

Citotoxicity and Immune Response induced by Organopalladium(II) Compounds in Mice bearing Ehrlich Ascites Tumour

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Os ciclometalados de paládio(II) são compostos inorgânicos reativos empregados em vários estudos biológicos devido a seu potencial antitumoral e interação com o sistema imune. Neste estudo, a resposta imune e citotóxica induzida por dois complexos organopaladados: [$\text{Pd}(\text{N},\text{C-dmba})_2(\mu\text{-NCS})_2$] (**1**), [$\text{Pd}(\text{C-dmba})(\text{NCS})(\text{dppp})$] (**2**) [dmba = *N,N'*-dimetilbenzilamina, dppp = 1,3-bis(difenilfosfina)propano] e cisplatina (*cis*-DDP), como padrão, foram investigados em camundongos portadores do tumor ascítico de Ehrlich. Os camundongos foram divididos em cinco grupos e inoculados com (**1**) ou (**2**) ou *cis*-DDP ou apenas veículo ou solução salina tamponada de fosfatos (PBS). Diversos parâmetros foram avaliados, tais como a porcentagem de células tumorais presentes no exsudato peritoneal, os níveis de óxido nítrico (NO) e fator de necrose tumoral (TNF- α) séricos e o aumento na expectativa de vida. Os dados obtidos demonstram que o composto (**2**) apresentou atividade similar à da *cis*-DDP, como, por exemplo, aumento na expectativa de vida, diminuição dos níveis séricos de TNF- α e aumento da produção de NO.

Cyclometallated palladium(II) complexes are reactive inorganic compounds employed in several biological studies because of their antitumour potential and interaction with immune system. In the present study, the immune and cytotoxic response induced by two organopalladated complexes: [$\text{Pd}(\text{N},\text{C-dmba})_2(\mu\text{-NCS})_2$] (**1**), [$\text{Pd}(\text{C-dmba})(\text{NCS})(\text{dppp})$] (**2**) [dmba = *N,N'*-dimethylbenzylamine, dppp = 1,3-bis(diphenylphosphino)propane] and cisplatin (*cis*-DDP), as standard, were investigated in mice bearing Ehrlich ascites tumour. The mice were divided into five groups and inoculated with the compounds (**1**) or (**2**) or cisplatin, or only vehicle or phosphate-buffered saline (PBS). Many parameters were evaluated, such as tumour cell percentage in the peritoneal exsudate, levels of seric nitric oxide (NO) and tumour necrosis factor-alpha (TNF- α) and increase in life span. Analysis of all data revealed, for compound (**2**), an activity similar to that presented by cisplatin, resulting in increased life span, lower levels of seric TNF- α and increase in NO production.

Keywords: palladium(II) complexes, Ehrlich ascites tumour, macrophages, nitric oxide, tumour necrosis factor-alpha.

Introduction

Cancer is a disease in which unremitting clonal expansion of somatic cells kills by invading, subverting, and eroding normal tissues.¹ Millions of people die every year from the metastatic spread of cancer that occurs

through blood and lymphatic vessels or directly into tissues and body cavities.² Between the several types of cancer, breast cancer is the most common malignancy in women worldwide.³

In some cases, immune cells constitute a prominent component of the host response to cancer, but their participation in tumour pathogenesis remains not completely understood. Dense intratumoral lymphocyte

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infiltrates in early stages of neoplasm are strongly related with reduced frequencies of metastasis and improved survival time of patients with several types of cancer.⁴ Macrophages play an important role in the host defence against the neoplastic disease, recognizing and killing tumour cells, which are activated with a variety of agents and produce a number of cytostatic or cytotoxic mediators upon stimulation.⁵ This process involves the release of reactive oxygen intermediates (ROI) during the respiratory burst, along with reactive nitrogen intermediates (RNI) and proinflammatory cytokines like tumour necrosis factor (TNF).⁶ When these inflammatory mediators are produced in high quantities, they mediate the tissue damage associated with inflammation and ischemic injury.^{7,8}

cis-Diamminedichloroplatinum(II),⁹ a clinically important antitumour drug, acts like a classical alkylating agent in chemotherapy against some types of cancers. It is a potent stimulator of macrophages and polymorphonuclear leukocytes in the human body. This drug has been proved to be effective alone or in addition to other drugs or cytokines for the treatment of a variety of human and animal malignancies,¹⁰ but its administration in humans shows many side effects such as nausea, nephrotoxicity and neurotoxicity.¹¹ In addition, its applicability is still limited to a relatively few number of tumours. This effect occurs because of the natural resistance to cisplatin or because of the resistance that happens after the initial treatment. These drawbacks are a strong reason for the development of new metal drugs containing other metal centres, for instance, cyclopalladated complexes, which in a previous research presented cytotoxic potential in tests *in vitro* against several tumour lineages.¹² However, currently *in vivo* investigations are very scarce, mainly evaluating effects of organopalladium solutions in cell viability and their ability to induce murine macrophages to recognize and to attack tumour cells.

