SUPPLEMENTARY INFORMATION

Study of lipid particle-encapsulated N-heterocyclic carbene platinum complexes on cancer cells and cancer stem cells as a potential breakthrough in glioblastoma therapy

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I. MATERIALS AND METHODS

1.1. Synthesis of ligand precursors and platinum derivatives

1.1.1. Proligand synthesis

1.1.1.1. Proligand: 3-(((3*r*,5*r*,7*r*)-adamantan-1-yl)methyl)-1-(3-(triphenylphosphonio)propyl)-1*H*-imidazol-3-ium bromide

A mixture of 1-(adamantan-1-yl) methylimidazole (474.6 mg, 2.19 mmol, 1.5 equiv.) (prepared following the reported procedure [1]) and (3-bromopropyl) triphenylphosphonium bromide (678.9 mg, 1.46 mmol, 1 equiv.) were heated at 120 °C in dry C_6H_5Cl (20 mL) under argon for 16 h. The solvent was subsequently removed in vacuo. After the crude residue had dried, it was washed with Et_2O (3 x 20 mL) using an ultrasonic bath, resulting in the formation of a white solid aggregate. Then, the obtained white powder was then dried under vacuum (0.992 g, 99 %). Spectroscopy data were found to be consistent with those reported in existing literature [2]. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.39 – 1.47 (m, 6H, H_{Ad}), 1.54 (d, *J* = 12.5 Hz, 3H, H_{Ad}), 1.68 (d, *J* = 12.4 Hz, 3H, H_{Ad}), 2.21 (s, 3H, H_{Ad}), 2.55 (q, *J* = 7.9 Hz, 2H, N-CH_2-$\underline{CH_2}$-CH_2-PPh_3), 3.62 – 3.77 (m, 2H, N-CH_2-CH_2-$\underline{CH_2}$-PPh_3), 3.80 (s, 2H, N-$\underline{CH_2}$-Ad), 5.06 (t, *J* = 7.4 Hz, 2H, N-$\underline{CH_2}$-CH_2-CH_2-PPh_3), 6.92 (t, *J* = 1.8 Hz, 1H, H_{imid}), 7.64 – 7.91 (m, 15H, H_{PPh3}), 8.63 (t, *J* = 1.8 Hz, 1H, H_{imid}), 10.21 (t, *J* = 1.7 Hz, 1H, $H_{imid(N=CH=N)}$) ppm.

1.1.1.2. Proligand: 1-methyl-3-(4-nitrobenzyl)-1 *H*-imidazol-3-ium bromide

A mixture of methylimidazole (300 mg, 3.65 mmol, 1 equiv.) and 1-(bromomethyl)-4-nitrobenzene (947.2 mg, 4.38 mmol, 1.2 equiv.) was heated at 75 °C in dry acetonitrile (5 mL) under argon overnight. The solvent was subsequently removed in vacuo. After the crude residue had dried, it was washed with Et_2O (3 x 20 mL) with an ultrasonic bath resulting in a white-yellowish aggregate. The desired product was purified by column chromatography on silica gel (gradient dichloromethane/methanol 90/10). The obtained white-yellowish powder was dried under vacuum (1.08 g, 99%). Spectroscopy data were found to be consistent with those reported in existing literature [3]. ^{1}H NMR (300 MHz, D_2O) δ 3.97 (s, 3H, N-CH_3), 5.60 (s, 2H, N-CH_2), 7.45 – 7.67 (m, 4H, H_{arom}), 8.28 (dt, *J* = 8.0, 3.0 Hz, 2H, 2H_{imid}).

1.1.1.3. Proligand: 3-(((3*r*,5*r*,7*r*)-adamantan-1-yl)methyl)-1-methyl-1 *H*-imidazol-3-ium iodide

A mixture of 1-(adamantan-1-yl) methylimidazole (453 mg, 2.09 mmol, 1 equiv.) and iodomethane (13.04 mL, 209.4 mmol, 100 equiv.) was refluxed under argon for 36 h. The

solvent was removed in vacuo. Once the crude residue was dry, it was washed with Et_2O (3 x 15 mL) thanks to an ultrasonic bath affording a white aggregate. The obtained white powder was dried under vacuum (665.2 mg, 89%). Spectroscopy data were found to be consistent with those reported in existing literature [4]. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.45 – 1.78 (m, 12H, H_{Ad}), 2.03 (s, 3H, H_{Ad}), 3.99 (s, 2H, N-CH_2), 4.16 (s, 3H, N-CH_3), 7.14 (s, 1H, H_{imid}), 7.35 (s, 1H, H_{imid}), 10.17 (s, 1H, H_{imid}).

1.1.2. NHC-Pt(II) complexes synthesis

1.1.2.1. Complex 3

During the first step of the reaction, a mixture of the proligand **1** (100 mg, 146.95 µmol, 1 equiv.), $PtCl_2(SMe_2)_2$ (68.82 mg, 176.34 µmol, 1.2 equiv.), KI (243.94 mg, 1.47 mmol, 10 equiv.) and K_2CO_3 (203.1 mg, 1.47 mmol, 10 equiv.) was heated at 40 °C in dry $(CH_3)_2CO$ (5 mL) under argon overnight. Subsequently, the solvent was removed in vacuo, resulting in a yellowish solid, which was then dissolved in DCM and filtered through celite to eliminate the salts. After filtration, DCM was evaporated. For the second step, 5 mL of extra-dry pyridine was added under argon and the reaction was stirred at room temperature overnight. The pyridine was subsequently removed in vacuo. The desired product underwent purification using the CombiFlash® Rf+ apparatus using RediSep disposable chromatography columns (gradient dichloromethane/methanol 100/0 → 95/5). The resulting yellow powder was dried under vacuum (128.5 mg, 74.4%). Spectroscopy data were found to be consistent with those reported in existing literature [2]. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.62 – 1.77 (m, 12H, H_{Ad}), 2.02 (s, 3H, H_{Ad}), 2.74 (s, 2H, N-CH_2-$\underline{CH_2}$-CH_2-PPh_3), 3.82 (s, 2H, N-CH_2-CH_2-$\underline{CH_2}$-PPh_3), 4.17 (s, 2H, N-$\underline{CH_2}$-Ad), 4.96 (s, 2H, N-$\underline{CH_2}$-CH_2-CH_2-PPh_3), 6.86 (s, 1H, H_{imid}), 7.27 – 7.35 (m, 2H, H_{pyr}), 7.61 – 8.00 (m, 17H, H_{arom}), 8.80 (d, *J* = 4.8 Hz, 2H, H_{pyr}) ppm.

1.1.2.2. Complex 5 – C-H insertion complex

A mixture of the proligand (50 mg, 80.34 µmol, 1 equiv.), $PtCl_2$ (21.37 mg, 80.34 µmol, 1 equiv.), KI (133.36 mg, 0.8 mmol, 10 equiv.) and K_2CO_3 (111.03 mg, 0.8 mmol, 10 equiv.) was heated at 110 °C in extra-dry (5 mL) under argon overnight. Subsequently, the solvent was removed in vacuo, resulting in a yellowish solid, which was then dissolved in DCM and filtered through celite to eliminate the salts. The DCM was evaporated. The desired product was purified by colum chromatography on silica gel (gradient chloroform/methanol 100/0 → 95/5). The resulting yellow powder was dried under vacuum and recrystallized using a dichloromethane/pentane mix (v/v; 1/2) (25 mg, 34.3%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.09

(t, *J* = 11.6 Hz, 2H, N-CH_2-$\underline{CH_2}$-CH-PPh_3), 3.21 (ddd, *J* = 10.7, 8.1, 3.9 Hz, 1H, N-CH_2-CH_2-$\underline{CH}$-PPh_3), 3.54 (dd, *J* = 13.3, 4.9 Hz, 2H, N-$\underline{CH_2}$-CH_2-CH-PPh_3), 5.81 (d, *J* = 5.0 Hz, 2H, H_{arom}), 6.54 (d, *J* = 1.8 Hz, 1H, Pt-CH-CH-P), 6.61 (d, *J* = 1.9 Hz, 1H, Pt-CH-CH-P), 6.92 – 7.01 (m, 2H, H_{arom}), 7.25 (s, 2H, N-$\underline{CH_2}$-Ph), 7.27 – 7.31 (m, 1H, H_{imid}), 7.40 – 7.45 (m, 3H, H_{arom}), 7.55 – 7.65 (m, 3H, H_{arom}), 7.68 – 7.76 (m, 4H, H_{arom}), 7.91 (ddd, *J* = 11.7, 8.3, 1.3 Hz, 3H, H_{arom}), 8.97 – 9.00 (m, 1H, H_{imid}) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) δ 26.49 (d, *J* = 1.5 Hz, N-CH_2-CH_2-$\underline{CH}$-PPh_3), 51.71 (d, *J* = 4.8 Hz, N-CH_2-$\underline{CH_2}$-CH-PPh_3), 53.57 (N-$\underline{CH_2}$-CH_2-CH-PPh_3), 56.38 (2x$CH_{Pt\text{-}\underline{Ph}\text{-}P}$), 120.03 ($CH_{Pt\text{-}\underline{Ph}\text{-}P}$), 120.16 ($CH_{Pt\text{-}\underline{Ph}\text{-}P}$), 123.39 (d, *J* = 13.2 Hz, CH_{Ph}), 126.08 (d, *J* = 85.0 Hz, 3xC_{Ph}), 127.70 (CH_{2Bn}), 128.64 (d, *J* = 9.0 Hz, CH_{Ph}), 128.92 (d, *J* = 11.6 Hz, CH_{Ph}), 129.67 (d, *J* = 10.0 Hz, CH_{Ph}), 130.93 (CH_{imid}), 132.76 (d, *J* = 49.5 Hz, CH_{Ph}), 133.12 (d, *J* = 8.3 Hz, CH_{Ph}), 134.60 (d, *J* = 8.9 Hz, CH_{Ph}), 137.60 (C_{NHC}-Pt), 144.01 (CH_{imid}), 171.70 (C_{Bn}), 176.36 (C_{Ph}-Pt) ppm. ^{31}P NMR (202 MHz, $CDCl_3$) δ 30.47 ppm. HRMS ESI: calculated for $C_{31}H_{28}N_2P^{195}Pt$ 654.1638, found 654.1634. ν (ATR): 908.82 (s), 997.91 (w), 1106.81 (s), 1454.79 (m), 1570.00 (m), 2068.02 (w), 3029.1 (w).

