Identification and characterization of novel palladium based compounds as potential anticancer drugs
Introduction

- Cancer is a leading cause of death worldwide and remains a therapeutic enigma.

- Breast cancer and Melanoma among the most aggressive forms of cancers.
Breast Cancer:
- 30% of all cancers in women occur in the breast making it the most commonly diagnosed female cancer.
- 1 of 8 women (12.2%) will develop breast cancer in their lifetime.
- One third of breast cancer patient die.

Melanoma:
- The incidence of malignant melanoma is rising globally faster than for any other form of cancer and has not been associated with significantly better therapeutic options.
Treatment strategies for cancer

1- Surgery
2- Radiation
3- Immunological Therapy
4- Chemotherapy:
5- Hormone Therapy: Breast and Prostate cancers
Chemotherapy

Alkylating Agents:

Electrophilic molecules that covalently modify nucleophilic molecules in the cell.

Ex. Cisplatin

- Is considered to be the platinum agent of choice in combination chemotherapy for many cancer types. (lung, ovarian, cervix, testicular, esophageal and Breast)
Cisplatin mechanism of action

- It can bind to various cellular components specifically the N7 atom of guanine.

- Elicits DNA crosslinks, causing a kink to the DNA molecule in the major groove.
Cisplatin mechanism of action

Tamoxifen, Doxorubicin, 5 FU and other chemotherapeutics

Necrosis, Necroptosis, Autophagy, Mitotic catastrophe
Drug resistance

1- Diminished drug accumulation.

2- Apoptosis defects such as a lack of caspase 3 (ex. mcf7 cells)

3- Enhanced expression of anti-apoptotic genes, and inactivation of the intrinsic apoptosis.

3- DNA repair or elevated DNA damage tolerance.

- Moreover most of the current chemotherapies cause: Nephrotoxicity, ototoxicity, neurotoxicity, and haematological toxicity.

- What is required?

- More efficient drugs: Have more than one mechanism of cell death.

- Less toxic drugs and minimal side effects
Candidates

- Other metal drugs have been suggested such as titanium, gallium, ruthenium, iron and are of *particular interest to this study, palladium.*
Palladium [Pd(II)] compounds properties

• The Pd(II) ions are capable of interacting with DNA thus enabling cross bindings and inhibiting its synthesis as well as inducing apoptosis.

• In recent years, numerous palladium(II) (Pd) complexes with promising activity against tumor cell lines have been synthesized and published.

• Previous studies reported that Pd complexes demonstrated significant anti-tumor activity comparable with cisplatin.
Mononuclear palladium complexes

• Aromatic N-containing ligands like pyridine, and its derivatives have been used to stabilize palladium

• $[\text{Pd(dmnp)}_2\text{Cl}_2]$ where (dmnp) = 2,6-dimethyl-4-nitro-pyridine is more effective than cisplatin in three of the cell lines tested (T47D, HCV 29T and A549)
Dinuclear palladium complexes

- A dinuclear (Pd(II) chelate with a spermine ligand) effect on breast cancer cell lines has been compared with cis-DDP effect.

- Pd$_2$-Spm and cis-DDP have good antiproliferative effect, but Pd$_2$-Spm was more effective against the estrogen ER(-) cell line MDA-MB-231.

- Interesting because ER(-) breast cancers are notoriously unresponsive to current treatments
Palladium complexes mechanisms of action

• The biologic activity of a dinuclear Pd(II)–spermine complex toward human breast cancer involves inducing DNA damage and apoptosis.

• A cyclopalladated complex interacts with mitochondrial membrane thiol-groups and induces the apoptotic intrinsic pathway in murine and cisplatin-resistant human tumor cells.

• However very little is known about how palladium compounds exert its anticancer effect.
Aims of the study

- Identification of new Palladium-based chemotherapeutic compounds

- Characterization of the mechanism of action of identified compounds in breast cancer and melanoma.

- To compare -in vitro and in vivo- the efficiency and the possible side effects of palladium compounds with cisplatin.
Chapter 3

Anti-tumor effect of palladium based compounds on breast cancer cells
Cytotoxic effect of the identified palladium compounds on breast cancer cells

- **Mononuclear palladacycles**: Has a single palladium center (AJ-1, 2,3,4)

- **Binuclear palladacycles**: Has two palladium centers (AJ-5,6,7 and 8) and (C5,6,7 and 8)
Cytotoxic effect of the identified palladium compounds on breast cancer cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ (µM) on MCF7</th>
<th>IC$_{50}$ (µM) on MDA-MB-231</th>
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<tr>
<td>C5</td>
<td>46.22 ± 1.3960</td>
<td>54.11 ± 3.524</td>
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<td>C6</td>
<td>18.75 ± 1.01</td>
<td>17.43 ± 1.61</td>
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<td>C7</td>
<td>11.17 ± 1.9465</td>
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<td>C8</td>
<td>15.51 ± 5.292</td>
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<tr>
<td>AJ-5</td>
<td>0.175 ± 0.047806</td>
<td>0.1938± 0.0146</td>
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Among 12 palladium based compounds tested on breast cancer cells AJ-5 displays an exceptionally strong cytotoxic effect.
Cytotoxic effect of AJ-5 on cancer cell lines and normal cells

Compared to normal control cells AJ-5 displays a high cytotoxic activity in the MDA-MB-231 triple negative and MCF7 estrogen receptor positive breast cancer cell lines.

