

Structural Characterization and Biological Evaluation of a Clioquinol–Ruthenium Complex with Copper- Independent Antileukaemic Activity

Supporting Information

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0. Abbreviations

Ac-DEVD-AFC, *N*-acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin; AMC, 7-amino-4-methylcoumarin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CqH, clioquinol; DMEM, Dulbecco's modified Eagle's medium; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; PBS, phosphate-buffered saline; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid); SEAP, secreted embryonic alkaline phosphate; TNF α , tumor necrosis factor alpha; zVAD.fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp(*O*-Me) fluoromethyl ketone.

1. Materials and methods

Attenuated total reflectance (ATR) infrared spectra were recorded on a Perkin-Elmer Spectrum 100 spectrometer. The measurements were made in the range from 4000 cm⁻¹ to 600 cm⁻¹.

Elemental analyses were performed on a Perkin-Elmer 2400 CHN Elemental analyzer.

X-ray diffraction data were collected on an Oxford Diffraction SuperNova diffractometer with a Cu microfocus X-ray source, with mirror optics and an Atlas detector. The structures were solved using SIR92.¹ Full-matrix least-squares refinement on the *F* magnitudes with anisotropic displacement factors for all of the non-hydrogen atoms used SHELXL.² The drawings and the analysis of bond lengths, angles and intermolecular interactions were carried out using Mercury and Platon.³ Hydrogen atoms were placed in geometrically calculated positions and were refined using a riding model.

1. Altomare, A.; Cascarano, G.; Giacovazzo, C.; Guagliardi, A. C.; Burla, M. C.; Polidori, G.; Camalli, M., *SIR92 – a program for automatic solution of crystal structures by direct methods. J. Appl. Cryst.* **1994**, *27*, 435.
2. Sheldrick, G. M., A short history of SHELX. *Acta Crystallogr., Sect. A* **2008**, *64*, 112-122.
3. Macrae, C. F.; Edgington, P. R.; McCabe, P.; Pidcock, E.; Shields, G. P.; Taylor, R.; Towler, M.; van De Streek, J., Mercury: visualization and analysis of crystal structures. *J. Appl. Cryst.* **2006**, *39*, 453-457.

Mono-dimensional (¹H [500 MHz], ¹³C [126 MHz]) **and bi-dimensional** (¹H-¹H COSY, ¹H-¹³C HSQC, ¹³C DEPT 135, ¹H-¹³C HMBC, ¹H-¹⁵N HMBC) **NMR spectra** were recorded on a Bruker Avance III 500 spectrometer. ¹H chemical shifts in D₂O were referenced to the internal standard 2,2-dimethyl-2,2-silapentane-5-sulfonate (DSS) at δ = 0.00, while CDCl₃ and DMSO-*d*₆ were referenced to the peak of residual nondeuterated solvent (δ = 7.26 and 2.50, respectively); ¹³C chemical shifts were referenced to the peak of residual non-deuterated chloroform (δ = 77.4).

Cell culture. Raji, Thp-1, Jurkat, HOS, PC-3 and MCF-7 cells were from ATCC (LGC Standards, UK), Ramos-Blue™ cells were from Invivogen (San Diego, CA, USA). Raji, Thp-1 and Jurkat cells were cultured in RPMI 1640 medium with 50 μ M 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), PC3 cells were cultured in advanced DMEM/ F-12

(1:1, Gibco, Grand Island, NY, USA), HOS cells were cultured in DMEM (Sigma), and MCF-7 cells were cultured in advanced DMEM/ F-12 with insulin (10 µg/mL), epidermal growth factor (20 ng/mL) and hydrocortisone (0.5 µg/mL) (all from Sigma). Each media was supplemented with 10% fetal bovine serum (Gibco), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Ramos-Blue™ cells were cultured according to the manufacturer instructions. All cells were grown in a humidified chamber at 37 °C and 5% CO₂.

Metabolic activity assay. The cells (100 µL; 1 ×10⁵ cells/mL) were treated with the appropriate concentrations of the compounds of interest or the corresponding vehicle (control cells), in triplicate in 96-well plates. The metabolic activity was assessed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA), according to the manufacturer instructions.

Solutions of compounds for all bioassays were prepared immediately before each experiment. Compounds were dissolved in DMSO and were subsequently diluted in the appropriate culture medium (with DMSO never exceeding 0.5%). In the assays, concentrations of compounds were only used where there was no formation of crystals in the culture media, as examined under a microscope.

Cell cycle analysis. The assay was performed according to the manufacturer instructions. Briefly, Raji cells (5 ×10⁵) were treated with the compound of interest for 24 h. The cells were then washed with PBS and fixed with 80% EtOH at –20 °C for 15 min. The fixed cells were pelleted by centrifugation and resuspended in 5 mL PBS for 15 min at room temperature. The collected cells were resuspended in 500 µL staining buffer (3 µM propidium iodide, 100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 0.1% Nonidet P-40). After a 15-min incubation, the samples were analyzed on a FACScalibur and evaluated using the FlowJo software (BD Bioscience, Franklin Lakes, NJ, USA).

Annexin V assay. Annexin V-FITC Apoptosis Detection kits (Sigma) were used, according to the manufacturer instructions. Briefly, following their treatment with the compounds of interest, the Raji cells were washed with PBS and resuspended in binding buffer at 5 ×10⁵ cells/mL. The cell suspension was transferred to a 5-mL tube and Annexin V and propidium iodide were added. The suspension was gently vortexed and incubated for 15 min at room temperature in the dark. The samples were then analyzed using a FACScalibur flow cytometer.

