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Enhanced selectivity, cellular uptake, and *in vitro* activity of an intrinsically fluorescent copper-tirapazamine nanocomplex for hypoxia targeted therapy in prostate cancer

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Abstract

In the present work, a copper-tirapazamine (TPZ) nanocomplex [Cu(TPZ)₂] was synthesized as a selective hypoxia-targeted therapy. The nanocomplex revealed a crystalline form, and exhibited higher lipophilicity, compared to TPZ. Furthermore, its stability was confirmed in different media, with minimum dissociation in serum (~20% up to 72 h). In contrast to other hypoxia-targeted agents, our intrinsically fluorescent nanocomplex offered an invaluable tool to monitor its cellular uptake and intracellular distribution under both normoxia and hypoxia. The conferred higher cellular uptake of the nanocomplex, especially under hypoxia, and its biocompatible reductive potential resulted in superior hypoxia selectivity in two prostate cancer (PC) cell lines. More promisingly, the nanocomplex showed higher potency in three-dimensional tumor spheroids, compared to TPZ, due to its slower metabolism, and probably deeper penetration in tumor spheroids. Interestingly, the nuclear localization of the intact nanocomplex, combined with its higher DNA binding affinity, as evidenced by the DNA binding assay, resulted in significant S- phase cell-cycle arrest, followed by apoptosis in three-dimensional spheroids model. In conclusion, the presented findings suggested that Cu(TPZ)₂ nanocomplex can be a promising hypoxia-targeted therapeutic, which could potentiate the efficacy of existing chemo- and radiotherapy in PC.

1 Introduction

Bio-reductive hypoxic activated pro-drugs (HAP) have been widely exploited as selective cytotoxic compounds, driven by low oxygen conditions, showing promise as novel cancer therapeutics^{1, 2}. Several *N*-oxide compounds have been proposed as potential candidates, with some progressing into clinical trials. Of particular interest is the hypoxic cytotoxin 3-amino-1, 2, 4-benzotriazine-1, 4-dioxide (tirapazamine – TPZ) which exhibits 30-300 times higher toxicity to cells in low oxygen conditions, compared to other cytotoxic agents³⁻⁵. TPZ is the most advanced bioreductive pro-drug in clinical trials, owing to its one-electron reduction pathway, and hypoxia selective DNA damaging action³. Nonetheless, its clinical efficacy has been limited by its poor cellular uptake, restricted tissue diffusion and rapid metabolism⁴.

Copper has attracted special attention over the last years as a promising metal coordinate to target cancer hypoxia. The low molecular weight, lipophilicity and planar structure of its complexes allow rapid cellular internalization and targeting of hypoxia^{6, 7}. It also possesses unique properties conferred by the variety of coordination geometries, metal-ligand interactions and redox activity that allows functionalization of ligands and effective cellular targeting (DNA, proteins, enzymes and redox pathways)⁸. Its unique electronic structure, and cell compatible reduction potential (E^0 ($\text{Cu}^{2+}/\text{Cu}^+$) allows it to play a fundamental role in cellular growth and proliferation⁹. The coordination of metal ions to hypoxic cytotoxins is an interesting strategy to develop novel hypoxia-targeted therapeutics. These complexes have been exploited therapeutically with different organic ligands⁹, and in radiodiagnosis or radiotherapy¹⁰. Extensive work by J. Dearling, J. Holland and J. Dilworth has provided detailed analysis on the therapeutic and hypoxia imaging applications of copper-complexes^{7, 9-11}.

Copper-based complexes have now been widely exploited for anti-cancer therapy, and their potential thoroughly reviewed elsewhere¹². Most of these complexes presented improved chemical characteristics compared to the free ligand, contributing to their increased hypoxic uptake, thus more selective and/or potent biological activity^{11, 13-15}. Particular attention has been drawn to Cu(II) complexes including *N,N*-diimine ligands, thiosemicarbazones, bis(thiosemicarbazones), schiff base assemblies and pyridine-*N*-oxides and their ability to induce DNA damage, *via* binding or intercalation¹². Although this is the primary mechanism of action, new cellular targets such as DNA topoisomerases¹⁶, the proteasome complex^{17, 18}, endoplasmic reticulum¹⁹ and lysosome²⁰, have also become interesting pathways of cell death for copper-complex mediated therapeutics, highlighting the versatility and potency of these metal therapeutics.

Lin and Ho²¹ were the first to demonstrate the potential use of TPZ with copper to enhance DNA damaging in cancer cells. Next, they prepared a radioactive TPZ-copper complex for targeted cancer therapy²². These studies did not report the basic characterization of the complex, which, in our opinion, is considered essential to further understand its biological

relevance. Furthermore, data regarding its potential use as a hypoxia cytotoxic agent in more complexed hypoxia models is still warranted. In the present work, we aimed to synthesize and characterize a cupric-TPZ complex (abbreviated in the text as Cu(TPZ)₂). We sought to understand how complexation with copper could modulate TPZ's physicochemical properties (e.g. optical properties, solubility, and lipophilicity), and its biological activity. Ideally, this Cu(TPZ)₂ complex should maintain or improve TPZ hypoxia selectivity and potency while providing an innovative metal-complex strategy for hypoxia therapeutics. Therefore, it was essential to determine the selectivity and potency of this complex vs TPZ alone. Using both 2-dimensional (2D), and 3-dimensional (3D) prostate cancer models, the uptake and cytotoxicity of Cu(TPZ)₂ was determined in this study. Overall, Cu(TPZ)₂ complex should overcome some of the TPZ shortcomings *in vitro*, which could potentiate the efficacy of existing chemo- and radiotherapy *in vivo*.

75

76 Experimental

77 Materials

4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), sodium chloride (NaCl), phosphate buffer saline (PBS) tablets, tirapazamine ($\geq 98\%$ HPLC), copper(II) chloride (97%), methanol (HPLC grade) and Universal MALDI Matrix [1:1, 2,5-dihydroxybenzoic acid (DHB): α -cyano-4-hydrocinnamic acid] were obtained from Sigma (UK). Laboratory reagent dimethyl sulfoxide (DMSO), chloroform (HPLC grade, stabilized with amylene), methanol (HPLC grade), and ethanol (absolute, 99.8%) were bought from Thermo Fisher Scientific (UK). Milli-Q water was prepared using a water purification system (Millipore Corp., Bedford, MA, USA). All organic solvents were of analytical grade, unless otherwise stated. All starting materials were commercially available research grade chemicals and were used without further purification.

