Palladium complexes with 3-methyl-8-thia-1,3-diaza-spiro[4.5]decane-2,4-dione: experimental and theoretical studies

Emiliya D. Cherneva, Rossen T. Buyukliev, and Adriana G. Bakalova

Department of Chemistry, Faculty of Pharmacy, Medical University - Sofia, 2 Dunav Str., 1000 Sofia, Bulgaria
*Email: e.d.cherneva@gmail.com*

Abstract

Platinum complexes are a cornerstone of the treatment of various cancers. Their side effects, however, like nephrotoxicity and ototoxicity, have led to the investigation of alternative metal-based anticancer drugs such as palladium complexes, which show promising activities. New *cis*-palladium complexes with general formulas $[PdL_2Cl_2]$ and $[PdL_2Cl_4]$ and ligand 3-methyl-8-thia-1,3-diaza-spiro[4.5]decane-2,4-dione have been synthesized. The structures of the complexes were elucidated by elemental analyses and spectroscopic techniques as well as theoretical methods. Quantum chemical calculations confirm the square planar geometry of Pd(II) and a distorted octahedral coordination of Pd(IV) complexes. The *in vitro* cytotoxicity-assay results against K-562 and REH cell lines are also reported.
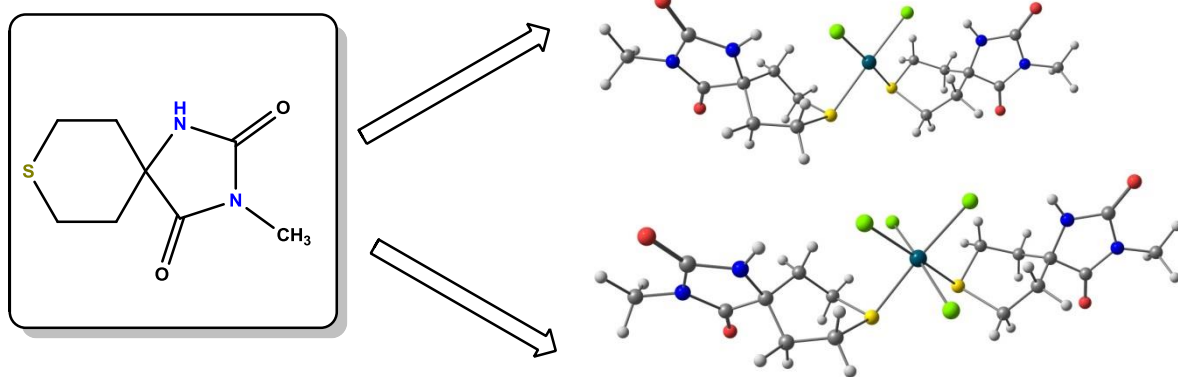
Introduction

Platinum compounds, particularly cisplatin ([cis-PtCl$_2$(NH$_3$)$_2$]), are at the core of the metal-based compounds used in cancer therapy.[1] The complexes are principally indicated for the treatment of cervical, ovarian, testicular, head and neck, breast, bladder, stomach, prostate and lung cancers. Their anticancer activities are also extended to Hodgkin's and non-Hodgkin's lymphomas, neuroblastoma, sarcoma, melanoma and multiple myeloma.[2] On this basis, alternative platinum compounds were derived. Carboplatin, oxaliplatin, satraplatin, ormaplatin, aroplatin, enloplatin, zeniplatin, sebriplatin, miboplatin, picoplatin, satraplatin and iproplatin are all products of the extensive research of platinum complexes.[3] While it is virtually impossible to deny the success of platinum drugs in chemotherapy, it is important to also note their limitations, such as drug resistance, poor water solubility, limited spectrum of activity, and worsening side effects.[4] To surmount these challenges, efforts have been made to critically consider and examine other metal-based complexes with cytotoxic properties, such as ruthenium, gold, gallium, iron complexes, etc. Since palladium(II) displays similar chemical features to Pt(II), the coordination chemistry of its compounds are similar to Pt(II) compounds.[5-6] In the past few years, a large number of palladium complexes have been designed and synthesized which have shown comparable or better antitumor activity than Pt(II) complexes against various types of tumors.[7] Although many Pd(II) complexes with tumor-treatment possibilities were illustrated, rapid hydrolysis and less stability raised concerns. As palladium(II) complexes exchange ligands 10^4-10^5 times faster than analogous Pt(II) compounds, and thus dissociate readily in solution forming very reactive species, they often do not reach their pharmacological targets. Mononuclear palladium complexes with aromatic N-containing ligands, amino-acid ligands, S-donor ligands, and P-containing ligands have their respective properties and qualities due to the different structures and properties of the ligands.[8]

