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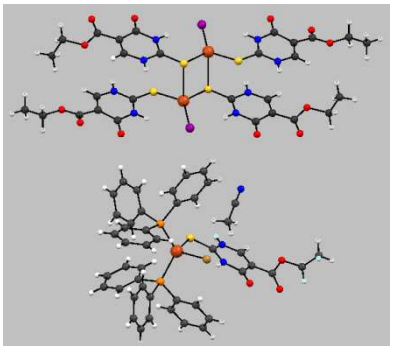
Graphical Abstract



5-carbethoxy-2-thiouracil as ligand in new dinuclear and mononuclear copper(I) halide complexes of composition $[CuX(eitotH_2)_2]_2$ and $[CuX(eitotH_2)(PPh_3)_2]$ respectively, with remarkably high *in vitro* cytotoxic activity against human pulmonary carcinoma cells, human fetal lung fibroblast and human epithelial carcinoma cells.

Highlights

- New copper(I) complexes containing 5-carbethoxy-2-thiouracil and triphenylphosphine.
- Remarkable *in vitro* cytotoxicity against two carcinoma cell lines.
- The mononuclear complexes possessing triphenylphosphine are highly cytotoxic.
- The phosphine-free dicopper(I) complexes are only moderately cytotoxic.

Copper(I) halide complexes of 5-carbethoxy-2-thiouracil: Synthesis, structure and *in vitro* cytotoxicity

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Abstract

5-carbethoxy-2-thiouracil (eitotH_2) reacts with copper(I) halides CuX (X = Cl, Br, I) to give dinuclear complexes of the formula [CuX(eitotH_2)$_2$]$_2$ while mononuclear mixed-ligand complexes of the formula [CuX(PPh_3)$_2$(eitotH_2)] result when the reactions are performed in the presence of two equivalents of triphenylphosphine (PPh_3). The molecular structures of representative compounds from each of the above types of complexes, namely [CuI(eitotH_2)$_2$]$_2$, [CuCl(PPh_3)$_2$(eitotH_2)] and [CuBr(PPh_3)$_2$(eitotH_2)] have been established by single-crystal X-ray diffraction. The new copper(I) complexes were evaluated for *in vitro* antitumor properties against two tumor cell lines, A549 (human pulmonary carcinoma cell line) and HeLa (human epithelial carcinoma cell line) and one normal immortalized cell line MRC5 (human fetal lung fibroblast). The mixed-ligand complexes possessing triphenylphosphine were found to be highly cytotoxic in contrast to the phosphine-free ones which inhibited cell proliferation only in relatively high concentrations.

Keywords: Copper(I); 5-carbethoxy-2-thiouracil; triphenylphosphine; cytotoxicity.

1. Introduction

The biochemical key role of copper both as an essential trace metal bound to several metalloenzymes and as a constituent of exogenously administered compounds in humans – mainly in form of complexes that can interact with biomolecules – is well known. Numerous copper enzymes and proteins, many of them containing a bimetallic copper center in their active site, play crucial roles in biological systems [1]. Thus, bimetallic copper complexes are of particular interest, since they can serve as models for a number of important biological systems. For example, the role of dicopper sites in electron transfer processes in the biological binding, activation and reduction of dioxygen is a subject of intense research during the last two decades [2–5]. Current interest in copper complexes includes their potential use as antimicrobial [6–8], anti-inflammatory [9, 10] and antitumor [11–14] agents, however, most of the compounds tested are copper(II) complexes. Similar studies on copper(I) derivatives are less widespread, and the complexes normally contain planar aromatic chelating ligands [15–17] or ligands capable of stabilizing the low oxidation state of the metal ion in aqueous media [18].

Orotic acid (6-carboxyuracil) is a biologically very important molecule, being the only effective precursor of the pyrimidine bases for nucleic acid formation [19, 20]. For this reason, orotic acid plus its derivatives, and metal complexes, have been the subject of intensive studies [21–24]. On the other hand, relatively less attention has been paid to the coordination chemistry of isoorotic acid (5-carboxyuracil), despite its anticancer, antibacterial and antihypertensive properties [25, 26]. This is even more remarkable when the notable and promising results of the few related studies are considered, for example, antibacterial activity was found for a copper(II) complex of isoorotic acid [27], while a platinum(II) complex of ethyl-isoorotate showed antitumor, antibacterial and antiviral properties [28].

Some reports have been concerned with the biological evaluation of not only thiolated orotic acid derivatives [29–31] but also coordination compounds with 2-thioorotic acid as ligand [32–34]. On the other hand, only a small number of reports are related to thioisoorotic acid and its derivatives. These are quite limited to some structural and biological studies on first row transition metal complexes containing 2-thioisoorotato ligands that appeared about two decades ago [35–38].

With these considerations in mind, we decided to include 5-carbethoxy-2-thiouracil (eitotH_2) [shown in Scheme 1], in our investigations. In preferring the ester instead of the free 5-carboxy-2-thiouracil we intended to ensure the well-known thione-S coordination to the soft copper(I) ion avoiding any possible side effects, for example, oxidation of the metal. Despite its biological importance [39], 5-carbethoxy-2-thiouracil has received little attention so far, as there are practically no references about its coordinating behavior.

In this work we report the synthesis and characterization of copper(I) halide complexes $[CuX(eitotH_2)_2]_2$ and $[CuX(eitotH_2)(PPh_3)_2]$ and the structural characterization of representative compounds of each type. Further we investigated the cytotoxic activity of the new compounds against two tumor cell lines, A549 (human pulmonary carcinoma cell line) and HeLa (human epithelial carcinoma cell line) and one normal immortalized cell line MRC5 (human fetal lung fibroblast).

(Scheme 1)

2. Experimental section

2.1. Materials for the Synthesis and Instrumentation

Commercially available copper (I) halides, triphenylphosphine and 5-carbethoxy-2-thiouracil were purchased as reagent grade and were used as supplied, whereas the solvents were purified according to standard procedures. Infra-red spectra in the region of 4000–200 cm^{-1} were obtained in

KBr discs with a Nicolet FT-IR 6700 spectrophotometer, while a Shimadzu 160A spectrophotometer was used to obtain the electronic absorption spectra. Melting points were determined in open tubes with a STUART scientific instrument and are uncorrected.

2.2. Synthesis

*Synthesis of complexes **1**–**3**:* To a solution of (0.5 mmol) of the copper(I) halide (49 mg for CuCl, 71.7 mg for CuBr, 95.2 mg for CuI) in 30 cm^3 of dry acetonitrile, a solution of 200,2 mg (1 mmol) of 5-carbethoxy-2-thiouracil in 20 cm^3 of methanol was added and the mixture was stirred for 1 h at ambient temperature. The resulting bright yellow solution was filtered off and left to stand at ambient, whereupon yellow crystals deposited which were filtered off and dried *in vacuo*.

*$[CuCl(eitotH_2)_2]_2$ (**1**):* Yellow powder (112 mg, 45 %), m.p. 270 ^oC; Anal. Calc. For $C_{28}H_{32}Cu_2Cl_2N_8O_{12}S_4$: C, 33.67; H, 3.23; N, 11.22. Found: C, 33.47; H, 3.12; N, 11.08. IR (cm^{-1}): 3119m, 3064m, 2934m, 1715vs, 1747vs, 1617s, 1562vs, 1464s, 1372m, 1293s, 1140vs, 1061m, 1010m, 802s, 746m, 584s; UV-Vis (λ_{max}, log ε): 264 (4.23), 311 (4.37).

