Supporting Information:

Highly Cytotoxic Cu(II) Terpyridine Complexes as Chemotherapeutic Agents

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EXPERIMENTAL SECTION

Materials

All chemicals were obtained from commercial sources and used without further purification. Solvents were dried over molecular sieves if necessary. The substituted 4'-(4'-substituted-phenyl)-2,2':6',2''-terpyridine ligands 4'-(4'-*ortho*-methoxy-phenyl)-2,2':6',2''-terpyridine (Q.-P. Qin, T. Meng, M.-X. Tan, Y.-C. Liu, X.-J. Luo, B.-Q. Zou, H. Liang *Eur. J. Med. Chem.* **2018**, *143*, 1387-1395), 4'-(4'-*meta*-methoxy-phenyl)-2,2':6',2''-terpyridine (J.-W. Liang, Y. Wang, K.-J. Du, G.-Y. Li, R.-L. Guan, L.-N. Ji, H. Chao *J. Inorg. Biochem.***2014**,*141*, 17-27), and 4'-(4'-*para*-methoxy-phenyl)-2,2':6',2''-terpyridine (J.-H. Li, H. Hao, Z.-Y. Wang, R.-P. Liu, B.-M. Luo, D.-F. Yang, H.-L. Chen, L.-X. Pan, Z. Ma *Dalton Trans.* **2021**, *43*, 16043-16060) were synthesised as previously reported.

Synthesis

$CuCl_2$ (0.30 mmol) was dissolved in 20 mL of dry methanol under N_2 atmosphere. A solution of the 4'-(4'-substituted-phenyl)-2,2':6',2''-terpyridine ligand (0.30 mmol) in dry methanol/dichloromethane (20 mL) was added dropwise. The mixture was stirred vigorously for 24 h at room temperature. The generated precipitate was isolated by filtration and washed with diethyl ether (10 mL) and dichloromethane (10 mL). The crude product was purified by recrystallisation from methanol.

Cu(4'-(4'-*ortho*-methoxy-phenyl)-2,2':6',2''-terpyridine)$(Cl)_2$: Yield: 67%; ESI-HRMS (pos. detection mode): calcd for $C_{22}H_{17}Cl_1CuN_3O$ *m/z* $[M\text{-}Cl]^+$ 437.0356; found: 437.0355; Elemental analysis calcd for $C_{22}H_{17}Cl_2CuN_3O$ (%): C 55.77, H 3.62, N 8.87; found: C 55.41, H 3.35, N 8.63.

Cu(4'-(4'-*meta*-methoxy-phenyl)-2,2':6',2''-terpyridine)$(Cl)_2$: Yield: 72%; ESI-HRMS (pos. detection mode): calcd for $C_{22}H_{17}Cl_1CuN_3O$ *m/z* $[M\text{-}Cl]^+$ 437.0356; found: 437.0353; Elemental analysis calcd for $C_{22}H_{17}Cl_2CuN_3O$ (%): C 55.77, H 3.62, N 8.87; found: C 55.37, H 3.54, N 8.77.

Cu(4'-(4'-*para*-methoxy-phenyl)-2,2':6',2''-terpyridine)$(Cl)_2$: Yield: 86%; ESI-HRMS (pos. detection mode): calcd for $C_{22}H_{17}Cl_1CuN_3O$ *m/z* $[M\text{-}Cl]^+$ 437.0356; found: 437.0355; Elemental analysis calcd for $C_{22}H_{17}Cl_2CuN_3O$ (%): C 55.77, H 3.62, N 8.87; found: C 55.50, H 3.78, N 8.48.

Cell Culture

All cell lines were obtained from the American Type Culture Collection (ATCC). The cell lines were maintained in DMEM media supplemented with fetal bovine serum (10%), penicillin (100 units/mL), streptomycin (50 units/mL) at 37 °C in a humidified atmosphere with 5% CO_2. Before an experiment, the cells were passaged three times.

Cytotoxicity

A total of 6×10^3 cells were seeded onto 96-well plates and allowed to adhere overnight. The cells were treated with the sample which was diluted in cell media to a total volume of 200 µL. The cells were treated with increasing concentrations of the sample diluted in cell media achieving a total volume of 200 µL. The cells were incubated with the sample for 48 h at 37 °C and 10 % CO_2. After the specified incubation period, the culture medium was substituted with PBS buffer containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) with a final concentration of 12 µM. The cells were incubated for 2 h at 37 °C and 10 % CO_2 and the DPBS-MTT mixture was replaced by 200 µL DMSO. Using a 620 nm reference wavelength, the absorption of the formazan was measured at 550 nm with an Infinite M Nano plus Microplate reader (Tecan).

