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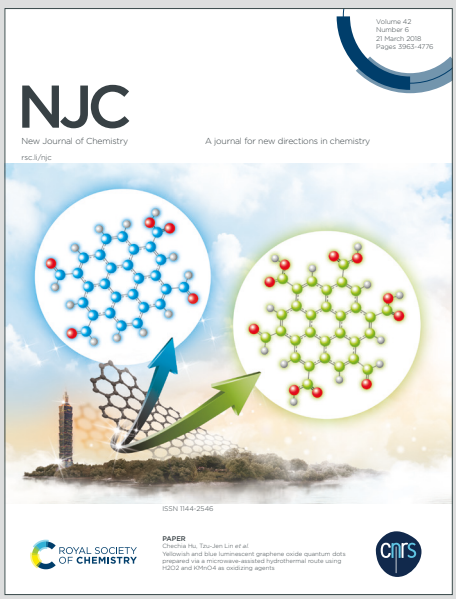

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ARTICLE

Palladium(II) complexes bearing 1-iminothiolate-3,5-dimethylpyrazoles: synthesis, cytotoxicity, DNA binding and enzymatic inhibition studies

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Four palladium(II) compounds of general formulae [PdCl(L$_n$)(PPh$_3$)] {**L$_1$** = 3,5-dimethylpyrazole-1-iminothiolate (**1**); **L$_2$** = 3,5-dimethyl-pyrazole-N-methyl-1-iminothiolate (**2**), **L$_3$** = 3,5-dimethylpyrazole-N-ethyl-1-iminothiolate (**3**); **L$_4$** = 3,5-dimethylpyrazole-N-phenyl-1-iminothiolate (**4**); PPh$_3$ = triphenylphosphine} have been synthesized. The novel synthesized compounds have been characterized by C, H and N elemental analysis, 1D (^{1}H and ^{13}C) and 2D (HSQC and HMBC) NMR, MS, FT-IR, and molar electrical conductivity measurements. The molecular structure of complex **3** has been solved by single-crystal X-ray crystallography. The stability of the complexes in solution was studied in a DMSO/D$_2$O (7:3) solution after 48h. The antiproliferative assay of all free ligands and the stable palladium complexes **2**-**4** were assayed using the human breast tumour cell line MCF-7, lung tumour cell line A549 and human fetal lung fibroblast cell line MRC-5. Complex **3** was more active than cisplatin against MCF-7 cells whilst palladium compounds **2**-**4** exhibited no drug response towards A549 cells on concentrations < 50 μM. The binding properties of compounds **2** and **3** on ct-DNA have been studied using circular dichroism and fluorescence spectroscopy. The Topoisomerase IIα inhibition has been studied for complex **2** and **3**. The ability of all complexes to inhibit the activity of cathepsin B and L has also been investigated in this work. Compound **4** inhibited more than 50% the cathepsin B activity at a concentration of 10 μM. Docking simulations have been carried out to gain more information about the interaction of the complexes and cathepsin B.

1. Introduction

Over the last three decades, much research efforts have been devoted to Pd^{II} complexes in the search for novel metal-based drugs.[1-8] Several biologically active Pd^{II} compounds have been described in the literature and particular attention has been drawn to complexes bearing N,S-chelating ligands, such as thiosemicarbazides and thiosemicarbazones.[9-13] The incorporation of such ligands into Pd^{II} compounds is a well-known strategy to enhance their kinetic stability in solution and consequently to reach their pharmacological targets in significant concentration.[14]

In previous studies we have described the synthesis of compounds of the type [PdX(tcz)(PPh$_3$)]X (tcz = 4-methylthiosemicarbazide, 4-phenylthiosemicarbazide; PPh$_3$ = triphenylphosphine; X = Cl, I, NCS).[11] These compounds were more cytotoxic than cisplatin against a panel of tumour cells with IC$_{50}$ values below 10 μM and showed a limited reactivity towards the model nucleobase guanosine and DNA,[11] suggesting that their mechanism of action may include not only interaction with DNA but also binding to other pharmacological targets. In fact, some of these Pd^{II} complexes induced the inhibition of human Topoisomerase IIα (hTop2) at 5 μM. Moreover, complexes [PdI(tcz)(PPh$_3$)]I induced the inhibition of cathepsin B activity with IC$_{50}$ values inferior to 10 μM.[11] Similar compounds of the general formulae [PdX(TSC)(PPh$_3$)] (X = Cl, NNN, NCS) and [PdI(TSC)(PPh$_3$)]I (TSC = (2E)-2-[(2E)-3-phenyl-1-ylidene]hydrazinecarbothioamide) demonstrated to be active against MCF-7 cells with IC$_{50}$ values ranging from 1.81–4.46 μM and able to prevent hTop2 activity at 6.25–25 μM.[12] These findings have prompted us to investigate the influence of the type N,S-chelating ligand on the antiproliferative activity and ability to inhibit hTop2 and cathepsins.

In this work, we sought to investigate the effects of replacing thiosemicarbazides/thiosemicarbazones by 1-(N-substituted)-thiocarbamoyl-3,5-dimethylpyrazoles on cytotoxicity and inhibition of hTop2 and cathepsins B and L. The

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† Electronic Supplementary Information (ESI) available: Relevant geometrical parameters for the complex, DNA Interaction (Spectroscopic Titration, Circular Dichroism and Hoechst 33258) and Topoisomerase IIα experimental methods, Docking studies and modelling calculation, vibrational (FTIR), NMR and ESI-MS spectroscopic data see DOI: 10.1039/x0xx00000x

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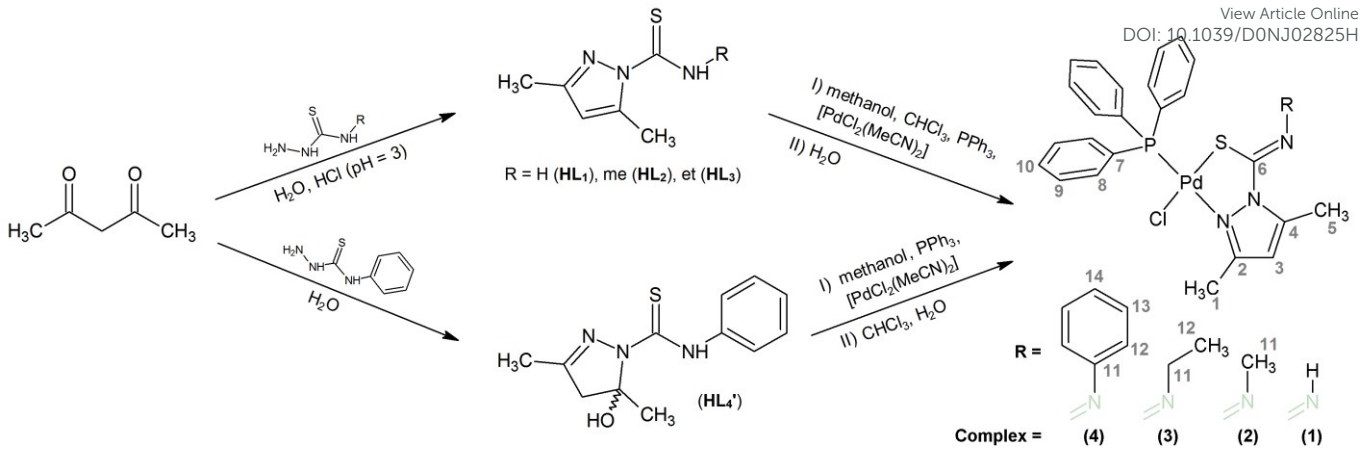

Scheme 1 Synthetic pathways for the preparation of complexes **1–4** and numbering adopted for the signals assignment observed in the NMR spectra.

choice of using, such ligands was motivated by our previous studies on the cytotoxic activity observed for their palladium derivatives.[15,16]

In the framework of our current research on the coordination and biological chemistry of metal-based compounds,[11,12,15,16] we describe herein the synthesis and characterization of the compounds $[PdCl(L_n)(PPh_3)]$ where L_1 = 3,5-dimethylpyrazole-1-iminothiolate (**1**), L_2 = 3,5-dimethyl-pyrazole-N-methyl-1-iminothiolate (**2**), L_3 = 3,5-dimethylpyrazole-N-ethyl-1-iminothiolate (**3**), L_4 = 3,5-dimethylpyrazole-N-phenyl-1-iminothiolate (**4**). Their antiproliferative activity against human breast and lung tumour cell lines (MCF-7 and A549, respectively) together with their ability to induce the inhibition of hTop2 and cathepsin B and L have been evaluated in this work.

