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Cytotoxic Triosmium Carbonyl Clusters: A Structure–Activity Relationship Study

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A structure–activity relationship (SAR) study of the triosmium carbonyl cluster $Os_3(CO)_{10}(NCCH_3)_2$ was carried out with a series of clusters of the general formula $Os_3(CO)_{12-n}L_n$, cationic osmium clusters and a hemi-labile maltolato-Os cluster. The SAR results showed that good solubility in DMSO and at least one vacant site are required for cytotoxicity. In vitro evaluation of these new compounds showed that some are selectively active against estrogen receptor (ER)-independent MDA-MB-231 breast cancer cell lines relative to ER-dependent MCF-7 breast cancer cells, suggesting that the compounds have a different biological target specific to MDA-MB-231 cells. In particular, the maltolato cluster exhibits strong antiproliferative activity, with an IC_{50} value of 3 μM after only 24 h incubation. Additionally, biochemical assays conducted with the cationic cluster show that it induces apoptosis, although a biological target has not yet been identified. Further research to establish the molecular targets of these compounds and to develop improved organometallic clusters as potential breast cancer therapeutics is underway.

Introduction

The emergence of organometallic compounds within the biological sciences has been gaining momentum, especially in the area of drug development. Much interest has been focused on organometallic compounds that possess anticancer activity, which can be generally classified into two groups: one group that involves modification of organic analogues, such as the ferroquines[1] and ferrocifens,[2] and another group exhibiting metal-based activity, such as the arenes[3] and titanocenes.[4]

The organometallic clusters are a class of organometallic compounds that was recently discovered to have potential as drug candidates.[5] We were the first to report a series of triosmium carbonyl clusters (including clusters **1** and **3a** below) that showed antiproliferative activity against both ER^+ and ER^- breast cancers. Mode of action studies for the most active compounds in the series, cluster species $Os_3(CO)_{10}(NCCH_3)_2$ (**1**), showed that these compounds hyperstabilized microtubules and induced apoptosis.[6] In this previous study, we hypothesized that the molecular requirement for cytotoxicity was the availability of two coordination sites. The study was, however, hampered by the low solubility of the compounds in DMSO, the vehicle used in the cytotoxicity assays. This problem was particularly acute with the monosubstituted derivatives, such as **3a**, which was not very soluble in DMSO and was found to be inactive. The effect of solubility on the anticancer activity of these compounds is therefore of interest.

In the present study, we decided to examine the structure–activity relationships (SAR) of a series of similar derivatives to assess the effect of solubility (e.g., protonation of **3a** to form **4a** increases its solubility in DMSO), number of vacant sites (e.g., comparison of activity for positive control **1** with **3b** and **3c**), and type of ligands (e.g., γ-pyrone of compound **5** in place of the nitrile ligand of the parent compound) on activity (Figure 1).

Results and Discussion

Syntheses of triosmium carbonyl clusters

Syntheses of osmium carbonyl clusters **1** and **3a** were carried out as previously reported[7] by heating $Os_3(CO)_{12}$ in excess acetonitrile in the presence of trimethylamine-*N*-oxide (TMNO).

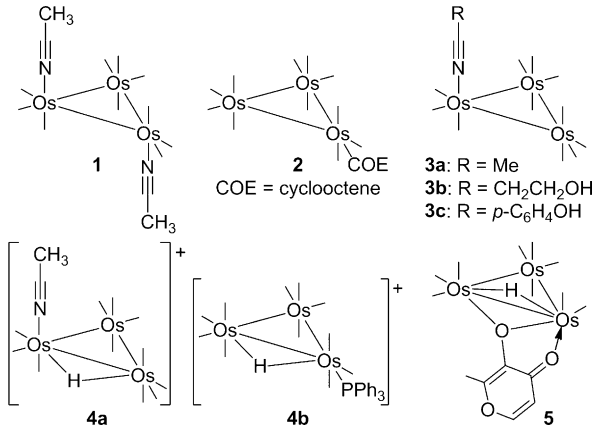

Figure 1. Molecular structures of osmium clusters **1**–**5**.