ROS Generation

A total of 2.5×10^5 cells were seeded onto 8-well plate and allowed to adhere overnight. The cells were treated with the sample (1 µM) which was diluted in cell media to a total volume of 200 µL for 4 h. The cells were incubated with PBS-Buffer containing 2',7'-dichlorodihydrofluorescein diacetate at a final concentration of 5 µM at 37 °C and 10 % CO_2 for 30 minutes. After incubation, the cells were washed with PBS and imaged by fluorescence microscopy. The camera was mounted on an Olympus IX81 inverted fluorescence microscope. The images were recorded with a 10x objective and 1x ocular. λ_{ex} = 460-490 nm, λ_{em} = 517-527 nm.

Cellular Uptake

The cellular uptake of the compound was determined by measuring the Cu content inside the cell. 20×10^6 cells were incubated with the compound (10 μM) for 4 h at 37°C. After this time, the cells were detached with trypsin and harvested. The number of cells was accurately counted. Each sample was digested using a 60% nitric acid solution for three days. The Cu content was determined using inductively coupled plasma optical emission spectroscopy. The Cu content was then associated with the number of cells. The naturally occurring content of Cu inside the respective organelle was subtracted.

Intracellular Distribution

The organelle-localization of the compound was determined by measuring the Cu content inside the cell. 20×10^6 cells were incubated with the compound (10 μM) for 4 h at 37°C. After this time, the cells were detached with trypsin and harvested. The number of cells was accurately counted. The amount was equally divided. In the first portion, the nucleus was extracted using a nucleus extraction kit (Thermo Scientific); in the second portion, the mitochondria was extracted using a mitochondria extraction kit (Thermo Scientific); in the third portion, the lysosome was extracted using a lysosome extraction kit (Sigma Aldrich); and in the fourth portion, the cytoplams was extracted using a cytoplasm extraction kit (Sigma Aldrich). Each sample was digested using a 60% nitric acid solution for three days. The Cu content was determined using inductively coupled plasma optical emission spectroscopy. The Cu content was then associated with the number of cells. The naturally occurring content of Cu inside the respective organelle was subtracted.

Cell Death Mechanism

The cell death mechanism assay was investigated by measuring the cell viability using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after preincubation with various cell death inhibitors. 3-Methyladenine (100 μM), Z-VAD-FMK (20 μM), cycloheximide (0.1 μM) and necrostatin-1 (60 μM) were pre-incubated with the cells for 1 h. The cells were incubated with the compounds with the IC_{50} concentration. The cells were incubated with the sample for 48 h at 37 °C and 10 % CO_2. After the specified incubation period, the culture medium was substituted with PBS buffer containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) with a final concentration of 12 μM. The cells were

incubated for 2 h at 37 °C and 10 % CO_2 and the DPBS-MTT mixture was replaced by 200 µL DMSO. Using a 620 nm reference wavelength, the absorption of the formazan was measured at 550 nm with an Infinite M Nano plus Microplate reader (Tecan).

SUPPORTING FIGURES

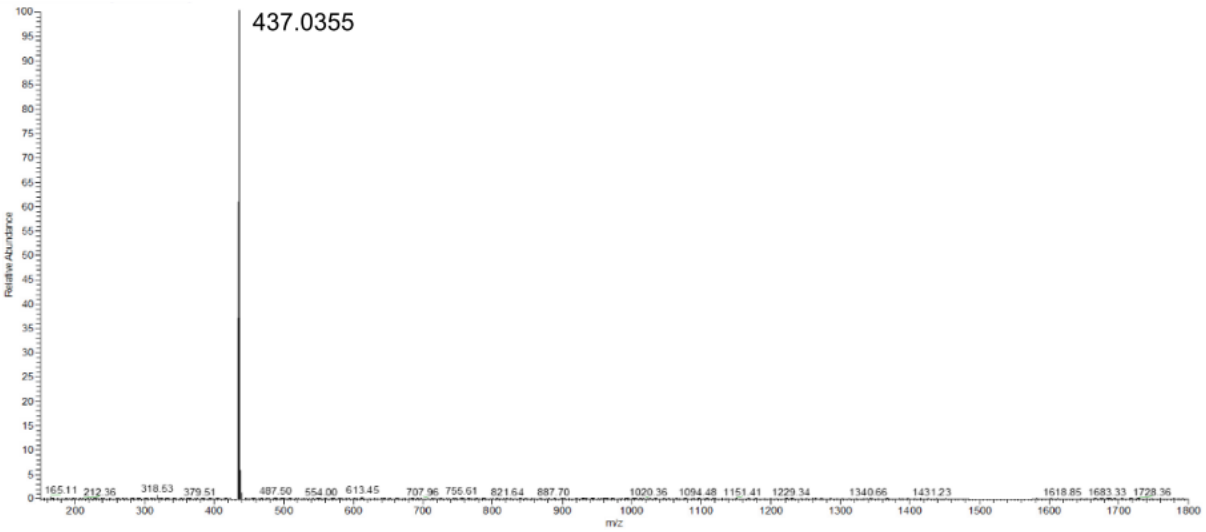

Figure S1. High-resolution electron spray ionization mass spectrum of **Cu1**.

