Chemistry Central Journal

RESEARCH ARTICLE Open Access

Cellular responses induced by Cu(II) quinolinonato complexes in human tumor and hepatic cells

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Abstract

Background: Inspired by the unprecedented historical success of cisplatin, one of the most important research directions in bioinorganic and medicinal chemistry is dedicated to the development of new anticancer compounds with the potential to surpass it in antitumor activity, while having lower unwanted side-effects. Therefore, a series of copper(II) mixed-ligand complexes of the type $[Cu(qui)(L)]Y \cdot xH_2O$ (1–6), where Hqui = 2-phenyl-3-hydroxy-4(1*H*)-quinolinone, $Y = NO_3$ (1, 3, 5) or BF_4 (2, 4, 6), and L = 1,10-phenanthroline (phen) (1, 2), 5-methyl-1,10-phenanthroline (mphen) (3, 4) and bathophenanthroline (bphen) (5, 6), was studied for their *in vitro* cytotoxicity against several human cancer cell lines (A549 lung carcinoma, HeLa cervix epitheloid carcinoma, G361 melanoma cells, A2780 ovarian carcinoma, A2780cis cisplatin-resistant ovarian carcinoma, LNCaP androgen-sensitive prostate adenocarcinoma and THP-1 monocytic leukemia).

Results: The tested complexes displayed a stronger cytotoxic effect against all the cancer cells as compared to cisplatin. The highest cytotoxicity was found for the complexes 4 ($IC_{50} = 0.36 \pm 0.05$ μM and 0.56 ± 0.15 μM), 5 ($IC_{50} = 0.66 \pm 0.07$ μM and 0.73 ± 0.08 μM) and 6 ($IC_{50} = 0.57 \pm 0.11$ μM and 0.70 ± 0.20 μM) against A2780, and A2780cis respectively, as compared with the values of 12.0 ± 0.8 μM and 27.0 ± 4.6 μM determined for cisplatin. Moreover, the tested complexes were much less cytotoxic to primary human hepatocytes than to the cancer cells. The complexes 5 and 6 exhibited significantly high ability to modulate secretion of the pro-inflammatory cytokines TNF-α (2873 ± 238 pg/mL and 3284 ± 139 pg/mL for 5, and 6 respectively) and IL-1β (1177 ± 128 pg/mL and 1087 ± 101 pg/mL for 5, and 6 respectively) tested on the lipopolysaccharide (LPS)-stimulated THP-1 cells as compared with the values of 1173 ± 85 pg/mL and 118.5 ± 4.8 pg/mL found for the commercially used anti-inflammatory drug prednisone. The ability of the tested complexes to interact with sulfur-containing biomolecules (cysteine and reduced glutathione) at physiological levels was proved by electrospray-ionization mass spectrometry.

Conclusions: Overall positive results of the biological activity studies revealed that the presented complexes may represent good candidates for non-platinum anticancer drugs, however, we are aware of the fact that further and deeper studies mainly in relation to the elucidation of their mechanisms of antiproliferative action will be necessary.

Keywords: Copper(II) complexes, *In vitro* cytotoxicity, Hepatotoxicity, Gene reporter assay, Inflammatory response, Quinolinone derivatives

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Background

One of the most important research directions in bioinorganic and medicinal chemistry is dedicated to the development of new anticancer compounds with the potential to be used either in monotherapy or in combination therapy with other approved anticancer drugs as chemosensitizers [1]. Within this scope of research, two main groups of antitumor active compounds have been developed: (a) platinum-based complexes derived from cisplatin, oxaliplatin or carboplatin [2], and (b) non-platinum metal complexes, for example, complexes of ruthenium, gold, copper and/or silver, which have proved their effectiveness against a variety of cancer cell lines [3]. Apart from the potential to achieve relatively high cytotoxicity or remarkable selectivity against specific cancer cells [4], the main reason for the use of the non-platinum metal complexes is the ability to overcome negative side-effects of cisplatin and other platinum-based anticancer drugs, *e.g.* nephrotoxicity, myelosuppression and intrinsic and acquired drug-resistance. Suppressions of these undesirable side-effects could be achieved by applying metallotherapeutic complexes based on biogenic transition metals, whose biological functions play an important role in different essential biochemical pathways, *e.g.* cell division, angiogenesis, inflammatory processes, regulation of redox processes, etc. Copper belongs among such essential trace elements which are important for most living species [5]. In the human body, it is critical for multiple biochemical processes, as it plays a fundamental role in the structure and function of a number of metalloenzymes. Additionally, it is a redox-active metal and thus participates in many redox-related transformations [6]. The influence on the metabolism of reactive oxygen species (ROS) by copper ions (dominantly the cuprous ions) has been shown to have an effect on the development of cancer [7] as well as the potential manifestation of cytotoxicity. Elevated levels of copper in blood have been recently proved to induce inflammation *in vitro* [8] as well as *in vivo* [9], at least partly, due to a higher production of ROS. However, this issue needs further investigation as, on the contrary, some copper(II) complexes have been shown to act as anti-inflammatory agents *via* the inhibition of the NF-κB activity and attenuation of its target gene expression [10,11]. Regarding these indispensable roles of copper for the human organism, it is not surprising that complexes containing copper(II) as the central metal atom together with a variety of different ligands are one of the fastest growing groups of non-platinum complexes with considerable anticancer effects [12-17]. Some of these complexes, like the mixed-ligand complexes involving the amino acids and 1,10-phenanthroline-based ligands, known as Casiopeinas®, received much more attention than the others due to their remarkable cytotoxicity [18].

Recently, we have been engaged in the preparation, characterization and *in vitro* cytotoxicity study of mixed-ligand copper(II) complexes, involving 2-phenyl-3-hydroxy-4(1*H*)-quinolinone (Hqui) and 1,10-phenanthroline derivatives [19,20]. These compounds showed high *in vitro* cytotoxic effects against the HOS and MCF7 human cancer cells, with $IC_{50} \approx 2–5$ μM. We proved that these complexes interact with DNA *via* a partial intercalation and act as effective chemical nucleases under the proper conditions (*i.e.* oxidative stress and the presence of reducing agent).