*$[CuBr(eitotH_2)_2]_2$ (**2**):* Yellow crystals (207 mg, 70 %), m.p. 271 ^oC; Anal. Calc. For $C_{28}H_{32}Cu_2Br_2N_8O_{12}S_4$: C, 30.92; H, 2.97; N, 10.30. Found: C, 31.17; H, 3.03; N, 10.18. IR (cm^{-1}): 3142m, 3050m, 2999m, 1733vs, 1622vs, 1552vs, 1529vs, 1460s, 1395s, 1302vs, 1210vs, 1145vs, 1010m, 886m, 792m, 602s, 510m; UV-Vis (λ_{max}, log ε): 263 (4.41), 310 (4.67).

*$[CuI(eitotH_2)_2]_2$ (**3**):* Yellow crystals (221 mg, 75 %), m.p. 274 ^oC; Anal. Calc. For $C_{28}H_{32}Cu_2I_2N_8O_{12}S_4$: C, 28.46; H, 2.73; N, 9.48. Found: C, 28.55; H, 2.78; N, 9.41. IR (cm^{-1}): 3129m, 3050m, 2911m, 1733vs, 1617vs, 1556vs, 1524vs, 1460vs, 139v0s, 1302vs, 1210vs, 1176s, 1149vs, 1010m, 886m, 866s, 792s, 602vs, 593vs, 514vs; UV-Vis (λ_{max}, log ε): 264 (3.94), 311 (4.11).

*Synthesis of complexes **4**–**6**:* To a solution of (0.25 mmol) of the copper(I) halide (24.5 mg for CuCl, 35.8 mg for CuBr, 47.6 mg for CuI) in 30 cm^3 of dry acetonitrile, triphenylphosphine (131

mg, 0.5 mmol) was added and the solution was stirred until a white precipitate was formed. A solution of 50 mg (0.25 mmol) of 5-carbethoxy-2-thiouracil in 20 cm^3 of methanol was added and the mixture was stirred for 1 h at room temperature. The resulting bright yellow clear solution was filtered off and kept at ambient, whereupon pale yellow crystals of the respective product deposited, which were filtered off and dried *in vacuo.*

[CuCl(PPh$_3$)$_2$(eitotH$_2$)]·0.5 CH$_3$C(O)CH$_3$ ***(4)***: Yellow crystals (134 mg, 65 %), m.p. 147 ^{o}C; Anal. Calc. For $C_{43.5}H_{40}CuClN_2P_2O_{3.5}S$: C, 62.67; H, 4.85; N, 3.28. Found: C, 62.47; H, 4.82; N, 3.32. IR (cm^{-1}): 3050m, 2984m, 1752vs, 1723vs, 1695vs, 1616s, 1565, 1540 vs, 1480s, 1461vs, 1435vs, 1410s, 1369s, 1296vs, 1226vs, 1147vs, 1093vs, 1061m, 1030m, 796s, 742vs, 694vs, 590s, 517vs, 501vs; UV-Vis (λ_{max}, log ε): 262 (4.48), 310 (4.39).

[CuBr(PPh$_3$)$_2$(eitotH$_2$)]·CH$_3$CN ***(5)****:* Yellow crystals (173 mg, 80 %), m.p. 154 ^{o}C; Anal. Calc. For $C_{45}H_{41}CuBrN_3P_2O_3S$: C, 59.44; H, 4.54; N, 4.62. Found: C, 59.17; H, 4.61; N, 4.64. IR (cm^{-1}): 3148m, 3049m, 2936m, 1752vs, 1730vs, 1619s, 1566, 1556vs, 1482vs, 1458s, 1435vs, 1407s, 1367s, 1293vs, 1223vs, 1150vs, 1097vs, 1061m, 1026m, 846m, 745vs, 694vs, 587s, 517vs, 498vs; UV-Vis (λ_{max}, log ε): 263 (4.56), 308 (4.49).

[CuI(PPh$_3$)$_2$(eitotH$_2$)] ***(6)****:* Yellow powder (176 mg, 77 %), m.p. 191 ^{o}C; Anal. Calc. For $C_{43}H_{38}CuIN_2P_2O_3S$: C, 56.43; H, 4.18; N, 3.06. Found: C, 56.15; H, 4.10; N, 3.01. IR (cm^{-1}): 3050m, 2932m, 1755vs, 1736vs, 1616s, 1556vs, 1477vs, 1457s, 1435vs, 1401s, 139v0s, 1293vs, 1226vs, 1144vs, 1093s, 1024m, 886m, 846m, 799s, 742vs, 694vs, 587s, 517vs, 501vs, 489s; UV-Vis (λ_{max}, log ε): 264sh (5.11), 309 (4.88).

2.3. Crystal Structure Determination

Single crystals of **3** suitable for crystal structure analysis were obtained by slow evaporation of the CH_3CN/CH_3OH mother liquid at room temperature. X-ray diffraction data were collected on a Rigaku Saturn 724+ diffractometer by the NCS [40] using *CrystalClear-SM Expert* [41] for cell

refinement, data collection and data reduction. The structure was solved with *SHELXS97* [42] and refined with *SHELXL97*. The program used for molecular graphics was *PLATON* [43]. Tables for publication were prepared with *SHELXS97* and *WINGX* [44]. Single crystals of compound **5** suitable for crystal structure analysis were obtained by slow evaporation of the CH_3CN/CH_3OH mother liquid at room temperature, whereas compound **4** was recrystallized from acetone. For the structure determination the single crystals of **4** and **5** were mounted on a Bruker Kappa APEX II diffractometer equipped with a triumph monochromator. Both structures were solved using SUPERFLIP package [45] and refined by full-matrix least-squares method on F^2 using the CRYSTALS package version 14.40 [46]. In structure of **4** five of the six atoms of one of the phosphane phenyl rings were found disordered over two positions with equal occupation factors. The whole acetone solvent molecule was also found disordered over two positions with equal occupation factors. The disordered atoms have been refined isotropically. All the remaining non-hydrogen atoms of the main residue have been found and refined anisotropically. All hydrogen atoms of the main residue were found at their expected positions and refined using soft constraints. All hydrogen atoms connected to the disordered atoms were positioned geometrically. By the end of the refinement all hydrogen atoms were positioned using riding constraints to the parent atoms. The crystals data and some details of the data collection and structure refinement for both compounds are given in Table 1.

2.4. Materials and methods for biological tests

Materials: Culture medium DMEM (Dulbecco's Modified Eagle Medium with L-glutamin), Fetal Bovine Serum, Antibiotic-Antimycotic and PBS (pH=7, 2) were from E.U Gibco BRL. Trypsin+EDTA 1x was purchased from Invitrogen. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) powder 1g, was purchased from Invitrogen. All other chemicals used were of high purity and available from commercial sources.

Cell cultures: Human cell lines from three different sources, namely **A549** (human pulmonary carcinoma cell line), immortalized **MRC5** (human fetal lung fibroblast) and **HeLa** (human epithelial carcinoma cell line) were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal bovine serum and antibiotics penicillin and streptomycin. Cell line was maintained in continuous culture at 37^{o}C, in 5% CO_2, in a fully humidified incubator, using standard aseptic techniques and cell growth was monitored by determining the cell number/ml with the use of a Coulter counter model ZBI.

MTT cytotoxicity assay: The growth inhibitory effect towards tumor cell lines was evaluated by means of MTT assay. Cell proliferation was assessed by monitoring the conversion of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to formazan. The reduction of MTT is catalyzed by mitochondrial dehydrogenase enzyme and is therefore a measure for cell viability [47]. 1x10^4 cells /well (A549) and 5 x 10^3 cells /well (HeLa and MRC5) were seeded in 96-well plates and after 24 hrs were treated with varying concentrations (5, 10, 15, 20, 30, 40, 60 and 120 μM) of copper complexes and their uncoordinated ligands(5-carbethoxy-2-thiouracil and triphenylphosphine) which were dissolved prior in DMSO.

