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Synthesis, Characterization, and Cytotoxicity of a Novel Gold(III) Complex with *O*,*O*′-Diethyl Ester of Ethylenediamine-*N*,*N*′-Di-2-(4-Methyl)Pentanoic Acid

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Abstract: A novel gold(III) complex, $[AuCl_2\{(S,S)\text{-}Et_2eddl\}]PF_6$, ((*S*,*S*)-$Et_2$eddl = *O*,*O*′-diethyl ester of ethylenediamine-*N*,*N*′-di-2-(4-methyl)pentanoic acid) was synthesized and characterized by IR, 1D (^{1}H and ^{13}C), and 2D (H,H-COSY and H,H-NOESY) NMR spectroscopy, mass spectrometry, and elemental analysis. Density functional theory calculations confirmed that (*R*,*R*)-*N*,*N*′ diastereoisomer was energetically the most stable isomer. In vitro antitumor action of ligand precursor $[(S,S)\text{-}H_2Et_2eddl]Cl_2$ and corresponding gold(III) complex was determined against tumor cell lines: human adenocarcinoma (HeLa), human colon carcinoma (LS174), human breast cancer (MCF7), non-small cell lung carcinoma cell line (A549), and non-cancerous cell line human embryonic lung fibroblast (MRC-5) using microculture tetrazolium test (MTT) assay. The results indicate that both ligand precursor and gold(III) complex have showed very good to moderate cytotoxic activity against all tested malignant cell lines. The highest activity was expressed by $[AuCl_2\{(S,S)\text{-}Et_2eddl\}]PF_6$ against the LS174 cells, with IC_{50} value of 7.4 ± 1.2 μM.

Keywords: gold(III) complex; R_2edda-type ligand; DFT; cytotoxicity

1. Introduction

Cancer is a disease in which a group of cells display uncontrolled growth, invasion, and sometimes metastasis [1]. Over the last fifty years, about 500,000 natural and synthetic chemical compounds have been tested for their anticancer activity, but only about 25 of these are in wide use today [2]. In particular, transition metal complexes offer potential advantages over the more common organic-based drugs.

Cisplatin has been proven through many years of successful implementation in the treatment of various types of cancers, such as ovarian, cervical, bladder, lung, head, and neck [3–10]. Despite the therapeutic success of cisplatin, severe toxicities, including oto-, nephro-, and neurotoxicity, its narrow spectrum of activity and low water solubility limit its clinical utility [11,12]. Therefore, great efforts have been made to develop new derivatives with improved pharmacological properties, and cisplatin has become the prototype of a unique class of antineoplastic agents [10].

Several families of non-platinum metal complexes have been studied extensively as potential cytotoxic and antitumor agents [13–16]. In the last 10–20 years, a number of gold(III) complexes that are highly cytotoxic towards cancer cells have been discovered [17,18]. Gold(III) complexes show chemical features that are very close to those of clinically employed platinum(II) complexes, such as the preference for square-planar geometry and d^8 electronic configuration [19,20]. However, in comparison with platinum(II) compounds, gold(III) analogues turned out to be relatively unstable and light-sensitive with high redox potential, making their use rather problematic under physiological conditions [21].

Gold(III) complexes differ significantly and are especially susceptible to reduction to gold(I) and colloidal gold [22]. Chemical strategies for imparting redox stability to gold(III) complexes typically involve the use of chelating and macrocyclic ligands containing strong neutral or anionic σ-donor atoms (C, N, and O to match the hard gold(III) ion). More recent studies have demonstrated that polydentate ligands (such as polyamines) enhance the stability of gold(III) complexes in biological environments, and despite the fact gold(III) complexes are often structural analogs of cisplatin, they are widely thought to impart tumor cell death via a different mechanism [23]. Up to now, very little is known concerning the molecular mechanisms underlying the pharmacological effects of gold(III)-based antitumor metallodrugs. Interest in the reactions of some biological *N*-donor nucleophiles with gold(III) complexes could be very important because there is evidence of direct interactions of gold(III) complexes with a different model of proteins [24]. Indeed, it has been shown that the therapeutic effect of gold therapies may originate within the mitochondria, with cell death possibly being initiated via the inhibition of the enzyme thioredoxin reductase [24].

