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Chiral cyclopalladated complexes derived from *N*,*N*-dimethyl-1-phenethylamine with bridging bis(diphenylphosphine)ferrocene ligand as inhibitors of the cathepsin B activity and as antitumoral agents

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Abstract—Chiral cyclopalladated complexes derived from *N*,*N*-dimethyl-1-phenethylamine and the coordinating ligand 1,1′-bis(diphenylphosphine)ferrocene were synthesized and studied as Cathepsin B inhibitors and antitumoral agents against solid tumors. Our results revealed that the palladium compound $[Pd_2(C^2,N\text{-}S_{(-)}dmpa)_2(\mu\text{-}dppf)Cl_2]$ (**2**) was able to inhibit Cathepsin B activity in a reversible fashion. This palladacycle compound binds to free cathepsin B (E) as well as to the enzyme–substrate complex (ES) with dissociation constants of $K_H = 12 \pm 1$ μM and $\alpha K_H = 2.4 \pm 0.3$ μM, respectively. The application of this complex, in Walker tumor-bearing rats, resulted in 90% inhibition of the tumor growth. Subcutaneous inoculations of 10^6 tumoral cells produced solid tumors with a mass of 4.0 ± 1.0 g in 12 days Walker tumor-bearing rats. However, when these animals were treated with one dose of the palladacycle compound (2.0 mg/kg), the tumoral mass was reduced to 0.3 ± 0.1 g. On the other hand, the same complex (**2**) did not afford any protection to mice bearing the non-metastatic Ehrlich Ascites tumor treated with doses of 0.5, 5.0, and 30 mg/kg for a period of four, three and one day, respectively, beginning 72 h after tumor inoculation. Toxicological studies using mice treated with one high dose of the complex (**2**) (100 mg/kg) did not show any alterations in red and white blood cell morphology 14 days after the drug administration. Similar results were obtained with hepatic, kidney, and spleen tissues. The results presented in this work introduce the title cyclopalladated complexes as promising antitumoral drugs with reduced toxicity in experimental studies.

1. Introduction

Cyclopalladated and cycloplatinated compounds are described in the literature as promising antitumoral agents.[1–6] In this regard, the monomeric cyclopalladated compounds coordinated to amines are biologically active against several human tumor cell lines.[1] The complexes derived from *N*-(4-Methoxyphenyl)-α-benzoyl-benzylideneamine and from *N*-(4-chlorophenyl)-α-benzoyl benzylideneamine presented antiproliferative activity in both, HL-60 leukemic cells and mammary cancer cells (MDA-MB 468) in vitro.[2,3]

The synthesis of a novel tetranuclear complex derived from *p*-isopropylbenzaldehyde thiosemicarbazone showed a cytotoxic activity against *cis*-DDP resistant tumor cell lines.[4] Moreover, the complexes that induced apoptosis in *cis*-DDP resistant cells,[5] the binuclear cyclopalladated complexes derived from *N*,*N*-dimethyl-1-phenethylamine, having a bridged dppe ligand were high effective against melanoma cell lines.[6]

In all of the cases cited above, the compounds act mostly by intercalation into DNA, suggesting that is the reason for their cytoxicity in many tumoral cell lines. However, there are no reports in the literature regarding the

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interaction of these compounds with proteolytic enzymes, such as the serine and cysteine proteinases, especially the cathepsin B,[7–9] which is also involved in tumor development.

The lysosomal cysteine proteinases cathepsins B and L have been implicated in a variety of pathological conditions, especially in diseases involving tissue-remodeling states, such as tumor metastasis.[7–10] The role of cathepsin B, which contributes to metastasis formation by degradation of several extra-cellular matrix components, has been studied by our group.[9,10] Recently, we began a research program involving the synthesis of new palladacycle compounds having biphosphinic ligands. Some of these compounds showed antitumoral activity in studies in vivo and in vitro.[11]

Based on the results described above, in the present paper we investigated the interactions of cathepsin B with some cyclopalladated compounds derivative from $R_{(+)}$ and $S_{(-)}$ enantiomers of *N*,*N*-dimethyl-1-phenethylamine and the ligand 1,1′-bis(diphenylphosphine)ferrocene (dppf). In vivo, assays with these complexes against Walker-256 carcinoma and with non-metastatic Ehrlich ascite tumor (EAT) revealed that these complexes were activity only for the Walker-256 carcinoma growth. Histopathological analyses of mouse kidney, spleen, and liver after administration of a high dose of the complex (**2**) (100 mg/kg) showed that the palladium complex does not cause lesions in these tissues, even when administrated in a dose 50-fold higher than the effective dose in Walker tumor-bearing rats (2 mg/kg). The results obtained in this study showed that the palladacycle compounds inhibited the Walker-tumor growth and cathepsin B activity in vivo and in vitro. Interestingly, the protective doses identified in this work produced no lesions for important organs such as those cited above. We concluded that the antitumoral activity of the palladacycle compounds presented here can be attributed, at least in part, to the inhibitory properties of these complexes on the cysteine–protease activity, such as cathepsin B.

