Supporting Information

Organometallic anticancer complexes of lapachol: metal centre-dependent formation of reactive oxygen species and correlation with cytotoxicity

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Table of contents

Materials and methods

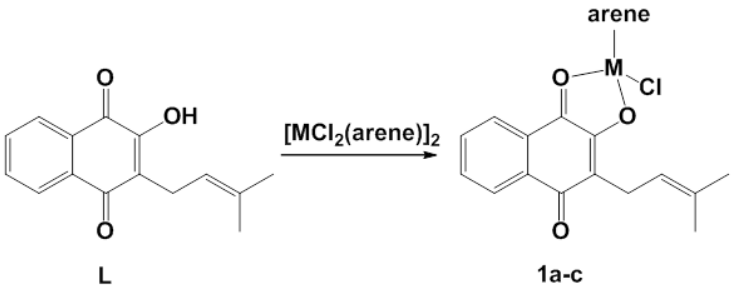
Absolute methanol was dried according to standard procedures. Lapachol, sodium methoxide and pentamethylcyclopentadiene were purchased from Sigma Aldrich, α-terpinene from Acros Fischer, rutheniumtrichloride trihydrate from Degussa, osmiumtetroxide and rhodiumtrichloride trihydrate from Johnson Matthey, hydrazinedihydrochloride from Fluka, and hydrochloric acid from Merck. All these chemicals were used without further purification.

The ruthenium- and osmium-arene starting compounds $[M(\eta^6\text{-}p\text{-cymene})(Cl)(\mu\text{-}Cl)]_2$($M = Ru^{II}$, Os^{II}) and the rhodium-arene starting compound $[Rh(Cp^*)(Cl)(\mu\text{-}Cl)]_2$ were prepared as described in literature.[1-3]

^1H-, $^{13}C\{^1H\}$- and two-dimensional NMR spectra were recorded at 298 K on a Bruker Avance III 500 MHz spectrometer at 500.10 (^1H) and 125.75 MHz (^{13}C). Elemental analyses were performed by the Microanalytical Laboratory of the Faculty of Chemistry of the University of Vienna, on a Perkin Elmer 2400 CHN elemental analyser. Electrospray ionisation mass spectrometry (ESI-MS) was carried out with a Bruker Esquire 3000 instrument (Bruker Daltonics, Bremen, Germany).

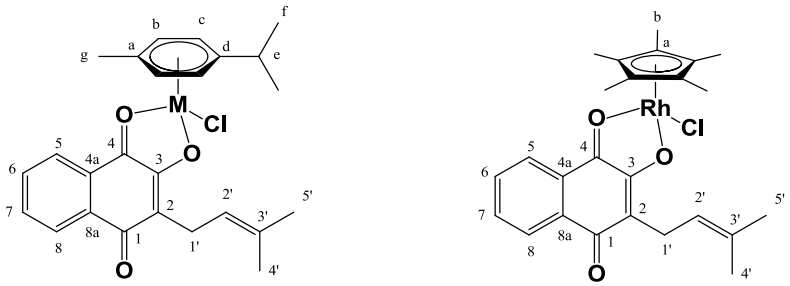
X-ray diffraction analyses of **1a** and **1b** were performed on a Bruker X8 APEX II CCD diffractometer at 150 K. Single crystals were positioned at 40 mm from the detector and 1144 frames for 5 s exposure time over 1° scan width were measured for **1a**, and 449 frames for 60 s over 1° scan for **1b**.

Synthesis of complexes – General procedure



Scheme S1. Synthetic pathway to the lapachol complexes **1a–c** (M = Ru^{II}, Os^{II}, Rh^{III}; arene = η^6-*p*-cymene for Ru^{II}, Os^{II} and η^5-pentamethylcyclopentadiene for Rh^{III}).

A solution of the corresponding arene dimer complex in dry dichloromethane was added to a solution of lapachol and methoxide in dry methanol. The reaction mixture was stirred for 4.5 or 24 h at room temperature and under argon atmosphere. The solvent was evaporated *in vacuo*, the residue was dissolved in dichloromethane and filtered. The filtrate was concentrated under reduced pressure to a volume of about 3 mL, and *n*-hexane was added to give the pure product (needles, crystals) in very good yields (75-96%).



Scheme S2. Numbering scheme for NMR signal assignment (M = Ru^{II}, Os^{II}).

Chlorido[(3-(3-methylbut-2-enyl)-2-oxo-κ*O*)-[1,4]-naphthoquinonato-κ*O*](η^6-*p*-cymene)ruthenium(II) (1a)

The reaction was performed according to the general procedure, using lapachol (88 mg, 0.36 mmol, 1 eq), NaOMe (22 mg, 0.40 mmol, 1.1 eq) and bis[(η^6-*p*-cymene)dichloridoruthenium(II)] (100 mg, 0.16 mmol, 0.9 eq). The reaction mixture was stirred for 4.5 hours. Yield: 92%; mp 148–150 °C. ^{1}H NMR (500.10 MHz, $CDCl_3$) δ: 8.00 (d, ^3J(H,H) =

