Synthesis, Characterization and Evaluation of the Antibacterial and Antitumor Activity of Halogenated Salen Copper(II) Complexes derived from Camphoric Acid

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Abstract

Platinum metal complexes are the most common chemotherapeutics currently used in cancer treatment. However, the frequent adverse effects, as well as acquired resistance by tumor cells, urge the development of effective alternatives. In the recent past, copper complexes with Schiff base ligands have emerged as good alternatives, showing interesting results. Accordingly, and in continuation of previous studies in this area, three new camphoric acid-derived halogenated salen ligands and their corresponding Cu(II) complexes were synthesized and their antitumor activity was evaluated in order to determine the influence of the type and number of halogens present (Br, Cl). The *in vitro* cytotoxic activity was screened against colorectal WiDr and LS1034 and against breast MCF-7 and HCC1806 cancer cell lines. The results proved the halogenated complexes to be very efficient, the tetrachlorinated Cu(II) complex being the most promising, presenting IC_{50} of 0.63-1.09 μM for the cell lines studied. The complex also shows selectivity to colorectal cancer cells compared to non-tumor colon cells. It is worth highlighting that the tetrachlorinated Cu(II) complex, our most efficient complex, shows a significantly more powerful antitumor effect than the reference drugs currently used in conventional chemotherapy.

The halogenated salen and corresponding complexes were also screened for their antimicrobial activity against four bacterial species-*Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa*-and four fungal species-*Candida albicans*, *Candida glabrata*, *Aspergillus fumigatus* and *Alternaria alternata*. The compounds were found to exhibit moderate to strong antibacterial activity against the bacterial strains studied.

NMR studies and theoretical calculations provided some insight into the structure of the ligands and copper complexes.

Considering the results presented herein, our work validates the potential use of copper-based chemotherapeutics as alternatives for cancer treatment.

1. Introduction

Cancer is one of the leading worldwide causes of death. Colorectal and breast cancer are two of the most incident and mortal types of cancer.[1] The discovery of platinum-based drugs in the twentieth century embodied an enormous breakthrough for cancer chemotherapy. Cisplatin, oxaliplatin and carboplatin are platinum metal complexes currently used in the treatment of cancer. However, these chemotherapeutic compounds, and others, namely, fluoropirimidines, irinotecan, taxanes and anthracyclines, besides having many adverse side effects, have led to the development of increased resistance of cancer cells to the pharmaceuticals used.[2]

A similar problem exists concerning antibiotic resistance. Although this is an ancient phenomenon that occurs naturally in the environment,[3] it has now reached alarming proportions, with the emergence and spreading of multidrug-resistant bacteria, making the treatment of infectious diseases increasingly difficult. Several approaches to circumvent this problem are currently being investigated and one of these is the development of new metal-based drugs with different modes of action that are not affected by the current resistance mechanisms, since their targets are expected to be different.

Hence, in order to solve these problems, it is of fundamental importance to search for alternative therapeutic agents, with improved pharmacological properties and minimal negative characteristics.

In the recent past considerable attention has been given to the use of metal complexes in medicine for various types of therapy and diagnosis. In cancer treatment, over the last decades, many non-Pt metal complexes have emerged and have been investigated as potential cancer treatments. Among these, copper complexes seem to be good alternatives to Pt complexes and those with Schiff base ligands have shown interesting results.[4]–[6] The biological activity of many Schiff base and salen metal complexes, Figure 1, has been recognized, specifically with respect to antimicrobial, antifungal, antiviral, antioxidant, anti-inflammatory, insecticidal and anticancer activities.[4][6]–[11]

Figure 1.

Among these complexes, the salen metal complexes are emerging as potential therapeutic agents. The excellent chelating ability of these compounds with many metals is responsible for their improved biological activity.[8] These complexes are very attractive alternatives due to the versatility of the salen ligands, which result from the condensation of amines with salicylaldehydes or aromatic hydroxyketones, and thus can be easily fine-tuned both sterically and electronically by varying one or both of the reagents used. The structural diversity and reactivity of these complexes also makes them very useful in organic synthesis, particularly in catalytic processes, namely, alkene epoxidations, hydroxylations, cyanations and alkylations, among others.

Increasing attention is being given to the study of the therapeutic activity of salen metal complexes, with greater focus on copper, iron and manganese complexes.[8][9][12][13]

Our previous studies on this topic involved the assessment of the *in vitro* cytotoxic activity of Cu(II), Fe(III) and Mn(III) metal complexes of (*1R,3S*)-*N,N'*-bis(salicylidene)-1,3-diamino-1,2,2-trimethylcyclopentane and of the corresponding dibrominated derivative, (*1R,3S*)-*N,N'*-bis(5-bromosalicylidene)-1,3-diamino-1,2,2-trimethylcyclopentane, against human melanoma, colorectal and breast cancer cell lines. Our studies showed that Cu(II) complexes presented higher antitumour activity, possibly due to the square planar geometry of the complexes which can favor their interaction with biological targets.[14] Our results further indicated that the copper (II) complex of the dibrominated derivative showed the highest cytotoxic activity towards all cell lines studied, with IC_{50} values of 0.95-2.32 μM, a 3-5 fold increase when compared to the non-halogenated counterpart,[10] a marked improvement relatively to current conventional chemotherapy. These results are in agreement with published studies, which refer that many pharmaceuticals that incorporate halogen atoms in their structures show enhanced biological activity concerning cytotoxic effects, as well as improved pharmacodynamic and pharmacokinetic properties.[15]–[17]

These results led us to question whether increasing the number of bromine atoms in the structure or exchanging bromine for chlorine would have any effect on the cytotoxicity of the complexes. With this in mind, we synthesized the analogous tetrabrominated, tetrachlorinated and dichlorinated salen ligands (*1R,3S*)-*N,N'*-bis(2,5-dibromosalicylidene)-1,3-diamino-1,2,2-trimethylcyclopentane, (*1R,3S*)-*N,N'*-bis(2,5-dichlorosalicylidene)-1,3-diamino-1,2,2-trimethylcyclopentane and (*1R,3S*)-*N,N'*-bis(5-chlorosalicylidene)-1,3-diamino-1,2,2-trimethylcyclopentane, as well as their corresponding Cu(II) complexes and screened them for their *in vitro* cytotoxic activity against two breast cancer cell lines, HCC1806 and MCF7, and two colon cancer cells lines, LS1034 and WiDr. It is worth emphasizing that LS1034 cells are multidrug resistant and that HCC1806 cells are triple negative, associated with poor prognosis.

