Synthesis, characterization, and biological activity of a new palladium(II) complex with deoxyalliin

Pedro P. Corbi, Antonio C. Massabni, Andréia G. Moreira, Francisco J. Medrano, Miriam G. Jasiulionis, and Claudio M. Costa-Neto

Abstract: Synthesis, characterization, and biological activity of a new water-soluble Pd(II)–deoxyalliin (*S*-allyl-L-cysteine) complex are described in this article. Elemental and thermal analysis for the complex are consistent with the formula $[Pd(C_6H_{10}NO_2S)_2]$. ^{13}C NMR, ^1H NMR, and IR spectroscopy show coordination of the ligand to Pd(II) through S and N atoms in a square planar geometry. Final residue of the thermal treatment was identified as a mixture of PdO and metallic Pd. Antiproliferative assays using aqueous solutions of the complex against HeLa and TM5 tumor cells showed a pronounced activity of the complex even at low concentrations. After incubation for 24 h, the complex induced cytotoxic effect over HeLa cells when used at concentrations higher than 0.40 mmol/L. At lower concentrations, the complex was nontoxic, indicating its action is probably due to cell cycle arrest, rather than cell death. In agreement with these results, the flow cytometric analysis indicated that after incubation for 24 h at low concentrations of the complex cells are arrested in G0/G1.

Key words: palladium(II), deoxyalliin, *S*-allyl-L-cysteine, tumor cells, cancer.

Résumé : On décrit la synthèse, la caractérisation et l'activité biologique d'un nouveau complexe, soluble dans l'eau, de Pd(II)–désoxyalliine (*S*-allyl-L-cystéine). Les analyses élémentaire et thermique du complexe sont en accord avec la formule $[Pd(C_6H_{10}NO_2S)_2]$. Les spectres de RMN du ^{13}C et du ^1H ainsi que la spectroscopie infrarouge permettent de montrer que la coordination du ligand au Pd(II) se fait par les atomes de soufre et d'azote dans une géométrie plan carré. Le résidu final du traitement thermique a été identifié comme un mélange de PdO et de Pd métallique. Des essais antiprolifération à l'aide de solutions aqueuses du complexe ont permis de démontrer l'activité prononcée du complexe contre les cellules cancéreuses HeLa et TM5, même à faibles concentrations. Après une incubation de 24 h, le complexe utilisé à des concentrations supérieures à 0,40 mmol/L induit un effet cytotoxique vis-à-vis des cellules HeLa. À des concentrations plus faibles, le complexe n'est pas toxique, ce qui indique que son action est probablement due à un arrêt du cycle cellulaire plutôt qu'à une mort de la cellule. En accord avec ces résultats, l'analyse de l'écoulement cytométrique indique que, après une incubation de 24 h à de faibles concentrations de complexe, les cellules de G0/G1 s'arrêtent.

Mots clés : palladium(II), désoxyalliine, *S*-allyl-L-cystéine, cellules cancéreuses, cancer.

[Traduit par la Rédaction]

Introduction

The most largely used metal-based drug is cisplatin (*cis*-diammindichloroplatinum(II)), which has been used for the treatment of several human cancers, particularly testicular, ovarian, bladder, head, and neck (1, 2). For a review see ref. 3. The anticancer properties of cisplatin were first observed by Barnet Rosenberg at the Michigan State University in 1965 (4), but only in 1978 was this compound approved worldwide for cancer treatment (5). Toxic side effects of cisplatin, mainly nephrotoxicity, neurotoxicity (2), and ototoxicity (6), have limited its use and led to the development of second generation drugs. Interest in developing new complexes with lower side effects than cisplatin, but at the same time with high activity against tumors, has stimulated syntheses of many new complexes of platinum(II) and

Received 12 July 2004. Published on the NRC Research Press Web site at http://canjchem.nrc.ca on 12 February 2005.

P.P. Corbi[1] and A.C. Massabni. Departamento de Química Geral e Inorgânica, Instituto de Química – UNESP, CP 355, CEP 14801–970, Araraquara, SP, Brazil.
A.G. Moreira. Departamento de Química Geral e Inorgânica, Instituto de Química – UNESP, CP 355, CEP 14801-970, Araraquara, SP, Brazil and Departamento de Bioquímica e Imunologia, Faculdade de Medicina de Ribeirão Preto – USP, 14049-900, Ribeirão Preto, SP, Brazil.
F.J. Medrano. Laboratório Nacional de Luz Síncrotron – LNLS, CP 6192, CEP 13084-971, Campinas, SP, Brazil.
M.G. Jasiulionis. Departamento de Micro-Imuno-Parasitologia, Escola Paulista de Medicina – UNIFESP, 04023-062, São Paulo, Brazil.
C.M. Costa-Neto. Departamento de Bioquímica e Imunologia, Faculdade de Medicina de Ribeirão Preto – USP, 14049-900, Ribeirão Preto, SP, Brazil.