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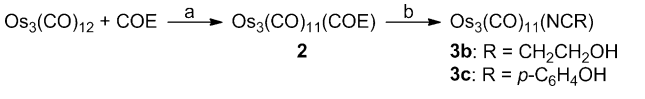
This route, however, was inadequate for the preparation of analogues in which the nitrile was a solid or a high boiling point liquid. In principle, **1** or **3a** could serve as precursors, and an excess of the desired nitrile could be used to displace the acetonitrile ligands; however, distinguishing the product and precursor spectroscopically was difficult as they showed almost identical patterns of carbonyl stretches in the IR spectrum. A synthetic route based on the cluster $Os_3(CO)_{11}(COE)$ (**2**) as the intermediate was thus used for the syntheses of $Os_3(CO)_{11}(NCCH_2CH_2OH)$ (**3b**) and $Os_3(CO)_{11}(NCC_6H_4OH\text{-}p)$ (**3c**) (Scheme 1). Cluster **2** had a distinct IR pattern that was different from the nitrile-substituted osmium clusters, and the cyclooctene (COE) ligand was more weakly bound than the acetonitrile ligand, making it a better leaving group for substitution reactions. The identity of **2** was established by its IR spectrum, which showed a similar pattern for the CO stretches as that of the ethene analogue $Os_3(CO)_{11}(C_2H_4)$, and by its reaction with PPh_3 to form the known derivative $Os_3(CO)_{11}(PPh_3)$.[8] $Os_3(CO)_{11}(NCCH_3)(\mu\text{-}H)^+BF_4^-$ (**4a**) was prepared by protonating **3a** with HBF_4 acid,[9] while $Os_3(CO)_9(\mu\text{-}H)(\mu\text{-}\gamma\text{-}C_6H_5O_3)$ (**5**) was synthesized by the reaction of **1** and 2-methyl-3-hydroxy-γ-pyrone.[10]



Scheme 1. Synthetic route to osmium clusters with nitrile ligands. *Reagents and conditions*: a) Me_3NO, CH_2Cl_2, 2 h, 40 °C, 98% yield; b) NCR, CH_2Cl_2, 1 h, room temperature, 87–93% yield; COE = *cis*-cyclooctene.

Biochemical studies

Cell viability was assessed using the MTS assay in a MDA-MB-231 cell line at a concentration of 20 μM for triosmium carbonyl clusters **3**–**5**, with **1** as the positive control (Figure 2). A factor that is expected to influence the cytotoxicity of a compound is its ability to permeate the cell membrane. This is usually measured as the water/octanol partition coefficient via reversed-phase HPLC. However, the instability of some of the compounds precluded this method. The cell permeability of these compounds was therefore estimated using their solubility in DMSO instead (Table S1).

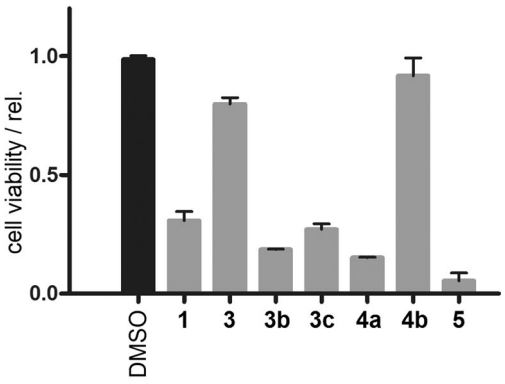


Figure 2. MTS assay of osmium clusters **1** and **3**–**5** on MDA-MB-231 breast cancer cells; 20 μM, 24 h. DMSO concentration: 0.2% (*v/v*).