On the basis of the encouraging preliminary results regarding the *in vitro* cytotoxicity against the mentioned human cancer cell lines reported in the case of $[Cu(qui)(L)]Y \cdot xH_2O$ (1–6), where Hqui = 2-phenyl-3-hydroxy-4(1*H*)-quinolinone, $Y = NO_3$ (1, 3, 5) or BF_4 (2, 4, 6), and L = 1,10-phenanthroline (phen) (1, 2), 5-methyl-1,10-phenanthroline (mphen) (3, 4) and bathophenanthroline (bphen) (5, 6), we decided to extend the spectrum of human cancer cell lines and to study the *in vitro* cytotoxicity of the complexes against next seven cell lines. Moreover, we also decided to study the cytotoxicity of the complexes on the primary culture of human hepatocytes and to evaluate their effect on the healthy cells, with the aim to estimate their therapeutic index. In an effort to further understand the mechanisms of the cytotoxic activity of the reported copper(II) complexes, the inflammation related processes were also addressed, since it has been shown that inflammation has an essential role in tumor progression, and multiple cancers arise from the sites of infection, chronic irritation and inflammation [21]. To evaluate whether complexes 1–6 have a rather pro-inflammatory or anti-inflammatory potential, their ability to modulate the proinflammatory cytokines TNF-α and IL-1β expressions on the lipopolysaccharide (LPS)-stimulated macrophage-like cell line THP-1 *in vitro* were tested. Additionally, to reveal possible drug-drug interactions that might dramatically influence the applicability of the studied complexes as drugs, the capability of the presented complexes to induce major drug-metabolizing cytochromes P450 was studied. Transcriptional regulation of these enzymes is controlled mainly by glucocorticoid receptor (GR) and aryl hydrocarbon receptor (AhR). Therefore, we studied the effects of the copper(II) complexes 1–6 on the transcriptional activity of GR and AhR, using stably transfected gene reporter cell lines [22].

Results

Cytotoxicity against human cancer cell lines

In an effort to deeper understand the cytotoxicity and uncover possible selectivity of the studied complexes, and in connection with the preliminary data presented in our recent publications [19,20], we performed *in vitro* cytotoxicity testing of complexes 1–6 against a panel of

Table 1 The results of *in vitro* cytotoxicity of the Cu(II) complexes 1–6

Compounds	A549	HeLa	G361	A2780	A2780cis	LNCaP	THP-1	HH1
[Cu(qui)(phen)]$NO_3 \cdot H_2O$ (**1**)	2.5 ± 0.4	2.9 ± 0.3	0.82 ± 0.15	0.65 ± 0.28	0.83 ± 0.30	2.4 ± 0.6	2.0 ± 0.1	>10
[Cu(qui)(phen)]BF_4 (**2**)	3.9 ± 1.2	2.7 ± 0.6	0.64 ± 0.20	0.52 ± 0.10	1.7 ± 0.6	1.9 ± 0.6	1.7 ± 0.2	20.4 ± 1.2
[Cu(qui)(mphen)]$NO_3 \cdot H_2O$ (**3**)	2.5 ± 0.6	2.6 ± 0.4	0.53 ± 0.22	0.38 ± 0.03	0.78 ± 0.07	2.1 ± 0.6	1.5 ± 0.3	>10
[Cu(qui)(mphen)]$BF_4 \cdot H_2O$ (**4**)	2.5 ± 0.6	2.4 ± 1.0	0.53 ± 0.09	0.36 ± 0.05	0.56 ± 0.15	1.5 ± 0.6	1.1 ± 0.4	9.3 ± 0.6
[Cu(qui)(bphen)]$NO_3 \cdot H_2O$ (**5**)	1.7 ± 0.3	1.0 ± 0.2	0.96 ± 0.03	0.66 ± 0.07	0.73 ± 0.08	0.74 ± 0.21	0.57 ± 0.08	3.6 ± 0.2
[Cu(qui)(bphen)]BF_4 (**6**)	1.3 ± 0.4	1.3 ± 0.4	0.72 ± 0.06	0.57 ± 0.11	0.70 ± 0.20	0.62 ± 0.08	0.56 ± 0.10	5.3 ± 0.3
cisplatin	25.8 ± 7.1	10.0 ± 2.6	3.4 ± 0.1	12.0 ± 0.8	27.0 ± 4.6	3.8 ± 1.5	2.0[a]	>50

The results of the *in vitro* cytotoxic study of the Cu(II) complexes **1**–**6** and *cisplatin* together with the results of the toxicity to primary human hepatocytes (HH1). Measurements were performed in triplicate, and cytotoxicity experiments were repeated in three different cell passages. The data are expressed as $IC_{50} \pm SD$ (μM).
[a] See Ref. [23].

seven human cancer cell lines (A2780 , A2780cis (*cisplatin*-resistant), G361, A549, HeLa, LNCaP, and THP-1) and compared the obtained results with those belonging to the widely used anticancer drug cisplatin (Table 1). The antiproliferative effect of the starting compounds, *i.e.* Hqui, $Cu(BF_4)_2 \cdot H_2O$, $Cu(NO_3)_2 \cdot 3H_2O$, phen, mphen and bphen, was assessed on all cancer cell lines and all the compounds showed insignificant *in vitro* cytotoxicity, with $IC_{50} > 50$ μM. The significance of individual differences between the selected groups was evaluated by the ANOVA method (the level of $p < 0.05$ was considered as significant, for further details See Additional file 1: Figure S1-S7).

Cytotoxicity to human hepatocytes

It is *"condicio sin qua non"* that an anticancer drug should be selectively toxic against cancer cells, but not against healthy, non-transformed cells. Therefore, we examined the cytotoxicity of the Cu(II) compounds 1–6 in primary cultures of human hepatocytes (Table 1). The toxicity against the human hepatic cells was expressed as a mean value of IC_{50}, calculated from three parallel determinations. The significance of individual differences between the selected groups was evaluated by the ANOVA method (the level of $p < 0.05$ was considered as significant, for further details See Additional file 1: Figure S8).

In vitro anti-inflammatory activity testing

In addition to testing of toxicity on cellular level, we decided also to investigate in greater detail the molecular mechanisms of action of studied complexes using the cellular model system based on the LPS-activated respiratory burst in phagocytes and its effect on the inflammatory cytokine expression. It is well known that reactive oxygen species (ROS) participate in a series of different processes causing the intracellular damage, including the modulation of transcription factor NF-κB activity (and production of inflammatory cytokines like TNF-α) and thus represent a link between the oxidative stress, inflammatory response and apoptosis caused by the external stimuli (environmental effects, xenobiotics, etc.) [24,25]. With the knowledge that the tested complexes are able to increase the ROS formation in cell-free systems, we decided to evaluate their ability to induce the NF-κB-dependent TNF-α and IL-1β expressions in the LPS-activated macrophages. Complexes 1, 2 and 3 slightly increased the secretion of TNF-α (by factor of ~ 1.25 in comparison to vehicle) (Figure 1). Similar effect was observed for the cells treated by the 0.1% DMSO solution of $CuSO_4 \cdot 5H_2O$, which increased the TNF-α expression by the factor of 1.4. On the other hand, complexes 4, 5 and 6 significantly increased the production of this cytokine by the factor of 1.6, 1.8, and 2, respectively.