2. Results and discussion

2.1. Synthesis and characterization of the complexes

The ligands and their palladium(II) derivatives have been synthetized via the process shown in Scheme 1. As reported previously, 1-thiocarbamoylpyrazoles **HL_1**-**HL_3** were obtained by the acid-catalyzed condensation of thiosemicarbazide and 2,4-pentadienone in water.[11,15,17,18] Complexes **1**-**3** have been prepared from the reaction between bis(acetonitrile)dichloropalladium(II), the suitable pyrazolyl ligand and triphenylphosphine in chloroform in a molar ratio of 1:1:1, respectively. Deprotonation of coordinated 1-thiocarbamoyl-3,5-dimethylpyrazoles **HL_1**-**HL_3** has been achieved by washing the organic reaction media with water until the aqueous solution gives a pH 7. Washing the organic solution with water allowed to segregate the HCl produced, avoiding the formation of cationic complexes.

Although the conventional methodology employing acid catalysis has been successful for obtaining **HL_1**-**HL_3**, several attempts to prepare the N-phenyl-1-thiocarbamoyl-3,5-dimethylpyrazole (**HL_4**) have proved fruitless. The acid conditions brings the product decomposition in phenyl isothiocyanate and 3,5-dimethylpyrazole. Taking into account that the synthesis of pyrazoles from 1,3-diketones with hydrazines involves the formation of hydroxypyrazoline intermediates,[19] we hypothesized that **HL_4** could be obtained *in situ* upon coordination and further dehydration of its corresponding hydroxypyrazoline **HL_4'**. Thus, we have prepared the intermediate **HL_4'** by the condensation reaction under neutral conditions[20] (see Scheme 1). The characterization of **HL_4**´ is discussed in the ESI file. The reaction between $[PdCl_2(MeCN)_2]$, **HL_4'** and PPh_3 has been carried out in methanol for 24 h. After evaporating the solvent almost to dryness, the residue was mixed with chloroform. The resulting organic mixture was washed with water until the aqueous phase gives a neutral pH. This procedure has not yielded the corresponding pyrazoline-based complex, but the pyrazole complex **4** instead.

Table 1 Selected signals in ^1H and ^{13}C DEPTQ NMR, in ppm, for **1**-**4**

	Complex 1		Complex 2		Complex 3		Complex 4	
Position	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C
1	2.71[s]	15.5	2.69[s]	15.5	2.69[s]	15.5	2.74[s]	15.5
2	-	156.1[d] ($^3J_{CP}$ = 3.3)	-	154.6[d] ($^3J_{CP}$ = 3.3)	-	154.5[d] ($^3J_{CP}$ = 2.2)	-	155.8 ($^3J_{CP}$ = 3.3)
3	5.99[d] ($^5J_{HP}$ = 2.0)	111.2[d] ($^4J_{CP}$ = 5.5)	5.92[d] ($^5J_{HP}$ = 2.6)	110.5[d] ($^4J_{CP}$ = 5.5)	5.91[d] ($^5J_{HP}$ = 1.8)	110.4[d] ($^4J_{CP}$ = 4.4)	6.02[d] ($^5J_{HP}$ = 2.2)	111.1[d] ($^4J_{CP}$ = 5.5)
6	-	164.3[d] ($^3J_{CP}$ = 5.5)	-	156.9[d] ($^3J_{CP}$ = 5.5)	-	154.4[d] ($^3J_{CP}$ = 5.5)	-	156.8[d] ($^3J_{CP}$ = 4.4)
8	7.77[m]	134.6[d] ($^2J_{CP}$ = 11.1)	7.79[m]	134.8[d] ($^2J_{CP}$ = 11.1)	7.79[m]	134.8[d] ($^2J_{CP}$ = 11.1)	7.73[m]	134.7[d] ($^2J_{CP}$ = 11.1)
11	8.38[br]		3.06[s]	38.7	3.27[q] ($^3J_{HH}$ = 7.2)	46.2	-	148.2

s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; br = broad.

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The dehydration of the hydroxypyrazoline **HL$_4$'** was clearly detected in the ^{1}H NMR spectrum of **4** by the following evidences (see Fig. S28-S32 in ESI): i) the disappearance of the doublets at 3.2 and 2.9 ppm (18.3 Hz) attributable to the methylene protons at C4 of hydroxypyrazoline nucleus; ii) the lack of the signal at 6.48 ppm assigned to -OH group at 5-position; iii) the appearance of a new doublet at 6.01 ppm ($^4J_{P-H}$ = 2.2 Hz) related to H-4 atom of the pyrazolyl nucleus. The dehydration of the pyrazoline ligand **HL$_4$'** has occurred *in situ*, merely on the complex shape. This dehydration is expected to occur by the H^+ byproduct issued from the thioamide group after coordination to palladium, catalyzing the final dehydration to bring the pyrazole moiety. All four compounds were isolated as air-stable and nonhygroscopic solids, soluble in chloroform and DMSO and showed colours varying from yellow to orange.

Complexes **1-4** were characterized by 1D (^{31}P, ^{1}H, DEPTQ) and 2D (HSQC, HMBC) NMR and IR spectroscopy, CHN analyses, molar conductance and X-ray crystallography (complex **3**). The CHN analyses of the complexes are consistent with the formulae [PdCl(L$_n$)(PPh$_3$)]. Molar conductivity results of all complexes in DMSO (1,0 mmol L^{-1}) were observed in the range of 2.23-4.50 S cm^2 mol^{-1}, indicating that the complexes are non-electrolytes. [21]

The obtained ^{1}H/^{13}C/^{31}P-NMR spectra showed good purity for all complexes. The ^{1}H NMR spectra of **1-4** in $CDCl_3$ showed only one set of signals for coordinated **L$_1$**-**L$_4$** and PPh_3 ligands, which evidence the existence of single species in solution. ^{1}H NMR spectra of **1-4** indicated the deprotonation of the thioamide group with the lack of N-H signal and its coupling with CH_2 or CH_3 ($^3J_{NH-CH}$ = 4.95 and 5.50 Hz for **L$_2$** and **L$_3$** respectively). Also, the shielding of H11 (**L$_2$**: 3.24 ppm; **L$_3$**: 3.74 ppm) and deshielding of H1 (**L$_2$** and **L$_3$**: 2.22 ppm) signals in comparison with the free ligands strongly suggests the *N,S*-anionic coordination mode in all cases. The coordination of PPh_3 is observed by the deshielding of phenyl signals in ^{1}H and ^{13}C NMR. Long-range couplings $^5J_{P-H3}$ (1.8-2.2 Hz) and $^4J_{P-C3}$ (4.4-5.5 Hz) in the spectra are typical of the formation of the H4–C–C=N–Pd–P system and therefore the Pd-pyrazole-PPh_3 complexes. Long-range ^{31}P-^{1}H spin-spin couplings is dependent on the extent of coplanarity of the linking bonds and are in agreement with the expected highly planar complexes structure.[22,23] The major changes in NMR spectra are showed in Table 1. The ^{31}P NMR spectra of complexes in DMSO-d_6 display a singlet in 32.51-32.69 ppm. The chemical shifts are consistent with a structure in which the phosphorus atom is trans to an iminic nitrogen atom.[22,24]

The infrared spectra of all compounds share the characteristic absorption bands of the N,S-anionic coordination mode in the complexes. Firstly, the lack of the strong and broad νNH band (3240-3325 cm^{-1}) indicates the deprotonation of the thiocarbamoyl moiety. Secondly, the shift of the absorption band attributed to ring stretching mode (ν_{ring}) to lower frequency (1435-1437 cm^{-1}) when compared with that one of the free ligands (1574–1593 cm^{-1}).[25] Thirdly, the shift to higher frequency of the thioamide I band (νCN + δNH) from ca. 1513-1523 cm^{-1} in free ligands **L$_2$**-**L$_4$** (1599 cm^{-1} for L$_1$) to ca. 1578-1610 cm^{-1} (2086 cm^{-1} for complex **1**) because of the greater double bond character of the CN bond on deprotonation of the thiocarbamoyl moiety and S-coordination.[26] Intense IR absorptions of the coordinated PPh_3 ligand were found at 1480 cm^{-1} (νCC), 1097 cm^{-1} (νP-C) and 747 cm^{-1} (νC-H).[27]