[1]Corresponding author (e-mail: pedrocorbi@yahoo.com).

 doi: 10.1139/V05-003

also of palladium(II) (7, 8). Pd(II) complexes containing S and N donor ligands have been prepared and biological assays showed their activities against several tumor lines (HL-60, HeLa, 3T3, JURKAT, Pam212, and Pam-ras) (7).

Deoxyalliin (*S*-allyl-L-cysteine, $C_6H_{11}NO_2S$), a sulfur-containing amino acid derived from cysteine, is a product of vegetal origin present in onion and garlic bulbs (9, 10). More recent studies have shown that deoxyalliin may be considered a biological antagonist of nitrosomorpholine, the substance responsible for the development of hepatic cancer in humans (11). It also exhibits the capacity to inhibit proliferation of malignant cells from the human nervous system and thorax (12, 13).

Complexes containing Pt(II) or Pd(II) with mixed ligands such as amino acids and 1,10-phenanthroline displayed cytotoxic activities in vitro against Molt-4, a human leukaemia cell line (1). A study of *S*-alkyl and *S*-alkylaryl cysteine complexes with platinum(II) characterized by ^{13}C NMR, ^{1}H NMR, and IR absorption spectroscopy has also been reported (14). Biological tests were not performed for the latter complexes. The present article describes the synthesis and characterization of a new palladium(II) complex with deoxyalliin and its biological action against proliferation of tumor cells.[2]

Experimental

Materials

L-Deoxyalliin of analytical purity was purchased from LKT Laboratories. Palladium(II) chloride and lithium chloride of analytical purity were purchased respectively from Acros and Mallinckrodt Laboratories. Elemental analyses for carbon, hydrogen, nitrogen, and sulfur were performed with a CHNS-O EA1110 analyzer (CE Instruments); cystine was used as a reference substance. IR spectra were recorded on a FT-IR spectrophotometer spectrum 2000 (PerkinElmer) with samples prepared as KBr or CsI pellets. ^{13}C NMR and ^{1}H NMR were recorded on a Varian 500 MHz spectrometer; samples were analyzed in deuterium oxide solutions. Thermal analyses were performed on a Thermoanalyzer TG/DTA simultaneous SDT 2960 (TA Instruments) in the following conditions: synthetic air, 100 cm^3/min and heating rate of 10 °C/min, from 40 to 1100 °C. Palladium content was determinated by analyzing the residue of the thermal treatment at 900 °C. Powder X-ray analysis was performed on a D 5000 Siemens diffractometer using $CuK_{\alpha 1}$ radiation (λ = 1.5406 Å) with a graphite diffracted beam monochromator. The sample was scanned over the 2θ range from 4° to 70° in 0.05° steps. The counting time was 1.0 s/step. Cisplatin was purchased from Acros and MTT from Sigma. Media (DMEM and RPMI) and antibiotics for cell culture were purchased from Life Technologies, Inc. (Gaithersburg, Maryland) and FCS from Cultilab (Campinas, Brazil). Flasks and 24-well plates for cell culture were purchased from Costar (Corning Inc., Corning, N.Y.).

Preparation of the Pd(II) complex

The potassium salt solution of deoxyalliin was prepared by adding 1.12×10^{-3} mol of potassium hydroxide to a methanolic solution containing 1.12×10^{-3} mol of deoxyalliin (molar proportion of 1:1). This reaction was carried out at room temperature with stirring for 1 h.

The Pd(II) complex was synthesized by adding 5.6×10^{-4} mol of a freshly prepared methanolic solution of Li_2PdCl_4 to the solution of the potassium salt of deoxyalliin previously prepared, containing 1.12×10^{-3} mol of the ligand (molar proportion Pd(II):deoxyalliin of 1:2). This reaction was carried out at room temperature with stirring. A pale yellowish solid of the complex was precipitated slowly. After 2 h of constant stirring, the complex was filtered, washed with cooled methanol, and dried in a desiccator under P_4O_{10}. Final yield of the synthesis was about 70%. Anal. calcd. for $[Pd(C_6H_{10}NO_2S)_2]$ (%): C 33.8, H 4.72, N 6.56, S 15.0, Pd 24.9; found: C 33.4, H 4.60, N 6.40, S 14.7, Pd 23.7. No single crystals of the complex were obtained for X-ray structure determination. The lithium tetrachloropalladate solution was prepared by the reaction of $PdCl_2$ and LiCl in methanol under stirring and reflux for 30 min.