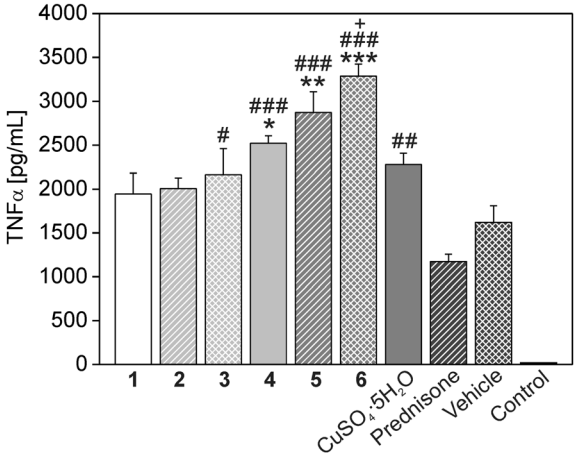

Figure 1 Effects of the Cu(II) complexes and prednisone on the LPS-induced TNF-α secretion. The cells were pre-treated with complexes 1–6 (0.2 μM), prednisone (0.2 μM) or the *vehicle* (DMSO) only. After 1 h of incubation, the inflammatory response was induced by LPS (except for the *control* cells). The secretion was measured 24 h after the LPS adding. The results are expressed as means ± S.D. for three independent experiments. * significant difference in comparison with the vehicle-treated cells ($p < 0.05$), ** significant difference in comparison with the vehicle-treated cells ($p < 0.01$), *** significant difference in comparison with the vehicle-treated cells ($p < 0.001$), # significant difference in comparison with the prednisone-treated cells ($p < 0.05$), ## significant difference in comparison with the prednisone-treated cells ($p < 0.01$), + significant difference in comparison to $CuSO_4$-treated cells ($p < 0.05$).

A similar situation as for TNF-α was observed in the case of the IL-1β secretion. The complexes 1, 2, and 3 had practically no influence on the IL-1β expression in the LPS-activated cells (Figure 2). Compound 4 moderately raised production of IL-1β by the factor of 1.6, similarly to the solution of $CuSO_4 \cdot 5H_2O$. Compounds 5 and 6, containing bphen as an N-donor ligand, significantly increased the amount of IL-1β by the factor of 6.9, and 6.4, respectively. The significance of the differences between the results was assessed by the ANOVA analysis, followed by a Tuckey's post-hoc test for multiple comparisons, with $p < 0.05$ considered to be significant.

Transcriptional activity of AhR and GR in gene reporter assays

Since AhR and GR receptors are master transcriptional regulators of drug-metabolizing enzymes, we tested the effects of the copper(II) complexes on the AhR and GR transcriptional activities in stably transfected human reporter cell lines AZ-AhR and AZ-GR. These series of experiments were performed to assess the potential of the studied compounds to cause the induction of xenobiotic metabolizing enzymes. The cells were treated for 24 h with the complexes (<5 μM), model activators (TCDD, dexamethasone) and vehicle (DMSO). The

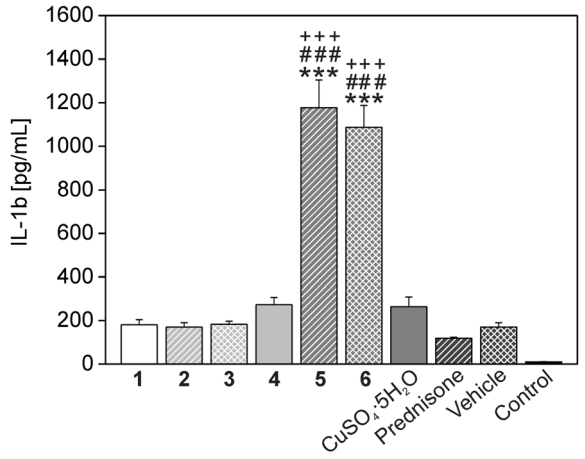

Figure 2 Effects of the Cu(II) complexes and prednisone on the LPS-induced IL-1β secretion. The cells were pre-treated with complexes **1**–**6** (0.2 μM), prednisone (0.2 μM) or the *vehicle* (DMSO) only. After 1 h of incubation, the inflammatory response was induced by LPS (except for the *control* cells). The secretion was measured 24 h after the LPS adding. The results are expressed as means ± S.D. for three independent experiments. *** significant difference in comparison with the vehicle-treated cells ($p < 0.001$), ### significant difference in comparison with the prednisone-treated cells ($p < 0.001$), +++ significant difference in comparison with the $CuSO_4$-treated cells ($p < 0.001$).

luciferase activity was strongly, dose-dependently induced by TCDD and DEX in the AZ-AhR and AZ-GR cells, respectively (Figure 3).

Interactions with sulfur-containing biomolecules

In an effort to mechanistically describe the interactions of three representative complexes 1, 3 and 5 with different relevant biomolecules (amino acids and small peptides) and to identify the biologically active species possibly responsible for the high cytotoxicity of the tested compounds, the electrospray-ionization mass-spectrometry (ESI-MS) measurements were carried out. The positive ionization mode was used with the solution of two most important sulfur-containing low-molecular biogenic compounds, *i.e.* cysteine (Cys) and reduced glutathione (GSH), containing the physiological levels of cysteine (290 μM) and reduced glutathione (6 μM) [26]. In order to find a possible effect of the concentration on the type and intensity of the ionic species formed by the interaction with cysteine and glutathione, two different concentrations (1 μM and 7 μM) of the tested complexes were used. The corresponding interaction systems contained two freshly prepared working solutions: (a) the solution of cysteine (the final concentration of 290 μM) and reduced glutathione (final conc. 6 μM)