8 Hz, 1H, H-5), 7.96 (d, ^{3}J(H,H) = 8 Hz, 1H, H-8), 7.68 (ddd, ^{3}J(H,H) = 8 Hz, ^{3}J(H,H) = 8 Hz, ^{4}J(H,H) = 2 Hz, 1H, H-7), 7.51 (ddd, ^{3}J(H,H) = 8 Hz, ^{3}J(H,H) = 8 Hz, ^{4}J(H,H) = 2 Hz, 1H, H-6), 5.80-5.73 (m, 2H, H-c), 5.54-5.46 (m, 2H, H-b), 5.31-5.26 (m, 1H, H-2'), 3.43-3.25 (m, 2H, H-1'), 3.03-2.96 (m, 1H, H-e), 2.40 (s, 3H, H-g), 1.82 (s, 3H, H-4'), 1.68 (s, 3H, H-5'), 1.42 (d, ^{3}J(H,H) = 8 Hz, 6H, H-f). $^{13}C\{^{1}H\}$ NMR (125.75 MHz, d_4-CH_3OH) δ: 196.20 (C_4), 182.65 (C_1), 169.11 (C_3), 136.10 (C_7), 132.60 (C_{8a}), 131.65 (C_6), 131.20 ($C_{3'}$), 127.91 (C_{4a}), 125.80 (C_5), 125.69 (C_8), 125.28 (C_2), 121.59 ($C_{2'}$), 100.48 (C_d), 96.92 (C_a), 81.03 (C_c), 78.97 (C_b), 31.14 (C_e), 24.29 ($C_{5'}$), 21.57 ($C_{1'}$), 21.02 (C_f), 17.07 (C_g), 16.57 ($C_{4'}$). (ESI$^+$): *m/z* 477.11 $[M - Cl]^+$. Anal. Calc. for $C_{25}H_{27}ClO_3Ru \cdot 0.5H_2O$: C 57.63%, H 5.42%. Found: C 57.57%, H 5.11%.

Chlorido[(3-(3-methylbut-2-enyl)-2-oxo-κ*O*)-[1,4]-naphthoquinonato-κ*O*](η^6-*p*-cymene)osmium(II) (1b)

The reaction was performed according to the general procedure, using lapachol (65 mg, 0.27 mmol, 1 eq), NaOMe (16 mg, 0.29 mmol, 1.1 eq) and bis[(η^6-*p*-cymene)dichloridoruthenium(II)] (100 mg, 0.13 mmol, 0.95 eq). The reaction mixture was stirred for 4.5 hours. Yield: 75%; mp 148–150 °C. ^{1}H NMR (500.10 MHz, $CDCl_3$) δ: 8.02 (d, ^{3}J(H,H) = 8 Hz, 1H, H-5), 8.00 (d, ^{3}J(H,H) = 8 Hz, 1H, H-8), 7.72 (td, ^{3}J(H,H) = 8 Hz, ^{3}J(H,H) = 8 Hz, ^{4}J(H,H) = 2 Hz, 1H, H-7), 7.54 (td, ^{3}J(H,H) = 8 Hz, ^{3}J(H,H) = 8 Hz, ^{4}J(H,H) = 2 Hz, 1H, H-6), 6.25-6.19 (m, 2H, H-c), 5.96-5.89 (m, 2H, H-b), 5.31-5.25 (m, 1H, H-2'), 3.40-3.29 (m, 2H, H-1'), 2.89-2.80 (m, 1H, H-e), 2.43 (s, 3H, H-g), 1.80 (s, 3H, H-4'), 1.67 (s, 3H, H-5'), 1.39 (d, ^{3}J(H,H) = 8 Hz, 6H, H-f). $^{13}C\{^{1}H\}$ NMR (125.75 MHz, $CDCl_3$) δ: 198.61 (C_4), 182.75 (C_1), 169.78 (C_3), 136.30 (C_7), 132.91 (C_{8a}), 132.05 ($C_{3'}$), 131.34 (C_6), 127.92 (C_{4a}), 127.01 (C_2), 126.71 (C_5), 126.48 (C_8), 121.71 ($C_{2'}$), 90.80 (C_d), 87.95 (C_a), 72.42 (C_c), 69.10 (C_b), 32.11 (C_e), 25.80 ($C_{5'}$), 22.77 ($C_{1'}$), 22.64 (C_f), 19.12 (C_g), 18.15 ($C_{4'}$). (ESI$^+$): *m/z* 567.16 $[M-Cl]^+$. Anal. Calc. for $C_{25}H_{27}ClO_3Os$: C 49.95%, H 4.53%. Found: C 49.94%, H 4.22%.

Chlorido[(3-(3-methylbut-2-enyl)-2-oxo-κ*O*)-[1,4]-naphthoquinonato-κ*O*](η^5-pentamethylcyclopentadienyl)rhodium(III) (1c)

The reaction was performed according to the general procedure, using lapachol (83 mg, 0.34 mmol, 1.00 eq), NaOMe (20 mg, 0.38 mmol, 1.10 eq) and bis[(η^6-*p*-cymene)dichloridoruthenium(II)] (100 mg, 0.16 mmol, 0.95 eq). The reaction mixture was

stirred for 24 hours. Yield: 96%; mp 144–146 °C. ^{1}H NMR (500.10 MHz, $CDCl_3$) δ: 7.99 (d, ^{3}J(H,H) = 7 Hz, 1H, H-5), 7.98 (d, ^{3}J(H,H) = 7 Hz, 1H, H-8), 7.69-7.64 (m, 1H, H-7), 7.51-7.46 (m, 1H, H-6), 5.36-5.30 (m, 1H, H-2'), 3.42-3.24 (m, 2H, H-1'), 1.82 (s, 3H, H-4'), 1.67 (s, 3H, H-5'). ^{13}C{^{1}H} NMR (125.75 MHz, CDCl3) δ: 194.79 (C_4), 182.94 (C_1), 168.54 (C_3), 135.68 (C_7), 133.48 (C_{8a}), 131.33 ($C_{3'}$), 131.04 (C_6), 129.08 (C_{4a}), 126.33 (C_5), 126.23 (C_8), 125.64 (C_2), 122.55 ($C_{2'}$), 92.90 (C_a), 25.83 ($C_{5'}$), 22.79 ($C_{1'}$), 18.21 ($C_{4'}$), 9.07 (C_b). (ESI^{+}): *m/z* 479.10 [M-Cl]$^{+}$. Anal. Calc. for $C_{25}H_{28}ClO_3Rh$: C 58.32%, H 5.48%. Found: C 58.59%, H 5.62%.