2. Materials and methods

2.1. Synthesis and characterization of the palladacycle compounds

2.1.1. General comments. All the syntheses were carried out at room temperature. The reagents were obtained from commercial suppliers and used without further purification. Elemental analysis was performed by the Central Analitica IQ-USP-SP-Brazil. ^{1}H and ^{31}P{^{1}H}NMR spectra were measured on a multinuclear Bruker *Avance DPX-300* spectrometer, at 300 and 81 MHz, respectively. The proton NMR data are listed in parts per million downfield from TMS at 0.00 ppm. Phosphorus-31 NMR chemical shifts were measured with respect to H_3PO_4. All the NMR measurements were obtained in the solvent $CDCl_3$. Molar conductivity measurements of the complexes were obtained in a conductometer *Metrohm mod.712* using nitromethane as solvent.

2.1.2. Preparation of starting complexes. Pd(C^2,N-dmpa)μ-Cl]$_2$; dmpa = enantiomers *R*(+) and *S*(−) of *N*,*N*-dimethyl-1-phenethylamine. These compounds were prepared as reported previously.[12,13] Elem. anal., found % (calcd $C_{20}H_{28}N_2Cl_2Pd_2$): (a) *R*(+): C, 40.4 (40.6); H, 4.8 (4.7); N, 4.8 (4.4). (b) *S*(−): C, 40.4 (40.9); H, 4.8 (4.2); N, 4.8 (4.3). ^{1}H NMR (ppm): CH–CH_3^* (6H, d, 1.60); –$N(CH_3)_2$ (6H, s, 2.60), –$N(CH_3)_2$ (6H, s, 2.95); –CH*–CH_3 (2H, m, 3.90), H-aromatic rings (8H, m, 6.74–7.24).

2.1.3. Preparation of the complexes with dppf ligand. Molecular compounds having a bridged biphosphinic ligand with the general structure [Pd_2(C^2,N-dmpa)$_2$ (μ-L)-Cl_2] (L = bridged dppf ligand) were synthesized from the reactions of the starting cyclopalladated complexes with the biphosphinic ligand (L).

A molar ratio of 1:1 of biphosphinic ligand to palladium complex was used. Thus, 0.2 mmol of the dimeric cyclopalladated compounds were partially dissolved in 50 mL of dichloromethane, and 0.2 mmol of biphosphinic ligand (L) were added to the resulting solution. The mixture was left under constant agitation at room temperature for 1 h. Subsequently the solvent was evaporated under reduced pressure to almost dryness and the reaction product was precipitated by the addition of hexane. This resulting solid was filtered, washed with Et_2O, and dried under vacuum. The following complexes were isolated and characterized.

2.1.4. Pd$_2$(C^2,N-*R*$_{(+)}$dmpa)$_2$(μ-dppf)Cl$_2$]. Yield: 95%. Elemental analysis, found % (calcd): C, 57.32 (57.10); H, 4.82 (4.97), N, 2.40 (2.46). ^{1}H NMR (ppm): CH–CH_3^* (3H, d, 1.60); CH–CH_3^* (3H, d, 1.61); –$N(CH_3)_2$ (6H, br s, 2.60); –$N(CH_3)_2$ (6H, br s, 2.90); Cp (2H, br s, 4.23), Cp (2H, br s, 4.57), Cp (3H, m, 4.96), Cp (3H, m, 5.00), –CH*–CH_3 (2H, q, 4.10); H-aromatic rings (24H, m, 6.25–7.57). ^{31}P{^{1}H} NMR (ppm): 1 singlet: 32.7. $\Lambda M = 2.4$ S cm^2 mol^{-1}.

2.1.5. Pd$_2$(C^2,N-*S*$_{(-)}$dmpa)$_2$(μ-dppf)Cl$_2$]. Yield: 97%. Elemental analysis, found % (calcd): C, 56.73 (57.10); H, 5.17 (4.97), N, 2.40 (2.46). ^{1}H NMR (ppm): CH–CH_3^* (3H, d, 1.60); CH–CH_3^* (3H, d, 1.61); –$N(CH_3)_2$ (6H, br s, 2.60); –$N(CH_3)_2$ (6H, br s, 2.90); Cp (2H, br s, 4.23), Cp (2H, br s, 4.57), Cp (3H, m, 4.96), Cp (3H, m, 5.00), –CH*–CH_3 (2H, q, 4.10); H-aromatic rings (24H, m, 6.25–7.57). ^{1}P{^{1}H NMR (ppm): 1 singlet: 32.7. $\Lambda M = 9.3$ S cm^2 mol^{-1}. Figure 1 shows the general scheme of synthesis of isolated complexes (**1**) and (**2**).