This paper presents the synthesis, chemical investigation and theoretical calculations of 3-methyl-8-thia-1,3-diaza-spiro[4.5]decane-2,4-dione and its palladium complexes. The cytotoxic activity of the compounds includes the preliminary screening *in vitro* against two human-tumor cell lines using the standard MTT-dye-reduction assay for cell viability.

Results and Discussion

Syntheses of 3-Methyl-8-thia-1,3-diaza-spiro[4.5]decane-2,4-dione (**2**) and the new palladium (Pd) complexes c*is*-bis(3-methyl-8-thia-1,3-diaza-spiro[4.5]decane-2,4-dione)-dichlorido palladium(II) − *cis*-[PdL$_2$Cl$_2$] (**3**), and c*is*-bis(3-methyl-8-thia-1,3-diaza-spiro[4.5]decane-2,4-dione)-tetrachlorido palladium(IV) − *cis*-[PdL$_2$Cl$_4$] (**4**), are presented in Scheme 1. Elemental analyses of the ligand **2** and the Pd complexes **3** and **4** correspond to the formulas: C$_8$H$_{12}$SN$_2$O$_2$ (**2**), [Pd(C$_8$H$_{12}$SN$_2$O$_2$)$_2$Cl$_2$] (**3**), and [Pd(C$_8$H$_{12}$SN$_2$O$_2$)$_2$Cl$_4$] (**4**). To evaluate the mode of coordination of the ligand to the metal ion, IR, ^{1}H and ^{13}C NMR spectra of the ligand and its palladium complexes were obtained and elucidated.
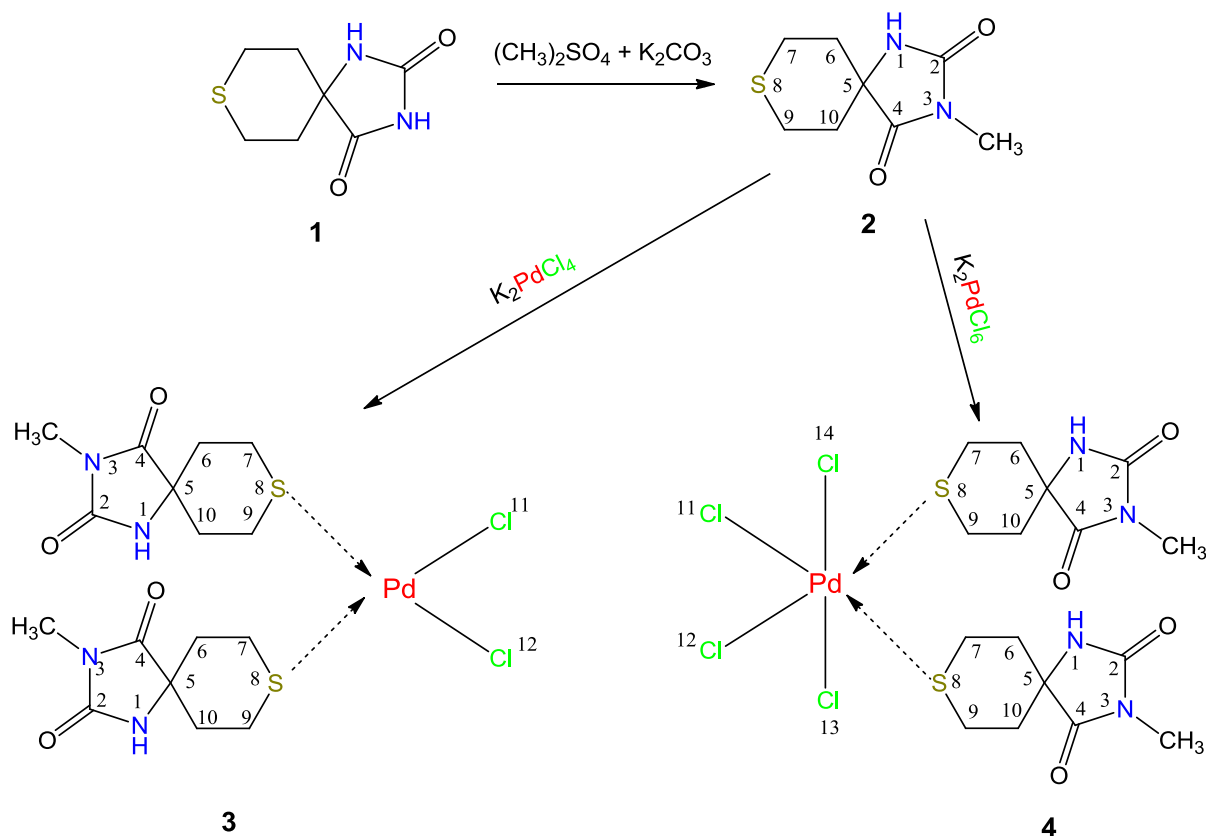
Scheme 1. Synthesis of **2** and its Pd(II) and Pd(IV) complexes **3** and **4**.