These new halogenated salen copper (II) complexes, as well as the analogous previously prepared dibrominated copper (II) complex were also screened for their antimicrobial and antifungal activity. For testing the antimicrobial activity, four bacterial species were used: *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa*. Four fungal species, *Candida albicans, Candida glabrata, Aspergillus fumigatus* and *Alternaria alternata,* were used to test the antifungal activity.

2. Experimental

All solvents were purified or dried prior to use following standard procedures. Sonication was performed in a Bandelin Sonorex RK100H cleaning bath with a frequency of 35 Hz and a nominal

power of 80/160 Watts. High-resolution mass spectra (HRMS) were obtained on a TOF VG Autospect M spectrometer with electrospray ionization (ESI). Melting points were determined using a FALC melting point apparatus (open capillary method). NMR spectra were recorded at room temperature on a Bruker Avance III 400 MHz (100 MHz for ^{13}C). TMS was used as the internal standard and chemical shifts are given in ppm. FTIR spectra were recorded Thermo Scientific Nicolet 6700 FTIR (solids were processed as KBr pellets). UV/visible were recorded on a T8 UV/VIS spectrophotometer (pg instruments) over the range 300-650 nm, using dichloromethane as solvent. Elemental analyses were carried out on an Elementar Vario Micro Cube analyser. The Cu(II) complex of (*1R,3S*)-*N,N'*-bis(5-bromosalicylidene)-1,3-diamino-1,2,2-trimethylcyclopentane was synthesized as previously described.[10]

2.1. Ligand Synthesis

Salen ligands **2a-c** were synthesized according to our previously described procedure:[18]

In a 25 mL Erlenmeyer flask, 1,3-diamino-1,2,2-trimethylcyclopentane **1** (5 mmol, 0.71 g) was dissolved in 20 mL of dry ethanol and the aldehyde (2,5-dibromosalicylaldehyde, 5-chlorosalicylaldehyde or 2,5-dichlorosalicylaldehyde) (10 mmol, 2.01 g) and silica (2.50 g) were added. The mixture was placed in an ultrasound bath until the reaction was complete, as monitored by thin layer chromatography (tlc), approximately 30 min. Dichloromethane (50 mL) was added and the silica was filtered off. The solvents were evaporated and the products were isolated by crystallization from dichloromethane/hexane to give yellow solids.

(*1R,3S*)-*N,N'*-bis(2,5-dibromosalicylidene)-1,3-diamino-1,2,2-trimethylcyclopentane (2a)

Yellow solid, yield 67%. m.p. 239-240 °C.

NMR ^{1}H ($CDCl_3$): 0.96(s, 3H); 1.00(s, 3H); 1.34 (s, 3H); 1.86-1.92 (m, 1H); 1.98-2.07 (m, 1H); 2.17-2.37 (m, 2H); 3.66 (t, 1H, J= 8 Hz); 7.35 (d, 2H, J= 4 Hz); 7.37 (d, 2H, J= 4 Hz); 7.70 (d, 2H, J= 4 Hz); 8.21 (s, 2H); 14.59 (s, 1H); 15.15 (s, 1H).

NMR ^{13}C ($CDCl_3$): 18.9, 20.8, 24.4, 27.9, 33.8, 48.6, 70.8, 75.4, 108.9, 109.4, 112.5, 113.0, 119.7, 119.8, 132.7, 133.0, 137.8, 158.4, 159.3, 160.1, 162.4.

IR (cm^{-1}): 3448, 2983, 2883, 1631, 1496, 1433,1385, 1213, 1153, 1095, 1051, 845, 766.

UV/vis [CH_2Cl_2], λ_{max} (nm) (relative intensity, %): 336 (100), 432 (42).

Elemental analysis: calculated for $C_{22}H_{22}Br_4N_2O_2$ (%): C, 39.67; H, 3.33; N, 4.21. Found: C, 39.45; H, 3.08; N, 4.00.

(*1R,3S*)-*N,N'*-bis(5-chlorosalicylidene)-1,3-diamino-1,2,2-trimethylcyclopentane (2b)

Yellow solid, yield 68%. m.p. 176.0–177.0 ºC

NMR ^{1}H ($CDCl_3$): 0.93 (s, 3H); 0.97 (s, 3H); 1.31 (s, 3H); 1.83-1.89 (m, 1H); 1.97-2.07 (m, 1H); 2.16-2.23 (m, 1H); 2.25-2.34 (m, 1H); 3.60 (t, 1H, J= 10.0 Hz); 6.91; (d, 2H, J= 12.0 Hz); 7.24-7.26 (m, 4H); 8.27 (s, 1H); 8.28 (s, 1H); 13.47 (s, 1H); 14.00 (s, 1H).

NMR ^{13}C ($CDCl_3$): 18.9, 20.9, 24.3, 28.2, 34.1, 48.5, 71.0, 76.4, 1118.6, 118.7, 119.5, 119.7, 123.0, 123.2, 130.4, 130.6, 132.0, 132.1, 195.8, 160.0, 160.3, 162.7.

IR (cm^{-1}): 3440, 2968, 1633, 1483, 1379, 1281, 1186, 1124, 1088, 1061, 827, 789, 646, 563, 474.

UV/vis [CH_2Cl_2], λ_{max} (nm) (relative intensity, %): 329 (100), 416 (12).

Elemental analysis: calculated for $C_{22}H_{24}Cl_2N_2O_2$ (%): C, 63.01; H, 5.77; N, 6.68. Found: C, 62.92; H, 5.40; N, 6.25.

(*1R,3S*)-*N,N'*-bis(2,5-dichlorosalicylidene)-1,3-diamino-1,2,2-trimethylcyclopentane (2c)

Yellow solid, yield 47%. m.p. 101-102ºC

NMR ^{1}H ($CDCl_3$): 0.97 (s, 3H); 1.00 (s, 3H); 1.34 (s, 3H); 1.86-1.93 (m, 1H); 1.99-2.08 (m, 1H); 2.19-2.27 (m, 1H); 2.29-2.38 (m, 1H); 3.66 (t, 1H, J= 8.0 Hz); 7.17; (d, 1H, J= 4 Hz); 7.20; (d, 1H, J= 4 Hz); 7.42 (d, 2H, J= 4.0 Hz); 8.24 (s, 1H); 8.25 (s, 1H); 14.47 (s, 1H); 15.05 (s, 1H).

NMR ^{13}C ($CDCl_3$): 18.9, 20.7, 24.4, 27.9, 33.8, 48.6, 75.5, 119.2, 119.3, 122.1, 122.6, 123.0, 129.0, 129.2, 132.4, 157.0, 157.9, 160.3, 162.6.

IR (cm^{-1}): 3438, 2970, 1629, 1454, 1375, 1292,1215, 1182, 1116, 1070, 866, 742, 567.

