Supporting Information for

A tri-metallic palladium complex with breast cancer stem cell potency

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Experimental Details

Materials and Methods. All synthetic procedures were performed under normal atmospheric conditions or under nitrogen. The infrared (IR) spectra were recorded with an IRAffinity-1S Shimadzu spectrophotometer. High resolution electron spray ionisation mass spectra were recorded on a BrukerDaltronics Esquire 3000 spectrometer by Dr. Lisa Haigh (Imperial College London). UV-Vis absorption spectra were recorded on a Cary 3500 UV-Vis spectrophotometer. ^{1}H and ^{31}P NMR spectra were recorded on a BrukerAvance 400 MHz Ultrashield NMR spectrometer. ^{1}H NMR spectra were referenced internally to residual solvent peaks, and chemical shifts are expressed relative to tetramethylsilane, $SiMe_4$ ($\delta = 0$ ppm). Elemental analysis of the compounds prepared was performed commercially by the Department of Chemical Sciences, Tezpur University (using a Perkin Elmer, 2400 Series II instrument). The palladium(II) complex, Pd(1,2-bis(diphenylphosphino)ethane)$(OSO_2CF_3)_2$, **1** was prepared according to a previously reported protocol.[1] Benzotriazole and sodium methoxide were purchased from Sigma Aldrich and used as received.

Synthesis of the tri-nuclear palladium(II) complex, 2. To a dry DMF solution (5 mL) containing benzotriazole (11.9 mg, 0.1 mmol) and sodium methoxide (5.4 mg, 0.1 mmol), a dry DMF solution (5 mL) containing **1** (80.0 mg, 0.1 mmol) was added. The resultant pale yellow mixture was stirred at 80°C for 3 h. The solution was then reduced and centrifuged. The clear centrifugate was collected and treated with diethyl ether to obtain **2** as a pale yellow solid (197 mg, 85%); ^{1}H NMR (400 MHz, DMSO-d_6) δ_H 8.36 (dd, 10H, H_{Ph}), 7.74 (t, 6H, H_{Ph}), 7.62-7.56 (m, 16H, H_{Ph}), 7.37 (t, 6H, H_{Ph}), 7.04-6.99 (m, 12H, H_{Ph}), 6.94-6.89 (m, 10H, H_{Ph}), 6.78 (dd, 6H, H_1), 6.39 (dd, 6H, H_2), 3.79-3.67 (m, 6H, H_3), 2.69-2.61 (m, 6H, H_4); ^{31}P NMR (400 MHz, DMSO-d_6) δ_P 64.14 (s, 6H); IR (solid, cm^{-1}): 1670 (C=C), 1491, 1441 (N=N), 1256 (N-N), 1222, 1144 (C-N), 1105 (C-N), 1027 (P-C), 999, 881, 814, 781, 747, 708 (C-H), 691 (P-C), 658, 636, 574, 529 (Pd-P); ESI-MS Calcd. for $C_{96}H_{84}N_9P_6Pd_3$ $[M\text{-}3SO_3CF_3]^{3+}$: 622.5955 a.m.u. Found $[M\text{-}3SO_3CF_3]^{3+}$: 622.7510 a.m.u., Calcd. for $C_{97}H_{84}F_3N_9O_3P_6Pt_3S$ $[M\text{-}2SO_3CF_3]^{2+}$: 1008.9578 a.m.u. Found $[M\text{-}2SO_3CF_3]^{2+}$: 1009.1423; Anal. Calcd. for $C_{99}H_{84}F_9N_9O_9P_6Pt_3S_3 \bullet 3DMF \bullet H_2O$: C, 50.80; H, 4.22; N, 6.58. Found: C, 51.03; H, 4.35; N, 6.76 (attempt 1); Anal. Calcd. for $C_{99}H_{84}F_9N_9O_9P_6Pt_3S_3 \bullet 3DMF$: C, 51.16; H, 4.17; N, 6.63. Found: C, 51.35; H, 4.35; N, 6.43 (attempt 2).

X-ray Single Crystal Diffraction Analysis. Standard procedures were used to mount the crystal on a Gemini diffractometer with graphite-monochromated Mo Kα radiation (λ = 0.71073 Å) at 293 K. The crystal structure was solved using direct methods in SHELXS and refined by full-matrix least-squares routines, based on F^2, using the SHELXL program.[2] All the H atoms were placed in geometrically idealised positions and constrained to ride on their parent atoms. The structure has been deposited with the Cambridge Crystallographic Data Centre (CCDC 1906804). This information can be obtained free of charge from www.ccdc.cam.ac.uk/data_request/cif.

Measurement of water-octanol partition coefficient (LogP). The LogP value for **2** 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 **2** (500 µL, 100 µM) was incubated with octanol (500 µL) in a 1.5 mL tube. The tube was shaken at room temperature for 24 h. The two phases were separated by centrifugation and the **2** content in each phase was determined by UV-Vis spectroscopy.

Cell Lines and Cell Culture Conditions. HEK 293T embryonic kidney cells were acquired from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% penicillin and 10% fetal bovine serum. The BEAS-2B bronchial epithelium cell line was acquired from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI 1640 medium with 2 mM L-glutamine supplemented with 1% penicillin and 10% fetal bovine serum. The human mammary epithelial cell lines, HMLER and HMLER-shEcad were kindly donated by Prof. R. A. Weinberg (Whitehead Institute, MIT). The human epithelial breast MCF10A cell line was acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA). HMLER, HMLER-shEcad, and MCF10A 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% CO_2.

Cytotoxicity MTT assay. The colourimetric MTT assay was used to determine the toxicity of **1**, **2**, and $PdCl_2$. HMLER, HMLER-shEcad, and MCF10A cells (5×10^3) were seeded in each well of a 96-well plate. After incubating the cells overnight, various concentrations of the compounds (0.2-100 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. 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, 20 μL of a 4 mg/mL solution of MTT in PBS was added to each well, and the plate was incubated for an additional 4 h. The MEGM/MTT mixture was aspirated and 100 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 versus % cell viability. IC_{50} values were extrapolated from the resulting dose dependent curves. The reported IC_{50} values are the average of three independent experiments, each consisting of six replicates per concentration level (overall n = 18).

Tumorsphere Formation and Viability Assay. HMLER-shEcad cells (5×10^3) 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 **2** and salinomycin (0-133 μM). Mammospheres treated with **2** and salinomycin (at their respective IC_{20} 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 (λ_{ex} = 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_{50} values were extrapolated from the resulting dose dependent curves. The reported IC_{50} values are the average of two independent experiments, each consisting of three replicates per concentration level (overall n = 6).

Cellular Uptake. To measure the cellular uptake of **2**, *ca.* 1 million HMLER-shEcad cells were treated with **2** (2 μM) at 37 ºC for 24 h. After incubation, the media was removed and the cells were washed with PBS (2 mL × 3), and harvested. The number of cells was counted

at this stage, using a haemocytometer. This mitigates any cell death induced by **2** at the administered concentration and experimental cell loss. The cells were centrifuged to form pellets. The cellular pellets were dissolved in 65% HNO_3 (250 μL) overnight. The cellular pellets were also used to determine the palladium content in the cytoplasmic, nuclear, and membrane fractions. The Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Kit was used to extract and separate the cytoplasmic, nuclear, and membrane fractions. The fractions were dissolved in 65% HNO_3 overnight (250 μL final volume). All samples were diluted 5-fold with water and analysed using inductively coupled plasma mass spectrometry (ICP-MS, PerkinElmer NexION 350D). Palladium levels are expressed as Pd (ppb) per million cells. Results are presented as the mean of five determinations for each data point.

