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New Copper (II), Palladium (II), and Platinum (II) 2-Acetylpyrazine Tert-Butylthiosemicarbazone Complexes: Inhibition of Human Topoisomerase IIα and Activity against Breast Cancer Cells

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Abstract

The new ligand, 2-acetylpyrazine-tertbutylthiosemicarbazone (APZ-tBTSC), and its Cu(II), Pd(II) and Pt(II) complexes have been synthesized. This ligand coordinates to the metal ions in a tridentate monoanionic fashion forming monometallic complexes with the formula [M(APZ-tBTSC)Cl]. The ligand and the three metal complexes [Cu(APZ-tBTSC)Cl], [Pd(APZ-tBTSC)Cl], and [Pt(APZ-tBTSC)Cl] were tested for anti-proliferative biological behavior with a panel of seven microbes, and the copper and palladium complexes were found to be highly active against Gram positive bacteria. The 4 compounds were also tested in human topoisomerase IIα DNA relaxation assays and all three metal complexes had topoisomerase inhibition at a concentration between 4 - 6 micro-molar. The 4 compounds were also tested for activity with the HEK293T cell line and also the breast cell cancer line, MDA-MB-231. The most effective compound for activity against the HEK293T cell line was the [Cu(APZ-tBTSC)Cl] complex, and the MDA-MB-231 breast cancer cell line was the [Pt(APZ-tBTSC)Cl] complex.

Keywords

Topoisomerase, Breast Cancer, α-(N)-Heterocyclic Thiosemicarbazones

1. Introduction

Chemical compounds called thiosemicarbazones are becoming a very sought-after

biological inhibitor for clinical uses, by themselves or when combined with transition metals [1] [2] [3] [4]. Certain thiosemicarbazones have even completed clinical tails. A specific subset of thiosemicarbazones known as α-(N)-heterocyclic thiosemicarbazones (3-AP; Triapine) have shown remarkable inhibitory activity against ribonucleotide reductase [5] [6] [7]. For example, Triapine has been extensively assessed in more than 20 phase I and II clinical trials primarily focusing on its anti-tumor activity. Accounts in the literature show that α-(N)-heterocyclic thiosemicarbazone molecules, with a variety of backbone substrates and with their metal complexes, produce potent anti-cancer agents [8] [9] [10]. In order for many of these transition metal complexes to be effective, and to inhibit the catalytic activity of human topoisomerase IIα, the ligand must act as a (N-N-S) monoanionic tridentate ligand for the transition metal center, and the metal center must adopt a square planar geometry [11]-[18].

These ligands coordinate transition metals such as Pd(II), Pt(II) ions in this square planar geometry. Also and most importantly, Cu(II) ions exhibit this same square planar geometry and the complexes of the formula [Cu(α-(N)-heterocyclic thiosemicarbazone)Cl] are the among the strongest inhibitors of Topo [14]-[18]. The mechanism of this inhibition is still being elucidated. The Cu(II) ion rarely exhibits the square planar geometry unless a ligand that is already planar itself facilitates the geometry. Additionally, the Cu(II) ion, even when it is square planar and 4-coordinate, can easily add ligands and become 5-coordinate or 6-coordinate. That property is not shared by Pd(II) or Pt(II), which prefers to stay 4-coordinate.

Our study focuses on comparing metal complexes of an α-(N)-heterocyclic thiosemicarbazone, specifically acetylpyrazine tert-butylthiosemicarbazone (APZ-tBTSC), with a consistent formula of [Cu(APZ-tBTSC)Cl], [Pd(APZ-tBTSC)Cl] and [Pt(APZ-tBTSC)Cl], as illustrated in **Figure 1**, so that the similarities and differences of the compounds can be compared directly.

The given capability to inhibit human topoisomerase IIα has the potential to prevent adverse effects often exhibited by TopoII-targeting agents, and the study of underlying differing inhibition mechanisms for compounds of systematically varying structures is of great importance to prevent these adverse effects. This particular research article attempts to explain the underlying links between a series of metal complexes and their microbial inhibitory responses, inhibition of topo IIα and their inhibition of HEK 293 T cells, and breast cancer cells. The structures of the 2-acetylpyrazine tert-butylthiosemicarbazone ligand and its metal complexes are depicted in **Figure 1**, shown below.