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1.1.2.3. Complex 2

A mixture of the proligand (50 mg, 167.7 µmol, 1 equiv.), $PtCl_2(SMe_2)_2$ (44.6 mg, 167.7 µmol, 1 equiv.), KI (278.4 mg, 1.68 mmol, 10 equiv.) and K_2CO_3 (231.8 mg, 1.468 mmol, 10 equiv.) was heated at 110 °C in extra-dry pyridine (5 mL) under argon overnight. Subsequently, the solvent was removed in vacuo. The resulting brownish solid was solubilized in DCM and then filtered through celite in order to remove the salts. The DCM was evaporated after the filtration. The desired product was purified using the CombiFlash® Rf+ apparatus using RediSep disposable chromatography columns (gradient dichloromethane/methanol 100/0 → 85/15). The obtained brown powder was dried under vacuum (120 mg, 96%). Spectroscopy data were found to be consistent with those reported in existing literature [5]. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.02 (s, 3H, N-CH_3), 5.86 (s, 2H, N-CH_2), 6.67 (d, *J* = 2.1 Hz, 1H, H_{imid}), 6.92 (d, *J* = 2.2 Hz, 1H, H_{imid}), 7.27 – 7.39 (m, 2H, H_{arom}), 7.54 – 7.84 (m, 3H, H_{arom}), 8.16 – 8.31 (m, 2H, H_{pyr}), 9.00 (dt, *J* = 5.1, 1.6 Hz, 2H, H_{pyr}) ppm.

1.1.2.4. Complex 1

A mixture of the proligand (103 mg, 330.9 µmol, 1 equiv.), $PtCl_2(SMe_2)_2$ (105.6 mg, 397.1 µmol, 1.2 equiv.), KI (549.3 mg, 3.31 mmol, 10 equiv.) and K_2CO_3 (457.3 mg, 3.31 mmol, 10 equiv.) was heated at 110 °C in extra-dry pyridine (5 mL) under argon overnight. Subsequently, the solvent was removed in vacuo. The resulting yellowish solid was solubilized in DCM and

then filtered through celite in order to remove the salts. The DCM was evaporated after the filtration. The desired product was purified using the CombiFlash® Rf+ apparatus using RediSep disposable chromatography columns (gradient cyclohexane/ethyl acetate 90/10 → 80/20). The obtained yellow powder was dried under vacuum (80 mg, 31.9%). Spectroscopy data were found to be consistent with those reported in existing literature [1]. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.62 – 1.83 (m, 12H, H_{Ad}), 1.98 – 2.09 (m, 3H, H_{Ad}), 4.01 (s, 3H, N-CH_3), 4.21 (s, 2H, N-CH_2), 6.86 (dq, *J* = 4.4, 2.2 Hz, 2H, H_{imid}), 7.27 – 7.38 (m, 2H, H_{pyr}), 7.66 – 7.77 (m, 1H, H_{pyr}), 8.92 – 9.11 (m, 2H, H_{pyr}).

1.2. Preparation of platinum derivatives for *in vitro* assays

The purity of all Pt complexes was validated by elemental analysis prior to the biological studies. NHC-Pt(II) complexes were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Quentin Fallavier, France) at a concentration of 5 mM. Oxaliplatin (Sigma-Aldrich, St. Quentin Fallavier, France) was dissolved in ultrapure water at a concentration of 5 mM platinum. The solutions can be stored at 4 °C for several weeks, with no change in activity.

1.3. Lipophilicity determination (LogP)

The octanol-water partition coefficient (LogP) was determined based on Baron *et al.* 2014 [6] using the shake-flask method. Stock solutions of the compounds were prepared in PBS buffer (Dulbecco's phosphate-buffered saline, Sigma-Aldrich, St. Quentin Fallavier, France) and n-octanol (Alfa-Aesar, Lancashire, UK) 1:1 v:v at a concentration of 1 mM. The solutions were stirred for 24 h at room temperature and then equilibrated for 24 h also at room temperature. The concentrations of the complexes in the organic and aqueous phases were determined using the UV-VIS-NIR Spectrometer (Perkin Elmer Lambda 950, Massachusetts, USA). LogP is defined as the logarithm of the ratio of the concentrations of the complex in the organic and aqueous phases ($\log P = \log \frac{[Concentration]octanol}{[Concentration]PBS} = \log \frac{(OD)octanol}{(OD)PBS}$)

1.4. Liposome formulation

Liposomes were prepared by thin lipid film hydration followed by sonication. Lipids used for formulation are egg yolk L-α-Phosphatidylcholine (PC) (Sigma-Aldrich, St. Quentin Fallavier, France) and egg yolk L-α-Phosphatidyl-DL-glycerol (PG) (Avanti Polar lipids, Alabama, USA). Cholesterol (Chol) was recrystallized in methanol before its use (Sigma-Aldrich, St. Quentin Fallavier, France). Negatively charged liposomes were formulated with PC:PG:Chol in 53.3:13.3:33.3 molar ratio, respectively [7]. Briefly, based on Célia Jacoberger-Foissac *et al*. 2019

[8], the corresponding amount of each stored lipid and cholesterol solution (lipids and cholesterol were solubilized in chloroform/methanol, 9/1, v/v) for a final phospholipid concentration of 10 mM and the necessary volume of the compound to be encapsulated (dissolved in dichloromethane) for a final concentration of 0.5 mM were mixed in a round-bottom Pyrex tube. The solution was dried up under high vacuum for 1 hour. The resulting lipid film was then hydrated in an aqueous buffer (10 mM Hepes Pufferan (> 99.5 % Buffer grade, Carl Roth, Karlsruhe, Germany), 5% D-Sorbitol (Sigma-Aldrich, St. Quentin Fallavier, France), pH = 7.4 and filtered through a 0.22 µm filter) and vortexed until its complete homogenization leading to the formation of Multi Lamellar Vesicles (MLVs). This suspension was then sonicated, for 1 second cycle every 3 seconds, for 1 hour at room temperature under a continuous flow of argon with a Vibra Cell 75041 ultrasonicator (750 W, 20 kHz, Fisher Bioblock Scientific) equipped with a 3 mm-diameter tip probe set at 40% amplitude (sonication strength). The suspension was kept cooled in a water bath to avoid heat generated during sonication. The resulting Small Unilamellar Vesicles (SUVs) preparation was centrifuged at 10 000 g for 10 minutes to remove the titanium dust coming from the sonication probe. The different formulations were kept frozen by adding 4% D-glucose (Fluka) w/v, then by quickly freezing in liquid nitrogen and stored at -80 °C. After thawing, liposomes could be stored at 4 °C under argon up to 7 to 10 days.

1.5. Lipid nanocapsules (LNCs) formulation

Lipid nanocapsules (LNCs) were formulated as described by Heurtault *et al.* 2002 [9] using a phase inversion method of an oil-water system. First, the to be encapsulated compound was solubilized in dichloromethane and the corresponding volume to achieve a final concentration of 1 mM was transferred to a formulation vial to be dried up under high vacuum. Then, 85 mg of Solutol HS15® (BASF, Ludwigshafen, Germany), 7.5 mg of Lipoid® S75 (Lipoïd GMBH, Ludwigshafen, Germany), 103 mg of Labrafac® (Gattefossé, Saint-Priest, France) and 300 µl of the aqueous buffer (80 mM Hepes Pufferan (> 99.5 % Buffer grade, Sigma-Aldrich, St. Quentin Fallavier, France), 150 mM NaCl (Sigma-Aldrich, , St. Quentin Fallavier, France) in Milli-Q water, pH = 7.4 and filtered through a 0.2 µm filter) were mixed under magnetic stirring until everything was homogeneous. Next, three temperature cycles of heating up to 85 °C and cooling down to 60°C were done also under magnetic stirring. During the cooling down process, there is an inversion phase where the solution passes from “water in oil” to “oil in water” passing through a distinguishable phase inversion zone that can be observed with the

naked eye. In the final cooling cycle, an irreversible shock was induced at the beginning of the phase inversion zone by diluting with 1.2 ml of cold buffer (close to 0 °C). The resulting LNC suspension was stirred for 5 minutes at room temperature before further use. LNCs were stored at 4 °C for 1 month maximum.

1.6. Nanoparticle diameter measurement by dynamic light scattering

The average diameter of the formulated liposomes and lipid nanocapsules was measured by dynamic light scattering using the Zetasizer Nano-ZS (Malvern Panalytical, Orsay, France). The following parameters were used: sampling time, 120 seconds; viscosity, 0.8309 cP; refractive index, 1.43; scattering angle 90°; temperature, 25 °C. Liposomes and LNCs were diluted 100 times in their respective formulation buffer. The results were the average of three consecutive measurements. The parameters were expressed in intensity. These measurements represent the distribution of the scattered light intensity as a function of particle size. The samples were considered monodispersed when the PDI (polydispersity index) was below 0.300.

1.7. Zeta potential of liposomes by electrophoretic light scattering

The zeta potential of all formulated liposomes was measured using a Zetasizer Nano-ZS (Malvern Panalytical, Orsay, France). The following parameters were used: sampling time, 120 seconds; viscosity, 0.8309 cP; refractive index, 1.43; scattering angle 90°; temperature, 25 °C. Liposomes were diluted at 1/10 in their formulation buffer. The results were the average of three consecutive measurements. The zeta potential was calculated from the electrophoretic mobility based on the Smoluchowski approximation.

1.8. Cell culture

1.8.1. NCH421K cells

The Pr. Christel Herold-Mende (Division of Neurosurgical Research, Department of Neurosurgery, University of Heidelberg, Germany) provided the NCH421K glioblastoma stem cells (GSCs) [10]. GSCs were grown as spheroids in DMEM-F12 (Dulbecco's Modified Eagle Medium, PAN-Biotech, Aidenbach, Germany) supplemented with 20% of BIT100 (Bovine Serum Albumin, Insulin, Transferrin) (Provitro, Berlin, Germany), 20 ng/mL of Fibroblast Growth Factor (FGF, Reliatech, Wolfenbüttel, Germany), 20 ng/mL of Epidermal Growth Factor (EGF, Reliatech, Wolfenbüttel, Germany), 100 U/mL of penicillin and 0,1 mg/mL of streptomycin (Sigma-Aldrich, St. Quentin Fallavier, France) at 37 °C, 95% humidity and 5% CO_2. Spheroid cultures were maintained by passaging once a week by recuperating the spheroids,

PBS washing, and accutase (Sigma-Aldrich, St. Quentin Fallavier, France) treatment for 5 minutes at room temperature. Then, after a single wash, dissociated cells were resuspended in culture medium and counted.

