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Cu(II) phenanthroline–phenazine complexes dysregulate mitochondrial function and stimulate apoptosis†

Garret Rochford, [a] Zara Molphy, [b] Kevin Kavanagh, [c] Malachy McCann,[d] Michael Devereux, [a] Andrew Kellett [b] and Orla Howe *[a]

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Herein we report an in-depth study on the cytotoxic mechanism of action of four developmental cytotoxic copper(II) complexes: $[Cu(phen)_2]^{2+}$ (Cu-Phen); $[Cu(DPQ)(Phen)]^{2+}$ (Cu-DPQ-Phen); $[Cu(DPPZ)(Phen)]^{2+}$; and $[Cu(DPPN)(Phen)]^{2+}$ (where Phen = 1,10-phenanthroline, DPQ = dipyrido[3,2-*f*:2′,3′-*h*]quinoxaline, DPPZ = dipyrido[3,2-*a*:2′,3′-*c*]phenazine, and DPPN = benzo[*i*]dipyrido[3,2-*a*:2′,3′-*c*]phenazine). This complex class is known for its DNA intercalative properties and recent evidence—derived from an *in vivo* proteomic study—supports the potential targeting of mitochondrial function. Therefore, we focused on mitochondrial-mediated apoptosis related to cytotoxic activity and the potential impact these agents have on mitochondrial function. The Cu(II) complexes demonstrated superior activity regardless of aromatic extension within the phenazine ligand to the previously demonstrated activity of cisplatin. Unique toxicity mechanisms were also identified in prior demonstrated cisplatin sensitive and resistant cell lines. Double strand breaks in genomic DNA, quantified by γH2AX foci formation, were then coupled with apoptotic gene expression to elucidate the mechanisms of cell death. These results indicate that while DNA damage-induced apoptosis by *BAX*, *XIAP* and *caspase-9* and *-3* expression is moderate for the Cu(II) complexes when compared to cisplatin, protein targets independent of DNA exert a multimodal mechanistic effect. Significantly, mitochondrial gene expression of oxidative stress, protease expression, and fission/fusion processes—upregulated *HMOX*, *DRP1* and *LON*, respectively—indicated an increased oxidative damage associated with compromised mitochondrial health upon exposure to these agents. These data support a unique mode of action by these complexes and provide valuable evidence of the developmental potential of these therapeutic inorganic complexes.

Significance to metallomics

The attachment of copper(II) phenanthroline to three different extended phenazine π-backbones gives novel cationic complexes multimodal biological effects. The DNA-binding and cytotoxic properties of these complexes were probed further to show that the mitochondria are a key player in their biological effects. Our work presents the importance of mitochondrial function and apoptotic cell death as additional mechanisms of action in their multi-modal nature.

Introduction

Copper(II) complexes are part of a large array of therapeutically active inorganic-based small molecules.[1–3] The multimodal cellular activity of Cu(II) complexes reported in the literature includes: association with production of reactive oxygen species,[4] and knock-on antioxidant effects, lipid peroxidation,[5,6] DNA cleavage,[7–9] mitochondrial dysfunction and apoptosis induction.[10,11] Cu(II) complexes with different ancillary ligands have shown strong cytotoxicity profiles against a wide spectrum of cancer cell lines.[12–16] The Casiopeinas® are a family of Cu(II) ternary complexes that are capable of activation of apoptosis and genotoxicity in a range of medulloblastoma, glioma and colorectal adenocarcinoma cells.[17–20] The Cu(II) complexes in this study were synthesised and characterised by Molphy and colleagues.[21,22] The Cu(II) complexes presented in this study are Cu(II) phenanthroline

[a] *FOCAS Research Institute and School of Biological & Health Sciences, Technological University Dublin, City Campus, Dublin 8, Ireland. E-mail: orla.howe@TUDublin.ie*
[b] *School of Chemical Science and The National Institute for Cellular Biotechnology, Dublin City University, Dublin 9, Ireland*
[c] *Department of Biology, Maynooth University, Maynooth, Kildare, Ireland*
[d] *Department of Chemistry, Maynooth University, Maynooth, Kildare, Ireland*
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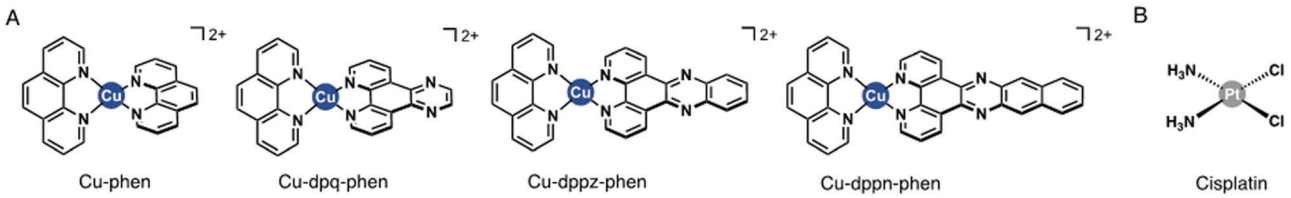

Fig. 1 (A) Molecular structures of the Cu(II) phenanthroline–phenazine complexes used in this study. (B) Molecular structure of the clinical anticancer drug cisplatin.

(Cu-Phen) (**1**) (Fig. 1) along with their three phenazine π-backbone extended cationic complexes Cu(II) phenanthroline–phenazine $[Cu(DPQ)(Phen)]^{2+}$ (where DPQ = dipyrido[3,2-*f*:2′,3′-*h*]quinoxaline) (Cu-DPQ-Phen) (**2**) (Fig. 1), Cu(II) phenanthroline-phenazine $[Cu(DPPZ)(Phen)]^{2+}$ (where DPPZ = dipyrido[3,2-*a*:2′,3′-*c*]phenazine) (Cu-DPPZ-Phen) (**3**) (Fig. 1) and Cu(II) phenanthroline-phenazine $[Cu(DPPN)(Phen)]^{2+}$ (where DPPN = benzo[*i*]dipyrido-[3,2-*a*:2′,3′-*c*]phenazine) (Cu-DPPN-Phen) (**4**) (Fig. 1). These Cu(II) complexes were previously biophysically characterised in addition to a preliminary biological screening using the cisplatin resistant SKOV-3 cell line. Both of these studies determined that the complexes induced metal-hydroxo and free hydroxyl species and result in 8-oxo-2′-deoxyguanosine (8-oxo-dG) lesion formation.[22] Additionally, the DNA binding constants of the complexes were the highest observed in phenanthrene based complexes and were comparable to the transcription inhibitor actinomycin D. The preliminary biological screening demonstrated cytotoxicity in cisplatin resistant SKOV-3 cells equivalent to adriamycin.[21] A more recent study with these complexes in the *in vivo* model *Galleria mellonella* details a series of proteomic changes which strongly suggest changes in antioxidant and mitochondrial related responses.[23] The *in vivo* study also determined that the increased DNA binding previously established by DNA binding studies was accompanied by increases in enzymes associated with P450 detoxification, glycolysis, purine metabolism and nitrogen detoxification. Interestingly, the *in vivo* model demonstrated that superior nuclease action was associated with increased toxicity in the model, while greater DNA binding increased the complexity of the multimodal response mechanism.[23] The proteomic study evidenced substantial mitochondrial-related protein involvement in response to the Cu(II) complexes which strongly suggested that mitochondrial-mediated apoptosis was one of the key mechanisms of action. These Cu(II) phenanthroline–phenazine complexes have previously demonstrated strong DNA binding activity, therapeutic activity against cisplatin resistant SKOV-3 cells and compelling evidence of antioxidant and mitochondrial-related dysfunction in *G. mellonella*. The comparison of these complexes to the activity of the highly successful clinical drug cisplatin is an important comparator for evaluating the therapeutic activity of these complexes.[24] The activity of these complexes was compared to cisplatin in MCF-7 and SKOV-3 cells lines.[13,14,21] Both MCF-7 and SKOV-3 cell lines have been used in previous studies and have demonstrated different values to those presented in this report. A strong motivation in presenting the isogenic A2780 cell lines along with its therapeutically resistant daughter cell is to address the variability inherent in the use of MCF-7 and SKOV-3 cell lines.