Comparative analysis of the infrared spectra of the metal-free ligand **2** and the complexes **3** and **4** revealed that the absorption bands characteristic for the stretching vibrations of C-S bonds were shifted from 617 cm^{-1} of the ligand to 597 and 584 cm^{-1} of the complexes, respectively, which were very close to the theoretical shifts from 616 cm^{-1} of the ligand to 591 and 584 cm^{-1}, respectively. The shifting of the frequencies characteristic for C-S bonds of 20 and 33 cm^{-1} in the complexes shows that the sulfur atom is coordinated with the palladium ion. A good correlation between theoretical calculations and experimental results was observed.

In the ^{1}H NMR spectra of the Pd(II) and Pd(IV) complexes with 3-methyl-8-thia-1,3-diaza-spiro[4.5]decane-2,4-dione, the signal of the N-H proton was not influenced. The protons of N-CH$_3$ in the Pd complexes were also not shifted in comparison with the metal-free ligand. The S-CH$_2$ and C-CH$_2$ protons are shifted 0.08 – 0.13 ppm for Pd-complex **3** and 0.13-0.21 ppm for Pd-complex **4**. This confirms the bonding of sulfur with the palladium ion. The six-membered ring orientation is fixed because the sulfur is bonded with the palladium ion; therefore, the axial and equatorial hydrogen atoms are easily discerned in the ^{1}H NMR spectra.

Geometries of the ligand and its complexes were analyzed by theoretical calculations using density-functional theory (DFT) studies, due to the difficulties to obtain crystals suitable for X-ray analysis. The LANL2DZ effective-core potential was used for palladium and the atomic 6-311++G(g,p) basis set for all non-metal atoms. DFT calculations were performed using the Becke, 3-parameter, Lee-Yang-Parr (B3LYP) functional. Theoretical methodology was applied to evaluate the structural characteristics and spectroscopic properties of the synthesized compounds. The geometries of the ligand and palladium complexes are shown in Figures 1-3. Selected geometry parameters are summarized in Table 1.
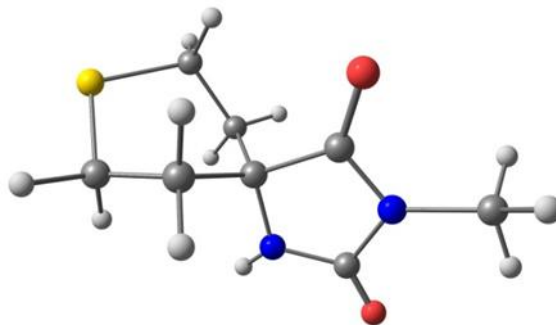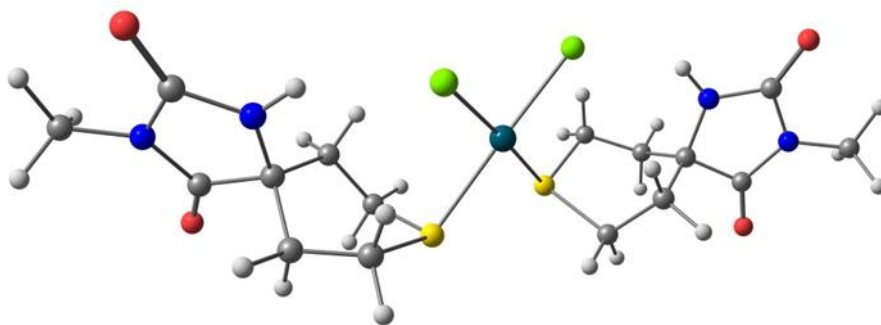
Figure 1. Optimized structure of the ligand **2**.