IC\textsubscript{50} (µM)

- MCF7 = 0.17
- MDA-MB-231 = 0.19
- DNB = 0.4631
- FG0 = 0.4049
- CT-1 = 0.4296
How is AJ-5 leading to decrease numbers of breast cancer cells?

A- Cell cycle analysis

Cell cycle analysis

MDA-MB-231 cells

Plate 3 $\times$ $10^5$ cells/well in 6 well plates

48 hours settle

Treat with 0.2 $\mu$m AJ-5 For 24 and 48 hours

Trypsinize and fix with cold ethanol for at least 30 min

FACS process and analysis
AJ-5 induces a G1 arrest and apoptosis (sub-G1 peak) in breast cancer cells
Does AJ-5 induce apoptosis in breast cancer cells?

**Annexin V staining**

MDA-MB-231 cells

- Plate 3 X 10^5 cells/well in 6 well plates
- 48 hours settle
- Treat cells with 0.2 µm AJ-5 for 24 and 48 hours
- Trypsinize and stain cells with ( Annexin V and PI)
- FACS analysis

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**Viable cells**

**Late apoptosis**

**Early apoptosis**

**Necrosis**

**FACS analysis**
Does AJ-5 induce apoptosis in breast cancer cells?

AJ-5 induces apoptosis in breast cancer cells in accordance with the cell cycle analysis.
Confirmation of apoptosis: PARP cleavage and nuclear fragmentation

MCF7

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<tr>
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<th>24 h</th>
<th>48 h</th>
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<tr>
<td>0.0 μM AJ-5</td>
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<td>0.1 μM AJ-5</td>
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<td>0.2 μM AJ-5</td>
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MDA-MB-231

<table>
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<tr>
<th></th>
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<td></td>
</tr>
<tr>
<td>0.2 μM AJ-5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Nuclear fragmentation after treatment with 0.2 μM AJ-5 for 24 h followed by Hoescht staining.

AJ-5 treatment leads to an increase in PARP cleavage level and nuclear fragmentation confirming that it induces apoptosis.
Which apoptosis pathway is induced by AJ-5?
Which apoptosis pathway is induced by AJ-5?

Check for intrinsic and extrinsic apoptosis pathways markers

AJ-5 activates both intrinsic and extrinsic as early as 1h of the treatment
Morphological changes: Formation of large vacuoles

Does AJ-5 induce autophagy?
Autophagy

- **Autophagy**: is an intracellular process whereby double-membrane structures termed autophagosomes deliver cellular components to lysosomes for their degradation.

When it does happen?
- Normal (basal level) clearance.
- Starvation
- Infected cells (bacterial and fungal infections)
- Cancer, DNA damage, UV, and other mutagenesis.
- Chemotherapy
Autophagy and cancer

• Autophagy was found to be deregulated in many tumors.

• Beclin1, an autophagy gene that is monoallelically deleted in up to 40–75% of sporadic breast, ovarian and prostate cancer, acts as an effective haploinsufficient tumor suppressor in mice.

• Autophagy can lead to growth arrest and reduction in cell number and several death forms are associated with appearance of autophagic vesicles.

• However, only few data directly connect autophagy to cell death. For example “Vitamin D analog EB1089 triggers dramatic lysosomal changes and Beclin 1-mediated autophagic cell death”
Autophagy and cancer

• It should be noted that autophagy can serve as a protective mechanism against starvation and apoptosis by recycling macromolecules and removing damaged mitochondria and other organelles, respectively.
Autophagy and Autophagy markers

1- Molecular marker

2- Morphological Marker
Autophagy and Autophagy markers

• It is important to be aware of the difference between monitoring the *steady-state level of Atg8/LC3 and autophagic flux*, the latter can be determined by following Atg8/ LC3 in the absence and presence of autophagy inhibitors. (bafilomycin A1)

• If flux is occurring, the amount of LC3-II will be higher in the presence of the inhibitor.
Does AJ-5 induce autophagy?

1- Transmission electron microscopy: Check for autophagosomes

AJ-5 treated cells showed high levels of vacuolization, swollen mitochondria and a decrease in healthy intracellular organelles indicating that AJ-5 does induce autophagy.
Confirmation of autophagy

2- LC3II punctate assay

LC3-GFP plasmid

12-24h Check for fluorescence GFP expression

12-24h Check whether AJ-5 increased the fluorescent dots as a punctate which is part of the autophagosomes membranes.