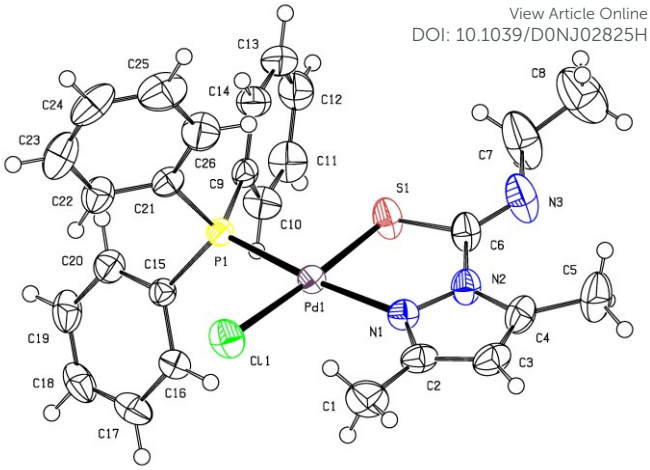

Fig. 1 ORTEP representation of cis-[PdCl(N,S-**L$_3$**)(PPh$_3$)] (**3**), showing the labelling of the atoms. Displacement ellipsoids are drawn at the 50% probability level.

The ESI-MS study confirmed the molecular formula proposed for all complexes. In the positive mode a main peak assigned to the singly charged form of (M^+) or its chloride-eliminated species (M-Cl^-) were obtained.

2.2. X-ray crystal structure of complex 3

Crystals suitable for X-ray diffraction studies were obtained in a vapour diffusion system, using chloroform as solvent and methanol as antisolvent.[28] The crystal structure of **3** has been determined by single-crystal X-ray diffraction, and its ORTEP representation with the atom labeling scheme is illustrated in Fig. 1. Selected interatomic bond distances and angles with their estimated standard deviations are shown in Table 2.

Complex **3** crystalizes in the orthorhombic space group Pbca. The palladium(II) center is coordinated in slightly distorted square-planar geometry with one deprotonated 3,5-dimethylpyrazole-N-ethyl-1-iminothiolato acting as *N,S*-bidentate ligand via its sulfur (Pd-S = 2.2542(11) Å) and pyridine-

Table 2 Selected geometric parameters (Å, °) for cis-[PdCl(N,S-**L$_3$**)(PPh$_3$)] (**3**)

Bond distances	
Pd1—S1	2.2542(11)
Pd1—P1	2.2482(10)
Pd1—N1	2.121(3)
Pd1—Cl1	2.3376(11)
Cl···H1–C1	2.74 (Cl···C: 3.285(5))
Cl···H16–C16	2.79 (Cl···C: 3.353(4))
Bond angles	
S1—Pd1—P1	91.44(4)
S1—Pd1—N1	83.83(9)
N1—Pd1—Cl1	99.97(9)
P1—Pd1—Cl1	84.75(4)
P1—Pd1—N1	174.49(9)
Cl1—Pd1—S1	176.18(4)
C6—N3—C7	117.8(4)

like nitrogen (Pd-N = 2.121(3) Å) atoms, forming a five-membered metallocycle with a bite angle N(1)-Pd-S(1) of 83.83(9)°. The chlorido group is oriented *trans* to the sulfur atom, while the triphenylphosphine ligand is *trans* to the coordinated nitrogen (Fig 1). The short exocyclic C(6)-N(3) bond length of 1.258(6) Å is consistent with an enhanced double bond character, confirming that the thiocarbamoylpyrazolyl ligand is in the thiolate form.[29,30] In addition, the C(6)-S(1) bond distance of 1.741(4) Å is indicative of an increased single bond nature. The Pd–N bond length of 2.121(3) Å is longer than the predicted single bond value of 2.011 Å, based on the sum of covalent radii for N(sp^2) and Pd, 0.701 and 1.31 Å, respectively,[31] and is attributed to the *trans* effect of the phosphine ligand. The Pd–P bond distance of 2.2482(10)Å is smaller than the values found for a number of neutral complexes of the type [PdCl(N,S-TSC)(PPh_3)] (2.2526-2.2707 Å) {N,S-TSC: **MTPTSC**;[32] **PY4MeTSC'**;[33] **4MeBTSC**[34]}.

2.3. Stability in solution

Time-dependent experiments in solution phase were carried out aiming at evaluating the stability of palladium complexes under biologically occurring conditions. The behavior of **1**-**4** in DMSO-d_6 solution and phosphate buffered saline solution ([$H_2PO_4^-$] = 10 mM, pH 7.3; deutered solvent: D_2O 3:7 DMSO-d_6) was studied by monitoring the changes in $^1H/^{31}P$ NMR spectra at 10 min, 24 and 48 h after sample preparation (Figs. S33-S39 in ESI). With exception of complex **1**, all palladium compounds demonstrated to be stable over a period of 48 h in the presence of DMSO, and only a minor additional signal (less than 5%, obtained by measuring the relative heights of their corresponding ^1H NMR signals) occurs in a phosphate buffered saline solution. Taking into account the low stability of complex **1** in DMSO-d_6 (see ^{31}P NMR in Fig. S33, ESI), its biological effects were not studied in this work.

2.4. Antiproliferative assay in vitro of 2-4

The antiproliferative effect of complexes **2**, **3**, and **4** was evaluated against the tumor cell lines MCF-7 and A549 using the MTT cell survival assay,[35] cisplatin was used as a positive control. The EC_{50} values of the compounds were calculated and are listed in Table 3. The ligands **L_2**-**L_4** exhibited to be inactive towards drug concentrations <200 µM for both tumor cell lines.

For breast tumor cells (MCF-7), compound **3** displayed medium EC_{50} value of 31.94 µM, being up to ca. 1.5 fold more active than **2** and cisplatin. Complex **4** demonstrated to be inactive towards MCF-7. With respect to the antiproliferative effects on lung tumor cells (A549), compounds **2**-**4** exhibited no drug response at drug concentrations <50 µM, and therefore were considered inactive. As a comparison, the effect of complexes **2**-**4** on non-tumor human lung fibroblasts (MRC-5) was examined. The compounds **2** and **3** and cisplatin showed comparable EC_{50} values, whereas complex **4** was inactive.

From the inspection of Table 3, it was noticed that the replacement of thiosemicarbazides/thiosemicarbazones by 3,5-dimethylpyrazole-N-substituted-1-iminothiolato ligands into the molecular structure of [PdCl(N,S-ligand)(PPh_3)] resulted in a decrease of the cytotoxicity against MCF-7. For instance, complex **3** (31.94 ± 3.35 µM) was less active than [PdCl(PPh_3)(cinnamaldehydethiosemicarbazonato)] (4.46 ± 1.23 µM)[12] and [PdCl(PPh_3)(4-methyl-3-thiosemicarbazide)]Cl (5.92 ± 0.59 µM).[11] It is important to emphasize that this structure-activity relationship is only preliminary taking into account the limited number of Pd^{II} compounds synthesized.

Table 3 EC_{50}(µM) for Cisplatin and Compounds **2**–**4**

	EC_{50} (µM)		
Compounds	MCF-7	A549	MRC-5
2	47.57 ± 1.16	69.37 ±4.32	26.31 ± 0.38
3	31.94 ± 3.35	71.795 ± 7.15	26.32 ± 0.15
4	>78	>78	>78
Cisplatin	>50	44.39 ± 0.17	19.86 ± 1.22

In order to shed some light on the ability of compounds **2** and **3** to interact with some possible pharmacological targets, preliminary DNA binding studies and enzymatic inhibition assays were performed.