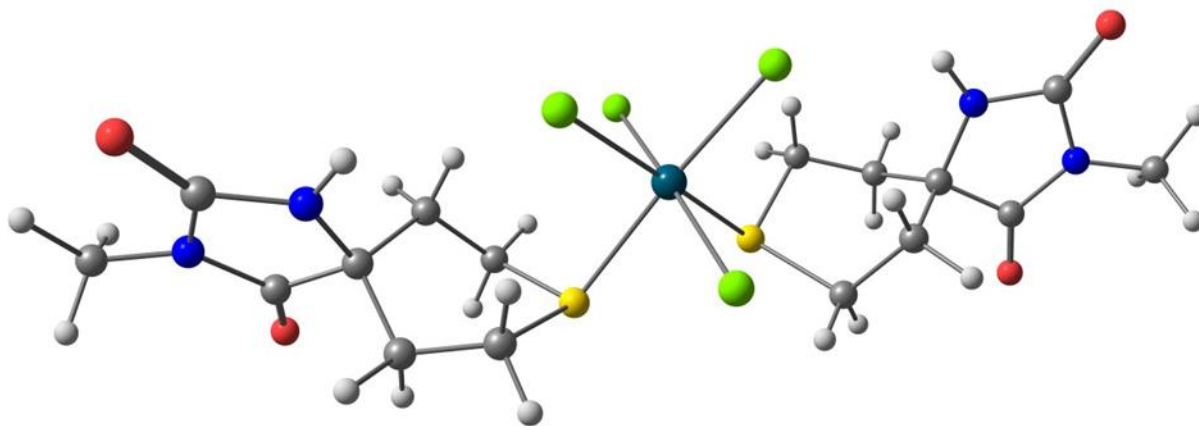**Figure 2**. Optimized structure of the *cis*-[PdL$_2$Cl$_2$] (**3**).

Figure 3. Optimized structure of the *cis*-[PdL$_2$Cl$_4$] (**4**).

The complexes involve a two-molecules of ligand which form complexes *via* the S-atom of the tetrahydrothiopyrane moiety. The location of the ligands around the metal ions leads to formation of square-planar geometry of the Pd(II) complex and distorted octahedral-coordination environment of Pd(IV).

Upon complexation, the C-S bond lengths for the ligand are elongated by 0.07 and 0.08 Å in the Pd complexes. This confirms that the coordination is through the sulfur atom. The Pd-S bond distance in the PdL$_2$Cl$_2$ complex is slightly shorter than that in the PdL$_2$Cl$_4$ complex by 0.04 Å according to the calculations. The Pd-S bond lengths are similar to those found in the literature for other optimized Pd complexes.[9-10] The Pd-Cl bond lengths are slightly shorter in Pd(II) than in Pd(IV). One of the valence angles of Pd-S-C is increased by 6.9° in the Pd(IV) complex while another one is decreased by 5°.

Table 1. Calculated geometry parameters of the ligand **2** and its complexes **3** and **4** using atom numbering in Scheme 1

Parameters	Ligand(**2**)	*cis*-[PdL$_2$Cl$_2$] (**3**)	*cis*-[PdL$_2$Cl$_4$] (**4**)
μ(D)	0.75	9.67	8.73
Bond lengths (Å)			
C$_7$-S$_8$	1.84	1.91	1.91
C$_9$-S$_8$	1.84	1.92	1.92
Pd-Cl$_{11}$	-	2.39	2.41
Pd-Cl$_{12}$	-	2.39	2.41
Pd-Cl$_{13}$	-	-	2.42
Pd-Cl$_{14}$	-	-	2.42
Pd-S$_8$	-	2.48	2.52
Angles (°)			
C$_7$-S$_8$-C$_9$	99.2	99.2	99.4
S$_8$-C$_9$-C$_{10}$	112.1	111.4	110.6
Pd-S$_8$-C$_9$	-	110.5	105.5
Pd-S$_8$-C$_7$	-	101.9	108.8
S$_8$-Pd-Cl$_{13}$	-	-	83.7
S$_8$-Pd-Cl$_{14}$	-	-	92.7
Dihedral angles (°)			
Cl$_{11}$-Pd-S$_8$-C$_7$	-	90.8	72.8
Cl$_{11}$-Pd-S$_8$-C$_9$	-	-13.9	-33.1
Pd-S$_8$-C$_7$-C$_6$	-	-67.4	-71.4
Pd-S$_8$-C$_9$-C$_{10}$	-	118.6	133.8