1.8.2. U87-MG TMZ-Sensitive cells and HCT116

The U87-MG TMZ-Sensitive cells (obtained from D. Monique Dontenwill's laboratory, UMR7021, Strasbourg, France) come from a cell line, with epithelial morphology, isolated from a malignant human glioblastoma. Temozolomide (TMZ) is toxic at 50 µM to these cells. Human colon cancer cells HCT116 were purchased from ATCC®. Both cell lines are adherent cells and were grown in RPMI-1640 medium (Roswell Park Memorial Institute, Sigma-Aldrich, St. Quentin Fallavier, France) supplemented with 10% Fetal Bovine Serum (FBS, Gibco, Brazil), 100 U/mL of penicillin and 0.1 mg/mL of streptomycin (Sigma-Aldrich, St. Quentin Fallavier, France) at 37 °C, 95% humidity and 5% CO_2. They were maintained by passaging at least two times a week by washing with PBS and trypsin (Sigma-Aldrich, St. Quentin Fallavier, France) treatment for 5 minutes at 37 °C. Then, after a single wash, cells were resuspended in culture medium and counted.

1.8.3. U87-MG, U251-MG and GL261

U87-MG, U251-MG and GL261 are glioblastoma differentiated cell lines. They were obtained from Dr. Emmanuel Garcion's laboratory, Inserm U1232 CRCINA équipe 17 (GLIAD), Angers, France. U87-MG is a human glioblastoma cell line derived from a malignant glioma from a female patient by explant technique. U251-MG is a human glioblastoma cell line derived from a malignant glioblastoma tumor by explant technique. GL261 is a murine glioblastoma cell line established in the mid 1990s derived from glioblastoma induced tumor by the intracranial injection of 3-methylcholanthrene into C57BL/6 mice. They are adherent cells and were cultivated in DMEM-High Glucose (Dulbecco's Modified Eagle Medium, Sigma-Aldrich, St. Quentin Fallavier, France) supplemented with 10% Fetal Bovine Serum, 100 U/mL of penicillin and 0.1 mg/mL of streptomycin at 37 °C, 95% humidity and 5% CO_2. They were maintained by passaging between two to three times a week by washing with PBS, followed by treatment with trypsin for 5 minutes at 37 °C. Cells were then washed once, resuspended in culture medium, and counted.

1.9. Cell viability assays

1.9.1. Non-adherent cells (NCH421K)

96-well flat solid bottom plates (Greiner Bio-One, Monroe, USA) were seeded with 7 500 NCH421K cells per well in 100 µl of their specific culture media. They were incubated for 4 days at 37 °C, 95% humidity and 5% CO_2 to allow them to form spheres before any treatment.

To achieve the necessary concentrations for the cell viability assay, 10 µl of each compound diluted in NCH421K culture medium, 11 times more concentrated (going from 550 µM to 2,15 µM), were added to each well to reach the targeted concentration (50 µM to 195 nM). The cells, in the presence of the compounds, were incubated for 24 h at 37 °C, 95% humidity and 5% CO_2.

1.9.2. Adherent cells (U87-MG TMZ sensitive, U87-MG, U251-MG, GL261 and HCT116 cells)

96-well flat solid bottom plates were seeded with 18 000 U87-MG TMZ-Sensitive, U87-MG, U251-MG, GL261 or HCT116 cells per well in 100 µl of their specific culture media. They were incubated for 24h at 37 °C, 95% humidity and 5% CO_2 to allow the cells to adhere and acquire their spindle shape before any treatment. The old medium was removed and replaced with 100 µl of fresh culture medium containing the compound under evaluation at the targeted concentration (going from 50 µM to 195 nM). The cells, in the presence of the compounds, were incubated for 24 h at 37 °C, 95% humidity and 5% CO_2.

1.10. Revelation – Cell Titer-Glo® 3D

Cell Titer-Glo® 3D (Promega, Madison, USA) reagent, was employed v/v to assess cell viability based on ATP quantification, which serves as an indicator of metabolically active cells. Cells were incubated in the dark for 25 minutes at room temperature. Spectrofluorometer (SAFAS Monaco SP2000, Genius 5801) was used to read the bioluminescence. To calculate cell viability, the bioluminescence of blank wells (containing only the culture medium and Cell Titer-Glo® 3D without cells) was subtracted from each well value. The bioluminescence of untreated cells was considered as 100% viable. Thus, viability was determined using the following formula: % viable cells = $\frac{OD\ (treated)}{OD\ (non-treated)} * 100$. Half maximal inhibitory concentration (IC_{50}) values were calculated using GraphPad Prism 8 software through a non-linear regression analysis.

1.11. Inductively coupled plasma mass spectrometry (ICP-MS)

1.11.1. Encapsulation efficiency (EE) and drug loading (DL)

For sample preparation Vivaspin® Turbo 4 membrane 100 000 MWCO (Sartorius, Göttingen, Germany) were hydrated with 500 µl of 80 mM HEPES and 150 mM NaCl buffer for LNCs or with 500 µl of 10 mM HEPES and 5% D-sorbitol for liposomes and centrifuged for 20 minutes at 0.3 rcf. Then, 300 µl of the LNCs or liposomes (containing the encapsulated complexes) were added in the Vivaspin® and centrifuged for 30 minutes or 10 minutes, respectively at 0.3 rcf. The eluates are recovered which contain the non-encapsulated complex for analysis. Also, some of the stock solutions, where LNCs and liposomes were previously dissolved using 0.1% Triton X100 (Acros Organics, Fair Lawn, USA) [11], were used as controls for analysis which contain the encapsulated and non-encapsulated compound. Analysis was done by the Analytical Platform for Inorganics at the Ecole européenne d'ingénieurs de Chimie, Polymères et Matériaux (ECPM) in Strasbourg. the mass of encapsulated complex was evaluated directly from loaded liposomes. The encapsulation efficiency (EE) was calculated using the equation : $EE\ \% = \frac{mass\ of\ encapsulated\ complex}{mass\ of\ encapsulated\ complex + mass\ of\ non\ encapsulated\ complex} * 100$. The drug loading (DL) was calculated using the equation : $DL\ \% = \frac{mass\ of\ encapsulated\ complex}{mass\ of\ the\ system\ comprising\ the\ complex} * 100$.

1.11.2. Platinum uptake by HCT116 cells

Sample preparation was executed based on Chekkat *et al.* 2016 [12]. $1x10^6$ cells HCT116 cells were seeded in a T75 flask (SPL Life Sciences, Geumgang-ro, Korea) and incubated for 3 days at 37 °C, 95% humidity and 5% CO_2. When cells were at approximately 80% of confluence and at a concentration of roughly $10x10^{10}$ cells/mL the compounds tested were added, diluted with the corresponding medium, at a concentration of 5 µM in the flask and incubated for 4 h or 6 h in their non encapsulated and encapsulated version (1 flask per condition) at 37 °C, 95% humidity and 5% CO_2. Afterwards, the medium was recovered. The cells were washed with PBS, trypsinized and collected by centrifugation (0.3 rcf for 5 minutes). ICP-MS measurements were performed by the Analytical Platform for Inorganics at the Ecole européenne d'ingénieurs de Chimie, Polymères et Matériaux (ECPM) (Strasbourg, France) on the cell pellets and the supernatants to assess the quantity of platinum present. Concomitantly, a

small amount of the cell pellets of each sample were lysed with a lysis buffer (0.6% Triton X100, 1 mM dithiothreitol (DTT) (Sigma-Aldrich, St. Quentin Fallavier, France), 1 mM EDTA (Alfa Aesar, Lancashire, UK), 15% glycerol (Sigma-Aldrich, St. Quentin Fallavier, France), 8 mM $MgCl_2$ (Sigma-Aldrich, St. Quentin Fallavier, France), and 25 mM Tris-phosphate (Sigma-Aldrich, St. Quentin Fallavier, France) buffer pH 7.8) for 5 minutes at room temperature. Then, cells were centrifuged for 6 min at 10 000 rpm and protein contents of the different cell pellets were measured by Bradford dye-binding using the Biorad protein assay (Bio-Rad protein assay kit, Hercules, USA) for normalization of the ICP-MS data. 4 µl of each pellet sample were diluted with 200 µl of the Bradford reagent diluted five times in Milli-Q water. The optical density was measured at 595 nm using the spectrofluorometer (SAFAS Monaco SP2000, Genius 5801). The amount of protein present in each sample was determined using a calibration curve with bovine albumin as a model protein, with concentration ranging from 0 to 0.035 mg/mL (7 concentrations).

1.12. *In vivo* studies

In vivo studies were carried by the OPTIMAL platform at the Institute of Advanced Biosciences at the University of Grenoble Alpes (INSERM U1209) on 6-week-old female NMRI mice obtained from Janvier labs. The procedures were performed in accordance with the authorization of animal experiments delivered by the Grenoble Ethics Committee and the French Ministry of Higher Education and Research (reference: APAFIS#33137-202110411585349v2).

Mice were injected with 5.10^6 U87-MG cells in their right flank. Tumor volume was followed by caliper measurement. The xenographs were left to develop until they reached approximately 100 mm^3. Mice were sorted into four treatment groups (10 mice per group): non treated, 0.2 mg/kg of oxaliplatin, 0.6 mg/kg of NHC-Pt-Ad-PPh_3^+ within liposomes and 0.6 mg/kg of NHC-Pt-Ad-PPh_3^+ diluted in the presence of DMSO. The administration of each treatment was done by intra-tumoral injections every 48 h over the course of 20 days (10 injections). Liposomes were stored at -80 °C and were thawed as needed. The non-formulated complex was pre-diluted in DMSO at a stock concentration of 5 mM (6 mg/mL) and stored at 4 °C. Compounds were diluted in a 0.9% w/v NaCl solution, and the final volumes were adjusted to achieve final volume injections of 30 µl. At the end of the study, mice were euthanized. However, if ethical limits were reached (i.e., tumor volume reaching 2000 mm^3), they were sacrificed before the end of the study. The monitoring consisted on the clinical

supervision of body weight and tumor volume (3 times a week), the general body aspect, and the behavior and potential signs of pain of the animals (every day). If total scores were ≥ 3 for 24 h, the corresponding animal was euthanized.