Cells which were not exposed to copper complexes served as the control. The complexes incubation with the cells for 48 hrs was followed by medium removal from each well and its replacement with 100 μl fresh medium and 10μl MTT 12 mM (5 mg/ml) per well. After another short incubation for 4 h at 37 °C, until the development of the purple-colored formazan product, 100 μl of SDS10%-HCl 0,01 M was added to the cells and left under the same growth conditions for 15–18 hrs.

The Organon Teknika@ Enterprise Ireland plate was used to measure the absorbance of the plates at 570 nm. Values shown are the mean values of at least three measurements. IC50 values represent the copper complexes concentrations that reduced the mean absorbance at 570 nm to 50% of those in the untreated control wells.

3. Results and Discussion

3.1. Synthesis

As expected, the interaction of heterocyclic thiones, which are versatile S- and/or N-donor ligands, with a soft Lewis acid like monovalent copper is highly flexible, resulting in the formation of a rich variety of coordination compounds ranging from mono- and dinuclear complexes to polynuclear networks. In the specific case of copper(I) halides, the result of the reactions depends on the stoichiometry and the conditions chosen, usually leading to four-coordination which is most favoured for copper(I). Although steric demands of the ligated molecules are expected to influence both the local and the overall structure of a coordination compound, during our previous work we have never noticed a decrease of the coordination number in copper(I) complexes by introducing bulky ligands. Quite to the contrary, we recently observed the formation of three-coordinated mononuclear species $[CuX(th)_2]$ for the sterically undemanding 2-thiohydantoin [48], which implies that electronic interactions should also be taken into consideration.

Further, dimerization within complexes of monovalent copper halides with heterocyclic thioamides is quite common as it allows the copper ion to obtain a pseudotetrahedral environment. This was also the case for the reactions within the present study, which in addition turned out to be independent from the ratio of reactants chosen (CuX: eitotH_2 = 1:2 or 1:3) the composition of compounds **1**–**3** being always the same, namely $[CuX(eitotH_2)_2]_2$.

Aiming to obtain phosphine/thione mixed-ligand complexes, we treated a 1:2 mixture of CuX and PPh_3 in dry acetonitrile with a methanolic solution of one equivalent of 5-carbethoxy-2-thiouracil. Stirring this mixture at 50^{o}C produced a clear yellow solution, from which, on slow evaporation, the corresponding desired microcrystalline compound was deposited. Interestingly, while compounds **4**–**6** were easily obtained and in high yield by this simple one-top two-step synthetic procedure, attempts to prepare complexes using compounds **1**–**3** as precursors remained

unsuccessful. All prepared compounds are air stable diamagnetic solids, moderately soluble in acetonitrile, chloroform or acetone.

3.2. Spectroscopy

The electronic absorption spectra of complexes **1**–**6**, recorded in acetonitrile at room temperature, show two intense broad bands with maxima at ~264 and ~310 nm. With reference to the absorption spectrum of the uncoordinated 5-carbethoxy-2-thiouracil, the high energy band can be attributed to intraligand π→π* transitions on the thione ligand, whereas the lower energy band can be considered as a thione originating CT transition at the C=S bond [49, 50], which may possess partial CT character, as it lies in the region where the free ligand absorbs, expressing a small red shift as a consequence of the coordination to copper. In the spectra of compounds **4**–**6** the absorption due to the intraligand transition within the triphenylphosphine completely overlaps with the high energy band assigned to the intraligand π→π* transitions on the 5-carbethoxy-2-thiouracil ligand, resulting in a broad band of increased intensity relatively to that observed in the spectra of compounds **1**–**3**. The absence of any d-d transitions confirms the diamagnetic nature of the complexes.

The infrared spectra of compounds 1–6, recorded in the range 4000-250 cm^{-1} contain all the characteristic bands of the 5-carbethoxy-2-thiouracil ligand with shifts indicating an exclusive S-coordination. In particular, the intense bands at 1755 cm^{-1} and 1733 cm^{-1} in the spectrum of free eitotH_2, attributed to ν(C=O) stretching vibrations, appear slightly shifted to higher energies in the spectra of all complexes. On the other hand, the very strong band at 1565 cm^{-1} known as "thioamide I" band, having contributions from ν(C–N) and δ(C–H), remains almost unshifted, but the band at 1163 cm^{-1} assigned to the "thioamide III", which involves major contributions from ν(C=S), appears clearly shifted (by ca. 20 cm^{-1}) towards lower energies upon coordination. Finally, shifts of the ν(N–H) vibration band towards lower wavenumbers, in some cases accompanied by a

broadening, indicates involvement of this group in N–H···X hydrogen bridges, a fact that is also clearly confirmed by the crystal structure investigations.

3.3. X-ray structural investigations

The structures of $[CuI(eitotH_2)_2]_2$ (**3**), $[CuCl(PPh_3)_2(eitotH_2)]$ (**4**) and $[CuBr(PPh_3)_2(eitotH_2)]$ (**5**) (details of crystal and structure refinement are given in Table 1) were established by single crystal X-ray diffraction.

Compound **3** crystallizes in the triclinic system Pī, with one discrete molecule in the unit cell. Main bond lengths and angles are given in Table 2 and a perspective drawing showing the atom numbering is shown in Figure 1. The basic structural unit is a dimer in which the two copper atoms, separated by 3.555(1) Å, are doubly bridged by two S atoms of two thione ligands to form a strictly planar four-membered Cu_2S_2 core. The highly distorted tetrahedral coordination around each copper is completed by the S atom of a terminally bonded thione ligand and one iodine atom.

(Figure 1)

(Table 2)

Each of the two *trans* positioned iodine atoms stabilize the complex with two strong intramolecular hydrogen bonds [N(1)···I(1) = 3.809(2) Å, H(1)···I(1) = 2.794 Å, N(1)–H(1)···I(1) = 169.6^o and [N(4)···I(1) = 3.636(2) Å, H(4)···I(1) = 2.76 Å, N(4)–H(4)···I(1) = 174.3^o]. Further strong intramolecular hydrogen bonds exist between pairs of bridging and terminal thione units (see Table 2), which are essentially parallel to each other.

Within the Cu_2S_2 core, there is an extremely large asymmetry in the two bridging Cu–S bonds distances [2.2598(9) and 2.9447(11) Å]. By comparison, the corresponding distances are 2.2727(9) and 2.5634(10) Å in bis[μ-S(pyrimidine-2-thione)(pyrimidine-2-thione)copper(I)bromide] [51] and 2.3348(9) and 2.4879(7) Å in bis[μ-S(pyridine-2-thione)(tmtp)copper(I)chloride] [52]. The two terminally bonded thione ligands are *trans* disposed with a Cu−S bond distance of 2.2475(9) Å, which is close to the "short" one in the Cu−S−Cu bridge. The Cu−I bond distance of 2.5089(7) Å is somewhat shorter than the values observed in dimeric copper(I) iodide compounds with double bridging sulphur atoms or in monomeric copper(I) iodide complexes bearing terminally bonded thione ligands [53, 54]. Finally, the S–Cu–S and Cu–S–Cu angles of 103.89(3) and 85.08(3)^o respectively deviate from the ideal values (109.5 and 70.5^o) predicted for symmetric dimers, reflecting the distortions induced by the intramolecular hydrogen bridges, but are close to the ideal geometry for $Y_2MX_2MY_2$ dimers according to theoretically proposed criteria [55].

Pale yellow crystals of [$CuCl(PPh_3)_2(eitotH_2)$] (4) and [$CuBr(PPh_3)_2(eitotH_2)$] (5) have been obtained by slow evaporation at room temperature of their saturated acetone and acetonitrile solutions respectively. Both compounds crystallize in the monoclinic system C2/c, with 4 discrete molecules in the unit cell. Main bond lengths and angles are given in Tables 3 and 4 and drawings showing the atom numbering are shown in Figures 2 and 3 respectively.