The present study describes a comparative evaluation of the cytotoxic effects of two newly synthesized Pd(II) and Pd(IV) complexes with 3-methyl-8-thia-1,3-diaza-spiro[4.5]decane-2,4-dione vs. the metal-free ligand, and reference antineoplastic agent, cisplatin, on two human-tumor cell lines, K-562 and REH, using the standard MTT-dye-reduction assay for cell viability. The compounds exerted cytotoxic effects after 72 h continuous exposure. The IC$_{50}$ values are summarized in Table 2. The ligand has some cytotoxic effect in the REH cell line. The tested compounds displayed cytotoxic effects in a concentration-dependent manner. As a result, the ligand and the newly-synthesized palladium complexes were found to be less active than the reference, cisplatin. These findings are in line with our previous reports on the *in vitro* biological performance of related platinum and palladium species of a different series.[11,12] The complexes **3** and **4** are typical "rule-breaking" compounds, i.e., they do not retain the antitumor properties of the classical platinum therapeutic compounds.

Table 2. Cytotoxicity of the ligand **2** and its metal complexes **3** and **4** in comparison to cisplatin

Compound	$IC_{50}(\mu M)$	
	K-562[a]	REH[b]
Ligand	> 200	121.5
cis-[PdL$_2$Cl$_2$]	> 200	> 200
cis-[PdL$_2$Cl$_4$]	> 200	> 200
cisplatin	36.9	1.07

[a]K-562 (Chronic myeloid leukemia); [b] REH (acute lymphoblastic leukemia).

Conclusions

A new organic compound, 3-methyl-8-thia-1,3-diaza-spiro[4.5]decane-2,4-dione, and its two palladium complexes, *cis*-bis(3-methyl-8-thia-1,3-diaza-spiro[4.5]decane-2,4-dione)-dichlorido palladium(II) – *cis*-[PdL$_2$Cl$_2$] and c*is*-bis(3-methyl-8-thia-1,3-diaza-spiro[4.5]decane-2,4-dione)-tetrachlorido palladium(IV) – *cis*-[PdL$_2$Cl$_4$], were synthesized and studied. The molecular formulas of the compounds were determined by elemental analyses, IR, ^{1}H and ^{13}C NMR spectra. The coordination mode of the ligand and its Pd(II) and Pd(IV) complexes was confirmed by theoretical calculations using density-functional theory because of the difficulties to prepare crystals suitable for X-ray analysis. The mode of the coordination of the ligand to the metal ion in the complexes is realized through the sulfur atom from the six-membered ring. The complexes were tested for antiproliferative activity *in vitro* on two leukemic cell lines – K-562 and REH.

Experimental Section

General. All the chemicals used were of analytical grade. The starting hydantoin is available by means of Bucherer-Berg reaction from $(NH_4)_2CO_3$, NaCN and tetrahydro-4H-thiopyran-4-one (Aldrich).[13] Potassium tetrachloropalladate(II) was purchased from Merck, Germany, and potassium hexachloropalladate(II) from Aldrich, USA. The microchemical analyses (C, H, N) of the compounds were carried out with a EuroEA 3000 – Single, EuroVectorSpA. Infrared spectra were recorded on a Thermo Scientific Nicolet iS10 spectrophotometer in the range of 4000-400 cm^{-1} using Attenuated Total Reflection Fourier Transform Infrared Spectroscopy (ATR-FTIR). The ^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6 on Bruker DRX 250 (250 MHz) and Bruker WM 500 (500MHz) spectrometers. Corrected melting points were determined using a Buchi 535 apparatus.