2.5. DNA binding studies

UV–visible absorption spectroscopy is one of the most employed methods for detecting the binding of metal complexes with DNA by monitoring the spectral changes in the CT bands upon addition of increasing concentrations of DNA.[36] The interaction of **2** and **3** with DNA was firstly verified by spectrophotometric titration using fixed concentrations of the complexes and varying the concentration of the ct-DNA. Successive additions of both complexes led to a hypochromism (up to 14%) without significant red shift of the CT band at 258 nm as shown in Fig. 2a-b. The intrinsic constant K_b was calculated through the Wolfe-Shimer[37] relationship, with values found in the order of $6.05x10^4$ M^{-1} (*log* K_b 4.78) for **2** and $7.12x10^4$ M^{-1} (*log* K_b 4.85) for **3**. Such K_b values are significantly lower than those ones found for ethidium bromide (4.94×10^5 M^{-1}) and other classical metallointercalators (10^5 - 10^6 M^{-1}).[38,39] Aiming at providing additional evidence of the DNA binding mode of **2** and **3**, circular dichroism (CD) and competitive binding experiments were performed.

Circular dichroism (CD) is a powerful method to investigate the interaction between metal complexes and DNA.[40,41] As can be seen in Fig. 2c-d, no appreciable changes in the typical CD bands of ct-DNA with increasing concentration of of **2** and **3** were observed, indicating that B-DNA structure remains unchanged upon the interaction of complexes and DNA.

Competitive binding experiments with Hoechst 33258 were also carried out. Hoechst 33258 binds to the minor groove of DNA, providing fluorescence at 458 nm when excited around 350 nm. The quenching of the intrinsic fluorescence of Hoechst-bound DNA system with increasing concentration of metal-based compounds can be employed to investigate whether the metal complex can interact with DNA and displace the Hoechst 33258.[42-44] As shown in Fig. 2e-f, the emission intensity of Hoechst-bound DNA decreased by ca. 75% upon addition of

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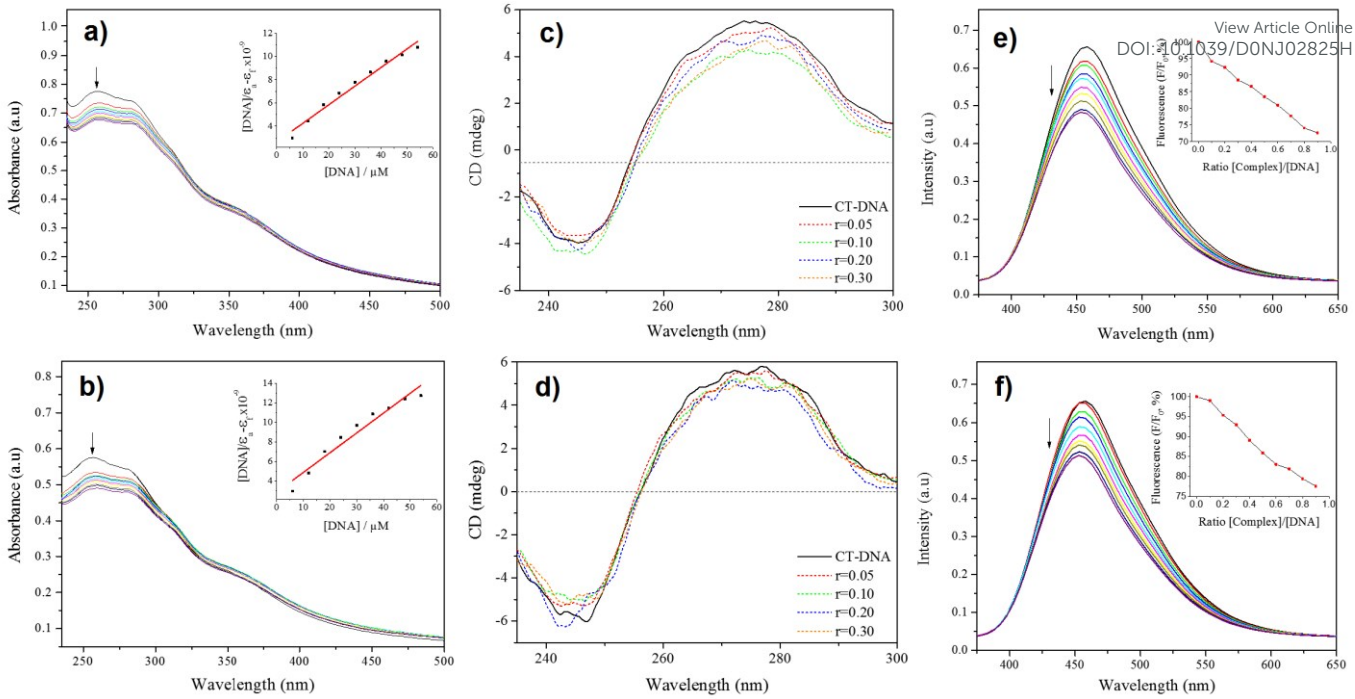

Fig. 2 **a,b)** Absorption spectra of compounds **2** (**a**) and **3** (**b**) with increased amounts of DNA at pH 7.3 in tris-HCl buffer. The arrows show the decreasing of the absorbance with respect to an increase in the DNA concentration (inset: Plot of [DNA]/($\varepsilon_a - \varepsilon_f$) [DNA] vs at λ=258 nm); **c,d)** Circular dichroism spectra of ct-DNA in increased amounts of compound **2** (**c**) and compound **3** (**d**) at pH 7.3 in tris-HCl buffer; **e,f)** Fluorescence emission spectra ($\lambda_{excitation}$= 340 nm) for the Hoechst 33258-DNA solution ([Hoechst]= 6 μM, [DNA]= 60 μM) with increased amounts of complex **2** (**e**) and **3** (**f**) at pH 7.3 in tris-HCl buffer. The arrows show the decreasing of the fluorescence intensity with respect to an increase in the compound concentration (inset: Plot of relative fluorescence intensity (F/F0, %) vs ratio [Complex]/[DNA] at $\lambda_{emission}$= 458 nm).

palladium complexes. The quenching constants (K_{sv}) values calculated for compounds **2** and **3** were 6.86x10^3 M^{-1} (*log* Ksv 3.84) and 5.36x10^3 M^{-1} (log Ksv 3.73), respectively. This finding indicates that complexes **2** and **3** can interact with DNA and can displace the Hoechst 33258 from the minor groove. It is important to point out that other agents that bind elsewhere on the DNA can also replace Hoechst.[45] Although **2** and **3** are able to displace Hoechst, it seems reasonable to suggest that both complexes are a less strong binder, since the Hoechst-bound DNA fluorescence is not totally quenched even in presence of a 9-fold excess of the palladium complexes.

2.6. Human topoisomerase II inhibition assay

Type II topoisomerases are a class of ubiquitous enzymes that alter DNA topology by catalyzing the passing of an intact DNA double helix through a transient double-stranded break made in a second helix. Since hTop2 is highly up-regulated in some cancer cells, it is considered to be the primary pharmacological target for some of the most active drugs currently available for the treatment of human malignancies, as etoposide and doxorubicin.[46] The ability of compounds **2** and **3** to induce the *in vitro* inhibition of hTop2 was also evaluated in this work by agarose gel electrophoresis experiments (Fig. 3).

Circular pBR322 plasmid DNA was incubated with the enzyme in the presence of 12.5, 25, 50 and 100 μM of the complexes. None of the compounds was able to inhibit hTop2 in the tested concentrations. This findings may indicate that the replacement of thiosemicarbazides/thiosemicarbazones from the structure of [PdCl(N,S-ligand)(PPh_3)] by 3,5-dimethylpyrazole-N-substituted-1-iminothiolato ligands afforded inactive compounds towards hTop2.

