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Does cytotoxicity of metallointercalators correlate with cellular uptake or DNA affinity?

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Abstract

The cytotoxicity of the metallointercalators, [Pt(5,6-dimethyl-1,10-phenanthroline)(trans-1R,2Rdiaminocyclohexane)]²⁺ ([56MERR]) and [Pt(5,6-dimethyl-1,10-phenanthroline)(trans-1S,2Sdiaminocyclohexane)]²⁺ ([56MESS]), towards A549 human lung cancer cells was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The IC₅₀ value obtained following exposure of A549 cells to [56MESS] for 4 h was approximately three times smaller than that obtained when [56MERR] was administered under the same conditions, indicating that the former complex displayed greater cytotoxicity. Both IC₅₀ values were greater than that obtained after exposure of A549 cells to cisplatin, demonstrating that the latter compound was the most cytotoxic of the three examined. Microprobe synchrotron radiation X-ray fluorescence (SR-XRF) analyses of metallointercalator-treated A549 cells showed that platinum became localised in DNA-rich regions of the nucleus. In contrast, when the same cells were treated with cisplatin the metal became distributed throughout the cell. Determination of the maximum concentration of platinum present inside the cells using graphite furnace atomic absorption spectrophotometry (GFAAS) of platinum-treated cells suggested that there was greater uptake of [56MERR] compared to [56MESS] by the A549 cells, and that platinum uptake did not account for the greater toxicity of [56MESS], as assessed by the MTT assay. Electrospray ionization mass spectrometric (ESI-MS) and circular dichroism (CD) spectroscopic studies of solutions containing either [56MERR] or [56MESS], and a duplex hexadecamer molecule, showed the two metallointercalators displayed very similar affinity towards the nucleic acid. Overall these results indicate that the difference in cytotoxicity of the two platinum metallointercalators is probably the result of variations in their interactions with other cellular components.

Keywords

metallointercalators, does, cytotoxicity, affinity, correlate, dna, uptake, cellular, CMMB

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PAPER

Does cytotoxicity of metallointercalators correlate with cellular uptake or DNA affinity?†

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The cytotoxicity of the metallointercalators, [Pt(5,6-dimethyl-1,10-phenanthroline)(*trans*-1*R*,2*R*-diaminocyclohexane)]²⁺ ([56MERR]) and [Pt(5,6-dimethyl-1,10-phenanthroline)(*trans*-1*S*,2*S*-diaminocyclohexane)]²⁺ ([56MESS]), towards A549 human lung cancer cells was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The IC₅₀ value obtained following exposure of A549 cells to [56MESS] for 4 h was approximately three times smaller than that obtained when [56MERR] was administered under the same conditions, indicating that the former complex displayed greater cytotoxicity. Both IC₅₀ values were greater than that obtained after exposure of A549 cells to cisplatin, demonstrating that the latter compound was the most cytotoxic of the three examined. Microprobe synchrotron radiation X-ray fluorescence (SR-XRF) analyses of metallointercalator-treated A549 cells showed that platinum became localised in DNA-rich regions of the nucleus. In contrast, when the same cells were treated with cisplatin the metal became distributed throughout the cell. Determination of the maximum concentration of platinum present inside the cells using graphite furnace atomic absorption spectrophotometry (GFAAS) of platinum-treated cells suggested that there was greater uptake of [56MERR] compared to [56MESS] by the A549 cells, and that platinum uptake did not account for the greater toxicity of [56MESS], as assessed by the MTT assay. Electrospray ionization mass spectrometric (ESI-MS) and circular dichroism (CD) spectroscopic studies of solutions containing either [56MERR] or [56MESS], and a duplex hexadecamer molecule, showed the two metallointercalators displayed very similar affinity towards the nucleic acid. Overall these results indicate that the difference in cytotoxicity of the two platinum metallointercalators is probably the result of variations in their interactions with other cellular components.

1. Introduction

Cisplatin (*cis*-diamminedichloroplatinum(II)) is a clinically-used metaldrug that is extremely effective for the treatment of testicular cancer, against which cure rates of 90% have been reported.^{1,2} Unfortunately, the administration of cisplatin and its analogues can also result in severe side effects, which include neurotoxicity, ototoxicity and nephrotoxicity.³ In addition, acquired resistance resulting from increased drug efflux from cancer cells, mutations/alterations in drug targets, and deactivation of the drug, may also result.^{4,5} These issues have prompted an enormous effort directed towards developing new platinum compounds that display similar or greater levels of anticancer

activity to cisplatin, but with reduced side effects. Despite the preparation and testing of thousands of platinum compounds,^{4–6} only a handful have entered clinical use. Most of the latter compounds, such as carboplatin and oxaliplatin, display a similar spectrum of anticancer activity to cisplatin, albeit in some cases with lower toxicity to the patient. There is now general awareness that most so-called second generation platinum anticancer agents interact with DNA in a similar fashion to cisplatin, and that this is the origin of their similar profiles of biological activity.^{5,7,8} As a consequence there is now growing interest in developing new cytotoxic metalodrugs with different structures to that of cisplatin, which enable their interaction with nucleic acids by alternative DNA binding mechanisms (Fig. 1).^{7–9}

Intercalation is a non-covalent method of binding to DNA which results in unwinding of the double helix in order to facilitate π -stacking interactions between the π electron clouds of the base pairs and the intercalating compound.^{10–12} Metallointercalators are metal complexes that possess planar aromatic ring systems which can be inserted between DNA base-pairs, and usually also possess an overall positive charge, which enables additional stabilizing interactions with DNA.^{12–15} Owing to the

