Supplementary Information

In vitro and *In vivo* Inhibition of amyloid β aggregation by a Ru(II)-naphthalene diimide complex

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Materials and Methods

Reagents: $RuCl_3.xH_2O$, 1,10-phenanthroline, 1,4,5,8-Naphthalenetetracarboxylic dianhydride, phenanthroline-5-amino, lithium chloride and tetrabutylammonium hexafluorophosphate (NH_4PF_6) were purchased from Sigma-Aldrich. Acetone and DMF were distilled before used.

pNDIp synthesis: The pNDIp ligand and RuNDI complex was synthesized following literature procedures.[1] For this purpose, 100 mg (0.37 mmol) of naphthalene-1,4,5,8-tetracarboxylic dianhydride was added to 3 mL of deaerated DMF in a sealed system under N_2 flux. The temperature was raised to 130 °C in a silicone bath, followed by dropwise addition of 225 mg (1.15 mmol) of 5-aminophenanthroline dissolved in 3 mL DMF and 215 µL (1.53 mmol) of triethylamine. The reaction proceeded under reflux with constant stirring for 3 hours. After cooling to room temperature, the precipitated solid was filtered through a silica filter, washed with 10 mL of cold ethanol, and dried under vacuum. The pale yellow product was further purified with hot DMF (50 mL) to remove unreacted naphthalene precursor and cold ethanol (50 mL), yielding 50% after vacuum drying.

RuNDI synthesis: The complex was prepared by reacting *cis*-$[Ru(phen)_2Cl_2]$ (100 mg, 0.18 mmol) with pNDIp ligand (170 mg, 0.25 mmol) in 15 mL of deaerated DMF under N_2 atmosphere and light-free conditions. The reaction mixture was refluxed with constant stirring for 24 hours. After cooling, the solution was filtered hot to remove insoluble ligand excess, followed by addition of NH_4PF_6 (62 mg, 0.37 mmol). The product was precipitated with 250 mL diethyl ether and refrigerated overnight. The red solid was collected by silica filtration, dissolved in acetone (50 mL), and centrifuged (12,000 RPM, 10 min) to remove residual pNDIp. The dissolution-centrifugation cycle repeated until no

precipitate was observed. Final purification yielded a dark red solid after rotary evaporation and vacuum drying. MALDI-TOF MS m/z for $C_{62}H_{34}N_{10}O_4RuP_2F_{12}$; calcd: 622.1390; found 1084.1798 $(M)^{2+}$.

Characterization: Optical spectra were recorded in an Agilent 8453A spectrophotometer, and emission spectra were obtained using a Shimadzu RF-5301PC spectrofluorometer. ^{1}H and ^{13}C NMR spectra were recorded on BRUKER DXR spectrometers operating at 400 and 600 MHz, using 3 mg of the compound dissolved in 600 μL of DMSO-d_6 at 298 K. Chemical shifts (δ) are reported in parts per million (ppm) relative to residual solvent signals (δ = 2.50 ppm for ^{1}H and 39.5 ppm for ^{13}C).

High-resolution mass spectrometry (HRMS) analyses were carried out on an Agilent 6545 QToF instrument (Agilent Technologies, Santa Clara, CA, USA) equipped with a Jet Stream electrospray ionization (ESI) source operating in positive ion mode. The RuNDI complex were dissolved in 100 μL of HPLC-grade DMSO and 900 μL of LC-MS-grade methanol (MeOH) and subsequently diluted to a final concentration of 5 ppm using LC-MS-grade MeOH.

Spectra were recorded by direct infusion over m/z ranges of 100–1000 Da and 400–800 Da, with a scan rate of 3 spectra.s^{-1}. The mass accuracy (error in ppm) was determined from the difference between theoretical and experimental monoisotopic masses. All data were acquired and processed using MassHunter Workstation Software version B.08.00.

Cell culture and cytotoxicity assay

The in vitro cytotoxicity assays of the **RuNDI** complex was assayed against human lung fibroblast MRC-5 (ATCC No. CCL-171). The cells were routinely maintained at 37 °C in a humidified 5% CO2 in Dulbecco's Modified Eagle (DMEM) medium containing fetal bovine serum (FBS) 10%, penicillin (100 UI/mL), streptomycin (100 mg/mL) and

L-glutamine (2 mM). For the cytotoxic assays, the cells (1×10^4 cells / 100 µL) were seeded in sterile 96-well plates and kept at 37 °C and 5% CO_2 for 24 hours in a cell culture incubator. The next day, the culture medium was removed from the wells, and different concentrations of complex diluted in DMSO (1.56 to 200 µM) were added to the wells. The cells were incubated for 24 hours in the same conditions as described above, and after the incubation, a new culture media containing 0.5 mg / mL of MTT [3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide] was added (50 µL / well) and the plates were incubated for additional 4 hours at 37 °C. After this time, the crystals formed were diluted in DMSO (100%) and the absorbance of the conversion of MTT to formazan by metabolically viable cells was read on microplate reader (Biotek Synergy HT) at a wavelength of 570 nm, according to Mosmann.[2] The viability of treated cells was normalized to that of negative control cells (without treatment). The complex IC50 (concentration that induces 50% cell death) was determined from a concentration curve using the GraphPad Prism software, version 9. The selectivity index (SI) was calculated as the ratio of the IC50 (non-tumor cell) / IC50 (tumor cell).