Recently, our research group reported the synthesis, characterization, and in vitro biological evaluation of gold(III) complexes with *N*,*N*′-ethylenediamine bidentate ester ligands [19,20]. In this work, we report the synthesis, characterization, and cytotoxic activity of novel gold(III) complex, $[AuCl_2\{(S,S)\text{-}Et_2eddl\}]PF_6$, ((*S*,*S*)-$Et_2$eddl = *O*,*O*′-diethyl ester of ethylenediamine-*N*,*N*′-di-2-(4-methyl)pentanoic acid). To elucidate the features that determine the preferred configuration of (*S*,*S*)-Et_2eddl ligand coordinated to the gold(III) complex, density functional theory analyses was performed. This newly synthesized complex, together with already-reported ligand precursor [25], were tested against tumor cell lines: HeLa human adenocarcinoma, human colon carcinoma (LS174), human breast cancer (MCF7), non-small cell lung carcinoma cell line (A549), and MRC-5 normal human embryonic lung fibroblast cell line.

2. Experimental

2.1. Materials and Methods

$[(S,S)\text{-}H_2Et_2eddl]Cl_2$ was synthesized according to the method described in [25]. $Na[AuCl_4]$ was synthesized by the standard procedure [26]. Elemental analyses were performed on an Elemental Vario EL III microanalyzer. A Nicolet 6700 FT–IR spectrometer and ATR (attenuated total reflection) technique were used for recording mid-infrared spectra (400–4000 cm^{-1}). ^1H, ^{13}C, H,H-COSY and H,H-NOESY NMR spectra were recorded on a Bruker Avance III 500 spectrometer. High resolution mass spectrum of the complex was recorded with an Orbitrap LTQ XL instrument (Thermo Scientific, Bremen, Germany) in MeOH. Reagents and solvents were of commercial reagent grade quality and used without further purification.

2.2. Synthesis of Complex $[AuCl_2\{(S,S)\text{-}Et_2eddl\}]PF_6$

First, 0.126 mmol (0.053 g) of $[(S,S)\text{-}H_2Et_2eddl]Cl_2$ was suspended in a minimum amount of MeOH (3 mL), and $LiOH{\cdot}H_2O$ (0.011 g, 0.252 mmol) was added. After 1 h of stirring, deprotonated ligand dissolved completely. Then, 4 mL of $Na[AuCl_4]{\cdot}2H_2O$ (0.050 g, 0.126 mmol) solution in MeOH was introduced in the flask, followed by addition of solid NH_4PF_6 (0.062 g, 0.378 mmol). Reaction was performed in the dark at room temperature. The solution was evaporated under vacuum and the

yellow product was washed with an excess of water. After filtration, the complex was recrystallized in MeOH and air-dried.