UV/vis [CH_2Cl_2], λ_{max} (nm) (relative intensity, %): 335 (100), 431 (41).

HRMS (ESI): calculated for $C_{22}H_{23}Cl_4N_2O_2$ $[M+H]^+$: 487.05082; found 487.05059.

Elemental analysis: calculated for $C_{22}H_{22}Cl_4N_2O_2 \cdot 0.5H_2O$ (%): C, 53.14; H, 4.66; N, 5.63. Found: C, 53.30; H, 4.24; N, 5.25.

2.2. Synthesis of the copper (II) complexes (3a-c)

The metal salt, $Cu(OAc)_2 \cdot H_2O$, (1 mmol) and the ligand (1 mmol) were dissolved in methanol (20 mL). The reaction mixture was refluxed until the disappearance of the ligand as observed by tlc, usually 2-4 h. the complex was isolated as described below.

2.2.1. [CuL] (**3a)**

The complex precipitated upon cooling and the resulting solid was filtered and dried under vacuum, to give the pure product.

Brown solid. Yield 87%. m.p. > 300 ºC

IR (cm^{-1}): 3446, 2954, 1624, 1606, 1510, 1440, 1421, 1412, 1388, 1321, 1215, 1164, 1147, 1052, 874, 754, 708, 534.

UV/vis [CH_2Cl_2], λ_{max} (nm) (relative intensity, %): 383 (100%)

HRMS (ESI): calculated for $C_{22}H_{21}Br_4CuN_2O_2$ $[M+H]^+$: 723.76271; found: 723.76269.
Elemental analysis: calculated for $C_{22}H_{20}Br_4CuN_2O_2$ (%): C, 36.32; H, 2.77; N, 3.85. Found (%): C, 36.46; H, 2.50; N, 3.81.

2.2.2. [CuL] (**3b)**
The solvent was partially evaporated and the resulting solid was filtered and dried under vacuum, to give the pure product.
Brown solid. Yield 95%. m.p. > 300 ºC
IR (cm^{-1}): 3448, 3076, 2983, 2900, 1637, 1452, 1385, 1288, 1180, 1093, 1043, 887, 850, 733, 704.
UV/vis [CH_2Cl_2], λ_{max} (nm) (relative intensity, %): 377 (100%)
HRMS (ESI): calculated for $C_{22}H_{23}Cl_2CuN_2O_2$ $[M+H]^+$: 480.04271; found: 480.04296.
Elemental analysis: calculated for $C_{22}H_{22}Cl_2CuN_2O_2 \cdot H_2O$ (%): C, 52.96; H, 4.85; N, 5.62. Found: C, 53.18; H, 5.01; N, 5.28.

2.2.3. [CuL] (**3c)**
The complex precipitated upon cooling and the resulting solid was filtered and dried under vacuum, to give the pure product.
Brown solid. Yield 58%. m.p. > 300 ºC
IR (cm^{-1}): 3460, 2958, 1626, 1608, 1523, 1444, 1425, 1412, 1392, 1321, 1211, 1176, 870, 758, 596.
UV/vis [CH_2Cl_2], λ_{max} (nm) (relative intensity, %): 381 (100%)
HRMS (ESI): calculated for $C_{22}H_{21}Cl_4CuN_2O_2$ $[M+H]^+$: 547.96477; found:547.96496.
Elemental analysis: calculated for $C_{22}H_{20}Cl_4CuN_2O_2$ (%): C, 48.06; H, 3.67; N, 5.10. Found (%): C, 47.67; H, 3.73; N, 5.05.

2.3. Nuclear Magnetic Resonance Studies

The ^1H and ^{13}C NMR spectra were obtained on a Bruker Avance III HD 500 MHz NMR spectrometer. The residual signal of the solvent, $CDCl_3$, was used as the internal reference for ^1H (δ 7.27) and ^{13}C (δ 77.23) shifts. 2D NMR spectra, COSY, NOESY, HSQC and HMBC were recorded on the same spectrometer. The ^{13}C spectra were recorded using proton decoupling techniques, taking advantage of the nuclear Overhauser effect.

2.4. Theoretical Studies

All calculations were carried out with the ORCA electronic structure package version 4.0.0.2.[19] Structures were optimized at the density functional theory (DFT) level using the meta-GGA exchange-correlation functional of Tao *et al.* (TPSS) with the flexible multiply polarized triple-

ς Def2-TZVP basis set.[20]–[22] Dispersion corrections to the TPSS functional were added using Grimme's D3 method.[23][24] Solvation effects (water) were treated with the SMD continuum solvent model.[25][26]

The same level of theory as was in the geometry optimizations was also employed in a subsequent ab-initio molecular dynamics (AIMD) simulation to ensure compatibility with the minimized structures. The AIMD simulation used a time-step of 0.5 fs and was run for 1000 steps with thermostatic coupling to a 335K heat-bath. The Berendsen thermostat was employed to regulate the temperature with the coupling parameter τ set to 10 fs.

In order to evaluate the effects of halogenation on the global characteristics of the complexes the total electronic dipole moment, ⟨μ⟩, and the isotropically averaged polarizability, ⟨α⟩, were calculated at the TPSS-D3/Def2- TZVP level of theory with SMD aqueous solvation. The polarizability ⟨α⟩ was calculated using the coupled-perturbed SCF approach. The local effects of halogenation were evaluated by fitting condensed atomic charges to the molecular electrostatic potential using the CHELPG method.[19]

2.5. Biological Activity Studies

Cell Culture

Two breast cancer cell lines [HCC1806 (ATCC® CRL-2335™) and MCF7 (ATCC® HTB-22™)] and two colorectal cancer cell lines [LS1034 (ATCC® CRL-2158™) and WiDr (ATCC® CCL-218™)] were purchased from American Type Culture Collection (Rockville, MD, USA). As control, CCD-841 CoN (ATCC® CRL1790™) and HFF1 (ATCC® SCRC-1041™) cell lines were used. The normal human colon epithelial cell line, CCD-841 CoN, was kindly provided by Instituto de Investigación Sanitaria de la Fundación Jiménez Díaz (Madrid, Spain). Cell lines were propagated as previously described.[10]

Metabolic Activity

Cancer cells were seeded in 48-well plates in a concentration of 50-70x10^5 cells/mL. After 24 hours, cells were treated with the synthesized complexes (0.5 to 5 μM) for 48 hours. Cell proliferation was then assessed by the colorimetric assay MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), which allows to measure metabolically active cells, through the quantification of formazan crystals produced, due to the relationship of direct proportionality between the quantity of crystals and the cells' metabolic activity. After the dissolution of the formazan crystals, absorbances were measured in a spectrophotometer at 570 and 620 nm, and the data processed using Origin Pro 8.5, making it possible to establish dose-response curves and to determine the respective IC_{50} (half-maximal inhibitory concentration) values.

