Supporting information for:

Copper-dependent cytotoxicity of 8-Hydroxyquinoline derivatives correlates with their hydrophobicity and does not require caspases activation.

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Stability constants determination. The thermodynamic stability of Cu(II) complexes with the 8-HQ and 5-SO_3-8-HQ ligands was studied in DMSO:water 1:1 (*w/w*) (T = 25 °C, I = 0.1 M (KCl)) by potentiometric titrations, using an apparatus already described in the literature.[1] Freshly boiled, N_2-saturated doubly distilled water and N_2-fluxed DMSO (heated at 180 °C) were used for the preparation of the DMSO:water mixtures. The Cu(II) stock solution (C_{Cu} = *ca.* 0.016 M) was prepared by weight from $CuCl_2 \cdot 2H_2O$, and its titre was determined by complexometric titrations with standard EDTA solutions using pyrocatechol violet as the indicator. HCl aqueous solutions (*ca.* 0.2 M) and KOH solutions (*ca.* 0.2 M in DMSO:water 1:1 (*w/w*)) were prepared by diluting concentrated Merck Titrisol ampoules with the proper amounts of CO_2-free water and DMSO, and standardized with the usual procedure of this laboratory.[1] Solutions of the ligands 8-HQ and 5-SO_3-8-HQ (*ca.* 0.1 M) were prepared by weight in DMSO:water 1:1 (*w/w*) using the pure ligands and adding a stoichiometric amount of HCl (aqueous 10 N solution). Their titre (ligand content and excess of acid) was checked by potentiometric titration with standard KOH solutions. The titrations were carried out at T = 25.0 ± 0.1 °C and I = 0.1 M (KCl) under a stream of N_2, using 50 ml samples. Prior to its use, the reference compartment of the Hamilton combined glass electrode (P/N 238000) was filled with a 0.1 M KCl solution in DMSO:water 1:1 (*w/w*), and left to equilibrate overnight. The electrode was calibrated in terms of $[H^+]$ by titrating HCl solutions with standard KOH solutions and the calculated pK_w value resulted to be 15.25(5).

The protonation constants of the ligands were determined by alkalimetric titration of three samples (6.1-9.1 × 10^{-3} M for 8-HQ, 3.3-3.8 × 10^{-3} M for 5-SO_3-8-HQ) in the pH range 2-11. The complex formation constants of the Cu^{2+}/8-HQ or 5-SO_3-8-HQ systems were determined by alcalimetric titrations of three samples (Cu^{2+}:8-HQ = 1:1.0-1.2, C_{Cu} = 7.0-7.9 × 10^{-4} M; Cu^{2+}:5-SO_3-8-HQ = 1:2.0-2.2, C_{Cu} = 1.5-1.6 × 10^{-3} M) in the pH range 2-9. The 8-HQ/Cu^{2+} ratio was limited to 1.2 and pH 4.5 because of the poor solubility of the $[Cu(8\text{-}HQ)_2]$ complex in the solvent mixture.

Table S1. Logarithms of proton dissociation and of complex formation constants of the [Cu(L)] and $[Cu(L)_2]$ complexes for the ligands 8-HQ and 5-SO_3-8-HQ in DMSO:water 1:1 (*w/w*) (T = 25.0 ± 0.1 °C and I = 0.1 M (KCl). The pK_a values refer to the ligands in the H_2L form. Charges are omitted for simplicity.

Ligand	**pK_{a1}, pK_{a2}**	**Log β_1, Log β_2**
8-HQ	3.99(1), 10.53(1)	11.26(4), 22.31(3)
5-SO_3-8-HQ	3.14(4), 9.26(2)	11.72(9), 22.76(7)

For both ligands, the first proton dissociation occurs with a pK_a of 3.1-4 and is associated to the release of the proton from the protonated quinolinic nitrogen atom. The second dissociation, occurring with pK_a values of 9.2-10.5, is associated to the release of protons from the hydroxyl group. Due to its low basicity, the sulfonate group results always deprotonated in the pH ranges where the measurements have been performed (2-11). On the basis of these protonation constants, it results that the predominant forms of the ligands at pH 7.4 are the neutral form HL for 8-HQ and the negative charged HL^- for 5-SO_3-8-HQ. These protonation constants are quite similar to those reported for alcoholic solutions of the two ligands, at a 0.1 M ionic strength and T = 25 °C (8-HQ: 4.97, 9.65; 5-SO_3-8-HQ: 3.90, 8.37).[2] In agreement with literature data, the presence of sulfonate group in 5 position produces a decrease in the pK_{a2} value.

Both ligands form 1:1 and 1:2 Cu(II):ligand complexes in solution, with remarkably similar stabilities. These two values match pretty well with the values determined in other aqueous/organic

solvent mixtured (Log β_1 11-14 and Log β_2 22-25) for analogous ionic strength and temperature.[2-4] This similarity is particularly interesting if it is considered that our measurements is performed in 50% (weight) DMSO, which might act as a competing ligand towards the complexation of copper(II) through its sulfur atom. The data confirm the moderate/high stability of the complexes in solution.