Crystallographic Structure Determination

Table S1. Crystal data and details of data collection for **1a** and **1b**

	1a	**1b**
Chemical formula	$C_{25}H_{27}ClO_3Ru$	$C_{25}H_{27}ClO_3Os$
M (g mol^{-1})	511.99	601.12
Temperature (K)	150(2)	150(2)
Crystal size (mm)	0.60 × 0.10 × 0.10	0.12 × 0.10 × 0.02
Crystal color, shape	blue, block	violet, block
Crystal system	monoclinic	monoclinic
Space group	$P2_1/n$	$P2_1/n$
a (Å)	12.8956(5)	13.4764(4)
b (Å)	11.8913(4)	8.4120(2)
c (Å)	14.2756(5)	21.0225(5)
α (°)	90	90
β (°)	98.501	107.9860
γ (°)	90	90
V (Å^3)	2165.05(13)	2266.72(10)
Z	4	4
D_c (g cm^{-3})	1.571	1.761
μ (mm^{-1})	0.872	5.767
F (000)	1048	1176
θ range (°)	1.99 – 30.07	2.14 – 28.00
h range	-18/18	-17/17
k range	-16/16	-9/11
l range	-20/17	-27/27
no. reflections	50326	17682
no. parameters	274	274
R_{int}	0.0382	0.0426
R_1[a]	0.0225	0.0247
wR_2[b]	0.0595	0.0507
GOF[c]	1.000	0.997
Residuals (e$^-$ Å^{-3})	0.645, -0.394	0.806, -0.648

[a] $R_1 = \Sigma||F_o| - |F_c||/\Sigma w|F_o|$, [b] $wR_2 = \{\Sigma[w(F_o^2 - F_c^2)^2]/\Sigma[w(F_o^2)^2]\}^{1/2}$, [c] $S = \{\Sigma[w(F_o^2 - F_c^2)^2] / (n - p)\}]^{1/2}$,where n is the number of reflections and p is the total number of parameters refined.

ESI-MS stability and biomolecule interaction studies

Materials. Dimethylsulfoxide (DMSO) was obtained from Acros; formic acid (98%) and L-cysteine (Cys) from Fluka; glycine (Gly) and L-histidine (His) from Merck; ubiquitin (ub, bovine erythrocytes) and 9-ethylguanine (EtG) from Sigma; L-methionine (Met) from Sigma-Aldrich and tetramethylammonium acetate from TCI Europe. MilliQ water (18.2 MΩ; Millipore Advantage A10, 185 UV Ultrapure Water System, Molsheim, France) and methanol (HPLC grade, Fisher) were used for ESI-MS studies.

Instrumentation. Electrospray ionisation mass spectra were recorded on a Bruker AmaZon SL ion trap (ESI-IT) and a UHR MaXis time-of-flight (ESI-TOF) mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany). Experimental data was acquired and processed using Compass 1.3 and Data Analyis 4.0 (Bruker Daltonics GmbH, Bremen, Germany). In-source collision-induced dissociation (ISCID) was performed on the ESI-TOF-MS using collision energies of 90 eV. Deconvolution was obtained by automatic data point spacing and 30000 instrument resolving power. The accuracy of the ESI-TOF measurement was calculated in Δppm, while the measurements on the ESI-IT include a standard deviation of $m/z \pm 0.05$. ESI-IT-MS spectra were recorded in the positive ion mode using the following parameters: capillary -4.5 kV, nebulizer 8 psi, dry gas 6 L/min, dry temperature 180 °C, accumulation time 0.1 ms and trap drive 57%. Similar parameters were used for recording ESI-TOF mass spectra: capillary -4.5 kV, nebulizer 5.8 psi, dry gas 6 L/min and dry temperature 180 °C. Finally, samples for ESI-MS were introduced by direct-infusion into the mass spectrometer at a flow rate of 4 µL/min.

Sample preparation. Stock solutions of **1a–c** (50–100 µM) were prepared in water using 1% DMSO. Additionally, stock solutions were prepared of ub (200 µM) and a mixture of EtG : Cys : Gly : His : Met (1 : 1 : 1 : 1 : 1) containing 400 µM of each compound in water. The three compounds were independently incubated with ub at a 2 : 1 metal-to-protein ratio and with the amino acid/model nucleobase mixture at a 1 : 1 molar ratio giving final metal concentrations of 25–50 µM. All mixtures were incubated in the absence of light. Experiments in buffered solution contained tetramethylammonium acetate buffer (2.5 mM, pH ≈ 8.5). Mass spectra of the incubation solutions were recorded directly after mixing and after 1, 3, 6 and 24 h. Small

molecule samples were diluted with H_2O : MeOH (1 : 1), while the protein samples were diluted with H_2O : MeOH : formic acid (50 : 50 : 0.2) to a final metal concentration of 1 – 5 µM prior to infusion into the mass spectrometer. The protein samples were therefore analysed under denaturing conditions leading to the rupture of the protein tertiary structure.

Table S2. Experimental and theoretical *m/z* vlaues of the observed metal-containing species in incubation mixtures of **1a–c** in aqueous solution and in the presence of biological nucleophiles. The measured *m/z* values on the ESI-IT-MS include a standard deviation of *m/z* ± 0.05.