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Fig. 1 Structures of the complexes examined in this study.

presence of the metal centre, metallointercalators often exhibit geometries not found amongst organic compounds. This broadens the range of possibilities for formulating new drugs that can bind in unique or targeted ways to specific DNA sequences and structures.¹⁵

Investigations into metallointercalators date back to the pioneering studies of Lippard and co-workers in the late 1970s, which centered on the binding of platinum complexes containing terpyridine to calf thymus DNA.^{16–18} More recently, a number of studies into the biological activity of platinum(II) metallointercalators containing phenanthroline, or one of several methylated phenanthroline ligands, have been conducted by Aldrich-Wright and co-workers.^{19–22} For the complexes, [Pt(en)(Me_xphen)]²⁺ ($x = 1, 2$ or 4), it was found that the number and position of the methyl groups on the phenanthroline ligand affect both DNA affinity and activity toward L1210 leukemia cells. Even greater cytotoxicity was displayed by complexes containing the bulky *trans*-1,2-diaminocyclohexane (dach) ancillary ligand, which is also present in the clinically used anticancer agent oxaliplatin ([Pt(*trans*-*R,R*-1,2-diaminocyclohexane)(oxalate)]). The stereochemistry of the dach ligand was found to have a significant effect on cytotoxicity, with platinum metallointercalators containing the enantiomeric *S,S*-dach ligand exhibiting greater cytotoxicity than those containing *R,R*-dach.²² For example, in cytotoxicity assays performed with the 5637 human bladder cancer cell line and the murine leukemia L1210 cell line, [Pt(*S,S*-dach)(phen)](ClO₄)₂ exhibited IC₅₀ values of 0.091 and 0.13 μM, respectively, while for [Pt(*R,R*-dach)(phen)](ClO₄)₂ these values were 0.54 and 1.50 μM, respectively, following a 96 h treatment period.²² Both complexes displayed cytotoxicity that was comparable to, or better than that exhibited by cisplatin, for which the corresponding IC₅₀ values were 0.31 and 0.50 μM, respectively. Methylation of the achiral ligands in these complexes also increased cytotoxicity, with [Pt(*S,S*-dach)-(5-methyl-1,10-phenanthroline)]²⁺ and [Pt(*S,S*-dach)(5,6-dimethyl-1,10-phenanthroline)]²⁺ ([56MESS]) displaying IC₅₀ values of 0.033 μM and 0.0092 μM, respectively against the L1210 leukemia cell line.²⁰

While there have been a multitude of studies that have shown that metallointercalators can bind to isolated pieces of DNA in a test tube, only a small number have demonstrated that they can actually enter cells and reach the nucleus.^{23–25} Consequently, it was decided to explore the cellular fate of metallointercalators, and the role that DNA binding may play in their mechanism of action, by comparing the results of experiments performed using a suite of spectroscopic studies and biological assays, with the metallointercalators [56MESS] and [56MERR] ([Pt(*R,R*-dach)-(5,6-dimethyl-1,10-phenanthroline)]²⁺) shown in Fig. 1. The overall cytotoxicity of the complexes was first evaluated using

the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay,²⁶ while graphite furnace atomic absorption spectrophotometry (GFAAS) and microprobe synchrotron radiation X-ray fluorescence (SRXRF) imaging were used to compare the cellular uptake and localization, respectively, of the platinum intercalators and cisplatin by A549 human lung cancer cells. Microprobe SRXRF is a highly sensitive analytical technique that can detect elements such as platinum in cells at sub-micron resolution, without the need for radioactive or fluorescent labeling.^{27–29} It can be used to provide a qualitative measure of the amount of platinum entering cells. The elemental mapping abilities of the technique can provide information regarding platinum co-localization with phosphorus, zinc, calcium or other elements naturally present in cells, thereby providing clues as to whether the metallointercalator localizes primarily in the nucleus or the cytoplasm of cells, or becomes distributed throughout. The studies reported here were performed to compare the cellular fate of each of the three platinum complexes (Fig. 1), and to see if any correlations could be drawn with respect to differences in their cytotoxicity.

The relative DNA binding affinity of the two metallointercalators was also assessed, using electrospray ionization mass spectrometry (ESI-MS) and circular dichroism (CD) spectroscopy, to see if any correlations existed between this property and cytotoxicity. We have shown that ESI-MS can provide information about the number, relative amounts, and stoichiometry of non-covalent complexes formed in solutions containing double stranded DNA (dsDNA) and metal complexes.^{30–33} ESI-MS was used to examine the stability of the metallointercalators in the cell medium employed in the cytotoxicity assays. Circular dichroism (CD) spectroscopy has been widely employed in the past to study non-covalent interactions of metal complexes with calf thymus (CT) DNA, or other DNA molecules such as poly(dA-dT)₂ and poly(dG-dC)₂.^{34–38} However, there have been relatively few studies that have used CD spectroscopy to investigate interactions between metal complexes and short duplex DNA molecules with discrete base sequences. It was, therefore, of interest to see whether addition of the platinum metallointercalators discussed herein produce similar or different changes to the CD spectrum of a duplex molecule, D2, to those observed previously on addition of octahedral metallointercalators containing nickel(II) or ruthenium(II) to the same DNA.^{32,33}