3. Enzymatic activity studies

3.1. Cathepsin B activity

Cathepsin B and papain were purchased from Calbiochem Co. The concentrations of the active enzymes were determined by titration using the cysteine proteinase inhibitor E-64.[14] These enzymes were stored at 4 °C in 50 mM sodium acetate buffer (pH 5.0) containing

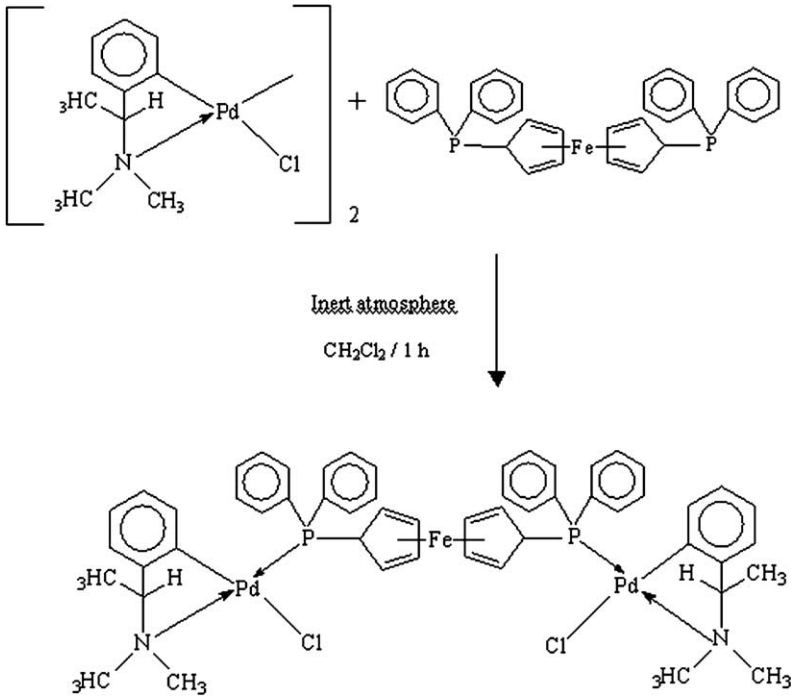


Figure 1. Schematic representation of the synthesis of the cyclopalladated complexes $[Pd_2(C^2,N\text{-}R_{(+)}dmpa)_2(\mu\text{-}dppf)Cl_2]$ (**1**) and $[Pd_2(C^2,N\text{-}S_{(-)}dmpa)_2(\mu\text{-}dppf)Cl_2]$ (**2**).

10 μM MMTS (methylmethane thiosulfonate). The fluorogenic amidomethylcoumaryl substrate Z-Phe-Arg-MCA, trypsin, and the papain irreversible inhibitor E-64 were purchased from Sigma–Aldrich Co.

The influence of organometallic compounds upon cathepsin B endopeptidase activity was spectrofluorometrically determined using the substrate Z-Phe-Arg-MCA. This peptide isa substrate for cathepsin B with $k_{cat}/K_s = 4.5 \times 10^5\ M^{-1}\ s^{-1}$. According to Schechter and Berger (1967), which described the nomenclature for substrate-binding site of enzymes,[15] the Phe and Arg residues occupy P_2 and P_1, respectively, and the substrate covers the main substrate-binding sites S_2, S_1, and S_1' in papain-like cysteine proteinases.[16] Fluorescence intensity was monitored on a thermostatic *Hitachi F-2000 spectrofluorometer*. The wavelength was set to 380 nm for excitation and 440 nm for emission for all assays. The enzyme was activated by incubation for 5 min at 37 °C in 50 mM sodium phosphate (pH 6.4) containing 200 mM NaCl, 1 mM EDTA, and 2 mM DTT.

Measurements were performed in the same buffer and kinetic parameters determined by measuring the initial rate of hydrolysis at various substrate concentrations in the presence or absence of different concentrations of the organometallic compounds. The resulting data were analyzed by non-linear regression using the program GraFit 3.01 (Erithacus Software Ltd). Figure 2 shows the curves obtained in the cathepsin B kinetics assays in the presence of cyclopalladated complex (**2**).

4. Biological assays

4.1. General comments

Studies in vivo have demonstrated that the cyclopalladated compound (**2**) presents no toxicity to granulocyte/macrophage colony forming cells (CFU-GM) as measured by the clonal culture technique in the presence of 1.5 μM of the complex. Since toxicity to bone marrow cells is an important side effect to be considered during the development of new drugs, we decided to initiate biological assays by studying the effect of complex (**2**) on two experimental tumor models according to the following descriptions.

4.2. Walker-256 mammary carcinoma

For the assays with Walker tumors the compounds were diluted in phosphate buffer, pH = 7.4, containing 1% dimethylsulfoxide (DMSO) and administrated to rats. Based on the kinetics results, which provided an estimate of the minimum effective dose, molar quantities of the cyclopalladated compounds corresponding to a concentration of 10 μM were used. These experiments were performed by inoculations consisting of 10^6 tumor cells together with the palladium compounds. Thus, single doses of 2.0 mg/kg were administered to the rats. This dose corresponds to a concentration of 14.7 μM, considering the mean weight of a cavy (0.25 kg) and an average blood volume of 0.03 L. Rats with two tumoral implants were used, one in each thigh. The left side implant was treated with the palladium complex and the right side implant did not receive treatment.

