Supporting Information for

The Breast Cancer Stem Cell Potency of Copper(II) Complexes Bearing Nonsteroidal Anti-Inflammatory Drugs and Their Encapsulation Using Polymeric Nanoparticles

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**References**
Experimental Details

Materials and Methods. All synthetic procedures were performed under normal atmospheric conditions. High resolution electron spray ionisation mass spectra were recorded on a BrukerDaltronics Esquire 3000 spectrometer by Dr. Lisa Haigh (Imperial College London). Fourier transform infrared (FTIR) spectra were recorded with a IRAffinity-1S Shimadzu spectrophotometer. Elemental analysis of the compounds prepared was performed commercially by London Metropolitan University. 3,4,7,8-Tetramethyl-1,10-phenanthroline and 4,7-diphenyl-1,10-phenanthroline were purchased from Sigma Aldrich and used as received. The methoxy poly(ethylene glycol)-b-poly(D,L-lactic-co-glycolic) acid (PEG-PLGA) copolymer used in the nanoparticle studies was purchased from PolySciTech (USA).

Synthesis of Cu(3,4,7,8-tetramethyl-1,10-phenanthroline)(naproxen)_2 (2a): KOH (30 mg, 0.53 mmol) was added to a solution of naproxen (97 mg, 0.42 mmol) dissolved in methanol (4 mL). This solution was stirred at room temperature for 1 h, after which 3,4,7,8-tetramethyl-1,10-phenanthroline (50 mg, 0.21 mmol) was added, followed by a methanolic solution (4 mL) of CuCl_2·2H_2O (32 mg, 0.19 mmol). The resulting mixture was stirred at 50 °C for 20 h. The solution obtained was evaporated to dryness and the resulting solid was washed thoroughly with water (3 x 20 mL) and diethyl ether (3 x 20 mL). The isolated product was a blue-green solid. (127 mg, 89 %); IR (solid, cm⁻¹): 1600, 1561, 1504, 1482, 1453, 1425, 1418, 1378, 1364, 1289, 1265, 1252, 1230, 1212, 1168, 1161, 1121, 1031, 925, 895, 882, 853, 832, 793, 769, 747, 734, 703; HR ESI-MS Calcd. for C_{47}H_{46}CuN_2O_7 [M+Na]^+: 816.4079 a.m.u. Found: 816.4376 a.m.u.; Anal. Calcd. for 2a, C_{44}H_{42}CuN_2O_6: C, 69.69; H, 5.58; N, 3.69. Found: C, 69.61; H, 5.65; N, 3.46.

Synthesis of Cu(3,4,7,8-tetramethyl-1,10-phenanthroline)(tolfenamic acid)_2 (2b): KOH (30 mg, 0.42 mmol) dissolved in methanol (4 mL). This solution was stirred at room temperature for 1 h, after which 3,4,7,8-tetramethyl-1,10-phenanthroline (50 mg, 0.21 mmol) was added, followed by a methanolic solution (4 mL) of CuCl_2·2H_2O (32 mg, 0.19 mmol). The resulting mixture was stirred at 50 °C for 20 h. The precipitate obtained was filtered off, and the filtrate was evaporated to dryness. The solid obtained was washed thoroughly with water (3 x 20 mL) and diethyl ether (3 x 20 mL). The product isolated was a bright green solid. (95 mg, 64 %); IR (solid, cm⁻¹): 1603, 1585, 1560, 1507, 1459, 1431, 1382, 1352, 1318, 1305, 1284, 1259, 1246, 1205, 1180, 1154, 1139, 1089, 1080, 1040, 1012, 965, 923, 910, 894, 869, 849, 832, 812, 803, 777, 764, 737, 722; HR ESI-MS Calcd. for C_{46}H_{44}Cl_2CuNaO_6S [M+Na]^+: 922.2502 a.m.u. Found: 922.1638 a.m.u.; Anal. Calcd. for 2b, C_{44}H_{38}Cl_2CuNaO_4: C, 64.35; H, 4.66; N, 6.82. Found: C, 64.02; H, 4.81; N, 6.72.