Determination of caspase 3/7 activity. Caspase 3/7 activity was measured in total cell lysates using a fluorescent DEVD substrate as described previously.⁴ Briefly, after incubation with the compounds of interest, the cells (2 ×10⁶) were washed twice in PBS and resuspended in 200 µL ice-cold caspase lysis buffer (0.1% Triton X-100, 100 mM phosphate buffer, pH 6.0, 1.3 mM EDTA, 100 mM NaCl), sonicated, and left on ice for 30 min. After centrifugation (14,000× g, 15 min, 4 °C), the total protein in the supernatants was measured using BioRad Protein Assay kits (Bio-Rad, Hercules, CA, USA). Cell lysates (20 µg protein) were incubated for 30 min at 37 °C in caspase reaction buffer (20 mM PIPES, pH 7.2, 10%

sucrose, 0.1% CHAPS, 1 mM EDTA, 100 mM NaCl), after which 100 μ M Ac-DEVD-AFC peptide substrate (Bachem, Bubendorf, Switzerland) was added. Immediately following the addition of the substrate, the fluorescence intensity was monitored continuously for 30 min using a fluorescence microplate reader (Synergy HT, BioTek) at the excitation and emission wavelengths of 405 nm and 535 nm, respectively. The data are expressed as the increase in fluorescence as a function of time ($\Delta F/\Delta t$).

4. Murn, J.; Urleb, U.; Mlinarič-Raščan, I., Internucleosomal DNA cleavage in apoptotic WEHI 231 cells is mediated by a chymotrypsin-like protease, *Genes to Cells*, 2004, **9**, 1103-1111, doi: 10.1111/j.1365-2443.2004.00794

Quanti-blue assay. Ramos-Blue™ cells (Invivogen), which stably express an NF κ B/AP-1-inducible secreted embryonic alkaline phosphatase (SEAP) reporter construct, were assayed for NF κ B transcriptional activity changes following their pre-treatment with the compounds of interest for 1 h, with their subsequent stimulation with recombinant TNF- α (50 ng/mL). After 16 h, the supernatants were collected and the SEAP activity was determined according to the manufacturer instructions.

20S proteasome kinetic assay. Purified human 20S proteasome (1 μ g/mL) (Boston Biochem, Inc., Cambridge, MA, USA) was incubated in the absence and presence of the compounds of interest at 37 °C for 30 min in the assay buffer (20 mM Tris, pH 8.0, 0.5 mM EDTA, 1 mM dithiothreitol). Then, 100 μ M Suc-Leu-Leu-Val-Tyr-AMC (Bachem, Bubendorf, Switzerland) was added to determine the chymotrypsin-like activity. Fluorescence of the AMC released was continuously monitored at the excitation and emission wavelengths of 320 nm and 420 nm, respectively, at 37 °C, using an automated microplate reader (Synergy HT, BioTek). The measured fluorescence values (RFU) were plotted *versus* time.⁵

5. Gobec, M.; Obreza, A.; Prijatelj, M.; Brus, B.; Gobec, S.; Mlinarič-Raščan, I., Selective Cytotoxicity of Amidinopiperidine Based Compounds Towards Burkitt's Lymphoma Cells Involves Proteasome Inhibition, *PLOS One*, 2012, **7**, doi: 10.1371/journal.pone.0041961

ctDNA intercalation assay. The DNA binding assay was performed as previously described.⁴ Briefly, calf-thymus DNA (ctDNA, type I, fibers) was dissolved in 10 mM phosphate buffer with 50 mM NaCl (pH 7.01) overnight at 4 °C. The absorbance titrations were performed at room temperature and at a fixed concentration of the clioquinol–ruthenium complex (**1**) (15 μ M). Varying concentrations of the ctDNA were added (0 μ M to 150 μ M, ϵ_{260} , ctDNA = 6600 M⁻¹ cm⁻¹ per nucleotide). After every addition, the samples were incubated at room temperature for 10 min before the UV–visible spectra was recorded. A reference cell loaded with buffer was necessary, in which only DNA was added each time, to minimize the changes in absorption due to the DNA.

2. Experimental section

Crystallographic data.

The crystal structure of **1** has been deposited to the Cambridge structural database (CSD) and has been allocated the number CCDC 977981.

Table S1: Crystallographic data for the clioquinol–ruthenium complex **1**.

	1
Empirical formula	C ₁₉ H ₁₈ Cl ₂ INORu
M _w	575.21
T, K	150(2)
Crystal system	<i>monoclinic</i>
Space group	<i>P 2₁/n</i>
a, Å	15.2671(2)
b, Å	8.0205(2)
c, Å	15.8597(3)
α, deg.	90
β, deg.	100.0050(10)
γ, deg.	90
V, Å ³	1912.48(6)
Z	4
D _{calc} , g/cm ³	1.998
μ, mm ⁻¹	21.935
F(000)	1112
Crystal size, mm	0.20×0.20×0.30
Color	orange
Data collected / unique	15988 / 3889
R _{int}	0.0574
Data / restr. / param.	3889 / 0 / 229
S	1.032
R _I , wR ₂ [I>2σ(I)]	0.0418 / 0.1132
R _I , wR ₂ (all data)	0.0453 / 0.1183
Largest diff. peak / hole (e Å ⁻³)	1.365 / -1.185

Spectroscopic data.

