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

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ABSTRACT

Breast cancer is the most common malignancy amongst women worldwide but despite enormous efforts to address this problem, there is still limited success with most of the current therapeutic strategies. The current study describes the anti-cancer activity of a binuclear palladacycle complex (AJ-5) in oestrogen receptor positive (MCF7) and oestrogen receptor negative (MDA-MB-231) breast cancer cells as well as human breast cancer stem cells. AJ-5 is shown to induce DNA double strand breaks leading to intrinsic and extrinsic apoptosis and autophagy cell death pathways which are mediated by the p38 MAP kinase. This study provides evidence that AJ-5 is potentially an effective compound in the treatment of breast cancer.

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Introduction

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 lifetime. More than 70% of human breast cancers (BCs) are hormone-dependent and approximately 15% are hormone-receptor-negative which includes BCs lacking oestrogen receptor (ER) expression. The most commonly used anti-tumour drug for treating ER-positive BCs is tamoxifen which functions by blocking oestrogen receptors. Approximately 30% of breast cancer patients, however, fail to respond to endocrine therapy including tamoxifen and many patients eventually develop resistance to endocrine therapy. Increasing evidence suggests that breast cancer stem cells (BCSCs), a small subset of cells with the cell surface marker signature CD44+CD24−, play a major role in this resistance. 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.

Recently palladium (Pd) complexes have attracted a lot of interest as chemotherapeutic agents. While early palladium based compounds showed little anti-tumour activity due to poor stability, the use of stabilising ligands has improved the efficacy of these compounds. 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. Importantly, this palladium compound was more effective against the ER-negative cell line MDA-MB-231 than the ER-positive MCF7 cell line. This is particularly significant because ER-negative breast cancers are notoriously unresponsive to current treatments. Furthermore, we recently reported on the anti-tumour activity of a novel binuclear cyclometalated Pd(II) complex [Pd(C^N)Cl(dppe)], AJ-5, in advanced human melanoma cells. Using in vitro and in vivo nude mice studies we showed that AJ-5 induced cell death by both apoptosis and autophagy at very low concentrations (IC50 0.2 μM) while having negligible effects on normal cells. Our results showing that AJ-5-induced autophagy contributes to cell death suggest that it may be effective in the treatment of breast cancer because autophagy induced by tamoxifen has been described as an efficient cell death mechanism. The current study therefore tested this in the ER-negative MDA-MB-231 and ER-positive MCF7 breast cancer cells.
Our results demonstrate that AJ-5 exhibits robust anti-tumour effects in both cell lines due to a G1 cell cycle arrest and cell death by intrinsic and extrinsic apoptosis and autophagy. We provide a detailed molecular mechanism involving the p38 MAP kinase by which AJ-5 exerts this anti-tumour activity. Moreover, we show that AJ-5 is effective against breast cancer stem cells as demonstrated by the decrease in the number of CD44<sup>hi</sup>CD24<sup>lo</sup> cells and inhibition of mammosome formation. Together these results provide strong support for the potential use of palladium complexes as anti-cancer drugs and in particular that AJ-5 holds lots of promise for the treatment of ER-positive and -negative breast cancers.

### Materials and methods

#### Cell culture and treatments

The normal fibroblast cell lines FGO, DNB and human breast adenocarcinoma MDA-MB-231 (triple negative) were maintained in DMEM (Highwold Biological, Lyndhurst, United Kingdom (UK)). Human breast adenocarcinoma MCF7 (oestrogen receptor positive) cell lines were maintained in RPMI 1640 (Highwold Biological, Lyndhurst, UK). All media were supplemented with 10% foetal bovine serum (FBS), 100U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37 °C in a 5% CO₂–95% air-humidified incubator. Autophagy inhibitors bafilomycin A (10 nM) and wortmannin (1 μM) (Sigma, St. Louis, MO, USA) and p38 inhibitor SB203580 (10 μM) (Calbiochem, USA) were added 1 hour prior to treatment with AJ-5.

