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Ramu, V., Gill, M.R., Jarman, P.J. et al. (4 more authors) (2015) A cytostatic ruthenium(II)-platinum(II) bis(terpyridyl) anticancer complex that blocks entry into S phase by up-regulating p27KIP1. Chemistry - A European Journal, 21 (25). pp. 9185-9197. ISSN 0947-6539

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Experimental details

General. All chemicals were purchased from Sigma Aldrich unless otherwise indicated. ^{1}H NMR spectra were recorded on a Bruker 500 MHz FT NMR (model: Advance-DPX 500) spectrometer at room temperature (RT, 25 °C). ESI MS measurements were carried out on a Waters QTof-Micro instrument. Microanalyses (C, H, N) were performed using a Perkin-Elmer 4100 elemental analyzer. Infrared spectra were recorded as KBr pellets using a Perkin Elmer Spectra GX 2000 spectrometer. UV-Vis spectra were obtained using a Cary 500 Scan UV-Vis-NIR spectrometer.

Partition Coefficients. n-octanol-saturated water and water-saturated n-octanol were obtained using Millipore water stirred with n-octanol for 24 h before the two layers were separated by centrifugation (3000 rpm, 5 min). Cisplatin and chloride salts of VR52, VR54 and VR63 were dissolved in n-octanol-saturated water. This was then mixed with water-saturated n-octanol in the ratio of 1:1 (v/v). Resulting solvent mixtures were vortexed for 30 min at room temperature, and then were subjected to centrifugation (3000 rpm, 5 min) to obtain two distinct separate layers. Samples from each layer were obtained using a fine-gauge needle and the absorbance of respective complex in each phase determined using high performance liquid chromatography (HPLC) coupled to a UV-vis detector. The concentration in each phase was calculated using reference to calibration absorbance/concentration graphs in each phase and the octanol/water partition coefficient (log P) for each respective complex was then calculated. The value obtained for cisplatin by this method was in agreement with previously published data.[1]

DNA Binding Studies. Solutions of VR52 (3.7×10^{-5} M) or VR54 (1.0×10^{-5} M) (5 mM Tris-HCl, 25 mM NaCl, pH 7.2, 0.5% DMSO) in 1 cm path length optical glass cuvettes were prepared and maintained at 25 °C. The absorption spectra were recorded before 2 µL of a concentrated, known, DNA solution was added, mixed by pipette and allowed to equilibrate for 5 min. Spectra after mixing were recorded. The titration process was repeated until there was no change in the spectrum for at least four titrations indicating binding saturation had been achieved. Absorption data were used to

construct nonlinear Scatchard plots (r/C_f versus r) and fitted to the McGhee-von Hippel model,[2] in which neither the site size nor binding constant was set. Viscosity measurements were carried out in a Cannon-Manning semi-micro viscometer (size 50) immersed in a thermostat bath maintained at 27 °C. The concentration of DNA was kept constant at 0.5 mM (base pairs), and samples were prepared by adding ligand to the DNA solution to give an increase in the ligand/bp ratios. The flow times were measured after thermal equilibration of at least 20 minutes. Each sample was measured three times and the averaged time was used in calculations.

Irreversible binding studies followed the protocol described by Wheate *et al.*.[3] Briefly, each complex was pre-dissolved in DMF and added to guanosine in D_2O (final concentrations 200 μM, 1:1 ratio). The solutions were incubated at 25 °C and the ^1H NMR spectrum of each was recorded at 0, 6, 24 and 48 h timepoints.

Cell Culture. A2780 and A2780CIS cells were cultured in RPMI1640 supplemented with 10% FBS and penicillin/streptomycin. Cell lines were maintained at 37 °C in an atmosphere of 5% CO_2 and routinely sub-cultured. A2780CIS growth media was supplemented with 2 μM cisplatin every fourth passage to maintain cisplatin-resistance.

Cell proliferation (MTT) assay. A2780 or A2780CIS cells were seeded in 48-well plates at a seeding density of $2x10^4$ cells/well and allowed to proliferate for 24 h. Cell cultures were treated with 0 – 200 μM solutions of VR52, VR54, VR63 (final medium composition = 90% cell media, 9.5% PBS, 0.5% DMSO) or cisplatin (90 % cell media, 10 % PBS) in triplicate for 48 h. After incubation, the solutions were removed and 0.5 mg mL^{-1} MTT (thiazolyl blue tetrazolium bromide) dissolved in serum-free media added for 30 min.[4] The formazan product was eluted using acidified isopropanol and the absorbance at 540 nm quantified by plate reader (reference peak 620 nm). An average absorbance for each concentration was obtained and the metabolic activity of the cell population was determined as a percentage of the relative solvent negative control.

Nuclear morphology. Cells were incubated with VR54 (40 μM) or cisplatin (30 μM) for 24 or 48 h, washed with PBS and fixed with ethanol. Slides were stained with 5 μg /mL DAPI and visualized with a Zeiss LSM 510 inverted confocal microscope using a 405 nm diode laser for excitation and emission detected using a 420-480 nm BP filter. At least 200 cells were counted for each experiment.

Cell growth and cell viability (Trypan Blue). A2780CIS cells were treated with VR54 or cisplatin at 0, 40 or 100 μM for 48 h. Cells were detached by scraping and concentrated via centrifugation. Pellets were re-suspended in 1 ml of serum-free medium, a sample of which was stained with Trypan Blue solution (0.4 %) and the number of total cells and Trypan Blue-positive cells counted by haemocytometer. A minimum of 200 cells were counted. For each independent experiment, counts were made in triplicate and an average value used.

Cellular uptake quantification (ICP-MS). A2780CIS cells were treated with 50 μM solutions of VR52, VR54, VR63 or cisplatin or an untreated control in triplicate for 24 h. Cells were washed in PBS and detached with Trypsin. Cell numbers were counted by haemocytometer and each sample then solubilized using 60% nitric acid at 60 °C overnight. Samples were made up to exactly 10 mL using deionized water and the levels of Pt and Ru determined by ICP-MS (see reference [5] for details on machine calibration). For data expressed in ng/mg protein format, the ICP-MS data was plotted against a cell number/protein concentration calibration subsequently determined. Briefly, a range of cell concentrations were lysed ($2.5x10^5$ – $1 x10^6$ cells, lysis protocol as described below), the protein content determined using Bradford reagent and a protein content/cell number plot (R^2 value = 0.99) constructed.