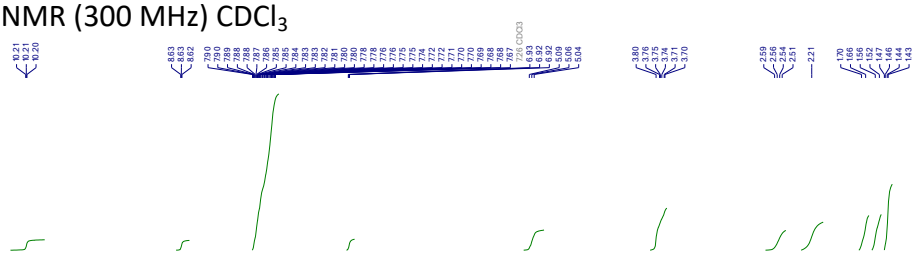
II. GENERAL CHEMICAL REMARKS

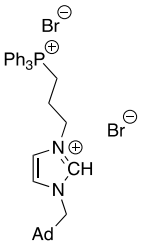
All manipulations were carried out under an inert atmosphere of argon using standard Schlenk techniques unless otherwise stated. (3-Bromopropyl)triphenylphosphonium bromide and all the other reagents were purchased from commercial chemical suppliers (mainly Acros, Aldrich, Alfa Aesar, TCI Europe and Strem) and used without further purification. Solvents were dried and degassed according to standard procedures. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 300 spectrometer and a Bruker Avance III HD - 500 MHz. ^{1}H-NMR spectra were referenced using the residual solvent peak ($CDCl_3$: δ H = 7.26 ppm; D_2O: δ H = 4.79 ppm) at 295K. Chemical shifts δ are given in ppm whereas coupling constants *J* are stated in Hertz (Hz). The following abbreviations are used to classify the multiplicity of the observed signals: s = singlet, d = doublet, t = triplet, q = quartet, quint = quintuplet, dd = doublet from doublet, dt = doublet from triplet, m = complex multiplet or broad signal.

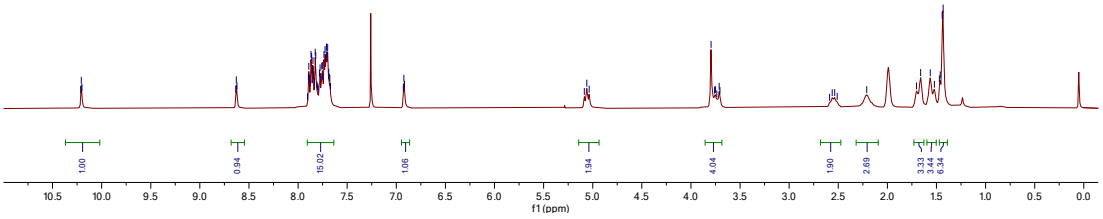
III. ^{1}H,^{13}C & ^{31}P NMR SPECTRA OF IMIDAZOLIUMS

Proligand 1

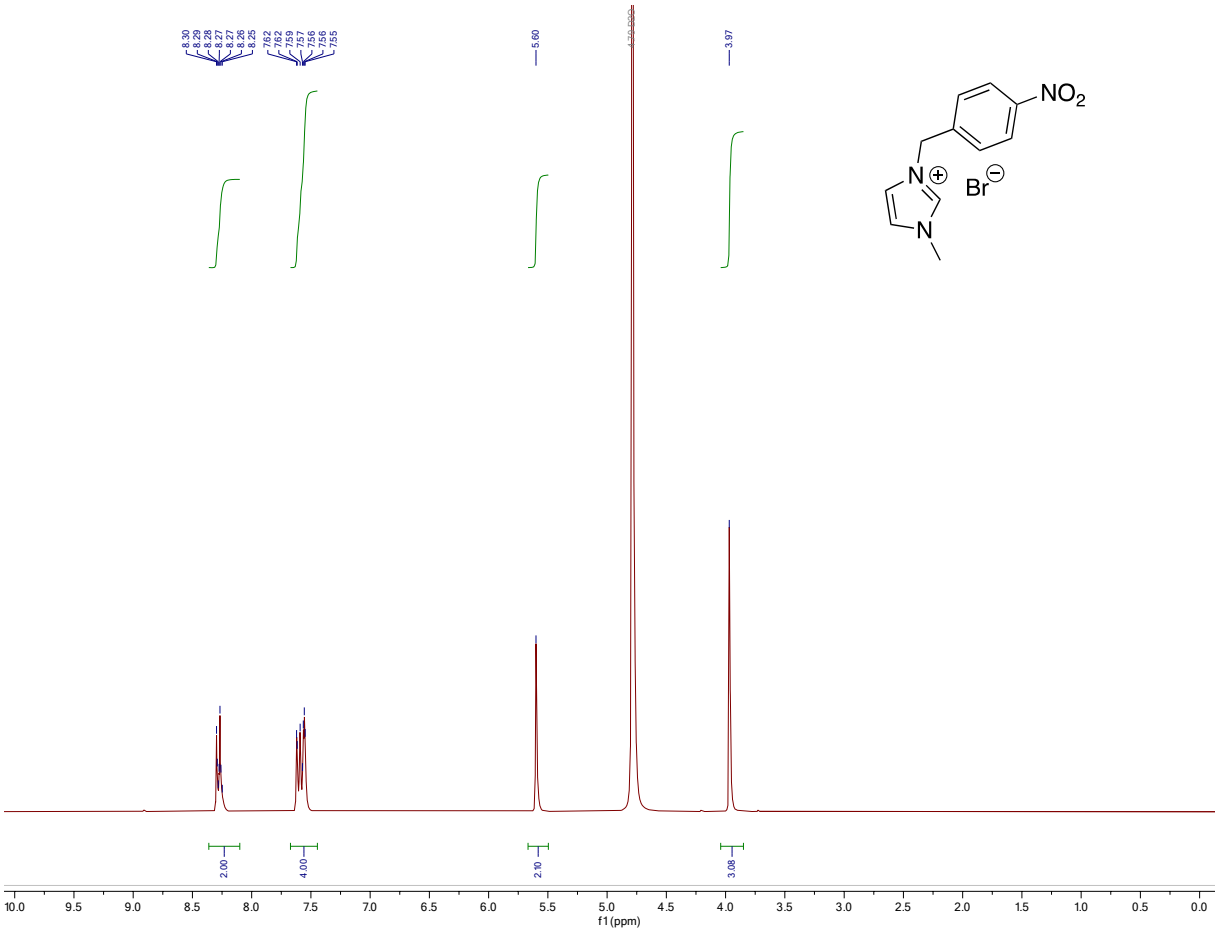
^{1}H NMR (300 MHz) $CDCl_3$





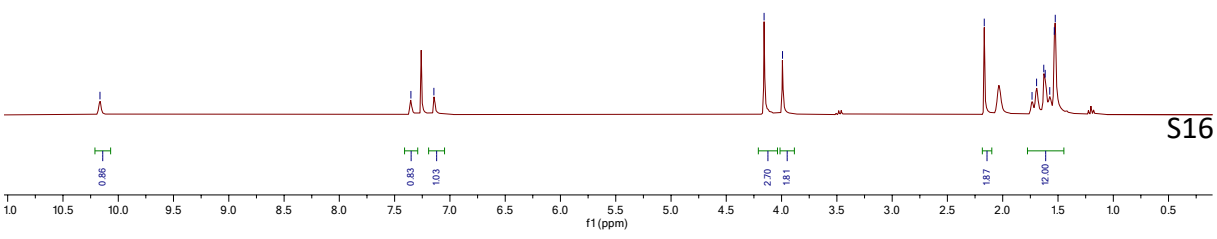


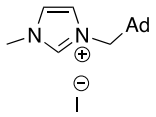
Proligand 5a
^{1}H NMR (300 MHz) D_2O

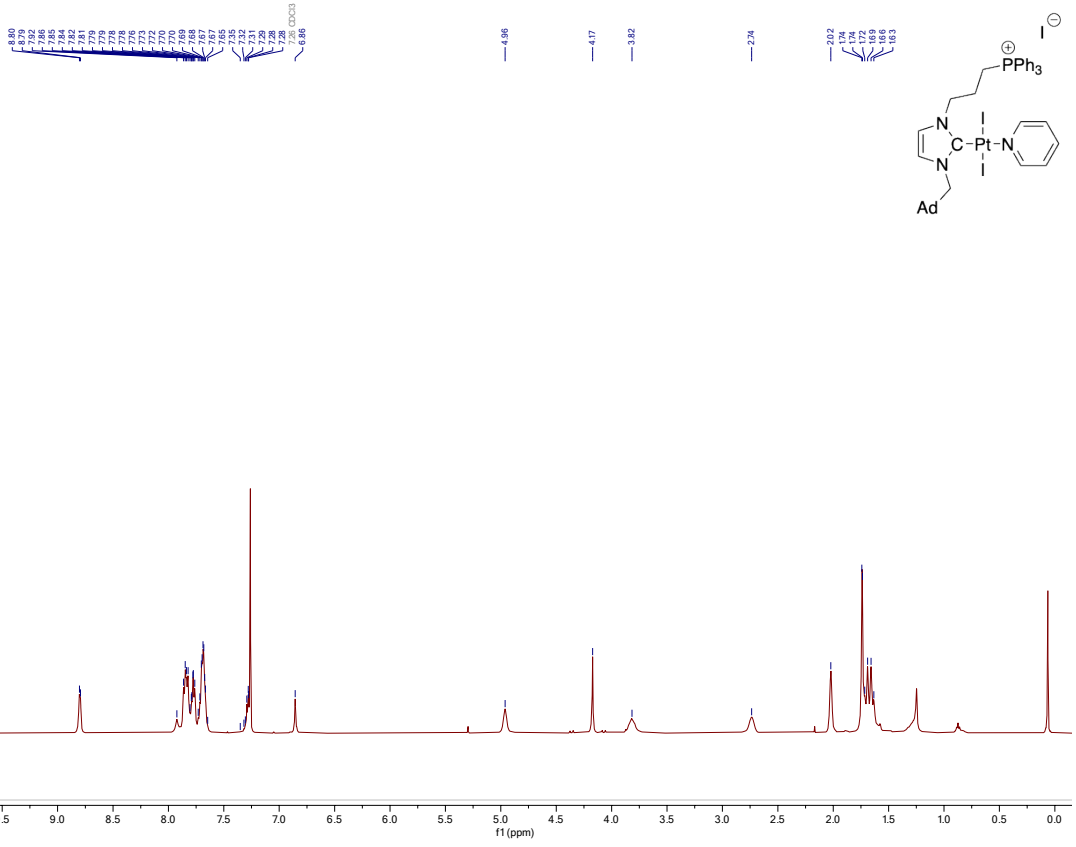

8.30
8.29
8.28
8.27
8.27
8.26
8.25
7.762
7.762
7.759
7.757
7.756
7.756
7.755
5.60
3.97
NO2
Br
2.00
4.00
2.10
3.08
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