#### Cytotoxicity assays

AJ-5 was dissolved in DMSO to give a stock concentration of 5 mM and stored at room temperature for no more than 7 days. Cells (3 × 10<sup>3</sup>–6 × 10<sup>4</sup>) were seeded in 96-well plates and two days later treated with a range of AJ-5 concentrations (0–10 μM) or vehicle for 48 hours. Cytotoxicity of this compound was assessed by MTT assay (Roche, USA) [20]. Absorbance (585 nm) was determined and the mean cell viability was calculated as a percentage of the mean vehicle control.

#### Clonogenic survival assays

Cells were pre-cultured and treated with the indicated concentrations of AJ-5 for 24 hours, washed, collected and re-plated at 1000 cells per well in 6-well plates. Cells were allowed to grow for 14 days and surviving cells were fixed and stained with crystal violet (Sigma-Aldrich, USA).

#### Cell cycle measurements

Log-phase cells were exposed to AJ-5 (0.1 and 0.2 μM) or vehicle for 24 hours and 48 hours and then trypsinised, washed with PBS and fixed in 95% ethanol at 4 °C overnight followed by RNase treatment and propidium iodide (PI) staining. The DNA content was analysed by flow cytometry with a 488 nm Coherent laser. The CellQuest Pro version 5.2.1 software was used for data acquisition and analyses performed using Modfit version 2.0 software.

#### Detection of apoptosis

Log-phase cells were exposed to AJ-5 (0.1 and 0.2 μM) or vehicle for 24 hours and 48 hours and both adherent and floating cells were collected and double-labelled with Annexin V-FITC and PI (Sigma, St. Louis, MO, USA). Annexin V conjugated to FITC was used to quantitatively determine the percentage of cells in the population that are undergoing apoptosis and PI was used to stain all dead cells. Cells were analysed by flow cytometry with a 488 nm Coherent laser equipped with FACStation running CellQuest software (Becton Dickinson, South San Francisco, CA).

#### Cytochrome c release assay

Cells were grown on glass coverslips and treated with 0.2 μM AJ-5 or vehicle for 24 hours and stained with mitochondria green (Invitrogen, M7514, UK) according to the manufacturer’s instructions. The cells were then fixed and incubated with the cytochrome c antibody (sc-13560, Santa Cruz, California, USA) (1:500) at 4 °C in a humidifying chamber. After 16 hours cells were washed and incubated with the Cy3-conjugated goat anti-mouse secondary antibody (1:1000) (Jackson Immunoresearch Laboratories, Inc., USA) and the DNA was stained with Hoechst 33342, sigma, USA) for 10 min. The slides were visualised by confocal microscopy (Zeiss, Germany) and images were captured at 600×.