92 Preparation of Cu(TPZ)₂ complexes

Two equivalents of TPZ (13.2 mg, 0.074 mmol) were added slowly to a total of 5 mL stirred ethanolic solution of CuCl₂ (5 mg, 0.037 mmol), resulting in the formation of a deep red precipitate. Following overnight stirring, the dark red precipitate was isolated, and washed with small volumes of ethanol by centrifugation at 5000 $\times g$ for 10 min, until a colorless supernatant was obtained. The red complex was then left to dry under vacuum at 25°C, and resuspended in DMSO until complete solubility was obtained (~ 2.5 mM stock solution). The complex was then sterile filtered through 0.2 μm membrane filter. The stoichiometry of the compound was [Cu(TPZ)₂], and the yield $\sim 70\%$ (11 mg, 0.026 mmol). The results of the elemental analysis (%) obtained with a Carlo Erba EA 1108 analyzer were: (C₁₄H₁₀CuN₈O₄) Found/Calcd.: C, 39.85/40.32; N, 25.66/26.87; H, 2.39/2.21. IR (ATR) analysis: (ν/cm^{-1}) - ($\nu\text{N-H}_2$), 3360-3311; ($\delta\text{N-H}_2$), 1570; ($\nu\text{N} \rightarrow \text{O}$), 1358; ($\nu\text{C-N}/\nu\text{C-C}$), 1111, 1091, 1165, 1215. MALDI-MS: m/z (%) [Cu(TPZ)₂-H]⁺, Found/Calcd (%): 418.0199/418.0202 (100). Full details of TPZ and Cu(TPZ)₂ characterization can be found in the experimental section of the supplementary information.

113

114 Biological studies

115 *In vitro* cytotoxicity of TPZ and Cu(TPZ)₂ complex in PCA 116 monolayers

117 LNCap (CRL-1740™) and C4-2B cells were purchased from ATCC®
118 and MD Anderson Cancer Center (Texas, USA), respectively. The
119 cells were maintained in Advanced RPMI-1640 (1x) medium
120 supplemented with 10% heat inactivated FBS, 1%
121 penicillin/streptomycin and 1% GlutaMax (200 mM). Cells were
122 maintained in Corning® CellBIND® 75 cm³ canted-neck tissue
123 culture flasks (Fisher Scientific, UK) to enhance cell adherence.
124 For normoxic condition, cell cultures were maintained in a
125 humidified incubator at 37°C in 5% CO₂/95% air. For hypoxia
126 treatment, cells were placed in a hypoxic chamber (Incubator
127 chamber Billups Rothenberg Inc., MIC-101) with a mixture of 1%
128 O₂, 5% CO₂, and 94% N₂ (BOC, UK). Hypoxia was validated using
129 the CYTO-ID® Hypoxia/Oxidative Stress Detection kit (Enzo Life
130 Sciences, UK) according to the manufacturer manual ²³.

131

132 Cell culture media and drug stock solutions were pre-purged
133 with the hypoxic gas mixture to minimize oxygen fluctuations.
134 The cytotoxicity of TPZ and Cu(TPZ)₂ was determined using the
135 resazurin assay, as previously described ²⁴. Resorufin
136 fluorescence intensity (FI) was measured using a BMG
137 microplate reader (FLUOstar Omega, BMG Labtec) at 544 nm
138 excitation and 590 nm emission. The average percentage cell
139 viability was calculated by normalizing the values to the
140 untreated cells, using the following formula:

141

$$142 \quad \text{Cell Viability (\%)} = \frac{FI_{\text{treated}}}{FI_{\text{control}}} \times 100$$

143 Uptake and cellular localization of TPZ and Cu(TPZ)₂ complex

144 For quantitative uptake studies, confluent C4-2B cells were
145 harvested by trypsinization and seeded onto pre-coated poly-D-
146 lysine (100 µg/mL) 24 flat-bottom well plates (Triplered, UK) at
147 5×10^4 cells/well. The cells were incubated overnight under
148 either normoxia or hypoxia to allow adherence. Next, cells were
149 incubated with free TPZ or its complex (100 µM equivalent TPZ)
150 at 37 °C for 1, 4, 8 and 24 h to assess uptake. At specific time
151 periods, the medium was removed and the wells were washed
152 (3X) with 200 µl of cold PBS to remove any free drug. After that,
153 cells were harvested by trypsinization and pelleted by
154 centrifugation (500 x g, 10 mins). The pellet was resuspended in
155 200 µL of DMSO (to guarantee solubility of released drug) and
156 cells were then lysed by mechanical disruption using a bath
157 sonicator for about 15 min (Ultrasound cleaning baths, USC,
158 VWR). This ensured full disruption of cell membrane and
159 intracellular organelles. The fluorescence intensity of each
160 sample well was measured using a microplate reader (FLUOstar
161 Omega, BMG Labtec) at 485 nm excitation and 590 nm emission.
162 The percentage of drug uptake was calculated by dividing the
163 fluorescence of each well by the fluorescence of the initial drug
164 concentration used.

165

166 For fluorescence microscopy, C4-2B cells were seeded at $5 \times$
167 10^4 /well onto pre-coated poly-D-lysine (100 µg/mL) Coverslips
168 (Fisher Scientific, UK), placed in 24 well assay plates (Triplered,
169 UK) and left to adhere overnight at 37 °C under normoxia or
170 hypoxia. Next, the cells were incubated with TPZ or its complex
171 for 1, 4, 8 and 24 h, under both normoxia or 1% hypoxia. Cells

172 were then washed with cold PBS and fixed with 4% PFA at room
173 temperature for 20 min, then washed (3X) with cold PBS. The
174 nuclei were stained with Hoechst 33258 (5 µg/mL) (Sigma, UK)
175 for 30 min at 37°C. Finally, the coverslips were washed (3X) with
176 PBS, and mounted using ProLong™ Gold Antifade mounting
177 media (Fisher Scientific, UK). Images were obtained using a Zeiss
178 Axioplan 2 epifluorescence Axiovision 4.1.8 software. Images
179 were processed and analysed using ImageJ (NIH, Bethesda, MD,
180 USA: <http://imagej.nih.gov/ij>). For each endpoint, duplicates of
181 two independent experiments (n=2) resulted in the analysis of
182 over 50 cells. For some experimental conditions a z-stack with
183 1.75 µm intervals was generated using the Apotome to form a
184 3D reconstruction and evaluate co-localization *via* orthogonal
185 projections. All images were acquired with a 10x/0.45 air
186 objective and Axiovision 4.1.8 software, processed and analysed
187 using ImageJ (NIH, Bethesda, MD, USA:
188 <http://imagej.nih.gov/ij>).