3-Methyl-8-thia-1,3-diaza-spiro[4.5]decane-2,4-dione (2). 8-Thia-1,3-diaza-spiro[4.5]decane-2,4-dione (1.86 g) is dissolved in 20 mL aqueous ethanol, 1.4 g K_2CO_3 is added, and 1 mL $(CH_3)_2SO_4$ is added drop-wise. The resulting mixture was stirred and heated at 60 °C for 3 hours. After cooling, the resulting crystals of 3-methyl-8-thia-1,3-diaza-spiro[4.5]decane-2,4-dione were filtered off and recrystallized from aqueous ethanol, giving **2** as a white solid (1.34 g, 67%). mp 252-253°C. IR (solid, ATR, $v_{max,}$ cm^{-1}, cm^{-1}): 3280, 3115, 1773, 1708, 617. ^{1}H NMR (500 MHz, DMSO-d_6): δ_H 8.72 (1H, s, NH), 2.79 (3H, s, N-CH$_3$), 2.78 (2H, m, CH$_2$-S(a)), 2.62 (2H, m, CH$_2$-S(e)), 1.89 (2H, m, CH$_2$-C(a)), 1.76 (2H, m, CH$_2$-C(e)). ^{13}C NMR (DMSO-d_6, 125 MHz): δ_C 176.4 (C=O – 4'), 156.2

(C=O − 2'), 60.2 (C-5'), 34.7 (N-CH$_3$), 24.6 (CH$_2$-S), 23.1 (CH$_2$-C). Mas: M$^+$=200. (Scheme 1). Anal. calc. for C$_8$H$_{12}$N$_2$O$_2$S (200): C, 48.00; H, 6.00; N, 14.00. Found: C, 48.49; H, 5.54; N, 14.28.

***cis*-Bis(3-methyl-8-thia-1,3-diaza-spiro[4.5]decane-2,4-dione)-dichlorido palladium(II) − *cis*-[PdL$_2$Cl$_2$] (3).** An aqueous ethanol solution of (**2**) was mixed with a water solution of K$_2$PdCl$_4$. The obtained solutions were stirred with a magnetic stirrer under dark conditions for 3-4 hours. The yellow crystals were filtered off and dried under КОH and P$_2$O$_5$. The new complex of Pd(II) with formula *cis*-[PdL$_2$Cl$_2$] was dissolved in ethanol and DMSO. The purity was checked by thin-layer chromatography with the eluent CH$_3$COOC$_2$H$_5$/C$_2$H$_5$OH - 2:1, and elemental analyses. Yellow solid (0.0761 g, 56%). Mp 93°C (dec.). IR (solid, ATR, cm^{-1}, ν$_{max,}$ cm^{-1}): 3257, 1763, 1704, 597. ^{1}H-NMR (500 MHz, DMSO-*d$_6$*): δ$_H$ 8.75 (1H, s, NH); 2.86 (2H, m, CH$_2$-S(a)); 2.81 (3H, s, N-CH$_3$); 2.75 (2H, m, CH$_2$-S(e)); 2.04 (2H, m, CH$_2$-C(a)); 2.00 (2H, m, CH$_2$-C(e)). ^{13}C-NMR (DMSO-*d$_6$*, 125 MHz): δ$_C$ 176.4 (C=O − 4'), 156.2 (C=O − 2'), 60.2 (C-5'), 34.7 (N-CH$_3$), 24.6 (CH$_2$-S), 23.1 (CH$_2$-C). Anal. calc. for C$_{16}$H$_{24}$N$_4$O$_4$S$_2$Cl$_2$Pd (577.3): C, 33.26; N, 9.70; H, 4.16. Found: C, 33.69; N, 9.87; H, 4.02.

