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Original article

3,5-Dimethyl-1-thiocarbamoylpyrazole and its Pd(II) complexes: Synthesis, spectral studies and antitumor activity

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ABSTRACT

Complexes of the type $[PdX_2(tdmPz)]$ {$X = Cl^-$(**1**), Br^-(**2**); I^-(**3**); SCN^-(**4**); tdmPz = 1-thiocarbamoyl-3,5-dimethylpyrazole} have been synthesized and characterized. Compound **1** was formed from the reaction between $[PdCl_2(CH_3CN)_2]$ and 1-thiocarbamoyl-3,5-dimethylpyrazole. Complexes **2**, **3** and **4** were obtained by metathesis of the chloro groups from **1** by bromide, iodide and thiocyanate ions, respectively. All the compounds and cisplatin have been tested *in vitro* by MTT assay for their cytotoxicity against three murine cancer cell lines: mammary adenocarcinoma (LM3 and LMM3) and lung adenocarcinoma (LP07) as well towards normal murine peritoneal exudate cells (PEC). Promising cytotoxic effect against LM3 has been found for **3** showing IC_{50} equal to 24.5 μM which is comparable to the value obtained for cisplatin (30.3 μM).

1. Introduction

Although cisplatin is one of the five antitumor agents most used in clinical therapy [1], it has several limitations including toxicity (nephrotoxicity, neurotoxicity, ototoxicity, and emetogenesis) and intrinsic or acquired resistance [2]. To achieve lower toxicity and tumor selectivity, a continuing effort has been devoted to the design of new complexes of platinum and of other metals [3]. Among the non-platinum compounds for cancer treatment, palladium derivatives have attracted considerable attention because of the notable analogy between the coordination chemistry of Pd(II) and Pt(II) complexes. The design of Pd(II) compounds with anticancer activity represents a great challenge since Pd(II) compounds exchange ligands 10^5 times faster than analogous Pt(II) compounds [4]. Due to this rapid exchange, palladium(II) derivatives do not maintain their structural integrity long enough to reach the pharmacological targets. In order to overcome the high lability of Pd(II) compounds, chelating ligands have been used to afford high thermodynamic stabile and kinetically inert complexes [5,6]. Particularly, *N*,*S*-chelating ligands have been successfully employed by many authors to achieve a limited lability of the Pd(II) complexes, and consequently, to enhance the activity of these species in the cellular medium [7].

The 1-thiocarbamoylpyrazole derivatives are well known *N*,*S*-chelating ligands towards palladium(II) ions and coordinate to the metal ion through the sulfur atom and the pyridine-like nitrogen (N_2) at pyrazolyl moiety, yielding a stable five-membered ring [8–12] (Scheme 1).

For this reason, this class of ligands would be particularly suitable to prepare active Pd(II) complexes with promising biological activities. Recent studies have described that some 1-thiocarbamoylpyrazolyl chelates of palladium(II) displayed antiamoebic [9–11] and antitumor properties [12]. Recently, we have reported the synthesis and DFT studies on the compounds of general formulae $[PdX_2(tdmPz)]$ (X = Cl, SCN; tdmPz = 1-thiocarbamoyl-3,5-dimethylpyrazole) [13]. However, to the best of our knowledge, antitumor studies on this class of compounds have not been described yet in literature.

Motivated by the aforementioned findings, and as a part of our continuing research program in the field of coordination and biological chemistry of azolyl complexes [13–22], we present herein the evaluation of the *in vitro* cytotoxic activity of the compounds $[PdCl_2(tdmPz)]$ (**1**), $[PdBr_2(tdmPz)]$ (**2**), $[PdI_2(tdmPz)]$ (**3**) and $[Pd(SCN)_2(tdmPz)]$ (**4**) (tdmPz = 1-thiocarbamoyl-3,5-dimethylpyrazole) against murine mammary adenocarcinoma (LM3 and LMM3) and lung adenocarcinoma (LP07) as well towards

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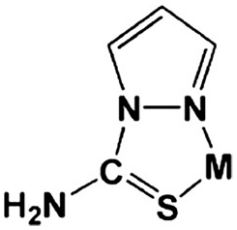
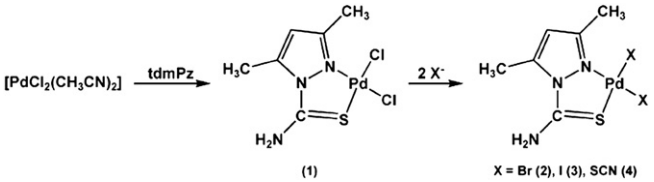


Scheme 1. *N,S*-coordination mode of 1-thiocarbamoylpyrazole-type ligands.