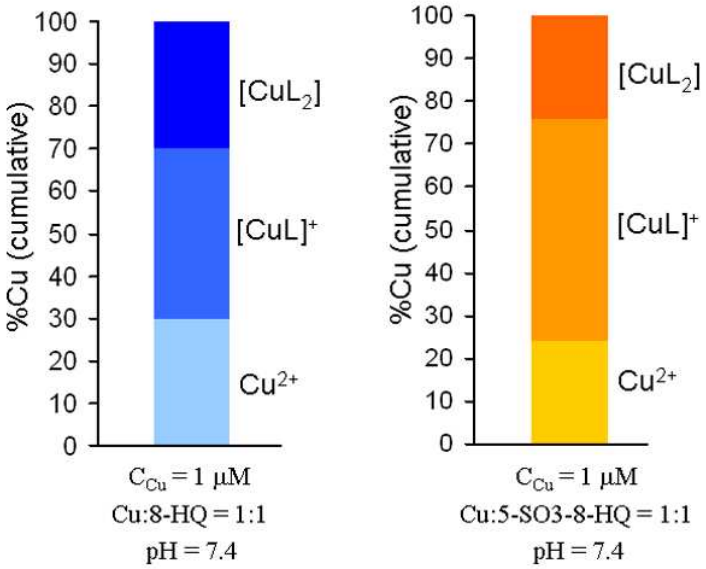


Figure S1. Expected concentrations of the indicated species, calculated in accordance with the reported Log β_1 and Log β_2, at the concentration of 1 µM in the absence of other competitors for copper binding.

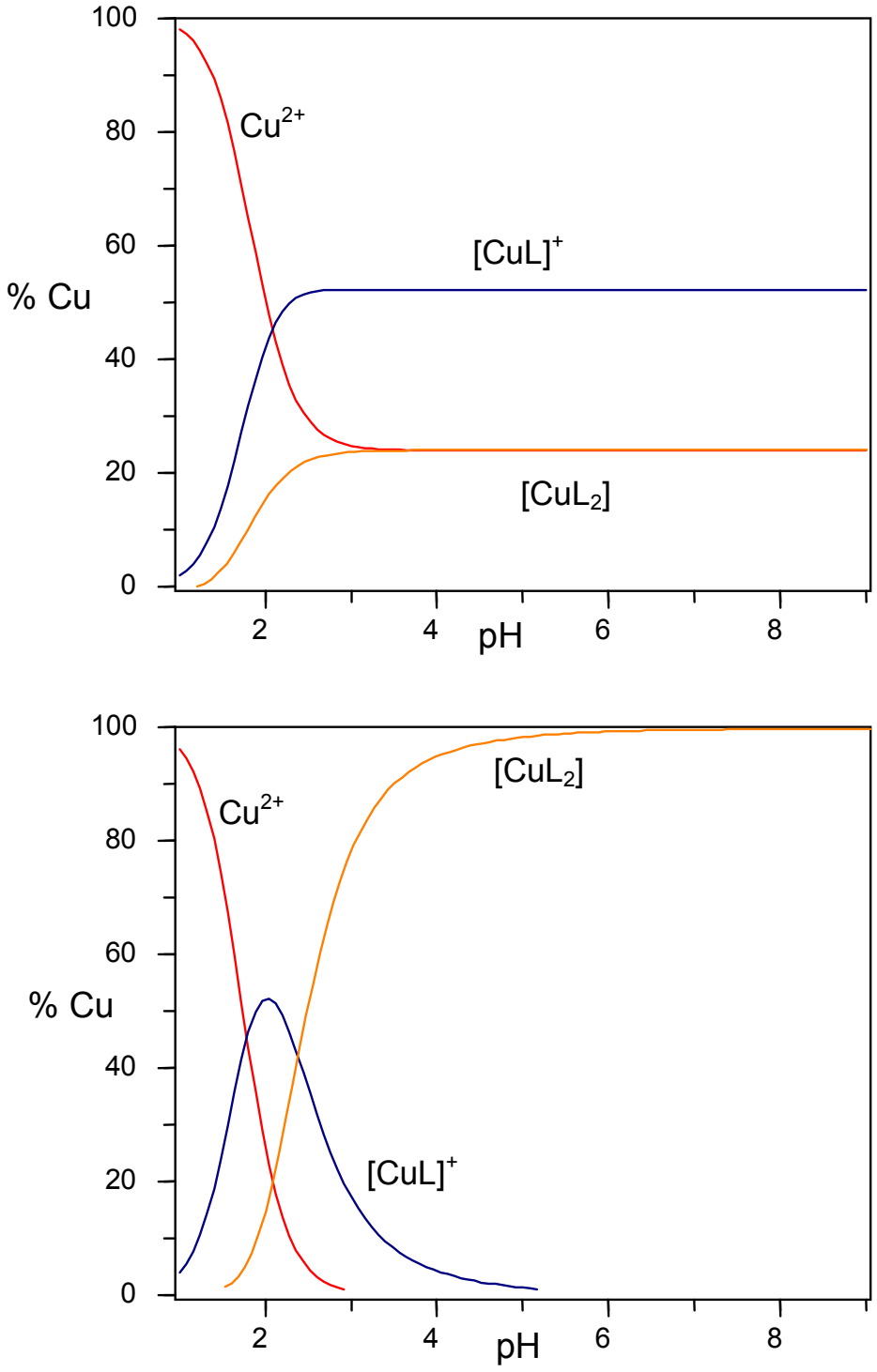


Figure S2. Distribution diagrams of the system Cu^{2+}/5-SO_3-8-HQ. Above, Cu^{2+}/5-SO_3-8-HQ = 1/1, $C_{Cu} = 1 \times 10^{-3}$ M. Below, Cu^{2+}/5-SO_3-8-HQ = 1/2, $C_{Cu} = 1 \times 10^{-3}$ M.