2.7. Preliminary studies on Cathepsin B and L inhibition

Cathepsin B and L belong to a family of cysteine peptidase of the papain family. Besides their involvement in normal physiological processes, abnormal cathepsin B or L activities are linked in many serious diseases such as cancer, autoimmune disorders and osteoporosis.[48,49]

In this work, complexes **1**–**4** were initially evaluated for their inhibitory activity against human cathepsin B and L in

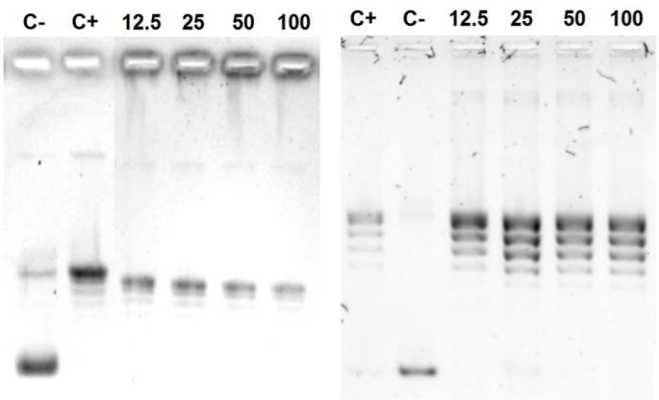

Fig. 3 Topoisomerase IIα relaxation assay. C+ (supercoiled plasmid, topoisomerase and ATP), C– (supercoiled plasmid) and different concentrations (μM) of complex **2** (left) and **3** (right)

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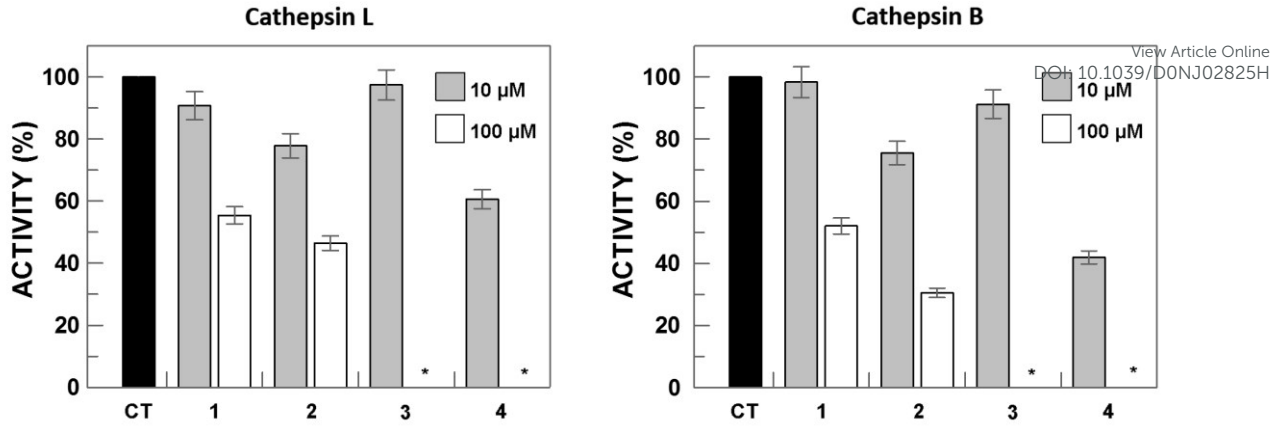

Fig. 4 Cathepsin L and B resisual activity in presence of **1**-**4** in 10 and 100 µM. *insoluble at 100 µM in 100 mM sodium acetate buffer as item 3.3

concentrations of 10 and 100 µM after 5 min of incubation (data are reported in Fig. 4).

At high concentration (100 µM), compounds **1** and **2** induced a reduction of cathepsins B and L activity to ca. 30-55 % whilst complexes **3** and **4** were insoluble. With respect to the inhibitory effects on cathepsins B and L at 10 µM, a progressive decrease on the residual enzymatic activity was observed according to the R-substituent group at thioamide moiety, following the order:

Cathepsin L: R = Et (97%) > H (91%) > Me (78%) > Ph (61%)
Cathepsin B: R = Et (98%) > H (91%) > Me (75%) > Ph (42%)

Compound **4** demonstrated to be the most active among all complexes tested at 10 µM, inhibiting the activity of cathepsins B and L to ca. 58 and 39 %, respectively. It is worth mentioning that inhibition of cathepsin B by **4** was comparable to that of observed for similar complexes of the type [PdI(tscz)(PPh_3)]I (tscz = 4-methyl-3-thiosemicarbazide; 4-phenyl-3-thiosemicarbazide) with IC_{50} < 10 µM.[11]

The design of new cathepsin B inhibitors is of particular interest since its aberrant activity is associated with tumor invasion and metastasis.[49,50] Thus, cathepsin B is considered to be an attractive target for the control of tumor progression. Taking into account that the active site of cathepsin B contains a thiolate-imidazolium ion pair vital for the catalytic activity, a strategy to inhibit the proteolytic activity of cathepsin B consists of designing molecules able to establish reversible or irreversible bonds with the reactive thiolato group.[51,52] Due to its high affinity for sulfur-based ligands, Pd^{II} center has been investigated as potential cathepsin B inhibitor.[53-60] As hypothesized by Spencer et al.,[59] the thiolato group is expected to replace a labile ligand from the palladium complex whereas the co-ligand(s) may establish additional attractive contacts within the active site pocket. However, more detailed studies are required in order to confirm the binding of a Pd^{II} center to the catalytic cysteine residue.

In order to gain additional information about the possible non-covalent interactions between complexes **1**-**4** and cathepsin B, complementary theoretical studies have been undertaken by molecular docking.

2.10. *In silico* approach

The 3-D molecular structures of **1**-**4** were built with chloride ligand given the previous stability studies. The geometry optimized molecules were achieved out by means of the QM/MM calculations.[61,62] The root-mean-square deviation (RMSD) retrieved from an overlay of heavy atoms from the theoretical and molecular crystal structure of **3**, which amounts to 0.4884 Å, endorses the quality of the modelling. See ESI for more details (**Fig S2**).

The active site of the two-folded cathepsin B covers an active cysteine (Cys29), from the left domain, keeping in contact with a right one histidine (His199) through an ion pair. Based on cysteine protease studies,[63-66] it is well-known that residues localized in the S-site are responsible for the enzyme specificity, notable S_2 since S_n' is restricted by the occluding loop. Herein based on docking studies, the ranked binding modes for **1** and **2** were similar, exhibiting only slight changes in the position (Fig 5a-b). These complexes were not able to interact with active Cys29, and **1** is in unfavourable proximity to the side chain of Met196 (*N*42…*O*Met196, *d* = 2.80 Å). However, the presence of a *N*-methyl group at thioamide in **2** favoured a hydrogen bonding interaction with Met196. According to our model, pyrazolyl nucleus of all complexes seemed to play an important role for protein-ligand recognition in S-binding pocket by the establishment of π–lone pair hydrophobic interactions with Gly198 (Fig 5a-c) and Glu122 (Fig 5d). Likewise, as the nonpolar character increases at *N*-terminal thioamide position, the ligand conformations into the binding site changes. Thus, **3** and **4** afforded hydrophobic lone pair-π interactions with side-chain of Cys29 by the aromatic rings of triphenylphosphine. Only complex **4** established additional interactions with the side-chains of Gln23 and Gly73, which were as important as for the inhibitor conformation viewed from the crystal structure[67] (Fig 5d and S1). Accordingly, these computational outcomes agree well with the enhanced *in vitro* inhibition of cathepsin B by compound **4**.

3. Experimental

All solvents and reagents were purchased commercially and used without further purification unless otherwise noted. Calf thymus DNA sodium salt (CT DNA) was obtained from Sigma