Scheme 2. Preparation of compounds **1**–**4**.

normal murine peritoneal exudate cells (PEC). In addition, this paper also describes the synthesis of two new compounds [$PdBr_2$(tdmPz)] (**2**) and [PdI_2(tdmPz)] (**3**), which were characterized by means of elemental analysis, IR, UV-Vis, ^{1}H and ^{13}C{^{1}H} NMR spectroscopy.

2. Results and discussion

2.1. Chemistry

The precursor [$PdCl_2$(MeCN)$_2$] reacts with 1-thiocarbamoyl-3,5-dimethylpyrazole (tdmPz) in methanol to afford [$PdCl_2$(tdmPz)] (**1**). Compounds [$PdBr_2$(tdmPz)] (**2**), [PdI_2(tdmPz)] (**3**), and [Pd(SCN)$_2$(tdmPz)] (**4**) are readily obtained by metathesis of the chloro groups in **1** by bromide, iodide and thiocyanate salts, respectively. A general scheme which represents the strategy employed for the synthesis of the complexes is illustrated in Scheme 2

The syntheses were carried out at room temperature with constant magnetic stirring. The complexes are air-stable powders and exhibit color that varies from yellow to brown. The molar conductivities of all complexes in DMSO are between 10 and 13 $ohm^{-1}cm^2\,mol^{-1}$, in agreement with their nonelectrolytic nature [23]. The complexes are soluble in DMSO, sparingly soluble in CH_3CN but insoluble in most of the organic solvents, therefore, all the attempts to grow crystals suitable for structure determination were unsuccessful. Analytical results are in agreement with the proposed formulae (Table 1).

2.2. Spectroscopic studies

In our previous paper, we have performed an infrared theoretical analysis on compounds **1** and **4** [13]. The FT-IR spectra of the complexes **2** and **3** were interpreted using the assignments for tdmPz [24], **1** and **4** [13]. The frequencies (cm^{-1}) of the more significant bands that appear in the IR spectra of tdmPz, **1**–**4** are given in Table 2 along with their assignments.

The formation of the *N,S*-chelated products **1**–**4** is strongly supported by IR spectroscopic data. Coordination of the pyridine-like nitrogen from pyrazole nucleus is inferred by the appearance of a new band assigned to the ring N–N stretching mode at *ca.* 1205 cm^{-1} in the IR spectra of the complexes [13,24]. The lack of large systematic shifts of $\nu_{as}NH_2$ and ν_sNH_2 absorptions to lower frequencies rules out any possibility of interaction between the Pd(II) center and the –NH_2 from thioamide group. The coordination via the thione sulfur atom is suggested by the decrease of intensity and shift to lower frequency of the $\nu C=S$ absorption from 879 cm^{-1} (free ligand) to 872 cm^{-1} (**1**), 862 cm^{-1} (**2**), 867 cm^{-1} (**3**), and 864 cm^{-1} (**4**). The terminal *S*-bonded coordination mode of the thiocyanate ligand in **4** is characterized by the presence of two overlapped sharp ν_{as}SCN bands at 2114 cm^{-1} and 2098 cm^{-1} [25,26]. The appearance of two ν_{as}SCN bands agrees well with the *cis* configuration adopted by the complex **2**.

The electronic absorption spectra of tdmPz and their Pd(II) derivatives in acetonitrile solutions are depicted in Fig. 1.

The electronic spectrum of tdmPz features two absorptions at 244 nm ($\log\epsilon = 3.9$) and 287 nm ($\log\epsilon = 4.2$), attributed to a $\pi \rightarrow \pi^*$ transition of the pyrazole ring and $n \rightarrow \pi^*$ transition of the thione moiety, respectively. The UV-vis spectra of all the palladium(II) complexes exhibit a similar pattern, showing one strong band at about 245 nm ($\pi \rightarrow \pi^*$) and one absorption in the region of 314–343 nm are attributed to a combination of S→ Pd, *X*→ Pd charge transfers (*X* = Cl, Br, I, SCN) and *d*–*d* transitions. These observations have also been noticed earlier in other 1-thiocarbamoylpyrazole derivatives of palladium(II) [11].