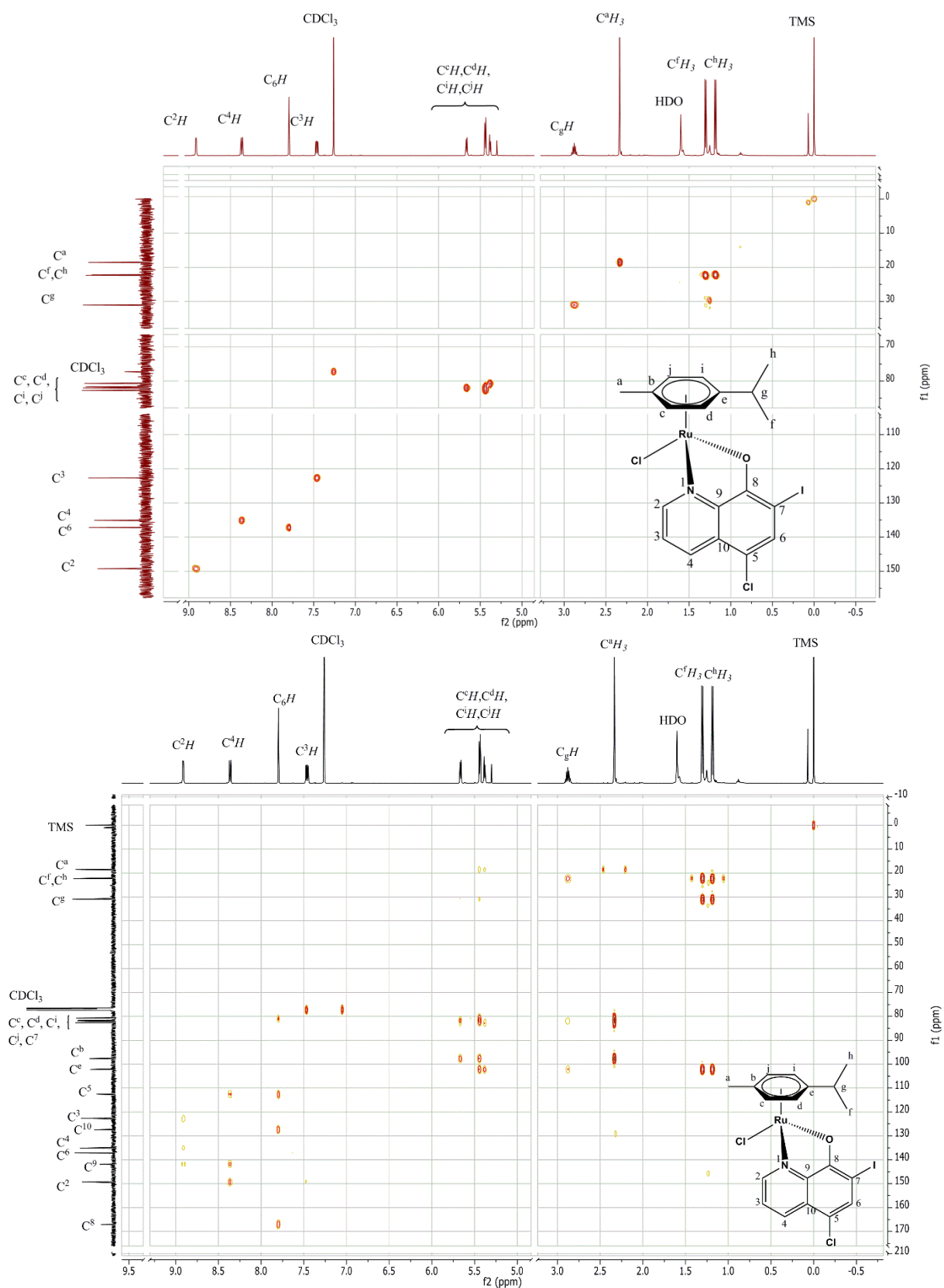


Figure S1: The 2D ^1H - ^{13}C HSQC (top, horizontal trace ^1H spectrum, vertical trace ^{13}C DEPT 135) and ^1H - ^{13}C HMBC NMR spectra of **1** in CDCl_3 at 25.0 $^\circ\text{C}$.

