Electronic Supplementary Material (ESI) for Medical Chemical Communications

Supporting Information

Accompanying the manuscript

Water-soluble bis(1,10-phenanthroline) octanedioate Cu^{2+} and Mn^{2+} complexes with unprecedented nano and picomolar *in vitro* cytotoxicity: promising leads for chemotherapeutic drug development

Andrew Kellett,*[a] Mark O'Connor,[b] Orla Howe,[b] Alan Casey,[b] Malachy McCann,[c] Pauraic McCarron,[b,c] Mary McNamara,[b] Sean Kennedy,[b] Donald D. May,[d] Philip Skell,[e] Denis O'Shea[b] and Michael Devereux[b*]

List of Contents:

S-1: Competitive ethidium displacement and quenching experiments.

Competitive Ethidium Displacement.[1] A working solution containing 1 μ*M* CT-DNA (ε_{260} = 12,824 $M(bp)^{-1}$ cm^{-1}) (Sigma) along with 1.26 μ*M* ethidium bromide (EtBr) at neutral pH in TES buffer (10 m*M* TES, 0.1 m*M* Na_2EDTA, pH = 7.0) was prepared. Stock solutions of complexes **1** and **2** were perpared at 2.0 m*M* in 100% ethanol. Two millilitres of DNA-Et solution were placed in a 10-mm quartz cuvet (3 mL) and positioned in a temperature controlled (20°C) spectrofluorometer (Perkin Elmer LS55B Luminescence Spectrometer). Excitation and emission wavelengths were set to 546 and 595 nm, respectively. After thermal equilibrium was established the emission and excitation slits were changed to give a fluorescence reading of 50 arbitrary units with measurements being recorded over a 20-s interval. An aliquot of complex or drug solution was taken (0.5 – 10 μL), added to the cuvette and after equilibration the fluorescence reading was recorded. Repeated aliquots were added until the fluorescence was 20-40% of the initial control. Triplicate titrations were preformed and the apparent binding constants were calculated using $K_{app} = K_e$ x $1.26/C_{50}$ where $K_e = 9.5 \times 10^6$ $M(bp)^{-1}$.

DNA-Ethidium Fluorescence Quenching.[1] A working solution containing 20 μ*M* CT-DNA (ε_{260} = 12,824 $M(bp)^{-1}$ cm^{-1}) along with 2.0 μ*M* Et in NaOAc buffer (2 m*M* NaOAc, 9.3 m*M* NaCl, 0.1 m*M* Na_2EDTA, pH = 5.0) was prepared. Subsequent experimental steps are identical to the displacement experiment with the exception that a fluorescence reading of 200 arbitrary units with measurements being recorded over a 20-s interval was used. From a plot of the fluorescence versus added drug concentration, the *Q* values are given by the concentration required to effect 50% removal of the initial fluorescence.

S-2: DNA cleavage experiments.

Nuclease Activity. Reactions were carried out according to the following general procedure; in a total volume of 15 μL using 20 m*M* sodium phosphate buffer (pH = 7.2) with 20 m*M* NaCl with 1-50 μ*M* of complex, which were initially prepared in ethanol, then diluted in buffer, with 0.75 μL of 0.25 μg/μL pUC18 (Roche). Samples were incubated at 37^{o}C. Quench buffer (3 μL; 0.25% bromophenolblue, 0.25% xylene cyanole and 30% glycerol) was then added and samples were loaded onto agarose gel (1.2%) containing 1.5 μL of solution of GelRed™ (10,000X). Electrophoresis was completed at 80 V for 2 h in 1XTAE buffer.

Experiments were carried out as follows

(*a*) 20 h incubation in the absence of added oxidant or reductant, lane 1: DNA alone; lanes 2-6: 1, 5, 10, 20, 50 μ*M* complex **1**; lanes 7-10: 5, 10, 20, 50 μ*M* complex **2**.

(*b*) 2 h incubation in the presence of added ascorbate (at twice complex concentration), lane 1: DNA alone; lanes 2-5: 1, 5, 10, 20 μ*M* complex **1**; lanes 6-9: 1, 5, 10, 20 μ*M* complex **2**.

(*c*) 20 h incubation of 20 μ*M* complex **1** in the absence of added oxidant or reductant, lane 1: + 100 m*M* Na_2EDTA; lane 2: saturated in an argon atmosphere.