The chemical shifts and assignment of NMR experiments are summarized in Table 3.

In the ^{1}H NMR spectrum of 1-thiocarbamoyl-3,5-dimethylpyrazole, the H-4, 3-CH_3 and 5-CH_3 protons (see numbering in Scheme 3) appeared as singlets at 6.18, 2.17 and 2.66 ppm, respectively.

Although the overall pattern of the ^{1}H NMR spectra of **1**–**4** resemble very closely to that of the free ligand, all the signals have been shifted upon coordination. The most significant shifts of the signals of the coordinated ligand compared to the free tdmpz were those of H-4 and 3-CH_3 hydrogen atoms. The H-4 resonance changed from 6.18 ppm (free ligand) to *ca.* 6.53 ppm in the complexes whereas the ^{1}H NMR signal of the 3-CH_3 hydrogen was displaced *ca.* 0.5 ppm to downfield. On the other hand, the signals of the –NH_2 protons remain almost unchanged at the same position (see Table 3) in both ^{1}H NMR spectra of the tdmPz ligand and complexes. This fact strongly supports that the amino group from the thioamide moiety does not participate in coordination.

In the ^{13}C{^{1}H} NMR spectra of complexes **1**–**4**, the resonances of the 3-CH_3 and C-4 atoms are shifted to *ca.* 2.0 ppm downfield with respect to the free ligand. This deshielding is attributed to the removal of the electron density from the ligand to the palladium center via σ-charge donation from the pyridine-like N donor atom.

Table 1
Analytical and physico-chemical data on palladium(II) complexes [$PdCl_2$(tdmPz)] (**1**), [$PdBr_2$(tdmPz)] (**2**), [PdI_2(tdmPz)] (**3**) and [Pd(SCN)$_2$(tdmPz)] (**4**).

Complex	M.p. (°C)	UV-Vis (λ/nm)	Carbon		Nitrogen		Hydrogen	
			Found	Calc.	Found	Calc.	Found	Calc.
			%					
$C_6N_3H_9SCl_2Pd$ (**1**)	217 dec.	244 (4.2), 314 (3.5)	21.85	21.67	12.89	12.66	2.85	2.73
$C_6N_3H_9SBr_2Pd$ (**2**)	212 dec.	244 (4.4), 331 (3.7)	17.53	17.10	10.37	9.97	2.28	2.15
$C_6N_3H_9SI_2Pd$ (**3**)	178 dec.	247 (4.3), 343 (3.3), 510 (2.8)	14.25	13.98	8.50	8.15	2.08	1.76
$C_8N_5H_9S_3Pd$ (**4**)	206 dec.	246 (4.3), 318 (3.8)	25.29	25.43	18.25	18.54	2.64	2.40

Table 2
Vibrational data (cm^{-1}) for compounds tdmPz, [$PdCl_2$(tdmPz)] (**1**), [$PdBr_2$(tdmPz)] (**2**), [PdI_2(tdmPz)] (**3**) and [$Pd(SCN)_2$(tdmPz)] (**4**) along with assignments.