Based on previous studies and following from the *in vivo* investigation of the activity of these Cu(II) complexes, apoptosis and the mitochondria are a central focus of the mechanistic investigation of these complexes in the *in vitro* mammalian model. Previous studies have investigated the presence of apoptosis as a mechanism of regulated cell death following exposure to a range of Cu(II) complexes.[11,25,26] In this study we investigated using gene expression to examine a number of processes in relation to the apoptotic cascade and additionally to genes related to different functions in the mitochondria which are key interrelated processes in apoptosis. Many proteins are involved in the process of apoptosis (Table 1). The B cell lymphoma-2 family (*BCL-2*) function in both pro- and anti-apoptotic manners. Pro-apoptotic proteins, such as BCL-2 associated X protein (*BAX*), are activated when mitochondria-mediated apoptosis is signalled, upon which they form monomers of pores that localize to the mitochondrial membrane so that cytochrome *c* and inhibitors of X-linked Inhibitor of Apoptosis Protein (*XIAP*), such as second mitochondria-derived activator of caspases (*Smac*) or serine protease HTRA2/Omi, can potentiate or inhibit the process.[27] Anti-apoptotic proteins such as *BCL-2* prevent this process occurring by interacting with a BH3 regulatory repressor protein such as *Bad*. Deficiencies in *BCL-2* associated X protein (*BAX*)/*BCL-2* homologous antagonist killer (*BAK*) have been shown to confer resistance in *in vitro* models, and over expression in *BCL-2* and B-cell Lymphoma extra-large ($BCL\text{-}X_L$) is known to increase resistance *in vitro*.[28–31] Caspases are a series of serine proteases with a pivotal role in the initiation (*caspase-9*) and execution (*caspase-3*) of apoptosis. The caspase cascade can be initiated in response to DNA damage and mitochondrial dysfunction in addition to other cellular stimuli.[32]

Multiple mitochondrial-related genes and associated signalling events that are reported to characterise mitochondrial function were also selected for this study (Table 1). Heme oxygenase (*HMOX-1*) is a member of the phase II antioxidant enzymes and is primarily involved in the removal of iron based porphyrin, heme from the non-protein moiety of haemoglobin. Nuclear factor (erythroid-derived 2)-like 2 (*Nrf-2*) is closely involved with the induction of *HMOX-1* through the Antioxidant Response Element (ARE) transcription region.[33] *Nrf-2* also forms part of the transcriptional regulation of Transcription factor B1/2, mitochondrial (*TFB1M* and *TFB2M*) which in turn regulates the transcription of the general mitochondrial transcription factor, Transcription Factor A (*TFAM*).[34–36] Genes including ATP-dependent Clp protease proteolytic subunit (CLPP), Lon protease homolog 1 (*LON1*), paraplegin matrix AAA peptidase subunit (*SPG7*) and ATP-dependent

Table 1 Genes, their functions and associated cellular location

Gene	Function	Cellular location
Caspase-9	Apoptosis initiator	Cytoplasm
Caspase-3	Apoptosis executioner	
BCL-2	Pro- and anti-apoptotic function	
BAX	Pro apoptotic function, associated intrinsic apoptosis	
XIAP	Inhibits the activity of caspases	
HMOX1	Reduces oxidative stress through catabolization of haem	Mitochondria
TFAM	Mitochondrial related transcription factor, associated with action of *TFB1M* and *TFB2M*	
TFB1M	Mitochondrial related transcription factors, associated with *Nrf-2* action	
TFB2M	Mitochondrial related transcription factors, associated with *Nrf-2* action	
NRF-2	Transcription element of the ARE complex, activated through oxidative stress	
CLPP	Mitochondrial protease, associated with mitochondrial quality control	
LON		
SPG7		
YME1L1		
DRP1	Mitochondrial fission regulator	
MFN1	Responsible for mitochondrial fusion and cristae remodelling	
MFN2		
OPA1		
Actin	Assembly of microfilaments	Cytoplasm (reference genes)
β-Tubulin	Polymerization of microtubules	

metalloprotease (*YME1L1*) are involved in the regulation of mitochondrial quality control. *LON* and *CLPP* are present in the mitochondrial matrix and are involved in the proteolysis of oxidised proteins from ROS damage and impaired, misfolded protein due to damage respectively.[37–39] Dynamin-Related Protein (*DRP1*), Mitofusin-1/-2 (*MFN1*), (*MFN2*) and Dynamin-like 120 kDa protein (OPA1) are involved in the control of mitochondrial fission and fusion. The process of mitochondrial fission is largely controlled by *DRP1*, which is translocated to the outer membrane of the mitochondria to bring about fission.[40] The process of mitochondrial fusion involves *OPA1* in concert with *MFN1* and *MFN2* which regulate mitochondrial fusion from the inner mitochondrial membrane and are essential for cristae remodelling, which also has a regulatory function in oxidative phosphorylation.[40,41]

This study explores the mitochondrial and apoptotic mechanisms of action of these complexes in *in vitro* cell models.

Materials and methods

Mammalian cell culture conditions and maintenance

The MCF-7, SKOV-3, A2780 and A2780cis cell lines were obtained from the internal cell bank at Technological University Dublin (TUDublin City Campus) and commercially through the European Collection of Authenticated Cell Cultures (ECACC), Public Health England. Each cell line was grown in Roswell Park Memorial Institute media (RPMI-1640) (Sigma, R8758) supplemented with 12% Foetal Bovine Serum (Sigma, F2442) and 2% L-glutamine (200 mM, Sigma, G7513) in a T75 cell culture flask (Sigma, CLS3276). Cells cultures were incubated at 37 °C in a 5% CO_2 atmosphere. Cells were kept in cell culture media until 80% confluent and subcultured using trypsin solution (Thermo, 15090046) : EDTA (Sigma) (1 : 1).