Yield 57 mg, 59%. Anal. Calcd. for $C_{18}H_{36}N_2O_4AuCl_2PF_6$: C, 28.55; H, 4.79; N, 3.70%. Found: C, 28.35; H, 4.88; N, 3.65%. ^{1}H NMR (500 MHz, $CDCl_3$): **Isomer A:** 1.00 (m, $C^{6,7}H_3$, 6H), 1.34 (m, CH_3CH_2–OOC–, 6H), 1.75 (m, C^5H, 2H), 1.87 (m, C^4H_2, 4H), 3.61 (m, C^1H_2, 4H), 4.21 (m, C^2H, 2H), 4.34 (m, CH_3CH_2–OOC–, 4H), 5.21 (s, N*H*, 2H). **Isomer B:** 1.00 (m, $C^{6,7}H_3$, 6H), 1.34 (m, CH_3CH_2–OOC–, 6H), 1.87 (m, C^5H, 2H), 1.87(m, C^4H_2, 4H), 4.02 (m, C^1H_2, 4H), 4.34 (m, CH_3CH_2–OOC–, 4H), 4.56 (m, C^2H, 2H), 5.15 (s, N*H*, 2H). ^{13}C NMR (125 MHz, $CDCl_3$): **Isomer A:** 14.0 (CH_3CH_2–OOC–), 22.1 ($C^{6,7}$), 24.9 (C^5), 39.0 (C^4), 47.9 (C^1), 59.8 (C^2), 63.1 (CH_3CH_2–OOC–), 169.9 (C^3); **Isomer B:** 14.0 (CH_3CH_2–OOC–), 22.4 ($C^{6,7}$), 23.6 (C^5), 38.3 (C^4), 44.1 (C^1), 59.8 (C^2), 63.5 (CH_3CH_2–OOC–), 170.7 (C^3). Isomer ratio: 3/1 (A/B). IR (ATR, cm^{-1}): ν_{max} = 2976, 2875, 1736, 1471, 1241, 1213, 863, 489. HR ESI–MS (CH_3OH), m/z: 611.1688 $[M]^+$.

2.3. Computational Details

Gaussian 09 package was used to perform geometry optimizations [27]. B3LYP functional [28] was used for structure optimizations, and the Stuttgart/Dresden (SDD) basis set was employed for all atoms in the calculations [29,30]. Optimizations of all systems were done without symmetry restrictions. Resulting geometries were characterized as equilibrium structures by analysis of force constants of normal vibrations.

2.4. Biological Studies

2.4.1. Preparation of Drug Solutions

The solutions of the investigated gold(III) complexes were prepared in DMSO (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 1 mM, and diluted by nutrient medium to various working concentrations. Nutrient medium was RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Biochrom AG, Berlin, Germany) and penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA).

2.4.2. Cell Lines

Cervix adenocarcinoma cell line (HeLa), human colon carcinoma (LS174), human breast cancer (MCF7), non-small cell lung carcinoma cell line (A549), and a non-cancerous cell line human embryonic lung fibroblast (MRC-5) were grown in RPMI-1640 medium (Sigma). Media were supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1% penicillin–streptomycin (Sigma).

2.4.3. Determination of Cell Survival

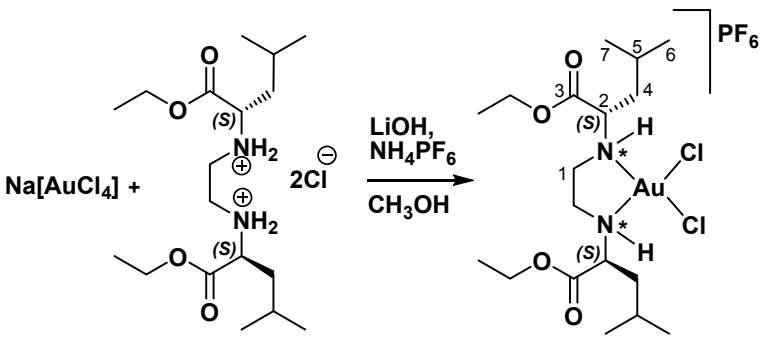
Target cells HeLa (2000 cells/well), LS174 (7000 cells/well), MCF7 cells (3000 cells/well), A549 (5000 cells/well), and non-cancerous MRC-5 (5000 cells/well) were seeded into the wells of a 96-well flat-bottomed microtitre plate. Twenty-four hours later, after the cell adhesion, different concentrations of investigated compounds were added to the wells, except for the controls, where only the complete medium was added. The final concentration range used in the experiments was 1–200 μM (12.50, 25, 50, 100, and 200 μM). The final concentration of DMSO never exceeded 0.5%, which is a non-toxic concentration for the cells. All experiments were performed in technical and biological triplicates. Culture medium with corresponding concentrations of investigated compounds, but without cells, was used as blank, also in triplicate. The cultures were incubated for 72 h, and the effects of the investigated compounds on cancer cell survival were determined using the microculture tetrazolium test (MTT), according to Mosmann [31] with modification by Ohno and Abe [32], 72 h after the addition of the investigated compounds. Briefly, 20 μL of MTT solution (5 mg/mL of phosphate-buffered saline, PBS) was added to each well. Samples were incubated for an additional 4 h at 37 °C in a humidified atmosphere of 5% CO_2 (v/v). Afterward, 100 mL of 100 g/L sodium dodecyl sulfate (SDS) were added

