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Biologic Activity of a Dinuclear Pd(II)–Spermine Complex Toward Human Breast Cancer

Sónia M. Fiuza[1]*, Jon Holy[2], Luis A. E. Batista de Carvalho[1] and Maria P. M. Marques[1,3]

[1]*Química-Física Molecular, Departamento de Química, FCTUC, Universidade de Coimbra, P-3004-535 Coimbra, Portugal*
[2]*Department of Anatomy and Cell Biology, University of Minnesota School of Medicine, Duluth, 1035 MN 55812, USA*
[3]*Departmento de Bioquímica, FCTUC, Universidade de Coimbra, Ap. 3126, P-3001-401 Coimbra, Portugal*
**Corresponding author: Sónia M. Fiuza, sonia.mfiuza@gmail.com*

A dinuclear palladium-based complex (Pd_2-Spm) was synthesized and compared with cisplatin (cDDP) on two different human breast cancer cell lines (MCF-7 and MDA-MB-231) as well as toward an untransformed cell line (BJ fibroblasts). The results obtained show that Pd_2-Spm is more effective against the estrogen receptors [ER(−)] cell line MDA-MB-231, while cDDP displayed better results for the ER(+) MCF-7 cell line. It was shown that, like cDDP, Pd_2-Spm triggers phosphorylation of H2AX, indicating that this compound damages DNA. Apart from DNA, Pd_2-Spm also targets the cytoskeleton having a greater impact on cell morphology than cDDP. Pd_2-Spm and cDDP have opposite antiproliferative activities in the presence of the PI3K inhibitor wortmannin. Furthermore, Pd_2-Spm at an optimized concentration displays a rapid antiproliferative effect as opposed to cDDP, which seems to have a slower kinetics. The results point to a distinct mechanism of action for each of these complexes, which may explain their synergistic action when coadministrated.

Key words: gamma-H2AX, human breast cancer, Pd(II) complex, polynuclear, spermine, wortmannin

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Breast cancer is the most common cancer among women and one of the main causes of death in women in Portugal (1–3). Evolution of human breast cancer is related with cells' dependence on ovarian estrogens, with the presence (+) or absence (−) of estrogen receptors (ER) being an important marker for the prognosis and choice of therapeutic strategies. Generally, patients suffering from ER(+) breast cancer have better life prospects than those with breast cancer lacking ER expression, which tend to be more aggressive (66-month survival rate) (4). For advanced stages of breast cancer, chemotherapy becomes an important therapeutic option. While cisplatin [*cis*-diamminedichloroplatinum(II), *cis*-$Pt(NH_3)_2Cl_2$, cDDP, Figure 1A] is still among the most widely used drugs in cancer chemotherapy, patients that are treated with cDDP suffer from severe side-effects and, very often, develop resistance mechanisms. These facts urge for the pursuit of improved antitumor agents, displaying lower toxicity coupled to a broader spectrum of activity. Hundreds of new cisplatin-based compounds have been synthesized to date, to overcome cisplatin's harmful side-effects while retaining efficacy. Other inorganic agents, comprising different transition metals, have also been studied (5). Pd(II) complexes are particularly interesting because although structurally similar to Pt(II), their reactivity is fairly distinct. In fact, reactions involving Pd(II) are reported to be about 10^4–10^5 faster than those with Pt(II) (6,7). This increased lability is thought to be the main reason for the biologic inactivity of some Pd(II) agents, namely, *cis*-diamminedichloropalladium(II) (*cis*-$Pd(NH_3)_2Cl_2$, cDDPd). However, despite the initial belief that Pd(II) compounds were inactive as antineoplastic agents, many have been synthesized and shown to be not only more active than cisplatin (8–10) but also more effective than their Pt(II) counterparts (11–13).

Because it is broadly accepted that one of the main targets of this type of metal-based compounds is DNA, new strategies to increase their activity range are strongly correlated to their ability to act through a distinct mechanism than cisplatin, even if aiming at the same molecular target. In this regard, multinuclear Pt(II) polyamine complexes comprising cisplatin-like moieties (either $[PtCl(NH_3)_2]$ or $[PtCl_2(NH_3)]$) linked by variable length alkanediammine chains were synthesized and constitute a promising class of anticancer agents (14–16). In fact, the trinuclear complex BBR3464 ([(*trans*-$PtCl(NH_3)_2)_2(\mu$-*trans*-$Pt(NH_3)_2(NH_2(CH_2)_6NH_2)_2]](NO_3)_4$) has already entered phase II clinical trials (17). These multinuclear Pt(II) polyamine chelates display DNA binding properties distinct from those of cisplatin, because their flexible linkers allow the formation of 'long-distance' inter- and intrastrand cross-links unavailable to conventional Pt(II) drugs such as cisplatin or its mononuclear first- and second-generation analogs (18). The biogenic polyamine spermine is able to chelate with metal ions providing such flexible linkers and conferring hydrophobic character to the molecule which is important for drug uptake. In addition, it was previously shown that spermine synergizes with cDDP by modulating cDDP influx through cell membranes (19).

The present work reports a study on the biologic activity of a dinuclear Pd(II) chelate with a spermine ligand (20), Pd_2-Spm [$(PdCl_2)_2$(spm), (spm = spermine, $H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$)) – Figure 1B]. toward two different human breast cancer cell

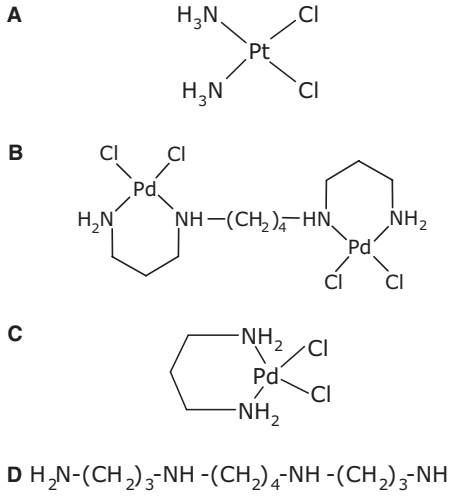

Figure 1: Schematic representation of the compounds used in this study. (A) Cisplatin (cDDP); (B) $(PdCl_2)_2$Spm (Pd_2-Spm); (C) Pd(dap)Cl_2; and (D) Spermine (Spm).

lines – MCF-7 cell line with functional ER and MDA-MB-231 which lacks the expression of ER and is therefore insensitive to estrogen and antiestrogens drugs such as tamoxifen and benzothiophene (21). The BJ cell line was used as a non-tumorigenic model. This study comprises the evaluation of the Pd(II) complex antiproliferative profile, its capacity to induce DNA damage, and the importance of DNA repair on this induced damage.

