Complexes of Cu (II) with α-Ketoglutaric Acid and 1-(o-tolyl) Biguanide

Synthesis, characterization and biological Activity

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The three copper complexes having α-ketoglutaric acid (H,A) and 1-(o-tolyl) biguanide (TB) ligands have been synthesized and characterized. The proposed formulas for these complexes are: [Cu(TB)(H,A)Cl]Cl (C1), [Cu(TB)(H,A)]CH3COO•H2O (C2) and [Cu(TB)(H,A)]NO3 (C3) where HA represents deprotonated H,A.

The complexes obtained were tested for antibacterial activity against Staphylococcus aureus ATCC 25923 and Pseudomonas aeruginosa ATCC 27853, antifungal activity on Candida albicans ATCC 10231 and antitumor activity on HeLa tumor cells. Due to the antitumor, antifungal, antimicrobial activity and inhibition of inert substrate adhesion, complexes synthesized could be used for potential therapeutic applications.

Keywords: 1-(o-tolyl)biguanide, α-ketoglutaric acid, copper complexes, antibacterial activity, cellule HeLa, antifungal activity.

α-ketocarboxylic acids are very important agents in the synthesis and degradation of amino acids and proteins in the metabolism of lipids and carbohydrates [1]. Studies on α-ketocarboxylic acids with biological activity have shown that they can produce different effects on the biological tissue, interacting at different cellular levels at different pH, temperatures enzyme-controlled and environment [2-4]. α-ketoglutaric acid plays an essential role in the Krebs cycle; it antagonizes the toxic effects against both HCN and NaCN [5-6]. It has also a neuroprotective effect, helping to heal and regenerate tissues and to set up gastrointestinal tract; in lung disorders and some types of cancer [1].

Several complexes of lanthanides containing α-ketoglutaric acid: [MIII(NO3)6(OH)2]6[NO3] with antibacterial and antioxidant activity, have been synthesized; where MIII = Ce and MII = Cu, Co, Ni and L is α-ketoglutaric acid [7-8].

Biguanides are compounds exhibiting important biological properties, e.g. antibacterial, antifungal, hypoglycaemic, antimalarial and antitumor activity [9-16].

Many of the complex combinations in which the ligand is part of this class of compounds have biological properties, which explains the particular interest in their study.

Complexes of Cu (II), Fe (III) and Ni (II) with N, N-dimethylbiguanide ligands and derivatives thereof[17-19], [Cu(HTBG)]Cl2, [Cu(TBG)]-3H2O where HTBG is 2-dimethylbiguanide ligands and derivatives thereof [17-19], which explains the particular interest in their study.

Chemical and spectral analysis

Elemental analysis to determine the carbon, nitrogen and hydrogen content has been done by microcombustion using an Organic Elemental Flash 2000 Analyzer. For copper determination atomic absorption spectrometry using a Perkin Elmer Aanalyst 400 Atomic Absorption Spectrophotometer have been used.

The presence of chlorine in the C1 complex was marked with AgNO3.

Thermal analyses were performed with a simultaneous thermogravimetric analysis/differential scanning calorimetry (STA/DSC) 449F1 Jupiter working in a dynamic air atmosphere at a flow rate of 20 mL/min and at a heating rate of 10 °C/min. From RT to 900°C.

The electron spectra of complexes C1-C3 and H2A and TB ligands were recorded with a Jasco V670 spectrometer by diffuse reflectance, in the range of 200-1500nm using MgO standard.

The EPR spectra for complexes synthesized were determined on powders at room temperature in the X band (frequency = 9.4 GHz) using a ADANI CMS 8400 spectrometer. The experimental set up was chosen to obtain the best ratio signal/noise.

Experimental part

Synthesis of copper complexes

For the synthesis of the three complexes, high purity substances has been used: α-ketoglutaric acid - C5H6O5 (Alfa Aesar), 1-(o-tolyl) biguanide - C9H13N5, CuCl2•2H2O, Cu(NO3)2•6H2O, Cu(CH3COO)2•H2O and CH3OH (Sigma Aldrich). In the first step, the metal salts and the two ligands were dissolved in ethanol.

For all three complexes the metal salt molar ratio: α-ketoglutaric acid: 1-(o-tolyl) biguanide was 1:1:1, using 1mmol of each substance (0.1461g C5H6O5, 0.1913g C9H13N5, 0.1705g CuCl2•2H2O, 0.2956g Cu(NO3)2•6H2O and 0.1997g Cu(CH3COO)2•H2O).