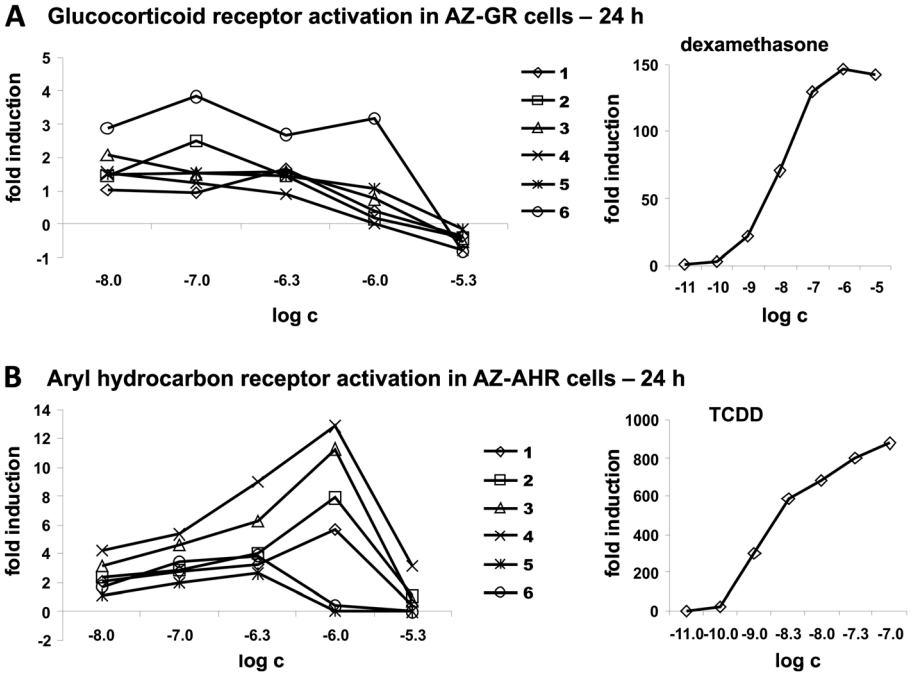

Figure 3 Effects of the Cu(II) complexes on the transcriptional activity of AhR and GR. AZ-GR (Panel **A**) and/or AZ-AHR (Panel **A**) cells were seeded on 96-well plates and stabilized for 16 h. Thereafter, the cells were treated for 24 h with the tested complexes (10 nM – 5 μM), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 10 pM – 10 μM), dexamethasone (DEX; 10 pM – 100 nM) and/or vehicle (DMSO; 0.1% V/V). After the treatments, the cells were lysed and the luciferase activity was measured. The data are mean from triplicate measurements and are expressed as a fold induction over the DMSO-treated cells. Experiments were performed in two different passages of the AZ-AHR and AZ-GR cells. The differences between the individual measurements were lower that 5%.

dissolved in an aqueous solution of 50 mM ammonium acetate in water, and (b) the solutions of 1, 3 and 5 in methanol, to give the final concentration of 1 μM or 7 μM. All the ESI-MS spectra were compared to the control, containing only the solution of cysteine (the final concentration of 290 μM) and reduced glutathione (the final concentration of 6 μM) in the equibalanced mixture of 50 mM ammonium acetate in water and methanol. The resulting interacting solutions were measured immediately after the preparation and visually controlled for further 2 hours. The immediately mixed interacting systems were slightly opalescent during the measurements of ESI-MS spectra and white precipitate formed during the next two hours, leaving the colorless or slightly yellowish transparent supernatant. The ESI-MS experiments revealed diverse combinations of species in all the measured systems (see Figure 4). In the control sample, containing only the mixture of cysteine and reduced glutathione, the following ions were identified: (see Figure 4, red line (a); *m/z*, [the corresponding pseudomolecular ion]$^+$): 122.06, [Cys + H]$^+$; 143.98, [Cys + Na]$^+$; 240.96, [Cys-S-S-Cys + H]$^+$; 262.96, [Cys-S-S-Cys + Na]$^+$; 308.09, [GSH + H]$^+$; 330.09, [GSH + Na]$^+$; and 427.01, [GS-S-Cys + H]$^+$. An example showing the interactions of the most cytotoxic complex 5 (in the final concentration of 1 μM) with the mixture of cysteine and reduced glutathione is depicted in Figure 4, green line (b). The ESI-MS spectrum of this system confirmed the redox-based interactions, as can be seen from the appearance of the peaks associated with the oxidation of cysteine and glutathione and their intermolecular disulphides: (*m/z*, [the corresponding pseudomolecular ion]$^+$): 427.07, [GS-S-Cys + H]$^+$; 449.02, [GS-S-Cys + Na]$^+$; 480.84, [GS-S-Cys + H + 3H_2O]$^+$; 502.67, [GS-S-Cys + Na + 3H_2O]$^+$. Moreover,