Immunoblotting Analysis. HMLER-shEcad cells (1×10^6 cells) were incubated with **2** (at various concentrations, 5, 10, and 20 μM) for 72 h at 37 ^oC. Cells were washed with PBS, scraped into SDS-PAGE loading buffer (64 mM Tris-HCl (pH6.8)/ 9.6% glycerol/ 2%SDS/ 5% β-mercaptoethanol/ 0.01% Bromophenol Blue), and incubated at 95 ^oC for 10 min. Whole cell lysates were resolved by 4-20 % sodium dodecylsulphate polyacylamide 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 analysed using a chemiluminescence imager (Amersham Imager 600).

ct-DNA precipitation assay. A solution of ct-DNA (0.25 mM, nucleotide) was incubated with **1**, **2**, or $PdCl_2$ at a base pair:palladium ratio of 5:1 at 37 ºC in PBS at 37 ^oC. After 4 h incubation, a 250 μL aliquot was removed and the DNA was precipitated by adding 10 μL of KCl (5 M) and 1 mL of cold EtOH (pre-stored at 4 °C). The solution was centrifuged at 4 °C to isolate the DNA. The isolated DNA samples were dissolved in 65% HNO_3 overnight (250 μL final volume). All samples were diluted 5-fold with water and analysed using inductively coupled plasma mass spectrometry (ICP-MS, PerkinElmer NexION 350D). Palladium levels were expressed as Pd (ppm), and corresponded to the concentration of covalently bound **1**, **2**, or $PdCl_2$ on DNA. Results are presented as the mean of four determinations for each data point.

Ethidium Bromide Displacement Studies. To a mixture of ethidium bromide (1 μM) and ct-DNA (20 μM) in 5 mM Tris–HCl (pH 7.4) buffer (with a total volume of 2 mL) an increasing amount of the palladium(II) complex, **2** (0-10 μM) was added in aliquots, from a 10 mM stock solution of the palladium(II) complex, **2**. The solution was incubated at room temperature for 30 seconds after each aliquot addition of the palladium(II) complex, **2** and then the emission spectrum was recorded between 550 and 800 nm with an excitation wavelength of 526 nm. The fluorescence intensity at λ_{max} was used to determine the quenching constant of the palladium(II) complex, **2**. The fluorescence studies were performed on a Varian Cary Eclipse spectrometer.

The quenching constant (K_q) was determined using the Stern-Volmer equation: $F^o/F = K_q[Q] + 1$, where F^o is the emission intensity of ct-DNA and ethidium bromide in the absence of the palladium(II) complex, **2**, F is the emission intensity in the presence of palladium(II) complex, **2**, K_q is the quenching constant, and [Q] is the concentration of the palladium(II) complex, **2**. The quenching constant was extrapolated from F^o/F versus [Q] plots. The

experiment was conducted three times, independently. The reported quenching constant is the average of the three values obtained from the three independent experiments.

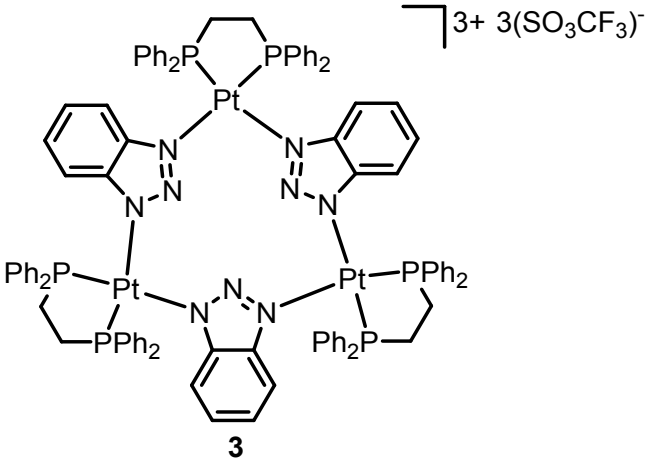

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Fig. S1 Chemical structure of the previously reported tri-nuclear platinum(II) complex, **3**.

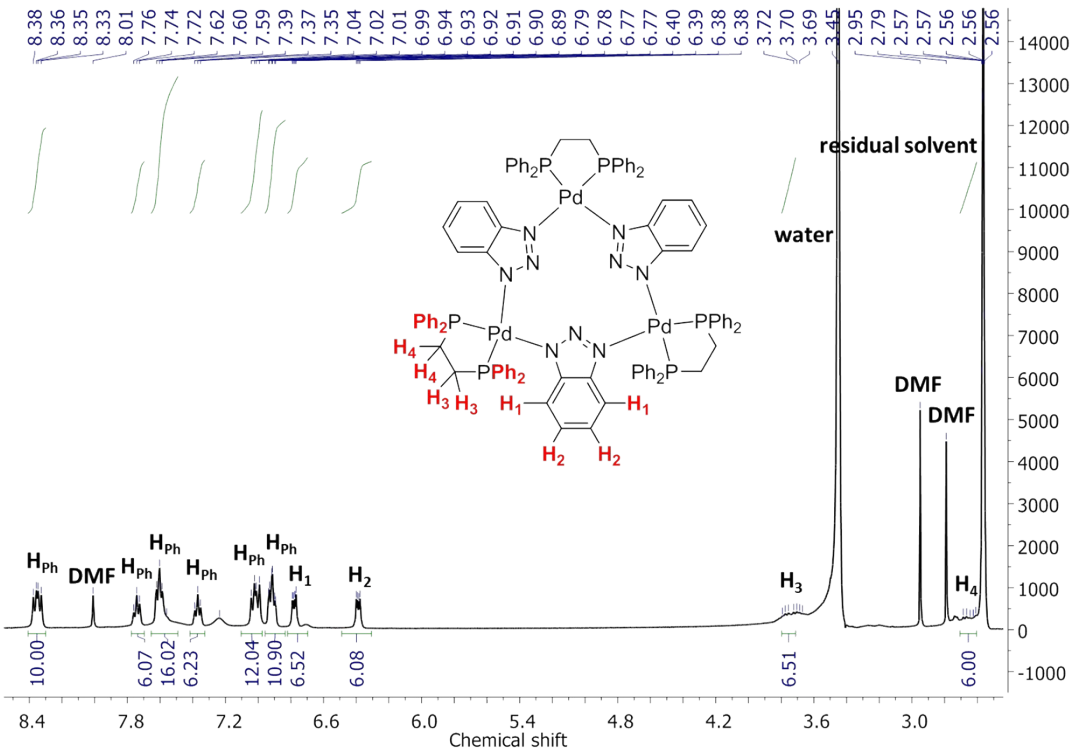

Fig. S2 ^{1}H NMR spectrum of **2** in DMSO-d_6.