	Detected Ion	***m/z***	$\mathbf{m_{theor}}$
ESI-IT-MS	[**1a** – Cl]$^+$	477.11	477.10
	[(cym)$_2$Ru$_2$(μ-OH)$_2$(μ-OCH$_3$)]$^+$	537.04	537.05
	[(cym)$_2$Ru$_2$(μ-OH)(μ-OCH$_3$)$_2$]$^+$	551.04	551.07
	[(cym)$_2$Ru$_2$(μ-OCH$_3$)$_3$]$^+$	565.06	565.08
	[(cym)Ru(Met) – H$^+$]$^+$	386.06	396.06
	[(cym)Ru(His) – H$^+$]$^+$	390.07	390.08
	[(cym)Ru(L)(EtG)]$^+$	656.17	656.18
	[**1b** – Cl]$^+$	567.16	567.16
	[(cym)$_2$Os$_2$(μ-OH)$_3$]$^+$	701.12	701.15
	[(cym)$_2$Os$_2$(μ-OH)$_2$(μ-OCH$_3$)]$^+$	715.11	715.16
	[(cym)$_2$Os$_2$(μ-OH)(μ-OCH$_3$)$_2$]$^+$	729.15	729.18
	[(cym)$_2$Os$_2$(μ-OCH$_3$)$_3$]$^+$	743.19	743.19
	[(cym)Os(Cys) – H$^+$]$^+$	446.04	446.08
	[(cym)Os(Met) – H$^+$]$^+$	474.10	474.11
	[(cym)Os(His) – H$^+$]$^+$	480.11	480.13
	[(cym)Os(L)(EtG)]$^+$	746.21	746.24
	[**1c** – Cl]$^+$	479.10	479.11
	[(Cp*)$_2$Rh$_2$(μ-Cl)$_3$]$^+$	580.93	580.95
	[(Cp*)Rh(Met) – H$^+$]$^+$	386.05	386.07
	[(Cp*)Rh(His) – H$^+$]$^+$	392.07	392.08
	[(Cp*)Rh(L)(Cys)]$^+$	600.11	600.13
	[(Cp*)Rh(L)(Met)]$^+$	628.14	628.16
	[(Cp*)Rh(L)(His)]$^+$	634.15	634.18

Table S3. Accurate and exact masses of the metal-containing species obtained by incubation of **1a–c** with ubiquitin (ub). The accuracy of the ESI-TOF-MS measurements is given in Δppm.

	Deconvoluted Ion	m_{acc}	m_{ex}	Δppm
ESI-TOF-MS	ub^+	8564.6255	8564.6299	0.5
	$[ub + (Cp^*)Rh]^+$	8801.6571	8801.6450	1.4
	$[ub + Ru(OH)]^+$	8683.5649	8683.5444	2.4
	$[ub + (cym)Ru]^+$	8797.6239	8797.6280	0.5
	$[ub + 2(cym)Ru]^+$	9031.6104	9031.6282	2.0
	$[ub + (cym)Os]^+$	8886.6776	8886.6821	0.5

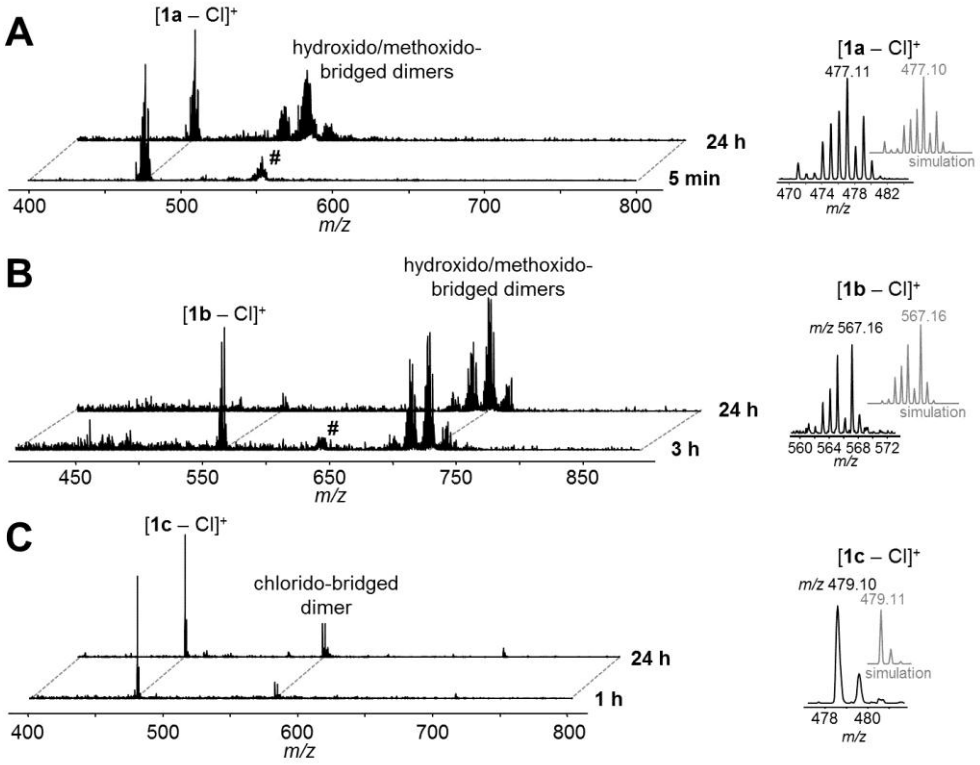


Figure S1. The stability of **1a** (**A**), **1b** (**B**) and **1c** (**C**) in water was determined by ESI-IT-MS and representative mass spectra are shown. The different types of dincuclear species in **A** and **B** stem from the dilution with H_2O : MeOH (1 : 1) prior to infusion into the mass spectrometer and correspond to $[(cym)M(\mu\text{-}OH)_{n\text{-}3}(\mu\text{-}OCH_3)_n]^+$, where M is Ru (n = 1 – 3) or Os (n = 0 – 3). DMSO adducts of the general formula [**1a,b** – Cl + DMSO]$^+$ were only detected in small amounts during the first hours of incubation and the signals are annotated with #.