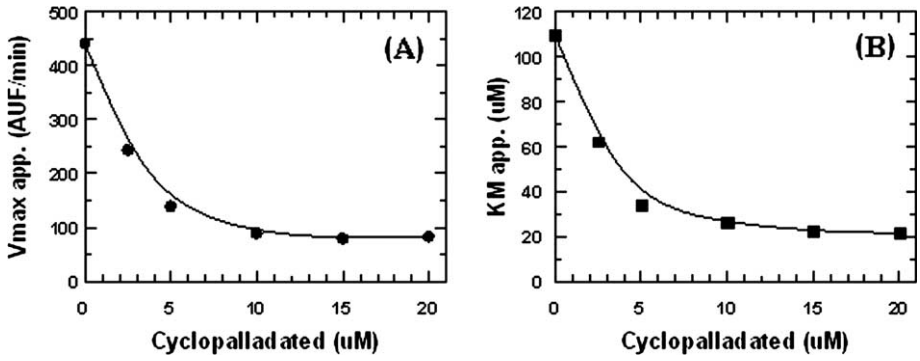


Figure 2. Effect of cyclopalladated complex (**2**) upon cathepsin B activity. In A, the presence of the organometallic compound results in a decrease in k_{cat} for the hydrolysis of Z-Phe-Arg-MCA. B shows that the compound also markedly increases the affinity of cathepsin B for the substrate *Z-Phe-Arg-MCA*.

In a second experiment, the compound (**2**) was also studied against Walker tumors using the intraperitoneal or subcutaneous routes. In these experiments, the palladacycle (**2**) was administered after the tumoral implant. Daily doses of the complex were administered over a consecutive period of 10 days using concentrations varying from 0.67 up to 5.36 mg/kg. The results of this second experiment are shown in Figures 4–6.

4.3. Non-metastatic Ehrlich ascites tumor (EAT)

Male Swiss mice, 8–10 weeks old, inbred in our laboratory were matched for body weight before use. The animals were housed 10/cage and were allowed free access to laboratory chow and water. All mice were raised under specific pathogen-free conditions and were maintained in conventional animal rooms before use.

Non-metastatic Ehrlich ascites tumor was maintained in Swiss mice in ascites form by serial transplantation. Tumoral cell suspensions were prepared in balanced salt solution at pH 7.4. For the experiments, mice were injected (ip) on day 0 with 6×10^6 viable tumor cells suspended in a volume of 0.1 mL. The viability, assessed by Trypan blue dye exclusion was always found to be more than 95%.

For the assays with EAT, the compounds were diluted in phosphate buffer, pH = 7.4, containing 1% dimethylsulfoxide (DMSO). The antitumoral activity of cyclopalladated compounds was evaluated by measuring survival time. The compounds were injected into the mice subcutaneously at concentrations of 0.5, 5.0, and 30 mg/kg for a period of four, three, and one day, respectively, beginning 72 h after tumor inoculation. Mice were checked daily for survival. The results are presented in Figure 7.

5. Acute toxicity investigation

For the evaluation of acute toxicity, mice were treated (ip) with a dose of 100 mg/kg of palladacycle compound, which was dissolved in phosphate buffer, pH = 7.4, containing 1% DMSO (at a final concentration of 1%). Control animals received diluent only. After 14 days of complex (**2**) administration, the animals were sacrificed and the following analyses performed.

5.1. Hematological evaluation

Blood from the Swiss mice was collected by caudal puncture using heparinized microcapillaries for total leukocyte counts. Blood films were prepared directly from venous blood and stained with Leishman's stain for differential leukocytes analysis and red blood morphology.[18] Table 1 shows the results obtained.

5.2. Histopathological analyses

Histopathological analyses were performed according to the literature.[17] Swiss mice were treated with one dose of 100 mg/kg of palladacycle complex (**2**). Fragments of spleen, liver, and kidney tissue were extracted from treated and non-treated Swiss mice and fixed in a 10% buffered formaldehyde suspension. After mounting in paraffin blocks they were cut to a thickness of 4 μm using a microtone Spencer 820 American Optical and subsequently stained with the HE technique. The tissue analyses were performed using a Nicon Eclipse E-200 mod. Olympus BH-2 microscope.

6. Results and discussion

The new *ortho*-chloro-cyclopalladated complexes (**1** and **2**) containing the dppf ligand were synthesized from the $R_{(+)}$ and $S_{(-)}$ enantiomers of *N*,*N*-dimethyl-1-phenethylamine. According to the molar conductivity measurements[19] in nitromethane, the complexes have been classified as molecular binuclear compounds. The gen-

Table 1. Effects of palladacycle (**2**) (100 mg/kg) on differential leukocyte counts of mice after 14 days of drug inoculation

Groups	Granulocytes (%)	Lymphocytes (%)
Control	14.3 ± 1.52	81.3 ± 3.0
Palladacycle compound (**2**)	24.2 ± 10.2[a]	72.2 ± 1.0[a]

[a] $P = 0.03$ in relation to control—ANOVA, Tukey–Kramer.

eral molecular structures of these complexes are presented in Figure 1.