Preparation of stock and working solutions: Stock solutions of the RuNDI complex (1 mM) were prepared by dissolving the solid compound in dimethyl sulfoxide (DMSO, analytical grade). The complex was initially solubilized in a small aliquot of DMSO corresponding to 2% of the final solution volume (e.g., 20 µL of DMSO in a final volume of 1 mL) to ensure complete dissolution.

For *in vitro* assays, stock solutions were diluted with the appropriate buffer to the desired concentrations. The highest concentration used was 100 µM, corresponding to a RuNDI:Aβ molar ratio of 1:5 and a final DMSO content of 0.2% (v/v).

For *in vivo* assays, stock solutions of the RuNDI complex (1 mM, 2% DMSO) were prepared in biological-grade dimethyl sulfoxide (DMSO) and subsequently diluted with

phosphate-buffered saline (PBS) to obtain a 4 mL working solution at 25 μM, corresponding to a final DMSO concentration of 0.05% (v/v). Mice received daily intraperitoneal injections of this solution, corresponding to a dose of 0.1 mg kg^{-1} day^{-1}. Body weight was monitored weekly to adjust the administered volume when necessary. Control animals received vehicle (PBS containing equivalent DMSO concentration). The final DMSO content did not exceed 0.2% (v/v) in any preparation, a level considered non-toxic for in vivo use.

Expression and Purification of $A\beta_{42}$: The plasmid containing the sequence for the fusion protein NT*FlSp-$A\beta_{42}$ was kindly provided by the group of Henrik Biverstal (Karolinska Institutet). The expression and purification protocol followed the previously stablished protocol by Biverstal's group. In short, the plasmid transformed into chemically competent *E. coli* BL21 (DE3) cells and expressed as described previously. For cell culture, 4 mL of pre-culture were independently incubated in six sterile flasks containing LB medium (400 mL) and kanamycin (70 μg/mL). These cultures were incubated at 30 ºC, 120 rpm and induced by adding IPTG at a final concentration of 0.1 mM individually to each flask. Flasks containing bacterial cells were incubated at 20 ºC, 120 rpm overnight. The next day, cells were isolated by centrifuging the culture medium at 5000xg using a Beckman JLA-81000 centrifuge for 20 minutes at 4 ºC. The supernatant was discarded, and cell pellets were resuspended in 20 mM Tris-HCl buffer, pH 8, 8M urea. The solution was then sonicated three times (3 x 1 minute – 15 seconds pulse, 45 seconds pause). The lysed cells were centrifuged for 15 minutes at 21,000 rpm at 4 ºC. The supernatant was isolated and filtered using a 0.45 μm pore size vacuum filter. The filtered cell lysate was loaded onto an immobilized nickel affinity column (3 x 5 mL HisTrap Excel – GE Healthcare) and elution was performed with a high concentration of imidazole buffer (300 mM imidazole, 20 mM Tris-HCl, 8M urea, pH 8). The same

process was repeated for the flow-through. SDS-PAGE was performed to identify fractions containing the NT*FISp-Aβ protein (~20 kDa), and these fractions were combined and dialyzed overnight in 20 mM Tris-HCl buffer, pH 8 at 4 ºC. Cleavage of the NT*FISp was carried out by dialysis, adding TEV protease to the fusion protein solution at a ratio of 20 NT*FISp-Aβ: 1 TEV in 3 kDa pore size dialysis bags in 4 liters of Tris-HCl buffer, pH 8, 0.5 mM EDTA, and 1 mM DTT at 4 ºC. SDS-PAGE confirmed the cleavage in the NT*FISp-Aβ protein. After dialysis, solutions were centrifuged, and the supernatant was lyophilized. The lyophilized cleavage product was solubilized in 6 M guanidine-HCl, and the protein was loaded onto a Superdex 30 26/600 pg size exclusion column (GE Healthcare) and eluted in 25 mM Na_2HPO_4, 0.2 mM EDTA, pH 8.0 phosphate buffer. Fractions corresponding to the pure Aβ peptide were combined, lyophilized and stored at -80 ºC.

$Aβ_{42}$ Preparation: Lyophilized recombinant $Aβ_{42}$ peptide was dissolved in 6 M guanidine-HCl and incubated for 3 hours to dissociate pre-formed aggregates. The peptide was then purified by size-exclusion chromatography using a preparative column (Cytiva Superdex 200 Increase 10/300 GL), eluting with degassed NaPi buffer (20 mM sodium phosphate, 0.2 mM EDTA, pH 8.0). Protein concentration was determined by UV detection (ÄKTA purification system), calculated from the integrated peak area of the eluted protein using the formula: Concentration = (peak area/0.2) × 1490.

Nephelometry: Freshly prepared $Aβ_{42}$ (10 μM) solutions were incubated for 30 minutes at low temperature with varying molar ratios of RuNDI complex (1:1, 1:2, and 1:5 $Aβ_{42}$:RuNDI). Following pre-incubation, samples were transferred to 96-well half-area plates (Corning 3881) and subjected to real-time aggregation monitoring at 37°C for 4 hours using a nephelometer (NEPHELOstar Plus, BMG Labtech).

Circular Dichroism (CD) Spectroscopy: Freshly prepared $A\beta_{42}$ (10 μM) peptide samples were incubated with the RuNDI complex at molar ratios of 1:1 and 1:5 ($A\beta_{42}$:RuNDI) for 24 hours at 37 °C in a static incubator (HeraTherm, Thermo Fisher). Following incubation, samples were centrifuged at 21,000 × *g* and 4 °C for 90 minutes. The pellet was resuspended in deionized water and analyzed using a Jasco J-815 spectropolarimeter equipped with a 1 mm pathlength depolarized quartz cuvette.