2. Results and discussion

Prior to performing platinum uptake and cellular distribution studies, the MTT assay was used to determine the IC₅₀ values for the metallointercalators and cisplatin with the A549 cancer cell line. These concentrations were then used as a guide for determining appropriate platinum concentrations for GFAAS and microprobe SRXRF analyses in order to minimise cell death but ensure detection of platinum within the cells. Table 1 shows the results obtained from the MTT assays. The IC₅₀ value for cisplatin was 150 ± 40 μM, which indicates that this complex is approximately 3.5 times more toxic than [56MESS] (IC₅₀ = 520 ± 160 μM) and 10 times more toxic than [56MERR] (IC₅₀ = 1540 ± 480 μM). The greater toxicity of cisplatin is probably due to it being the only one of the three platinum complexes

Table 1 Summary of the IC₅₀ values determined from the MTT assay of A549 cells treated with platinum complexes for 4 h^a

Complex	IC ₅₀ (μM)
Cisplatin	150 ± 40
[56MESS]	520 ± 160
[56MERR]	1540 ± 480

^a Results represent the mean and standard deviation obtained from triplicate assays

able to interact covalently with DNA. [56MESS] was found to be three times more toxic than the isomeric [56MERR], indicating that the chirality of the dach ligand significantly affects the cytotoxicity of these complexes toward A549 cancer cells. While the IC₅₀ values were higher than those reported by Fisher *et al.*²² and Wheate *et al.*,²⁰ this is expected due to shorter exposure times (4 h vs. 96 h), the use of a different end point for measuring cell viability (MTT as opposed to crystal violet), and the use of a different cell line. Importantly, however, the general trend in the observed toxicity of the isomers mirrors that reported earlier for a series of square planar(II) metallointercalators containing different derivatives of phenanthroline, as well as either *SS*-dach or *RR*-dach.³⁹ For the latter complexes the IC₅₀ values determined using the L1210 murine leukemia cell line were between 3 and 10 times smaller when the ancillary ligand present was *SS*-dach.

There are several possible explanations for the difference in cytotoxicity displayed by [56MERR] and [56MESS] towards the A549 cancer cell line. These include differences in cellular uptake, variations in the subsequent distribution of the complexes or their metabolites within organelles and throughout the cytoplasm, and/or differences in how the complexes interact with chiral biomolecules such as DNA and proteins.

GFAAS was used to investigate the first of these possibilities whereby platinum uptake in the A549 cells was measured following treatment with cisplatin, [56MERR], or [56MESS]. Initially a preliminary uptake experiment was performed to determine the ideal platinum dose (150 μM, 500 μM, or 750 μM) and exposure period(s) (2, 4, 24 and 48 h) that could be used for comparison of all three complexes. It was found that a dose of 500 μM Pt, facilitated platinum detection and allowed discernment between the treatments. The 2 h exposure period resulted in very little uptake, while the 48 h exposure period resulted in a substantial loss (by >90%) in cell numbers and cell integrity resulting in the predominance of cell debris. As such the 4 and 24 h treatment periods have been reported.

Fig. 2 shows the results obtained from GFAAS analysis of cells treated with DMEM (only), cisplatin, [56MERR], or [56MESS] (all Pt complexes 500 μM Pt in DMEM). Fig. 1S† shows the complimentary cell populations associated with each treatment and the proportion of cells that stained positive for trypan blue (*i.e.*, loss of membrane integrity). It is apparent that there was a drop in cell numbers associated with platinum treatment; approximately 5–10% for the 4 h exposure period and approximately 60% for the 24 h exposure period. Of note is the similarity between the cell populations resulting from treatment with [56MERR] or [56MESS]. This suggests that the MTT assay is either a more sensitive toxicity assay or that the observations in Table 1 stem from more specific interactions.

Fig. 2 GFAAS detection of platinum in A549 cells following exposure to the specified platinum complexes (500 μM Pt) for 4 h or 24 h. Columns represent the mean value and error bars represent the standard deviations obtained from triplicate samples.

The platinum uptake, reported in Fig. 2, has been presented as the platinum detected per cell, which includes the total cell count. Importantly, those cells that stained positive for trypan blue represent only a small proportion of the total cells for each treatment, such that it is unlikely that the loss of membrane integrity has significantly contributed to the observed platinum uptake.

Statistical analysis (ANOVA, Tukey-Kramer) of the platinum associated with the 4 h treatment group showed significant uptake ($P < 0.001$) in each of the platinum-treated cell samples compared with the control cells. It was also evident that the cellular platinum was highest following the 4 h exposure to cisplatin compared to [56MERR] and [56MESS]. Following 24 h exposure there was, once again, significant platinum uptake in the platinum-treated cells *versus* the control cells (cisplatin, $P < 0.001$; [56MERR], $P < 0.001$; [56MESS], $P < 0.01$). Interestingly, platinum uptake was greatest in [56MERR]-treated cells following 24 h exposure whereby the platinum concentration per cell was 1.8 times higher than that of cisplatin ($P < 0.001$). Importantly, the platinum uptake in [56MERR]-treated cells was approximately 2 times higher than that associated with [56MESS]-treated cells following 4 h treatment, and increased to 2.7 times higher than that of [56MESS] following the 24 h treatment period ($P < 0.001$). While this result contrasts that expected based on the MTT assays, whereby [56MERR] was less toxic than [56MESS], the result was confirmed by two individual GFAAS experiments (triplicate samples in each experiment) and it was also consistent with the trend observed in preliminary microprobe SRXRF analyses of A549 cells treated with equimolar (1 mM) [56MERR] or [56MESS] (ESI Fig. S2†).

