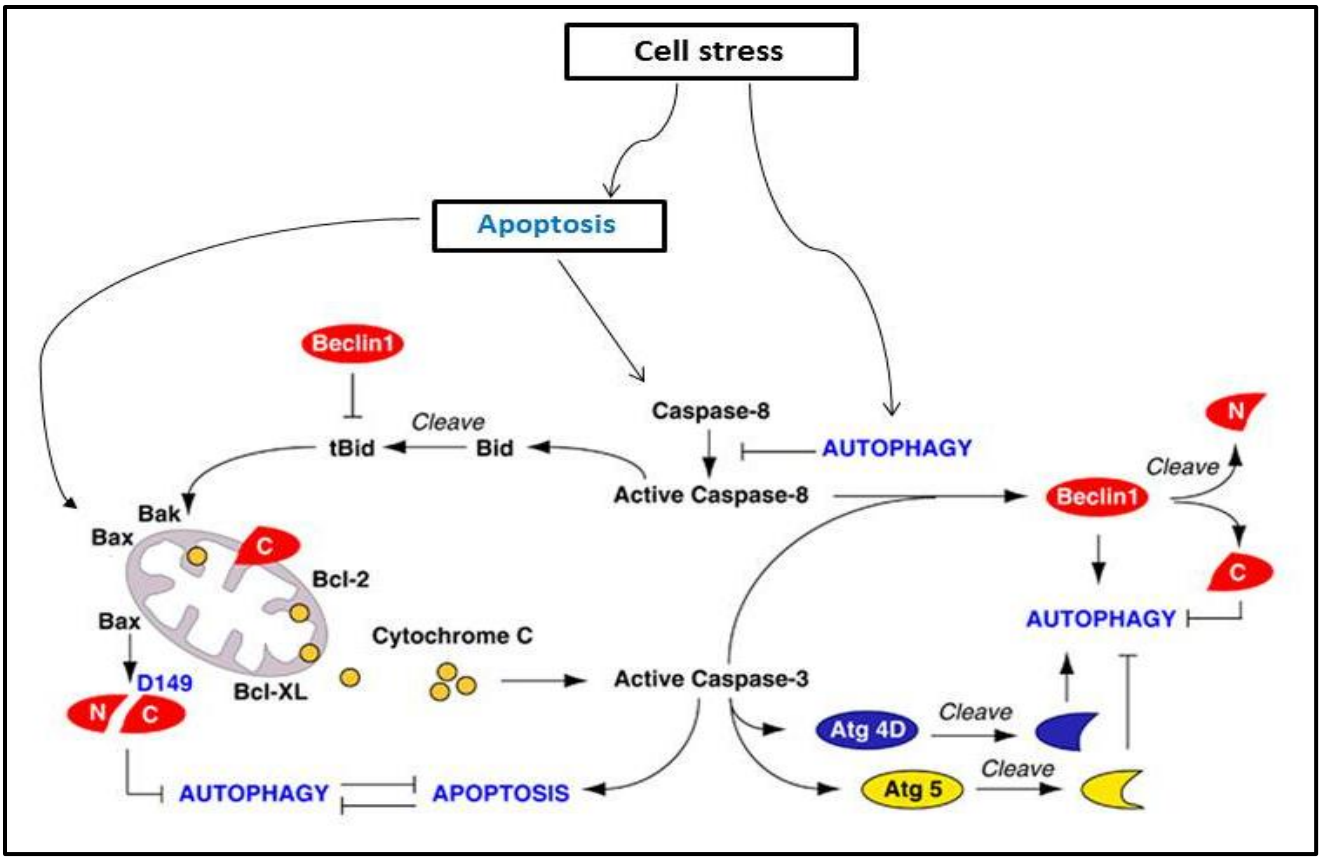


Figure 1.3 Crosstalk between apoptosis and autophagy. Autophagy and apoptosis share common signalling pathways, and exhibit some degree of mutual inhibition. During sustained cellular stress, caspase-mediated cleavage of Beclin 1 generates fragments ('N' and 'C') that are unable to induce autophagy. The C-terminal fragment translocates to the mitochondria and sensitizes cells to apoptotic signals. Although apoptosis-associated cleavage of Beclin 1 and Atg5 inactivates autophagy, the cleavage of Atg4D by caspase-3 generates a fragment with increased autophagy activity. Moreover, autophagy inhibits apoptosis partly by degrading active caspase-8 or preventing activation of Bid by Beclin 1. Figure modified from R Kang, et al 2011 [173].

Results obtained from several studies indicate that the balance between cleaved and full-length Beclin1 may also determine the equilibrium between apoptosis and autophagy [174, 175]. While full length Beclin1 mediates autophagy, the cleaved Beclin1 (C-terminal fragment) localizes predominantly in the mitochondria and sensitizes the cells to apoptosis [175]. Caspase 3 cleaves Atg4 which is required for autophagy but cleaved Atg4 was also shown to localize to the mitochondria and it is unclear whether it plays a role in apoptosis [176]. Other apoptotic regulators upstream of caspase 8 also appear to impact on autophagy. It has been reported that death-inducing signalling complex (DISC) can activate autophagy when caspase 8 is inactive [170]. Furthermore activation of FADD, an extrinsic apoptotic pathway regulator, resulted in high levels of autophagic cell death in cells with defective extrinsic apoptotic machinery [177, 178]. Taken together these observations may suggest that apoptosis regulate autophagy negatively and that the inhibition of apoptosis leads to autophagic cell death as an alternative cell death mechanism.

Autophagy on the other hand can modulate apoptosis and several studies have focused on its negative regulation of apoptosis. For example, the failure of TRAIL treatment to kill aggressive colon cancers is thought to result, in part, from the cytoprotective effects of autophagy which may have resulted from the autophagy inhibiting apoptosis because when autophagy was inhibited apoptosis was induced [179]. Although different mechanisms have been proposed for how TRAIL-induced autophagy may modulate apoptosis, the exact mechanism remains to be elucidated. It has, however, been suggested that one mechanism by which autophagy can inhibit apoptosis is by the degradation of apoptotic proteins in autophagic vacuoles. All these

conflicting observations indicate that further investigations are required to gain greater insight into the crosstalk between the apoptotic and autophagic pathways and more efforts in this field will be critical in developing new approaches and therapies for cancer. The current study investigates a novel chemotherapeutic agent to treat melanoma and breast cancer and the following sections of this chapter will therefore focus on these two cancers and the current chemotherapies used to treat them.

1.5 Breast cancer

Breast cancer is the most common malignancy worldwide and it is estimated that one out of eight women will develop breast cancer in their life time [180]. In Europe alone, approximately 430,000 new cases occur every year, and of the new cases 132,000 die in the same period of time. In spite of the fact that most of the patients are diagnosed in early and curable stages, metastatic breast cancer occurs in one third of patients affecting bone, liver and lung, ultimately leading to death [181].

To understand breast cancer development it is important to know the structure of the normal breast. The female breast consists mostly of fatty and fibrous connective tissues and is divided into about 20 sections called lobes. Each lobe is further subdivided into a group of lobules which are the structures that contain milk-producing glands. Once milk has been produced, a complex system of tiny ducts carries it through the breast to the collecting chamber located below the nipple [182]. Breast cancers mostly originate from either the ducts or the lobules in which case they are referred to as ductal carcinomas or lobular carcinomas, respectively. When

confined to the site of origin, they are further classified as non-invasive (or in situ breast cancer) but when they spread beyond the basement membrane and invade the underlying connective tissue they are classified as invasive breast cancer [183]. There are also less common breast cancers such as medullary, papillary and mucinous carcinoma.

While the transition from non-invasive to invasive breast cancer has been reported, breast cancer progression remains a point of debate and two prevailing models (linear and parallel) have been proposed. The linear model proposes that cells in the primary (non-invasive) tumour accumulate progressive mutations in genes regulating cell division and growth [184, 185]. The most common mutations identified in the primary tumour include BRCA1, BRCA2, p53 and RB and amplification of the HER-2 receptor [186–189]. Some of these cells eventually acquire the ability to proliferate autonomously; they expand clonally and leave the primary site to travel the vascular systems to a distant organ where they develop into a secondary metastatic growth. This model also suggests that metastasized cells in the secondary site should also be able to leave that site to set up at a tertiary site [185, 190]. The parallel progression model proposes that cancer cells may leave the primary tumour site at a very early stage and may be subsequently genetically modified in the metastatic niche where they later settle [191]. However, these tumour cells may also differ genetically from cells that eventually develop into a metastasis in the same patient which could reflect the requirements for these cells to adapt and be able to successfully grow in the new microenvironment [192].

Recently, another hypothesis has emerged which suggests that breast cancer can originate from cancer stem cells. This theory suggests that a subset of cancer cells have the characteristics of stem cells which give rise to progeny with different differentiation states [190]. In line with this proposal, several studies showed that breast cancer stem cells with the cell surface marker $CD44^{hi}CD24^{lo}$ are responsible for initiating and facilitating tumour metastasis. Experimentally, as few as 100 of these cells could generate a tumour, while tens of thousands of cells from the rest of the population were unable to do so [193]. Furthermore, compared to the other heterogeneous cells in tumours, breast cancer stem cells are more resistant to different cancer treatments [194, 195] and are responsible for tumour resistance to anti-cancer agents [193, 196].

Enormous efforts are invested to find a successful therapy for breast cancer, but unfortunately limited success has been achieved with most of the current therapeutic strategies [197]. Several studies showed that histologically similar breast tumours have different prognoses and respond differently to cancer therapy [198]. There is evidence that these differences are due to molecular variations between breast tumours. More than 70% of human breast cancers (BCs) are hormone-dependent and approximately 15% are hormone-receptor-negative which includes BCs lacking estrogen receptor expression [199]. Immunohistochemical studies showed that breast cancers that express the estrogen receptor, progesterone receptor or both respond well to hormone therapy. For example, five years of adjuvant tamoxifen safely reduces 15-year risks of breast cancer recurrence and death [200]. The same line of therapy however had little or no effect on patients with estrogen receptor negative (ER-) breast cancer. Another important

progress in breast cancer therapy was identifying and targeting the Her2 subtype of epidermal growth factor receptors (EGFR) which improved the outcome of Her2 positive patients. Triple negative breast cancers (TNBCs) are called such because they lack receptors for estrogen, progesterone and Her2. TNBCs are highly aggressive and resistant to conventional chemotherapy and is more common in individuals of African descent [201–204]. It is important to note that more than 70% of TNBCs overexpress genes implicated in metastasis and invasion as well as genes involved in proliferation and resistance to apoptosis including AKT, PI3K, RAS and NF-κB [205–207]. More importantly, mutations in p53 is reported to be one of the most common features of TNBCs and several studies indicate that these mutant p53 proteins enhance tumourigenesis and treatment resistance [208–210].

Thirty years ago anthracyclines and taxanes entered clinical trials for the treatment of breast cancers and because of their high levels of toxicity they are usually suggested for patients with TNBC and rapidly progressive cancers [8]. Currently capecitabine, ixabepilone and eribulin are in use for patients with breast cancers that are resistant to anthracycline and taxanes [211]. Generally, this cytotoxic therapy is used in combination with other lines of therapies such as anti-HER2 therapy for patients with HER2/neu over expression. It has been reported that up to 10 % of patients with metastatic breast cancer may survive beyond 10 years or longer if there has been an objective response to the cytotoxic therapy [211].However there are studies which showed that concomitant use of cytotoxic therapy and hormone therapy is no more effective than cytotoxic therapy alone [212]. Other therapies to treat BC include Pt(II) analogues (CDDP, carboplatin), anti-metabolites (gemcitabine) and topoisomerase I inhibitors (irinotecan) which

are also used either alone or in combination with other cytotoxic agents [213–215]. In spite of the promising response usually observed in most patients with metastatic breast cancer treated with cytotoxic therapy, resistance regularly develops [211].

1.6 Melanoma

Malignant melanoma is an aggressive skin cancer and worldwide its incidence is increasing faster than any other cancer [216]. Presently, less than 10% of patients diagnosed with metastatic melanoma have a 10-year survival rate [217]. While the exact origin of melanoma remains unclear, it is generally agreed that they arise from melanocytes which are specialized cells that synthesize pigments known as melanins [218]. Melanocytes are found in different parts of the body such as the skin, leptomeninges, ear and the choroid layer of the eye and melanoma can arise in any of these regions [218, 219]. Based on histopathological features, Clarke and his colleagues proposed that melanoma progression can be represented as 5 stages [220]. The first stage, referred to as a benign nevus, is a selective accumulation of structurally normal melanocytes, also commonly referred to as a mole. While the growth control of these nested neval melanocytes is disrupted, the growth of a nevus is limited and it rarely becomes cancerous. In the second stage a dysplastic nevus, or atypical mole, forms and is characterised by aberrant melanocyte growth in a pre-existing benign nevus or a new lesion [219]. Phenotypically a dysplastic nevus can be differentiated from a benign nevus as it is often not uniform in colour and has irregular and undefined borders. Once the cells acquire the ability to spread within the epidermis, the lesion is referred to as a radial growth phase (RGP) melanoma, which is regarded as an early and less aggressive melanoma. RGP cells fail to form colonies in

soft agar and show no tumourigenic potential when injected into immunodeficient mice [221, 222]. Increased tumourigenic potential is a characteristic of the vertical growth phase (VGP) melanoma, as cells have the ability to migrate deep into the dermis by breaking through the basement membrane and form tumours in vitro and in vivo [221]. Importantly, while VGP melanomas are aggressive, they require additional genetic alterations to metastasise. Metastatic melanoma cells enter the blood and lymphatic system, travel to a distant site and colonise the tissue to form a secondary tumour. The most common sites of melanoma metastases include the liver, brain and bones [223]. At a molecular level, melanoma progression is less well defined but it has been associated with specific germline and sporadic genetic alterations.

The first chemotherapeutic agent approved by the FDA to treat melanoma was dacarbazine, however, only 2% of patients treated with this drug showed a significant response and only 11.2% showed a partial response [224]. Importantly, despite its moderate effect, dacarbazine continues to be the standard treatment for metastatic melanoma simply because no other chemotherapy has yet been shown to have a significant survival benefit over dacarbazine. Recently temozolomide, an imidazotetrazine analogue, has been investigated in a randomized multi-centre phase III trial in the treatment of metastatic melanoma [225]. In comparison to a median overall survival of 9.1 months with dacarbazine, temozolomide treatments resulted in a median overall survival of 9.4 months. Pt(II) analogues such as CDDP and carboplatin have also been used in metastatic melanoma treatment either alone or in combination with other treatments [216]. While CDDP treatment of metastatic melanoma resulted in a positive

response in less than 10% of the patients, carboplatin treatment showed a response in approximately 19% of patients [226, 227]. However, comparing the efficiency of CDDP/IL-2/temozolomide to CDDP/IL-2/dacarbazine, there was no advantage of temozolomide over dacarbazine in the prevention of brain metastasis [225].

Until as recently as 2011, only dacarbazine and high-dose IL-2 were approved by the FDA in the treatment of melanoma. But neither drug increases the median survival period for patients with metastatic melanoma [228]. There is a growing body of evidence suggesting that the activation of the RAS-RAF-MEK-ERK signalling pathway is important in the pathogenesis of melanoma [229, 230] and at least 60% of all melanomas harbour activating mutations in the B-RAF gene. Currently the only one of two agents approved for the treatment of advanced melanoma is the BRAF inhibitor, vemurafenib [231]. Vemurafenib pointedly increased the median overall survival of patients with advanced melanoma containing the BRAF V600E mutation from 9 to 13-16 months [232, 233]. However, more than 50% of patients who showed a promising response to vemurafenib eventually reactivated the RAF-MEK-ERK pathway which resulted in vemurafenib resistance [231]. Several studies have described the mechanisms leading to this reactivation and resistance. One study showed that 20% of the patients treated with vemurafenib have high levels of BRAF expression and/or splice-variants of BRAF [234]. Other studies showed that vemurafenib resistance is associated with overexpression of the MEK-activating kinase COT1 [235, 236].

The immune cytotoxic therapy ipilimumab (T lymphocyte antigen 4 antibody) was also approved by the FDA in 2011 for the treatment of metastatic melanoma [237]. Patients treated with a combination of ipilimumab and dacarbazine, had a longer median overall survival than patients treated with dacarbazine plus placebo [238]. However, different combination therapies containing ipilimumab have improved the median overall survival only by 10% more than the control. Other cytotoxic agents which work as microtubular assembly or dissembly inhibitors such as vindesine, vinblastine, taxanes or paclitaxel have been shown to exert modest activity in patients with metastatic melanoma [239–242]. Together the above studies stress the fact that the efficacy of the current melanoma chemotherapies is very limited and there has therefore been a drive to developing more effective treatment for melanoma.

1.7 Aims of this study

Advanced stages of melanoma and breast cancer are horrific diseases with little chance of a cure with available modern treatments. Recently Pd(II) complexes have been reported to exert a significant cytotoxic effect on cancer cells. Compared with Pt(II) based drugs, Pd(II) compounds seem to display a broader spectrum of activity and a lack of cross-resistance. Importantly, unlike Pt(II) complexes, many Pd(II) complexes are thought to be non-mutagenic and seem to have lower side effects. Therefore Pd(II) based compounds may be potentially effective anti-cancer therapies. This thesis explores the possible anti-cancer activities of several late transition Pd(II) compounds which were synthesised in the laboratory of my co-supervisor, Prof Selwyn Mapolie, by addressing the following aims:

1. To test a panel of Pd(II) compounds for their anti-proliferative and cytotoxic effects on advanced melanoma and breast cancer cells compared to non-tumourigenic cell lines. Only compound(s) identified to have anti-proliferative and cytotoxic effects on cancer cells will be further investigated in greater detail as described in aims 2 and 3.
2. To determine the molecular mechanism including the signalling pathway(s) through which compound(s) identified in aim 1 mediate their anti-cancer activity in (a) ER-positive and ER-negative breast cancer cells and (b) vertical growth phase and metastatic melanoma cells.
3. To determine whether silencing TBX3, which is overexpressed and oncogenic in breast cancer cells, will potentiate the cytotoxicity of the compound(s) identified in aim 1.

Chapter 2: Materials and Methods

2.1 Cell culture

The normal fibroblast cell lines FG0 and DNB [243] and X-radiation transformed WI-38 fibroblast cells called CT-1 [244] and human breast adenocarcinoma MDA-MB231 (triple negative) were maintained in DMEM (Highveld Biological, Lynd-hurst, United Kingdom (UK)). The following human cell lines- ME1402 vertical growth phase (VGP), WM1158 metastatic melanoma cell lines [245] and MCF7 breast adenocarcinoma (estrogen receptor positive) cells were maintained in RPMI 1640 medium (Highveld Biological, Lynd-hurst, UK). The human melanocytes Nohm-6 were maintained in RPMI supplemented with 200 nM TPA (12-O-tetradecanoylphorbol 13-acetate), 10ng/ml human stem cell factor (SCF), 10nM endothelin 1 (EDN1) and 200 pM cholera toxin. All media were supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were maintained at 37°C in a 5% CO_2 and 95% air-humidified incubator. Media was replaced every 2-3 days and cells were routinely subjected to mycoplasma tests. Only mycoplasma free cells were used in experiments.

2.2 Mycoplasma test

Cells grown on a coverslip for a minimum of 24 hours in antibiotic-free medium were fixed in a 1:3 mixture of glacial acetic acid and methanol for 5 sec, washed briefly with water to remove the fixing solution and then air-dried at room temperature for 5 min. Once dried, the DNA was stained with 0.5 µg/ml Hoechst 33258 (Sigma, St Louis, MO, USA) for 30 sec, washed briefly with water to remove excess stain and then mounted on a slide with mounting fluid (see

appendix, section 6.1). The cells were viewed immediately by fluorescence microscopy. Mycoplasma negative cells stained positive with Hoechst 33258 only in the nucleus, while cells infected with mycoplasma showed staining in both the nucleus and the cytoplasm.

2.3 Treatments

2.3.1 Palladium based compounds treatments

The laboratory headed by my co-supervisor, Prof Selwyn Mapolie, synthesized several palladium based compounds (AJ-1, AJ-2, AJ-3, AJ-4, AJ-5, C5, C6, C7, and C8) (see appendix, section 6.2) with potential anti-cancer activities. The compounds used in this study were dissolved in DMSO to give a final concentration of 5 mM and stored at room temperature for no more than 7 days. In order to get the final concentration, subsequent dilutions in the appropriate media for each cell line were prepared. Vehicle treated cells were incubated in normal media with DMSO (the vehicle in which AJ-5 was dissolved in).

2.3.2 Cisplatin treatment

Cisplatin (CDDP) (Pfitzer, South Africa) was used as a positive control for melanoma treatment [113, 246]. Cells were treated with CDDP at specific concentrations as indicated in each experiment. In all cases untreated cells were incubated with 150 mM NaCl (the vehicle in which CDDP was dissolved in). Cells were incubated in the dark at 37°C.

2.3.3 Inhibitors

2mM Caffeine, 10 nM bafilomycin, 1 or 10 µM wortmanin (Sigma, St Louis, MO, USA), 10 µM p38 inhibitor (SB203580), 10 µM MEK-1 inhibitor (PD98095), 10 µM AKT VIII inhibitor (Calbiochem, USA) or JNK inhibitor SP600125 (20 M) (Calbiochem, San Diego, CA, USA) were added 1 hour prior to treatment with AJ-5. All inhibitors were incubated in the dark.

2.4 Cell morphology

Cells were plated at suitable numbers in order to obtain 60-70% confluency on the day of treatment. After treating the cells with the palladium compounds, drugs or pharmacological inhibitors the morphological changes were monitored and photographed using an inverted light microscope (Olympus 1X71, USA) and camera (Zeiss AxioCam, Germany) respectively. Any morphological changes were photographed using a light microscope.

2.5 Cytotoxicity assays

To determine the cytotoxic effect of the indicated compounds and drugs, cells were seeded (3000-6000 cells/well) in quadruplicate in a 96-well plate and treated after two days with a range of the indicated concentrations of specific compounds or vehicles for 48 hours. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide (MTT) assay according to the manufacturer‘s instructions (Roche, USA). Briefly, 10 µL of MTT solution was added to each well and incubated for 4 hours at 37°C. This was followed by the addition of 100 µL solubilization buffer (10% SDS in 0.01 M HCl) and incubated overnight at 37°C. Absorbance (585 nm) was then determined for each well and the mean cell viability was calculated as a percentage of the control. Three separate experiments were performed to

determine the concentration of AJ-5 required to kill 50% of the cells (IC_{50}). The IC_{50} values were calculated from sigmoidal plots with GraphPad Prism version 5.

2.6 In vitro cell migration assay

Cell migration was measured using a two-dimensional in vitro scratch motility assay. Cells were grown to confluence in 6 well plates, and a linear wound was made by scratching through the monolayer using a sterile 200 µl pipette tip. To prevent cell proliferation, mitomycin C (Sigma, USA) was added at a final concentration of 10 µg/mL. Several markings were made along the edges of the scratch line that were used as reference points. The areas of the wounds were measured using Axiovert software (Zeiss, Germany) at the time of the scratching (0 hours) and at different time points after AJ-5 treatment as indicated on figures and expressed relative to zero time and pictures were taken using (10 x; Olympus 1X71).

2.7 Mammosphere assay

Anchorage-independent growth was assessed by mammosphere assay as previously described [247]. Briefly, cells were lifted with 0.25% (v/v) trypsin before passing through a 40 µM cell strainer (BD Biosciences) to obtain a single cell suspension. Cells were seeded at a density of 1000 cells per well in 96-well ultralow attachment plates (Nunc) containing DMEM supplemented with 2 mM L-glutamine, 1% (v/v) PSA, 2% (v/v) B-27 supplement, 20 ng/ml EGF, 20 ng/ml bFGF, 4 ng/ml heparin and 10 µg/ml insulin. Treatment with either 0.1% (v/v) dimethyl sulphoxide (DMSO) vehicle control or AJ-5 was carried out either upon seeding or at Day 4 for quadruplicate samples. Medium was changed every 48 hours and the mammospheres

were photographed after 7 days in culture at 100x magnification. Quantification of mammosphere formation as Sphere Forming Efficiency (SFE) was carried out by counting of mammospheres under an Olympus DSZ5000X inverted microscope at 40x magnification and reported as the number of mammospheres formed in 96 wells divided by the original number of single cells seeded and expressed as a percentage. Statistical significance between treatments was assessed using a Student's t-test where p values less than 0.05 were deemed statistically significant. Cell viability of mammospheres following treatment with AJ-5 was assessed by means of a WST-1 assay according to manufacturer's instructions. In this assay, percentage viability was calculated relative to the DMSO vehicle control (taken as 100% viability) with equal cell numbers being seeded. After treatment and growth of the mammospheres for 7 days, 10 µL of a 5 mg/ml WST-1 reagent was added to each well and incubated for a further 4 hours before reading the absorbance at 450 nm. Viability assays were carried out in quintuplicate.

2.8 Flow cytometry

2.8.1 Cell cycle analysis

Cells were collected by trypsinisation, washed twice with 1XPBS, resuspended in 2 ml of cold 1XPBS and counted on a haemocytometer to determine the volume of propidium iodide (PI) solution that will be added. Cells were fixed in 8 ml of 70% cold ethanol for at least 30 min at -20°C. Fixed cells were collected by centrifugation at 1500 rpm for 5 min at room temperature, washed twice with 1XPBS and centrifuged at 6000 rpm for 1 min at room temperature. Before flow cytometry analyses, the samples were treated with RNase A (50 µg/ml) for 15 min at 37°C

and immediately stained for 30 min at room temperature with PI solution (see appendix, section 6.3), to a final concentration of 1 X 10^6 cells/ml. A minimum of 50 000 cells/sample were subjected to analysis using a Beckman Coulter FACSCalibur flow cytometer (Beckman Coulter, USA).

2.8.2 Measurement of apoptosis

Cells were collected by trypsinization, and washed twice with cold 1 x PBS. Cells were harvested by centrifugation at 15000 rpm for 5 min and counted. The supernatant was discarded, and the pellet was resuspended in 1 x binding buffer at a density of 1.0 x 10^5 cells per ml. One hundred µl of the sample was transferred to a 10 ml culture tube, and incubated with Annexin V-FITC/PI (Sigma, USA) as per the manufacturer's instructions. Annexin V conjugated to FITC was used to quantitatively determine the percentage of cells in a population that are undergoing apoptosis and PI was used to stain all dead cells. Four hundred µl of 1 x binding buffer was added to each sample, and analysis was performed by FACS in a Beckman Coulter Cytomics FC500 flow cytometer (Beckman, USA) using the BD CellQuestTM Flow Cytometry Software (BD Biosciences, USA).

2.8.3 Analysis of CD44/CD24 marker expression

The effect of AJ-5 on the CD44/CD24 expressing cells in the MCF7 cell line was tested according to protocol reported by Gupta and his colleagues [248]. MCF7 cells were treated with DMSO vehicle control or AJ-5 (at concentrations of 0.05 and 0.1 µM) for 4 days in complete medium, after which the medium was replaced with complete medium lacking AJ-5, and the cells

allowed to recover for an additional 4 days. The percentage of $CD44^{hi}CD24^{lo}$ cells was determined by flow cytometry on a FACSAria II after double staining using CD44-APC and CD24-FITC antibodies or isotype matched fluorescently conjugated control antibodies. Data was analysed using FlowJo software (Treestar Inc) and percentage of $CD44^{hi}CD24^{lo}$ cells established from the live population by gating based on the isotype control staining. Each treatment was conducted in triplicate for duplicate experiments.

2.9 Fluorescent microscopy

2.9.1 Nuclear fragmentation

Cells were treated with 0.2 µM AJ-5 for 24 hours and then stained with Hoechst 33342 for 10 min. The cells were viewed by fluorescence microscopy (Zeiss, Germany).

2.9.2 Determination of cytochrome c release into the cytosol

Cells were grown on glass coverslips were treated with AJ-5 or the vehicle for 24 hours and stained with MitoTracker® Green FM dye (Invitrogen, M7514) according to the manufacturer's instructions. This probe is a carbocyanine-based MitoTracker which emits green fluorescence and can be visualized at emission maxima ~516 nm. After 30 min incubation with MitoTracker, cells were viewed by a fluorescent microscope (Zeiss, Germany) to test the MitoTracker stain. If the stain was successful, the cells were washed with PBS and fixed in 4% paraformaldehyde (see appendix, section 6.4) for 20 min at room temperature. Cells were then permeabilised in 0.2% Triton X-100 in PBS for 10 min at room temperature. Cells were blocked for 1 hour with blocking buffer (5% swine serum in PBS) at room temperature and incubated with the

cytochrome c primary antibody (sc-13560, Santa Cruz, California, USA) diluted in blocking buffer at 4°C overnight (O/N) in a humidifying chamber. Cells were washed in PBS and incubated with the Cy3-conjugated goat anti-mouse secondary antibody (1:1000) (Jackson ImmunoResearch Laboratories, Inc., USA) for 1 hour at room temperature in the dark. Cells were again washed in PBS and the DNA was stained with Hoechst 33342 for 10 min at room temperature in the dark. Cells were washed, the coverslips mounted onto glass slides with Movial mounting medium (Hoechst, Germany) containing n-Propyl gallate (Sigma, USA) to prevent the signal fading and the cells visualised by fluorescence microscopy using an Axiovert fluorescent microscope (Zeiss, Germany). Finally the cells visualised by a confocal microscope (Zeiss LSM 510 Meta with NLO, Software: ZEN 2009, Lasers: Argon 488 green, solid state laser: 561 nm Red, MaiTai 2 photon laser: 750 nm for DAPI/ Hoechst, Germany). Negative controls were as for above, but the primary antibody was excluded.

2.9.3 Quantification of GFP-LC3 puncta

Cells were plated on coverslips in 35 mm dishes and were transiently transfected with 2 mg/ml GFP-LC3 plasmid (a gift from Dr Lester Davids, Department of Human Biology, University of Cape Town) using Lipofectamine (Invitrogen, Carlsbad, CA, USA) and FuGENE HD (Roche, Germany) according to manufacturers' instructions. Twenty four hours post-transfection, cells were visualised by fluorescence microscopy using an Axiovert fluorescent microscope (Zeiss, Germany) in order to test GFP expression. If the expression was successful, the cells were then treated with 0.2 µM AJ-5 for 24 hours, after which they were fixed with 4% paraformaldehyde and stained with Hoechst 33342 for nuclei observation. Autophagy was quantified by counting

the GFP-LC3II puncta at 400x magnification in twenty fields of view using an Axiovert fluorescent microscope (Zeiss, Germany) and divided by the total number of transfected cells within these fields and the number of GFP-LC3 puncta/cell are presented as means ± SEM of three independent experiments. Autophagy flux induced by AJ-5 was confirmed using the autophagy inhibitor bafilomycin A (10 nM) and estimating the accumulation of fluorescent puncta of autophagosomes in GFP-LC3 transfected cells.

2.10 Electron microscopy

Transmission electron microscopy was used in this study to identify autophagosomes. Briefly, cells or tumour sections were fixed in 0.1 M sodium cacodylate buffer (pH 7.4) containing 2.5% glutaraldehyde (see appendix, section 6.5) for 16 h at 4^{O}C. Ultrathin sections of 100 nm were obtained using a Reichert Ultracut S ultramicrotome (Leica, Germany) and collected on 200-mesh copper grids. Sections were stained with uranyl acetate and lead citrate and analysed using a F20 transmission electron microscope (Tecnai F20, USA). The samples were submitted to the electron microscopy unit (University of Cape Town, South Africa) for further processing for imaging. Briefly, the samples were washed and treated with 0.1% Millipore-filtered cacodylate buffered tannic acid, post-fixed with 1% buffered osmium tetroxide for 30 min, and stained with 1% Millipore-filtered uranyl acetate. The samples were dehydrated in increasing concentrations of ethanol, infiltrated, and embedded in resin medium. The samples were polymerized for 2 days in a 70°C oven.

2.11 Western blot analysis

Cells were washed twice with ice-cold PBS and collected by scraping with a 1 ml plunger. Whole cell extracts were prepared using 2X Laemmli sample buffer (see appendix, section 6.6), boiled for 10 min and stored at -20°C. Alternatively, whole cell extracts prepared from cells using RIPA buffer (see appendix, section 6.6) were stored on ice for 30 min and collected by centrifugation at 12 000 rpm for 20 min at 4°C. The protein concentration for each cell extract was determined using the BCA Protein Assay kit (Pierce, USA), with bovine serum albumin as the standard. Equal amounts of protein were loaded in each lane and resolved on 6-15 % SDS-PAGE gels (see appendix, section 6.6) and then transferred electrophorectically to a Hybond ECL nitrocellulose membrane (Amersham Biosciences, USA). Membranes were blocked for 1 hour at room temperature with PBS containing 5% non-fat dry milk and probed with appropriate primary antibodies O/N at 4°C with shaking. Membranes were washed in PBS containing 0.1% Tween 20 (PBS/T) and incubated with either donkey anti-goat (Santa Cruz Biotechnology, CA, USA), goat anti-mouse or goat anti-rabbit IgG peroxidase-conjugated secondary antibodies (1:5000) (BioRad, Hercules, CA,USA) in blocking solution at room temperature with shaking for 1 hour. Membranes were again washed in PBS/T and visualised by enhanced chemiluminescence (Pierce, USA). The following dilutions of these antibodies and dilutions were used: 1:1000 rabbit polyclonal anti-PARP1/2 (sc-7150), mouse monoclonal anti-p53 (sc-126), mouse monoclonal anti-cyclin B1 ((sc-752), rabbit polyclonal anti-cyclin A (sc-751), rabbit polyclonal anti-p21 (sc-756), mouse monoclonal anti-tubulin (sc-8035), rabbit polyclonal anti-Bax (sc-7480), 1: 1500 rabbit polyclonal anti-p21 (sc-756), 1:1000 mouse monoclonal anti-ATM (sc-23921), (Santa Cruz, California, USA), 1:1000 rabbit polyclonal PUMA antibody supplied by AbCam (ab9643,

Cambridge, MA, USA), 1:5000 rabbit polyclonal anti-p38 (M0800)(Sigma, St. Louis, MO, USA) or the following antibodies which were obtained from Cell Signalling (Boston, MA, USA): 1: 1000 rabbit polyclonal anti-phospho- AKT (#9271), rabbit polyclonal anti phospho-H2AX (#2577), rabbit polyclonal anti-p44/42 MAPK (Erk1/2) (#9102), rabbit polyclonal anti-phospho-p44/42 MAPK (Erk1/2)(#4370), rabbit polyclonal anti-phospho-p38 MAP Kinase (#9211), rabbit polyclonal anti-LC3II (# 2775), rabbit polyclonal anti-Beclin1 (#3738), rabbit polyclonal anti-BCL-2 (#2876), rabbit polyclonal anti-phospho-CHK2 (#2661), rabbit polyclonal anti-Caspase-3 (#9661), rabbit polyclonal anti-Caspase-7 (#9492), mouse monoclonal anti-Caspase-8 (#9746), rabbit polyclonal anti-p70S6 kinase (#9202) mouse monoclonal anti-phospho-ATM (#4526) and 1:500 rabbit polyclonal anti phospho-SAPK/JNK (#9251). If necessary, the expression of these proteins was quantified as the densitometry value analysed by UN-SCAN-IT gel 6.1 software and normalised to the appropriate loading control.

2.12 Transfection assays

Prior to performing transfections, the concentration and quality of all DNA constructs were assessed using a NanoDrop® ND-1000 spectrophotometer (Agilent Technologies, Boeblingen, Germany) and confirmed by agarose gel electrophoresis (data not shown). If not otherwise stated, cells were plated in 12 well plates and transfected when 70% confluent. Three different transfection reagents were used:

FuGENE HD (Roche, Germany): For transient transfections, 1.2 µl of FuGENE HD (Roche, Germany) was added to 48.8 µl serum-free medium (no antibiotic), incubated for 5 min at room

temperature and then added to the DNA and incubated for a further 15 min at room temperature. In each case, the transfection reagent: DNA complex was added drop-wise to the cells and incubated for 30 hours at 37^{O}C. FuGENE HD was used for all transfections for the luciferase assay in this study and for the transfection of MCF7 cells with GFP-LC3B for monitoring autophagy.

Lipofectamine™ 2000 (Invitrogen Life Technology, San Diego, CA, USA): For transient transfections, 2.5 µl of Lipofectamine™ 2000 was added to 47.5 µl serum-free medium (no antibiotic), incubated for 5 min at room temperature and then added to the DNA or siRNA and incubated for a further 15 min. In each case, the transfection reagent and DNA/siRNA complex were added drop-wise to the cells and incubated for 30 h at 37^{O}C. Lipofectamine™ 2000 was used for transient knock-Down of p38, p53 and TBX3 expression in MDA-MB-231, ME1402 and WM1158 cells with GFP-LC3B for monitoring autophagy. The concentrations of siRNA used were: 50 nM of si-p38, p53, 10nM of TBX3 or equal concentration of a control (non-silencing) siRNA (Qiagen, USA).

DharmaFECT4 and DharmaFECT 1 (Dharmacon, USA)

Transient knock-down of LC3II and MAPK1/3 (ERK1/2) expressions were achieved by siRNA that specifically targets LC3II (si-LC3) or ERK1/2 (si-ERK1/2) mRNA. The cells were transfected with 50 nM of si-LC3, si-ERK1/2 or a control (non-silencing) siRNA (Qiagen, USA) using 2 µl of DharmaFECT4 for melanoma cells and MDA-MB-231 cells and DharmaFECT1 for MCF7 cells according to manufacturer's instructions.

2.13 Luciferase assays

Cells were transfected as described (see section 2.10), cultured for 30 hours and extracts were then assayed for firefly and renilla luciferase activity using the dual luciferase assay system (Promega, USA) according to the manufacturer's instructions. The vector pRL-TK, containing the thymidine kinase promoter which drives the expression of a renilla reporter, was used as an internal control for transfection efficiency in all luciferase assays (50 ng per transfection). Briefly, cells cultured in a 12-well plate were lysed using 100 µl 1X Passive Lysis Buffer. Cell lysates subjected to a freeze-thaw cycle were collected by centrifugation at 13 000 rpm for 1 min at room temperature. The supernatant was transferred to Eppendorf tube and 10 µl of cell lysate was assayed immediately for reporter gene activity. Luciferase activities were measured using the Luminoscan Ascent luminometer (Thermo Labsystems, Franklin, MA, USA). To normalise transfection efficiency, firefly luciferase values were divided by the renilla luciferase activity, where renilla activity did not respond to the treatment. Where renilla activity did respond, renilla values were scrutinised to ensure similar levels across untreated samples as a measure of transfection efficiency. Promoter activity was calculated as a ratio of the luciferase activity generated by (a) the empty vector to that obtained in the presence of the appropriate expression vector or (b) untreated samples as compared to treated. All luciferase assays were performed in duplicate and at least three independent experiments were done to confirm reproducibility. The Microsoft Excel program was used to calculate the standard deviation and statistically significant differences between samples using the Student *t* test. *P* values of <0.05 were considered statistically relevant.