2. Experimental

2.1. Material and Methods

All reagents and solvents used to synthesize the ligands and the metal complexes were purchased from Sigma-Aldrich or Alfa-Aesar and used without further preparation unless otherwise noted. Recombinant human Topoisomerase IIα

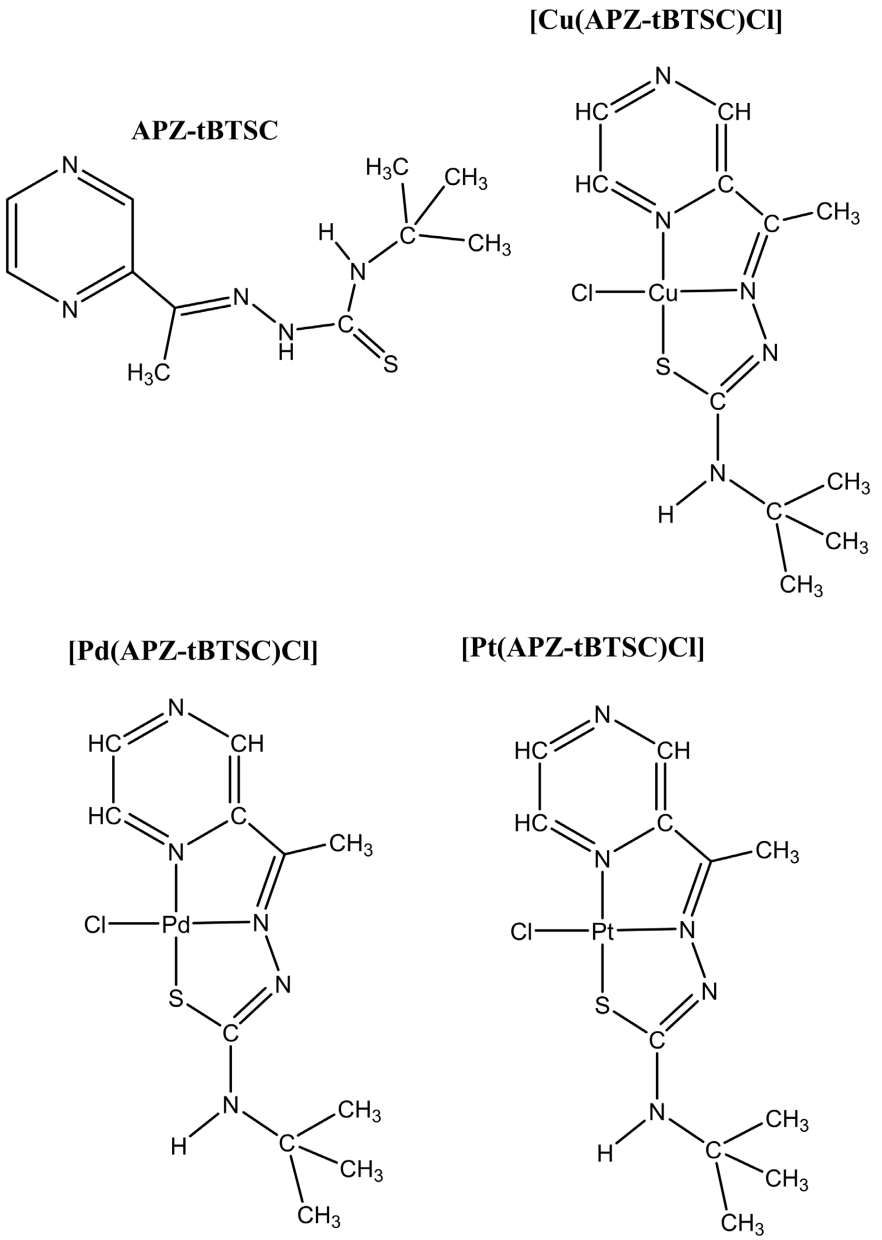

Figure 1. The structures of the 2-Acetylpyrazine tert-butylthiosemicarbazone ligand and its metal complexes.

was overexpressed and purified from yeast *Saccharomyces cervisiae* as described [17]. The enzyme was stored in the buffer with 50 mM Tris (pH 7.8), 750 mM KCl, 40% Glycerol and 0.5 mM DTT (dithiothreitol) as 1 mg/mL stock in liquid nitrogen. Recombinant pBR322 plasmid was amplified and purified following the protocol of Qiagen™ Plasmid Mega Kit.

2.2. Instrumentation

The melting points were taken with a Stanford Research Systems Digimelt MPA160, and TLCs were taken with Whatman 250 μm layer PE SIL G/UV polyester-backed plates. The thiosemicarbazone ligands were characterized by ^{1}H,