***cis*-Bis(3-methyl-8-thia-1,3-diaza-spiro[4.5]decane-2,4-dione)-tetrachlorido palladium(IV) − *cis*-[PdL$_2$Cl$_4$] (4).** An aqueous ethanol solution of (**2**) was added to a water solution of K$_2$PdCl$_6$. The obtained mixtures were stirred under dark conditions for 3-4 hours. The yellow crystals were filtered off and dried under and P$_2$O$_5$. The new complexes of Pd(IV) with formula *cis*-[PdL$_2$Cl$_4$] are soluble in ethanol and DMSO. The purity was checked by thin-layer chromatography with the eluent CH$_3$COOC$_2$H$_5$/C$_2$H$_5$OH - 2:1, and elemental analyses. Yellow-brown solid (0.0988 g,61%). mp. 285°C (dec.); IR (solid, ATR, cm^{-1}, ν$_{max,}$ cm^{-1}): 3260, 1763, 1706, 584. ^{1}H-NMR (500 MHz, DMSO-*d$_6$*): δ$_H$ 8.75 (1H, s, NH-1); 2.96 (2H, m, CH$_2$-S(a)); 2.80 (3H, s, N-CH$_3$); 2.79 (2H, m, CH$_2$-S(e)); 2.02 (2H, m, CH$_2$-C(a)); 1.90 (2H, m, CH$_2$-C(e)). ^{13}C-NMR (DMSO-*d$_6$*, 125 MHz): δ$_C$ 176.4 (C=O − 4'), 156.2 (C=O − 2'), 60.2 (C-5'), 34.7 (N-CH$_3$), 24.6 (CH$_2$-S), 23.1 (CH$_2$-C). Anal. calc. for C$_{16}$H$_{24}$N$_4$O$_4$S$_2$Cl$_4$Pd (648.2): C, 29.62; N, 8.64; H, 3.70. Found: C, 29.93; N, 8.90; H, 3.51.

Computational details. All theoretical calculations were performed using the Gaussian 03 package of programs.[14] Optimization of the structures of the ligand and its complexes was carried out by hybrid DFT calculations, employing the B3LYP (Becke's three-parameter non-local exchange)[15,16] correlation functional and 6-311++G(g,p) basis set for all non-metal atoms, and LANL2DZ basis set for the palladium center.

Pharmacological screening. The present study describes a preliminary screening of the cytotoxic effects of a new organic compound, 3-Methyl-8-thia-1,3-diaza-spiro[4.5]decane-2,4-dione, used as a ligand, in comparison to two new ligand-palladium complexes and the reference cytotoxic agent, cisplatin, on two human-tumor cell lines, K-562 and REH, using the standard MTT-dye-reduction assay for cell viability.

Cell culture conditions. The cell lines used for the experiments were: K-562 (Chronic myeloid leukemia, derived from a 53-year-old woman with chronic myeloid leukemia, (CML) from a blast crisis in 1970) and REH (acute lymphoblastic leukemia, established from the peripheral blood of a 15-year-old North African girl with acute lymphoblastic leukemia in 1973). The cell lines were obtained from the DSMZ German Collection of Microorganisms and Cell Cultures and were well validated in our laboratory as a proper test system for metal complexes. Their DSMZ catalogue numbers are as follow: K-562 (ACC 10) and REH (ACC 22).

Cytotoxicity assessment. The cytotoxicity of the tested compounds was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye-reduction assay as described by Mossman[17] with some modifications.[18] Exponentially-growing cells were seeded in 96-well microplates (100 μL/well at a density of 3.5 x 10^5 cells/mL for the adherent, and 1 x 10^5 cells/mL for the suspension cell lines), and allowed to grow for 24 h prior to exposure to the studied compounds. Stock solutions of the organic compound and its palladium complexes were freshly dissolved in DMSO, and then promptly diluted in RMPI-1640 growth medium immediately before treatment of the cells. Our prior experience with water-insoluble metal complexes, including cisplatin, has indicated that the dose-response curves following dissolution in water or

stock solution in DMSO (which is then promptly diluted in aqueous phase) overlap, and there is no significant modulation of the individual cell lines chemosensitivity. At the final dilutions, the solvent concentration never exceeded 0.5%. Cells were exposed to the tested agents for 72 h. A set of 8 separate wells were used for each concentration. Every test was run in triplicate, i.e., in three separate microplates. After incubation with the tested compounds, MTT-solution (10 mg/mL in PBS) aliquots were added to each well. The plates were further incubated for 4 h at 37 $^{\circ}$C and the formazan crystals formed were dissolved by adding 110 µL of 5% HCOOH in 2-propanol. Absorption of the samples was measured by an ELISA reader (UniscanTitertec) at 580 nm. Survival fraction was calculated as a percentage of the untreated control. The experimental data were processed using GraphPad Prizm software and fitted to a sigmoidal concentration/response. The IC$_{50}$ values were calculated using non-linear regression analysis.