Transmission Electron Microscopy (TEM): Freshly prepared $A\beta_{42}$ peptide solutions (10 μM) were incubated with the RuNDI complex (1:5 molar ratio) at low temperature for 30 minutes. Samples were centrifuged at 21,000 × *g* and 4 °C for 30 minutes, and the resulting pellet was resuspended in deionized water. Aliquots were deposited onto 400-mesh copper grids coated with 3 nm carbon film. After washing with deionized water, samples were negatively stained with 1% (w/v) uranyl acetate for 2 minutes, followed by two additional deionized water washes. Grids were air-dried and imaged using a Thermo Scientific Talos F200X G2 transmission electron microscope at the Cambridge Advanced Imaging Center (Dr. Heather Greer, operator).

Animals and Treatment: APP/PS1 (Tg (APPswe, PSEN1dE9) 85Dbo, stock no: 004462 from Jackson Laboratories) mice were used in this study. Animals were subjected to two types of treatment. (1) RuNDI group received daily intraperitoneal injections of the $[Ru(phen)_2(pNDIp)]^{2+}$ complex (0.1 $mg \cdot kg^{-1} \cdot day^{-1}$ in phosphate-buffered saline, PBS), while (2) Control group received vehicle (PBS). Treatments were administered from 20 to 30 weeks of age.

The animals were euthanized by an overdose of pentobarbital (100 mg/kg) at 30 weeks of age. Brains were immediately collected, and the right hemisphere was dissected and flash-frozen at −80 °C, while the left hemisphere was fixed in 4% paraformaldehyde for two weeks prior to sectioning. All animal procedures were performed in accordance with

the Animal Care and Use Guidelines of the University of Cádiz and complied with European (Directive 2010/63/EU) and Spanish (Royal Decree 53/2013) regulations. The protocol was approved by the corresponding Ethics Committee (protocol no. 4/2021/138).

Aβ40 and Aβ42 levels: Cortical tissue samples were homogenized in 50 µL Pierce™ IP Lysis Buffer (Thermo Fisher Scientific) supplemented with Halt™ Protease and Phosphatase Inhibitor Cocktail. Homogenates were centrifuged at 12,000×g for 10 minutes at 4 °C, and the resulting supernatants were aliquoted for soluble Aβ40 and Aβ42 quantification. The remaining pellet was resuspended in 50 µL of 70% formic acid, centrifuged (14,500×g, 12 min), and the supernatant was neutralized with 1:50 dilution in Tris buffer (pH 11) for insoluble Aβ40 and Aβ42 measurement. Soluble and insoluble Aβ40 and Aβ42 levels were measured by colorimetric ELISA kits (Wako, Japan), following the manufacturer's instructions. Absorbance was measured at 450 nm using a spectrophotometer (MQX200R2, BioTek Instruments, Burlington, VT, USA). Data were expressed as pmol/g tissue.

Amyloid Plaque Immunostaining (4G8/Thioflavin-S): The left hemisphere was sectioned coronally (30 µm, −25 °C) using a Thermo Fisher HM525 cryostat and stored in PBS/glycerol (1:1) at 4 °C until use. Sections spaced 1 mm apart were selected, ranging from 1.5 mm to −3.5 mm from Bregma. The selected sections included representative regions of the cortex (n = 6) and hippocampus (n = 3).

The tissues were pre-treated with 70% formic acid for 10 minutes and blocking solution (3% BSA + 0.5% Triton-X100 in PBS) for 1 hour, then incubated overnight at 4 °C with mouse monoclonal anti-Aβ 4G8 primary antibody (BioLegend, UK; 1:1000 dilution in 0.5% BSA)], followed by a 2-hour incubation with Alexa Fluor 594 goat anti-mouse IgG secondary antibody (Molecular Probes, USA)] in the dark. Finally, sections were stained with 0.05% Thioflavin-S (TS) for 10 minutes and mounted on Thermo Scientific™

SuperFrost™ Plus slides. Images were acquired using an Olympus U-RFL-T laser fluorescence microscope (Olympus, Japan) with MMICellTools software. ImageJ software was used for quantitative analysis of plaque size (μm^2), density (number of plaques/mm^2), and plaque burden (% area) in cortical and hippocampal regions. Quantification was performed under identical acquisition and thresholding parameters for all samples. For each animal, measurements obtained from multiple sections were averaged to generate a single biological replicate prior to statistical analysis (n = 4 animals per group)

Statistical Analysis: Differences were analyzed using two-way ANOVA and Student's t-test for independent samples. All statistical analyses were performed using SPSS software, version 29.