^{13}C DEPTQ-135, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, ^{1}H-^{15}N HSQC, ^{1}H-^{13}C HMBC, and ^{1}H-^{15}N HMBC NMR techniques as well as mass spec. NMR spectroscopy was carried out at the Center for Structural Chemistry, Tennessee Technological University (USA). The spectra reported here were measured with a Bruker Avance III HD 500 spectrometer at 500.13 MHz (^{1}H), 50.69 MHz (^{15}N) and 125.03 MHz (^{13}C) at 25˚C equipped with a PRODIGY cryoprobe. For these measurements, the substances were dissolved in the appropriate deuterated solvent, and the chemical shifts were referenced to the solvent residual peak. Coupling constants (J) are given in Hertz. The ^{1}H NMR experiments were acquired using Bruker's standard PROTON (zg30) NMR pulse sequence with the following parameters: Relaxation delay, 1 s; 90˚ pulse, 12.0 μs; spectral width, 10,000 Hz; number of data points, 32 K; and digital resolution, 0.153 Hz/point. The UV-Vis data was gathered using a Cary Varian 3E UV-Vis Spectrophotometer. Each sample was diluted to 10 ppm and scanned over a range of 200 - 800 nm. All mass spectrometry data was taken with a Varian 300/310/320-MS LC/MS Quadrupole Mass Spectrometer in negative mode using APCI. The corona current was set to −5.00 μA, while the shield potential was set to −600.00 volts. The housing, drying gas, and vaporizer gas temperatures were set to 50˚C, 150˚C, and 350˚C respectively. The drying, nebulizing, and vaporizer gas pressures were each set to 12.0 psi, 55.0 psi, and 17.0 psi. For the mass spectrometry data, each sample was dissolved in minimal amounts of dimethyl sulfoxide and then diluted to 10 ppm in methanol.

2.3. Synthesis and Characterization

2.3.1. Synthesis of the APZ Ligand

APZ-tBTSC: *N-tertbutyl-2-[1-(2-pyrazinyl)ethylidene]-hydrazinecarbothioamide*

The ligand, APZ-tBTSC, was synthesized by combining 0.508 g (4.20×10^{-3} mol) of acetylpyrazine and 0.612 g (4.20×10^{-3} mol) of 4-tert-butyl-3-thiosemicarbazide with 20 mL of isopropanol solvent in a small Erlenmeyer flask equipped with a magnetic stir bar. One drop of concentrated H_2SO_4 was added as a catalyst. The mixture was covered, stirred, and heated at 60˚C for 24 hours. The white precipitate was filtered, washed with 5 mL of isopropanol, and dried. Yield: 0.4035 g (1.60×10^{-3} mol), 38.2%. MP: 147.8˚C - 149.7˚C. Theoretical MS *m/z* (relative intensity) 100% = 250.11. Actual m/z (relative intensity) 100% = 250.01.

^{1}H NMR (500 MHz, DMSO-*d*6) δ 10.37 (s, 1H), 9.29 (d, J = 1.5 Hz, 1H), 8.66 - 8.60 (m, 2H), 7.90 (s, 1H), 2.38 (s, 3H), 1.56 (s, 9H). ^{13}C NMR (126 MHz, DMSO) δ 176.34, 150.15, 145.34, 144.00, 143.28, 142.34, 52.99, 39.52, 28.35, 12.17.

2.3.2. Synthesis of the Copper Complex

[Cu(APZ-tBTSC)Cl]: *chloro[N-tert-butyl-2-[1-(2-pyrazinyl-κN^{1})ethylidene] hydrazinecarbothioamidato-κN^{2},κS]-copper(II)*

The copper complex was synthesized by combining 0.2570 g (1.00×10^{-3} mol) of APZ-tBTSC and 0.1757 g (1.00×10^{-3} mol) of copper (II) chloride dihydrate

with 40 mL of ethyl alcohol solvent into a 50 mL Erlenmeyer flask. The mixture was equipped with a stir bar, covered, and heated to 60˚C for 24 hours and stirred for 48 hours. The precipitate was filtered, washed with 5 mL of ethyl alcohol solvent, and dried. The precipitate was a dark green microcrystalline powder. Yield: 0.3467 g (9.92×10^{-4} mol), 97.06%. Theoretical MS *m/z* (relative intensity) 100% = 348.01. Actual m/z (relative intensity) 100% = 347.89.

2.3.3. Synthesis of the Palladium Complex

[Pd(APZ-tBTSC)Cl]: *chloro[N-tert-butyl-2-[1-(2-pyrazinyl-κN^1)ethylidene] hydrazinecarbothioamidato-κN^2,κS]-palladium(II)*

The palladium complex was synthesized by combining 0.1011 g (2.6×10^{-4} mol) of dichlorobis (benzonitrile) palladium (II) and 0.0669 g (2.7×10^{-4} mol) of the ligand, APZ-tBTSC with 15 mL of acetone solvent into a 25 mL Erlenmeyer flask accompanied by a small stir bar. The mixture was covered and stirred for 48 hours with no heat. The red precipitate was filtered, washed with 5 mL of acetone solvent, and dried. Yield: 0.0815 g (2.1×10^{-4} mol), 79.92%. Theoretical MS *m/z* (relative intensity) 100% = 390.98. Actual m/z (relative intensity) 100% = 390.79.