tdmPz[b]	**1**[c]	**2**	**3**	**4**[c]	Assignment
3387 s	3510 s	3500 m	3431 m	3440 m	$\nu_{as}NH_2$
3240 s	3410 ms	3259 ms	3271 ms	3141 m	$\nu_{s}NH_2$
3132 ms	3130 w	3132 w	3136 w	[a]	νCH
2980 w	2947 m	[a]	2982 w	[a]	$\nu_{as}CH_3$
–	–	–	–	2114 s, 2098 s	ν_{as}SCN
1601 s	1647 s	[a]	1660 m	[a]	$\nu_{py} + \delta NH_2$
1576 s	1614 s	1618 s	[a]	1614 s	$\delta_{sci}NH_2 + \nu$CN
1489 m	1520 m	1500 m	1500 ms	[a]	$\nu_{py} + \delta_s CH_3 + \nu$C–$CH_3$
1454 m	1466 m	1466 m	1469 m	1444 m	$\nu_{as}CH_3 + \nu_{py}$
1389 ms	1386 ms	1389 ms	1381 ms	1380 m	$\delta_s CH_3 + \nu_{py}$
1342 s	1356 m	1358 s	1350 s	1317 m	$\delta_r NH_2 + \nu CN_{py} + \nu_{C=S}$
–	1273 m	1271 m	1273 w	12943 m	βCH + ν_{py}
–	1211 w	1198 w	1209 w	1201 w	νNN_{py}
1147 m	1157 m	1157 m	[a]	1155 w	βCH + νC–CH_3
1093 m	1118 w	1119 w	1124 w	1103 m	$\nu NN_{py} + \delta_r CH_3$
1030 m	1053 m	1055 m	1067 w	1051 m	$\delta_r CH_3$
972 m	993 w	993 m	989 w	995 w	$\nu_{py} + \delta_r CH_3$
–	956 w	957 w	959 w	933 w	$\nu_{py} + \delta_r CH_3$
879 s	872 w	862 w	867 w	864 w	$\nu_{C=S} + \delta_r NH_2$
808 m	829 w	808 m	800 m	810 m	γCH
727 ms	748 w	746 w	754 w	748 w	νC–CH_3 + βCH + $\nu_{C=S}$
656 m	629 w	627 w	649 w	694 m	τ_{py}
590 w	582 w	582 w	584 w	588 w	$\tau_{py} + \beta_{py} + \nu_{C=S}$
496 w	500 w	498 w	497 w	460 w	$\beta C–NH_2 + \nu_{C=S} + \beta$C–$CH_3$

Intensity: s = strong, ms = medium-strong, m = medium, w = weak.
[a] Not observed.
[b] Ref. [20].
[c] Ref. [13].

An upfield shift of *ca.* 18 ppm of the C = S resonance in the $^{13}C\{^1H\}$ NMR spectra of the complexes indicates a π-back bonding from the palladium to the thione sulfur atom and gives a clear evidence of Pd–S bond formation. Besides the expected signals of coordinated tdmPz, the $^{13}C\{^1H\}$ NMR spectrum of **4** showed a peak at 118.85 ppm which is characteristic of *S*-thiocyanato bonding mode [26,27].

In agreement with the spectroscopic data, we assume that 1-thiocarbamoyl-3,5-dimethylpyrazole is attached to the palladium(II) ion in the bidentate *N*,*S*-chelating fashion ($N_{pyrazole}$ and $S_{thioamide}$) and the anionic groups chloro (**1**), bromo (**2**), iodo (**3**) or *S*-thiocyanato (**4**) occupy the remaining coordination sites, in a *cis* configuration (Scheme 2). Similar structures involving Pd(II) compounds bearing 1-thiocarbamoylpyrazole-type ligands have already been described in literature [9–12].

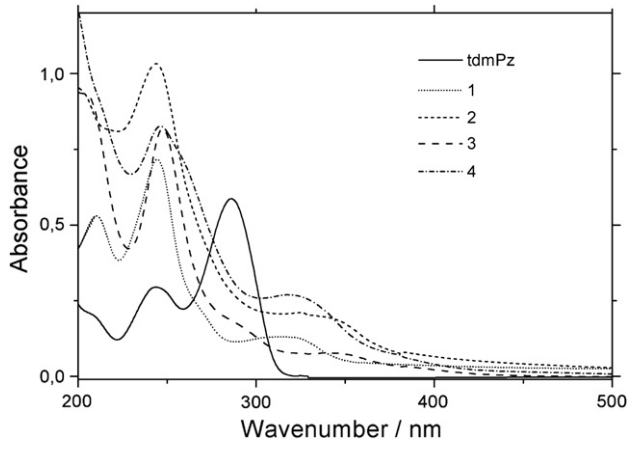


Fig. 1. UV-Vis spectra of tdmPz, [$PdCl_2$(tdmPz)] (**1**), [$PdBr_2$(tdmPz)] (**2**), [PdI_2(tdmPz)] (**3**) and [$Pd(SCN)_2$(tdmPz)] (**4**), in CH_3CN solutions.