(Figure 2 + Figure 3)

(Table 3 + Table 4)

The geometrical characteristics of both the four-coordinate monomers generally refer to those of other copper(I) halide complexes tetrahedrally coordinated by thione and phosphine ligands. Three of the interbond angles around the central copper atom deviate moderately from the idealized tetrahedral geometry while the P1–Cu1–P2 angle is opened up to a value of 122.71(3)^o and 123.47(3)^o in **4** and **5** respectively, reflecting the steric imposition of the bulky phosphine ligands.

The Cu–S distance of 2.4083(8) Å and 2.3931(8) Å are quite large, but the Cu–Cl and Cu–Br bond lengths as well as the two individual Cu–P distances in both complexes are comparable to the corresponding values previously found in related four coordinate complexes with terminal chlorine, bromine and thione-S donors [41, 56, 57].

3.4. Cytotoxicity assay

The antitumor activity of the compounds has been estimated by assessing their ability to inhibit the growth of the tumor cells in the culture medium DMEM (Dulbecco's Modified Eagle Medium with L-glutamin) supplemented with 10% fetal bovine serum and the antibiotics penicillin and streptomycin. The copper compounds **1**–**6** and the corresponding free ligands eitotH_2 and PPh_3 were subjected to MTT assay and their cytotoxic properties were investigated against a panel of two tumor cell lines, **A549** (human pulmonary carcinoma cell line) and **HeLa** (human epithelial carcinoma cell line) and furthermore in one immortalized normal cell line **MRC5** (human fetal lung fibroblast). IC50 values, calculated from the dose-survival curves obtained after 48 h complex solution treatment from MTT are shown in Table 5.

(Table 5)

As it can be clearly seen from Table 5 and through Figures 4–9, free 5-carbethoxy-2-thiouracil (eitotH_2) and triphenylphosphine (PPh_3) proved to be quite ineffective in all cell lines used. In particular PPh_3 gave detectable IC50 values only in higher concentrations. On the contrary, all the tested complexes showed a growth inhibitory potency in the micromolar range towards the different cell lines. Especially the three mixed-ligand complexes [CuI$(PPh_3)_2$(eitotH_2)], [CuCl$(PPh_3)_2$(eitotH_2)] and [CuBr$(PPh_3)_2$(eitotH_2)] (Figures 5, 7 and 9) were appreciably more effective against all cell lines and noteworthy against HeLa cell line exhibiting IC50 values of 2.55 μM, 3.63 μM and 3.36 μM, respectively (Table 5). Interestingly, the copper halide complexes of 5-

carbethoxy-2-thiouracil (compounds **1**–**3**) are appreciably less effective towards HeLa, A549 cancer cell lines as well as against MRC5, which is a non cancer cell line, than their triphenylphosphine containing counterparts (compounds **4**–**6**) which are highly toxic even at low concentrations.

Among the three homoleptic copper (I) halide complexes **1**–**3** the cytotoxic action follows the order of $[CuI(eitotH_2)_2]_2$ > $[CuBr(eitotH_2)_2]_2$ > $[CuCl(eitotH_2)_2]_2$ with half minimum inhibitory concentrations (IC_{50}) ranging from 45.5–86.6 μM for HeLa, to 54.25–89.56 for MRC5 and to 41.3–110 for A549.

Considering the three heteroleptic complexes **4**–**6**, the values of the half minimum inhibitory concentrations (IC_{50}) are significantly lower ranging from 2.55–3.6 μM for HeLa, 4.77–6.84 μM for MRC5 and 4.65–5.0 for A549. The *in vitro* cytotoxicity of all three complexes against A549 exceeds that recently reported for cisplatin by a factor of about 2 [58]. In contrast with the case of compounds **1**–**3**, there is no clear trend in the cell killing effect observed on going from chloro to bromo to iodo derivative.

(Figures 4 – 9)

Currently, extensive search for anticancer agents as alternative to platinum drugs is in full swing. In the quest for new metal-based drugs, a variety of copper-based drugs have been examined and proved to be very promising due to their selective cytotoxicity against tumor cells [59]. The use of phosphine containing complexes as potential anticancer agents is well documented [60–62]. In an early study the cytotoxicity/anti-tumor activity of a large number of auronofin analogues was evaluated [63]. It was observed that in most cases the presence of a phosphine in these gold thiolates caused an enhanced cytotoxicity compared with the other phosphine free species. Meanwhile, research on potential cytotoxic compounds has been focused, among others, on the use

of copper(I) compounds bearing tertiary phosphine ligands [64–66]. It is obvious that the particularly high cytotoxicity of the mixed-ligand complexes **4**–**6**, compared to that of compounds **1**–**3**, is due to the presence of the triphenylphosphine ligand. This finding is in good agreement with earlier observations on gold(I) compounds [5] but also recently on phosphine containing mixed-ligand complexes of copper(I) [57]. For these later copper(I) phosphine complexes the cytotoxic activity was correlated with their ability to act as endoplasmic reticulum stress inducers, and a nonapoptotic mechanism of programmed cell death (paraptosis) was proposed to be likely [67].

It is known that, like other established proteasome inhibitors, copper binding compounds are only effective in inducing ubiquinated protein accumulation and apoptosis in tumor, but not in non-trasformed cells. The anti-angiogenesis effects, as well as the potential use of proteasome inhibitors in cancer therapies, have been extensively reviewed [68, 69]. However, the ability of some copper binding compounds to inhibit the proteasome and induce apoptosis by themselves in copper-enriched cancer cells is promising for their development as anticancer compounds in a nontoxic chemotherapeutic strategy [70]. The mechanism of action of the copper complexes presented in this work has not been elucidated at the molecular level. Investigations involving DNA breakage or copper substitution of copper harbouring enzymes, ROS production enhancement or alteration in copper metabolism in general need to be performed. However, our results along with recent studies concerning copper(I) derivatives are encouraging as we can consider these new derivatives as lead compounds to develop new potential drugs against cancer.

Conclusions

5-carbethoxy-2-thiouracil (eitotH_2) coordinates to copper(I) halides exclusively via the “soft” thione-S atom forming dinuclear complexes of composition [CuX(eitotH_2)$_2$]$_2$ with the two metal ions in a strongly distorted tetrahedral environment, doubly bridged by thione sulfur atoms. These

dimers are used as precursors for the preparation of mononuclear four-coordinate phosphine/thione mixed-ligand complexes of the type [$CuX(eitotH_2)(PPh_3)_2$].

Evaluation of the cytotoxicity results shows that the presence of triphenylphosphine in complexes **4**–**6** results in a significant increase of cytotoxic activity when compared to the activity of the phosphine-free compounds **1**–**3.** Moreover, the cytotoxicity of all the above complexes is higher in all the cell lines that were tested, in comparison to that of each of the free ligands. With reference to literature information available so far, the remarkable antitumor activity of [$CuCl(PPh_3)_2(eitotH_2)$], [$CuBr(PPh_3)_2(eitotH_2)$] and [$CuI(PPh_3)_2(eitotH_2)$], which is due to the presence of PPh_3 , could be considered indicative of their ability to induce apoptosis.

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Appendix. Crystallographic data

Crystallographic data (without structure factors) for the structures reported in this paper have been deposited with the Cambridge Crystallographic Data Centre (CCDC) as supplementary publication no. CCDC-945117 (**3**), CCDC-944792 (**4**) and CCDC-944791 (**5**). Copies of the data can be obtained free of charge from the CCDC (12 Union Road, Cambridge CB2 1EZ, UK; Tel.: +44-1223-336408; Fax: +44-1223-336033; e-mail: deposit@ccdc.cam.ac.uk; Web site: http://www.ccdc.ca.ac.uk).

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Scheme/Figure Captions

Scheme 1. The heterocyclic thione 5-carbethoxy-2-thiouracil (eitotH_2) used as ligand.

Figure 1. A view of compound **3** with atom labels. Displacement ellipsoids are shown in the 50% probability level.