TBX3 repress PUMA: MCF7 cells were plated at 1 X 10^5 cells per well in a 12-well plate 2 days before transfection. Cells were co-transfected with 200 ng of the Fragment 1 or Fragment 2 *PUMA* promoter reporter plasmid (see appendix, section 6.7) plus the empty pCMV vector, the PCMV TBX3 (WT-TBX3), the human TBX3 DNA-binding domain mutant (TBX3 DBM) or the TBX3 N terminal (TBX3 N-terminal) expression constructs and normalized by Renilla. The TBX3 DBM construct contains a disrupted DNA-binding domain due to a conversion of an arginine to a glycine at position 133 (R133G) and generated by site-directed mutagenesis. The TBX3 N terminal (TBX3 N-terminal) which lacks the C-terminal was generated by and restriction enzyme digest. Both TBX3 DBM and TBX3 N-terminal constructs were generated previously in our laboratory by Dr. Jade Peres and a PhD student Aretha Cooper.

2.14 Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using the RNeasy Plus Mini kit (Qiagen, USA). The quality and concentration of RNA was determined by spectrophotometry. Only samples exhibiting an A260/A280 ratio equal to or greater than 1.8 were selected and stored at -80°C for further applications. Reverse transcription of RNA was performed according to the manufacturer's instructions using the InProm-IITM reverse transcription system (Promega, USA). Briefly, 1 µg of RNA was combined with 0.5 µg of Oligo (dT)15 primer in a 5 µl volume and denatured at 70°C for 5 min, chilled on ice and combined with reverse transcription reaction mix (1X ImProm-IITM Reaction buffer, 3 mM $MgCl_2$, 0.5 mM dNTP mix, 20 units RNasin® ribonuclease inhibitor and 1 µl of ImProm-II TM reverse transcriptase) to a final volume of 20 µl. After a brief annealing at 25°C for 5 min, the reactions were incubated at 42°C for 1 hour, followed by 15 min incubation

at 70°C to inactivate the reverse transcriptase prior to PCR. qRT-PCR was conducted in 96 well sealed plates on an Applied Biosystems StepOne Plus thermal cycler using 2x SYBR green master mix (Applied Biosystems, Carlsbad, CA, USA), a final concentration of 0.3 µM of each primer and 2 µl of cDNA in a total volume of 10 µl. PCR cycle parameters were: denaturation for 15 min at 95°C, combined annealing and extension for 35 cycles at 60°C for 1 min. Each DNA sample was quantified in triplicate and a negative control without cDNA template was run with every assay to assess the overall specificity. Melting curve analyses was carried out to ensure product specificity. Relative mRNA expression levels were normalised to human glucuronidase beta (GUSB) or human beta-actin using the 2-ΔΔCt method. The Microsoft Excel program was used to calculate the standard deviation and statistically significant differences between samples using the Student *t* test. *P* values of <0.05 were considered statistically relevant. Primers used are listed in table 2.1.

Table 2.1: Sequences (5' to 3') or catalogue numbers of qRT-PCR primers used in this study.

Gene name	**Sequence/Catalogue number**	**Manufacturer**
Human GUSB	QT00046046	Qiagen, USA
Huamn Beta-Actin	F: CGGCATCGTCACCAACTG	IDT, USA
	R: AACATGATCTGGGTCATCTTCTC	
Human TBX3	QT00022484	Qiagen, USA
Human PUMA	F: GTAGAGATGGGGTTTTACCATGATGG	IDT, USA
	R: CCTCAGCCTCCCTAGTAGC	

2.15 Chromatin immunoprecipitation (ChIP) assay

Cells were plated in 2 X 15 cm tissue culture dishes at a total number of 10–20 million cells. On the day of harvest, cells were harvested by trypsinisation in a total of 40 ml PBS and treated with 1% formaldehyde at room temperature for 10 min, to crosslink proteins to DNA. The reaction was quenched with 125 mM glycine for 5 min at room temperature and cells were pelleted at 1500g at 4°C for 5 min, washed once in PBS, once in buffer 1 (see appendix, section 6.8) and once in buffer 2 (see appendix, section 6.8) prior to lysis in 500 µl lysis buffer (see appendix, section 6.8) plus protease inhibitors. Samples were sonicated to obtain DNA fragments of 300–500 base pairs, then centrifuged at 13 000 rpm for 10 min at 4°C to pellet cell debris and the supernatant removed to fresh tubes. For each sample, 300 µl of sonicated lysate was diluted with immunoprecipitation buffer (see appendix, section 6.8) plus protease inhibitors to a final volume of 1.5 ml and pre-cleared three times with protein A/G beads (Santa Cruz Biotechnology, USA) rotating at 4°C for 1 hour each time. Pre-cleared supernatant was then split into two tubes, with 3 µg goat polyclonal anti-TBX3 antibody (sc-17871) (Santa Cruz Biotechnology, USA) added to one tube and an equal amount of normal rabbit control IgG (sc-2027, Santa Cruz Biotechnology, USA) added to the other, with 1% of the supernatant volume reserved as input. Samples were rotated at 4°C O/N, followed by incubation with 50 µl of protein A/G beads rotating at 4°C for 3.5 hour. The beads were then washed twice in each of the wash buffers 1, 2, 3 and 4 (see appendix, section 6.8) by centrifuging at 2000g at 4°C for 1 min to pellet the beads, adding 1 ml of wash buffer and rotating for 10 min at 4°C. The beads were then incubated in 100 µl extraction buffer (see appendix, section 6.8) for 15 min at room temperature, then pelleted at 2000g for 1 min and the supernatant removed to fresh tubes.

The extraction was repeated to yield 200 µl supernatant in total and input samples were made up to this volume with extraction buffer. Samples were incubated at 65°C O/N to reverse the crosslink between DNA and proteins, purified using a PCR purification kit (Qiagen, USA) according to the manufacturer's instructions and DNA eluted in 20 µl of elution buffer. Quantitative real time PCR was set up as described above (see section 2.13). Crossing values (Ct) of PUMA or IgG precipitated DNA were normalised against the Ct values of 1% input DNA. Fold enrichment was determined using the ΔΔCt method: Fold enrichment= $2^{-(\Delta Ct1-\Delta Ct2)}$, where ΔCt 1 is the ChIP of interest and ΔCt2 the control ChIP. Statistical differences were determined using a student *t* test. Significance was accepted at $p < 0.05$. Quantitative real time PCR was conducted using primer pairs specific for the PUMA promoter: (5'-GTAGAGATGGGGTTTTACCATGATGG -3' and 5'- CCTCAGCCTCCCTAGTAGC -3'), or a non-specific region in the GAPDH gene: (5'-CAGCCAGACGAGGACACA-3' and 5'-CCTTTCTGGGATTGCCTTTC -3').

2.16 Nude mice study

2.16.1 Acute toxicity study

4-6 week old nude mice were obtained from the University of Cape Town (UCT) Animal Facility and the experiments approved by the Animal Research Ethics Committee of UCT. We examined the acute toxicity profile of 10 mg/kg AJ-5. Three mice were injected intraperitoneally (i.p) with a single dose of either vehicle or AJ-5, and the mice were checked for signs of toxicity such as decreased food uptake, convulsion, collapse, non-responsiveness to stimuli, weakness, weight loss, decreased activity and diarrhoea.

2.16.2 Sub- acute toxicity study

Because we observed some signs of toxicity at 10 mg/kg AJ-5, we therefore decided to perform the sub-acute study at 2 mg/kg. For this part of the study three groups of 4 to 6 week-old mice containing 6 mice per group were divided as follows: Control group (blank) received no treatment, control group received the vehicle (DMSO) and test groups received AJ-5 at concentration of 2 mg/kg of body weight. The mice were injected i.p daily and checked for signs of toxicity such as decreased food uptake, convulsion, and collapse, non-responsiveness to stimuli, weakness, weight loss, decreased activity and diarrhoea over 14 days.

2.16.3 Anti-tumour activity study

The flanks of nude mice were injected subcutaneously with 5×10^6 ME1402 cells and 10 days later the animals were randomly divided into five groups. The mice in the AJ-5 or CDDP groups were injected i.p three times a week for 2 weeks with AJ-5 (2 mg/kg of body weight) dissolved in DMSO/PBS or CDDP (2 mg/kg of body weight) dissolved in sodium chloride respectively. The control groups received no treatment or were treated with an equal volume of AJ-5 or CDDP vehicle (NaCl). Tumour growth was measured three times a week using the formula Volume mm^3 = (length) × ($width^2$) × 0.5. After 2 weeks of treatment mice were euthanized and organs including tumours were taken for further analyses.

2.17 Statistical analysis

Data presented are mean ± SEM (Standard error of the means) of three independent experiments. Statistical significance was assessed between the animal groups using the Student's t-test. A value of $P< 0.05$ was accepted as statistically significant.

Chapter 3: Results

Introduction

Cancer is a major health problem worldwide and deaths from cancer are projected to continue rising, with an estimated 13.5 million deaths in 2030 [1]. For most tumours, current therapies including surgery radiotherapy, and chemotherapy are insufficient at providing a cure, prolonging survival and improving quality of life [5, 6]. The main reason for failure of anti-cancer therapies is tumour resistance to several groups of drugs [13] and severe side effects such as neuro/nephrotoxicity and myelosuppression which is associated with platinum based chemotherapies [83]. Efforts for overcoming these limitations have focused on identifying novel chemotherapeutic agents and understanding the mechanisms by which they function.

Recently palladium (Pd) complexes have attracted a lot of interest as chemotherapeutic agents because they have been shown to exert a significant cytotoxic effect on cancer cells [65]. While early palladium based compounds showed little anti-tumour activity due to poor stability, the use of stabilizing ligands have improved the efficacy of these compounds [249, 250]. Indeed, a recent study showed that a dinuclear Pd(II) chelate with a spermine ligand displayed strong anti-tumour activity in breast cancer cell lines [71]. Importantly Pd complexes have been shown to exert anti-tumour activity in CDDP resistant cells and to have less side effects than CDDP [66, 78]. This led to suggestions that Pd(II) compounds may have different mechanisms of action to that of CDDP but this is still unresolved [251]. It is however generally accepted that the cytotoxic effects exerted by most metal based compounds result from them inducing DNA

double-strand breaks which trigger a canonical DNA damage signalling pathway through activating ataxia telangiectasia mutated (ATM) [29]. Active ATM together with ataxia telangiectasia mutated and Rad3 related (ATR) drives the accumulation of the phosphorylated form of the histone variant H2AX (γH2AX) to the area surrounding the break [29, 30]. γH2AX facilitates the activation of the checkpoint kinase CHK2 and the multifunctional transcription factor p53 that mediate phenotypic responses to DNA damage including cell cycle arrest, DNA repair and/or the induction of programmed cell death [31, 252]. p53 plays an important role in deciding cell fate in response to DNA damage through trans-activating the cyclin-dependent kinase inhibitor p21 as well as pro-apoptotic proteins [32]. While the above are well established DNA damage pathways, recent studies have also implicated the ERK1/2 and p38 MAPK pathways in the DNA damage response where they have also been shown to activate p53 and p21 [43].

While most chemotherapeutic agents have been described to induce cell death via apoptosis there is increasing evidence that they can also function by initiating mitotic catastrophe and autophagy. Indeed, several studies have confirmed a complex crosstalk between apoptosis and autophagy [253] but while some studies indicate that autophagy inhibits the process of apoptosis [254], others suggest a role for autophagy in the induction of cell death [173]. It would however appear that these opposing roles of autophagy depend, in part, on both the cell type and the chemotherapy used.

This chapter of the study investigates the possible anti-cancer activity of a group of palladium based compounds and the one which exerted the most cytotoxicity was chosen for further investigation as a possible drug to treat breast cancer and melanoma. Finally, the role of the developmentally important T-box factor, TBX3, in sensitizing breast cancer cells to the priority drug was explored.

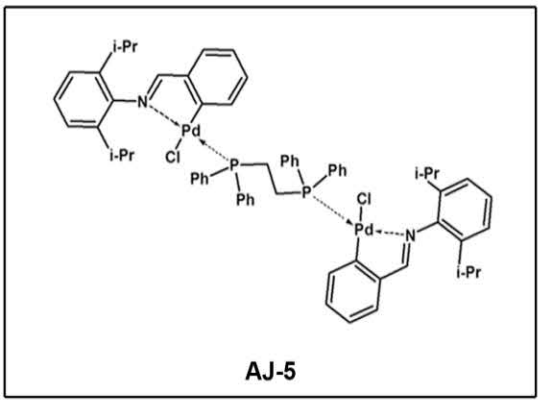
3.1 Screening of a group of palladium based compounds in melanoma and breast cancer cell lines

A panel of palladium based compounds was synthesised by the laboratory headed by my co-supervisor, Prof Selwyn Mapolie. This panel included mononuclear (AJ-1, AJ-2, AJ-3, and AJ-4) and binuclear (AJ-5, AJ-6, AJ-7, AJ-8, C5, C6, C7, and C8) palladium complexes. The chemical structure of the compounds and the ligands used in the synthesis are shown in (appendix, section 6.2). All compounds were first screened for possible cytotoxic effects in MCF7 and MDA-MB-231 breast cancer cell lines as well as the ME1402 VGP melanoma cell line. The cells were treated with a range (0 to 20 µM) of the compounds or vehicle for 48 hours. Cell viability was determined using the MTT assay, and the concentration that inhibits cell growth by 50% (IC_{50}) was calculated from sigmoidal plots using GraphPad Prism version 5 **(Fig. 3.1a).** Compared to all the compounds investigated, AJ-5, a binuclear palladium metallacycle complex with 1,2-*bis*(dipenylphosphino)ethane as co-ligand **(Fig. 3.1b)**, showed a very strong cytotoxic effect with a low IC_{50} (less than 0.2 µM) in the breast cancer and melanoma cells lines tested and was therefore chosen for further study.

a

Compound	IC_{50} (μM) ± SEM		
	MCF7	MDA-MB-231	ME1402
AJ-1	17.17 ± 1.19	21.93 ± 0.93	23.67 ± 1.17
AJ-2	17.03 ± 1.99	19.31 ± 0.98	20.00 ± 2.71
AJ-3	31.19 ± 2.33	21.02 ± 1.52	33.11 ± 3.73
AJ-4	23.92 ± 2.14	20.65 ± 0.72	32.85 ± 2.26
AJ-5	**0.175 ± 0.04**	**0.193 ± 0.01**	**0.1945 ± 0.08**
AJ-6	20.65 ± 2.14	18.96 ± 1.07	29.60 ± 2.38
AJ-7	15.03 ± 1.23	15.60 ± 2.97	13.63 ± 1.52
AJ-8	13.01 ± 1.84	17.77 ± 1.29	16.76 ± 0.55
C5	46.22 ± 1.39	54.11 ± 3.52	21.02 ± 2.51
C6	18.75 ± 1.01	17.43 ± 1.61	6.03 ± 1.27
C7	11.17 ± 1.94	7.45 ± 1.03	8.37 ± 1.37
C8	15.51 ± 5.29	7.63 ± 3.48	9.65 ± 2.97

b



AJ-5

Figure 3.1 The cytotoxic effects of a panel of palladium based compounds on breast cancer and melanoma cell lines. (a) The indicated cell lines were plated in 96-well plates and after 48 hours the cells were treated with increasing concentrations of the indicated compounds (0-20 μM). Cell viability was assessed by the methylthiazoltetrazolium (MTT) assay after 48 hours of treatment. The table shows the concentrations of the compounds required for killing 50% of the cells (IC50) ± SEM (standard error of the mean) which was calculated from sigmoidal plots using GraphPad Prism version 5. The data represents pooled results of at least three independent experiments performed in quadruplicate. **(b)** Chemical structure of dppe—1,2-*bis*(diphenylphosphino)ethane (AJ-5).

3.2 The palladacycle, AJ-5, exhibits anti-tumour and anti-cancer stem cell activity in breast cancer cells

Breast cancer is the most common malignancy worldwide amongst women and it is estimated that one out of eight women will develop breast cancer in their life time [180, 255, 256]. Despite enormous efforts to address this problem, there is still limited success with most of the current therapeutic strategies. More than 70% of human breast cancers (BCs) are hormone-dependent and Estrogen receptor (ER)-positive and approximately 15% are hormone-receptor-negative which includes BCs lacking ER expression [199]. The most commonly used anti-tumour drug for treating ER-positive BCs is tamoxifen which functions by blocking estrogen receptors [257]. Approximately 30% of breast cancer patients, however, fail to respond to endocrine therapy including tamoxifen and many patients with endocrine-responsive breast cancer eventually develop resistance to endocrine therapy [258, 259]. Increasing evidence suggest that breast cancer stem cells (BCSCs), a small subset of cells with the cell surface marker signature $CD44^{hi}CD24^{lo}$, play a major role in this resistance [260]. Indeed, BCSCs are more resistant to chemotherapeutic agents and radiation than other heterogeneous cells in tumours and there is therefore a need to develop efficient drugs to treat breast cancers and in particular which are effective against BCSCs [193–196, 261]. This study therefore tested the effect of AJ-5 on the ER-negative MDA-MB-231 and ER-positive MCF7 breast cancer cells and investigated the molecular mechanism of its action. Moreover, the anti-cancer stem cells activity of AJ-5 was also addressed.

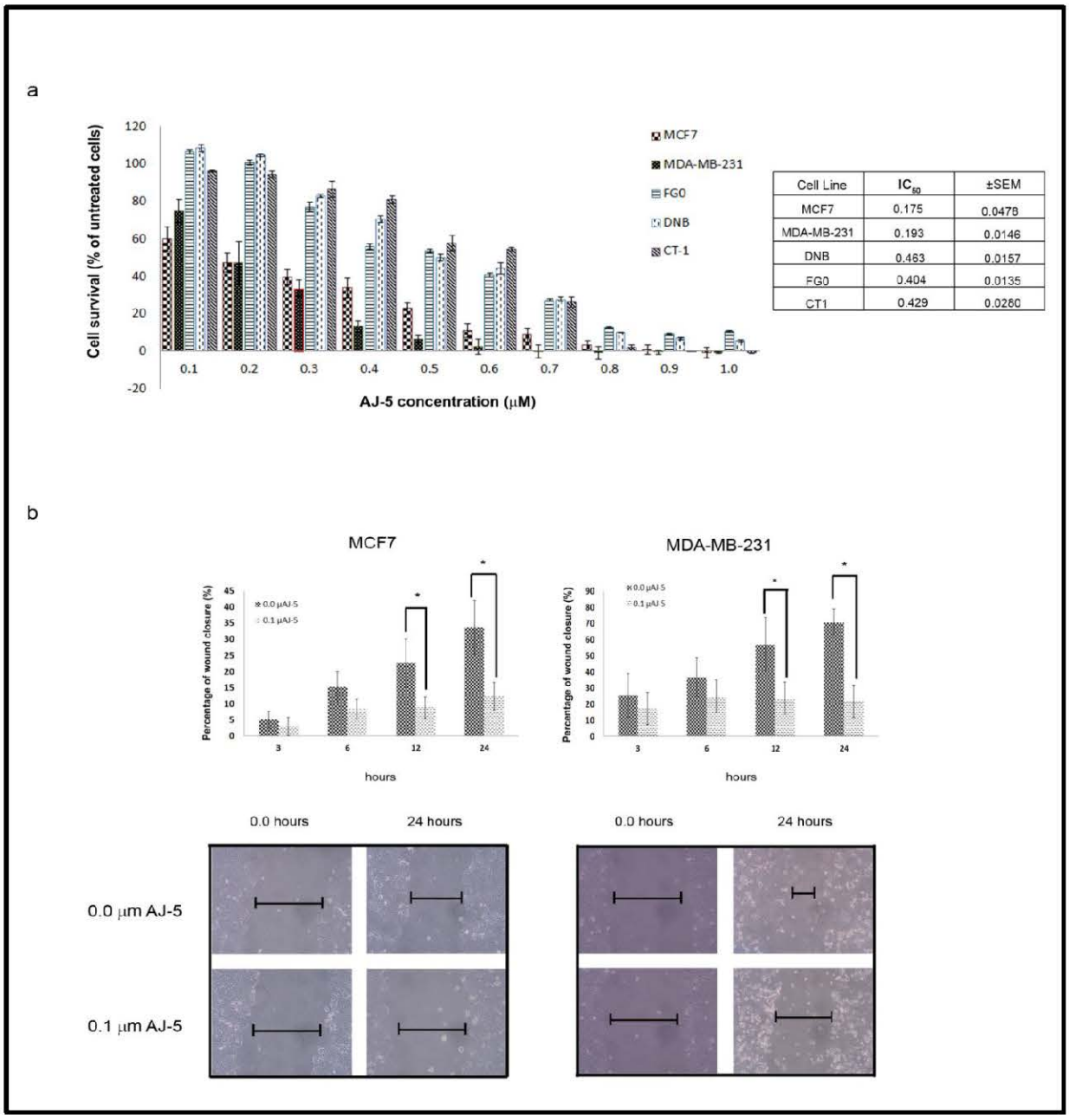
3.2.1 AJ-5 inhibits cell proliferation and migration of human breast cancer cells

The cytotoxic effect of AJ-5 on the ER-positive MCF7 and ER-negative MDA-MB-231 breast cancer cell lines was examined using the MTT assay. After 48 hours of AJ-5 treatment a strong dose dependent inhibition of cell proliferation was observed in both breast cancer cell lines **(Fig. 3.2a)**. This effect was less pronounced in three non-malignant fibroblast cell lines (DNB, FG-0, and CT-1) which were included as controls. Indeed, the IC_{50} values obtained for MCF7 and MDA-MB-231 cells were 0.175 µM and 0.193 µM respectively whereas they were more than 0.4 µM for the normal cells. Importantly while 0.4 µM AJ-5 killed more than 60% of breast cancer cells, on average approximately 70% of fibroblast cells continued to survive and more than 20% were still viable at 0.7 µM AJ-5. These results show that AJ-5 exerts potent cytotoxic effects specifically on the breast cancer cell lines compared to the control cells.

To further explore the anti-tumour activity of AJ-5, a scratch motility assay was performed and a significant reduction in cell migration was observed for both cell lines exposed to 0.1 µM AJ-5 for 12 and 24 hours **(Fig. 3.2b)**. Taken together these data demonstrate that at low concentrations AJ-5 has potent cytotoxic and anti-migratory effects on ER-positive and ER-negative breast cancer cells.

3.2.2 AJ-5 has putative anti-cancer stem cell activity

Cancer stem cells (CSCs) are a biologically distinct subpopulation of tumour cells with stem cell characteristics that have been isolated in vitro from both clinical samples and established cell lines [195, 262–264].



Cell Line	IC_{50}	±SEM
MCF7	0.175	0.0478
MDA-MB-231	0.193	0.0146
DNB	0.483	0.0157
FG0	0.404	0.0135
CT1	0.429	0.0280

Figure 3.2 AJ-5 induces cytotoxicity and inhibits migration of breast cancer cells. (a) Cell survival rate (as measured by MTT assay) of indicated cell lines cell lines treated with increasing concentrations of AJ-5 (0-1.0 μM) or vehicle for 48 hours. Results show the mean percentage ± SEM of untreated cells and represent the pooled results of at least three experiments performed in quadruplicate. The concentrations of AJ-5 required for killing 50% of the cells (IC_{50}) are shown in the table and were calculated from sigmoidal plots with GraphPad Prism version 5. **(b)** AJ-5 inhibits the migratory ability of breast cancer cells in an in vitro scratch assay. Cells were grown to 100% confluence and a linear wound created through the cell monolayer. Cell motility was assayed at the indicated times after addition of either vehicle (control) or AJ-5 (0.1 μM) for 24 hours. Cells were pre-treated with mitomycin C to prevent de novo cell proliferation. At specified time points (x-axis) cells were photographed using (10x; Olympus 1X71) and the area migrated was measured and expressed relative to zero time (y-axis). Assays were done in duplicate and two independent experiments were performed (*P <0.001, student's t test).

In breast cancer, as few as 100 cells with the cell surface marker signature $CD44^{hi}CD24^{lo}$ could generate a tumour, while tens of thousands of cells from the rest of the population were unable to do so [193]. Breast cancer stem-like cells can be identified on the basis of this marker profile, as well as the ability to grow in serum-free anchorage independent cultures known as mammospheres [194, 248, 265]. Due to their role in cancer development, metastasis and drug resistance, CSCs are thought to have clinical significance, and therefore treatments that target these cells have potential therapeutic applications [263, 266–268]. The putative anti-CSC activity of AJ-5 was assessed in the MCF7 cell line by determining its effect on the proportion of $CD44^{hi}CD24^{lo}$ cells and mammosphere formation **(Fig. 3.3)**. Cultures treated with AJ-5 showed a significant dose dependent reduction in the proportion of $CD44^{hi}CD24^{lo}$ cells at concentrations lower than the IC_{50} value determined for adherent cells **(Fig. 3.3a)**. These data suggested that $CD44^{hi}CD24^{lo}$ cells are more sensitive to AJ-5 treatment and therefore are reduced relative to the rest of the tumour population. In addition, AJ-5 had a dose-dependent inhibitory effect on the number, size and viability of MCF7 mammospheres **(Fig. 3.3b, c)**. This effect was greater when AJ-5 was added to existing mammospheres on day 4 post seeding, than when treatment occurred upon seeding. Indeed, as little as 0.025 µM AJ-5 on day 4 caused a statistically significant decrease in sphere forming efficiency which was greater than the effect produced when three times the dose (0.075 µM) of AJ-5 was added upon seeding **(Fig. 3.3c)**. This suggest that mammosphere derived MCF7 cells are more sensitive to AJ-5 inhibition than the bulk MCF7 cells. It has been demonstrated that MCF7 mammospheres are enriched in cells bearing the putative cancer stem-like marker profile, $CD44^{hi}CD24^{lo}$ [247] and therefore the enhanced sensitivity of the day 4 mammospheres to AJ-5 may be explained by the greater sensitivity of

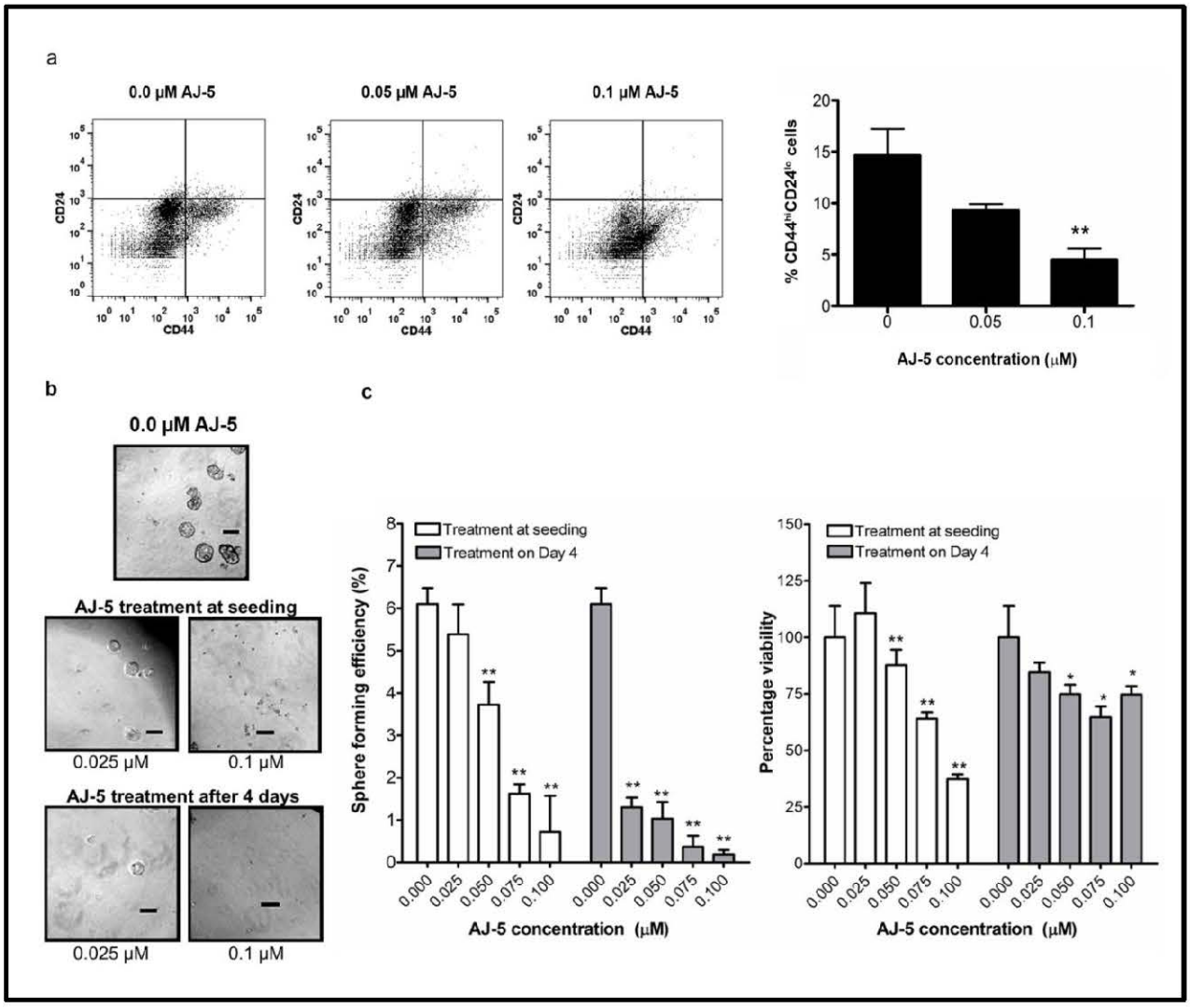


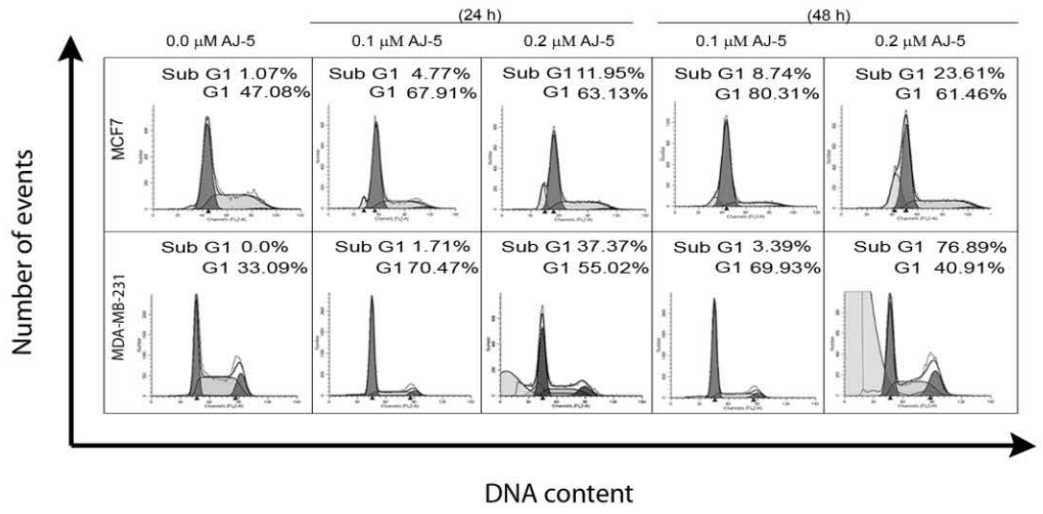
Figure 3.3 AJ-5 displays activity against cancer stem-like cells from the MCF7 cell line. (a) Proportion of MCF7 cells with the cancer stem-like surface profile $CD44^{hi}CD24^{lo}$ was determined by flow cytometry after treatment with 0.05 µM and 0.1 µM AJ-5. Results represent the mean percentage ± SEM of at least two experiments performed in triplicate (** P<0.01, one way ANOVA). **(b)** Mammosphere formation in MCF7 cells treated with AJ-5 at seeding or 4 days post seeding under mammosphere conditions (1000 cells/well). Mammosphere formation after 7 days following treatment with AJ-5 was assessed by microscopy. Images were captured under an Olympus inverted microscope at 100x magnification and are representative of three randomly selected fields for each treatment. Scale bars indicate 100 µm. **(c)** Mammosphere formation was quantified as sphere forming efficiency (SFE), which is calculated as the number of mammospheres formed per number of single cells seeded and expressed as a percentage. The viability of mammospheres was assessed by WST-1 assay on day 7. The percentage viability after treatment was calculated relative to the DMSO vehicle control (taken as 100%) in which equal numbers of cells were seeded. Data shown are mean ± SEM for at least 4 replicates and two independent experiments were performed (**P < 0.01, *P < 0.05, student's t test).

$CD44^{hi}CD^{lo}$ cells to AJ-5 **(Fig. 3.3a)**. These data are consistent with observations of other groups who have demonstrated the anti-breast cancer stem cell activity of the compounds salinomycin [248] and niclosamide [269]. Taken together, these data suggest that AJ-5 has in vitro activity against MCF7 derived cancer stem-like cells.

3.2.3 AJ-5 induces DNA damage and G1 cell cycle arrest

Palladium compounds have previously been shown to exert its cytotoxicity by inducing DNA double strand breaks (DSBs) leading to cell cycle arrest and cell death [78, 88]. To investigate the mechanism by which AJ-5 inhibits breast cancer cell growth its effect on the cell cycle profile and DNA damage response was therefore tested. Flow cytometry analyses reveal that 0.1 and 0.2 µM AJ-5 treatment for 24 hours and 48 hours induced a G1 cell cycle arrest in both breast cancer cell lines which occurred mostly at the expense of S phase cells **(Fig. 3.4a, b).** Sub-G1 peaks, generally accepted to represent dead cells [270], were present in both breast cancer cell lines treated with AJ-5. Particularly striking was the 72.4% sub-G1 population of MDA-MB-231 cells treated with 0.2 µM AJ-5 for 48 hours. These results are consistent with previous study reported by Ulukaya et al. for another palladium compound in prostate cancer cells [65]. Palladacycles have previously been shown to exert cytotoxicity by inducing DNA double strand breaks (DSBs) which can be measured by the levels of γ-H2AX, a variant form of histone H2A that is directly phosphorylated at Ser139 in response to DSBs. To begin to identify the molecular mechanism underpinning AJ-5 cytotoxicity, immunoblotting was performed with antibodies to γ-H2AX as well as to a number of proteins involved in the DNA damage response. The results show that AJ-5 does indeed induce DNA damage as evidenced by an induction of

a



b

Cell Line	Cell Cycle Phase	0.0 µM AJ-5	24 hours		48 hours	
			0.1 µM AJ-5	0.2 µM AJ-5	0.1 µM AJ-5	0.2 µM AJ-5
MCF7	Sub G1 ± SEM	0.57 ± 0.70	2.36 ± 0.14	12.89 ± 1.33	13.49 ± 6.72	25.77 ± 3.05
	G1 ± SEM	45.34 ± 2.46	53.13 ± 0.23	60.22 ± 4.10	78.95 ± 1.91	59.50 ± 2.76
	S ± SEM	54.15 ± 2.085	44.38 ± 1.80	35.02 ± 0.45	20.51 ± 2.31	37.20 ± 1.33
	G2/M ± SEM	0.50 ± 0.37	2.48 ± 1.56	4.76 ± 3.64	0.53 ± 0.39	3.2 ±1.42
MDA-MB-231	Sub G1 ± SEM	0.06 ± 0.01	2.08 ± 0.53	36.60 ± 1.08	16.45 ± 2.53	72.41 ±6.33
	G1 ± SEM	31.84 ± 1.76	71.38 ± 1.29	56.17 ± 1.62	38.75 ± 1.20	41.23 ± 0.45
	S ± SEM	52.12 ± 0.71	21.75 ± 0.90	29.78 ± 0.62	51.82 ± 3.78	37.80 ± 0.49
	G2/M ± SEM	16.03 ± 1.04	6.86 ±0.38	14.05 ± 1.00	9.425 ± 2.58	20.97 ± 0.94

Figure 3.4 AJ-5 induces sub-G1 peak and G1 cell cycle arrest. (a) Cell cycle profile of breast cancer cells exposed to AJ-5 (0.1 µM and 0.2 µM) or vehicle for 24 hours and 48 hours was determined by flow cytometry. The proportion of cells at each phase of the cell cycle was expressed as a percentage of the total number of cells analysed and the percentage of sub G1 and G1 phases are shown. **(b)** The table shows the pooled results of three independent experiments ± SEM.

γ-H2AX and phosphorylation of ATM (Ser1981) and its substrate CHK2 (Thr68) in both breast cancer cell lines **(Fig. 3.5)**. Under all conditions tested, there was a robust p53 response in MCF7 cells, which in general correlated with an increase in levels of the cell cycle regulator p21. Importantly, while MDA-MB-231 cells have high levels of a mutant p53 [271] their p21 levels were markedly increased at 24 hours of AJ-5 treatment suggesting a p53-independent induction of p21 in these cells **(Fig. 3.5)**. Together these results suggest that AJ-5 induced DNA damage and that the G1 cell cycle arrest observed in **figure 3.4** is probably p21 dependent.