Proligand 5b

^{1}H NMR (300 MHz) $CDCl_3$







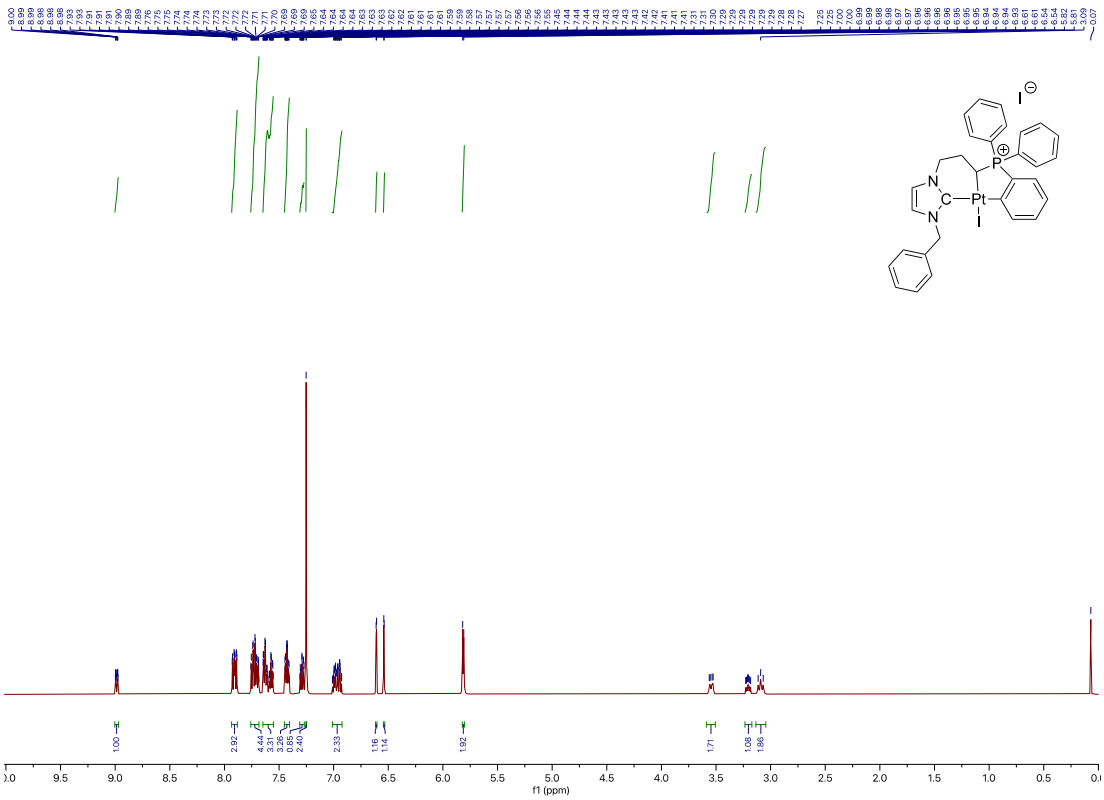
IV. $^{1}H,^{13}C$ & ^{31}P NMR SPECTRA OF NHC-Pt COMPLEXES (

Complex 3

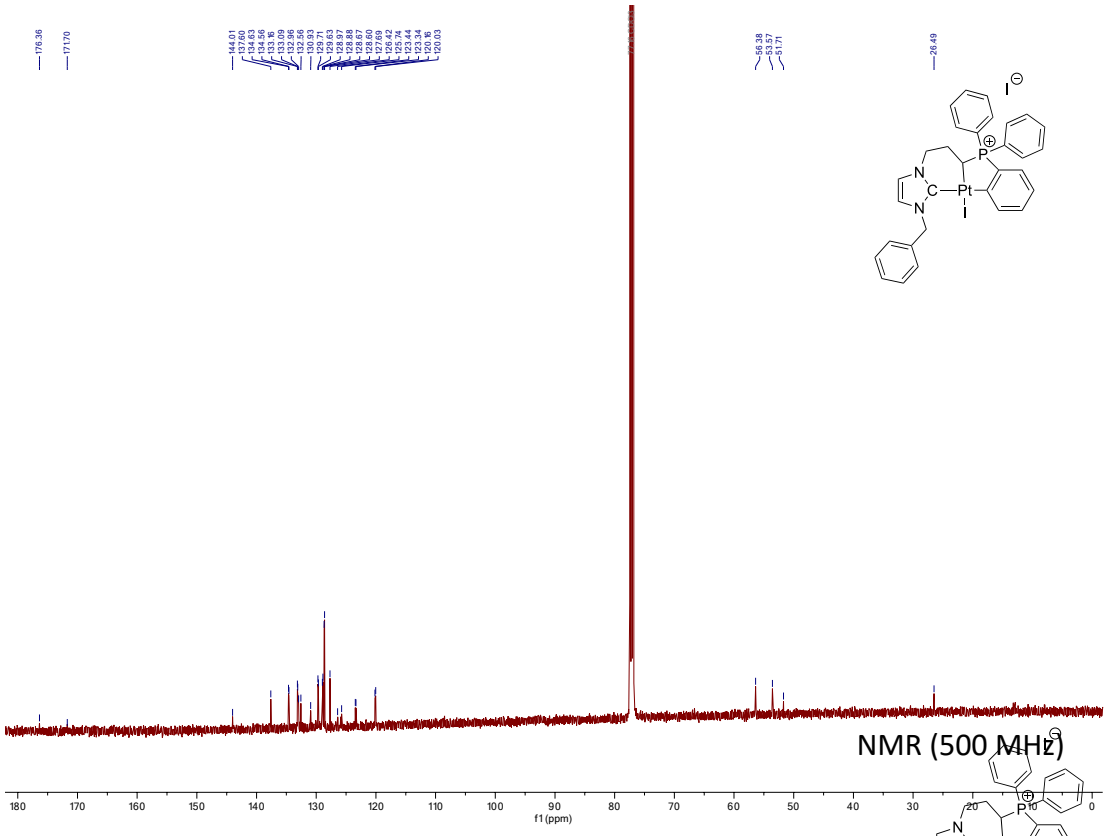
^{1}H NMR (500 MHz) $CDCl_3$

Complex 4 – C-H-insertion

^{1}H NMR (500 MHz) $CDCl_3$

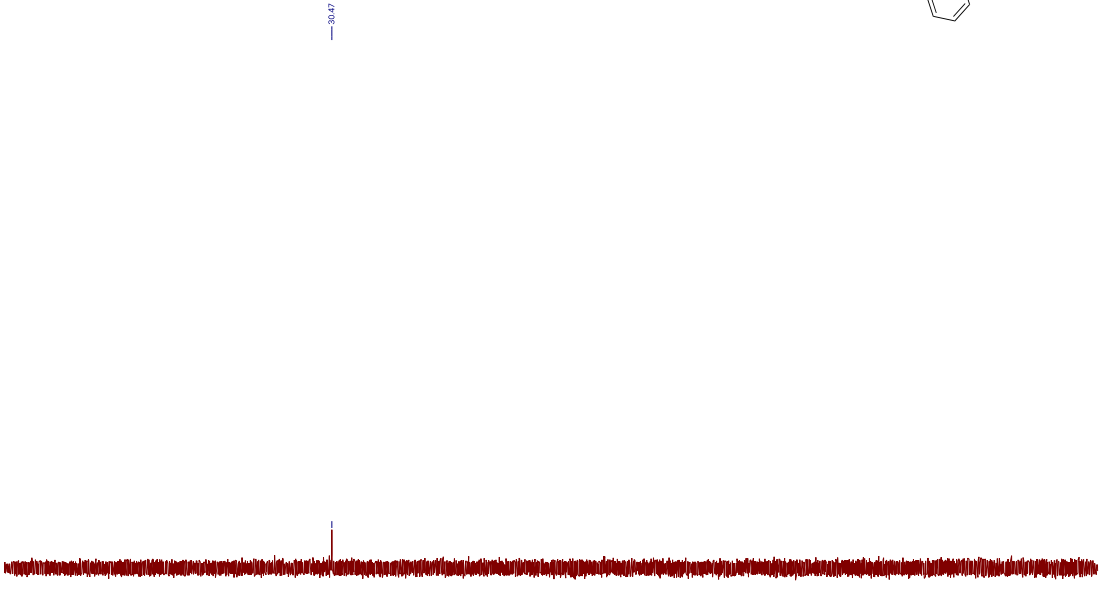

I
P
N
C
Pt
N
I
1.00
2.92
4.44
3.31
2.26
0.85
2.40
2.33
1.16
1.14
1.92
1.71
1.08
1.88
f1 (ppm)