Cytotoxicity assessment by MTT viability

MCF-7, SKOV-3, A2780 and A2780cis cells were subcultured and seeded at 1×10^5 cells in 96-well plates and incubated for 24 h prior to complex exposure. Cell lines were exposed to Cu-Phen (**1**), Cu-DPQ-Phen (**2**), Cu-DPPZ-Phen (**3**) and Cu-DPPN-Phen (**4**). The cell lines were also treated with cisplatin (Sigma, P4394). The Cu(II) complexes and cisplatin were dissolved in DMSO prior to subsequent dilution with aqueous cell culture media. Cell treatment with complexes **1–4** was performed with an initial stock in DMSO (Sigma, 276855) (5–10 mM) prior to dilution to a range of 0.25–10 μM with aqueous cell culture media. The cells were treated with the Cu(II) complexes (0.25–10 μM) and cisplatin (0.5–200 μM) for 24 h. Drug-treated cell culture media was removed and 100 μl working solution of MTT (methylthiazolyldiphenyl-tetrazolium bromide) (5 mg ml^{-1}) (Sigma, M5655) was added to the cells and incubated for 3 h, as described in the method by *Mosmann*, 1983.[42] The cells were washed (×3) with sterile PBS (Sigma, 806544) and formazan solubilised in 100 μl DMSO. The absorbance was recorded at 595 nm using a 1420 Multilabel Counter Victor^{3}V spectrophotometer (PerkinElmer, USA). Treatments were performed on three separate occasions in triplicate.

Immuno-detection of γH2AX foci with flow cytometry and confocal microscopy

A2780 and A2780cis cell lines were selected due to their respective sensitivity and resistance to cisplatin as determined by the MTT viability assay. Additionally, immunostaining analysis was conducted on MCF-7 and SKOV-3 cells and data are included in Supplementary S1 (ESI†). A2780 and A2780cis cells were subcultured and plated at 1×10^5 cells in T25 flasks (Sigma, CLS430639) and incubated for 24 h to allow for attachment and growth. After 24 h the A2780 and A2780cis cells were exposed (at IC_{25} concentration values as determined by the MTT viability assay) to the Cu(II) complexes and cisplatin for 24 h. The cells were fixed using 2% paraformaldehyde (Sigma, F8775) and permeabilised using 0.25% Triton X-100 (Sigma, T8787). The cells were blocked using 2% Bovine Serum Albumin (Sigma, A9418) and

subsequently incubated with the primary antibody, anti-phosphohistone H2A.X (Ser 139), clone JBW301 (Cat# 05-636) (Lot# 2068177) (1 : 500) (Milipore, Ireland) and fluorescently labelled using Alexa Fluor 488 FITC conjugate (1 : 200) (ThermoFisher, A-11029). The cells were then washed with PBS and counterstained using propidium iodide (1 μg μl^{-1}) (Sigma, P4864). The Median Fluorescence Intensity (MFI) was calculated from the gated region experimentally determined from controls using the BD Accuri C6 (BD Biosciences, USA) flow cytometer using the whole cell population. The experimental measurements were conducted on three separate occasions. Confocal microscopy was performed using an LSM© 510 Meta Confocal Microscope (Zeiss, Germany) with slides mounted in an inverted configuration. Images were recorded at 620× magnification with controls used to establish the minimum laser gain, amplitude and offset setting in addition to identifying the upper limits of image saturation and fluorophore excitation using argon and helium–neon laser scanning simultaneously. All images were analysed using ImageJ software (open source). A2780cis images were contrast enhanced by 5% using an equalized histogram of the image.

Apoptosis and mitochondrial-related gene expression

The molecular gene targets of apoptosis, inhibition of apoptosis (*XIAP*) and mitochondrial-related function were analysed by gene expression in real-time (Table 1). These selected candidate genes can characterise the state of the cell specifically related to the mitochondria after exposure to the Cu(II) phenanthroline–phenazine complexes and cisplatin. Well-characterised cisplatin sensitive and resistant cells were adopted to observe mechanistic trends related to sensitivities and resistance, respectively. The primer sequences for each gene of interest and reference gene are presented in Table 2. Primer sequences were designed using NCBI nucleotide sequences (https://www.ncbi.nlm.nih.gov/nucleotide/) and Primer3PLUS and were synthesised by Sigma, Ireland.

A2780 and A2780cis cell lines were seeded at 1×10^4 cells in T25 flasks and allowed 24 h to attach and grow before the cells were treated with the complexes at their IC_{25} concentration values for 24 h. Similarly, A2780 and A2780cis were treated with cisplatin in the same manner as the Cu(II) complexes. The Cu(II) complexes, cisplatin and untreated controls were incubated in triplicate. The RNA extraction was performed using Tri-Reagent (Sigma, T9424), washed in 75% ethanol and stored in 30 μl of 0.1% diethylpyrocarbonate (DEPC) treated water (Sigma, 40718). Extracted RNA was quantified using a MaestroNano™ spectrophotometer (MaestroGen, USA). Samples were normalized to 150 ng and were converted to cDNA using the qScript cDNA synthesis kit (Quanta Biosciences, 733-1173) employing the following thermal program: 5 min at 22 °C, 30 min at 42 °C, 5 min at 85 °C followed by holding at 4 °C. Synthesised cDNA templates were amplified and quantified using SYBR Green I (Thermo, 4309155) on the 7500 Fast Real-Time PCR System (Applied Biosystems, USA). The final reaction volume of 20 μl was amplified and quantified using the following program: (i) pre-incubation (1 cycle) 95 °C, 5 min, ramp 4.4 °C; (ii) quantification (45 cycles) 95 °C 10 s ramp 4.4 °C, 60 °C 10 s ramp 2.2 °C, 72 °C 10 s ramp 4.4 °C; (iii) melting curve (1 cycle) 95 °C 5 s ramp 4.4 °C, 65 °C 1 min ramp 2.2 °C, 97 °C continuous ramp 0.11 °C acquisition 10 per °C and (iv) cooling (1 cycle) 40 °C 10 s ramp 1.5 °C. Gene amplification of target genes was determined in relation to the reference genes (N = 2–4). Template controls were used to confirm the absence of autofluorescence. Relative expression values were calculated using geometric means of the