Synthesis of Cu(3,4,7,8-tetramethyl-1,10-phenanthroline)(indomethacin)_2 (2c): KOH (41 mg, 0.73 mmol) was added to a solution of indomethacin (225 mg, 0.63 mmol) dissolved in methanol (6 mL). This solution was stirred at room temperature for 1 h, after which a methanolic solution (4 mL) of CuCl_2·2H_2O (25 mg, 0.15 mmol) was added. This resulted in the formation of a green precipitate. This mixture was left to stir at 70 °C for 15 minutes. 3,4,7,8-Tetramethyl-1,10-phenanthroline (50 mg, 0.21 mmol) was then added and the resulting mixture was left to stir at 70 °C for 24 h. A blue green solution was obtained, and this was evaporated to dryness. The solid obtained was washed thoroughly with water (3 x 20 mL) and diethyl ether (3 x 20 mL). The product isolated was a blue-green solid. (106 mg, 71 %); IR (solid, cm⁻¹): 1673, 1590, 1477, 1354, 1315, 1223, 1147, 1088, 1014, 934, 832, 754;
HR ESI-MS Calcd. for C_{54}H_{47}Cl_{2}CuN_{4}O_{8} [M+H]^+: 1014.3840 a.m.u. Found: 1014.2081 a.m.u.; Anal. Calcd. for 2c, C_{54}H_{46}Cl_{2}CuN_{4}O_{8}: C, 64.00; H, 4.85; N, 5.53. Found: C, 63.65; H, 4.80; N, 5.42.

**Synthesis of Cu(4,7-diphenyl-1,10-phenanthroline)(naproxen)_{2} (3a):** KOH (21 mg, 0.37 mmol) was added to a solution of naproxen (70 mg, 0.30 mmol) dissolved in methanol (4 mL). This solution was stirred at room temperature for 1 h, after which 4,7-diphenyl-1,10-phenanthroline (50 mg, 0.15 mmol) was added, followed by a methanolic solution (4 mL) of CuCl_{2}•2H_{2}O (23 mg, 0.14 mmol). The resulting mixture was stirred at 50 °C for 20 h. The precipitate obtained was filtered off, and the filtrate was evaporated to dryness. The solid obtained was washed thoroughly with water (3 x 20 mL) and diethyl ether (3 x 20 mL). The product isolated was a dark green solid. (96 mg, 62.52 mmol). The product was isolated as a dark green solid. (26 mg, 0.14 mmol). The resulting mixture was stirred at 50 °C for 20 h. The precipitate obtained was filtered off, and the filtrate was evaporated to dryness. The solid obtained was washed thoroughly with water (3 x 20 mL) and diethyl ether (3 x 20 mL). The product isolated was a blue-green solid. (96 mg, 850, 833, 792, 769, 747, 735, 703; HR ESI-MS Calcd. for C_{53}H_{44}CuN_{2}O_{8} [M+CH_{3}O_{2}]^{+}: 900.4374 a.m.u. Found: 899.1279 a.m.u.; Anal. Calcd. for 3a: C, 67.12; H, 4.18; N, 5.05. Found: C, 66.94; H, 4.25; N, 5.17.

**Synthesis of Cu(4,7-diphenyl-1,10-phenanthroline)(tolfenamic acid)_{2} (3b):** KOH (21 mg, 0.37 mmol) was added to a solution of tolfenamic acid (79 mg, 0.30 mmol) dissolved in methanol (4 mL). This solution was stirred at room temperature for 1 h, after which 4,7-diphenyl-1,10-phenanthroline (50 mg, 0.15 mmol) was added, followed by a methanolic solution (4 mL) of CuCl_{2}•2H_{2}O (23 mg, 0.14 mmol). The resulting mixture was stirred at 50 °C for 20 h. The precipitate obtained was filtered off, and the filtrate was evaporated to dryness. The solid obtained was washed thoroughly with water (3 x 20 mL) and diethyl ether (3 x 20 mL). The isolated product was a dark green solid. (26 mg, 21 %); IR (solid, cm⁻¹): 1660, 1563, 1520, 1504, 1482, 1453, 1426, 1417, 1378, 1364, 1289, 1265, 1251, 12330, 1211, 1160, 1120, 1087, 1063, 1030, 1020, 925, 895, 882, 832, 792, 769, 747, 735, 703; HR ESI-MS Calcd. for C_{53}H_{44}CuN_{2}O_{8} [M+CH_{3}O_{2}]^{+}: 900.4374 a.m.u. Found: 899.1279 a.m.u.; Anal. Calcd. for 3b: C, 65.00; H, 4.85; N, 5.53. Found: C, 64.00; H, 4.85; N, 5.53.