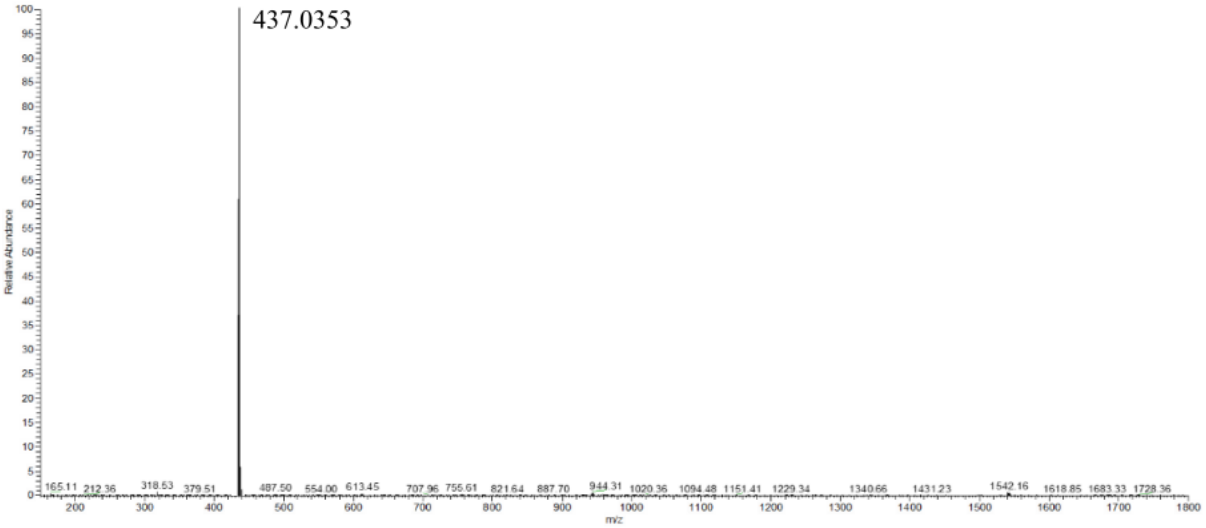

Figure S2. High-resolution electron spray ionization mass spectrum of **Cu2**.

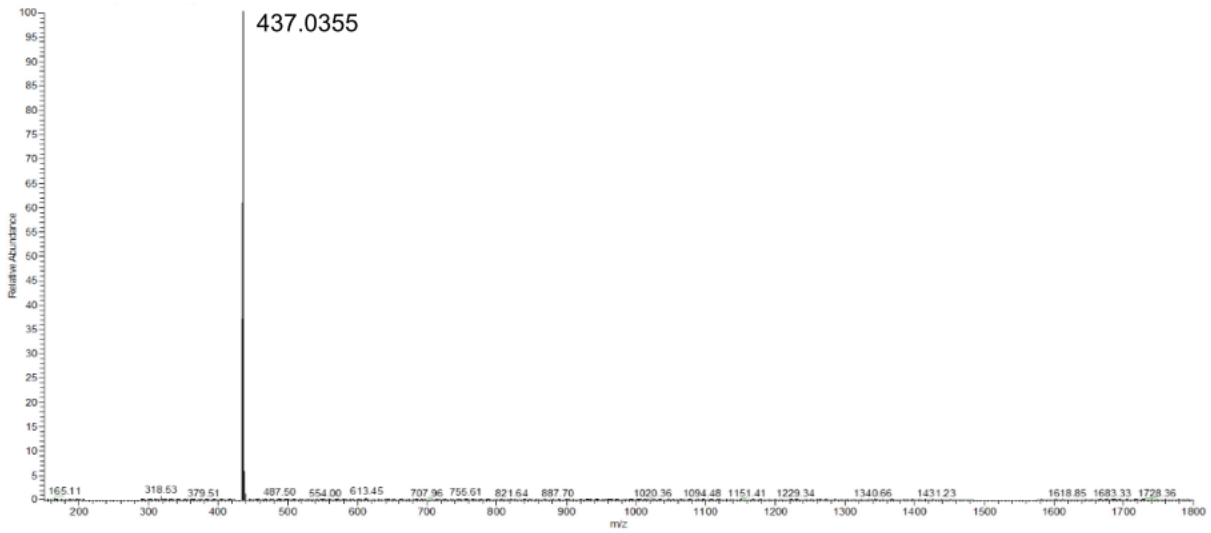

Figure S3. High-resolution electron spray ionization mass spectrum of **Cu3**.