Flow Cytometry

The effect of the synthesized compounds on cell viability and cell cycle was evaluated through flow cytometry, using the cytometer FACSCalibur. $1x10^6$ cells were used per experience, for three conditions: the control, the concentration corresponding to the IC_{50} of each cell line and 5 µM. All cell lines were labelled with annexin V bound to the fluorochrome fluorescein isothiocyanate (AV-FITC, Immunostep, ANXVF-200T) and propidium iodide (Immunostep, PI), as previously described.[27] In order to evaluate alterations on the cell cycle, tumor cell lines were fixed and labelled with a solution of PI and RNAse (Immunostep, PI/Rnase), as previously described.[28] The number of events obtained through the CellQuest™ program, corresponding to the number of cells, was $1x10^4$ for the annexin and propidium iodide (AV/IP) double labelling and $2.5x10^4$ for cell cycle study. The analysis and quantification of the information was made using a specific software that processes in a dedicated computer (Paint-a-Gate 3.02, Macintosh Software).

Antimicrobial Activity

Fresh stock solutions of salen ligands **2a-c** and copper(II) complexes **3a-c** were prepared in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/mL (eq. to 15 mM for **2a**; 14 mM for **3a**; 20 mM for **2c**; 18 mM for **3c**; 24 mM for **2b**; 21 mM for **3b**).

The bacteria and fungi used in this study belong to the collection of the Institute of Microbiology, FMUC and were obtained from clinical samples with the exception of *Enterococcus faecalis*, that was purchased from American type culture collection (ATCC® 29212™).

The antibacterial activity of all compounds was tested against two Gram-positive bacteria - *S. aureus* and *E. faecalis* - and two Gram-negative bacteria - *E. coli* and *P. aeruginosa* - according to standard M07-A10 of the Clinical Laboratory Standards Institute.[29] Briefly, overnight cultures of each strain were transferred into a tube containing 2 mL of normal saline (NaCl 0.9%), until the turbidity standard 0.5 McFarland.

A 96-well microtiter plate was inoculated with 100 µl of tested compounds ten-fold serial diluted in Muller-Hinton broth medium (over the range 1 ng/mL to 100 µg/mL) and 10 µL of the bacterial suspensions at a final concentration of $5x10^4$ CFU/well. To ensure that DMSO had no effect on microbial growth, a control test was performed with Mullher-Hinton medium supplemented with DMSO. Plates were incubated at 37 ºC for 24 h and the bacterial growth was determined by absorbance at 600 nm using a microplate reader (SpectraMax® Plus384, Marshall Scientific, Hampton, EUA). The minimum inhibitory concentration (MIC) is defined as the lowest concentration of the compounds at which the microorganisms do not demonstrated visible growth. All the determinations were done in triplicate.

The decimal reduction ratio (RD) of the microbial population was determined by aspirating 0.1 mL of suspensions from the wells which did not show any growth after incubation during MIC assays and plated on Columbia agar medium followed by counting the CFU after 18-24 h of incubation at 37 ºC. RD was calculated according the formula:

$RD = \log N_0/N$ [30]

wherein N_0 is the CFU of untreated bacteria and N the CFU after antimicrobial treatment. A condition that causes one log10 reduction of the initial population corresponds to 90% kill of the initial population.[31]

The antifungal activity was evaluated against two yeasts, *C. albicans* and *C. glabrata*, by M44.P standard of the National Committee for Clinical Laboratory Standards[32] using the paper disk diffusion method in Mueller-Hinton agar supplemented with 2% glucose and methylene blue and against two filamentous fungi, *Asp. fumigatus* and *Alt. alternata*, according to the method described by EUCAST standard 9.3.1 (2017) using the microdilution assay in RPMI medium.

Statistical Analysis

Statistical analysis was performed using the IBM™ SPSS™ 24.0 software (IBM Corporation, USA). Normality of the quantitative variable distribution and variance homogeneity was assessed using Shapiro-Wilk and Levene tests, respectively. Student T-test or one-factor ANOVA were used in parametric analysis and Mann-Whitney or Kruskal-Wallis, in non-parametric analysis. For more than 2 groups, post-hoc analysis was also made using the Games-Howel test (in case of variances homogeneity) and using the Bonferroni correction (without variances homogeneity). A *P* value of less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Synthesis of the Salen Ligands and their Metal Complexes

(*1R,3S*)-1,3-diamino-1,2,2-trimethylcyclopentane (**1**) was obtained from (*1R,3S*)-camphoric acid in a simple one-step procedure.[18] Subsequent condensation of (**1**) with 2,5-dibromosalicylaldehyde, 5-chlorosalicylaldehyde or 2,5-dichlorosalicylaldehyde in ethanol, in the presence of activated silica gel, under ultrasound irradiation for 30 minutes at room temperature, gave the halogenated salen ligands **2a-2c**, Scheme 1.

Scheme 1.

The reaction of ligands **2a-2c** with $Cu(OAc)_2 \cdot H_2O$ under methanol reflux, originated the corresponding copper complexes **3a-3c**, respectively.

Mass spectrometry and elemental analysis are in agreement with a 1:1 metal:ligand stoichiometry for all three complexes synthesized.

3.2. Nuclear Magnetic Resonance Studies

A study of the structure of the salen ligands and complexes was carried out by NMR using the tetrachlorinated derivatives **2c/3c** as models, Scheme 2.

Scheme 2.

Assignments were made using homonuclear and heteronuclear 2D correlations (Figures S1-S4, supplementary material). The ^{1}H and ^{13}C NMR spectra of **2c/3c** were obtained in $CDCl_3$-d_1 solution, Figures 2 and 3, respectively. The complete ^{1}H and ^{13}C NMR spectral parameters are given in Tables 1 and 2, respectively. ^{1}H NMR chemical shifts together with ^{13}C and metal ion NMR can provide valuable structural information, including the type of metal center and nature of ligand coordination present in the complexes. Broadening and/or coordination induced shifts of the ^{1}H and ^{13}C signals of the ligand in the presence of the metal ions, compared with those of the free ligand, can provide clear indications of metal-ligand binding sites, as illustrated in our previous studies of the complexation between various metal ions and relevant ligands,[33]–[41] including the paramagnetic copper(II)[38] and chromium(III)[37] ions.

Figure 2.

Figure 3.

Table 1.

Table 2.