The resulted metallic complexes have been further washed with ethanol and ethyl ether. The compounds obtained were pure and therefore no further purification was required.

α-ketoglutaric acid and 1-(o-tolyl) biguanide have antibacterial and antioxidant activity, have been synthesized; where MIII = Ce and MII = Cu, Co, Ni and L is α-ketoglutaric acid [7-8].

Given the biochemical role of copper and the biological properties of α-ketoglutaric acid and 1-(o-tolyl) biguanide, we have proposed to synthesize and characterize new complex combinations of Cu (II) with these ligands.

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For spectra FT-IR, 4000-200 cm⁻¹ was used using a Nicolet IS 50 FT-IR spectrophotometer. The molar electrical conductivity was determined with a CybemScan PCD 6500 conductivity meter at 25°C in a solution of N,N-dimethylformamide at a concentration of 0.001 mol/L.

Biological activity

Antimicrobial activity was determined for both C1-C3 and H2A and TB ligands. The tests were performed in vitro and the bacterial strains were *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853. Antifungal activity was tested on *Candida albicans* ATCC 10231.

To determine the minimal inhibitory concentration, (MIC), a quantitative method based on binary serials microdilutes in a broth, steriley distributed into 96-well plates, was used. For each tested compound, working solutions of 10 mg/mL in DMSO were prepared.

By serial binary dilutions, eight solutions were prepared with concentrations ranging from 5 mg/mL to 0.0391 mg/mL. Eight wells were pipetted with 150 µL of liquid medium (broth). Into the first well were added 150 µL of 10 mg/mL compound, into the second 150 µL from the first well, into the third 150 µL from the second, and so on to the eight well.

After microdilutions, 20 µL of 0.5 McFarland microbial suspension was added to each well. The same was done for the solvent used in the dilutions, (DMSO). A microbial culture witness (a well of medium containing culture medium inoculated with microbial suspension) and an environmental sterility test for each assay were also run. The so-seeded plates were incubated at 37 °C for 24h.

After incubation for each compound, the macroscopic MIC value was determined as the latter concentration at which the environmental turbidity (microbial growth) was not observed, but also by spectrophotometric reading of the microbial culture absorbance developed in the liquid medium at a length of 620 nm wave.

In the following wells, including the growth control well, the medium was turbidity due to microbial growth. In the sterility control well, the liquid content remained transparent because there was no bacterial growth.

To determine the influence of the tested compounds on the adhesion of microbial biofilm to the inert substrate, microbial cells were instilled in 96-well plate with nutrient broth. The preparation of solutions of each compound (H,A, TB, C1-C3, DMSO) is the same as for MIC determination.

The seedlings were also incubated at 37 °C for 24 h. After incubation the plates were emptied and washed with physiological sterile water. Fixation of the adherent cells was done with 0.1 mL of 80% methanol for 5 min. The methanol solution was removed by overturning and the adherent cells were stained with 1% (0.1 mL/well) purple crystallium (hexamethyl-para hydrochloride) for 15 min. The staining solution was removed by washing the plates with water; platelet microbial biofilms were resuspended by bubbling in a 33% acetic acid solution. The intensity of the suspension color was evaluated by measuring the absorbance at 492 nm with a Max F5 Multi-Mode Microplate Reader Spectrophotometer.

Antitumor activity for C1-C3 complexes and H2A and TB ligands was performed on HeLa cells (MTT assay). The concentration of each compound was 500 µg/mL in DMSO, the incubation time was 24h at 37°C in a 5% CO₂ atmosphere. For incubation, Heraetherm Microbiological Incubator - Thermo Scientific - static incubator for microorganisms has been was used.

The test was also performed for the solvent used dilutions (HeLa control). MTT Method Principle: The MTt cell viability assay is based on the ability of viable cell-dependent NAD (P) H oxidoreductases to reduce MTT formazan, a violet insoluble compound, of whom the absorbance can be spectro-photometrically determined (Scheme 1).

To assess the color intensity of formazan, optical density was measured with a Max F5 Multi-Mode Microplate Reader Spectrophotometer at 570 nm. This method is often used to measure the cytotoxicity of some compounds and cell proliferation. The reaction is carried out in the dark because the MTT reagent is light sensitive.