Table 3
^1H and $^{13}C\{^1H\}$ NMR data for tdmPz, [$PdCl_2$(tdmPz)] (**1**), [$PdBr_2$(tdmPz)] (**2**), [PdI_2(tdmPz)] (**3**) and [$Pd(SCN)_2$(tdmPz)] (**4**) at 298 K, in DMSO-d_6, given as ppm, multiplicity, [integration].

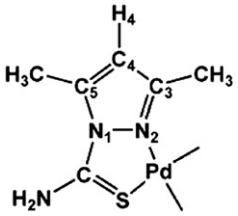
nuclei	Compound				
	tdmPz	**1**	**2**	**3**	**4**
^1H NMR					
3-CH_3	2.17 s, [3H]	2.61 s, [3H]	2.58 s, [3H]	2.57 s, [3H]	2.64 s, [3H]
H-4	6.18 s, [1H]	6.53 s, [1H]	6.54 s, [1H]	6.53 s, [1H]	6.53 s, [1H]
5-CH_3	2.66 s, [3H]	2.62 s, [3H]	2.59 s, [3H]	2.58 s, [3H]	2.58 s, [3H]
NH_2	9.51 s, 9.09 s	9.50 s, 9.09 s	9.50 s, 9.07 s	9.49 s, 9.08 s	9.47 s, 9.07 s
$^{13}C\{^1H\}$ NMR					
C-3	144.41	145.47	145.49	145.45	145.41
C-4	112.02	114.82	114.89	114.80	114.13
C-5	148.69	145.47	145.49	145.45	145.41
3-C_{methyl}	13.14	15.50	15.51	15.48	15.13
5-C_{methyl}	16.98	13.50	13.52	13.49	13.62
C=S	178.41	161.53	161.51	161.50	160.28
SCN	–	–	–	–	118.85

s = singlet.

2.3. Cytotoxic activities against murine tumor cell lines and peritoneal exudate cells (PEC)

The cytotoxic activities of the tdmPz and its palladium(II) complexes were tested against murine mammary adenocarcinoma cell lines (LM3 and LMM3) and lung adenocarcinoma (LP07) as well towards normal peritoneal exudate cells (PEC). Cells were exposed to a range of drug concentrations (140–2 μM) for 24 h and cell viability was analyzed by MTT assay. IC_{50} values (the concentration that inhibited in 50% the cellular proliferation) are presented in Table 4. For comparison purposes, the cytotoxicity of cisplatin, a standard antitumor drug, was also evaluated under the same conditions.

The free tdmPz showed no drug response at drug concentrations <140 μM in all the tested cultures, and thus has to be considered inactive. However, in most of the cases, coordination of this ligand on palladium center resulted in higher proliferative activities. After treatment of LM3 cells with compounds **1**–**4** and cisplatin, a comparable effect of [PdI_2(tdmPz)] (**3**) to that of cisplatin was noticed whereas **1** and **2** were approximately threefold less toxic. On the other hand, compound [$PdBr_2$(tdmPz)] (**2**) showed the highest cytotoxic activity against LMM3 cell line (IC_{50} value of 20.73 μM) among all tested compounds. With respect to the cytotoxic effects on LP07 cells, a progressive increase on the cytotoxic activity of [PdX_2(tdmPz)] complexes was observed according to the anionic group, following the order Cl ≈ SCN < Br < I. However, complexes **1**–**4** were considerably less active than cisplatin. At this point, it is very difficult to rationalize the obtained IC_{50} values in terms of structure–activity relationship. These results indicated that further syntheses should be undertaken to determine the possible potentiating effect of anionic group *X* coordinated to Pd(II) as well studies on DNA binding properties of these complexes will be necessary to understand the mechanism of action.



Scheme 3. Numbering scheme used in assignments of NMR data.