**Synthesis of Cu(4,7-diphenyl-1,10-phenanthroline)(indomethacin)_{2} (3c):** KOH (29 mg, 0.52 mmol) was added to a solution of indomethacin (150 mg, 0.42 mmol) dissolved in methanol (4 mL). This solution was stirred at room temperature for 1 h, after which 4,7-diphenyl-1,10-phenanthroline (70 mg, 0.21 mmol) was added, followed by a methanolic solution (4 mL) of CuCl_{2}•2H_{2}O (36 mg, 0.21 mmol). The resulting mixture was stirred at 50 °C for 20 h. The precipitate obtained was filtered off, and the filtrate was evaporated to dryness. The solid obtained was washed thoroughly with water (3 x 20 mL) and diethyl ether (3 x 20 mL). The product was isolated as a dark green solid (91 mg, 39%); IR (solid, cm⁻¹): 1674, 1588, 1560, 1478, 1354, 1317, 1223, 1067, 1015, 922, 831, 754, 702, 633; HR ESI-MS Calcd. for C_{52}H_{47}Cl_{2}CuN_{4}O_{8} [M+H]^+: 1110.4719 a.m.u. Found: 1110.2162 a.m.u.; Anal. Calcd. for 3c: C, 67.12; H, 4.18; N, 5.05. Found: C, 66.94; H, 4.25; N, 4.94.

**Measurement of Water-Octanol Partition Coefficient (Log P):** The log P value of 2a-3c was determined using the shake-flask method and UV-Vis spectroscopy. The octanol used in this experiment was pre-saturated with water. An aqueous solution of 2a-3c (500 μL, 100 μM) was incubated with pre-saturated octanol (500 μL) in a 1.5 mL tube. The tube was shook at room temperature for 48 h. The two phases were separated by centrifugation and 2a-3c content in each phase was determined by UV-Vis spectroscopy.
Cell Lines and Cell Culture Conditions. The human mammary epithelial cell lines, HMLER and HMLER-shEcad were kindly donated by Prof. R. A. Weinberg (Whitehead Institute, MIT). HMLER and HMLER-shEcad cells were maintained in Mammary Epithelial Cell Growth Medium (MEGM) with supplements and growth factors (BPE, hydrocortisone, hEGF, insulin, and gentamicin/amphotericin-B). The cells were grown at 310 K in a humidified atmosphere containing 5% CO₂.

Cytotoxicity MTT assay. The colourimetric MTT assay was used to determine the toxicity of 2a-3c, naproxen, tolfenamic acid, and 3c NP⁵. HMLER or HMLER-shEcad cells (5 × 10³) were seeded in each well of a 96-well plate. After incubating the cells overnight, various concentrations of the compounds/ nanoparticle formulation (0.0004-100 µM), were added and incubated for 72 h (total volume 200 µL). Stock solutions of the compounds were prepared as 10 mM solutions in DMSO and diluted using media. Stock solutions of the nanoparticle formulation, 3c NP⁵ were prepared as 1-5 mM solutions in ddH₂O and diluted using media. For compounds diluted from DMSO stock solutions, the final concentration of DMSO in each well was 0.5% and this amount was present in the untreated control as well. After 72 h, the medium was removed, 200 µL of a 0.4 mg/mL solution of MTT in MEGM was added to each well, and the plate was incubated for an additional 4 h. The MEGM/MTT mixture was aspirated and 200 µL of DMSO was added to dissolve the resulting purple formazan crystals. The absorbance of the solutions in each well was read at 550 nm. Absorbance values were normalized to (DMSO-containing) control wells and plotted as concentration of test compound/ nanoparticle formulation versus % cell viability. IC₅₀ values were interpolated from the resulting dose dependent curves. The reported IC₅₀ values are the average of three independent experiments, each consisting of six replicates per concentration level (overall n = 18).

Cellular Uptake. To measure the cellular uptake of 1, 2a-3c and 3c NP⁵ ca. 1 million HMLER-shEcad cells were treated with 1, 2a-3c, or 3c NP⁵ (0.5 µM) at 37 ºC for 4 or 12 h. In the case of 3c NP⁵ the experiment was also conducted at 4 ºC, and in the presence of endocytosis inhibitors, NH₄Cl (50 mM) and chloroquine (100µM). After incubation the media was removed, the cells were washed with PBS (2 mL × 3), harvested, counted using a hemocytometer, and centrifuged. The cellular pellets were dissolved in 65% HNO₃ (250 µL) overnight. The samples were diluted 5-fold with water and analysed using inductively coupled plasma mass spectrometry (ICP-MS, PerkinElmer NexION 350D). As copper is an endogenous metal, the copper concentration in untreated HMLER-shEcad cells was also measured. Copper levels are expressed as Cu (ppb) per million cells. Results are presented as the mean of five determinations for each data point.