Table 2 List of genes and oligonucleotides

Cellular region	Gene	Oligonucleotides (5′–3′)	Gene	Oligonucleotides (5′–3′)
Mitochondrial	*HMOX1*	CAGGGCCATGAACTTTGTCC (F) GAGAGGGACACAGTGAGAGG (R)	*TFAM*	GGGATCTTGCTATGTTGCCC (F) TTATGGTCAAGCATGGTGGC (R)
	TFB1M	AGTCGCCTCTCTGTTATGGC (F) ATGGCTGCTCTATCTTGGGC (R)	*TFB2M*	ACCAAGTAGACCTCCACACC (F) GTTTTGTCACTTTCGAGCGC (R)
	NRF-2	ACCAAAACCACCCTGAAAGC (F) AGGCCAAGTAGTGTGTCTCC (R)	*CLPP*	CCTTGTTATCGCACAGCTCC (F) TCGGGTTGAGGATGTACTGC (R)
	LON	GAAGGAAAGTTCGTCTCGCC (F) CTCATGGATCTGGGCAAACG (R)	*SPG7*	ATATCGAGGCCAAGGACAGG (F) GGCCAGACGGAAAACATACC (R)
	YME1L1	CTTTTCCGGAGCAGAGTTGG (F) CACTTCTTCTTTCAGGCCCC (R)	*DRP1*	CCAATTATGCCAGCCAGTCC (F) CTCACAATCTCGCTGTTCCC (R)
	MFN1	GGAAGAGGCCAGTTTTGCC (F) GCATTTCCACCAGCTCAAGG (R)	*MFN2*	CTTGTGTGTGTCCATCTCGC (F) AGCATCCTTTCTACCACCCC (R)
	OPA1	CCCGCTTTATGACAGAACCG (F) GTCCTCCGCAAAGTCATTCC (R)		
Cytoplasmic	*Casp 9*	TCCAGATTGACGACAAGTGC (F) CCTACAAGTCGTGACAGGGA (R)	*Casp 3*	TTTGTTTGTGTGCTTCTGAG (F) TATAAGAACCGCTTTAAGT (R)
	BCL-2	AAGCGGTCCCGTGGATAGA (F) TCCGGTATTCGCAGAAGTCC (R)	*BAX*	AGGATGCGTCCACCAAGAAG (F) CCAGTTGAAGTTGCCGTCAGA (R)
	XIAP	GCACGGATCGTTACTTTTGGACA (F) GTGGAAGCACTTCACTTTATCGCC (R)		
Reference genes	***Actin***	**ACTCTTCCAGCCTTCCTTCC (F)** **GTTGGCGTACAGGTCTTTGC (R)**	***B-Tubulin***	**GCTTCTTGGTTTTCCACAGC (F)** **CGTTCTTCAGGTTCGACCTC (R)**

Highlighted in bold are the primers of reference genes used to establish the relative expression of the target genes. (F) refers to forward primer while (R) refers to reverse primer.

reference genes and target genes were normalized according to the Livak and Schmittgen formula: $2^{-\Delta Ct1(test)-\Delta Ct2(control)}$.[43]

Statistical analysis

The MTT assay raw data were normalized to the negative control within each experiment. The statistical package, Prism (ver. 5.0) (GraphPad, USA) was used to establish the 25% inhibitory concentration (IC_{25}) using least squares non-linear regression analysis. The statistical significance of the changes between treatment with the Cu(II) complexes and the cisplatin was determined using one-way ANOVA with Tukey's *post hoc* test. The flow cytometry data analysis was performed using the BD Accuri C6 software package and Microsoft® Excel (USA) to establish the mean of the MFI and percent positive data. Prism was used to perform one-way ANOVA significance testing with *post hoc* Bonferroni correction between test complexes and the control. For the analysis of gene expression, the geometric mean Ct values for both actin and tubulin reference genes were used for normalization as per the Vandesompele method.[44] Target gene expression values were calculated along with a standard deviation as per the Nordgård, Livak and Schmittgen formula.[43,45] Both the positive and negative error bars display the 95% confidence interval around the mean normalized gene expression value.

Results

Cytotoxicity assessment by MTT viability

The MTT assay was used to establish the activity of Cu-Phen (**1**), Cu-DPQ-Phen (**2**), Cu-DPPZ-Phen (**3**) and Cu-DPPN-Phen (**4**) in both cisplatin-sensitive (MCF-7 and A2780) (Table 3) and cisplatin-resistant (SKOV-3, A2780cis) cells relative to the established clinical therapeutic; cisplatin (Table 3). Cisplatin was tested over the concentration range of 0.5–200 μM and Cu-Phen (**1**), Cu-DPQ-Phen (**2**), Cu-DPPZ-Phen (**3**) and Cu-DPPN-Phen (**4**) were tested over 0.25–10 μM. Cisplatin displayed a varied level of activity across the range of tested cell lines with SKOV-3 cells presenting the indicative profile of platinum resistance.[46] Cisplatin displayed differential activity in both A2780 and A2780cis cell lines, with the resistant variant demonstrating a higher concentration in relation to the sensitive variant at 24 h (>200 μM and 63.10 μM, respectively). The Cu(II) complexes (**1–4**) all showed IC_{25} values of <10 μM. The A2780 and the cisplatin-resistant counterpart A2780cis demonstrate classic cisplatin resistance, whereas SKOV-3 cells demonstrate cisplatin resistance but to a lower extent. The diminished level of cisplatin resistance observed in SKOV-3 cells has previously been documented in the evaluation of other novel Cu complexes.[12,14,16] Cu(II) phenanthroline phenazine complexes demonstrate superior activity (as denoted by *) to cisplatin in all cell lines while the cisplatin sensitive cell lines (MCF-7 and A2780) demonstrate higher toxicity towards the Cu(II) complexes in comparison to both known cisplatin resistant cell lines (SKOV-3 and A2780cis). Exposure to Cu(II) phenanthroline–phenazine complexes demonstrated consistently increased toxicities irrespective of the ligated phenazine structure in comparison to cisplatin with Cu-DPPN-Phen (**4**) showing slightly lower toxicity possible due to larger size and lipophilicity. Overall the inclusion of cisplatin resistant cell lines demonstrates that cisplatin resistance is not a confounding factor for the activity of the Cu(II) phenanthroline–phenazine complexes.

Immunodetection of γH2AX foci with flow cytometry and confocal microscopy

The induction of γH2AX foci was detected using flow cytometry and confocal microscopy after A2780 and A2780cis cells were exposed to the IC_{25} concentrations of cisplatin and the Cu(II) phenanthroline–phenazine complexes for 24 h and compared to the unexposed control. Foci formation was determined using MFI (Fig. 2).

Following the exposure of A2780 to cisplatin and the Cu(II) complexes, a significant increase ($p \leq 0.05$) in foci formation was observed, with the exception of Cu-DPPN-Phen (**4**). Induction of γH2AX foci in A2780 cells occurred to a small extent after exposure to Cu-Phen (**1**), Cu-DPQ-Phen (**2**) and Cu-DPPZ-Phen (**3**) and is absent upon treatment with Cu-DPPN-Phen (**4**). The induction of γH2AX foci in the A2780cis cells occurs to a larger extent following exposure to Cu-Phen (**1**) and Cu-DPQ-Phen (**2**) and is almost entirely absent after exposure to Cu-DPPZ-Phen (**3**) and Cu-DPPN-Phen (**4**). The exposure of A2780cis cells to cisplatin induces non-significant variable increases in foci formation. The induction of γH2AX foci was also detected using confocal microscopy imaging to record the incidences of foci formation using the FITC labelled antibody and the nuclear counterstain, propidium iodide (Fig. 3). Overall, the results of confocal microscopy complement the flow cytometry results.