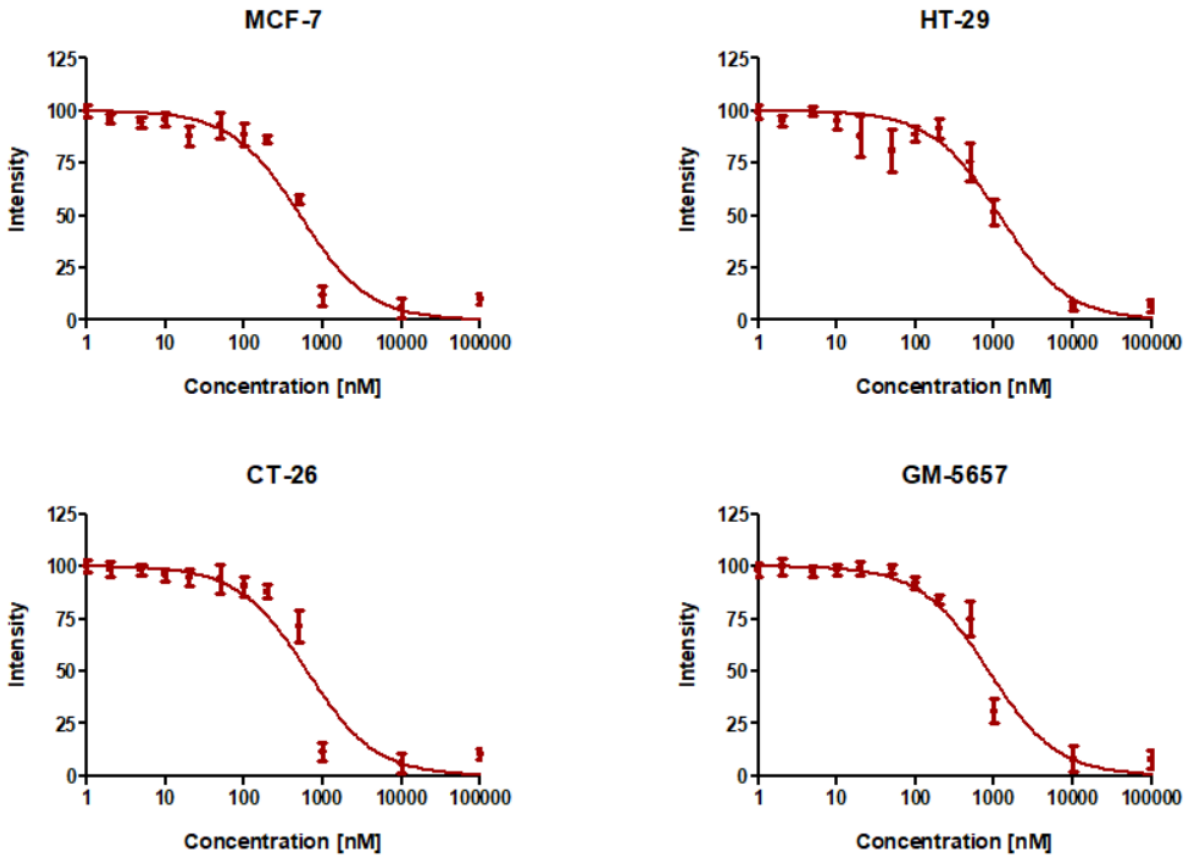

Figure S4. Drug-response curves of **Cu1** against cancerous human breast adenocarcinoma (MCF-7), colon adenocarcinoma (HT-29), mouse colorectal carcinoma (CT-26) cells and non-cancerous human fibroblasts (GM-5657).