Sub-cellular fractionation. A2780 cells were treated with 50 μM VR54 or control solvent for 24 h prior to subcellular fractionation using standard procedures.[6] Briefly, cells were harvested as above and resuspended in 3 volumes of hypotonic buffer (210 mM sucrose, 70 mM mannitol, 10mM Hepes, pH 7.4, 1 mM EDTA) containing protease inhibitor cocktail (see below). After gentle

homogenization with a Dounce glass homogenizer, cells were subjected to a freeze thaw cycle, and cell lysates were either retained for total lysate analysis by ICP-MS, or centrifuged at 1,000 x *g* for 5 min (Eppendorf 5417 centrifuge) to isolate a nuclear fraction. The supernatant was collected and centrifuged at 10,000 x *g* (Beckman TLS100 Ultracentrifuge, TLS-55 rotor) to pellet the mitochondria-enriched membrane fraction. The supernatant was collected and utilized as a cytosolic fraction. Aliquots of each fraction were retained for protein content analysis by Bradford assay. Total cell lysates or individual subcellular fractions were individually processed by ICP-MS to determine levels of Pt and Ru as described above. Recovery of metals after fractionation compared to unfractionated controls were 102% (Ru) and 106% (Pt).

Cell cycle analysis (Flow Cytometry). Cells were treated with solutions of VR54 or solvent control for 24 h. Samples were harvested by trypsinization, washed with PBS and fixed with 70 % cold ethanol. Cells were RNAase treated, stained with 30 µg /mL propidium iodide (1 h at room temperature) and the DNA content analyzed using a Biosciences LSRII Flow Cytometer. Data were processed for cell cycle phase apportionment using FloJo software.

Immunoblot Analysis (Western Blotting). A2780 or A2780CIS cells were treated for indicated times with solutions of each compound at the stated concentration. After treatment, unless otherwise indicated, the cells were collected and lysed in lysis buffer (20 mM Tris, pH 7.5, 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, protease inhibitors (10 µg/mL leupeptin, 2 µg/mL pepstatin, 50 µg/mL antipain, 2 µg/mL aprotinin, 20 µg/mL chymostatin and 2 µg/mL benzamidine), and phosphatase inhibitors (50 mM NaF, 1 mM Na_3VO_4 and 20 mM Na β-glycerophosphate)). Aliquots of cell lysates were resolved by 8-15% SDS-PAGE, transferred onto nitrocellulose membrane and probed with the appropriate antibodies. Antibodies against cleaved caspase 3, p-Chk1 (Ser345), p-H2AX (Ser139), p-Chk2 (Ser516), Chk2, p-p53 (Ser20), p27, p-Rb (Ser780), Rb, and Lamin A/C were obtained from Cell Signaling and used at the recommended dilution. Anti-β-actin and α-tubulin monoclonal antibodies (1/5000 dilution) were obtained from Sigma. Antibodies against p21 (1/1000 dilution) were obtained from Santa Cruz Biotech. Anti-Tom20 antibodies were a kind gift from Prof. O.

Bandmann (University of Sheffield). Anti-Chk1 was generated in-house.[7] HRP-conjugated anti–rabbit or anti–mouse antibodies (Santa Cruz Biotech.) were used at 1/5000 dilution. Blots were visualized using ECL or ECL+ chemiluminescence reagents (GE Healthcare Life Sciences) with X-ray development (Fuji medical film and Optimax 2010 processor).

Supporting Figures.

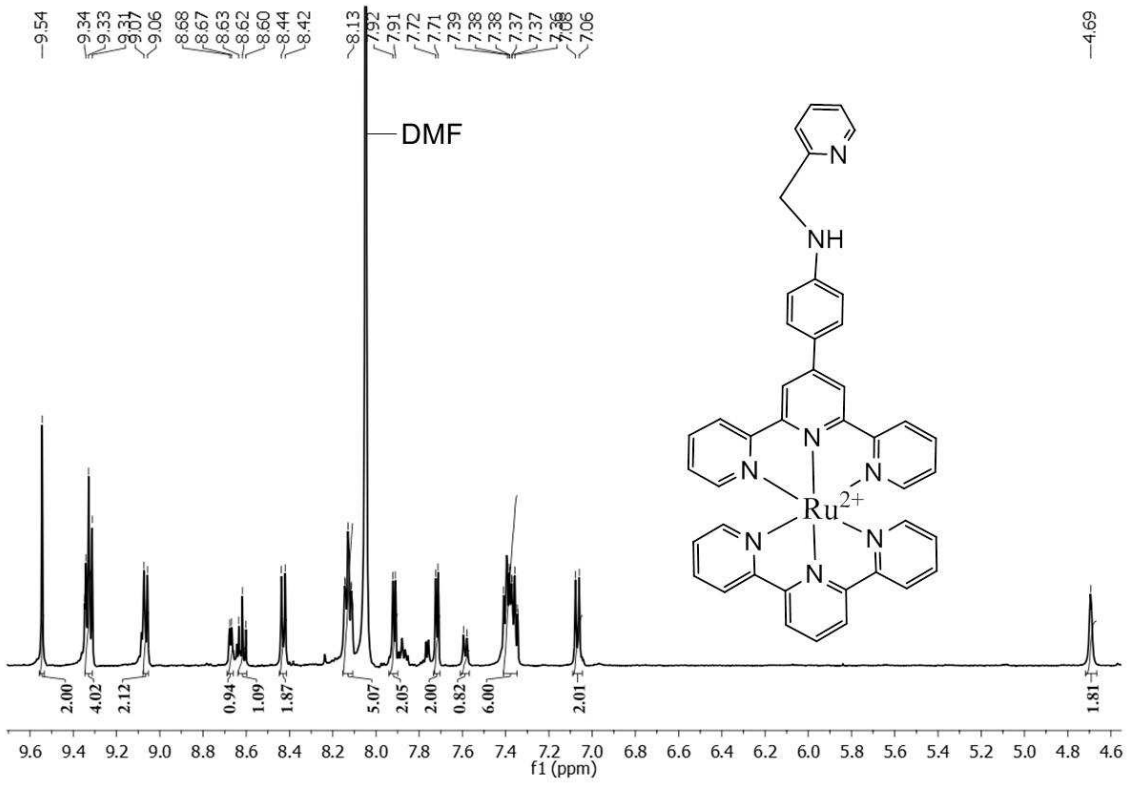

Figure S1 ^{1}H NMR spectrum of VR52 recorded in DMF-d_7.