3.2.4 AJ-5 induces intrinsic and extrinsic apoptosis in breast cancer cells

To investigate whether the cytotoxicity of AJ-5 involves the induction of apoptosis, breast cancer cells were treated as in **figure 3.4** and the percentage of apoptosis was quantified using AnnexinV/PI staining. Annexin V is an impermeable dye which binds to phosphatidylserine on the cell membrane when cells undergo apoptosis and apoptotic cells can therefore be measured by positive staining for both PI and FITC [272]. **Figure 3.6a** shows that AJ-5 induced apoptosis in both breast cancer cell lines which correlates with the sub-G1 peaks detected in **figure 3.4**. Interestingly the level of MDA-MB-231 cells undergoing apoptosis was significantly higher than the apoptotic population of MCF7 cells. Indeed, MDA-MB-231 cells undergoing apoptosis when treated with 0.2 μM AJ-5 increased significantly from 43.5% at 24 hours to 75.4% at 48 hours compared to 28.1% and 51.6% respectively for MCF7 cells. Furthermore AJ-5 treated cells showed increasing levels of poly(ADP-ribose) polymerase (PARP) cleavage, a molecular marker of apoptosis, under all conditions tested **(Fig 3.6b).**

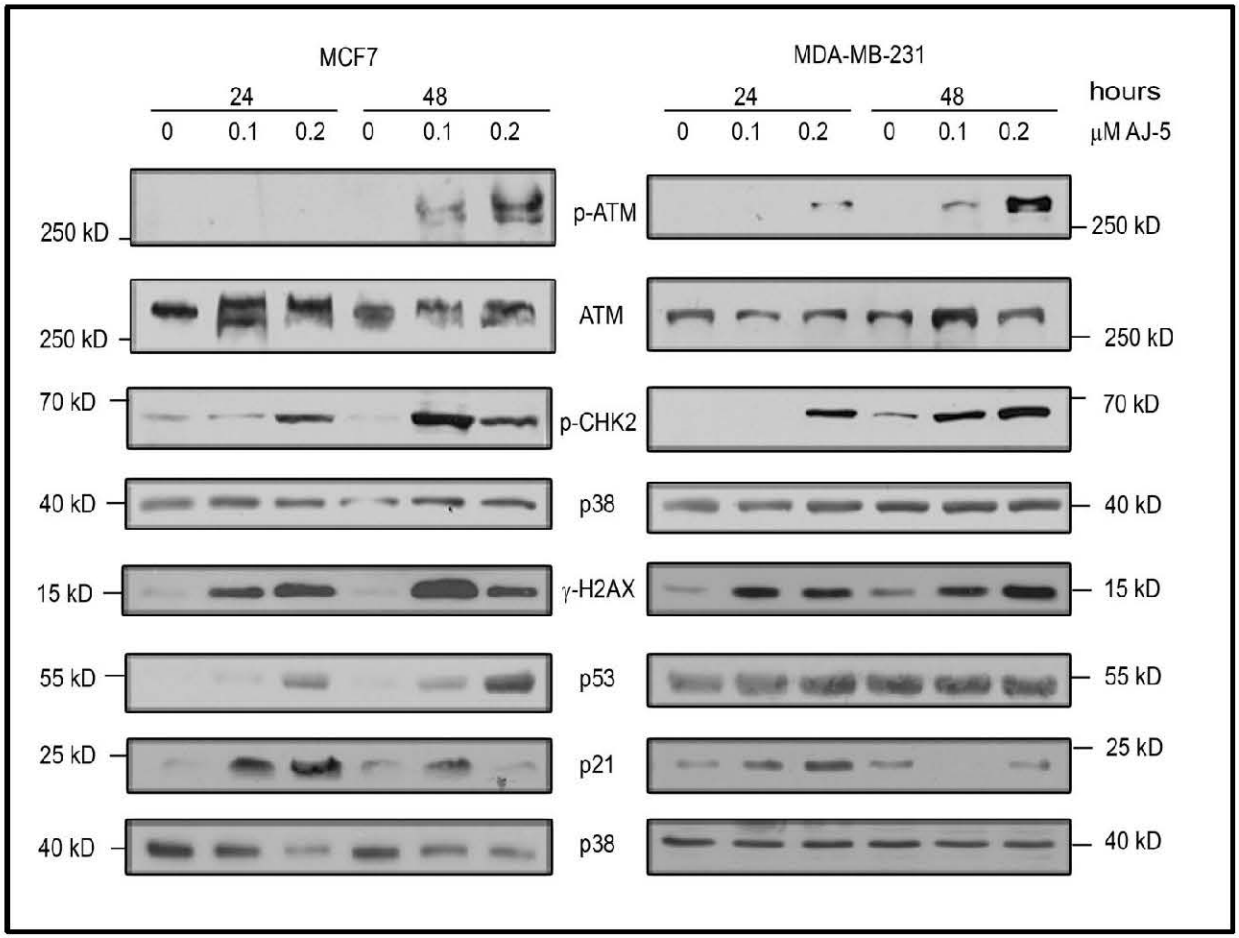


Figure 3.5 AJ-5 activates the DNA damage response. MCF7 and MDA-MB-231 cells were treated with vehicle or 0.2 µM AJ-5 for 24 and 48 hours. Protein extracts analysed by SDS-PAGE (6-15%) and western blotting using antibodies to p-ATM, p-CHK2, γ-H2AX, p53 and p21 showing that AJ-5 induces a DNA damage response. Total ATM and p38 were used as loading controls.

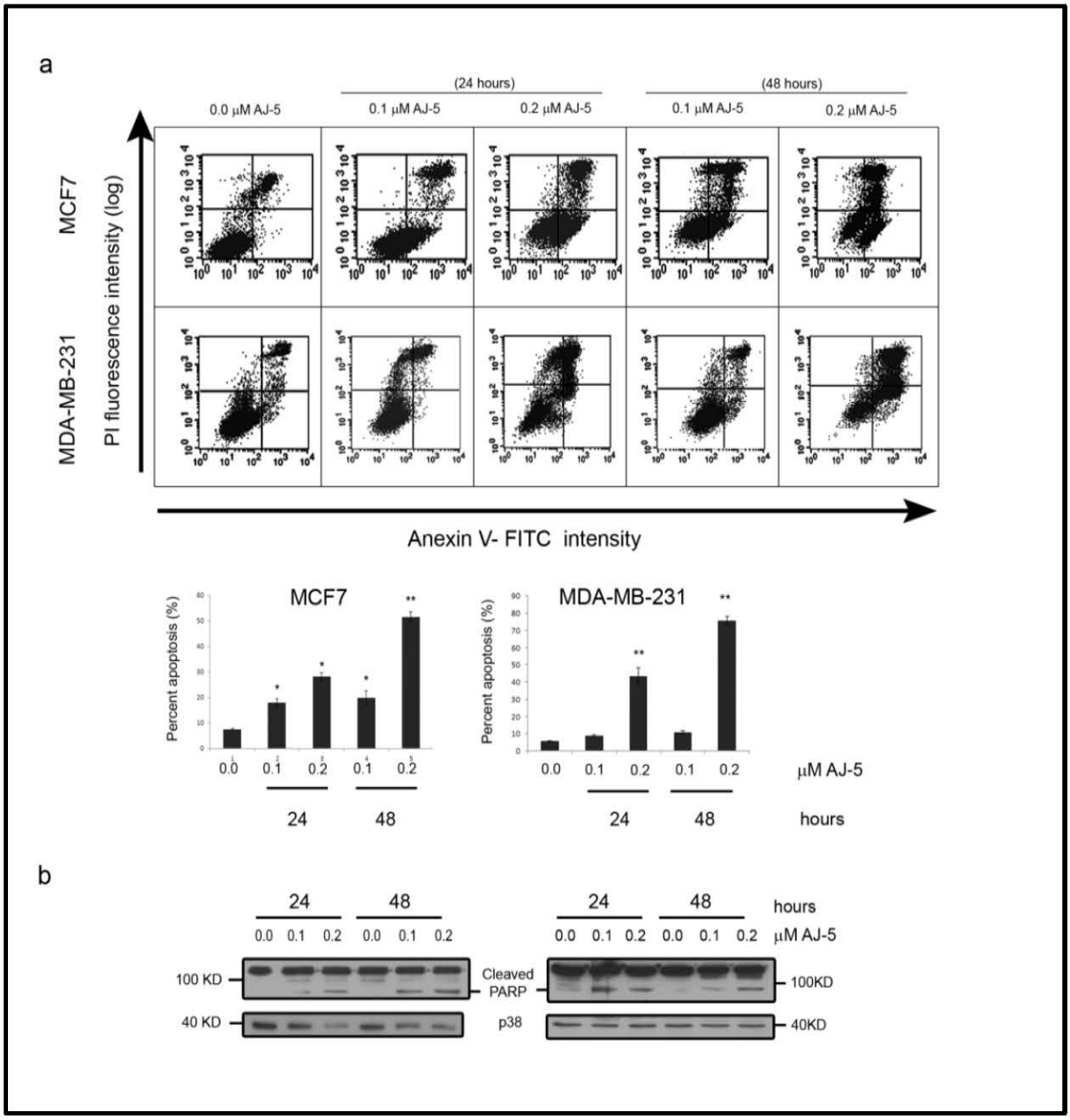


Figure 3.6 AJ-5 induces apoptosis in breast cancer cells. (a) Flow cytometric analyses of Annexin V–FITC/propidium iodide staining show the induction of apoptosis in cells treated with AJ-5 (0.1 μM and 0.2 μM) for 24 and 48 hours. The graphs represent the percentage of cells undergoing apoptosis (early and late) and the results are presented as the mean of three independent experiments ± SEM versus control (*$P < 0.05$, **$P < 0.01$, student's *t* test). **(b)** MCF7 and MDA-MB-231 cells were treated with 0.1 and 0.2 μM AJ-5 or vehicle for 24 and 48 hours. Protein extracts harvested from the indicated cells were analysed by SDS-PAGE (8%) and western blotting using antibody to PARP cleavage. p38 was detected as loading control.

Transient activation of PARP causes poly(ADP-ribose) accumulation in early apoptotic cells which is followed by PARP cleavage by caspases in late stages of apoptosis [273].

Apoptosis can be activated through two main pathways namely, the extrinsic and intrinsic pathways [102]. While extrinsic apoptosis is mediated by death receptors and characterized by caspase 8 activation [104], intrinsic apoptosis is mitochondrial mediated and usually triggered by intracellular signals such as hypoxia and DNA damage [121]. Overexpression of pro-apoptotic BCL-2 proteins disrupts the ratio of pro-and anti-apoptotic Bcl-2 family members and eventually leads to the release of cytochrome c from the mitochondria [274]. To determine if AJ-5 activated either or both apoptotic pathways in breast cancer cells, the levels of intrinsic and extrinsic apoptotic molecular markers were measured by western blotting **(Fig. 3.7)**. The results reveal that the cleaved caspase 8 products 43/41 and 18 kDa increased in MCF7 and MDA-MB-231 cell lines from as early as 1 hour of AJ-5 treatment. In addition, the intrinsic pro-apoptotic factors, PUMA and Bax, were induced as early as 1 hour of AJ-5 treatment in both breast cancer cell lines. Not surprisingly, the levels of the anti-apoptotic protein, BCL-2, decreased with AJ-5 treatment. Importantly, whereas MDA-MB-231 cells showed cleavage of caspase 3 from 3 hours of AJ-5 treatment, MCF7 cells, reported to lack this caspase, displayed an increase in total and cleaved caspase 7 levels. These results would suggest that caspase 7 can substitute for caspase 3 in MCF7 cells. Furthermore, **figure 3.8** shows that while cytochrome c is highly localized to the mitochondria of vehicle treated cells, cytochrome c was abundant in the cytoplasm of AJ-5 treated cells. Taken together these observations show that AJ-5 induces the intrinsic and extrinsic apoptotic pathways in both MCF7 and MDA-MB-231 cell lines.

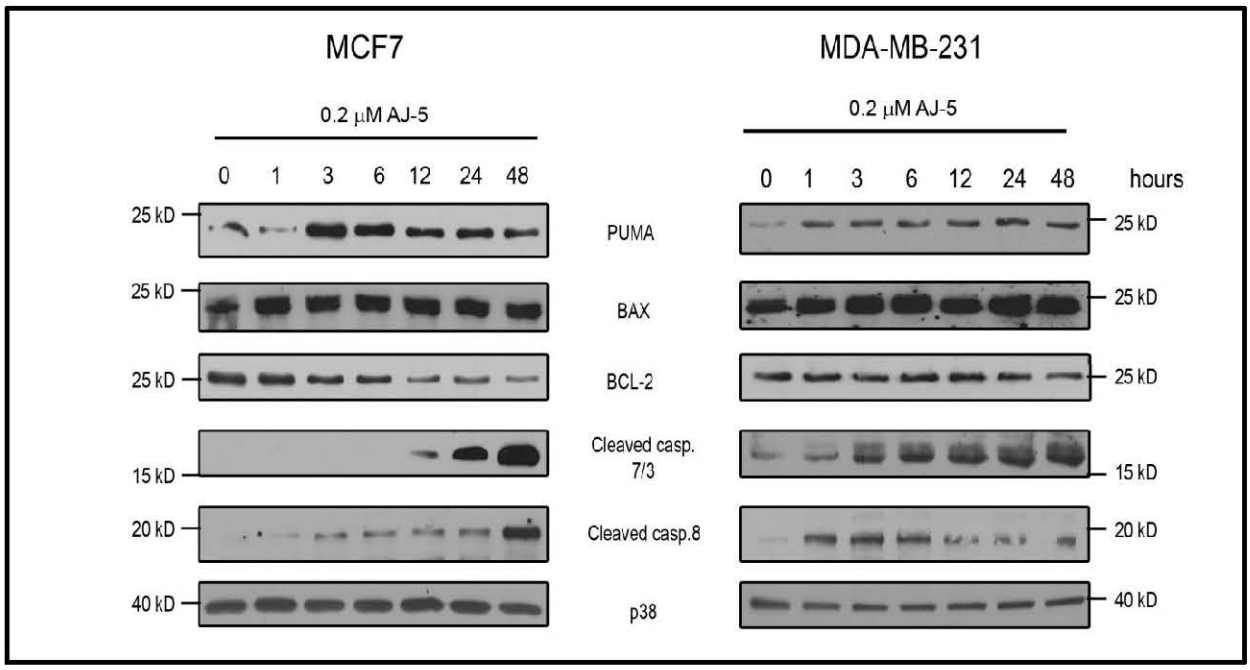


Figure 3.7 AJ-5 induced apoptosis involves both intrinsic and extrinsic apoptosis. MCF7 and MDA-MB-231 cells were treated with either vehicle or 0.2 μM AJ-5 for up to 48 hours. Protein extracts were harvested and analysed by SDS-PAGE (8-15%) and western blotting using antibodies to PUMA, Bax, BCL-2, caspase 7/3 and caspase 8. p38 was detected as loading control.

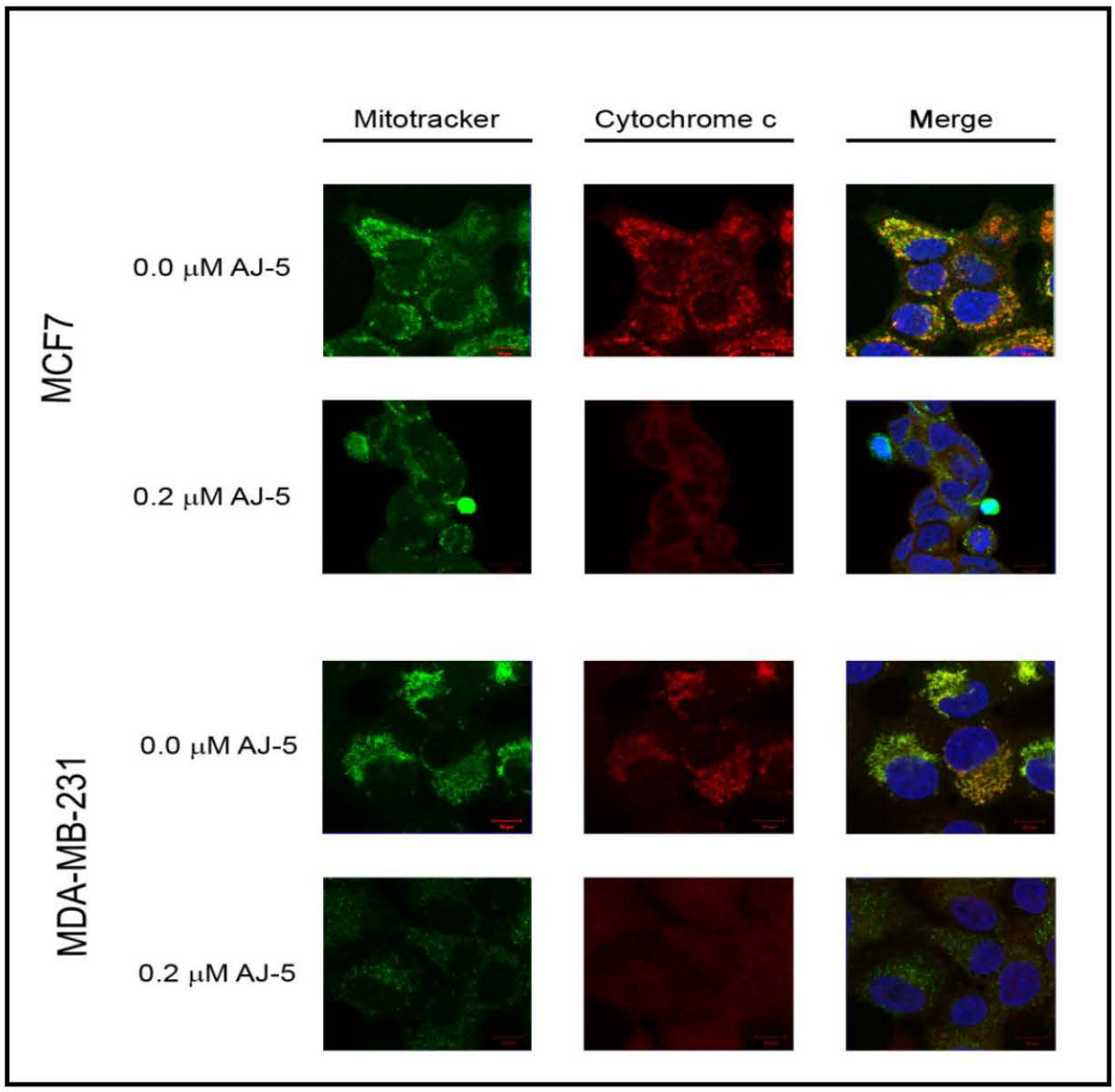


Figure 3.8 AJ-5 induces cytochrome c release from the mitochondria to the cytoplasm. MCF7 and MDA-MB-231 cells were grown on glass coverslips and treated with AJ-5 or vehicle for 24 hours and stained for cytochrome c (red fluorescence), mitochondria (mitotracker green; green fluorescence) and nuclei (Hoechst 33258; blue fluorescence). Images were captured under a confocal microscope (Zeiss, Germany) at 600x magnification and are representative of three randomly selected fields for each treatment (scale bars indicate 10 μm). In untreated cells cytochrome c was primarily localized in mitochondria as revealed by yellow-orange staining due to the merging of green (mitochondria) and red fluorescence (cytochrome c). AJ-5 treatment caused translocation of cytochrome c from mitochondria to the cytosol as evidenced by the appearance of red fluorescence in the cytoplasm.

3.2.5 AJ-5 induces autophagy and inhibits the mTOR pathway

Increasing evidence has shown that autophagy is activated in response to anti-cancer chemotherapies [119] and, compared to vehicle-treated cells **(Fig. 3.9a, b)**, large vacuoles were observed in breast cancer cells treated with AJ-5 for 24 hours **(Fig. 3.9c, d)**. These were confirmed by transmission electron microscopy to be autophagosomes and autolysosomes **(Fig. 3.10)**. Moreover AJ-5 treated cells displayed swollen mitochondria **(Fig. 3.10c- f, white arrows)**, a decrease in healthy intracellular organelles and increasing levels of organelles localized in autophagic vacuoles **(Fig. 3.10e- f, black arrows).**

While the accumulation of autophagosomes is one of the most important morphological features of autophagy, it is not always indicative of autophagy induction since blocking of autophagosomal maturation can also increase the amount of autophagosomes [275]. To therefore conclusively show that a chemotherapeutic agent is inducing autophagy one would need to monitor autophagic flux which can be demonstrated by LC3-II turnover using florescent microscopy and/or western blot analysis in the absence and presence of a lysosomal inhibitor such as bafilomycin A (Baf). If autophagic flux is occurring, LC3-II level will increase in the presence of the lysosomal inhibitor because the degradation of LC3-II will be blocked. To therefore confirm that AJ-5 induces autophagic flux, MCF7 and MDA-MB-231 breast cancer cells were transiently transfected with a GFP-LC3 expression vector and treated with AJ-5 in the presence or absence of Baf. Treatment with AJ-5 led to an increase in cytoplasmic vacuoles and high levels of GFP-LC3 puncta in both breast cancer cell lines and in the presence of Baf there

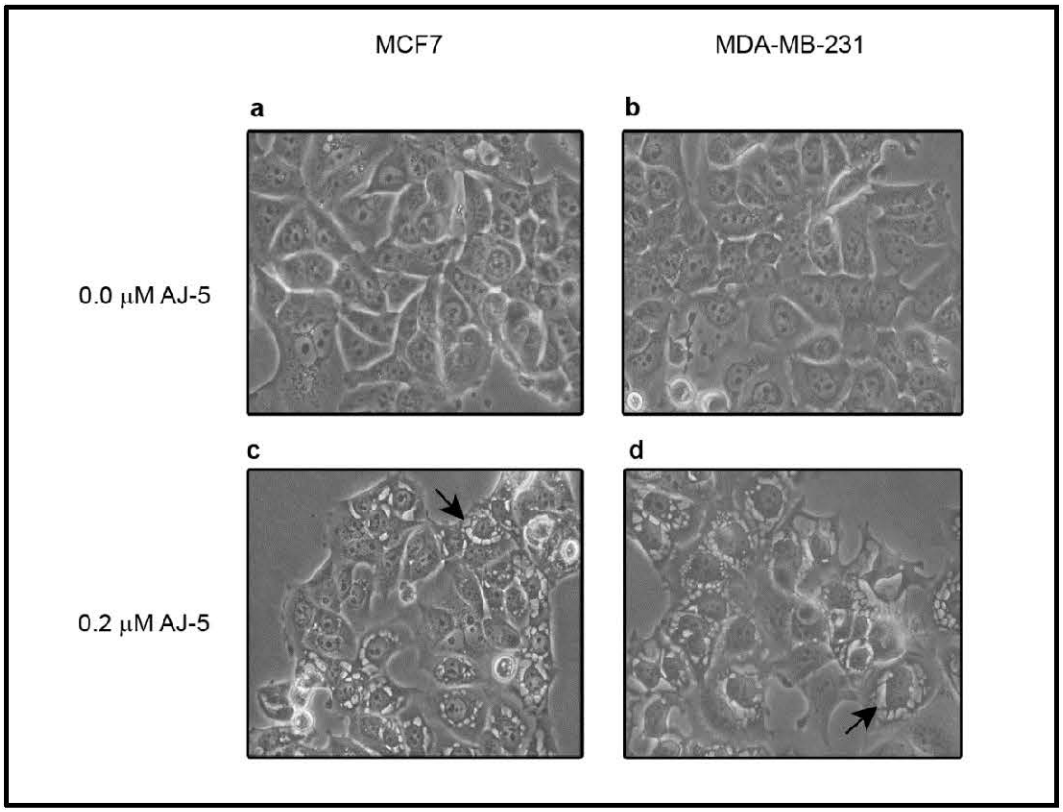


Figure 3.9 AJ-5 induces vacuoles in breast cancer cells. Representative phase-contrast photomicrographs (400x; Olympus 1X71) of MCF7 and MDA-MB-231 cells treated with vehicle **(a, b)** or 0.2 μM AJ-5 for 24 hours **(c, d)**.

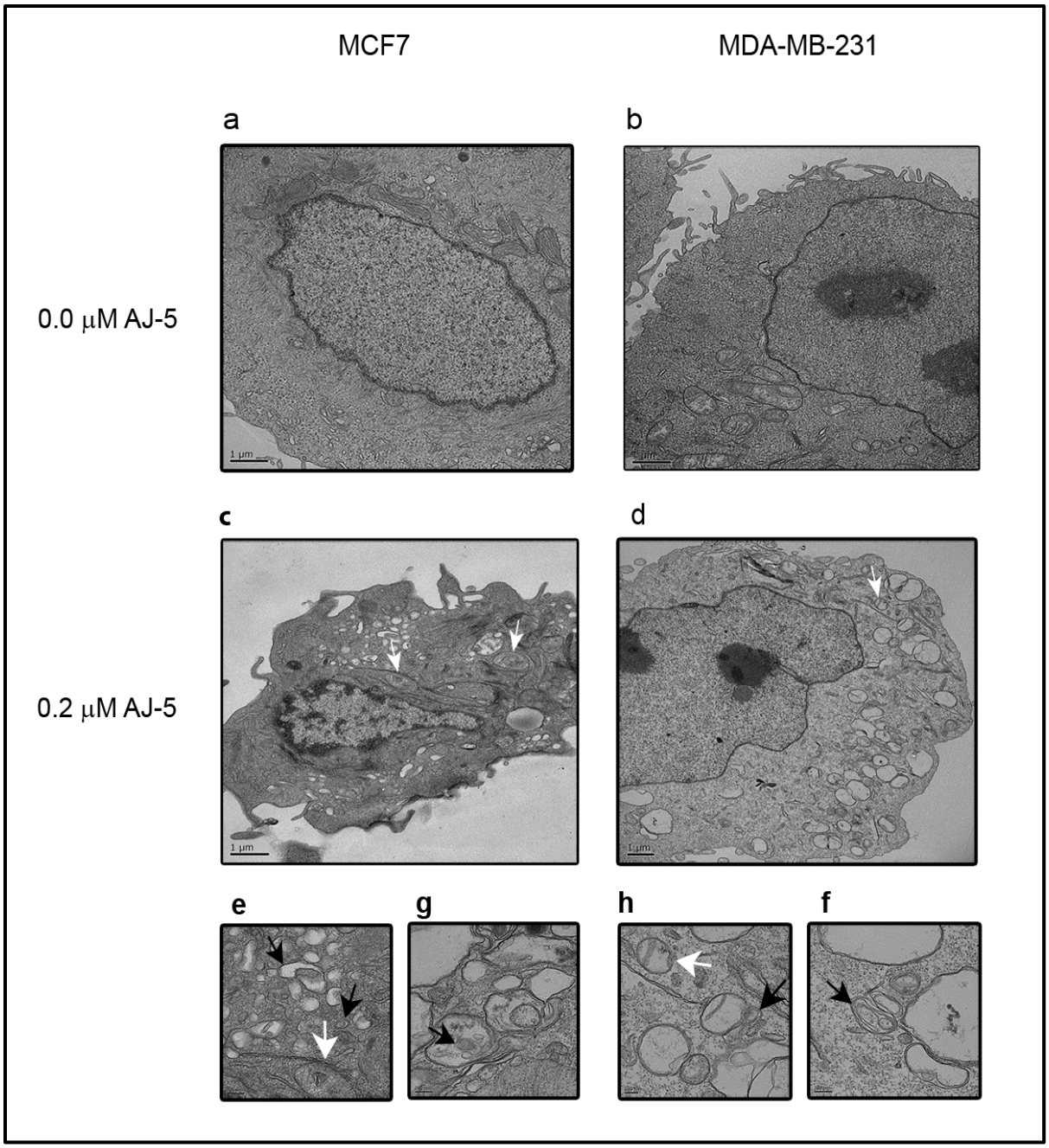


Figure 3.10 AJ-5 induces autophagy in breast cancer cells. (a-d) Representative transmission electron photomicrographs showing MCF7 and MDA-MB-231 cells treated as in fig 3.9 Higher magnifications of AJ-5 treated MCF7 **(e, g)** and MDA-MB-231 cells **(h, f)**. Black arrows in **e-f** indicate typical autophagosomes containing cytoplasmic inclusions; white arrows indicate swollen mitochondria.

was an accumulation of these puncta **(Fig. 3.11a)**. The induction of autophagic flux by AJ-5 was confirmed by western blotting for LC3II **(Fig. 3.11b)**.

Several molecular pathways have been implicated in the regulation of autophagy with the PI3K/mTOR pathway having a well-established role in preventing autophagy initiation [276, 277]. To determine whether AJ-5 induces autophagy by inhibiting this pathway, the effect of AJ-5 on levels of p-mTOR and its direct substrate p70 S6 kinase was assessed by western blotting. The results obtained show that AJ-5 severely inhibited levels of p-mTOR and p70 S6 kinase in the both breast cancer cell lines **(Fig. 3.12)** which was accompanied by a dramatic increase in markers of autophagy (LC3II). Importantly this correlated with an increase in cleaved PARP and cleaved Beclin1 (35/37kDa) which plays a role in releasing cytochrome c from the mitochondria to the cytoplasm and is therefore considered a pro-apoptotic factor [174, 278]. Together these results suggest that AJ-5 induced autophagy and apoptosis by a mechanism involving the inhibition of the mTOR pathway.

3.2.6 AJ-5 induced cytotoxicity involves autophagy

In light of the above data, it was next investigated whether the AJ-5 induced autophagy observed in this study is a cell death or cell survival mechanism. Results from MTT and Annexin V assays show that pharmacological inhibition of autophagy by wortmannin significantly reduced the cytotoxicity and the total cell death induced by AJ-5 **(Fig 3.13a, b)**. Furthermore when AJ-5 induced autophagy was inhibited using siRNA specific to LC3 or wortmannin the level

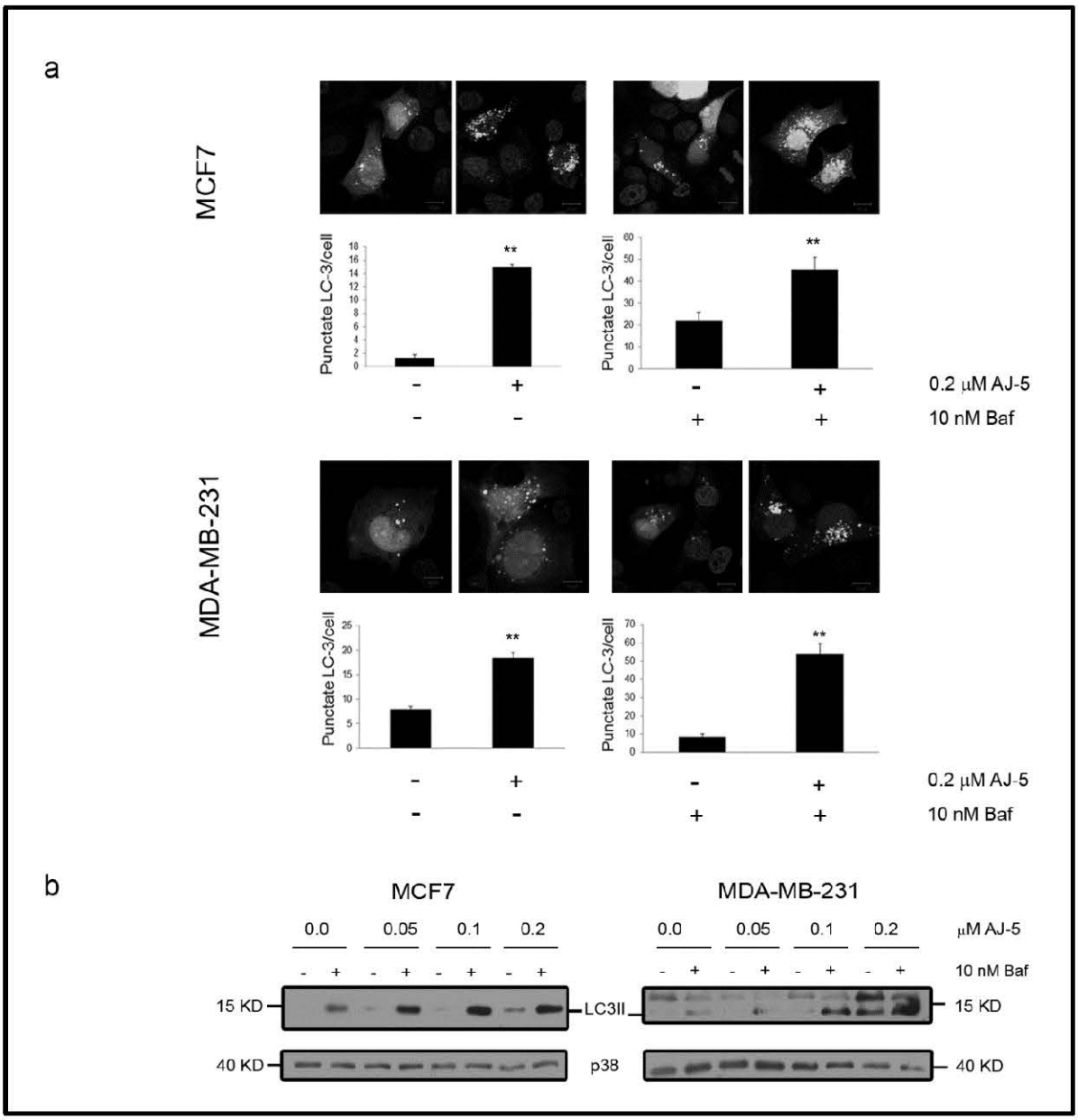


Figure 3.11 AJ-5 induces autophagy flux in breast cancer cells. (a) MCF7 and MDA-MB-231 cells were transiently transfected with a GFP-LC3 plasmid and treated with 0.2 μM AJ-5 for 24 hours in the presence or absence of bafilomycin A (Baf). Autophagy was quantified by counting the GFP-LC3 puncta at 400x magnification in twenty fields of view and divided by the total number of transfected cells within these fields. The number of GFP-LC3 puncta/cell are presented in the graphs as means ± SEM of three independent experiments (**P <0.001, student's t test). **(b)** Western blotting of protein from cells pre-treated with 10 nM Baf and treated with the indicated concentrations of AJ-5 for 24 hours. Blots were probed with antibodies to LC3II and p38 was used as a loading control.

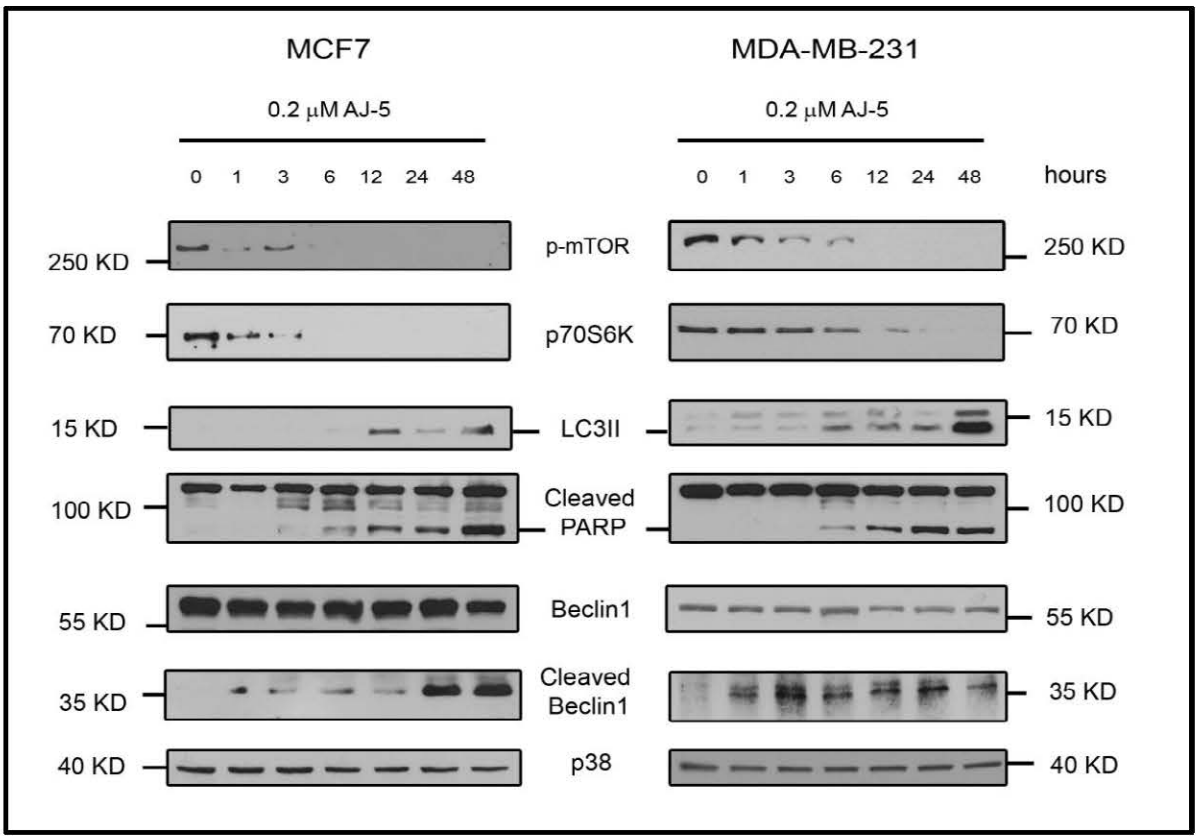


Figure 3.12 AJ-5 inhibits the mTOR pathway and activates concurrent apoptosis and autophagy. MCF7 and MDA-MB-231 cells were treated with either 0.2 μM AJ-5 or vehicle for 1- 48 hours. Protein extracts were harvested and analysed by SDS-PAGE (8-15%) and western blotting using antibodies to p-mTOR, p70S6K, LC3II, Beclin1 and PARP. p38 was used as a loading control.