Tumorsphere Formation and Viability Assay. HMLER-shEcad cells (5 × 10³) were plated in ultralow-attachment 96-well plates (Corning) and incubated in MEGM supplemented with B27 (Invitrogen), 20 ng/mL EGF, and 4 µg/mL heparin (Sigma) for 5 days. Studies were also conducted in the presence of 2a, 2b, 3c, naproxen, tolfenamic acid or indomethacin (0-66 µM). Mammospheres treated with 2a, 2b, 3c, naproxen, tolfenamic acid or indomethacin (at their respective IC₂₀ values, 5 days) were counted and imaged using an inverted microscope. The viability of the mammospheres was determined by addition of a resazurin-based reagent, TOX8 (Sigma). After incubation for 16 h, the solutions were carefully transferred to a black 96-well plate (Corning), and the fluorescence of the solutions was read at 590 nm (λₑₓ = 560 nm). Viable mammospheres reduce the amount of the oxidized TOX8 form (blue) and concurrently increases the amount of the fluorescent TOX8 intermediate (red), indicating the degree of mammosphere cytotoxicity caused by the test compound. Fluorescence values were
normalized to DMSO-containing controls and plotted as concentration of test compound versus % mammospheres viability. IC\textsubscript{50} values were interpolated from the resulting dose dependent curves. The reported IC\textsubscript{50} values are the average of two independent experiments, each consisting of three replicates per concentration level (overall n = 6).

RNAi Signatures.\textsuperscript{2-4} The copper(II) complex, 3c was dosed to achieve an LD80-90 in \(E_{\mu}^{\mu}p^{19\text{arf}}\) cells by propidium iodide exclusion as determined by flow cytometry after 48 h incubation. GFP enrichment/depletion was then determined by flow cytometry at 72 h. Linkage ratios (LR) and p-values were generated as described previously. All flow cytometry was conducted using a FACSScan flow cytometer (BD Biosciences).

GFP Competition Assays. \(E_{\mu}^{\mu}p^{19\text{arf}}\) lymphoma cells were infected with GFP-tagged shRNAs such that 15-25% of the population were GFP positive. An eighth of a million cells in 250 \(\mu\)L B-cell media (BCM) were then seeded into 24-well plates. For wells that would remain untreated as a control, only 1/16th of a million cells were seeded. Next, 250 \(\mu\)L of media containing the active agent was added to the cells. After 24 h, 300 \(\mu\)L of cells from untreated wells are removed and replaced by 300 \(\mu\)L fresh BCM. All wells then received 500 \(\mu\)L BCM before being placed by in the incubator for another 24 h. At 48 h, cells transduced with the control vector, MLS, were checked for viability via flow cytometry on a FACScan flow cytometer (BD Biosciences) using propidium iodide as a live/dead marker.

Intracellular ROS Assay. HMLER-shEcad cells (5 \(\times\) 10\(^3\)) were seeded in each well of a 96-well plate. After incubating the cells overnight, they were treated with 3c, 3c NP\textsuperscript{5}, or \(H_2O_2\) (0.5, 0.5 and 6 \(\mu\)M for 6, 12, and 48 h), in the presence or absence of \(N\)-acetylcysteine (1.5 mM), and incubated with 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (20 \(\mu\)M) for 30 min. The intracellular ROS level was determined by measuring the fluorescence of the solutions in each well at 529 nm (\(\lambda_{ex} = 504\) nm).

