A shotgun metalloproteomic approach enables identification of proteins involved in the speciation of a ruthenium anticancer drug in cytosol of cancer cells†

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Electronic supplementary information

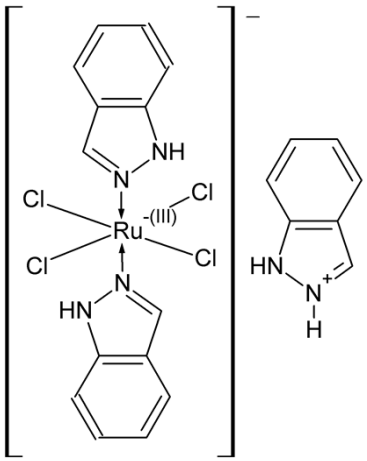

Fig. S1 Structural formula of the Ru drug.

Table S1 Operational parameters of SEC-ICP-MS and μHPLC-ESI-QqQ-MS

SEC-ICP-MS	
Column	Superdex 200 10/300 GL
Column temperature	20 °C
Injection volume	100 μL
Mobile phase	10 mM ammonium acetate, pH 6.0
Flow rate	0.5 mL min^{-1}
RF power	1360 W
Plasma, auxiliary and nebulizer gas flow	15.0, 1.0 and 1.05 L min^{-1}, respectively
Cones	sampler – Pt, skimmer – Pt
Monitored isotopes	^{102}Ru, ^{57}Fe
Dwell time	100 ms
μHPLC-ESI-QqQ-MS	
Column	Zorbax SB-C18
Column and autosampler temperature	37 °C
Injection volume	0.5 μL
Mobile phase	0.1% (*v/v*) formic acid in water (A), methanol (B)
Flow rate	6 μL min^{-1}
Elution program	0–5 min – 0% B 5–20 min – 0–100% B 20–30 min – 100% B 30–32 min – 100–0% B
Polarity	positive (+)
Mode	SCAN, Product Ion
Mass range	*m/z* 50 – 1500
Ionization voltage	2000 V (+)
Orifice voltage	90 V
Drying gas flow	5 L min^{-1}
Drying gas temperature	300 °C
Nebulizer pressure	35 psi
Sheath gas flow	10 L min^{-1}
Sheath gas temperature	350 °C
Nozzle voltage	500 V
Collision energy	5–40 eV

Determination of total protein concentration in cytosol of cancer cells

In order to assess the proteomic procedure parameters, such as the proper amounts of *DL*-dithiothreitol (DTT), iodoacetamide (IAM), or trypsin (from bovine pancreas) to be added to the total concentration of proteins in cytosol of cancer cells was determined using Bradford test. As a standard the cytosol from human liver was taken (pooled fraction, Sigma-Aldrich), in which the protein concentration is known (20 g L^{-1}). Three solutions were prepared: 0.1 M NaCl as blank, cytosol standard, and cancer cell cytosol, both diluted 20-times in 0.1 M NaCl. To 0.1 mL of the resultant samples, the Bradford reagent (3 mL) was added. After 10 min the

absorbance of cytosol samples (in comparison to blank solution) was measured at a wavelength of 595 nm.

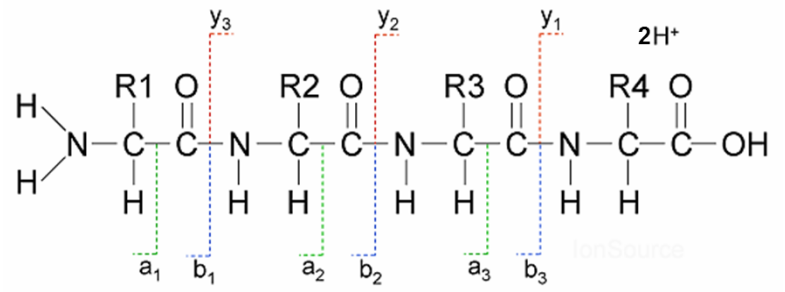

Fig. S2 Biermann's nomenclature of peptide fragmentation ions.