Methods and Materials

All chemicals and solvents used were reagent grade (Sigma and Aldrich, Sintra, Portugal) and were used without further purification. K_2PdCl_4 (98%), spermine (≥97%), and cisplatin (≥99.9%) were acquired from Sigma (Sintra, Portugal) and used without further purification. Cisplatin was solubilized in PBS and filtered prior to cell treatment. Wortmannin (≥98%) was obtained from Sigma as powder, reconstituted in DMSO, and stored at −20 °C. This solution was diluted in water prior to addition to the cell cultures so that the DMSO concentration never exceeded 1% *(v/v)*. DMEM-HG medium containing phenol red and lacking sodium bicarbonate (99.5%) were obtained from Sigma. Fetal bovine serum (FBS; Gibco, Alfagene, Carcavelos, Portugal) and Trypsin-EDTA (0.05%) were obtained from Gibco. The primary monoclonal anti-β-tubulin antibody E7 was from Developmental Studies Hybridoma Bank, Iowa City, IA, USA, the goat anti-mouse secondary fluorescein-conjugated antibodies from Jackson ImmunoResearch, West Grove, PA, USA, Hoechst 33258 from Sigma, and rhodamine-conjugated phalloidin from Molecular Probes, Eugene, OR, USA. SDS–polyacrylamide gels and nitrocellulose membranes were purchased from Bio-Rad, Hercules, CA, USA. The primary monoclonal antibody anti-γH2AX was obtained from Upstate, Cell Signalling, Piscataway, NJ, USA and the ECF detection system from Amersham, UK.

Synthesis

Pd_2-Spm synthesis was carried out according to Codina *et al.* (20). Briefly, 2 mmol of K_2PdCl_4 were dissolved in a minimal amount of water, and an aqueous solution containing 1 mmol of spermine was added dropwise under continuous stirring (which was kept for about 24 h). This reaction yielded a yellow powder of $(PdCl_2)_2$(spm) which was filtered and washed with pure acetone. The elemental analysis was carried out at the Atlantic Microlab, Inc., Georgia, USA. The vibrational analysis, carried out by both Raman and Inelastic Neutron Scattering (INS) spectroscopies, evidenced the presence of the bands characteristic of these particular metal-amine chelates $\nu_{s(Pd\text{-}N)}$ = 501/cm; $\nu_{as(Pd\text{-}N)}$ = 449/cm; $\nu_{s(Pd\text{-}Cl)}$ = 324/cm; $\nu_{as(Pd\text{-}Cl)}$ = 309/cm.Yield:68%. Calculated – C: 21.56%; H: 4.70%; N: 10.06%, Cl: 25.46% and Found: C: 21.22%; H: 4.68%; N: 9.60%, Cl: 25.88%. Pd_2-Spm was solubilized in PBS and filtered prior to cell treatment.

Cell lines and cell culture

The MDA-MB-231 cell line (human Caucasian estrogen-independent breast adenocarcinoma) was purchased from the European Collection of Cell Cultures (ECCAC, Salisbury, UK), while the BJ line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The MCF-7 line (human Caucasian estrogen-dependent breast adenocarcinoma) was kindly made available by the Biochemistry Service of the Faculty of Medicine of the University of Coimbra.

All the cell lines were cultured as monolayers in tissue culture petri dishes, at 37 °C, in a humidified atmosphere of 5% CO_2. Cultures were grown in DMEM-HG medium containing phenol red supplemented with 10% *(v/v)* fetal bovine serum and sodium bicarbonate. Trypsin-EDTA was used for passaging cells at near-confluence. Under these growing conditions, the duplication time was found to be 26, 51, and 167 h for the MDA-MB-231, MCF-7, and BJ cell lines, respectively, [which is in accordance with previous findings (21)].

Proliferation assays

Simple proliferation assays

For the determination of the antiproliferative activity of the Pd_2-Spm complex, cultures were established in 24-well plates (1 mL/well) at a density of 5×10^3 cells/mL and were allowed to attach for about 24 h. Triplicate cultures were treated for different incubation periods and different concentrations of the test compounds (from 1 to 16 μM). Because the three cell lines studied have different population doubling times, the results were compared both for equal time-points (an early 24-h time-point and a mid-time-point of 72 h), as well as a late time-point that reflected a similar number of population doublings for each cancer line (about four population doublings after the addition of the compounds, corresponding to the 96-h time-point for MDA-MB-231 and 168 h for MCF-7). Because of the low growth rate of the non-tumorigenic BJ cells, the 168-h time-point simply comprises one population doubling, and therefore, these results are interpreted as cell survival. At the end of each time-point, the growth media was aspirated, the wells were washed, and the cells were fixed with ice-cold methanol [1% *(v/v)* acetic acid] and stored at −20 °C. After this fixation process, cell proliferation was evaluated through the Sulforhodamine B (SRB) staining assay that determines cellular protein content interpreted as cell number (22–26).

Two different schedules of drug treatment were used: (i) continuous exposure of the cells to the compounds under study; (ii) non-continuous exposure, having the drugs removed and replaced by fresh media, after one population doubling.

Cisplatin was used in all experiments for comparison purposes. The results obtained for Pd_2-Spm and cDDP can be compared in terms of potency, for either equal doses of each agent or using twice the concentration of cDDP for each Pd_2-Spm dosage, i.e., considering an equivalent number of metals centers.

Proliferation assays in the presence of wortmannin

The proliferation assays in the presence of wortmannin were performed in the continuous presence of the inhibitor. All the cells, including the control samples, were preincubated with 10 μM wortmannin for about 1 h prior to administration of the test compounds. These experimental conditions were based on a previously published study (27).

Proliferation assays of Pd_2-Spm combined with cDDP

For this experiment, the cells were seeded in a 24-well plate (1 mL/well) at a density of 5×10^3 cells/mL and were allowed to attach for 24 h. To test for two different drugging schedules, (i) the cells were exposed simultaneously to either 2 or 4 μM of each compound (ii) the cells were treated with an initial dose of Pd_2-Spm (4 μM) for 24 h, after which the media was removed and the wells were washed with PBS. Fresh media was added, and cisplatin was administered at 1 and 2 μM concentrations. The end-points were collected from this time forward.

For the experiment where the compounds were coadministrated, the drug interactions were assessed using the methods described by Berenbaum (28). Synergism was evaluated using the formula (29):

$$\frac{a}{A} + \frac{b}{B} = I \qquad (1)$$

where A and B represent the IC_{50} values of compounds Pd_2-Spm and cDDP, respectively, a is the IC_{50} calculated for the coadministration and b is the concentration (μM) of cDDP used in combination with Pd_2-Spm. If $I < 1$, there is synergy; if $I = 1$, there is an additive effect only and when $I > 1$, an antagonist interaction occurs.