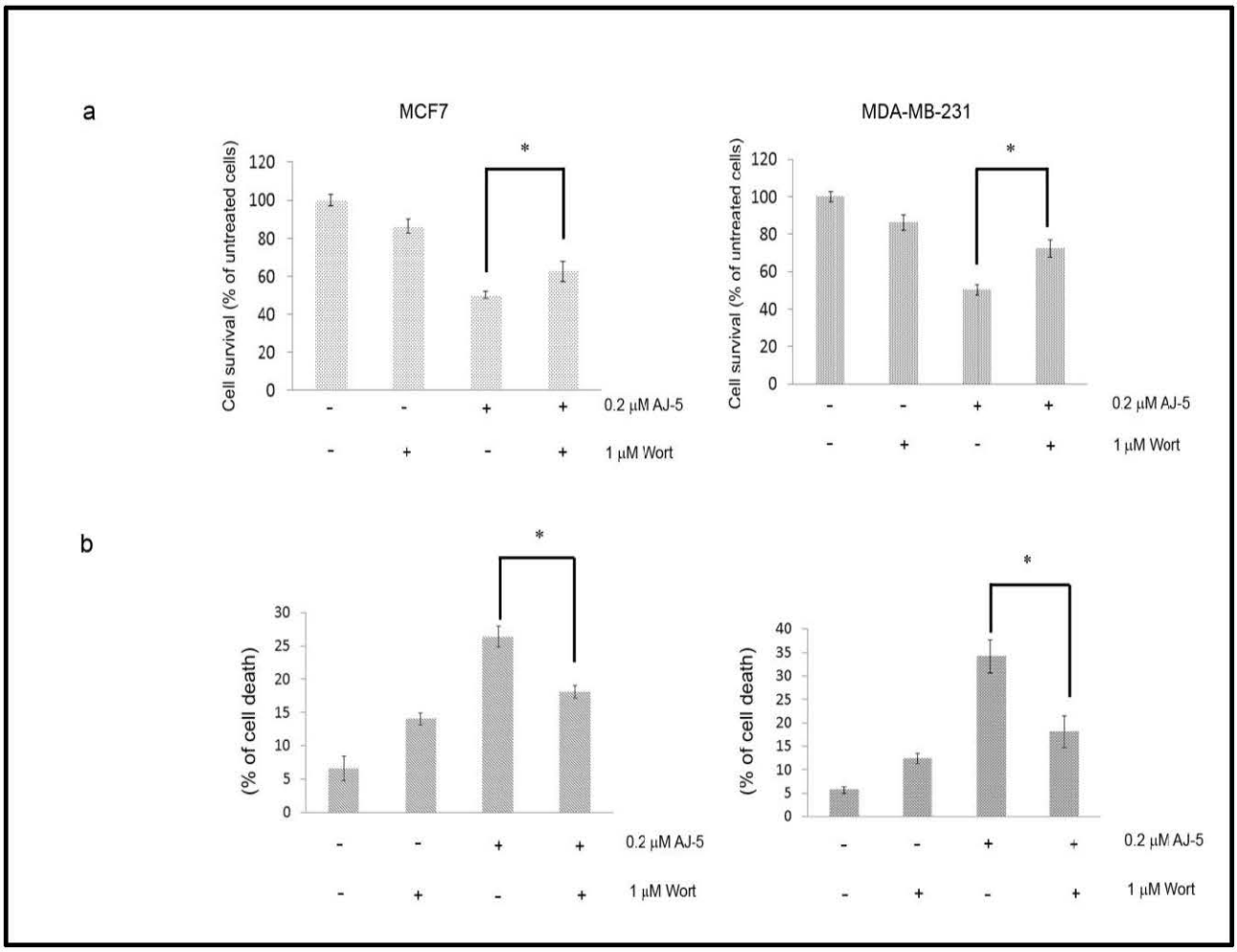


Figure 3.13 Inhibition of autophagy decreases the cytotoxic effect of AJ-5 in MCF7 and MDA-MB-231 cells. (a) MTT assay showing percentage of cell viability and **(b)** Annexin V/PI double staining assay showing percentage of AJ-5 induced cell death when autophagy was inhibited by 1 μM wortmannin (Wort) and the cells treated with 0.2 μM AJ-5 for 24 hours. A Microsoft Excel student's *t* test was performed to calculate statistical significance (*P <0.05).

of cleaved PARP decreased **(Fig 3.14a, b)**. These results suggest that AJ-5 induced autophagy favours apoptosis and that it contributes to cytotoxicity which are in line with previous studies showing that autophagy induced by paclitaxel and tamoxifen in MCF7 cells is a cell death mechanism [279–281].

3.2.7 The p38 MAPK pathway mediates AJ-5 cytotoxicity

The MAPK pathways are activated during autophagy and apoptosis [282, 283] and to determine whether these pathways play a role in mediating AJ-5 induced apoptosis and autophagy, the levels of p-p38, p-ERK1/2 and p-JNK1/2 were determined. In response to AJ-5 treatment, there was a robust and sustained increase in only p-p38 levels in both MCF7 and MDA-MB-231 cell lines **(Fig. 3.15a)** and inhibition of this pathway reduced cleaved LC3II and PARP levels **(Fig. 3.15b, c)**. Furthermore, MTT assays show that inhibition of p-p38 activity decreased AJ-5 cytotoxicity **(Fig. 3.15d)** which is consistent with reports indicating that the p38 MAPK pathway mediates cell death induced by a number of chemotherapeutic compounds [44, 283].

The above results show that the palladacycle AJ-5 has a strong cytotoxic effect against breast cancer stem cells and that it displays selective anti-cancer activity in ER-positive and ER-negative breast cancer cells by activating both apoptosis and autophagy. At a molecular level, AJ-5 is shown to function by inducing DNA damage and stimulating the p38 MAPK pathway which mediates its impact on apoptosis and autophagy. Together this provides compelling evidence to suggest that AJ-5 may be an effective chemotherapeutic drug in the treatment of breast cancer.

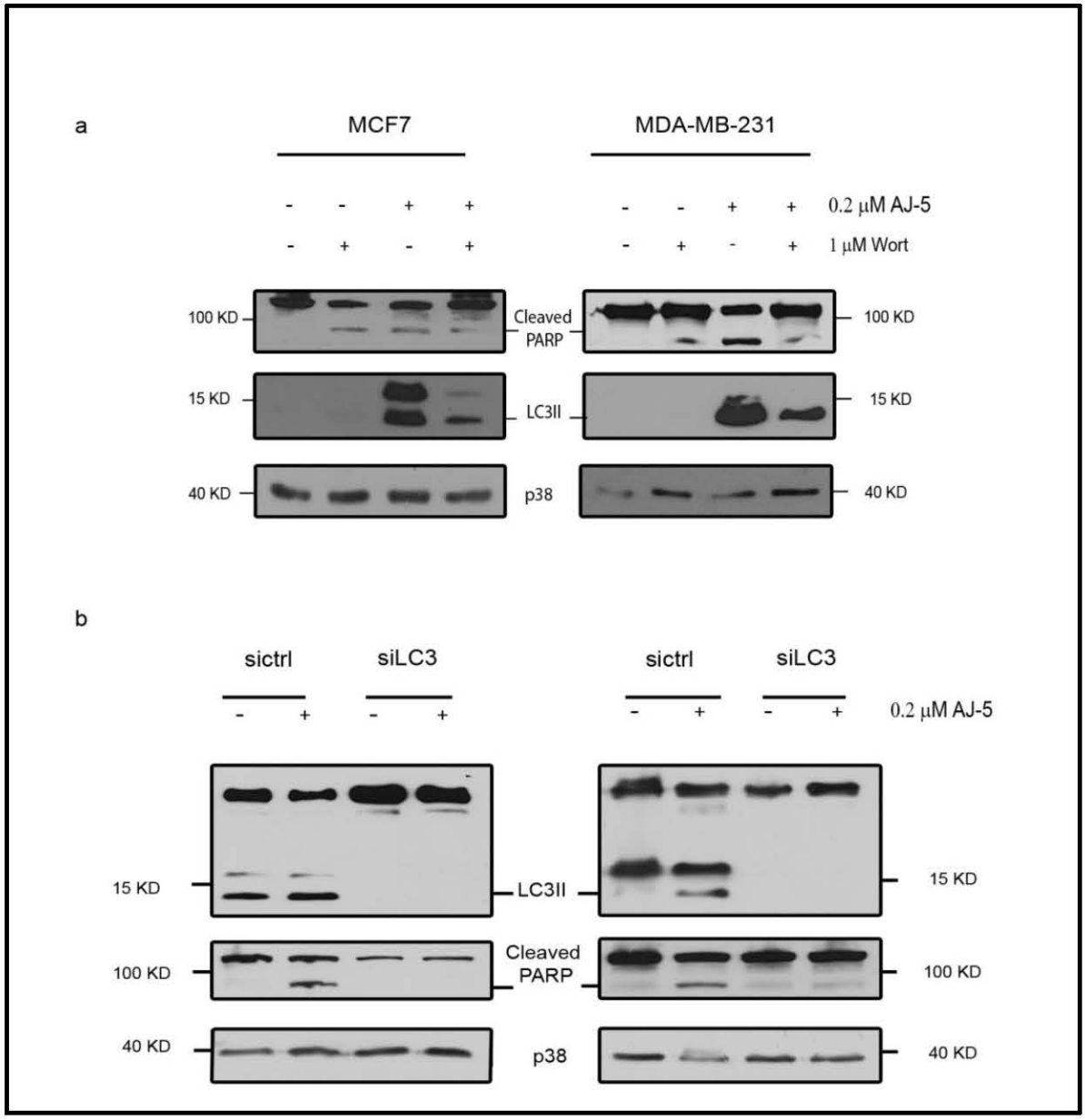


Figure 3.14 Inhibition of autophagy reduces AJ-5 induced apoptosis. (a) Representative western blots showing levels of cleaved PARP and LC3II in MCF7 and MDA-MB-231 cells pre-treated with 1 μM wortmannin (Wort) and treated with AJ-5 or vehicle for 24 hours. **(b)** Cells transfected with non-silencing siRNA (sictrl) or LC3 specific siRNA (siLC3) and treated with AJ-5 for 24 hours. Protein extracts were harvested and analysed by SDS-PAGE (8-15%) and western blotting using antibodies to LC3II expression and PARP. p38 was used as a loading control.

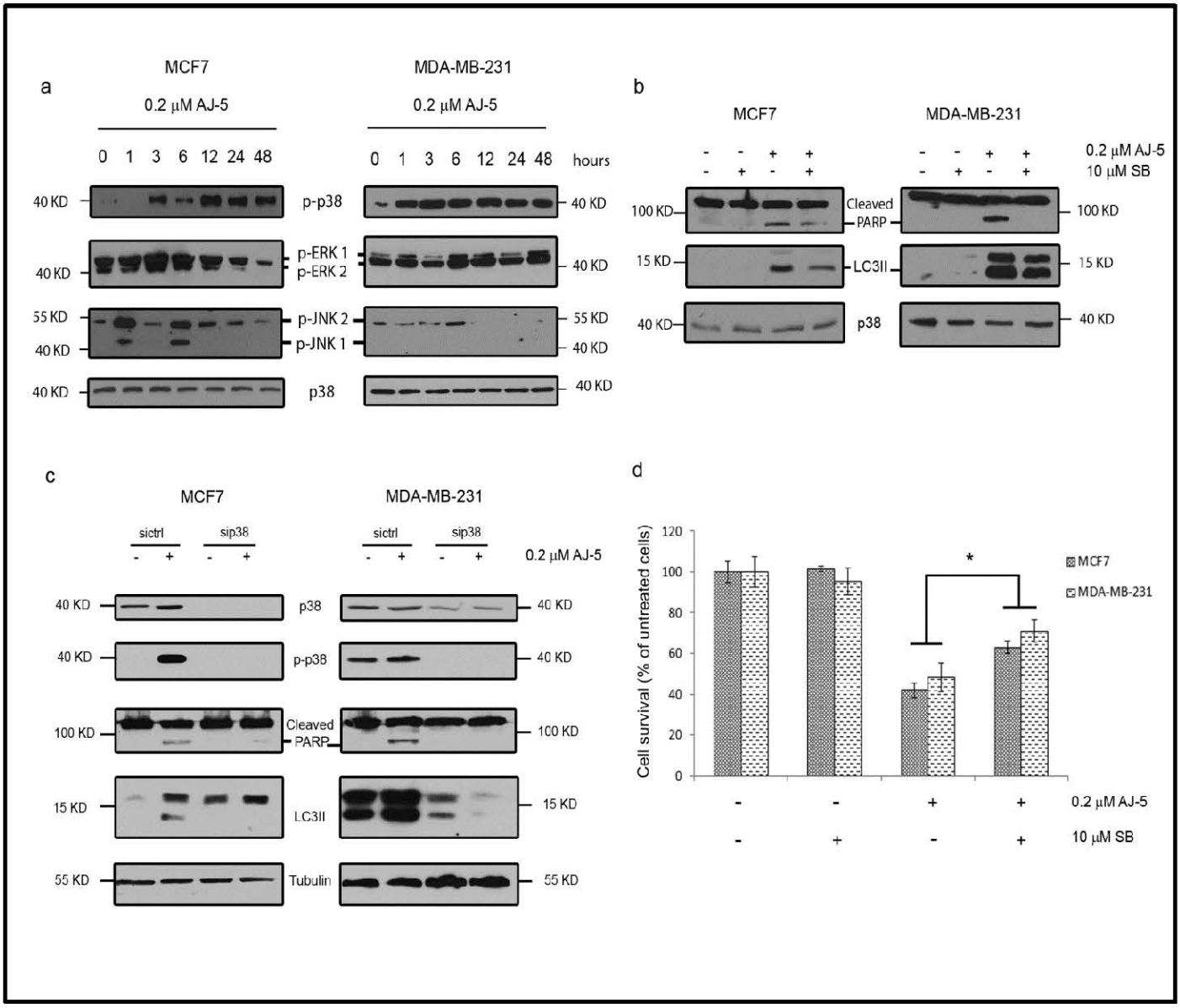


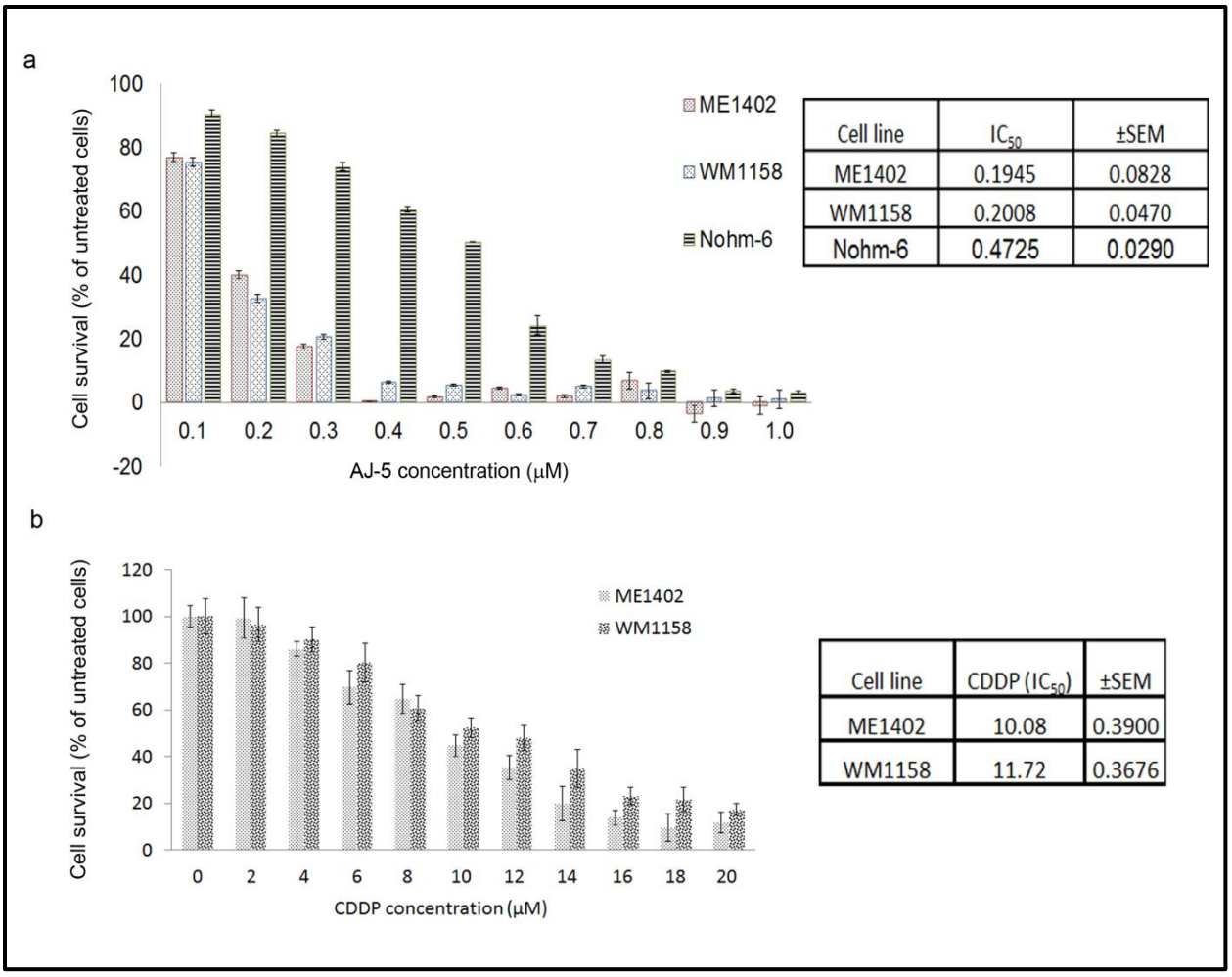
Figure 3.15 p38 MAPK mediates AJ-5 induced autophagy and apoptosis in breast cancer cells. (a) Cells treated with vehicle or 0.2 μM AJ-5 for up to 48 hours and protein extracts were harvested and analysed by SDS-PAGE (8%) and western blotting using antibodies to p.p38, p-RKE1/2 and p-JNK1/2. p38 was used as a loading control. **(b, c)** Representative western blots of p38, p-p38, PARP and LC3II in breast cancer cells treated with AJ-5 or vehicle for 24 hours **(b)** pre-treatment with the p38 inhibitor SB203580 (10 μM) and **(c)** transfection with non-silencing siRNA (sictrl) or p38-specific siRNA (sip38). Tubulin and p38 were used as loading controls. **(d)** Pharmacological inhibition of p38 reduces AJ-5 cytotoxicity. MTT assays for breast cancer cells pre-treated with the p38 inhibitor SB203580 (10 μM) and followed by treatment with 0.2 μM AJ-5 for 24 hours (*P <0.05, student's t test).

3.3 AJ-5 inhibits melanoma growth in vitro and in vivo through apoptosis and autophagy

In light of the data showing that AJ-5 is cytotoxic in an advanced melanoma cell line at low concentrations **(Fig. 3.1a)** as well as the above data showing that AJ-5 holds great promise as an anti-cancer drug the following section of this thesis investigates the anti-tumour activity of AJ-5 in the vertical growth phase (ME1402) and metastatic (WM1158) melanoma cell lines in vitro and in vivo.

3.3.1 AJ-5 exerts potent anti-proliferative activities against human melanoma cells

To investigate the cytotoxic effect of AJ-5 on melanoma cells, the ME1402 VGP and the WM1158 were treated with a range of AJ-5 (0 to 1.0 µM) for 48 hours and the MTT assay was used to determine cell viability. As controls, normal human melanocytes (Nohm-6) [245] were included in these experiments. The concentration that inhibits cell growth by 50% (IC50) was calculated and concentrations of 0.194, 0.200, and 0.472 µM AJ-5 were obtained in ME1402, WM1158 and Nohm-6 cell lines, respectively **(Fig. 3.16a)**. These results indicate that the melanoma cell lines were more sensitive to AJ-5 than the normal human melanocytes. Importantly, while 0.4 µM AJ-5 killed more than 90% of melanoma cells, more than 70% of normal cells continued to survive at this concentration and 13.3% were still viable at 0.7 µM AJ-5 **(Fig. 3.16a)**. Importantly, while cisplatin (CDDP) exerted a dose-dependent cytotoxic effect on ME1402 and WM1158 melanoma cell lines, as much as 10 µM CDDP was required to kill 57.97% and 48.52% of the cells, respectively **(Fig. 3.16b)**. These results show that AJ-5 displayed potent cytotoxicity at much lower concentrations than CDDP.



Cell line	IC_{50}	±SEM
ME1402	0.1945	0.0828
WM1158	0.2008	0.0470
Nohm-6	0.4725	0.0290

Cell line	CDDP (IC_{50})	±SEM
ME1402	10.08	0.3900
WM1158	11.72	0.3676

Figure 3.16 AJ-5 induces a potent cytotoxic effect on melanoma cell lines. The indicated cell lines were plated in 96-well plates and after 48 hours the cells were treated with increasing concentrations of AJ-5 (0-1.0 μM) in **(a)** or CDDP (0-20 μM) in **(b).** Cell viability was assessed by the methylthiazoltetrazolium (MTT) assay after 48 hours of treatment. The graphs represent the mean percentage ± SEM of surviving treated cells relative to control cells of at least three independent experiments performed in quadruplicate. The tables show the concentrations of AJ-5 in **(a)** or CDDP in **(b)** required for killing 50% of the cells (IC_{50}) which was calculated from sigmoidal plots with GraphPad Prism version 5.

3.3.2 AJ-5 induces S and G2/M cell cycle arrests

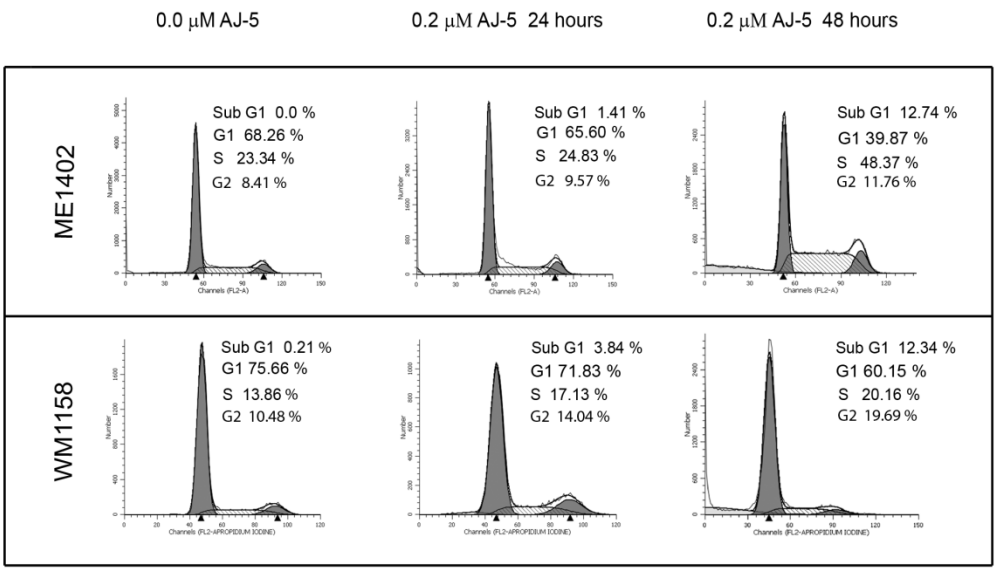
To identify the mechanism by which AJ-5 induces cytotoxicity in melanoma cells, the effect of AJ-5 on cell cycle progression was first analysed by treating ME1402 and WM1158 cells with 0.2 µM AJ-5 for 24 and 48 hours and subjecting the cells to flow cytometry. In both cell lines, AJ-5 induced significant S and G2/M cell cycle arrests as well as sub-G1 peaks which were particularly evident after 48 hours **(Fig. 3.17a, b)**.

3.3.3 AJ-5 induces intrinsic and extrinsic apoptosis in melanoma cells

To assess whether AJ-5 induced cell death by apoptosis, melanoma cells were treated as above and stained with propidium iodide (PI) and Annexin V-FITC for flow cytometry. The results correlated well with the appearance and size of the sub-G1 peaks observed in **figure 3.18** with more WM1158 cells undergoing apoptosis compared with the ME1402 cells **(Fig. 3.18a)**. Indeed, 24 and 48 hours of AJ-5 treatment led to 21.43% and 61.17% of WM1158 cells undergoing apoptosis respectively and 15.71% and 35.58% respectively for ME1402 cells. Furthermore, when the cells were stained with Hoechst 33342 and visualized by fluorescence microscopy AJ-5 treated ME1402 and WM1158 cells displayed fragmented chromatin typical of apoptotic cells **(Fig. 3.18b)** [284].

To confirm that AJ-5 induced apoptosis at a molecular level western blotting with an antibody to PARP was performed and the results show that PARP cleavage increases in ME1402 and WM1158 cell lines at both times of AJ-5 treatment **(Fig. 3.19)**. While CDDP treatment induced PARP cleavage in both melanoma cell lines, the level of cleaved PARP was more obvious in AJ-5

a



b

Cell Line	Cell cycle phase	0.0 μM AJ-5	0.2 μM AJ-5 (24 hours)	0.2 μM AJ-5 (48 hours)
ME1402	Sub G1 ± SEM	0.03 ± 0.02	1.10 ± 0.04	11.90 ± 1.152
	G1 ± SEM	70.03 ± 2.50	66.69 ± 1.54	34.96 ± 3.21
	S ± SEM	21.50 ± 2.59	24.35 ± 0.67	44.62 ± 1.06
	G2/M ± SEM	9.03 ± 0.87	9.235 ± 0.47	11.10 ± 0.92
WM1158	Sub G1 ± SEM	0.47 ± 0.59	2.81 ± 0.34	11.99 ± 1.48
	G1 ± SEM	77.09 ± 2.02	70.33 ± 2.12	49.07 ± 2.94
	S ± SEM	13.11 ± 1.05	16.12 ± 1.42	22.16 ± 4.02
	G2/M ± SEM	9.59 ± 1.25	13.54 ± 1.70	17.27 ± 3.41

Figure 3.17 AJ-5 induces sub-G1 peak and S and G2/M cell cycle arrest. (a) Representative results of the cell cycle distribution of ME1402 and WM1158 cells exposed to vehicle or AJ-5 (0.2 μM) for 24 and 48 hours was determined by staining cells with propidium iodide and measuring their DNA content by flow cytometry. The proportion of cells at each phase of the cell cycle was expressed as a percentage of the total number of cells analysed. **(b)** The table shows pooled results from three independent experiments ± SEM.

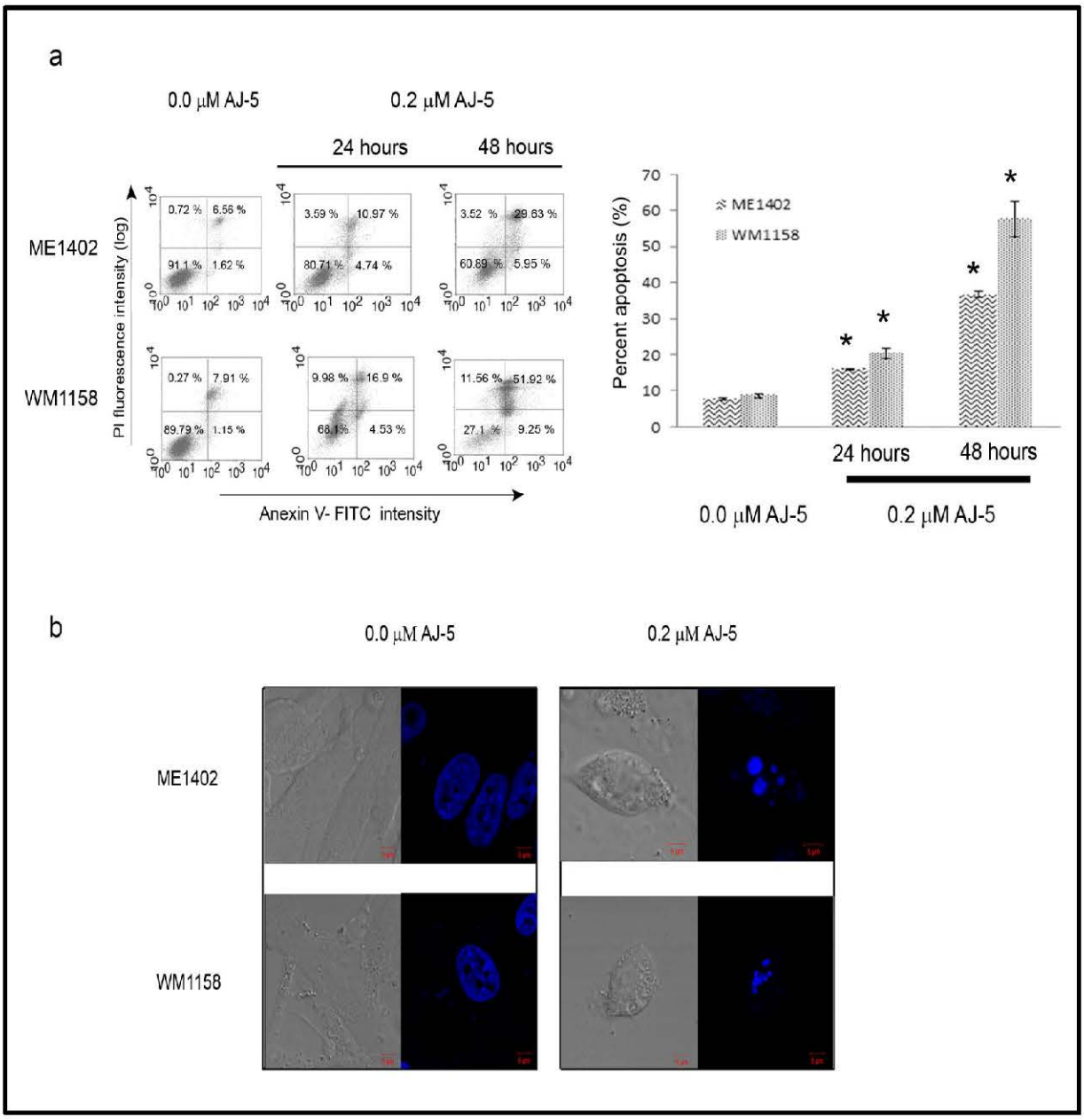


Figure 3.18 AJ-5 induces apoptosis in melanoma cells. (a) Flow cytometric analyses of Annexin V–FITC/propidium iodide (PI) staining show the induction of apoptosis in melanoma cells treated with 0.2 μM AJ-5 for 24 and 48 hours. The graph represents the percentage of cells undergoing apoptosis (early and late) and the results are presented as the mean ± SEM versus control of three independent experiments. A Microsoft Excel student's *t* test was performed to calculate statistical significance (**P* < 0.05; ***P* < 0.01). **(b)** Fluorescence microscopy showing that when ME1402 and WM1158 cells were treated with 0.2 μM AJ-5 for 24 hours and stained with Hoechst 33342 they display fragmented chromatin typical of apoptotic cells. Images were captured under a confocal microscope (Zeiss, Germany) at 600x magnification and are representative of three randomly selected fields for each treatment (scale bars indicate 5 μm).

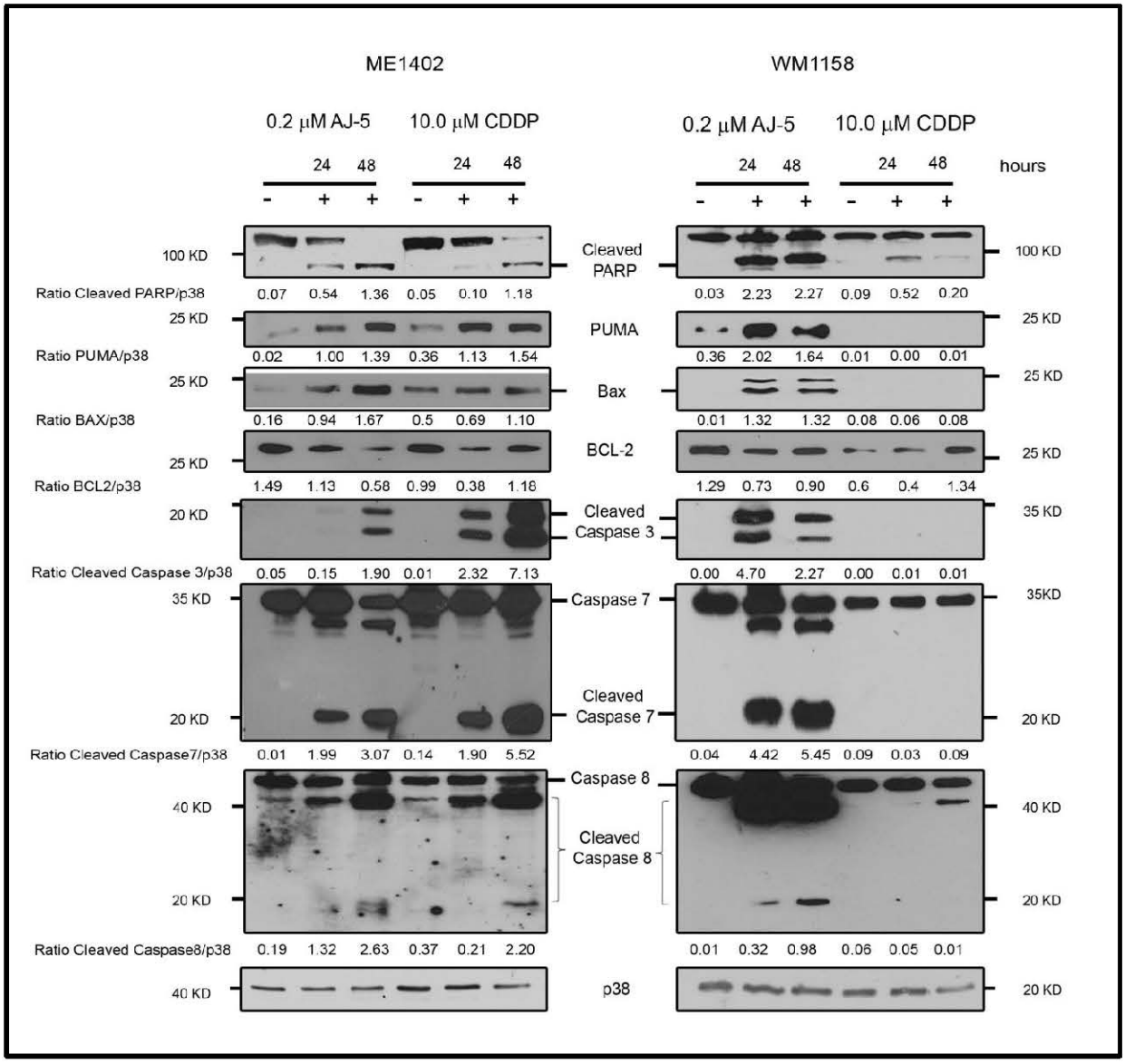


Figure 3.19 AJ-5 induces molecular markers of apoptosis in melanoma cells. ME1402 and WM1158 cells were treated with 0.2 μM AJ-5 or 10 μM CDDP for 24 and 48 hours. Protein extracts harvested from the indicated cells were analysed by SDS-PAGE (8-15%) and western blotting using antibodies to cleaved and total PARP, PUMA, Bax, BCL-2, active caspase 3, caspase 7 and caspase 8. p38 was detected as loading control and the numbers below each western indicate the ratio/p38.

treated cells. To investigate whether AJ-5 induced intrinsic and/or extrinsic apoptosis the levels of key proteins involved in these pathways were determined **(Fig. 3.19)**. The results demonstrate that following AJ-5 treatment of both ME1402 and WM1158 cells, the levels of intrinsic pro-apoptotic PUMA and Bax increased while the anti-apoptotic protein BCL-2 decreased. Furthermore, AJ-5 treatment of both melanoma cell lines activated caspase 3, caspase 7 and the extrinsic caspase 8 as measured by cleaved levels of these proteins. Although similar results were obtained for CDDP treatment of ME1402 cells, in WM1158 cells CDDP only slightly increased levels of the cleaved caspase 8 product 43/41 kDa and it had no impact on proteins involved in the intrinsic apoptotic pathway.

Taken together these results showed that AJ-5 induces the intrinsic and extrinsic apoptotic pathways more efficiently than CDDP in both melanoma cell lines.

3.3.4 AJ-5 also induces autophagy in melanoma cells

In the course of this study, large vacuoles, reminiscent of autophagic vacuoles, was observed in AJ-5 treated ME1402 and WM1158 cells **(Fig. 3.20 black arrow heads in c, d)**. To confirm this, transmission electron microscopy was performed and results **(Fig. 3.21)** show that while cells treated with vehicle had normal mitochondria and very few vacuoles **(Fig. 3.21a, b)**, AJ-5 treated cells showed high levels of vacuolization, a decrease in healthy intracellular organelles and swollen mitochondria **(Fig. 3.21, white arrows in c-f)**. Moreover organelles localized both within double- (AVI) and single- (AVII) membrane autophagy vacuoles in AJ-5 treated cells were observed **(Fig. 3.21e-f, black arrows)**.

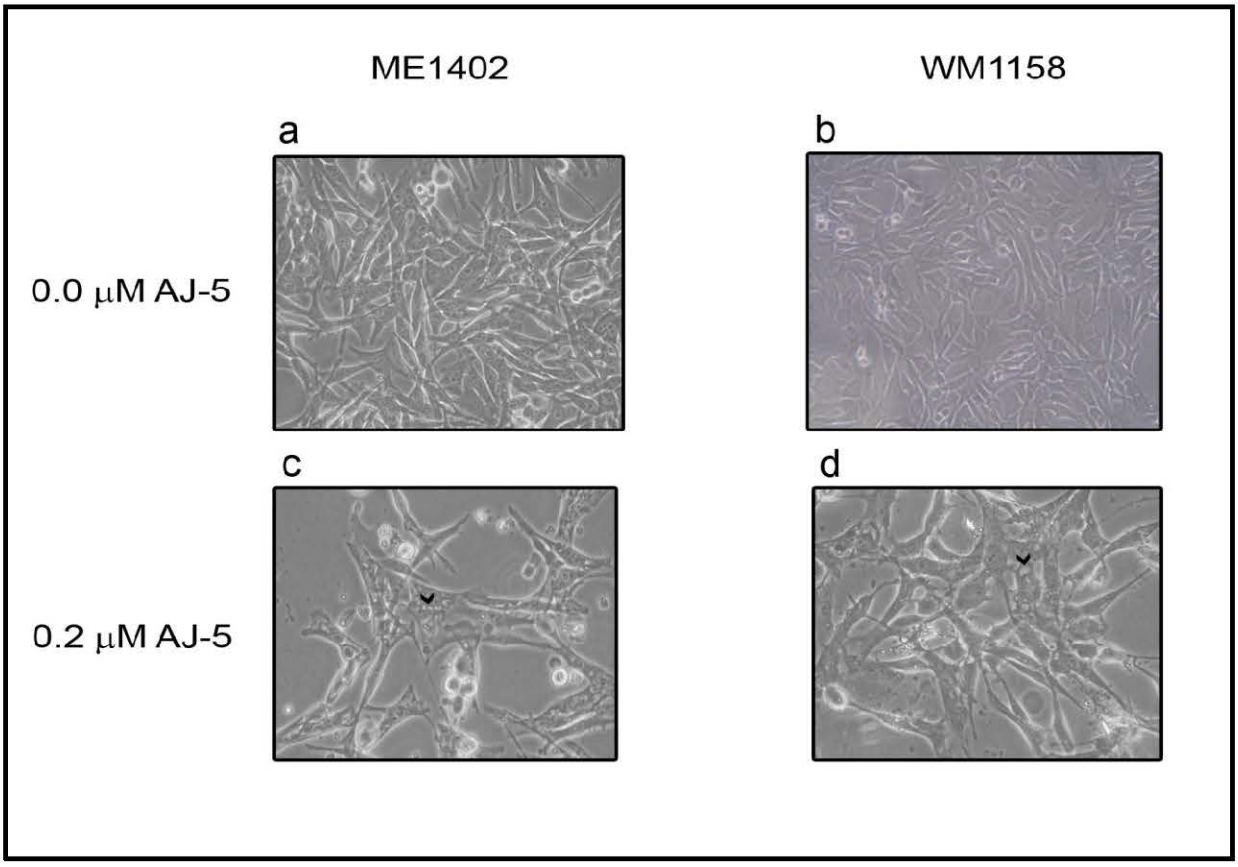


Figure 3.20 AJ-5 induces high levels of vacuolization in melanoma cells. Representative phase-contrast photomicrographs (400x; Olympus 1X71) showing ME1402 and WM1158 cells treated with vehicle **(a, b)** or 0.2 μM AJ-5 for 24 hours **(c, d)**. Black arrow heads indicate vacuoles in AJ-5 treated cells.