The complex is paramagnetic and, because of the rapid nuclear relaxation induced by the paramagnetic properties of the Cu(II) metal ion, ^{1}H NMR signals of bound ligands (inner sphere) are broadened and/or shifted to lower and higher frequencies (Figure 2 and Table 1). The large widths of the bound ligand ^{1}H resonances (metal ion inner sphere) of the complex preclude an unambiguous assignment of some of the signals. The probable assignment for ^{1}H signals of the bound ligand is presented in Table 1. The

large shifts and linewidths observed for the signals from the ^{1}H nuclei near the binding sites of the ligand (H-3, H-9 and H-16), together with the severe broadening of the signals attributed to the protons OH-11 and OH-18 suggest that the ligand is coordinated to the metal ion by the N atoms and the deprotonated OH groups; the complex is assumed to have a metal:ligand stoichiometry of 1:1. These findings are in agreement with similar effects observed in previous studies on the paramagnetic 8-hydroxyquinoline complex of Cr(III)[37] and other mononuclear complexes of the Cu(II) metal ion.[38] Accordingly, the ^{13}C NMR signals of the ligand in the complex are broadened (Figure 3), spanning the ranges from 8.5 to 60.8 ppm and from 129.0 to 149.0 ppm, in the aliphatic and in the aromatic regions, respectively. Both the N=C-H-9 and N=C-H-16 carbon nuclei are shifted and broadened and the COH-11 and COH-18 signals are not detected, probably because of severe broadening. These findings are in complete agreement with the proposed binding sites based on the ^{1}H NMR spectra.

Shifts as a result of ligand binding to paramagnetic metals may come either from through-space dipolar interactions (pseudo-contact), or from direct delocalization of unpaired electron spin-density from the metal (contact shift). The Cu(II) ($3d^9$) metal ion in the complex will have both contact shift and pseudo-contact shift contributions, resulting in very broad signals for mononuclear complexes. In contrast, binuclear copper(II) complexes show relatively sharp NMR line widths which are two orders of magnitude narrower than the mononuclear analog.[42] The observed broad signals support the hypothesis that the copper(II)/ligand complex is a mononuclear CuL species.

3.3. Theoretical Studies

Geometries

To provide something to gauge the properties of the halogenated complexes against, it was decided to include in the analysis the Cu(II)complex of the non-halogenated ligand along with complexes **3a-c**. Initial geometry optimizations began with the Cu(II)ion in an octahedral complexation geometry with the four ligand N and O atoms forming the central plane and two water molecules filling the upper and lower positions. The result of optimization of this complex is shown in Figure 4 (left) where it can be seen that the lower aquo ligand has been ejected from the metal center and relocated to a position hydrogen-bonded to the two ligand oxygen atoms.

This water molecule was removed and the system re-optimized to give the structure shown in Figure 4 (right). Loss of the lower water had essentially no effect on the structure of the complex and in particular the metal environment suggesting that in this complex the preferred metal environment is square-pyramidal.

Figure 4.

In order to investigate the stability of the square pyramidal aquo complex an ab-initio molecular dynamics (AIMD) simulation was performed. Figure 5 shows two snapshots from the AIMD simulation. At 100 fs the axial water ligand has already dissociated from the metal center and is moving away from the complex. 200 fs into the simulation the water has moved further away and the remaining complex has lost its symmetry and the metal center is transitioning towards a distorted square planar geometry. Subsequent removal of the dissociated upper water molecule and re-optimization of the remaining complex resulted in only minor changes to the geometry

Figure 5.

Initial geometry optimizations for octahedral **3a-c** with two aquo ligands showed the same ejection of the lower water. Repeats of the AIMD simulations for all complexes were not feasible with the available computational hardware, however given the evidence that the preferred coordination geometry of the non-halogenated Cu(II)complex excludes water ligands it was decided to optimize the geometries of **3a-c** without explicit waters and only using the SMD continuum model. The results of the optimizations are shown in Figure 6. The non-halogenated geometry (fully re-optimized without aquo ligands) is included for reference.

Figure 6. Optimized geometries of the salen-Cu(II) complexes.

Electronic Properties

The electronic properties of the Cu(II) complexes were investigated using the previously optimized geometries for **3a-c** and the non-halogenated complex. CHELPG charges for the Cu(II) center and the four phenyl ring substituent atoms at the halogen substitution positions are shown in Table 3 (see Scheme 2 for atomic numbering).

Relative to the non-halogenated complex, the positive charge on Cu(II) is very slightly reduced in the tetrabrominated **3a** whereas in both of the chlorinated complexes **3b** and **3c** the positive change increases suggesting increased electron-withdrawing power in the chlorine-substituted phenyl moieties. The four hydrogens at positions 14, 12, 19 and 21 (this ordering represents moving from left to right in the figures) in the non-halogenated complex carry positive charges of similar magnitude suggesting that a reasonably uniform charge distribution would be presented to any other molecular species interacting with this part of the complex.

This reverses to being negative in all four positions in complexes **3a** and **3c** and due to the much greater size of the halogens compared with adjacent hydrogen atoms this suggests a uniform negatively charged surface on the edges of the phenyl rings which would lead to very different behavior in intermolecular interactions than would be expected for the non-halogenated complex. Complex **3b**, having only two chlorine substituents, displays a more oscillatory charge distribution amongst the four phenyl positions considered with larger positive and negative charges than seen in any of the other complexes.

Table 3.

Several global electronic properties for the complexes are shown in Table 4. The energies of the open-shell singly-occupied molecular orbital (SOMO) and the lowest unoccupied molecular orbital (LUMO) can be seen to be relatively insensitive to the various halogen substitutions, however each of the halogenated complexes display values that are significantly more negative than those of the non-halogenated complex. This behavior is mirrored in the gap energy (E_{gap}) with a reduction in E_{gap} relative to the non-halogenated complex suggesting enhanced reactivity in the case of the halogenated complexes.

The total electric dipole moment $\langle\mu\rangle$ undergoes a small increase on going from the non-halogenated complex to the dichlorinated **3b** but can only be expected to produce a small change in the polarity of the complex. However, in both of the tetrahalogenated complexes **3a** and **3c** a much larger increase of approximately 50% is seen indicating significantly more polar character for these complexes.

The orientationally-averaged polarisability, $\langle\alpha\rangle$ also increases with the degree and type of halogen substitution. Here, an increase of 6% and 12% are seen for the dichloro- and tetrachloro-complexes, respectively indicating a stepwise augmentation of $\langle\alpha\rangle$ with the number of Cl atoms. As might be expected, the tetrabrominated complex **3a** displays the largest $\langle\alpha\rangle$ value with a 20% increase over that of that of the non-halogenated complex.

Table 4.

