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Bidentate Ligands on Osmium(VI) Nitrido Complexes Control Intracellular Targeting and Cell Death Pathways

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Abstract

The cellular response evoked by anti-proliferating osmium(VI) nitrido compounds of general formula OsN(N^N)Cl$_3$ (N^N = 2,2′-bipyridine **1**, 1,10-phenanthroline **2**, 3,4,7,8-tetramethyl-1,10-phenanthroline **3**, or 4,7-diphenyl-1,10-phenanthroline **4**) can be tuned by subtle ligand modifications. Complex 2 induces DNA damage, resulting in activation of the p53 pathway, cell cycle arrest at the G2/M phase, and caspase-dependent apoptotic cell death. In contrast, **4** evokes ER stress leading to the upregulation of proteins of the unfolded protein response pathway, increase in ER size, and p53-independent apoptotic cell death. To the best of our knowledge, **4** is the first osmium compound to induce ER stress in cancer cells.

The antiproliferative activity of cisplatin created a new paradigm in the field of anticancer drugs.[1] The platinum-based drugs cisplatin, carboplatin, and oxaliplatin now make up the first line of defense against many systemic malignancies.[2] Drawbacks associated with platinum therapy,[3] such as acquired or inherent resistance, toxic side effects, and tumor recurrence after initial treatment, have prompted researchers to investigate alternative transition metal-based anticancer drugs.[4] Ruthenium[5] and titanium[6] compounds have undergone clinical trials and much time has been devoted to understanding their mechanisms of action. In contrast, the anticancer properties of Os-containing compounds are relatively unexplored, perhaps because of the reputation of osmium as being highly toxic.[7] Nevertheless, several half-sandwich "piano-stool" osmium(II) arene complexes have emerged with promising in vitro activity and no cisplatin cross-resistance.[8] More recently, DNA-targeting osmium(VI) nitrido compounds with tridentate Schiff bases[9] and monodentate azole heterocycle ligands[10] have displayed encouraging in vitro and in vivo properties. Here we report the anti-cancer properties of osmium(VI) nitrido compounds bearing bidentate ligands in which small changes to the ligand periphery evoke completely different cellular responses. This discovery further highlights the great, unexplored potential of metal coordination chemistry in the design of therapeutic agents.

The complexes investigated in this study are depicted in Fig. 1. The bidentate ligands used were 2,2′-bipyridine, 1,10-phenanthroline, 3,4,7,8-tetramethyl-1,10-phenanthroline, and 4,7-

diphenyl-1,10-phenanthroline. The complexes were synthesized by reacting (Bu$_4$N) [OsNCl$_4$] with the appropriate ligand in acetone or dichloromethane. Characterization of **1-4** and the crystal structure of **4** are reported in the SI. Prior to carrying out cellular studies, the stability of **2**, taken as a representative member of the family, in phosphate buffered saline (PBS) and DMEM cell culture media was established by UV-vis spectroscopy (Figs. S1-2). Its thermal stability was demonstrated by variable temperature ^{1}H NMR spectroscopy (Fig. S3).

The anti-proliferative properties of **1-4** against a panel of human cancer cell lines were assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Cisplatin was included as a control. The IC$_{50}$ values, concentrations required to induce 50% viability, were derived from dose–response curves and are summarized in Table S1. The complexes all display micromolar toxicities comparable to, and in some cases, better than that of cisplatin. Compounds **2** and **4** are the most potent osmium(VI) compounds investigated in vitro. None of the Os complexes exhibits cross-resistance with cisplatin, as shown by their ability to indiscriminately kill cisplatin-sensitive and -resistant ovarian cancer cell lines, A2780 and A2780CP70. As a measure of therapeutic potential, we conducted cytotoxicity studies with healthy lung fibroblast MRC5 cell. In general **1-4** are less potent toward MRC5, indicating selective toxicity for cancerous over healthy cells. Notably, **2** displays an 8-fold higher potency for the ovarian cancer cell line A2780 than for the normal lung fibroblast, MRC5 cell line.