in order to extract the insoluble formazan, which represents the product of the conversion of the MTT dye by viable cells. The number of viable cells in each well is proportional to the intensity of the absorbance (A) of light, which was measured in an enzyme-linked immunosorbent assay (ELISA) plate reader at 570 nm, 24 h later. To determine cell survival (%), the A of a sample with cells grown in the presence of various concentrations of the investigated compounds was divided by the control optical density (the A of control cells grown only in nutrient medium) and multiplied by 100. The A of the blank was always subtracted from the A of the corresponding sample incubated with the target cells. IC_{50} is defined as the concentration of an agent inhibiting cell survival by 50% compared with the vehicle-treated control. Cisplatin was used as positive control. All experiments were performed in triplicate.

3. Results

3.1. Synthesis and Characterization

In the reaction of $Na[AuCl_4]\cdot 2H_2O$ and an equimolar amount of corresponding ligand (Scheme 1), previously deprotonated with LiOH, upon precipitation in the presence of PF_6^- ions, the desired complex was obtained as a yellow product. The prepared complex is soluble in methanol, ethanol, acetone, dichloromethane, chloroform, dimethyl sulfoxide, and acetonitrile.



Scheme 1. Synthesis of $[AuCl_2\{(S,S)\text{-}Et_2eddl\}]PF_6$ complex.

High-resolution electrospray ionization mass spectrometry (HR ESI-MS) was recorded in positive ion mode, and the $[M-PF_6]^+$ peak was detectable. Additionally, the proposed stoichiometric formula of synthesized complex is in agreement with elemental analysis. The IR spectra of synthesized complex show the characteristic absorption for aliphatic esters COOR strong absorption stretching band ν(C=O) at 1736 cm^{-1}, similarly to the ligand precursor $[(S,S)\text{-}H_2Et_2eddl]Cl_2$ [25], indicating that coordination of the carbonyl oxygen atom to the metal center is excluded. Additionally, a band arising from ν(C–O) was found at 1241 cm^{-1}. Asymmetric $\nu(CH_3)$, $\nu(CH_2)$, and ν(CH) stretching vibrations were found at around 2976 and 2875 cm^{-1}. A characteristic band for secondary amines identified at 3180 cm^{-1} was confirmed in the IR spectra of the complex. Coordination through the nitrogen atom can be supposed on the basis of changes in values of asymmetric C–N vibrations from 803 (ligand precursor) to 863 cm^{-1} for complex.

In ^{1}H NMR spectra, the hydrogen atoms belonging to the secondary amino groups of the complex appeared at around 5.2 ppm (compared to the ligand precursor, approximately 10 ppm). The resonances of ethylene hydrogen atoms (C^1H_2) showed coordination-induced shifts (*ca.* 0.4 ppm), which also indicated that coordination occurred via nitrogen atoms. In the ^{13}C NMR spectrum, carbonyl atoms show resonances at expected chemical shifts for this class of compounds where oxygen is not participating in coordination (C^3, 169 ppm) [33,34]. The resonances of carbon atoms from the ethylenediamine moiety (C^1) in the complex is shifted downfield relative to that of the ligand precursor [25]. Two sets of signals were found for the synthesized gold(III) complex, indicating the formation of diastereoisomers arising out of new nitrogen stereocenters formed due to coordination.