^{1}H NMR (500 MHz, DMSO-*d6*) δ 9.07 (d, J = 1.2 Hz, 1H), 8.86 (d, J = 2.8 Hz, 1H), 8.49 (dd, J = 2.8, 1.2 Hz, 1H), 8.00 (s, 1H), 2.41 (s, 3H), 1.34 (s, 9H). ^{13}C NMR (126 MHz, DMSO) δ 175.46, 153.79, 152.98, 147.59, 145.44, 140.55, 54.54, 39.52, 28.21, 13.06.

2.3.4. Synthesis of the Platinum Complex

[Pt(APZ-tBTSC)Cl]: *chloro[N-tert-butyl-2-[1-(2-pyrazinyl-κN^1)ethylidene] hydrazinecarbothioamidato-κN^2,κS]-platinum(II)*

The platinum complex was synthesized by combining 0.151 g (0.32×10^{-4} mol) of dichlorobis (benzonitrile) platinum (II), 99% and 0.080 g (0.32×10^{-4} mol) of APZ-tBTSC. Each reactant was individually dissolved in 10 mL of ethanol solvent. The reactants were combined by pipetting each into one 50 mL flask. The mixture was equipped with a stir bar, condenser, and submerged in an oil bath at approximately 75˚C for 24 hours. After the first few seconds of stirring a color change occurred from light to dark red. The red precipitate was filtered, washed with 10 mL of ethanol solvent, and dried. Yield: 0.1388 g (2.9×10^{-4} mol), 90.69%. Theoretical MS *m/z* (relative intensity) 100% = 480.05. Actual m/z (relative intensity) 100% = 479.88.

^{1}H NMR (500 MHz, DMSO-*d6*) δ 8.97 (d, J = 1.2 Hz, 1H), 8.86 (d, J = 3.0 Hz, 1H), 8.75 (dd, J = 3.0, 1.2 Hz, 1H), 8.11 (s, 1H), 2.54 (s, 0H), 2.37 (s, 3H), 1.35 (s, 9H). 13C NMR (126 MHz, DMSO) δ 177.82, 154.29, 153.78, 149.08, 146.02, 138.52, 54.76, 39.52, 28.45, 13.12.

2.4. Microbial Studies

Concentrations that inhibit 90% of bacterial growth (IC90) were determined as described in the literature [19]. The microbial minimum inhibitory concentra-

tion studies were performed by dissolving 200 mg of the thiosemicarbazone ligand in 10 mL of DMSO, and 100 mg of the metal compounds in 10 mL of DMSO and performing serial dilutions. All microbes used were grown in petri dishes in the biology department of Tennessee Technological University.

TopoII*α* Mediated Relaxation Assay

The assay has been described previously [17]. A 20-ul reaction is set up with 0.2 μg of TopoII*α*, 0.3 μg of DNA pBR322, 2 mM ATP and DMSO (ND) or different concentrations (1 - 10 μM) of thiosemicarbazone compounds. The reactions were incubated at 37˚C for 30 min and terminated by addition of 3 μL of stop solution (77.5 mM EDTA, 0.77% SDS). Then proteinase K was added to the reaction for incubation at 45˚C for 30 min. The products were subject to electrophoresis in 1% agarose gel in 1× TBE buffer. The results were imaged with BioRad Gel Doc XR+ imaging system.

2.5. Methods for Alamar Blue Viability Assay of Breast Cancer Cells Exposed to Topoisomerase II Inhibitors

2.5.1. Cell Culture and Plating

Cell lines used were MDA-MB-231 (cancerous, epithelial-type human breast adenocarcinoma cells), and MCF-7 (cancerous, epithelial-type human breast adenocarcinoma cells). Adherent cells were cultured in 25 cm^2 or 75 cm^2 tissue culture flasks within a humidified incubator at 37˚C and 5% CO_2. DMEM containing L-glutamine (Lonza) supplemented with penicillin/streptomycin and 10% FBS was used as the complete culture medium. Confluent cells were trypsinized, cell clumps dissociated by gentle pipetting, and split using a 1:10 ratio into fresh flasks containing pre-warmed complete media. Cell counts were performed by staining 100 μL of cells with 100 μL of trypan blue (Hyclone) and then counted on a hemocytometer. With this number determined, cells were diluted in complete media to a concentration 25,000 cells/mL, and 200 μL/well of cell solutions was added to a 96-well plate using a multichannel pipette. Plated cells were placed in the humidified incubator at 37˚C and 5% CO_2 to incubate for 24 hr prior to the addition of the drug.