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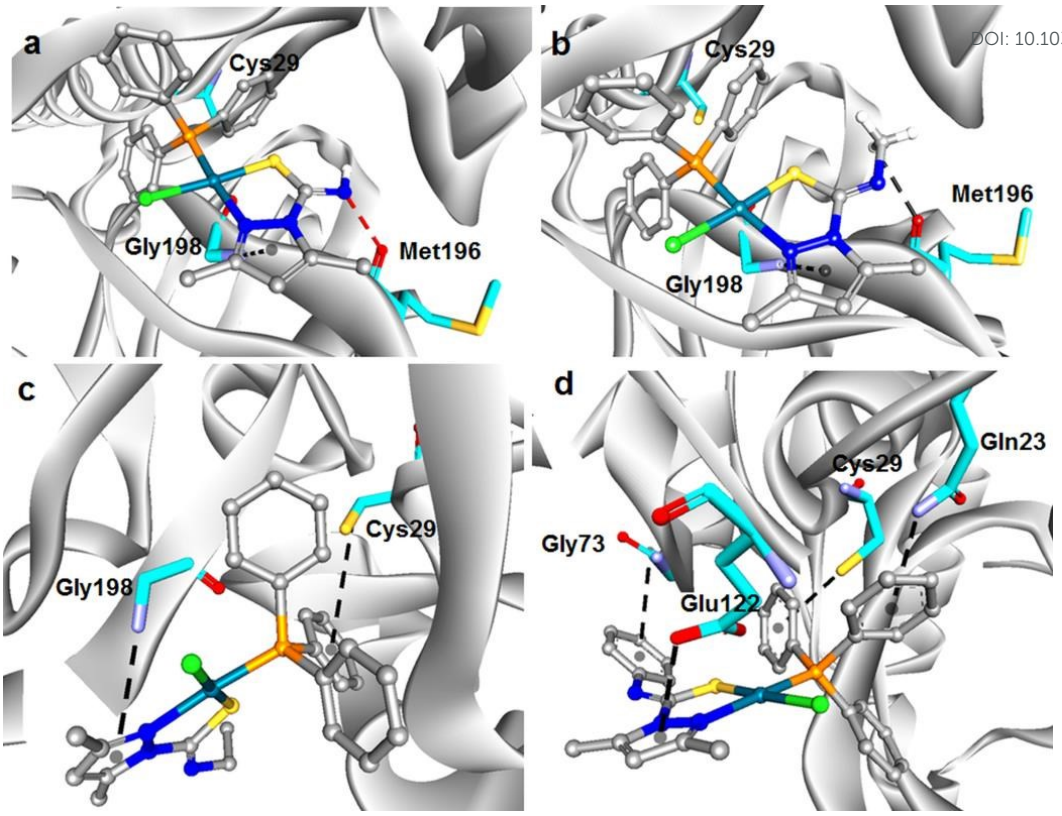

Fig. 5 The best docking pose of the 1-4 at S-site from cathepsin B. The a-d poses are 1-4, respectively. Interacting residues are shown as sticks. The key side-chains are shown as C-atom in light blue, O-atom in red, N-atom in royal blue, S-atom in yellow. The complexes are shown as C-atom in grey, Cl-atom in green, P-atom in orange and Pd-atoms in cyan. Non-interacting H-atoms were omitted for clarity.

Aldrich. Cell lines of MCF-7 were obtained from *Banco de Células do Rio de Janeiro* (Rio de Janeiro, RJ, Brazil), A549 and MRC-5 were generously donated by Dr. Alzir Azevedo Batista, from DQ - UFSCar (São Carlos, SP, Brazil), and by Dr. Fernando Rogério Pavan, from FCF/UNESP (Araraquara, SP, Brazil), respectively. Mass spectra were recorded on a 3200QTRAP (AB Sciex) spectrometer. The data of elemental analysis were carried out on a Perkin Elmer 2400 series II. IR spectra of solid samples (KBr pellets) were obtained on a Nicolet IS5 Thermo Scientific spectrometer in the 4000–400 cm^{-1} region. The NMR spectra were recorded on a Bruker Avance III HD 600 spectrometer with DMSO-d_6 or $CDCl_3$ as solvent at room temperature. The chemical shifts are reported on the δ-scale in parts per million relative to tetramethylsilane (TMS) as internal standard for protons and relative to H_3PO_4 as an external reference for the ^{31}P nuclei

3.1. Synthesis

5-hydroxy-3,5-dimethyl-(N4-phenylthiosemicarbazone)pyrazoline (L4'). 2,4-pentanedione (133.0 mg, 1.328 mmol, 1.1 equiv.) were treated with 4-phenyl-3-thiosemicarbazide (201.8 mg, 1.207 mmol, 1.0 equiv.) in ethanol (20 mL) at room temperature. After stirring for 14 h, the solvent was removed under reduced pressure and the resulting residue was washed three times with diethyl ether (3 x 3 mL). All the extracts were then combined and filtered off. After removing the solvent under vacuum, a viscous oil was obtained. Thin layer chromatography showed just a single spot, indicating the presence of a single product. The compound was stored in the freezer (T < 0° C). yield 137.1 mg (97%) of **L4'**. ^1H NMR (600 MHz, $CDCl_3$): δ = 8.97^{br} ([1], NH), 7.51^{m} (7.8 Hz [2H], *ortho*-phenyl), 7.37^{t} (^3J = 7.8 Hz [2H], *meta*-phenyl), 5.92^{t} (^3J = 7.8 Hz [1H], *para*-phenyl), 6.48^{br} ([1H], OH), 3.22^{d} (^2J = 18.3 Hz [1H], CH_2), 2.92^{d} (^2J = 18.3 Hz [1H], CH_2) 2.09^{s} ([3H], CH_3, H1) 2.05^{s} ppm ([3H], CH_3, H5). DEPTQ-135 NMR (125 MHz, $CDCl_3$): δ = 172.9 (C=S), 155.0 (C2), 137.7 (C7), 128.7 (C9), 126.1 (C10), 125.4 (C8), 94.2 (C4), 52.8 (C3), 27.6 (C5), 16.3 ppm (C1). IR (KBr): 3325 (νOH), 3250(νNH), 1593 (νCN_{ring}), 1513 ($\nu CN_{thioamide}$), 1245 (δC-N), 1123 (δC-O), 1054 (νNN_{ring} + δrCH3) 801 cm^{-1} (νC-S). Calcd. for $C_{12}H_{15}N_3OS$ (249.3): C, 57.81; H, 6.06; N, 16.85; found: C, 57.45; H, 6.51; N, 17.17.

Chlorido(triphenylphosphine)(3,5-dimethylpyrazole-1-imino-thiolate-κ²N²,S)palladium(II) hydrate, [PdCl(L1)(PPh3)]·H2O (complex 1). Triphenylphosphine (101.1 mg, 0.3854 mmol, 1.0 equiv.) and **L1** (59.8 mg, 0.385 mmol, 1.0 equiv.) were treated with $[PdCl_2(MeCN)_2]$ (100.0 mg, 0.3852 mmol, 1.0 equiv.) in chloroform/methanol (9:1, v/v, 10 mL). After stirring for 2 h, 10 mL of water was added to the reaction media. The organic layer was washed three times with 10 mL of water until the aqueous solution gives a pH 7. The organic layer was evaporated under reduced pressure to a final volume of ca. 1 mL and pentane (30 mL) was added to precipitate the compound. The yellow solid was filtered off, washed with pentane, and finally dried under vacuum to yield 143.5 mg (67%) of complex **1**. ^1H NMR (600 MHz, $CDCl_3$): δ = 8.34^{s} ([1H], NH), 7.77^{ddd} ($^3J_{P-H}$ = 12.1 Hz, ^3J = 7.15 Hz, ^4J = 1.3 Hz, [6H], *ortho*-phenyl), 7.51^{m} ([3H], *para*-phenyl), 7.45^{m} ([6H], *meta*-phenyl), 5.99^{d} ($^4J_{P-H}$ = 2 Hz, [1H], CH),

2.71^{s} ([3H], CH_3), 2.55 ppm^{s} ([3H], CH_3). DEPTQ-135 NMR (600 MHz, $CDCl_3$): δ = 164.3^{d} ($^{3}J_{P-C}$ = 5.5 Hz, N=C-S), 156.1^{d} ($^{3}J_{P-C}$ = 3.3 Hz, C2), 144.4 (C4), 134.6^{d} ($^{2}J_{P-C}$ = 11 Hz, *ortho*-PPh_3), 131.1^{d} ($^{4}J_{P-C}$ = 2.2 Hz, *para*-PPh_3), 128.5^{d} ($^{1}J_{P-C}$ = 57.5 Hz, *ipso*-PPh_3), 128.2^{d} ($^{3}J_{P-C}$ = 12,1 Hz, *meta*-PPh_3), 111.2^{d} ($^{4}J_{P-C}$ = 5.5 Hz, CH), 15.50 (CH_3), 14.44 ppm (CH_3). ^{31}P NMR: 32.55 ppm. IR (KBr): 3295 (νN-H), 1436 (νC-N_{ring}), 2086 (νC-$N_{thioamide}$), 1481 (νC-C_{PPh3}), 1273 (δC-N), 1051 (νNN_{ring} + δrCH_3) 691 cm^{-1} (νCS). Calcd. for $C_{24}H_{23}ClN_3PPdS{\cdot}H_2O$ (558.4): C, 50.01; H, 4.37; N, 7.29; found: C, 50.41; H, 4.01; N, 6.74. ESI-MS: base peak at m/z = 522 (M - Cl^-); molecular ion at m/z = 558. Conductivity data (10 mM in DMSO): ΛM = 2.62 ohm^{-1} cm^2 mol^{-1}