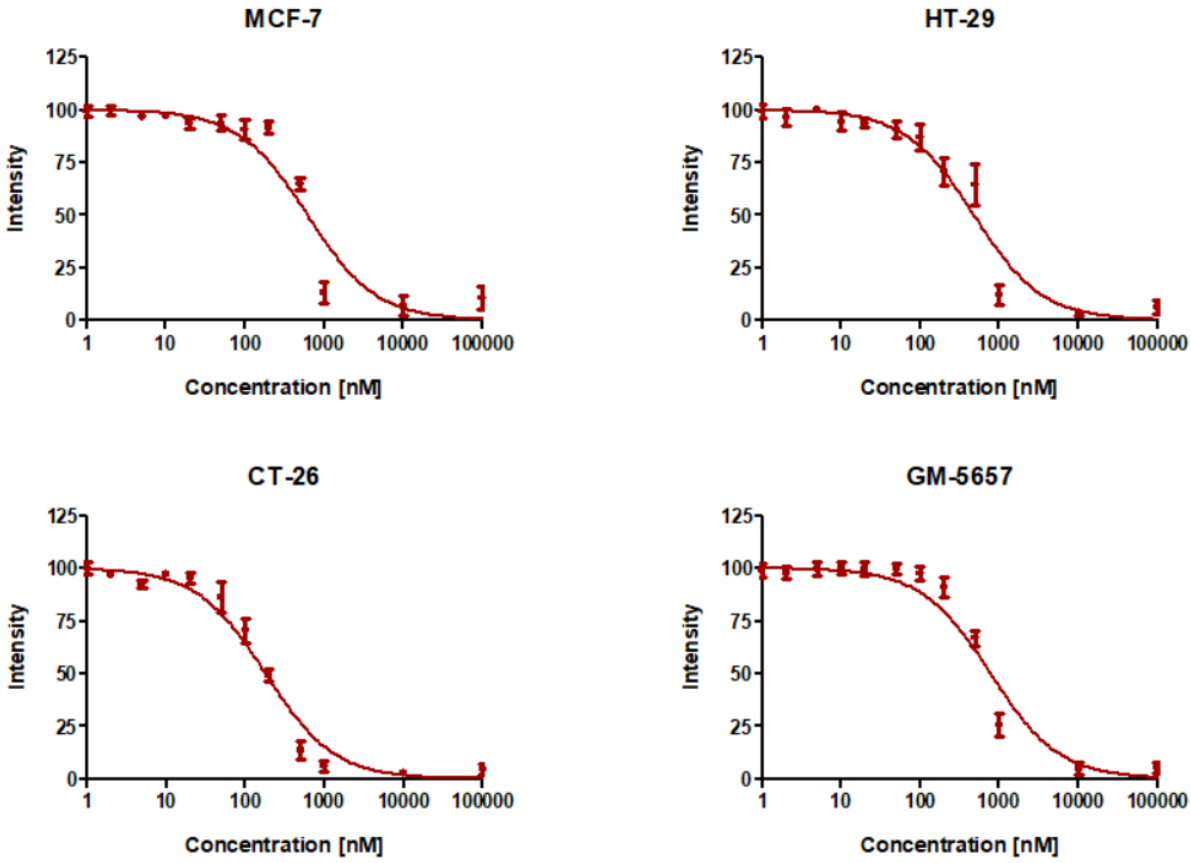

Figure S5. Drug-response curves of **Cu2** against cancerous human breast adenocarcinoma (MCF-7), colon adenocarcinoma (HT-29), mouse colorectal carcinoma (CT-26) cells and non-cancerous human fibroblasts (GM-5657).