As part of a program directed at the synthesis of complexes having ligands with noteworthy biological properties, such as aromatic amines and diphenylphosphines, the present research evaluated the cytotoxic activity of the organopalladated compounds [$\{Pd(N,C-dmba)\}_2(\mu-NCS)_2$] (1) and [Pd(dmba)(NCS)(dppp)] (2), dmba = *N,N'*-dimethylbenzylamine, dppp = 1,3-bis(diphenylphosphino)propane, Figure 1, against Ehrlich ascites tumour (EAT), evaluating their cytotoxic potential *in vitro* (IC₅₀), besides their effects in the inflammatory response, the mean survival time and the increase in life span of groups of Swiss mice. In all experiments, *cis*-diamminedichloroplatinum(II) (*cis*-DDP) was used as a standard drug.

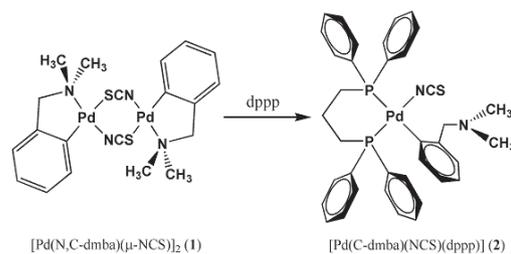


Figure 1. Structures of cyclopalladated compounds [$\{Pd(N,C-dmba)\}_2(\mu-X)_2$] (1) and [Pd(C-dmba)(X)(dppp)] (2), X = NCS.

Experimental

Synthesis

All the syntheses were carried out at room temperature and the reagents were employed without further purification.

The compound [$\{Pd(N,C-dmba)\}_2(\mu-NCS)_2$] (1) was obtained and characterized according to literature methods.¹³ The [Pd(C-dmba)(NCS)(dppp)] (2) was obtained as described: to a yellow suspension of 250 mg (0.42 mmol) of [$\{Pd(N,C-dmba)\}_2(\mu-NCS)_2$] (1) in 30 mL of acetone, 71.90 mg (0.84 mmol) of 1,3-bis(diphenylphosphino)propane (dppp) were added. The resulting solution was stirred for 1 h and then concentrated under reduced pressure. Addition of dichloromethane and pentane gave a white solid. The solid was filtered off, washed thoroughly with pentane and dried *in vacuo*. Yield: 90% (Found: C, 62.25; H, 5.78; N, 4.06; S, 4.32. Calc. for C₃₇H₃₆N₂SP₂Pd: C, 62.40; H, 5.52; N, 3.93; S, 4.50%).

We have employed commercial cisplatin Fauldiscipla[®] 1 mg mL⁻¹ in this investigation as standard.

Instrumental

IR spectra were recorded on a NICOLET IMPACT 400 spectrophotometer in the 4000-400 cm⁻¹ range with the samples in the form of KBr pellets.

Tumour cell line

The tumour cell line used in this experiment was Ehrlich ascites tumour (EAT). The maintenance of tumour was made according to literature methods.¹⁴ This procedure was carried out at the Laboratory of Clinical Immunology of "Faculdade de Ciências Farmacêuticas de Araraquara" - Universidade Estadual Paulista, Araraquara, SP, Brazil. Cell viability was evaluated by trypan blue exclusion test, and only the cell suspensions that showed 95% of viability were employed in the studies.

Animals

Seventy-six male Swiss mice weighing 18 to 25g were supplied by the Faculdade de Ciências Farmacêuticas, Universidade Estadual Paulista, Araraquara, SP, Brazil. They were maintained in polycarbonate boxes at 23 ± 2 °C, $56 \pm 2\%$ humidity, 10-18 cycles/h and a 12 h light/dark cycle, with water and food available *ad libitum*.

In vitro and *in vivo* assays from mice bearing EAT

Animals were sacrificed five days after tumour transplantation, considering as pure culture those containing 95% of tumour cells, without significantly inflammatory cells influx.¹⁴

Cell suspensions were collected in an sterile conic tube (Corning Inc.) and were then centrifuged with 15 parts of NH_4Cl 0.17 mol L^{-1} followed by phosphate-buffered saline (PBS), pH 7.2, for 5 min at 2000 rpm and 25 °C.¹⁵ Cell concentration was adjusted in RPMI medium (Sigma), using a Neubauer haemocytometer (Boeco) and Trypan blue solution. For *in vitro* assays, 1.0 mL of RPMI-1640-Complete, RPMI-1640-C (containing 5% fetal calf serum, mercaptoethanol, penicillin and streptomycin) was added to the cells, adjusting the concentration to 1×10^6 cell/mL.¹⁶ For *in vivo* assays 1.0 mL of phosphate-buffered saline (PBS), pH 7.2 was employed, adjusting the concentration to 1×10^3 cell/mL.¹⁴

Assessment of cytotoxicity using MTT assay for EAT cells

100.0 μL samples of tumour peritoneal cell suspension (1×10^6 cell/mL, adjusted in RPMI-1640-C), obtained from 10 EAT-bearing mice, were added to each well of a 96-well tissue culture plate (Corning) containing 100.0 μL of the compounds (1), (2) and *cis*-DDP in different concentrations (between 100.0 and 5.0 $\mu\text{mol L}^{-1}$) or RPMI 1640-Complete medium with 0.3% of dimethylsulphoxide (DMSO) or only RPMI 1640-Complete as cell control of viability, using the Mossman assay.¹⁷ The effects of the compounds on the cells were determined 48h after culture incubation.