Figure 2. A view of compound **4** with atom labels. Displacement ellipsoids are shown in the 50% probability level.

Figure 3. A view of compound **5** with atom labels. Displacement ellipsoids are shown in the 50% probability level.

Figure 4. Dose-effect survival plots for the three homoleptic complexes [**1**–**3**] against A549 cell line 48 h after the administration of the agents. Cytotoxicity was estimated via MTT assay (each point represents mean of three replicate wells).

Figure 5. Dose-effect survival plots for the three heteroleptic complexes [**4**–**6**], against A549 cell line 48 h after the administration of the agents. Cytotoxicity was estimated via MTT assay (each point represents mean of three replicate wells).

Figure 6. Dose-effect survival plots for the three homoleptic complexes [**1**–**3**] against HeLa cell line 48 h after the administration of the agents. Cytotoxicity was estimated via MTT assay (each point represents mean of three replicate wells).

Figure 7. Dose-effect survival plots for the three heteroleptic complexes [4–**6**], against HeLa cell line 48 h after the administration of the agents. Cytotoxicity was estimated via MTT assay (each point represents mean of three replicate wells).

Figure 8. Dose-effect survival plots for the three homoleptic complexes [**1**–**3**] against MRC5 cell line 48 h after the administration of the agents. Cytotoxicity was estimated via MTT assay (each point represents mean of three replicate wells).

Figure 9. Dose-effect survival plots for the three heteroleptic complexes [**4**–**6**], against MRC5 cell line 48 h after the administration of the agents. Cytotoxicity was estimated via MTT assay (each point represents mean of three replicate wells).

Table 1. Crystal data and structure refinement details for compounds **3**, **4** and **5**

Compound Structural formula	**3** $C_{28}H_{32}Cu_2I_2N_8O_{12}S_4$	**4** $C_{43}H_{38}ClCuN_2O_3P_2S$, **1/2**($C_3H_6O$)	**5** $C_{43}H_{38}BrCuN_2O_3P_2S$, C_2H_3N
Formula weight	1181.74	852.84	909.30
Crystal system	Triclinic	Monoclinic	Monoclinic
Temperature	100(2) K	295 K	295 K
Wavelength	0.71073 Å	0.71073 Å	0.71073 Å
Space group	Pī	C 2/c	C 2/c
Unit cell dimensions	a = 7.853(2) Å *b* = 11.2722(15) Å *c* = 12.403(2) Å *α* = 64.425(14)° *β* = 85.40(2)° *γ* = 83.02(2)°	a = 24.867(3) Å *b* = 10.0343(11) Å *c* = 35.478(4) Å *α* = 90° *β* = 105.678(2)° *γ* = 90°	a = 25.088(4) Å *b* = 10.1036(18) Å *c* = 35.742(6) Å *α* = 90° *β*□= 106.720(5)° *γ* = 90°
Volume	982.5(3) $Å^3$	8523.1(16) $Å^3$	8677(2) $Å^3$
Z	1	4	4
Absorption coefficient (μ)	2.935 mm^{-1}	0.742 mm^{-1}	1.589 mm^{-1}
Density (calculated)	1.997 Mg/m^3	1.33 Mg/m^3	1.391 Mg/m^3
F(000)	1060	3536	3728
Reflections collected	8931	62728	87736
Independent reflections	4414	14218	12845
Completeness up to theta	97.7%	99.5%	99.3%
Data / restraints / parameters	4414/0/256	7762/28/489	7981/2/509
Goodness-of-fit on F^2	0.990	0.999	1.000
Final R indices [I>2□(I)]	R_1=0.0260, wR_1=0.0554	R_1=0.0515, wR_1=0.1051	R_1=0.0433, wR_1=0.0749
R indices (all data)	R_2=0.0319, wR_2=0.0567	R_2=0.0636, wR_2=0.1097	R_2=0.0904, wR_2=0.0948
Largest diff. peak and hole	0.804, – 0.650 $e.Å^{-3}$	0.59, – 0.54 $e.Å^{-3}$	0.69, – 0.50 $e.Å^{-3}$

Table 2. Selected bond distances [Å] and angles [°] in $[CuI(eitotH_2)_2]_2$ (**3**)

I(1)–Cu(1)	2.5089(7)	S(1)–C(1)	1.682(3)
Cu(1)–S(1)	2.2475(9)	S(2)–C(8)	1.682(3)
Cu(1)–S(2)	2.2598(9)	Cu(1)–S(2)#1	2.9447(11)
Cu(1)–S(2)#1	2.9447(11)		
S(1)–Cu(1)–S(2)	103.89(3)	I(1)–Cu(1)–S(2)#1	99.79(3)
S(1)–Cu(1)–I(1)	128.88(3)	C(1)–S(1)–Cu(1)	110.76(10)
S(2)–Cu(1)–I(1)	123.91(3)	C(8)–S(2)–Cu(1)	112.09(9)
S(1)–Cu(1)–S(2)#1	92.46(3)	C(8)–S(2)–Cu(1)#1	100.80(10)
S(2)–Cu(1)–S(2)#1	94.92(3)	Cu(1)–S(2)–Cu(1)#1	85.08(3)

Hydrogen bonds

D–H···A	d(D–H)	d(H···A)	d(D···A)	<(DHA)
N(1)–H(1)···I(1)	0.88	2.94	3.809(2)	169.6
N(4)–H(4)···I(1)	0.88	2.76	3.636(2)	174.3
N(2)–H(2)···O(5)#2	0.88	1.90	2.764(3)	164.9
N(3)–H(3)···O(2)#3	0.88	1.94	2.658(3)	137.5

Symmetry transformations used to generate equivalent atoms: #1 -x+1,-y+1,-z #2 x-1,y+1,z #3 x+1,y,z-1

Table 3. Selected bond distances [Å] and angles [^{o}] in [$CuCl(PPh_3)_2(eitotH_2)$] (**4**)

Cu(1)–Cl(1)	2.3317(8)	Cu(1)–P(2)	2.2851(7)
Cu(1)–S(1)	2.4083(8)	S(1)–C(1)	1.670(3)
Cu(1)–P(1)	2.2992(8)		
S(1)–Cu(1)–P(1)	104.63(3)	P(1)–Cu(1)–Cl(1)	109.82(3)
S(1)–Cu(1)–P(2)	105.15(3)	P(2)–Cu(1)–Cl(1)	107.81(3)
P(1)–Cu(1)–P(2)	122.71(3)	Cu(1)–S(1)–C(1)	111.02(10)
S(1)–Cu(1)–Cl(1)	105.31(3)		

Table 4. Selected bond distances [Å] and angles [^{o}] in [CuBr(PPh_3)$_2$(eitotH_2)] (**5**)

Cu(1)–Br(1)	2.4709(6)	Cu(1)–P(2)	2.2857(8)
Cu(1)–S(1)	2.3931(8)	S(1)–C(1)	1.679(3)
Cu(1)–P(1)	2.2962(8)		
S(1)–Cu(1)–P(1)	105.51(3)	P(1)–Cu(1)–Br(1)	108.54(2)
S(1)–Cu(1)–P(2)	105.76(3)	P(2)–Cu(1)–Br(1)	105.19(3)
P(1)–Cu(1)–P(2)	123.47(3)	Cu(1)–S(1)–C(1)	111.71(10)
S(1)–Cu(1)–Br(1)	107.52(2)		

Table 5. IC50 values (in μM) of complexes **1**–**6** and the uncoordinated eitotH_2 and PPh_3 in all three cell lines

Cell line	IC50							
	eitotH_2	**PPh_3**	**1**	**2**	**3**	**4**	**5**	**6**
HeLa	-	105	45.5	49.55	86.6	3.63	3.36	2.55
MRC5	-	115.2	54.25	58.16	89.56	5	4.77	6.84
A549	-	118	41.3	44.3	110	5.55	4.65	5