#### Western blotting

Cells were harvested and protein prepared as described previously [18]. Primary antibodies used were: anti-PARP1/2 (sc-7150), anti-p53 (sc-126), anti-p21 (sc-756), anti-Bax (sc-7480), anti-ATM (sc-23921) and anti-α-Tubulin (sc-8035) (Santa Cruz, USA, California, USA), anti-phospho-H2AX (#2577), anti-phospho-p38 MAP Kinase (#9211), anti-phospho-p44/42 MAP Kinase (#9101), anti-phospho-SAPK/JNK (#9251), anti-LC3II (#2775), anti-Beclin1 (#3738), BCL-2 (#2876), anti-phospho-CHK2 (#9261), anti-Caspase-3 (#9661), anti-Caspase-7 (#9492), anti-Caspase-8 (#9746), anti-phospho-p38 MAP Kinase (#3824) and anti-phospho-ATM (#4526) (Cell Signaling, Boston, Massachusetts, USA). Secondary antibodies used were: anti-PARP1/2 (sc-7150), anti-p53 (sc-126), anti-p21 (sc-756), anti-Bax (sc-7480), anti-ATM (sc-23921) and anti-α-Tubulin (sc-8035) (Santa Cruz, USA, California, USA), anti-phospho-H2AX (#2577), anti-phospho-p38 MAP Kinase (#9211), anti-phospho-p44/42 MAP Kinase (sc-7150), anti-phospho-SAPK/JNK (#9251), anti-LC3II (#2775), anti-Beclin1 (#3738), BCL-2 (#2876), anti-phospho-CHK2 (#9261), anti-Caspase-3 (#9661), anti-Caspase-7 (#9492), anti-Caspase-8 (#9746), anti-phospho-p38 MAP Kinase (#3824) and anti-phospho-ATM (#4526) (Cell Signaling, Boston, Massachusetts, USA). Anti-PARP1/2 (sc-7150), anti-p53 (sc-126), anti-p21 (sc-756), anti-Bax (sc-7480), anti-ATM (sc-23921) and anti-α-Tubulin (sc-8035) (Santa Cruz, USA, California, USA), anti-phospho-H2AX (#2577), anti-phospho-p38 MAP Kinase (#9211), anti-phospho-p44/42 MAP Kinase (#9101), anti-phospho-SAPK/JNK (#9251), anti-LC3II (#2775), anti-Beclin1 (#3738), BCL-2 (#2876), anti-phospho-CHK2 (#9261), anti-Caspase-3 (#9661), anti-Caspase-7 (#9492), anti-Caspase-8 (#9746), anti-phospho-p38 MAP Kinase (#3824) and anti-phospho-ATM (#4526) (Cell Signaling, Boston, Massachusetts, USA).

#### Mammosphere assay

Anchorage-independent growth was assessed by mammosphere assay as previously described [21]. 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 ultra-low attachment plates (Nunc) containing DMEM supplemented with 2 mM L-glutamine, 1% (v/v) FBS, 2% (v/v) B-27 supplement, 20 ng/ml EGF, 20 ng/ml bFGF, 4 ng/ml heparin and 10 ng/ml insulin. Treatment with either 0.1% (v/v) dimethyl sulfoxide (DMSO) vehicle control or AJ-5 was carried out either upon seeding or at Day 4 for quadruplicate samples. The medium was changed every 48 hours and the mammospheres were photographed after 7 days in culture at 100× magnification. Quantification of mammosphere formation as Sphere Forming Efficiency (SFE) was carried out by counting of mammospheres under an Olympus DSZ5000X inverted microscope at 40× 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 one way ANOVA comparing treated samples to the relevant untreated control and where P values less than 0.05 were deemed statistically significant.

#### 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 the protocol of Gupta [22]. 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<sup>hi</sup>CD24<sup>loc</sup> 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 were analysed using Flowjo software (TreeStar Inc) and percentage of CD44<sup>hi</sup>CD24<sup>loc</sup> cells established from the live population by gating based on the isotype control staining. Each treatment was conducted in triplicate for duplicate experiments.

#### Statistical analysis

Data presented are mean ± SEM (Standard error of the means) of three independent experiments and a value of P < 0.05 was accepted as statistically significant. One way ANOVA was used to compare means of more than two samples/groups and the t test was used to compare two samples/groups.

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Results

AJ-5 inhibits cell proliferation and survival 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. 1A). This effect was less pronounced in the normal fibroblast cell lines FG0 and DNB which were included as controls. Indeed, the IC50 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. These results show that AJ-5 exerts potent anti-proliferative activity against breast cancer cells at very low concentrations. Furthermore, FG0, DNB, MCF7 and MDA-MB-231 cells were treated with AJ-5 and cell viability assessed using clonogenic survival assays. Fig. 1B and C shows that while the normal fibroblast cell lines survived all the doses of AJ-5, the MCF7 and MDA-MB-231 breast cancer cells had statistically significant decreased survival rates when treated with 0.1 μM and 0.2 μM AJ-5. Taken together these data demonstrate that at low concentrations AJ-5 has potent cytotoxic and anti-survival effects on ER-positive and ER-negative breast cancer cells.