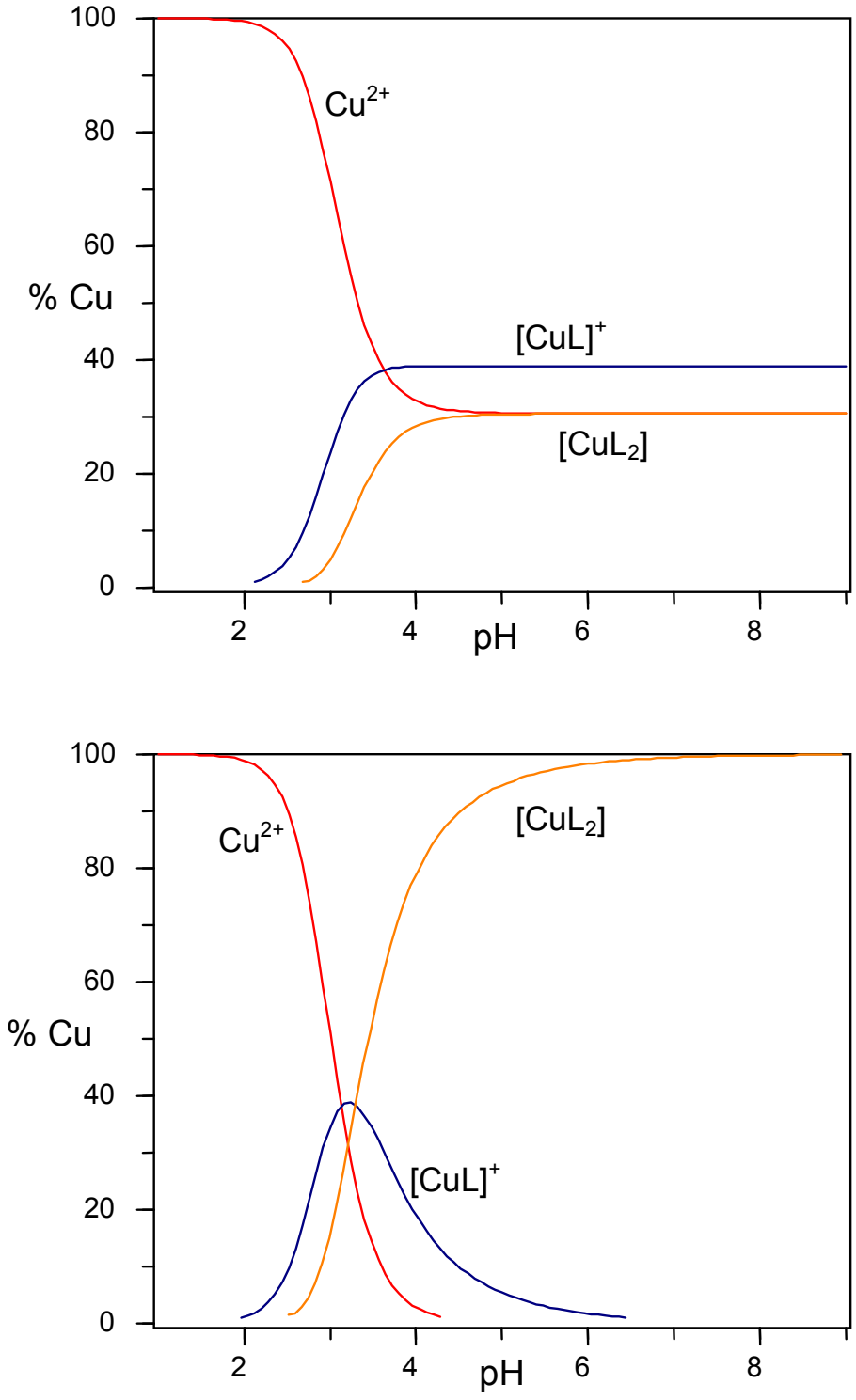


Figure S3. Distribution diagrams of the system Cu^{2+}/8-HQ. Above, Cu^{2+}/8-HQ = 1/1, $C_{Cu} = 1 \times 10^{-3}$ M. Below, Cu^{2+}/8-HQ = 1/2, $C_{Cu} = 1 \times 10^{-3}$ M.

Copper/8-hydroxyquinolines interaction with human serum albumin

The circular dichroism spectra of HSA in absence and in presence of $CuCl_2$, $[Cu(8\text{-}HQ)_2]$ or $[Cu(5\text{-}SO_3\text{-}8\text{-}HQ)(H_2O)_2]$ were performed on samples containing HSA (85 μM) in DMSO:H_2O 1:9 *v:v*, HEPES 50mM, pH 7.4. The concentration of the copper compounds was 37 μM ($CuCl_2$, $[Cu(8\text{-}HQ)_2]$) or 59 μM ($[Cu(5\text{-}SO_3\text{-}8\text{-}HQ)(H_2O)_2]$). The samples were prepared by mixing proper aliquots of stock solutions of HSA (*ca*. 0.5 mM, DMSO:H_2O 1:9 *v:v*, HEPES 50mM, pH 7.4) and of the copper compounds (40-70 μM in DMSO:H_2O 1:9 *v:v*, unbuffered). The pH was checked after preparations of the samples. The spectra were collected on a Jasco J715 spectropolarimeter provided with a Peltier thermostatting device, using quartz cells of 1 cm path length.