Immunoblotting Analysis. HMLER-shEcad cells (5 \(\times\) 10\(^5\) cells) were incubated with 3c (0.25-0.5 \(\mu\)M), 3c NP\textsuperscript{5} (0.5-1 \(\mu\)M), or indomethacin (20 \(\mu\)M) for 72 h at 37 \(^\circ\)C. Cells were washed with PBS, scraped into SDS-PAGE loading buffer (64 mM Tris-HCl (pH6.8)/ 9.6% glycerol/ 2% SDS/ 5% \(\beta\)-mercaptoethanol/ 0.01% Bromphenol Blue), and incubated at 95 \(^\circ\)C for 10 min. Whole cell lysates were resolved by 4-20 % sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE; 200 V for 25 min) followed by electro transfer to polyvinylidene difluoride membrane, PVDF (350 mA for 1 h). Membranes were blocked in 5\% (w/v) non-fat milk in PBST (PBS/0.1% Tween 20) and incubated with the appropriate primary antibodies (Cell Signalling Technology). After incubation with horseradish peroxidase-conjugated secondary antibodies (Cell Signalling Technology), immune complexes were detected with the ECL detection reagent (BioRad) and analyzed using a chemiluminescence imager (Amersham Imager 600).

COX Inhibition Enzyme Immunoassay (EIA). The cyclooxygenase-2 (COX-2) inhibitory properties of 3c and indomethacin was determined using the “COX (ovine) Inhibitor Screening Assay (Cayman Chemicals).” The assay was performed according to the manufacturer’s instructions. Inhibitory activity was determined at 0.05, 0.5 \(\mu\)M, 5, and 50 \(\mu\)M for 3c and indomethacin. Absorption was recorded using a microplate reader at 412 nm.

Flow Cytometry. HMLER-shEcad cells were seeded in 6-well plates (at a density of 5 \(\times\) 10\(^5\) cells/ mL) and the cells were allowed to attach overnight. The cells were treated with lipopolysaccharide (LPS) (2.5 \(\mu\)M for 24 h), and then treated with 3c (0.5 \(\mu\)M), 3c NP\textsuperscript{5} (0.5 \(\mu\)M), and NM104 (10 \(\mu\)M) for 24 h.
μM), or indomethacin (20 μM), and incubated for a further 48 h. The cells were then harvested by trypsinization, fixed with 4% paraformaldehyde (at 37 °C for 10 min), permeabilized with ice-cold methanol (for 30 min), and suspended in PBS (200 μL). The Alexa Fluor® 488 nm labelled anti-COX-2 antibody (5 μL) was then added to the cell suspension and incubated in the dark for 1 hr. The cells were then washed with PBS (1 mL) and analysed using a FACSCanto II flow cytometer (BD Biosciences) (10,000 events per sample were acquired). The FL1 channel was used to assess COX-2 expression. Cell populations were analysed using the FlowJo software (Tree Star).

Nanoparticle Encapsulation. A 0.5 mL DMF solution containing 10 mg of PEG-PLGA (5000:30000 Da, 1:1 LA:GA) and an appropriate amount of 3c to give the desired feed (2.5-50%), defined as (mg of 3c /mg of polymer) × 100, was prepared. This solution was added in a dropwise manner over the course of 10 min to 5 mL of rapidly stirring ddH₂O water. The DMF solutions were added by a mechanical pipet, and the nanoprecipitations were carried out in 20 mL glass scintillation vials. The water was stirred magnetically using a 0.5 cm stir bar at approximately 1000 rpm. After addition of the DMF solution, the water acquired a milky blue coloration owing to Tyndall scattering of the nanoparticles that formed. ddH₂O then added along the edge of the vial to bring the final volume to 10 mL. This suspension of nanoparticles was stirred for an additional 20 min and then loaded into an Amicon Centrifugal Filtration Device (100 kDa MWCO regenerated cellulose membrane). The loaded device was centrifuged at 2000 g for 20 min, concentrating the nanoparticle suspension to approximately 1 mL. This concentrated material was suspended in an additional 10 mL of fresh ddH₂O water and centrifuged again under identical conditions. Each sample was washed three times in this manner. The final concentrated suspension was diluted to 1.0 mL with Milli-Q water for use in further experiments. All nanoprecipitations were carried out in duplicates. The metric used to evaluate encapsulation efficiency was the amount of copper present in the final colloidal suspension relative to the amount of polymer (loading efficiency) or 3c (encapsulation efficiency) used. The copper concentration was determined by ICP-MS (PerkinElmer NexION 350D). Results are presented as the mean of five determinations for each data point.

Nanoparticle Characterisation. Aqueous solutions of the nanoparticle formulation, 3c NP⁵ were diluted to concentrations within the linear dynamic range of the spectrometer using ddH₂O. The nanoparticle size (diameter in nm), polydispersity, and zeta-potential were recorded using a Zetasizer Nanoseries Malvern instrument. The data was processed and analysed using the Zetasizer software package.