Presentation of the proliferation assay results and statistical analysis

Proliferation data were obtained from experiments in which both controls and cultures exposed to the test compounds were established and processed in parallel. All the results are expressed in terms of percentages of the control value. The IC_{50} values were calculated from dose–response studies for each compound in a range of 0–50 μM (data not shown). The data presented are an average of at least three independent experiments, with the corresponding standard error of the mean (SEM) having been calculated in all cases. The statistical significance of the differences from the control was assessed using Newman–keuls *post*-test. All the calculations were performed with the GraphPad Prism 4 Software (GraphPad Software, La Jolla, CA, USA).

Immunocytochemistry

MDA-MB-231 were grown on glass coverslips and treated with 2 and 4 μM of both Pd_2-Spm and cDDP. After a 24-h exposure time, the media was removed, the wells were washed with PBS, and the cells were fixed in the appropriate solution. For microtubules labeling, the cells were fixed in ice-cold methanol and kept at −20 °C for an hour. After rehydration in PBST (50 mM Tris–HCl, pH 8; 154 mM NaCl and 0.1% Tween 20), coverslips were blocked with 1% powdered milk in PBST for 30 min at 37 °C and subsequently washed three times with PBST for 5 min. The primary monoclonal anti-β-tubulin antibody E7 was then incubated for 1 h at 37 °C. Following primary antibody incubation, coverslips were washed three times with PBST for 5 min each and treated with the goat anti-mouse secondary fluorescein-conjugated antibodies. All secondary antibodies were diluted 1:50 with PBST and used at 37 °C for 1 h. DNA was fluorescently stained with 5 μg/mL Hoechst 33258. After a final set of three washes with PBST of 5 min each, coverslips were mounted in antifade medium (90% glycerol, 10% CAPS (*N*-cyclohexyl-3-aminopropanesulfonic acid) buffer, 0.1% phenylenediamine, pH 9) to retard photobleaching and examined and photographed with a Nikon TE-300 inverted epifluorescence microscope equipped with a Photometrics CoolSnap ES CCD camera.

For microfilament labeling, the same procedure was carried out, with the exception that the cells were fixed with 4% paraformaldehyde at 4 °C and were labeled with rhodamine-conjugated phalloidin for 2 h at 37 °C, according to manufacturer's instructions.

Western blot analysis

Phosphorylated H2AX histone (γ-H2AX) quantity was analyzed by Western blot. Cell culture petri dishes (25 cm^2) with confluent MDA-MB-231 cells were exposed for 6 h to 20 μM of each Pd_2-Spm and cDDP and the same amount of vehicle solution (PBS) added to the control cells. The cells were harvested, and Laemmli buffer (20% SDS, 0.1% bromphenol blue dye, 13 M glycerol, 1 mL β-mercaptoethanol) was added at a proportion of 1×10^5 cells/10 μL Laemmli buffer, sonicated, and denaturated at 95 °C for 5 min. The samples were tested for equal amount of protein by Coomassie and Ponceau dye staining as well as by immunolabeling the membranes with β-Actin to confirm equal protein loading in each lane. The samples were loaded in the gel (20 μL), separated by electrophoresis on 8% SDS–polyacrylamide gels (SDS–PAGE), and electrophoretically transferred to a nitrocellulose membrane. After blocking with 5% milk in PBST for 2 h at room temperature, membranes were incubated with the antibodies directed against the phosphorylated form of histone H2AX for 1 h at 37 °C. Membranes were washed with PBST and further incubated with horseradish peroxidase-conjugated secondary antibodies, for 1 h at 37 °C. Membranes were reacted with the ECF detection system and were exposed to Kodak X-Ray film.

Results

Simple proliferation assays

Continuous exposure experiments

To accomplish the proposed objectives, several experiments were performed, starting with the investigation of the simple antiproliferative profile of the Pd_2-Spm complex. SRB assays indicate that Pd_2-Spm inhibits MDA-MB-231 proliferation more strongly than MCF-7 cells (Figure 2, Table 1). Indeed, MCF-7 cells are able to recover from the effect of Pd_2-Spm at 2 and 4 μM. Although the Pd_2-Spm complex is not very effective at lower concentrations (2 μM for MDA-MB-231 and 2 and 4 μM for MCF-7), for the maximum dosage tested (8 μM), it has a dramatic effect within 24 h. In contrast to Pd_2-Spm, cisplatin more effectively inhibits the proliferation of MCF-7 than MDA-MB-231 cells (but requires more than 3 days of treatment to do so). In fact, for the latter, at 96 h of incubation, the effect of 8 μM of Pd_2-Spm is only reproduced using twice the dose of cDDP (16 μM). Nevertheless, cDDP is more effective than Pd_2-Spm at 2 μM for the MDA-MB-231 cell line and 2 and 4 μM for the MCF-7 cell line after four population doublings. Regarding the MCF-7 line, cDDP's maximum activity is only verified after an incubation time of 168 h (about four population doublings) which is reflected in its IC_{50} values (Table 1 – 24 versus 72 h for both cell lines). Overall, cDDP presented a certain lag time relative to Pd_2-Spm for both cell lines, corresponding to a quite low growth inhibition profile for the early first time-point (24 h).

Survival of BJ fibroblasts as a non-tumorigenic model

To examine how Pd_2Spm compared with cDDP in targeting non-malignant cells, the effects of these compounds were tested on normal (untransformed) fibroblasts. Both compounds behave very similarly (Figure 2), and for the lowest concentrations used (2 and 4 μM), BJ cell survival is never lower than 80%, with higher