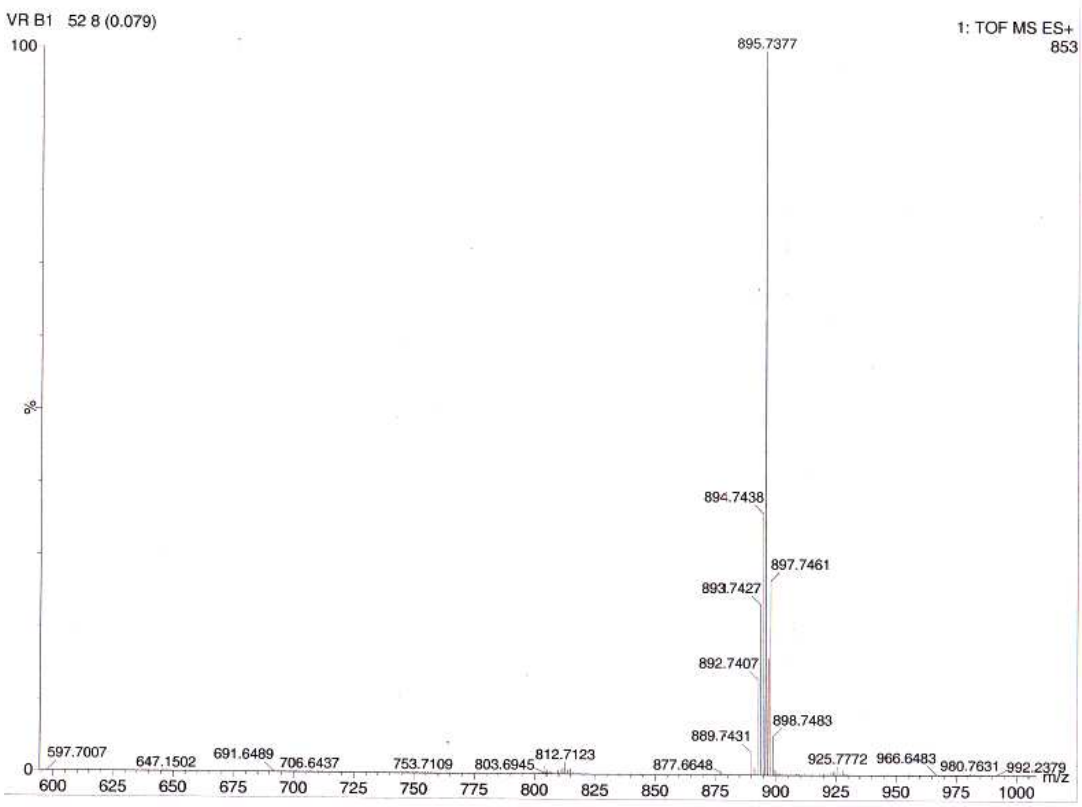

Figure S2 ESIMS mass spectrum of VR52.

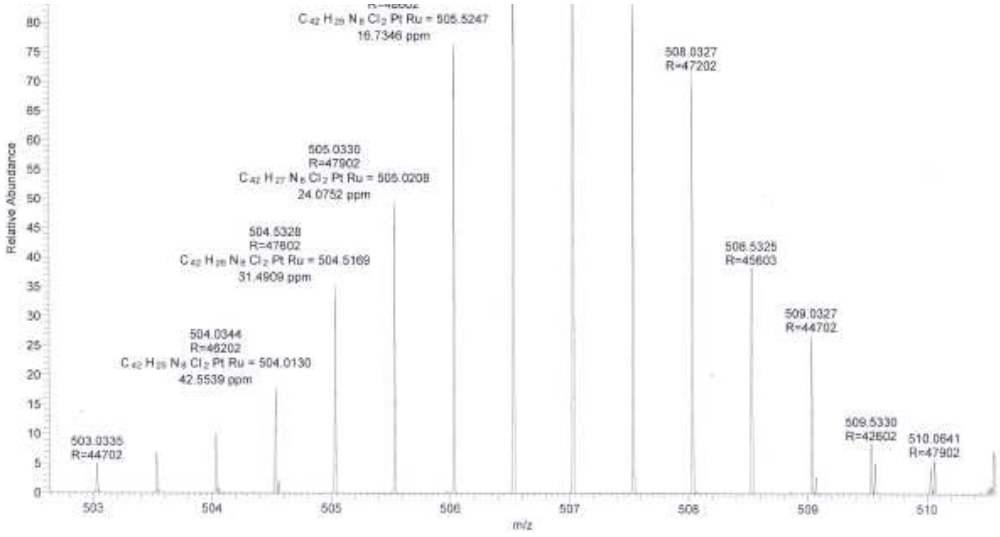

Figure S3 ESIMS mass spectrum of intermediate complex $[Ru(tpy)(tpypma)Pt(Cl)_2]^{2+}$ (VR53).