^{13}C NMR (500 MHz) $CDCl_3$



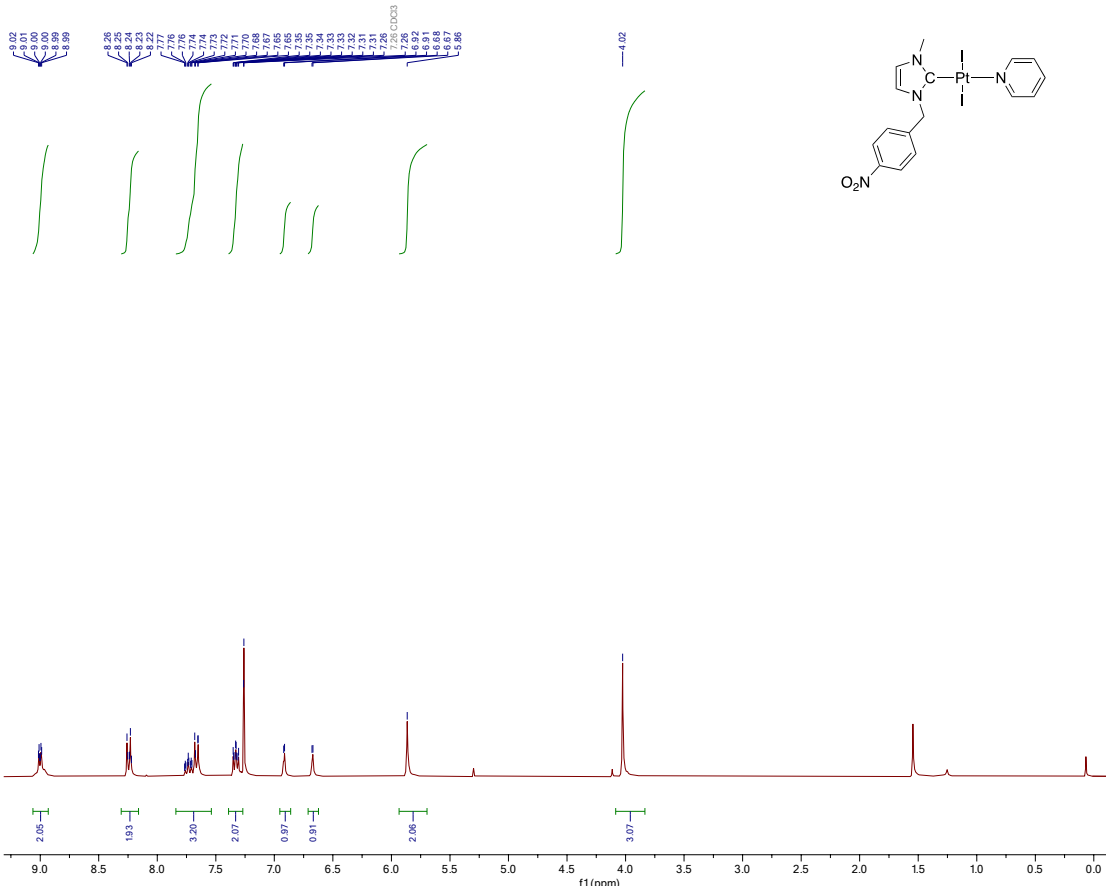
^{31}P NMR (500 MHz)

$CDCl_3$



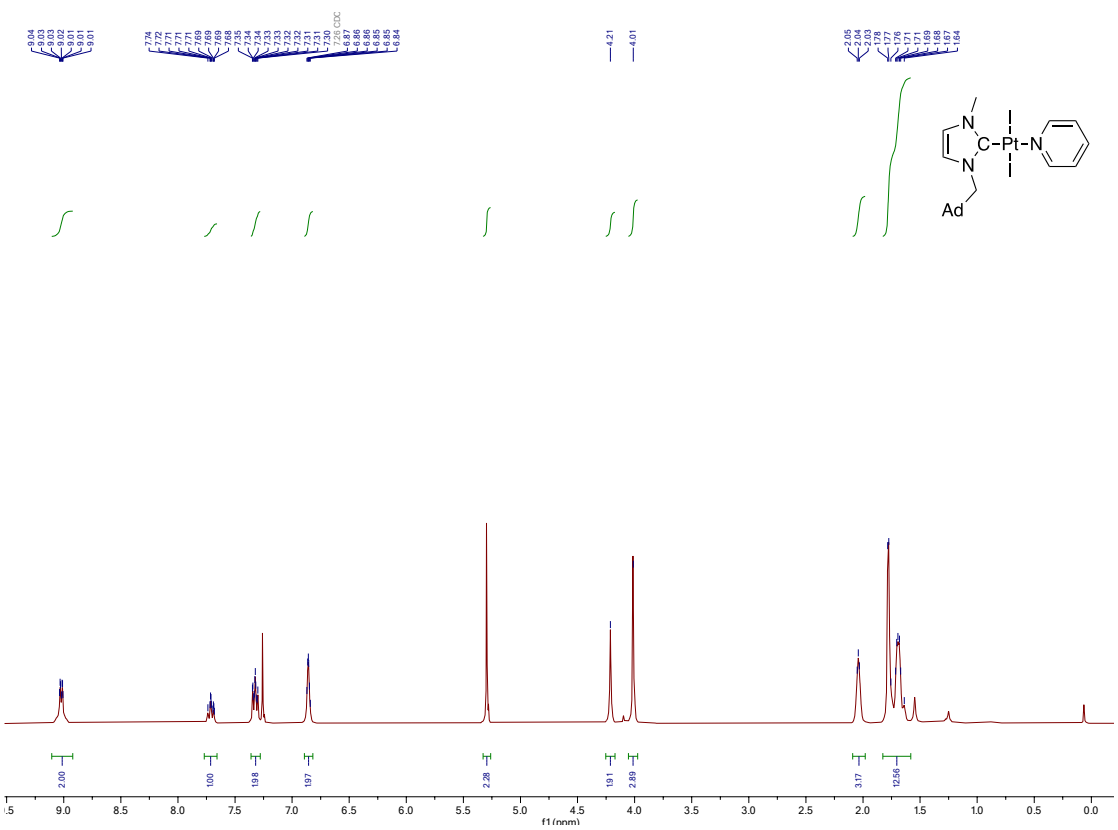
Complex 2

^{1}H NMR (300 MHz) $CDCl_3$


9.02
9.01
9.00
9.00
8.99
8.99
8.26
8.25
8.24
8.23
8.22
7.77
7.76
7.76
7.74
7.74
7.73
7.72
7.71
7.70
7.68
7.67
7.66
7.65
7.35
7.35
7.34
7.33
7.33
7.32
7.31
7.31
7.26 CDCl3
7.26
6.92
6.91
6.68
6.67
5.86
4.02
N
C
Pt
N
N
I
I
O2N
2.05
1.93
3.20
2.07
0.97
0.91
2.06
3.07
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

Complex 7

^{1}H NMR (300 MHz) $CDCl_3$


9.04
9.03
9.03
9.02
9.01
9.01
9.01
7.74
7.73
7.71
7.71
7.71
7.71
7.70
7.69
7.69
7.68
7.68
7.35
7.34
7.34
7.33
7.33
7.32
7.32
7.31
7.31
7.31
7.30
7.26 CDC
6.87
6.86
6.86
6.86
6.85
6.85
6.84
4.21
4.01
2.05
2.04
2.03
1.78
1.77
1.76
1.71
1.71
1.69
1.68
1.67
1.64
N
C
Pt
N
N
Ad
2.00
1.00
1.98
1.97
2.28
1.91
2.89
3.17
12.56
f1 (ppm)

V. SIZE MEASUREMENTS

Content	Carrier	Before freezing		Post-thawing	
		Diameter ± SD (nm)	PDI	Diameter ± SD (nm)	PDI
2	**Lp(+)**	52 ± 8	0.275	42 ± 5	**0.563**
	Lp(N)	68 ± 9	0.217	71 ± 10	0.255
3	**Lp(+)**	49 ± 7	0.214	54 ± 7	**0.474**
	Lp(N)	61 ± 7	0.214	70 ± 7	**0.908**

Table S1. Physicochemical characterization of the positively charged liposomes (Lp(+)) and non-charged liposomes (Lp(N)) loaded by complexes **2** or **3** measured by DLS before freezing at -80°C and post-thawing.

