



The cytotoxic and growth inhibitory effects of palladium(II) complexes on MDA-MB-435 cells

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Abstract

The antitumorigenic potential of two palladium(II) complexes, [Pd(ca₂-o-phen)Cl₂] – C1 and [Pd(dmba)(dppp)Cl] – C2, was evaluated, using MDA-MB-435 cells, a human breast adenocarcinoma cell-line that does not express the estrogen receptor α (ER-). Growth inhibition and induced alterations in cell-morphology were analyzed. The sulforhodamine B test showed that, compared to control cells, both C1 and C2 significantly inhibited ($p < 0.5$) cell growth. The maximum effect with both was achieved with 1 μ M complexes, after 24 h of treatment. No further cell-growth inhibition was achieved by increasing concentration or incubation time. Cell morphology was analyzed after staining with hematoxylin-eosin (HE). The morphological changes noted in the treated cells were cell rounding-up, shrinkage, nuclear condensation and reduction of cell length ($p < 0.05$), thereby indicating that both C1 and C2 are cytotoxic to breast adenocarcinoma cells. All together, there was every indication that, by decreasing cell growth and inducing morphological changes, the tested complexes are cytotoxic, hence their potentiality as promising candidates for antineoplastic drug development.

Key words: adenocarcinoma cells, antitumor, breast cancer, chemotherapy, coordination complexes.

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Introduction

Globally, 12.7 million new cases of cancer and 7.6 million deaths were estimated to have occurred in 2008. Worldwide, the second most commonly diagnosed type is breast cancer, with an estimated 1.4 million new cases (10.9% of all cancers diagnosed) and 458,000 deaths in 2008 (Jemal *et al.*, 2010). For 2010, 49,000 new cases of breast cancer were expected for Brazil alone and 1.5 million worldwide, with the highest mortality rates in low- and middle-income countries (Anderson, 2010; Brazil, 2011).

Through breast carcinoma development being multifactorial in a multistep mutational process, cells acquire a set of genetic lesions, leading to a cancerous state with the subsequent forming of phenotypically different tumors. Phenotypic changes during breast-cancer progression reflect aberrant gene expression and pathways supporting deregulated growth. The various forms of expression of estrogen receptor (ER), progesterone receptor, and human epithelial growth factor receptor-2, are important molecu-

lar markers used in clinical practice for disease diagnosis, prognosis and therapy. ER expression is one of the most important markers for the selection of adequate breast cancer therapy (Giacinti *et al.*, 2006; Oldenhuis *et al.*, 2008; Sahab *et al.*, 2011). Around 60% of all breast cancers diagnosed are ER positive (ER+), a noninvasive differentiated breast cancer which responds to antiestrogen therapy. Nevertheless, some breast cancer cells, through losing the ability to express ER, give rise to a therapy-resistant cancer. ER negative (ER-) breast cancers are a highly invasive, dedifferentiated form of breast carcinoma, which have been associated with focal myoepithelial disruption, a lack of expression of tumor suppressors, and a higher rate of cell proliferation, itself increasing metastatic potential, thereby posing a challenge to treatment (Sahab *et al.*, 2011).

Despite favorable prognosis if early detected, and recent progress in drug therapy, there are no effective drugs for treating and curing most types of breast cancers, especially metastatic ER-tumors (Oldenhuis *et al.*, 2008; Brough *et al.*, 2011). Serious side effects have become obstacles in treatment when using current compounds with anticancer activity (Rybak *et al.*, 2009), whereby the necessary search for new potential antitumor substances to im-

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prove treatment efficacy with smaller doses and less toxic drugs. The development of drugs that selectively eliminate cancer cells without harming normal ones, also remains a considerable challenge (Brough *et al.*, 2011).

The first step in the search for new anticancer drugs is *in vitro* screening to detect the cytotoxic and cytostatic effects of a compound. Collectively, cytotoxicity comprises a set of biochemical and genetic alterations that disrupt cell homeostasis, and it could be estimated by enzyme release, cell viability, survival and death assays, among others (Sumantran, 2011).

A cytotoxic compound induces short-term loss of cell viability either by triggering cell-death or decreasing cell-survival. In contrast, a cytostatic elicits long-term cell survival or cell proliferation without affecting eventual, viable cell-number (Sumantran, 2011). The first and most readily observed effects, following cell exposure to toxicants, are morphological alterations in the cell-layer and/or cell-shape in monolayer cultures. Another indicator of cytotoxicity is altered cell growth (Ekwall *et al.*, 1990).