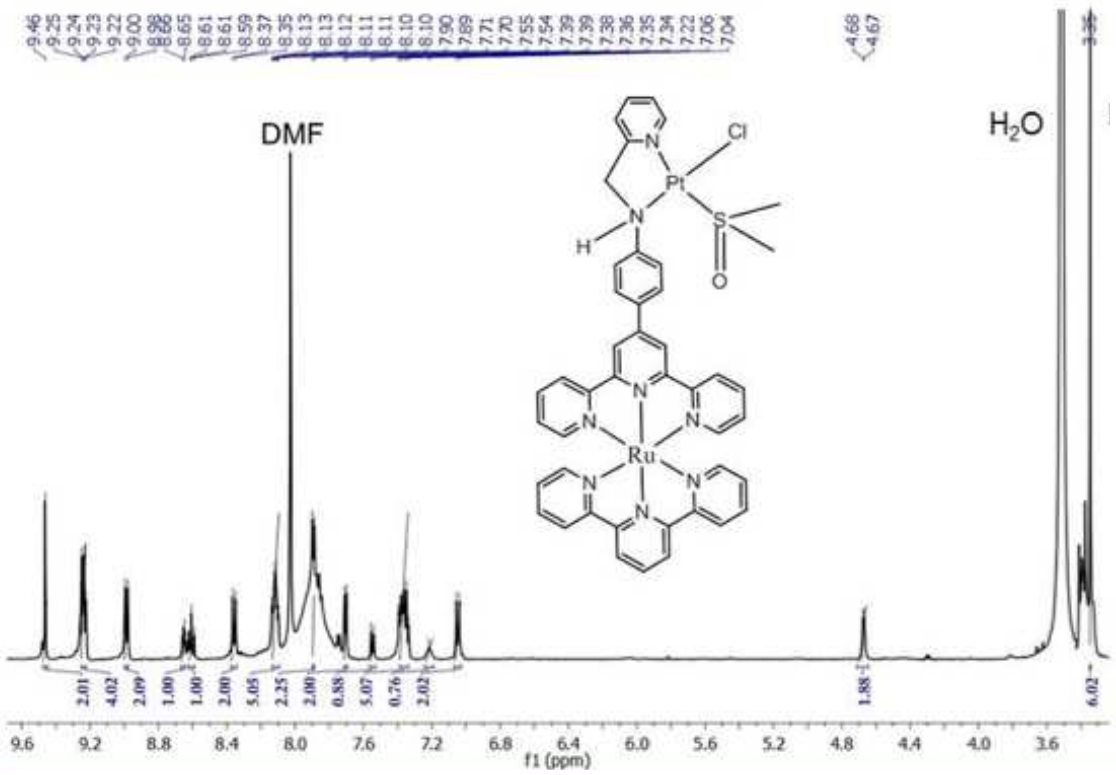

Figure S4 ^{1}H NMR spectrum of VR54 recorded in DMF-d_7. Solvent impurities are indicated.

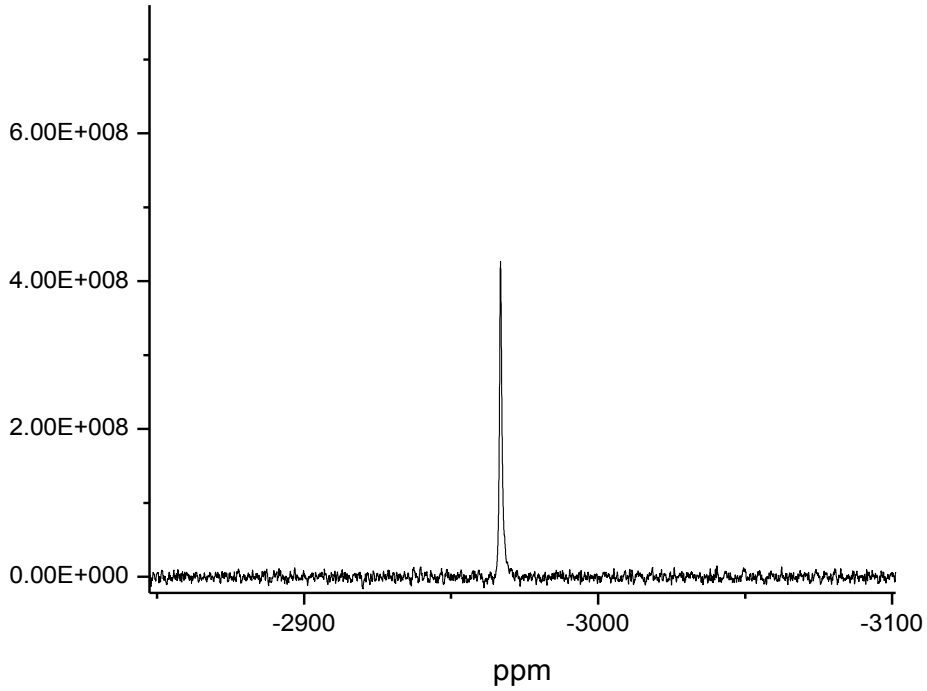

Figure S5 ^{195}Pt NMR spectrum of VR54.

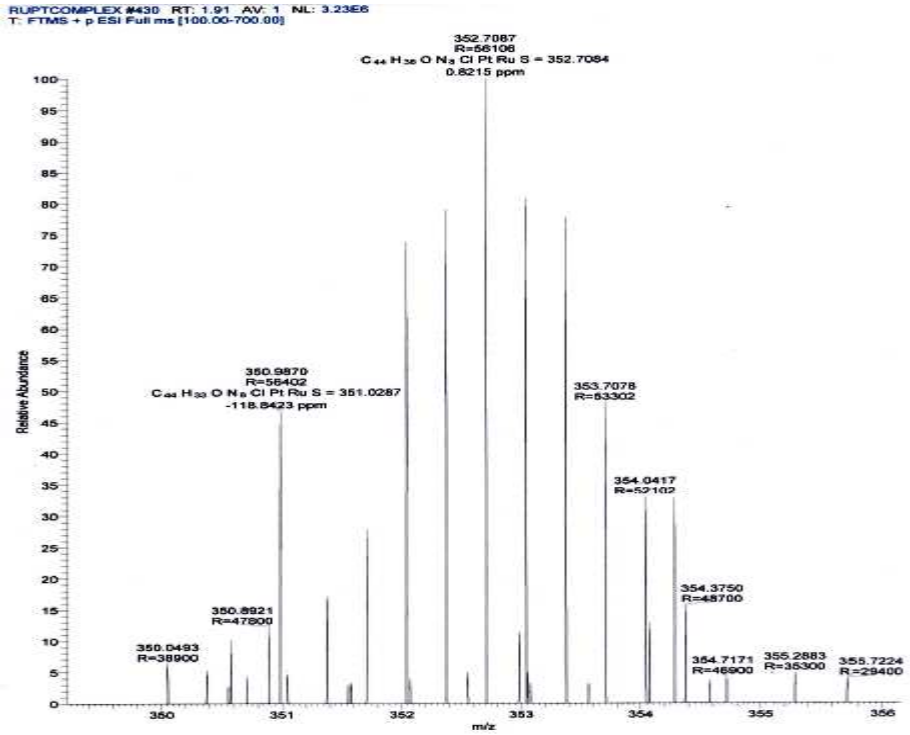

Figure S6 ESIMS mass spectrum of VR54.

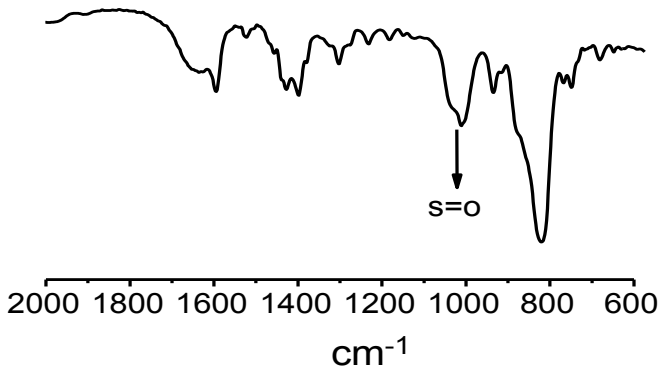

Figure S7 IR spectrum of VR54. The *v*(SO) stretching frequency is highlighted.