Identification of proteins presented in cytosol of cancer cells

In order to identify the main proteinaceous binding partners for Ru complex under cytosolic conditions, the proteins presented in the sample should be first detected. The HMM fraction of cancer cytosol was subjected to a proteolytic treatment including the reduction, alkylation, and digestion steps, before sample ultrafiltration (in order to separate only digested peptides) and analyzed by μHPLC-ESI-MS. As was mentioned in Experimental part, analyses of blanks were also carried out (prepared as the sample but containing only 5 mM ammonium acetate, pH 6.0 or a mixture of DTT, IAM, and trypsin in ammonium acetate solution). A chromatogram of resulting proteinaceous cytosolic fraction is shown in Fig. S3.

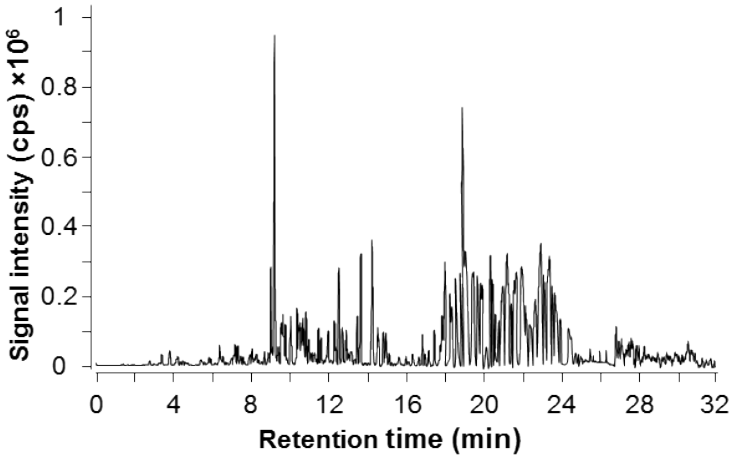

Fig. S3 The fingerprinting total-ion-current chromatogram of digested HMM fraction of cytosol of cancer cells after subtracting the signals from DTT, IAM, trypsin, and ammonium acetate buffer (registered in positive ionization mode). For analysis parameters, see Experimental.

Based on the obtained chromatographic signals the parent ions were chosen to collision induced dissociation experiments. When at least three specific digested peptides of a given protein were identified on the basis of their fragmentation ions, the protein was regarded as presenting in the cytosol. The applied methodology allowed for identification of 15 cytosolic proteins: pro-apoptotic – Apaf1, cytochrome c (CytC), p53; inhibitors of apoptosis – APC and BCL2; regulators of cell progression – p16, p21, RB; heat shock proteins – HSP27 and HSP70; DNA repair proteins: BLM, BRCA1, BRCA2, XP-C, SMS. The detailed information about the observed fragmentation ions and identified specific peptides for the detected proteins is summarized in Table S2. The exemplary scanning and fragmentation spectra for the cytochrome c peptide (EER) (at the retention time of 12 min; *m/z* 455) are presented in Fig. S4.

Table S2 Proteins identified in cytosol of cancer cells by μHPLC-ESI-QqQ-MS method