Most synthetic metal complexes used as antitumor chemotherapeutic compounds are structural analogs of cisplatin. Recently, the palladium (II) complexes were included among the newly introduced structural types of metallic complexes, designed for increased antitumor efficiency and decreased toxicity to normal cells (Caires *et al.*, 1999; Kontek *et al.*, 2011). The first of these had little or no application as antitumor compounds, due to poor stability and rapid hydrolysis in biological environments. However, the use of chelating ligands in their synthesis was opportune in improving their stability, which was further improved by the generation of cyclopalladated compounds (Caires, 2007).

Previous studies by our group have shown that the palladium(II) complexes are cytotoxic to tumor cells (Caires *et al.*, 1999; Rocha *et al.*, 2007), although there are no available data on their toxicity to ER- breast tumor cells.

The aim of the present study was to evaluate the cytotoxic activity of two synthesized palladium(II) complexes in the human breast adenocarcinoma cell line, MDA-MB-435 (ER-), a highly invasive, estrogen independent and dedifferentiated breast carcinoma cell line, by analyzing their effect on cell growth and morphology.

Materials and Methods

Reagents

All reagents, solvents and ligands (dmbs = dimethylbenzylamine and dppp = (diphenylphosphine)propane) were purchased from Sigma-Aldrich, St Louis, USA (ACS quality) and employed without further purification.

Ligand synthesis

The iminic ligand or Schiff's base (ca₂-o-phen = bis(cinnamaldehyde)-o-phenylenediimine) was synthe-

sized as follows: A solution of *trans*-cinnamaldehyde 1.14 g (8.6 mmol) in ethanol (2 mL) was added dropwise to an ethanolic solution (20 mL) of *o*-phenylenediamine 0.47 g (4.3 mmol). The reaction mixture was stirred at room temperature for 3 h. After cooling for 24 h, a white solid took form. The pellet was filtered off, washed with ethanol, water and ethyl ether, and finally vacuum-dried.

Palladium(II) complex synthesis

[Pd(ca₂-o-phen)Cl₂] (C1)

The complex [Pd(ca₂-o-phen)Cl₂] (C1) was synthesized as follows: A solution of imine ca₂-o-phen 0.15 g (1.16 mmol) in methanol (20 mL) was slowly added dropwise to a solution containing palladium(II) chloride 0.411 g (2.32 mmol) and lithium chloride 0.197 g (4.46 mmol) dissolved in methanol (50 mL). The resulting solution was stirred at room temperature for 8 h. The pale yellow pellet thus formed was filtered off and washed with ethanol, water and ethyl ether, and then vacuum-dried.

[Pd(dmbs)(dppp)Cl] (C2)

Synthesis of [Pd(dmbs)(dppp)Cl] (C2) has already been described (Caires *et al.*, 1999). Planar molecular geometry of the two palladium(II) complexes synthesized is shown in Figure 1. Both were dissolved in DMSO (1 mg.mL⁻¹) and diluted with distilled water to obtain the desired concentration.

Cell line culture

Human breast adenocarcinoma MDA-MB-435 cells were purchased from the Rio de Janeiro Cell Bank (CR 117), and cultured in RPMI 1640, supplemented with 20%

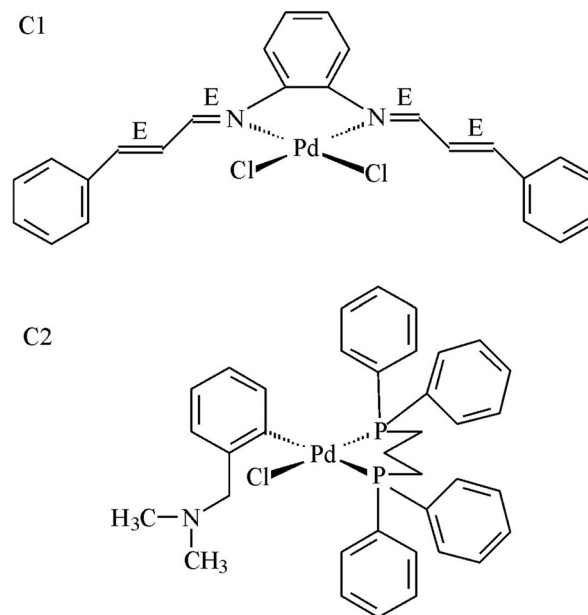


Figure 1 - Structures of palladium(II) complexes. C1, [Pd(ca₂-o-phen)Cl₂]; C2, [Pd(dmbs)(dppp)Cl].