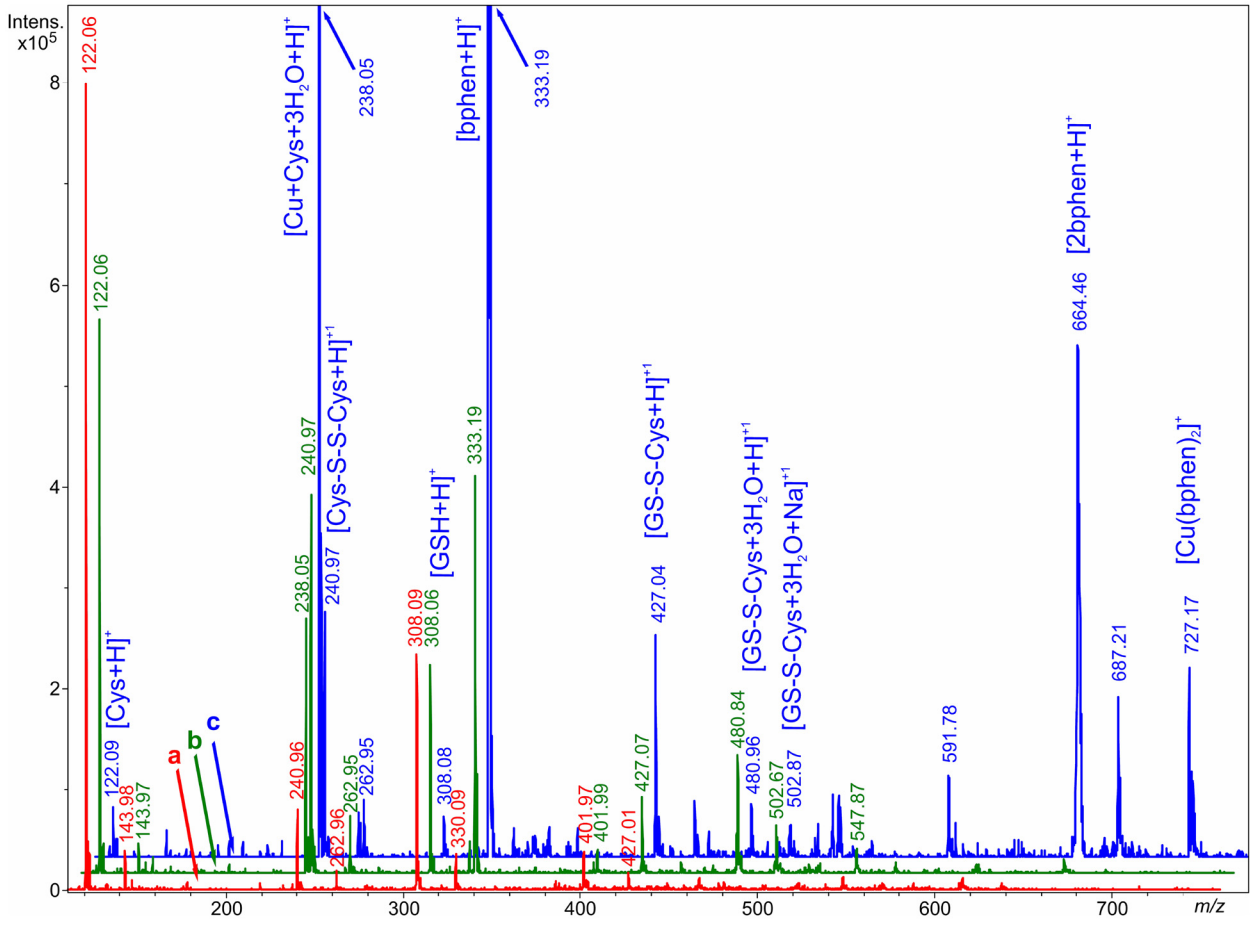

Figure 4 ESI-MS spectra of interacting systems, involving the Cu(II) complexes, L-cysteine, and reduced glutathione. ESI-MS spectrum of the mixture of physiological levels of cysteine (290 μM) and glutathione (6 μM) in 50 mM ammonium acetate in water (a; red line); the interacting system containing cysteine (290 μM) + glutathione (6 μM) + complex **5** (1 μM) in the mixture of 50 mM ammonium acetate and methanol (1:1, v/v) immediately after the preparation (b; green line); the interacting system containing cysteine (290 μM) + glutathione (6 μM) + complex **5** (7 μM) in the mixture of 50 mM ammonium acetate and methanol (1 : 1, v/v) immediately after the preparation (c; blue line), both (*i.e.* lines b and c) showing the appearance of the specific ions, connected either with the oxidative mechanism of the interaction or the ligand-exchange mechanism (for more details See Section Interactions with sulfur-containing biomolecules).

the ionic species confirming the direct interaction of the copper(II) ion from the complex 5 with cysteine was identified as well: (*m/z*, [corresponding pseudomolecular ion]$^+$): 238.05, $[Cu + Cys + H + 3H_2O]^+$, which was accompanied by the release of the N-donor ligand (bphen), as proved by the detection of the peaks observed at 333.19, $[bphen + H]^+$ and 664.40, $[2bphen + H]^+$ (see Figure 4, green line (b)). In an effort to uncover the influence of the studied complexes concentrations on the type and intensity of the ionic species, we performed the same experiments, described in the spectrum labeled as (b), however, with the concentration of complex 5 of 7 μM (see Figure 4, blue line (c)). In this interaction system, the considerable decrease in the intensities of the peaks corresponding to the disulphide-species, formed by the oxidation of cysteine and glutathione, *i.e.* $[GS\text{-}S\text{-}Cys + H + 3H_2O]^+$ and $[GS\text{-}S\text{-}Cys + Na + 3H_2O]^+$, was observed. Moreover, a new peak associated with the presence of the $[Cu(bphen)_2]^+$ species was observed at *m/z* 727.17 (see Figure 4, blue line (c)). On the other hand, the intensities of the peaks corresponding to the ionic species $[bphen + H]^+$ at *m/z* 333.19, $[2bphen + H]^+$ at *m/z* 664.46, and $[2bphen + Na]^+$ at *m/z* 687.21 were increased considerably. The identical interaction patterns and similar ionic species were also found in the ESI-MS spectra of the interacting systems, involving both complex 1 and 3.

Discussion

The results of *in vitro* cytotoxicity studies against the panel of seven human cancer cell lines uncovered the significant differences of the $IC_{50} \pm SD$ (μM) values (as presented in Table 1) between all the tested complexes 1–6 and the reference drug cisplatin against the A549, HeLa, G361, A2780 and A2780cis cell lines. Moreover, all the tested complexes showed remarkable selectivity towards the G361 and A2780 cell lines, but importantly also towards cisplatin-resistant A2780cis cell line, almost in all the cases with sub-micromolar IC_{50} values. Additionally, it can be said that the calculated IC_{50}(A2780cis)/IC_{50}(A2780) ratio is practically two times lower for 5 and 6 in comparison with cisplatin (Figure 5). The cytotoxic effects for complexes 4, 5 and 6 towards the LNCaP and THP-1 cell lines were identified to be significantly better than those for cisplatin, making them the most effective antiproliferative agents within the tested compounds.

As can be seen, the studied complexes may be divided into two groups differing in the counter ion only, *i.e.* either the NO_3^- (1, 3, and 5) or BF_4^- (2, 4, and 6) counter ion was used. Taking into consideration the same complex cation and different anions, there are three pairs of such complexes which can be compared, *i.e.* 1 vs. 2, 3 vs. 4, and 5 vs. 6. The comparison of the cytotoxic effect within these pairs leads to the conclusion that the type of anionic species does not have an effect on the antiproliferative action, because there was found no statistical significance between the IC_{50} values of the corresponding pairs against all the studied cell lines. Moreover, the lipophilicity of the heterocyclic N-donor co-ligands (phen < mphen < bphen) in the studied complexes correlates well with the cytotoxicity in the case of the A549, HeLa, LNCaP and THP-1 cell lines, *i.e.* the increase in lipophilicity of the ligands is accompanied by the decrease in the IC_{50} values. These observations are in an agreement with the recent reports on significant toxicity of copper(II) mixed-ligand complexes [12,27] involving the heterocyclic N-donor ligands of the 1,10-phenanthroline-type.

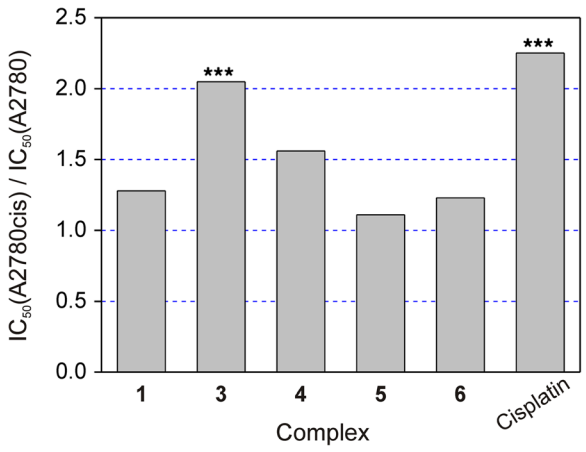

Figure 5 The resistance factors of Cu(II) complexes, expressed as the IC_{50}(A2780cis)/IC_{50}(A2780) ratios. The graphical view of the IC_{50}(A2780cis)/IC_{50}(A2780) ratios calculated from the obtained IC_{50} (μM) values of the *in vitro* cytotoxicity for complexes **1** and **3**–**6**, and cisplatin against the ovarian carcinoma (A2780) and ovarian carcinoma cisplatin resistant (A2780cis) human cancer cell lines. The symbol (***) denotes the statistically significant differences ($p < 0.001$) between the values of IC_{50}(A2780cis) and IC_{50}(A2780).