(*d*) 2 h incubation of 20 μ*M* complex **2** with added ascorbate (at twice complex concentration), lane 1: + 100 m*M* Na_2EDTA; lane 2: saturated in an argon atmosphere.

S-3: Superoxide dismutase (SOD) experiments.

NBT Assay. The SOD mimetic activities of the complexes **1** & **2** was determined using an indirect method in which the xanthine/xanthine-oxidase system served as the source for superoxide radicals ($O_2^{\bullet -}$).[3] The quantitative reduction of NBT to blue formazan by the $O_2^{\bullet -}$ was followed spectrophotometrically using a thermostatically controlled Varian Cary 50 Scan single beam spectrophotometer at 550 nm at 25^{o}C. Reagents were obtained from Sigma-Aldrich Chemical Co. Ltd and assays were run in a total volume of 3 mL. Tabulated results were derived from linear regression analyses and are given as the concentration (μ*M*) equivalent to 1U of bovine erythrocyte SOD activity, where one unit of SOD activity is described as the concentration required to effect a 50% inhibition in the reduction of Nitro-bluetetrazolium (NBT) (IC_{50} value). Triplicate inhbition assays were preformed at each equivalent concentration for both complexes **1** and **2**.

S-4: Cytotoxicity experiments.

Cell Culture. For cytotoxicity evaluation HT29 (ATCC, USA), SW480 (ATCC, USA) and SW620 (ATCC, USA) cell lines were employed; all the cell lines were grown in RPMI 1640 media (Fischer). All HaCaT cells a normal human keratinocytes (kindly provided by Prof. Dr. Boukamp, Heidelberg), were grown in Dubeccos's modified minimum essential medium F-12 (DMEM F-12, Lonza). All media were supplemented with 10% foetal bovine serum (FBS) and 45 IU/ml penicillin and 45 g/ml streptomycin and cells were maintained at 37◦C in a 5% CO_2 humidified incubator.

Test Sample Preparation. For sterilisation samples were filtered with a 0.2 micron cellulose acetate filter. For cytotoxicological evaluation all samples were prepared fresh on the day of exposure. A stock concentration of test compound was prepared in the exposure media (10% DMEM F-12) necessary for the cell line under test. This sample was vortexed to ensure a uniform dispersion of the compound under test.

MTT Assay. This method is based on the reduction of the tetrazolium salt, methylthiazolyldiphenyl-tetrazolium bromide (MTT) into a crystalline blue formazan product by the cellular oxidoreductases of viable cells.[4] The resultant formazan crystal formation is proportional to the number of viable cells. For cytotoxicity assays cells were seeded in 96-well microplates (Nunc, Denmark) at a density of $1x10^5$ cells/ml for the 24 h test and 3×10^4 cells/ml for the 96 h test. These densities were found to be optimal to achieve the desired confluence at the end of the exposure period. After 24 h of cell attachment, plates were washed with 100 µL/well phosphate buffered saline (PBS) and the cells were treated with increasing concentrations of each compound for 24 h. Six replicate wells were used for each control and test concentration per microplate. Following the desired time of compound exposure, control medium or test exposures were removed, cells were then rinsed with PBS and 100 µL of fresh medium (without FBS or supplements) was added to each well. 10 µL of MTT (5 mg/mL) prepared in PBS was then added to each well and the plates were incubated for 3 h at 37°C in a 5% CO_2 humidified incubator. After this incubation period the medium was discarded, the cells were washed with 100 µL of PBS and 100 µL of DMSO was added to each well to extract the dye. The plate was shaken at 240 rpm for 10 min and the absorbance was measured at 570 nm.

Data Analysis. At least three independent experiments were conducted in triplicate for each cell line and toxicity endpoint. Test results for each test were expressed as percentage of the unexposed control ± standard deviation (SD). Control values were set as 100%. Differences between samples and the control were evaluated using the statistical analysis package SPSS 14.0. Statistically significant differences were set at $p \leq 0.05$. One-way analysis of variances (ANOVA) followed by Dunnett's multiple comparison tests were carried out for normally distributed samples with homogeneous variances. The average cytotoxicity data obtained and its associated SD was then fitted to a sigmoidal curve and a four parameter logistic model used to calculate the LD50 values for each test sample at various time points, which was the concentration of tested compound which caused a 50% cellular lethality in comparison to untreated controls. The LD50 values are reported ± 95 % Confidence Intervals (± 95% CI).