189

190 Analysis of Cu(TPZ)₂ metabolism using FTIR and NMR

191 C4-2B cells were seeded on pre-coated poly-D-lysine (100
192 µg/mL) at 5×10^4 /well in 24 well assay plates (Triplered, UK) and
193 left to adhere overnight at 37 °C under hypoxia. Next, the cells
194 were incubated with TPZ in its free or complex form (100 µM
195 equivalent TPZ) for 1, 4, 8 and 24 h, under 1% hypoxia using a
196 hypoxic chamber (Ruskin Invivo2 400 Hypoxia Workstation). At
197 specific time periods, the medium was removed and the wells
198 were washed (3X) with PBS to remove any free drug, and cells
199 were lysed with 200 µL of RIPA buffer. Cell lysate samples were
200 stored at -20 °C until further FTIR and NMR analysis.
201 200 µL of cell lysates was diluted in 200 µL D₂O (Fisher, UK) and
202 probed by nuclear magnetic resonance (NMR). The Spectra were
203 recorded at 297 K, on an ultra-shield 400 instrument (¹H
204 frequency: 400 MHz; Bruker, Germany). Then, all the samples
205 were dried and were dissolved in 400 µL CDCl₃ in presence of
206 Tetramethyl silane (TMS) and run at the same conditions.
207 Finally, the samples were dried and dissolved in DMSO in the
208 presence of TMS. The samples were analyzed using IR
209 spectroscopy (Spectrum Two FTIR, Perkin Elmer, USA) over the
210 range 650–4000 cm⁻¹ with a resolution of 4 cm⁻¹.

211 *In vitro* cytotoxicity of TPZ and Cu(TPZ)₂ in C4-2B spheroids

212 C4-2B spheroids were cultured and characterized as described
213 in the supporting information. Five days post spheroid seeding,
214 100 µL of media was carefully removed and replaced with pre-
215 diluted fixed concentrations of TPZ in free and complexed form
216 (TPZ equivalent: 0.002 up to 20 µM). Spheroids were treated for
217 48, 72 and 96 h using 10% DMSO as a positive control. Spheroids
218 were carefully washed with PBS 1x and replenished with fresh
219 media or pre-treated with EDTA (5 mM) for 30 min, with shaking
220 (70 rpm) to ensure resazurin penetration and reliable
221 quantification ²⁴. Resazurin solution (20 µl) was then added to
222 each well and the spheroids were further incubated overnight,
223 at 37°C to determine cell viability. To complement the viability
224 assay, representative spheroid images were taken to monitor
225 spheroid size changes before and after treatment. The average
226 percentage cell viability for each drug concentration was
227 expressed as mean ± S.D of six replicates of three independent
228 experiments (≥18 spheroids, n≥2) and calculated by normalizing
229 the FI values to the untreated cells. Dose-response curves were
230 generated, where possible, and the IC₅₀ values for drug
231 inhibition were determined by nonlinear regression analysis of

the data fit to a four-parameter equation (GraphPad Prism version 7, La Jolla California USA www.graphpad.com).

Cell cycle and apoptosis analysis using Flow Cytometry

C4-2B spheroids were cultured at a density of 5×10^3 cells/well, as described above and on day 5 of growth, were treated with 2 and 20 μ M (equivalent TPZ content) in free and copper-complexed form. Untreated spheroids were used for population gating to avoid the undesired noises from debris and singlet gating to minimize debris and cell aggregates. 72 h post-treatment, spheroids were harvested, using a 1 mL pre-cut micropipette tip, into 1.5 mL micro centrifuge tubes (Fisher Scientific, UK) and centrifuged at $500 \times g$ for 5 min, to sediment the spheroids. The cells were then washed $3 \times$ with cold PBS, with repeated centrifugation and resuspended in 100 μ L of PBS. Thereafter, a single cell suspension was formed by mechanical dissociation, using a micropipette, and cells were fixed in 70% ethanol for at least 1 h, on ice. To achieve this, 400 μ L of ice-cold 70% ethanol was added dropwise to each sample, while vortexing to ensure complete fixation and avoid cell clumping. Cells were then washed $3 \times$ with PBS (first spin at $800 \times g$, 10 min, to recover all cells) and the cell pellet was resuspended in 0.5 mL of propidium iodide/RNase solution (FxCycle™ Solution, Molecular Probes, UK). The samples were incubated for 30 min at room temperature, protected from light and further analysed, without washing, by flow cytometry using a Becton Dickinson CytoFLEX (Beckman Coulter, UK). Cell doublets were separated from single cells in G2/M phase using pulse-width/pulse-area signal. At least 10,000 cells were acquired in a histogram and cell cycle data were exported as FSC files and analysed using FlowJo software. Each data measurement was made up from at least ten pooled spheroids, repeating the whole procedure independently two times ($n=2$). Results were expressed as mean $\pm$ S.D, after population deconvolution, peak integration and calculation of population percentage.

DNA binding studies

The interaction between Cu(TPZ)₂ (25 μ M) and calf-thymus DNA (CT-DNA) was analysed by UV/Vis spectroscopy. The CT-DNA stock solution, was prepared in Tris-buffer (containing 5 mM Tris-HCl and 50 mM NaCl at pH 7.2). The UV absorbance at 260 and 280 nm of the CT-DNA gave a ratio of 1.84, indicating that DNA was sufficiently free of protein contamination. DNA stock concentration (8.71 mM) was determined spectrophotometrically at 260 nm, by using the molar extinction coefficient value of $6600 \text{ M}^{-1} \text{ cm}^{-1}$.²⁵ Cu(TPZ)₂ (25 μ M) solutions were also prepared in Tris-buffer, where red dispersion was observed, indicating good dispersion of the complex in this buffer. We further performed electronic absorption titrations, which were carried out with a constant concentration of the copper(II) complex (25 μ M) and varying concentrations of CT-DNA (0–500 μ M) in Tris-buffer. The complex and DNA solutions were incubated at 37 °C for 24 h. Subsequently, the spectra were recorded using a UV/Vis spectrophotometer at ambient temperature. Solutions of free CT-DNA (namely in the absence of copper compound) at the corresponding concentrations (0–500 μ M) were used as blanks before recording the absorption band of each sample. The titrations of the corresponding concentration of TPZ (50 μ M) were also performed, with varying concentrations of CT-DNA (0–500 μ M). Solvent samples without DNA or drug were used as baseline references. The results were presented as mean

absorbance of triplicate samples of two independent experiments ($n=2$).