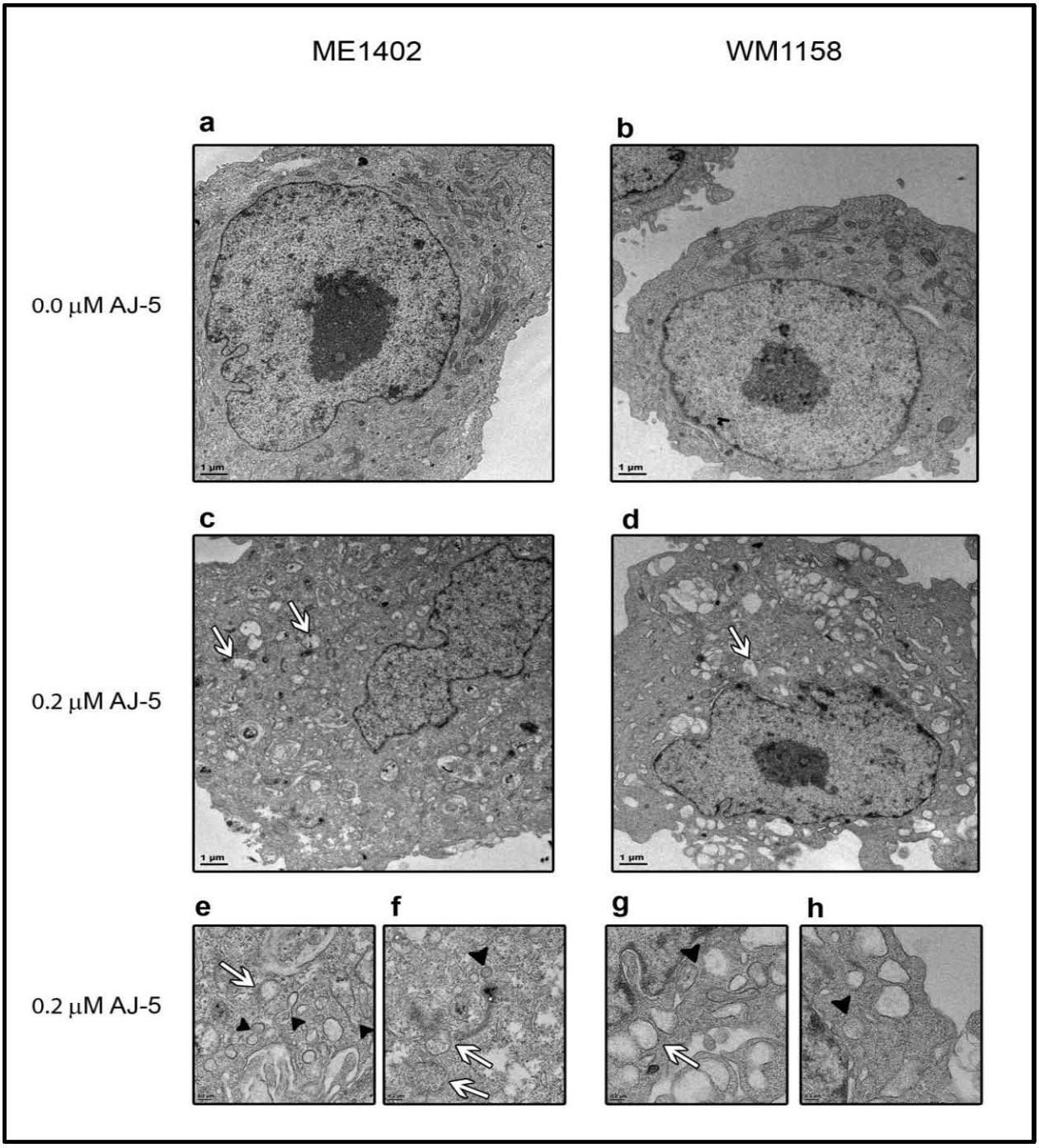


Figure 3.21 AJ-5 induces autophagosomes formation in melanoma cells. Representative transmission electron photomicrographs of ME1402 and WM1158 cells treated with vehicle **(a, b)** or 0.2 μM AJ-5 for 24 hours **(c, d)**. Higher magnifications of AJ-5 treated ME1402 **(e, f)** and WM1158 **(g, h)** cells. Black arrow heads indicate typical autophagosomes containing cytoplasmic inclusions; white arrows indicate a swollen mitochondrion.

To test whether AJ-5 induces autophagic flux in melanomas, the ME1402 and WM1158 cells were transiently transfected with a GFP-LC3 expression vector and autophagosome formation monitored by confocal microscopy as previously described for breast cancer cells. Indeed, AJ-5 treatment led to an increase of GFP-LC3 puncta from basal levels of 2.1 to 14.5% in ME1402 cells and 5.0 to 23.5% in WM1158 cells **(Fig. 3.22)**. Furthermore, co-treatment of the cells with AJ-5 and the lysosomal inhibitor, bafilomycin A (Baf) resulted in additional accumulation of GFP-LC3 puncta **(Fig. 3.22)** and LC3II protein levels **(Fig. 3.23a)**. These results show that AJ-5 induces autophagic flux in the melanoma cell lines tested in this study, and this was further confirmed using wortmannin which inhibits the formation of LC3II and the early stages of autophagy processes **(Fig. 3.23b)**.

3.3.5 AJ-5 regulates proteins involved in the DNA damage response and cell cycle progression through p53 independent manner

As seen in breast cancer cell lines above, immunoblotting performed with antibodies to γ-H2AX, p-ATM(Ser1981) and p-CHk2(Thr68) revealed that AJ-5 induced a DNA damage response in both ME1402 and WM1158 cells **(Fig. 3.24)**. Furthermore, consistent with reports for other chemotherapeutic agents, AJ-5 treatment led to a decrease in levels of cyclin A and cyclin B which are required for S and G2/M progression [285, 286]. Interestingly, in both melanoma cell lines tested, the results obtained for p53 levels were not reproducible. While, as shown in **Figure 3.24**, p53 levels sometimes increased in response to AJ-5 treatment, in other experiments there was either no change in p53 levels or p53 levels went down. The levels of p21 however consistently increased in AJ-5 treated cells suggesting that p21 may be regulated

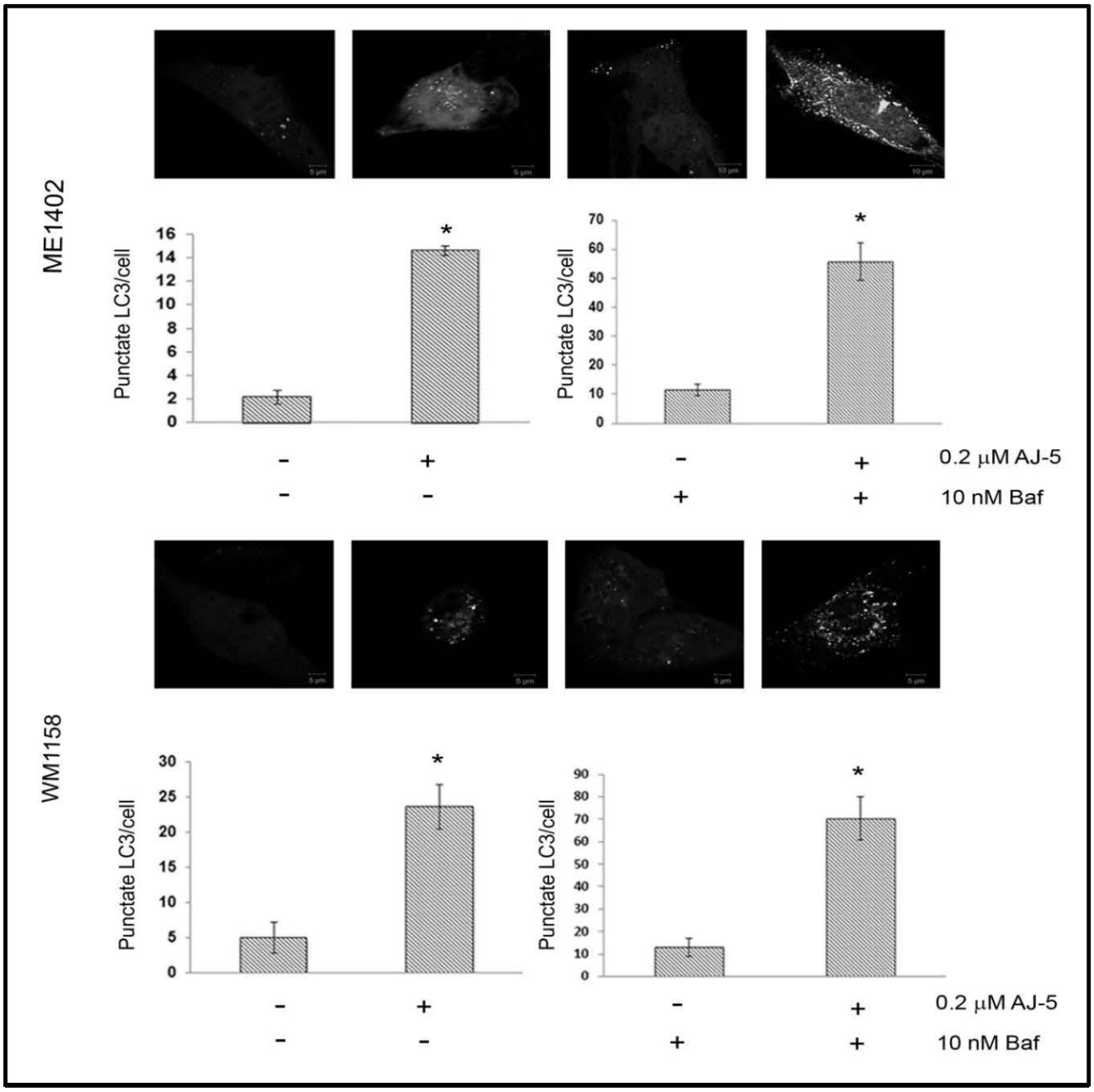


Figure 3.22 AJ-5 induces continuous autophagy flux in ME1402 and WM1158 cells. Cells were transiently transfected with a GFP-LC3 plasmid and then treated with vehicle or 0.2 μM AJ-5 for 24 hours with or without bafilomycin A (Baf). Quantitation of autophagy was done by counting the GFP-LC3 puncta at 400x magnification in twenty fields of view and divided by the total number of transfected cells within these fields. The number of GFP-LC3 puncta/cell are presented in the graphs as means ± SEM of three independent experiments (**P <0.001, student's t test). Images were captured under a confocal microscope (Zeiss, Germany) at 600x magnification and are representative of three randomly selected fields for each treatment.

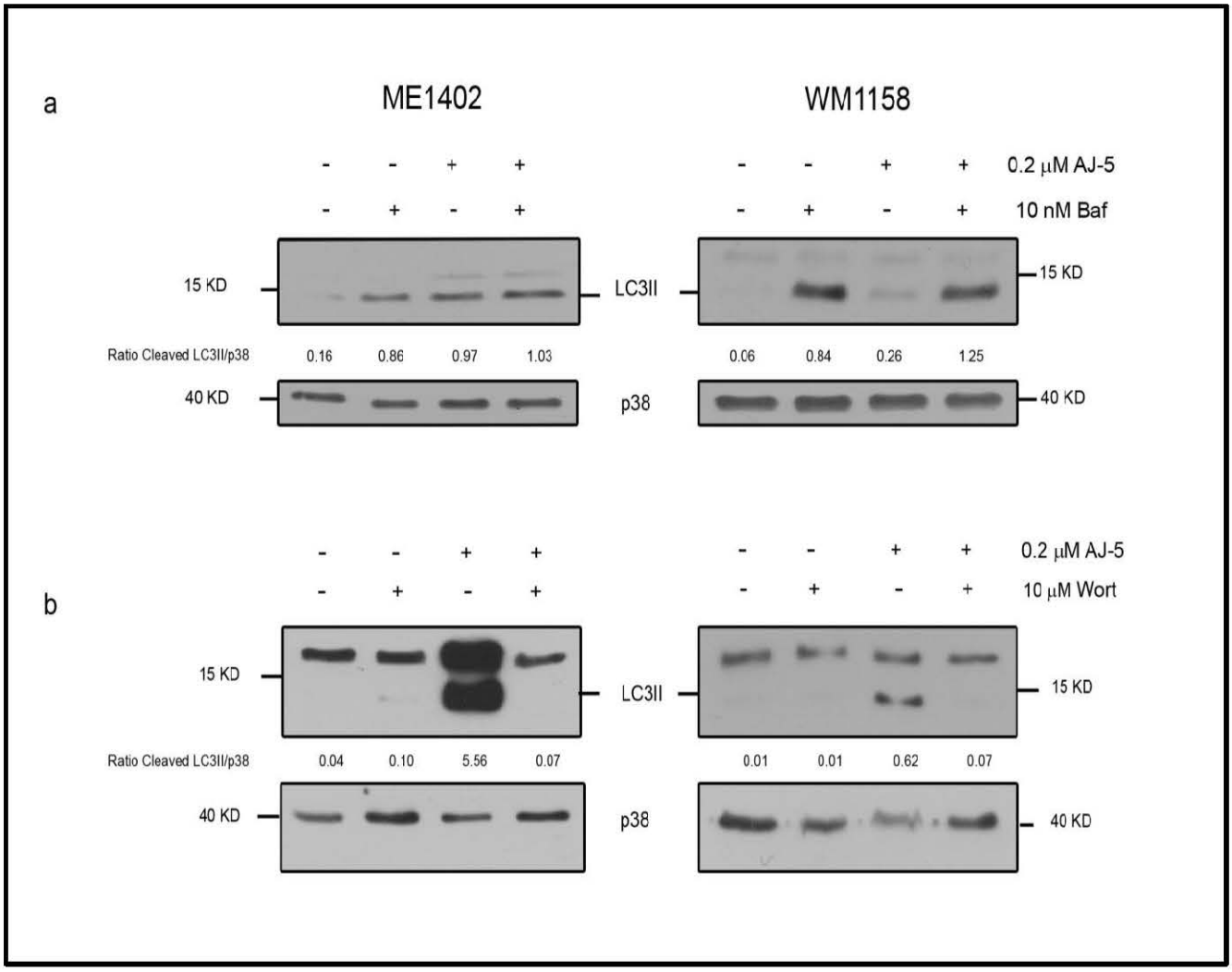


Figure 3.23 Confirmation of AJ-5- induced autophagy flux. ME1402 and WM1158 cells pre-treated with 10 nM bafilomycin A (Baf) **(a)** or 10 μM wortmannin (Wort) **(b)** and treated with vehicle or 0.2 μM AJ-5 for 24 hours. Protein extracts were harvested and analysed on 8-15% SDS-PAGE and by western blotting using antibodies to LC3II protein. p38 was used as a loading control and the numbers below each western indicate the ratio/p38.

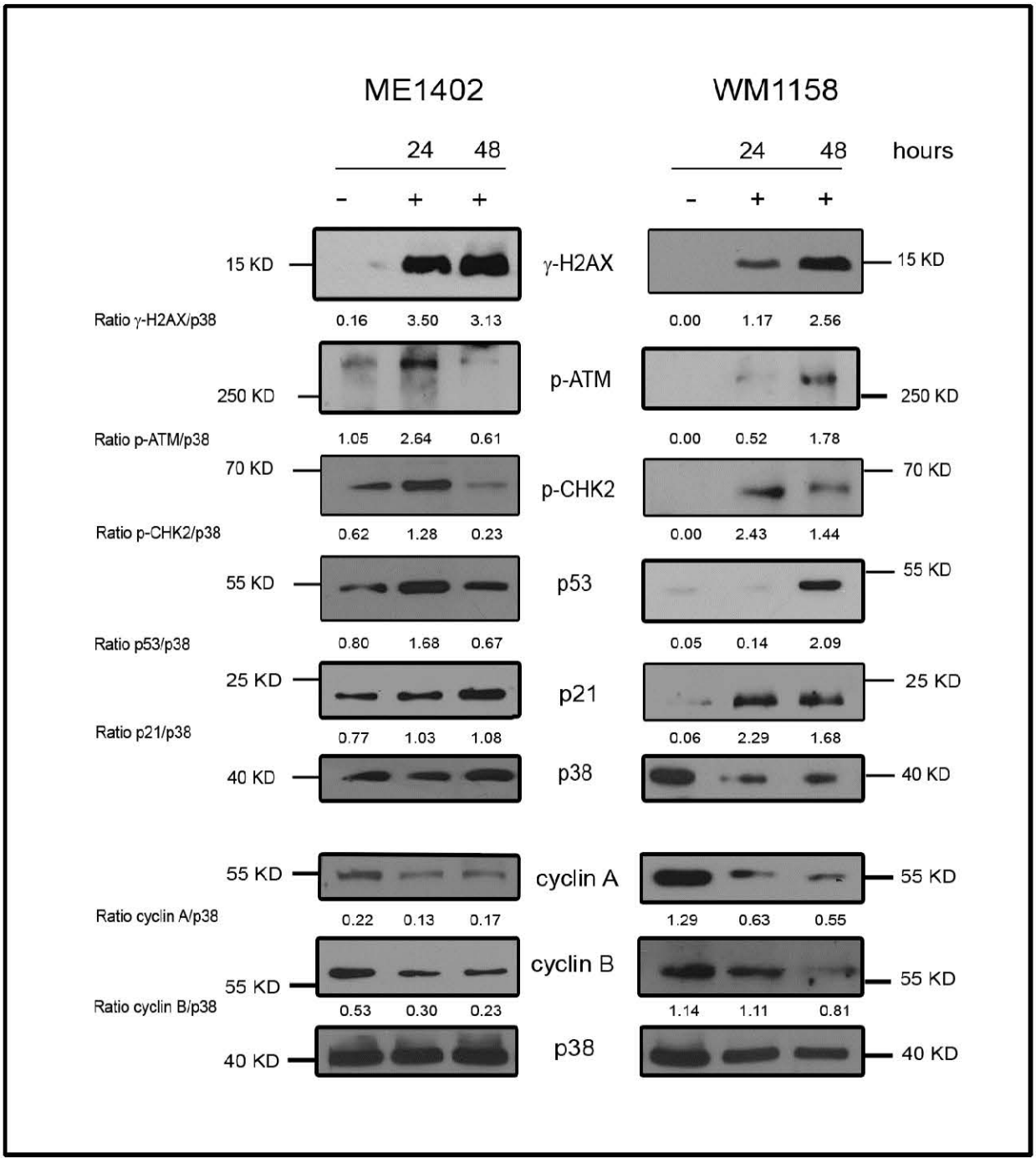


Figure 3.24 AJ-5 regulates DNA damage and cell cycle proteins. ME1402 and WM1158 cells were treated with vehicle or 0.2 μM AJ-5 for 24 and 48 hours. Protein extracts analysed by SDS-PAGE (6-15%) and western blotting using antibodies to γ-H2AX, p-ATM, p-CHK2, p53, p21, cyclin A and cyclin B show that AJ-5 treatment induces markers of DNA damage and important cell cycle regulators. p38 was used as a loading control and the numbers below each western indicate the ratio/p38.

in a p53-independent manner in these cells **(Fig. 3.24)**. This is consistent with increasing evidence that the transcriptional activity of p53 is compromised in many melanomas [287–289] and implies that AJ-5 induced its cytotoxicity in ME1402 and WM1158 cells independent of p53. To investigate this ME1402 and WM1158 cells were transiently transfected with siRNA specific to p53 (sip53) or a non-specific siRNA control (sictrl), the cells treated with AJ-5 for 24 hours and markers of cell cycle arrest, apoptosis and autophagy analysed by western blotting. **Figure 3.25** indeed shows that there was a robust p21 response to AJ-5 treatment in both melanoma cell lines which was independent of p53. Furthermore this corresponded with an increase in PARP cleavage and LC3II levels which confirms that AJ-5-induced cytotoxicity is p53-independent.

3.3.6 AJ-5 induced cytotoxic effect is mediated by the activation of p38 and ERK1/2 MAPK pathways

The MAPK pathway plays an important role in the DNA damage response and in the activation of autophagy and apoptosis [49, 290] and therefore the possibility that it may mediate the downstream effects of AJ-5 was considered. To determine this, the effect of AJ-5 on levels of p-p38, p-ERK1/2 and p-JNK1/2 was measured in the ME1402 and WM1158 melanoma cell lines. The results show that all three MAPKs are active in both cell lines and their levels further increased over, at the very least, 6 hours of AJ-5 treatment **(Fig. 3.26a)**. Importantly, chemical inhibition of p38 (SB203580) and MEK/ERK (PD98059) pathways resulted in a significant decrease in AJ-5 cytotoxic effect as demonstrated by the MTT assay **(Fig. 3.26b)**. Furthermore, inhibition of p38 with either a chemical (SB203580) or biological (siRNA) inhibitor attenuated

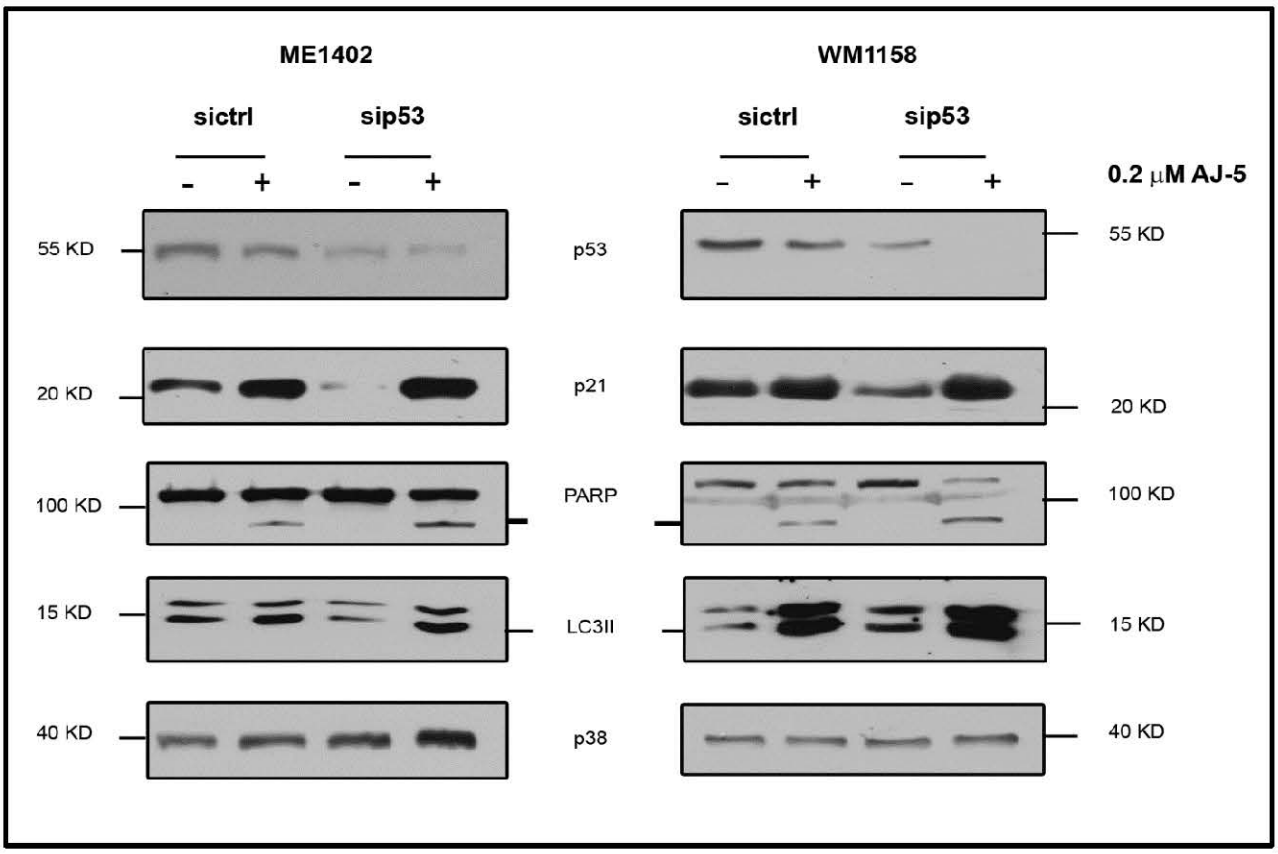


Figure 3.25 AJ-5 cytotoxicity is p53-independent. Melanoma cells were transiently transfected with non-silencing siRNA (sictrl) or p53-specific siRNA (si-p53). Transfected cells were treated with vehicle or 0.2 μM AJ-5 for 24 hours and protein extracts were analysed by SDS-PAGE (8 and 15%) and western blotting using antibodies to p53, p21, PARP and LC3II . p38 was used as a loading control.

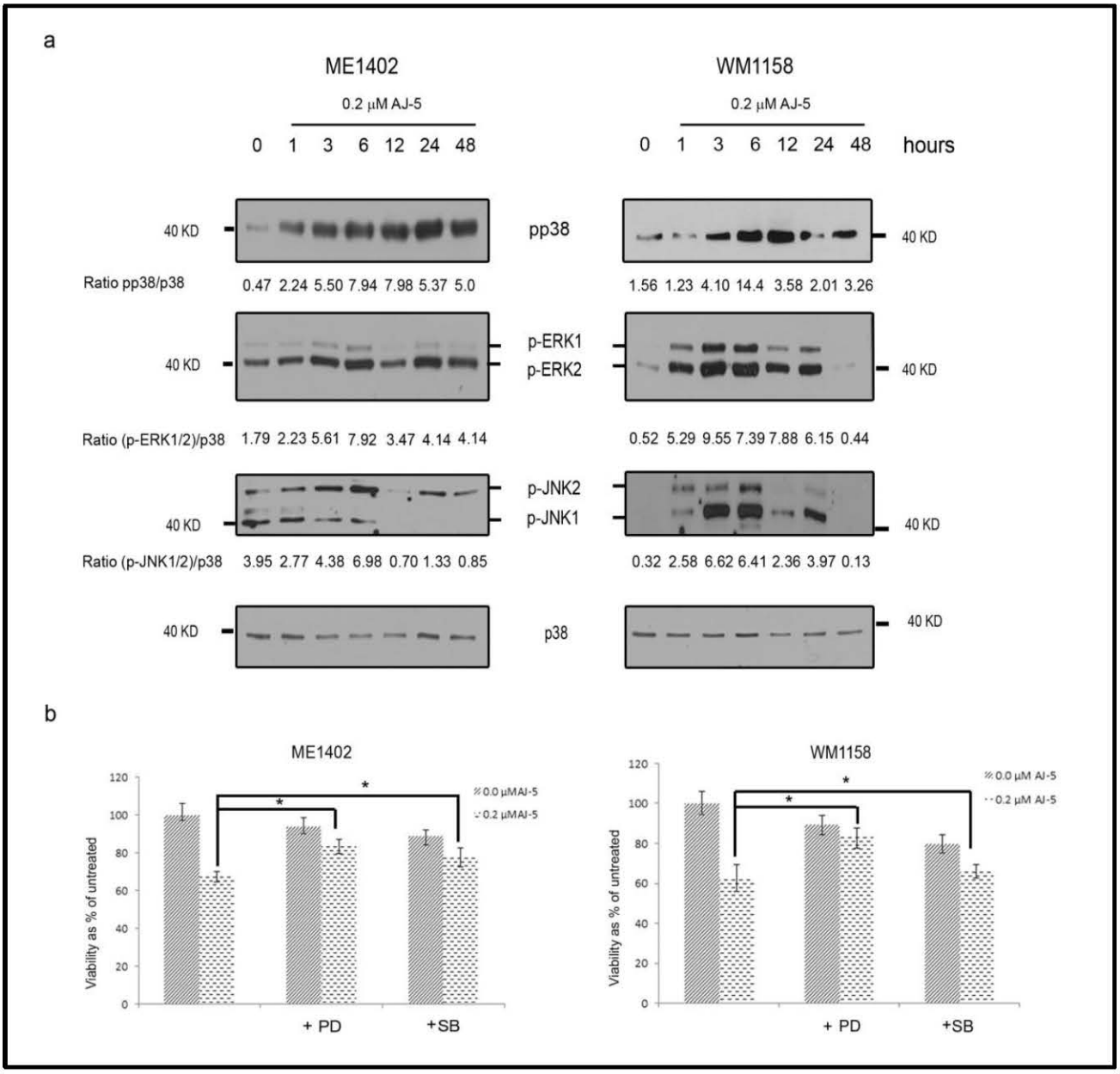


Figure 3.26 AJ-5 cytotoxic effect involves the activation of p38 and ERK1/2 MAPKs. (a) AJ-5 induces a sustained activation of p38 and ERK1/2 MAPKs. ME1402 and WM1158 cells were treated with either vehicle or 0.2 μM AJ-5 for up to 48 hours. Protein extracts were harvested and analysed by SDS-PAGE (8-15%) and western blotting using antibodies to phosphorylated p38, ERK1/2 and JNK1/2. p38 was detected as loading control and the numbers below each western indicate the ratio/p38. **(b)** Pharmacological inhibition of p38 and MEK/ERK1/2 pathways reduces AJ-5 cytotoxic effect. Melanoma cells were pre-treated with the p38 inhibitor SB203580 (10 μM) or MEK-1 inhibitor PD98095 (10 μM) and then treated with 0.2 μM AJ-5 for 24 hours. Cell viability was measured using the MTT assay. A Microsoft Excel student's *t* test was performed to calculate statistical significance (**P* <0.05).

AJ-5 induced apoptosis and autophagy as judged by cleaved PARP and LC3II levels **(Fig. 3.27a, b)**. While the same results were obtained when MEK/ERK was inhibited chemically by (PD98059) or biologically by (siRNA) **(Fig. 3.28a, b)**, the JNK inhibitor, SP600125, had no effect on AJ-5 induced apoptosis and autophagy (data not shown). Together the above results show that the activation of p38 and ERK plays an important role in AJ-5 induced cytotoxicity.

3.3.7 AJ-5 induced apoptosis and autophagy involves the inhibition of the AKT/mTOR pathway

The AKT/mTOR signalling pathway is a negative regulator of autophagy and apoptosis [291, 292] and reports indicate that it's inhibition leads to the induction of both these processes in several cancers including melanoma [63, 148, 292–295]. Therefore the effect of AJ-5 on this pathway was examined. Indeed, at the later time points of AJ-5 treatment, the levels of p- AKT were noticeable reduced and the levels of p-mTOR and its direct substrate p70 S6 kinase (p70S6K) were almost undetectable in both melanoma cell lines **(Fig. 3.29a)**. Importantly, this corresponded with a dramatic increase in markers of both autophagy (LC3II) and apoptosis (PARP) which was comparable to that seen when the AKT pathway was inhibited with the chemical inhibitor, AKT VIII **(Fig. 3.29b)**. While Beclin1 levels are frequently regarded as a marker of autophagy when it is cleaved by caspases 3, 7 and 8 it sensitizes cells to apoptosis [173, 175]. It is therefore important to note that the inhibition of the AKT/mTOR pathway was associated with a decrease in total Beclin1 levels and an increase in cleaved Beclin1 (35/37 kDa) levels **(Fig. 3.29a)**. These results show that AJ-5 induced cytotoxicity is also mediated by the inhibition of AKT/mTOR pathway.

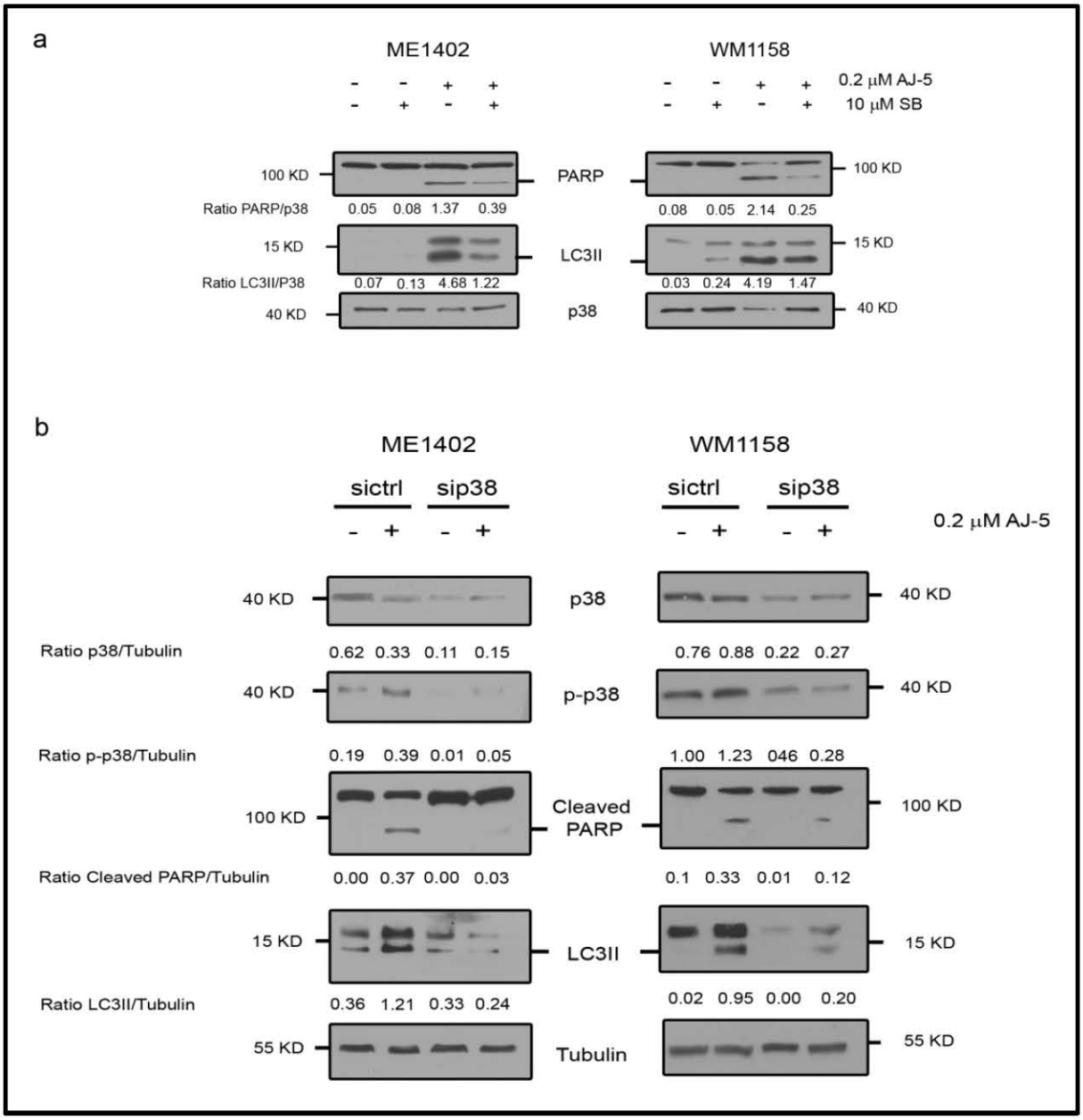


Figure 3.27 The p38 MAPK pathway mediates AJ-5 induced apoptosis and autophagy. Western blot analyses of melanoma cells pre-treated with the p38 inhibitor SB203580 (10 μM) in **(a)** or transfected with non-silencing siRNA (sictrl) or p38-specific siRNA (sip38) in **(b)** then treated with 0.2 μM AJ-5 for 24 hours. Representative western blots of PARP and LC3II show the effect of p38 inhibition on apoptosis and autophagy. Blots for p38 and p-p38 in **(b)** show the successful inhibition of p38 kinase. p38 in **(a)** and tubulin in **(b)** were detected as loading controls and the numbers below each western indicate the ratio/p38 in **(a)** and ratio/Tubulin in **(b)**.