The cationic cluster **4a**, which has better solubility in DMSO, shows enhanced activity over its neutral analogue **3a** (saturated concentrations of 19.9 and 3.7 mM, respectively). This also appears to be a factor for the cytotoxicity of **1** (saturated concentration of 15.0 mM). On the other hand, the cationic cluster **4b**, which contains a non-labile phosphine ligand, is inactive. The importance of this is further supported by the observation that **3b** and **3c**, which contain labile nitrile ligands with hydroxy groups that improve their solubility (saturated concentrations of 14.0 mM and 9.5 mM, respectively), also show good antiproliferative activity. Good solubility (cell permeability) alone, however, is insufficient; a labile ligand is also required. Furthermore, neither a positive charge on the cluster nor on the BF_4^- anion contributes to the activity. The activity of cluster **5**, in which the weak bonding interaction between the carbonyl group of the pyrone and the cluster core can lead to the formation of a vacant coordination site, also demonstrates that activity is not limited to compounds with nitrile ligands; the availability of a vacant coordination site at the metal center is the only requirement.

Table 1. Inhibition of cell growth by triosmium carbonyl clusters after 24 h, as determined by MTS assay.

Compd	IC_{50} [μM][a]		
	MCF-7	MDA-MB-231	MCF-10A
1	30 ± 9	14 ± 6[b]	18 ± 3
3b	16 ± 2	8 ± 1	> 30
3c	28 ± 6	13 ± 3[b]	19 ± 2
4a	10 ± 3	7 ± 1	22 ± 1
5	19 ± 2	3 ± 1	23 ± 10

[a] Data represent the mean ± SD of $n = 9$ independent experiments performed in triplicate. [b] Calculated from the results of a separate assay.

The IC_{50} values of **1**, **3b**, **3c**, **4a**, and **5** were determined and showed significant antiproliferative activity at 20 μM in three cell lines, namely, MCF-7 (ER^+ breast cancer), MDA-MB-231 (ER^- breast cancer) and MCF-10A (normal breast epithelial) cell lines (Table 1). These results showed that clusters **3b**, **3c**, **4a**, and **5** have either similar or higher cytotoxicity (lower IC_{50} values) than **1**. In particular, cluster **5**, the most active compound, has an IC_{50} value of 3 μM in a short incubation time of 24 h. One clear structural difference between cluster **5** and the other clusters is that it contains a maltolato instead of a nitrile ligand. Maltolato–metal complexes, especially those that contain iron, have been found to generate reactive oxygen species (ROS) in cells. In many cancer models, it has been found that this causes DNA strand breakage, leading to antineoplastic activity.[11] It is therefore possible that, in addition to Os-induced activity, the activity of **5** may be enhanced by release of the maltolato ligand upon reaching the target. This may then bind to any metal ions, such as iron, that are present in the cell to form ROS species. This hypothesis requires further investigation.

