Supporting Information

Aqueous Chemistry and Antiproliferative Activity of a Pyrone-based Phosphoramidate Ru(Arene) Anticancer Agent

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Experimental

Materials

All reactions were carried out in dry solvents and under inert atmosphere. Chemicals obtained from commercial suppliers were used as received. Methanol and dichloromethane were dried using standard procedures. $RuCl_3 \cdot 3H_2O$ (40.4%) was purchased from Johnson Matthey; L-cysteine from Fluka; horse heart cytochrome c, dimethylsulfoxide (DMSO) and 9-ethylguanine and glycine from Sigma; α-terpinene from Acros; L-histidine from Merck and sodium methoxide and triphenylphosphite from Sigma-Aldrich. Methanol (VWR Int., HiPerSolv CHROMANORM), formic acid (Fluka) and milliQ H_2O (18.2 MΩ, Advantage A10, 185 UV Ultrapure Water System, Millipore, France) were used for ESI-MS experiments. The dimer bis[dichlorido(η^6-p-cymene)ruthenium(II)][1,2] and the ligand 2-(azidomethyl)-5-hydroxy-*4H*-pyran-4-one[3] were synthesized as previously described.

Instrumentation

NMR spectra were recorded at 25 °C on a Bruker FT NMR spectrometer Avance III^{TM} 500 MHz at 500.10 (^{1}H), 202.63 MHz (^{31}P{^{1}H}) and 125.75 MHz (^{13}C{^{1}H}) and 2D NMR data were collected in a gradient-enhanced mode. Hydrogen and carbon atoms were numbered according to crystal structure numbering. Elemental analysis was carried out on a Perkin–Elmer 2400 CHN Elemental Analyzer by the Laboratory for Elemental Analysis, Faculty of Chemistry, University of Vienna. Electrospray ionization mass spectra were recorded on a Bruker AmaZon SL ion trap (IT) and on a UHR MaXis time-of-flight (TOF) mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany). Data was acquired and processed using Compass 1.3 and Data Analysis 4.0 (Bruker Daltonics GmbH, Bremen, Germany). Deconvolution was obtained by applying the maximum entropy algorithm with a 0.1 *m/z*

mass step and 0.5 *m/z* instrument peak width for the IT and automatic data point spacing and 30'000 instrument resolving power for the TOF. X-ray diffraction measurements of single crystals were performed on a Bruker X8 APEX II CCD diffractometer at 100 K. The crystals were positioned at 35 mm from the detector and 607 frames for 50 sec over 1° were measured. The data was processed using the SAINT Plus software package.[4] Crystal data, data collection parameters, and structure refinement details are given in Table S1. The structures were solved by direct methods and refined by full-matrix least-squares techniques. Non-hydrogen atoms were refined with anisotropic displacement parameters. Hydrogen atoms were inserted at calculated positions and refined with a riding model. The following software programs and tables were used: SHELXS-97,[5] SHELXL-97[5] and Mercury CSD 3.0.[6]

Methods

Interaction with biomolecules

Samples for ESI-MS experiments were analyzed by direct infusion at a flow rate of 3 µL/min and at typical concentrations of 1–5 µM. Protein samples were analyzed under denaturing conditions by diluting with water : methanol : formic acid (50 : 50 : 0.2), while the reference complexes and small molecule samples were diluted with water : methanol (50 : 50). Stock solutions of compound **1A** were prepared in H_2O containing 1% DSMO. The following molar ratios were used for interaction studies at 37 °C and pH 5.5: cyt : **1A** (1 : 3), amino acid : **1A** (1 : 1) and EtG : **1A** (2 : 1). Mass spectra were recorded after 0, 1, 3, 6, 24 and 48 h. In general, relative intensities correspond to percentages of the area under peaks of the sum of all assignable ruthenium signals in the spectrum.