Identified protein (molecular mass in kDa)	Identified characteristic peptide	Monoisotopic mass of peptide (Da)[a]	Ion observed in scanning mode (*m/z*)[b]	Fragmentation ions (*m/z*)
Apaf1, (141.8)	SFR	408.2	$[L + H]^+$ (409.2)	y_2 (322.2), b_2 (235.1), y_1 (175.2), b_1 (88.0)
	MHK	414.2	$[L + H]^+$ (415.2)	y_2 (284.3), b_2 (269.2), y_1 (147.3), b_1 (132.1)
	DFR	436.2	$[L + H]^+$ (437.2)	y_2 (322.3), b_2 (263.1), a_2 (235.1), y_1 (175.1), b_1 (116.1)
	LQAK	458.3	$[L + H]^+$ (459.3)	y_3 (346.3), b_2 (242.3), y_2 (218.3), y_1 (147.3)
	AAMLIK	645.4	$[L + H]^+$ (646.4)	y_5 (575.4), y_4 (504.4), y_3 (373.3), b_3 (274.2), b_2 (143.1)
	IFQSR	649.4	$[L + H]^+$ (650.4)	y_3 (390.3), b_3 (389.3), y_2 (262.3), b_2 (261.3)
APC, (311.6)	GSK	290.2	$[L + H]^+$ (291.2)	y_2 (234.3), y_1 (147.3), b_2 (145.1), b_1 (58.1)
	GPR	328.2	$[L + H]^+$ (329.2)	y_2 (272.2), b_1 (58.1)
	QIR	415.3	$[L + H]^+$ (416.3)	y_2 (288.3), b_2 (242.2), a_2 (214.2), y_1 (175.2), b_1 (129.1)
	APSR	429.2	$[L + H]^+$ (430.2)	y_3 (359.2), y_2 (262.2), y_1 (175.2), b_2 (169.2)
	NADSK	533.2	$[L + H]^+$ (534.2)	y_4 (420.3), y_3 (349.2), b_3 (301.2), y_2 (234.3), b_2 (186.2)
	SYGSR	568.3	$[L + H]^+$ (569.4)	y_3 (319.3), b_3 (308.2), y_2 (262.3), b_2 (251.1)
	DNQAK	574.3	$[L + H]^+$ (575.3)	b_3 (358.2), y_3 (346.3), b_2 (230.1), y_2 (218.2)
	VSTWR	647.3	$[L + H]^+$ (648.3)	y_3 (462.3), y_2 (361.3), b_3 (288.2), b_2 (187.2), y_1 (175.2)
	LSQLPR	712.4	$[L + H]^+$ (713.4)	b_4 (442.4), y_2 (272.2)
	ALVAQLK	741.5	$[L + H]^+$ (742.5)	y_5 (558.4), y_4 (459.4), y_3 (388.3), b_4 (355.3)
	NASSIPR	743.4	$[L + H]^+$ (744.4)	b_5 (473.3), y_2 (272.2), b_3 (273.2)
	IQQIEK	757.4	$[L + H]^+$ (758.4)	y_4 (517.4), y_3 (389.4), b_3 (370.2), b_2 (242.1)
	NGISPPNK	825.4	$[L + H]^+$ (826.4)	b_7 (680.5), y_4 (455.4), b_4 (372.2)
BCL2, (26.3)	DPVAR	556.3	$[L + H]^+$ (557.3)	b_3 (312.2), y_2 (246.3), a_3 (284.2)
	EIVMK	618.3	$[L + H]^+$ (619.3)	y_4 (490.3), y_3 (377.3), b_3 (342.2), y_2 (278.3)
	MAHAGR	641.3	$[L + H]^+$ (642.3)	y_4 (440.3), b_3 (340.2), y_3 (303.3), y_1 (175.2)
	TGYDNR	724.3	$[L + H]^+$ (725.3)	b_4 (437.2), y_4 (567.4), y_3 (404.3), b_3 (322.2), y_2 (289.3)
	DGVNWGR	802.4	$[L + H]^+$ (803.4)	y_5 (631.3), y_4 (532.3), y_3 (418.4), b_4 (386.2), y_2 (232.2)
	QAGDDFSR	894.4	$[L + H]^+$ (895.4)	y_6 (696.3), y_5 (639.4), y_4 (524.3), b_4 (372.2), y_3 (409.3)
	FATVVEELFR	1209.6	$[L + H]^+$ (1210.6)	y_6 (792.4), y_5 (693.5), b_5 (518.4),