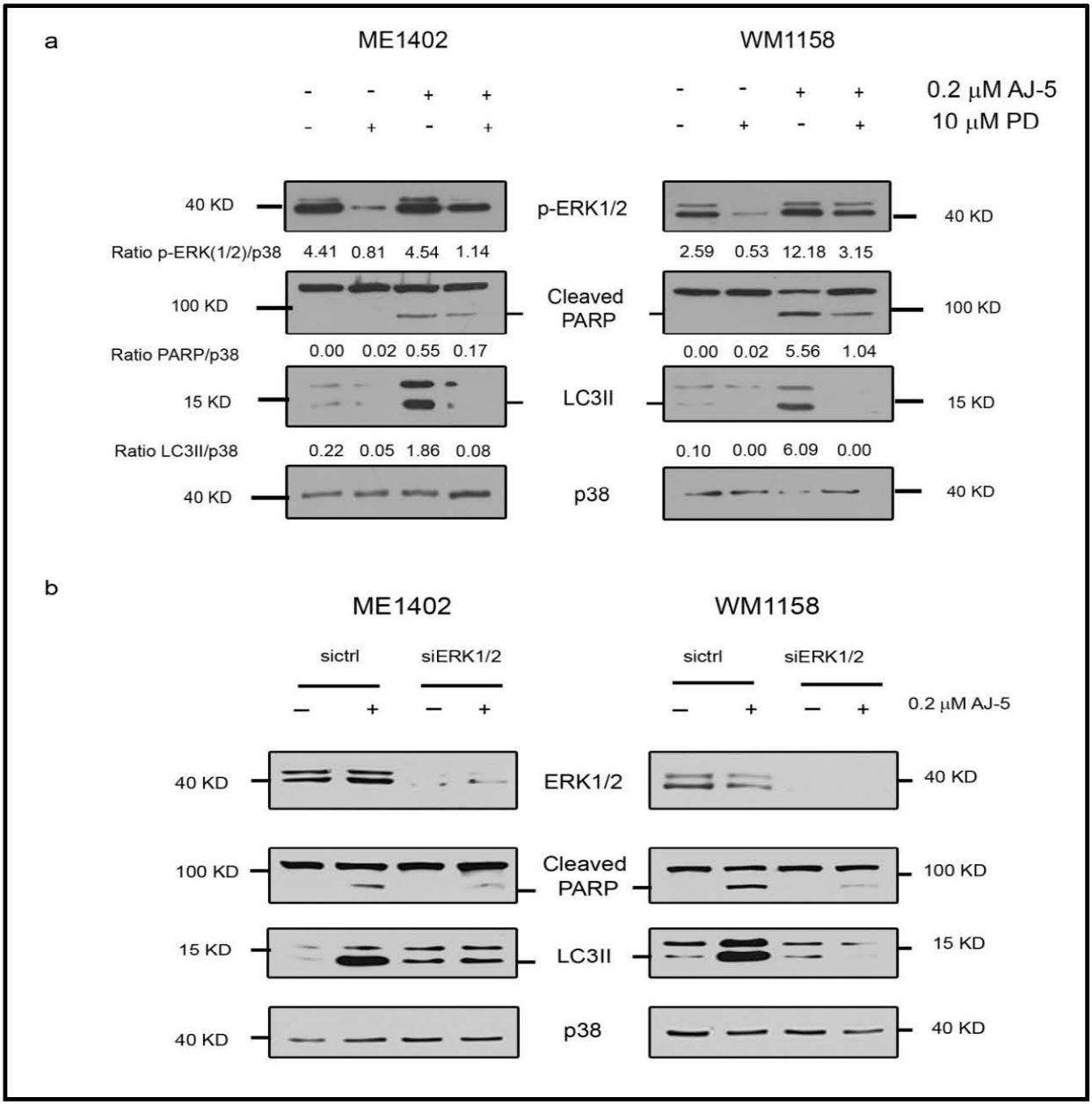


Figure 3.28 The ERK1/2 MAPK pathway mediates AJ-5 induced apoptosis and autophagy. Western blot analyses of melanoma cells pre-treated with the MEK-1 inhibitor PD98095 (10 μM) in **(a)** or transfected with non-silencing siRNA (sictrl) or ERK1/2-specific siRNA (siERK1/2) in **(b)** then treated with 0.2 μM AJ-5 for 24 hours. Representative western blots of PARP and LC3II show the effect of ERK1/2 inhibition on apoptosis and autophagy. Blots for p-ERK1/2 in **(a)** and total ERK1/2 in **(b)** show the successful inhibition of ERK1/2 kinases. p38 was detected as loading control and the numbers below each western in **(a)** indicate the ratio/p38.

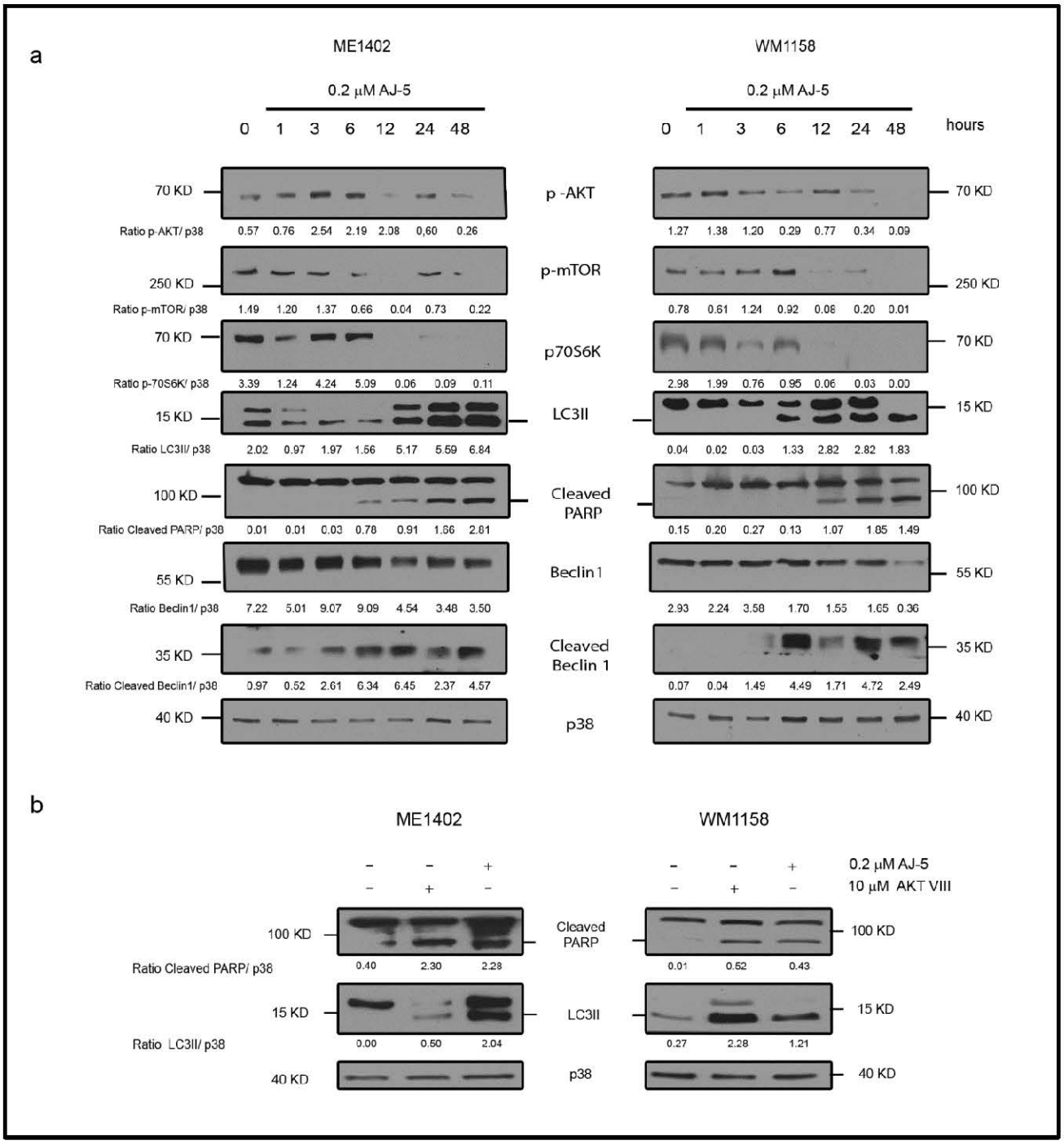


Figure 3.29 AJ-5 cytotoxicity involves the inhibition of AKT/mTOR signalling pathway in melanoma cells (a) ME1402 and WM1158 cells were treated with either vehicle or 0.2 μM AJ-5 for 1-48 hours. Protein extracts were harvested and analysed by SDS-PAGE (8-15%) and western blotting using antibodies to p- AKT, p-mTOR, p70S6K, LC3II, PARP and Beclin1. **(b)** Inhibition of the AKT pathway leads to induction of autophagy and apoptosis in ME1402 and WM1658 melanoma cells. Cells were treated with 10 μM AKTVIII or 0.2 μM AJ-5 for 24 hours. Protein extracts were harvested and analysed by SDS-PAGE (8-15%) and western blotting using antibodies to PARP and LC3II. p38 was used as a loading control and the numbers below each western indicate the ratio/p38.

3.3.8 Inhibition of autophagy decreases AJ-5 induced cell death

While previous studies have suggested that chemotherapeutic agents may induce cell death via apoptosis and autophagy [296], others have shown that they can induce autophagy which attenuates apoptosis leading to drug resistance [49]. The current study shows that compared with AJ-5 treatment only, co-treatment of AJ-5 with Baf resulted in a significant reduction in cell death and enhanced the cell viability as measured by the Annexin V/PI double staining and MTT assays **(Fig. 3.30a, b)**. Importantly, in the presence of the autophagy inhibitors Baf or wortmannin the levels of cleaved PARP induced by AJ-5 treatment decreased **(Fig. 3.31a, b)**. These results were reproducible when autophagy was inhibited with small interfering RNA specifically to LC3II **(Fig. 3.32c)**. Together these data indicate that AJ-5 induced autophagy contributes to melanoma cell death and not survival.

3.3.9 AJ-5 inhibits melanoma tumour growth in vivo

While the above results suggested that AJ-5 may be a potentially good chemotherapeutic agent for treating advanced melanomas, they were generated using in vitro assays and therefore the anti-tumour activity of AJ-5 was investigated in melanoma bearing nude mice (6-week-old). To address this, the possibility that AJ-5 may have adverse effects in vivo had firstly to be determined by acute and sub-acute toxicity tests. Three (3) mice were injected intraperitoneally with AJ-5 (10 mg/kg of body weight) and 3 days later signs of toxicity including weight loss and behavioural changes were observed. Based on this, the mice were euthanized and a lower dose (2 mg/kg of body weight) of AJ-5 was chosen for the sub-acute study as described below.

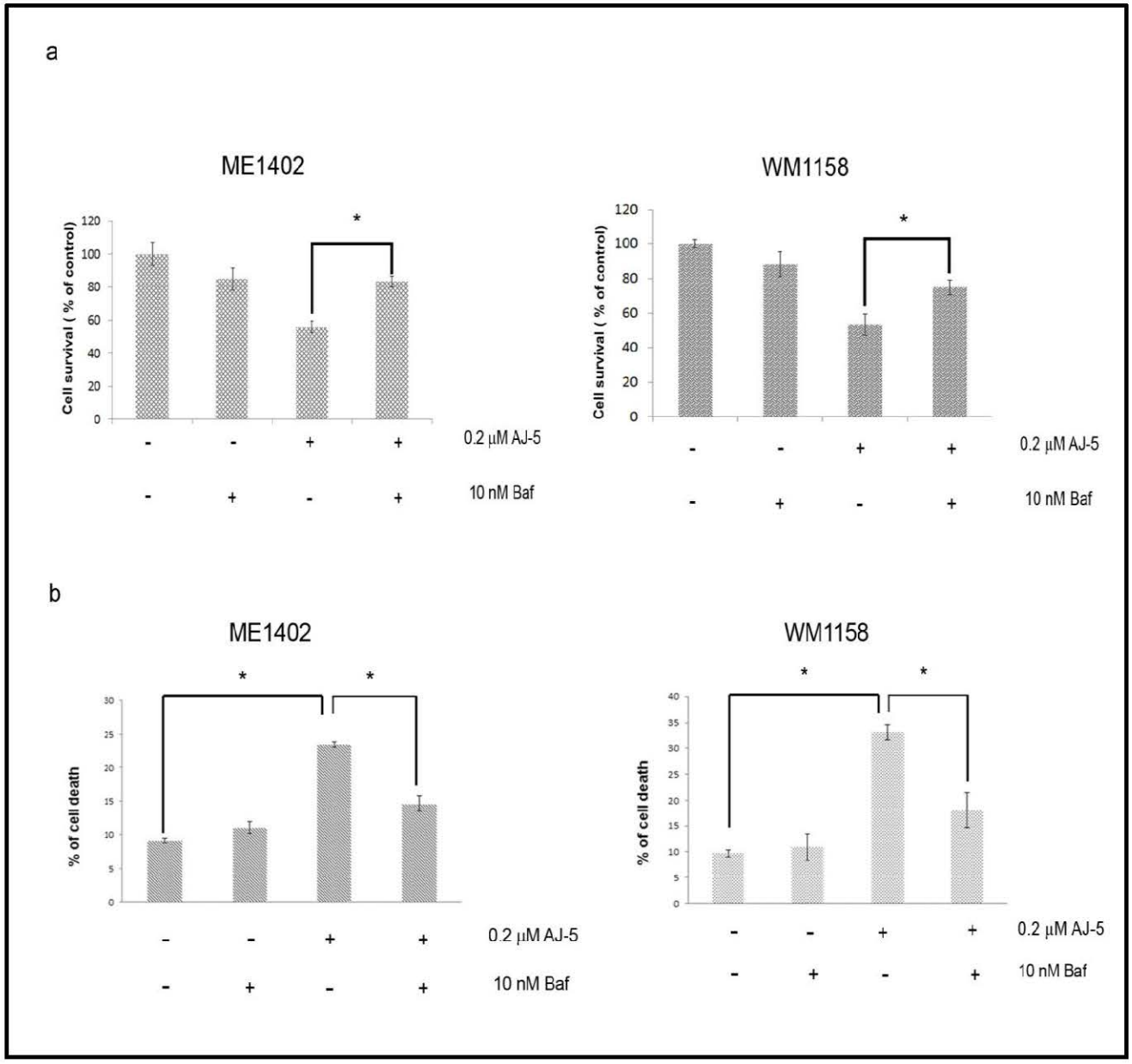


Figure 3.30 AJ-5 induced autophagy contributes to AJ-5 cytotoxicity. (a) Percentage of cell survival as measured by MTT assay for melanoma cells pre-treated with 10 nM bafilomycin A (Baf) and treated with 0.2 μM AJ-5 or vehicle for 24 hours. **(b)** Percentage of cell death as measured by the Annexin V/PI double staining assay of melanoma cells pre-treated with 10 nM bafilomycin A and then treated with 0.2 μM AJ-5 or vehicle for 24 hours. A Microsoft Excel student's *t* test was performed to calculate statistical significance (*P <0.05).

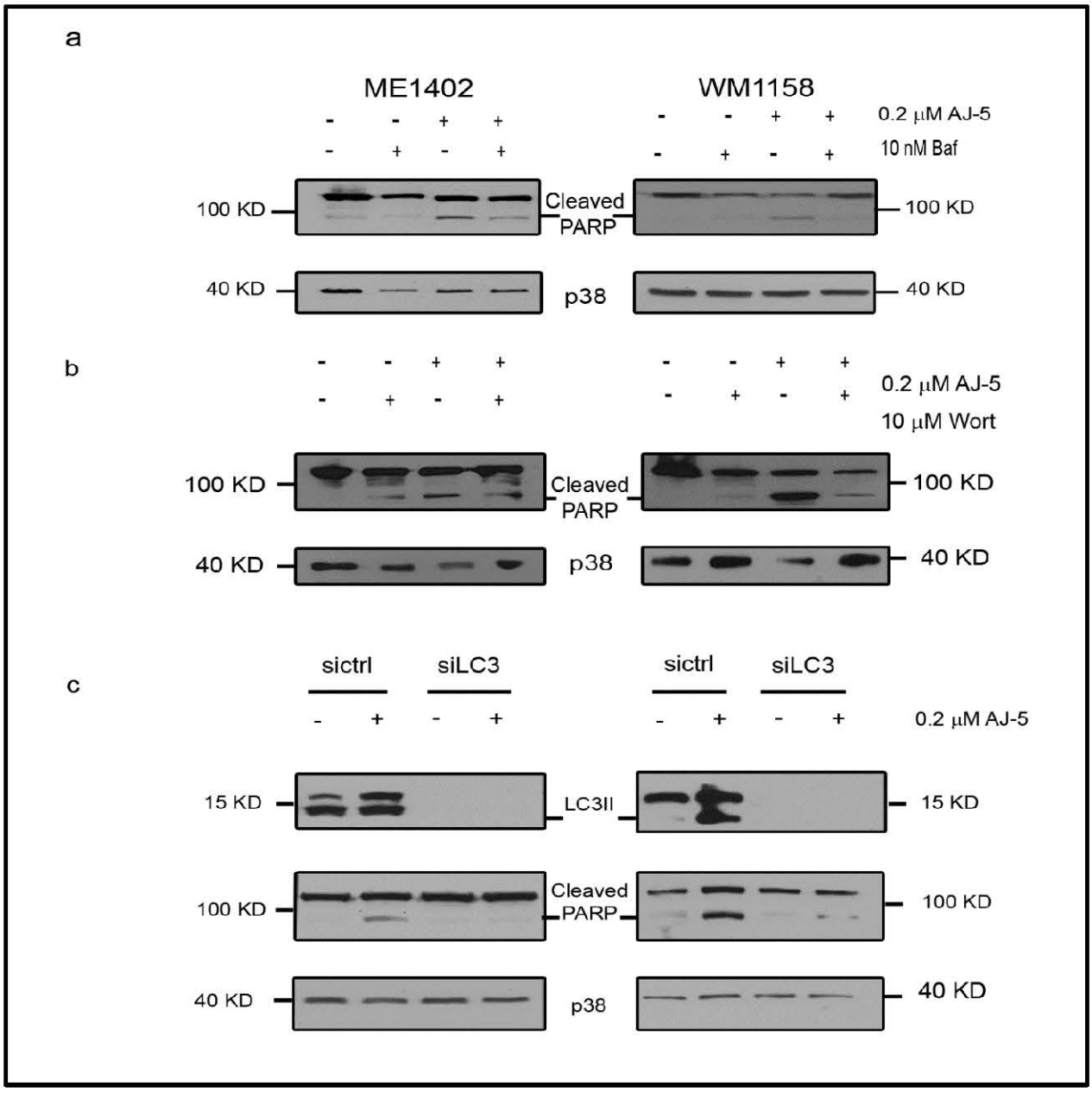


Figure 3.31 inhibition of autophagy reduces AJ-5 induced apoptosis. (a) Western blotting of proteins from ME1402 and WM1158 cells pre-treated with 10 nM bafilomycin A (Baf) **(a)** or 10 μM wortmannin (Wort) **(b)** and treated with AJ-5 or vehicle for 24 hours. Representative western blots of PARP show that inhibition of autophagy decreased apoptosis induced by AJ-5. **(c)** Melanoma cells transfected with non-silencing siRNA (sictrl) or LC3 specific siRNA (siLC3) and treated with AJ-5 for 24 hours. Protein extracts were harvested and analysed by SDS-PAGE (8-15%) and western blotting using antibodies to LC3II expression and PARP. p38 was used as a loading control.

The sub-acute study involved three groups of nude mice each consisting of six mice. One group received no treatment (untreated); the second group was injected intraperitoneally daily for 2 weeks with vehicle only (AJ-5 vehicle) and the third group was injected intraperitoneally daily for 2 weeks with AJ-5 (2 mg/kg of body weight). Signs of toxicity were monitored by observing the mice twice daily for any behavioural changes and by recording their total body weight every three days. Mice from the control and AJ-5 treated groups behaved normally and as shown in **Figure 3.32a** there was no significant difference between their total body weight. The liver/body weight ratio is a significant indicator of toxicity [297, 298] and when this was measured it was comparable for the control and AJ-5 treated groups **(Fig. 3.32b)**. Furthermore, upon dissection of mice from all groups, no macroscopic injury to any organs was observed (data not shown). Together these results suggest that AJ-5 at the dose of 2 mg/kg of body weight of nude mice is not toxic.

To assess the effect of AJ-5 on tumour growth in vivo, ME1402 cells were injected subcutaneously in 6-week-old nude mice (5×10^6 cells/mouse) and 10 days later they were separated into four groups of 6 mice each which were injected twice a week with one of the following treatments: AJ-5, AJ-5 vehicle, CDDP or CDDP vehicle. A fifth group which was not treated served as a control for the study. While the control groups (untreated and vehicle only) showed a rapid tumour growth, AJ-5 and CDDP treatment attenuated tumour development **(Fig. 3.33a)**. Compared to their vehicles, 15 days of AJ-5 treatment led to more than 90% and 85% reduction in tumour size and weight respectively and CDDP treatment to 56.4% and 63.6% respectively **(Fig. 3.33b-d)**. It is however important to note that no weight loss was observed in

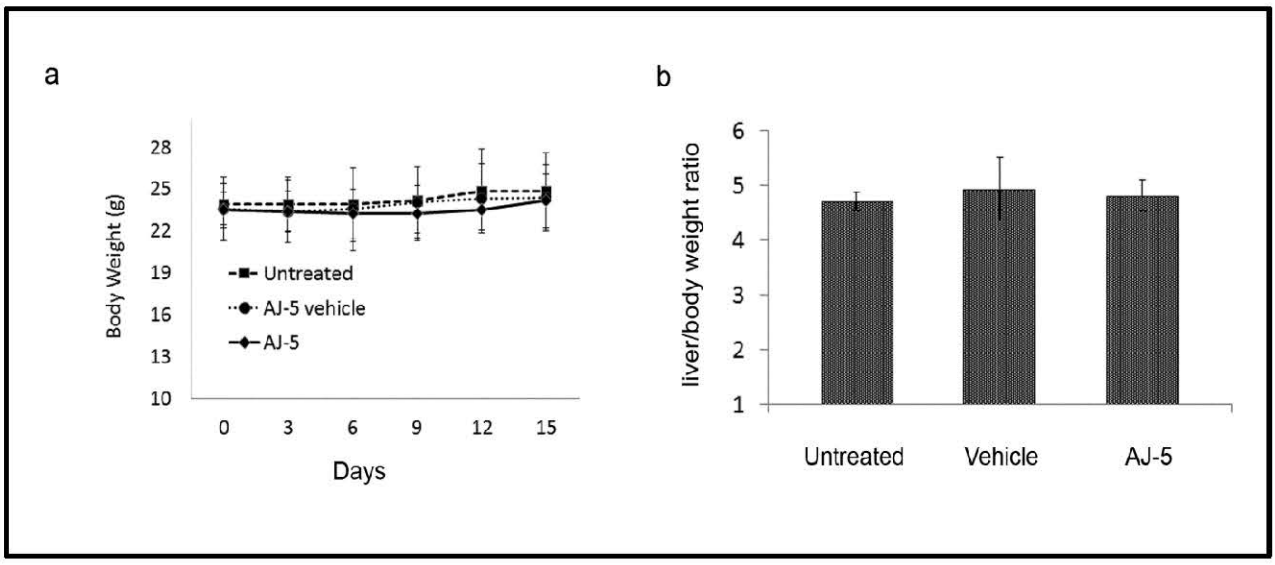


Figure 3.32 AJ-5 has no adverse effects on the total body weight and liver/body ratio. Eighteen of six weeks old nude mice were assigned to each of the three groups; untreated, vehicle or AJ-5. The vehicle and AJ-5 groups were injected intraperitoneally once daily for 14 days. The body weight was recorded every 3 days and the liver weights were recorded at the end of the experiments. **(a)** Graph showing the average weight of the six animals per group. **(b)** Graph showing the liver/body weight ratio of each group. Data are means ± SEM (n =6).

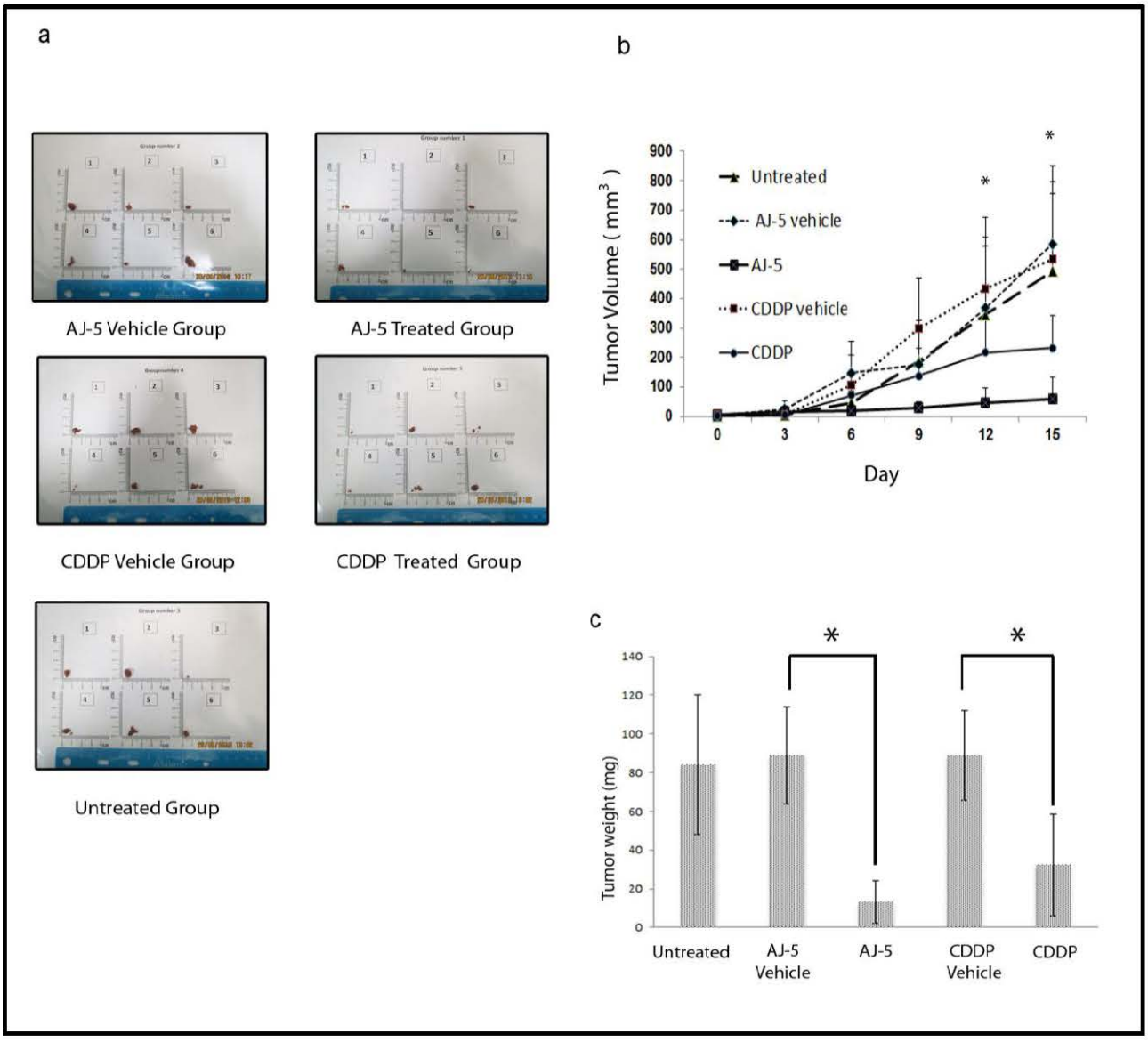


Figure 3.33 AJ-5 inhibits melanoma growth in vivo. Micrographs showing measurements of tumours from all groups. **(b)** Graph showing average tumour size (mm^3) measured at 0 to 15 days after starting the indicated treatments. Data presented are average ± SEM. Statistical significance was assessed between the animal groups using the Student's *t* test. **(c)** Average tumour weight (mg) at the end of indicated treatments $*P< 0.05$.

AJ-5 or CDDP treated mice **(Fig. 3.34)**.These data clearly show that AJ-5 has anti-melanoma activity in vivo and the next set of experiments were performed to determine whether this also occurs through the induction of apoptosis and autophagy as observed in vitro. In these experiments protein was extracted and pooled from 3 tumour samples per group and subjected to western blotting. The results show an increase in levels of cleaved PARP and LC3II in tumours of mice treated with AJ-5 or CDDP **(Fig. 3.35a)**. Furthermore transmission electron microscopy confirmed that tumours from AJ-5 treated mice have a high level of vacuolization and double membrane autophagosomes **(Fig.3.35b)**.

Together these results show that AJ-5 inhibited melanoma growth in vitro and in vivo by the induction of apoptosis and autophagy and the data presented suggest that AJ-5 may be an effective drug for treating melanoma.

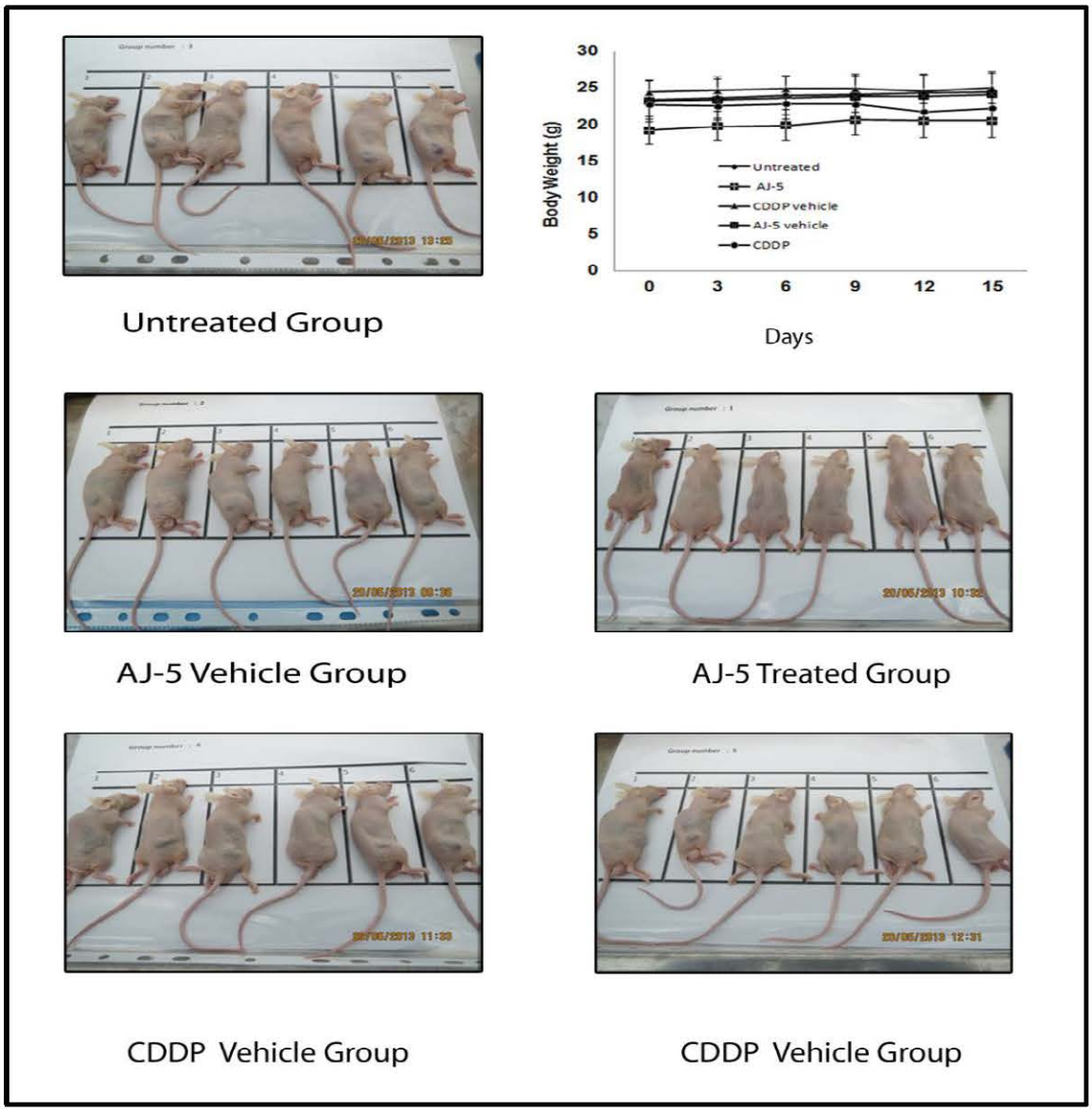


Figure 3.34 AJ-5 had no effect on mouse body weight. The photographs show the euthanized mice at the end of the experiment. The graph shows the average of the total body weight measured at 0 to 15 days after starting the indicated treatments. Data presented are mean ± SEM. There was no significant difference between the groups at a value of ($P < 0.05$).

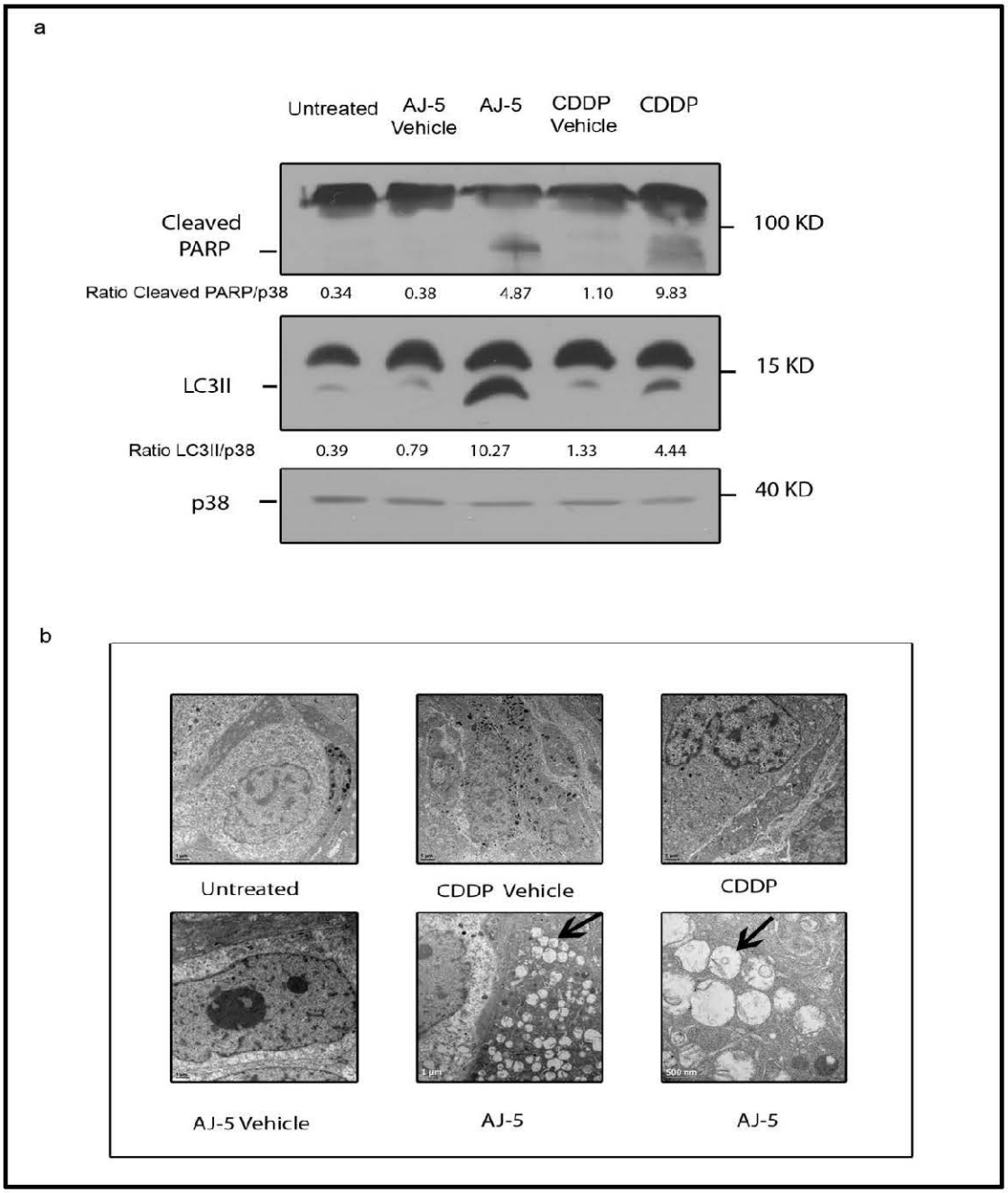


Figure 3.35 AJ-5 inhibits melanoma growth in vivo through induction of apoptosis and autophagy. (a) Western blotting of proteins from each group (three tumours per group) shows cleaved PARP and LC3II levels. p38 was used as a loading control. **(b)** Representative transmission electron photomicrographs of tumour sections of each indicated group showing autophagosomes. Black arrows indicate autophagosomes containing cytoplasmic inclusions.

3.4 The role of the developmentally important T-box factor, TBX3, in AJ-5 induced cytotoxicity

The T-box family of transcription factors are important developmental regulators of a wide range of tissues and organisms, and have been shown to contribute to several human syndromes [299]. In addition to their key role in development, extensive investigations suggest that overexpression of some T-box factors, including TBX3 may drive cancer [300–307]. TBX3 is up-regulated in a number of cancers including breast cancer [308] and melanoma [309] where it was shown to be required for tumour formation and cell migration [304, 310–312]. Importantly, knocking down TBX3 was shown to reverse key features of the melanoma and breast cancer phenotype suggesting that it may be a useful target in the development of novel anti-cancer drugs to treat these cancers. Furthermore, silencing Tbx3 in rat bladder carcinoma cells rendered the cells sensitive to doxorubicin-induced apoptosis and the overexpression of TBX3 was associated with a chemotherapy-resistant phenotype [313]. Similar findings showed that knocking down TBX3 sensitized human colorectal carcinoma cells to doxorubicin via activating the p14- p53 pathway [314]. Taken together these findings suggest that depleting TBX3 in cancers where it is overexpressed might enhance the anti-cancer activity of chemotherapeutic drugs. The following section of this thesis investigates this possibility in MCF7 breast cancer cells treated with AJ-5.

3.4.1 AJ-5 up-regulates TBX3 post-transcriptionally

Based on the above reports we hypothesized that in response to chemotherapeutic agents TBX3 levels may be up-regulated resulting in cancer cells being less sensitive to these anti-

cancer treatments. To explore this possibility the effect of AJ-5 on TBX3 levels was firstly determined. Briefly, MCF7 cells were treated with 0.2 µM AJ-5 for 24 and 48 hours and total protein harvested and subjected to western blotting with an antibody to TBX3. The results obtained revealed that in response to AJ-5 treatment TBX3 levels increased in a time-dependent manner in MCF7 cells **(Fig. 3.36.a)**. To investigate whether AJ-5 up-regulates TBX3 transcriptionally or post-transcriptionally, MCF7 cells were treated with 0.2 µM AJ-5 for 6 hours and TBX3 expression was measured by quantitative real time PCR (qRT–PCR) analysis. The results show that AJ-5 had no effect on mRNA TBX3 level which suggests that AJ-5 may up-regulate TBX3 post-transcriptionally **(Fig. 3.36b)**. To confirm these observations, cells were pre-treated with 5 µM Actinomycin D, a transcriptional inhibitor, and then treated with 0.2 µM AJ-5 for 12 hours. **Figure 5.36c** displays that Actinomycin D did not affect the AJ-5-mediated increase of TBX3 protein which confirms that AJ-5 increases TBX3 levels in a post-transcriptional manner.