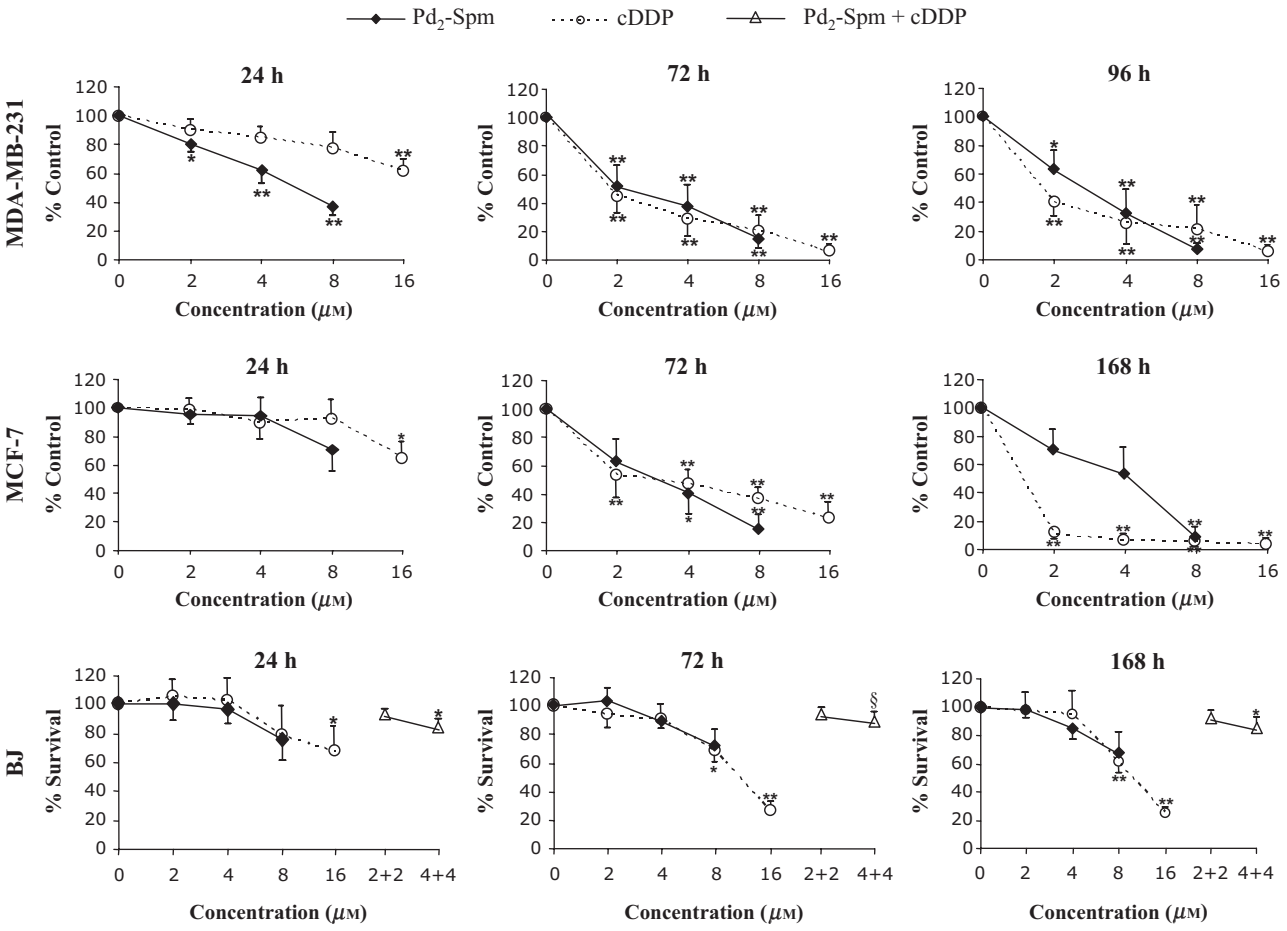

Figure 2: Continuous exposure experiment proliferation results for the MDA-MB-231, MCF-7, and BJ cell lines exposed to Pd_2-Spm and cDDP. The results are presented as percentage of the control ± SEM and are compared for equal time periods (24 and 72 h) and in terms of population doublings (about four population doublings at 96 h for MDA-MB-231 and at 168 h for MCF-7 cell line; BJ cell line takes about 167 h to duplicate, and therefore, the results reflect only one population doubling are only presented as a percentage of survival instead of proliferation). The one-way ANOVA statistical analysis was used, and the Neuman–keuls post-test was carried out to verify the significance of the obtained results (**$p < 0.001$; *$p < 0.01$; $^{\S}p < 0.05$ versus control for the same time-point).

Table 1: Calculated IC_{50} values for the different experiments presently performed. For the proliferation assays of Pd_2-Spm combined with cDDP, a synergy parameter (eqn 1) is also included

	IC_{50}			
	MCF-7		MDA-MB-231	
Experiment	24 h	72 h	24 h	72 h
Simple proliferation assays	Pd_2-Spm (μM)			
	10.9	3.3	4.7	2.8
	cDDP (μM)			
	22.3	2.8	20.2	3.2

	MDA-MB-231	
Proliferation assays in the presence of wortmannin	24 h	72 h
	Pd_2-Spm (μM)	
	5.2	2.6
	cDDP (μM)	
	37.2	8.0
Proliferation assays of Pd_2-Spm combined with cDDP. Schedule (i)	Pd_2-Spm + cDDP (μM)	
	1.9	1.1
Proliferation assays of Pd_2-Spm combined with cDDP. Schedule (ii)	cDDP (μM)	
	0.83	0.66

Proliferation assays of Pd_2-Spm combined with cDDP	I[a]	Observation
2 μM Pd_2-Spm + 2 μM cDDP	0.6	Synergy
4 μM Pd_2-Spm + 4 μM cDDP	0.9	Synergy

[a]For details, refer to Experimental Section (Proliferation assays – Proliferation assays of Pd_2-Spm combined with cDDP).

dosages leading to a lower survival rate. In general, BJ cells appear to be more resistant to both Pd_2-Spm and cDDP than MDA-MB-231 and MCF-7. These results are important, because they provide promising data for the selectivity of the Pd_2-Spm complex.

Simple proliferation assays

Non-continuous exposure experiments

To determine the irreversibility of the antiproliferative effect of the compounds under study, experiments were also performed in a non-continuous manner, by removing the culture medium after continuous exposure to the drug for a period equal to one population doubling time (26 h for MDA-MB-231 and 51 h for MCF-7).

In general, the data for this non-continuous treatment evidence lower cell density values than the ones observed for the continuous treatment (Figure 3), with most of the variations lying within the experimental error range. This is possibly due, at least partially, to the experimental protocol, because the removal of the culture media followed by the extra washing step may dislodge and remove some cells, especially those that are dividing (which are rounded up and weakly attached). The main conclusion to withdraw from this experiment is that the damage induced by these compounds occurs mainly at the first population doublings and that it is not reversible for the experimental conditions tested.

In sum, the simple proliferation experiments allowed to conclude for the tested cell lines that: (i) Pd_2-Spm is more active than cDDP in rapidly suppressing the ER(–) MDA-MB-231 cell line growth; (ii) Pd_2-Spm is less active than cDDP in long-term suppression of MCF-7 cells; (iii) compared to cisplatin, Pd_2-Spm is more effective at early time-points; (iv) the responses of the normal fibroblast cell line were similar to both Pd_2-Spm and cDDP and (v) overall, both of these malignant cell lines appeared to be more sensitive to both compounds than normal BJ fibroblasts, especially for longer time periods and higher doses of the compounds.