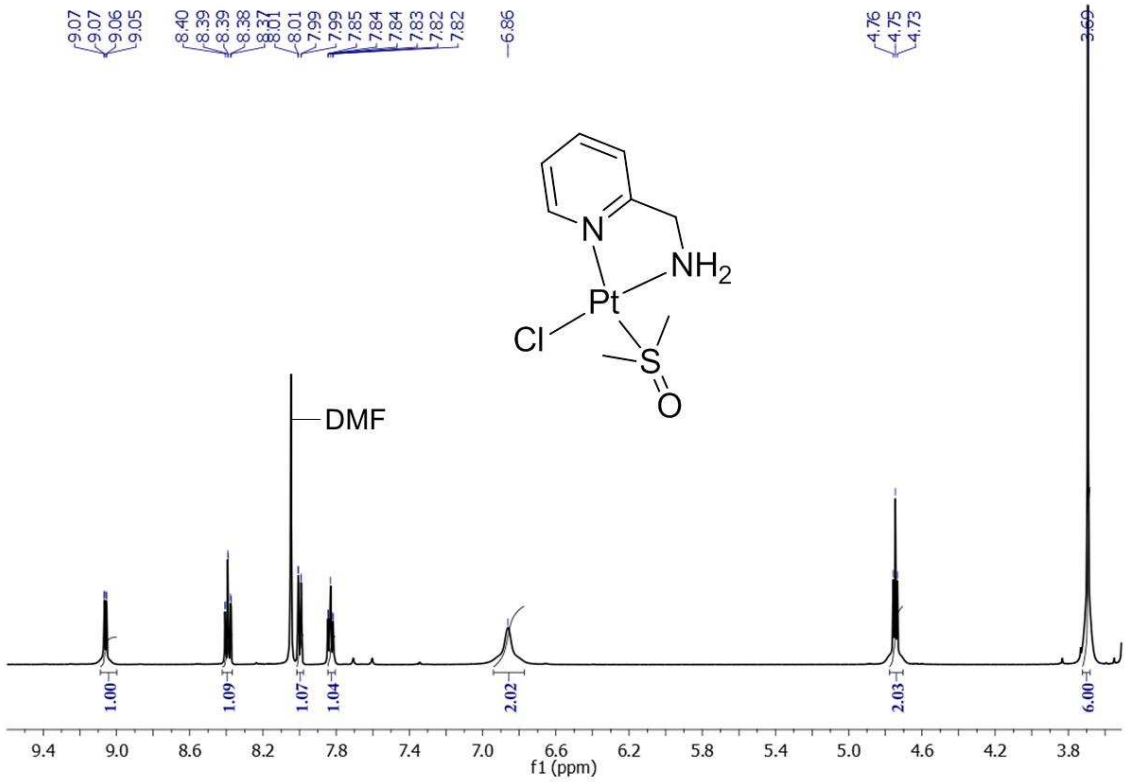

Figure S8 ^{1}H NMR spectrum of VR63 recorded in DMF-d_7.

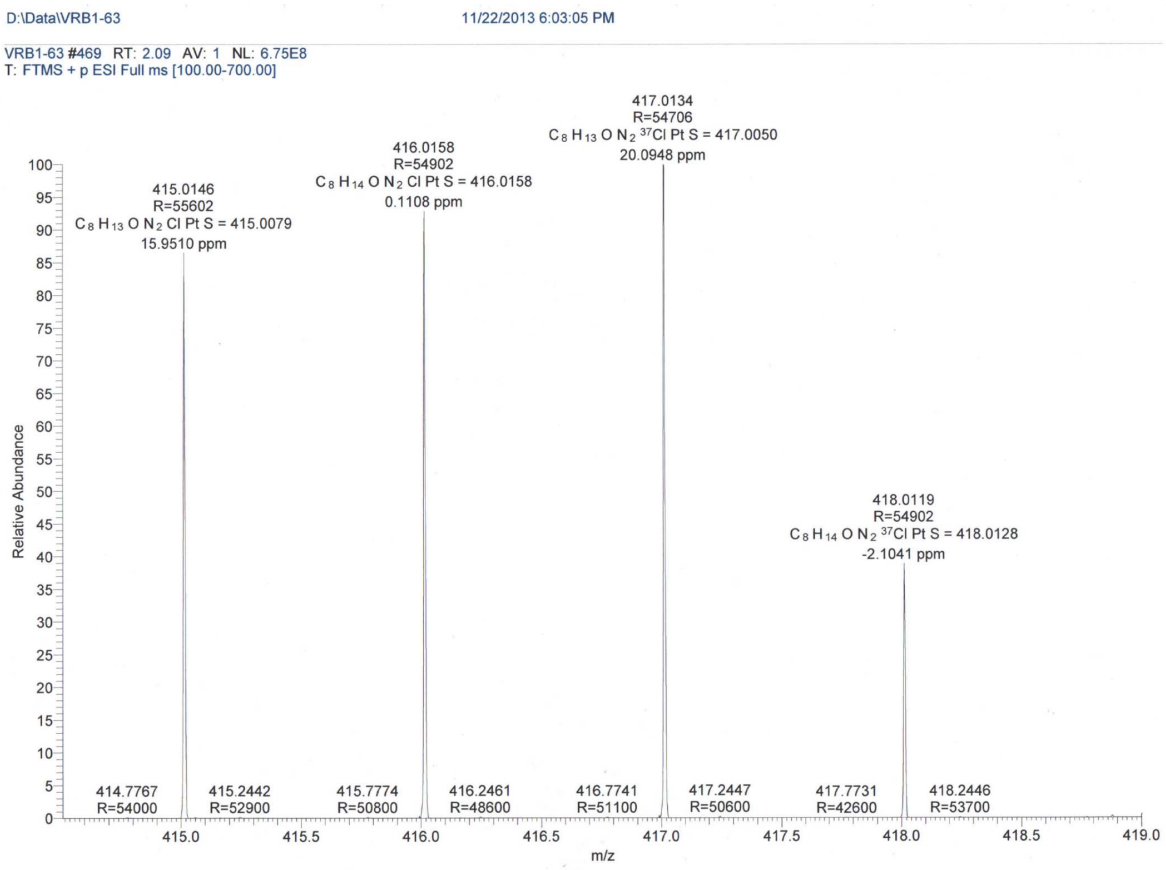

Figure S9 ESIMS mass spectrum of VR63.