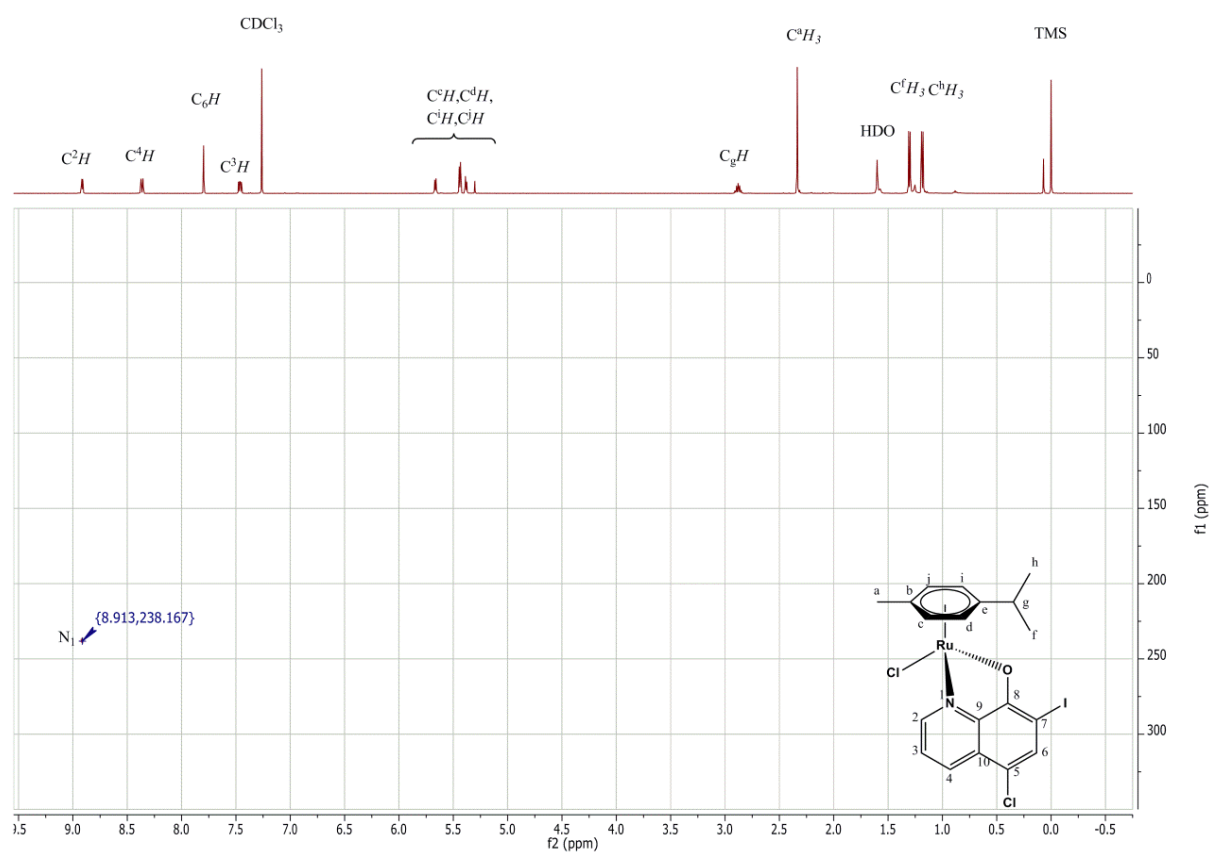


Figure S2: ^1H - ^{15}N HMBC NMR spectrum of **1** in CDCl_3 at 25.0°C .

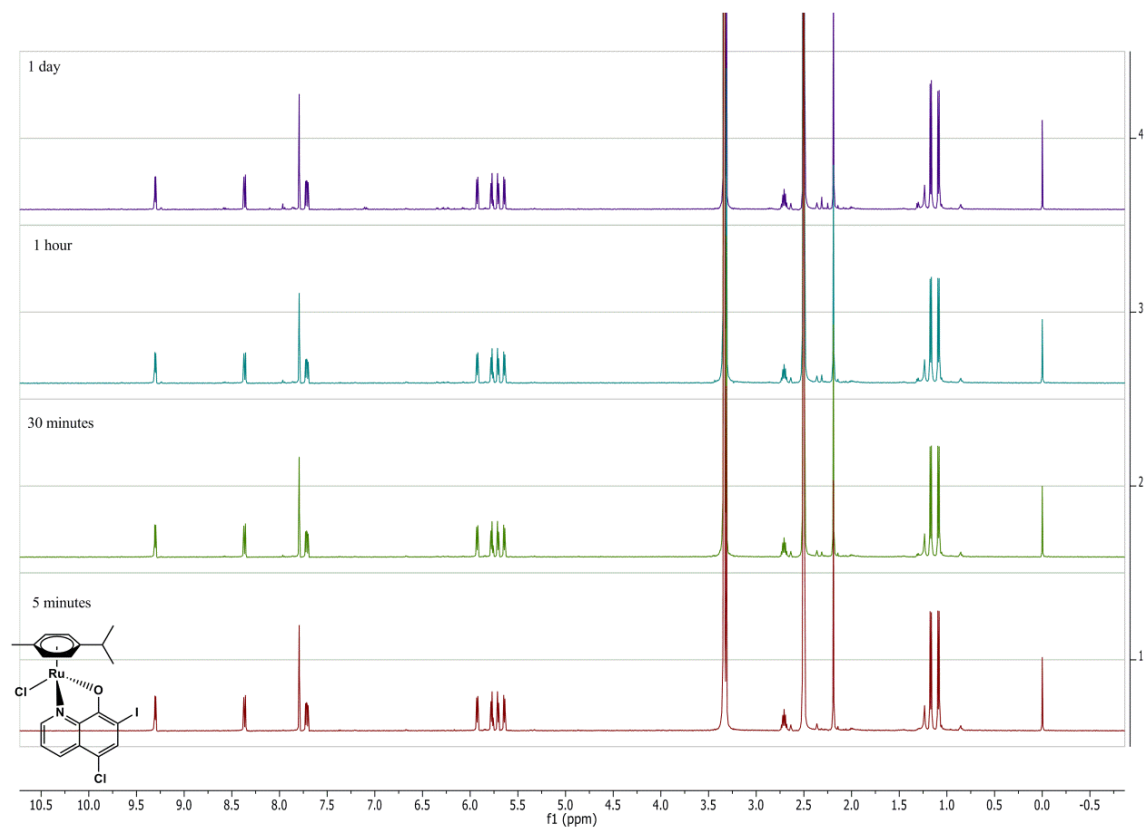


Figure S3: ^1H NMR spectra of **1** in $\text{DMSO}-d_6$ at 25.0°C .