Table 3 IC_{25} values for MCF-7, A2780, SKOV-3 and A2780cis cells over 24 h drug exposure

	IC_{25} (μM) ± S.D.			
	Cisplatin-sensitive		Cisplatin-resistant	
Treatment	MCF-7	A2780	SKOV-3	A2780cis
Cisplatin	0.45 ± 0.07	63.10 ± 13.91	21.52 ± 4.54	>200
Cu-Phen (**1**)	0.97 ± 0.10	0.39 ± 0.07***	2.56 ± 0.12***	0.83 ± 0.00***
Cu-DPQ-Phen (**2**)	1.27 ± 0.40*	0.34 ± 0.09***	2.51 ± 0.09***	0.72 ± 0.02***
Cu-DPPZ-Phen (**3**)	0.63 ± 0.01	0.31 ± 0.04***	1.61 ± 0.74***	0.39 ± 0.07***
Cu-DPPN-Phen (**4**)	1.37 ± 0.32**	0.36 ± 0.10***	5.71 ± 0.92***	0.53 ± 0.02***

Mean ± standard deviation ($N = 3$). * denotes $p < 0.05$ in comparison to cisplatin. ** denotes $p < 0.01$ in comparison to cisplatin. *** denotes $p < 0.001$ in comparison to cisplatin.

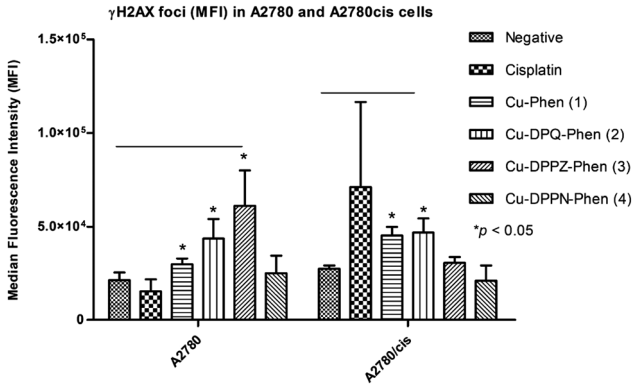

Fig. 2 Immunodetection using MFI of γH2AX foci in A2780 and A2780cis cells after 24 h treatment with cisplatin and the Cu(II) complexes. Statistical significance was determined between the negative control and the Cu(II) complexes using one-way ANOVA with Bonferroni correction.

Apoptosis and mitochondrial-related gene expression

The level of gene expression in the target genes was represented in relation to the geometric mean of the reference genes. The variance for target genes was error propagated and log(2) transformed and a 95% confidence interval was plotted around the ΔΔCt value. Grouped columns in Fig. 4–6 display the log(2) transformed mean expression value relative to the biological negative control, with the broken line at $y = 1$ indicating lower expression below and higher expression above.

Differential expression of *Bax* and *XIAP* expression was observed between A2780 (Fig. 4A) and A2780cis cells (Fig. 4B) when each was exposed to cisplatin. The increase in expression of *BCL-2*, an anti-apoptotic gene in A2780cis cells, demonstrated a classic mechanism of cisplatin resistance. The apoptotic response following exposure to the Cu(II) complex was markedly different to that of cisplatin. The apoptotic response to the Cu(II) complexes is characterised by increase of *BAX* and with markedly increased expression in the anti-apoptotic gene, *XIAP*. *Caspase 9, caspase 3*, *BCL-2* and *BAX* characterise the response to the Cu(II) complexes, and particularly to exposure to Cu-DPPZ-Phen (**3**) and Cu-DPPN-Phen (**4**) in the cisplatin-resistant A2780cis cells. The differential apoptotic response between the Cu(II) complexes and cisplatin in the A2780cis resistant cells demonstrates that the cisplatin resistance is not an impeding factor in the activity of the Cu(II) complexes.

The mitochondrial related gene expression profile produced by the A2780 cells (Fig. 5) demonstrates that the mitochondrial protease gene, *CLPP* and to a smaller extent the mitochondrial fusion gene, *OPA1* are elevated following exposure to cisplatin. This profile is in contrast to that caused by exposure to Cu-Phen (**1**) where only a small increase in the oxidative stress, transcription, protease and fission/fusion genes was detected. All of the other Cu(II) complexes produce no significant increase except for a strong increase in the mitochondrial fission regulator, *OPA1* after exposure to Cu-DPPN-Phen (**4**). The gene expression profile in the A2780cis cells (Fig. 6) showed significant changes in comparison to the A2780 cells. Cisplatin action features prominently in the mitochondrial fission/fusion regulation in the resistant cells in contrast to the sensitive A2780 cells. In contrast to A2780 cells, exposing A2780cis cells to the Cu(II) complexes produced substantial increases in oxidative stress, transcription, protease and fission/fusion genes. An increase was observed in oxidative stress/transcription and protease genes after exposure of A2780cis cells to Cu-DPQ-Phen (**2**), which contrasts with the response of the A2780 cells. Considerable increases in the gene groups in A2780cis cells was recorded following treatment with Cu-DPPN-Phen (**4**), which again differs to the behaviour of the A2780 cells. In contrast to the performance of the A2780 cells, the *DRP1* fission regulator has increased expression after exposing the resistant cells to cisplatin and the Cu(II) complexes, demonstrating a similar therapeutic response to both types of metal complex. The use of the cisplatin-resistant A2780cis cells

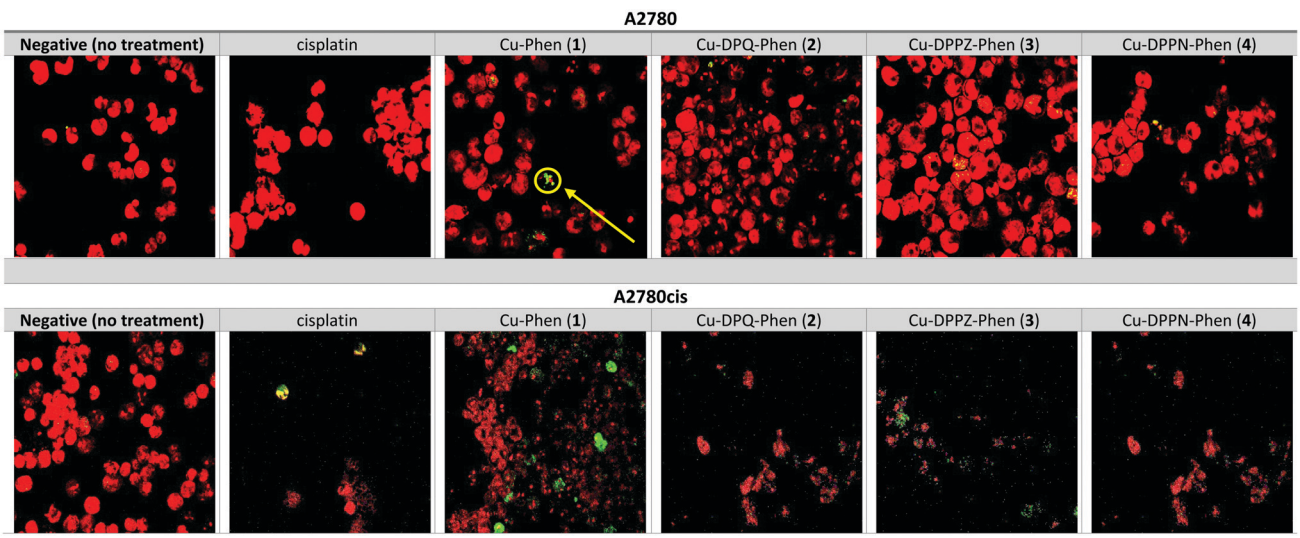

Fig. 3 Confocal microscopy images of γH2AX foci (indicated by yellow arrow and circle) arising from treatment of A2780 and A2780cis cells with cisplatin and the Cu(II) complexes. Red staining indicates nuclear material with green foci representing double stranded DNA break formation (γH2AX).