2.5.2. Viability Assay of the Ligands and Metal Complexes

After cells were cultured for 24 hr, the existing culture media was replaced with complete media containing dilutions of compounds APZ-tBTSC, [Cu(APZ-tBTSC)Cl], [Pd(APZ-tBTSC)Cl], and [Pt(APZ-tBTSC)Cl], ranging from 1000 μM down to 0.001 μM. The dilutions were generated using serial dilutions of prepared DMSO stock solutions of 10 mM in ACS-grade DMSO (Fischer) which were stored at −20˚C until use. Wells were also prepared with 200 μL of complete media only or 200 μL of complete media with 1:100 dilution of DMSO as positive controls. Both positive controls were found to be statistically equivalent showing no added toxicity of DMSO at the highest applied concentration of DMSO. The 96-well plate was then incubated (humidified, 37˚C, 5% CO_2) for 24 hr after application of the compounds. Alamar Blue (Thermo

Scientific) was then added to each well using complete media as a carrier to attain 8% Alamar blue per well and applied using a multichannel pipette. The plate was then placed back into the incubator and allowed to incubate for approximately 5 hr, after which fluorescence measurements were made at 560 nm excitation and 590 nm emission using the Tecan infinite M200 Pro reader.

2.5.3. Data Analysis

Initial analysis of fluorescence data was performed with Microsoft Excel 2013 normalized by the fluorescence signal from positive controls. Statistical analysis, data fitting, and plot generation was performed with GraphPad Prism 6; normalized data were fit using log(inhibitor) vs. response with variable slope (four parameter) model. This model calculated EC50 and Hill slope values for each curve.

3. Results and Discussion

3.1. Synthesis of Ligand and Metal Complexes

The synthesis of the APZ-tBTSC ligand proceeds in good yield to produce a clean, crystalline off-white solid. This ligand, along with its metal complexes, has some solubility in polar organic solvents such as methanol, ethanol, and isopropanol; but they are not appreciably soluble in water.

The thiosemicarbazone hydrazinic N-H hydrogen H(1) appears at 10.37 ppm for the APZ-tBTSC ligand, which is lost in coordination with the metals, as evidenced in the loss of this proton resonance in the ^{1}H NMR spectra of [Pd(APZ-tBTSC)Cl] and [Pd(APZ-tBTSC)Cl] as seen in **Figure 2**. The [Cu(APZ-tBTSC)Cl] complex is paramagnetic (Cu^{2+} is d^{9}) so the ^{1}H NMR spectrum of this complex was unattainable.

3.2. The Microbial Inhibition Studies

The compounds [1] [2] [3] [4] were tested for activity with a series of microbes that were gram-positive (Bacillus subtilus and Staphylococcus aureus) or gram-negative (Pseudomonas aeruginosa and Escherichia coli). A minimum inhibitory concentration was determined for each at which microbial growth was terminated the results of which are shown below in **Table 1**.

The ligand [1] is similar to triapine in structure, is able to cross the cell membrane of the gram-positive bacteria and inhibits cell proliferation down to 0.97 ug/ml. The Cu and Pd complexes [2] and [3] were shown to have complete inhibition of all gram-positive microbes down to 0.48 ug/ml which was the lowest concentration studied. The Pt complex [4] was shown to have little/no inhibition toward the microbes.

3.3. The Cu(II) Complexes Inhibit TopoIIα-Mediated DNA Plasmid Relaxation

Human Topoisomerase II is an enzyme that is capable of relaxing supercoiled DNA. During this study a control of just DNA was used to indicate relaxed

