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Half-sandwich Os(II) and Ru(II) bathophenanthroline complexes: anticancer drug candidates with unusual potency and cellular activity profile in highly invasive triple-negative breast cancer cells

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Experimental

Starting materials

Complexes [Os(η^6-*p*cym)(bphen)(dca)]PF_6 (**Os-dca**), [Ru(η^6-*p*cym)(bphen)(dca)]PF_6 (**Ru-dca**) (Fig. 1) and [Os(η^6-*p*cym)(bphen)Cl]PF_6 (**Os-Cl**), and [Ru(η^6-*p*cym)(bphen)Cl]PF_6 (**Ru-Cl**)) were prepared and characterized as described previously.[1] Cisplatin [*cis*-diamminedichloridoplatinum(II)], dimethyl sulfoxide (DMSO), 6-thioguanine (6-TG),), sulforhodamine B, trichloracetic acid (TCA), Matrigel, crystal violet, glutaraldehyde, methylene blue, Lactate Assay Kit, penicillin and streptomycin were obtained from Sigma–Aldrich s.r.o. (Prague, Czech Republic). Stock solutions of metal-based compounds were freshly prepared in DMSO and diluted with water or cell culture medium to an appropriate concentration just before use. RPMI 1640 medium and Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and trypsin/EDTA were from PAA (Pasching, Austria). JC-1 dye was purchased from Cayman Chemical (MI, USA). Aquaporin 5 antibody was from Abcam (Cambridge, UK). Porimin antibody was purchased from Santa Cruz Biotechnology, Inc. (Texas, USA).

Cell lines

The human ovarian carcinoma A2780 (cisplatin sensitive) and A2780R (with acquired resistance to cisplatin) cell lines were kindly supplied by Professor B. Keppler (University of Vienna, Austria). Invasive breast epithelial carcinoma cells MDA-MB-231 were purchased from the European collection of authenticated cell cultures (ECACC) (Salisbury, UK). Chinese hamster lung fibroblast cells V79 were obtained from Dr. M. Pirsel (Cancer Research Institute, Slovak Academy of Sciences, Bratislava, Slovakia). Human prostate carcinoma DU-145 cells were kindly supplied by V. Mašek, Palacky University, Olomouc, Czech Republic. Human skin fibroblasts (primary cell culture) was a kind gift from Professor T. Adam, Laboratory of Inherited Metabolic Disorders, Department of Clinical Chemistry, Palacky University and Hospital, Olomouc, Czech Republic. Human embryonic kidney HEK293 cells were purchased from ECACC. The A2780 and A2780R cells were grown in RPMI 1640 medium supplemented with streptomycin (100 μg mL^{-1}), penicillin (100 U mL^{-1}) and heat inactivated FBS (10%). The resistance of A2780R cells was maintained by the addition of 1 μM cisplatin to culture medium every second subculture. The MDA-MB-231 cells were grown in DMEM medium supplemented withstreptomycin (100 μg mL^{-1}), penicillin (100 U mL^{-1}), heat inactivated FBS (10%) and 1% nonessential amino acids (NEAA) (Sigma-Aldrich). The DU-145, HEK293 and primary skin fibroblasts were grown in DMEM medium supplemented with streptomycin (100 μg mL^{-1}), penicillin (100 U mL^{-1}) and heat inactivated FBS (10%). The cells were cultured in a humidified incubator at 37 °C in the atmosphere of 5% CO_2. Confluent cells were subcultured 2–3 times per week according to standard procedure.

Cytotoxicity testing

The cytotoxicity of Ru and Os complexes and cisplatin was determined against human ovarian carcinoma cell lines A2780 (cisplatin sensitive) and A2780R (with acquired resistance to cisplatin) cell lines, human prostate carcinoma (DU 145) and invasive ductal carcinoma MDA-MB-231 cell lines, nontumorigenic primary skin fibroblast and human embryonic kidney HEK293 cell lines. Cells were seeded on 96-well tissue culture plates at a density of 10^4 cells/well in 100 μL of growth medium and incubated at 37 °C in a humidified 5% CO_2 atmosphere for 16 h (overnight). After the incubation period, the cells were treated with the compounds and kept in the incubator for additional 72 h. The stock solutions of compounds were always freshly prepared in DMSO before use. The final concentration of DMSO in cell culture medium did not exceed 0.1% (v/v). After the treatment period, 100 μl of a 0.33% solution of neutral red (40 μg ml^{-1}) in culture medium (w/o FBS, ATB) was added to each well with adherent cells and the plate was incubated at 37 °C in a humidified 5 % CO_2