This is also confirmed with H,H-COSY NMR spectroscopy. Moreover, H,H-COSY NMR spectroscopy excluded the presence of a (*R*,*S*)-*N*,*N*′-isomer. Thus, for this diastereoisomer, two resonances expected are for the chiral CH as well as for NH hydrogen atoms, which were not detected. However, NMR spectroscopy could not provide strong evidence for (*R*,*R*)-*N*,*N*′ and (*S*,*S*)-*N*,*N*′ isomers assignment, because of the absence of well-defined resonances in ^{1}H and a similar correlation pattern in the H,H-NOESY spectrum.

3.2. Quantum Chemical Calculations

Quantum chemical calculations of similar gold(III) complexes have been performed and reported before [19,20]. Herein, density functional theory (DFT) calculations were used to estimate the most energetically-favorable isomers obtained for the [$AuCl_2${(*S*,*S*)-Et_2eddl}]PF_6 complex. The calculated results for [$AuCl_2${(*S*,*S*)-Et_2eddl}]PF_6 showed that the (*R*,*R*)-*N*,*N*′ diastereoisomer is the most stable. The difference in the total electronic energies between diastereoisomers (*R*,*R*)-*N*,*N*′ and (*S*,*S*)-*N*,*N*′ was ΔE_{tot} = 2.71 kcal/mol. The energy of the third isomer, (*R*,*S*)≡(*S*,*R*) was 5.65 kcal/mol higher than the energy of the (*R*,*R*) isomer. These results are in agreement with those obtained for platinum(IV) complexes with the same type of ligands [35]. Due to a small difference in energy and as shown by NMR spectroscopy, DFT also points out the formation of (*R*,*R*)-*N*,*N*′ and (*S*,*S*)-*N*,*N*′ diastereoisomers. *ORTEP* (The Oak Ridge Thermal Ellipsoid Plot) presentations of isomers are given in Figure 1.

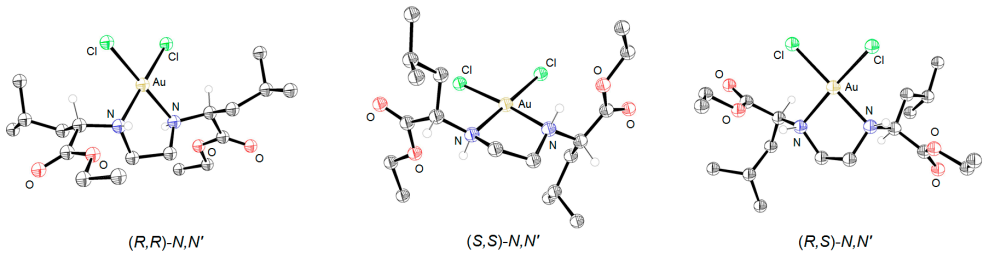


Figure 1. Calculated structures of complex [$AuCl_2${(*S*,*S*)-Et_2eddl}]PF_6. Only H atoms bonded to chiral atoms are shown.

3.3. Biological Activity

In our previous research, gold(III) complexes with various (*S*,*S*)-R_2edda type ligands were examined for their activities as anticancer agents [19,20]. In this work, the cytotoxic potential of ligand precursor, [(*S*,*S*)-H_2Et_2eddl]Cl_2, and a new synthesized gold(III) complex [$AuCl_2${(*S*,*S*)-Et_2eddl}]PF_6 were studied in a panel of malignant cell lines, originating from solid tumors as well as against a normal, non-cancerous cell line. In Figure 2, graphs showing the survival of HeLa, LS174, MCF7, A549, and non-cancerous MRC-5 cells in the presence of different concentrations of [(*S*,*S*)-H_2Et_2eddl]Cl_2 and [$AuCl_2${(*S*,*S*)-Et_2eddl}]PF_6 are presented. The IC_{50} values for compounds against these cell lines are summarized in Table 1.