3.4. Biological Activity Studies

In Vitro Cytotoxic Activity

In order to determine the cytotoxicity of complexes **3a-c**, their effects on the proliferation of four human cancer cell lines were first investigated. Cell lines were incubated with increasing concentrations of the complexes (0.5 to 5 μM), and subsequently, their metabolic activity was

evaluated using MTT assay for 48 h. Cell proliferation was determined as a measure of metabolic activity relative to the control experiments. A sigmoid fitting was performed to determine the IC_{50} values. Results are presented in Figure 7 as dose-response curves.

In all human cancer cell lines, the increase in concentration of the Cu(II) complexes led to cell proliferation inhibition. Also, cell proliferation inhibition is dependent on the Cu(II) complex tested. Tetrachlorinated Cu(II) complex **3c** has the strongest anti-proliferative activity against HCC1806, MCF-7 and WiDr cells. Cell proliferation inhibition in LS1034 cell line was more evident when cells were treated with the dichlorinated Cu(II) complex **3b**.

Figure 7.

Table 5 shows IC_{50} values for the three complexes studied. Included in the table, for comparative purposes, are the results of the analogous non-halogenated and dibrominated complexes previously studied by us.[10] Complex **3c** is the most cytotoxic against three of the four cell lines studied, with IC_{50} values at the submicromolar range of 0.66 μM for HCC1806, 0.63 μM for MCF-7 and 0.65 μM for WiDr. Complex **3b** has an IC_{50} value of 0.94 μM for LS1034, being the lowest value for this cell line.

The effect of the presence of halogen atoms in the structures of the Cu (II) complexes is evident, since the halogenated complexes have an antiproliferative effect 3 to 6 times more efficient than the non-halogenated Cu (II) complex previously studied. This may be due to the lipophilic characteristics that this type of atoms can confer to the metal complexes, thus facilitating the cell membrane permeability and consequent interaction with the biological target. The type of halogen may also be important, since the chlorinated structures generally have a more expressive antiproliferative effect, presenting lower IC_{50} values than the brominated ones. Also, the influence of the number of chlorine atoms in the complex on the antiproliferative capacity of tumor cells is evident, when the results of complexes **3b** and **3c** are compared. The presence of 4 chlorine atoms promoted a higher inhibition of cell proliferation compared to the presence of 2 chlorine atoms (with the exception of LS1034 cell line). On the other hand, the influence of the number of bromine atoms is not as obvious: the tetrabrominated complex **3a** only presented higher antiproliferative effect than the dibrominated Cu(II) complex in LS1034 and HCC1806 cell lines.

Table 5.

Comparison of IC_{50} values of reference drugs currently used in conventional chemotherapeutic protocols, with our most efficient complex, **3c**, Table 6, prove this complex to be very promising. It shows a significantly more powerful cytotoxic effect than 5-FU, oxaliplatin and irinotecan against

WiDr cells. A more effective cytotoxicity is also observed with **3c** than with 5-FU and irinotecan against LS1034 cells and the complex is more potent than cisplatin against MCF-7 cells.

Being **3c** the most promising complex, the antiproliferative activity of the corresponding free ligand **2c** was evaluated, in order to ascertain the influence of metal on the antitumor effect. Figure 8 shows the results of this study. The importance of the metal, Cu(II), is obvious since the free ligand shows little or no effect on tumor cell proliferation ($p<0.001$ for all conditions above 1 μM). This data is in agreement with our previous studies concerning the dibrominated ligand and its corresponding Cu(II) complex.[10] It also coincides with reports referring that Cu(II) plays a crucial role in antitumor activity, inducing tumor cell death through various mechanisms, among which are ROS production, DNA damage and angiogenesis inhibition.[45]

Table 6.

Figure 8.

Cell Viability and Cell Cycle Assessment

Cell viability and the types of cell death induced after incubation with two different concentrations of **3c** (IC_{50} and 5 μM) were assessed through double staining with AV-FITC/PI. From the analysis of Figure 9, it can be seen that in all cancer cell lines, as the concentration of **3c** increases, cell viability decreases. When cells are treated with 5 μM of **3c**, viable cell population decreases by 73% for HCC1806 cells, 71% for MCF-7 cells, 53% for LS1034 cells and 28% for WiDr cells ($p<0.001$ for all cell lines compared with control).

Figure 9.

The WiDr cell line was found to be the less sensitive to the complex. The reduction in cell viability was accompanied by an increase in cell death, normally by late apoptosis/necrosis and necrosis, except for LS1034 cells whose predominant type of cell death was early apoptosis. WiDr cells and HCC1806 cells die predominantly by necrosis, as indicated from the largest population of dead cells observed when they are exposed to 5 μM of **3c**.

When analyzing data from non-tumor cells, the results showed that HFF1 had no response to **3c** in IC_{50} concentration. On the other hand, with 5 μM the results show an increase in cell death, reaching 60% necrosis ($p<0.01$) and 28% late apoptosis/necrosis ($p<0.001$). Concerning the non-tumor cell line CCD-841 CoN, it had almost no noticeable effect when submitted to both **3c** concentrations, compared to an increased effectiveness on cancer cell lines WiDr and LS1034 from the same organ.

Cell cycle studies were assessed using incubation of cell lines with IC_{50} and 5 μM of **3c** through staining with IP/RNAase. Figure 10 shows that **3c**, in a concentration of 5 μM, induced a significant increase of the apoptotic peak in MCF-7 ($p<0.05$) and HCC1806 ($p<0.05$) cell lines.

Figure 10.

In addition to the increase of the apoptotic peak, results also show a cell cycle blockage in the S phase regarding MCF-7 ($p<0.01$) cell line, also observed with the IC_{50} concentration in HCC1806 cells. No significant and biologically relevant differences were obtained after incubation of both colorectal tumor cell lines with **3c**.

Metal complexes are described as being capable of inhibiting enzymes, interacting with intracellular biomolecules, enhancing lipophilicity, altering the cell membrane functions, and blocking the cell cycle and other functions.[8] The ability of copper complexes to interact with DNA is well documented. However, recent studies indicate other cell constituents as possible targets, namely topoisomerases and the proteasome multiprotein complex.[13] In fact, the almost absence of alterations in cell cycle analysis of treated cells reveals that DNA may not be the main target of our halogenated Cu(II) complexes. The antitumor activity of **3c** was clearly demonstrated by the evidence of the apoptotic peak in cell cycle analysis, along with the induction of cell death by apoptosis and/or necrosis, dependent on the cell line. Although **3c** was effective in inhibiting cell proliferation of both colorectal and breast tumor cells, cell viability results showed that breast cancer cells are the most sensitive to the compound. Other authors also reported that Cu(II) complexes with chiral Schiff-base ligands are capable of activating different mechanisms of apoptosis in different cell lines.[4][46][47] Another advantage of **3c** is its selectivity towards the colorectal cancer chemoresistant cell line (LS1034) when compared to the results obtained with the non-tumor cell line (CCD-841 CoN).