Payload Release from Nanoparticle. To determine the amount of payload, 3c, released from the nanoparticle formulation, 3c NP⁵, the nanoparticles were incubated in PBS (pH 7.4) or sodium acetate buffer (pH 5.2) at 37 °C for 72 h. At certain time points, the nanoparticle suspension were filtered using 100 kDa MWCO centrifugal filters (Amicon; Millipore) and resuspended in fresh PBS. The copper concentration of the filtrates was measured by ICP-MS (PerkinElmer NexION 350D), giving an indication on the amount of 3c released.
Table S1. Experimentally determined log P values for 2a-3c.

<table>
<thead>
<tr>
<th>Compound</th>
<th>log P&lt;sub&gt;o/w&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>0.89</td>
</tr>
<tr>
<td>2b</td>
<td>0.97</td>
</tr>
<tr>
<td>2c</td>
<td>1.00</td>
</tr>
<tr>
<td>3a</td>
<td>0.98</td>
</tr>
<tr>
<td>3b</td>
<td>0.97</td>
</tr>
<tr>
<td>3c</td>
<td>1.01</td>
</tr>
</tbody>
</table>

**Fig. S1** UV-vis spectrum of 3c (50 μM) in PBS with HMLER-shEcad cell lysate (5 x 10<sup>3</sup> cells) over the course of 24 h at 37 °C.
**Fig. S2** UV-Vis spectrum of 3c (50 μM) in PBS in the presence of ascorbic acid (0.5 mM) over the course of 24 h at 37 °C.

**Fig. S3** UV-Vis spectrum of 3c (50 μM) in MEGM cell media over the course of 24 h at 37 °C.
Fig. S4 Representative dose-response curves for the treatment of HMLER-shEcad cells with 2a-3c after 72 h incubation.

Fig. S5 Representative dose-response curves for the treatment of HMLER cells with 2a-3c after 72 h incubation.
Table S2. IC\textsubscript{50} values of naproxen and tolfenamic acid against HMLER and HMLER-shEcad cells. \textsuperscript{[a]}Determined after 72 h incubation (mean of three independent experiments ± SD).

<table>
<thead>
<tr>
<th>Compound</th>
<th>HMLER IC\textsubscript{50} [\mu M]\textsuperscript{[a]}</th>
<th>HMLER-shEcad IC\textsubscript{50} [\mu M]\textsuperscript{[a]}</th>
</tr>
</thead>
<tbody>
<tr>
<td>naproxen</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>tolfenamic acid</td>
<td>30.77 ± 4.69</td>
<td>57.95 ± 1.62</td>
</tr>
</tbody>
</table>

Fig. S6 Representative dose-response curves for the treatment of HMLER and HMLER-shEcad cells with salinomycin after 72 h incubation.
Fig. S7 Representative dose-response curves for the treatment of HMLER and HMLER-shEcad cells with cisplatin after 72 h incubation.

Fig. S8 Representative dose-response curves for the treatment of HMLER-shEcad cells with naproxen and tolfenamic acid after 72 h incubation.
Fig. S9 Representative dose-response curves for the treatment of HMLER cells with naproxen and tolfenamic acid after 72 h incubation.

Fig. S10 Quantification of mammosphere formation with HMLER-shEcad cells untreated and treated with 2a, 2b, 3c, naproxen, tolfenamic acid, and salinomycin at their respective IC$_{20}$ values for 5 days. Error bars represent standard deviations and Student $t$ test, * = p < 0.05.
Fig. S11 Representative dose-response curves for the treatment of HMLER-shEcad mammospheres with 2a, 2b, and 3c after 5 days incubation.

Fig. S12 Representative dose-response curves for the treatment of HMLER-shEcad mammospheres with 1 after 5 days incubation.
Fig. S13 Representative dose-response curves for the treatment of HMLER-shEcad mammospheres with dichloro(1,10-phenanthroline)copper(II) after 5 days incubation.