Data acquisition. For cytotoxicity evaluation fluorescence measurements were all quantified using a microplate reader (TECAN GENios, Grödig, Austria).

S-5: Cellular reactive oxygen species (ROS) experiments.

ROS activity. Intra-cellular oxidative stress.

Intra-cellular oxidative stress was quantified with the aid of 2′,7′-Dichlorofluorescin diacetate (DCFH-DA).[5] Briefly, healthy confluent HT29 cells were harvested and seeded (1000 cells/well) into black bottomed 96 well plates (Nunc, Denmark) and allowed to attach for a period of 24 h prior to exposure. For ROS quantification three independent experiments were performed for each independent experiment eight replicate wells were used for control, eight replicate wells were employed for the positive control and eight replicate wells were used for each test concentration per micro-plate. A working stock of 20 μ*M* DCFH-DA in PBS was prepared and all test concentrations, unexposed controls and positive controls were prepared and exposed to the cells in this working stock. The first control consisted of the working stock solely namely a 20 μ*M* DCFH-DA solution in PBS, the positive control consisted of a 0.5 μ*M* Hydrogen Peroxide (H_2O_2) prepared in the 20 μ*M* DCFH-DA /PBS working stock solution and finally the test concentrations consisted of a concentration range of the test compounds prepared in the working stock a 20 μ*M* DCFH-DA /PBS solution.

After the initial 24 h attachment period the media was removed, the cells were subsequently washed with 100 μL of PBS and treated with 100 μL of the control, positive control and the test concentrations and incubated. The rate of intracellular oxidative stress was then monitored by the emission at 529 nm (after 504 nm excitation) of the DCFH-DA dye at time intervals of 30 to 300 minutes (the exposure plates were re-incubated for the remaining time after each measurement had been recorded).

Data acquisition. Fluorescence measurements were all quantified using a microplate reader (TECAN GENios, Grödig, Austria).

S-6: *In vivo* drug tolerance experiments

Galleria Mellonella toxicity. *Galleria mellonella* larvae in the 6th developmental stage were used to determine the *in vivo* cytotoxicity of the complexes **1**, **2,** phen and cisplatin. Thirty healthy larvae between 0.200-0.400 g in weight with no cuticle discolouration were used for each experiment.

Fresh solutions of the test complexes were prepared immediately prior to testing under sterile conditions. Each of the tested compounds (0.05 g) were dissolved in DMSO (1 cm^3) and added to sterile water (9 cm^3) to give a stock solution (5000 µg cm^{-3}). Each compound was tested across the range; 5000 µg cm^{-3} - 200 µg cm^{-3}. Test solutions (20 µL) were administered to the larvae by injection directly into the haemocoel through the last pro-leg. The base of the pro-leg can be opened by applying gentle pressure to the sides of the larvae and this aperture will re-seal after removal of the syringe without leaving a scar.

Larvae were placed in sterile Petri dishes and incubated at 30°C for 72 h. The survival of the larvae was monitored every 24 h. Death was assessed by the lack of movement in response to stimulus together with discolouration of the cuticle. Three controls were employed in all assays. The first consisted of untouched larvae maintained at the same temperature as the test larvae. The second was larvae with the pro-leg pierced with an inoculation needle but no solution injected. The third control was larvae that were inoculated with 20 µL of sterile water.

References

[1] T. C. Jenkins and K. R. Fox (Ed.), *Methods in Molecular Biology; Drug-DNA Interaction Protocols*, Chapter 14, **90**, 195-218

[2] T. Mosmann, *J. Immunol. Meth.*, 1983; **65**, 55–63

[3] Goldstein, S. and Czapski, G., *Free Radicals - A Practical Approach*, Oxford University Press, UK, 1996, 252.

[4] T. Mosmann, *J. Immunol. Meth.*, 1983; **65**, 55–63

[5] O. Myhre, J. M. Andersen, H. Aarnes and Frode Fonnum, *Biochem. Pharm.*, 2003, **65**, 1575-1582