Then, supernatants were removed and 100.0 μL of solution of [3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyl-tetrazolium bromide] (MTT) were added to each well containing the samples. MTT assay was performed and the plates were incubated for 3h. Then, absorbances were measured and the cytotoxic midpoint value, the concentration of chemical agent needed to reduce the spectrophotometric absorbance to 50%, was determined by linear regression analysis with 95% of confidence

limits. The IC_{50} was defined as the average result of three independent experiments through the equation of graphic line obtained (Microcal Origin 5.0™).

In vivo assays

Healthy male Swiss mice were divided in five groups each one containing 10 animals. They were inoculated intraperitoneally (i.p.) with cell suspension at 1×10^3 cells mL^{-1} of PBS. After 3 days of tumour development, the compounds [$\{\text{Pd}(\text{dmba})\}_2(\mu\text{-NCS})_2$] (1), $[\text{Pd}(\text{dmba})(\text{NCS})(\text{dppp})]$ (2) and *cis*-DDP were inoculated, according to literature methods.¹⁸ Groups were named according to the compounds inoculated:

Group 1 or G1: *cis*-DDP (1 mL, i.p., 10 mg kg^{-1}); Group 2 or G2: $[\text{Pd}(\text{dmba})(\text{NCS})(\text{dppp})]$ (1 mL, i.p., 1 mmol L^{-1}); Group 3 or G3: [$\{\text{Pd}(\text{dmba})\}_2(\mu\text{-NCS})_2$] (1 mL, i.p., 1 mmol L^{-1}); Group 4 or G4: Vehicle control (1mL, i.p., DMSO 0.3%); Group 5 or G5: Tumour control (1mL, i.p., PBS).

EAT cells score

Six animals of each group ($n=12$) were sacrificed six days after the inoculation with the complexes, *i.e.*, on the ninth day of tumour development.¹⁴ Determination of the total number of EAT cells was carried out in a Neubauer haemocytometer (Boeco). Differentiation of cells was made by May-Grünwald Giemsa (Reagen) with subsequent observation in an optical microscope under immersion lens (100X).¹⁸

Cell viability by trypan blue exclusion

100.0 μL samples of the cell suspension were added to 100.0 μL of Trypan blue solution (1:10) and the samples were dropped into glass sheets and observed in a microscope under 40X lens. We have considered dead the cells which absorbed Trypan blue solution.

Spreading of macrophages

The spreading of peritoneal macrophages from tumour bearing mice was assessed according to the technique adapted from Rabinovich *et al.* (1977).¹⁹ Briefly, aliquots of 200.0 μL of peritoneal cell suspensions were placed over glass cover slips (Corning Inc.) and incubated for 15 min at 37 °C. The non-adherent cells were removed by washing with PBS and the adherent cells were incubated in RPMI-C culture medium, at 37 °C, in a humidified chamber containing 7.5% of CO_2 (Forma Scientific). Subsequently, the medium was removed and the cells were

fixed with 2.5% glutaraldehyde (Sigma Chemical Co.) and examined under an optical microscope to determine the percentage of spread cells.

Nitric oxide assay

Nitric oxide (NO) measurements were made in the mice serum after conversion of NO_3^- to NO_2^- by bacterial nitrate reductase.²⁰ 100.0 μL of mice serum samples from all test groups were incubated at room temperature in 83.0 μL of Milli-Q water, 15.0 mL of NADPH (reduced dinucleotide adenine β -nicotinamide phosphate - 0.5 mg mL^{-1}) and 2.0 mL of nitrate reductase (5.0 U mL^{-1} NADPH reductase) for 2h. Later, nitrite concentrations were indirectly measured by a quantitative colorimetric assay using Griess reagent system: 50.0 μL aliquots of serum were added to 50.0 μL of Griess reagent [1% sulphanimide (Merck), 0.1% naphthylethylenediamine (Merck) and 3% H_3PO_4 (Mallinckrodt)], incubated at room-temperature for 10 min., and the absorbance was measured at 540 nm in an Elisa reader (Multiskan Ascent Labsystems). Samples in quadruplicate were assayed in four experiments and reported as μmol NO / 100.0 μL of serum \pm SD quantified from the standard curve.