(v/v) inactivated fetal bovine serum, 100 U.mL⁻¹ penicillin and 100 µg.mL⁻¹ streptomycin. Cells were maintained in a humidified atmosphere with 5% CO₂ at 37 °C. Before beginning each experiment, cell viability was assessed by the trypan blue-dye exclusion method and 2.10⁴ viable cells.mL⁻¹ were used in all experiments.

Cell growth analysis

The effect of C1 and C2 complexes on cell growth was determined by the sulforhodamine B (SRB) colorimetric assay (Vichai and Kirtikara, 2006). Briefly, cells were seeded onto 96-well plates, and then left for 24 h to reach exponential growth. The medium was replaced, whereupon the cells were treated with vehicle (negative control), 5.0 µM cisplatin (positive control), or various concentrations of both complexes (0.1, 0.5, 1.0, 5.0 and 10.0 µM). Cells were then incubated for 24 and 48 h and fixed with 10% trichloroacetic acid (w/v) for 30 min at 4 °C. After careful removal of the plate content, each well was washed with distilled water. The plate was then dried for 24 h and stained with 0.4% SRB (w/v) in 1% acetic acid (v/v) for 30 min, after which, excess SRB was removed, and the wells washed 4 times with 1% acetic acid (v/v). The bound SRB was dissolved by adding 100 µL of 10 µM Tris pH 10.5 for 10 min and absorbance read at 510 nm.

Morphological analysis

Morphological analysis, with modifications, was as described by Pedro *et al.* (2006). In order to reach exponential growth, cells were cultured on coverslips for 24 h to achieve the exponential growth, whereupon the medium was replaced, and the cells treated with various concentrations of the C1 and C2 complexes (0.1, 0.5, 1.0 and 5.0 µM) for 24 h. Cells treated with the vehicle were the negative control, and with 5.0 µM cisplatin, the positive. After treatment, cells were fixed with 70% acetone and stained with hematoxylin-eosin. Slides were mounted in Entellan and observed by light microscopy. Cell digital images were acquired with an Olympus BX52 microscope and Motic Images Plus 2.0 software. Fifteen aleatory fields were analyzed per treatment, to describe cell morphology. In each treatment, 30 cells (two per field) were measured with Motic Images Plus 2.0 software, in order to determine cell length.

Statistical analysis

Obtained data were compared by one-way analysis of variance (ANOVA), followed by the Tukey test, when $p < 0.05$. Data are shown as the mean \pm SEM of three independent experiments.

Results and Discussion

After completing synthesis, the palladium(II) complexes were investigated for *in vitro* antitumor activity when using the hormone-insensitive human breast-cancer cell line, MDA-MB-435. This cell-line does not express ER α , and has been used as the model in metastatic breast-cancer studies. Even though some previously synthesized palladium(II) complexes have proved to be cytotoxic to tumor cells, their effect on breast-cancer cells remains unknown. It is also important to evaluate the cytotoxicity of newly synthesized complexes with increased stability, the case of C1.

We evaluated the cytotoxicity of C1 and C2 complexes using the SRB assay (Figure 2). SRB, in moderated acid conditions, binds stoichiometrically to basic protein aminoacids. The OD observed is well correlated to cell number, thus being an indicator of the cell growth (Vichai and Kirtikara, 2006). The results depicted in Figure 2 show that both complexes hindered cell growth and the maximum effect was obtained with 1 µM C1 and C2 after 24 h incubation. No further significant ($p > 0.05$) hinderance was achieved by increasing C1 and C2 concentration or incubation time. Thus, it is possible to sustain the positive cytotoxic role of these synthesized complexes in the inhibition of MDA-MB-435 cell growth and spread.