Cell lines and culture conditions

CH1 (human ovarian adenocarcinoma) cells were provided by Lloyd R. Kelland (CRC Centre for Cancer Therapeutics, Institute of Cancer Research, Sutton, UK). SW480 (human adenocarcinoma of the colon) and A549 (human non-small cell lung cancer) cells were provided by Brigitte Marian, and HL-60 (human promyelocytic leukemia) cells by Walter Berger (both Institute of Cancer Research, Department of Medicine I, Medical University of Vienna, Austria). All cell culture reagents were purchased from Sigma-Aldrich Austria and plastic ware from Starlab Germany. Cells were grown in 75 cm^2 culture flasks as adherent monolayer cultures in Minimum 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). Cultures were maintained at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2.

Cytotoxicity tests

Cytotoxicity was determined by the colorimetric MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) microculture assay. For this purpose, cells were harvested from culture flasks by trypsinization and seeded in 100 µL/well aliquots of complete MEM into 96-well microculture plates. Cell densities of 1.0×10^3 cells per well (CH1), 2.0×10^3 cells per well (SW480) and 3.0×10^3 cells per well (A549) were chosen in order to ensure exponential growth of untreated controls throughout the experiment. Cells were allowed to settle and resume exponential growth for 24 h. The test compound was dissolved in DMSO, then serially diluted in MEM and added to the plates (100 µL/well) where the final DMSO content did not exceed 0.5% (v/v). Compound **1A** displayed a solubility of *ca.* 0.5 mM in MEM containing 1% DMSO. After 96 h exposure, the medium was replaced with 100 µL per well of a 1:7 MTT/RPMI 1640 solution (MTT solution, 5 mg/mL of MTT reagent in phosphate-buffered saline; RPMI 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum and 4 mM L-

glutamine). After incubation for 4 h, the MTT/RPMI 1640 solution was removed, and the formazan product formed by viable cells was dissolved in 150 µL DMSO per well. Optical densities at 550 nm were measured with a microplate reader (BioTek ELx808), by 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 three independent experiments, each comprising three replicates per concentration level.

Flow cytometric detection of apoptotic cells

Induction of cell death was analyzed by fluorescence-activated cell sorting (FACS) using FITC-conjugated annexin V (BioVision, USA) and propidium iodide (PI; Fluka) double staining. HL-60 cells were seeded into 12-well plates (Starlab) in a density of 5×10^4 cells per well in complete medium (as described above) and allowed to settle for 24 h. The cells were exposed to the test compound in different concentrations for 48 h at 37 °C. Stausporine (Sigma-Aldrich Austria) was used as a positive control. After the incubation, cells were gently trypsinized, washed with PBS, and suspended with FITC-conjugated annexin V (0.25 µg/mL) and PI (1 µg/mL) in binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at 37 °C for 15 min. Stained cells were analysed with a Guava 8HT EasyCyte Flow cytometer (Merck Millipore, Guava, USA) using InCyte software. Three independent experiments were conducted, and 5000 cells per analysis were measured.

Western blot detection of PARP cleavage

The cleavage of the apoptosis-associated protein PARP was detected in CH1 cells. Cells were seeded in densities of 2×10^5 cells per well into 6-well plates (Starlab) and allowed to resume proliferation for 24 h. The cells were exposed to the test compound and staurosporine (positive control) in different