TNF- α bioassay

L929 mouse tumour cells were used to measure TNF- α levels in mice serum. Killing of L929 mouse tumour cells was used to measure the citotoxicity of soluble factor(s) present in the serum.²¹ Briefly, L929 cells were seeded in RPMI-1640-Complete medium at 4×10^4 cells per well in 96-well microlitre plates (Corning) and incubated overnight at 37 °C in an atmosphere of air/7.5% CO_2 . 100 μL of serum samples previously obtained were added, or serum of normal mice, or cells only, as control, plus the same volume of medium containing 1.0 mg Actinomycin D mL^{-1} in triplicate. The next day, cell survival was assessed fixing and staining the cells with crystal violet (0.2% in 20% methanol), dissolving the stained cells with 0.1 mL 1% SDS (sodium dodecyl sulphate) (v/v) per well and reading the absorbance of each well at 490 nm with an Elisa reader (Multiskan Ascent Labsystems). TNF- α units were calculated using a standard curve obtained with recombinant TNF- α constructed for each test run. The criterion employed in this bioassay is that fifty percent of L929 cells death corresponds to one unit of TNF- α , approximately one picogram of recombinant TNF- α .²² In order to confirm the presence of TNF- α in the culture supernatants, these preparations were previously incubated with rabbit anti-r

TNF- α immune serum, and the test samples were added to L929.

Analysis of the effect of compounds on mice survival

The mean survival time (MST) of each group containing 6 mice was monitored for 90 days by recording the mortality daily. Based on the values of MST found we calculated the increase in life span (ILS). These parameters were calculated²³ according to the following equations: $\text{MST} = [(\text{Day of first death} + \text{day of last death}) / 2]$; $\text{ILS} (\%) = \{[(\text{MST of the treated group} / \text{MST of the control group}) - 1] \times 100\}$.

Statistical analysis

Results are representative of three independent experiments and they are presented as Average Values \pm Standard Deviation from quadruplicate observations. Data were statistically analyzed by the variance test in ANOVA and by the Tukey-Kramer post-test, using significance level $p < 0.05$, in the Graph Instat PadTM software.

Results and Discussion

IR spectra

Treatment of the thiocyanate-bridged dimer [$\{\text{Pd}(N, C\text{-dmdba})\}_2(\mu\text{-NCS})_2$] (**1**) with dppp gave $[\text{Pd}(C\text{-dmdba})(\text{NCS})(\text{dpppp})]$ (**2**), in which the diphosphine behaves as a chelating ligand. The IR spectrum of (**2**) showed ν_{CN} , ν_{CS} and δ_{NCS} bands at 2048, 840 and 440 cm^{-1} , respectively, characteristic of a terminal *N*-thiocyanate group, together with the $\nu_{\text{P-C}}$ and δ (C-P-C_{ar}) bands at 696 and 1096 cm^{-1} , respectively, characteristic of the diphosphine coordinated to Pd.

These characteristics indicate that compound (**2**) has the *N*-coordination of thiocyanate. Taking into account that the coordination geometry around the Pd atom is square planar, it is suggested that two phosphorus atoms of the phosphine ligand and one carbon atom of dmdba, leaving the nitrogen atom of dmdba uncoordinated, occupy the three remaining coordination sites. Thus, the compound may be formulated as $[\text{Pd}(C\text{-dmdba})(\text{NCS})(\text{dpppp})]$ (**2**).

Biological activities

Ehrlich ascites tumour (EAT) has been used for evaluating both the citotoxic properties of many classes of substances and the influence of substances in immune response.^{16,24,25} Since *cis*-diamminedichloroplatinum(II)

(*cis*-DDP) emerged as the mostly employed antitumour metal-drug,⁹ many novel complexes containing metals of platinum group are being investigated.

Some cyclopalladated complexes²⁶ of the type $[\text{Pd}(\text{C}-\text{N})(\text{X})(\text{L})]$, where C-N is an aromatic or aliphatic amine coordinated via C and N atoms to Pd; L=nitrogen ligand; X= chloride or acetate, have shown to be citotoxic towards seven tumour lines²⁷, e.g., $[\text{Pd}(\text{dmba})(\text{Cl})(\text{py})]$; dmba = *N,N'*-dimethylbenzylamine and py = pyridine.²⁶ Cyclopalladated complexes containing thiosemicarbazones ligands have also shown antitumour properties when coordinated to palladium atoms.^{27,28}

In the MTT assay,¹⁷ the compound $[\{\text{Pd}(\text{N},\text{C}-\text{dmba})\}_2(\mu\text{-NCS})_2]$ (**1**) has not shown a considerable activity when compared to the citotoxic behaviour of the complex $[\text{Pd}(\text{C}-\text{dmba})(\text{NCS})(\text{dppp})]$ (**2**) as presented in Figure 2. So, we have verified that complex (**2**) showed lower IC_{50} value ($5.29 \pm 3.89 \mu\text{mol L}^{-1}$) than those values of *cis*-DDP (33.77 ± 2.29) and (**1**) (47.86 ± 4.32). These results suggest that structural differences between compounds (**1**) and (**2**), such as the number of metal centres and the presence of diphenylphosphine in (**2**) have influenced their citotoxic effects towards EAT cells *in vitro*. DMSO has the ability to suppress the growth of Ehrlich carcinoma in the solid form.²⁹ In this study the vehicle, DMSO 0.3% diluted in RPMI-Complete medium, was not toxic to EAT cells *in vitro* (99% of cell viability, data not shown).