In Vitro Antimicrobial Activity

Over the years, it has been shown that copper has the ability to kill bacteria, yeast and viruses, with the assumption that copper ions are released, leading to a disruption of the bacterial cell membrane and causing cell death. The entry of these ions into the bacteria leads to the production of reactive oxygen species (ROS) and the degradation of plasmid and genomic DNA.[48]

Complexes **3a-c** as well as the corresponding free salen ligands **2a-c** were screened for their antibacterial activity against *S. aureus*, *E. faecalis*, *E. coli* and *P. aeruginosa* and for their antifungal effect against *C. albicans*, *C. glabrata*, *Asp. fumigatus* and *Alt. alternata*. The tests were carried out at different concentrations, between 1 ng/mL to 100 μg/mL.

In the presence of free ligands **2a-c** and copper complexes **3a-c** no significant antifungal activity was observed against the fungal species tested.

Antibacterial activity was observed with **2a-c** and **3a-c**, as indicated by the minimal inhibitory concentration (MIC) and decimal reduction ratio (RD) values obtained, Table 7. **2a** and **3a** recorded the highest MIC values (100 µg/mL). **2b** and **3b** exhibit a MIC value of 1 µg/mL against all the tested bacteria. **2c** and **3c** display a MIC value of 1 µg/mL against *S. aureus* and *P. aeruginosa* and a MIC value of 0.1 µg/mL against *E. faecalis.* Regarding the *E. coli* only **2b** exhibits antibacterial activity (MIC= 1 µg/mL).

Table 7.

In relation to the decimal reduction ratio, the highest activity was found for *P. aeruginosa* with a decrease of 99.99999% (RD 7.95- 7.22) of the initial population due to compounds **3c**, **2c**, **3b** and **2a** and a 99% (RD 2.4-1.88) decline in response to the treatment with **2b** and **3a**. For *S. aureus* a remarkable effect was observed with a decrease of 99.999% (RD 5.3) of the initial population when treated with **2a**; a reduction of 99% (RD 2.85) after the treatment with **3b** and a decrease of 90% (RD 1-0.96) with **2c** and **3a**. Concerning *E. faecalis* a moderate effect was observed corresponding to a reduction of 99% (RD 2.22) of the initial population under the action of the compound **3b** and a diminishment of 90% (RD 1.5) after the treatment with **2c**.

From the *in vitro* antimicrobial assays it can be concluded that the tested compounds exhibit mild to strong antibacterial activity. In fact, among the bacteria studied, the most remarkable effect was observed in *P. aeruginosa*, followed by *S. aureus*. Only in *E. coli* was no antimicrobial activity found. Our results are corroborated by several works that evaluated the antimicrobial efficacy of copper complexes and their corresponding free ligands. In the studies of Gaballa, Anacona and colleagues and Khan *et al*., good antibacterial activity was reported for both Gram positive and Gram negative bacteria.[49]–[51]

Similarly to our results, Azam and colleagues observed insignificant antifungal effect with copper complexes and free ligand.[52] This same result was also described by Khanmohammadi *et al.* that in addition also reported no activity against *E. coli*.[53]

The metal complex **3b** shows higher bactericidal activity than its free ligand **2b**, probably due to stronger lipophilic properties of the metal complexes, which can be explained on the basis of Tweedy's chelation theory and Overtone´s concept.[51] According to Tweedy's theory,[54] chelation could favor the lipophilic nature of the central metal, which consequently increases its permeation through the lipid membrane of the bacterial cell, thus blocking the metal binding sites on enzymes leading to impairment of some normal biological processes. In Overtone´s concept of cell

permeability, the lipid membrane that surrounds the bacterium favors the passage of molecules which are mostly soluble in lipids.[55]

Regarding complex **3a** and the corresponding free ligand **2a,** we found that the latter clearly has greater antibacterial activity than the corresponding copper complex. This has also been observed by Anacona and colleagues,[50] which suggests a mechanism of action independent of the increased ability to cross biological membranes. One hypothesis is that the antimicrobial activity may be linked with the ability of chemical compounds to generate reactive oxygen species which can damage the DNA.[56]

4. Conclusions

Herein the synthesis of three new halogenated salens derived from camphoric acid and the corresponding copper (II) complexes is described.

NMR studies using the tetrachlorinated derivative allowed for a better understanding of the structures of both the free ligands and their complexes. The obtained results suggest that the ligand is tetracoordinated to the copper ion by the two nitrogen and the deprotonated OH groups, adopting a 1:1 metal:ligand stoichiometry.

Theoretical studies established some geometrical and electronic characteristics of the complexes, which vary according to the type and degree of halogen substitution. The complexes are proposed to have a distorted square planar geometry. Enhanced biological activity appears to be correlated with increases of dipole moment and polarizability and reduction in the SOMO-LUMO gap in the Cu(II) complexes. Halogenation causes the edges of the ligand phenyl rings to become more negatively charged, which also corresponds to increased activity. Whilst the geometry and electronic properties of **3a** and **3c** do not differ greatly, their biological activities are significantly different suggesting that the increased steric bulk of the four bromine atoms in **3a** compared with the chlorines in **3c** may alter some aspect of the binding properties of the complex in an adverse way.

The cytotoxicity of the copper complexes was evaluated against colorectal WiDr and LS1034 and against breast MCF-7 and HCC1806 cancer cell lines. The halogenated complexes were found to be very efficient, the most promising being the tetrachlorinated Cu(II) complex with IC_{50} of 0.63-1.09 μM for the cell lines studied. Additionally, the complex shows selectivity to colorectal cancer cells compared to non-tumor colon cells. cells. The antitumor effect exhibited by this complex is significantly more powerful than the reference drugs currently used in conventional chemotherapy.

Antimicrobial activity against four bacterial species-*Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa*-and four fungal species-*Candida albicans*, *Candida*

glabrata, *Aspergillus fumigatus* and *Alternaria alternata* was also evaluated for the complexes and the free ligands. The results showed that the compounds exhibit moderate to strong antibacterial activity.

The results of our studies allowed for a better structural understanding of the halogenated salens and the corresponding copper complexes synthesized as part of this work. The results of the biological studies, both antitumoral and antimicrobial, are very promising, justifying additional studies and the potential use of copper-based chemotherapeutics as valid alternatives for cancer treatment.