AJ-5 has putative anti-cancer stem cell activity

The putative anti-CSC activity of AJ-5 was assessed in the MCF7 cell line by determining its effect on the proportion of CD44hiCD24lo cells and mammosphere formation (Fig. 2). MCF-7 cells were treated with DMSO (vehicle control) or AJ-5 (0.05 and 0.1 μM) for 4 days, allowed to recover in fresh medium for 4 days and subsequently stained for the expression of markers CD44 and CD24. The isotype control was used to establish the negative gates for the CD44 and CD24 staining. The proportion of CD44hiCD24lo cells was established by copying the isotype control quadrant gates from the DMSO control was used to establish the negative gates for the CD44 and CD24 staining. The proportion of CD44hiCD24lo cells was established by copying the isotype control quadrant gates from the DMSO control sample on to all other analyses to identify the positively stained cells. The CD44hiCD24lo cells are indicated in the lower right quadrant and the percentage of cells in this quadrant (as a proportion of the total live population) is indicated on the graph (Fig. 2A). Cultures treated with DMSO had an average CD44hiCD24lo population of 15.4 ± 1.0%. AJ-5 treatment showed a dose dependent reduction in the proportion of CD44hiCD24lo cells (average values of 10.3 ± 1.9% and 6.2 ± 1.5% in cells treated with 0.05 μM and 0.1 μM AJ-5, respectively) at concentrations lower than the IC50 value determined for adherent cells (Fig. 2A). These data suggested that CD44hiCD24lo cells are reduced relative to the rest of the tumour population upon AJ-5 treatment. In addition, AJ-5 had a dose dependent inhibitory effect on the number and size of MCF7 and MDA-231 mammospheres (Fig. 2B and 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. 2C). This suggested that mammosphere derived MCF7 cells are more sensitive to AJ-5 inhibition than the bulk MCF7 cells. We have previously demonstrated that MCF7 mammospheres are enriched in cells bearing the putative cancer stem-like marker profile, CD44hiCD24lo [21] and therefore the enhanced sensitivity of the day 4 mammospheres to AJ-5 may be explained by the greater sensitivity of CD44hiCD24lo cells to AJ-5 (Fig. 2A). These data are consistent with observations of other groups who have demonstrated the anti-breast cancer stem cell activity of the compounds salinomycin [22] and niclosamide [23]. Taken together, these data suggest that AJ-5 has in vitro activity against MCF7 derived cancer stem-like cells.

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 [24,25]. To investigate the mechanism by which AJ-5 inhibits breast cancer cell growth we therefore tested its effect on the cell cycle profile and DNA damage response. 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. 3A). Sub-G1 peaks, generally accepted to represent dead cells, 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 our previous findings for AJ-5 in melanoma cells [18] and with that reported by Ulukaya et al. for another palladium compound in prostate cancer cells [14]. Furthermore, 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 MCF7 and MDA-MB-231 cells (Fig. 3B). 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 [26] their p21 levels were markedly increased at 24 hours of AJ-5 treatment suggesting a p53-independent induction of p21 in these cells (Fig. 3B). This is in agreement with other studies in MDA-MB-231 cells which showed that cell cycle arrest and apoptosis are mediated by increasing levels of p21 but not p53 in these cells [27,28]. Together these results suggest that AJ-5 induced DNA damage and that the G1 cell cycle arrest observed at all time points tested for MCF7 cells and at 24 hours for MDA-MB-231 cells is probably p21 dependent.

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 Fig. 3A and the percentage of apoptosis was quantified using AnnexinV/PI staining. The results demonstrate that AJ-5 induced apoptosis in both breast cancer cell lines and they showed a comparable trend to that observed by the sub-G1 peaks detected in Fig. 3A. Interestingly, when treated with 0.2 μM AJ-5, the level of MDA-MB-231 cells undergoing apoptosis was significantly higher than the apoptotic population of MCF7 cells (Fig. 4A). 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 PARP cleavage, a molecular marker of apoptosis, under all conditions tested (Fig. 4B).