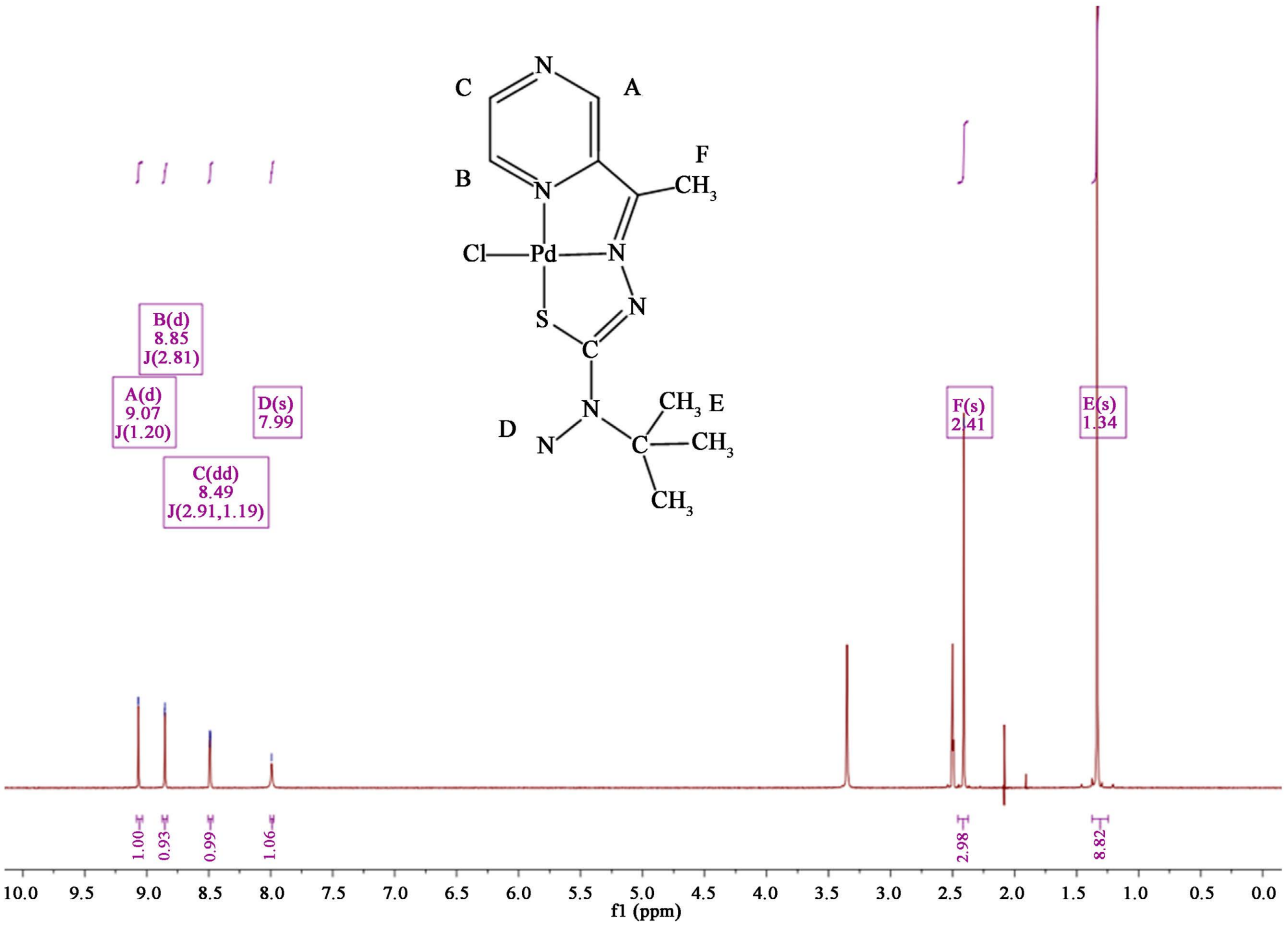

Figure 2. The ^{1}H NMR of [2] Pd(APZ-tBTSC)Cl] in d6-DMSO.

Table 1. Minimum Inhibitory Concentrations of Compounds [1] [2] [3] [4] in μg/mL.

COMPOUND	Gram Positive Bacteria		Gram Negative Bacteria	
	Bacillus subtilis	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Escherichia coli*
1) APZ-tBTSC	0.97	0.97	*NA*	*NA*
2) [Cu(APZ-tBTSC)Cl]	0.48	0.48	250	0.97
3) [Pd(APZ-tBTSC)Cl]	0.48	0.48	*NA*	125
4) [Pt(APZ-tBTSC)Cl]	*NA*	*NA*	*NA*	*NA*

DNA. Then topoisomerase and DNA were combined and used to indicate supercoiling of DNA (topo activity). The ligand and metal complexes were then tested with the topoisomerase and DNA and results were recorded. The ligand did not inhibit topoisomerase. However, all three metal complexes had topoisomerase inhibition at a concentration between 4 - 6 micro-molar.

As shown in **Figure 3**, concentrations of APZ-tBTSC, [Cu(APZ-tBTSC)Cl], Pd(APZ-tBTSC)Cl] and [Pt(APZ-tBTSC)Cl] were examined from 1 - 10 μM. The results show that [Cu(APZ-tBTSC)Cl], Pd(APZ-tBTSC)Cl], and [Pt(APZ-tBTSC)Cl]