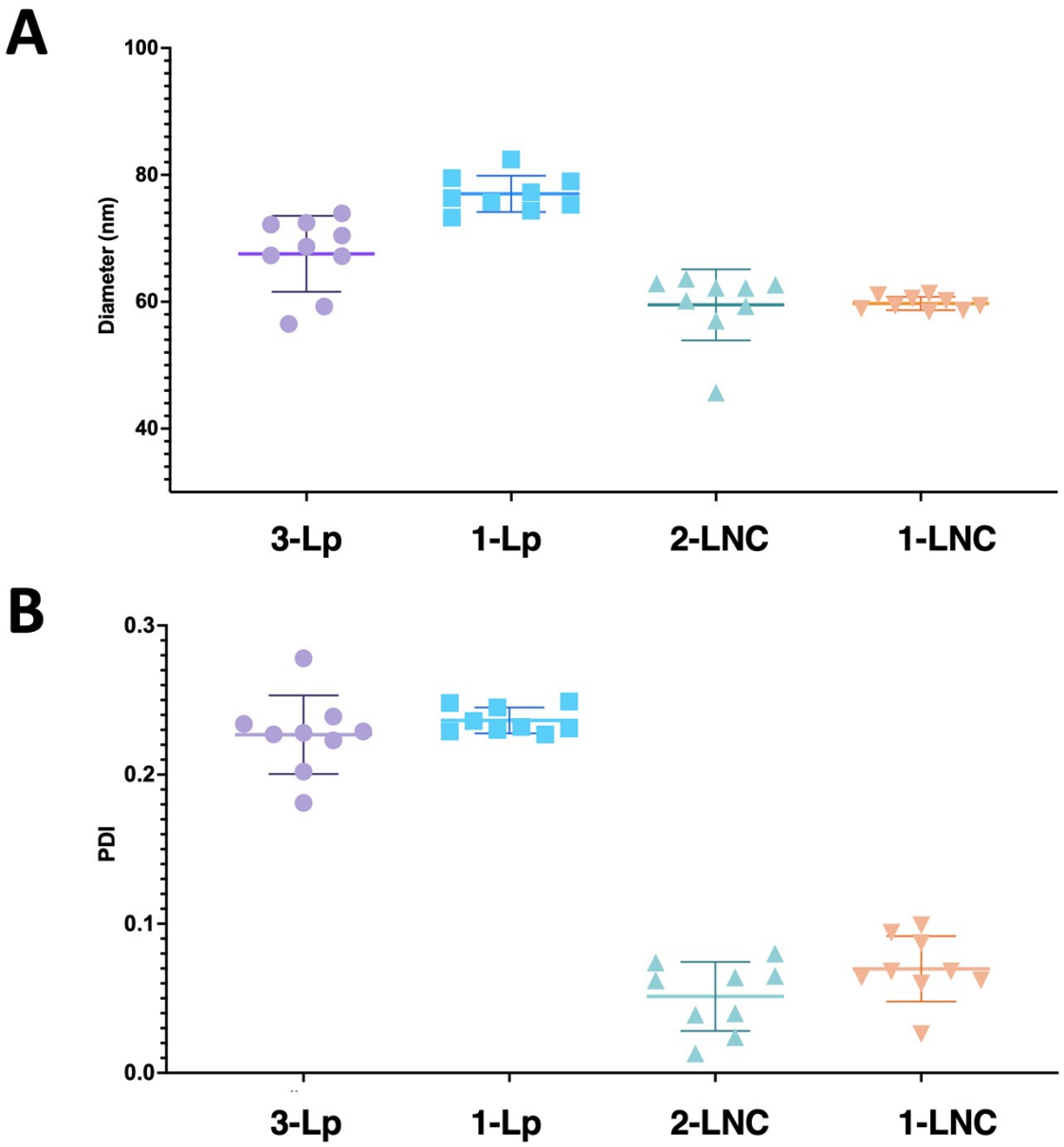


Figure S1. Size characterization of the liposomes and the LNCs. A) Diameters in nanometers. B) Polydispersity index (PDI) of the nano-delivery systems (no units). All values are represented by a symbol and the mean and standard deviation values are represented by horizontal lines. Parameters were acquired by DLS. Lp = Liposome; LNC = Lipid nanocapsule. *GraphPad Prism 8 software*.

VI. CYTOTOXICITY MEASUREMENTS

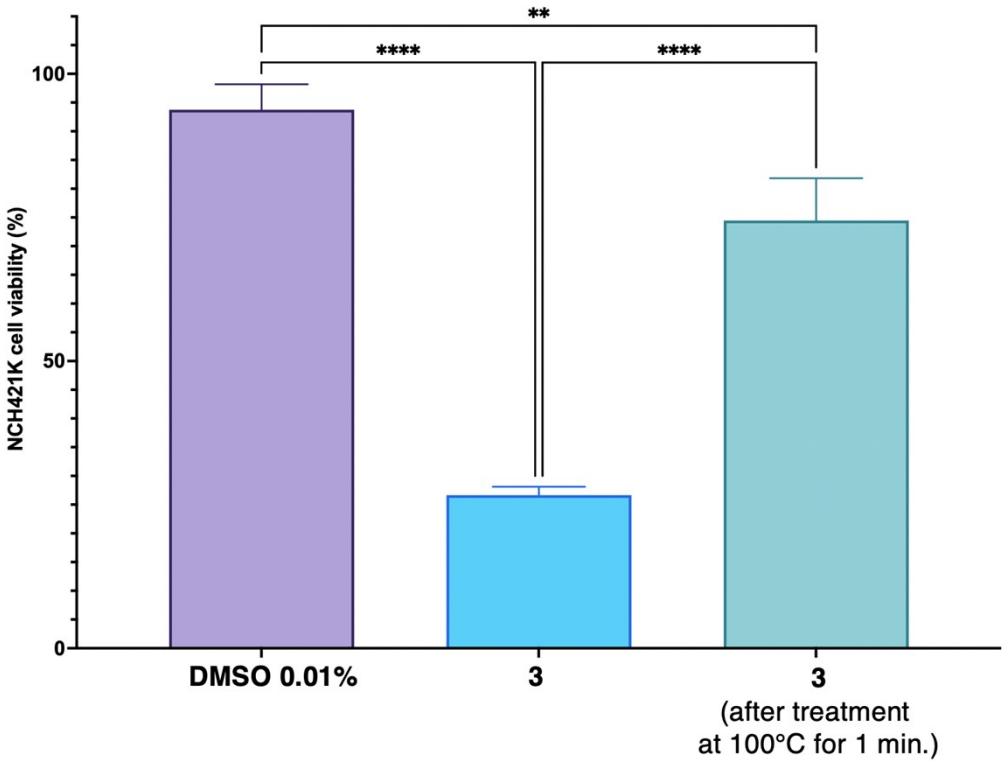


Figure S2. Cytotoxicity activity on glioblastoma stem cells (NCH421K) after 24 h of treatment at 37 °C with complex **3** after being heated at 100 °C for one minute. The heated compound is compared to the non-heated one, at a concentration of 0.5 µM, and to the vehicle control (DMSO 0.01%). This graphic shows the results of one assay carried out in technical triplicates for each experimental condition. Data, obtained via Cell Tier Glo® 3D cell viability assay, is expressed in percentage of cell viability. The statistical analysis was done using GraphPad Prism 8 software by performing an ordinary one-way anova test (** = $p < 0.01$, **** = $p < 0.0001$).

VII. *IN VIVO* STUDY

Toxicity assay:

The formulation of complex **3** into the liposomes was tested *in vivo* on a 6-week-old NMRI nude female mice U87-MG glioblastoma model. These are mutant immunodeficient hairless albino mice. Before starting the study, a toxicity assay was undertaken to evaluate which concentration of the complex could be injected into the mice without having secondary effects. Three concentrations were tested of the formulated complex via intraperitoneal injections: 6 mg/kg, 1.5 mg/kg and 0.6 mg/kg. To this purpose just 3 mice were used for each treatment. To begin with, 6 mg/kg of the encapsulated complex **3** were tested. After the first injection, the weight loss observed was comprised between 10% and 15% of the total body weight of the mice, and after the second injection, on day 3, they had lost between 15% and 20%, which was physically noticeable (Fig. S3). The mice showed clear signs of distress and the autopsy showed nearly abolished peristaltic activity in the intestines. Intestinal dysfunction is a side effect that has already been established for chemotherapeutic agents, such as oxaliplatin.

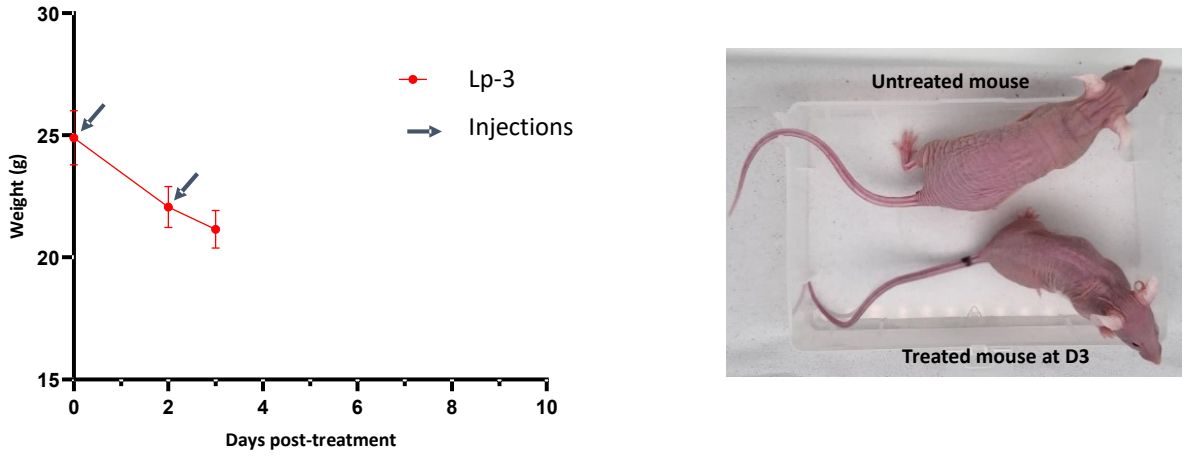


Figure S3. *In vivo* toxicity results after injection of 6 mg of the **3** complex encapsulated within liposomes (Lp-3) per kg of NMRI nude mice. On the left, the graph shows the mean weight of the mice (n = 3) over time (in days). The injections are indicated by arrows. The graph was generated using GraphPad Prism 8 software. On the right, the image shows the difference between a treated and a non-treated healthy mouse on day three after the first injection.

Subsequently, two lower concentrations were assessed: 1.5 mg/kg and 0.6 mg/kg. In both cases all ten planned injections were carried out over the course of 20 days. For the highest dose, one mouse was found dead on day 7 after the first injection. The two remaining mice lost weight between day 7 and 9 but they were able to gain it back. In comparison, with the

lowest dose (0.6 mg/kg), the mice didn't lose weight and they started putting on some following the 5th injection (Fig. S4). In both cases, the autopsy showed no abnormalities or lesions on the organs. The concentration of 0.6 mg/kg was selected for the *in vivo* study as it didn't demonstrate any adverse effect on the mice.