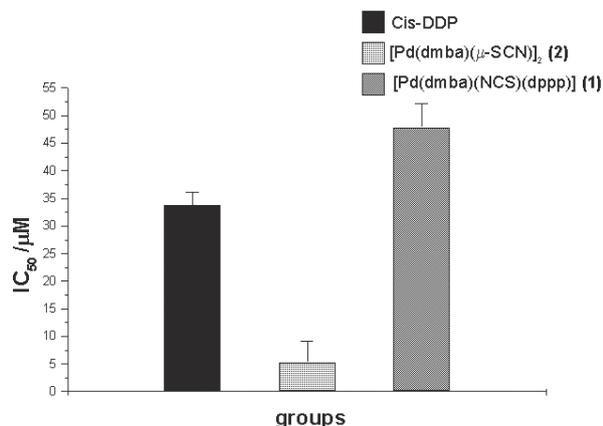


Figure 2. IC_{50} values (mmol L^{-1}) for compounds, determined by concentration versus cell viability, assay *in vitro* after 48 h exposition - 1×10^6 cells mL^{-1} ; $n = 10$ mice.

Besides the tests *in vitro*, in this study we have performed tests *in vivo*, evaluating the inflammatory response of the peritoneal cavity of EAT-bearing mice, inoculated intraperitoneally with solutions of compounds (**1**), (**2**) and *cis*-DDP as standard drug.

As reported in the literature,¹⁴ it has been verified that the inoculation of 1×10^3 cells mL^{-1} maintained the tumour

growth unchanged till the ninth day. During this time the number of polymorphonuclear (PMN) and mononuclear (MN) cells in the cavity was very low. It was observed that the tumour cells did not induce a significant influx of inflammatory cells in this period. Taking into consideration that the spreading due to tumour inoculation occurs in the three first days after inoculation but not later on (nine days), the spreading verified could be due to the action of the compounds. Based on all information above, the complex solutions were injected three days after tumour transplantation, taken as day zero, and the animals were sacrificed on the ninth day of tumour development.

The intensity of the inflammatory reaction evoked by tumour cells is of fundamental importance in the evolution of tumour growth. In clinical pathology, tumours presenting an intense inflammatory infiltrate are considered to have a good prognostic.¹⁴

Organopalladated solutions of (**1**) and (**2**) were responsible for a significant increase of inflammatory influx of macrophages (Figure 3). *Cis*-DDP and (**2**) showed values equal to $58.28\% \pm 7.52$ ($p < 0.001$) and $36.71\% \pm 13.2$, respectively, both different from that of tumour control group. However, compound (**1**) has not showed a statistically significant behaviour, $32.85\% \pm 3.97$.

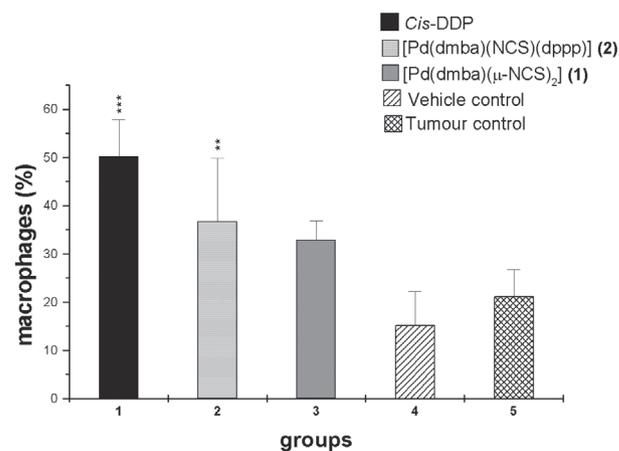


Figure 3. Influence of compounds on the percentage of macrophages in the peritoneal exsudate. The values are represented as mean \pm SD. *** $p < 0.001$, significantly different of Tumour control group.

According to other report, PMN citotoxic activity *in vitro* against EAT is significantly high in Swiss mice. The data indicate that PMN acts against EAT controlling tumour development *in vivo*.³⁰

Table 1 shows better performance of complex (**2**) when compared to (**1**) and similar to *cis*-DDP. Polymorphonuclear cells determination suggests that *cis*-DDP and complex (**2**) have promoted local inflammatory influx of PMN cells of 22.5 ± 4.39 and $38.43\% \pm 11.7$, respectively

($p < 0.001$, significantly different of tumour control group), and complex (1) did not cause the same effect in peritoneal cavity ($9.57\% \pm 3.36$). The tumour cell percentage has showed equivalent results for *cis*-DDP ($27.14\% \pm 4.10$) and complex (2) ($24.86\% \pm 4.26$), while (1) showed a higher value 57.57 ± 5.22 , demonstrating lower citotoxicity *in vivo*. The percentage of cell death observed in all groups ranged from 18.75 ± 4.85 to 26.25 ± 4.78 , that is, the difference is statistically non-significant ($p > 0.05$).