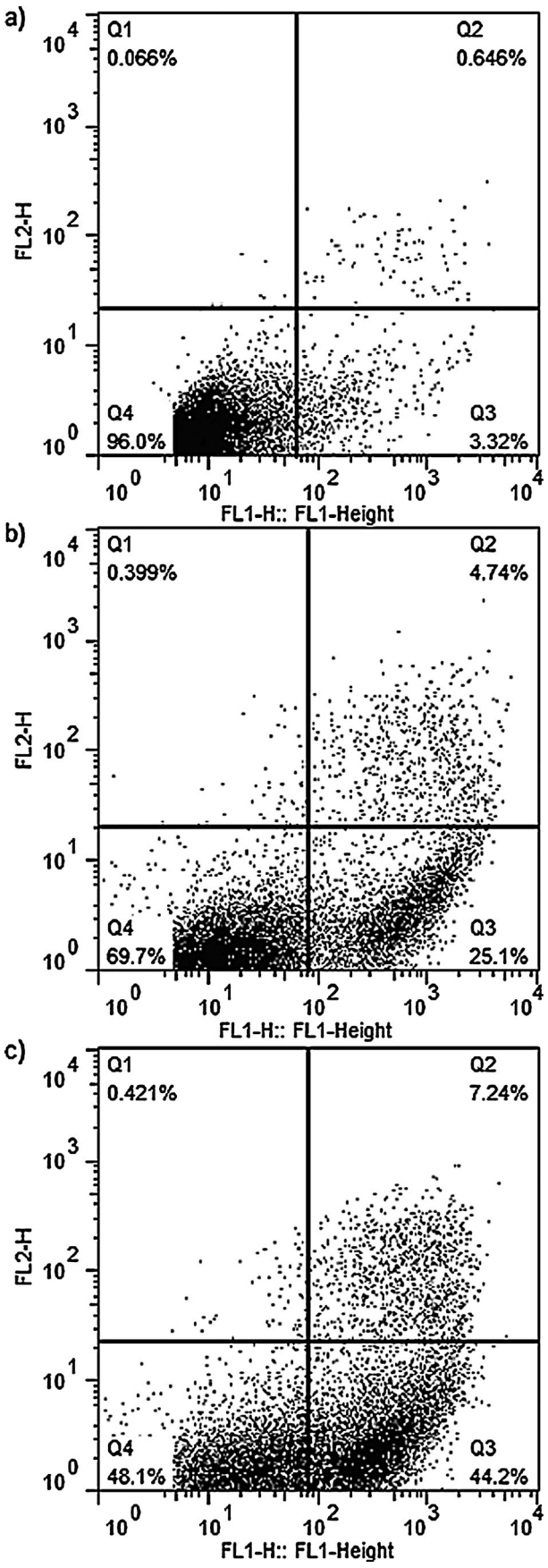


Figure 3. MDA-MB-231 cells stained with Annexin V–FITC and PI after 15 h incubation with a) vehicle only (control), b) 10 μM **1** (positive control), and c) 10 μM **4a**. FL1-H and FL2-H refer to Annexin V–FITC and PI, respectively. Q2: necrotic cells (stained with both Annexin V–FITC and PI); Q3: apoptotic cells (stained with Annexin V–FITC only); Q4: live cells (no stain).

Interestingly, all of the compounds were also found to be more active against MDA-MB-231 than against MCF-7 cells. This is in contrast to tamoxifen, the drug currently used for the treatment of breast cancer, which is active only against MCF-7 cells. It would therefore appear that these compounds do not act through estrogen receptors but rather act on a different biological target that is specific to MDA-MB-231 cells.

The cytotoxicity exhibited by cationic cluster **4a** was particularly interesting as, unlike its unprotonated analogue **3a**, it did not lose its acetonitrile ligand easily. For example, **4a** failed to react upon stirring with triphenylphosphine, even up to 80 °C. Charged species are known to be able to induce necrosis through rupture of the cell membrane. This was unlikely in the case of **4a**, however, as **4b**, which is also charged, was not cytotoxic. Nevertheless, assays to show that **4a** induced apoptosis were carried out. For instance, flow cytometric analysis, using FITC-conjugated annexin V and propidium iodide (PI) staining of MDA-MB-231 cells that were incubated for 15 h with 10 μM of **4a** showed that 50% of the cells were dead, of which 80% were in early apoptosis (Figure 3). Induction of apoptosis was further supported by significant changes in chromatin distribution of MDA-MB-231 cells treated with a 5 μM solution of **4a** relative to the control (Figure 4; more images in the Supporting Information), as well as observation of the characteristic sub-G_1 peak in the DNA content frequency histogram for cells treated with a 10 μM solution of **4a** for 15 h (14.8%, relative to 2.11% with control).[12]

A possible biological target for **4a** is the mitochondria, reminiscent of other lipophilic cationic compounds such as the alkyltriphenylphosphine (TPP) cations, which have been employed to target the mitochondria.[13] TPP cations are able to pass directly through the phospholipid bilayer of the cell membrane because of their large hydrophobic surface area. They also tend to accumulate in the mitochondria because of the large mitochondria membrane potential (higher negative charge inside the mitochondria);[14] selective targeting of cancer cells rests on the fact that these cells exhibit higher mitochondrial transmembrane potential than normal cells. Mode of action studies to test this hypothesis for **4a** are currently underway.