Cell viability studies were also carried out with quiescent A549 lung cancer cells (see Fig. S4 for cell cycle profiles). Comparison of the IC$_{50}$ values for quiescent and proliferating A549 cells revealed that **1-4** selectively target the latter (Fig. S5). This property is highly desirable in preclinical studies.

Most previously reported osmium compounds are assumed to induce cell death through DNA interactions.[7] We therefore investigated the interaction of **2** or **4** with pUC19 plasmid DNA using gel electrophoresis (Fig. S6). As the concentration of **2** increases, there is a clear increase in the amount of nicked circular, and a concomitant decrease in the amount of supercoiled, plasmid DNA. At concentrations > 250 μM, the bands in the gel smear and then disappear. Surprisingly, **4** had no visible effect on the migration pattern of pUC19 DNA. The gel images clearly indicate that **2** induces conformational changes and degradation of circular DNA whereas **4** has no such effect. The DNA binding kinetics of **2** and **4** were studied by measuring the amount of osmium bound to precipitated ct-DNA (Fig. S7). The half-life of this reaction for **2** was 2 h. The half-life for **4** could not be calculated because a large portion remained unbound after a 16-h incubation. These results illustrate the differing DNA-binding abilities of **2** and **4**..

To determine how the osmium compounds behave intracellularly, the most active compounds **2** and **4** were analyzed using a recently developed strategy whereby RNAi is used to classify the mechanism of cytotoxic drug action.[11] This RNAi-based methodology relies on a fluorescence competition assay involving lymphoma cells that are partially infected with one of eight distinct short hairpin RNAs (shRNAs). shRNA-bearing cells will either enrich or deplete relative to the uninfected population based on drug survival advantage or disadvantage conferred by a given shRNA. The responses of these cells comprise signatures, which have been obtained for all classes of clinically used cytotoxic agents. The signature of a novel compound is compared to those of a reference set of drugs using a probabilistic K-nearest neighbors algorithm to determine whether it belongs to a class in the reference set or a new category that is not represented in the reference set. Interestingly, neither **2** nor **4** classified as belonging to any category of drug mechanism present in the reference set and thus represent novel mechanisms of drug action (Fig. 2).

Individual shRNA responses can, however, give clues about broader mechanistic aspects of action. For instance, all DNA-damaging drugs in the reference set have a roughly 1:1 log2(RI) shp53:shChk2 ratio with the two values being over 3.0. For **2**, however, shp53 and shChk2 values were 2.7 and 2.6, respectively, indicating that DNA damage may be a part of a pleiotropic mechanism of **2** induced cell death. Conversely, for **4**, shp53 and shChk2 values of 1.32 and 0.73, respectively, indicate that this compound does not kill cells by damaging DNA.

To gain further insight into the modes of action of **2** and **4**, immunoblotting analyses were conducted to monitor changes in expression of biomarkers related to the DNA damage pathway (Fig. S8). A2780 cells incubated for 72 h with **2** showed a marked increase in expression of the phosphorylated forms of H2AX (H2AX), CHK2, CHK1, and p53 (Ser15), indicative of DNA damage.[12] In contrast, cells treated with **4** displayed little signs of DNA damage. Additionally upon extraction of genomic DNA from A2780 cells, significantly higher levels of osmium were detected in samples dosed with **2** as compared to **4** (Fig. S9). Taken together the results indicate that, in cells, **2** targets DNA more readily than **4**. This difference could be related to steric effects imposed by the phenyl groups in **4**.