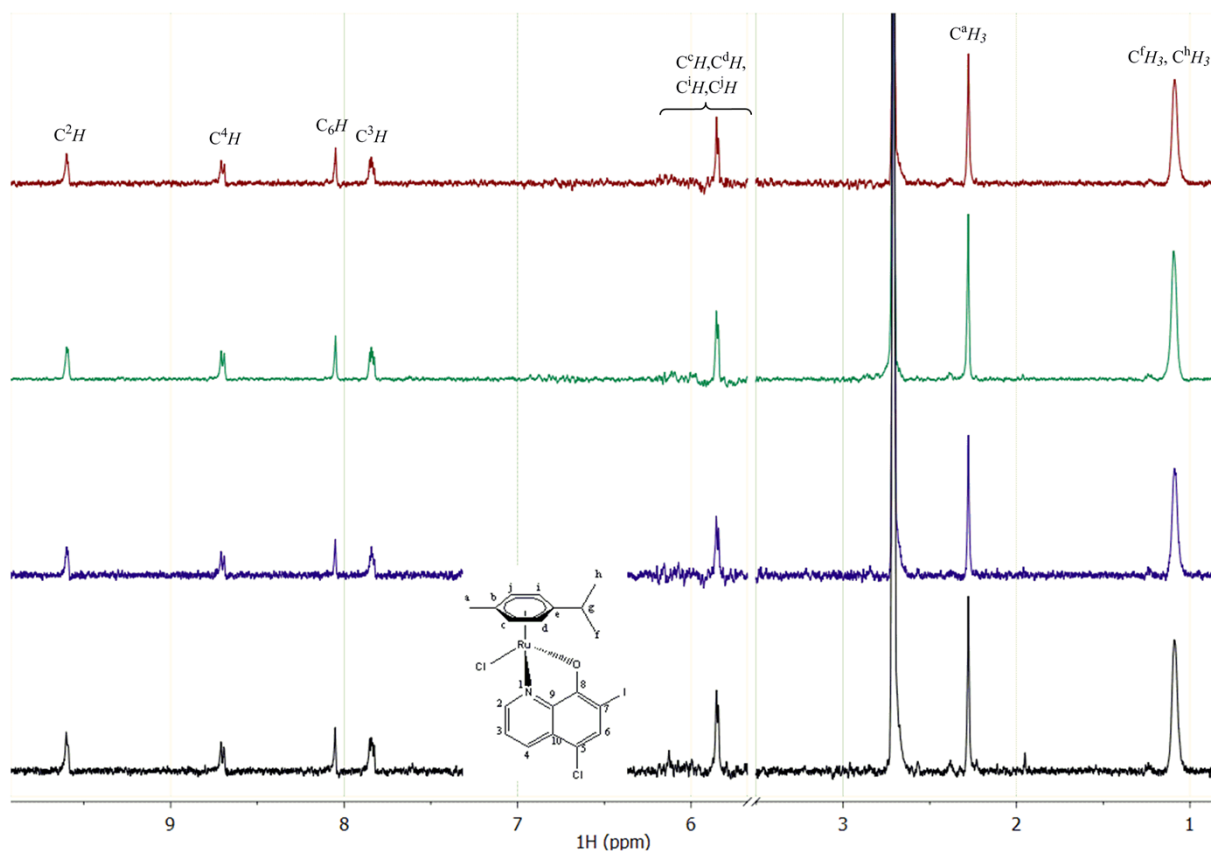


Figure S4: ^1H NMR spectra of **1** in 10% $\text{DMSO-}d_6$ / 90% H_2O at 25.0 $^\circ\text{C}$.

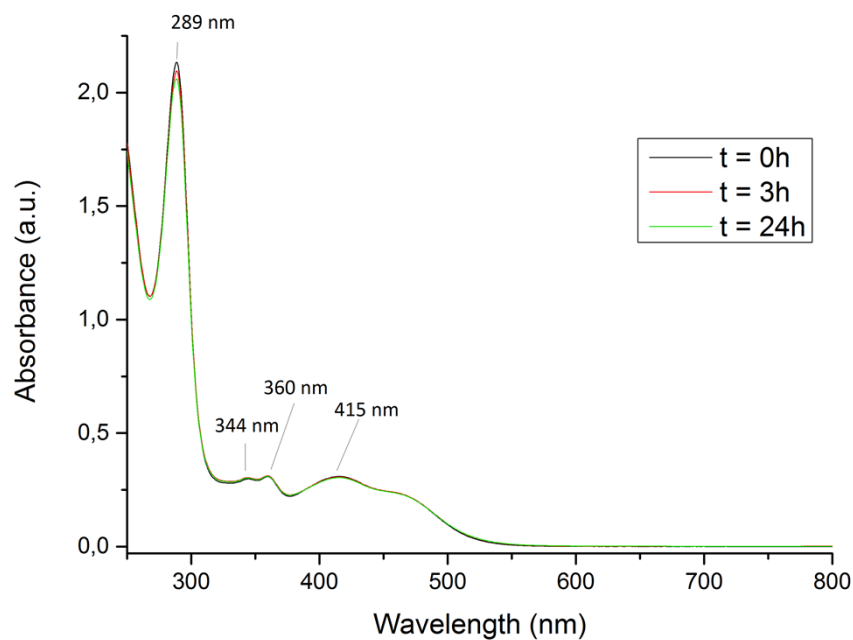


Figure S5: UV-Vis spectrum of **1** ($c = 1 \times 10^{-4} \text{ M}$) in 5% DMSO in phosphate buffer pH = 8.

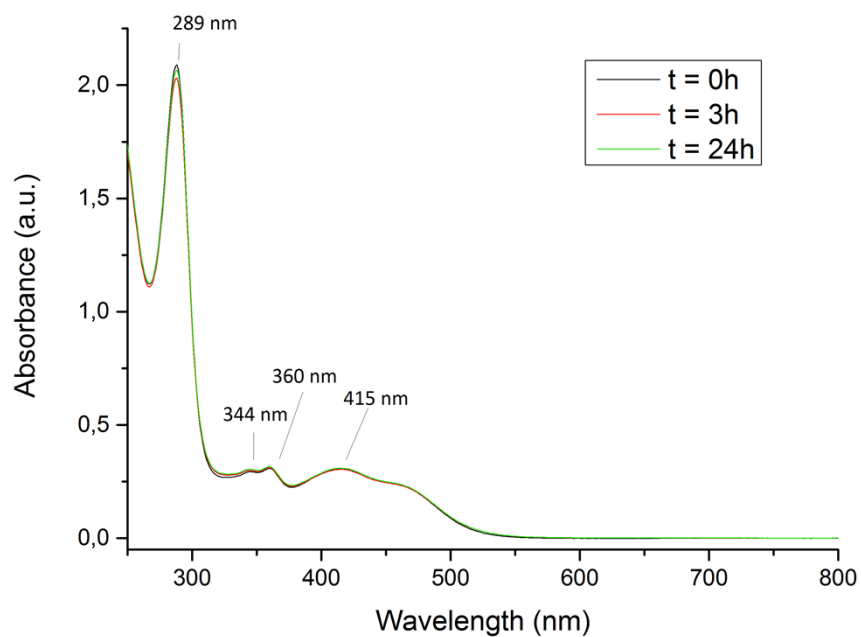


Figure S6: UV-Vis spectrum of **1** ($c = 1 \times 10^{-4}$ M) in 5% DMSO in phosphate buffer pH = 8.5.