Table 4
Cytotoxicity (IC_{50}) of the tdmPz, complexes **1**–**4** and cisplatin against murine LM3, LMM3 and LP07 cell lines and murine peritoneal exudate cells (PEC)

Compound	IC_{50} (μM) ± SD			
	LM3	LMM3	LP07	PEC
tdmPz	>140	>140	>140	>140
[$PdCl_2$(tdmPz)] (**1**)	90.24 ± 2.55	98.76 ± 5.64	62.45 ± 1.19	33.15 ± 9.19
[$PdBr_2$(tdmPz)] (**2**)	100.51 ± 0.94	20.73 ± 4.99	42.45 ± 2.70	29.30 ± 2.61
[PdI_2(tdmPz)] (**3**)	24.54 ± 2.27	>140	28.70 ± 3.40	50.24 ± 3.06
[$Pd(SCN)_2$(tdmPz)] (**4**)	41.69 ± 0.98	83.34 ± 5.64	59.63 ± 2.33	83.98 ± 3.71
Cisplatin	30.30 ± 3.72	>140	4.34 ± 0.45	62.88 ± 2.14

Since some of the synthesized Pd(II) compounds described in this work showed interesting IC_{50} values in the range 20–30 μM, their cytotoxicity was assayed against normal murine PEC cells. According to the results in Table 3, the Pd(II) compounds appeared to be also toxic with IC_{50} ranging from 29 to 84 μM. Particularly, the biological activity displayed by compound [PdI_2(tdmPz)] (**3**) deserves further comment. The iodo derivative was the most toxic Pd complex against LM3 cells, and is statistically similar in cytotoxicity to cisplatin. Besides that, **3** exhibited a slightly higher toxicity than cisplatin to normal peritoneal exudate cells. This result appears to be promising if we take into account that a limited spectrum of side effects is required for a new metal-based drug [28].

3. Conclusions

In conclusion, the results indicated that neutral chelated palladium complexes with 1-thiocarbamoyl-3,5-dimethylpyrazole might be a promising source of new metal-based antitumor agents. Currently studies are ongoing in our laboratories in order to gain a better insight in the mechanism of action of these Pd(II) pyrazolyl compounds, which may be employed in the design of new improved derivatives for antitumor assays.

3.1. Experimental protocols

3.1.1. Reagents

The materials employed in the syntheses were all commercially available and were used without purification. All solvents were dried and stored over molecular sieves prior to use. Literature procedures were followed for the synthesis of 1-thiocarbamoyl-3,5-dimethylpyrazole (tdmPz) [29] and [$PdCl_2(MeCN)_2$] [30].

3.1.2. Chemical synthesis

Compounds dichloro(1-thiocarbamoyl-3,5-dimethylpyrazole)palladium(II) (**1**) and (1-thiocarbamoyl-3,5-dimethylpyrazole)dithiocyanatopalladium(II) (**4**) were prepared as previously described [13].

*3.1.2.1. Synthesis of dibromo(1-thiocarbamoyl-3,5-dimethylpyrazole)palladium(II) (**2**).* To a brick yellow suspension of [$PdCl_2$(tdmPz)] (100 mg; 0.18 mmols) in 10 mL of MeOH, 75 mg of KBr (0.37 mmols) dissolved in 1 mL of water was added. The entire mixture became brownish yellow. After stirring for 5 min., the mixture was filtered off and the obtained solid was washed with methanol and dried under vacuum. (Yield 85%).

*3.1.2.2. Synthesis of diiodo(1-thiocarbamoyl-3,5-dimethylpyrazole)palladium(II) (**3**).* To a brick yellow suspension of [$PdCl_2$(tdmPz)] (100 mg; 0.18 mmols) in 10 mL of MeOH, 105 mg of KI (0.37 mmols) dissolved in 1 mL of water was added. The entire mixture became brown. After stirring for 5 min., the mixture was filtered off and the obtained solid was washed with methanol and dried under vacuum. (Yield 70%).

3.1.3. Instrumental

C, H, and N analyses were performed by the Central Analítica at IQ-University of São Paulo. Conductivities were measured with a Digimed-DM-31 conductometer using 1×10^{-3} mol L^{-1} solutions in DMSO. Infrared spectra were recorded as KBr pellets on a Nicolet FT-IR-Impact 400 spectrophotometer in the spectral range 4000–400 cm^{-1}. ^1H and $^{13}C\{^1H\}$ NMR spectra were recorded in DMSO-d_6 solutions at room temperature on a Varian INOVA 500 spectrometer, using $SiMe_4$ as internal standard. Electronic absorption spectra in UV-Vis range were registered on a Perkin–Elmer Lambda 14 P spectrophotometer using a 1.00 cm cuvette and solutions 4×10^{-5} mol L^{-1} of the complexes in acetonitrile.