The results indicate that ligand precursor and complex showed very good to moderate cytotoxic activity against all tested malignant cell lines. It is evident that ligand precursor, [(*S*,*S*)-H_2Et_2eddl]Cl_2, and the complex, [$AuCl_2${(*S*,*S*)-Et_2eddl}]PF_6, showed less toxicity than the Na[$AuCl_4$] complex. Comparing the IC_{50} values, it is found that complex [$AuCl_2${(*S*,*S*)-Et_2eddl}]PF_6 shows a cytotoxicity several times stronger than the ligand precursor and Na[$AuCl_4$]. The most promising activity was found for [$AuCl_2${(*S*,*S*)-Et_2eddl}]PF_6 against the LS174 cell line, which was five times higher than that for cisplatin.

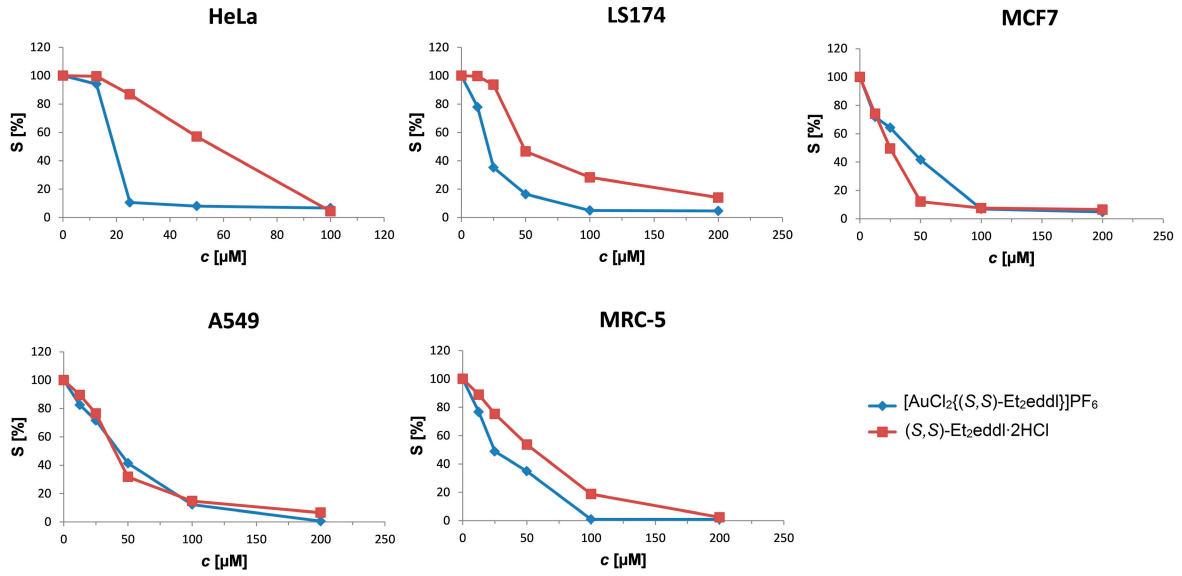


Figure 2. The survival of HeLa, LS174, MCF7, A549, and MRC-5 cells incubated for 72 h with different concentrations of investigated compounds (microculture tetrazolium test (MTT) assay).

Table 1. Concentrations of [(*S*,*S*)-H_2Et_2eddl]Cl_2, [$AuCl_2${(*S*,*S*)-Et_2eddl}]PF_6, Na[$AuCl_4$], and cisplatin that were able to induce a 50% decrease in cell survival (IC_{50} [μM]), after 72 h of incubation (mean ± SD).

Compounds	IC_{50} (μM)				
	HeLa	LS174	MCF7	A549	MRC5
[(*S*,*S*)-H_2Et_2eddl]Cl_2	53.97 ± 2.72	53.23 ± 4.12	46.97 ± 2.19	38.44 ± 1.39	55.91 ± 0.61
[$AuCl_2${(*S*,*S*)-Et_2eddl}]PF_6	18.25 ± 0.87	7.44 ± 1.19	41.10 ± 1.96	36.35 ± 1.75	24.94 ± 0.43
Na[$AuCl_4$]	52.80 ± 2.93	39.89 ± 3.60	75.70 ± 0.38	45.66 ± 2.35	54.60 ± 3.11
cisplatin	6.90 ± 1.71	22.40 ± 0.44	18.13 ± 0.57	17.20 ± 0.82	14.21 ± 1.54