AJ-5

bafilomycin A1
Confirmation of autophagy

2- LC3II punctate assay

- AJ-5 treatment led to a significantly increase of GFP-LC3 puncta.
- Furthermore, cotreatment with bafilomycin A (BAF) resulted in additional accumulation of GFP-LC3 puncta.
- Confirming that AJ-5 induces an autophagy flux in breast cancer cells.
Confirmation autophagy

Checking the endogenous LC3II to confirm autophagy and autophagy flux

- AJ-5 increases endogenous LC3II level.
- Co-treatment with BAF accumulates LC3II more than AJ-5 does alone.
- Co-treatment with Wort decreases LC3II level in AJ-5 treated cells.
- Taken together AJ-5 induces an autophagy flux and both intrinsic and extrinsic apoptosis.

Which one of these two cellular processes is triggered first, or whether they are induced concurrently?
Which one of these two cellular processes is triggered first, or whether they are induced concurrently?

Check for apoptosis and autophagy markers at different time points.

AJ-5 induces both apoptosis and autophagy simultaneously.
1- Is AJ-5 induced autophagy a cell death or cell survival mechanism?

2- What is the impact of autophagy inhibition on AJ-5 induced apoptosis?
1- Is AJ-5 induced autophagy a cell death or cell survival mechanism?

A- Measuring the cytotoxic effect- by MTT assay- of AJ-5 with or without autophagy inhibitors (Wortmanin, PI3K inhibitor and Bafilomycin A).

In MCF7 cells inhibition of autophagy significantly decreased the cell death induced by AJ—5
1- Is AJ-5 induced autophagy a cell death or cell survival mechanism?

A- Measuring the cytotoxic effect-by MTT assay- of AJ-5 with or without autophagy inhibitors (Wortmanin, PI3K inhibitor and Bafilomycin A).

In MDA-MB-231 cells inhibition of autophagy by Bafilomycin decreased the cell death induced by AJ-5.
1- Is AJ-5 induced autophagy a cell death or cell survival mechanism?

B- Measuring the total cell death (using Annexin V assay) - of AJ-5 with or without autophagy inhibitors (Wortmanin)

In both cell lines inhibition of autophagy reduced the total cell death by AJ-5 (but not significantly statistics).
- These results indicate that AJ-5 contribute to some degree in AJ-5 cell death.
2- What is the impact of autophagy inhibition on AJ-5 induced apoptosis?

Measuring the level of AJ-5 induced apoptosis with or without autophagy inhibitor (wortmanin) using:
1- Western blot analysis to check for PARP cleavage level.
2- Annexin V assay

1- In MCF7 cells inhibition of autophagy led to decrease apoptosis level as measured by Annexin V and PARP level.
2- In MDA-MB-231 cells autophagy inhibition by wortmanin didn’t show a significant difference on apoptosis level as measured by PARP and Annexin V assay.
• However due to the toxicity shown by Wortmanin and Bafilomycin, I need to inhibit autophagy by a biological inhibitor SiRNA for LC3B and to check apoptosis markers.
Identification and characterize of the molecular pathways involved in AJ-5 induced apoptosis and autophagy.
Signaling pathways involved in other Metal based chemotherapeutics
Signaling pathways involved in AJ-5 cytotoxic effect

1- Signaling pathways involved in DNA damage response.

AJ-5 induces DNA damage and activates ATM pathway
Confirming DNA damage by ICC

0.0 μM AJ-5

0.2 μM AJ-5

MCF7

MDA-MB-231
Confirmation of ATM activation

- Check for p-ATM and downstream targets in AJ-5 treated cells with or without caffeine (ATM inhibitor)

MCF7

- - + + + + +
- - + - + - +
250 Kd
70 Kd
40 Kd

MDA-MB-231

- - + + + + 0.2 µM AJ-5
- + - + - - +
2mm Caf

p-ATM
p-CHK2
p-p38
p38

Inhibition of p-ATM decreased p-p38 in MCF7 and p-chk2 levels in MDA-MB-231 cells.
2- MAPKs signaling pathways

AJ-5 Activates p38, ERK and JNK MAPKs pathways

Which MAPK pathway is involved in the cytotoxic effect of AJ-5?
Which MAPK pathway is involved in the cytotoxic effect of AJ-5?

Pretreat with every MAPK inhibitor and measure the cytotoxic effect – MTT assay – of AJ-5 with / without the inhibitors.

Inhibition of p38 and ERK pathways significantly reduced the cytotoxic effect of AJ-5 which indicate the involvement of these tow pathways in AJ-5 induced cell death.
Confirmation that p38 and ERK pathways are involved in AJ-5 induced cell death.

- 1- p38 MAPK pathway:

Inhibition of p38 MAPK significantly reduced both apoptosis and autophagy markers which confirms the contribution of p38MAPK in apoptosis and autophagy induced by AJ-5.
Confirmation that p38 and ERK pathways are involved in AJ-5 induced cell death.

2- ERK MAPK pathway:

Inhibition of ERK MAPK significantly reduced both apoptosis and autophagy markers which confirms the contribution of ERK MAPK in apoptosis and autophagy induced by AJ-5.
AJ-5 → DSB → ATM

- Cell cycle arrest
  - p53
  - P38/ERK MAPK

  - Apoptosis
    - Cell death
  - Autophagy

  Cell death