Results and discussions

Elemental analysis

For C1-C3 complex combinations, elemental analysis was performed to determine the corresponding formulations. The percentages of experimentally determined carbon, nitrogen, hydrogen and copper is in good concordance with the calculated ones.

Found: C, 38.81; H, 4.06; N, 15.98; Cu, 14.39%. Calculated for [Cu(TB)(HA)]Cl: C, 38.63; H, 4.17; N, 16.09; Cu, 14.60%. Found: C, 40.13; H, 4.72; N, 14.81; Cu, 13.51%. Calculated for [Cu(TB)(HA)CH₃COO]•H₂O: C, 40.29; H, 4.86; N, 14.68; Cu, 13.32%.

Found C, 36.21; H, 3.78; N, 18.37; Cu, 13.84%. Calculated for [Cu(TB)(HA)](NO₃): C, 36.40; H, 3.93; N, 18.20; Cu, 13.76%.

Molar conductivity

For C1-C3 complexes molar conductivity at 25 °C was determined in N,N-dimethylformamide solution at a concentration of 0.001 mol/L. The molar conductivity for the complexes are: C1 72.1 S·cm²·mol⁻¹, C2 36.5 S·cm²·mol⁻¹ and C3 81.1 S·cm²·mol⁻¹. The values obtained indicate a 1:1 type electrolyte for C1 and C3 complexes, while C2 is a non-electrolyte [25].
Thermal analysis

From the results of the thermogravimetric analysis, the information obtained contributed of the three proposed complexes formulations, namely the presence of water molecules and the thermal effects accompanying the mass loss processes.

Thermogravimetric (TG) and differential scanning calorimetry (DSC) curves of the three complexes are presented in Figure 1(a-c).

From the analysis of the C1 complex TG and DSC curves, the first decomposition step (220-260 °C) indicates the loss of chlorine anion in the form of HCl (exp. loss 8.07%, calc. loss 8.38%). The ligand decomposition takes place in the range 260-660°C. In the C2 complex, it is observed that below 160°C there is a mass loss corresponding to a crystallization water molecule (3.72% exp. loss, 3.77% calc. loss). The acetate ion is removed as CO2 and H2O to about 250 °C (12.61% exp. loss, 12.37% calc. loss) and ligand degradation occurs up to 540 °C. Analyzing the C1 complex TG and DSC curves, it is observed that above 250 °C takes place the elimination of nitrate ions as nitrogen oxides and the oxidative degradation of the ligand. Ligand degradation for all three complexes is accompanied by strong exothermic effects. For all analyzed complexes, the residue obtained is CuO. The percentage of the determined copper is: for C1 (14.81% exp., 14.60% calc.), for C2 (13.05% exp., 13.32% calc.), and for C3 (13.58% exp., 13.76% calc.).

Between the spectrophotometrically and the residue from termogravimetric analysis the metal content is in a good agreement.

Ultraviolet-visible-near-infrared spectra

The correlation of the results obtained on the basis of the EPR spectra and the bands observed in the UV-Vis NIR electron spectra led to important information regarding the stereochemistry of the complexes obtained and the character of the metal-ligand bonds [26-34]. The spectra corresponding to the C1-C3 complexes are shown in Figures 2-4, and the assignments of the bands corresponding to the d-d transitions are shown in Table 1.

The three complexes have absorption bands in the range of 240-370 nm that may be attributed to the π→π* and n→π* transitions of organic ligands H2A and TB, slightly displaced by coordination of the ligand to metal ions.

In the case of C1 complex, the values of the g-factor, in the presence and perpendicular (g⊥) direction to the external field, are gII = 2.1960 and g⊥ = 2.0669. Using the gII value and the energy of transition...
B_{2g} \rightarrow B_{2g}$ results the $K_{II}$ value (for the $\sigma$ bonds from the plan); for the out of plane bonds, using $g_\perp$ and the energy of transition $2B_{1g} \rightarrow 2E_g$ we get $K_{\perp}$. $K_{II}$ and $K_{\perp}$ are the orbital factors of the spin-orbit coupling constant of the free ion).