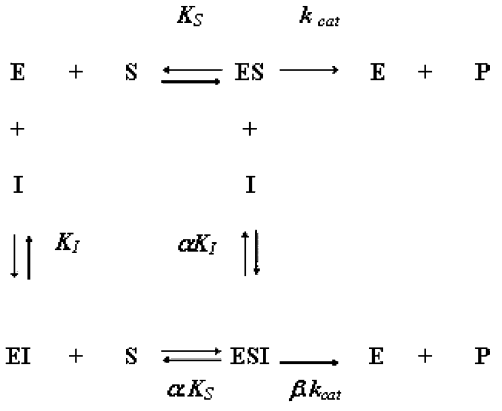
The biphosphinic complexes (**1**) and (**2**) were tested as inhibitors of cathepsin B. Both complexes inhibited the enzyme activity in a similar and reversible way, presenting an IC_{50} of 4 μM. Detailed kinetic studies were performed with the cyclopalladated complex $[Pd_2(C^2,N\text{-}S_{(-)}dmpa)_2(\mu\text{-dppf})Cl_2]$ (compound **2**) and the results are presented in Figure 2. As depicted in Figure 2A, the presence of the complex (**2**) in the cathepsin B kinetic assays resulted in a decrease in the k_{cat} values for the hydrolysis of *Z-Phe-Arg-MCA*. On the other hand, Figure 2B shows that the compound markedly increases the affinity of cathepsin B for the substrate *Z-Phe-Arg-MCA*. The effect of the organometallic upon cathepsin B endopeptidase activity can be described by a hyperbolic mixed type inhibition as depicted in Scheme 1. The system efficiency for substrate hydrolysis can be altered by changing either K_S (parameter α) or V_{max} (parameter β).

The data were fitted to Eq. (1) by using non-linear regression and the values for the constants determined.

$$v = \frac{V_{max}[S]}{Ks\dfrac{\left(1+\dfrac{[I]}{Ki}\right)}{\left(1+\dfrac{\beta.[I]}{\alpha.Ki}\right)}+[S]\dfrac{\left(1+\dfrac{[I]}{\alpha.Ki}\right)}{\left(1+\dfrac{\beta.[I]}{\alpha.Ki}\right)}} \quad (1)$$

The kinetic model depicted in Scheme 1 describes the effect of cyclopalladated compounds on the hydrolysis of Z-Phe-Arg-MCA by cathepsin B, where S stands for the Z-Phe-Arg-MCA substrate; I for the cyclometallated compound (**2**), E for cathepsin B; K_S for the substrate dissociation constant; K_I for the apparent organometallic dissociation constant; α for the K_S perturbation parameter; and β for the V_{max} (k_{cat}) perturbation parameter.

The results show that the palladacycle compound binds to free cathepsin B (E) with a dissociation constant K_H of 12 ± 1 μM, and also binds to the enzyme–substrate complex (ES) with a dissociation constant αK_H of 2.4 ± 0.3 μM. The complex also induced a 5.3-fold increase in the affinity of cathepsin B for the substrate



Scheme 1.

Z-Phe-Arg-MCA as shown by the reduction in the K_S value from 110 ± 15 to 21 ± 2 μM in presence of the organometallic compound, corresponding to $\alpha = 0.19 \pm 0.02$ (Fig. 1B). On the other hand, the k_{cat} value in the presence of the organometallic complex (**2**) was also decreased 5.6-fold corresponding to $\beta = 0.18 \pm 0.02$. It is interesting to observe that the cyclopalladated complex decreased the rate constant for product formation ($\beta = 0.18 \pm 0.02$) to the same extent that it presented an increased in the affinity of cathepsin B for the substrate Z-Phe-Arg-MCA ($\alpha = 0.19 \pm 0.02$), that is, $\alpha = \beta$. Despite the fact that cathepsin B was strongly inhibited by the organometallic complex (81%), its catalytic efficiency for the substrate was not altered, $\beta/\alpha = 1.1 \pm 0.1$. The second order rate for substrate hydrolysis was the same, $k_{cat}/K_S = 4.5 \times 10^5\ M^{-1}\ s^{-1}$, in the absence or in the presence of the palladacycle compound.

This inhibitory effect was also observed in other cysteine proteinases of the papain-family, for example, papain ($IC_{50} = 1.3 \pm 0.1$ μM), cathepsin B ($IC_{50} = 4.5 \pm 0.6$ μM), and cathepsin L ($IC_{50} = 1.6 \pm 0.2$ μM). Thus, these results show that cyclopalladated compounds with bridging biphosphine–ferrocene ligand are effective inhibitors of papain-like cysteine proteinases. This result can be involved in the tumor growth delay observed in Walker tumor-bearing rats treated with cyclopalladated complexes, since several studies have demonstrated that the cathepsin B is involved in metastatic tumor development.[7–10]

Cathepsin B is structurally related to the papain superfamily.[20] These enzymes bind peptide substrates and use a thiolate–imidazolium ion pair in order to carry out hydrolysis. This close association of the catalytic cysteine with the side chain of a vicinal histidine confers high nucleophilicity to the active-site.[21] Cleavage of a substrate amide-linkage involves the formation of an acyl–enzyme intermediate. After formation of the noncovalent Michaelis complex, the active-site thiolate attacks the peptide linkage to form an oxyanion, which is stabilized in the so-called '*oxyanion hole*' by a glutamine residue.[22] The collapse of the tetrahedral intermediate results in the acyl–enzyme and releases the first product. Subsequent hydrolysis of the acyl enzyme regenerates the catalytic ion pair and releases the new carboxylic acid product.