concentrations for 24 h at 37 °C. After the treatment the cells were washed with PBS and lysed by adding 100 µL per well of RIPA lysis buffer (150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulphate), 50 mM Tris, pH 8.0). Cells were carefully scratched and sonicated for 10 sec to shear DNA and reduce sample viscosity. The protein content of lysates was measured with Pierce Micro BCA Protein Assay (Thermo Scientific). The appropriate volume of cell lysates (20 µg protein content per gel pocket) was mixed with 6× loading buffer (12% w/v SDS, 30% 2-mercaptoethanol, 60% glycerol, 0.012% bromophenol blue, 0.375 M Tris HCL, pH 6.8) and heated at 95° C for 5 min. The proteins were separated by 8% SDS-polyacrylamide gel electrophoresis and subsequently transferred onto nitrocellulose membranes (Millipore) by using a semi-dry blotting apparatus (Biorad). The membranes were blocked with blocking buffer (1× TBS, 0.1% Tween-20 with 5% BSA) and immunoblotted with the relevant primary PARP rabbit antibody diluted in TBS/T buffer in a ratio of 1:1000 (Cell Signaling Technology). Primary antibodies were detected by using anti-rabbit IgG HRP-linked antibody diluted in TBS/T buffer in a ratio of 1:3000 (Cell Signaling Technology) and visualized with a chemiluminescence detection system Fusion SL (Vilber Lourmat) using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Afterwards the membranes were cleaned by stripping buffer (15 g glycine, 1 g SDS, 10 mL Tween 20, pH 2.2) and immunoblotted with the β-actin rabbit antibody diluted in TBS/T buffer in a ratio of 1:3000 (Cell Signaling Technology) to check the quality of gel loading procedure. Results are based on three independent experiments.

Synthesis

Diphenyl{(5-hydroxy-4-oxo-4*H*-pyran-2-yl)methyl}phosphoramidate (1). 2-(Azidomethyl)-5-hydroxy-*4H*-pyran-4-one (200 mg, 1.2 mmol) was suspended in dry DCM (3 mL) under argon atmosphere and triphenylphosphite (627 µL, 4.8 mmol) was slowly added. The reaction mixture was stirred for 24 h at room temperature. Diethyl ether was added and the formed colorless crystals were separated by filtration and dried *in vacuo*. Yield: 216 mg (77%). MS (ESI^+): *m/z* 374.04 [**1** + H^+]$^+$ (m_{ex} = 374.08). ^{1}H NMR (500.10 MHz, d_6-DMSO): δ = 9.05 (s, 1H, –O*H*), 7.91 (s, 1H, H-6), 7.39 (dd, ^3J(H,H) = 7 Hz, ^3J(H,H) = 7 Hz, 4H, $H_{Ar\,3',5'}$), 7.23–7.19 (m, 6H, $H_{Ar\,2',4',6'}$), 6.57 (dt, ^2J(P,H) = 14 Hz, ^3J(H,H) = 7 Hz, 1H, –N*H*), 6.26 (s, 1H, H-3), 4.01 (dd, ^3J(P,H) = 14 Hz, ^3J(H,H) = 7 Hz, 2H, H-7) ppm. ^{13}C{^{1}H} NMR (125.75 MHz, d_6-DMSO): δ = 174.2 (C-4), 165.7 (C-2), 150.9 ($C_{Ar\,1'}$), 146.2 (C-5), 139.8 (C-6), 130.3 ($C_{Ar\,3',5'}$), 125.4 ($C_{Ar\,4'}$), 120.6 ($C_{Ar\,2',6'}$), 111.5 (C-3), 42.5 (C-7) ppm. ^{31}P{^{1}H} NMR (202.63 MHz, d_6-DMSO): δ = –0.02 ppm.

[Chlorido(η^6-p-cymene){5-oxo-κO-2-[(diphenylphosphoramido)methyl]-4*H*-pyronato-κO}ruthenium(II)] (1A). Ligand **1** (201 mg, 0.54 mmol) and sodium methoxide (32 mg, 0.59 mmol) were suspended in dry methanol under argon atmosphere at room temperature for 15 min. Bis[dichlorido(η^6-p-cymene)ruthenium(II)] (149 mg, 0.25 mmol) in methanol (10 mL) was added and the clear, orange colored reaction mixture was stirred for further 18 h. The solvent was removed under reduced pressure. The residue was dissolved in dichloromethane, filtered and the filtrate was concentrated to a final volume of ca. 2–3 mL. The product was precipitated by addition of *n*-hexane, filtered and dried *in vacuo*. The product **1A** was isolated as brownish crystals. Yield: 125 mg (40%). Elemental analysis calculated for $C_{28}H_{29}ClNO_6PRu{\cdot}0.3CH_2Cl_2$: C, 50.84; H, 4.46; N, 2.10; O, 4.63; found: C, 50.84; H, 4.44; N, 2.18; O, 4.71. MS (ESI^+): *m/z* 608.0745 [**1A** – Cl]$^+$ (m_{ex} = 608.0778, 5 ppm). ^{1}H NMR (500.10 MHz, d_6-DMSO): δ = 7.72 (s, 1H, H-6), 7.40 (dd, ^3J(H,H) = 7 Hz, ^3J(H,H) =