The computational relationships used are:

$\lambda_{II}, \lambda_\perp$ are coupling constants for Cu$^{2+}$ in complex in the direction parallel to the field, respectively perpendicular, $\lambda_0$ = -828 cm$^{-1}$ and is the spin-orbit coupling constant for the Cu$^{2+}$ free ion. The following values are obtained:

$\lambda_{||} = -298.8$ cm$^{-1}, \lambda_{\perp} = 471$ cm$^{-1}$ and $\lambda_0 = 0.57$

The value of the hyperphin coupling constant is $A_{II} = 125.16 \times 10^4$ cm$^{-1}$.

The $\alpha_2^{2}, \beta_1^{2}, \beta_2^{2}$ coefficients characterizing the $\sigma$ bonds in the plan, the $\pi$ bonds in the plan and $\pi$ bonds outside the plane respectively for the Cu$^{2+}$ metal ion are calculated with the relations 5-7:

$\alpha_2 = |A_{II}| / P + (g_{II} - 2.0023) + 3/7 (g_\perp - 2.0023) + 0.04$

$K_{||} = \alpha_2 \beta_1^{2}$

$K_{\perp} = \alpha_2 \beta_2^{2}$

The following values were obtained: $\alpha_2 = 0.61$ which means that the sigma bonds in the plane are strong with predominantly covalent character; $\beta_1^{2} = 0.57$ implies strong $\pi$ bonds in the xoy plane and $\beta_2^{2} = 0.93$ indicates weak $\pi$ bonds outside the plane.

The exchange interaction parameter $G$ is calculated by the relation (8)

$G = (g_{II} - 2) / (g_{\perp} - 2)$

The $G$ value obtained is 2.93 < 4 and suggests strong spin-spin interactions between Cu$^{2+}$ ions [35]. These values
are in agreement with axial symmetry, with the fundamental state $d_{x^2-y^2}$, $D_{4h}$ (square planar). The appearance and position of the bands in the electronic spectrum of the C2 complex, corresponds to a $C_4v$ (pentacoordinated) symmetry. From the RPE spectrum, $g_\perp = 2.0856$ and with relations (2) and (4) we calculate $\lambda_\perp = -7.48 \text{ cm}^{-1}$ and $K_\perp = 0.85$. The value of $K_\perp$ indicates almost ionic metal-ligand bonds. The symmetry of the C3 complex is the square plane being determined based on the two types of spectra. For C3 $g_\perp = 2.081$, $g_\perp = -2 = \frac{-\lambda_\perp}{\lambda_\perp} = 0.62$, which implies a predominantly covalent character of the ML bond.

**FT-IR Spectra**

In order to determine how copper ligands were coordinated, the FT-IR spectra of the three complexes were compared with H$_2$A and TB ligands [36, 37]. Spectral bands characteristic of complexes and ligands as well as their assignments are shown in Table 2.

To determine the coordination of $\alpha$-ketoglutaric acid the IR spectrum of sodium $\alpha$-ketoglutarate was also analyzed. The difference between the wavelengths corresponding to the $\nu(C=O)$ band below 1720 cm$^{-1}$. This displacement is in agreement with the coordination of 1-(o-tolyl) biguanide to the copper ion by the pair of electrons not participating in the imine nitrogen; the movement of this band to larger wavelengths is explained by the destruction of the electron delocalisation [38]. Coordination TB to copper ion by iminic nitrogen atoms is also supported by the band movement due to $\delta(NH) + \nu(C-N)$ coupled vibration.

In the complex C2 the intense bands at 1555 cm$^{-1}$ and 1365 cm$^{-1}$ indicate monodentated coordination of the acetate and the stretched band at 3380 cm$^{-1}$ is attributed to the crystallization water.

The presence of a single absorption band for the characteristic vibration modes $\nu_\text{as} - \nu_\text{ss}$ in the spectrum of the C3 complex indicates the presence of the nitrate anion in the ionization sphere. Bands between 527-548 cm$^{-1}$ and 588-597 cm$^{-1}$ were assigned to the formation of Cu-O and Cu-N bonds.

In all three complexes, both H$_2$A and TB function as bidentate ligands; the TB ligand coordinates to the copper ion through the imine nitrogen atoms and H$_2$A coordinates to the deprotonated metal ion through the oxygen atom in the ketone group at the alpha and the adjacent carboxyl group.