Apoptosis can be activated through two main pathways namely, the extrinsic and intrinsic pathways [29]. While extrinsic apoptosis is mediated by death receptors and characterised by caspase 8 activation [30], intrinsic apoptosis is mitochondrial mediated and usually triggered by intracellular signals such as hypoxia and DNA damage [31]. 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 [32]. 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. 4C). 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.
Fig. 1. AJ-5 induces cytotoxicity and inhibits survival of breast cancer cells. (A) Cell survival rate (as measured by MTT assay) of ER-positive (MCF7) and ER-negative (MDA-MB-231) breast cancer cell lines and normal fibroblast FGO and DNB cells 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\textsubscript{50}) are indicated and are calculated from sigmoidal plots with GraphPad Prism version 5. Assays were done in duplicate and two independent experiments were performed. (B and C) Cell survival rate (as measured by clonogenic survival assay) of FGO, DNB, MCF7 and MDA-MB-231 cell lines. Cells were treated with 0, 0.05, 0.1 and 0.2 μM AJ-5 for 24 hours, re-plated at low density and incubated for 14 days. Results show the mean percentage ± SEM of untreated cells and represent the pooled results of at least three experiments performed. The results of the duplicate experiments were analysed and show the average percentage of surviving colonies (*\textit{P} < 0.05, **\textit{P} < 0.003, Student’s t test).
Fig. 2. 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\textsuperscript{hi}CD24\textsuperscript{lo} (percentage indicated on the charts) was determined by flow cytometry after treatment with DMSO (vehicle control) or 0.05 μM and 0.1 μM AJ-5. MCF7 cells were treated for 4 days and then allowed to recover in fresh medium for a further 4 days before analysis. The panel marked ‘isotype’ indicates the staining for the isotype controls, while the panels marked Rep1, Rep2 and Rep3 indicate the staining obtained for triplicate independently treated samples. The subpopulation gates for CD44 and CD24 staining of live cells were established based on the DMSO isotype control staining and then copied on to all other analyses. Data shown are representative of at least two experiments performed in triplicate. (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 inverted microscope at 100× 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. Data shown are mean ± SEM for at least 4 replicates (**P < 0.01, *P < 0.05, one way ANOVA with Tukey post-test comparing treated samples to the relevant untreated control).
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, Fig. 5 shows that while cytochrome c is highly localised 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.

AJ-5 induces autophagy and inhibits the mTOR pathway

Increasing evidence has shown that autophagy is activated in response to anti-cancer chemotherapies [33] and we observed that compared to vehicle-treated cells (Fig. 6A and B), large vacuoles were present in breast cancer cells treated with AJ-5 for 24 hours (Fig. 6C and D). These were confirmed by transmission electron microscopy to be autophagosomes and autolysosomes (Fig. 6F–H). Moreover AJ-5 treated cells displayed swollen mitochondria (Fig. 6H–L, white arrows), a decrease in healthy intracellular organelles.
Fig. 4. AJ-5 induces intrinsic and extrinsic 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 and C) Western blotting of protein from AJ-5 treated breast cancer cells shows the levels of PARP cleavage, PUMA, BAX, BCL-2, caspase 3/7 and caspase 8 and total p38 were used as loading control. The expression of each protein was quantified as the densitometry value analysed by UN-SCAN-IT gel 6.1 software and is normalised to p38 levels.
and increasing levels of organelles localised in autophagic vacuoles (Fig. 6I–L, black arrows). LC3 is a marker of autophagosomes and to 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 bafilomycin A (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 was an accumulation of these puncta (Fig. 7A). The induction of autophagic flux by AJ-5 was confirmed by western blotting for LC3II (Fig. 7B).