7 Hz, 4H, $H_{Ar\ 3',5'}$), 7.24–7.19 (m, 6H, $H_{Ar\ 2',4',6'}$), 6.56 (dt, ^{2}J(P,H) = 14 Hz, ^{3}J(H,H) = 7 Hz, 1H, –N*H*), 6.47 (s, 1H, H-3), 5.69–5.66 (m, 2H, $H_{Cym\ 3',5'}$), 5.41–5.39 (m, 2H, $H_{Cym\ 2',6'}$), 4.03 (dd, ^{3}J(P,H) = 14.5 Hz, ^{3}J(H,H) = 7 Hz, 2H, H-7), 2.76 (sept, ^{3}J(H,H) = 7 Hz, 1H, $H_{Cym\ b}$), 2.15 (s, 3H, $H_{Cym\ a}$), 1.25 (d, ^{3}J(H,H) = 7 Hz, 6H, $H_{Cym\ c}$) ppm. $^{13}C\{^{1}H\}$ NMR (125.75 MHz, d_6-DMSO): δ = 185.1 (C-4), 165.1 (C-2), 159. 5 (C-5), 150.2 ($C_{Ar\ 1'}$), 139.9 (C-6), 129.8 ($C_{Ar\ 3',5'}$), 124.8 ($C_{Ar\ 4'}$), 120.0 ($C_{Ar\ 2',6'}$), 107.5 (C-3), 97.9 ($C_{Cym\ 1'}$), 94.8 ($C_{Cym\ 4'}$), 79.8 ($C_{Cym\ 3',5'}$), 76.9 ($C_{Cym\ 2',6'}$), 41.9 (C-7), 30.5 ($C_{Cym\ b}$), 21.9 ($C_{Cym\ c}$), 17.9 ($C_{Cym\ a}$) ppm. $^{31}P\{^{1}H\}$NMR (202.63 MHz, d_6-DMSO): δ = –0.11 ppm.

Table S1. Details on X-ray diffraction measurements and associated crystal cell parameters of **1A**.

compound	**1A**
CCDC N°	902336
chemical formula	$C_{28}H_{29}NO_6PClRu$
M (g mol^{-1})	643.03
temperature (K)	100(2)
crystal size (mm)	0.02 × 0.12 × 0.30
crystal color, habit	red, block
crystal system	monoclinic
space group	$P2_1/c$
a (Å)	15.998(3)
b (Å)	16.226(4)
c (Å)	10.5737(18)
V (Å^3)	2647.92
α (deg)	90.00
β (deg)	105.258(6)
γ (deg)	90.00
Z	4
D_c (g cm^{-3})	1.61
μ (mm^{-1})	0.80
F(000)	1312.0
Θ range (deg)	2.36–25.35
h range	-19/19
k range	-18/19
l range	-12/12
no. unique refls.	4760
no. parameters	344
R_{int}	0.1618
R_1 (obs.)	0.0783
wR_2 (all data)	0.1316
S	1.079

Table S2. Selected bond lengths (Å), angles (°) and torsion angles (°) of **1A**.