BLM, (159.0)	KPK	371.3	$[L + H]^+$ (372.3)	y_2 (244.3), b_1 (129.1), a_1 (101.2)
	GPGR	385.2	$[L + H]^+$ (386.3)	y_3 (329.3), b_3 (212.2), y_1 (175.1),
	ALVAK	500.3	$[L + H]^+$ (501.3)	y_3 (317.3), b_3 (284.2), y_2 (218.2), b_2 (185.2), y_1 (147.3)
	TASSGSK	636.3	$[L + H]^+$ (637.3)	y_4 (378.4), b_3 (347.2), y_3 (291.3), y_2 (234.3)
BRCA1, (67.3)	IIR	400.3	$[L + H]^+$ (401.3)	y_2 (288.2), b_2 (227.2), a_2 (199.2), y_1 (175.1), b_1 (114.1)
	EPVSTK	659.3	$[L + H]^+$ (660.3)	y_5 (531.4), b_4 (413.2), y_3 (335.3), b_3 (326.2), b_1 (130.1)
	SVESNIEDK	1019.5	$[L + H]^+$ (1020.5)	y_8 (933.4), y_6 (705.3), b_3 (316.2), b_1 (88.0)
BRCA2, (384.2)	YI	294.2	$[L + H]^+$ (295.2)	b_1 (164.1), a_1 (136.1), y_1 (131.0)
	NGR	345.2	$[L + H]^+$ (346.1)	y_2 (232.1), y_1 (175.2), b_2 (172.1), b_1 (115.1)
	ETAK	447.2	$[L + H]^+$ (448.2)	b_3 (302.2), b_2 (231.2), y_1 (147.3), $_{y2}$ (218.2)
	INSK	460.3	$[L + H]^+$ (461.3)	b_3 (315.3), a_3 (287.3), y_2 (234.3), b_2 (228.1), y_1 (147.3)
	EEEK	533.2	$[L + H]^+$ (534.2)	b_3 (388.2), y_2 (276.2), b_2 (259.2), y_1 (147.3)
	SCGTK	551.2	$[L + H]^+$ (552.2)	y_4 (406.1), b_3 (305.2), y_2 (248.2), y_1 (147.1)
	MFFK	571.3	$[L + H]^+$ (572.3)	b_3 (426.2), y_2 (294.4), b_2 (279.2), y_1 (147.3)
	MPIGSK	631.3	$[L + H]^+$ (632.6)	y_5 (501.4), b_3 (342.2), y_3 (273.3), b_1 (132.2)
	EFANR	635.3	$[L + H]^+$ (636.3)	y_3 (360.3), b_3 (348.2), y_2 (289.2), b_2 (277.2), y_1 (175.2)
	ENNENK	746.3	$[L + H]^+$ (747.3)	y_4 (504.4), y_3 (390.3), b_3 (358.2), b_2 (244.2), y_2 (261.2)
	YETPIK	749.4	$[L + H]^+$ (750.4)	b_5 (604.3), b_3 (394.2), y_3 (357.4), y_1 (147.1)
	VFVPPFK	832.5	$[L + H]^+$ (833.5)	b_6 (687.5), y_4 (488.6), b_3 (346.2), y_1 (147.1)
	TSSADTQK	836.4	$[L + H]^+$ (837.4)	b_6 (563.3), y_4 (491.4), b_5 (462.4), y_3 (376.3), b_4 (347.3)
CytC, (11.7)	EER	432.2	$[L + Na]^+$ (455.2)	y_2 (304.2), b_2 (259.2), y_1 (175.2), b_1 (130.2)
	IFIMK	650.4	$[L + Na]^+$ (673.4)	y_3 (391.4), b_4 (505.3), b_3 (374.3), y_2 (278.3), b_2 (261.2), y_1 (147.2)
	ADLIAYLK	905.5	$[L + H]^+$ (906.5)	y_4 (494.3), b_4 (413.2), y_2 (260.2), b_2 (187.1)
HSP27, (22.8)	MTER	535.2	$[L + H]^+$ (536.2)	y_3 (405.3), y_2 (304.2), b_2 (233.1), y_1 (175.2), b_1 (132.1)
	HTADR	598.3	$[L + H]^+$ (599.4)	y_3 (361.3), b_3 (310.2), y_2 (290.2), b_2 (239.1), y_1 (175.2)
	DWYPHSR	959.4	$[L + H]^+$ (960.5)	b_5 (699.3), y_4 (496.6), b_3 (465.2), y_2 (262.2)
HSP70, (54.8)	NSAK	418.2	$[L + H]^+$ (419.2)	y_3 (305.3), y_2 (218.2), b_2 (202.1), y_1 (147.3), b_1 (115.1)
	DILVK	586.4	$[L + H]^+$ (587.4)	y_3 (359.4), b_3 (342.2), y2 (246.3), b_2 (229.1), y_1 (147.3)
	SNILVFK	819.5	$[L + H]^+$ (820.5)	b_5 (527.4), y_4 (506.6), b_4 (428.3), y_3 (393.5), b_3 (315.2), y_1 (147.3)
p16, (16.5)	AGAR	373.2	$[L + Na]^+$ (396.1)	y_2 (246.1), b_3 (200.2), y_1 (175.2), b_2 (129.1), a_2 (101.1)
	YLR	450.3	$[L + H]^+$ (451.3)	y_2 (288.3), b_2 (277.1), y_1 (175.2), b_1 (164.1)
	GSNHAR	640.3	$[L + H]^+$ (641.3)	b_4 (396.2), y_3 (383.4), b_3 (259.1), y_2 (246.3),
p21, (18.1)	KP	243.2	$[L + H]^+$ (244.2)	b_1 (129.1), y_1 (116.1)
	LIFSK	606.4	$[L + H]^+$ (607.4)	b_4 (461.3), y_3 (381.5), b_3 (374.2), y_2 (234.3), b_2 (227.2), y_1 (147.3)
	QNPCGSK	789.3	$[L + H]^+$ (790.3)	y_5 (548.4), b_4 (500.2), y_3 (291.3), b_2 (243.3)
	LYLPTGPR	915.5	$[L + H]^+$ (916.5)	b_6 (645.4), y_5 (527.7), b_3 (390.2), y_2 (272.3),
	MSEPAGDVR	960.4	$[L + H]^+$ (961.4)	y_6 (614.4), b_5 (516.3), y_4 (446.3), b_3 (348.2)
p53, (43.7)	NTFR	536.3	$[L + H]^+$ (537.4)	b_3 (363.3), y_2 (322.3), b_2 (216.1), y_1 (175.2), b_1 (115.1)
	VCACPGR	818.4	$[L + H]^+$ (819.4)	b_6 (645.2), b_4 (491.1), y_3 (329.6), y_1 (175.2)
	NSFEVR	750.4	$[L + H]^+$ (751.4)	b_4 (478.4), y_3 (403.4), b_3 (349.2), y_2 (274.4)
	CPHHER	834.4	$[L + H]^+$ (835.3)	y_5 (675.4), y_3 (441.5), b_3 (395.2), y_2 (304.3), b_1 (161.2)
	VEYLDDR	908.4	$[L + H]^+$ (909.4)	y_4 (518.7), b_4 (505.4), y_3 (405.6), b_3 (392.2)
RB (106.2)	SPK	330.2	$[L + H]^+$ (331.3)	y_2 (244.2), b_1 (88.1)
	SPYK	493.3	$[L + H]^+$ (494.3)	y_3 (407.3), b_3 (348.2), y_1 (147.3), b_1 (88.1)
	MTPR	503.3	$[L + H]^+$ (504.3)	y_2 (272.3), b_2 (233.2)
	LYYR	613.3	$[L + H]^+$ (614.6)	b_3 (440.2), y_2 (338.4), b_2 (277.1), y_1 (175.2)
	LAYLR	634.4	$[L + H]^+$ (635.3)	y_3 (451.5), b_3 (348.2), y_2 (288.4), b_2 (185.1), y_1 (175.2)