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 [34,35]. We show that AJ-5 severely inhibits levels of p-mTOR and its direct substrate p70S6 kinase in both breast cancer cell lines (Fig. 7C) which was accompanied by a dramatic increase in LC3II, a marker of autophagy. Importantly this correlated with an increase in cleaved PARP and cleaved Beclin1 (35/37 kDa) which play roles in releasing cytochrome c from the mitochondria to the cytoplasm and are therefore considered pro-apoptotic factors [36,37]. Together these results suggest that AJ-5 induced autophagy and apoptosis by a mechanism involving the inhibition of the mTOR pathway.

**AJ-5 induced cytotoxicity involves autophagy**

In light of the above data, we 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 wortmannin, a PI3K kinase and DNA repair inhibitor frequently used as an autophagy inhibitor, significantly reduced the cytotoxicity and the total cell death induced by AJ-5 (Fig. 8A and B). Furthermore, wortmannin treatment decreased the levels of cleaved PARP induced by AJ-5 which was also seen when AJ-5 induced autophagy was

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**Fig. 5.** 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 an 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 localised in mitochondria as revealed by yellow-orange staining due to 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Fig. 6.** AJ-5 induces autophagy in breast cancer cells. (A–D) Representative phase-contrast photomicrographs (400x; Olympus 1X71) of MCF7 and MDA-MB-231 cells treated with vehicle (A and B) or 0.2 μM AJ-5 for 24 hours (C and D). (E–L) Representative transmission electron photomicrographs showing MCF7 and MDA-MB-231 cells treated as above. Higher magnifications of AJ-5 treated MCF7 (I and J) and MDA-MB-231 cells (K and L). Black arrows in I, J, K and L indicate typical autophagosomes containing cytoplasmic inclusions; white arrows indicate swollen mitochondria.
Fig. 7. AJ-5 induces autophagy and inhibits the mTOR pathway. (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-LC3II puncta at 400× 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. (C) Western blotting of protein from cells treated with 0.2 μM AJ-5 for the indicated time points and analysed with antibodies to p-mTOR, p70S6K, LC3II, Beclin1 and PARP. p38 was used as a loading control. The expression of each protein was quantified as the densitometry value analysed by UN-SCAN-IT gel 6.1 software and is normalised to p38 levels.
inhibited using siRNA specific to LC3 (Fig. 8C and D). Together these results suggest that Aj-5 induced autophagy favours apoptosis and it is tempting to speculate that it contributes to Aj-5 induced cytotoxicity which is in line with previous studies showing that autophagy induced by paclitaxel and tamoxifen in MCF7 cells is a cell death mechanism [19,38,39].

The p38 MAPK pathway mediates Aj-5 cytotoxicity

The MAPK pathways are activated during autophagy and apoptosis [40,41] and to determine whether these pathways play a role in mediating Aj-5 induced apoptosis and autophagy we determined the levels of p-p38, p-ERK and p-JNK. 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. 9A) and inhibition of this pathway reduced cleaved LC3II and PARP levels (Fig. 9B and C). Furthermore, MTT assays show that inhibition of p-p38 activity decreased Aj-5 cytotoxicity (Fig. 9D) which is consistent with reports indicating that the p38 MAPK pathway mediates cell death induced by a number of chemotherapeutic compounds including cisplatin and wogonin [41,42].

![Fig. 8. Inhibition of autophagy decreases the cytotoxic effect of Aj-5 in breast cancer 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 by 1 μM wortmannin and the cells treated with 0.2 μM Aj-5 for 24 hours (*P < 0.05, Student’s t test). (C and D) Representative western blots showing levels of cleaved PARP and LC3II in cells treated with Aj-5 or vehicle for 24 hours after (C) pre-treatment with 1 μM wortmannin and (D) transfection with non-silencing siRNA or LC3 specific siRNA. The expression of each protein was quantified as the densitometry value analysed by UN-SCAN-IT gel 6.1 software and is normalised to p38 levels.](image-url)
Breast cancer continues to be the leading cause of cancer deaths amongst women and its treatment is constantly evolving as new technologies, drugs, and strategies are discovered [43]. One approach to dealing with this 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 [25,44]. This study has investigated the synthetic binuclear palladacycle, AJ-5, for anti-tumour activity in breast cancer and breast cancer stem cells. Its potential is demonstrated by observations that AJ-5 may be an effective drug in the treatment of oestrogen receptor (ER)-positive and ER-negative breast cancer for which there is currently no effective treatment [18]. The results obtained from this study show that AJ-5 cytotoxicity involves the induction of cell cycle checkpoints and cell death by apoptosis and autophagy which is triggered by double strand DNA breaks and activation of the p38 MAPK signalling pathway.