C1, C2 and cisplatin treatment induced alterations in cell morphology, such as nuclear condensation, cell rounding-up and shrinkage indicating cytoskeleton disruption (Figure 3). Untreated control cells presented irregular spindle-shaped morphology, were homogeneously stained, with the cytoplasm less stained than the nucleus and the nucleolus remained plainly visible. On the other hand, when exposed to C1 and C2 complexes, cells became rounded

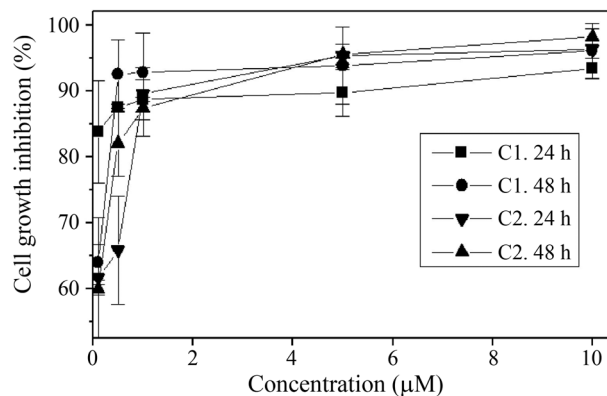


Figure 2 - Cell growth inhibition (%) after treatment with different palladium(II) complex (C1 and C2) concentrations for 24 and 48 h, estimated by the sulforhodamine B assay. C1, [Pd(ca₂-o-phen)Cl₂]; C2, [Pd(dmba)(dppp)Cl]. Growth inhibition was calculated relative to the negative control. The results are expressed as mean \pm SEM of three independent experiments. Note that the results were similar in both cases.

and individually separated, with cell shrinkage, nuclear condensation and wrinkled cytoplasmic membranes.