Activated macrophages present a large number of morphologic, functional, and metabolic differences from resting cells. They are bigger and display pronounced ruffling of the plasma membrane, and increased capacity for adherence and spreading on surfaces, increased formation of pseudopods, and increased number of pinocytotic vesicles, as well as functional differences.³¹ The results obtained for spreading macrophages have shown that complex (1) presents an expressive value of $62.0\% \pm 22.81$, different from that found in the tumour control group and similar to *cis*-DDP value of $78.4\% \pm 16.82$ ($p < 0.001$), while complex (2) did not present the same effect: $37.4\% \pm 11.78$.

Our research group has demonstrated that organopalladated compounds containing diphenylphosphine ligands have activated macrophages *in vitro*³² by the immunological activation, *in vitro*, determining the H_2O_2 released in cultures of peritoneal macrophage cells from Swiss mice in the presence of organopalladated compounds of the type $[Pd(dmba)(X)(dppp)]$, *dmba* = *N,N*-dimethylbenzylamine, *dppp* = 1,3-bis(diphenylphosphine)propane, X = Cl, N_3 , NCO, NCS. An excellent activation of macrophages by the $[Pd(dmba)(X)(dppp)]$ compounds was observed and the influence of the X ligand on the immune response could be verified.

Activated macrophages secrete several substances that are directly involved in tumour cell killing, *i.e.*, tumour necrosis factor (TNF) and nitric oxide (NO). Taken together, TNF and NO are considered as the most important mediators directly involved in tumour cell killing.⁵

NO is formed in activated phagocytes as a product of the conversion of *L*-arginine to *L*-citrulline by an inducible isoform of nitric oxide synthase. This reaction, like the oxidative burst, involves O_2 uptake. Nitrite (NO_2^-) and nitrate (NO_3^-) are generally believed to be the end products of the macrophage reactive nitrogen intermediates generating system.³³ Although many other agents are citotoxic to target cells, NO is often found to be the primary mediator of macrophage-induced citotoxicity.³⁴

Serum NO release, Figure 4, has showed that macrophages were activated with the three substances, (1), (2) and *cis*-DDP, presenting similar results: 7.61 ± 0.68 for (1); 9.18 ± 1.06 for (2) and 7.36 ± 1.25 , (*cis*-DDP), $p < 0.001$, significantly different of tumour control group 2.05 ± 0.47 .

These findings can be considered a good prognostic because the citotoxicity as a result of a substantial NO-formation is established to initiate apoptosis, characterized by up-regulation of the tumour suppressor p53, changes in the expression of pro- and anti-apoptotic Bcl-2 family members, cytochrome c relocation, activation of caspases, chromatin condensation, and DNA fragmentation.³⁵

It has been reported that murine macrophages treated with cisplatin acquire enhanced capacity to lyse tumour

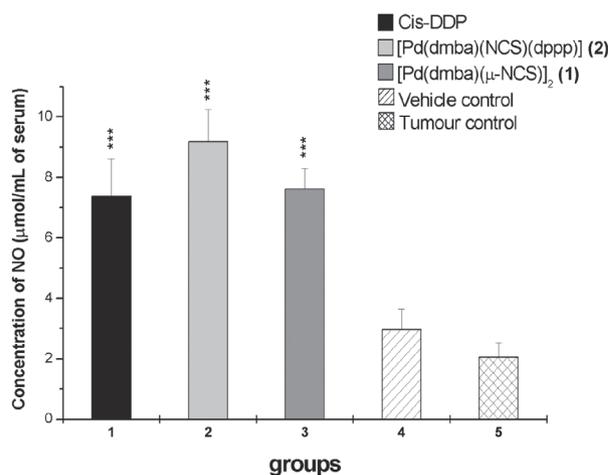


Figure 4. Influence of compounds on NO serum levels. The values are represented as mean \pm SD. *** $p < 0.001$, significantly different of Tumour control group.

Table 1. Effect of organopalladated compounds in mice bearing Ehrlich ascites tumour

Parameters	$[Pd(dmba)]_2(\mu-NCS)_2$ (1)	$[Pd(dmba)(NCS)(dppp)]$ (2)	<i>cis</i> - $[Pt(Cl)_2(NH_3)_2]$ (<i>Cis</i> -DDP)	Vehicle Control	Tumour control
EAT cells (%) ^a	$57.57 \pm 5.22^{***}$	$24.86 \pm 4.26^{***}$	$27.14 \pm 4.10^{***}$	81.25 ± 6.58	73.87 ± 6.92
PMN (%) ^b	9.57 ± 3.36	$38.43 \pm 11.7^{***}$	$22.57 \pm 4.39^{***}$	2.50 ± 1.52	2.80 ± 1.92
Cell death (%) ^c	26.25 ± 4.78	18.75 ± 4.85	22.50 ± 2.88	25.50 ± 3.32	25.50 ± 3.32
Spread macrophages (%) ^d	$62.0 \pm 22.81^{***}$	37.40 ± 11.78	$78.40 \pm 16.82^{***}$	15.14 ± 5.55	17.71 ± 7.06