Scheme 1

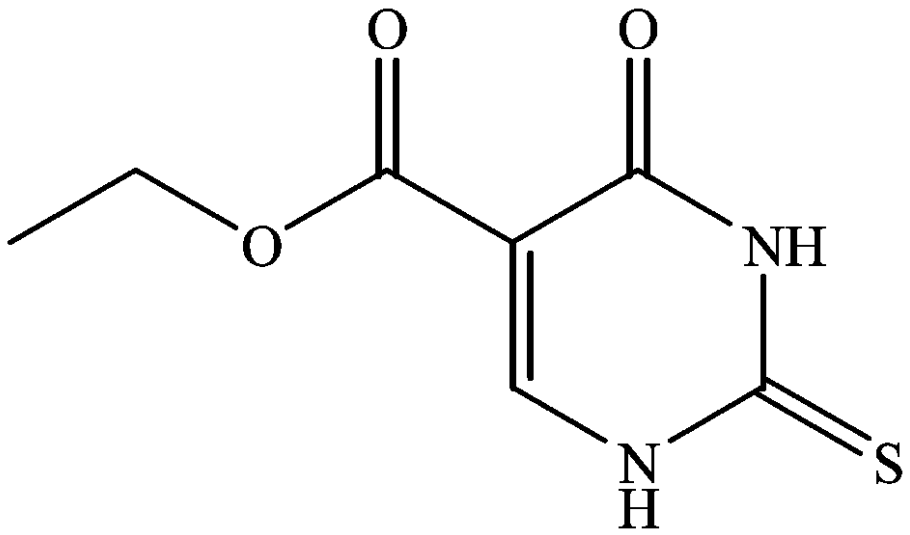

Figure 1

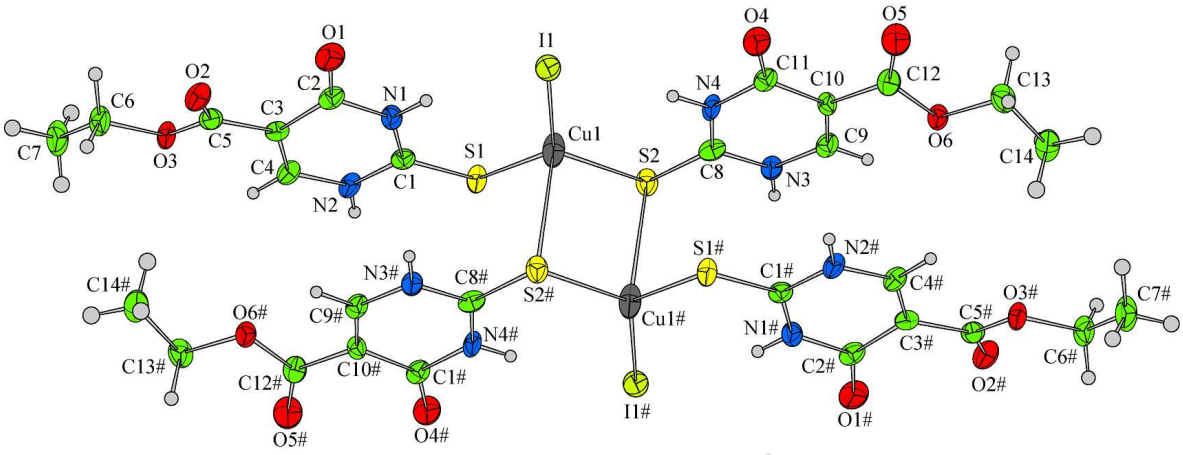

Figure 2

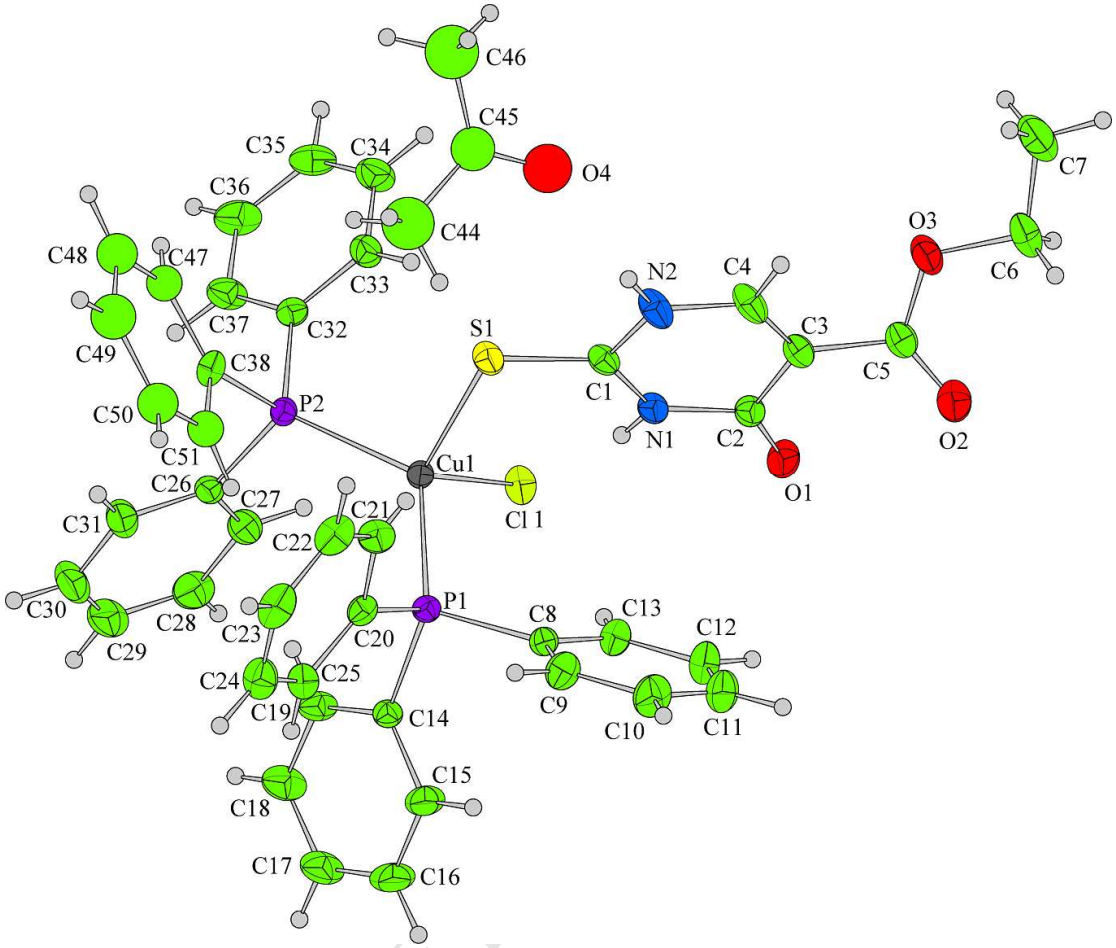