References

1. Ndagi, U.; Mhlongo, N.; Soliman, M.E. *Drug Design, Development and Therapy* **2017**, *11*, 599—616. https://doi.org/10.2147/DDDT.S119488
2. Florea, A-M.; Büsselberg, D. *Cancers (Basel)*. **2011**, *3*(1), 1351–1371. https://doi.org/10.3390/cancers3011351
3. Gao, E.; Liu, C.; Zhu, M.; Lin, H.; Wu, Q.; Liu, L. *Anti-Cancer Agents in Medicinal Chemistry* **2009**, *9*(3), 356-368, https://doi.org/10.2174/187152061090903356.
4. Page S. *Educ Chem*. **2012**, 10, 26–29.
5. Montaña, A.M.; Batalla, C. *Curr. Med. Chem*. **2009**, *16*, 2235-2260. https://doi.org/10.2174/092986709788453087
6. Fanelli, M.; Formica, M.; Fusi, V.; Giorgi, L.; Micheloni, M.; Paoli, P. *Coord. Chem. Rev.* **2016**, *310*, 41-79. https://doi.org/10.1016/j.ccr.2015.11.004
7. Pranczk, J.; Jacewicz, D.; Wyrzykowski, D.; Chmurzyński, L. *Current Pharmaceutical Analysis* **2014**, *10*, 2-9 https://doi.org/10.2174/1573412910011140102103324
8. Jia, P.; Ouyang, R.; Cao, P.; Tong, X.; Zhou, X.; Lei, T.; Zhao, Y; Guo, N.; Chang, H.; Miao, Y.; Zhou, S. *Journal of Coordination Chemistry* **2017**, 2175-2201. http://doi.org/10.1080/00958972.2017.1349313.
9. Ahmad, S.; Nadeem, Sh.; Anwar, A.; Hameed, A.; Tirmizi, S. A.; Zierkiewicz, W.; Abbas, A. A.; Isab, A.; Alotaibi, M. A. *J. Mol. Str*. **2017**, *1141*, 204-212. https://doi.org/10.1016/j.molstruc.2017.03.098
10. Mukherjee, S.; Reddy B., V.; Mitra, I.; Moi, S. Ch. *Polyhedron* **2017**, *124*, 251-261. https://doi.org/10.1016/j.poly.2016.12.046
11. Cherneva, E.; Buyukliev, R.; Burdzhiev, N.; Michailova, R.; Bakalova, A. *Bul. Chem. Comm*. **2017**, *49*, 164-170
12. Bakalova, A.; Buyukliev, R.; Momekov, G. *J. Mol. Struc*. **2015**, *1091*, 118–124. http://dx.doi.org/10.1016/j.molstruc.2015.02.055
13. Bakalova, A.; Nikolova-Mladenova, B.; Buyukliev, R.; Cherneva, E., Momekov, G., Ivanov, D. *Chem. Papers* **2016**, *70*(1), 93-100. https://doi.org/10.1515/chempap-2015-0194.
14. Gaussian 03, Revision C.02, Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Montgomery, Jr., J. A.; Vreven, T.; Kudin, K. N.; Burant, J.C.; Millam, J. M.; Iyengar, S. S.;

Tomasi, J.; Barone, V.; Mennucci, B.; Cossi, M.; Scalmani, G.; Rega, N.; Petersson, G. A.; Nakatsuji, H.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Klene, M.; Li, X.; Knox, J. E.; Hratchian, H. P.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Ayala, P. Y.; Morokuma, K.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Zakrzewski, V. G.; Dapprich, S.; Daniels, A. D.; Strain, M. C.; Farkas, O.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Ortiz, J. V.; Cui, Q.; Baboul, A. G.; Clifford, S.; Cioslowski, J.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Gonzalez, C.; and Pople, J. A.; Gaussian, Inc., Wallingford CT, 2004.

15. Stephens, P. J.; Devlin, F. J.; Chabalowski, C. F.; Frisch, M. J. *J. Phys. Chem*. **1994**, *98*, 11623-11627. https://doi.org/10.1021/j100096a001

16. Lee, C. T.; Yang, W. T.; Parr, R. G. *Phys. Rev. B* **1988**, *37*, 785-789. http://dx.doi.org/10.1103/PhysRevB.37.785.

17. Mosmann, T. *J. Immunol. Methods.* **1983**, *65*, 55-63. https://doi.org/10.1016/0022-1759(83)90303-4

18. Konstantinov, S.; Eibl, H.; Berger, M. Br. *J. Haematol.* **1999**, *107*, 365-374. https://doi.org/10.1046/j.1365-2141.1999.01700.x