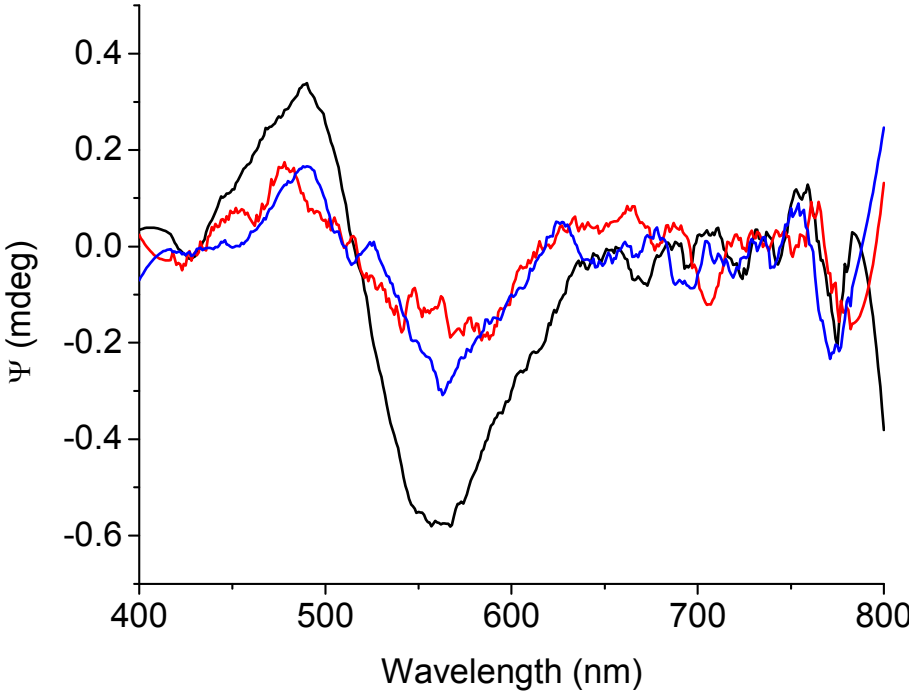


Figure S4. Cirular dichroism spectra of human serum albumin (HSA) (85 μM, DMSO:H_2O 1:9 *v:v*, HEPES 50mM, pH 7.4) in the absence (black dotted) and in presence of $CuCl_2$ (blue, 37 μM), $[Cu(8\text{-}HQ)_2]$ (red, 37 μM) or $[Cu(5\text{-}SO_3\text{-}8\text{-}HQ)(H_2O)_2]$ (green, 59 μM).

The stability of the complexes $[Cu(8\text{-}HQ)_2]$ and $[Cu(5\text{-}SO_3\text{-}8\text{-}HQ)(H_2O)_2]$ in the presence of HSA was evaluated by means of circular dichroism.[5] In Figure S4 we report the CD spectra of solutions of HSA (85 μM) in DMSO:H_2O (1:9 *v:v*, HEPES 50mM, pH 7.4) in presence and in absence of $CuCl_2$, $[Cu(8\text{-}HQ)_2]$ and $[Cu(5\text{-}SO_3\text{-}8\text{-}HQ)(H_2O)_2]$. The HSA concentration in the samples was 85 μM, close to those used for the viability tests (HSA ca. 60 μM). The CD spectra in presence of $CuCl_2$, $[Cu(8\text{-}HQ)_2]$, and $[Cu(5\text{-}SO_3\text{-}8\text{-}HQ)(H_2O)_2]$/HSA show a Cotton effect in the range 450-650 nm related to the Cu(II) d-d transitions and indicative of the coordination of Cu(II) to the high affinity binding site at the N-terminus of the protein. In the $CuCl_2$/HSA sample all Cu(II) is bound to HSA as the result of the Cu-HSA high affinity and this system was used as the control. By comparing the CD spectra derived by the $CuCl_2$/HSA sample with those derived by the $[Cu(8\text{-}HQ)_2]$/HAS and $[Cu(5\text{-}SO_3\text{-}8\text{-}HQ)(H_2O)_2]$/HSA samples, it can be noted that the latter systems presents a lower Cotton effect. This indicates that a fraction of Cu(II) forms the Cu-HSA adduct but that a significant fraction of $[Cu(8\text{-}HQ)_2]$ or $[Cu(5\text{-}SO_3\text{-}8\text{-}HQ)(H_2O)_2]$ is still present in solution, even in the presence of an excess of the protein.[6] Altogether, this data demonstrate that the complexes are stable to a significant extent in presence of serum albumin at the concentration used for the biological tests.

Single Crystal X-ray Structures. Single crystal data were collected with a Bruker Smart APEXII area detector diffractometers (Mo Kα; λ = 0.71073 Å). Cell parameters were refined from the observed setting angles and detector positions of selected strong reflections. Intensities were integrated from several series of exposure frames that covered the sphere of reciprocal space.[7] A multiscan absorption correction was applied to the data using the program SADABS.[8] The structures were solved by direct methods (SIR2004[9]) and refined with full-matrix least-squares (SHELXL-97),[10] using the Wingx software package.[11] Graphical material was prepared with the Mercury 3.0[12] programs. CCDC 891695 contains the supplementary crystallographic data.