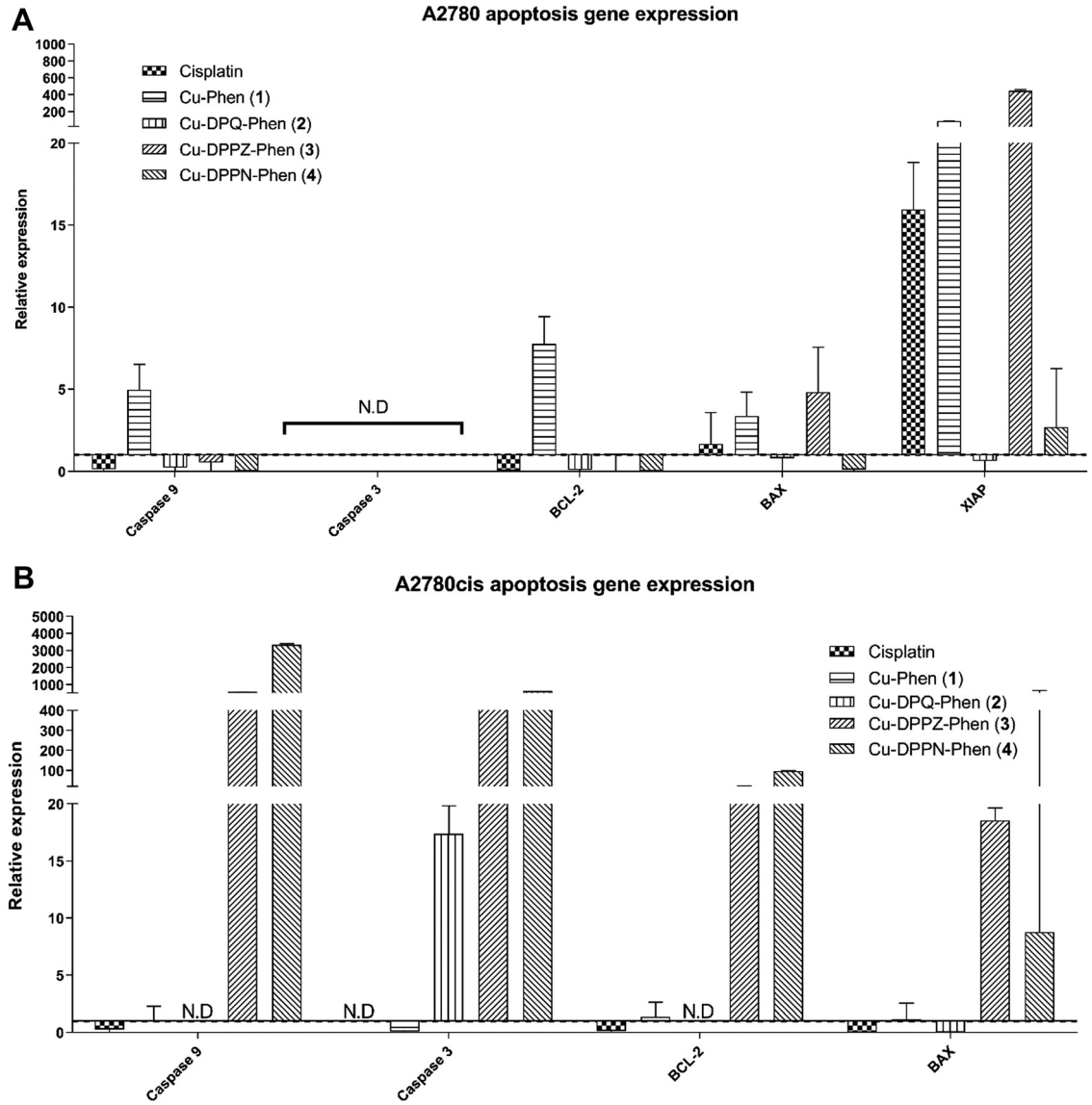

Fig. 4 A/B Gene expression by A2780 and A2780cis cells following treatment with cisplatin and the Cu(II) complexes. Error bars represent normalized standard deviation. Values below $y = 1$ (dotted line) represent negative expression. Target genes normalized to the geometric mean of actin and tubulin. N.D. = not detected.

demonstrates that the Cu(II) complexes operate through a different mechanistic mode of action to cisplatin and, furthermore, the Cu(II) complexes have substantially different mechanism(s) of action against cisplatin-resistant cells compared to the cisplatin-sensitive phenotype.

Discussion

Having recently established, the multimodal effects against the *in vivo* model, *G. mellonella* in a proteomic study,[23] and the previously reported oxidative chemical nuclease and DNA binding properties of the Cu(II) phenanthroline–phenazine complexes,[21,22] the aim of this study was to use *in vitro* models to investigate the mechanism(s) of action of these complexes. Cancer cell lines that confer sensitivity and resistance to the clinical drug, cisplatin, were used so that the mechanisms could be contrasted to cisplatin. Both the MCF-7 (breast) and SKOV-3 (ovarian) cells have been cited in the literature as cisplatin-sensitive and -resistant cell lines, respectively, and are part of the NCI developmental therapeutics program.[47–53] Additionally, the use of MCF-7 and SKOV-3 cell lines previously,[13,14,21] demonstrated the variable nature of the response to cisplatin in cells derived from different tissues. The A2780 (ovarian) cell line represents the parental lineage of a number of daughter cell lines, which are made resistant to various therapeutics such as doxorubicin (A2780ADR) and cross-resistant to melphalan, doxorubicin and cisplatin (A2780cis). The generation of resistant profiles in the A2780 parent cell lines is brought about through the supplementation of sub-toxic dose of the drug in question in the culture media in order to confer resistance over multiple passages of the cell. While MCF-7 and SKOV-3 cells have previously demonstrated cisplatin-sensitive