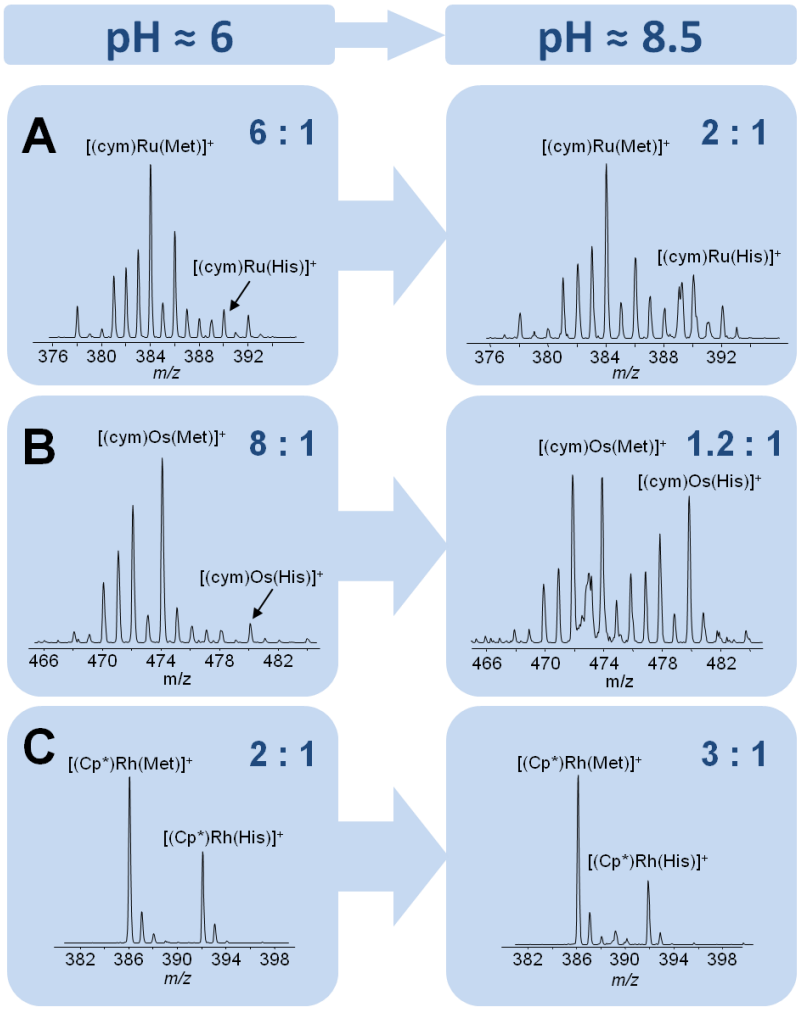


Figure S2. pH-Dependent adduct formation of **1a** (**A**), **1b** (**B**) and **1c** (**C**) with Met and His. The ratio between the abundance of the Met and the His adducts is given.

Table S4. Selected metallated peptide fragments obtained by top-down mass spectrometry of mono-adduct formed from the reaction between **1a–c** and ubiquitin using ISCID fragmentation on the ESI-TOF-MS. The relative abundance (%) refers to the most abundant mass signal in the ISCID spectrum.

Fragment	z	m_{acc}	m_{ex}	Δppm	%
[ub + (cym)Ru]					
$^{(cym)Ru}A_1$	1	338.0502	338.0514	3.7	6.8
$^{(cym)Ru}B_7$	2	541.7401	541.7384	3.3	8.1
$^{(cym)Ru}B_{13}$	3	565.9543	565.9549	1.1	6.7
$^{(cym)Ru}A_{14}$	3	590.3070	590.3059	1.9	14.7
$^{(cym)Ru}A_{15}$	3	627.9995	628.0006	1.8	12.2
$^{(cym)Ru}B_{16}$	3	680.3498	680.3465	4.9	42.8
$^{(cym)Ru}B_{18}$	3	756.3869	756.3836	4.3	18
[ub + (cym)Os]					
$^{(cym)Os}B_1$	1	456.1029	456.1030	0.2	11.8
$^{(cym)Os}B_2$	1	584.1602	584.1616	2.3	3.8
$^{(cym)Os}A_{13}$	3	586.6448	586.6419	5.1	4.0
$^{(cym)Os}B_{14}$	3	629.6585	629.6561	3.9	6.8
$^{(cym)Os}B_{18}$	3	786.4048	786.4021	3.4	6.4
[ub + (Cp*)Rh]					
$(Cp^*)Rh(\dot{C}H_2SCH_3)(NH_2)$	1	312.0286	312.0288	0.7	42.5
$^{(Cp^*)Rh}B_3$	1	609.1981	609.1976	0.8	3.7
$^{(Cp^*)Rh}A_7$	3	352.8323	352.8323	0.2	1.4
$^{(Cp^*)Rh}B_{12}$	3	528.9297	528.9292	0.9	12.5
$^{(Cp^*)Rh}A_{13}$	3	557.2945	557.2922	4.0	16.8
$^{(Cp^*)Rh}A_{14}$	3	590.9767	590.9748	3.2	15.2
$^{(Cp^*)Rh}B_{15}$	3	638.3350	638.3354	0.8	20.1
$^{(Cp^*)Rh}A_{16}$	3	672.0169	672.0180	1.6	9.9
$^{(Cp^*)Rh}B_{16}$	3	681.3501	681.3496	0.6	47.3
$^{(Cp^*)Rh}B_{18}$	3	757.3824	757.3866	5.5	14