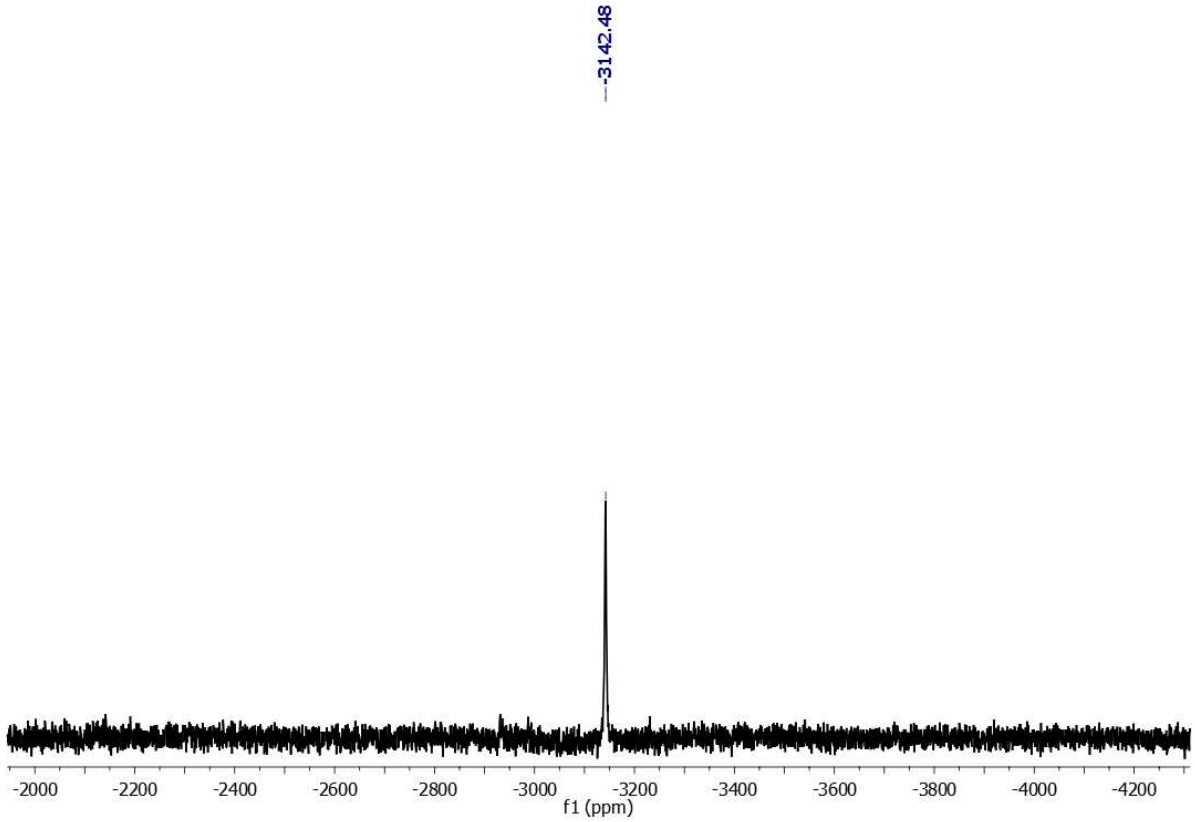

Figure S10 ^{195}Pt NMR spectrum of VR63.

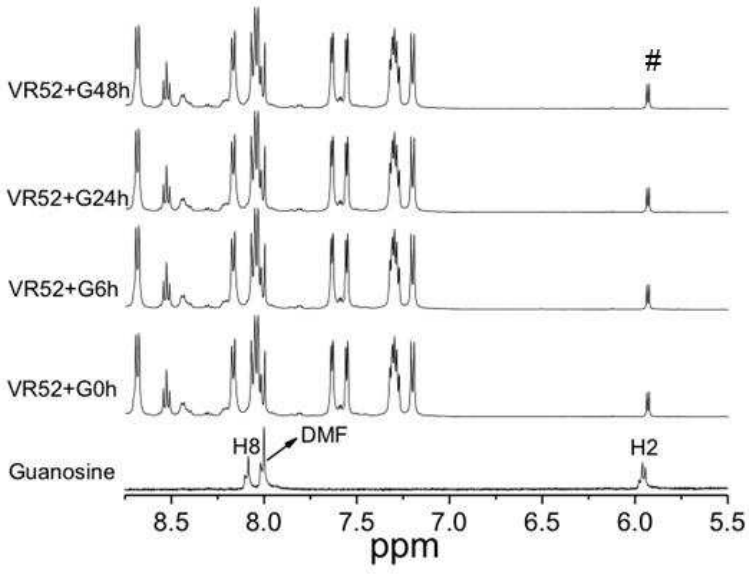

Figure S11. ^{1}H NMR spectra showing the extent of reaction of guanosine (G) with VR52. Based on reference [3], peaks labelled * are bound guanosine and peaks labelled # are free guanosine. The residual DMF solvent peak used for complex dissolution before addition to guanosine is indicated.