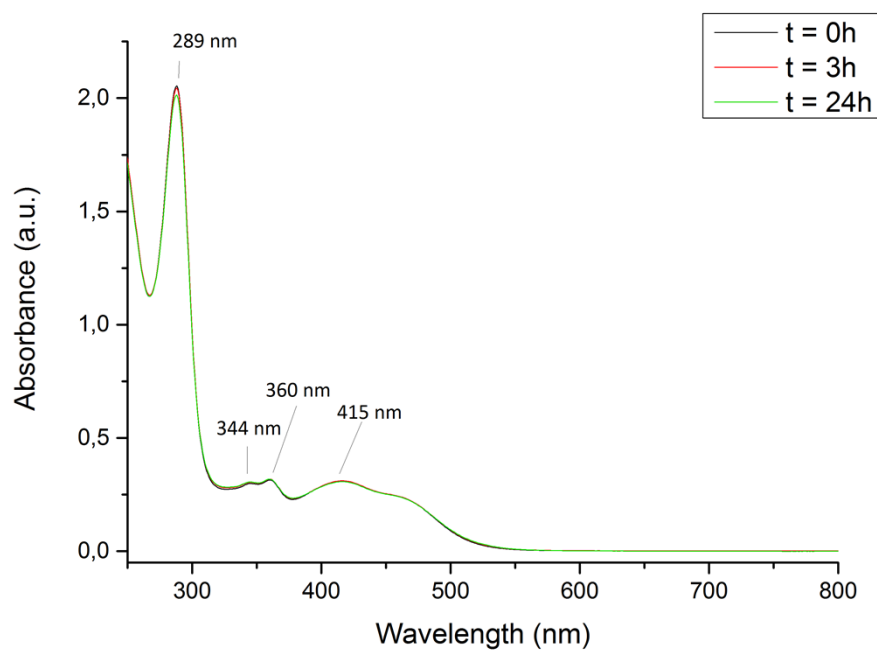


Figure S7: UV-Vis spectrum of **1** ($c = 1 \times 10^{-4}$ M) in 5% DMSO in phosphate buffer pH = 9.

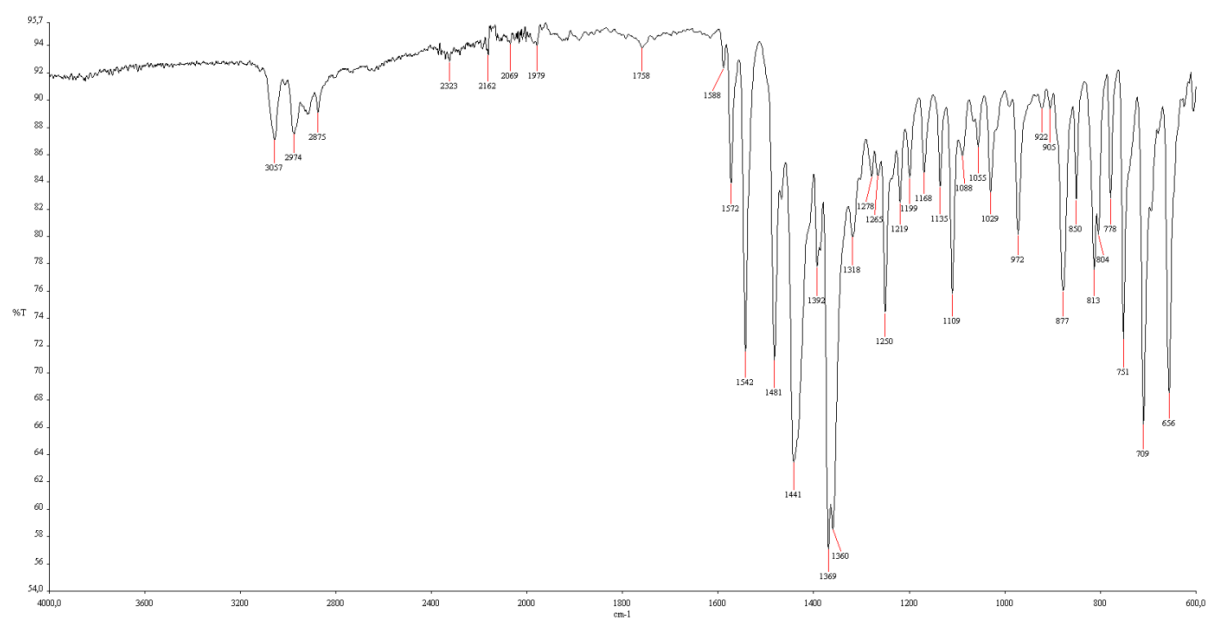


Figure S8: ATR IR spectrum of **1**.