Microprobe SRXRF imaging was performed on thin sections obtained from A549 cells treated with each of the platinum complexes (equitoxic dose, IC₅₀ values determined by the MTT assay) to shed light on whether the differences in the cytotoxicity

of the platinum complexes might be attributed to their differing targeting ability. Fig. 3a shows the elemental maps for sulfur, phosphorus, zinc, and platinum generated from microprobe SRXRF analysis of a typical untreated A549 cell. The range of concentrations of each element found within the cell is included above the elemental map. Inspection of the elemental map for sulfur reveals the overall size and shape of the cell, since high concentrations of this element are found throughout the cell owing to its presence in the toluidine blue stain (that targets DNA) and the proteins and enzymes found in both the nucleus and cytoplasm. There are distinct regions within the cell where both phosphorus and zinc are present in high concentrations. These can be used to locate the nucleus which contains DNA as well as zinc-rich proteins involved in DNA recognition and binding. The highest concentrations of phosphorus are present in specific regions of the nucleus where heterochromatin is present,

since this consists of densely packed DNA molecules.⁴⁰ Not surprisingly, the microprobe SRXRF results indicate that only very low amounts (background) of platinum were present within the control cell.

Due to beamtime limitations, only the 4 h platinum exposure periods were analysed, with the emphasis on establishing the initial targeting ability of the platinum complexes before the cells became saturated with platinum. Significantly greater amounts of platinum were present within cells treated with cisplatin. For example, inspection of the platinum elemental map for a typical cisplatin-treated cell shown in Fig. 3b reveals a maximum platinum concentration of $0.0665 \mu\text{g cm}^{-2}$, which is four times greater than that in the untreated cell ($0.016 \mu\text{g cm}^{-2}$). Comparison of the elemental map for platinum, with those for zinc and, in particular, phosphorus indicates that cisplatin and/or its metabolites become distributed throughout the cell,

Fig. 3 Microprobe SRXRF elemental maps for A549 cells following treatment (4 h) with: (a) cell medium only; (b) cisplatin; (c) [56MERR] and (d) [56MESS]. The elements are specified at the bottom of each column. Operating conditions include: Beam energy = 11.9 keV, Beam size = $0.2 \times 0.15 \mu\text{m}$; Stepsize = $0.3 \mu\text{m}$; Dwell time = 3 s/pt and Scan dimensions ($H \times V$) = (a) $12 \times 10 \mu\text{m}$; (b) $11 \times 11 \mu\text{m}$; (c) $10 \times 10 \mu\text{m}$; (d) $10 \times 10 \mu\text{m}$.

and not confined to the nucleus. A widespread distribution of platinum throughout the cell after treatment with cisplatin was found in each of the four A549 cells examined (further example shown in Fig. S3†), and is attributable to the formation of covalent compounds with DNA in the nucleus, as well as with various small peptides (e.g. glutathione) and proteins inside and outside the nucleus. This result is consistent with previous results obtained by Hall *et al.*²⁷ who used microprobe SRXRF analysis of A2780 ovarian cells treated with 20 μ M cisplatin (24 h) to show platinum accumulated in both the nucleus and the cytoplasm. In addition, transmission electron microscopy of thin sections prepared from cisplatin-treated A2780 ovarian cells revealed that platinum was in contact with the plasma membrane, nuclear envelope, cytoplasm and nuclear matrices.⁴¹

Cells treated with either [56MERR] or [56MESS] showed a cellular distribution of platinum that was distinctly different to that of cells treated with cisplatin, with typical examples of elemental distribution maps shown in Fig. 3c and d for both complexes. There is significant co-localization of platinum with both zinc and phosphorus, suggesting that the metallointercalator accumulates in the nucleus within as little as a 4 h exposure period. Close inspection of the elemental maps for these three elements reveals a very uniform distribution of zinc throughout the nucleus, whereas both platinum and phosphorus were found in the same specific regions of this organelle (Fig. S3† shows further results). This is consistent with the conclusion that the metallointercalator is interacting primarily with DNA present in the regions of the nucleus containing the heterochromatin. This result is in contrast to that obtained by microprobe SRXRF analysis of HepG2 hepatoma cells treated with arsenite, a non-intercalating metal complex that exhibits a low overall affinity towards DNA in other assays.⁴² In the latter case arsenite was found to be localised in the euchromatin region of the nucleus where the DNA is less densely packed and available to undergo transcription.

The above results also suggest that the platinum metallointercalators probably remain intact after entering the cell, as loss of either of the bidentate ligands would be expected to result in additional, covalent binding interactions with proteins in the cytoplasm. In order to provide further evidence that this assertion was correct, ESI-MS was used to examine the stability of [56MERR] and [56MESS] in DMEM over the time course of the cellular uptake and cytotoxicity experiments. The results obtained in the experiment performed with [56MESS] are presented in the ESI (Fig. S4†), and clearly demonstrate that this complex remained intact over a period of 4 h. During this time the abundance of ions from intact [56MESS] remained approximately the same, with all other ions present being attributable to components of the cell medium, or non-covalent complexes formed with the metallointercalator. Identical results were obtained when the experiment was performed using [56MERR], indicating that it too remained intact throughout the course of cell toxicity studies. The stability of the metallointercalators is due to the presence of two chelating ligands in the platinum coordination sphere. In contrast, ESI-MS analysis of reaction mixtures containing cisplatin and DMEM indicated that this complex underwent reactions with components of the cell medium.