Table S3. IC$_{50}$ values of 1, 2a, 2b, 3c, cisplatin, dichloro(1,10-phenanthroline)copper(II), and salinomycin against HMLER-shEcad mammospheres determined after 5 days incubation (mean of three independent experiments ± SD). *Data previously reported.$^5$

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mammosphere IC$_{50}$ [μM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.71 ± 0.45</td>
</tr>
<tr>
<td>2a</td>
<td>0.79 ± 0.39</td>
</tr>
<tr>
<td>2b</td>
<td>0.42 ± 0.01</td>
</tr>
<tr>
<td>3c</td>
<td>0.67 ± 0.01</td>
</tr>
<tr>
<td>cisplatin$^a$</td>
<td>8.24 ± 0.08</td>
</tr>
<tr>
<td>dichloro(1,10-phenanthroline)copper(II)</td>
<td>9.49 ± 0.20</td>
</tr>
<tr>
<td>salinomycin$^a$</td>
<td>14.05 ± 1.58</td>
</tr>
</tbody>
</table>
**Table S4.** Euclidian distances between the 3c RNAi signature and the those of dichloro(1,10-phenanthroline)copper(II) and a rhenium(V)-oxo-based necroptosis inducer.

<table>
<thead>
<tr>
<th></th>
<th>dichloro(1,10-phenanthroline)copper(II)</th>
<th>rhenium-based necroptosis inducer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euclidian distances from 3c</td>
<td>1.62</td>
<td>2.06</td>
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</tbody>
</table>

**Table S5.** Intra-category and inter-category Euclidian distances of the reference set relative to the 3c RNAi signature.

<table>
<thead>
<tr>
<th>Reference set Euclidian distance relationships</th>
<th>Intra-category</th>
<th>Inter-category</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.99 ± 0.38</td>
<td>2.69 ± 0.44</td>
</tr>
</tbody>
</table>

**Table S6.** Euclidian distance between the 3c RNAi signature and that of 1.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euclidian distances from 3c</td>
<td>2.94</td>
</tr>
</tbody>
</table>

**Fig. S14** RNAi signatures derived from the treatment of Eμ-Mycp19arf−/− lymphoma cells with a) 3c and b) 1 at the LD80–90 concentration for each compound.
Fig. S15 Normalised ROS activity in untreated HMLER-shEcad cells (control) and HMLER-shEcad cells treated with 3c (0.5 μM for 6 h), H₂O₂ (6 μM for 6 h); co-treated with 3c (0.5 μM for 6 h) and N-acetylcysteine (1.5 mM for 6 h), and H₂O₂ (6 μM for 6 h) and N-acetylcysteine (1.5 mM for 6 h). Error bars represent standard deviations and Student t test, * = p < 0.05.

Fig. S16 Normalised ROS activity in untreated HMLER-shEcad cells (control) and HMLER-shEcad cells treated with 3c (0.5 μM for 12 h), H₂O₂ (6 μM for 12 h); co-treated with 3c (0.5 μM for 12 h) and N-acetylcysteine (1.5 mM for 12 h), and H₂O₂ (6 μM for 12 h) and N-acetylcysteine (1.5 mM for 12 h). Error bars represent standard deviations and Student t test, * = p < 0.05.
**Fig. S17** Normalised ROS activity in untreated HMLER-shEcad cells (control) and HMLER-shEcad cells treated with 3c (0.5 μM for 48 h), H_2O_2 (6 μM for 48 h); co-treated with 3c (0.5 μM for 48 h) and N-acetylcysteine (1.5 mM for 48 h), and H_2O_2 (6 μM for 48 h) and N-acetylcysteine (1.5 mM for 48 h). Error bars represent standard deviations and Student *t* test, * = p < 0.05.

**Fig. S18** Immunoblotting analysis of proteins related to the p38, JNK, and apoptosis pathways. Protein expression in HMLER-shEcad cells cells following treatment with 3c (0.25 and 0.5 μM) after 72 h incubation. Whole cell lysates were resolved by SDS-PAGE and analyzed by immunoblotting against phos-p38, phos-MAPKAPK, phos-JNK, phos-c-Jun, cleaved caspase 3, cleaved caspase 7, and β-actin (loading control).
**Fig. S19** Representative dose-response curves for the treatment of HMLER-shEcad mammospheres with 3c only, and 3c in the presence of NAC (1.5 mM), LPS (2.5 μM), and PGE2 (20 μM) after 5 days incubation.