To certify that the verified antiproliferative effect was because of the complex as a whole as well as to aid the SAR's (Structure–Activity Relationships) investigation, the Pd(II) complex was considered as the sum of different chemical entities that could, by themselves, be significant for the overall biologic activity of the compound. For this reason, the spermine ligand (Figure 1D) was studied at equivalent concentrations of the complex (one spermine molecule *per* one complex unit), and the smaller palladium complex $Pd(dap)Cl_2$ (which is a good model of the metal co-ordination environment in Pd_2-Spm; Figure 1C) was screened as to its antiproliferative profile using twice the concentration of Pd_2-Spm to attain an equivalent number of metals centers. It was verified that neither spermine nor $Pd(dap)Cl_2$ displayed any antiproliferative effect against the cell lines studied and for the concentrations tested (data not shown) and were therefore not considered on further experiments.

Because one of the goals of this study is to assess the possible targets of Pd_2-Spm, the following experiments were only performed for the MDA-MB-231 cell line, which was found to be more sensitive to this compound.

DNA damage – γH2AX quantification by Western blot analysis

DNA damage can be divided in two general classes: single-base alterations and structural distortions. Cisplatin damages DNA by structural distortion through the formation of bulky adducts that result from covalent binding to the bases. These distortions are mainly because of intrastrand cross-links with the double helix (~90%) with only a small portion of the lesions being because of monoadducts and about 2% as a result of interstrand cross-links (ICLs) (30). These lesions block replication and transcription and might cause replication-mediated double-strand breaks (DSBs) (31). As cDDP, metal-based drugs such as Pd(II) and Pt(II) complexes are expected to interact with DNA (32,33). Because Pd_2-Spm presented good antiproliferative results, the next step was to assess whether Pd_2-Spm damages DNA, specifically by the induction of DSBs that are considered to be the most damaging biologic lesion that can take place in the cell (34).

The results obtained are depicted in Figure 4 and show that Pd_2-Spm induces H2AX phosphorylation to a high extent, evidencing a

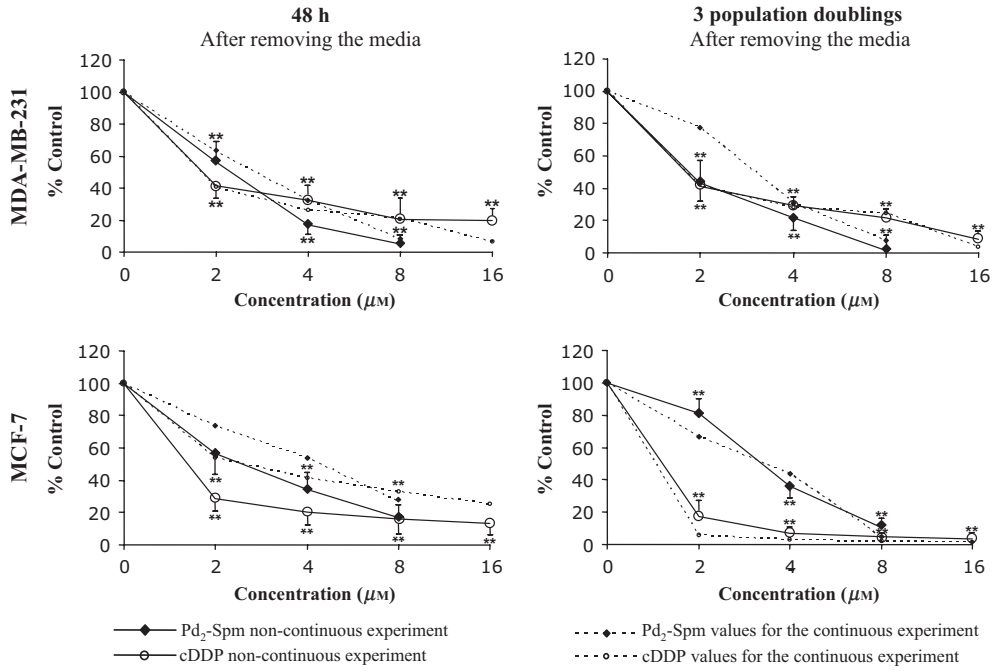

Figure 3: Non-continuous exposure experiment for MDA-MB-231 and MCF-7 cell lines. The results are presented as percentage of the control ± SEM. After the initial exposure to either Pd_2-Spm or cDDP during one population doubling (26 h for MDA-MB-231 and 51 h for MCF-7), the media containing the drug was removed, the cells were washed with PBS, and fresh media was added to the cells. The cells were collected 48 h and three population doublings after this procedure. Data obtained for equivalent time-points were collected from Figure 2 (Continuous exposure treatment) for comparison purposes and are presented as dashed lines. The error bars of this experiment are not presented for simplicity purposes. The one-way ANOVA statistical analysis was used, and the Neuman–keuls post-test was carried out to verify the significance of the obtained results (**$p < 0.001$ versus control for the same time-point).

damaging interaction with DNA. This damage occurs relatively rapidly (within 6 h of drug administration), and the effect is higher for Pd_2-Spm than for cDDP. The early time-point and the lack of morphological features of apoptosis exclude the possibility of apoptosis-induced H2AX phosphorylation. The increased levels of DNA damage (implied by H2AX phosphorylation) induced by Pd_2-Spm could explain the significantly greater inhibition of proliferation measured for this compound when compared to cDDP at the 24-h time-point. However, further experiments are needed to establish a relation between DNA damage and cell growth inhibition.

DNA-PK-mediated DNA repair – proliferation assays in the presence of Wortmannin

In light of the results yielded by the H2AX experiment, proliferation assays were performed in the presence of the phosphoinositide 3-kinases (PI3K's) (35) inhibitor wortmannin, which suppresses H2AX phosphorylation (36) and DNA and DSB repair. DNA repair is known to be one of the causes of resistance displayed by cancer cells to the effect of DNA-damaging antineoplastic drugs. The fungal furanosteroid metabolite wortmannin was shown to inhibit DSBs repair processes and can therefore potentiate the DNA-damaging effect of anticancer drugs (29,37,38).

There are five recognized DNA repair pathways: nucleotide excision repair (NER), mismatch repair (MMR), double-strand break repair

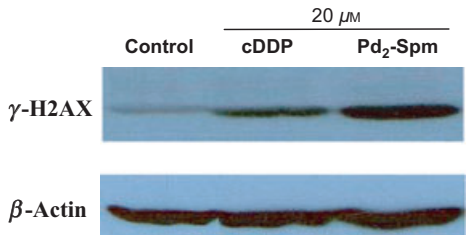

Figure 4: Western blot detection of H2AX phosphorylation (γH2AX) for the MDA-MB-231 cell line. Cells were exposed for 6 h to 20 μM of each Pd_2-Spm and cDDP with the same amount of vehicle solution (PBS) added to the control cells. The cells were collected and processed as described in the Experimental Section. The β-Actin immunolabeling is presented to verify the equal amount of protein.