The intrinsic binding constant (K_b) for the interaction of Cu(TPZ)₂ with CT-DNA was calculated using the following equation:

$$\frac{[\text{DNA}]}{\epsilon_a - \epsilon_f} = \frac{[\text{DNA}]}{\epsilon_b - \epsilon_f} + \frac{1}{K_b (\epsilon_b - \epsilon_f)}$$

where ϵ_a , ϵ_f and ϵ_b correspond to, the apparent molar extinction coefficient - $A_{\text{obsd}}/[\text{Cu}]$, the molar extinction coefficient for the free Cu(TPZ)₂, and the molar extinction coefficient for the Cu(TPZ)₂ in the fully bound form, respectively. By plotting $[\text{DNA}]/(\epsilon_a - \epsilon_f)$ vs $[\text{DNA}]$, K_b was then determined by calculating the ratio of the slope to the intercept.

Statistical analysis

Data was presented as mean $\pm$ SD. Two-way Analysis of variance (ANOVA) and Bonferroni multiple comparisons post-hoc test was performed when three or more groups of data were analysed. P-values < 0.05 were considered significant and statistical differences amongst groups was appropriately denoted in the figure captions. The IC₅₀ values for inhibition were determined by nonlinear regression analysis of the data fit to a four-parameter equation. All analyses and graphs were generated using GraphPad Prism version 7.0 (GraphPad Software, La Jolla California USA, www.graphpad.com).

Results

Cu(TPZ)₂ synthesis and characterization

In this study, Cu(TPZ)₂ complex was prepared by mixing TPZ with CuCl₂ salt in an ethanolic solution (Figure. 1A). The red precipitate was collected, and its chemical structure was confirmed using elemental analysis, FTIR (Figure. S1), and mass spectrometry (MS) (Figure. S2). The relevant physicochemical properties (molecular mass, pKa and logP values) of TPZ²⁶ and Cu(TPZ)₂²⁷ were summarized in Figure. 1B. Based on the literature, Cu(TPZ)₂ complex exhibited a biocompatible reductive potential (−0.75V vs. Ag/AgCl), which was more electronegative than that presented by TPZ (−0.65V vs. Ag/AgCl), suggesting superior selectivity under hypoxia^{10, 19, 20, 28}.

The morphology and the structure of the isolated Cu(TPZ)₂ complex were also analyzed using TEM and X-ray powder diffraction (XRD), respectively. Due to the higher hydrophobic nature of the complex (solubility $\sim$ 2.5mM in DMSO), some intermolecular interactions occurred at high concentrations, resulting in highly organized nanostructures in more polar solvents (e.g. ethanol). Interestingly, TEM examination revealed that the complex morphology was concentration-dependent, where needle-like structures (300–400 nm in length) were present at high concentrations (2 mM), and tiny and homogenous spherical nanoparticles (3–4 nm in diameter) were observed at lower concentrations (20 μ M) (Figure. 1C), similar to the concentration range used in this study (20 μ M). The large needle structures possibly result from $\pi \rightarrow \pi$ stacking and van

der Waals interaction between molecules^{14, 17}, which strongly agrees with the proposed 3D structure (**Figure. 1A**). Single-crystal X-ray diffraction measurement was not possible to perform due to the small size of the complex crystals, however, the XRD result confirmed that the complex is in the crystalline form, with the following diffraction reflections 8.3°, 10.9°, 13.4°, 15.6°, 18.9°, 25.6° and 28.7° (**Figure. S4**). All the complex characterization results are described in detail in the supporting information.

Cu(TPZ)₂ stability and dissociation in different media

TPZ complexation was further confirmed using UV-Vis spectrophotometry. Both free and complexed TPZ showed similar optical properties in DMSO, with two characteristic absorption peaks at $\lambda_{\max}$ 280 nm and 500 nm (**Figure. 1D**). In HBS TPZ presented a less intense band at $\lambda_{\max}$ 280 nm and a more intense band at $\lambda_{\max}$ 462 nm (**Figure. 1E**), arisen from the $\pi \rightarrow \pi^*$ transitions in the phenyl group and $\pi \rightarrow \pi^*$ transitions from the pyridine-*N*-Oxide, respectively. Cu(TPZ)₂ maintained a ligand centered band at $\lambda_{\max}$ 280 nm and a broad absorption band in the visible region at 500-600 nm, which is characteristic for copper (II) d-d transition band (band I: $d_{xy, yz} \rightarrow d_{x^2-y^2}$)²⁹. The optical properties in different aqueous media were also studied, as described in the supporting information (**Figure. S5**).

To fully understand the dissociation mechanism of the complex, a simple titration study was performed by diluting the formed complex in aqueous buffers with a wide range of pH (**Figure. 1F**). An immediate color change was observed by decreasing the pH from 7.4 to 5. This was confirmed by a visible shift in the complex absorption (555 nm and 595 nm) to a lower wavelength (462 nm), indicating the dissociation of the complex at lower pH. A single sigmoidal curve was fitted to the absorbance at 462 nm and plotted against pH, providing a pKa value of 2.7 (**Figure. S5D**), comparable to that obtained before for TPZ²⁷. Furthermore, the stability of the complex was assessed in 50% serum, where minimum dissociation was evidenced (~20% up to 72 h) (**Figure. S6**), indicating high stability of the complex under physiological conditions. It is possible that a small number of Cu(TPZ)₂ molecules could have undergone copper-translocation *via* serum albumin⁴⁷, leading to free TPZ liberation.

Cellular uptake and metabolic analysis of intrinsically fluorescent Cu(TPZ)₂ complex

Poole *et al.* previously reported weak fluorescence of TPZ in solutions³⁰. This agrees with our findings, where TPZ was fluorescent a wide range of aqueous media (**Figure. S7A**), except at pH 12.5 (**Figure. S7B**)³⁰. Our current results showed that complexing TPZ to copper did not affect TPZ fluorescence emission in DMSO (**Figure 2A, left**). On the other hand, a significant enhancement in intensity was observed in HBS buffer pH 7.4 (**Figure. 2A, right**), with a 50-nm red shift upon coordination to the metal center. Interestingly, as the complex dissociated at low pH, a two-fold reduction in the fluorescence intensities was observed, reaching the same level of free TPZ. This strongly indicates that the fluorescent enhancement was metal-induced (**Figure. S7C**). Despite the strong quenching capacity of Cu(II), such fluorescence enhancement following copper complexation was previously reported²⁸.