^{a-d} data are reported as MEAN \pm SD for at least three independent experiments carried out in triplicate. ^adifferentiate score by Giemsa methods, *** $p < 0.001$ different when compared to Tumour control group $n = 6$; ^bdifferentiate score by Giemsa methods, *** $p < 0.001$ different when compared to Tumour control group; $n = 6$; ^cresults obtained by trypan blue methods, $p > 0.05$, similar to Tumour control group $n = 6$; ^dspreading of macrophages, *** $p < 0.001$ different when compared to Tumour control group, $n = 6$.

cells *in vitro*, produce increased amount of interleukin-1 (IL-1) and tumour necrosis factor (TNF), reactive oxygen metabolites (ROI), reactive nitrogen intermediates (RNI), lysozyme and arginase.³⁶

Tumour necrosis factor alpha (TNF- α) is an immunomodulatory cytokine that has exhibited an antitumour activity in a variety of tumour cell lines, including breast cancer cell lines.³⁷ On the other hand, it is becoming more evident that many aspects of tumour promotion arise from persistent and unresolving inflammation. Clinically, elevated serum concentrations and increased expression of TNF-alpha are present in various pre-neoplastic and malignant diseases, compared with serum and tissue from healthy individuals.³⁸

TNF- α serum levels (Figure 5) in the group treated with *cis*-DDP have presented 48.45 ± 3.68 U mL⁻¹, and complex (2) 67.27 ± 5.89 U mL⁻¹ ($p < 0.001$, significantly different of tumour control, 330.57 ± 8.12). Complex (1) have presented results similar to those given by the tumour group (318.14 ± 14.44 U mL⁻¹).

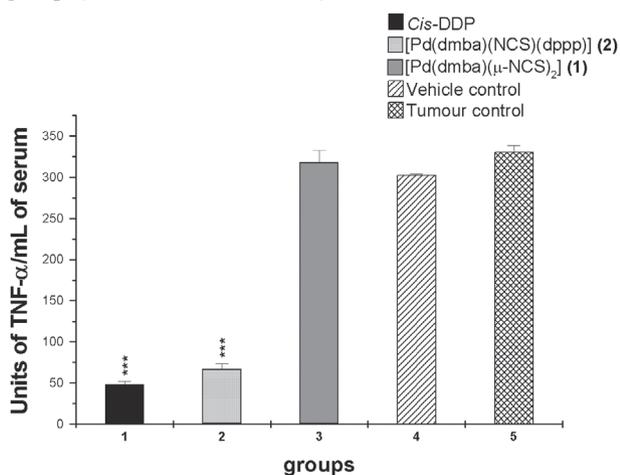


Figure 5. Influence of compounds on serum levels of TNF- α . The values means mean \pm SD. *** $p < 0.001$, significantly different of Tumour control group.

The decrease of TNF- α serum levels in the groups treated with compound (1) may be a good prognostic because the TNF over-expression may lead to malignancy as described above. Besides, a potential tumour-promoting effect of TNF- α has been demonstrated in study, showing that mice deficient in TNF- α were resistant to skin carcinogenesis.³⁹ Other data have demonstrate that over-expression of TNF- increased metastatic activity of tumour lines,⁴⁰ and treatment of mice with TNF- α promoted development of liver metastasis.⁴¹ In a study involving patients with prostate cancer, the results suggest that TNF-alpha directly correlate with the extent of disease.⁴²

Despite described in literature, in this paper mice treated with *cis*-DDP presented decrease in TNF- α levels. IFN- γ is a cytokine that stimulates the synthesis of inducible nitric oxide synthase (iNOS) and the release of NO from

macrophages.⁴³ Based on *in vivo* and *in vitro* observations, many anti-tumour mechanisms have been attributed to IFN- γ in several tumour lineages, including against breast cancer cells.^{37, 44, 45}

In this paper, the increase in NO concentration was observed concomitantly with the decrease of the TNF- α level. In future, experiments IFN- γ determinations deserve to be performed in order to verify its levels in mice treated with the compounds and the correlation with the NO levels.

A reliable criterion for judging the value of any anticancer agent is the increase of life span of animals.²³ Regarding this point, compound (2) has presented the best performance values, showing mean survival time superior to 90 days, equivalent to more than 100% of ILS, as with *cis*-DDP (Table 2).

Table 2. Effect of organopalladated compounds in the mean survival time (MST) and increase in life span (ILS) of the EAT-bearing mice

Groups	MST(days)	ILS (%)
G1	90*	100***
G2	90*	100***
G3	36	5.88
G4	34	—
G5	34	—

$n = 6$ mice in each group, * time that the mice were monitored daily, *** $p < 0.001$ related to Tumour control group; average values. Group 1 or G1: Cis-DDP; Group 2 or G2: [Pd(dmba)(NCS)(dppp)] (2); Group 3 or G3: [Pd(dmba)(μ -NCS)₂] (1); Group 4 or G4: PBS/DMSO 0,3% (Vehicle control); Group 5 or G5: PBS (Tumour control).