The microprobe SRXRF results described above demonstrate that [56MERR] and [56MESS] accumulate specifically in the nucleus of cells, and probably in greatest amounts in those regions where heterochromatin is present. In contrast, cisplatin becomes more widely distributed throughout cells, as a result of being able to interact covalently with both proteins and DNA. It is important to note that these results do not provide information about the mechanisms by which the platinum complexes interact with DNA. Both [56MERR] and [56MESS] are likely to interact with DNA using a variety of mechanisms, including electrostatic attraction, dipole–dipole interactions, and intercalation. However, neither complex contains a ligand such as dppz (dipyrido[3,2a:2',3'-c]phenazine), which is renowned for its ability to confer on metal complexes the ability to be inserted into the DNA base stack. We have previously used microprobe SRXRF to investigate the cellular distribution of $[\text{Ni}(\text{phen})_2(\text{dppz})]^{2+}$.⁴³ In many respects the nickel distribution map resembled those shown for platinum in Fig. 3c and d, in that it revealed a high degree of co-localization between the metal on the one hand, and both zinc and phosphorus. This suggests that $[\text{Ni}(\text{phen})_2(\text{dppz})]^{2+}$ also has a high propensity to interact with DNA, but does not yield any specific information about the nature of those interactions.

The absence of a correlation between the IC_{50} values derived from the MTT assays (Table 1), the extent of metal uptake for [56MERR] and [56MESS] (Fig. 2), and the demonstration that both platinum metallointercalators accumulate in the cell in regions where DNA is expected to be present (Fig. 3), led to the consideration as to whether or not the difference in cytotoxicity between these complexes is due to variations in their affinity towards the nucleic acid. This prompted investigations into the binding of [56MERR] and [56MESS] towards a discrete duplex DNA molecule (D2) using a combination of ESI-MS and CD spectroscopy.

Fig. 4 shows the negative ion ESI mass spectra of free D2 (Fig. 4a) and solutions containing a 1:3 ratio of D2 with [56MESS] (Fig. 4b) or [56MERR] (Fig. 4c). It is apparent from comparison of Fig. 4a with Fig. 4b and c that the proportion of free D2 (responsible for ions at m/z 1626.4 and 1951.9) decreases significantly following incubation with the platinum complexes, as a result of the formation of non-covalent complexes. However, the ESI mass spectra of solutions containing the different platinum isomers are qualitatively very similar to each other. In both cases, the most abundant non-covalent complexes present in solution are those containing one or two platinum molecules bound to D2. Furthermore the relative abundances of non-covalent complexes containing different numbers of platinum molecules bound to D2 were very similar between the two spectra. This indicates that the affinities of [56MESS] and [56MERR] towards D2 are very similar. An identical conclusion was reached after comparing the ESI mass spectra of solutions containing a 6:1 ratio of either [56MESS] or [56MERR], and D2.

Fig. 5 shows the induced CD spectra obtained between 200 and 360 nm, for solutions containing either [56MESS] or [56MERR], and D2, with Pt:D2 ratios ranging from 0:1 to 10:1. The two principal components of the spectrum of a solution containing only D2 (not shown) consisted of a large positive CD band centred at 268 nm, and a smaller negative CD

Fig. 4 Negative ion ESI mass spectra of free D2 and solutions containing a 3 : 1 ratio of metal complex and D2: (a) free D2; (b) D2 + 56MESS; (c) D2 + 56MERR. ● = free D2; ▲ = D2 + 1 metal complex; ■ = D2 + 2 metal complexes; ◆ = D2 + 3 metal complexes; X = D2 + 4 metal complexes.

Fig. 5 Induced circular dichroism spectra of solutions containing different ratios of either 56MESS or 56MERR, and D2: (a) solutions containing 56MESS; (b) solutions containing 56MERR.

band at 245 nm. These values are consistent with those reported previously for B-form DNA.⁴⁴ Addition of the platinum complexes resulted in almost identical induced CD spectra. At low

Pt : D2 ratios a positive CD band centred above 260 nm was no longer evident, while the negative CD band at 245 nm increased in ellipticity and split into two separate negative bands. At the highest Pt : D2 ratio examined (10 : 1), both platinum complexes gave a spectrum containing a positive CD signal with low ellipticity and a peak maximum at either 282 nm for [56MERR] or 284 nm for [56MESS]. In addition, both spectra contained strong, negative CD bands centred at 232 and 252 nm.

The above spectral changes contrast dramatically with what has been reported previously for solutions in which octahedral metal complexes non-covalently interact with D2. For example, addition of $[M(\text{phen})_3]^{2+}$ ($M = \text{Ni}, \text{Ru}$), or complexes such as $[M(\text{phen})_2(\text{L})]^{2+}$ ($M = \text{Ni}, \text{Ru}$; $\text{L} = \text{dpqC}, \text{dppz}$), which are capable of interacting more strongly by intercalation to D2, resulted in the positive CD band shifting to lower wavelengths and increasing significantly in ellipticity.^{32,33} Furthermore the magnitude of the changes in ellipticity afforded an order of relative binding affinity that matched that obtained by several other techniques.³² Addition of other complexes, such as $[\text{Ru}(\text{Me}_4\text{phen})_2(\text{dpqC})]^{2+}$ and $[\text{Ru}(\text{Me}_4\text{phen})_2(\text{dpqC})]^{2+}$, resulted in shifts to lower energy for the positive CD signal of D2 that were accompanied by major enhancements in ellipticity.³² Therefore the changes in CD spectra caused by addition of the square planar complexes examined here are distinct from those seen previously with octahedral complexes, and reflect the sensitivity of this technique to changes in DNA structure (*e.g.* changes to base stacking, degree of helical unwinding) brought about by small, non-covalent binding molecules. Further evidence that addition of the square planar platinum complexes resulted in different distortions to the structure of D2, than those elicited by octahedral complexes is provided by a comparison of the changes to the negative CD band at 245 nm in the spectrum of D2. Whilst addition of octahedral metal complexes generally had very little effect on the position or magnitude of the ellipticity of this CD band,^{32,33} addition of either [56MESS] or [56MERR] resulted in the band splitting into two separate components, both of which grew significantly in ellipticity.