Table S2. Summary of X-ray crystallographic data for [Cu(5-Me-7-Me-8-HQ)$_2$].

Empirical	$C_{66}H_{60}Cu_3N_6O_6$
Formula weight	1223.82
Colour, habit	Green, block
Crystal size, mm	0.13x0.12x0.07
Crystal system	Triclinic
Space group	*P*-1
a, Å	9.242(3)
b, Å	10.288(3)
c, Å	14.494(4)
α, deg.	77.219(5)
β, deg.	84.291(4)
γ, deg.	80.289(5)
V, Å^3	1321.9(7)
Z	1
T, K	293(2)
ρ (calc), Mg/m^3	1.537
μ, mm^{-1}	1.259
θ range, deg.	1.44 to 25.72
No.of	15119 / 5043
GooF	1.047
*R*1	0.0546
*wR*2	0.1273

$R1 = \Sigma||F_O|-|F_C||/\Sigma|F_O|$, $wR2 = [\Sigma[w(F_O^2-F_C^2)^2]/\Sigma[w(F_O^2)^2]]^{½}$, $w = 1/[\sigma^2(F_O^2) + (aP)^2 + bP]$, where $P = [\max(F_O^2,0) + 2F_C^2]/3$

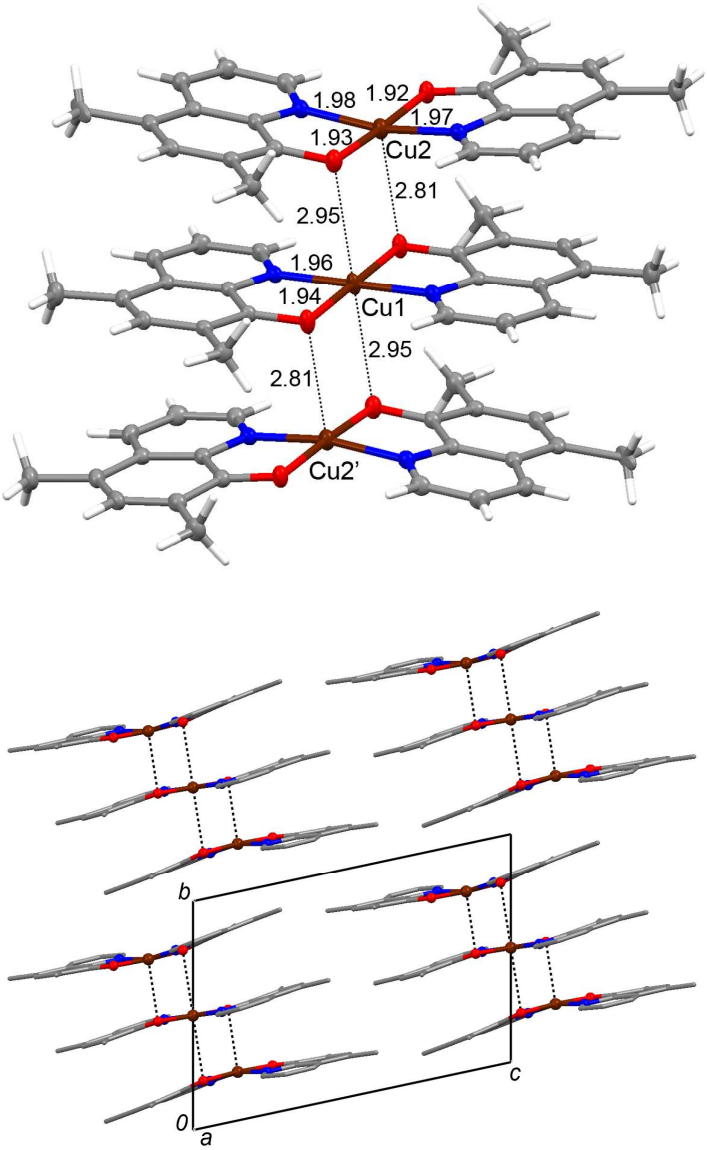


Figure S5. Above, molecular structures of [Cu(5-Me-7-Me-8-HQ)$_2$]. The Cu(1) atom lies on an inversion center, symmetry code ' = -x; 1-y; -z. Selected bond distances are reported in Å. C (grey), H (white), N (blue), O (red), Cu (brown). Below, crystal packing of [Cu(5-Me-7-Me-8-HQ)$_2$] projected along the *a* crystallographic axis.

The complex crystallizes in the *P*-1 space group. The unit cell contains three [Cu(5-Me-7-Me-8-HQ)$_2$] complex molecules, and the Cu(1) atom lies on an inversion center. The metal coordination is square planar with the Cu-N bond distances that are significantly longer than the Cu-O ones. The arrangement of the two 5-Me-7-Me-8-HQ surrounding each metal gives rise to a *trans* geometry. The three molecular entities comprising the unit cell may be described as a trimer according to the presence of very weak interactions between the metal and the oxygen atoms of superimposed molecules.