Cellular uptake studies revealed that **2** localized essentially evenly between the nucleus and cytoplasm (Fig. S10). Complex **4**, on the other hand, was predominantly found in the cytoplasm, at levels12-fold higher than in the nucleus. This finding led us to investigate the possibility that **4** may induce endoplasmic reticulum (ER) stress. Co-administration of **4** and salubrinal (10 μM), a known ER stress inhibitor,[13] reduced the cytotoxicity of **4** in A2780 cells. The IC_{50} value increased 13.3-fold compared to treatment with **4** alone, suggesting ER stress as a component of the cytotoxic mechanism of **4** (Fig. 3a). Salubrinal had limited effect on the toxicity of DNA-targeting compounds like **2** and cisplatin. To further validate the ER as the main target for **4**, we monitored the expression of proteins related to the unfolded protein response (UPR). Upon incubation of A2780 cells with **4** for 72 h, phosphorylated eukaryotic initiation factor 2 (eIF2) and C/EBP homologous protein (CHOP) were upregulated, indicative of ER stress (Figs. S11-12).[14] Notably, **2** had little effect on eIF2 and CHOP expression. ER membrane expansion is widely associated with ER stress.[15] With this fact in mind we examined the ER size of A2780 cells upon incubation with **4** using fluorescence microscopy. Relative to untreated controls (Fig. 3b), cells exposed to **4** (10 μM) for 4 h had 25.1% larger ER coverage (Fig. 3c), the quantitation of which is reported in Fig. S13. These results clearly show that **4** targets the ER and induces ER stress.

Having established the intracellular targets of **2** and **4**, we investigated the role of p53, a cell cycle and apoptosis regulator,[16] in the cellular responses evoked. A2780 cells treated with **2** displayed higher p53 expression compared to untreated cells (Fig. S14). A dose-dependent increase in the downstream effectors p21 and BAX was also observed (Fig. S11). Therefore, p53 plays a fundamental role in coordinating the cellular response to **2**-induced DNA damage.[17] p53 expression did not increase markedly upon incubation with **4**, however (Fig. S15). Additionally, BAX and p21 expression remained fairly constant (Fig. S15), indicating that p53 is not a major determinant in the cellular response induced by **4**. To relate p53 to potency, cytotoxicity studies were conducted in the presence of p53 inhibitors pifithrin-α[18] and pifithrin-μ[18] (both 10 μM) (Figs. S16-17). The IC_{50} value for **2** increased significantly in the presence of either p53 inhibitor, suggesting p53-dependent cell death. Conversely, the toxicity profile of **4** was unaffected by the p53 inhibitors. Compounds capable of inducing cell death independent of p53 status are clinically desirable because p53 is associated with tumorigenesis and is inactivated in many cancers.[19]

DNA flow cytometric analysis (Fig. S18) revealed that **2** stalled the cell cycle at the G2/M phase in a time-dependent manner (~3-fold increase in G2/M population after 72 h

incubation). This property may be related to upregulation of p53 and phosphorylation of CHK1.[20] Complex **4** induced relatively small perturbations to the cell cycle; changes in phase populations remaining < 5% even after a 72-h incubation (Fig. S19).

To have a more complete understanding of the cellular response induced by **2** or **4**, we evaluated their mode of cell killing. Many clinically approved cancer drugs exert their cytotoxic effects through apoptosis, and we therefore monitored features related to this pathway.[21] Apoptotic cells undergo morphological changes that lead to cell membrane disorientation. This process results in the translocation of phosphatidylserine residues to the membrane exterior, which can be detected by Annexin V.[22] Using a dual Annexin V staining/PI flow cytometry assay, we explored the occurrence of apoptosis in A2780 cells treated for 72 h with **2** or **4** (Fig. S20). Both osmium complexes induced large populations of cells to undergo early and late stage apoptosis. Immunoblotting studies revealed that cells treated with **2** or **4** expressed apoptosis positive proteins, cleaved caspase 3, 7, 9, and poly ADP ribose polymerase (PARP) (Figs. S21-22). These results provide validation for the apoptosis pathway. Cells can undergo other forms of cell death such as necrosis and autophagy. To probe these pathways, cytotoxicity assays were carried out in the presence of necrosis and autophagy inhibitors, chloroquine[23] and IM-54, respectively (Figs. S23-24). Because both chloroquine and IM-54[24] had little effect on the potency of either of the osmium complexes, necrosis and autophapy were ruled out as possible death pathways. Overall our cellular data show that **2** induces DNA damage which leads to G2/M phase arrest and apoptosis. On the other hand, **4** initiates ER stress, culminating in p53 independent, caspase directed apoptosis (Fig. 4). Most osmium complexes reported thus far, regardless of oxidation state (II or VI), induce cell death through DNA interactions; here we describe, to the best of our knowledge, the first osmium complex to induce cell death via ER stress.