Comparison of the two spectra in Fig. 5 corresponding to the solutions containing a Pt : D2 ratio of 10 : 1 shows that CD bands are centred at almost identical wavelengths, and have almost identical ellipticities. This was true for each of the other Pt : D2 ratios examined, and is consistent with the conclusion that [56MESS] and [56MERR] have very similar DNA binding affinities.

3. Experimental

3.1. Materials

MilliQ™ (18.2 MΩ, Millipore) water was employed for the preparation of all solutions. Cisplatin was obtained from Sigma-Aldrich, while [56MESS] and [56MERR] (as their diperchlorate salts) were prepared using a procedure described previously.²⁰ $[\text{Ni}(\text{phen})_2(\text{dppz})]^{2+}$ was synthesised by Jihan Talib, University of Wollongong.³³ Glutaraldehyde (25% (v/v) in H_2O) was obtained from Fluka while 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St Louis, USA). Ammonium acetate (98%, NH_4OAc), methanol (99.8%), ethanol (99.5%) and dimethylsulfoxide (DMSO, 99.9%) were purchased from Ajax Finechem

(Seven Hills, Australia). Dulbecco's modified Eagle's medium (DMEM, 11885-084) was sourced from GIBCO Invitrogen, while foetal bovine serum (FBS), penicillin and streptomycin were purchased from Thermo Electron Corporation. Phosphate buffered saline (PBS) tablets were purchased from Bacto Labs (New South Wales, Australia) and trypsin was purchased from Thermo Scientific (Utah, USA). Disposable pipettes and 96 well plates were sourced from BD Falcon (Victoria, Australia), and cell culture flasks were purchased from Greiner Bio-One GmbH (Frickenhhausen, Germany). Spurr's resin and gold FINDER grids were purchased from ProSciTech (Queensland, Australia).

3.2. Cell studies

A549 human lung cancer cells were supplied by Associate Professor A. Ammit (University of Sydney) and were grown from frozen semi-permanents following rapid thawing. Typically, cells were grown in cell culture flasks with 75 cm² surface area, and incubated in a 5% CO₂ incubator at 37 °C (Revo Ultima, USA) to maintain growth. The growth medium (GM) employed for cell studies contained DMEM, FBS (10% (v/v)), penicillin (100 IU mL⁻¹) and streptomycin (100 µg mL⁻¹). The cells were subcultured twice a week to prevent overcrowding and cell death.

3.3. MTT assays

The cytotoxicity of the platinum complexes towards the A549 cells was assessed using the MTT assay.²⁶ Cells (1 × 10⁵ in 100 µL GM) were seeded into a 96-well plate which was incubated in a 5% CO₂ incubator at 37 °C for 24 h. The cells were then treated with freshly prepared solutions of the platinum complexes prepared in DMEM (2 mL). The treatment protocol involved the addition of platinum solution (100 µL of the appropriate concentration) to each well, and then returning the plate to the 5% CO₂ incubator at 37 °C for 4 h. After this period, the treatment solution was removed by aspiration and each well was washed twice with PBS solution (200 µL). GM was then added to the wells, and the cells were re-incubated at 37 °C under a 5% CO₂ atmosphere for 24 h to allow them to recover. At the end of this period the GM was removed by aspiration and MTT solution (50 µL, 2 mg mL⁻¹ in PBS) was added to each well. The plate was then incubated in 5% CO₂ at 37 °C for 4 h, after which the MTT solution was carefully removed by aspiration to ensure that none of the purple formazan crystals were also removed. DMSO (200 µL) was added to each well to dissolve the crystals, and the plate was agitated for 15 s, after which the absorbance of the resulting solutions was measured at 570 nm using a Spectromax 250 microplate reader (Molecular Devices Corporation, USA) with Softmax PRO 4.0 software. Absorption measurements at 630 nm were also recorded for background subtraction. The measured absorbances were then converted to percentage cell viabilities using eqn (1). All cell viabilities are reported as a mean and standard deviation obtained from triplicate assays each of which were generated from 6 replicate measurements performed for each concentration of each metal complex and control.

$$\text{Cell viability (\%)} = \frac{A_{570} - A_{630} (\text{treated cells})}{A_{570} - A_{630} (\text{control cells})} \times 100 \quad (1)$$

3.4. GFAAS

A549 cells were seeded (1 × 10⁶ cells per dish, 5 mL GM) into 60 mm cell culture dishes after which they were incubated for 48 h at 37 °C, 5% CO₂. Following this period the GM was removed and the cells were washed twice with PBS. Treatment solutions of cisplatin, [56MERR] or [56MESS] (500 µM Pt in 5 mL DMEM) were applied to the cells for 4 h and 24 h at 37 °C. Control cells were also prepared in a similar manner but were treated solely with DMEM for 4 and 24 h. At the completion of the treatment period, the treatment solution was collected and centrifuged to collect any floating cells. These cells were combined with the adherent cells which were harvested from the dishes using trypsin (2 mL, 0.25% in PBS). The resultant cell pellet was washed twice with PBS by centrifugation (300 g, 5 min). The final cell pellets were resuspended in 2 mL of PBS, and the cells associated with each sample were scored by removing 50 µL of cell suspension and adding to trypan blue solution (50 µL, 0.5% w/v in PBS). After 5 min the proportion of intact cells (those that appeared colourless) and those with compromised membrane integrity (those that appeared blue) was determined using a haemocytometer.