Several studies realized by Ryabov and co-workers[23] have demonstrated that cyclometalated complexes of Pd(II) and Pt(II) are functioning mimetics of metallopeptidases. Among the features traditionally ascribed to enzymes, metalacycles display noticeable rate of accelerations and stereoselectivity They manifest the catalytic activity due to facile generation of the aqua:hydroxo ligand. Thus, we believe that these cited properties can be involved in the biological activity of palladacycles (**1**) and (**2**) presented here.

It is important to mention that the substitution of the dppf ligand by other biphosphines, such as 1,2-bis(diphenylphosphine)ethane (dppe), produces

cyclopalladated complexes, which are unable to inhibit the cathepsin B activity. We observed also that the substitution of chloride ion by azide in the complexes produce potently cytotoxic compounds, which do not promote enzyme inhibition. Nevertheless, they are able to intercalate into DNA molecules promoting apoptosis in tumoral cells.[6] The palladacycle (**1**) presented here, also inhibits cathepsin B activity. However, this compound showed greater cytotoxicity in vivo in relation to complex (**2**), suggesting that the metabolism of the drug may be enantiomer dependent. Based on these results, we have chosen the complex (**2**), derived from the enantiomer *S*(−) of *N*,*N*-dimethyl-1-phenethylamine for studying the details of its antitumoral activity.

In relation to the enzymatic reactions, during the kinetic experiments, an elevated DTT concentration was employed. DTT is a reducing agent necessary for maintaining the thiolate ion in solution. Under these conditions, a reversible inhibition of Cathepsin B by the palladium complexes was observed. Similarly, the intra-cellular medium also contains strong reducing agents such as NADPH/Glutathione, which suggest that the in vivo inhibition of the enzyme might be expected to be reversible. In this context, the fluxional behavior of the dppf ligand[24] allied to the breakage of the N–Pd bond exhibited by palladacycle in the presence of the strong nucleophilic agents[27,28] can be an important mechanism of the enzyme inhibition and biological activity of the complexes studied in this work.

Considering the results cited here and in the fact that another biphosphinic complex has exhibited no enzyme activity inhibition as that observed with the complexes containing the dppf ligand, we suggest that the interaction of the palladacycle compounds (**1** and **2**) with the complex (ES) occurs with the participation of the two metallic centers presented in the organometallic complex.

When the antitumoral activity of complex (**2**) against Walker tumor-bearing rats was analyzed, we verified in 90% of the animals studied that the tumor growth was totally inhibited (Fig. 3). This figure, which is representative of 10 animals studied presents the results obtained from one of them that received 106 Walker-256 mammary carcinoma tumor cells in the right thigh and the same implant of 106 tumoral cells plus 2.0 mg/kg of the palladacycle complex (**2**) in the left thigh. Our results demonstrate that in the non-treated thigh of these rats (control) the solid tumor growth reached a maximal mass of 4.0 ± 1.0 g 12 days after Walker tumor cell inoculation. However, in the left leg, which received the palladacycle compound during inoculation, the tumoral mass was reduced to 0.3 ± 0.1 g. The effectiveness of the palladacycle compound was confirmed when it was verified that it reverted installed Walker tumors in 90% of the 10 cases studied.

Figure 4 shows a rat with an implant of Walker-256 mammary carcinoma tumor cells. In normal non-treated animals, the tumor reached a weight of 5 g in 10 days. However, when these rats were treated daily (ip) with

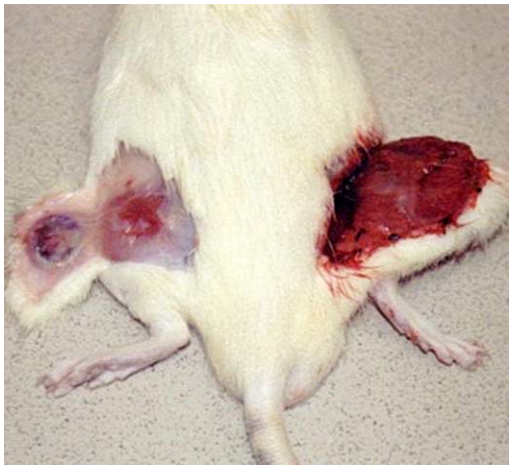


Figure 3. Rat showing two implants of tumoral cells (Walker-256 mammary carcinoma) left side implant (treated with compound (**2**) at a single dose of 2.0 mg/kg) shows a tumoral growth of 0.3 ± 0.1 g in 12 days. Right side implant (untreated) shows tumoral growth of 4.0 ± 1.0 g in 12 days.

doses of 0.67 mg/kg of the palladacycle complex for 12 consecutive days, the tumor did not grow in 20% of the cases studied ($n = 10$; Fig. 5). All of these results are presented in Figure 6. The green and red bars correspond to the number of rats with absence of tumoral growth.