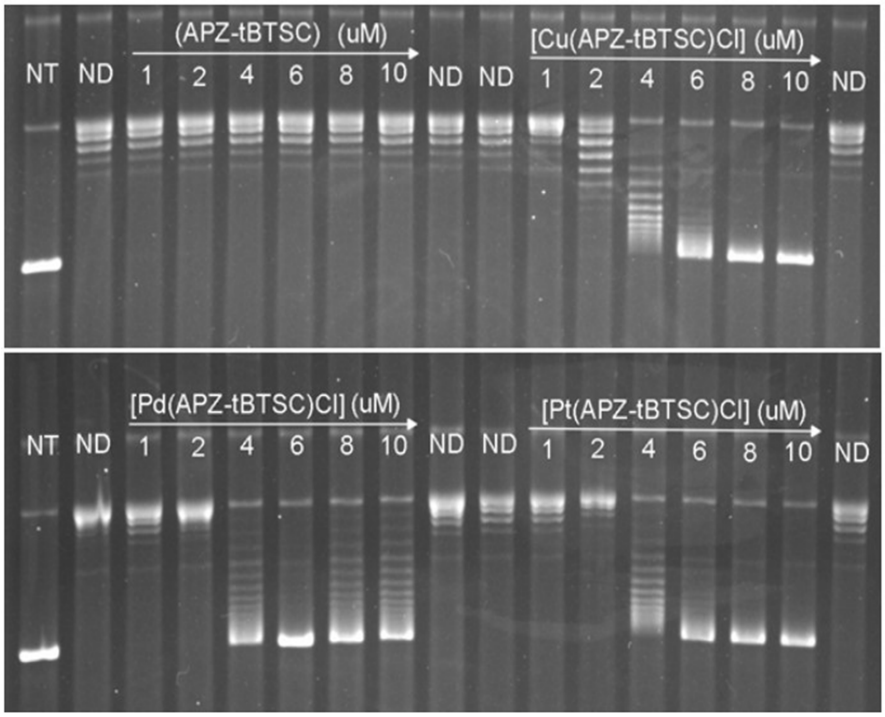

Figure 3. Dose-dependence inhibition of TopoII*α* by [Cu(APZ-tBTSC)Cl], Pd(APZ-tBTSC)Cl]. and [Pt(APZ-tBTSC)Cl].

all inhibit TopoII*α* at ~4 μM, while APZ-tBTSC does not inhibit. Our data suggest that [Cu(APZ-tBTSC)Cl], Pd(APZ-tBTSC)Cl] and [Pt(APZ-tBTSC)Cl] all exhibit excellent inhibitory activities on TopoII*α*.

3.4. *In Vitro* Viability Assays

The *in vitro* viability assay of compounds APZ-tBTSC, [Cu(APZ-tBTSC)Cl], Pd(APZ-tBTSC)Cl] and [Pt(APZ-tBTSC)Cl] were determined using cancerous, epithelial-type human breast adenocarcinoma cell line MDA-MB-231 and the human embryonic kidney cell line HEK 293T.

3.4.1. HEK293T Cells Assays

Cells were initially plated at a target density of 5000 cells/well in a 96-well plate. Inhibitors were added to the wells after 48 hours of incubation. Then, the cells were allowed to incubate another 24 hours with the inhibitors before assaying with Alamar Blue. Here, cell viability refers to the fraction of Alamar Blue signal relative to a positive control of healthy (no inhibitor) cells. Viability is proportional to the fluorescence signal from the conversion of Alamar Blue--greater signal means greater viability. As shown in **Figure 4** for one experiment of six of cellular viability using the four new compounds (each experiment had 4 replicates done for each concentration) the most effective compound was the [Cu(APZ-tBTSC)Cl] complex. The EC50 values for the HEK293T breast cancer cell line were 0.08 μM for (APZ-ETSC), 0.02 μM for [Cu(APZ-ETSC)Cl], 0.82 μM for [Pd(APZ-tBTSC)Cl], and 5.26 μM for [Pt(APZ-tBTSC)Cl].

3.4.2. Breast Cancer Cell Assays

The MDA-MB-231 cells were treated with ligands or copper complexes after a 48 hr recovery time. Then, the cells were allowed to incubate another 24 hr with the inhibitors before assaying with Alamar Blue. Concentration of inhibitors and ligands ranged from 0.001 μM to 1000 μM. All fluorescence data were collected after 4 hr of incubation with Alamar Blue. Each concentration was tested in replicates of 4. As shown in Figure 5 for one experiment of six of cellular viability using the four new compounds (each experiment had 4 replicates done for each concentration) the most effective compound was the [Pt(APZ-tBTSC)Cl] complex. The EC50 values for the MDA-MB-231 breast cancer cell lines were 21.43 μM for (APZ-ETSC), 13.82 μM for [Cu(APZ-ETSC)Cl], 8.05 μM for [Pd(APZ-tBTSC)Cl], and 1.05 μM for [Pt(APZ-tBTSC)Cl].