Cell culture

HeLa (human cervix cancer, ATCC CCL-2) and TM5 (murine melanoma, (15)) cell lines were cultured at 37 °C in a humidified atmosphere containing 5% CO_2, using, respectively, DMEM supplemented with 10% of Fetal Calf Serum (FCS) and RPMI at pH 6.9 with 5% FCS. Penicillin (100 U/mL) and streptomycin (100 µg/mL) were used as antibiotics.

Determination of the cell number and cell viability

A stock sample solution was prepared by dissolving the Pd(II) complex in a phosphate-buffered saline solution (PBS). PBS was used as the vehicle for the Pd(II) complex in all biological experimental procedures. Final different concentrations of the complex were achieved by dilution of the stock solution directly into the cell's medium. Cells were plated (25×10^4 cells/60 mm dish) 24 h prior to the beginning of the experiment. 48 h after addition of the complex or the vehicle, cells were detached and counted in individual samples using an impedance-based automated counter (CELM, Barueri, Brazil). To evaluate the immediate cytotoxic action of the Pd(II) complex, the MTT method (16) was applied to the cells after incubation for 24 h. Briefly, 24 h after plating 2.5×10^4 cells/well in a 24-well plate, the complex or the vehicle was added and the cells incubated for 24 h in culture conditions. After that, 100 µL of a MTT solution (5 mg/mL in PBS) were added and the cells were incubated for another period of 3 h. After washing with PBS, 200 µL of isopropanol were added, and cell viability was determined by absorbance measurements at 570 nm.

Cell cycle analysis by flow cytometry

Flow cytometric analysis of the Pd(II)-complex-treated TM5 cells was performed using a FACScan (Becton Dickinson). For synchronization, the cells were submitted to

[2] Abbreviations: phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute medium (RPMI), fetal calf serum (FCS), American type cell collection (ATCC).

a serum depletion for 24 h to induce M block, followed by addition of fresh serum to stimulate growth in the presence (0.20 mmol/L) or absence of the Pd(II) complex. After 24 h, cells were washed, fixed with ice-cold ethanol (70% final concentration), resuspended in 0.5 mL of a solution of propidium iodide (20 µg/mL) and 200 µg/mL of Dnase-free Rnase in PBS, and incubated for 30 min at 37 °C. For flow cytometric analysis, at least 10 000 cells were evaluated using a FACSCalibur™. Cell cycle distribution and pre-G1 fraction were determined and quantified using the CellQUEST™ program.

Effect of the complex on melanoma growth in C57BL/6 mice

Groups of six C57BL/6 mice weighing between 25 and 30 g were selected. For tumor induction, melanoma TM5 cells (2×10^5) were injected subcutaneously into the dorsal-side of each mouse. In the subsequent 5 days, the complex (24 mg/kg and 72 mg/kg) or the vehicle (PBS) was administrated subcutaneously into the dorsal-side of each animal at a distance (1 cm) from the site of the cells injection. Detection of tumors were followed for 30 days, after which animals were sacrificed. C57BL/6 mice were kept at Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia (CEDEME, UNIFESP, SP, Brasil) under the International Guiding Principles for Biomedical Research Involving Animals (CIOMS), Genebra (1985), and the Guide to the Care and Use of Experimental Animals, Canadian Council on Animal Care (www.ccac.ca).

Results and discussion

IR spectroscopy

The IR spectra of deoxyalliin, potassium *S*-allyl-L-cysteinate, and $[Pd(C_6H_{10}NO_2S)_2]$ complex are shown in Fig. 1.

Two very well resolved bands in the range 3400–3000 cm^{-1} are an indication of coordination of the amino group to Pd(II) (17). The IR spectrum of potassium *S*-allyl-L-cysteinate shows a broad band at 3350–2575 cm^{-1}, which corresponds to the NH_2 vibrational frequencies, while in the spectrum of deoxyalliin, the frequencies related to the amino group are observed in the range 3160–2675 cm^{-1} (broad band). Enlargement and shifting of the band show that the two hydrogen atoms of the amino group are involved in the formation of hydrogen bonds, which occur with cysteine and its derivatives, as deoxyalliin (17). The amino group involvement in the ligand coordination is attested by the presence of two very well resolved bands at 3220 and 3079 cm^{-1} for the Pd(II) complex (17). Moreover, the absence of the band at 2050–2150 cm^{-1} in the complex spectrum, in comparison to the free ligand and its potassium salt, confirms coordination through the amino group. The band at 2050–2150 cm^{-1} is assigned to a noncoordinated NH_2 group, as it occurs for the free ligand (18).