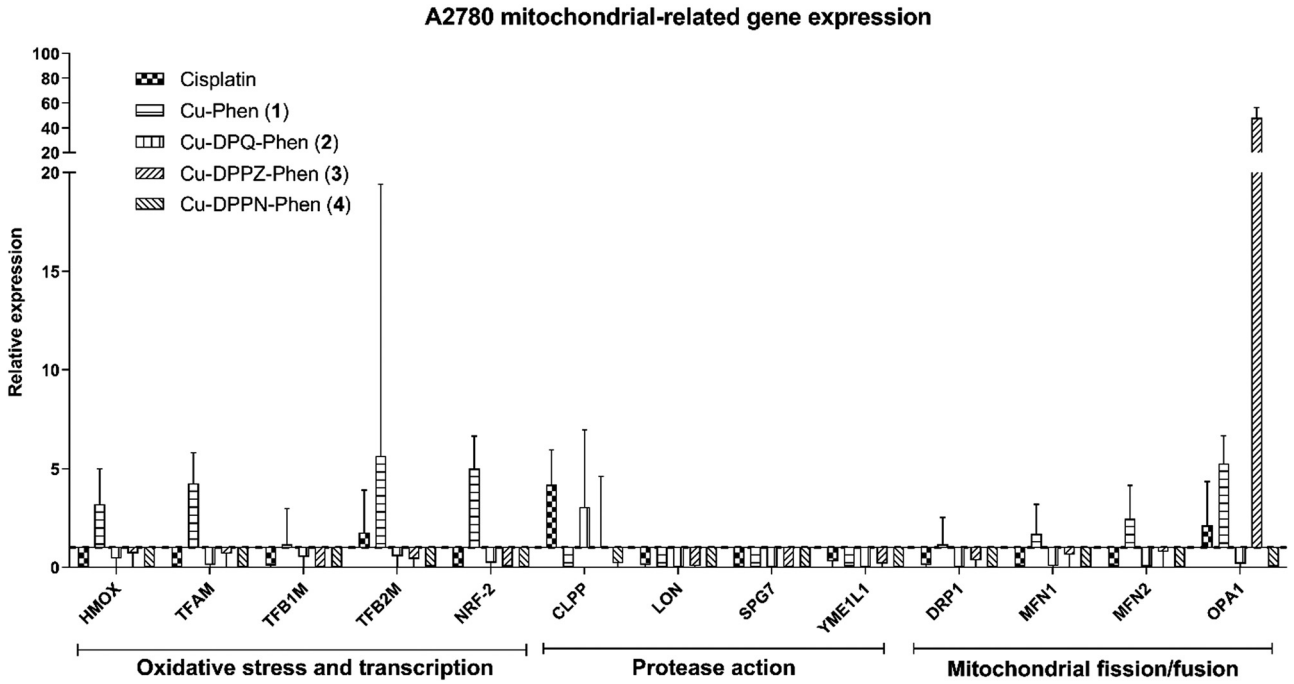

Fig. 5 Gene expression by A2780 cells after 24 h exposure to cisplatin and the Cu(II) complexes. Error bars represent normalized standard deviation. Values below $y = 1$ (dotted line) represent negative expression. Target genes normalized to the geometric mean of actin and tubulin.

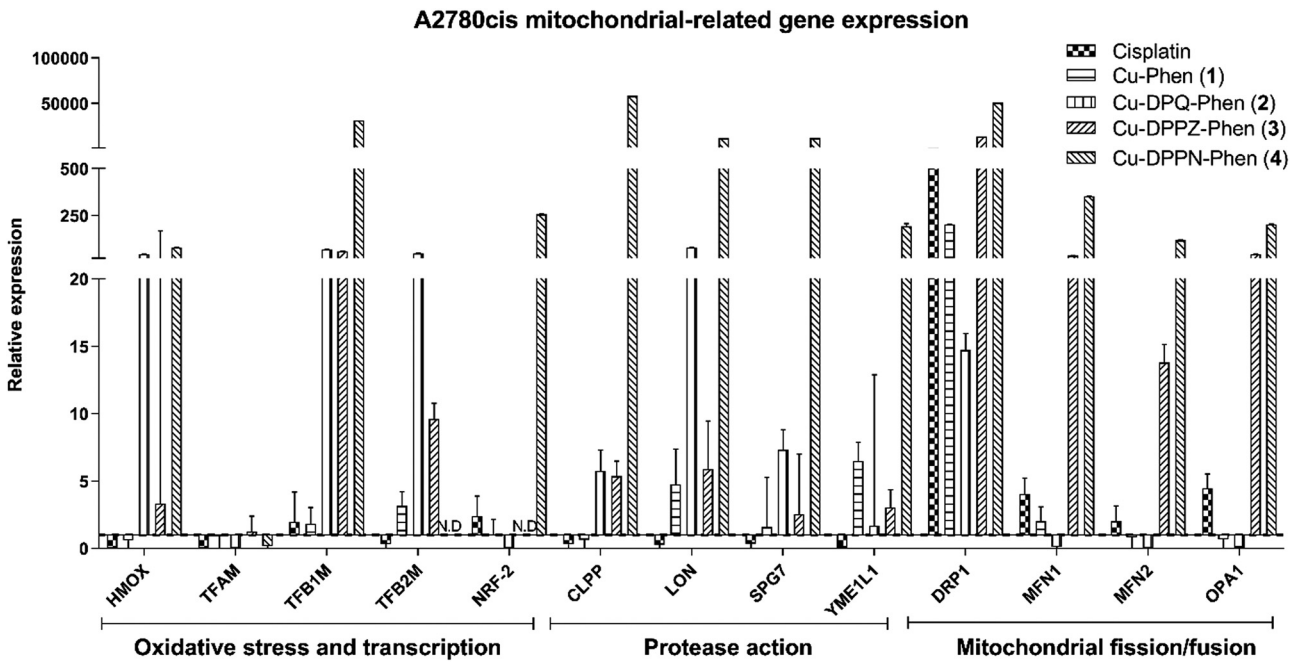

Fig. 6 Gene expression by A2780cis cells after 24 h exposure to cisplatin and the Cu(II) complexes. Error bars represent normalized standard deviation. Values below $y = 1$ (dotted line) represent negative expression. Target genes normalized to the geometric mean of actin and tubulin. N.D. = not detected.

and -resistant profiles respectively,[13,14] the direct comparison between the cell types is limited by their different tissue of origin and subsequently differing gene and protein expression profile. The A2780 and A2780cis cisplatin-resistant daughter cells have transcriptional and protein expression profiles that are comparative, and have been employed in multiple studies examining ovarian cancer and the biological mechanisms that contribute to therapeutic resistance.[48–53] The use of the isogenic A2780 parent and resistant A2780cis phenotype has gained popularity as a more homogeneous platform where sub-toxic levels of cisplatin are added to the culture media of A2780cis cells to generate and retain the resistance profile.[54]

These cells were used to compare the biological effects of the Cu(II) complexes and cisplatin on the MCF-7 and SKOV-3 traditional cells to the effects on the A2780 and A2780cis cells.