The importance of the results achieved on a panel of human cancer cell lines has been also emphasized by the results on primary culture of human hepatocytes. Considerably lower cellular toxicity of the studied complexes to primary human hepatocytes was identified and demonstrated by the values of IC_{50}, which were approximately an order of magnitude higher in most cases than those representing the cytotoxicity to the human cancer cell lines (Table 1). Hence, the *in vitro* cytotoxicity profile of the tested compounds is promising in terms of potential therapeutic use.

Although the most important mechanism, i.e. a direct effect of the studied Cu(II) complexes onto DNA, involving intercalation of the complexes into DNA structure and initiation of the oxidation stress leading to the oxidative DNA damage, has been already discussed in our previous paper [19,20], the next mechanism helping damage cell homeostase and contributing to cytotoxic effect of the studied Cu(II) complexes has been

suggested. The *in vitro* inflammation-modulating activity testing revealed significant augmentation of TNF-α cytokine expression induced by complexes 4, 5 and 6 could be caused by a higher pro-oxidant ability of these compounds. The graph in Figure 1 shows that the increase in lipophilicity of the N-donor co-ligands (phen < mphen < bphen, *i.e.* 1, 2 < 3, 4 < 5, 6) leads to the increase in the TNF-α secretion. This is supposedly caused by better penetration of lipophilic compounds to the cells. The type of counter ion (NO_3^- vs. BF_4^-) played a significant role in the TNF-α production. The complexes 2, 4, and 6 involving the BF_4^- counter anion non-significantly increased the amount of the cytokine in comparison with the NO_3^- counter anion complexes (1, 3, and 5 respectively). The level of the secreted TNF-α correlated with the rate of cytotoxicity well. It is possible, that observed cytotoxic effect is caused, at least in part, by activation of TNF receptor 1 (TNFR1) by secreted TNF-α leading to induction of apoptosis.

In the case of the IL-1β cytokine, the reason why complexes 5 and 6 increase its secretion so high may be related to the fact that they probably activate the NLRP3/NALP3 inflammasome via a higher ROS production or directly via the activation of the inflammasome itself. This enzyme complex is highly regulated by ROS and reacts on this danger signal by processing pro-IL-1β into active IL-1β [28]. A similar effect to compounds 5 and 6 was described for doxorubicin and daunorubicin [29]. The mentioned anticancer drugs were able to increase the production of IL-1β in the LPS-stimulated (not in unstimulated) murine bone marrow-derived macrophages via the NLRP3/NALP3 inflammasome activation. But recent studies have indicated that higher production of IL-1β is connected with tumor progression [30,31]. Because commonly used anticancer drugs doxorubicin and daunorubicin show similar effect as herein tested Cu(II) complexes, the particular role of IL-1β during cancerogenesis should be elucidated *in vivo* in the presence of these chemical agents. The effect of a different counter ion was not observed for the IL-1β secretion.

In connection with cytotoxic tests on healthy human hepatocytes, which revealed a relatively advantageous ratio between an effective concentration in relation to cancer cell lines and toxic concentration in relation to human hepatocytes, successful experiments were performed to assess the potential of the studied compounds to cause the induction of xenobiotic metabolizing enzymes as one of the substantive parameters determining the applicability of compounds as potential drugs. Gene reporter assays, assessing the transcriptional activity of AhR and GR in transfected cells proved that the tested compounds did not induce the luciferase activity in AZ-GR, but in the highest concentration of the complexes, the luciferase activity was decreased, probably due to the toxic effects of complexes (Figure 3). The luciferase activity in the AZ-AHR cells was slightly increased by complexes 1–4, but the induction was about 1% of the potency achieved by TCDD (Figure 3). Overall, the tested compounds may be considered inactive towards GR and AhR signaling pathways.

On the basis of the results following from the ESI-MS experiments, it was clearly shown that complexes 1, 3 and 5 are able to interact with the sulfur-containing biogenic biomolecules (cysteine and reduced glutathione) at the physiological levels. These interactions may be associated with at least two simultaneously proceeding mechanisms. The first mechanism is based on the oxidation of biomolecules, leading to the formation of disulphide bonds between cysteine and glutathione, while the second one is related to the direct reaction of copper species with cysteine or glutathione. It is evident, that the quinolinonate ligand is the first ligand in the corresponding complex undergoing the ligand-exchange, while the second bidentate N-donor ligand (a phenanthroline derivative, e.g. phen, mphen, or bphen) is involved in the dynamic equilibrium, enabling it to participate in the formation of much more complicated complex species, as was documented by the peaks in the mass spectra of interacting systems, involving complexes 1, 3, and 5, corresponding to the $[CuL_2]^+$ ion, where L stands for a 1,10-phenanthroline derivative. In an effort to find a relationship between the described mechanisms of interactions and the observed biological activity of the studied complexes, we performed a comparison of our findings with the published results regarding the mechanisms of action of copper(II) complexes involving the 1,10-phenanthroline (phen) moiety in combination with other types of ligands. The theories, presented in the literature [32-34], may lead to the conclusion that the $[CuL_2]^+$ ion, where L stands for a 1,10-phenanthroline derivative, can be considered as biologically relevant species which is able to reversibly bind onto the polynucleotide chain of DNA. Moreover, these ions are able to participate in the Fenton's reaction in proper conditions and produce a whole set of reactive oxygen species *in situ* [35], leading to the oxidative damage of nucleobases, oxidation-related mutagenesis, or cleavage of the polynucleotide backbone. The above-mentioned mechanism of the cytotoxic action of copper(II)-phen complexes is in agreement with our findings observed in the interaction study of complexes 1, 3 and 5 with a mixture of sulfur-containing low-molecular biogenic compounds, cysteine and glutathione, under physiological conditions.

Conclusions

A series of mixed-ligand copper(II) complexes of the type $[Cu(qui)(L)]Y \cdot xH_2O$ (1–6), where Hqui = 2-phenyl-3-

hydroxy-4(1*H*)-quinolinone, Y = NO_3 (1, 3, 5) or BF_4 (2, 4, 6), and L = 1,10-phenanthroline (phen) (1, 2), 5-methyl-1,10-phenanthroline (mphen) (3, 4) and bathophenanthroline (bphen) (5, 6), was studied for their *in vitro* cytotoxicity against seven human cancer cell lines (A549, HeLa, G361, A2780, A2780cis, LNCaP and THP-1). The tested complexes displayed a stronger cytotoxic effect against all the cancer cell lines as compared to cisplatin. The best results were achieved for complexes 4, 5 and 6 against A2780 and A2780cis, with the IC_{50} values in the range of 0.4 – 0.7 μM. On the other hand, the tested complexes were much less cytotoxic to primary human hepatocytes than to the cancer cells showing the ratio between the effective concentration against the cancer cells and the toxic concentration to primary human hepatocytes up to 1:40 (for complex 3). Moreover, complexes 5 and 6 exhibited a significantly higher ability to modulate the secretion of the pro-inflammatory cytokines TNF-α and IL-1β tested on the lipopolysaccharide (LPS)-stimulated THP-1 cells. The pro-inflammatory activity of the Cu(II) complexes represents next of possible pathways influencing cell processes leading to manifestation of cytotoxicity of the studied compounds. The ability of the tested complexes to interact with sulfur-containing biomolecules (cysteine and reduced glutathione) at physiological levels was proved by ESI-MS experiments and the results led to the proposal of several possible mechanisms of direct interaction with biomolecules in relation to the cytotoxic action. Overall positive results of the biological activity studies revealed that the presented complexes may represent good candidates for non-platinum anticancer drugs, however, we are aware of the fact that further and deeper studies mainly in connection with the elucidation of their mechanisms of antiproliferative action and acute toxicity will be necessary.