atmosphere for 2 h. Afterwards, the dye containing medium was carefully removed and the cells were washed with phosphate buffered saline (PBS). The incorporated dye was then solubilized in neutral red de-stain solution [ethanol (50%), deionized water (49 %), acetic acid (1%)], allowed to stand for 10 min at room temperature and the absorbance was measured at λ = 540 nm with an absorbance reader Synergy Mx (Biotek, USA). Compound concentrations that induce 50 % cell death (IC_{50}) were calculated from curves constructed by plotting cell survival (%) versus drug concentration (μM). The reading values were converted to the percentage of the control (percentage cell survival). Cytotoxic effects were expressed as IC_{50}.

Cellular accumulation

The MDA-MB-231 cells were seeded on Petri dishes at a density of $3x10^6$ cells/dish. After overnight incubation, the cells were treated with the investigated compounds at equimolar concentrations for 3 h (5 μM or 0.6 μM). Then the cells were carefully washed with PBS, treated with trypsin, collected, counted and the cell suspension washed four-times with ice cold PBS. Viability of the cells was assessed by tryphan blue exclusion assay. Cell pellets were digested by a high pressure microwave digestion system (MARS5, CEM) with HCl (11 M) to give a fully homogenized solution, and final metal content was determined by ICP-MS (Agilent 7500, Agilent, Japan).

Cell invasion assay

MDA-MB-231 were seeded on 40 mm Petri dishes at a density of 1.5×10^5 cells/dish and incubated at 37 °C in a humidified 5% CO_2 atmosphere for 48 h, followed by 24 h incubation in serum-free medium containing 0.1% w/v BSA. Cells were treated with metal-based compounds in PBS with either equitoxic concentrations corresponding to 4 x $IC_{50,72\ h}$ or equimolar (2 μM) concentrations and incubated for 1 h. Invasion assay was performed using a dual chamber system. Cells were seeded on BD Falcon™ cell culture inserts with transparent PET membranes (8.0 μm pore size) coated with Matrigel at a density of 5×10^4 cells/insert. The inserts were added to the upper chamber and incubated at 37 °C in a humidified 5% CO_2 atmosphere for 96 h. The bottom chamber was filled with complete growth medium containing 10% FBS as an attractant. For the negative control the bottom chamber was filled with serum-free medium supplemented with 0.1% w/v BSA. Invaded cells were fixed with glutaraldehyd and stained with crystal violet. The absorbance was measured at 590 nm using an absorbance reader Synergy Mx (Biotek, USA). Values of absorbance were expressed as normalized invasion (% of control).

Wound healing assay

Confluent MDA-MB-231 cells grown in 6-well culture plates were left to starve overnight in serum reduced medium (1% FBS) and scratched vertically with P200 pipet tip. The cells were washed twice with PBS to remove the peeled cells and treated with the investigated compounds at their equitoxic concentrations corresponding approximately to 65% the values of IC_{50} found for these compounds in MDA-MB-231 cells treated for 72 h. Alternatively, cells were treated with equimolar (0.32 μM) concentration of metallodrugs. Artificial wounds (scratches) were photographed immediately after addition of the investigated compounds and after 24 h using NIB 100 inverted microscope with Canon EOS 1200D camera. Digital images were acquired by QuickPHOTO MICRO 3.1 program (PROMICRA, Prague, Czech Republic) and processed with Tscratch analysis software (ETH Zürich, Switzerland). The extent of migration in each sample was measured as the area covered by the cells after 24 h and expressed as percentage of control.