SMS, (41.3)	AKP	314.2	$[L + H]^+$ (315.3)	y_2 (244.2), b_2 (200.1), a_2 (172.1), y_1 (116.1)
	EGR	360.2	$[L + H]^+$ (361.3)	y_2 (232.1), b_2 (187.1), y_1 (175.2), a_2 (159.2), b_1 (130.1)
	QDGK	446.2	$[L + H]^+$ (447.2)	b_3 (301.2), b_2 (244.1), y_2 (204.1), y_1 (147.3)
	GGAIDR	587.3	$[L + H]^+$ (587.4)	y_4 (474.3), y_3 (403.2), b_3 (186.2), y_1 (175.2), b_2 (115.1)
	AIMGSGK	662.3	$[L + H]^+$ (663.2)	b_5 (460.3), b_4 (373.3), y_4 (348.4), b_3 (316.2), y_3 (291.3)
	YWPTADGR	964.4	$[L + H]^+$ (965.5)	y_6 (616.4), b_4 (548.3), y_4 (418.5), b_2 (350.2), y_2 (232.2)
XP-C (106.0)	VAR	344.2	$[L + H]^+$ (345.3)	y_2 (546.2), y_1 (175.2), b_2 (171.1), b_1 (100.1)
	FNK	407.2	$[L + H]^+$ (408.3)	b_2 (262.1), y_2 (261.3), a_2 (234.2), b_1 (148.1), y_1 (147.3)
	THR	412.2	$[L + H]^+$ (413.2)	y_2 (312.2), b_2 (239.2), y_1 (175.2), b_1 (102.2)
	AHHLK	604.3	$[L + H]^+$ (605.4)	y_3 (397.4), b_3 (346.2), y_2 (260.3), b_2 (209.1), y_1 (147.3)
	AAGGEPR	656.3	$[L + H]^+$ (657.4)	y_5 (515.4), b_5 (386.2), y_2 (272.2), b_2 (143.2)
	DFPSDLK	820.4	$[L + H]^+$ (821.5)	y_5 (559.3), b_4 (447.3), y_3 (375.5), b_2 (263.2)