Encouraged by the intrinsic fluorescence of the complex, and its high serum stability, we further monitored its cellular

localization and accumulation under normoxia (21% O₂) and 1% hypoxia, where the latter was validated using a hypoxia marker (**Figure. S8**). Representative images were then taken at 1, 4, 8 and 24 h under normoxia (**Figure 2B, left**) and hypoxia (**Figure 2B, right**), which showed the cellular accumulation of Cu(TPZ)₂ (green fluorescence) in C4-2B cells. Interestingly, the results depicted a rapid uptake (1 h) of the complex, with strong green fluorescent signals in the cytosol and perinuclear region, under both normoxia and 1% hypoxia. Furthermore, some images evidenced nuclear localization of the intact fluorescent complex, presented as diffuse green signals in the nucleus (**Figure 2B**) or as punctate signals (**Figure. S9**). At 4 h, the appearance of bright green punctate structures (depicted by white arrows), particularly in hypoxic conditions, suggested that copper complex can also accumulate in lysosomal compartments or autophagic structures^{6, 10, 11, 14}, however, the exact mechanism of the complex internalization should be confirmed. After 8 h, the fluorescent signals were clearly lower, suggesting complex efflux, dissociation or metabolism, as we further confirmed. A slight increase in fluorescence was observed after 24 h, possibly due to reoxidation of a small number of Cu(TPZ)₂ molecules *via* copper recycling pools. Further studies are required to investigate this observation.

TPZ's weak fluorescence was not reliable to evaluate its cellular uptake and localization under the same conditions (**Figure. S10**). Therefore, we developed a quantitative method to assess the complex uptake overtime. We compared the total uptake of TPZ and Cu(TPZ)₂ up to 24 h, under both normoxia and 1% hypoxia conditions (**Figure. 2C**). As evidenced before⁵, TPZ showed low cellular uptake, with no significant differences under both normoxia and hypoxia. On the other hand, Cu(TPZ)₂ showed a significant improvement in cellular uptake, under normoxia (4 h, $p < 0.001$; 8 h, $p < 0.01$ and 24 h, $p < 0.0001$) and hypoxia (4 h, $p < 0.0001$; 8 h, $p < 0.05$ and 24 h, $p < 0.0001$), compared to TPZ (**Figure. 2C**). These observations could be attributed to the higher lipophilicity of the complex (LogP 2.88) compared to TPZ (LogP -0.31). More importantly and in line with previous reports^{10, 13, 14}, the complex demonstrated selective hypoxia uptake at 4 h with higher uptake efficiency compared to normoxia ($p < 0.001$). The bioreductive cycle in the lysosome ($E_{\text{red}} -0.75$), could be responsible for this high intracellular accumulation, with decreased complex efflux under hypoxia¹⁵. The full mechanism underlining this hypoxia selectivity is detailed in the discussion section. Furthermore, the slight decrease in cell uptake at 8 h, could be attributed to complex dissociation in acidic compartments, such as lysosomes, with consequent loss of fluorescence intensity, which agrees with the fluorescent imaging and previous results (**Figure S7C**).

Drug metabolism could be responsible for the decreased intracellular concentrations of the complex. To verify this, we assessed the metabolism of TPZ and the complex following cell internalization, under hypoxia, using non-fluorescent-based techniques, namely FTIR and NMR. Our qualitative FTIR results, revealed a fast metabolism of TPZ, in contrast to the complex, where free TPZ was metabolized and an intermediate was formed within 1 h of cell internalization (**Figure. S11**). This fast TPZ metabolism is in agreement with the literature³¹. On the other hand, neither free TPZ, nor its intermediate metabolite was detected in the cell lysate samples incubated for 1 h with the complex (**Figure. S12**), re-emphasizing the stability of the

complex intracellularly, and its slow metabolism, respectively. Between 4 and 24 h similar metabolites were present in all samples. Moreover, as anticipated, the metabolite levels in the TPZ samples were much higher compared to cells incubated with the complex (**Figure. 3C**), indicating slower metabolism of the complex, as confirmed by NMR. These quantitative results showed that the levels of the complex metabolites, in contrast to free TPZ, fluctuated over time, with the highest level detected after 4 h. The metabolism decreased slightly around 8 h, before increasing again after 24 h (**Figure. 3C**). It is worth mentioning that the complex was present in the cell lysate (pellet) samples (**Figure. 3B**), but could not be detected using NMR, since it was precipitated from the supernatant due to a solubility issue. Interestingly, these results could justify the loss of fluorescence signal at 8 h (**Figure. 2B& 2C**), followed by some signal recovery at 24 h. Several *in vitro* studies reported TPZ benzotriazine radical and mono-*N*-oxide (SR4317) as major TPZ metabolites³², however, in presence of DNA, identifying the exact metabolites becomes more challenging, since the interaction of TPZ benzotriazine radicals with the DNA results in DNA damage, and different products are formed³³. The latter observation could possibly explain the unreported metabolite peaks detected by NMR (**Figure. 3**), however, the exact metabolites formed in our study remain to be identified.

491 Hypoxia selectivity of Cu(TPZ)₂ complex in PCa cells

To assess hypoxia selectivity, the cytotoxicity of free TPZ and Cu(TPZ)₂ complex was studied under normoxia (21% O₂) and 1% hypoxia in two prostate cancer (PCa) cell lines. The inhibitory concentrations (IC₅₀) were determined under both conditions and used to calculate the hypoxia cytotoxicity ratio (HCR, IC₅₀ normoxia/IC₅₀ hypoxia), which expresses hypoxia selectivity. The IC₅₀ and HCR values obtained in the PCa cell lines are summarized in **Table S1**.

Our results demonstrated that the cytotoxicity of TPZ and its complex was cell-, and time-dependent (**Figure. 4A**). C4-2B, an androgen-independent³⁴, derived bone metastatic PCa cell model, exhibited high sensitivity to the hypoxia pro-drugs, with clear dose-response shifts to the left at 1% hypoxia (**Figure. S13**). The effect was especially pronounced in terms of hypoxia selectivity, confirmed by the overall increased HCR values of Cu(TPZ)₂, compared to TPZ (**Table S1**). The HCR values were consistently higher for the complex and achieved exceptional selectivity for this cell line at 72 h post-treatment (HCR: 72 h, ~70). Furthermore, the cytotoxic effects under normoxia revealed a significant 2.03, 2.61 and 3.78-fold increase in IC₅₀ (at 48, 72 and 96 h, respectively) for Cu(TPZ)₂ compared to TPZ alone (**Figure. 4A, top panel**). More promisingly, enhanced potentiation of the complex under hypoxia (IC₅₀: 1.61 ± 1.0), compared to TPZ (IC₅₀: 2.831 ± 1.04 μM) was also obtained at 72 h time-point, confirming the augmented selectivity and therapeutic potency of the complex in C4-2B. Similar finding were obtained in LNCap, an androgen-dependent PC cells (**Figure. 4A, bottom panel**), which showed high sensitivity to both agents under hypoxia (**Figure. S14**), and superior hypoxia selectivity with the Cu(TPZ)₂ (**Table S1**). LNCap cells have been shown to readapt under hypoxia, through a metabolic shift, which may sensitize these cells to hypoxia-selective treatments³⁵. It is therefore plausible to argue that oxygenation is only one parameter influencing the activity of these drugs and may well be a simplistic view of their MoA. All cell lines were cultured

under the same conditions, yet they exhibit differences in toxicity and selectivity. Hypoxia adaptation and the reductive capacity of these cell lines will certainly influence the sensitivity to TPZ and Cu(TPZ)₂.