(DSBr), base excision repair (BER), and direct repair (DR). Two general types of mechanisms exist for DSB repair: homologous recombination (HR) and non-homologous end-joining (NHEJ) with the latter been described as the predominant DSB repair mechanism in mammalian cells (39–41). NER and MMR appear to be major repair pathways of cisplatin-induced DNA damage (42), with the NHEJ pathway being activated for DSB repair in cisplatin-treated cells (39,43). The NHEJ pathway that involves the DNA-PK holoenzyme

(44,45) is greatly inhibited by Wortamnnin (27,29,37) by interaction with DNA-PKcs subunits that belong to the PI3K family.

Considering that Pd_2-Spm induces DSBs in DNA, we wanted to test whether the proliferation of MDA-MB-231 cells in the presence of Pd_2-Spm was affected if this DSB repair pathway was inhibited. An enhancement of the growth inhibition effect was previously detected in the presence of wortmannin for etoposide and alkylating agents such as chlorambucil (29,37), which induce different adducts with DNA (including ICLs), as well as for ionizing radiation also known to cause DSBs (38).

Proliferation assays in the presence of wortmannin were performed toward the MDA-MB-231 cell line, exposing both the control and the test cells to 10 μM of wortmannin for 1 h prior to drug administration as described in the experimental section.

The results obtained, depicted in Figure 5, evidence that wortmannin hardly affects the antiproliferative profile of Pd_2-Spm evidencing that DSB repair does not appear to play a key role in Pd_2-Spm mechanism of action. In fact, most alterations are within the experimental error range, and only a greater enhancement effect is verified for 2 μM of Pd_2-Spm at the 96-h time-point. It is interesting to notice that this improvement is occurring for the lowest concentration of Pd_2-Spm, which is the only dosage for which recovery is verified on the simple proliferation assays (Figure 2, for the MDA-MB-231 cell line in the presence of 2 μM of Pd_2-Spm at 72 h versus 2 μM at 96 h). This may suggest that the cell recovery observed is at least in some part because of DNA repair. The fact that this type of DNA repair is not related to the high amount of DSBs induced, can be as a result of different reasons one of them being that for higher dosages of Pd_2-Spm (4 and 8 μM in this case), the cell DNA repair capacity might be insufficient to maintain, and it does not become significant to inhibit DNA repair under these conditions. In fact, at higher concentrations, the compound might start targeting other components of the cell important for viability. To further evaluate the importance of DNA repair, other studies could be performed such as checking for more evident effects of wortmannin with lower doses of Pd_2-Spm and testing the effects of Pd_2-Spm on cell lines with known differences in their ability to repair DNA.

Cisplatin's antiproliferative effect that was measured to compare with Pd_2-Spm was surprisingly found to drastically decrease in the presence of Wortmaninn (Figure 5 and Table 1). This is in accordance with a previous study performed with fibroblasts and colon carcinoma cells (HT-29) which were protected against the cytotoxic effect of cisplatin in the presence of this inhibitor (46). This may be because of an alternative and efficient repair route (47) adopted by these cells which results in an adaptative and more efficient response to cisplatin (48,49), leading to a high cell survival rate in the presence of cDDP but not Pd_2-Spm. It should however be taken into account that wortmannin is a PI3K inhibitor with a broad spectrum of activity and can have a large effect on a variety of different cellular mechanisms, including the DNA damage–sensing Fanconi anemia/BRCA pathway that is sensitive to cross-linking agents (50–52). However, Jensen and Glazer (53) using mouse fibroblasts specifically mutant on DNA-PK subunits ($Ku80^{-/-}$ and $DNA\text{-}PK_{cs}$)

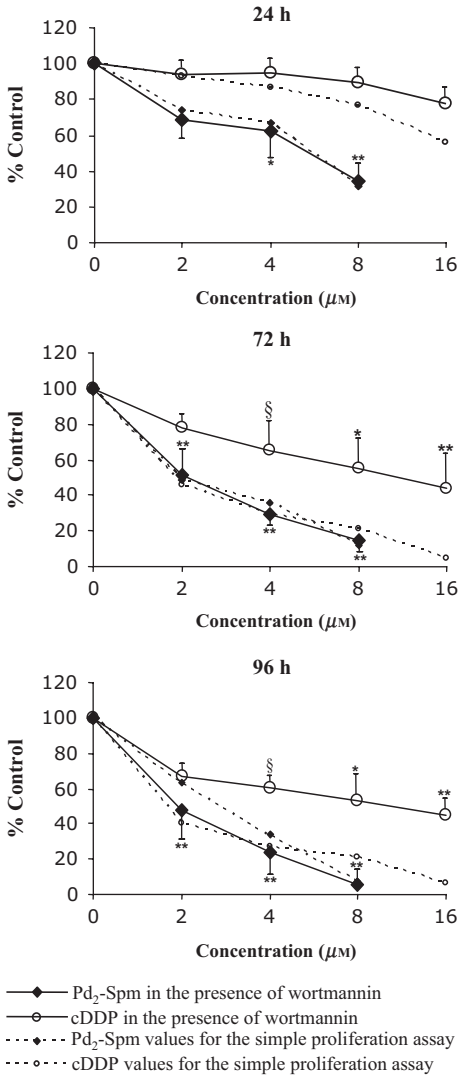

Figure 5: Proliferations assays toward MDA-MB-231 cell line in the presence of wortmannin. The results are presented as percentage of the control ± SEM. After the initial exposure of the control and test cells to 10 μM of wortmannin for an hour, Pd_2-Spm (2, 4, and 8 μM) and cDDP (2, 4, 8 and 16 μM) were added to the test wells. The cells were collected at 24-, 72-, and 96-h time-points. Data obtained for equivalent time-points were collected from Figure 2 (Continuous exposure treatment) and presented for comparison purposes and are presented as dashed lines. The error bars of this experiment are not presented for simplicity purposes. The one-way ANOVA statistical analysis was used, and the Neuman–keuls post-test was carried out to verify the significance of the obtained results (**$p < 0.001$; *$p < 0.01$; §$p < 0.05$ versus control for the same time-point).

showed that DNA-PK-mediated DNA repair is, in fact, important for cisplatin's mode of action.