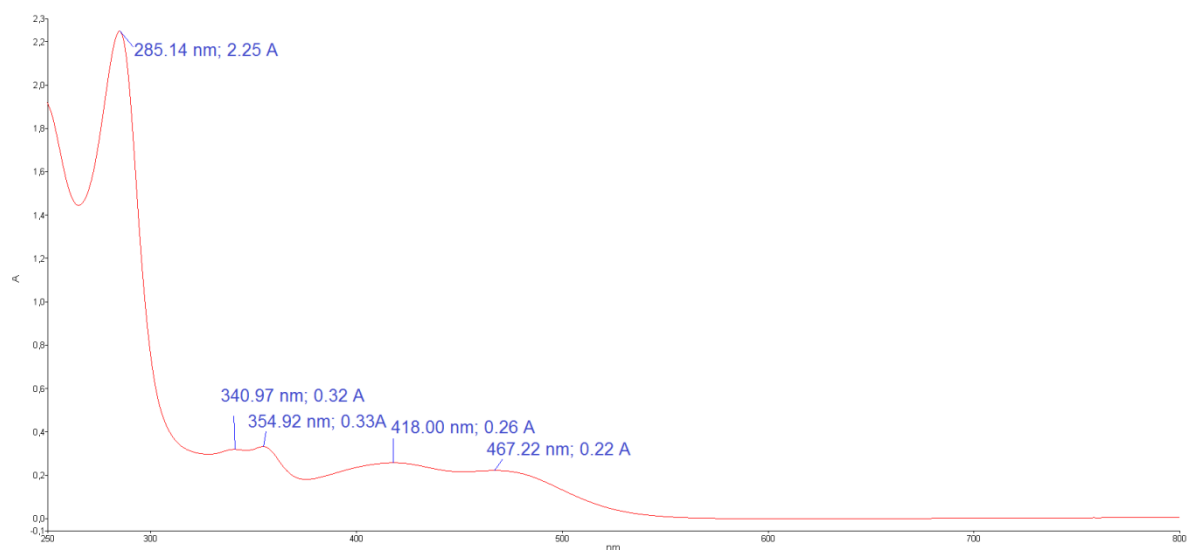


Figure S9: UV-Vis spectrum of compound **1** in MeOH, $c = 1 \times 10^{-4} \text{ mol L}^{-1}$.

Bioassays – supporting figures.

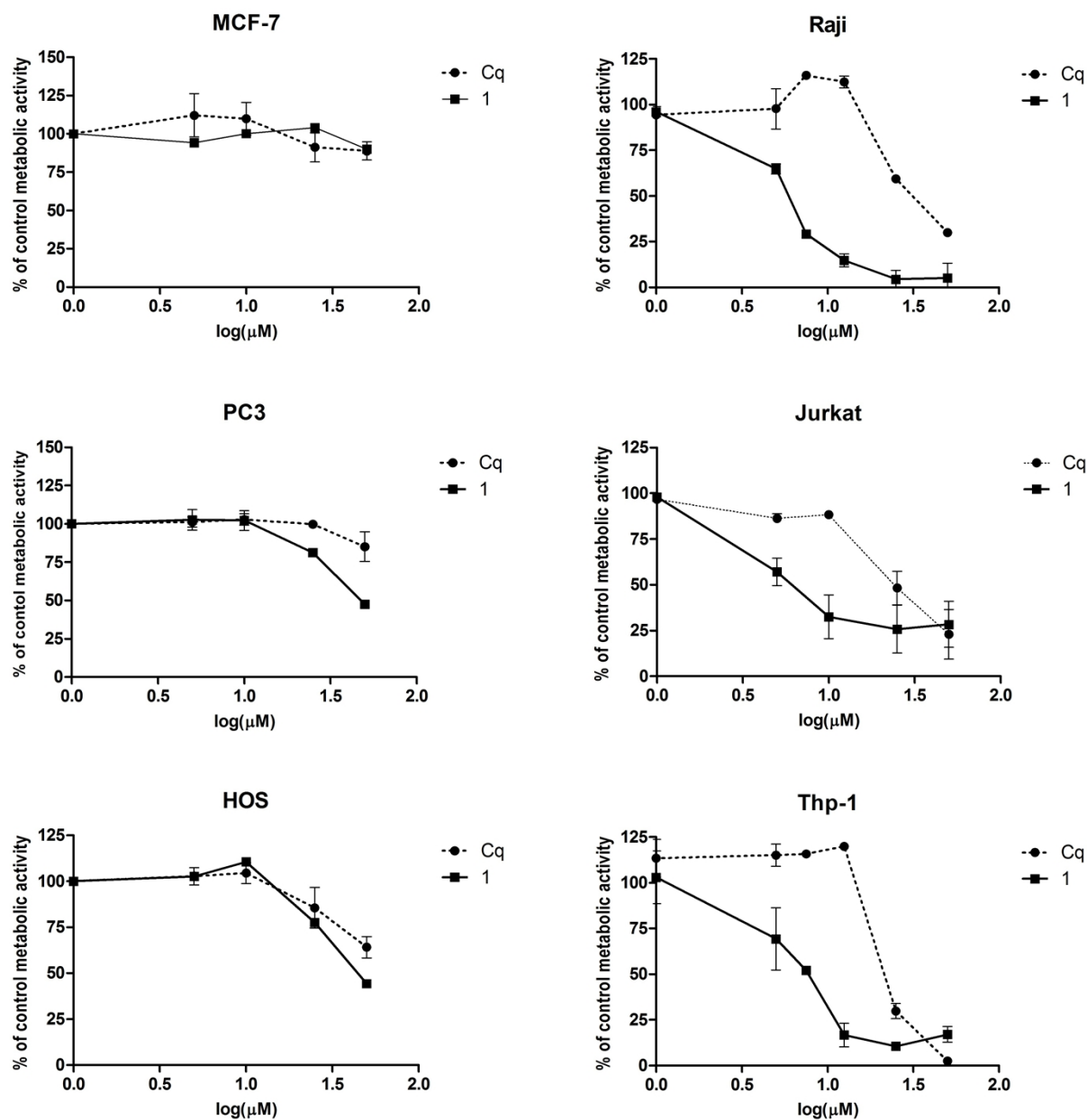


Figure S10: Cytotoxicity of clioquinol and the clioquinol–ruthenium complex (1) on MCF-7, PC-3, HOS, Raji, Thp-1 and Jurkat cells, measured using the MTS assay.