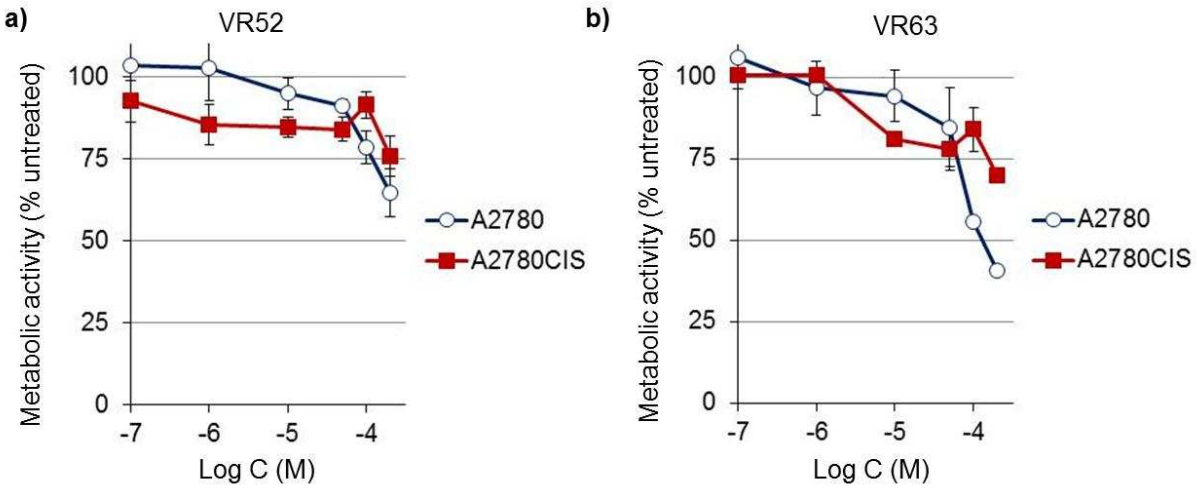

Figure S12 Effect of VR52 (a) or VR63 (b) (0-200 µM, 48 h incubation time) on metabolic activity of A2780 and A2780CIS cells, as assessed by MTT assay (n = 2, ± SEM). See Table 2 for details on the derived IC_{50} values (where applicable).

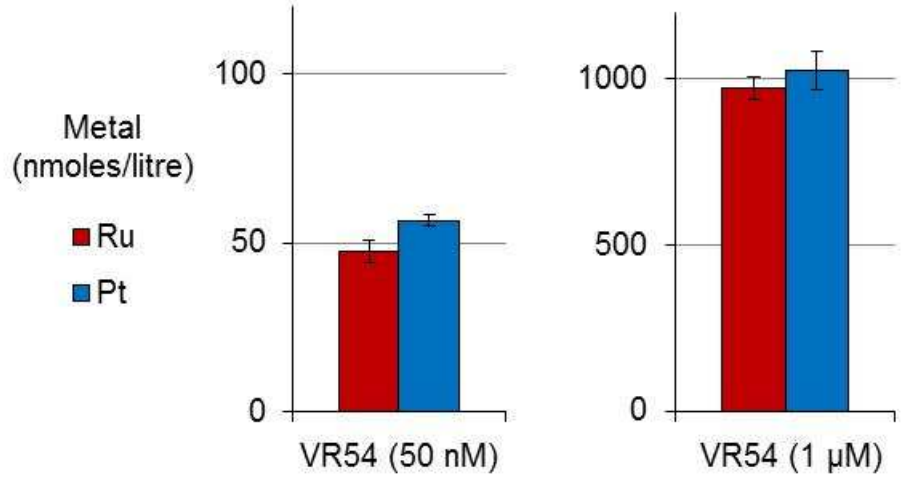

Figure S13 ICP-MS analysis of Ru and Pt content of VR54 stock solutions (0.5% DMSO, 99.5% PBS).

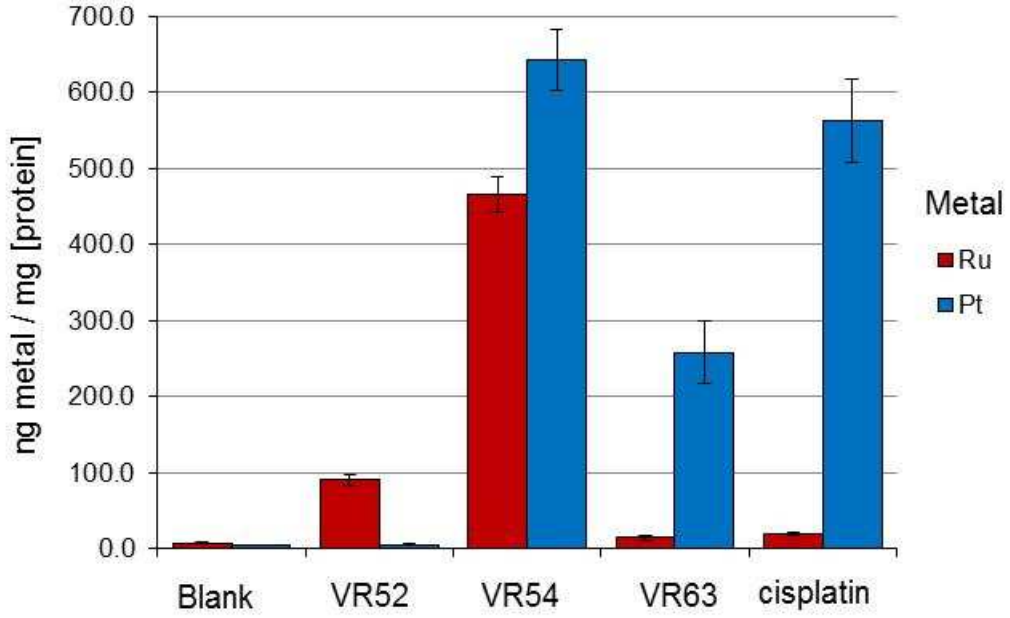

Figure S14 Relative uptake of VR52, VR54, VR63 and cisplatin. ICP-MS data of Ru and Pt content of A2780CIS cells after incubation with VR52, VR54, VR63 or cisplatin (50 µM, 24 hrs) subsequently normalized to ng (metal) per mg cellular protein, determined by Bradford assay.