The IR spectrum of $[Pd(C_6H_{10}NO_2S)_2]$ also shows a strong absorption band at 1617 cm^{-1}, which can be assigned to a free ionized carboxylate group. In the case of Pt(II) complexes with *S*-alkyl cysteine derivatives, in which the carboxylate group was not coordinated to the metal ion, the band attributed to the free ionized carboxylate group appears at 1620 cm^{-1} (14).

Fig. 1. IR absorption spectra for: (*a*) deoxyalliin, (*b*) potassium *S*-allyl-L-cysteinate, and (*c*) the Pd(II) complex.

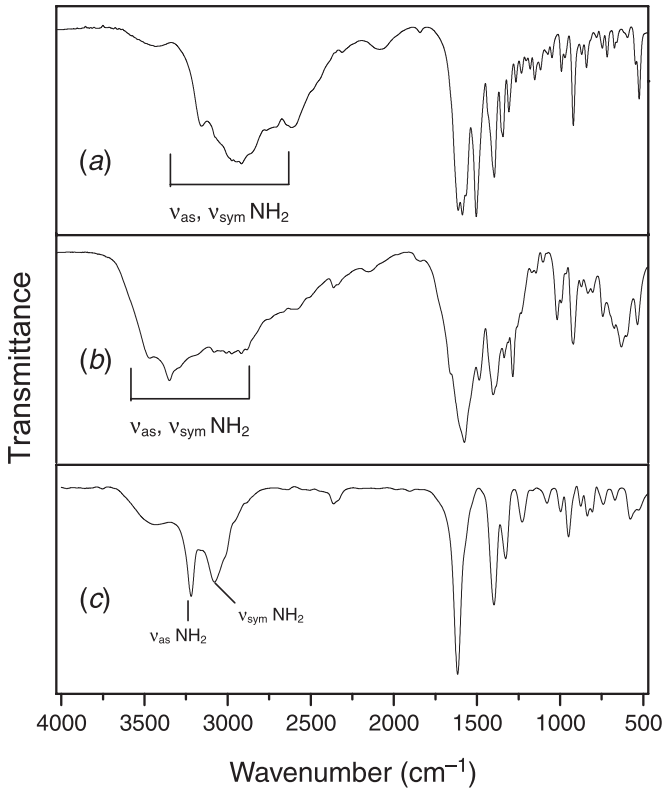
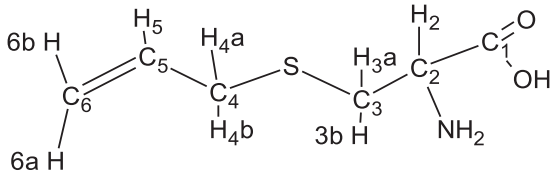


Fig. 2. The schematic structure of deoxyalliin.