Re-adhesion test

To study the effect of the investigated complexes on the cell ability to readhere to a growth surface, MDA-MB-231 cells were cultured for 48 h at 37 °C in a 5% CO_2 humidified atmosphere in a complete medium containing 10% FBS. Subsequently, the medium was replaced with the serum-free medium supplemented with 0.1% w/v BSA for 24 h before treatment. The cells were treated with the investigated compounds at equimolar concentrations (10 μM) or roughly equitoxic concentrations (4-fold the IC_{50} value, 72 h) for 2 h at 37 °C in a 5% CO_2 humidified atmosphere. At the end of the treatment, cells were gently scrapped and resuspended in a serum-free medium with 0.1% w/v BSA. Cells were left for 15 min at room temperature to allow surface receptor reconstitution and then seeded in 96-well tissue culture plate at a density of 3×10^4 cells/well in 100 μL of medium. Cells were left to adhere for 30 min at 37 °C in a 5% CO_2 humidified atmosphere. Thereafter, the medium containing nonadhered cells was removed and the adhesion of cells was detected by the sulforhodamine B (SRB) test. Briefly, cells were fixed with 10% v/v cold trichloroacetic acid (TCA) at 4 °C for 1 h. TCA was discarded and wells washed twice with distilled water and air-dried. SRB solution (0.4% w/v in 1% acetic acid) was added to the wells and samples were incubated for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid and wells were left to air-dry. The bound SRB was dissolved with 10 mM Tris base (pH 10.5). The absorbance was measured at λ = 570 nm with an absorbance reader Synergy Mx (Biotek, USA). The reading values were normalized to untreated control (percentage cell re-adhesion activity).

Analysis of changes in the mitochondrial membrane potential ψ

The effect of the investigated compounds on the mitochondrial membrane potential ψ in MDA-MB-231 cells was investigated by using JC-1 fluorescent probe (Cayman Chemical, MI, USA) and analyzed on confocal laser-scanning microscope Leica TCS SP8 SMD (Leica microsystems GmbH, Wetzlar, Germany). MDA-MB-231 cells were seeded on a 35 mm glass bottom culture dishes (MatTek Co., MA, USA) at the density 3 x 10^5 cells/dish and incubated overnight (16 h). Then, the cells were treated with equitoxic concentrations of the investigated compounds corresponding to 4 x $IC_{50,72\ h}$ and incubated for 2 h. The cells were subsequently stained with JC-1 cationic dye and visualized on confocal microscope. JC-1 dye was excited by argon laser at 488nm. J-monomers were detected at 510-550 nm, whereas J-aggregates were detected at 590-640 nm. Images were analyzed for the mean fluorescence intensities in the corresponding fluorescence channels by using ImageJ software. Data were expressed as the mean fluorescence ratio J-aggregates/J-monomers. Samples visualized in this experiment were used for the cell size analysis.

Lactate production assay

Lactate concentration in cell culture medium was measured using an enzymatic colorimetric Lactate Assay Kit (Sigma-Aldrich). MDA-MB-231 cells were seeded in a 6-well culture plates at a density of 2 x 10^6 cells/well. After overnight incubation in a complete growth medium at 37 °C in a 5% CO_2, the cells were treated with the investigated complexes at final, roughly equitoxic concentrations corresponding to $IC_{50,\ 72\ h}$ values in a serum-free medium for 16 h. Subsequently, the plates were spun down at 2500 rpm at room temperature for 10 min. Lactate dehydrogenase was removed from samples by using 10 kDa cutoff spin filter column. Soluble fraction (50 μL) was added into a 96-well plate and mixed with 50 μL of Lactate Assay Master Reaction Mix. The plates were shaken for 30 min at room temperature. After this time absorbance was measured at 570 nm using an absorbance reader Synergy Mx (Biotek, USA). The amount of lactate acid present in samples was determined from a standard curve plotted for the appropriate lactate standards and the lactate concentration was normalized to the number of cells in each sample.

Zymography

The effect on the activity and/or production of matrix metalloproteinase 9 (MMP-9) was assessed by SDS–PAGE zymography as already described [2]. Briefly, MDA-MB-231 cells (1.0 x 10^5/well) were seeded into 6-wells culture plates and cultured in a medium containing 10% FBS to near confluence of the cell monolayer. Then, the medium was removed, the cells were washed with PBS. The cells were incubated for 24 h in serum-starved medium before being treated with **Os-dca**, **Ru-dca** and cisplatin at their eqiumolar (2 μM) or equitoxic (2x $IC_{50;}$ i.e. 1 μM, 1.7 μM and 112 μM, respectively) for 1 h . At the end of the treatment, metal-complexes containing medium was removed and replaced by serum-free medium, and cells were incubated for additional 24 h. Culture media were then collected, and concentrated ca. 15 times using Centricon Ultracel YM-30 (30,000 molecular weight cut-off) Centrifugal Filter Devices (Millipore, Bedford, MA). Equal amounts of proteins for each sample, as determined by the Bradford method, were analysed by SDS–PAGE on a 7% polyacrylamide gel containing 0.2% w/v gelatine. At the end of the electrophoresis, the gels were washed for 30 min at 4°C in 2.5% v/v Triton X-100 and subsequently in water to remove SDS. The gels were then incubated for 18 h at 37°C in collagenase buffer [200 mM NaCl, 50 mM tris(hydroxymethyl)aminomethane, 5 mM $CaCl_2$, pH 7.4] to reactivate enzyme activity and, afterwards, stained with Coomassie brilliant blue G-250 (EZBlue Gel Staining Reagent, Sigma). The gelatinolytic regions were observed as white bands against a blue background. The intensities of the bands were quantitatively evaluated by using AIDA image analyzer software.