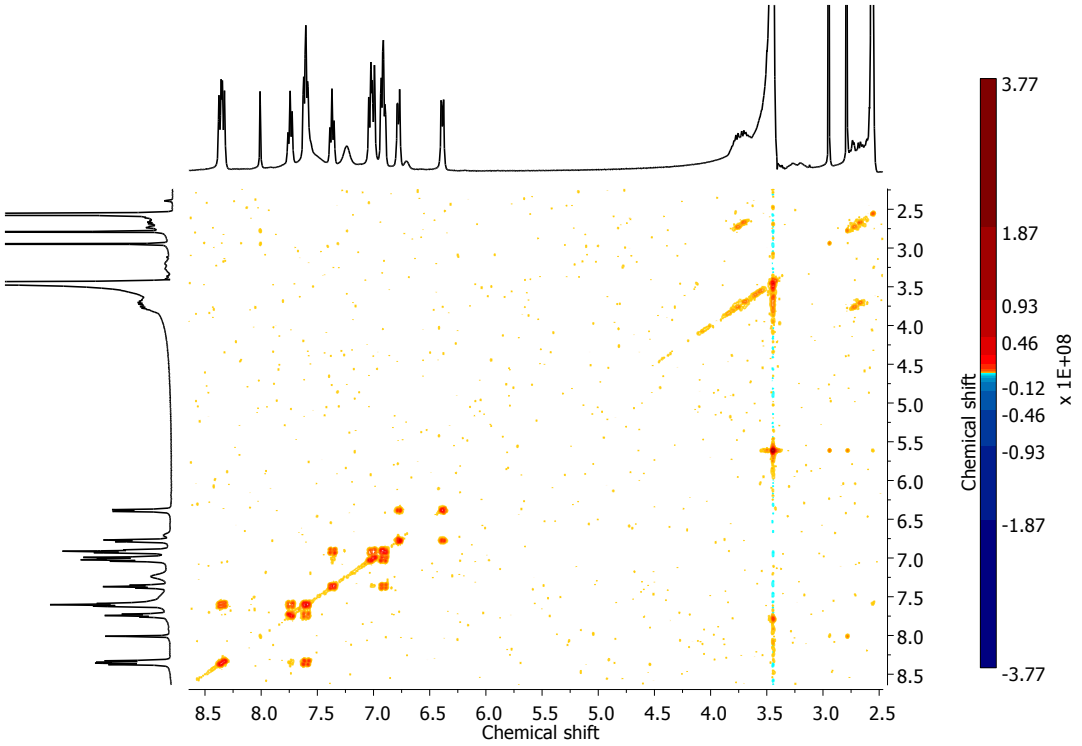

Fig. S3 ^{1}H-^{1}H COSY NMR spectrum of **2** in DMSO-d_6.

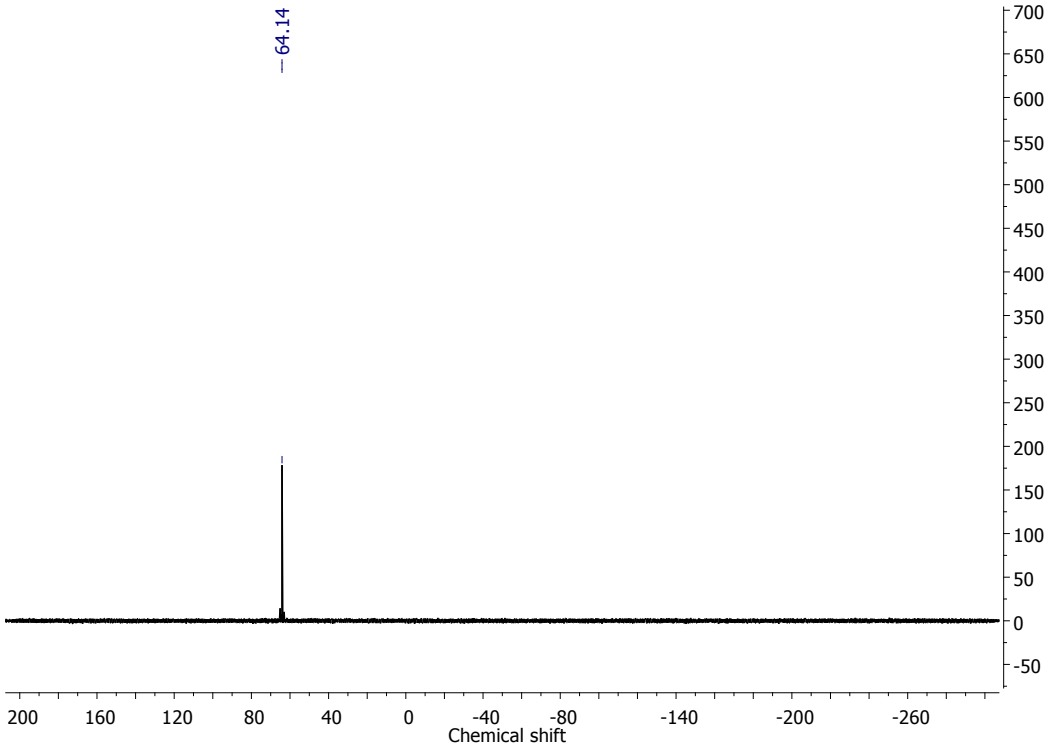

Fig. S4 ^{31}P NMR spectrum of **2** in DMSO-d_6.

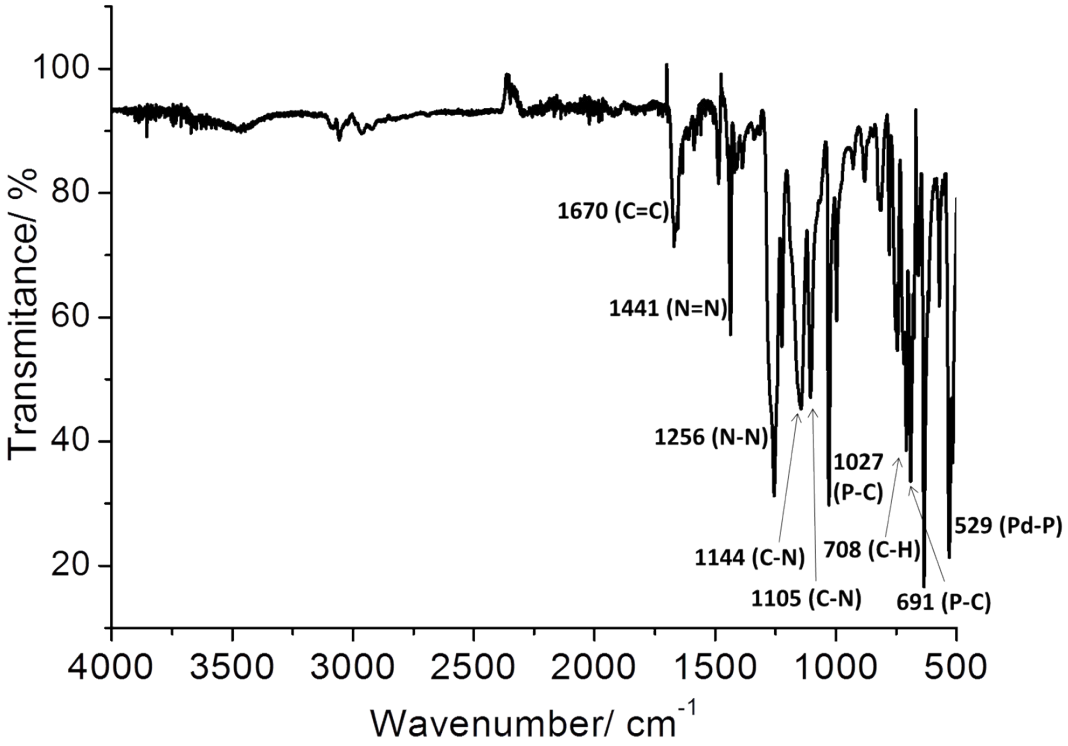

Fig. S5 IR spectrum of **2** in the solid form.