Obviously, the mechanisms of DNA repair and cellular response are a result of intricate processes based on a multifactor balance strongly dependent on the type of cell line. The final outcome of

the cell is a result of a cross talk between different signal transduction pathways. Although the use of wortmannin when using proliferation assays is not the best tool to asses DNA repair unambiguously, the experiments are interesting as they constitute evidence of a different behavior of Pd_2-Spm relative to cDDP. Therefore, whichever the route involved in the different behavior of cDDP and Pd_2-Spm in the presence of wortmannin, the data presently gathered suggest that there might be a significant mechanistic difference between these compounds as verified for other Pt(II) versus Pd(II) systems (54).

We hypothesized that this divergence could be because of the ability of Pd_2-Spm to induce DNA ICLs to a higher extent than cDDP (~2%) (30). ICLs are more difficult to repair than intrastrand ones, and cells seem to use several repair pathways in a co-ordinate manner to eliminate them, with different tumor types differing widely in their ICLs repair mode (55). This hypothesis, however, remains to be further verified because the experiments performed for evaluating the formation of ICLs (56) by Pd_2-Spm were not conclusive (data not shown).

At this point, it was possible to conclude that: (i) Pd_2-Spm leads to DNA DSBs; (ii) Pd_2-Spm induces DSBs to a higher extent than cDDP; (iii) the inhibition of DSB repair does not seem to play a key role in Pd_2-Spm mechanism of action; (iv) wortmannin experiments suggest that Pd_2-Spm and cDDP have a different mechanism of action.

Proliferation assays of Pd_2-Spm combined with cDDP

Considering the apparent mechanistic difference between Pd_2-Spm and cDDP, it was questioned if their combined effect would lead to improved efficacy. The investigation of their combined effect was assessed in two different ways: (i) by drugging the cells simultaneously with equal amounts of Pd_2-Spm and cDDP (2 and 4 μM of each) using drug concentrations that were not too harmful for the fibroblasts at the single-agent experiment (Figure 2) and (ii) by administering the compounds in an alternate schedule, with an initial higher dose of Pd_2-Spm (4 μM) having been given at the beginning of the experiment before dropping to lower maintenance doses of cDDP (1 and 2 μM). This experiment was designed to profit from the key advantages of each compound – Pd_2-Spm is more effective at early time-points and cDDP for longer ones.

The results obtained for the combined experiment (i) (Figure 6A) evidence that the coadministration of the test compounds yields better results than the single-agent experiments (Figure 2 versus Figure 6). In fact, the antiproliferative profile obtained in the former is more similar to the ones obtained for the higher doses tested individually (8 and 16 μM of Pd_2-Spm and cDDP, respectively) with the advantage of keeping BJ survival above 80% (Figure 2).

In view of better analyzing the data obtained by this simultaneous coadministration of cDDP and Pd_2-Spm, an interaction parameter was calculated, and the results are presented in Table 1. These are indicative of a synergistic effect between both compounds, rather than an additive one for this particular assay, reinforcing the idea that these compounds display at least some differences in their mechanism of action. The type of synergistic interaction, either anticounteractive, complementary or facilitating, is however unknown (57).

The second combined experiment with alternate drugging schemes yielded a good antiproliferative profile as well (Figure 6B). After 96 h of Pd_2-Spm·cDDP (2 μM) exposure, the obtained antiproliferative profile is similar to the ones of 8 and 16 μM of Pd_2-Spm and cDDP, respectively, for the single-agent experiments. Nevertheless, the toxicity toward BJ cell line is much lower (8 μM of Pd_2-Spm and 16 μM of cDDP versus 2 μM of cDDP, 4 μM of Pd_2-Spm or even 4 μM cDDP + 4 μM Pd_2-Spm – Figure 2). When comparing these results with the antiproliferative profile of 4 μM of Pd_2-Spm for the non-continuous experiments, it can be seen that the antiproliferative profile of the maintenance dose of 1 μM cDDP is slightly better than the one observed for the 4 μM of Pd_2-Spm and when using 2 μM of cDDP, there is an accentuated effect and a greater improvement of the antiproliferative profile.

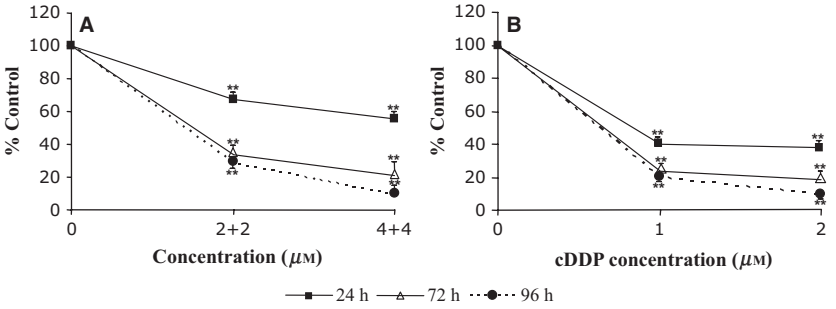

Figure 6: Proliferation assays of combined Pd_2-Spm and cDDP administration toward MDA-MB-231 cell line. The results are presented as percentage of the control ± SEM. (A) Pd_2-Spm and cDDP were coadministrated with 2 and 4 μM of each for 24-, 72-, and 96-h time-points. (B) Combined experiment of Pd_2-Spm and cDDP with an alternate drugging scheme. Cells were exposed for 24 h to 4 μM of Pd_2-Spm. After this time-point, the media was removed and the cells were washed with PBS. Fresh media was added to the cells, and a maintenance dose (1 or 2 μM) of cDDP was administrated to the cells. Incubation times of 24-, 72-, and 96-h counting after the addition of cDDP were considered. The one-way ANOVA statistical analysis was used, and the Neuman–keuls post-test was carried out to verify the significance of the obtained results (**$p < 0.001$; *$p < 0.01$ versus control for the same time-point).

Any of these combined schemes seem to be a good alternative to the use of higher dosages of the compounds used individually at a low toxicity cost and benefiting from both their intrinsic advantages.

An individual experiment has not been performed simultaneously for the BJ cell line because the individual toxicity found for the maximum doses used in this experiment was considered to be rather low (4 μM of Pd_2-Spm with BJ survival above 80% and 2 μM cDDP yielded BJ survival values above 90%; Figure 2). It was also observed that the combined use of cDDP and Pd_2-Spm at 4 μM + 4 μM also allowed a BJ survival above 80% (Figure 6).

The combined experiments allowed to shown that: (i) Pd_2-Spm acts synergistically with cDDP; (ii) a staggered or combined drugging scheme can improve both compounds efficacy; (iii) this data reinforce the idea of a different mechanism of action of these compounds.