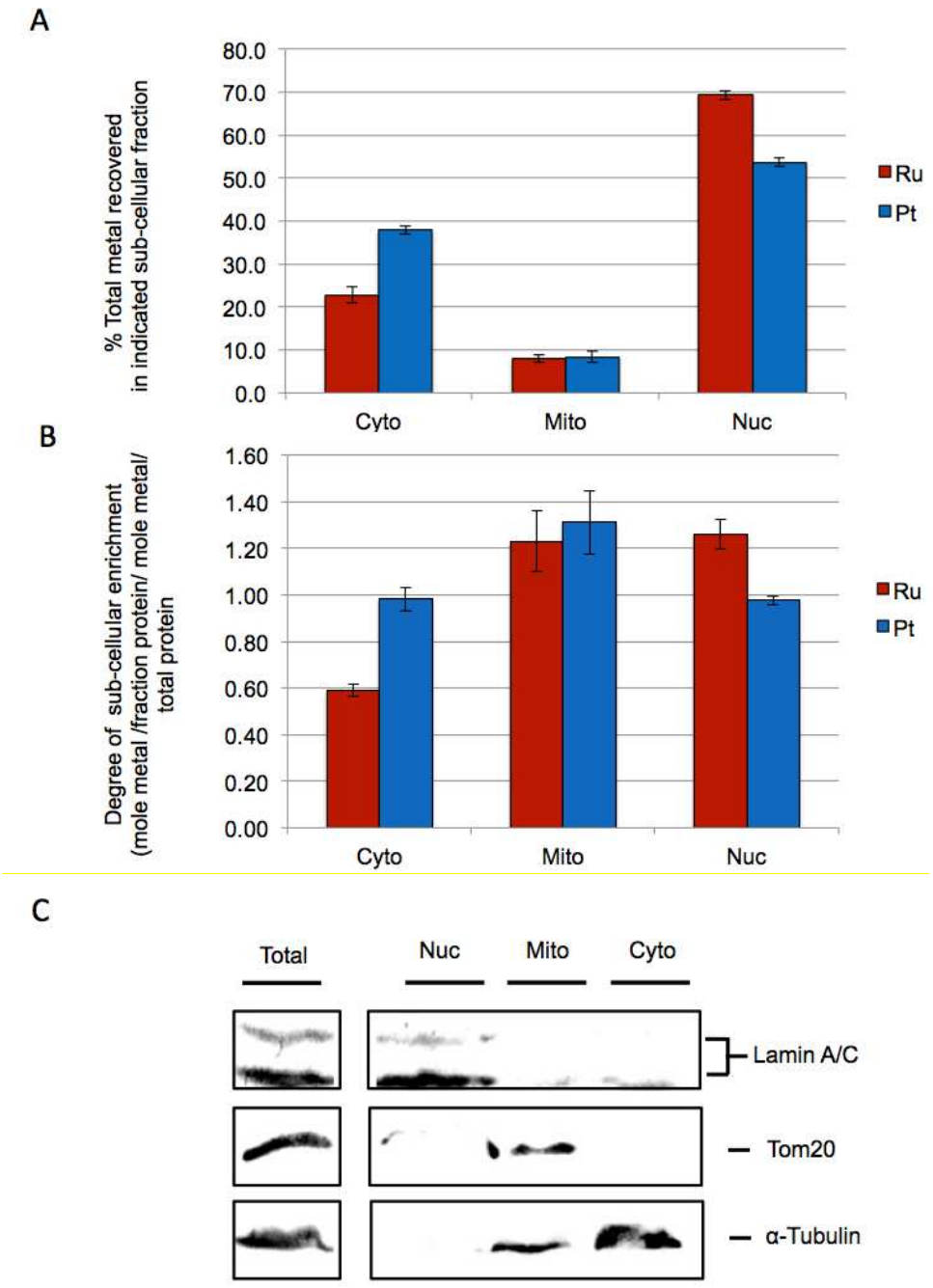

Figure S15 Sub-cellular distribution of VR54. (a,b) ICP-MS data showing relative Ru and Pt content of sub-cellular fractions from A2780 cells after incubation of cells with VR54 (50 μM, 24 hrs). (N = 2, +/- SD). (a) Data shown is the fraction of Pt and Ru in each sub-cellular fraction expressed as a proportion of the total amount of metal taken up in unfractionated cells. Recovery of metals after fractionation compared to unfractionated controls were 102% (Ru) and 106% (Pt). (b) Data in (a) subsequently expressed as amount of metal per mg protein in each fraction, normalised to the amount of metal per mg of total cell protein. See reference [8]. (c) Immunoblot of unfractionated (Total) or equal protein loadings (determined by Bradford assay) of nuclear- (Nuc), mitochondrial- (Mito), and cytosolic- (Cyto) enriched subcellular fractions for the nuclear marker, lamin A/C,[9] the mitochondrial marker, Tom20,[10] and the cytoplasmic marker, α-tubulin.[11]

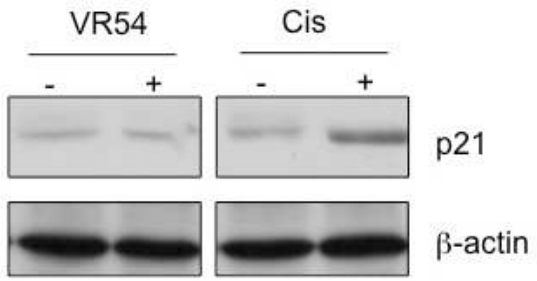

Figure S16. Exposure to VR54 has no effect on $p21^{CIP1}$ levels. A2780 cells growing in serum-containing medium were treated with 0.1% DMSO (control), 50 μM VR54, or 10 μM Cisplatin for 24 h prior to lysate preparation and immunoblotting with anti-p21 antibodies. β-actin levels were monitored as a loading control.

References

[1] J. A. Platts, S. P. Oldfield, M. M. Reif, A. Palmucci, E. Gabano, D. Osella, *J. Inorg. Biochem.* **2006**, *100*, 1199-1207.
[2] J. D. McGhee, P. H. von Hippel, *J. Mol. Biol.* **1974**, *86*, 469-489.
[3] N. J. Wheate, B. J. Evison, A. J. Herlt, D. R. Phillips, J. G. Collins, *Dalton Trans.* **2003**, 3486-3492.
[4] T. Mosmann, *J. Immunol. Methods* **1983**, *65*, 55-63.
[5] M. R. B. Binet, R. Ma, C. W. McLeod, R. K. Poole, *Anal. Biochem.* **2003**, *318*, 30-38.
[6] W. Hu, F. Wang, J. Tang, X. Liu, Z. Yuan, C. Nie, Y. Wei, *J. Biol. Chem.* **2012**, *287*, 68-80.
[7] C. Feijoo, C. Hall-Jackson, R. Wu, D. Jenkins, J. Leitch, D. M. Gilbert, C. Smythe, *J. Cell Biol.* **2001**, *154*, 913-924.
[8] C. de Duve, B. C. Pressman, R. Gianetto, R. Wattiaux, F. Appelmans, *Biochem. J.* **1955**, *60*, 604-617.
[9] J. Gotzmann, R. Foisner, *Crit Rev Eukaryot Gene Expr.* **1999**, *9*, 257-265.
[10] K. Terada, M. Kanazawa, M. Yano, B. Hanson, N. Hoogenraad, M. Mori, *FEBS Lett.* **1997**, *403*, 309-312.
[11] M. Osborn, K. Weber, *Proc. Natl. Acad. Sci. U. S. A.* **1976**, *73*, 867-871.