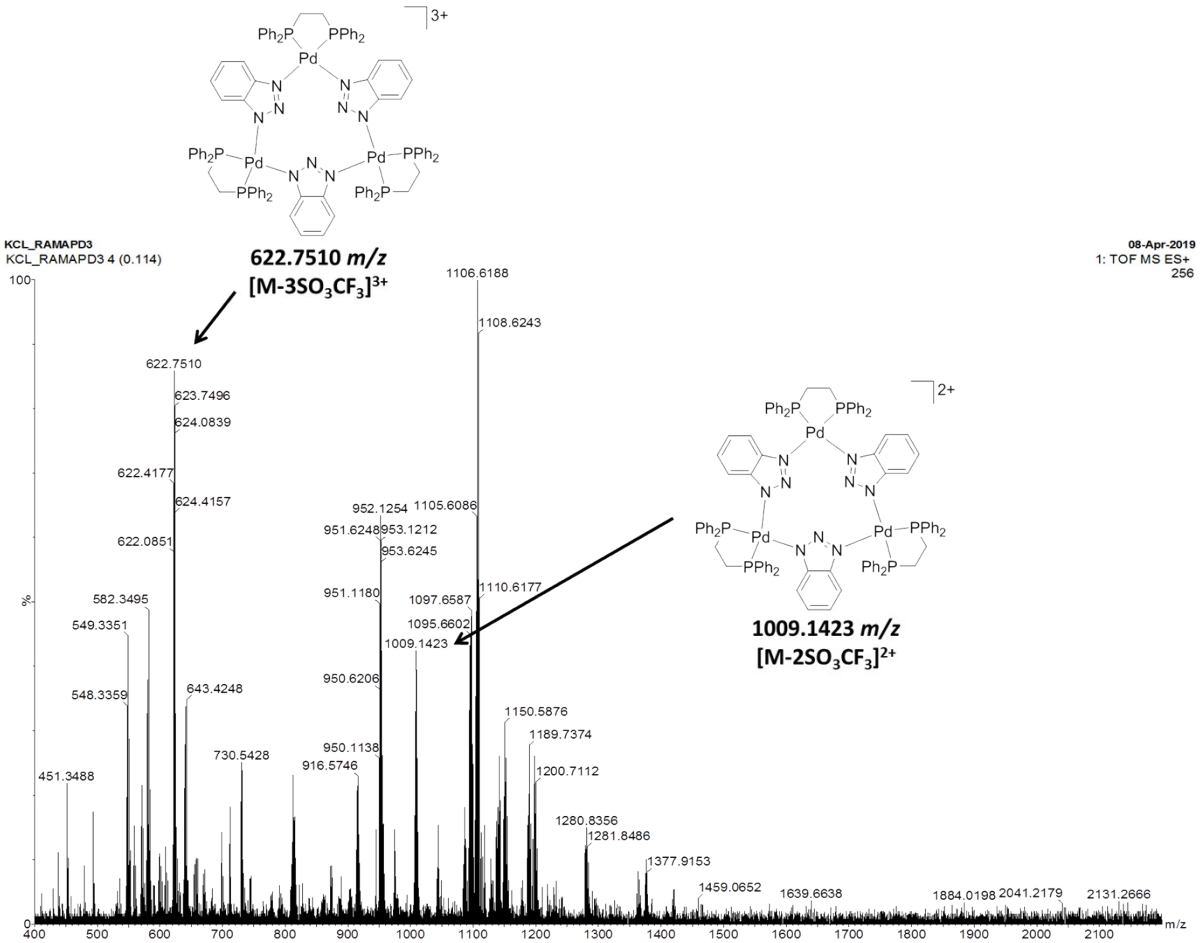

Fig. S6 High resolution ESI mass spectrum (positive mode) of **2**.

Table S1. Crystallographic data of **2**•3DMF•H_2O.

Identification code	**2** (CCDC 1906804)
Empirical formula	$C_{108}H_{107}F_9N_{12}O_{13}P_6Pd_3S_3$
Formula weight	2553.25
Temperature	296(2) K
Wavelength	0.71073 Å
Crystal system	Monoclinic
Space group	P 21/n
Unit cell dimensions	a = 14.9213(14) Å
	b = 28.264(2) Å
	c = 29.056(3) Å
	α = 90°
	β = 102.555(5)°
	γ = 90°
Volume	11960.9(19) $Å^3$
Z	4
Density (calculated)	1.418 Mg/m^3
Absorption coefficient	0.654 mm^{-1}
F(000)	5200
Crystal size	0.150 x 0.100 x 0.100 mm^3
Theta range for data collection	2.278 to 26.000°.
Index ranges	-18<=h<=18, -34<=k<=34, -29<=l<=35
Reflections collected	92748
Independent reflections	23457 [R(int) = 0.1008]
Completeness to theta = 25.242°	99.7 %
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	23457 / 1819 / 1566
Goodness-of-fit on F^2	1.061
Final R indices [I>2sigma(I)]	R1 = 0.0822, wR2 = 0.1983
R indices (all data)	R1 = 0.1329, wR2 = 0.2284
Largest diff. peak and hole	2.692 and -0.910 $e.Å^{-3}$

$R_1 = [\Sigma \, || F_0| - |F_c||/\Sigma|F_0|]$, $wR_2 = [\Sigma w(F_0^2 - F_c^2)^2/\Sigma w(F_0^2)^2]^{1/2}$

Table S2. Selected bond lengths (Å) and angles (°) for **2**•3DMF•H_2O.

N(1)-Pd(1)	2.091(6)	N(3)-Pd(2)	2.083(6)
N(4)-Pd(2)	2.088(6)	N(6)-Pd(3)	2.090(6)
N(7)-Pd(3)	2.098(6)	N(9)-Pd(1)	2.085(6)
P(1)-Pd(1)	2.248(2)	P(2)-Pd(1)	2.268(2)
P(3)-Pd(2)	2.2487(19)	P(4)-Pd(2)	2.258(2)
P(5)-Pd(3)	2.245(2)	P(6)-Pd(3)	2.257(2)
N(2)-N(1)-Pd(1)	118.4(5)	C(79)-N(1)-Pd(1)	132.2(5)
N(2)-N(3)-Pd(2)	120.1(4)	C(84)-N(3)-Pd(2)	129.8(5)
N(5)-N(4)-Pd(2)	124.0(5)	C(85)-N(4)-Pd(2)	125.5(5)
N(5)-N(6)-Pd(3)	125.3(5)	C(90)-N(6)-Pd(3)	123.0(5)
N(8)-N(7)-Pd(3)	113.5(5)	C(91)-N(7)-Pd(3)	137.2(6)
N(8)-N(9)-Pd(1)	120.9(5)	C(96)-N(9)-Pd(1)	129.5(5)
C(7)-P(1)-Pd(1)	116.2(4)	C(1)-P(1)-Pd(1)	110.6(3)
C(13)-P(1)-Pd(1)	106.7(3)	C(15)-P(2)-Pd(1)	112.3(3)
C(21)-P(2)-Pd(1)	115.2(3)	C(14)-P(2)-Pd(1)	108.3(3)
C(33)-P(3)-Pd(2)	114.2(3)	C(27)-P(3)-Pd(2)	116.3(3)
C(40)-P(3)-Pd(2)	105.5(3)	C(47)-P(4)-Pd(2)	112.6(3)
C(41)-P(4)-Pd(2)	113.6(3)	C(39)-P(4)-Pd(2)	110.0(3)
C(59)-P(5)-Pd(3)	105.0(4)	C(53)-P(5)-Pd(3)	122.9(3)
C(65)-P(5)-Pd(3)	108.4(3)	C(67)-P(6)-Pd(3)	119.8(3)
C(73)-P(6)-Pd(3)	108.8(3)	C(66)-P(6)-Pd(3)	109.6(4)
N(9)-Pd(1)-N(1)	87.1(2)	N(9)-Pd(1)-P(1)	92.58(19)
N(1)-Pd(1)-P(1)	177.57(18)	N(9)-Pd(1)-P(2)	177.90(19)
N(1)-Pd(1)-P(2)	94.96(18)	P(1)-Pd(1)-P(2)	85.32(8)
N(3)-Pd(2)-N(4)	86.7(2)	N(3)-Pd(2)-P(3)	96.53(17)
N(4)-Pd(2)-P(3)	174.28(18)	N(3)-Pd(2)-P(4)	178.30(18)
N(4)-Pd(2)-P(4)	93.22(17)	P(3)-Pd(2)-P(4)	83.42(7)
N(6)-Pd(3)-N(7)	86.6(3)	N(6)-Pd(3)-P(5)	95.04(19)
N(7)-Pd(3)-P(5)	171.57(19)	N(6)-Pd(3)-P(6)	170.7(2)
N(7)-Pd(3)-P(6)	95.52(18)	P(5)-Pd(3)-P(6)	84.18(8)