3.4. Selectivity Study

Against the non-cancerous lung fibroblasts (MRC-5), tested compounds were found to be moderately sensitive to toxic. In the case of [$AuCl_2${(*S*,*S*)-Et_2eddl}]PF_6, a selectivity greater than that for cisplatin was found against LS174 cells. Additionally, ligand precursor is also more selective against MCF7 cells than cisplatin. With an activity three times higher against LS174 cells and a selectivity 3.5 times higher than cisplatin, complex [$AuCl_2${(*S*,*S*)-Et_2eddl}]PF_6 could be a promising candidate for further stages of screening. Selectivity indices are given in Table 2.

Table 2. Selectivity indices.

Compounds	IC_{50} (MRC-5)/IC_{50} (cell line)			
	HeLa	LS174	MCF7	A549
[(*S*,*S*)-H_2Et_2eddl]Cl_2	0.93 ± 0.04	1.05 ± 0.08	1.19 ± 0.06	1.45 ± 0.05
[$AuCl_2${(*S*,*S*)-Et_2eddl}]PF_6	1.37 ± 0.07	3.35 ± 0.54	0.61 ± 0.03	0.69 ± 0.04
Na[$AuCl_4$]	1.03 ± 0.08	1.37 ± 0.15	0.72 ± 0.04	1.20 ± 0.09
cisplatin	2.06 ± 0.56	0.63 ± 0.07	0.78 ± 0.09	0.83 ± 0.10

4. Conclusions

The synthesis of a novel gold(III) complex with *O*,*O*′-diethyl ester of ethylenediamine-*N*,*N*′-di-2-(4-methyl)pentanoic acid is described. The compound was characterized by IR, ^{1}H, and ^{13}C NMR

spectroscopy, mass spectrometry, and elemental analysis. NMR spectroscopy showed the presence of two (*R*,*R*)- and (*S*,*S*)-*N*,*N*′ diastereoisomers, and DFT calculations indicate the formation of the same isomers. The newly synthesized complex $[AuCl_2\{(S,S)\text{-}Et_2eddl\}]PF_6$, as well as corresponding ligand precursor $[(S,S)\text{-}H_2Et_2eddl]Cl_2$, were tested against tumor cell lines (HeLa, LS174, MCF7, and A549) and the non-cancerous cell line human embryonic lung fibroblast (MRC-5) using the MTT assay. The complex showed up to three times stronger cytotoxicity than the ligand precursor against HeLa cells, and even up to seven times against LS174 cells (IC_{50} = 7.4 ± 1.2 μM), which is comparable to cisplatin activity. Additionally, $[AuCl_2\{(S,S)\text{-}Et_2eddl\}]PF_6$ showed 3.5 times higher selectivity in LS174 cells than cisplatin.

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Author Contributions: N. Pantelić, B.B. Zmejkovski and J. Vujić conceived, designed and performed experiments in chemical part of the work; D.D. Marković and T.P. Stanojković conceived, designed and performed the experiments of biological part of work. G.N. Kaluđerović performed DFT calculations; N. Pantelić, B.B. Zmejkovski and G.N. Kaluđerović wrote the manuscript; T.J. Sabo and G.N. Kaluđerović conceived the idea, supervised the work and revised the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

MeOH	Methanol
(*S*,*S*)-Et_2eddl	*O*,*O*′-diethyl ester of ethylenediamine-*N*,*N*′-di-2-(4-methyl)pentanoic acid
HeLa	Human adenocarcinoma cell line
LS174	Human colon carcinoma cell line
MCF7	Human breast cancer cell line
A549	Non-small cell lung carcinoma cell line
MRC-5	Non-cancerous cell line human embryonic lung fibroblast
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
SD	Standard deviation
IR	Infrared spectroscopy
NMR	Nuclear magnetic resonance spectroscopy
HR ESI-MS	High-resolution electrospray ionization mass spectrometry

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