533 Potency of Cu(TPZ)₂ against C4-2B tumor spheroids

In order to validate the previous cytotoxicity data in monolayers, 3D hypoxia models of C4-2B, were developed (**Figure. 5A & Figures. S15-S16**), and C4-2B spheroids were incubated with TPZ or Cu(TPZ)₂ up to 96 h. The resazurin assay was used to quantify cell viability, and spheroids imaging was used to monitor cell morphology post-treatment (**Figure. S17**). As expected, TPZ (**Figure 5B**) was less potent in spheroids (**Table S2**) compared to C4-2B monolayers (**Table S1**). Furthermore, TPZ toxicity in 3D cultures was not dose- or time- dependent (IC₅₀: 48h, 35.17 ± 1.17 μM; 72h, 26.21 ± 1.18 μM, 96h: 36.02 ± 1.89 μM) (**Figure. 5B & Table S2**). This data indicated that TPZ's toxicity is possibly restricted to the spheroid surface layer, since it is metabolized too rapidly^{26, 31, 36-39}, as we previously showed. On the other hand, Cu(TPZ)₂ showed dose and time-dependent cytotoxicity in C4-2B spheroids (**Figure 5C and Table S2**) (IC₅₀: 48h, 41.78 ± 5.67 μM; 72h, 25.58 ± 3.78 μM, 96h: 13.33 ± 1.43 μM). The results correlated well with the different metabolic rates of the complex compared to TPZ (**Figure. 3**). At 48 h, spheroids were slightly more resistant to Cu(TPZ)₂ (IC₅₀: 41.78 ± 5.67 μM) compared to TPZ (IC₅₀: 35.17 ± 1.17 μM), but reached a pronounced therapeutic activity at 96h (Cu(TPZ)₂, IC₅₀: 13.33 ± 1.43 μM; TPZ, IC₅₀: 36.02 ± 1.89 μM, p<0.0001).

Furthermore, in order to determine the cellular responses after TPZ and Cu(TPZ)₂ drug testing, cell cycle and apoptosis analyses were carried out. 5-days post seeding, C4-2B spheroids were treated for 72 h with 20 and 2 μM equivalent TPZ in both free and complex forms. Cell cycle histograms were generated to evaluate cell cycle distribution (**Figure. S18**). Interestingly, data showed marked arrest in S-phase population, followed by decrease in G1 for Cu(TPZ)₂ treated spheroids at both 10 (p<0.05) and 1 μM (p<0.01), compared to untreated or TPZ-treated spheroids (**Figure. 5D**). Furthermore, this significant S-phase arrest was accompanied by increased propidium iodide (PI)-positive apoptotic cells following Cu(TPZ)₂ (10 μM) treatment, compared to untreated and TPZ groups (**Figure. 5E**).

571 Enhanced DNA binding of Cu(TPZ)₂

The cell cycle analysis results suggested that TPZ cytotoxicity in C4-2B spheroids may be mediated by non-selective mitochondrial and ROS induced cell death⁴⁰, while Cu(TPZ)₂ may exert an increased DNA selective cytotoxic activity. To confirm the latter, UV-Vis absorption spectra of free TPZ were recorded in the absence and presence of increasing concentrations of CT-DNA solution (**Figure. 5F**). No spectral changes were observed, indicating that TPZ did not interact with DNA in the absence of its radical counterpart⁴¹. On the other hand, Cu(TPZ)₂ (**Figure. 5G**) showed a significant hyperchromic effect in the entire spectrum, with a small bathochromic shift, which indicates a non-intercalative binding mode through DNA groove binding, resulting in damaging of the DNA double-helical structure²⁵. The DNA binding constant (K_b 0.18 × 10² M⁻¹) (**Figure. 5G, inset**), was in agreement with other DNA groove binding agents and much lower than standard DNA intercalators⁴². This indicates that Cu(TPZ)₂ complex has a moderate DNA binding capacity,

possibly through DNA groove binding, but intercalation cannot be ruled out, supporting previous evidence on the enhanced DNA damaging capacity of this complex²¹. Further studies on DNA viscosity, damage and ROS production can further elucidate the mechanisms of action of this cupric-TPZ complex.

Discussion

Cancer hypoxia has been highly associated with treatment resistance and metastasis; therefore, a wide range of hypoxia-activated prodrugs have been developed to targeting hypoxia^{1,2}. TPZ is the most advanced bioreductive pro-drug in clinical trials, however, its clinical efficacy has been limited by its poor cellular uptake, restricted tissue diffusion and rapid metabolism⁴. Several structure-activity relationship (SAR) studies with TPZ analogues have provided substantive correlation between specific substituents (and their position) with improved pharmacological outcomes⁴³. Moreover, the use of transition metal complexes has shown to be a particularly suitable strategy to modify existing pro-drugs and develop a new biologically active molecule with improved hypoxia activity⁷. Copper(II) complexes have shown to coordinate well with 1,2,4-benzotriazine 1,4-di-*N*-oxides^{44, 45}, generating prodrug complexes that are stable under physiological conditions, but become kinetically labile with changes in the redox status or pH of the environment. Two studies reported TPZ-copper complex as a hypoxia-selective agent in 2D culture^{21,22}, however, these studies neither demonstrated any correlation between the complex characteristics and its biological activity, nor assessed its activity in 3D hypoxia culture models. In our work, we have revealed unreported characteristics of the complex, such as its lipophilicity (LogP 2.88), particulate nature, slow metabolism, as well as its superior intrinsic fluorescence, enabling its cellular trafficking and quantification under normoxia and hypoxia. Collectively, with the published reductive potential of the complex, we have been able, and for the first time, to provide better understanding of the biological activity of the complex in 2D and 3D hypoxia PC models.