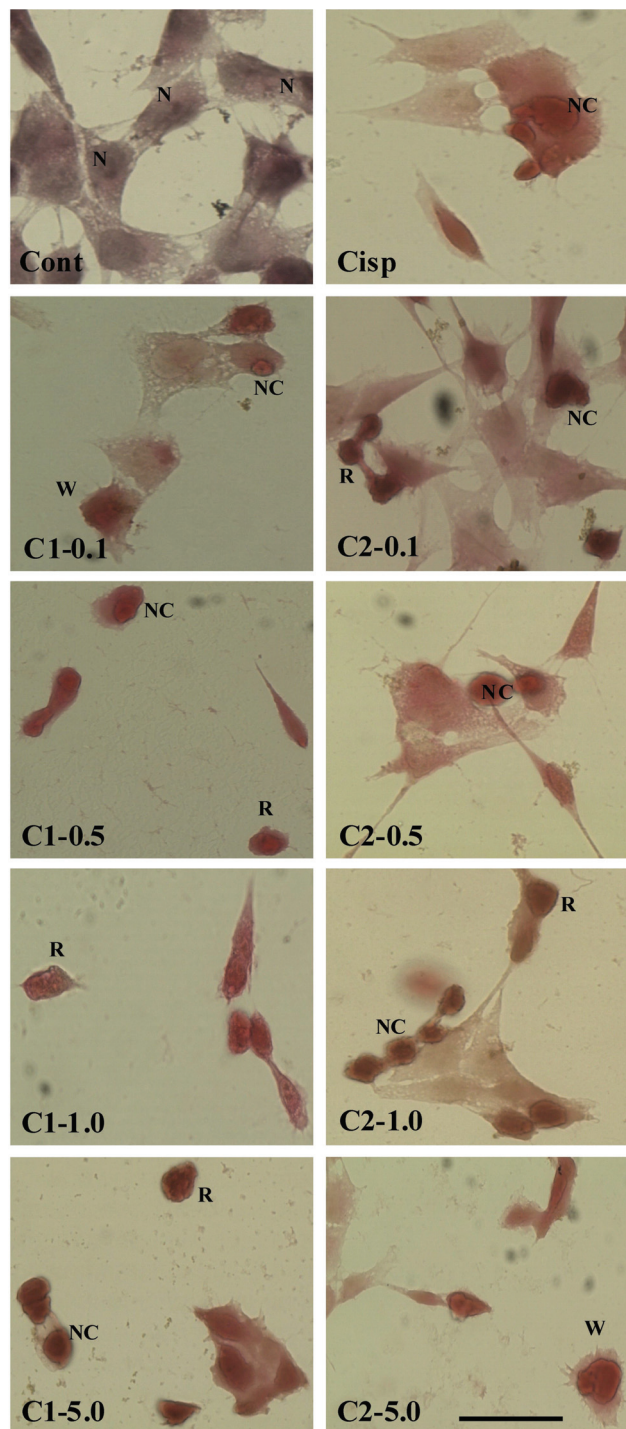


Figure 3 - Morphology of MDA-MB-435 cells stained with hematoxylin and eosin, after 24 h incubation. **Cont.**, control cells; **Cisp**, cells treated with 5 μ M cisplatin; **C1**, cells treated with $[\text{Pd}(\text{ca}_2\text{-o-phen})\text{Cl}_2]$; **C2**, cells treated with $[\text{Pd}(\text{dmba})(\text{dppp})\text{Cl}]$. Palladium(II) complexes were added to the cell culture at 0.1, 0.5, 1.0 and 5.0 μ M. All the pictures are typical of three independent experiments, each carried out under identical conditions. Bar = 20 μ m. N – nucleolus; R – rounding; NC – nuclear condensation; W – wrinkled cytoplasmic membranes.

When undergoing C1 and C2 treatment for 24 and 48 h, there was a significant ($p < 0.01$) decrease in cell-length in comparison to control cells. The complex effect proving to be similar to the cisplatin (Figure 4), even at lower concentrations. All told, these morphological alterations indicated C1 and C2 cytotoxicity.

The results herein corroborate the findings of Barbosa *et al.* (2006) showing palladium(II) complex cytotoxicity to cultured cells, with a decrease in cell number, chromatin condensation, and consequent viability reduction.

Caires *et al.* (1999), when studying C6, HeLa and Hep-2 cell lines, observed cell-growth inhibition upon palladium(II) complex treatment, with concentrations higher than those obtained in the present work. Here, the maximum effect on cell-growth inhibition was achieved with 0.1 μ M C1 and C2 complex treatments.

It has been demonstrated that palladium (II) complexes can bind to DNA in both the coordination and intercalative modes, thereby forming the DNA adducts involved in cytotoxic specificity and significant cancer-cell inhibitory activities (Gao *et al.* 2010, 2011). In the present work, the cytotoxic effect of palladium(II) complexes, possibly due to complex-DNA interaction, was clearly shown.

In conclusion, the tested C1 and C2 palladium(II) complexes showed growth inhibition activity and induced alterations in cell morphology, both compatible with cytotoxicity against human breast adenocarcinoma MDA-MB-435 cells. The cytotoxic effect is an indicative of the compound antitumor activity as the majority of clinical used antitumor drugs show cytotoxic activity (Le Tourneau *et al.*, 2010). This is the first report on the C1 and C2 complexes activity against breast cancer cells and they seem to be good candidates for antineoplastic drug development.

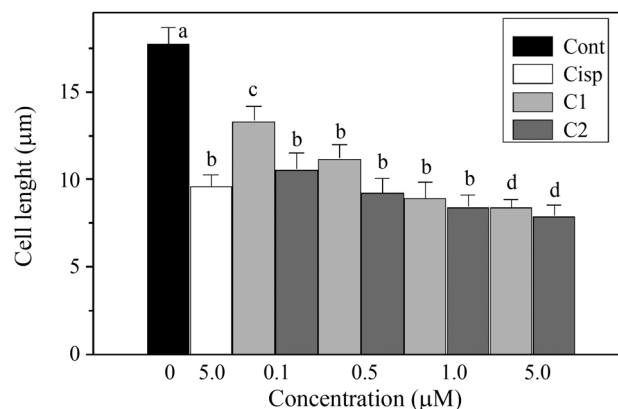


Figure 4 - MDA-MB-435 cell length (μ m) after 24 h incubation. **Cont.**, control cells; **Cisp**, cells treated with 5 μ M cisplatin; **C1**, cells treated with $[\text{Pd}(\text{ca}_2\text{-o-phen})\text{Cl}_2]$; **C2**, cells treated with $[\text{Pd}(\text{dmba})(\text{dppp})\text{Cl}]$. Note cell-length reduction after treatment, in comparison to control cells, thereby indicating Pd(II) complex cytotoxicity. Results are expressed as mean \pm SEM of three independent experiments. Different letters indicate significant differences ($p < 0.01$) by the Tukey test.

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