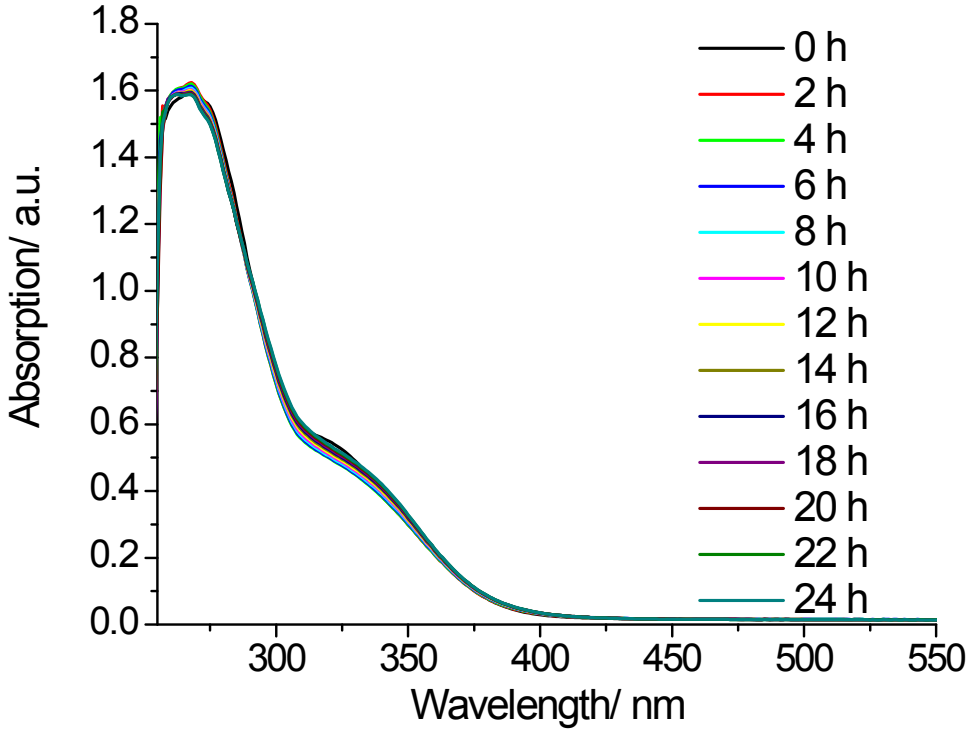

Fig. S7 UV-Vis spectrum of **2** in DMSO over the course of 24 h at 37 °C.

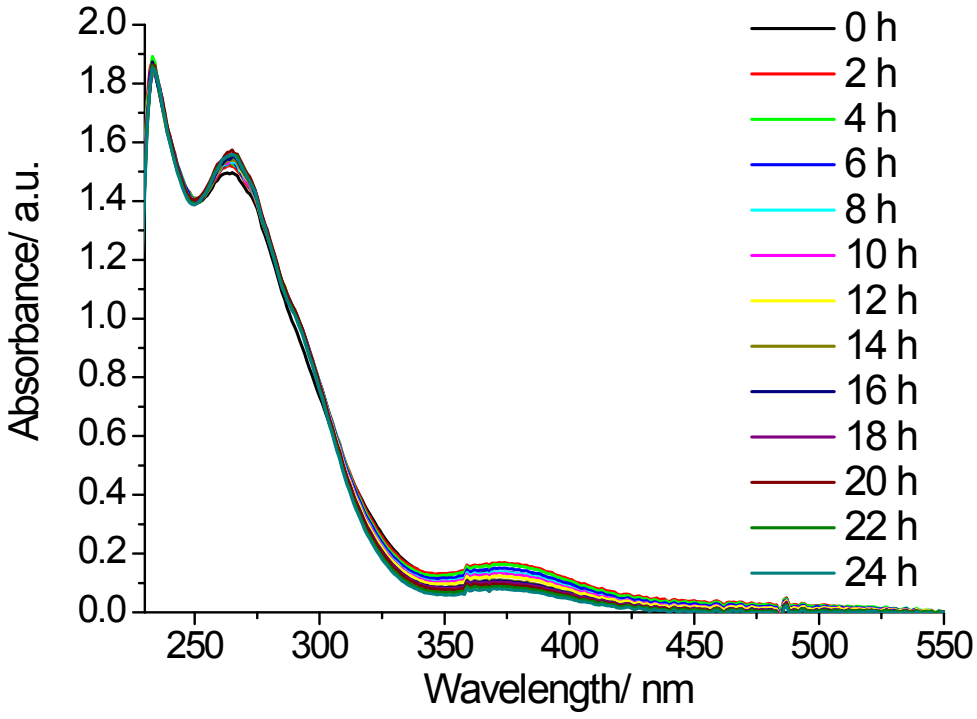

Fig. S8 UV-Vis spectrum of **2** in mammary epithelial cell growth medium (MEGM):DMSO (200:1) over the course of 24 h at 37 °C.

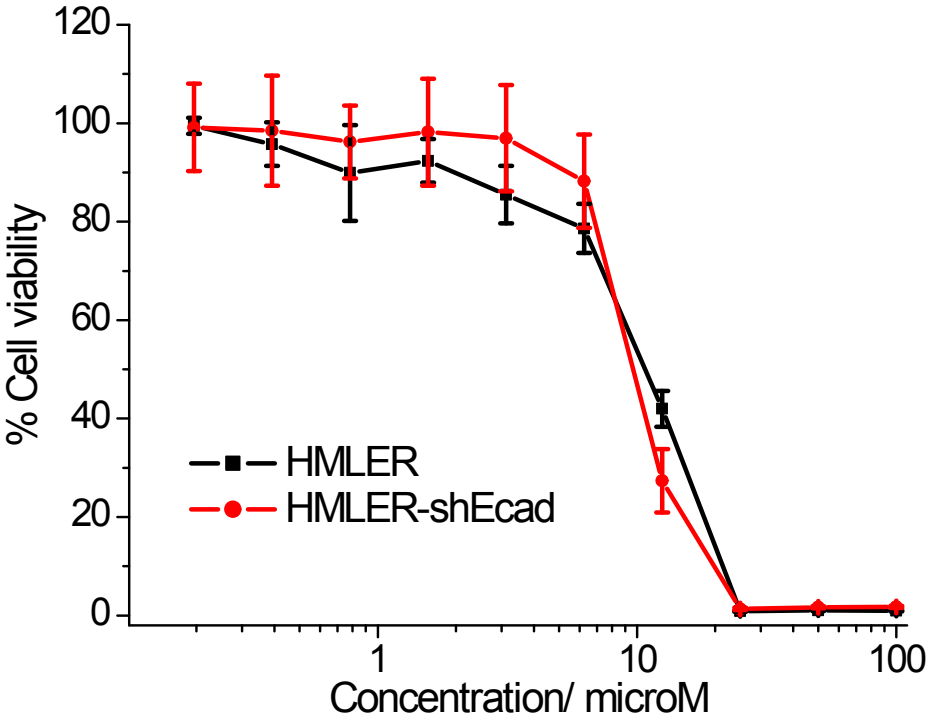

Fig. S9 Representative dose-response curves for the treatment of HMLER and HMLER-shEcad cells with **2** after 72 h incubation.