RuNDI Synthetic Route

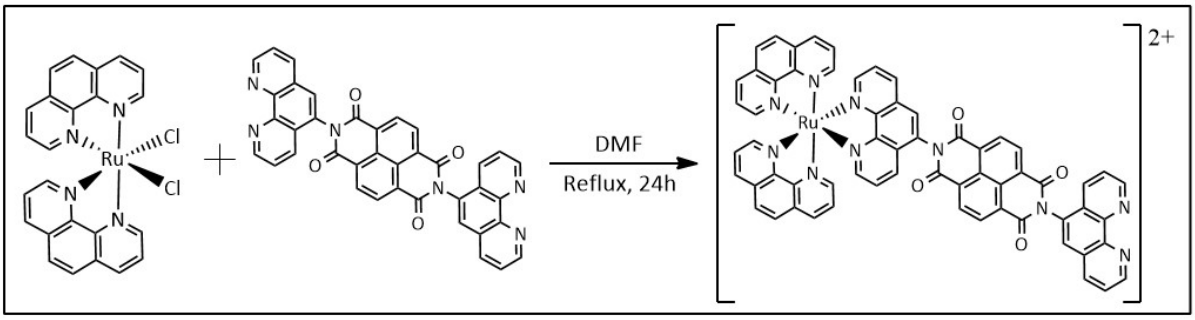

Figure S1. Synthetic pathway for the synthesis of the RuNDI complex

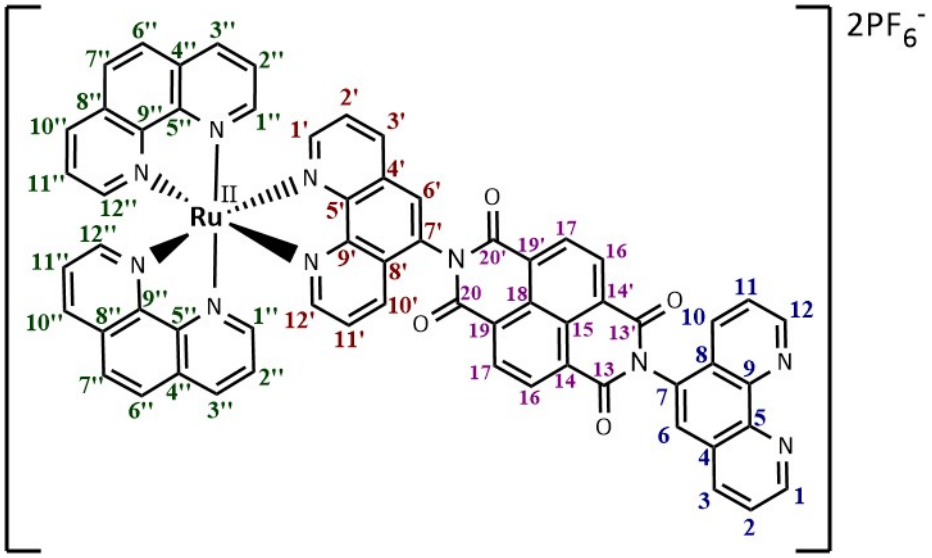

Figure S2. Structure of the RuNDI complex with assigned hydrogen and carbon atoms.

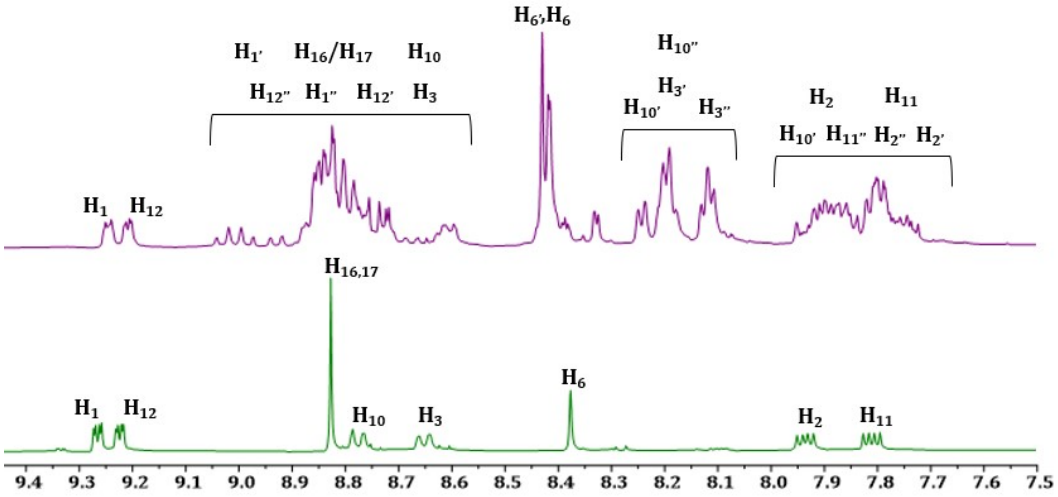

Figure S3. ^{1}H NMR spectra of the deshielded region for the RuNDI complex (purple) and the pNDIp ligand (green) in DMSO-d_6.

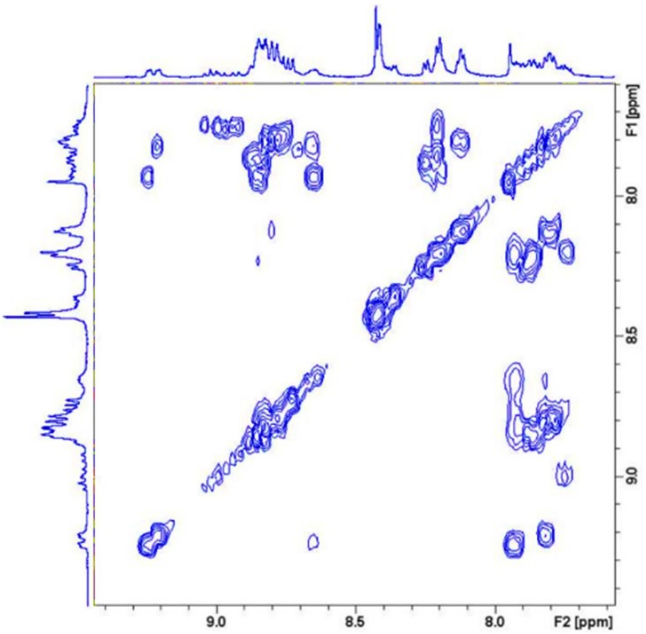

Figure S4. $^{1}H–^{1}H$ COSY NMR spectrum highlighting the deshielded region of the RuNDI complex in DMSO-d_6 at 298 K