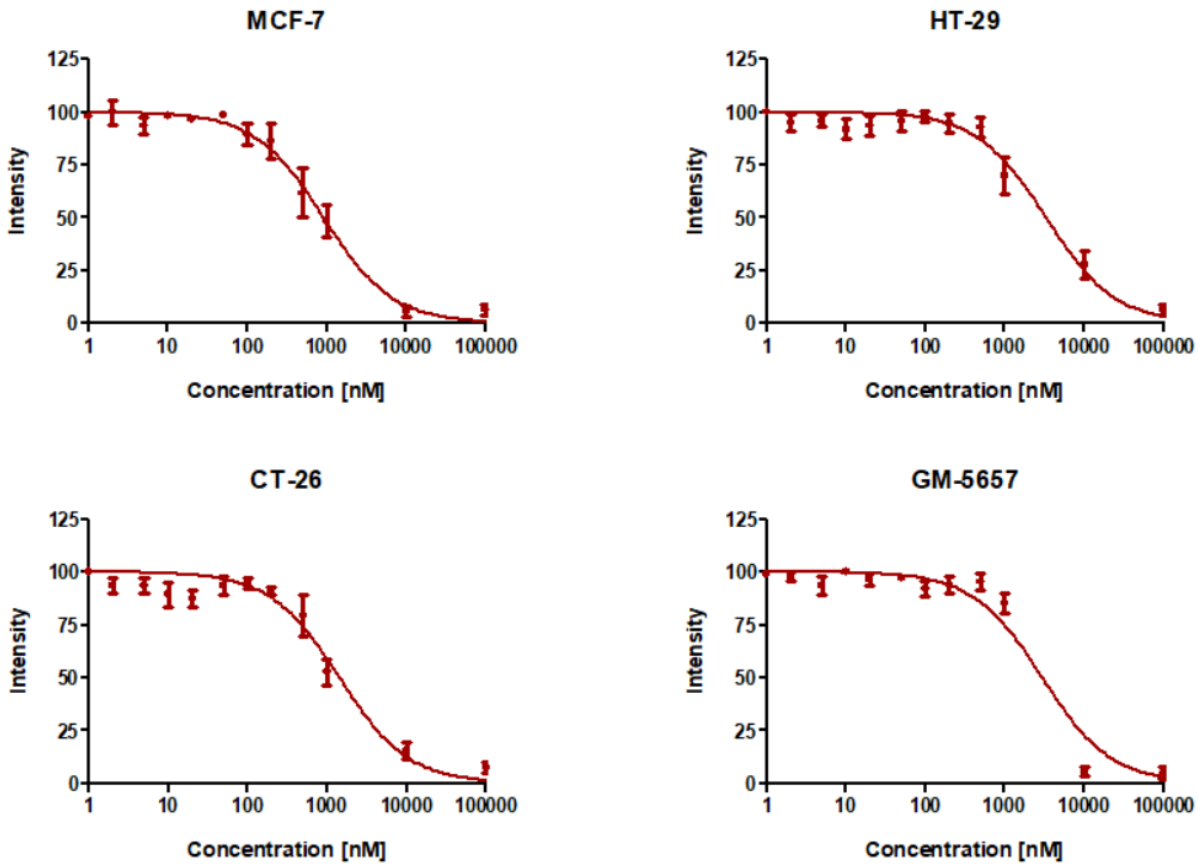

Figure S6. Drug-response curves of **Cu3** against cancerous human breast adenocarcinoma (MCF-7), colon adenocarcinoma (HT-29), mouse colorectal carcinoma (CT-26) cells and non-cancerous human fibroblasts (GM-5657).

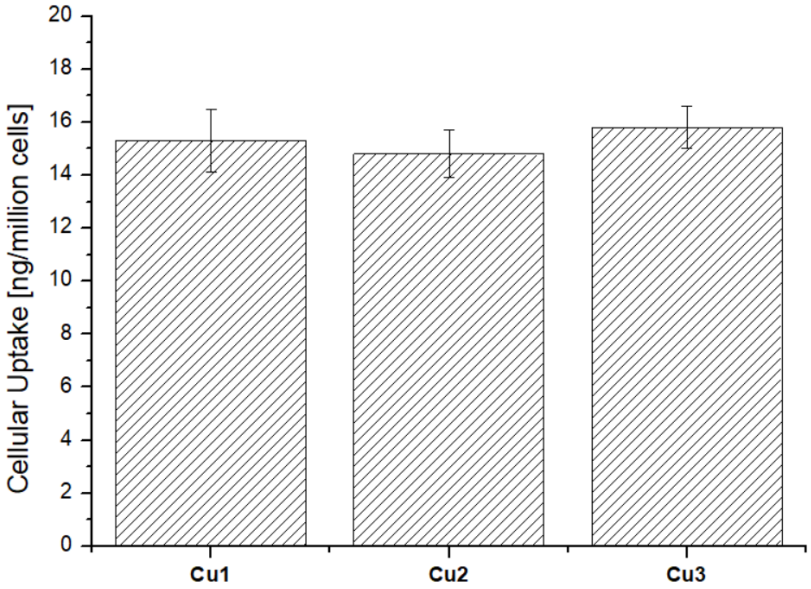

Figure S7. Comparison of the cellular uptake of **Cu1**-**Cu3** upon incubation in CT-26 cells for 4 h and determination of the metal content by inductively coupled plasma optical emission spectroscopy.