3.1.4. Tumor cell preparation

LM3 and LMM3 cell line was generously supplied by Prof. Elisa Bal De Kier Joffé from Cell Biology Department, Research Area, Institute of Oncology 'Angel H. Roffo', University of Buenos Aires, Buenos Aires, Argentina. LM3, LMM3 and LP07 cells were maintained in MEM (Sigma) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and 80 μg mL^{-1} gentamicin, defined as complete medium, in plastic flasks (Corning) at 37 °C in a humidified 5% CO_2 atmosphere [31,32]. Passages were made by trypsinization of confluent monolayers (0.25% trypsin and 0.02% EDTA in Ca^{2+}–Mg^{2+} free PBS). Cell number was counted by the Trypan blue dye exclusion method.

3.1.5. Animals

Female 6–8 weeks old BALB/c mice weighing 18–25 g were purchased from Universidade Estadual de Campinas (UNICAMP) central animal facilities CEMIB (Centro Multidisciplinar para Investigação Biológica), SP, Brazil. They were maintained in polycarbonate boxes at 23 ± 2 °C, 56 ± 2% humidity, at 12 h light/dark cycle, kept under specific-pathogen-free conditions (positive-pressure cabinet) and provided sterilized food and water *ad libitum* in accordance with the protocols of the Universidade Estadual Paulista (UNESP). All animal studies were conducted in accordance with the NIH 'Guide for the Care and the Use of Laboratory Animals' and following the Federal Government legislation on animal care.

3.1.6. Peritoneal exudate cells (PEC) preparation

Thioglycollate-elicited peritoneal exudate cells (PEC) were harvested from female BALB/c mice using 5.0 mL of sterile PBS, pH 7.4. The cells were washed twice by centrifugation at 200 rpm for 5 min at 4 °C and re-suspended in appropriate medium for each test.

3.1.7. Solutions

Test solutions of the compounds (1000 μM) were freshly prepared by dissolving the substance in 50 μL of DMSO completed with 4950 μL of culture medium. Afterwards, the tested

compounds were diluted in culture medium to reach the final concentrations ranging from 140 to 2 μM. The DMSO solvent in the concentrations used in test did not reveal any cytotoxic activity.

3.1.8. MTT assay

For the cytotoxicity evaluation, 200.0 μL samples of LM3, LMM3 and LP07 cells (5×10^4 cell mL^{-1}, adjusted in MEM), were added to each well of a 96-well tissue culture plate (Corning) and then preincubated in the absence of compounds for 24 h to allow adaptation of cells prior to the addition of the test agents. Then, supernatants were removed and 200.0 μL of the compounds in concentrations ranging from 2 to 140 μM or 200.0 μL of MEM-Complete as cell control of viability was added to each well. The effects of the compounds under the cells were determined 24 h after culture incubation. Then, supernatants were removed and 100.0 μL of solution of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) was added in each well containing the samples [33]. MTT assay was performed and the plates were incubated for 3 h. 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 medium of two independent experiments through the equation of graphic line obtained (Microcal Origin 5.0™). Each compound in a given concentration was tested in triplicates in each experiment.

The PEC were plated at a concentration of 5×10^6 per well in RPMI 1640 (Gibco), supplemented with 10% fetal bovine serum (FBS) (Cult-Lab), 100 U mL^{-1} of penicillin, 100 μg mL^{-1} of streptomycin, 2 mmol L^{-1} of L-glutamine, and 5×10^{-2} mol L^{-1} of 2-mercaptoethanol (Sigma); this mixture was named complete RPMI 1640 (RPMI 1640-C). Then, samples of 100 μL of peritoneal cells suspension (5×10^6 cell mL^{-1}) in RMPI-1640-C medium were added to each well of a 96-well tissue culture plate with 100 μL of the appropriate concentrations of the compounds tdmPz, **1**–**4** and cisplatin containing 0.1% DMSO and RPMI 1640-C medium. Cells were incubated for 24 h at 37 °C in a humidified atmosphere containing 7.5% of CO_2. After incubation, the medium was poured off, and macrophages were incubated with 100 μL of solution of MTT.

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