Cytotoxicity in cancer cell lines

Cell lines and culture conditions. CH1 cells (adenocarcinoma of the ovary, human) were provided by Lloyd R. Kelland (CRC Centre for Cancer Therapeutics, Institute of Cancer Research, Sutton, U.K). SW480 (adenocarcinoma of the colon, human), HL60 (acute promyelocytic leukemia) and A549 (non-small cell lung cancer, human) cells were purchased from ATCC. HCT-116 cells were provided by Dr. Vogelstein (John Hopkins University, Baltimore, MD) All cell culture reagents were purchased from Sigma-Aldrich. Cells were grown in 75 cm^2 culture flasks (Starlab) as adherent monolayer cultures in complete culture medium, *i.e.* Eagle's minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 4 mM L-glutamine, and 1% non-essential amino acids (from 100× ready-to-use stock) without antibiotics. Cultures were maintained at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2.

MTT assay conditions. Cytotoxicity was determined by the colorimetric MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide, purchased from Fluka) microculture assay. For this purpose, cells were harvested from culture flasks by trypsinization and seeded in 100 µL/well aliquots of complete culture medium into 96-well microculture plates (Starlab). Cell densities of 1×10^3 cells/well (CH1), 2×10^3 cells/well (SW480 and HCT-116), 3×10^3 cells/well (A549) and 5×10^4 cells/well (HL60) were chosen in order to ensure exponential growth of untreated controls throughout the experiment. For 24 h, cells were allowed to settle and resume exponential growth. The test compounds were dissolved in DMSO, serially diluted in complete culture medium (such that the DMSO content in actual test solutions did not exceed 0.5%) and added in 100 µL/well aliquots for an exposure time of 96 hours. At the end of exposure, the medium was replaced with 100 µL/well of a 7:1 mixture of RPMI1640 culture medium (supplemented with 10% heat-inactivated fetal calf serum) and MTT solution in phosphate-buffered saline (5 mg/ml). After incubation for 4 h, the supernatants were removed, and the formazan crystals formed by viable cells were dissolved in 150 µL DMSO per well. Optical densities at 550 nm were measured with a microplate reader (BioTek ELx808), using a reference wavelength of 690 nm to correct for unspecific absorption. The quantity of viable cells was expressed in terms of T/C values by comparison to untreated control microcultures, and 50% inhibitory concentrations (IC_{50}) were calculated from concentration-effect curves by

interpolation. Evaluation is based on means from at least three independent experiments, each comprising three replicates per concentration level.