Figure 3

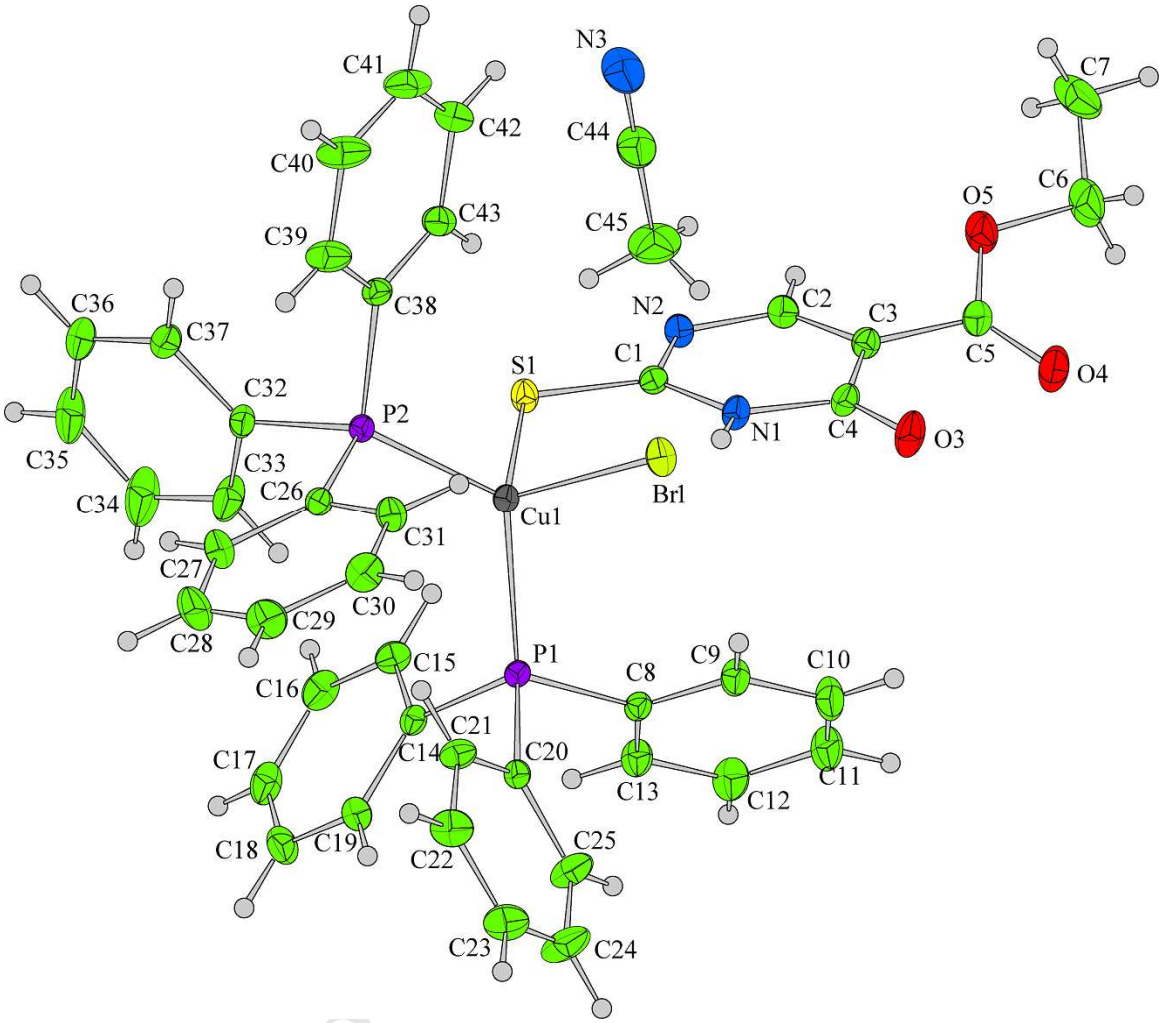

Figure 4

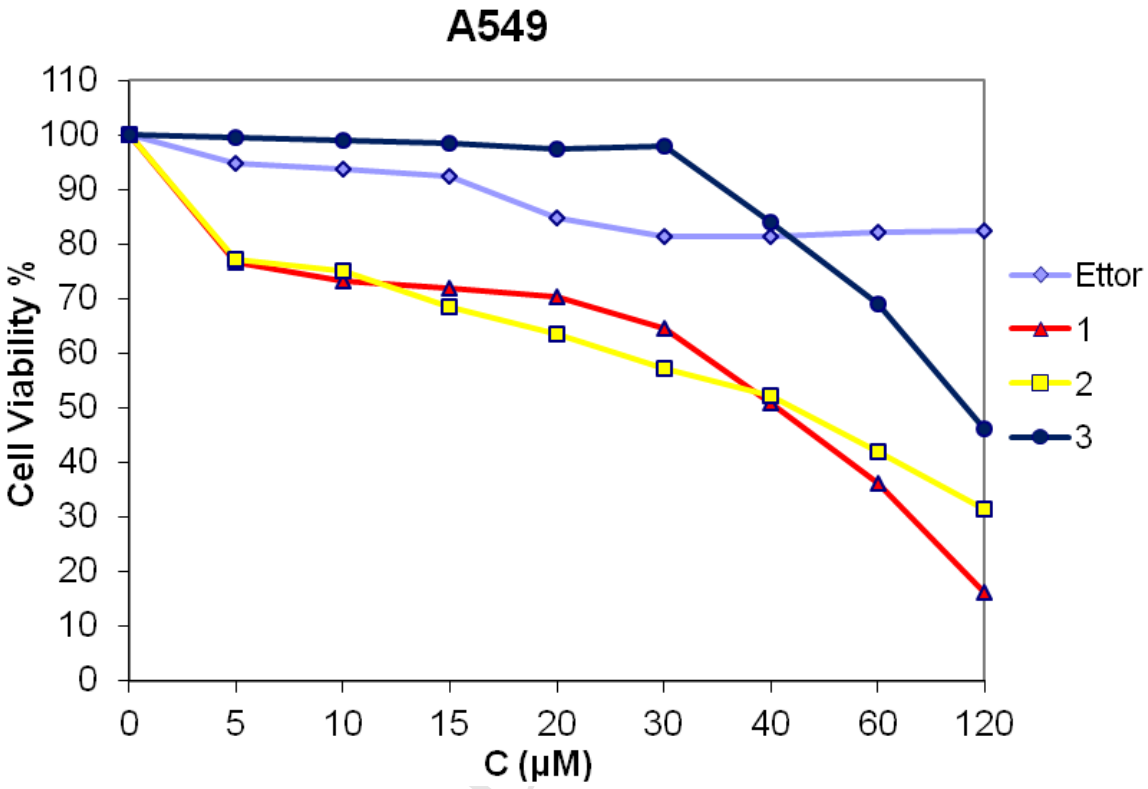

Figure 5

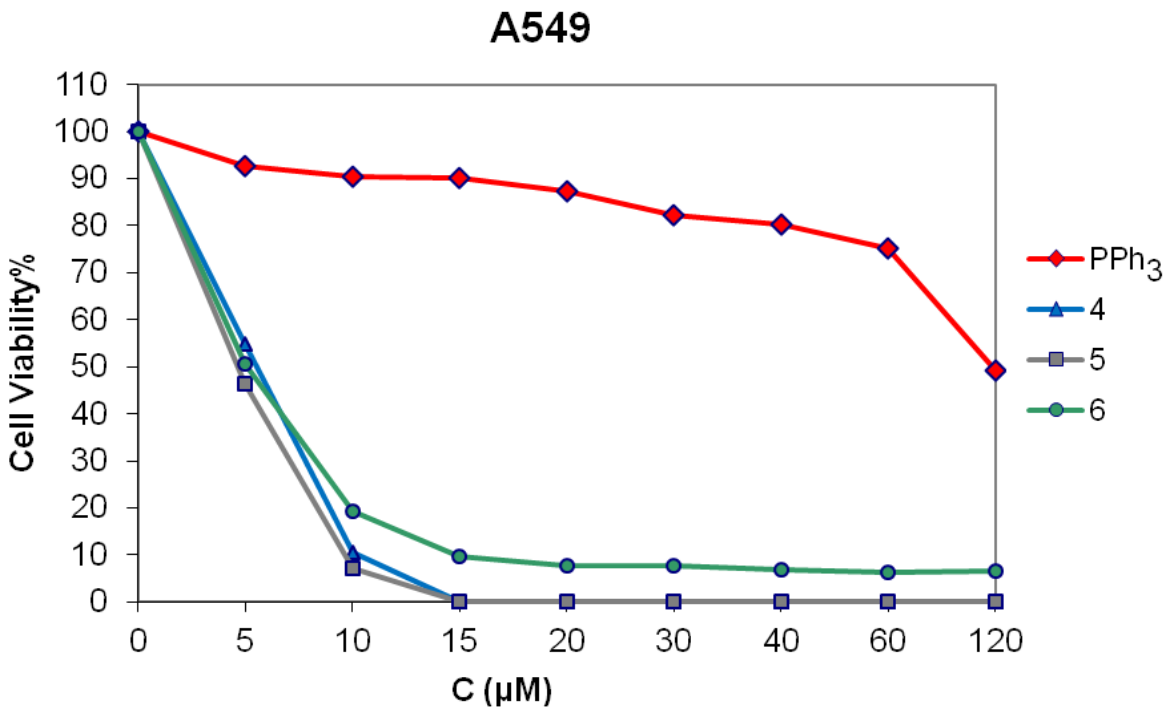

Figure 6