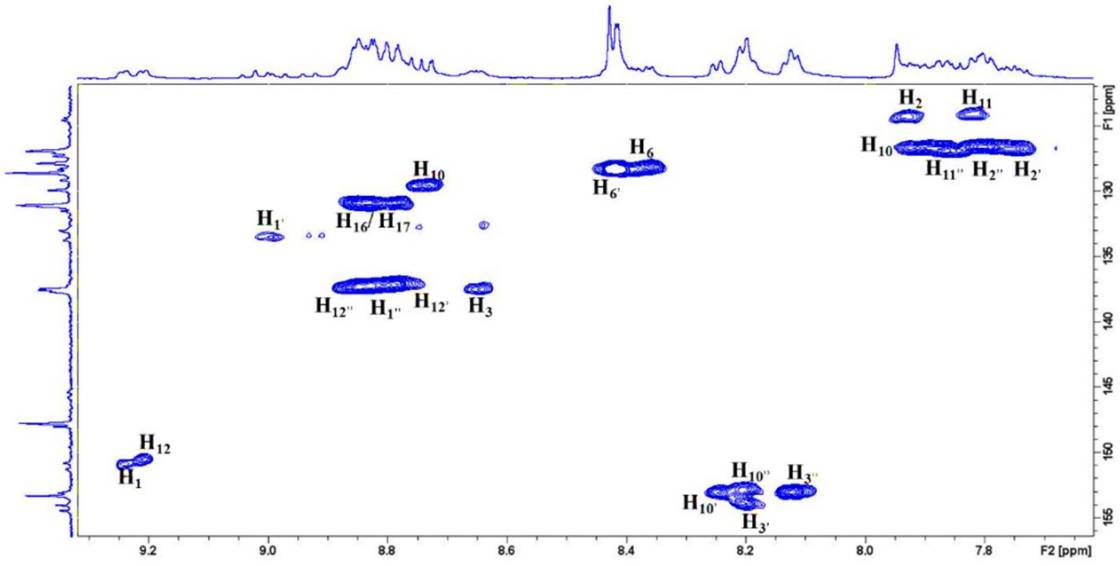

Figure S5. $^{1}H–^{13}C\{^{1}H\}$ HSQC NMR spectrum showing proton–carbon correlations in the deshielded region of the RuNDI complex in DMSO-d_6 at 298 K.

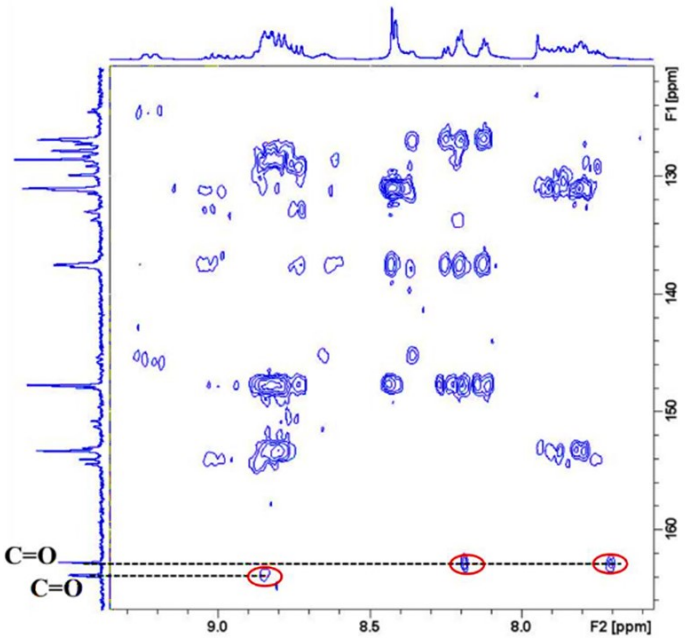

Figure S6. $^{1}H–^{13}C\{^{1}H\}$ HMBC NMR spectrum displaying long-range proton–carbon correlations in the deshielded region of the pNDIp ligand in DMSO-d_6 at 298 K.

Table S1. ^{1}H and ^{13}C NMR spectral assignments for the RuNDI complex in DMSO-d_6, including chemical shift (ppm), multiplicity and proton count. m - multiplet.

	Number	^{13}C - HSQC	^{1}H	^{1}H - ^{13}C – HMBC
phenanthroline	C1'', H1''	137.1	8.81 – 8.84; m; 2H	
	C2'', H2''	126.5	7.77 – 7.83; m; 2H	
	C3'', H3''	153.0	8.08 – 8.15; m; 2H	
	C4''	130.2 – 131.6		
	C5''	147.7		8.13 (H3'')
	C6'', H6''	128.5	8.40 – 8.44; m; 2H	
	C7'', H7''	128.5	8.40 – 8.44; m; 2H	
	C8''	130.2 - 131.6		
	C9''	147.7		8.21 (H12'')
	C10'', H10''	137.3	8.83 – 8.89; m; 2H	
	C11'', H11''	126.8	7.84 – 7.89; m; 2H	
	C12'', H12''	152.8	8.17 – 8.23; m; 2H	
pNDIp (coordinated phen)	C1', H1'	133.5	8.97 – 9.02; m; 1H	
	C2', H2'	126.7	7.71 – 7.78; m; 1H	
	C3', H3'	153.9	8.18 – 8.22; m; 1H	
	C4'	130.2 – 131.6		
	C5'	147.7		8.21 (H3')
	C6', H6'	128.2	8.40 – 8.44; m; 1H	
	C7'	Nd		
	C8'	130.2 – 131.6		
	C9'	147.7		8.25 (H12')
	C10', H10'	137.3	8.76 – 8.81; m; 1H	
	C11', H11'	127.1	7.89 – 7.95; m; 1H	
	C12', H12'	153.0	8.23 – 8.27; m; 1H	
Na	C20, C20'	163.9		8.19 (H12'), 7.70