Chlorido(triphenylphosphine)(3,5-dimethylpyrazole-N-methyl-1-iminothiolate-κ²N²,S)palladium(II), [PdCl(L_2)(PPh_3)] (complex 2). Triphenylphosphine (70.8 mg, 0.2698 mmol, 1.0 equiv.) and **L_2** (54.7 mg, 0.270 mmol, 1.0 equiv.) were treated with [$PdCl_2(MeCN)_2$] (70.0 mg, 0.2696 mmol, 1.0 equiv.) in chloroform/methanol (7:1, v/v, 8 mL). After stirring for 2 h, 10 mL of water was added to the reaction media. The organic layer was washed three times with 10 mL of water until the aqueous solution gives a pH 7. The organic layer was evaporated under reduced pressure to a final volume of ca. 1 mL and methanol (30 mL) was added to precipitate the compound. The yellow crystalline solid was filtered off, washed with methanol, and finally dried under vacuum to yield 137.1 mg (88%) of complex **2**. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.79^{ddd} ($^{3}J_{P-H}$ = 12.1 Hz, ^{3}J = 7.15 Hz, ^{4}J = 1.4 Hz, [6H], *ortho*-phenyl), 7.50^{m} ([3H], *para*-phenyl), 7.44^{m} ([6H], *meta*-phenyl), 5.92^{d} ($^{4}J_{P-H}$ = 2.6 Hz, [1H], CH), 3.06^{s} ([3H], CH_3 - thioamide) 2.69^{s} ([3H], CH_3 - pyrazole), 2.48^{s} ppm ([3H], CH_3 - pyrazole). DEPTQ-135 NMR (600 MHz, $CDCl_3$): δ = 156.9^{d} ($^{3}J_{P-C}$ = 5.5 Hz, N=C-S), 154.6^{d} ($^{3}J_{P-C}$ = 3.3 Hz, C2 - pyrazole), 143.4 (C4 - pyrazole), 134.7^{d} ($^{2}J_{P-C}$ = 11 Hz, *ortho*-PPh_3), 131.1^{d} ($^{4}J_{P-C}$ = 3.3 Hz, *para*-PPh_3), 129.2^{d} ($^{1}J_{P-C}$ = 57.5 Hz, *ipso*-PPh_3), 128.2^{d} ($^{3}J_{P-C}$ = 11,1 Hz, *meta*-PPh_3), 110.5^{d} ($^{4}J_{P-C}$ = 5.5 Hz, CH - pyrazole), 38.7 (CH_3 - thioamide) 15.5 (CH_3 - pyrazole), 14.8 ppm (CH_3 - pyrazole). ^{31}P NMR: 32.51 ppm. IR (KBr): 1437 (νCN_{ring}), 1610 (ν$CN_{thioamide}$), 1479 (νCC_{PPh3}), 1262 (δC-N), 1034 (νNN_{ring} + δrCH_3) 695 cm$_{-1}$ (νCS). Calcd. for $C_{25}H_{25}ClN_3PPdS{\cdot}0.5H_2O$ (572.4): C, 51.65; H, 4.51; N, 7.23; found: C, 51.46; H, 4.29; N, 7.28. ESI-MS: base peak at m/z (M - Cl^-) = 538; molecular ion at m/z = 572. Conductivity data (10 mM in DMSO): ΛM = 2.23 ohm^{-1} cm^2 mol^{-1}.

Chlorido(triphenylphosphine)(3,5-dimethylpyrazole-N-ethyl-1-iminothiolate-κ²N²,S)palladium(II), [PdCl(L_3)(PPh_3)] (complex 3). Triphenylphosphine (101.1 mg, 0.3854 mmol, 1.0 equiv.) and **L_3** (70.6 mg, 0.385 mmol, 1.0 equiv.) were treated with [$PdCl_2(MeCN)_2$] (100.0 mg, 0.3852 mmol, 1.0 equiv.) in chloroform/methanol (9:1, v/v, 10 mL). After stirring for 2 h, 10 mL of water was added to the reaction media. The organic layer was washed three times with 10 mL of water until the aqueous solution gives a pH 7. The organic layer was evaporated under reduced pressure to a final volume of ca. 1 mL and methanol (30 mL) was added to precipitate the compound. The orange crystalline solid was filtered off, washed with methanol, and finally dried under vacuum to yield 186.4 mg (83%) of complex **3**. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.78^{m} ([6H], ortho-phenyl), 7.49^{m} ([3H], para-phenyl), 7.44^{m} (m, [6H], meta-phenyl), 5.91^{d} ($^{4}J_{P-H}$ = 1.8 Hz, [1H], CH), 3.27^{q} (^{3}J = 7.2 Hz, [2H], CH_2) 2.69^{s} ([3H], CH_3 - pyrazole), 2.50^{s} ([3H], CH_3 - pyrazole), 1.15^{t} ppm (^{3}J = 7.2 Hz, [3H], CH_3). DEPTQ-135 NMR (600 MHz, $CDCl_3$): δ = 154.5^{d} ($^{3}J_{P-C}$ = 3.3 Hz, C2), 154.4^{d} ($^{3}J_{P-C}$ = 5.5 Hz, N=C-S) 143.5 (C4), 134.8^{d} ($^{2}J_{P-C}$ = 11.1 Hz, *ortho*-PPh_3), 131.1^{d} ($^{4}J_{P-C}$ = 2.2 Hz, *para*-PPh_3), 129.2^{d} ($^{1}J_{P-C}$ = 57.5 Hz, *ipso*-PPh_3), 128.2^{d} ($^{3}J_{P-C}$ = 12,2 Hz, *meta*-PPh_3), 110.4^{d} ($^{4}J_{P-C}$ = 4.4 Hz, CH), 46.2 (CH_2), 15.8 (CH_3), 15.5 (CH_3 - pyrazole), 15.0 ppm (CH_3 - pyrazole). ^{31}P NMR: 32.57 ppm. IR (KBr): 1436 (νCN_{ring}), 1600 (ν$CN_{thioamide}$), 1480 (νCC_{PPh3}), 1253 (δC-N), 1046 (νNN_{ring} + δrCH_3) 705 cm^{-1} (νCS). Calcd. for $C_{26}H_{27}ClN_3PPdS{\cdot}0.5H_2O$ (586.4): C, 52.45; H, 4.74; N, 7.06; found: C, 52.35; H, 4.64; N, 7.42. ESI-MS: base peak at m/z (M - Cl^-) = 550; molecular ion at m/z = 586. Conductivity data (10 mM in DMSO): ΛM = 4.50 ohm^{-1} cm^2 mol^{-1}