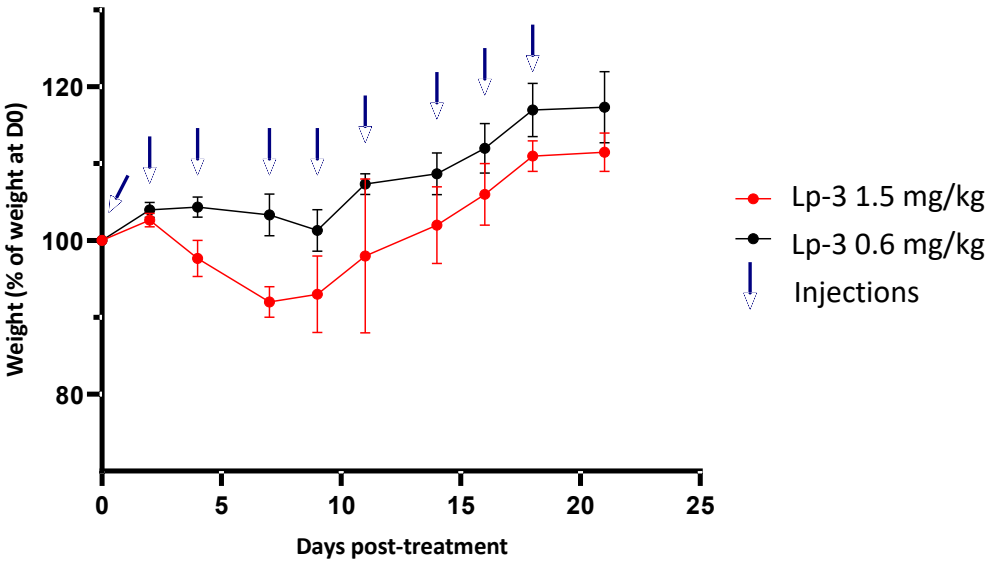
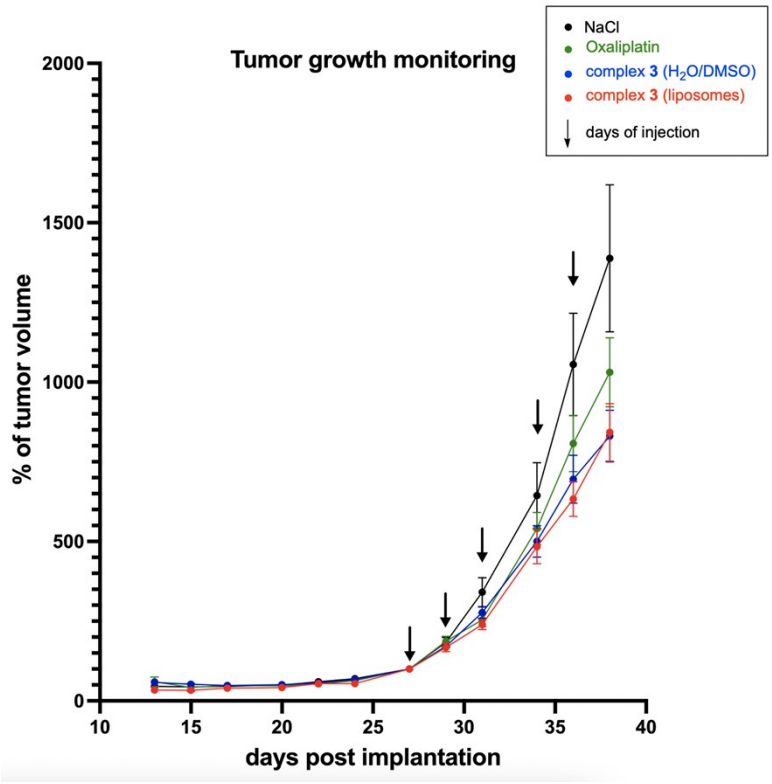


Figure S4. *In vivo* toxicity results after injection of 1.5 mg (red) or 0.6 mg (black) of the complex **3** encapsulated within liposomes per kg of NMRI nude mice. The graph represents the mean weight of the mice (n = 3) over time (in days). The injections are indicated by arrows. The graph was generated using GraphPad Prism 8 software.

Tumor growth *in vivo* results:



Two-way ANOVA		
Day 34	**P-value**	**Summary**
3-Liposomes vs. Oxaliplatin	0.9964	ns
3-Liposomes vs. 3-Water/DMSO	0.8859	ns
3-Liposomes vs. NaCl	0.1659	ns
Oxaliplatin vs. 3-Water/DMSO	0.9519	ns
Oxaliplatin vs. NaCl	0.2284	ns
3-Water/DMSO vs. NaCl	0.5387	ns
Day 36		
3-Liposomes vs. Oxaliplatin	0.8430	ns
3-Liposomes vs. 3-Water/DMSO	0.1137	ns
3-Liposomes vs. NaCl	< 0.0001	****
Oxaliplatin vs. 3-Water/DMSO	0.4525	ns
Oxaliplatin vs. NaCl	< 0.0001	****
3-Water/DMSO vs. NaCl	0.0082	**
Day 38		
3-Liposomes vs. Oxaliplatin	0.9986	ns
3-Liposomes vs. 3-Water/DMSO	0.0747	ns
3-Liposomes vs. NaCl	< 0.0001	****
Oxaliplatin vs. 3-Water/DMSO	0.0425	*
Oxaliplatin vs. NaCl	< 0.0001	****
3-Water/DMSO vs. NaCl	< 0.0001	****

Figure S5. Tumor growth (%) kinetic *in vivo* results over time (in days post-implantation of the tumors). The intra-tumoral injections are indicated with an arrow. The graph was generated using GraphPad Prism 8 software. Below, statistics represent one-way anova test. ns = non significative values, $p > 0.05$. complex 3-loaded liposomes: n = 9; Oxaliplatin: n = 10; complex 3 in water/DMSO: n = 9; NaCl: n = 9.

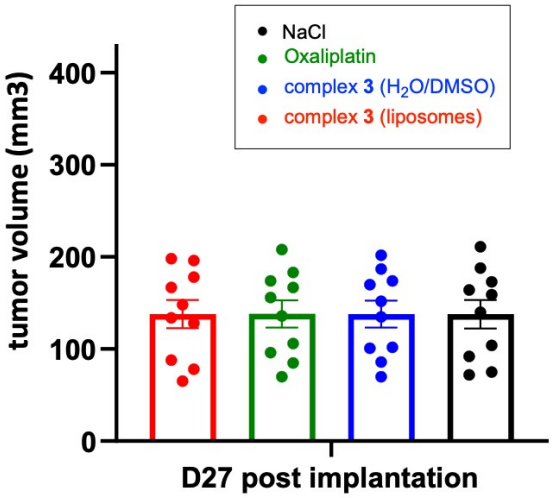


Figure S6. Mean tumor volumes (in mm^3) on day 27 after implantation of the U87-MG glioblastoma differentiated cells. The mice are sorted into four groups randomly (n = 10 per group) to start the treatment with the vehicle (NaCl), oxaliplatin, the encapsulated complex (Lp-(NHC-Pt-Ad-PPh_3^+)) and the complex solubilized in the presence of DMSO (DMSO-(NHC-Pt-Ad-PPh_3^+)). The means and standard deviations are similar between the four groups. The graph was generated using GraphPad Prism 8 software.

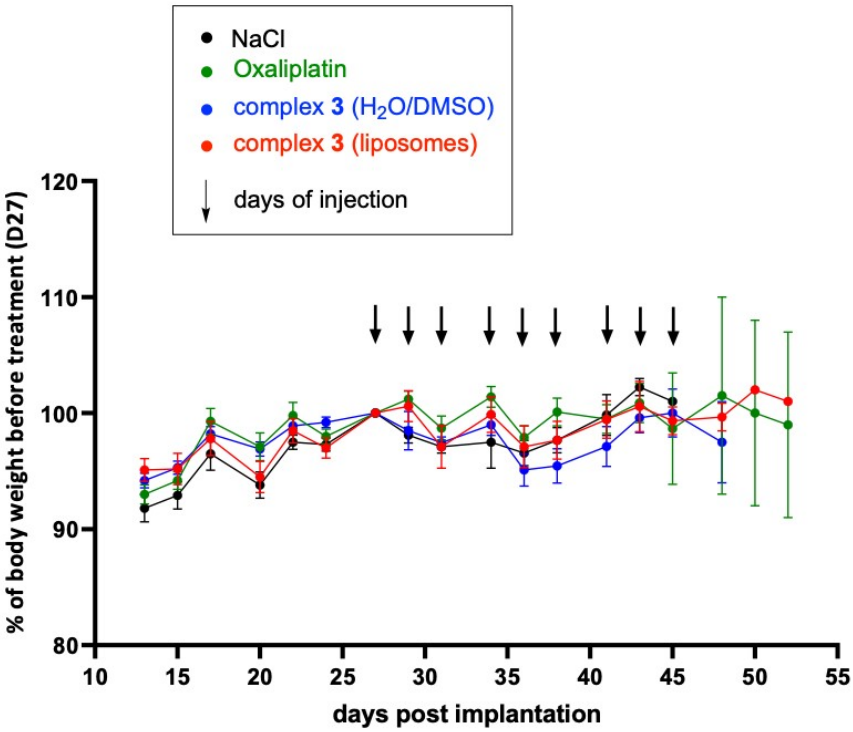
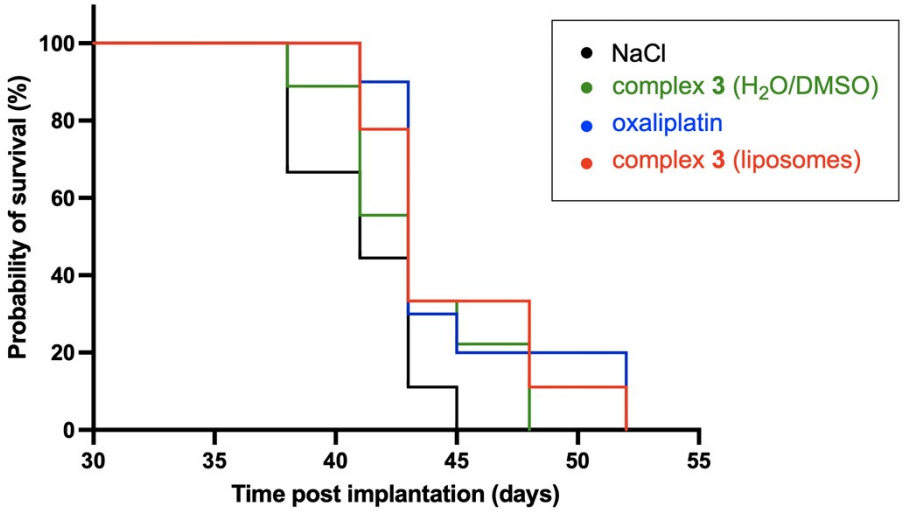


Figure S7. Body weight monitoring after implantation of the U87-MG glioblastoma differentiated cells. The treatment injections are represented by arrows and start on day 27. From day 38, some mice were killed due to their tumor volume which reached ethical limits. The reduced number of animals at the latest time points induce large standard deviations. The graph was generated using GraphPad Prism 8 software.



Log-rank (Mantel-Cox) test				
Comparison	**Chi square**	**df**	***p*-value**	**Summary**
Group 1 vs. Group 4	4.281	1	0.0385	*
Group 2 vs. Group 4	4,689	1	0.0304	*
Group 3 vs. Group 4	4,737	2	0.0936	ns

Figure S8. Survival curves. The graph was generated using GraphPad Prism 8 software. Below, statistics represent log-rank (Mantel-Cox) test. ns = non significative values, $p > 0.05$.

VIII. Bibliography

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