	(C=O)			(H2')
	C19, C19'	Nd		
	C18	Nd		
	C17, H17	130.8	8.77 – 8.81; m; 2H	
	C16, H16	130.8	8.80 – 8.88; m; 2H	
	C15	Nd		
	C14/C14'	Nd		
pht hal	C13, C13'	163.8		8.85 (H16; H16')
pNDIp (non coordinated phen)	C1, H1	150.9	9.22 – 9.26; m; 1H	
	C2, H2	124.3	7.90 – 7.96; m; 1H	
	C3, H3	137.5	8.62 – 8.67; m; 1H	
	C4	130.2 – 131.6		
	C5	145.2		8. 65 (H3); 9.25 (H1)
	C6, H6	128.1	8.34 – 8.39; m; 1H	
	C7	Nd		
	C8	130.2 – 131.6		
	C9	145.1		8.36 (H6); 9.20 (H10)
	C10, H10	150.5	9.19 – 9.22; m; 1H	
	C11, H11	124.1	7.80 – 7.84; m; 1H	
	C12, H12	129.5	8.71 – 8.77; m; 1H	

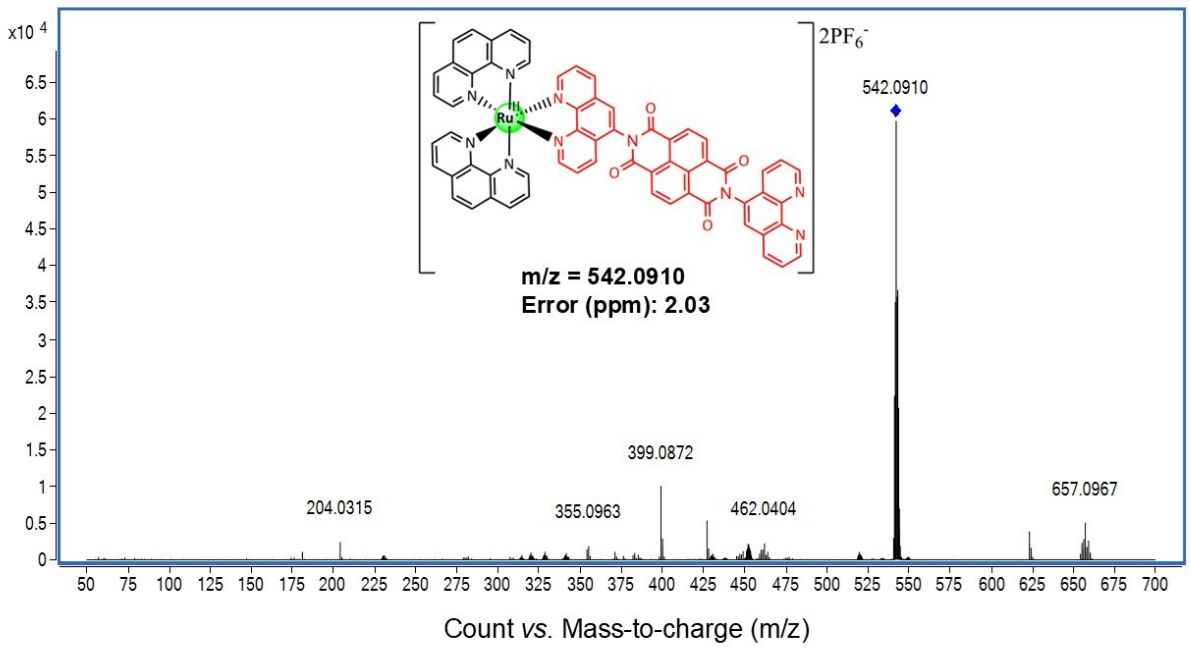

Figure S7. ESI(+)-MS spectrum of the RuNDI complex recorded in acetonitrile.