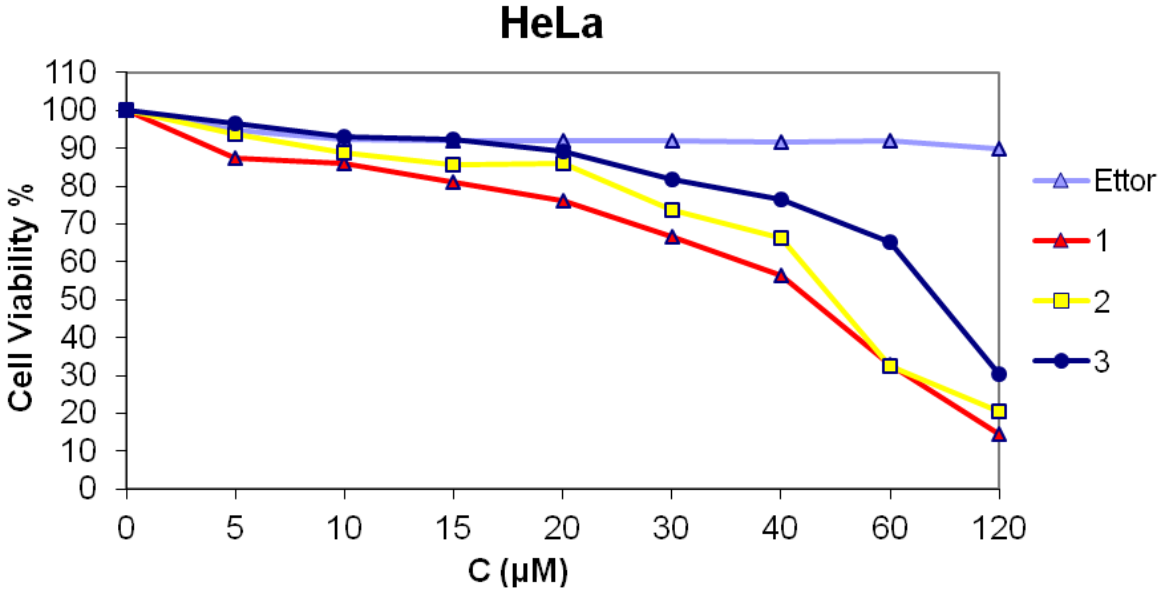

Figure 7

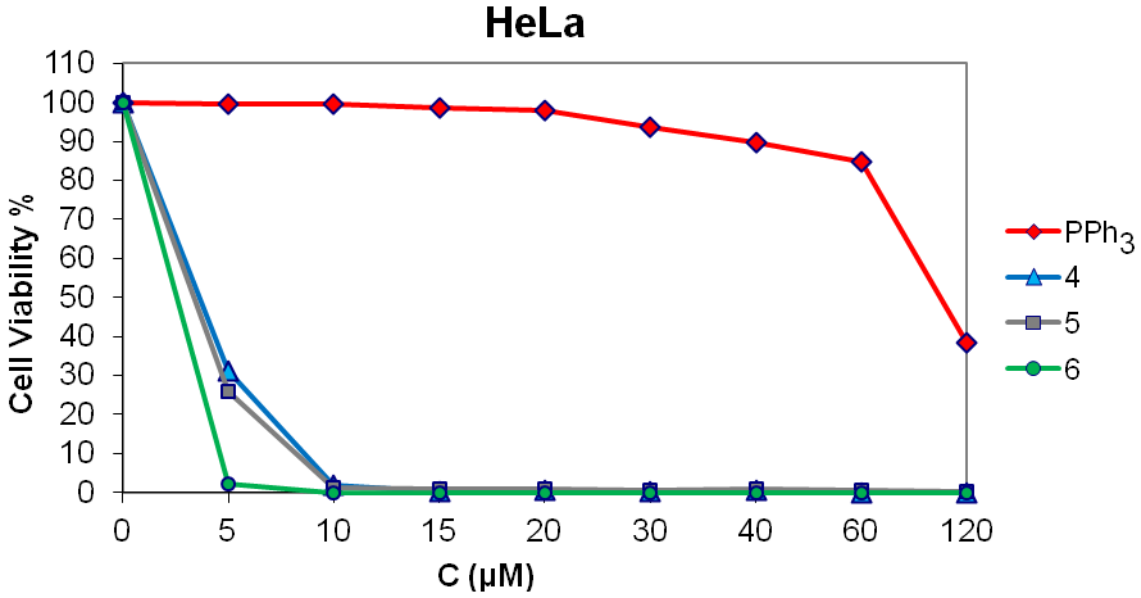

Figure 8

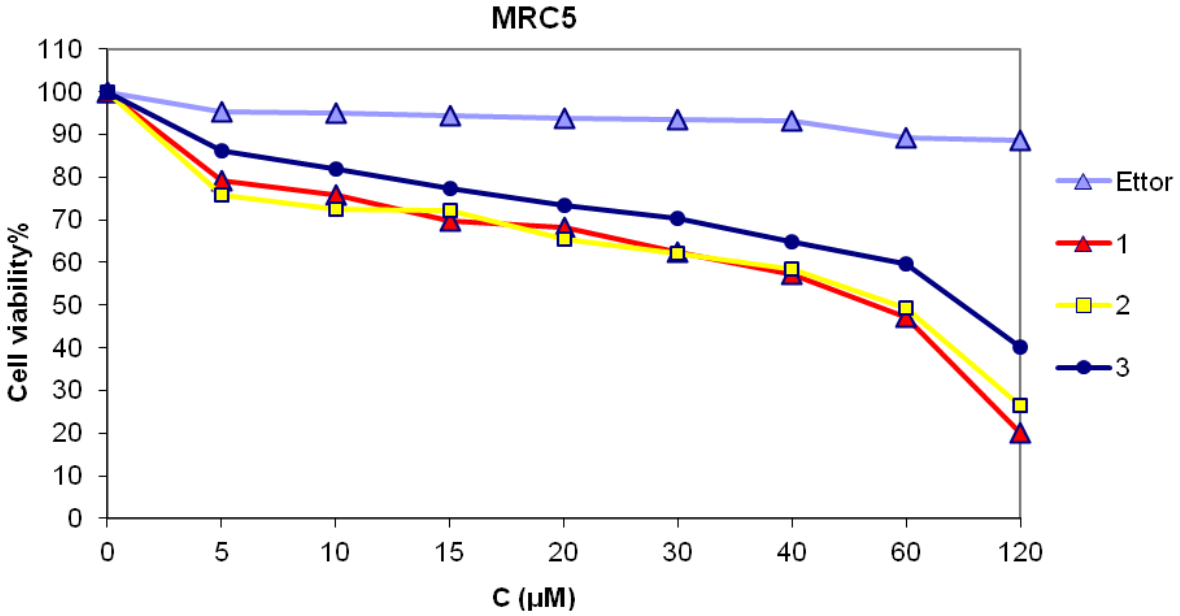

Figure 9