Pd_2-Spm effects on the cytoskeleton – immunocytochemistry

In addition to damaging DNA, cDDP has been shown to alter other aspects of cell function, including the organization of the cytoskeleton. Previous studies show that cisplatin arrests tubulin polymerization (58), induces MCF-7 cytoskeleton remodeling (59), and interferes with microtubule and intermediate filament organization (60). To compare Pd_2Spm and cDDP effects on cytoskeleton, microfilaments and microtubules were labeled in control and drug-treated MDA-MB-231 cells. Both compounds exhibited stronger effects on microtubules than on microfilaments (Figure 7C versus D). While 2 and 4 μM of cDDP seem to have a similar effects on microtubule organization, Pd_2-Spm is more damaging, and there is a greater effect for 4 μM relative to 2 μM. At higher Pd_2-Spm concentrations, the microfilaments also begin to look somewhat affected. Either this is a primary target for the Pd(II) complex or not remains under investigation.

Conclusions

A dinuclear palladium-based complex, Pd_2-Spm, was synthesized and compared with cisplatin on two different human breast cancer cell lines, MCF-7 and MDA-MB-231, as well as a normal, untransformed cell line (BJ fibroblasts). The results obtained show that both Pd_2-Spm and cDDP have good antiproliferative profiles against the human breast cancer cell lines tested. However, compared to cDDP, Pd_2-Spm is more effective against the ER(−) cell line MDA-MB-231, but less effective against the ER(+) MCF-7 cell line. Despite the fact that the results for cDDP against MCF-7 are very good, MCF-7 is ER(+) and this type of cancer has a diversity of possible compounds for treatment, namely estrogen antagonists. This raises the possibility that Pd_2-Spm could be of greater use than cDDP against more advanced, ER(−) breast cancers. It was shown

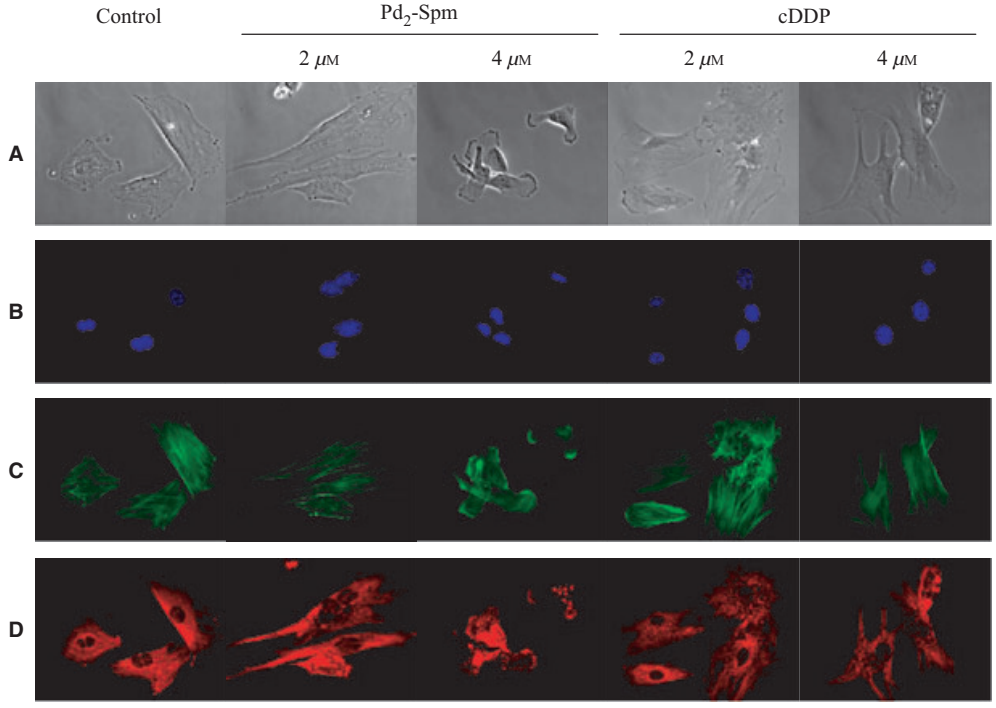

Figure 7: Immunocytochemistry results for the MDA-MB-231 cell line with labeled microtubules and microfilaments. Cells were grown in glass coverslips and exposed to 2 and 4 μM of either Pd_2-Spm or cDDP for 24 h and then collected and fixed in the appropriate solution. The monoclonal anti-β-tubulin antibody E7 was used to label micotubules, DNA was fluorescently stained with 5 μg/mL Hoechst 33258, and the microfilaments (F-actin) were labeled with rhodamine-conjugated phalloidin. Coverslips were mounted in antifade medium to retard photobleaching and examined and photographed with a Nikon Eclipse 3000 epifluorescence microscope. (A) Phase contrast; (**B**) Hoescht labeling; (C) Phalloidin labeling; (D) β-tubulin labeling.

that, like cDDP, Pd_2-Spm triggers phosphorylation of H2AX, indicating that this compound damages DNA. The exact nature of the DNA lesions imparted by Pd_2Spm has not been defined, but activation of H2AX suggests that the damage at least includes DSBs. Although the wortmannin experiments did not determine whether there were differences in the repair of cDDP- and Pd_2Spm-induced DNA damage, the fact that Pd_2-Spm antiproliferative activity was only slightly affected, while cDDP growth inhibition was highly antagonized seems to evidence that Pd_2-Spm must have other targets in the cell and that there is a marked difference in the cellular response to these two compounds, further arguing for distinct mechanisms of action. Also, at an optimized concentration, this agent has a rapid antiproliferative effect as opposed to cDDP, which seems to display a slower kinetics.

These results point to different mechanism of action of the two complexes which may explain their synergistic action when coadministered. Apart from DNA, Pd_2-Spm also targets the cytoskeleton having a greater impact on cell morphology than cDDP, because Pd_2-Spm was found to disrupt the microtubules to a larger extent. Despite the emphasis on the data that evidence that Pd_2-Spm and cDDP have distinct mechanistic pathways, the difference in their effectiveness appears to stem in the diverse modes of interaction on either different or shared molecular targets such as the DNA and the cytoskeleton which were presently found to be two important targets for Pd_2-Spm.

Furthermore, it can be concluded that the Pd_2-Spm complex as a whole entity displays a considerable cell growth inhibition effect, because neither the polyamine ligand nor the analogous metal complex Pd(dap)Cl_2 was shown to have significant antiproliferative profiles. Overall, this suggests that Pd(II) compounds are interesting enough to pursue as novel anticancer agents and may be developed to increase the efficacy of cDDP-type chemotherapeutics.

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