3.4.2 AJ-5 up-regulates TBX3 in an AKT dependent manner

Several observations indicate that the PI3K/AKT pathway is activated in response to chemotherapy [315–317] and this was reported to mediate CDDP resistance in different cancer types including the MCF7 breast cancer cell line [317–320]. In addition, it was recently proposed that TBX3 may be a substrate of the PI3K/AKT pathway in embryonic stem cells [321, 322]. To test whether AKT mediates TBX3 up-regulation in AJ-5 treatment, MCF7 cells were treated with 0.2 µM AJ-5 for different time points and p-AKT and TBX3 levels were determined by western blotting. Indeed, the expression pattern of p-AKT and TBX3 is quite similar especially from 3 to 24 hours of AJ-5 treatment which indicate that the increasing level of TBX3

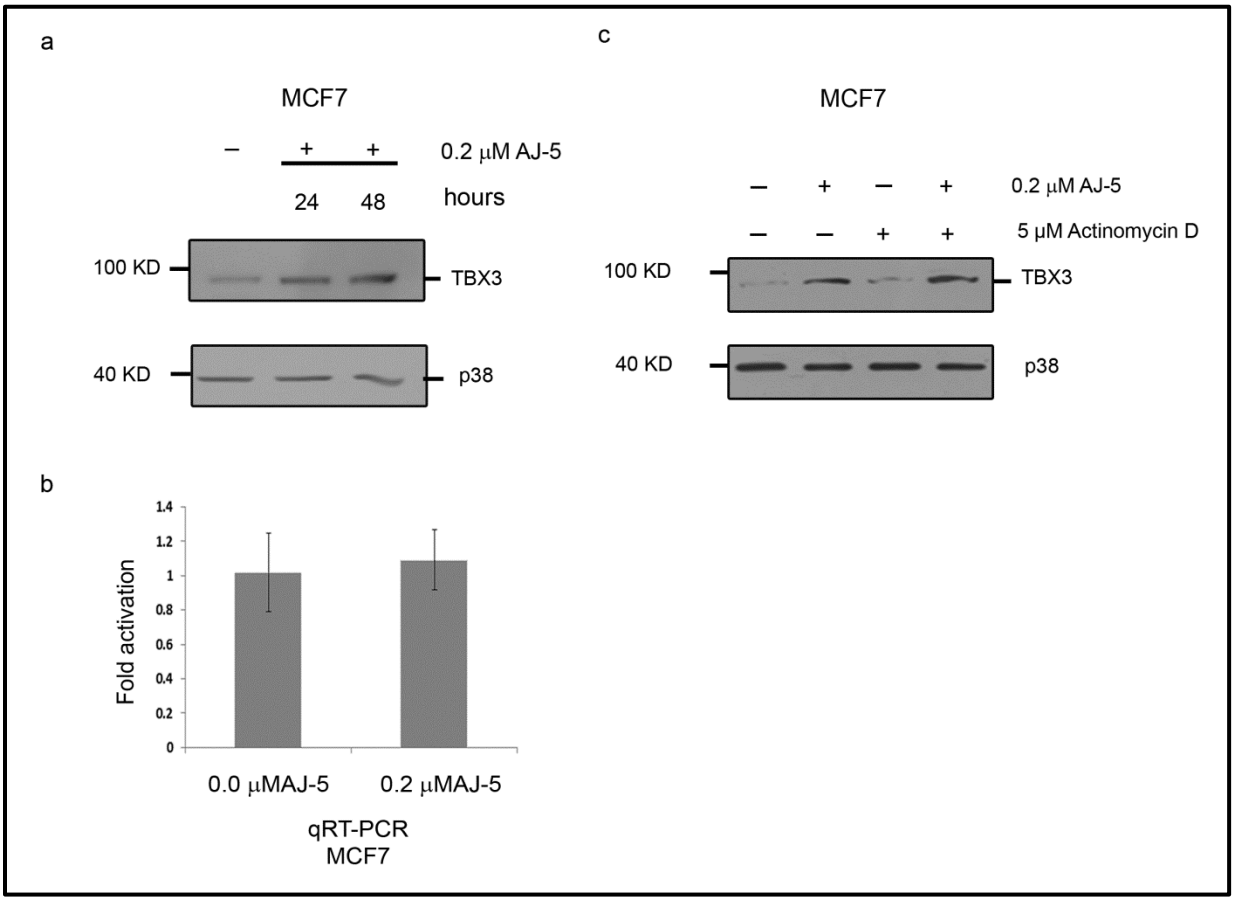


Figure 3.36 AJ-5 up-regulates TBX3 protein levels post-transcriptionally. (a) AJ-5 treatment increases TBX3 levels in MCF7 cells. Protein extract from MCF7 cells treated with vehicle or 0.2 μM AJ-5 for 24 and 48 hours were analysed by SDS-PAGE (8%) and western blotting using an antibody to TBX3. p38 was used as a loading control. **(b, c)** AJ-5 does not up-regulate TBX3 at a transcriptional level. **(b)** MCF7 cells were pre-treated with vehicle or 0.2 μM AJ-5 and relative *TBX3* mRNA levels were quantitated using qRT-PCR and normalized to human *GUSB*. Results represent the average of biological replicates. Error bars = ±SEM. **(c)** MCF7 cells were pre- treated with Actinomycin D (Act D) (5 μM) for 1 hour then treated with vehicle or 0.2 μM AJ-5 for 12 hours. The protein extract was harvested and analysed by SDS-PAGE (8%) and western blotting using an antibody to TBX3. p38 was used as a loading control.

by AJ-5 treatment might be mediated by p-AKT **(Fig. 3.37a)**. Importantly, pre-treatment with the AKT inhibitor, AKTVIII, completely abrogated this increase in TBX3 protein induced by AJ-5. Furthermore, inhibition of the AKT signalling pathway resulted in an increase in the levels of PUMA protein and markers of apoptosis and autophagy which were further enhanced by AJ-5 treatment **(Fig. 3.37b)**.

3.4.3 Knocking down TBX3 enhanced AJ-5 induced cytotoxicity

To investigate the potential role of TBX3 depletion in AJ-5-induced cytotoxicity, MCF7 cell lines stably expressing either a scrambled sh control (shctrl) or shTBX3 were treated with a range of AJ-5 doses (0 to 0.6 µM) for 24 hours and the MTT assay was used to determine cell viability. These cell lines were established by Jarod Li a PhD student in our laboratory and have previously been described [302]. **Figure 3.38a** confirms that the MCF7 shTBX3 cells used in this experiment did indeed have lower levels of TBX3 than the shctrl cells and **figure 3.38b** shows that the shTBX3 cells were more sensitive to AJ-5 than the shctrl cells. This was particularly striking at 0.1 and 0.2 µM AJ-5 which was previously shown in this study to represent the IC_{50} range for MCF7 cells. While 0.1 and 0.2 µM of AJ-5 killed 31.1% and 40.1% of shctrl cells respectively, the same concentrations killed 51% and 58.6% of shTBX3 cells. These results indicate that knocking down TBX3 might enhance the cytotoxic effect of AJ-5 in MCF7 cells especially at low concentrations.

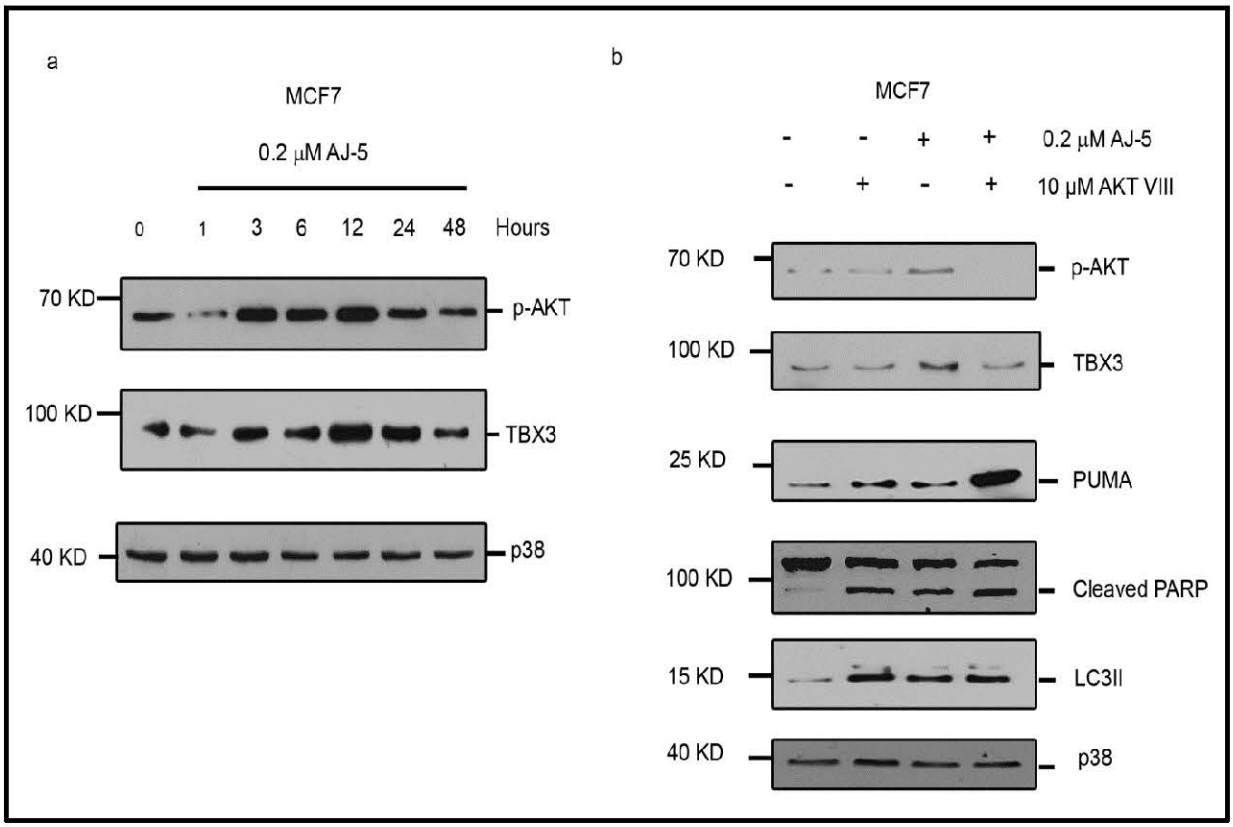


Figure 3.37 AJ-5 up-regulates TBX3 protein levels via activation of the AKT pathway. (a) AJ-5 increases p- AKT (Ser473) and TBX3 protein levels in a similar pattern. Cells were treated with vehicle or 0.2 μM AJ-5 for up to 48 hours and the protein was harvested and analysed by SDS-PAGE (8%) and western blotting using antibodies to TBX3 and p- AKT. p38 was used as a loading control. **(b)** Inhibition of AKT pathway abrogates AJ-5 induced TBX3 protein levels, increased PUMA protein levels and increased markers of apoptosis and autophagy. MCF7 cells were pre-treated with 20 μM AKTVIII (AKT inhibitor) for 1 hour then treated with vehicle or 0.2 μM AJ-5 and the protein was harvested and analysed by SDS-PAGE (8-15%) and western blotting using antibodies to TBX3, p- AKT, PUMA, PARP and LC3II. p38 was used as a loading control.

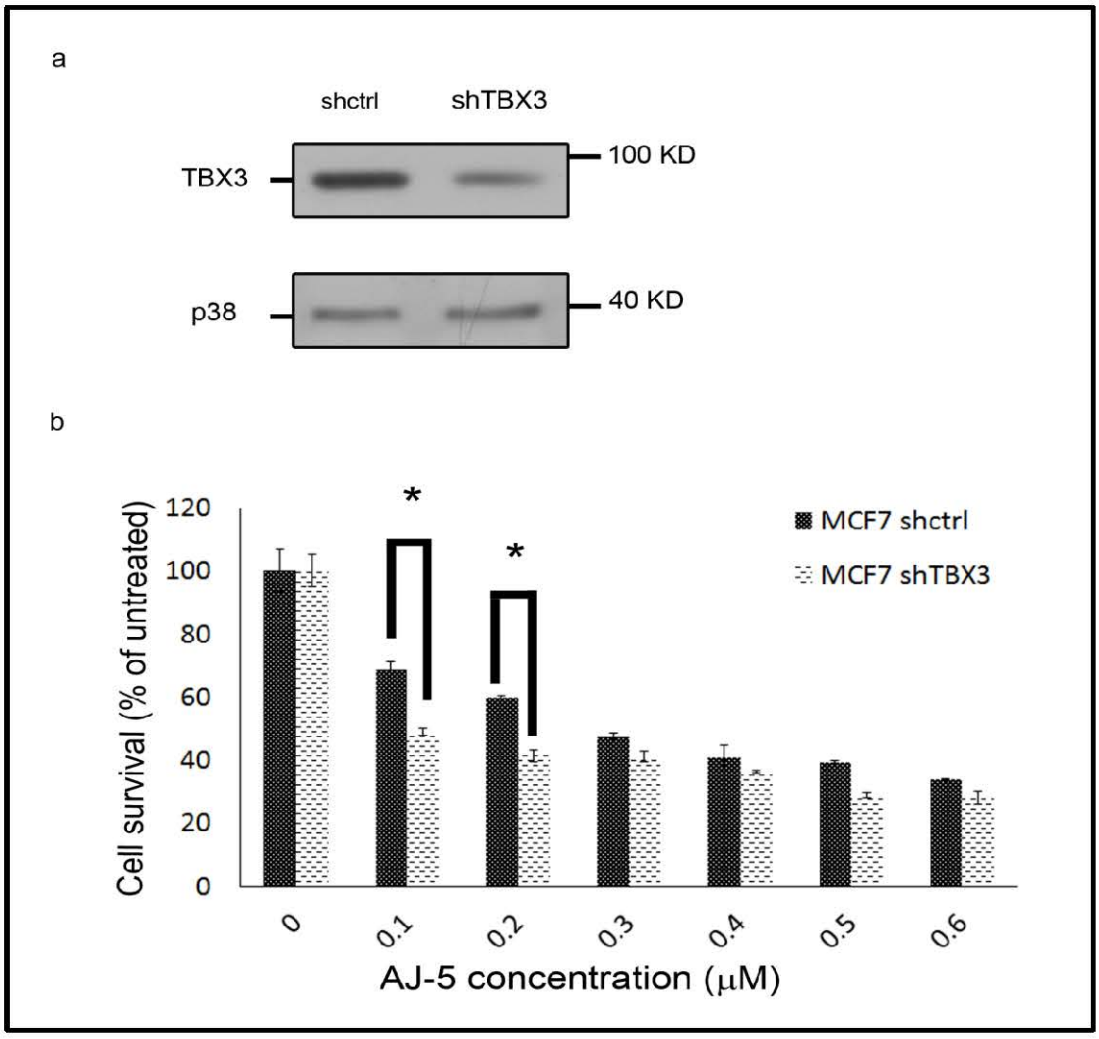


Figure 3.38 Knocking down TBX3 in MCF7 cells enhances AJ-5 cytotoxic effect. (a) Western blot of protein from MCF7 shctrl and MCF7 shTBX3 cells shows successful TBX3 knockdown in MCF7 shTBX3 cells. **(b)** MCF7 shTBX3 cells are more sensitive to AJ-5 than MCF7 shctrl cells. Cells were plated in 96-well plates and cell viability was assessed by the methylthiazol tetrazolium (MTT) assay over 24 hours of AJ-5 treatment. The graph represents the mean percentage ± SEM of untreated cells of at least three independent experiments performed in quadruplicate. A Microsoft Excel student's *t* test was performed to calculate statistical significance (*p<0.05).

3.4.4 Knocking down TBX3 increases apoptosis and autophagy in MCF7 cells

To investigate the role of TBX3 up-regulation on AJ-5 induced cell death, the effect of knocking down TBX3 on AJ-5 induced apoptosis and autophagy was analysed. Briefly, MCF7 cells in which TBX3 was either transiently (siTBX3) or stably (shTBX3) knocked down as well as their appropriate control cell lines, were treated with 0.2 μM AJ-5 for 24 hours and total protein harvested and subjected to western blotting with antibodies to PUMA, PARP and LC3II. **Figure 3.39a** shows that TBX3 levels in the knockdown cells are noticeably less than in the control cells confirming the successful knockdown of TBX3 in these cells. Importantly, AJ-5 treated shTBX3 and siTBX3 cells have higher levels of the pro-apoptotic protein PUMA, cleaved PARP and LC3II than control cells (shctrl and sictrl) confirming that depleting MCF7 cells of TBX3 increases their sensitivity to AJ-5 induced cell death pathways. It is worth noting that under unstimulated conditions PUMA and LC3II were induced in TBX3 depleted cells indicating that TBX3 plays an important role in regulating basal levels of these proteins. These results were particularly interesting in light of previous reports that TBX3 functions as an anti-apoptotic factor because it suggested that TBX3 may be doing so by directly repressing PUMA. To follow up on this possibility, *PUMA* mRNA levels were determined by qRT-PCR and indeed, in untreated cells *PUMA* mRNA levels were 9.3 fold higher in shTBX3 cells compared to shctrl cells **(Fig. 3.39b)**. Furthermore, while AJ-5 treatment led to *PUMA* mRNA levels being up-regulated by 5.3 folds in shctrl cells it had little effect on *PUMA* mRNA levels in shTBX3 cells. These results were exciting because it revealed for the first time that PUMA may be a TBX3 target gene and provided another possible mechanism by which TBX3 may be contributing to oncogenesis and

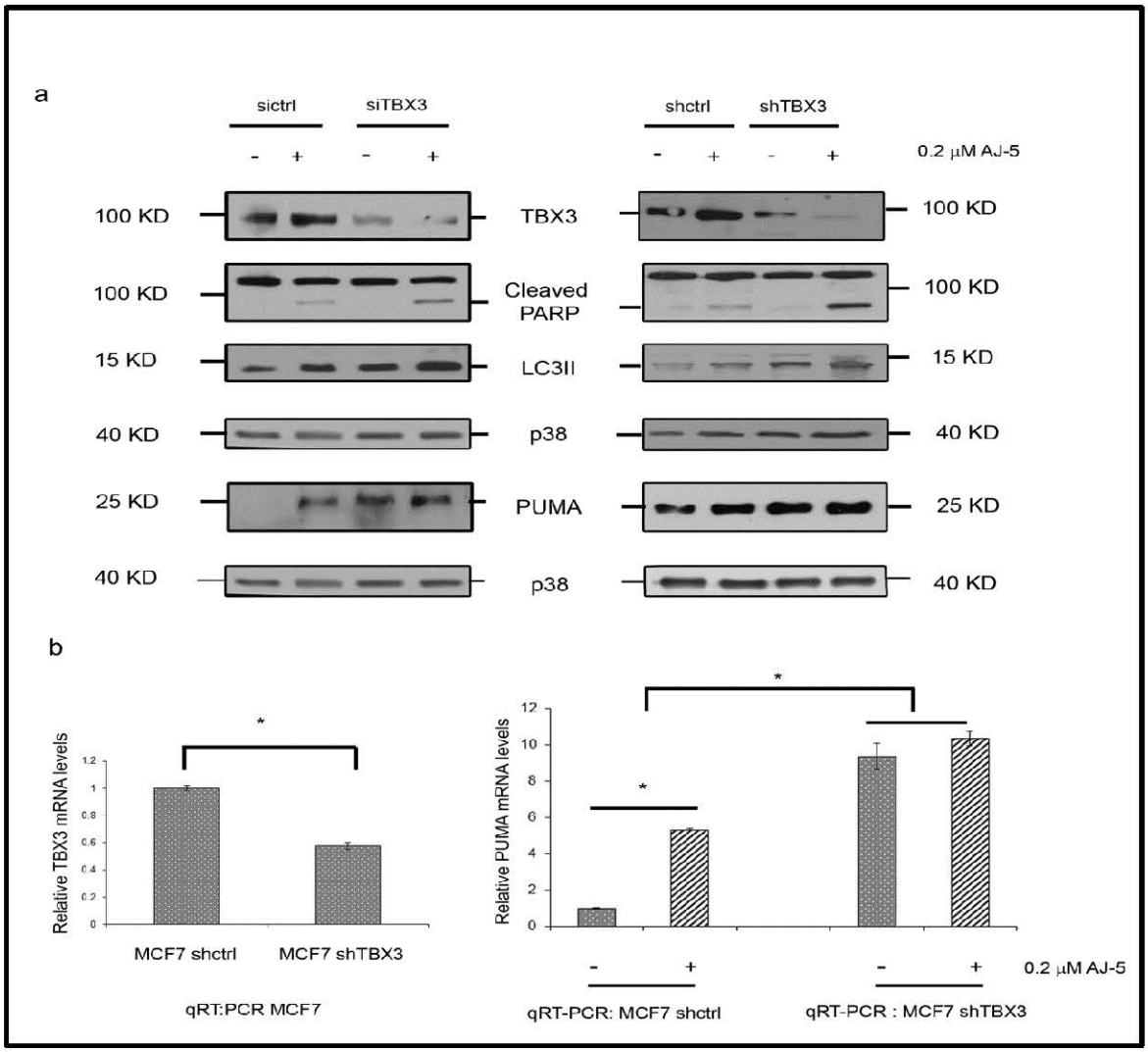


Figure 3.39 TBX3 knockdown sensitizes MCF7 cells to AJ-5 by up-regulation of PUMA protein and mRNA. (a) Knocking down TBX3 protein sensitizes the cells to AJ-5 as demonstrated by the increasing levels of PARP cleavage and LC3II proteins and up-regulation of PUMA protein. MCF7 sictrl and MCF7 siTBX3 cells **(left)** as well as MCF7shctrl and MCF7shTBX3 cells **(right)** treated with vehicle or 0.2 μM AJ-5 for 24 hours. Protein extracts were analysed by SDS-PAGE (8 and 15%) and western blotting using antibodies to TBX3, LC3II, PARP and PUMA. p38 was used as a loading control. **(b)** TBX3 knockdown increases *PUMA* mRNA levels. **Left** quantitative real-time PCR of *TBX3* mRNA extracted from MCF7 shctrl and MCF7 shTBX3 cells shows the successful knockdown of *TBX3* mRNA in MCF7 shTBX3 cells. The levels of *TBX3* mRNA expression were normalized to human *GUSB* mRNA levels. **Right** quantitative real-time PCR of *PUMA* mRNA extracted from MCF7 shctrl and MCF7 shTBX3 cells treated with vehicle or 0.2 μM AJ-5 for 6 hours was performed. The levels of *PUMA* mRNA expression were normalized to human *GUSB mRNA levels*. Results represent the average of biological replicates. A Microsoft Excel student's *t* test was performed to calculate statistical significance (**p*<0.05), Error bars = ±SEM.

chemotherapeutic drug resistance. Therefore while not directly linked to this project this possibility was further explored.

3.4.5 TBX3 transcriptionally down regulates *PUMA* promoter activity

To investigate whether *PUMA* is a direct target of TBX3, MCF7 cells were co-transfected with a luciferase reporter driven by a 0.4 kb human *PUMA* promoter (*PUMA* fragment 1-Luc, Addgene 16591) **(Fig. 3.40a)** and increasing amounts (100 ng-500 ng) of a TBX3 expression vector (WT-TBX3). Importantly, this *PUMA* promoter contains two consensus p53 binding sites, therefore to test whether TBX3 regulates *PUMA* promoter activity directly or indirectly via p53 the assay was also performed using a 0.2 kb human *PUMA* promoter (*PUMA* Fragment 2-Luc, Addgene 16592) **(Fig. 3.40a)** lacking these sites. The results show that TBX3 repressed both *PUMA* promoters in a dose-dependent manner **(Fig. 3.40b, c)** suggesting that TBX3 represses *PUMA* in a p53-independent manner.

3.4.6 The DNA-binding domain of the TBX3 protein is required for repression of *PUMA*

The DNA-binding domain (DBD) is important for the recognition and binding of T-box proteins to their target genes. Therefore to investigate whether *PUMA* is a direct target of TBX3, a TBX3 DBD mutant (DBM) was generated in which arginine was replaced with glycine at position 133 using site-directed mutagenesis **(Fig.3.41a)**. This mutation was previously shown to disrupt the highly homologous Tbx3 mouse DBD [301, 323, 324]. TBX3 has two dominant repression domains, R1, which resides in the C-terminus at amino acids 567-623 and a second repression

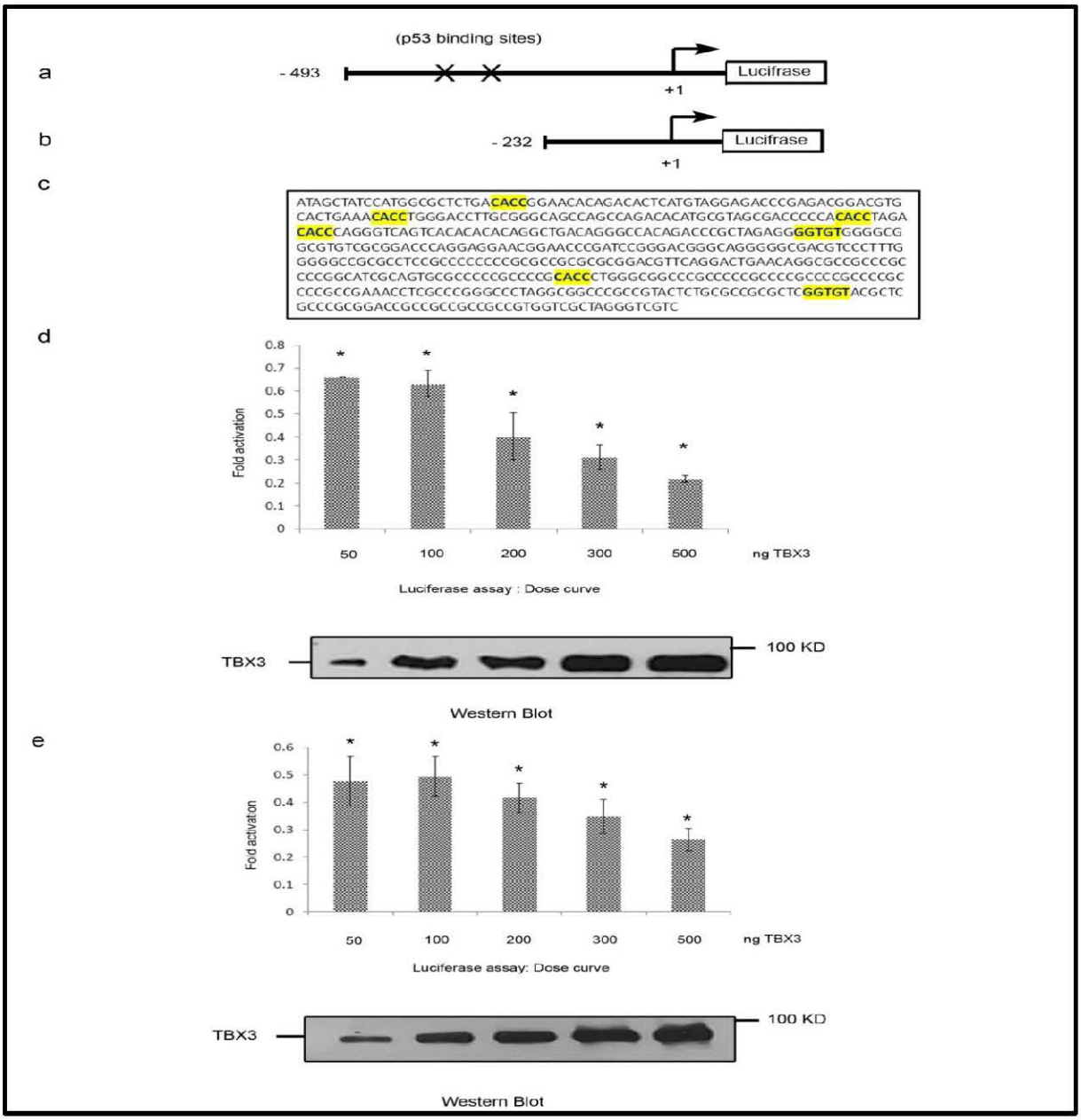


Figure 3.40 TBX3 transcriptionally down-regulates *PUMA* promoter activity. (a, b) Schematic diagrams of the human *PUMA* promoters fragment 1 **(a)** and fragment 2 **(b)** luciferase reporter constructs. **(c)** The human *PUMA* promoter fragment 1 luciferase reporter construct sequence was sourced from (http://www.addgene.org/16591/). Half consensus T-elements sites are highlighted. **(d, e)** TBX3 transcriptionally down-regulates *PUMA* promoter activities. **Upper panels in d and e**: MCF7 cells were co-transfected with 200 ng of the *PUMA* promoter fragment 1 in **(d)** or fragment 2 in **(e)** luciferase reporter constructs and either with human wild-type TBX3 expression construct (100-500 ng) or with the same concentration of the empty pCMV vector. Thirty hours following the transfection, cells were lysed and relative luciferase activity measured. Data was normalized against Renilla values and fold activation was calculated by setting *PUMA* promoter's activity of transfected cells with pCMV empty to 1. A Microsoft Excel student t-test was performed to calculate statistical significance (*p<0.05). Error bars represent standard error of the mean. **Lower panels in d, e**: Increase in levels of WT-TBX3 expression construct transfected corresponded to an increase in TBX3 protein expression. Equal volumes of luciferase lysates were analysed by SDS-PAGE (8%) and western blotting using an antibody to TBX3.

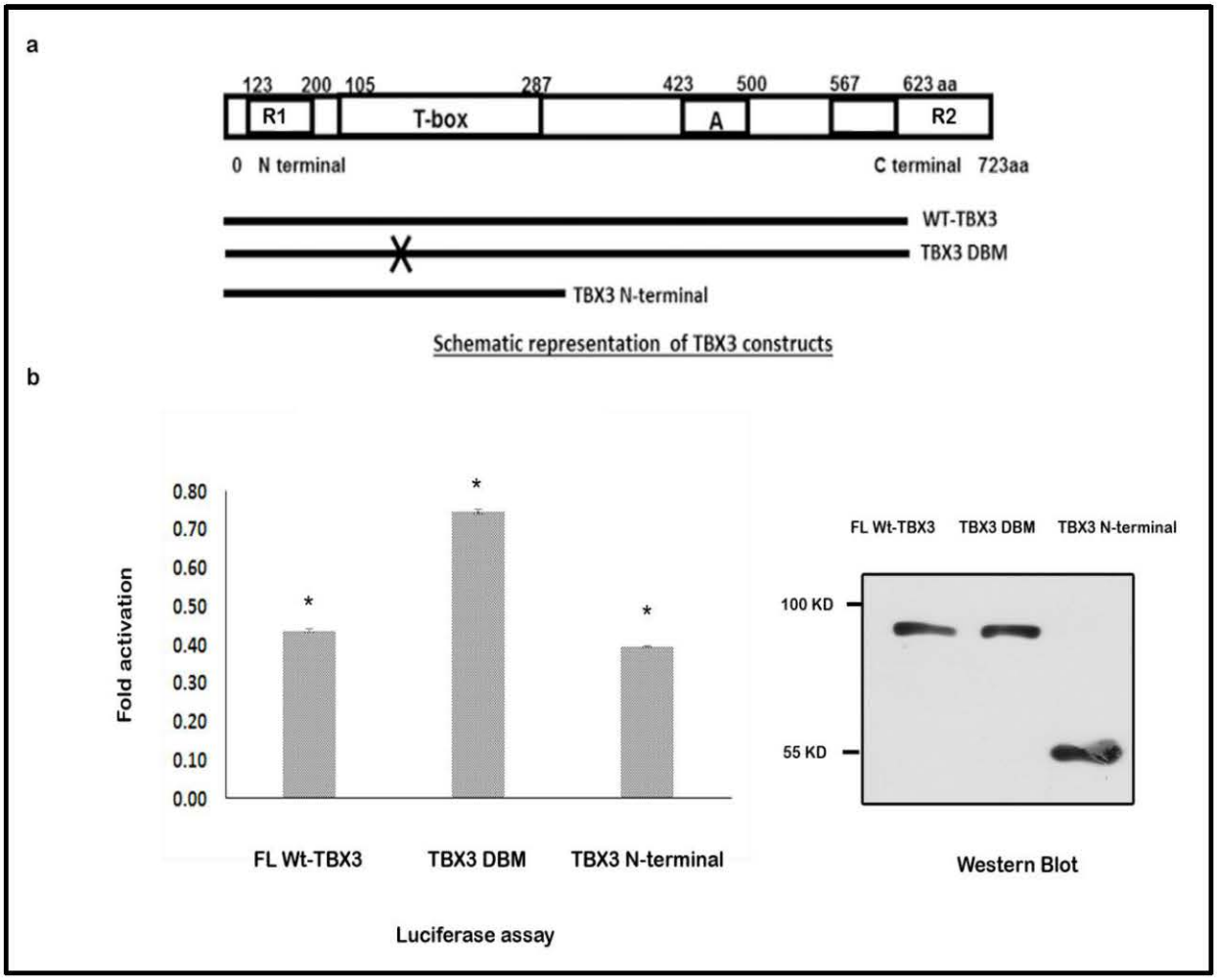


Figure 3.41 The DNA-binding domain and R1 repression domain of the TBX3 protein are required for repression of *PUMA*. (a) Schematic diagram of the human full length (FL) wild-type (WT) TBX3 protein (WT-TBX3), FL TBX3 DNA-binding domain mutant (TBX3 DBM) and TBX3 N terminal (TBX3 N-terminal) expression constructs. "A" and "R" denotes activation and repression domains respectively. **(b)** Left panel: MCF7 cells were co-transfected with 500 ng of *PUMA* promoter fragment 1 luciferase reporter construct together with 200 ng of pCMV empty as a control, 200 ng of WT-TBX3, TBX3 DBM and TBX3 N-terminal expression constructs. Thirty hours following transfection, cells were lysed and relative luciferase activity measured. Total amount of plasmid DNA transfected was held constant using the corresponding empty vector, pCMV. Data was normalised against Renilla values and fold repression was calculated. A Microsoft Excel student t-test was performed to calculate statistical significance (*$p<0.05$). Error bars represent standard error of the mean. Right panel: Western blot showing equal expression of transfected WT-TBX3, TBX3 DBM and TBX3 N-terminal proteins. Luciferase lysates were analysed by SDS-PAGE (8%) and western blotting using a TBX3 antibody.

domain, R2, in the N-terminus (position 123-200) [325]. R1 was previously reported to be the dominant repression domain and to determine if this is indeed the case for the repression of *PUMA* promoter activity, a TBX3 expression construct lacking this domain (referred to as TBX3 N-terminal; see **Figure 3.41a**) was cloned in the Prince Laboratory (see section 2.13). The ability of the TBX3 DBM and TBX3 N-terminal proteins to repress the *PUMA* promoter was compared to WT-TBX3 in luciferase reporter assays as described before. **Figure 3.41b** shows that while WT-TBX3 and TBX3 N-terminal significantly inhibited the *PUMA* promoter, when the DBD was mutated this effect was abrogated. Western blotting confirmed that all three TBX3 constructs tested in this assay were expressed at similar levels and hence loss of the ability of TBX3 DBM to repress *PUMA* was not due to lower levels of expression of this construct. These results suggested that the repression of *PUMA* required direct binding by TBX3 to the PUMA promoter through its DBD.

In addition, these results also suggest that in contrast to what was previously reported by Carlson et al (2001), other domains in the N-terminal portion of the protein, including the R2 domain, may also be playing a significant role in the repression of *PUMA* by TBX3.

3.4.7 TBX3 binds the *PUMA* promoter in vivo

T-box gene family members have been shown to regulate their target genes by binding to a core sequence GGTGTGA referred to as the T-element [326–330]. To determine if the regulation of PUMA by TBX3 is due to direct binding the *PUMA* promoter fragment 1 was screened for putative T-elements and several half consensus T-elements were identified **(highlighted in yellow in Fig. 3.40.a)**. To verify that TBX3 could bind the *PUMA* promoter in

vivo, chromatin immunoprecipitation (ChIP) assays were performed. Briefly, MCF7 cells were fixed with paraformaldehyde and the cross-linked chromatin was extracted and sheared by sonication and TBX3–bound chromatin immunoprecipitated with an anti-TBX3 antibody. After reversing the cross-link, a set of primers spanning the putative T-element shown in **figure 3.42a** in the *PUMA* promoter was used to amplify the immunoprecipitated DNA using qRT-PCR analysis. Importantly, this T-element is highly conserved amongst several species **(Fig. 3.42b)** and was described in another report as a full and perfect T-element [327, 331]. Primers specific to the *GAPDH* coding region were used as a negative control. The result obtained **(Fig. 3.42c)** revealed that relative to the nonspecific IgG control, there was an approximately 6.5 fold enrichment of TBX3 occupancy on the *PUMA* promoter but not the *GAPDH* gene. Together, these data suggest that TBX3 down-regulates the *PUMA* gene directly.

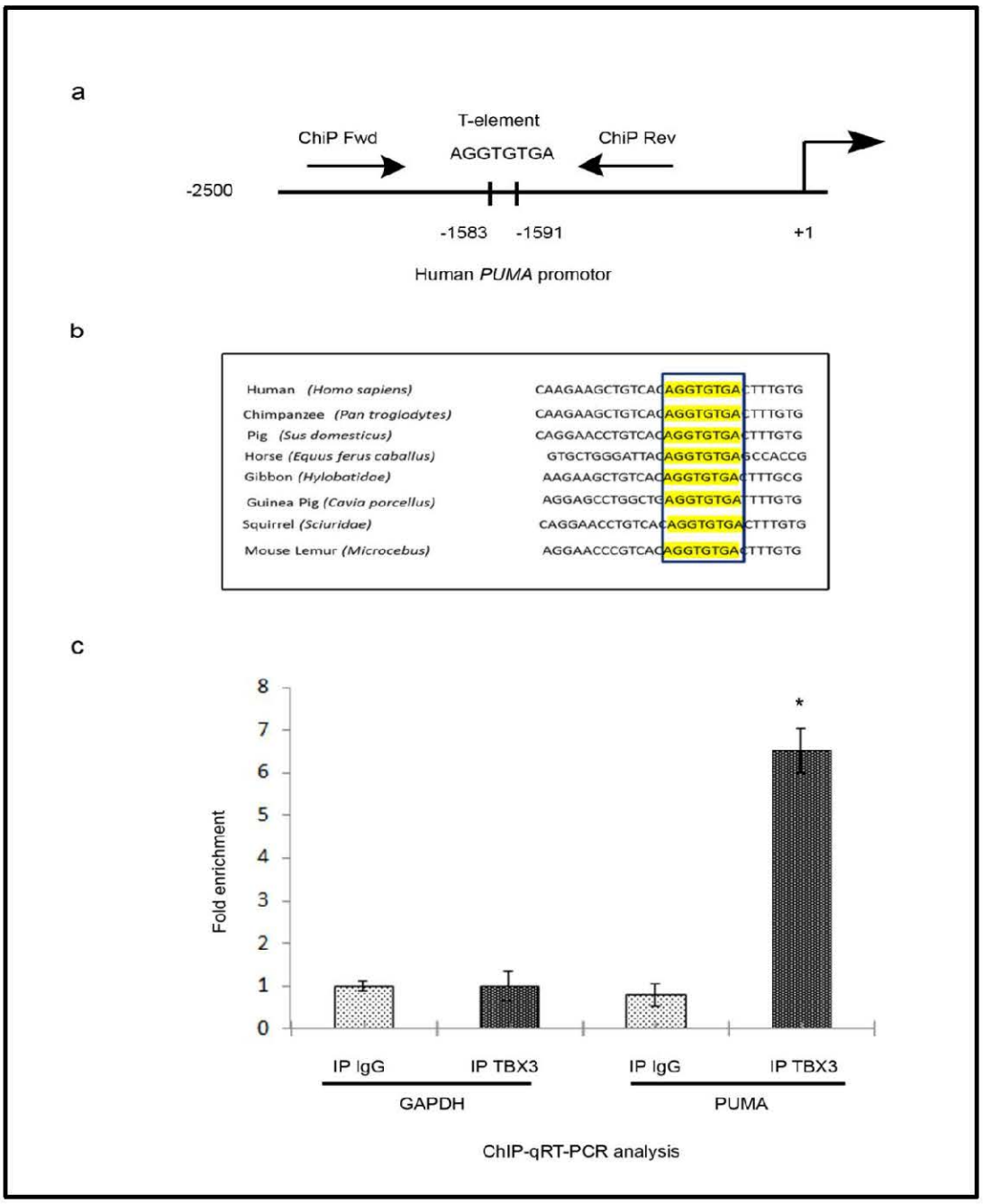


Figure 3.42 TBX3 binds the *PUMA* promoter in vivo. (a) Schematic representation of human *PUMA* promoter showing the T-element identified, forward (ChIP Fwd) and reverse (ChIP Rev) primers sites. **(b)** A full consensus T-element at the human *PUMA* promoter is conserved amongst several species. **(c)** MCF7 cells lysates were used in a ChIP assay performed with antibodies against TBX3 or IgG (negative control). Immunoprecipitated DNA was assayed by qRT-PCR with primers against the *PUMA* promoter or *GAPDH* (negative control). A Microsoft Excel student's *t* test was performed to calculate statistical significance (*p<0.05). The result of one ChIP experiment is shown which is representative of two independent experiments. Error bars = ±SD; * = p value< 0.001.