Bond Length (Å)	**1A**
Ru–Cl	2.4371(18)
Ru–O2	2.104(4)
Ru–O3	2.112(5)
Ru–centroid	1.654(7)
C6–O2	1.323(8)
C5–O3	1.277(8)
N1–P1	1.616(6)
Bond Angles (°)	
O2–Ru–O3	78.33(18)
O2–Ru–Cl	83.66(13)
O3–Ru–Cl	84.09(13)
Torsion Angle (°)	
Ru–O3–C4–C5	-8.3(7)
$C_{Cym\ 4'}$–Ru–O3–C4	-158.8(4)
C3–C2–C7–N1	89.2(8)

Table S3. The experimental and theoretical values of the assigned mass signals in the ESI-IT and –TOF MS experiments. Experimental values on the IT instruments include a standard deviation of *m/z* ± 0.03, except where noted. $\mathbf{1A_{hydr}} = [\mathbf{1A} - Cl]^+$.

	Detected Ion	***m/z***	$\mathbf{m_{theor}}$
IT MS	$[\mathbf{1} + H]^+$	374.04	374.08
	$\mathbf{1A_{hydr}}^+$	607.98	608.08
	$[Ru(cym)(Gly) - H]^+$	309.92	310.04
	$[Ru(cym)(Gly)_2 - H]^+$	387.90	388.09
	$[Ru(cym)(His) - H]^+$	389.96	390.08
	$[\mathbf{1A_{hydr}} + His]^+$	762	762.14
	$[Ru(cym)(Cys) - H]^+$	355.90	356.03
	$[\mathbf{1A_{hydr}} + EtG]^+$	786.98	787.16
	Deconvoluted Ion	***m/z***	$\mathbf{m_{theor}}$
	cyt^+	12356.7 ± 0.6	12358.3
	$[cyt + Ru(cym) - H]^+$	12591.4 ± 1.2	12593.4
	Deconvoluted Ion	$\boldsymbol{m_{acc}}$	$\mathbf{m_{ex}}$
TOF MS	cyt^+	12358.24	12358.35
	$[cyt + Ru(cym) - H]^+$	12592.23	12592.33
	$[cyt + \mathbf{1A_{hydr}}]^+$	12967.30	12967.43

Table S4. The table shows the relative intensity of each deconvoluted ion (%) of the reaction between **1A** and cyt after 48 h. Note that the extent of metallation is similar, but the nature of the adducts differs.

	cyt^+	$[cyt + Ru(cym)]^+$	$[cyt + \mathbf{1A_{hydr}}]^+$
IT MS	61	39	-
TOF MS	61	24	15

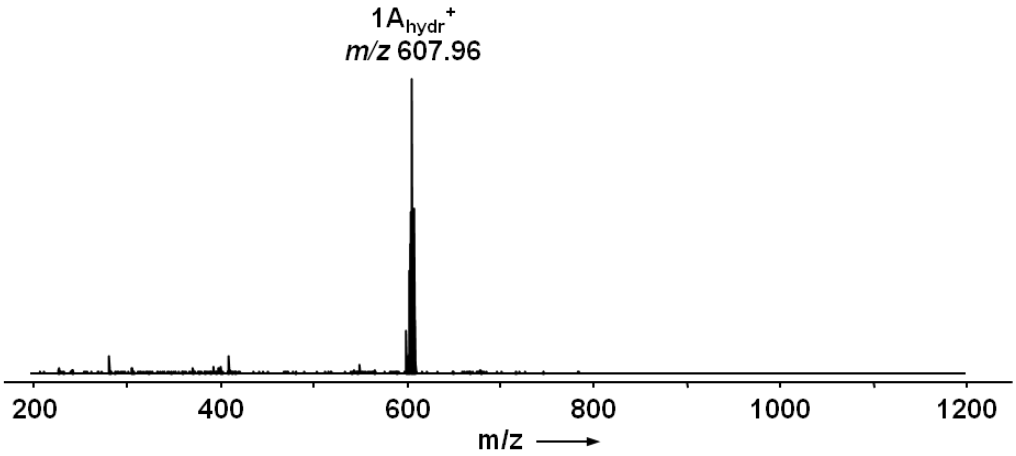

Figure S1. The ESI-IT mass spectrum of **1A** in aqueous solution recorded after 48 h shows that the organometallic compound is stable under these conditions ($\mathbf{1A_{hydr}}$ = $[\mathbf{1A} - Cl]^+$).