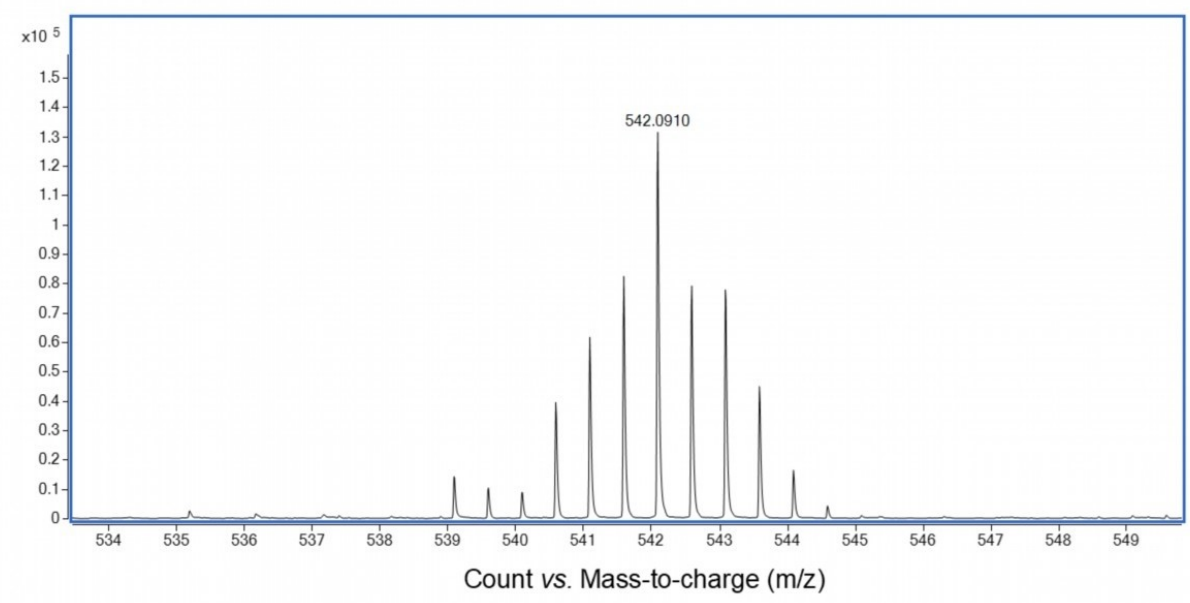

Figure S8. Expanded view of the $[M]^{2+}$ ion region (m/z = 542.0910), showing the characteristic ruthenium isotopic pattern.

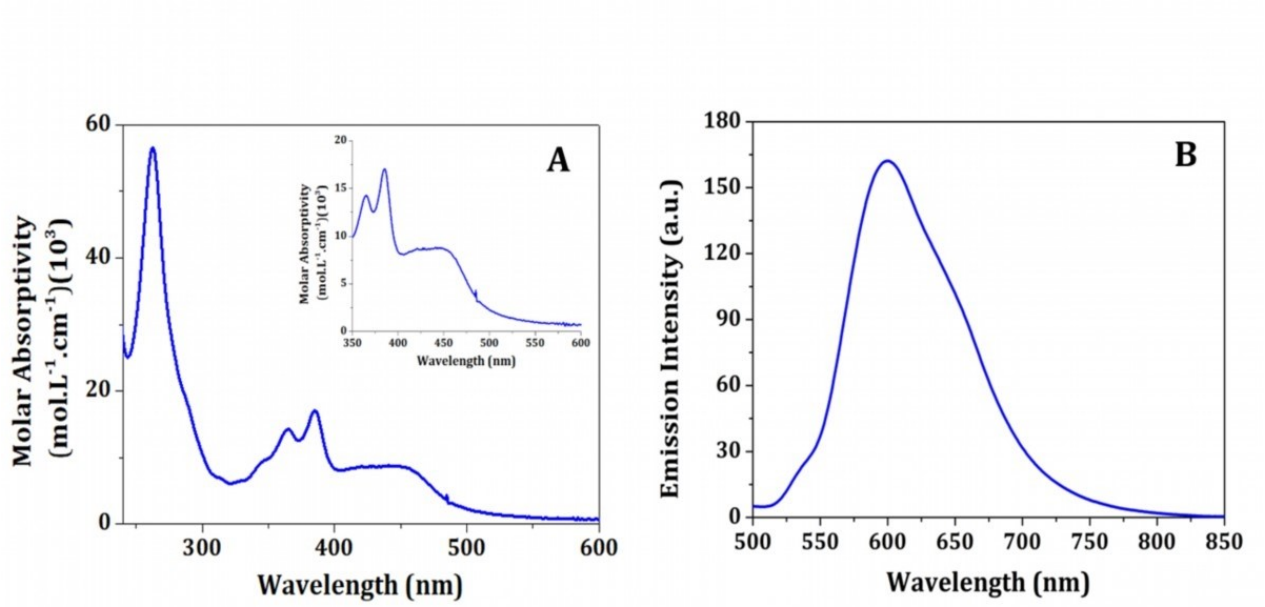

Figure S9. (A) UV–Vis absorption spectrum of the RuNDI complex in PBS, showing the characteristic π–π* and MLCT transition regions. *Inset:* expanded view highlighting the pNDIp and MLCT transitions. (B) Emission spectrum of the same solution (λexc = 450 nm), displaying typical MLCT-based luminescence of Ru(II)–phenanthroline complexes (λmax = 600 nm).

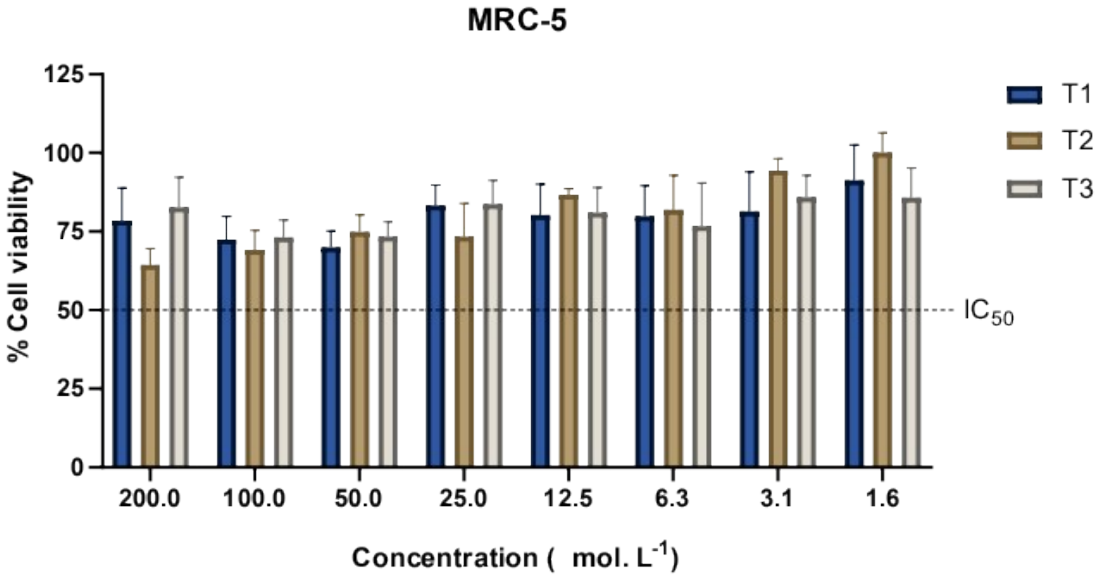

Figure S10. Cell viability graph of the complex in lung non-tumor cell lines (MRC-5) after 24 hours of incubation.