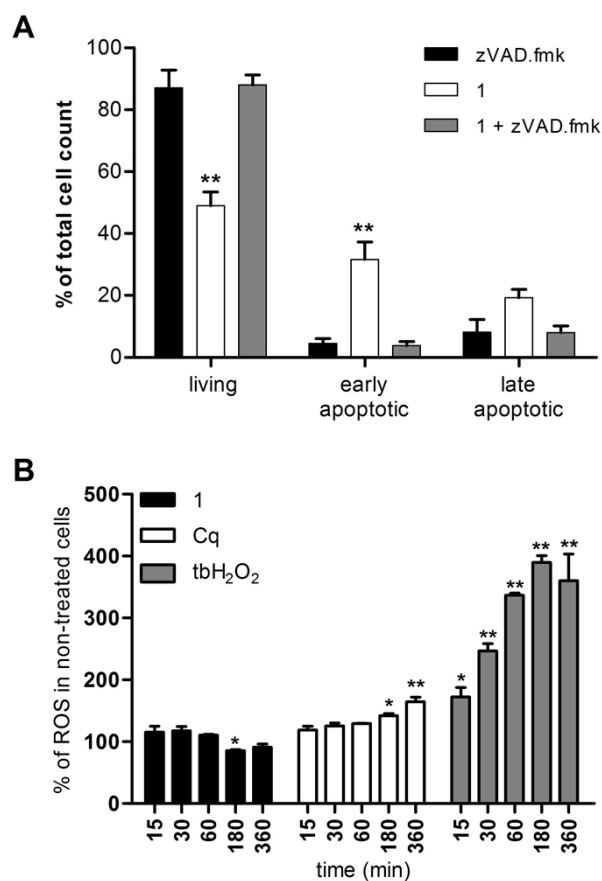


Figure S11: Caspase-dependent apoptosis with no accompanying reactive oxygen species production. (A) Analysis of Annexin V/ propidium-iodide-positive cells after 24 h treatment with **1** in the absence and presence of zVAD.fmk. (B) Raji cells were incubated for 15 min, 30 min, 1 h, 3 h or 6 h with clioquinol or **1**, both at 15 μ M. *Tert*-butyl peroxide (tbH₂O₂) was used as a positive control. * $p < 0.05$, ** $p < 0.01$; *versus* nontreated cells (B) or zVAd.fmk-treated cells (A).

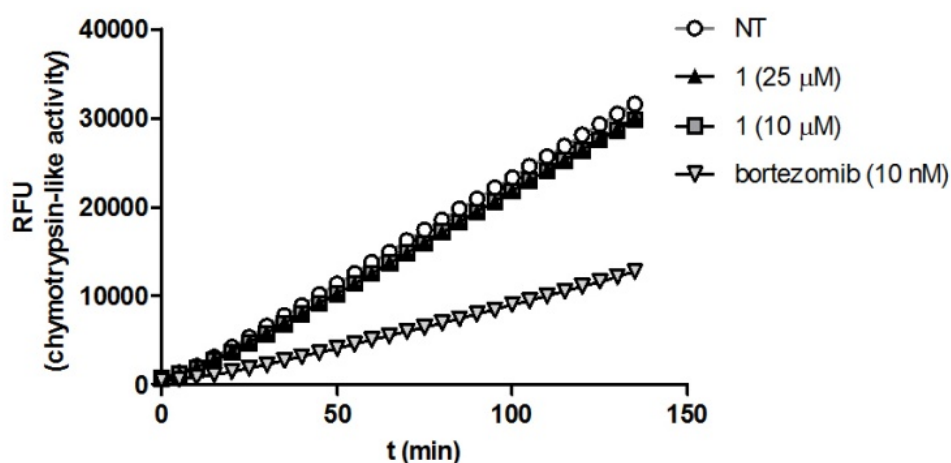


Figure S12: Cell-free proteasome activity assay. Chymotrypsin-like activity of the 20S proteasome in the absence and presence of **1** (10 μ M, 25 μ M). Bortezomib (10 nM) was used as a positive control.

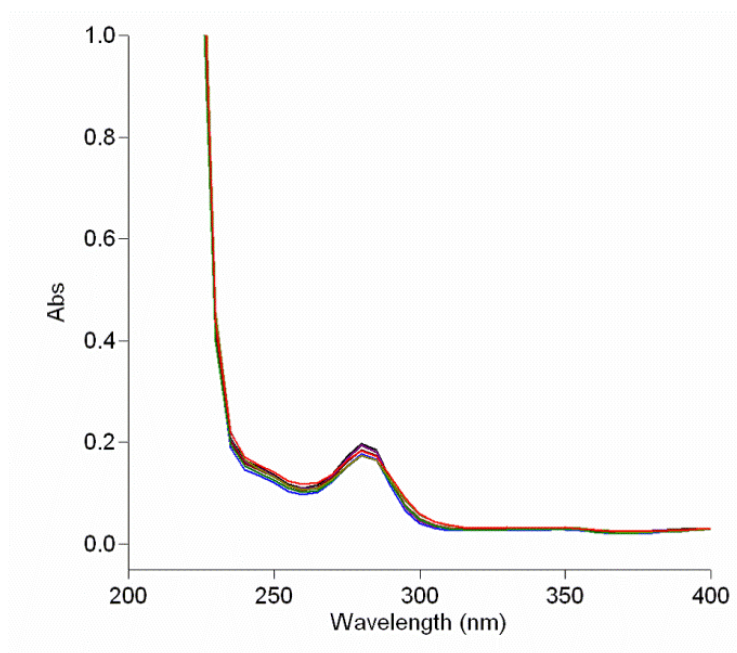


Figure S13: ctDNA binding. Changes in the absorption spectra of the clioquinol–ruthenium complex (**1**) (15 μM) with increasing concentrations of ctDNA, to $[\text{ctDNA}]/[\mathbf{1}] = 10$, were performed in 10 mM phosphate buffer with 50 mM NaCl (pH 7.01). A reference cell loaded with buffer and the appropriate amount of DNA (5 μM , 25 μM , 50 μM , 100 μM , 150 μM) was necessary each time to minimize the changes in absorption due to the DNA. The blue line represents the UV visible spectrum of **1** alone, the red line represents the spectrum at $10\times$ [ctDNA].