**Table S7.** IC₅₀ values of 3c against HMLER-shEcad mammospheres in the presence of NAC (1.5 mM), LPS (2.5 μM), and PGE2 (20 μM) determined after 5 days incubation (mean of three independent experiments ± SD).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mammosphere IC₅₀ [μM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>3c + NAC</td>
<td>3.13 ± 0.08</td>
</tr>
<tr>
<td>3c + LPS</td>
<td>1.68 ± 0.02</td>
</tr>
<tr>
<td>3c + PGE2</td>
<td>1.51 ± 0.02</td>
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</table>
**Fig. S20** Quantification of mammosphere formation with HMLER-shEcad cells untreated and treated with 3c (at the IC<sub>20</sub> value) only, and 3c (at the IC<sub>20</sub> value) in the presence of NAC (1.5 mM), LPS (2.5 μM), and PGE2 (20 μM) after 5 days incubation. Mammosphere formation with HMLER-shEcad cells treated with only NAC (1.5 mM), LPS (2.5 μM), and PGE2 (20 μM) after 5 days incubation is also shown. Error bars represent standard deviations and Student t test, * = p < 0.05.

**Fig. S21** Representative bright-field images (× 10) of HMLER-shEcad mammospheres in the absence and presence of 3c only (at the IC<sub>20</sub> value), NAC only (1.5 mM), LPS only (2.5 μM), PGE2 only (20 μM), 3c (at the IC<sub>20</sub> value) in the presence of NAC (1.5 mM), 3c (at the IC<sub>20</sub> value) in the presence of LPS (2.5 μM), and 3c (at the IC<sub>20</sub> value) in the presence of PGE2 (20 μM), after 5 days incubation.
Fig. S22 Cyclooxygenase-2 (COX-2) inhibitory properties of 3c (0.5-50 µM) and indomethacin (0.5-50 µM). Error bars represent standard deviations and Student t test, ** = p < 0.01.

Fig. S23 COX-2 expression in HMLER-shEcad cells pre-treated with lipopolysaccharide (LPS) (2.5 µM for 24 h), followed by treatment with 3c (0.25-0.5 µM), 3c NP5 (0.5-1 µM), or indomethacin (20 µM) after 72 h incubation. Whole cell lysates were resolved by SDS-PAGE and analysed by immunoblotting against COX-2 and β-actin (loading control).
**Fig. S24** (A) Representative histograms displaying the green fluorescence emitted by anti-COX-2 Alexa Fluor 488 nm antibody-stained HMLER-shEcad cells treated with LPS (2.5 μM) for 24 h (red) followed by 48 h in media containing 3c (0.5 μM, blue) or 3c NP5 (0.5 μM, orange) or indomethacin (20 μM, green). (B) Representative histograms displaying the green fluorescence emitted by anti-COX-2 Alexa Fluor 488 nm antibody-stained HMLER-shEcad cells treated with LPS (2.5 μM) for 24 h (red) followed by 48 h in media containing cisplatin (5 μM, blue) or dichloro(1,10-phenanthroline)copper(II) (8 μM, orange).

**Fig. S25** Graphical representation of the IC$_{50}$ values of 3c against HMLER-shEcad cells in the absence and presence of PGE2 (20 μM). Error bars represent standard deviations and Student $t$-test, * = $p < 0.05$. 
Fig S26. Dynamic light scattering size distribution of 3c NP5 suspended in water. Size refers to diameter of nanoparticles in nm.

Fig S27. Zeta potential distribution of 3c NP5 suspended in water after 12 zeta runs at 25 °C.
**Fig S28.** Variation in 3c NP⁵ diameter upon incubation in PBS with 10% FBS over the course of 72 h at 37 °C.

**Fig S29.** Copper content in HMLER-shEcad cells untreated and treated with 3c NP⁵ at 37 °C (0.5 μM for 4 h), 3c at 37 °C (0.5 μM for 4 h), and 3c NP⁵ at 4 °C (0.5 μM for 4 h). Error bars represent standard deviations and Student *t* test, * = p < 0.01.
**Fig S30.** Copper content in HMLER-shEcad cells untreated and treated with 3c NP5 only (0.5 μM for 12 h), and co-incubated with ammonium chloride (50 mM) and chloroquine (100 μM) at 37 °C. Error bars represent standard deviations and Student t test, * = p < 0.01.

**Fig S31.** Normalised ROS activity in untreated HMLER-shEcad cells (control) and HMLER-shEcad cells treated with 3c NP5 (0.5 μM for 48 h); co-treated with 3c NP5 (0.5 μM for 48 h) and N-acetylcysteine (1.5 mM for 48 h). Error bars represent standard deviations and Student t test, * = p < 0.05.
References