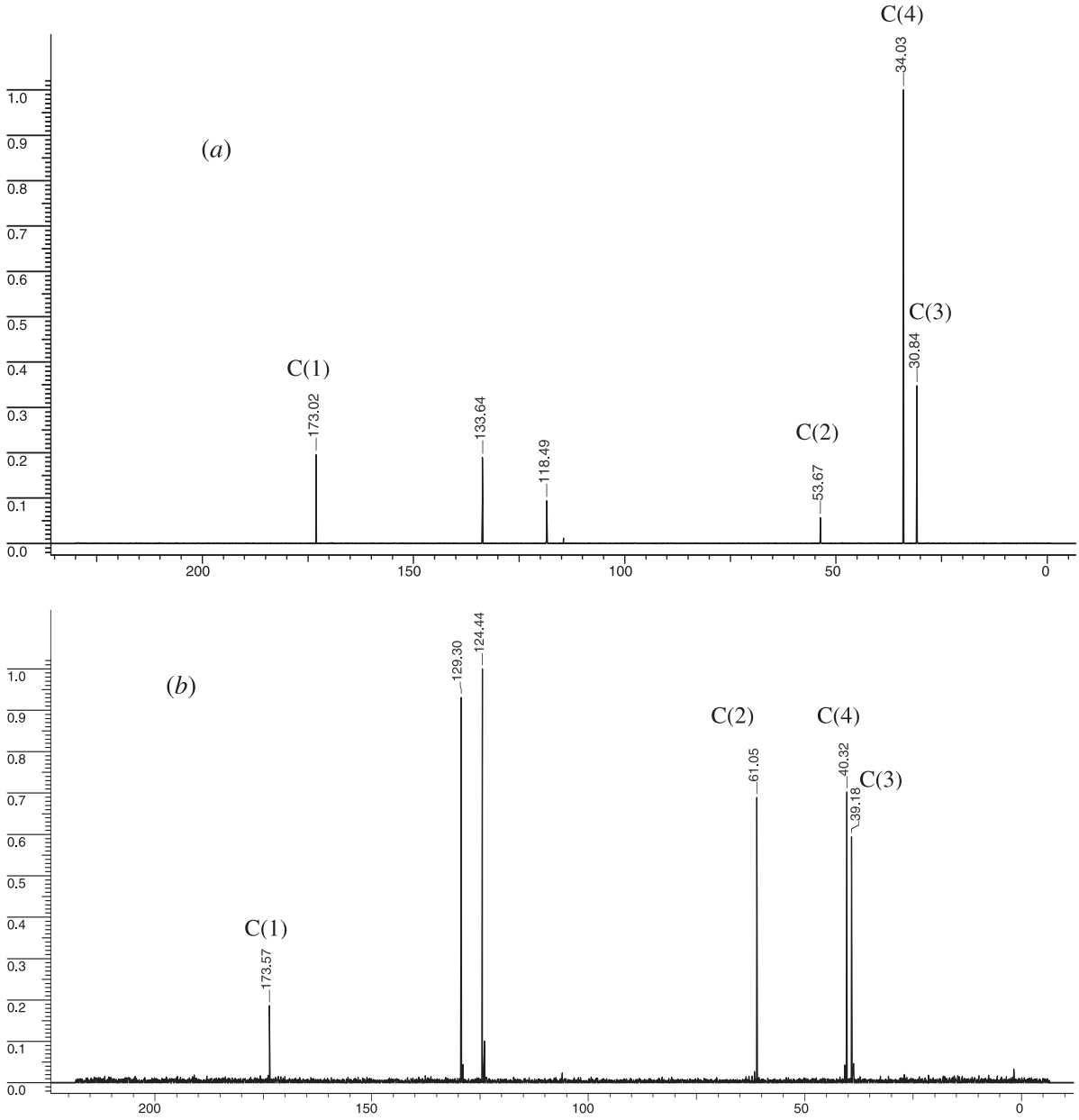
The IR spectra of the complexes were also measured in the region 700–150 cm^{-1} to identify frequencies related to M—S and M—N bonds. The IR spectrum shows frequencies at 342 cm^{-1} and at 554 cm^{-1}, which could be assigned to ν(M-S) and ν(M-N), respectively. These attributions are in agreement with the literature values, being similar to those found for an ethyl cysteinate complex of palladium(II) (19) and for other metal complexes with amino acids (14). Considering the presence of two well-defined bands for ν(M-N) and ν(M-S), a trans configuration could be proposed for the Pd(II) complex, as it occurs for complexes of the type $[Pt(L\text{-}H^+)_2]$, where L is an *S*-alkyl-L-cysteine derivative (14).

^1H and ^{13}C NMR spectrometry

^1H NMR and ^{13}C NMR spectra of the Pd(II) complex are useful for assigning ligand to metal bonding sites. The NMR spectra of the complex were analyzed in comparison to the spectra of the free ligand. The ligand structure is shown in Fig. 2.

The ^1H NMR spectrum of the Pd(II) complex with deoxyalliin is consistent with coordination of the ligand to

Fig. 3. ^{13}C NMR for: (*a*) deoxyalliin and (*b*) the Pd(II) complex.



the metal through the S and N atoms. Protons near the coordination sites are shifted downfield by 0.2–0.7 ppm. It was also observed that the chemical shifts for H(4a, 4b) (see Fig. 2) varies from 2.9 ppm for the ligand spectrum to 3.6 ppm for the complex. It was also observed that H(2a) varies from 3.8 ppm for the ligand to 4.0 ppm for the complex. These results are in agreement with the literature for Pd(II) and Pt(II) complexes involving amino acid derivatives showing *S*,*N*-coordination sites to the metal ions (14).

^{13}C NMR data are in agreement with S and N coordination proposed by analyzing the ^{1}H NMR and IR spectra. ^{13}C NMR chemical shifts for deoxyalliin and the Pd(II) complex are shown in Fig. 3.