[a] Generated by ExPASy base. [b] L denotes the respective characteristic peptide

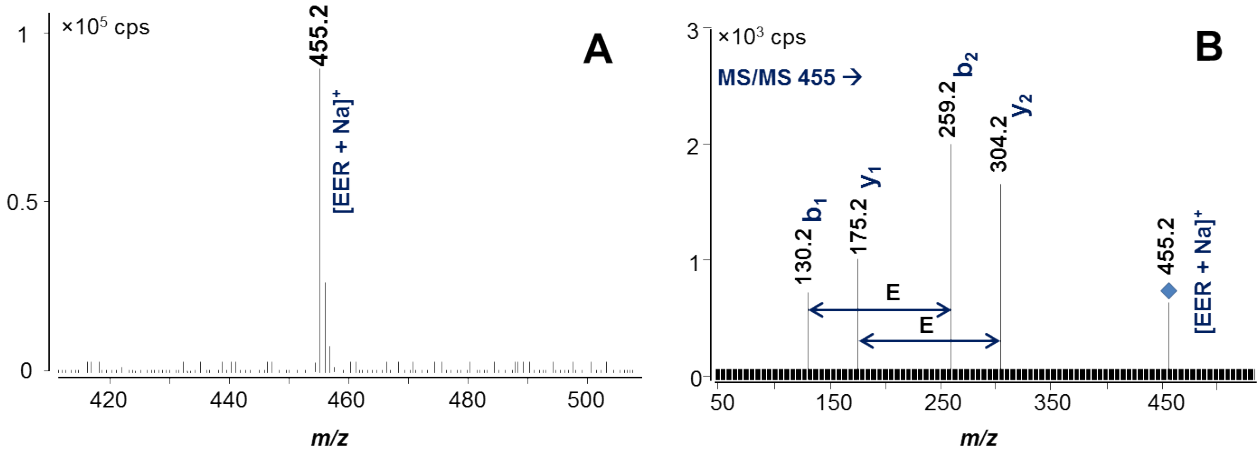

Fig. S4 Representative spectra used for identification of a specific cytochrome c peptide EER (positive ionization mode): A - scanning spectrum registered for the μHPLC signal at 12 min B – spectrum for the fragmentation ion of *m/z* 455 (collision energy 30 eV).