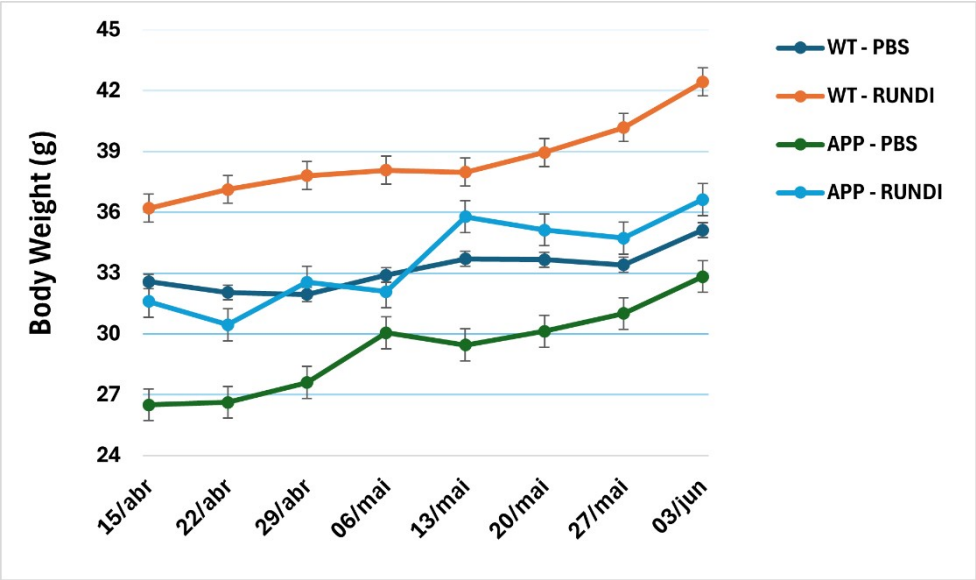

Figure S11. Body weight (g) monitored weekly throughout the treatment period in WT and APP mice receiving PBS or RuNDI: WT–PBS (dark blue), WT–RuNDI (orange), APP–PBS (green), and APP–RuNDI (light blue).

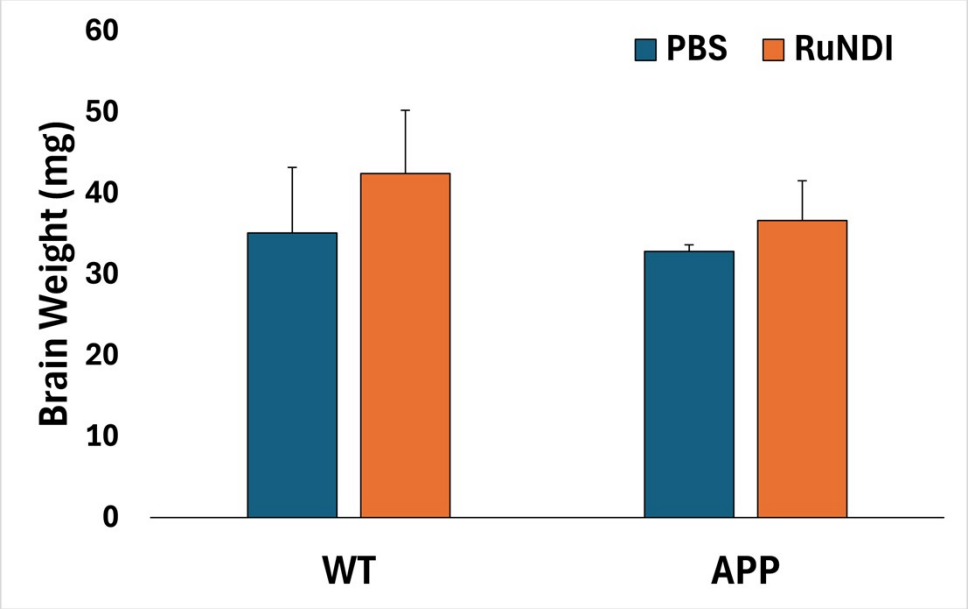

Figure S12. Brain weight (mg) measured at the experimental endpoint for WT–PBS, WT–RuNDI, APP–PBS, and APP–RuNDI.

1 L. Pereira, D. de Oliveira, M. Tiburcio, G. Ribeiro, C. Moraes, F. Neto, A. Camargo, L. De Boni, O. Nascimento, M. Homem and R. Carlos, Exploring the Reversible Equilibrium State between 3CS and 3CSS in a Ru(phen)-Naphthalene Diimide Dyad, *Inorg. Chem.*, DOI:10.1021/ACS.INORGCHEM.4C05443.

2 T. Mosmann, Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays, *J. Immunol. Methods*, 1983, **65**, 55–63.