Overall, the cytotoxicity profile of the Cu(II) phenanthroline–phenazine complexes demonstrated a cytotoxicity profile with the following cadence Cu-DPPZ-Phen (**3**) (most toxic) > Cu-Phen (**1**) ~ Cu-DPQ-Phen (**2**) > Cu-DPPN-Phen (**4**) (least toxic). Superior cytotoxicity activity of the Cu(II) phenanthroline–phenazine complexes to the clinical control, cisplatin, was noted in the cisplatin-resistant cell line (A2780cis). A previous study by Molphy *et al.* established a similar cytotoxic profile against the SKOV-3 cell line which accompanied the highest DNA binding constants and was also comparable to the oxidative DNA damaging agent doxorubicin.[21] The development of DNA damage is a key hallmark of cisplatin cytotoxicity and, as such, has been a valuable indicator in the profiling of new therapeutics. γH2AX foci formation is indicative of double stranded breaks (DSBs) in the DNA, and the induction of DSBs by the current Cu(II) complexes was observed. The MCF-7 and A2780 cell lines had less DSBs, which might indicate an alternative mode of action in the resistant cell lines. Additionally, the high levels of DSBs in SKOV-3 and A2780cis cells may be the driver of higher cytotoxicity of the Cu(II) complexes compared to cisplatin, this being despite the development of resistance to cisplatin-induced DNA damage by these two cell lines. This strongly suggests that the Cu(II) complexes are operating through biochemical mechanisms that are distinctly different to those of cisplatin. The identification of alternative mechanisms of action to cisplatin is indeed a developmental objective in metal-based anticancer chemotherapy. Recently our *in vivo* study in *G. mellonella* demonstrated increased levels of glutathione *S*-transferase (GST) in larvae exposed to the Cu(II) complexes.[23] The Cu(II) complexes contain polycyclic aromatic rings which have a similar cyclical structure to the DNA damaging agents naphthalene and benzo-[*a*]-pyrene. Additionally, GST is also upregulated in cells exposed to the chemical nuclease activity of cyclophosphamide and ifosfamide. The generation of oxidative radicals is a key mechanism through which DNA damage occurs. Due to the previously demonstrated nuclease cleavage ability of these Cu(II) complexes[22] and structural similarity to the intercalating ruthenium complex Λ-[Ru (phen)$_2$dppz]$^{2+}$,[55] it is likely that the Cu(II) complexes intercalate with the DNA in a similar fashion in addition to the nuclease capability of the complex facilitating DNA strand breakage. The *G. mellonella* study also determined that enzymes regulating purine synthesis were upregulated along with heat shock protein 90 which are present during increased purine turnover and the formation of the purinosome.[56] The high degree of cytotoxicity in SKOV-3 and A2780cis prompted further investigation of apoptosis, which is one of the most important mechanisms of regulated cell death.

Apoptotic cell death is related to loss in cellular viability accompanied by cytotoxicity.[27] Transition metal coordinated therapeutics have often been evaluated for their ability to induce apoptosis and other forms of regulated cell death with many evaluative studies on copper complexes using this as a therapeutic endpoint.[25,26,57–62] Additionally, cisplatin and other therapeutic compounds have been evaluated for their direct activity against *BCL-2*.[30,63] While these studies have used different markers of apoptosis to demonstrate the induction of this endpoint, the use of gene expression in this study allows multiple pathways in the apoptotic process to be investigated. Mitochondrial-mediated apoptosis can be initiated by the induction of DNA damage through *p53* or the action of *Bax* and *BCL-2* regulated by BH3 proteins of the *BCL2* family. The upregulation of *BCL-2* was observed in the cisplatin-resistant cell lines which is seen as a classic form of drug resistance.[64,65] This pattern was in direct contrast to that found when the cells were exposed to the Cu(II) complexes, where increased levels of *BAX* and *XIAP* were detected. Additionally, caspase-9 and caspase-3 genes were also expressed with the Cu(II) complexes, indicating a complete apoptotic cascade. The upregulation of *BAX* is a key progenitor of the formation of the mitochondrial outer membrane pores (MOMP) in mitochondria which then induces the caspase cascade (caspase-9 and caspase-3) for apoptosis.[66] The mitochondria are being increasingly recognised as a focal point for a multiple cellular process which proves pivotal in directing the fate of the cell. Critical amongst these are responses to inflammation,[67,68] ROS,[69–73] and apoptosis,[72,74–76] which are closely associated and dependent on the mitochondria. Indeed, substantial effort is being directed towards developing therapeutic targets in the mitochondria or processes associated with its action.[77–80]

The high toxicity profiles of the Cu(II) complexes with moderate to low *in vitro* DNA damage recorded by γH2AX foci coupled with mitochondrial-mediated pro-apoptotic gene expression led to further interest in the role and activity of the mitochondria beyond apoptosis. The mitochondrial related gene expression panel was examined through antioxidant enzyme production, *HMOX* (Table 1), while mitochondrial related transcription was monitored through *TFAM*, *TFB1M*, *TFB2M* and *NRF-2* (Table 1). Mitochondrial protease genes were monitored through *CLPP*, *LON*, *SPG7* and *YME1L1* (Table 1). The process of fission/fusion of the mitochondria was examined through *DRP1*, *MFN1*, *MFN2* and *OPA1* (Table 1). The groups of genes representing the mitochondrial, antioxidant, transcription and protease functions demonstrated the highest changes in expression between A2780 and A2780cis resistant cells. Exposure of resistant A2780cis cells to cisplatin induces a characteristic resistance to mitophagy through upregulation of *DRP1* and mitochondrial related transcription factors.[81,82] The role of *DRP1* in mitochondrial remodelling and its relationship to apoptosis has become clearer over the past few years. While *DRP1* is involved in mitochondrial membrane impairment and uncoupling of oxidative phosphorylation leading to its identification as a drug resistance factor,[83,84] new information has emerged about its direct role in bringing about apoptosis through accelerating cytochrome *c* release from the mitochondrial membrane,[85] and additional functions in the responses to oxidative stress,[86] and cellular differentiation.[87–89] The activity of the mitochondrial-related transcription factors and the fission/fusion regulator are also known to be involved in the drug resistance response.[84,90–92] As determined above, the differences in apoptosis between the Cu(II) complexes and cisplatin centre around the activation of *BAX* and *XIAP*, caspase-9 and caspase-3. In contrast to this, the

mitochondria fission regulator, *DRP1*, is commonly upregulated in all exposures. The upregulation of *DRP1* has recently been associated with cisplatin resistance[81,82,93–95] and apoptosis through cytochrome *c* release,[85] and with a similar upregulation after exposure to the Cu(II) complexes this may indicate the exploitation of a similar mechanism of action. While similarities in mechanisms can be shown with *DRP1* activity, the upregulation of all proteases particularly *LON*, following exposure of A2780cis cells to the Cu(II) complexes may indicate an increased rate of protein turnover due to misfolded, oxidized and otherwise denatured proteins, which may be contributing to the enhanced cytotoxicity of the Cu(II) complexes.[96–98] Additionally, the presence of *HMOX* and transcription factors related to the antioxidant response in the A2780cis cells treated with the Cu(II) complexes, builds on the evidence of upregulated protease factors to demonstrate potential increases in protein dysregulation as a potent factor in the mitochondrial toxicity.[36,99] Previously our *in vivo G. mellonella* study demonstrated increases in protein expression of metabolic enzymes associated with the mitochondria.[23] These changes also accompanied increase in pathways associated with protein detoxification and degradation, which complements the gene expression activity observed in this study indicating increased protein turnover potentially due to oxidative damage from mitochondrial dysregulation or the direct action of the Cu(II) complexes.

These Cu(II) complexes have a unique profile of cytotoxicity differing from that of cisplatin. The investigation into the effects of the mitochondria using gene expression has provided a wider context to the mechanistic activity in addition to demonstrating apoptosis. The results presented here demonstrate that Cu(II) phenanthroline–phenazine complexes are a promising therapeutic candidate which are highly potent against cisplatin-resistant cells. The superior activity, together with their multimodal activity against cisplatin-resistant cells offers a potentially novel therapeutic avenue for treating cisplatin-refractory cancers in combination with current chemotherapy.

Conflicts of interest

The authors have no financial conflicts to disclose.

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