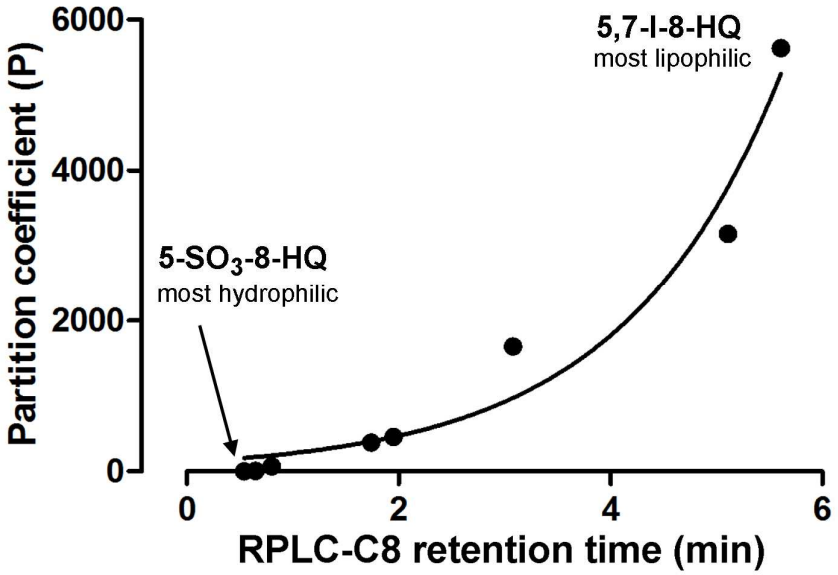
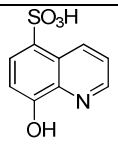
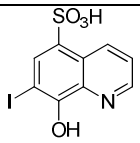
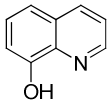
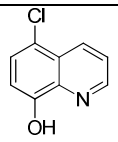
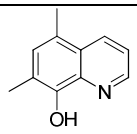
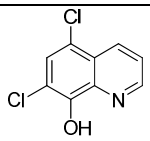
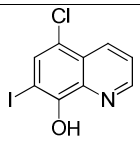
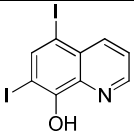


Figure S6. Correlation between the calculated partition coefficient values (P) and the RPLC-C8 retention time for the eight 8-HQ derivatives.

A DMSO mixture containing the ligands was analyzed by means of Reversed-Phase Liquid Chromatography (RPLC) and the analytes were detected with an electrospray ionization linear ion trap (ESI-LIT) mass spectrometry. LC separation was carried out on a C8 Ascentis Express (30 x 2.1 mm, 2.7 µm particles; Supelco, Sigma Aldrich) column thermostated at 25 °C using a gradient solvent elution system: (A) aqueous formic acid 0.1% solution (v/v), (B) 0.08% (v/v) formic acid in acetonitrile. Gradient elution was as follows: solvent B was set at 40% for 3 min, then delivered by a linear gradient from 40% to 60% for 1 min. Solvent B was maintained at 60% for 3 min before column reequilibration (3 min). The flow-rate was 300 µL/min. The mobile phase was delivered by the Surveyor chromatographic system (Thermoelectron Corporation, San Josè, CA, USA). Injection volume was 10 µL. ESI-MS^2 experiments were performed under product ion conditions (collision energy, 13 eV) in the 95-500 *m/z* range.

Table S3. Schematic depiction of the molecular structures of the ligands together with the IC_{50} values (μM) on HeLa and PC3 human tumor cell.

Ligand	Structure	cLogP	RPLC retention time (min.)	IC_{50} (HeLa)	IC_{50} (PC3)
5-SO_3-8-HQ		-0.21	0.54	n.d.	n.d.
5-SO_3-7-I-8-HQ		0.70	0.65	n.d.	n.d.
8-HQ		1.84	0.80	13.5	8.6
5-Cl-8-HQ		2.58	1.74	15.6	12.4
5,7-Me-8-HQ		2.66	1.95	13.8	7.6
5,7-Cl-8-HQ		3.22	3.08	16.8	17.8
(5-Cl-7-I-8-HQ)		3.50	5.11	26.1	23.7
5,7-I-8-HQ		3.75	5.61	25.2	25.7

Dose-response curves of CQ and Cu(CQ) in HeLa, PC3 and HUVEC cells in the presence of 10% FBS.