Methods

Chemistry

All chemicals and solvents were purchased from commercial sources (Sigma-Aldrich Co., Acros Organics Co., Lachema Co., Fluka Co.) and were used as received. The preparation and characterization of complexes 1, 3, 5 and 2, 4, 6 were already reported by our group [19,20]. The copper(II) mixed-ligand complexes of the types [Cu(qui)(phen)]$NO_3 \cdot H_2O$ (1), [Cu(qui)(phen)]BF_4 (2), [Cu(qui)(mphen)]$NO_3 \cdot H_2O$ (3), [Cu(qui)(mphen)]$BF_4 \cdot H_2O$ (4), [Cu(qui)(bphen)]$NO_3 \cdot H_2O$ (5) and [Cu(qui)(bphen)]BF_4 (6) (qui = 2-phenyl-3-hydroxy-4(1*H*)-quinolinonate anion, phen = 1,10-phenanthroline, mphen = 5-methyl-1,10-phenanthroline, bphen = bathophenanthroline) (Figure 6) were prepared and fully characterized previously [19,20]. Complexes 1–6 are soluble in polar solvents and their mixed water solutions with dimethylformamide (DMF), dimethyl sulfoxide (DMSO) or methanol (MeOH) were used in the tests of biological activities and interaction studies.

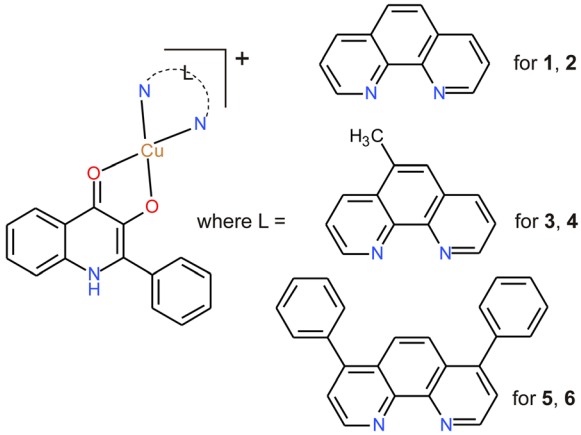

Figure 6 Schematic representations of the complex cations in [Cu(qui)(L)]Y•xH_2O (1–6). Schematic representations of the complex cations in [Cu(qui)(L)]Y · xH_2O (1–6), where Hqui = 2-phenyl-3-hydroxy-4(1*H*)-quinolinone, Y = NO_3 (1, 3, 5) or BF_4 (2, 4, 6).

In vitro cytotoxicity testing

Human cancer cell lines A2780 (ovarian carcinoma; ECACC No. 93112517), A2780cis (ovarian carcinoma *cisplatin*-resistant; ECACC No. 93112519), G361 (malignant melanoma; ECACC No. 88030401), A549 (lung carcinoma; ECACC No. 86012804), HeLa (cervix epitheloid carcinoma; ECACC No. 93021013), LNCaP (androgen-sensitive human prostate adenocarcinoma; ECACC No. 89110211) and THP-1 (human monocytic leukemia; ECACC No. 88081201) were purchased from the European Collection of Cell Cultures (ECACC). The culture medium and conditions were according to the ECACC instructions. Experiments were performed using the cell passage ranging from 5th to 30th. The cells were maintained at 37°C and 5% CO_2 in a humidified incubator. The human cancer cell lines A2780, A2780cis, G361, A549, HeLa and LNCaP were treated with the tested compounds (applied in the volume of 200 μL and in the concentrations ranging from 0.01 μM to 50 μM, unless their solubility was lower) for 24 h, using 96-well culture plates. In parallel, the cells were treated with vehicle (DMF; 0.1%, v/v) and Triton X-100 (1%, v/v) to assess the minimal (*i.e.* positive control) and maximal (*i.e.* negative control) cell damage, respectively. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was measured spectrophotometrically at 540 nm (TECAN, Schoeller Instruments LLC) [36]. The data were expressed as the percentage of viability, when 100% and 0% represent the treatments with DMF, and Triton X-100, respectively. The data were acquired from three independent experiments (conducted in triplicate) using

cells from different passages. The data are presented as mean IC_{50} ± SD (μM) values together with their standard deviations, calculated from viability curves. The obtained IC_{50} values were evaluated by the ANOVA analysis incorporated within the QC Expert program package [37].

The human monocytic leukemia cell line THP-1 (floating monocytes, 500 000 cells/mL) was incubated in 100 μL of the serum-free RPMI 1640 medium and seeded into 96-well plates in triplicate at 37°C. Measurements were taken 24 h after the treatment with the tested compounds in the concentrations of 6–0.074 μM dissolved in dimethylsulfoxide (DMSO). Viability was measured by the WST-1 test [Cell Proliferation Reagent WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolium]-1,3-benzene disulfonate) was obtained from Roche (Mannheim, Germany)] according to manufacturer's manual. The amount of created formazan (which correlates to the number of metabolically active cells in the culture) was calculated as a percentage of the control cells, which were treated only with DMSO and was set-up as 100% [38]. The IC_{50} values were calculated from viability curves by software GraphPad Prism 5.02 (GraphPad Software Inc., San Diego, CA). The data are presented as mean IC_{50} ± SD (μM) values of three independent experiments.

Primary cultures of human hepatocytes

Human hepatocytes were isolated from a liver tissue, resected from a multiorgan donor. A tissue acquisition protocol was in accordance with the requirements issued by an Ethical committee at the Faculty Hospital Olomouc in the Czech Republic. Human hepatocytes were isolated by two-step collagenase perfusion and the cells were plated on a collagen-coated culture dishes using cell density of 14×10^4 cells/cm^2 [39]. The culture medium was Williams and HAMs F-12 (1 : 1) supplemented with penicillin, streptomycin, ascorbic acid, linoleic acid, holo-transferin, ethanolamine, glucagon, insulin, dexamethasone, pyruvate, glucose, glutamine and amphotericin. The medium was enriched for plating with 2% of fetal calf serum (v/v). The medium was exchanged for a serum-free medium the next day and the culture was stabilized for additional 24 h. After that the cells were ready for the treatment. The cultures were maintained at 37°C and 5% CO_2 in a humidified incubator.