HPRT mutant frequency determination

The HPRT/V79 mutation assay was used for detection of 6-thioguanin (6-TG) resistant colonies in V79 cells in accordance with Robichova et al.[3] with some modifications. Briefly, V79 cells were seeded on 100 mm Petri dishes at a density of 5 x 10^5 cells/dish in duplicate for each sample and incubated at 37 °C in a humidified 5% CO_2 atmosphere for 48 h. Cells were exposed to the investigated Ru or Os complexes at the concentration of 1.5 μM or to 5 μM cisplatin for 2 h. After the treatment, the cells were trypsinized and plated (3.5 x 10^5 cells, in triplicate per sample) for further cultivation. For colony forming ability (CFA) the cells were plated on 60 mm Petri dishes (3 x 10^2 cells, in triplicate per sample). On the seventh day after treatment, the cells were stained with methylene blue and the number of colonies was counted. From the ratio of the number of colonies and the number of cells plated the percentage of CFA was calculated. The cells for further cultivation were kept by regular subculture at a certain cell density to avoid overcrowding. Each sample was processed as follows: the cells were plated: (i) on five Petri dishes (10 mm) at a density of 2 x 10^5 cells/dish in pentaplicate per sample for detection of 6-TG resistant mutations; after the cells were attached, the selective agent 6-TG was added at the final concentration of 37 μM; (ii) on the three Petri dishes (60 mm) at a density of 3 x 10^2 cells in triplicate per sample for estimation of viability of the cells. On the seventh day of expression the percentage of CFA was calculated. Colonies of mutations were stained by methylene blue and counted 10 days after adding 6-TG. The yield of 6-TG resistant mutations was calculated. The results were statistically evaluated by Student t-test.

Results

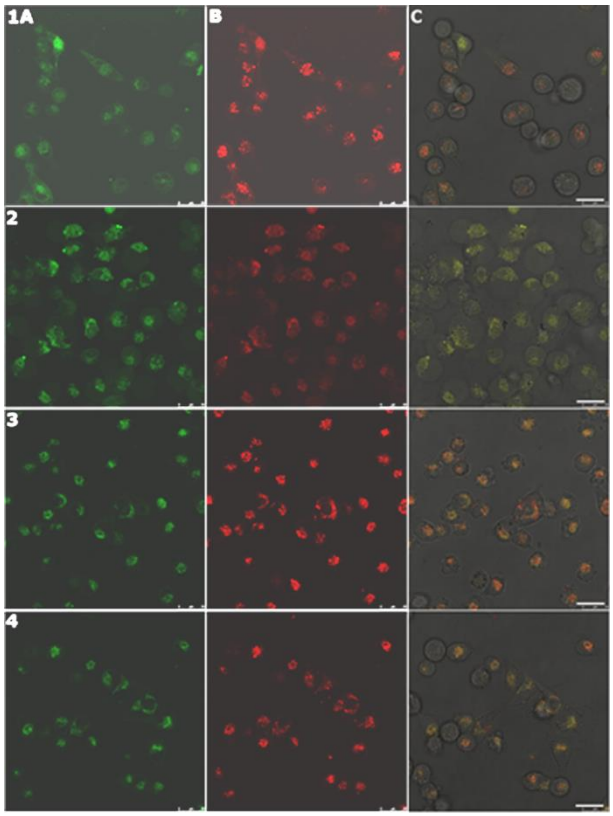

Fig S1 MDA-MB-231 cells stained with JC-1 and visualized by using confocal microscopy. Cells were treated for 2 h with equitoxic concentrations of the investigated compounds corresponding to 4 x IC_{50} (Table 1). Channels: A) green fluorescence (J-monomers); B) red fluorescence (J-aggregates); C) overlay of the signals from J-monomers and J-aggregates with the bright field channel. Panels: 1) **Ru-dca**, 2) **Os-dca**, 3) cisplatin, 4) control, untreated cells. Scale bars indicate 25 µm.

References

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