Chlorido(triphenylphosphine)(3,5-dimethylpyrazole-N-phenyl-1-iminothiolate-κ²N²,S)palladium(II), [PdCl(L_4)(PPh_3)] (complex 4). Triphenylphosphine (101.1 mg, 0.3854 mmol, 1.0 equiv.) and **L_4'** (96.1 mg, 0.385 mmol, 1.0 equiv.) were treated with [$PdCl_2(MeCN)_2$] (100.0 mg, 0.3852 mmol, 1.0 equiv.) in methanol (10 mL). After stirring for 24 h, the solution was evaporated under reduced pressure to a final volume of circa 2 mL and 20 mL of chloroform were added. In a separation funnel, the organic layer was washed three times with 10 mL of water until the aqueous solution gives a pH 7. The resulting red solution was then evaporated under reduced pressure to a final volume of ca. 1 mL and pentane (20 mL) was added to precipitate the compound. The brown solid was filtered off, washed with pentane, and finally dried under vacuum to yield 65.0 mg (27%) of complex **4**. ^{1}H NMR (600 MHz, $CDCl_3$):): δ = 7.72^{ddd} ($^{3}J_{P-H}$ = 12.1 Hz, ^{3}J = 7.15 Hz, ^{4}J = 1.3 Hz, [6H], *ortho*-PPh_3), 7.45^{m} ([3H], *para*-PPh_3), 7.38^{m} ([6H], *meta*-PPh_3), 7.19^{m} ([2H], *meta*-phenyl), 6.99^{tt} (^{3}J = 7.3 Hz, ^{4}J = 1.1 Hz, [1H], *para*-phenyl), 6.89^{m} ([2H], *ortho*-phenyl), 6.01^{d} ($^{4}J_{P-H}$ = 2.2 Hz, [1H], CH, pyrazole), 2.74^{s} ([3H], CH_3), 2.61^{s} ppm ([3H], CH_3). DEPTQ-135 NMR (600 MHz, CDCl3): δ = 156.8^{d} ($^{3}J_{P-C}$ = 4.4 Hz, N=C-S), 155.8 (C2), 148.2 (*ipso*-thioamide) 144.2 (C4), 134.8^{d} ($^{2}J_{P-C}$ = 11.1 Hz, *ortho*-PPh_3), 131.1^{d} ($^{4}J_{P-C}$ = 3.3 Hz, *para*-PPh_3), 128.8^{d} ($^{1}J_{P-C}$ = 57.5 Hz, *ipso*-PPh_3), 128.3 (*meta*-thioamide) 128.1^{d} ($^{3}J_{P-C}$ = 11,1 Hz, *meta*-PPh_3), 123.5 (*para*-thioamide) 121.7 (*ortho*-thioamide), 111.1^{d} ($^{4}J_{P-C}$ = 5.5 Hz, CH - pyrazole), 15.5 (CH_3), 15.2 ppm (CH_3). ^{31}P NMR: 32.69 ppm. IR (KBr): 1435 (νCN_{ring}), 1578 (ν$CN_{thioamide}$), 1480 (νCC_{PPh3}), 1273 (δC-N), 1050 (νNN_{ring} + δrCH_3) 692 cm^{-1} (νCS). Calcd. for $C_{30}H_{27}ClN_3PPdS{\cdot}0.5H_2O$ (634.5): C, 56.00; H, 4.39; N, 6.53; found: C, 55.99; H, 3.82; N, 6.45. ESI-MS: base peak at m/z (M - Cl^-) = 599; molecular ion at m/z = 635. Conductivity data (10 mM in DMSO): ΛM = 2.55 ohm^{-1} cm^2 mol^{-1}.

3.2. Cell proliferation

The free ligands and palladium complexes were assayed using the human breast tumor cell line MCF-7, human lung tumor cell line A549 and human fetal lung fibroblast cell line MRC-5. The

cells were routinely maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), gentamicin sulfate (50 mg L^{-1}) and amphotericin B (2 mg L^{-1}) at 310 K in a humidified 5% CO_2 atmosphere. Briefly, all cell lines were prepared at a concentration of 1.5×10^4 cells/100 µL, in complete medium, and plated on sterile 96 well plates for 24 h at 310 K in a humidified 5% CO_2 atmosphere. The compounds were added to the wells at different concentrations and incubated for 48 h under the same conditions as described above. The cell proliferation assay was performed compared to the wells where the vehicle (0.5% DMSO) was added instead of the tested compounds (at 0.5% DMSO). After incubation, the culture medium of each well was removed and a solution containing MTT (1 mg mL^{-1}) was added (50 µL/well).[35] The plates were then kept at 310 K for 4 h and the formed crystals were dissolved in isopropyl alcohol. The absorbance was read on an ELISA plate reader (BioTek, Epoch) at a wavelength of 570 nm and the IC_{50} (concentration of compound that induced 50% of cell death) value were determined using the GraphPad Prism software (www.graphpad.com/). Each assay were performed in triplicate.

3.3. Cathepsin B and L inhibition

Cathepsin B and L were expressed and purified according Linnevers *et al*.[68] The assays were performed in a spectrofluorometer Hitachi F-2700. The monitoring of the enzyme activity was followed at wavelengths λex=360 nm and λem=480 nm, under constant agitation, in 10 mm optical path quartz cuvettes at final volumes of 1 mL. Cathepsin B and L was incubated in 1000 µL of a preheated solution of sodium acetate buffer (100 mM); 5 mM EDTA; 100 mM NaCl; 0.01% Triton X-100 (only for cathepsin B); 20% glycerol, pH 5.5, 3 mM DTT, and 40 µM (cathepsin B) or 15 µM (cathepsin L) of Z-FR-AMC (Benzyloxycarbonyl-Phe-Arg-7-amido-4-methylcoumarin). The measurement of the enzyme activity was performed monitoring the product formation AMC (amino-4-methylcoumarin) by hydrolysis of substrate Z-FR-AMC used as fluorogenic probe after 5 minutes. The effects of the activity of the compounds on the enzymes were performed at concentration of 10 and 100 µM.

3.4. Computational Methodology

Molecular Modelling. The 3-D molecular structures of the complexes were handled as an in silico approach reported recently, but slightly modified. [12,53] As follows, the structures of **1**-**4** were constructed and primarily minimized by using molecular mechanics (MM) simulation in Biovia Discovery Studio.[69] Further, the semi-empirical PM7 Hamiltonian method was employed to acquire minimal energy structures.[70] The quality of the outcomes has been proved by root-mean-square deviation from the alignment between heavy atoms of the theoretical and molecular crystal structure of 3, see ESI for more details.

Molecular Docking. The crystal structure of a mature Cathepsin B, co-crystallized with a dipeptidyl nitrile inhibitor, was obtained from Protein Data Bank (PDB: 1GMY) and prepared for the modelling work.[71] Before docking, all hydrogen were added and the binding site was fixed at x = 28.8380, y = 37.2531 and z = 37.2500 coordinates (Cys29, S-donor atom). The docking radius was 15 Å and 50 runs were set to be calculated using GoldScore fitness function, which is embedded in CCDC GOLD software.[72,73] The docking results were evaluated using the top-ranked pose and the ability of that to reproduce the original intermolecular contacts as the co-crystallized inhibitor.

Conclusions

The synthesis and spectroscopic characterization of novel complexes of the type $[PdCl(L_n)(PPh_3)]$ {L_n = 1-iminothiolate-3,5-dimethylpyrazoles} have been reported in this work. The molecular structure of compound $[PdCl(\mathbf{L_3})(PPh_3)]$ (**L_3** = 3,5-dimethyl-pyrazole-N-ethyl-1-iminothiolate; complex **3**) was solved by single-crystal X-ray diffraction technique. The cytotoxicity of **2**-**4** against MCF-7 cells demonstrated to be dependent on the substituent at *N*-thioamide position. Complex **3** displayed the most potent cytotoxic effect, with an IC_{50} value of 31.94 ± 3.35 µM. In addition, compounds **1**-**4** did not induce inhibition of human topoisomerase II even at 100 µM, suggesting that other targets may be involved. Preliminary studies on the inhibition of cathepsins B and L activity showed that their potency seems to be also dependent on the type of R-substituent group at thioamide fragment. Compound **4** was the most potent inhibitor among all tested Pd^{II} complexes, resulting in a residual activity of 42% (cat B) and 61 % (cat L). *In silico* studies indicated that only complex **4** established additional interactions with the residues in the S-binding pocket. These findings agree well with the obtained results from *in vitro* inhibition assays. Thus, new palladium-based cathepsins inhibitors may be designed from a careful selection of 3,5-dimethyl-pyrazole-N-substitued-1-iminothiolate and phosphine ligands.

Authors' contributions

TRM conceived the study, synthesized and characterized all four complexes and wrote the manuscript; RDZ performed all DNA binding studies; DESS performed the experiments for EC_{50} determination; RLF performed the *in silico* experiments; MAL and FVR performed the topoisomerase assay; VMD performed the X-ray crystallography and structure elucidation of complex **3**; AAS, FSS and WASJ performed the cathepsins assays; JCMP wrote the manuscript; AVGN, AEM and WASJ participated in the coordination of the study, analysis of data and helped draft the manuscript. All authors read and approved the final version of the manuscript.

Conflicts of interest

There are no conflicts of interest to declare.

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Palladium(II) complexes bearing 1-iminothiolate-3,5-dimethylpyrazoles: synthesis, cytotoxicity, DNA binding and enzymatic inhibition studies

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TABLE OF CONTENTS ENTRY:

This work describes the enzymatic inhibitory activity of four novel Pd(II) complexes towards topoisomerase IIα and cathepsins B and L. *In silico* studies agree well with the enhanced *in vitro* cathepsin B inhibition induced by compound **4** (58% at 10 µM)