The cells were centrifuged to remove the PBS and washed twice with sterile saline solution (0.9%, 2 mL). The supernatants were removed and the cell pellets were freeze-dried for 2 h. Nitric acid (40 µL, Tracepur, 69% v/v) was added to each Eppendorf tube and the dehydrated pellets were digested overnight. The digested pellets were transferred to volumetric flasks (5.00 mL) and the volume was adjusted to produce a final concentration of 0.6% v/v HNO₃ (Tracepur, 2% v/v).

GFAAS was performed on the resultant solutions using a PerkinElmer AAnalyst 600 atomic absorption spectrometer equipped with the Winlab 32 AA Furnace program incorporating Zeeman-effect background correction, an AS-800 autosampler and an AA Accessory cooling system. A Pt hollow cathode lamp (PerkinElmer) was employed at 265.9 nm with a slit width of 0.7 nm, to give an instrument energy reading of approximately 63 W. A certified Pt standard solution (1003 mg L⁻¹ Pt, 2% HCl v/v, PerkinElmer) was used to prepare the stock Pt standard (100 µg L⁻¹, 0.6% HNO₃) for the calibration curve (correlation coefficients: 0.999301, 0.998997). A volume of 20 µL of analyte (standard or sample) was atomised from the surface of the pyrolytic graphite-coated tube using the furnace operating conditions presented in Table 2. The analyte reading was obtained during stage 4 (atomisation) and Ar gas was used during the analysis. Triplicate readings were measured for each of the triplicate cell samples prepared for each treatment condition. Statistical analysis of the results was performed using the One-way Analysis of

Table 2 Graphite furnace operating conditions used for analysis of Platinum in A549 cell digests

Stage	Temperature (°C)	Ramp (s)	Hold (s)	Gas flow (mL min ⁻¹)
Dry 1	110	1	30	250
Dry 2	130	15	30	250
Ash	1300	10	20	250
Atomisation	2200	0	5	0
Clean out	2450	1	3	250

Variance (ANOVA), Tukey-Kramer Multiple Comparisons Test using GraphPad InStat Version 3.10.

3.5. Microprobe SRXRF imaging

Microprobe SRXRF analysis of thin-sectioned cells was performed to determine the distribution of the metals and other selected elements at subcellular resolution following treatment with either cisplatin or one of the metallointercalators. Thin sections were prepared according to the procedure reported previously.^{29,45} Briefly, A549 cells were seeded into 75 cm² flasks containing GM (25 mL). Once cell adherence had occurred (after 24 h), the GM was removed and the cells were treated with solutions (20 mL) of the metal complexes in DMEM. The concentrations of metal complexes used corresponded to the IC₅₀ values obtained from the MTT assays. The cells and treatment solutions were then incubated for 4 h at 37 °C in a 5% CO₂ incubator. At the end of this period, the treated cells were collected (washings and harvested cells) and centrifuged at 300 g for 5 min (Labofuge 300, Heraeus) to produce a cell pellet. The pellet was washed twice with PBS, and the cells fixed in glutaraldehyde solution (2% in PBS) at room temperature for 2 h. The cells were centrifuged to form a pellet and stored in PBS overnight at 4 °C. The cells were dehydrated by washing with incrementally increasing concentrations of ethanol, followed by five washes with dry 100% EtOH. The cells were centrifuged and resuspended between each washing to ensure sufficient dehydration. The cell pellet was then infiltrated with 50% ethanol:50% Spurr's resin overnight at room temperature, followed by 100% Spurr's resin for 6–12 h (4 times) to ensure removal of all traces of water. Finally, the cell suspension contained in the Spurr's resin was transferred to a Beem capsule, and the cells were centrifuged to form a pellet. The Beem capsule was cured at 60 °C for 12 h, and the block was thin-sectioned (1 µm) using an UltraCut T microtome. The sections were stained with toluidine blue, washed four times with MilliQ water and mounted onto gold Finder grids.

Prior to analysis, the samples were inspected and imaged using a Leica DMXRE Epi-fluorescence/visual microscope (Leica, Germany) at 20× magnification. Specific cells were chosen for analysis according to the following criteria: (i) location away from the gold grid bars to reduce scatter effects; and (ii) the presence of a distinct nucleus.

Microprobe SRXRF analyses of thin-sectioned cell samples were performed under an atmosphere of helium at the 2-ID-D beamline at the Advanced Photon Source (APS), Argonne National Laboratory (Chicago, USA), using a monochromatic 11.9 keV X-ray beam. The beam was focused to ~0.2 µm² diameter using two stacked zone plates. The gold Finder grids (supporting the cell sections) were mounted on a high precision XYZ motorised stage mount (PM20076).