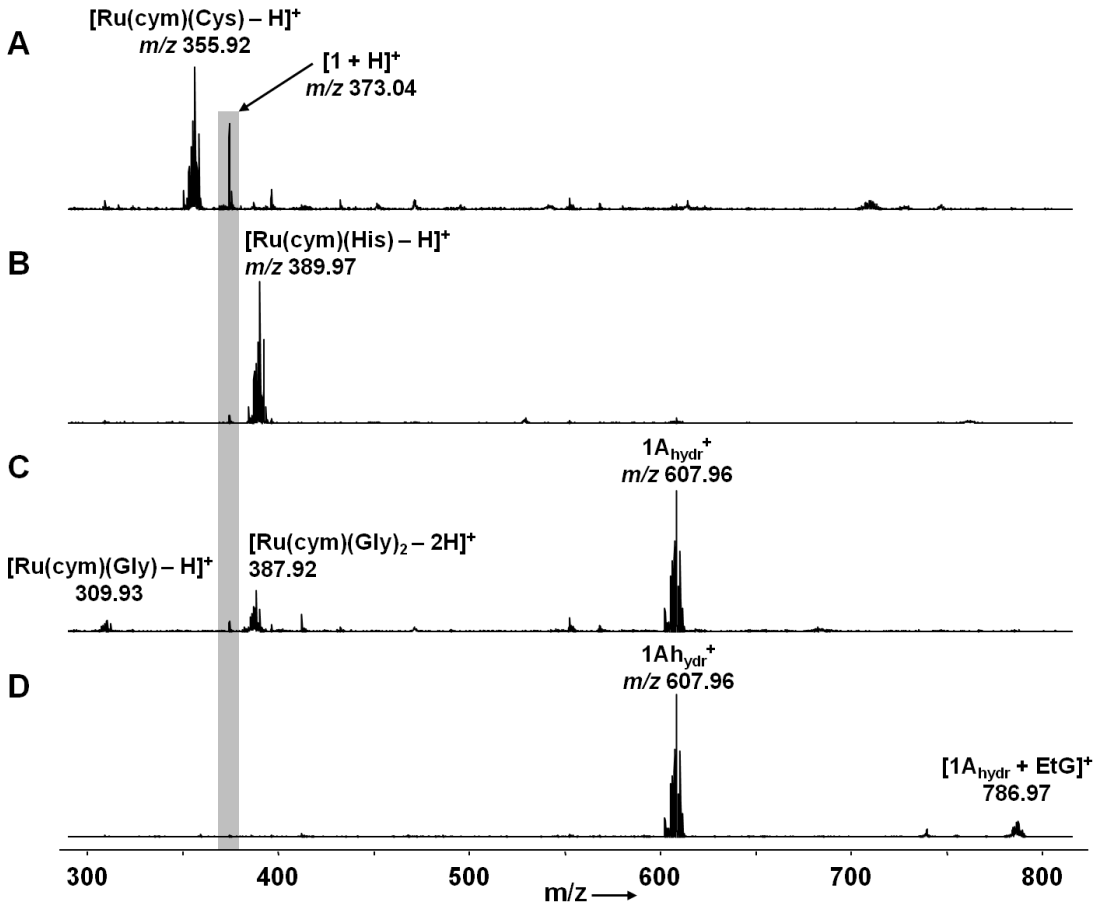

Figure S2. The ESI-IT mass spectra of the incubations of **1A** with L-cysteine (Cys, **A**), L-histidine (His, **B)**, glycine (Gly, **C**) and 9-ethylguanine (EtG, **D**). The metal-to-biomolecule ratios were 1 : 1 and 1 : 2 for the amino acids and EtG, respectively. The mass spectra in **A** and **B** were recorded after 6 h and the spectra **C** and **D** after 48 h (**1A$_{hydr}$** = [**1A** – Cl]$^+$).