TPZ has been shown to possess a pH-dependent cytotoxicity⁴⁶, where the slightly acidic microenvironment (pH 6.0) dramatically increased its aerobic cytotoxicity, thus contributing to the observed clinical side-effects (muscle cramping, peripheral neuropathy, and intestinal symptoms)^{4, 5}. Interestingly, our results suggest that the high pH stability of the complex could minimize TPZ systemic toxicity. Studies have also shown that the reductive hypoxic environment plays a key role in determining the dissociation rate of metal-complexes. Previous reports have shown that copper trans-chelation could occur with serum albumin⁴⁷, or thiol-rich molecules (glutathione, cysteine and ascorbate), which are capable of reducing these complexes intracellularly²⁷. The former was not observed with our complex since high serum stability was observed. Nevertheless, previous data²¹ showed that TPZ can compete with glutathione for copper, but not EDTA, once again confirming the stability of Cu(TPZ)₂. All these findings are important from a biological point of view, and the data presented here suggested that Cu(TPZ)₂ may be dissociated *via* a pH-dependent mechanism, intracellular thiol-mediated reduction, but not by serum albumin trans-chelation emphasizing its stability in the blood followings its systemic administration.

Previous reports, have shown high potentiation of TPZ under different hypoxia levels (HCR: 30-300) that was cell-line dependent⁴. Factors such as p53 status, expression of reductive enzymes, *e.g.* cytochrome c P450⁴⁸, CA-IX, acidic microenvironment and of homologous recombination proteins (XRCC2, Rad51D, BRCA1 and BRCA2) have been shown to influence TPZ sensitivity and cannot be ruled out⁴⁸. Interestingly, the PC cell lines used here showed differential metabolic profiles under hypoxia and aggressiveness is marked by defects in DNA repair mechanisms, p53 status and increased basal ROS levels³⁵. Previously, Adsule *et al.*¹⁸, developed a potent quinoline-2-carboxaldehyde copper complex, which inhibited proteasome activity in PC-3 and LNCaP cells (IC₅₀ of 4 and 3.2 μM, respectively). However, studies were only carried out under normoxic conditions, not specifying any hypoxia selectivity. In this work, Cu(TPZ)₂ complex exhibited low cytotoxicity under normoxia with IC₅₀ above *ca.* 50 μM for all cell lines, while under 1% hypoxia IC₅₀ values ranged from moderate (>10 μM) to potent activity (<10 μM). Cu(TPZ)₂ showed higher potency, compared to TPZ, in C4-2B, but higher selectivity was observed in both cells tested. The HCR heatmap results (Figure. 4B) showed a moderate effect at 48 h, for both drugs, against LNCaP, and C4-2B. HCR values were consistently higher for Cu(TPZ)₂ (>10) in LNCaP and C4-2B, compared to TPZ, with outstanding selectivity in C4-2B after 72 h (HCR of *ca.* 70), confirming the potential of this complex as HAPs in PC. This superior selectivity is attributed to the higher electronegativity of the complex compared to TPZ. **Most research surrounding hypoxia selectivity is based on the unique cellular trapping mechanism proposed for Cu(ATSM)⁴⁹. This copper(II) complex has been widely exploited *in vitro* and *in vivo*, due to its enhanced accumulation in hypoxic tissues.** Generally, complexes with reduction potential more electronegative than -0.57 V vs. Ag/AgCl, display optimum hypoxia selectivity. Upon reduction, under hypoxia, these copper(II) complexes will partially dissociate into an intermediary copper(I) compound, which is more labile and eventually undergoes complete dissociation, liberating the free ligand⁵⁰. Copper(I) is then bound to specific binding proteins and chaperones such as Ctr1 and Atx1, respectively, which mediate the copper cellular pool⁵⁰. Whilst, under normoxia, the intermediary copper(I) compound will be reversibly oxidize to its copper(II) counterpart and diffuse out of the cells. **Although the redox-properties provide reliable gold-standard in hypoxia selectivity, other factors influencing reduction rate should not be ruled out. Indeed, the degree of expression of intracellular reductases has shown to influence dissociation, as well as other biological reductants such as ascorbate, thiols or NADP(H), as discussed previously.**

TPZ's limited diffusion and incapacity to reach the relevant hypoxic cells, in tumor models have been previously reported^{26, 31, 36-39}. To overcome these limitations, analogues with increased lipophilicity (due to removal of H-bond donor of the 3NH₂ group), comparable to that of Cu(TPZ)₂, showed pronounced uptake, selectivity and tissue diffusion,^{26, 38, 43}. In agreement with these studies, the superior potency of the complex in C4-2B spheroids, compared to free TPZ, as confirmed by cell viability and cell cycle analysis, could be attributed to the high lipophilicity of the complex in combination with its good pH and serum stability, slow metabolism, and high electronegative potential (-0.75 V), resulting in decreased early onset toxicity to the outer layer of the spheroid and possible free ligand back-

diffusion from the inner spheroid to induce high cell toxicity throughout the spheroid environment⁵¹. Furthermore, its higher DNA binding as we demonstrated, and reported by others²¹ could contribute to higher potency. However, the exact mechanism of toxicity in spheroids has yet to be investigated.

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718 Conclusions

We successfully presented the synthesis and extensive characterization of a nano-sized Cu(TPZ)₂ complex that improved on TPZ's physicochemical properties and its hypoxia selectivity in PC. The synthesized copper-complex, exhibited high LogP, increased biocompatible redox-potential, slow metabolism, and high DNA binding, compared to TPZ. The intrinsic fluorescence of the complex enabled studying its cellular uptake, concluding on its significant hypoxia selective uptake and accumulation in the perinuclear area and in intracellular vesicles. In addition, our results reported for the first time the biological activity of Cu(TPZ)₂ complex in 3D hypoxia models. Interestingly, Cu(TPZ)₂ complex exhibited excellent hypoxia selectivity compared to TPZ, particularly in the castrate-resistant C4-2B PC monolayers. Furthermore, the improved structural activity of the complex correlated well with its enhanced efficacy in C4-2B spheroids. Further studies are still warranted to determine the Cu(TPZ)₂ exact mechanism of action *in vitro*. Finally, the limited solubility of the complex could be overcome using clinically relevant delivery systems, such as liposomes, to assess its therapeutic efficacy *in vivo*.

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740 Conflicts of interest

There are no conflicts to declare.