X-rays were detected in fluorescence mode using a Vortex EM (Radiant Detector Technologies, LLC 165-VTX-EM) silicon drift detector. Typically a low resolution map (2.5 µm stepsize, 40 × 40 µm² and 1 s per point dwell time) was initially collected to determine the exact coordinates and scan dimensions for cell maps. High resolution images were then collected using 0.2 µm steps and a 3 s per point dwell time. Elemental images were

prepared with the use of the MAPS version 1.6.0 software package provided by Dr Stefan Vogt at APS.⁴⁶ The full fluorescence spectra were fitted to modified Gaussians with the aid of corresponding measurements from the thin film standards, NBS-1832 and NBS-1833, obtained from the National Bureau of Standards (Gaithersburg, MD, USA). Selected elemental maps obtained after treatment of A549 cells with different metal complexes are presented in Fig. 3. Additional elemental maps are available in the ESI.†

3.6. ESI-MS stability studies of platinum complexes in cell media

The stability of the metallointercalators in DMEM was studied using positive ion electrospray ionization mass spectrometry to determine if they remain intact for the length of time employed for cytotoxicity and cell uptake studies (4 h). Mass spectra of the solutions were obtained immediately after preparation, as well as after 2 and 4 h.

3.7. DNA purification and preparation

Oligonucleotides (purchased in a deprotected 'trityl off' form) were obtained from Geneworks (South Australia) and purified as described previously.⁴⁷ The two complementary single strand sequences D2A and D2B were annealed to form the double stranded DNA (dsDNA) molecule which will be referred to as D2.

D2A = 5'-CCT CAT GGC CAT GAC C-3'

D2B = 5'-GGT CAT GGC CAT GAG G-3'

D2 = d(CCT CAT GGC CAT GAC C/ GGT CAT GGC CAT GAG G)

The concentration of stock solutions of the single strands D2A and D2B were determined by measuring their A₂₆₀ using UV spectrophotometry. Aliquots of the stock solutions were then mixed to prepare solutions with D2A : D2B ratios of 1 : 1, 1 : 1.5, 1 : 2, 1.5 : 1 and 2 : 1. These were annealed by first using a Speedivac freeze dryer to remove the solvent, adding 5 µL of 100 mM NH₄OAc (pH = 7.4), heating the resulting solutions at 63 °C for 15 min, and then allowing them to slowly cool to room temperature overnight. ESI-MS was used to determine which solution contained the highest proportion of dsDNA relative to its constituent single strands. The solution with a 1 : 1.5 ratio of D2A : D2B gave the greatest yield of dsDNA. This ratio was, therefore, used subsequently to prepare all stock solutions of D2 (1 mM D2 in 100 mM NH₄OAc (pH 7.4)).

3.8. ESI-MS studies of interactions between metallointercalators and DNA

Stock solutions of the diperchlorate salts of [56MESS] and [56MERR] were prepared by dissolving 0.2–1.0 mg of solid compound in 1.00 mL of 100 mM NH₄OAc (pH = 7.4). Reaction mixtures were prepared by adding 1 µL of stock D2 (1 mM) to appropriate volumes of stock solution of metal complex chosen to give metal complex to dsDNA ratios of 1 : 1, 3 : 1, 6 : 1 and 10 : 1. Finally, 100 mM NH₄OAc (pH 7.4) was added to produce a final volume of 100 µL, 200 µL or 500 µL.

Mass spectral analyses of solutions containing the metallo-intercalators and DNA were performed using a Waters Ultima ESI quadrupole time-of-flight (ToF) mass spectrometer, equipped with an electrospray ionization source set in negative ion mode. The instrumental conditions used in these experiments included: capillary voltage = 2.5 kV; cone voltage = 100 V; source temperature = 25 °C; desolvation temperature = 80 °C; desolvation gas flow = 100 L h⁻¹.

4. Conclusions

Each of the three platinum complexes examined was found to be stable in cell media, and toxic toward the A549 cell line. Of particular note was the observation that [56MESS] was about three times more toxic than [56MERR] (as determined by the MTT assay), in keeping with the results of previous cytotoxicity studies employing platinum complexes that differ only by the presence of the *S,S*-dach or *R,R*-dach ligand.³⁹ The even greater cytotoxicity of cisplatin is attributable to a different mechanism of action, in which the platinum becomes covalently attached to DNA. Microprobe SRXRF studies clearly showed a difference in cellular distribution between cisplatin, and both [56MERR] and [56MESS]. While the former complex was present throughout the cell, both [56MERR] and [56MESS] accumulated specifically in those regions of the nucleus where the heterochromatin is expected to be present. This is attributable to the very stable structure of the two metallointercalators, which prevents these complexes from undergoing aquation in a similar fashion to cisplatin, to form metabolites capable of binding covalently to peptides and proteins in the cytoplasm. In the absence of any initial hydrolysis reactions, [56MERR] and [56MESS] are capable of interacting with DNA by a variety of mechanisms that probably include partial intercalation.

Experiments were conducted in an attempt to identify an explanation for the greater cytotoxicity of [56MESS] compared to [56MERR] as determined by the MTT assay. Both ESI-MS and CD spectroscopy revealed only small differences in their DNA binding affinities, while microprobe SRXRF analysis showed similar cellular localization of platinum in cells treated with [56MERR] and [56MESS]. In contrast, the results of GFAAS experiments indicated that there was significantly greater uptake of [56MERR] compared to [56MESS]. As this is the opposite trend to the order of cellular toxicity for these two complexes, other factors must also play a part in determining the differences in cytotoxicity of [56MERR] and [56MESS]. For example, it will be important to probe the interactions of these complexes with other biomolecules such as proteins, most importantly the mitochondrial enzyme probed by the MTT assay (mitochondrial reductase), in order to develop a fuller understanding of the origin of the difference in their cytotoxicities.

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