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Table 1. ^{1}H NMR parameters[a] for dichlorinated salen **2c** and its corresponding complex **3c** (298 K)

	CH_3-6	CH_3-7	CH_3-8	CH_2-4	CH_2-5	CH-3	OH-11	OH-18	N=C-H-9/ N=C-H-16	C-H-13/ C-H-20	C-H-15/ C-H-22
Ligand											
δ	1.36	1.02/	0.98	2.05/2.25	2.34/1.91	3.68	11.38	9.87	8.27	7.21	7.44
Cu(II) complex[b]											
δ	1.26	0.99/	0.97	0.55/ -	1.91/-1.01	2.51	-[c]	-[c]	10.56[d]	7.29	7.31/
Δδ	-0.10	-0.03	-0.01	-1.50/ -	-0.43/-2.92	-1.17			2.29	0.08	-0.13

	$\lvert J_{5a/5b}\rvert$	$\lvert J_{4a/4b}\rvert$	$J_{4a/5a}$ /	$J_{4a/5b}$	$J_{4b/5a}$ /	$J_{4b/5b}$	$J_{3,4a}$	$J_{3,4b}$
Ligand								
	15.4	15.3	9.3	5.9	8.1	4.2	8.5	8.5
Cu(II) complex[b]								
	-[d]	-[d]	-[d]	-[d]	-[d]	-[d]	-[d]	-[d]

[a]δ values, in ppm, relative to Me_4Si as internal reference, using the solvent $CDCl_3$ (δ_H=7.27); *J* values in Hz. [b]complex synthesized according to the experimental procedure. [c]not detected. [d]broad.

Table 2. ^{13}C NMR parameters[a] for dichlorinated salen **2c** and its corresponding complex **3c** (298 K)

	CH_3-8	CH_3-7	CH_3-6	CH_2-5	CH_2-4	CH-3	C-1	C-2	N=C-H-9	N=C-H-16
Ligand										
δ	18.91	20.73	24.41	33.82	27.90	75.55	70.89	48.59	162.59	160.27
Cu(II) complex[b]										
δ	8.52/18.48/28.56/38.91/44.82/49.34/60.82								174.54/162.78[c]	
Δδ									11.95/	2.51

	C-10	C-17	CH-15	CH-22	CH-13	CH-20	CCl-14	CCl-21	CCl-12	CCl-19	COH-11	COH-18
Ligand												
δ	119.30	121.42	132.38	131.10	129.23	129.00	156.88/	157.89	156.26	155.94	195.03	195.03
Cu(II) complex[b]												
δ	129.04/148.96/162.54/174.54										-[d]	-[d]

[a]δ values, in ppm, relative to Me_4Si as internal reference, using the solvent $CDCl_3$ (δC=77.23). [b]complex synthesized according to the experimental procedure. [c]broad. [d]not detected.

Table 3. CHELPG charges for Cu(II)and the four halogen-substitution positions (see Scheme 2 for numbering; these refer to hydrogens when no halogen is present).

Complex	Cu(II)	14	12	19	21
Non-halogenated	0.63	0.16	0.18	0.18	0.16
3a	0.62	-0.12	-0.07	-0.07	-0.13
3b	0.66	-0.16	0.20	0.20	-0.16
3c	0.66	-0.14	-0.09	-0.10	-0.15

Table 4. Frontier orbital energies (eV), dipole moment ⟨μ⟩ (Debye) and isotropically averaged polarisability ⟨α⟩ (atomic units).

Complex	E_{SOMO}	E_{LUMO}	E_{gap}	⟨μ⟩	⟨α⟩
Non-halogenated	-4.62	-2.28	2.35	11.19	525
3a	-4.80	-2.58	2.23	15.87	633
3b	-4.70	-2.43	2.26	12.00	559
3c	-4.77	-2.55	2.22	15.89	591

Table 5. IC_{50} values obtained for the four tumor cell lines after incubation with Cu(II) complexes **3a-c** and the corresponding r^2 (coefficient of determination of the fitted curves).

Cell line	Non-halogenated Cu(II) complex [14]		Dibrominated Cu(II) complex [10]		**3a**		**3b**		**3c**	
	IC_{50} (μM)	*r^2*	*IC_{50} (μM)*	*r^2*	*IC_{50} (μM)*	*r^2*	*IC_{50} (μM)*	*r^2*	*IC_{50} (μM)*	*r^2*
WiDr	4.29	0.97	1.24	0.95	1.48	0.96	1.64	0.90	0.65	0.99
LS1034	--	--	2.32	1.00	1.52	0.96	0.94	0.98	1.09	0.94
MCF-7	4.66	0.99	0.95	0.97	1.26	0.99	1.07	0.98	0.63	0.89
HCC1806	--	--	1.28	0.75	1.07	0.99	0.82	0.97	0.66	0.94

Table 6. IC_{50} values obtained for WiDr, LS1034 and MCF-7 cell lines after incubation with tetrachlorinated Cu(II) complex **3c** and drugs used in conventional chemotherapy for 48 hours, and the corresponding r^2 (coefficient of determination of the fitted curves).

Cell line	**3c**		Conventional chemotherapy	
	IC_{50} (μM)	*r^2*	*IC_{50} (μM)*	*r^2*
WiDr	0.65	0.99	442.4[a] 21.0[b] 38.7[c]	> 0.90 > 0.90 > 0.90
LS1034	1.09	0.94	238.0[a] 5.9[b] 47.5[c]	> 0.90 > 0.90 > 0.90
MCF-7	0.63	0.89	32.5[d] 0.32[e]	- 0.93

[a]incubation of WiDr and LS1034 cells with 5-FU for 48h;[43] [b]incubation of WiDr and LS1034 cells with oxaliplatin for 48h;[43] [c]incubation of WiDr and LS1034 cells with irinotecan for 48h;[43] [d]incubation of MCF-7 cells with cisplatin for 48h;[44] [e]incubation of MCF-7 cells with epirrubicin for 48h.[10]

Table 7. MIC (μg/mL) of tested compounds and RD of the microbial population.

Microorganism	**2a**		**3a**		**2b**		**3b**		**2c**		**3c**	
	MIC	*RD*	*MIC*	*RD*	*MIC*	*RD*	*MIC*	*RD*	*MIC*	*RD*	*MIC*	*RD*
S. aureus	10	5.3	100	0.96	1	0.56	1	2.85	1	1	1	0.11
E. faecalis	100	0.7	100	0.43	1	0.8	1	2.22	0.1	1.5	0.1	0.41
E. coli	NE*		NE*		1	0.06	NE*		NE*		NE*	
P. aeruginosa	100	7.95	100	1.88	1	2.4	1	7.88	1	7.22	1	7.57

*NE= No effect