THP-1 cell culture and differentiation to macrophages

To determine the influence of the tested complexes on the TNF-α and IL-1β secretions, the human monocytic leukemia cell line THP-1 was used. The cells were cultivated at 37°C in the RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS), 100 U/mL of penicillin and 100 μg/mL of streptomycin in a humidified atmosphere containing 5% CO_2. The stabilized cells (3rd–20th passage) were split into microtitration plates to get a concentration of 100 000 cells/mL and the differentiation to macrophages was induced by a phorbol myristate acetate (PMA) as we described previously [40].

Drug treatment and induction of inflammatory response

Differentiated macrophages were pretreated for 1 h with 0.2 μM solutions of the tested complexes, prednisone or $CuSO_4 \cdot 5\ H_2O$ dissolved in DMSO (the final DMSO concentration was 0.1%) and with 0.1% DMSO solution itself (the experimental group called *vehicle*); the given concentrations of the tested compounds lack the cytotoxic effect. The inflammatory response was triggered by adding 1.0 μg/mL lipopolysaccharide (LPS) dissolved in water to pretreated macrophages, *control* cells were without the LPS treatment. Each experiment was repeated three-times [38].

Evaluation of cytokine secretion by ELISA

Macrophages pretreated with the tested compounds for 1 h were incubated with LPS for next 24 h. After this period, the medium was collected and the concentration of TNF-α and IL-1β was measured by Instant ELISA kit (eBioscience, Vienna, Austria) according to the manufacturer's manual.

Gene reporter assays

The transcriptional activity of AhR was assessed using the human hepatoma cells HepG2 stably transfected with plasmid containing dioxin-response elements fused to the luciferase reporter gene (AZ-AhR cells). The construction and characterization of the AZ-AhR cell line was described elsewhere [22]. The transcriptional activity of GR was assessed in human cervix carcinoma cells HeLa stably transfected with plasmid containing glucocorticoid-response elements fused to the luciferase reporter gene (AZ-GR cells). The cells were seeded in the medium supplemented with 10% charcoal/dextran-stripped foetal bovine serum on 96-well plates and stabilized for 16 h prior to the treatments. The cells were treated for 24 h with the tested compounds, TCDD (5 nM), dexamethasone (100 nM) and/or vehicle (DMSO; 0.1%, v/v). After the treatments, the cells were lysed using a luciferase detection system (Promega) and the luciferase activity was measured on a luminometer (Tecan, Schoeller, Prague).

Interactions of complexes 1, 3 and 5 with cysteine and glutathione assessed by electrospray-ionization mass spectrometry

The electrospray-ionization mass spectrometry (ESI-MS) is a widely used method for studies of covalent [41] or non-covalent [42,43] interactions of small molecules or

ions with biologically relevant molecules. Therefore we performed the interaction experiments between complexes 1–6 and cysteine or glutathione respectively on an Agilent HP1100 LC-MSD VL Ion-Trap mass spectrometer, using the positive ionization mode. The parameters set for the analyses were as follows: the measured solutions were infused directly to the sprayneedle by linear pump with the rate of 2000 μL/h, the nebulization gas (nitrogen) flow was set to 6 L/min and its pressure was 60 psi. The drying gas temperature was set to 250°C. The measured range of ions was set from *m/z* 50 to 1500. Before the analysis of the interacting systems, the instrument parameter set (such as skimmer voltages, trap drive, octopole RF amplitude, octopole delta, lenses voltages, capillary voltage, etc.) was automatically optimized for the ion m/z 308 ± 0.5, corresponding to the protonized form of reduced glutathione (*m/z* 308 ± 0.5; $GSH + H^+$).

Additional file

Additional file 1: Figure S1-S8. The Additional file 1 contains graphical representations of the results of *in vitro* cytotoxicity testing (Figs. S1–S8).

Abbreviations

A2780: Ovarian carcinoma; A2780cis: Ovarian carcinoma *cisplatin*-resistant; A549: Lung carcinoma; AhR: Aryl hydrocarbon receptor; bphen: Bathophenanthroline; DMF: Dimethylformamide; ESI-MS: Electrospray-ionization mass spectrometry; FBS: Fetal bovine serum; G361: Malignant melanoma; GR: Glucocorticoid receptor; HeLa: Cervix epitheloid carcinoma; HH1: Human hepatocytes; Hqui: 2-phenyl-3-hydroxy-4(1*H*)-quinolinone; HOS: Human osteosarcoma; IL-1β: Interleukin 1-beta; LNCaP: Androgen-sensitive human prostate adenocarcinoma; LPS: Lipopolysaccharide; MCF7: Breast adenocarcinoma; mphen: 5-methyl-1,10-phenanthroline; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB: Nuclear factor kappa; phen: 1,10-phenanthroline; PMA: Phorbol myristate acetate; ROS: Reactive oxygen species; TCDD: 2,3,7,8-tetrachlorodibenyodioxin; THP-1: Human monocytic leukemia; TNF-α: Tumor necrosis factor alpha; WST-1: 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolium]-1,3-benzene disulfonate.

Competing interests

The authors have no conflicts of interest.

Authors' contributions

RB prepared the tested compounds in sufficient quantities. ZD carried out the *in vitro* cytotoxicity experiments and experiments involving the human hepatocytes. JH carried out the *in vitro* cytotoxicity experiments on THP-1 cells and *in vitro* anti-inflammatory assays. JV carried out the interaction studies with sulfur-containing biomolecules. All authors participated on drafting of the manuscript. Additionally, ZT provided critical results evaluation and coordinated all works, contributed to the manuscript preparation, including the final approval of the manuscript before submitting. All authors read and approved the final manuscript.

Acknowledgements

The financial support from the Operational Program Research and Development for Innovations - European Regional Development Fund (CZ.1.05/2.1.00/03.0058), the Operational Program Education for Competitiveness - European Social Fund (CZ.1.07/2.3.00/20.0017), and Internal Grant Agency of the Palacký University in Olomouc (IGA PrF_2012_009) is greatly acknowledged.

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Received: 17 October 2012 Accepted: 18 December 2012
Published: 20 December 2012

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doi:10.1186/1752-153X-6-160
Cite this article as: Trávníček *et al.*: Cellular responses induced by Cu(II) quinolinonato complexes in human tumor and hepatic cells. *Chemistry Central Journal* 2012 **6**:160.