In summary, we present a small molecule platform that enables a switch between targeting of genomic DNA and the ER through minor modifications to the ligand scaffold. In addition to developing biologically active osmium(VI) compounds, a deeper understanding of osmium biology has been gained. Furthermore, the molecular design described constitutes a step towards discovering versatile small molecules that can target different cancer hallmarks with minimal synthetic effort.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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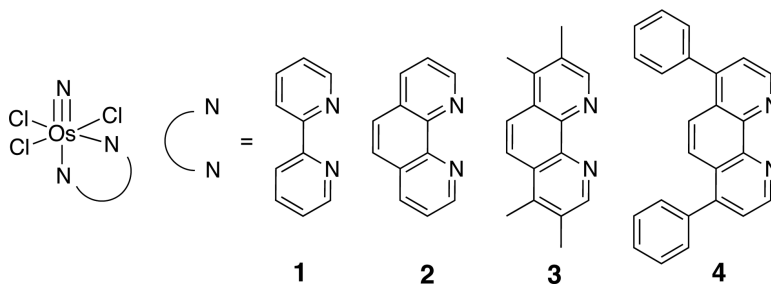
Figure 1.
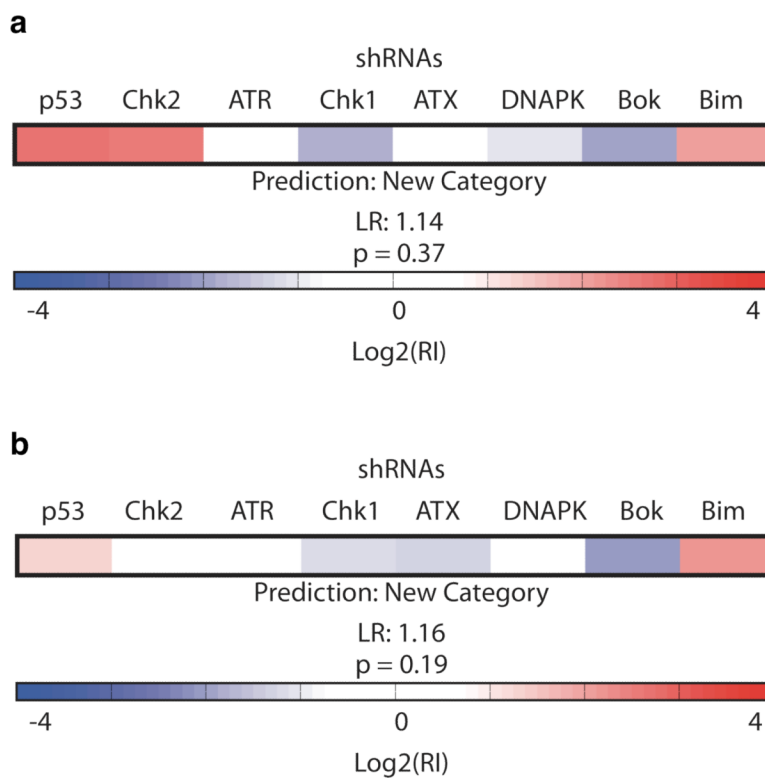Structures of the osmium(VI) nitrido complexes under investigation.