Conjunction of overall data, mainly those related to the serum NO and TNF- α release, showed that the mononuclear compound [Pd(*C*-dmba)(NCS)(dppp)] (2) presented a behaviour similar to that of *cis*-DDP. Organopalladated compounds deserve to be tested with other lines of mammary tumour cells, *in vitro* and *in vivo*, according to studies developed with many compounds of palladium(II) to confirm their antitumour properties.

The cyclometallated [Pd(*N,C*-dmba)(μ -NCS)₂] (1) has presented a good performance promoting inflammatory influx of macrophages and PMN cells to the peritoneal cavity. However, it has not showed high cytotoxic activity nor immunomodulatory effects as did [Pd(*C*-dmba)(NCS)(dppp)] (2), according to the elevated number of EAT cells percentage in the peritoneal cavity and the lower increase in the mice life span.

Conclusions

Both compounds, [Pd(*N,C*-dmba)(μ -NCS)₂] (1) and [Pd(*C*-dmba)(NCS)(dppp)] (2), have shown cytotoxic potential *in vitro* and *in vivo*, increasing the life span of the EAT-bearing mice, but (1) presented a lower cytotoxic

potential when compared to compound (2). Compound (2) has shown an activity similar to that of *cis*-DDP; this indicates that this compound is promising in the study of antitumour and immunological activity. The presence of dppp in complex (2), along with other structural characteristics, may be responsible for its better performance.

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Citotoxicity and Immune Response induced by Organopalladium(II) Compounds in Mice bearing Ehrlich Ascites Tumour

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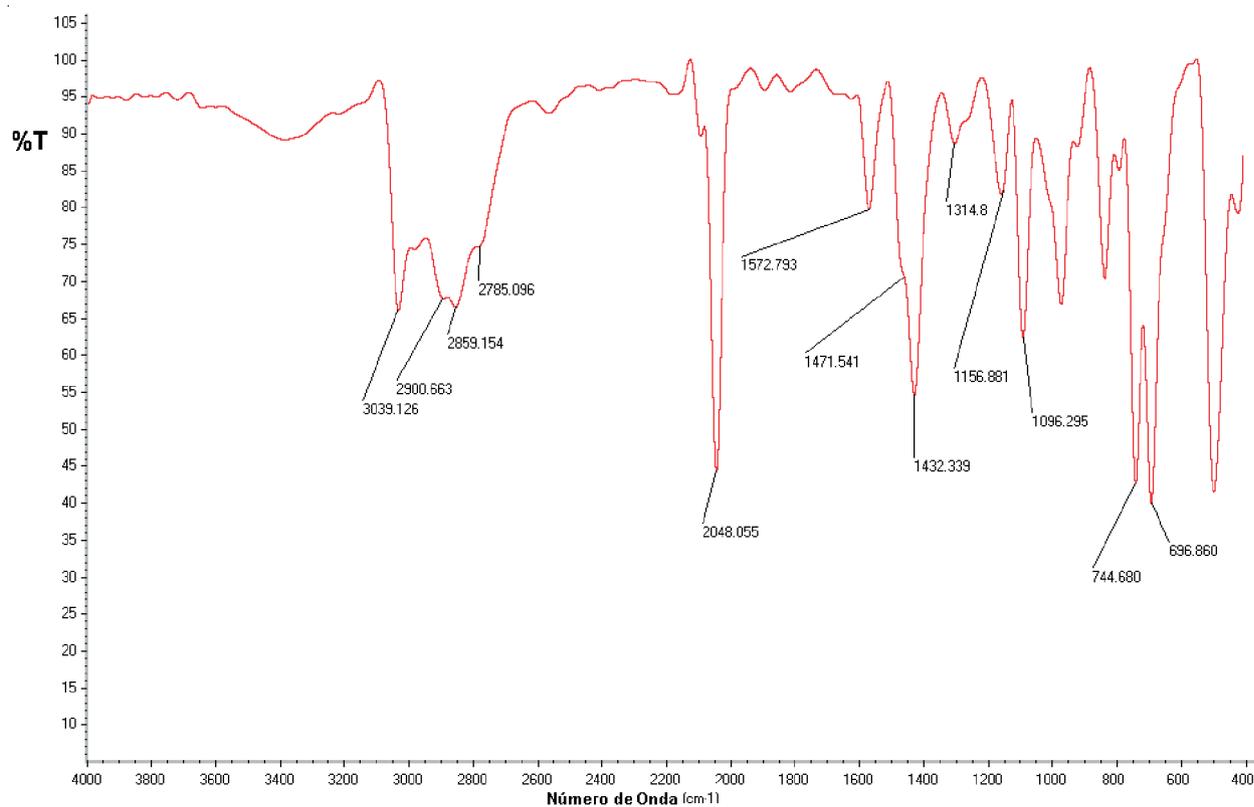


Figure S1. IR spectrum of the compound [Pd(C-dmba)(NCS)(dppp)] recorded as KBr pellets.

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