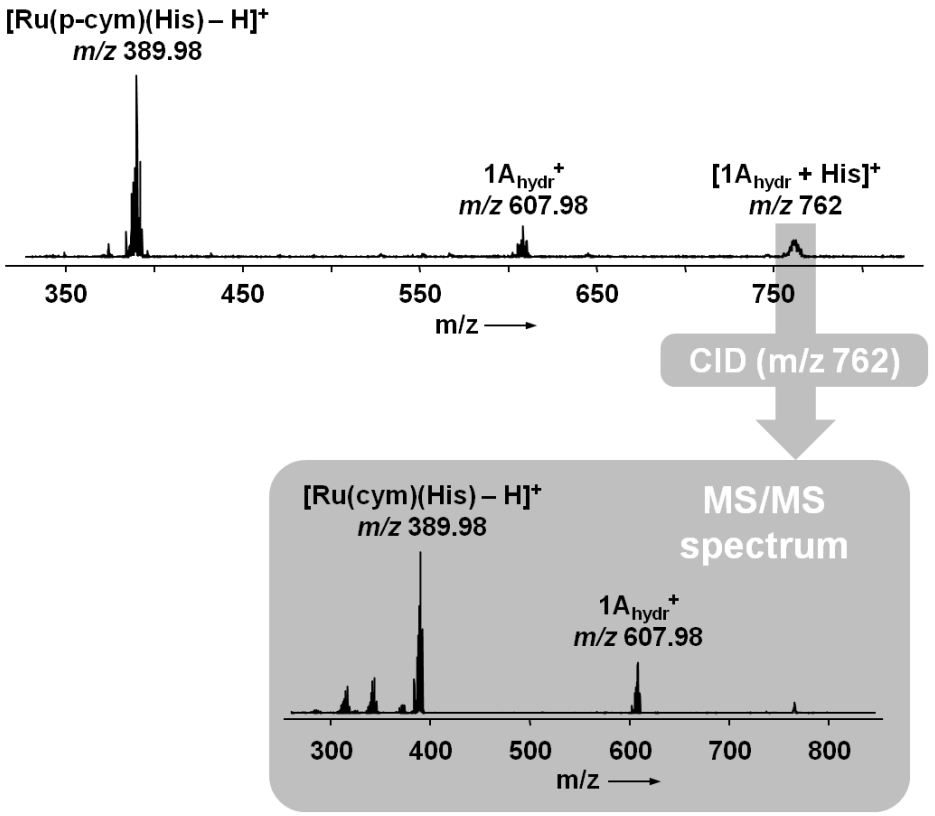

Figure S3. The ESI-IT mass spectrum of the reaction between **1A** and L-histidine (His) is shown after 3 h. The broad signal at *m/z* 762 indicates a mono-dentate approach of His to the free coordination site of the hydrolyzed **1A** prior to pyronato ligand cleavage. The MS/MS spectrum underlines His coordination to the metal ($\mathbf{1A_{hydr}}$ = $[\mathbf{1A} - Cl]^+$).