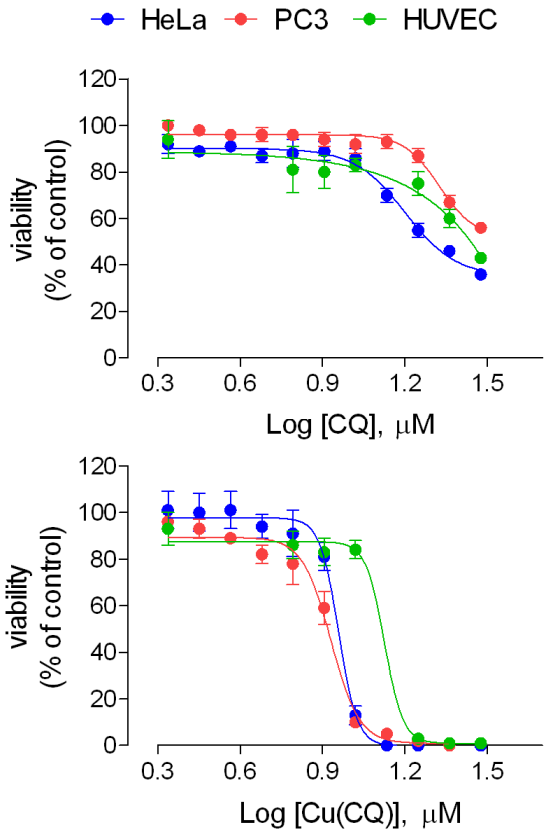


Figure S7. Different concentrations of CQ and Cu(CQ) were tested on HeLa, PC3 and HUVEC cells maintained in medium containing 10% FBS for 48 h. After the treatment, viability was assessed with the resazurin method. Data presented are means ± SEM of three independent experiments, each performed in quadruplicate. Error bars are visible when greater than the points.

Effect of Cu(CQ) on cell number and LDH release in human fibroblasts.

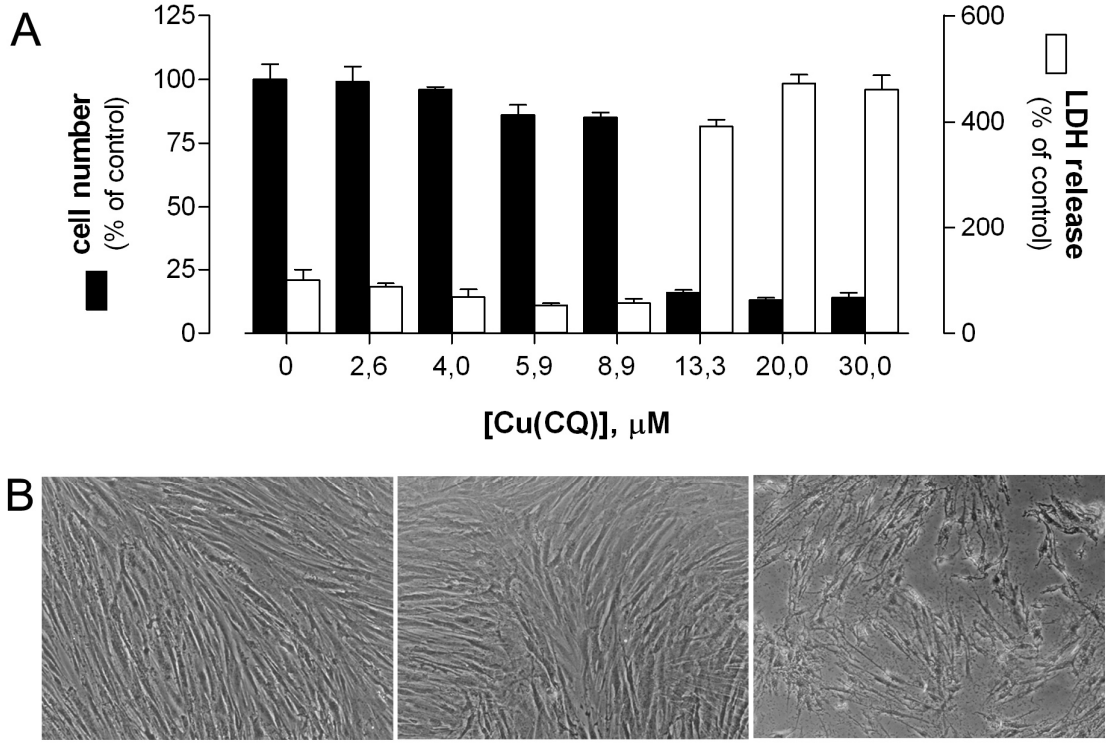


Figure S8. HF were treated for 48 h with the indicated concentrations of Cu(CQ). A. Cell number and LDH release were measured under each experimental condition. Data presented are means ± SEM of three independent experiments, each performed in triplicate. B. Phase contrast images of representative microscopic fields (100 x) are shown. Left: control, untreated cells; middle: 8.9 μM Cu(CQ); right: 30 μM Cu(CQ).

Effects of TM 40µM on Cu(CQ)-induced cytotoxicity in HeLa and PC3 cells.