**Antimicrobial and antifungal activity**

Testing ligands and complexes on *Staphylococcus aureus* ATCC 25923 it has been found that the best antimicrobial activity had C1 and C3 with a MIC of 0.3125 mg/mL. Complex C2 has a slightly weaker activity than C1 and C3, but better than ligands. H$_2$A ligand activity against *Pseudomonas aeruginosa* ATCC 27853 is weaker than the other ligand, TB and C1-C3 complexes. The best activity...
against this strain have C1 and C3 complexes (MIC is 0.625 mg/mL).

The solvent used in the dilutions does not influence the antimicrobial activity of the compounds tested at the working concentrations [39, 40]. The MIC for the compounds tested against the two bacterial strains are shown in Figures 5a and 5b.

Regarding the ability to inhibit the adhesion of microbial biofilm to inert substrate, all tested compounds inhibit this process depending on the used dose to a MBEC [41, 42]. The lowest MBEC is for C1-C3 complexes (0.0391 mg/mL), 0.1562 mg/mL for TB and 0.3125 mg/mL for H2A against Staphylococcus aureus. In the Pseudomonas aeruginosa strain, all the complexes and the TB ligand have biofilm inhibition capacity until a minimum biofilm eradication concentration of 0.0391 mg/mL is reached. This is not valid for H2A ligand having a MBEC of 0.0781 mg/mL.

The influence of the tested compounds on the inert substrate adhesion capacity is shown in Figures 6 and 7.

Antifungal activity was determined on Candida albicans ATCC 10231 (Figure 8). Ligands have the weakest activity, C1 and C3 have moderate activity and the best one is C2 having an MIC of 0.125 mg/mL.

**Antitumor activity**

Metabolism of HeLa cells depends on the type of material used. Percentages of viability for the test compounds (complexes and ligands) were calculated based on the untreated control. Figure 9 shows percent viability values for the samples and untreated control, indicating the metabolic activity of HeLa cell cultures. For test purposes, concentrations of 500 \( \alpha \)g/mL were used and incubation was done for twenty-four hours at 37 °C.

The H2A ligand has a very low effect on HeLa tumor cells under the conditions tested, whereas the TB ligand and the C2 complex have demonstrated a moderate cytotoxic effect on them, reducing their viability by 18% and 20%, respectively. Complexes C1 and C3 have a better cytotoxic effect against HeLa cells, decreasing their viability by 27% and 23%.

**Conclusions**

Three complex combinations of Cu (II) having alpha-ketoglutaric acid ligands and 1 - (o-tolyl) biguanide have been synthesized, characterized and tested for their biological activity against Staphylococcus aureus ATCC
25923, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* ATCC 10231, HeLa cells. The formulations of the C1-C3 complexes were based on the analyzes performed (elemental analysis, UV-Vis-NIR spectra, IR, RPE, thermal analysis, molar conductivity).

Both α-ketoglutaric acid and 1-(o-tolyl) biguanide function as bidentate ligands in the three complexes (their structures are shown in Figure 10 a-b).

For the synthesized complexes, the following formulas were proposed (Figure 11 a-c):

The best cytotoxic effect against HeLa tumor cells have complexes C1 and C3. Antibacterial activity against the *Pseudomonas aeruginosa* strain ATCC 27853 is comparable or better for complexes than for ligands (the best activity have C1 and C3).

With respect to ligands and complexes testing against the *Candida Albicans* ATCC 10231 and *Staphylococcus aureus* ATCC 25923 all complexes have better antifungal activity than ligands. Since the C1 and C2 complexes are electrolys type, their activity may result from the electrostatic interaction of the complex cation therewith with the negatively charged components of the membrane, which leads to their inactivation.

Antimicrobial/antifungal activity may also be associated with the complex combination stereochemistry but also with the combined effect of metal ion and ligand to inactivate a particular component involved in the pathogenesis of the microorganism.

The different activity of the C1-C3 complexes can be attributed to their different stereochemistry; C1 and C3 - square planar, C2 - square pyramid.

### Abbreviations:

- **TB**: 1-(o-tolyl)biguanide
- **H₂A**: a-ketoglutaric acid
- **HA**: deprotonated a-ketoglutaric acid
- **DMSO**: dimethylsulfoxide
- **NAD(P)H**: nicotinamide-adenine-dinucleotide phosphate
- **MTT**: 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide
- **MIC**: Minimum Inhibitory Concentration
- **MBEC**: Minimal Biofilm Eradication Concentration

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