In addition to its effect on ER-positive breast cancer cells, AJ-5 was also shown to be very effective against the MDA-MB-231 triple negative breast cancer cells (TNBC). Because of the lack of targeted therapies, patients with TNBC have an extremely poor prognosis and relapse and die quickly [45]. 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.

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 [13,46–48]. In breast cancer, as few as 100 cells with the cell surface marker signature CD44hiCD24lo could generate a tumour, while tens of thousands of cells from the rest of the population were unable to do so [9]. Breast cancer stem-like cells (BCSCs) 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 [12,22,49]. In addition to their role in cancer...
development, CSCs have also been linked to metastasis and drug resistance. For example, BCSCs have been shown to induce cisplatin resistance in mouse mammary tumours [50]. Therefore treatments that target these cells have potential therapeutic applications [47,51–53]. Here we show that low concentrations of AJ-5 reduced the proportion of CD44+CD24− cells in MCF7 cells and decreased the number and size of MCF7 mammospheres. This demonstrates that AJ-5 may target BCSCs which further highlights its potential as a chemotherapeutic drug to treat breast cancers [31]. These observations are interesting in light of the results from a very recent study that demonstrate that another Pd(II) compound, Pd-BENSPim, is able to reduce the CD44+CD24− population in JIMT-1 breast cancer cells [54]. It would be interesting to test whether other Pd(II) compounds also exhibit anti-BCSC activity because it may be a feature of these compounds.

This study shows that the anti-cancer activity of AJ-5 involves the induction of intrinsic and extrinsic apoptosis as well as autophagy. Intrinsic apoptosis was demonstrated by the upregulation of PUMA and Bax, the downregulation of BCL-2, and the release of cytochrome c from the mitochondria. Induction of extrinsic apoptosis was shown by the activation of caspase 8. Activation of both apoptotic pathways is significant for effective chemotherapeutics because resistance to many cancer treatments results from defects within one apoptotic pathway and the development of drugs that target both pathways may circumvent this problem [29,55,56]. AJ-5 treatment also activated markers of autophagy in both breast cancer cell lines and inhibition of autophagy significantly decreased the level of cell death induced by AJ-5. The induction of autophagic cell death together with apoptosis has also been shown for several other anti-cancer therapies [57–63]. For example, 5-FU, arsenic trioxide (As2O3) and paclitaxel induce cytotoxicity in many cancer types (colon, acute promyelocytic leukaemia, glioma and ovarian cancer) which is associated with the upregulation of autophagy markers and blocking autophagy reversed the cytotoxic effect of these drugs [38,39,57,64–69]. These findings are particularly important because autophagy in many cases serves as a pro-survival mechanism, which adapts cells to stress conditions and chemotherapies leading to drug resistance [31].

Taken together, our 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. This study, therefore, provides compelling evidence to suggest that AJ-5 may be an effective chemotherapeutic drug in the treatment of breast cancer. Furthermore, our previous findings that AJ-5 is also effective against malignant melanoma suggest that it may be useful in the treatment of many different cancers.

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Conflict of interest

All the authors on this manuscript have agreed to its submission to Cancer Letters. We declare that the content of the manuscript is original and that it has not been published or accepted for publication, either in whole or in part, in any form. We further declare that no part of the manuscript is currently under consideration for publication elsewhere and we declare no competing interest.

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