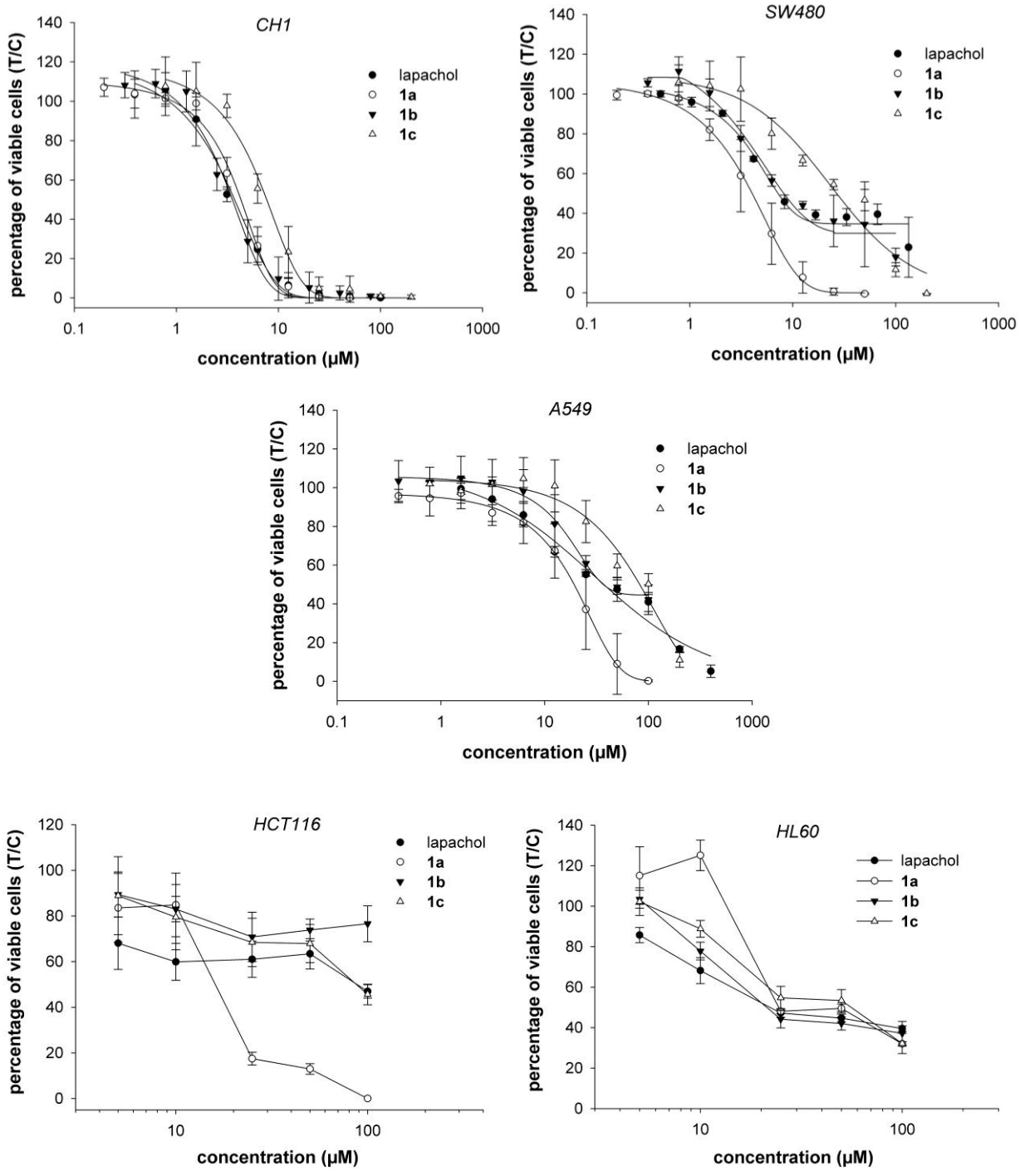


Figure S3. Concentration-effect curves determined for lapachol and its Ru, Os and Rh complexes in the human tumour cell lines CH1, SW480 and A549.

ROS Assay. For the fluorimetric analysis of reactive oxygen species (ROS) non-adherent HL60 cells (promyelocytic leukemia, human) were stained for 30 min at 37 °C and 5% CO_2 with 0.1 µM DCFH DA (2',7'-dichlorofluorescein diacetate) in Hank's Salt Solution supplemented with 1% bovine serum. $5 \times 10^4 - 8 \times 10^4$ cells/well were transferred to 96-well plates and treated directly with the test substances at different concentrations for 30 min at 37 °C, 5% CO_2. 500 µM freshly prepared H_2O_2 solution was used as a positive control and added 10 min before measurement. The generated ROS activity within the cells was measured on a Guava easyCyte 8HT device (Millipore). The resulting histograms of green fluorescence were quantified by FlowJo software (Tree Star). Green fluorescence intensity is defined as the ratio between the drug-treated sample and the untreated control.

Annexin V/PI Staining. SW480 cells (3×10^5) were exposed to the drugs for 24 h and were stained with Annexin V (Annexin V-FITC; BD Biosciences, San Jose, CA) and propidium iodide (200 ng/ml PI; Sigma-Aldrich) and analysed according to the manufacturer's protocol by flow cytometry using fluorescence-activated cell sorting (FACSCalibur; BD Biosciences). CellQuest Pro software (BD Biosciences) was used to analyse the data. Experiments were repeated twice.

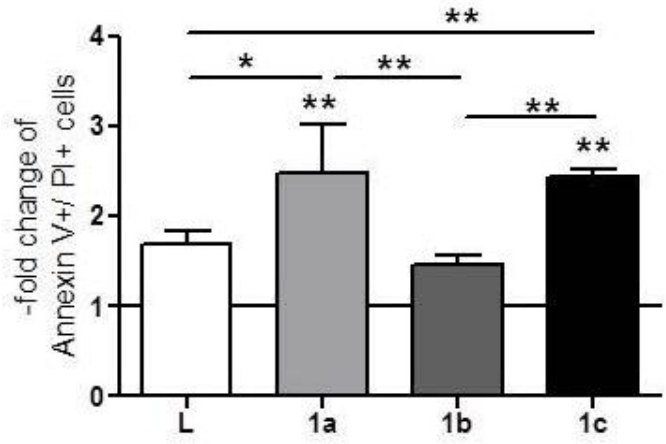


Figure S4. Extent of apoptosis induction by lapachol and its organo-Ru, -Os and -Rh complexes as determined by the annexin V assay in SW480 cells after drug treatment (25 µM) for 24 h. The line indicates levels of untreated control cells. Statistical analysis was performed from three independent experiments by one-way ANOVA with Bonferroni post-test (* $p < 0.05$; ** $p < 0.01$).

Cell Cycle Analysis (CH1 cells). HFS buffer (hypotonic fluorochrome solution) was prepared using 0.1% (V/V) Triton X-100, 0.1% (W/V) sodium citrate, in PBS. CH1 cells (1×10^5) in MEM medium were seeded into 12-well microculture plates (Starlab) and allowed to recover for 24 h. Cells were then treated with different concentrations of the test compounds for 24 h at 37 °C, 5% CO_2. Controls (negative: Eagle's minimal essential medium, positive: 0.01 and 0.05 µM gemcitabine) and drug-treated cells were collected, washed with PBS, and stained in 600 µL propidium iodide/HFS solution (50 µg/mL) for 24 h in the dark at 4 °C. Fluorescence was measured by flow cytometry by using a Guava easyCyte 8HT instrument (Millipore). The resulting histograms of red fluorescence were quantified by FlowJo software (Tree Star).