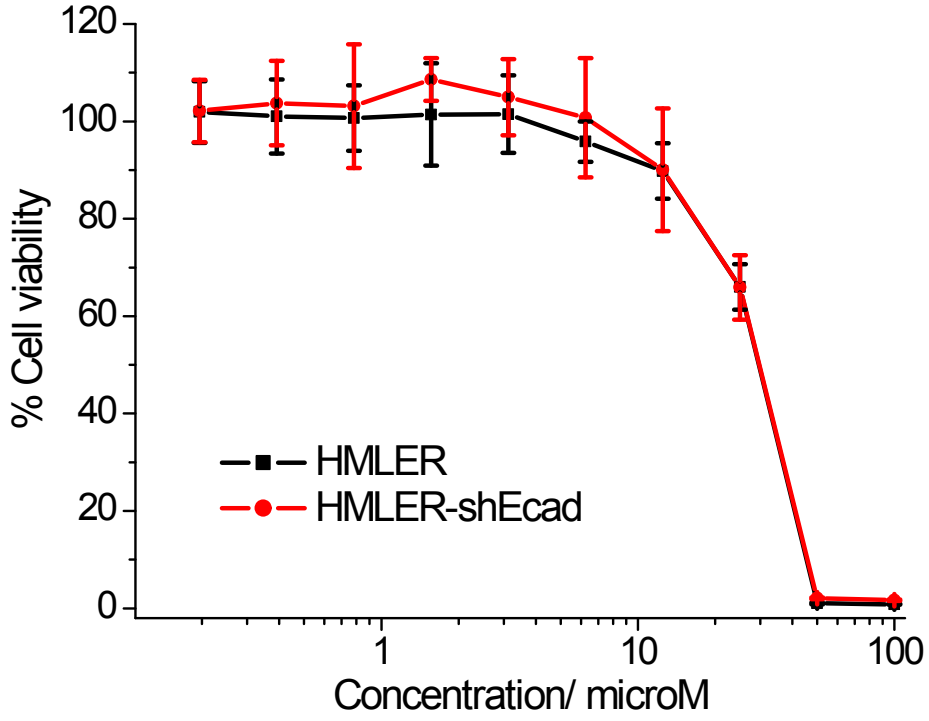

Fig. S10 Representative dose-response curves for the treatment of HMLER and HMLER-shEcad cells with **1** after 72 h incubation.

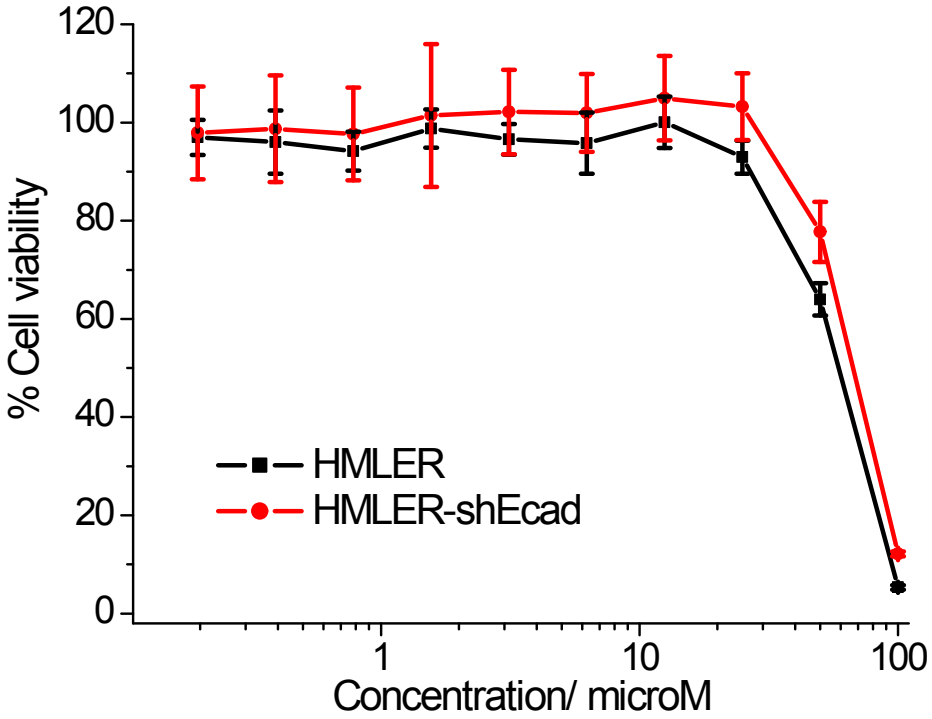

Fig. S11 Representative dose-response curves for the treatment of HMLER and HMLER-shEcad cells with $PdCl_2$ after 72 h incubation.

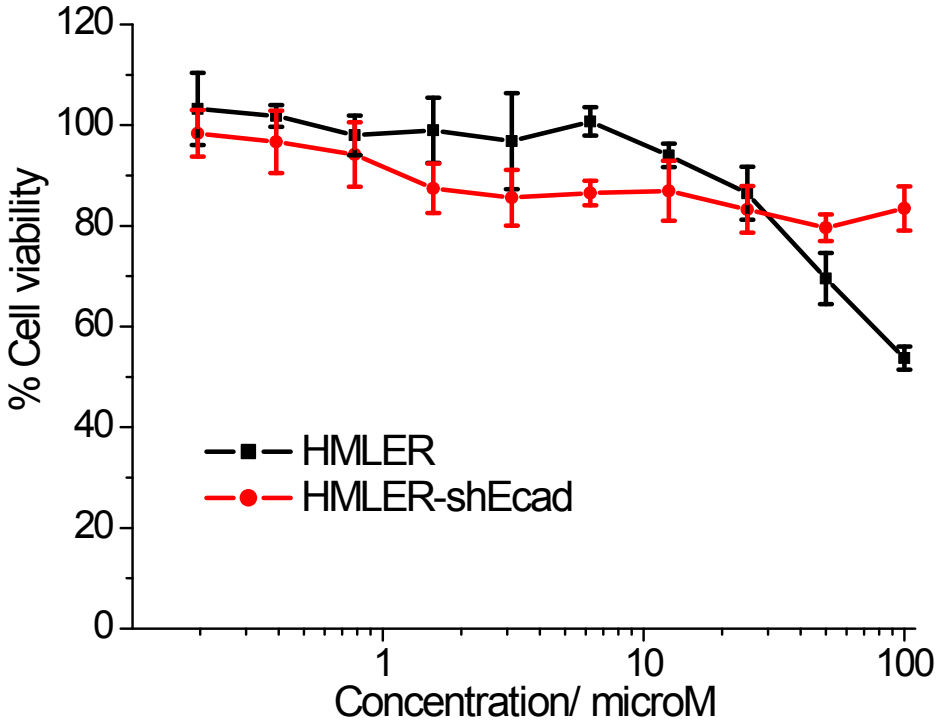

Fig. S12 Representative dose-response curves for the treatment of HMLER and HMLER-shEcad cells with benzotriazole after 72 h incubation.

Table S3. IC_{50} values of benzotriazole, cisplatin, and carboplatin against HMLER cells, HMLER-shEcad cells, and HMLER-shEcad mammospheres after 3 or 5 days incubation (mean of three independent experiments ± SD). n.d. not determined. [a] Reported in reference [3].