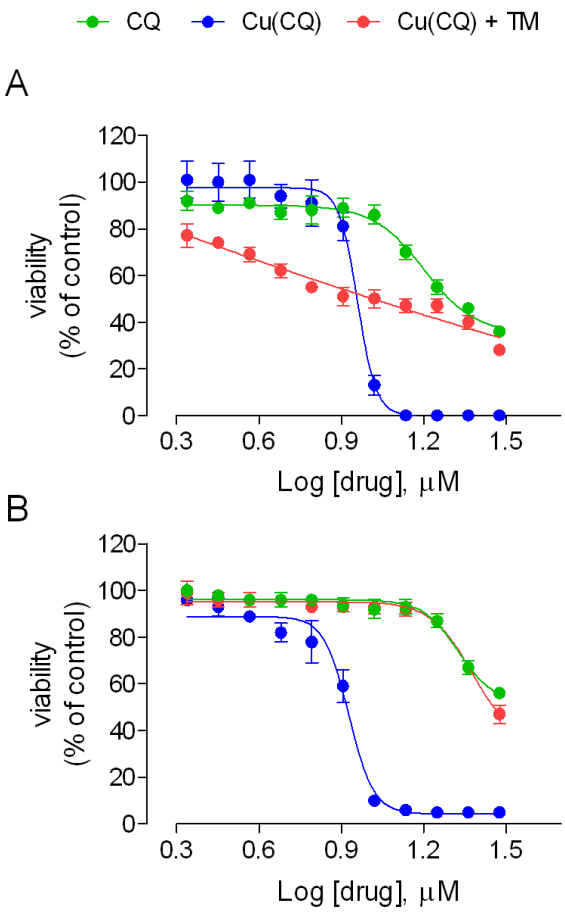


Figure S9. HeLa (A) and PC3 (B) cells were treated for 48 h with different concentrations of CQ or Cu(CQ), either in the absence or in the presence of 40 µM ammonium tetrathiomolybdate (TM). Cell viability was assayed with the resazurin method. Data presented are means ± SEM of two independent experiments, each performed in quadruplicate. Error bars are visible when greater than the points.

Effect of Cu(CQ) treatment on AIF localization in HeLa cells.

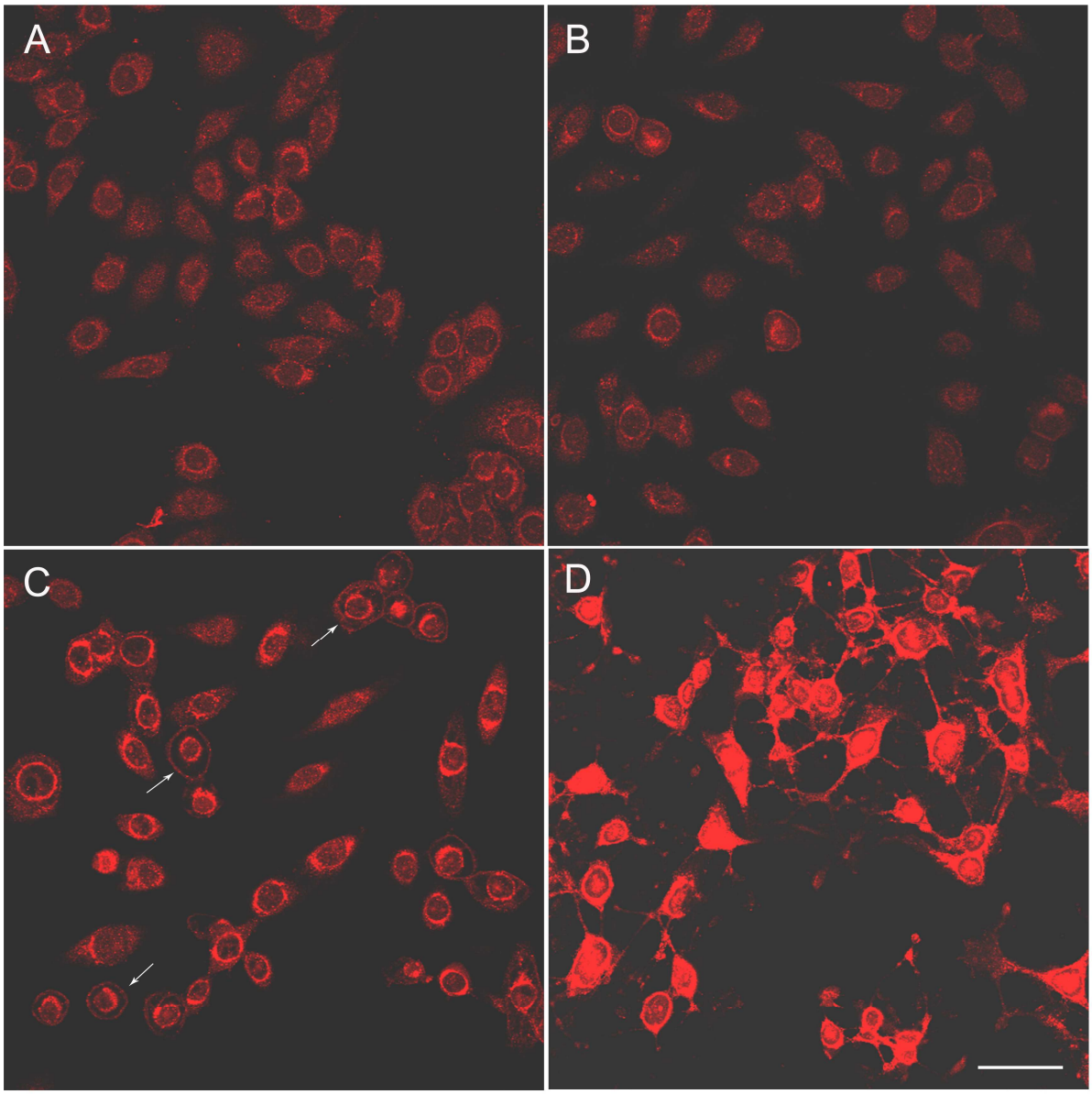


Figure S10. HeLa cells were left untreated (A), treated with 50 µM CQ (B) and 10 µM Cu(CQ) (C) for 18 h, or treated with 400 nM staurosporin (D) for 6 h. AIF immunostaining was performed as described in the experimental section. Confocal single sections of representative microscopic fields are shown. White arrows indicate cells showing extensive cytoplasmic vacuolization. Original magnification 400x, bar = 50 µm.

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