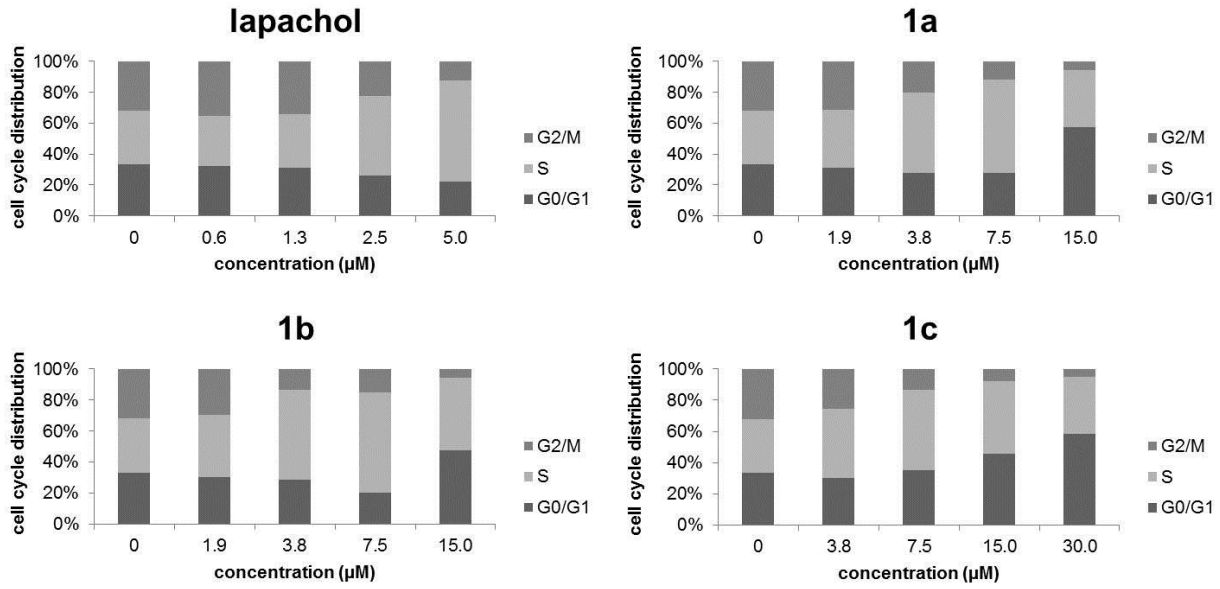


Figure S5. Concentration-dependent impact of lapachol and its Ru, Os and Rh complexes on the cell cycle distribution of CH1 cells after exposure for 24 h (values are means of three independent experiments).

Cell Cycle Analysis (SW480 and HCT-116). SW480 and HCT-116 Cells were exposed for 48 h to the drugs at 10 and 50 µM, respectively. To analyze cell cycle progression, cells were collected, washed with PBS, re-suspended in 0.9% NaCl solution, and fixed in 70% alcohol (–20 °C). Then, cells were incubated with RNAse (10 µg/µml; Sigma) for 30 minutes at 37 °C and stained with PI (10µg/µml PI, Sigma Aldrich) for 30 minutes at 4 °C. Cells were analyzed by flow cytometry using fluorescence-activated cell sorting (FACS Calibur, Becton Dickinson, Palo Alto, CA). Cell Quest Pro software (Becton Dickinson and Co., New York) was used to analyse the resulting DNA histograms. The experiments were repeated three times.

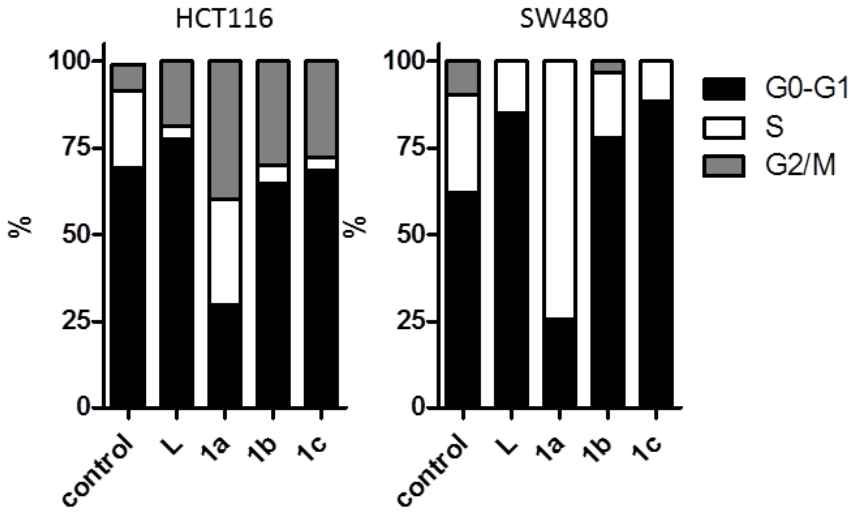


Figure S6. Impact of lapachol and its Ru, Os and Rh complexes on the cell cycle distribution of HCT-116 (50 µM) and SW480 (10 µM) cells after exposure for 48 h (values are means of three independent experiments).

Western Blot Analysis. HCT-116 cells (3×10^5) were exposed to **1a–c** for 1, 4 and 24 h. Total protein lysates were prepared, resolved by SDS-polyacrylamide gel electrophoresis, and transferred onto a polyvinylidene difluoride membrane for Western blotting.[5] The following antibodies were used: p53 (Neomarkers, CA, Clone DO-1), p21 (Cell Signaling Technology, #2946), cdc2 (Santa Cruz Biotechnology, #sc-137034), p-CDK1 (Tyr15) (Cell Signaling Technology, #9111), β-actin AC-15 (Sigma-Aldrich, #A1978), diverse Cyclins (Cell Signaling, Cyclin Antibody Sampler Kit #9869). Secondary horseradish peroxidase-labelled antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) were used at working dilutions of 1 : 10000. Western blot bands were quantified with QuantiScan software (Biosoft, Cambridge, UK).

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