Compound	HMLER IC_{50} [μM]	HMLER-shEcad IC_{50} [μM]	HMLER-shEcad mammosphere IC_{50} [μM]
benzotriazole	> 100	> 100	n.d.
cisplatin[a]	2.57 ± 0.02	5.65 ± 0.30	13.50 ± 2.34
carboplatin[a]	66.11 ± 0.50	72.59 ± 0.09	18.06 ± 0.40

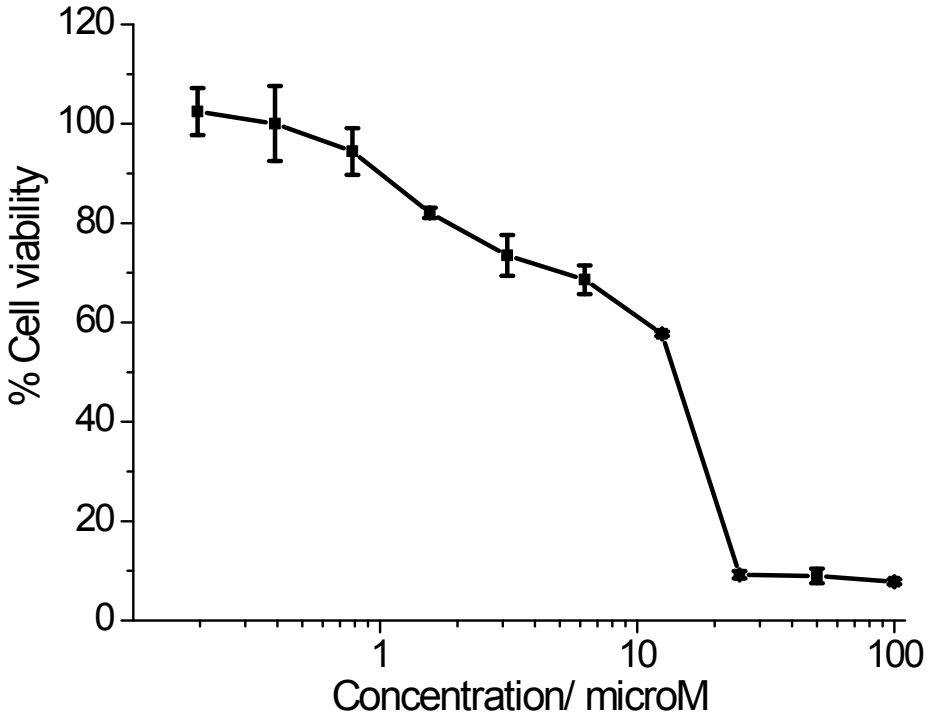

Fig. S13 Representative dose-response curve for the treatment of MCF10A cells with **2** after 72 h incubation.

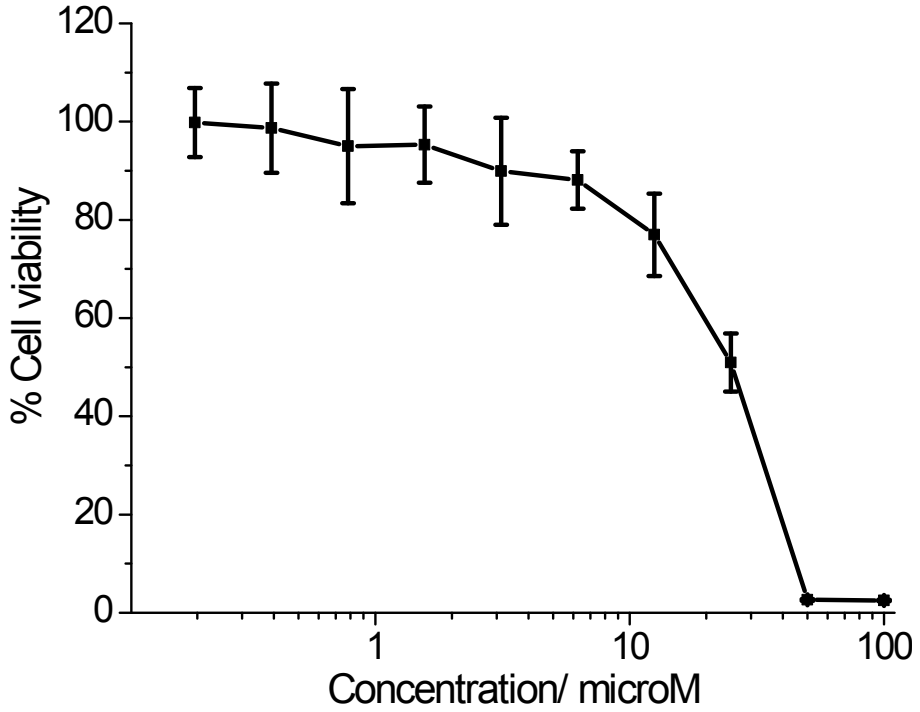

Fig. S14 Representative dose-response curve for the treatment of BEAS-2B cells with **2** after 72 h incubation.

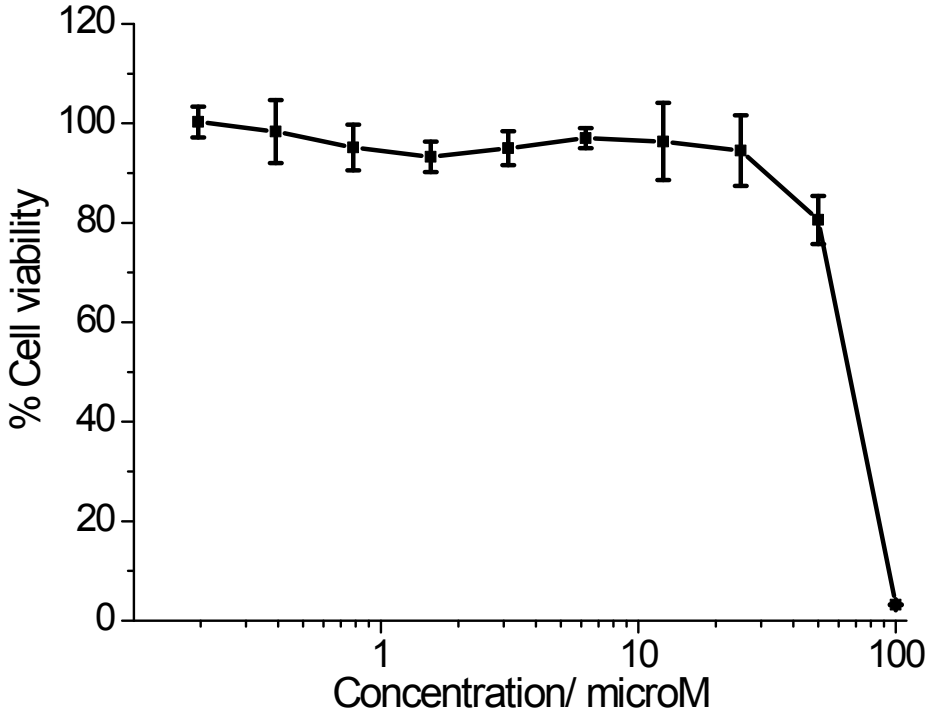

Fig. S15 Representative dose-response curve for the treatment of HEK 293T cells with **2** after 72 h incubation.