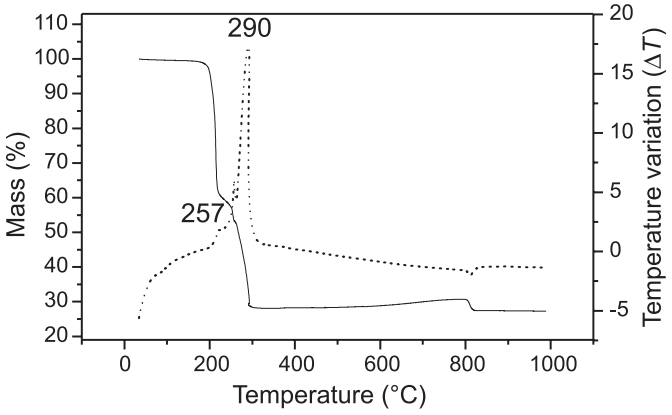
According to the ^{13}C NMR data, the chemical shift at 173 ppm in the spectrum of the ligand is assigned to the carbon atom of the COOH group (C1 in Figs. 2, 3*a*, and 3*b*). In the spectrum of the complex, no changes are observed for the chemical shift of the COOH group. Pronounced changes are observed for the chemical shifts of C2, C3, and C4 in the spectrum of the Pd(II) complex when compared to the free ligand (see Figs. 2, 3*a*, and 3*b*). These changes are other indications for coordination of deoxyalliin to Pd(II) through the N and S atoms of the amino acid structure.

Thermal analysis

TG and DTA curves for the Pd(II) complex are shown in Fig. 4. According to the thermogravimetric data, the composition of the complex formulated as $[Pd(C_6H_{10}NO_2S)_2]$ is confirmed. Oxidation of the coordinated ligand starts at temperatures near 200 °C. The residue formed after the thermal treatment of the Pd(II) complex at 900 °C was identified by powder X-ray diffractometry as a mixture of PdO (20) and metallic Pd (21).

DTA of the Pd(II) complex shows a strong exothermic

Fig. 4. TG (solid) and DTA (dot) curves for $[Pd(C_6H_{10}NO_2S)_2]$.



peak with a maximum at 290 °C and a weak peak at 257 °C (see Fig. 4). These effects are assigned to ligand oxidation of the complex in two steps.

Powder X-ray diffractometry

Powder X-ray diffraction data for $[Pd(C_6H_{10}NO_2S)_2]$ have been successfully indexed in supposition of an orthorhombic system with the following lattice cell parameters: a = 10.740 Å, b = 19.999 Å, and c = 5.2470 Å. A complete discussion of the powder X-ray indexation of this new complex has already been described (22).

Antiproliferative and cell viability analyses

The profile of the cell proliferation assays using HeLa and TM5 cells (Fig. 5) shows a blockage action of the Pd(II) complex starting at low concentrations such as 0.050 mmol/L, especially for HeLa cells. At 0.40 mmol/L, HeLa proliferation was totally eliminated and TM5 cell proliferation was only about 25% when compared to the control. Similar results were obtained when cisplatin was used: ~70% inhibition at 0.40 mmol/L (data not shown here).

The Pd(II) complex was also analyzed for HeLa cells after a longer exposure period of time (Fig. 6). The results showed that after 7 days of exposure to the Pd(II) complex (concentrations of 0.10 and 0.20 mmol/L), cell proliferation was about 75% inhibited. When the concentration was increased to 0.40 or 0.80 mmol/L, cell proliferation was completely inhibited.

The evaluation of the immediate cytotoxic action of the Pd(II) complex was analyzed after an incubation time of 24 h. After this period of incubation, a relevant effect was found only at high concentrations of the complex (0.40 and 0.80 mmol/L) suggesting that the antiproliferative action of the Pd(II) complex observed at lower concentrations is probably due to cell cycle arrest rather than cell death. In the case of cell death, cell viability would appear reduced at lower concentrations of the complex when compared to the control.

Flow cytometry analysis

As can be observed in Fig. 7, TM5 melanoma cells treated during 24 h with the Pd(II) complex at the concentration of 0.20 mmol/L did not present an apoptosis pattern, as seen by the low level of hypodiploid cells, which are detected when DNA fragmentation and (or) loss of DNA is occurring. On the other hand, cells were mainly arrested in G0/G1, when compared to the vehicle treated group. These results corroborate the data discussed here for the cytotoxic action of the compound after an incubation time of 24 h.

Fig. 5. Proliferation profiles for HeLa and TM5 cells after treatment with the Pd(II) complex in different concentrations for 48 h. The data represent an average of three to six independent experiments done in duplicate.