Chapter 4: Discussion

Cancer is the second leading cause of death worldwide and represents a tremendous emotional and economic burden on individuals and nations. Despite enormous advances in its diagnosis and treatment, deaths from cancer are still projected to rise, with an estimated 13.5 million deaths in 2030 [5]. One approach to dealing with the current cancer burden has been to rationally develop synthetic organometallic complexes as lead anti-cancer compounds. The most commonly used anti-tumour drugs include the platinum coordination complex, cisplatin (CDDP), and its analogues carboplatin and oxaliplatin. However the side effects and multi-drug resistance associated with this line of therapy necessitate the development of more efficient anti-tumour therapeutic drugs. Recently palladium Pd(II) complexes have attracted a lot of interest as chemotherapeutic agents because they were shown to exert anti-tumour activity in CDDP resistant cells and to have fewer side effects than CDDP [78, 332]. This study has screened synthetic palladacycles for selective cytotoxic activity in breast cancer and melanoma cells and identified a novel binuclear palladacycle, AJ-5, as an early lead compound. Its potential is demonstrated by observations that AJ-5 may be an effective drug in the treatment of estrogen receptor (ER)-positive and ER-negative breast cancer as well as advanced melanoma for which there is currently no effective treatment [333]. Furthermore this study demonstrates that AJ-5 induces double strand DNA damage breaks which triggers the ATM-CHK2 pathway and that the cytotoxic effect of AJ-5 is mediated by p38 and ERK1/2 MAPK signalling pathways leading to a p21-mediated cell cycle arrest and both apoptosis and autophagy. Importantly, these results were confirmed in melanoma-bearing nude mice without any noticeable side-

effects [333]. This study provides several lines of evidence that the binuclear palladacycle, AJ-5, holds a lot of promise as a novel chemotherapeutic drug to treat melanoma and breast cancers.

4.1 AJ-5 is an effective drug against estrogen receptor positive and triple negative breast cancer cells

Breast cancer continues to be the leading cause of cancer deaths among women and its treatment is constantly evolving as new technologies, drugs, and strategies are discovered [334]. The choice of therapy to treat breast cancers are, in part, guided by the status of the estrogen (ER), progesterone (PgR) and HER2 receptors [199]. Approximately 70% of breast cancers are classified as ER and/or PgR positive, which is a predictive tool for enhanced survival out-comes and responsiveness to endocrine therapies. The most widely used selective ER modulator is tamoxifen which inhibits proliferation by modulating the ER receptors on breast cancer cells [335, 336]. More recently tamoxifen has also been shown to target mitochondria to induce non-apoptotic cell death [335].

The second common hormone based therapy for ER-positive breast cancer is the aromatase inhibitors such as anastrazole and letrozole [334]. These compounds target the enzyme aromatase, which is responsible for the conversion of androgen to estrogen [337]. While the efficient dose of tamoxifen to inhibit proliferation of MCF7 cells is approximately 5-7 μM, it has been shown that 1 μM anastrazole inhibits the proliferation of MCF7 cells significantly [335, 338, 339]. Despite the verified efficacy of tamoxifen and aromatase inhibitors, acquired resistance and side effects constitute a major obstacle with their use. While aromatase

inhibitors are usually associated with increased bone loss, tamoxifen treatment causes hot flushes, vaginal dryness, sleep problems and weight gain [257, 340]. The current study shows that AJ-5 induces a strong cytotoxic effect in MCF7 cells at concentrations lower (IC_{50}=0.17 µM) than tamoxifen and anastrazole. Importantly, "normal" melanocytes and fibroblasts were less sensitive to AJ-5 with an IC_{50} higher than 0.4 µM. Furthermore, AJ-5 showed potent cytotoxicity against breast cancer stem cells (BCSCs) where low concentrations of AJ-5 reduced the proportion of $CD44^{hi}CD24^{lo}$ cells in MCF7 cells and decreased the number, size and viability of MCF7 mammospheres. This is particularly important because BCSCs seem to be a basis for multidrug resistance due to their ability to escape from chemotherapeutic cytotoxicity [269]. These observations are in agreement with results from a very recent study showing that a novel Pd(II) compound named Pd-BENSpm is able to reduce the $CD44^{hi}CD24^{lo}$ population in JIMT-1 breast cancer cells [341].

AJ-5 was also shown to be very effective against the MDA-MB231 triple negative breast cancer cells (TNBC). Increasing frequency of TNBC has been shown amongst breast cancer patients with American and African ancestry [342]. Because of the lack of targeted therapies, patients with TNBC have an extremely poor prognosis and relapse and die quickly [343]. Although initial responses to chemotherapies such as anthracycline, CDDP, and carboplatin have been reported in patients with TNBC, a high risk of relapse still remains. Therefore, there is an urgent need for novel chemotherapeutic agents that target this subtype of cancer and AJ-5 may be an effective agent in the treatment of this highly aggressive cancer.

4.2 AJ-5 is a potent cytotoxic agent in advanced melanoma cell lines

Malignant melanoma is an aggressive skin cancer and its incidence is increasing worldwide faster than any other cancer [217]. Presently less than 10% of patients diagnosed with metastatic melanoma have a 10-year survival rate [216]. While surgery is a successful treatment for primary melanomas the treatment of metastatic melanoma continues to be a challenge and indeed it is reported to be the most treatment-resistant human cancer [344]. Currently, systemic therapy remains the mainstay treatment for advanced stages of melanoma [216, 345]. For example, dacarbazine, an alkylating agent was approved to treat human metastatic melanoma since the 1970s. Although the response rates to dacarbazine are poor, it continues to be a drug of choice for many melanoma patients [346]. Furthermore, dacarbazine treatment is associated with many side effects which include nausea and vomiting, myelosuppression and fatigue and these side effects are escalated at the more effective doses [347]. The alkylating drugs CDDP and temozolomide have also been used as chemotherapeutic agents to treat metastatic melanoma but they have shown only a small advantage over dacarbazine [348]. Indeed, when used in combination with interferon alpha 2b (IFN·2b) as adjuvant therapy the median survival time for 37 patients enrolled in a clinical trial was only 48 weeks. There is therefore clearly a need to identify anti-cancer drugs that exhibit potent cytotoxicity at low doses in advanced melanoma.

The current study shows that compared to normal control cells, the binuclear palladacycle, AJ-5, displays potent cytotoxic activity in the ME1402 vertical growth phase (VGP) and WM1158 metastatic melanoma cell lines. Importantly, AJ-5 displayed an IC_{50} of approximately 0.2 µM for

the two advanced melanoma cell lines tested which was 50 fold less than that obtained for CDDP. These results suggest that at a much lower concentration AJ-5 is as effective as CDDP at killing melanoma cells and therefore that it may present with reduced side effects. Indeed, the in vivo results show that nude mice treated with AJ-5 did not display any obvious side effects as they did not show any weight loss or behavioural changes throughout the duration of the study. Furthermore, the high degree of cytotoxicity displayed by AJ-5 against the WM1158 is very encouraging given that metastatic melanoma cells are notoriously difficult to treat and have been shown to be resistant to CDDP and other DNA damaging agents [246].

4.3 AJ-5 triggers the intrinsic and extrinsic apoptotic pathways

While most chemotherapeutic agents are known to induce the intrinsic apoptotic pathway, this study shows that AJ-5 induces markers of both the intrinsic (up regulation of PUMA, Bax, down regulation of BCL-2 and releasing cytochrome c from the mitochondria) and extrinsic (active caspase 8) pathways. This is particularly interesting because resistance to many chemotherapeutic drugs results from defects within the intrinsic apoptotic pathway and the development of drugs that target the extrinsic pathway may circumvent this problem [102, 349, 350]. Furthermore, increased expression of anti-apoptotic BCL-2 family proteins is associated with drug resistance and poor clinical outcome and the results from this study show that AJ-5 is able to significantly inhibit BCL-2 in all breast cancer and melanoma cell lines tested [351]. It is worth noting that CDDP treatment, on the other hand, led to an increase in BCL-2 levels in WM1158 cells after 48 hours which may suggest a possible reason why AJ-5 was more effective than CDDP in these cells. These observations are consistent with results from other studies in

which WM1158 and BML metastatic melanoma cell lines were treated with CDDP [113, 246]. The effect of AJ-5 on BCL-2 levels is exciting in light of different strategies being proposed for targeting BCL-2 and its related anti-apoptotic proteins in the treatment of cancers.

4.4 AJ-5 induces autophagic cell death

Several currently used chemotherapies including doxorubicin, 5-fluorouracil (5-FU), CDDP, imatinib roscovitine have been shown to induce both apoptosis and autophagy in several different cancer cells [166, 352–357]. There is, however, a controversy as to whether the activation of autophagy in response to anti-cancer therapies promotes or inhibits cell death. Autophagy is a catabolic process involving the degradation and recycling of macromolecules and organelles and is considered a survival mechanism which is triggered under conditions of cellular stress [120]. A number of studies have also shown that autophagy serves as a survival mechanism in cancer cells and that it antagonizes or delays apoptosis in response to chemotherapeutic agents [292]. For example, inhibition of autophagy induced by 5-FU, camptothecin, ursolic acid and CDDP enhanced apoptotic cell death in several cancer cells [354, 358–361].

There is however also a growing body of evidence suggesting that autophagy can result in a mode of cell death called autophagic or type II cell death. It has been shown that 5-FU, arsenic trioxide (As2O3) and paclitaxel induce cytotoxicity in many cancer types (colon, acute promyelocytic leukemia, glioma and ovarian cancer) which is associated with markers of autophagy and that blocking autophagy reversed the cytotoxic effect of these drugs [211, 279,

280, 352, 362–366]. Furthermore, a study by Tomic et al (2011) showed that knocking down LC3II in A375 metastatic melanoma cells decreased metformin induced apoptosis. Interestingly, when apoptosis was inhibited in the same study it failed to affect LC3II expression mediated by metformin [296]. The results from the current study reveal that the inhibition of autophagy by several mechanisms significantly decreased the level of PARP cleavage and AJ-5 induced cell death. This suggests that autophagy induced by AJ-5 is a cell death mechanism and supports the theory that whether autophagy functions as a cell survival or cell death mechanism depends on the cancer cell type and/or the chemotherapy used.

4.5 AJ-5 inhibits the mTOR pathway

AKT/mTOR signalling is a survival pathway and one of the major pathways activated in melanoma cells and several reports have shown that it accelerates resistance to chemotherapeutic agents. Indeed, clinical reports showed that resistance to the melanoma MEK inhibitor, AZD6244, was mediated by the activation of AKT and inhibition of AKT or its target TORC1/2, resulted in cell death [367]. Similar findings were also observed after treating melanoma cells with vemurafenib (a BRAF inhibitor) and silencing AKT in combination with this therapy completely reversed the resistance [368]. It has been proposed that inhibitors of the AKT/mTOR pathway may be a successful strategy to treating melanoma [369]. The current study shows that AJ-5 strongly inhibited the AKT/mTOR pathway in advanced melanoma cells as demonstrated by the decrease in AKT, mTOR and p70S6 proteins levels which underscores its potential value in the treatment of this aggressive cancer.

Several breast cancer cell lines resistant to doxorubicin, trastuzumab and tamoxifen were also associated with high levels of AKT expression and inhibition of the AKT pathway sensitized them to these chemotherapies [370, 371]. Interestingly, the current study shows that AJ-5 does not inhibit AKT activity in the breast cancer cell lines tested but after 6 to 12 hours of treatment the levels of mTOR and its target p70S6 were reduced leading to autophagy in these cells. It would therefore appear that in the MCF7 and MDA-MB231 breast cancer cell lines AJ-5 compromises the AKT/mTOR pathway by directly regulating mTOR and/or its downstream targets.

4.6 AJ-5 cytotoxicity is mediated by the MAPKs

Investigations into the mechanisms by which chemotherapeutic drugs kill tumour cells have provided invaluable insight into the molecular basis of not only tumour cell death but also of cell death in general. This information has important implications for the development of chemotherapies that specifically target the machinery that regulate cell death. Here we show that AJ-5 induces both apoptosis and autophagy, which are distinct processes but have substantial overlap in the signalling pathways connecting them. Interestingly, while AJ-5 activates the ATM-CHK2 pathway, a key player in mediating both autophagy and apoptosis in response to DNA damage, inhibiting this pathway did not abrogate the effect of AJ-5 on either one of these two processes (data not shown). Importantly, the results reveal that AJ-5 activates the p38 and ERK MAPK pathways which are responsible for mediating AJ-5-induced apoptosis and autophagy. This is consistent with reports indicating that the MAPK pathway plays an important role in the DNA damage response and that it can activate the autophagic and apoptotic responses [44, 45]. Inhibition of p38, JNK and ERK MAPKs in different cell lines has

been shown to prevent apoptosis induced by CDDP and other treatments [217, 369]. Importantly, in contrast to these data another study showed that inhibition of MEK signalling pathway sensitized malignant melanoma cells to high concentrations of CDDP, as shown by the increasing levels of markers for both intrinsic and extrinsic apoptosis [113]. Furthermore, inhibition of p38 MAPK has been shown to reduce LC3II protein levels in response to capsaicin-induced autophagy [149] and many studies have confirmed that an increase in the levels of LC3II in response to chemotherapeutic agents are downstream of ERK [372, 373]. It is worth noting that there is evidence to suggest that the cellular response to chemotherapeutic agents depends on the kinetics of p38 MAPK activation [44]. For example, while short activations of the pathway lasting for up to 3 hours have been linked to CDDP resistance, sustained activation of the pathway for 8-12 hours has been associated with cellular sensitivity to this drug [45]. The current results show that AJ-5 activates p38 for up to 48 hours in the melanoma and breast cancer cells tested in this study suggesting that AJ-5 is a potent inducer of this pathway which is likely to ensure its cytotoxicity in these cells.

4.7 AJ-5 induced apoptosis is p53 independent

Several studies have shown that wild-type p53 is a key component of the anti-cancer response induced by chemotherapy and chemo-resistance has been shown to be associated with mutations in the p53 gene [374–377]. This is not surprising since in response to a number of cellular stresses p53 is an important mediator of apoptosis. For example, compared with breast cancer cells that express wild-type p53, those that express mutant p53 have been shown to be more sensitive to the chemotherapeutic drug, epirubicin [378]. The current study showed that

AJ-5 induces p53 in MCF7 cells which express wild-type p53 and this matched the AJ-5 induced apoptosis and autophagy in these cells. However, AJ-5 was also able to induce high levels of apoptosis and autophagy in the MDA-MB-231 cells which express mutant p53 suggesting that AJ-5 is able to kill breast cancer cells independent of p53 status. This is consistent with a very recent study which investigated the possible role of p53 in mediating apoptosis induced by a novel Pd(II) saccharinate complex in MDA-MB-231 and HeLa cell lines [379]. The study showed that knocking down p53 in both cell lines didn't rescue the cell death induced by this drug indicating that p53 is not essential for apoptosis induced by this compound.

There is also a controversy as to whether p53 is required for melanoma sensitivity to chemotherapy [287]. A study by Li et al. (1998) compared the response of four p53 wild-type and four p53 mutant melanoma cell lines to CDDP, camptothecin and vincristine and demonstrated that sensitivity to these chemotherapeutic agents required wild-type p53. Similar observations were reported for camptothecin in the wild-type p53 melanoma cell line, CRL-9607, compared to the p53 mutant melanoma cell line, SK-MEL-28 [380]. Furthermore ectopic expression of wild-type p53 in SK-MEL-28 cells significantly enhanced apoptosis in response to camptothecin. The current study also shows that AJ-5 treatment induces p53 and its target p21 protein in both ME1402 and WM1158 advanced melanoma cell lines. However, while the increasing levels of p21 level followed the up-regulation of p53 in ME1402 cells it preceded it in WM1158 cells. Furthermore knocking down p53 in both melanoma cell lines did not affect the AJ-5 induced p21 response and apoptosis. These results suggest that AJ-5 induced cell death in these melanoma cell lines were independent of p53. This is consistent with another study in

which eight melanoma cell lines expressing either wild-type or mutant p53 were treated with temozolomide [381]. The authors show that the p53 mutant melanoma cell lines were more sensitive to the treatment and more recently reported that functional p53 may confer resistance to chemotherapeutic drugs by activating efficient DNA damage repair and thus avoiding cell death [40]. Furthermore, previous studies have shown that CHK2 activated by DNA damage can induce p21 transcription in the absence of functional p53 and that this contributes to CHK2-mediated cell cycle arrest and apoptosis [382]. Interestingly, AJ-5 treatment activated CHK2 in all breast cancer and melanoma cells tested in this study and this matched the activation of p21 by AJ-5. Based on these findings it is tempting to speculate that AJ-5 induced cytotoxicity is p53 independent and that it is mediated by the CHK2-p21 pathway.

4.8 Knocking down TBX3 in a TBX3-driven breast cancer cell line sensitises them to AJ-5 treatment

The highly homologous TBX3 protein, a member of T-box family, has been implicated in the progression of a number of cancers including a subset of breast cancers [301, 302, 309, 383]. Tbx3 overexpression has been shown to be sufficient to immortalize mouse embryonic fibroblasts in a process involving the repression of the cell cycle regulator $p19^{ARF}$ [384, 385]. Two studies later showed that TBX3 interacts with histone deacetylases to repress $p14^{ARF}$, the human homolog of $p19^{ARF}$, in human breast cancer cells where it is overexpressed [386, 387]. Our laboratory has also previously reported that TBX3 is required for substrate independent proliferation and migration of MCF7 breast cancer cells [302]. The current thesis show that knocking down TBX3 led to the sensitisation of the MCF7 breast cancer cells to AJ-5. In

particular, depleting TBX3 in MCF7 cells resulted in an increase in apoptotic and autophagic cell death. This is in line with the results from a previous study which showed that TBX3 is overexpressed in a subset of drug resistant hepatoblastomas and that its inhibition enhanced doxorubicin-induced apoptosis [308]. Furthermore, Tbx3 was shown to suppress apoptosis by preventing the induction of p53 and its target $p19^{ARF}$ in mouse embryo fibroblasts overexpressing Myc and Ras [388]. Interestingly, the current study demonstrated that while knocking down TBX3 had no effect on p53 levels, it increased the levels of the pro-apoptotic protein, PUMA. This is particularly important because PUMA has been shown to play a significant role in apoptosis by activating Bax and mitochondrial outer membrane permeabilization [389]. In addition, PUMA has been reported to induce autophagy which leads to cytochrome c release and apoptosis [390]. In light of these observations, the current study explored the possibility that TBX3 may be inhibiting the apoptotic and autophagic response to AJ-5 by repressing *PUMA*. Using in vitro and in vivo assays, PUMA is indeed shown to be a novel direct target of TBX3. Furthermore, the ability of TBX3 to repress the PUMA promoter is demonstrated to involve the TBX3 N-terminal repression- and DNA binding- domains. These results are consistent with previous reports that TBX3 exerts its anti-apoptotic effect mainly by transcriptionally repressing genes such as $p19^{ARF}$ and suggest that TBX3 may inhibit apoptosis through a p53-dependent and –independent manner [388].

Several studies have proposed that TBX3 may be a substrate of the PI3K/ AKT pathway which, as mentioned earlier, has been implicated in chemo-resistance in several cell lines including the MCF7 breast cancer cell line [315–322]. In agreement with these reports, the current study

showed that inhibition of AKT resulted in TBX3 down-regulation, PUMA up-regulation and augmentation of cell death. Data from the current study thus confirm an anti-apoptotic function for TBX3 and provide evidence that it may exert this function by a mechanism involving the direct transcriptional inhibition of PUMA. Importantly, the data provide compelling evidence in support of targeting TBX3 in combination with AJ-5 as a viable option for treatment of TBX3-driven breast cancers.

4.9 Concluding remarks

This study describes the anti-tumour activity of AJ-5, a novel binuclear palladacycle complex and is the first study to provide a detailed mechanism by which a palladium-based compound exerts cytotoxicity in cancer cells. Based on the data generated from the in vitro and in vivo experiments of the anticancer activity of AJ-5, the following model is proposed **(Fig. 4.1)**. AJ-5 initially induces DNA double strand breaks which activates different molecular pathways including MAP kinases (p38 and ERK1/2) and p21 leading to cell cycle arrest and both intrinsic and extrinsic apoptosis. Furthermore, AJ-5 also activates autophagic cell death through mechanisms involving activation of the same MAP kinases and inhibition of AKT/mTOR. This study suggests that AJ-5 may be an effective chemotherapeutic drug in the treatment of, at the very least, ER positive and triple negative breast cancers as well as advanced melanoma.

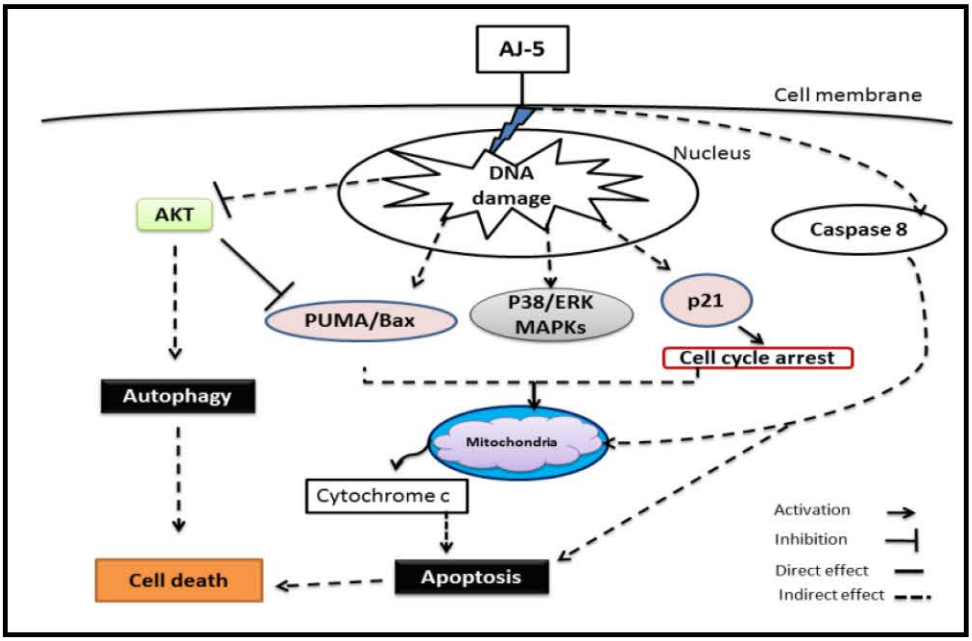


Figure 4.1 a proposed model for the mechanism by which AJ-5 exerts its anti-cancer activity.

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Chapter 6: Appendix

6.1 Mycoplasma Test

Mounting fluid

20 mM citric acid

55 mM $Na_2HPO_4.2H_2O$

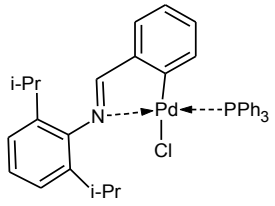
50% Glycerol

pH to 5.5 and store at 4°C

6.2 Palladium based compounds

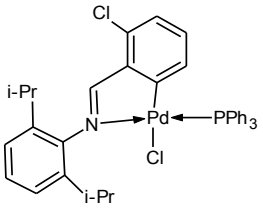
Mononuclear based compounds

AJ-1



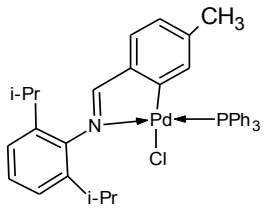
Molecular Formula: $C_{37}H_{37}ClNPPd$

AJ-2



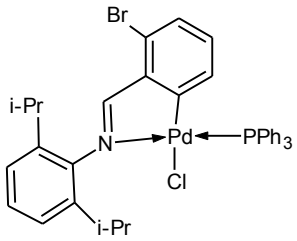
Molecular Formula: $C_{37}H_{37}Cl_2NPPd$

AJ-3



Molecular Formula: $C_{38}H_{39}ClNPPd$

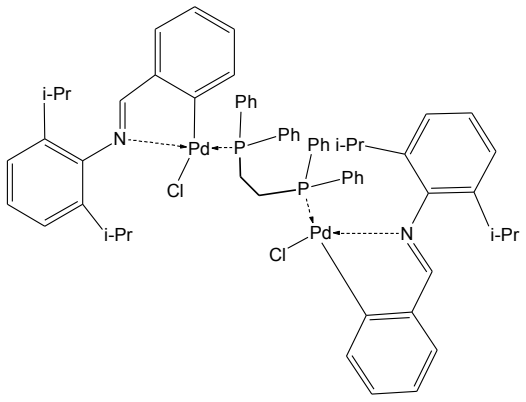
AJ-4



Molecular Formula: $C_{37}H_{36}BrClNPPd$

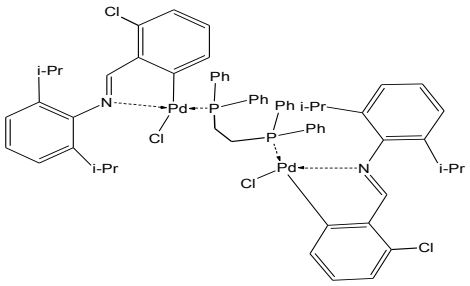
Binuclear based compounds

AJ-5



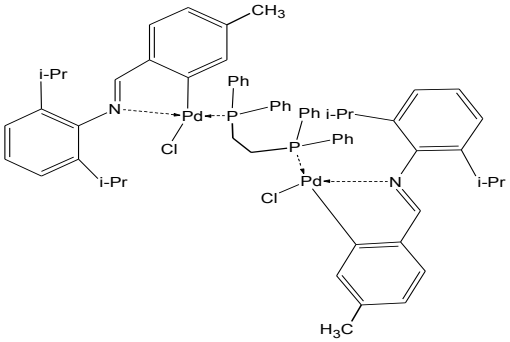
Molecular Formula: $C_{64}H_{68}Cl_2N_2P_2Pd_2$

AJ-6



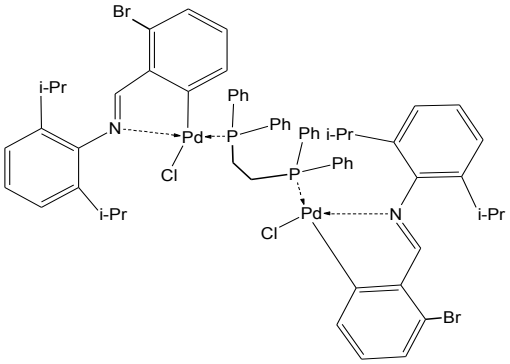
Molecular Formula: $C_{64}H_{66}Cl_4N_2P_2Pd_2$

AJ-7



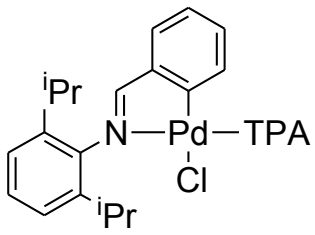
Molecular Formula: $C_{66}H_{72}Cl_2N_2P_2Pd_2$

AJ-8



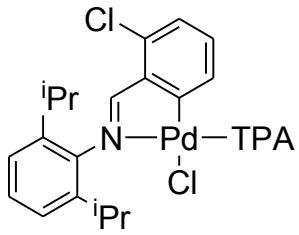
Molecular Formula: $C_{64}H_{66}Br_2Cl_2N_2P_2Pd_2$

C5



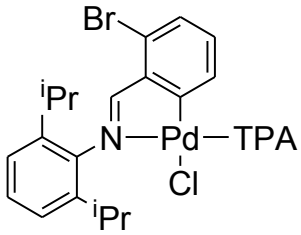
$[Pd(TPA)(C_6H_4)CH{=}N\{2,6\text{-}^{i}Pr_2\text{-}C_6H_3\}Cl]_2$

C6



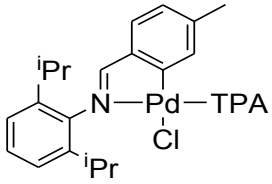
$[Pd(TPA)(2\text{-}Cl\text{-}C_6H_3)CH{=}N\{2,6\text{-}^{i}Pr_2\text{-}C_6H_3\}Cl]_2$

C7



$[Pd(TPA)(2\text{-}Br\text{-}C_6H_3)CH{=}N\{2,6\text{-}^{i}Pr_2\text{-}C_6H_3\}Cl]_2$

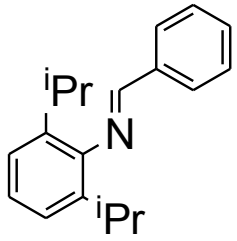
C8



$[Pd(TPA)(4\text{-}Me\text{-}C_6H_3)CH{=}N\{2,6\text{-}^{i}Pr_2\text{-}C_6H_3\}Cl]_2$

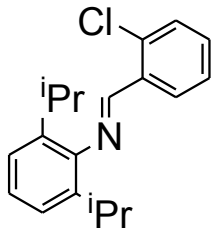
Ligands used in the synthesis of palladium compounds

L1



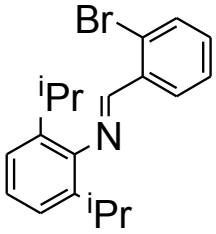
Benzylidene-2,6-diisopropylphenylamine

L2



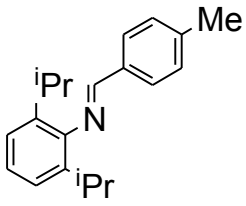
2-chlorobenzylidene-2,6-diisopropylphenylamine

L3



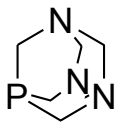
2-bromobenzylidene-2,6-diisopropylphenylamine

L4



4-methylbenzylidene-2,6-diisopropylphenylamine

TPA



1,3,5-triaza-7-phosphaadamantane

6.3 Flow Cytometry

Propidium Iodide solution
2 mM $MgCl_2$
10 mM Pipes buffer
0.1 M NaCl
0.1% Triton X-100
0.01 mg/ml Propidium iodide

6.4 Immunofluorescence

Paraformaldehyde (4%)
4 g paraformaldehyde
Dissolve in 100 ml PBS at 60°C, allow to cool and store at -20°C

6.5 Electron microscopy

0.2M Sorenson's Phosphate buffer: Stock solution
(A) 0.2M dibasic sodium phosphate ($Na_2HPO_4*2H_2O$)
(B) 0.2M monobasic sodium phosphate ($NaH_2PO_4*H_2O$)

2.5% Glutaraldehyde fixative
0.2M Sorenson's Phosphate buffer pH7.4 (stock)
25% Glutaraldehyde
dH_2O

6.6 Western Blot analysis

2X Laemmli sample buffer
4% SDS
20% glycerol
0.004% bromphenol blue
0.125 M Tris HCl, pH 6.8

10% β-mercaptoethanol

RIPA

150 mM NaCl
1% Triton X-100
0.1% SDS
20 mM Tris (pH 7.5)
1% deoxycholate
Protease inhibitors added prior to harvesting: 1X complete protease inhibitor tablets (Roche, Germany), aprotinin (1 µg/ml), pepstatin (1 µg/ml), phenylmethanesulphonyl fluoride (PMSF) (0.5 mM)

Sodium Dodecyl Sulphate (SDS)-polyacrylamide gels

Resolving gel:

Acryl-bisacryl-amide mix (30:08) (percentage depending on size of protein of interest)
0.375 M Tris (pH 8.8)
0.1% SDS
0.1% TEMED
0.1% Ammonium persulphate

Stacking gel:

5% Acryl-bisacryl-amide mix (30:08)
0.192 M Tris (pH6.8)
0.1% SDS
0.1% TEMED
0.1% Ammonium persulphate

Acryl-bisacryl-amide mix (30:08):

29 g acrylamide
1 g N,N`-methylenebisacrylamide
Make up to 100 ml, heating at 37°C to dissolve chemicals. Store at 4°C, protected from light

Running buffer:

1 g SDS
3.03 g Tris
14.41 g Glycine
Make up to 1 liter

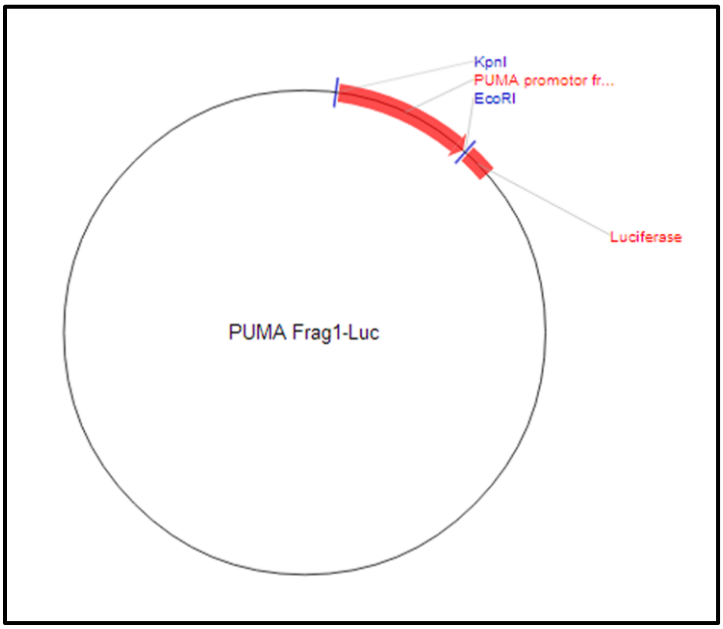
Transfer buffer:

2.9 g Glycine
5.8 g Tris
0.37 g SDS
200 ml isopropanol
Make up to 1 liter and store at 4°C.

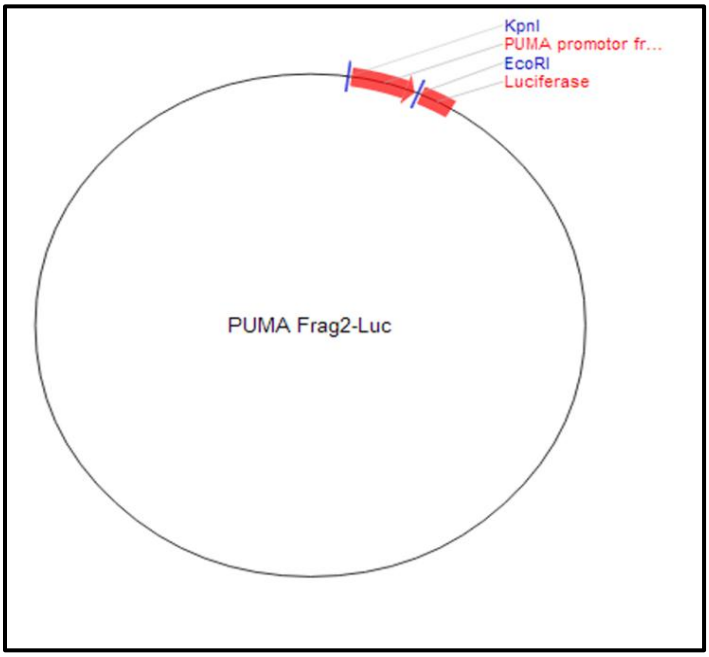
Stripping buffer

62.5 mM Tris-HCl (pH6.7)
2% SDS
100 mM β-mercaptoethanol

6.7 Plasmids and DNA constructs



PUMA Frag1-Luc



PUMA Frag2-Luc

6.8 Chromatin immunoprecipitation (ChIP) assay

Buffer 1
10 mM EDTA
0.5 mM EGTA
10 mM Hepes,
0.25% Triton X-100

Buffer 2
1 mM EDTA
0.5 mM EGTA
10 mM Hepes
200 mM (NaCl)

Lysis buffer
10 mM EDTA
50 mM Tris-Cl pH 8.1
0.5% Nonidet P-40
1% SDS

Immunoprecipitation Buffer
2 mM EDTA
150 mM NaCl
20 mM Tris-Cl pH 8.1
1% Triton X-100

Wash buffer 1
2 mM EDTA
20 mM Tris-Cl pH 8.1
0.1% SDS
1% Triton X-100
150 mM NaCl

Wash buffer 2
2 mM EDTA
20 mM Tris-Cl pH 8.1
0.1% SDS
1% Triton X-100
500 mM NaCl

Wash buffer 3
1 mM EDTA
10 mM Tris-Cl pH 8.1
250 mM LiCl
1% sodium deoxycholate
1% Nonidet P-40

Wash buffer 4
1 mM EDTA
10 mM Tris-Cl pH 8.1

Extraction buffer
100 mM $NaHCO_3$
1% SDS