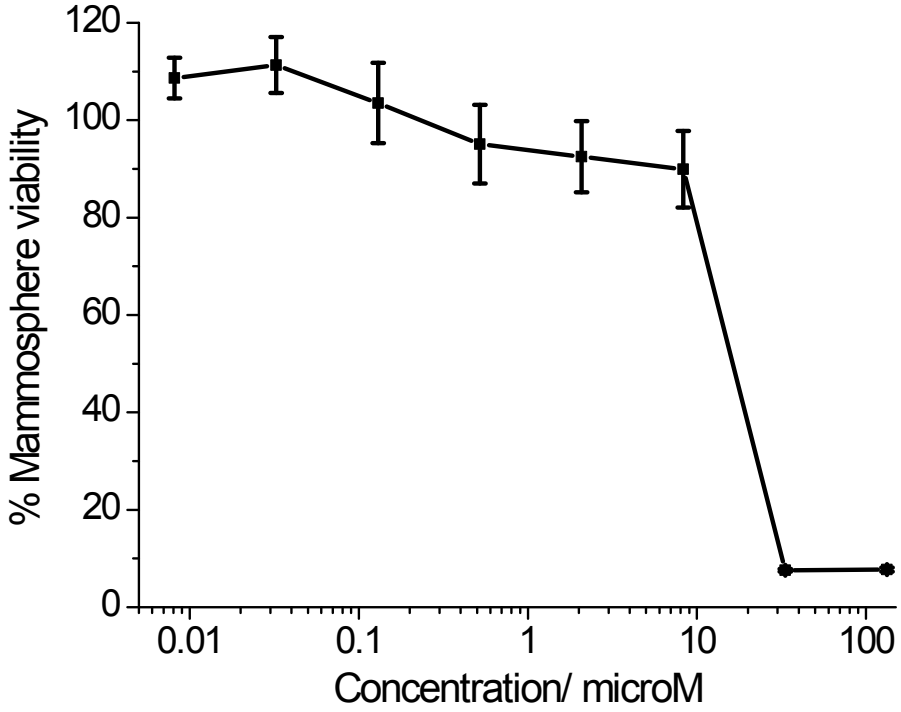

Fig. S16 Representative dose-response curve for the treatment of HMLER-shEcad mammospheres with **2** after 5 days incubation.

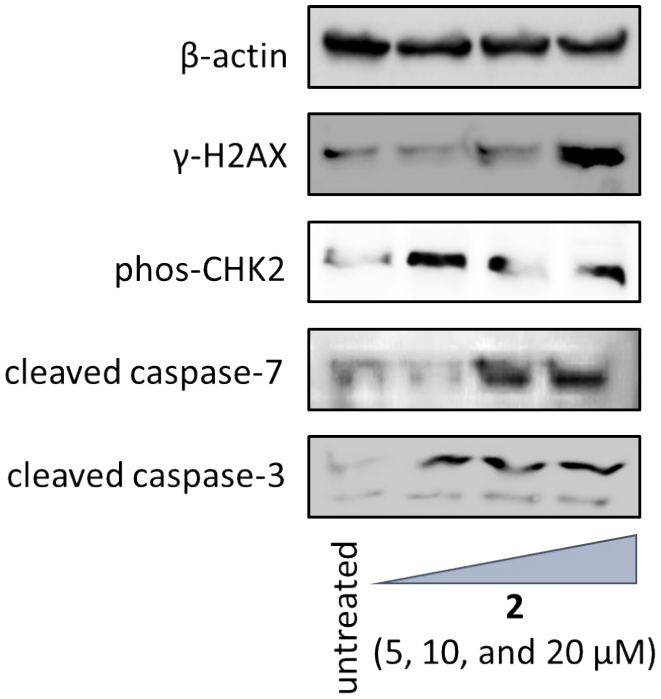

Fig. S17 Immunoblotting analysis of proteins related to the DNA damage and apoptosis pathway. Protein expression in HMLER-shEcad cells following treatment with **2** (5, 10 and 20 μM for 72 h).

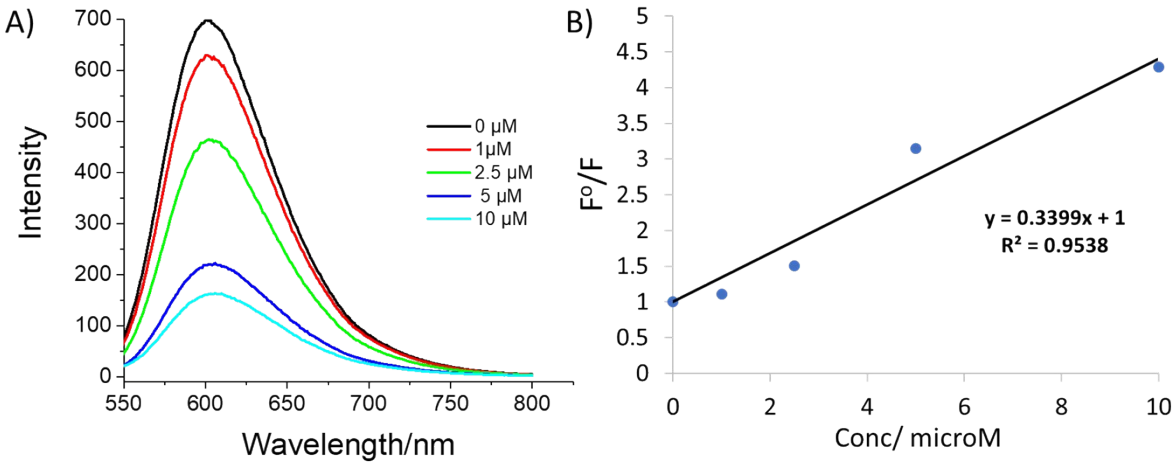

Fig. S18 (A) Representative emission spectrum for ethidium bromide (1 µM) bound to ct-DNA (1:20 ratio) upon addition of aliquots of **2** and (B) the corresponding F^o/F versus [Q] plots for **2**.

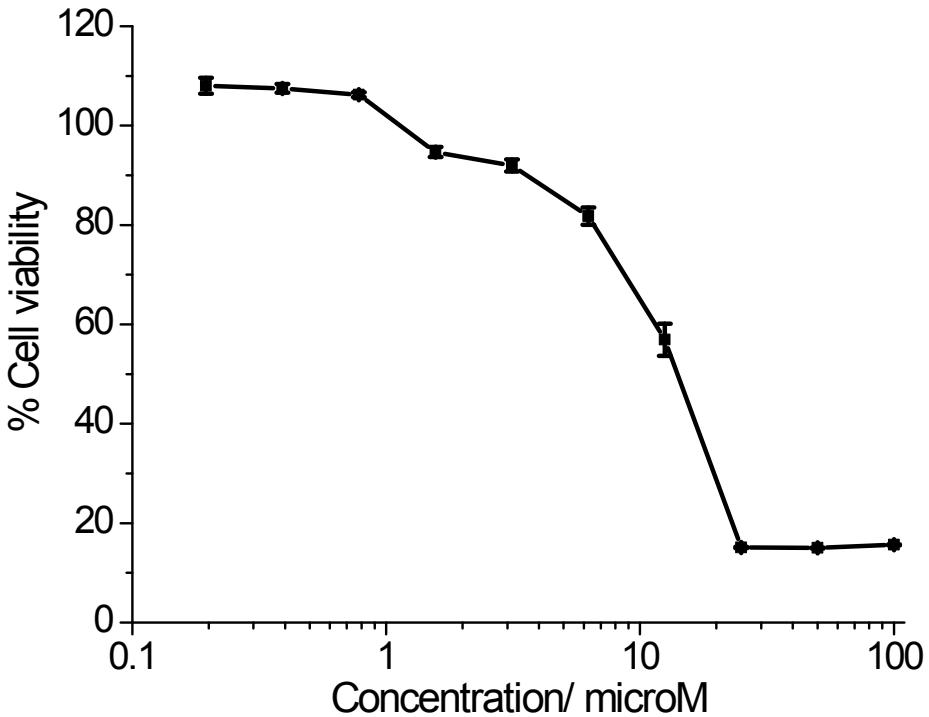

Fig. S19 Representative dose-response curve of **2** against HMLER-shEcad cells in the presence of z-VAD-FMK (5 µM) after 72 h incubation.

References

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