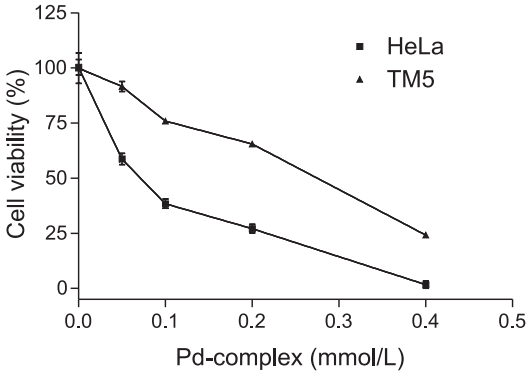
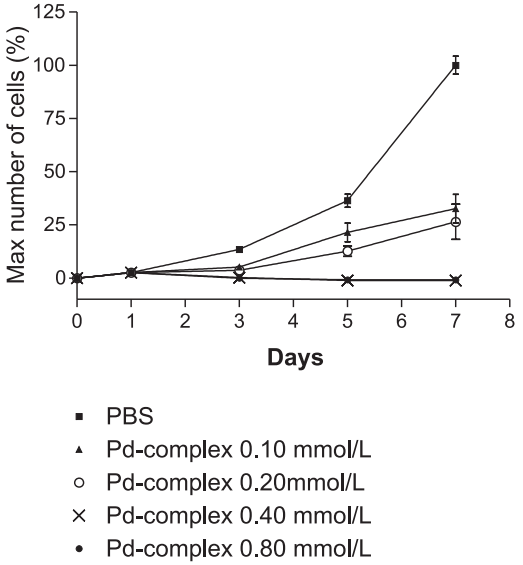


Fig. 6. Profiles for HeLa proliferation after treatment with the Pd(II) complex in different concentrations. The data represent an average of three independent experiments done in duplicate.



In vivo assays

The results obtained for the melanoma growth assay using C57/BL6 mice were considered nonsignificant due to the short delay (~48 h) observed for the appearance of tumors in the group treated with the complex (72 mg/kg body weight) when compared to the control group. No side effects, e.g., weight loss, were detected. This modest result warrants further in vivo investigation and optimization of dosage of the complex.

Final conclusions

Based on the chemical and spectroscopic results, a schematic structure for the Pd(II) complex with deoxyalliin is proposed in Fig. 8.

Fig. 7. Flow cytometric analysis of TM5 cells subjected to 0.20 mmol/L of Pd(II) complex or the vehicle (PBS) for 24 h. Hypodiploid cells correspond to cells that are undergoing apoptosis. The data represent an average of two independent experiments.

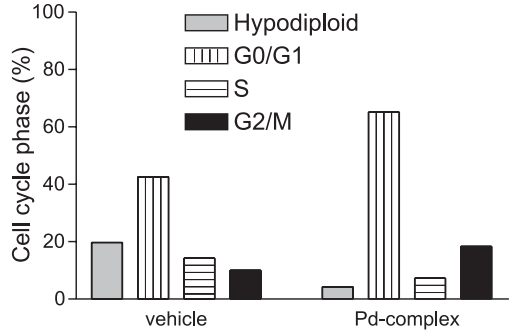
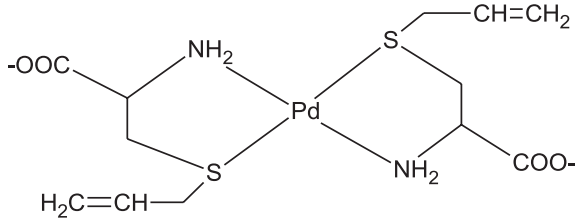


Fig. 8. The schematic structural formula proposed for $[Pd(C_6H_{10}NO_2S)_2]$.



Antiproliferative activity of the Pd(II) complex with deoxyalliin over HeLa and TM5 cells started at the concentration 0.050 mmol/L leading to a proliferation inhibition of about 40% for HeLa and 10% for TM5 cells. At the concentration 0.40 mmol/L, proliferation of HeLa cells was totally eliminated while inhibition of the proliferation of TM5 cells was about 75%. The MTT assay, used to evaluate the immediate cytotoxic effect of the complex, after an incubation of 24 h, indicated that at low concentrations, the Pd(II) complex action is probably due to cell cycle arrest rather than cell death, which was observed only under concentrations equal or higher than 0.40 mmol/L. In agreement with this conclusion, the flow cytometry analysis indicated that TM5 cells are mainly arrested at G0/G1 after a period of 24 h of incubation for the concentration 0.20 mmol/L of the complex. It is well-established that cisplatin treatment arrests cells in G2 with subsequent induction of apoptosis (3). In the present work, it was also possible to observe that, using the concentration 0.20 mmol/L of the Pd(II) complex, an apoptosis pattern was not triggered, as evidenced by a low percentage of hypodiploid cells (see Fig. 7). To our knowledge up to this date, a pattern of cell arresting by palladium complexes has not been described in the literature. We have also tested this Pd(II) complex against the development of melanoma tumors in mice in a preliminary trial. A slight delay (~48 h compared to untreated controls) in tumor appearance for the 72 mg/kg Pd(II)-complex-treated group was observed.