Another important biological activity verified in studies in vivo and in vitro, was the inhibitory effect of the palladacycle compound on the Cathepsin B activity, which is involved in the proliferation of endothelial cells in culture and angiogenesis.[7–10] Of note, the inhibitory effects of the complex (**2**) on the cathepsin B activity were verified in Walker tumor-bearing rats, which suggest a relationship between the antitumoral effects of the complex **2** with the inhibitory effects of the complex on the cathepsin B activity. These results suggest that the palladacycle(**2**) is a promising antimetastatic drug. It is also important to mention that we did not observe any collateral effects in the treated rats, corroborating the toxicological data showing no lesion in the kidney, liver and spleen of mice after 14 days of complex (**2**) treatment with one dose of 100 mg/kg (ip). These results allow us to conclude that this drug presents low systemic toxicity and specificity for tumoral tissues.

The specificity of the palladacycle compound was also verified by analyzing its antitumoral effects using the Ehrlich ascite tumor (EAT), a non-metastatic tumor model, which grows rapidly in almost any mouse strain,[25,26] killing its host even when given in extremely small doses. The administration of the cyclopalladated compound (**2**) for four days, beginning 72 h after tumor inoculation, did not present any protection to EAT-treated mice in relation to control (Fig. 7). These results could be explained, at least in part, by the fact that EAT is a non-metastatic tumor. In favor of this hypothesis is the result presented here, showing that the palladacycle compounds, especially complex (**2**), inhibited cathepsin B activity in vitro at low concentrations. As

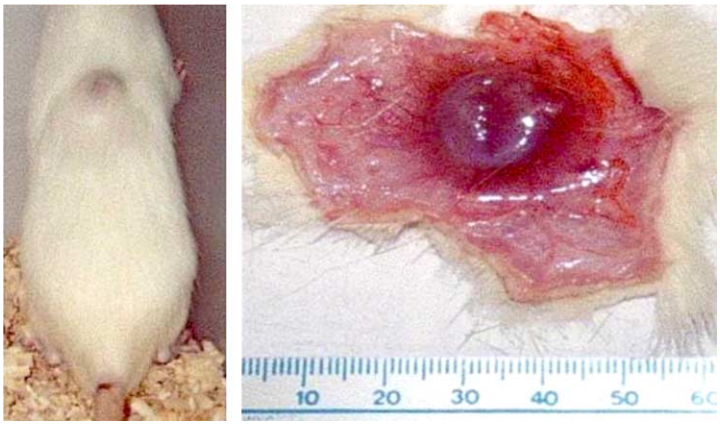


Figure 4. Rat showing an implant of tumoral cells (Walker-256 mammary carcinoma). The rat was untreated and the tumor reached a weight of 5 g in 10 days.

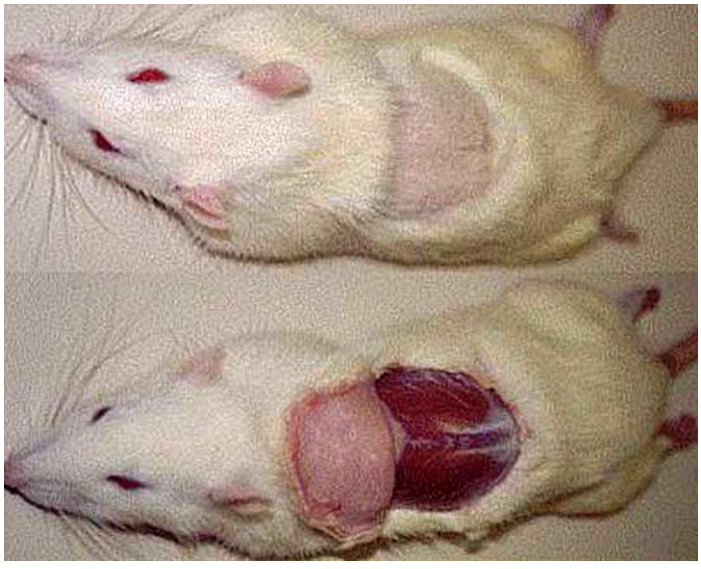


Figure 5. Rat showing an implant of tumoral cells (Walker-256 mammary carcinoma). The cavy was treated with intraperitoneal doses of 0.67 mg/kg of complex (**2**) daily for 12 days. In this dose no tumoral growth was observed for 20% of the cases as shown in Figure 6.

cited before, this enzyme is involved in the metastatic process, we also suggest that the palladacycle compounds are more selective for malignant tumors such as the Walker carcinoma.

Since the toxicity of many drugs limits their therapeutic application, we also analyzed here the acute toxicity of the palladacycle complex (**2**). For this purpose, histopathological analyses of kidney, spleen, and liver from mice treated with 100 mg/kg (ip) of the compound were performed after 14 days of drug administration. Our results demonstrate that compound (**2**) did not cause lesions in any of these tissues. Similar results were verified when we investigated the effects produced by the complex (**2**) on red and white blood cell morphology (Table 1). As can be seen from these results, the palladium complex (**2**) produces no effect on the total leukocyte numbers 14 days after drug inoculation. However, in the morphological analysis, an increase in the percentage of granulocytes was observed in mice exposed to 100 mg/kg of the metal complex ($P = 0.03$). With respect to red blood cells, our results demonstrated the presence of similar polychromasia in groups exposed to palladium complex when compared to untreated animals. There were no morphological alterations in the red blood cells in any of the groups studied.