742 Acknowledgements

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Figures and captions

Figure 1. Synthesis and characterization of hypoxia-selective Cu(TPZ)₂ complex. A) Schematic representation of the synthesis protocol of Cu(TPZ)₂ complex in ethanol (Inset: optimised structure); B) A summary of main physicochemical properties of TPZ and Cu(TPZ)₂; C) TEM images of Cu(TPZ)₂ at high (2 mM) and low (20 μM) concentrations, depicting a concentration-dependent, self-aggregation of the complex in ethanol; D) Absorbance spectra of TPZ (10 μM, solid line) and Cu(TPZ)₂ (5 μM, dotted line) in DMSO; E) Absorbance spectra of TPZ (60 μM, solid line) and Cu(TPZ)₂ (30 μM, dotted line) in HBS; and F) Absorbance of Cu(TPZ)₂ (50 μM) at different pH values, illustrating a pH-dependent dissociation of Cu(TPZ)₂. Inset shows a yellow color change of TPZ to a dark red solution upon complexation and vice-versa, upon dissociation.

Figure 2. Intrinsic fluorescence, uptake and cellular localization of Cu(TPZ)₂ complex in C4-2B prostate cancer cells. A) Fluorescence spectra of TPZ and Cu(TPZ)₂ diluted in DMSO (left) and HBS (right) (DMSO: $\lambda_{exc}/\lambda_{em}$ 490/600 nm; HBS: $\lambda_{exc}/\lambda_{em}$ 450/570 nm (TPZ) and $\lambda_{exc}/\lambda_{em}$ 500/600 nm (Cu(TPZ)₂). B) Qualitative uptake of Cu(TPZ)₂ (100 μ M equivalent TPZ) at 1, 4, 8 and 24 h under normoxia and 1% hypoxia. The white arrows depict intense green puncta signals, suggesting accumulation of the complex in intracellular vesicles. The signals were more prominent under hypoxic conditions and in the perinuclear area. Images are representative of at least 50 cells of two independent experiments (n=2). Green channel: Cu(TPZ)₂ treated cells; Blue channel: nuclei stained with Hoechst 33258. Scale bars, 20 μ m. C) Quantitative uptake of TPZ and Cu(TPZ)₂ in C4-2B cells. Cells were incubated with 25 μ M equivalent TPZ, and the cellular uptake was determined at 1, 4, 8 and 24 h under normoxia and 1% hypoxia. Fluorescence was measured at $\lambda_{exc}/\lambda_{em}$ 485/590 nm in DMSO. Results were expressed as mean $\pm$ SD for triplicates of three independent experiments (n=3). Statistical analysis using Two-way ANOVA multiple comparison with Bonferroni Post-test (GraphPad Prism version 7.0, GraphPad Software Inc., La Jolla, CA, USA) was performed to compare uptake of TPZ vs Cu(TPZ)₂: (**p $\leq$ 0.01, ***p $\leq$ 0.001 and ****p $\leq$ 0.0001, comparing drugs under normoxia; #p $\leq$ 0.05, and #####p $\leq$ 0.0001, comparing drugs under hypoxia; and \$\$\$p $\leq$ 0.001, comparing Cu(TPZ)₂ under hypoxia and normoxia.

Figure 3: ^1H -NMR in CDCl_3 of C4-2B cell lysates. Cells were incubated under 1% hypoxia with (A) TPZ and (B) $\text{Cu}(\text{TPZ})_2$ for different duration (1, 4, 8, 24 h). Untreated cells were used as a control. Samples were prepared for NMR analysis as described in the methods section. TPZ (1 mg/ 400 μl) was also analysed (peaks a,b,c), however the complex was not soluble in CDCl_3 and no peaks were detected. The photograph (inset in Figure. 3B) represents the pinkish cell lysate pellets, indicating the presence of $\text{Cu}(\text{TPZ})_2$ in the cell lysate samples. The main sharp peak (*) represents the solvent. (C) peak heights for the TPZ and $\text{Cu}(\text{TPZ})_2$ metabolites.

Figure 5. *In vitro* toxicity of Cu(TPZ)₂ in C4-2B spheroids and DNA binding assay. A) fluorescent images of 3D C4-2B spheroids (day 5) incubated with CYTO-ID® Hypoxia Detection probe (500 nM, Enzo Life Sciences, UK) for 4 h, taken using an inverted Zeiss Axiovert 200M with Texas Red (596/670 nm) filter and equipped with a Zeiss ApoTome (Carl Zeiss, UK) to create optical sections free of scattered light. For each spheroid, a Z-stack with 10 μm thickness was generated. Scale bar: 100 μm. IC₅₀ curves of B) TPZ and C) Cu(TPZ)₂ in C4-2B spheroids. 5 days post seeding, tumor spheroids were incubated with equivalent doses of Cu(TPZ)₂ and TPZ, and IC₅₀ values were determined after 48, 72 and 96 h. Cell viability was assessed by the resazurin assay, with spheroids pre-treated for 30 min with EDTA (5 mM). Results were normalized to untreated and expressed as mean ± SD for six replicates of at least two independent experiments (n≥2). The IC₅₀ values for inhibition were determined by nonlinear

regression analysis of the data fit to a four-parameter equation (GraphPad Prism version 7.0, GraphPad Software Inc., La Jolla, CA, USA). C4-2B spheroids were cultured for 5 days, then treated for 72 h with 20 and 2 μ M equivalent TPZ in both free and complex forms and analyzed for D) cell cycle distribution and E) the percentage of apoptotic cells. Each flow cytometry plot depicts the mean $\pm$ SD percentage of G1 (2n), S and G2/M (4n) fraction population. The data represents the mean of at least ten pooled spheroids of two independent experiments (n=2). F) Absorption spectra of the TPZ (50 μ M) in the absence (dashed line) and presence (solid line) of increasing amounts of CT-DNA at room temperature in 5 mM Tris/HCl/50 mM NaCl buffer, pH 7.2. Inset shows a zoom in of the visible spectral region, showing no changes in the spectrum. G) Absorption spectra of the complex (25 μ M) in the absence (dashed line) and presence (solid line) of increasing amounts of CT-DNA at room temperature in 5 mM Tris/HCl/50 mM NaCl buffer, pH 7.2. The arrow depicts the absorbance spectrum changes on increasing the CT-DNA concentration. Inset shows the plot of $[\text{DNA}]/(\epsilon_a - \epsilon_f) / (\epsilon_b - \epsilon_f)$ vs [DNA] and derived K_b value (M^{-1}); Statistical analysis: Two-way ANOVA multiple comparison Bonferroni Post-hoc test (* $p < 0.05$ ** $p < 0.01$, compared to control and # $p < 0.05$ ## $p < 0.01$, comparing TPZ vs $\text{Cu}(\text{TPZ})_2$, GraphPad Prism version 7.0).