a

b

Figure 2.
RNAi signatures derived from the treatment of Eμ-Myc$^{p19arf-/-}$ lymphoma cells with (a) **2** and (b) **4** at the LD80-90 concentration for each compound.
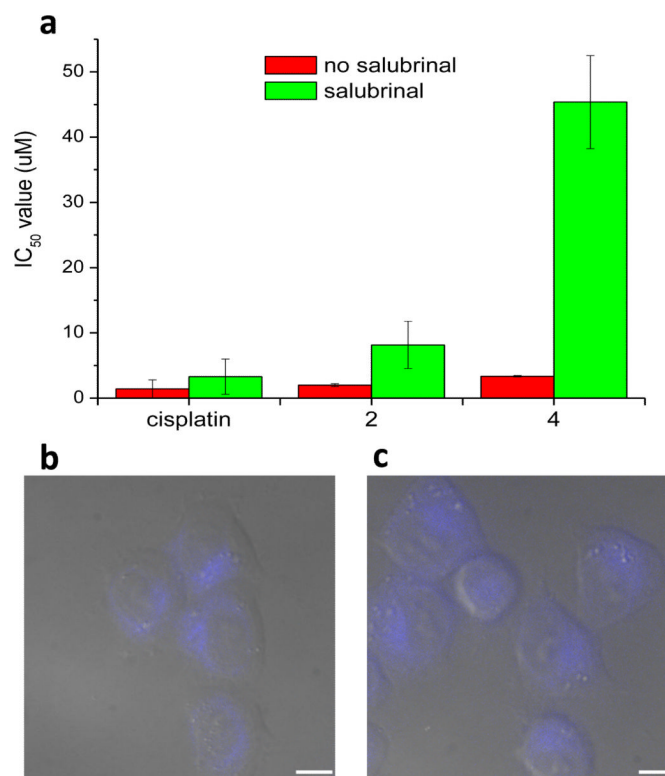
Figure 3.
(a) Graphical representation of the IC$_{50}$ values of cisplatin, **2**, and **4** in the absence and presence of ER stress inhibitor, salubrinal (10 μM). (b & c) Fluorescence microscopy of live A2780 cells incubated at 37 °C for 4 h without (b) and with (c) **4** (10 μM). Both panels contain the blue ER-tracker (5 μM) probe. Scale bar = 9 μm.
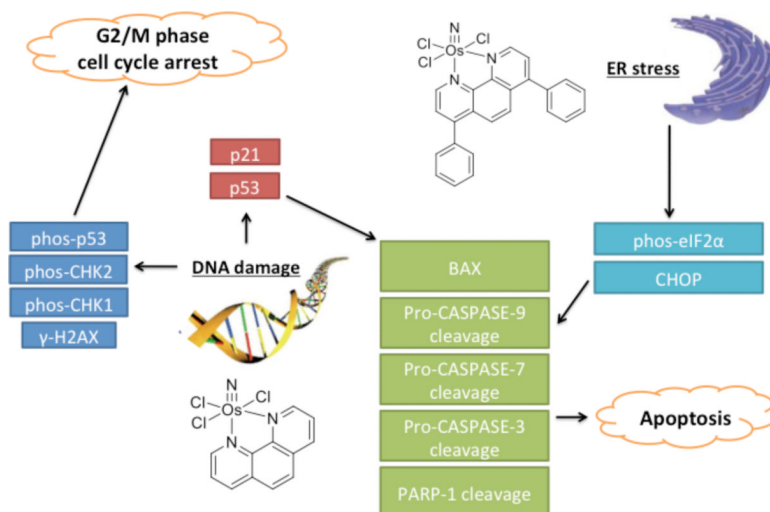
Figure 4.
Proposed cellular mechanism of action of **2** and **4**. Complex **2** targets genomic DNA, whereas **4** induces ER stress.