This work describes a new palladium(II) complex with in vitro biological activity in the μmol/L concentration. Its action is similar to other palladium complexes and also to cisplatin when tested against HeLa cells (8, 23–25).

Acknowledgments

This study was supported by grants from FAPESP (São Paulo State Research Foundation, Proc. 03/02616-7) and CNPq (Brazilian National Research Council). The authors are grateful to Dr. Nivaldo Boralle for the NMR spectra and also to Dra. Marisa S. Crespi for thermal analyses.

References

1. V.X. Jin and J.D. Ranford. Inorg. Chim. Acta, **304**, 38 (2000).
2. Y.S. Sohn, H. Baek, Y.H. Cho, Y. Lee, O. Jung, C.O. Lee, and Y.S. Kim. Int. J. of Pharm. **153**, 79 (1997).
3. M. Kartalou and J.M. Essigman. Mutat. Res. **478**, 1 (2001).
4. B. Rosenberg, L. Van Camp, and T. Krigas. Nature (London), **205**, 698 (1965).
5. D. Lebwohl and R. Canetta. Eur. J. Cancer, **34**, 1522 (1998).
6. J.L. Butour, S. Wimmer, and P. Castan. Chem. Biol. Interact. **104**, 165 (1997).
7. A.G. Quiroga and C.N. Ranninger. Coord. Chem. Rev. **248**, 119 (2004).
8. G.B. Onoa, V. Moreno, E. Freisinger, and B. Lippert. J. Inorg. Biochem. **89**, 237 (2002).
9. H.E. Renis and R.E. Henze. Food Res. **23**, 345 (1958).
10. T. Suzuki, M. Sugii, T. Kakimoto, and N. Tsuboi. Chem. Pharm. Bull. **9**, 251 (1961).
11. M.E. Dion, M. Agler, and A. John. Nutr. Cancer, **28**, 1 (1997).
12. C. Welch, L. Wuarin, and N. Sidell. Cancer Lett. **63**, 211 (1992).
13. G. Li, C.H. Quiao, R.I. Lin, J. Pinto, M.P. Osborne, and R.K. Tiwari. Oncol. Rep. **2**, 787 (1995).
14. V. Theodorou and N. Hadjiliadis. Polyhedron, **4**, 1283 (1985).
15. M. Correa, J. Machado, Jr., C.R.W. Carneiro, J.B. Pesquero, M. Bader, L.R. Travassos, R. Chammas, and M.G. Jasiulionis. Int. J. Cancer. In press.
16. T. Mosmann. J. Immun. Methods, **65**, 55 (1983).
17. K. Nakamoto. Infrared and raman spectra of inorganic and coordination compounds. Part B. 5th ed. John Wiley and Sons, New York. 1997. p. 385.
18. R.M. Silverstein and F.X. Webster. Spectrometric identification of organic compounds. 6th ed. John Wiley and Sons, New York. 1998. p. 482.
19. M. Chandrasekharan, M.R. Udupa, and G. Avaramudan. J. Inorg. Nucl. Chem. **36**, 1417 (1974).
20. The International Centre for Diffraction Data. Powder diffraction database. [CD-ROM]. File 30-1556 (JCPDS-ICDD). 1996.
21. The International Centre for Diffraction Data. Powder diffraction database. [CD-ROM]. File 5-0681 (JCPDS-ICDD). 1994.
22. P.P. Corbi, A.C. Massabni, and C.M. Costa-Neto. Powder Diffr. **19**, 270 (2004).
23. A.G. Quiroga, J.M. Perez, E.I. Montero, J.R. Masaguer, C. Alonso, and C. Navarro-Ranninger. J. Inorg. Biochem. **70**, 117 (1998).
24. A. Basu, B.A. Teicher, and J.S. Lazo. J. Biol. Chem. **265**, 8451 (1990).
25. G. Faraglia, D. Fregona, S. Sitran, L. Giovagnini, C. Marzano, F. Baccichetti, U. Casellato, and R. Graziani. J. Inorg. Biochem. **83**, 31 (2001).