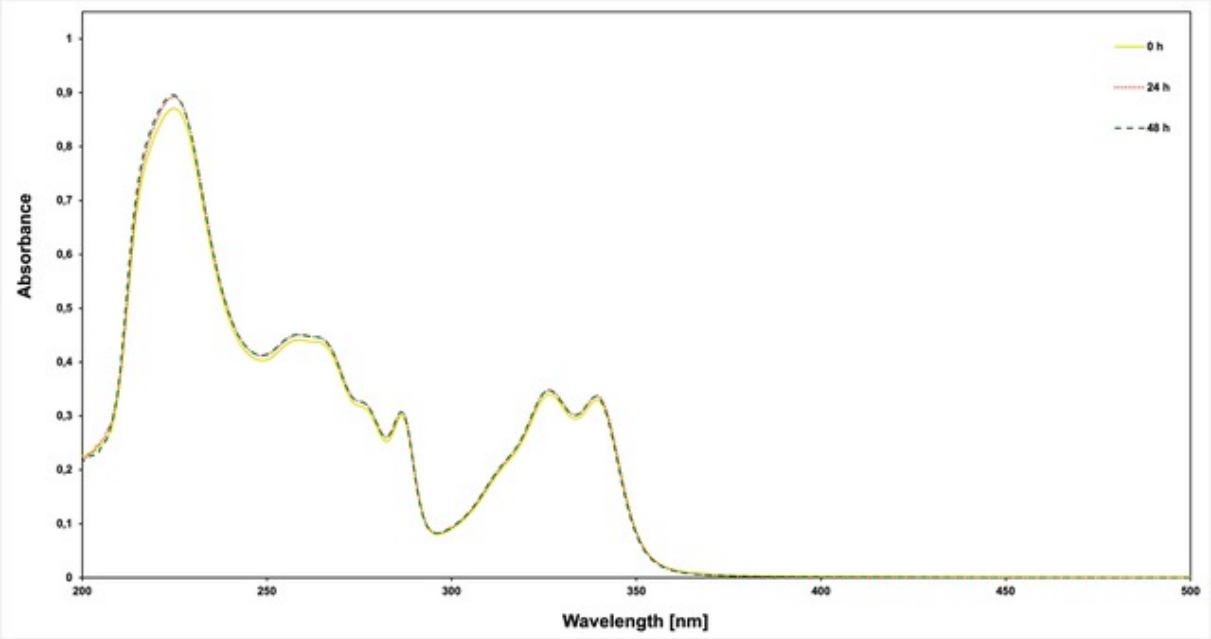

Figure S8. Time-dependent monitoring of the absorption spectrum of **Cu2** upon incubation in DMEM cell media.

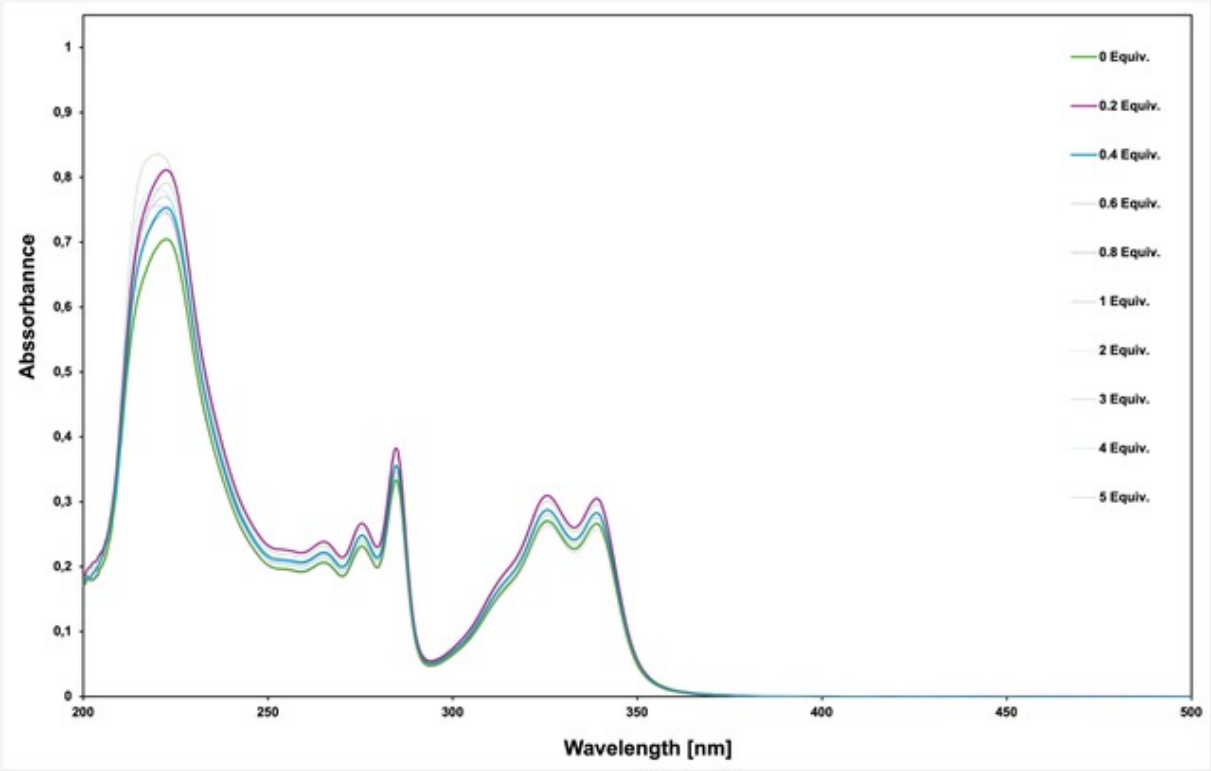

Figure S9. Monitoring of the absorption spectrum of **Cu2** upon incubation in DMEM cell media and titration of glutathione.