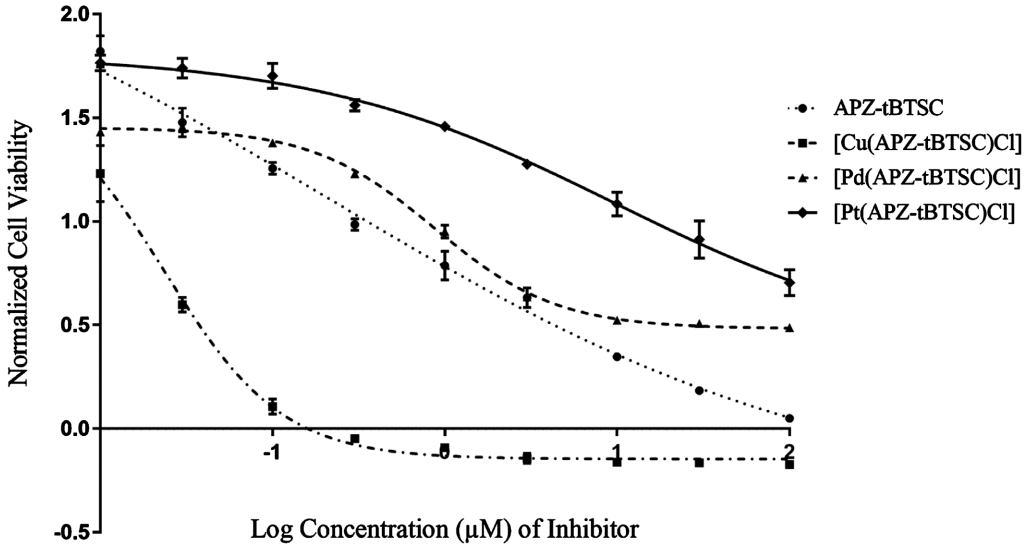

Figure 4. Viability curve for HEK293T cells with compounds APZ-tBTSC, [Cu(APZ-tBTSC)Cl] [Pd(APZ-tBTSC)Cl], and [Pt(APZ-tBTSC)Cl].

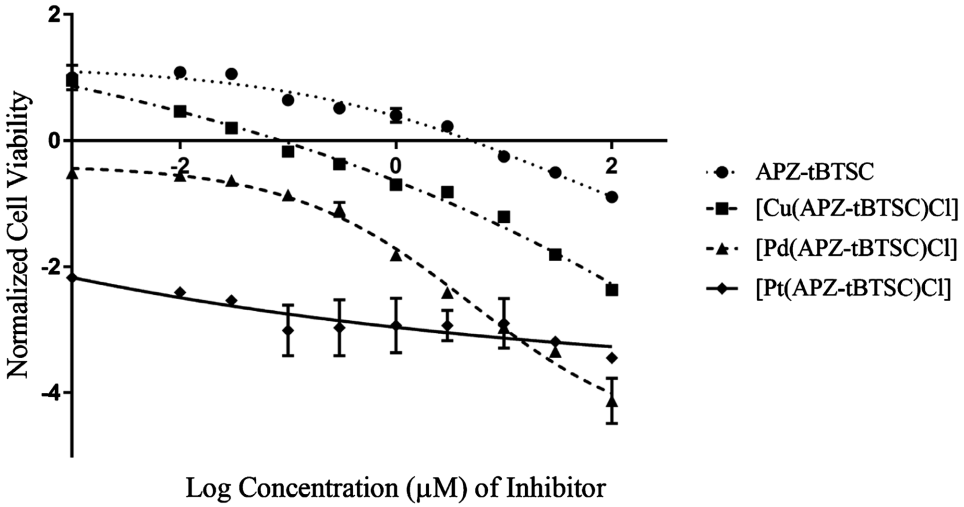

Figure 5. Breast Cancer Cell (MDA-MB-231) Viability Experiments with compounds APZ-tBTSC, [Cu(APZ-tBTSC)Cl] [Pd(APZ-tBTSC)Cl], and [Pt(APZ-tBTSC)Cl].

4. Conclusions

The APZ-tBTSC ligand and metal complexes in this experiment were synthesized by our traditional thiosemicarbazone synthesis and the ^{1}H NMR spectra, and mass spec. provides evidence that our proposed structures are correct. The palladium and platinum complexes are similar, and have no hydrazinic proton, confirming the tridentate monoanionic coordination mode.

Importantly, the copper, palladium, and platinum complexes of the ligand are far more effective at inhibition of the Topo enzyme than the ligands themselves, and also inhibit viability of the cancer cell line more than the ligand. The ligand itself will not inhibit Topo, however all of the metal complexes inhibit Topo at 4 - 6 uM.

Variation exists amongst the results of each biological inhibitory study completed. This gives rise to speculation that there are other factors contributing to the inhibitory properties of the ligand and its three metal complexes, besides Topo inhibition. For example, The Cu complex was most successful in the inhibition of the HEK 293 T cells however the Pt complex was least successful during this study. In contrast, the Pt complex had the highest inhibition towards the breast cancer cells and the Cu complex had the smallest amount of inhibition towards these malignant cells. In conclusion, we have to infer that more than one mechanism for the metal complexes must be involved in inhibition of growth besides Topo inhibition.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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