As hematotoxicity is a limiting factor in the treatment of cancer patients, its characterization is fundamental to the development of new therapeutic agents. Following a cytotoxic insult, for instance after chemotherapy, neutropenia, trombocytopenia and anemia are often nondesirable clinical effects.[27] Many of these undesirable effects were not observed in our present study in mice exposed to a high dose of the palladacycle complex (100 mg/kg). Thus, we suggest that hematotoxicity might not be a limiting factor for chemotherapy.

Based on the evidence that the failure of conventional cancer therapy in most neoplastic diseases is the result

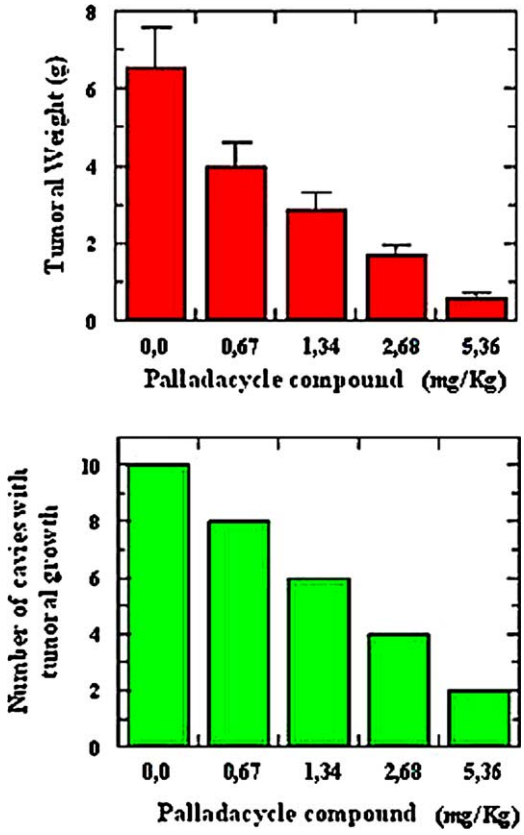


Figure 6. Dose response curve. Rats treated by intraperitoneal injections with increasing daily doses of the palladacycle (2). Red (above): tumoral weight (g) after 10 days. Green (below): number of cavies presenting tumoral growth after 10 days. In all of these assays 10 cavies were studied. The difference in the number of cavies in the comparison graphs corresponds to the number of cavies, which did not present evidence of tumoral growth.

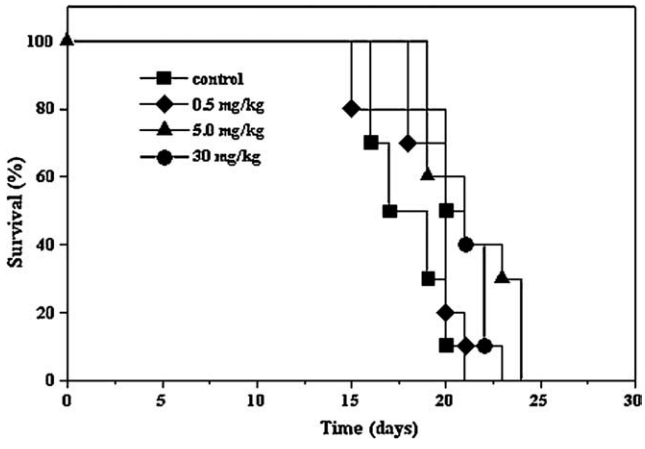


Figure 7. Effect of different doses of the palladacycle (**2**) on the survival of EAT-bearing mice treated with subcutaneous injections of 0.5, 5.0, and 30 mg/kg for four, three, and one day, respectively. Complex treatment started 72 h after intraperitoneal administration of 6×10^6 Ehrlich ascites tumor cells. Control mice received diluents only. Groups of 10 mice were checked daily for their survival rate. $P > 0.05$, compared with tumor group. *Statistical analysis*: survival curves were tested by comparing the cumulative percentage of survival using the Log-rank test.

of metastasis and on the fact that cathepsin B has been implicated in the metastatic process of several solid tumors including bladder, breast, lung, pancreas, prostate, stomach, and thyroid,[28] we suggest that the cyclopalladated compound (**2**) has antimetastatic effects and it should be considered for introducing into cancer chemotherapy after appropriate pre-clinical and clinical investigation.

As cancer chemotherapy becomes increasingly target-based, pharmacodynamics and not toxicology can be used to define doses, reducing the need for detailed pre-clinical toxicology experiments.[29,30] Toxicological studies should first and foremost predict a Phase I trial start dose that is safe, but not one that is so low that it is orders of magnitude away from therapeutic doses.[31] Considering these aspects, in this study we determine the minor active dose that protects the Walker-256 tumor-bearing rats, which was 50-fold less than the maximum dose administrated to animals in this work (100 mg/kg). It is important to mention that this dose produced no toxic effect for kidney, liver, and spleen tissues in histopathologycal evaluation. Further studies are in progress in our laboratory to better elucidate the antitumoral mechanism of this compound (**2**).

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Supplementary data

Supplementary data associated with this article can be found, in the online version at doi:10.1016/j.bmc.2005.01.057.

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