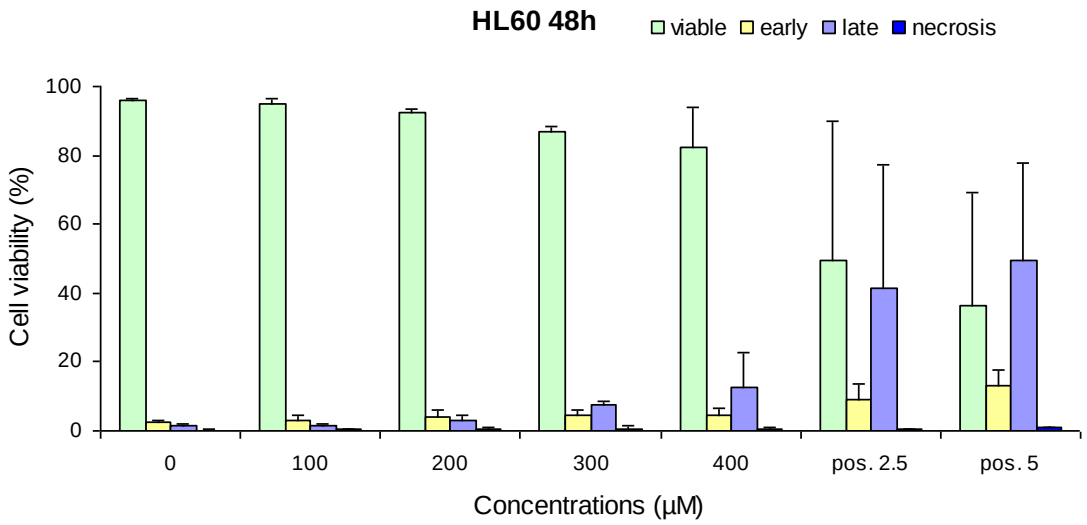

Figure S4. Apoptosis/necrosis induction in HL-60 cells after 48 h exposure to compound **1A**, measured by FACS using annexin V-FITC/propidium iodide double staining. The positive control (pos.) was treated with stausporine at concentrations of 2.5 and 5 µM.

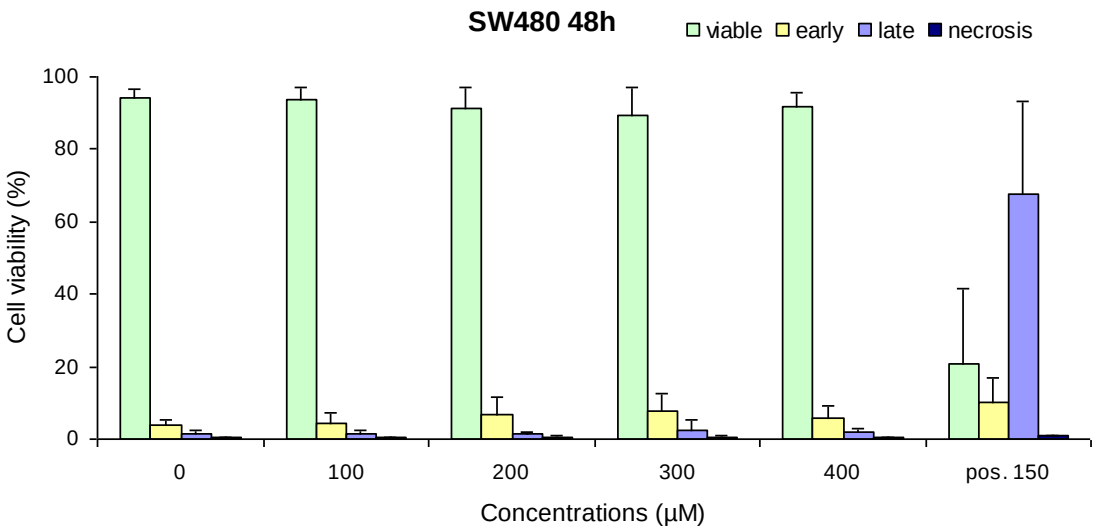

Figure S5. Apoptosis/necrosis induction in SW480 cells after 48 h exposure to compound **1A**, measured by FACS using annexin V-FITC/propidium iodide double staining. The positive control (pos.) was treated with the platinum(II)-oxime complex KP1988[7] at a concentration of 150 µM.

References

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