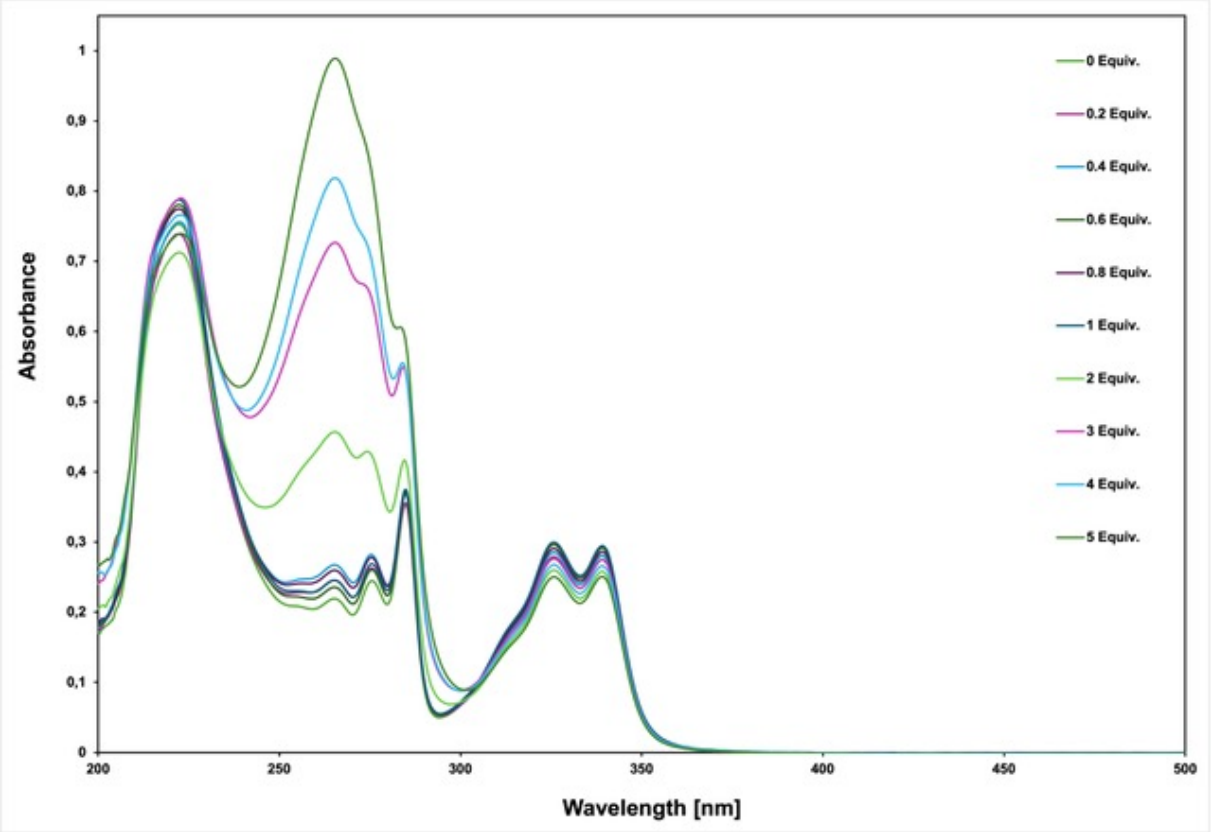

Figure S10. Monitoring of the absorption spectrum of **Cu2** upon incubation in DMEM cell media and titration of ascorbate.

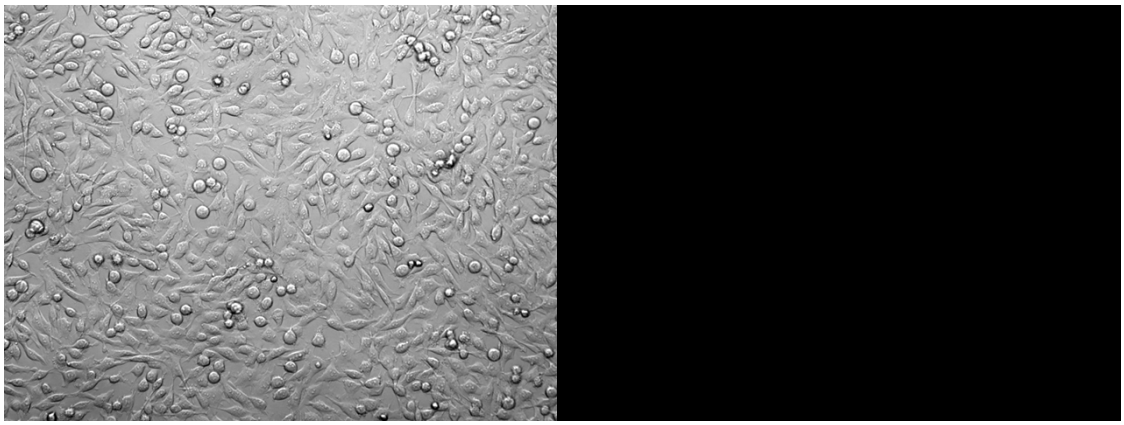

Figure S11. Left: Brightfield and Right: Fluorescence microscopy images of CT-26 cells upon incubation with the reactive oxygen species specific dye 2',7'-dichlorodihydrofluorescein diacetate. λ_{ex} = 460-490 nm, λ_{em} = 517-527 nm.