Conclusions

In this study, we have shown that the cytotoxicity of clusters of the structural type $Os_3(CO)_{12-n}(L)_n$ require good solubility and at least one labile ligand. We also found some clusters with different structural types that were cytotoxic. In particular, they were more cytotoxic against MDA-MB-231 ER^- breast cancer cells than against MCF-7 ER+ breast cancer cells or MCF-10A normal breast epithelial cells. This indicated that they did not act against the hormone receptor but via a different

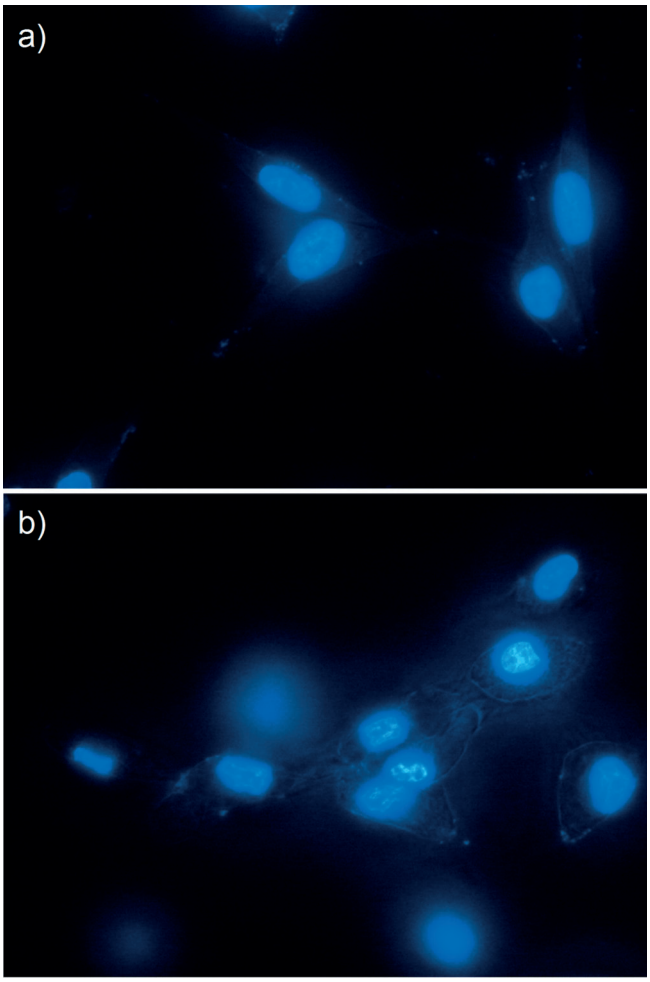


Figure 4. Fluorescence image (60×) of MDA-MB-231 cells stained with DAPI: a) control and b) after incubation with 5 μM **4a** for 3 h.

target specific to MDA-MB-231 cells. Although apoptotic assays confirmed that the cationic cluster **4a** induced apoptosis, its biological target has not been established, with further research on this aspect currently underway.

Experimental Section

Synthesis

General: Synthetic steps were carried out under an argon atmosphere using standard Schlenk techniques. ^{1}H NMR spectra were recorded on a Jeol 400 NMR spectrometer as $CDCl_3$ solutions unless otherwise stated; chemical shifts reported were referenced against the residual proton signals of the solvent. IR spectra were recorded on a Bruker Alpha FTIR spectrometer as CH_2Cl_2 solutions unless otherwise stated. The starting material, $Os_3(CO)_{12}$, was obtained from Oxkem Ltd. (Reading, UK); all other chemicals were purchased from other commercial sources and used as supplied. The clusters **1**,[7] **3a**,[7] **4a**,[9] **4b**,[15] and **5**[10] were synthesized according to published methods.

Synthesis of $Os_3(CO)_{11}$(COE) (2): A sample of $Os_3(CO)_{12}$ (20 mg, 22 μmol) and Me_3NO (2 mg, 26 μmol) were added to CH_2Cl_2 (10 mL) and *cis*-cyclooctene (1 mL) in a reaction vessel equipped with a magnetic stir bar. The reaction mixture was stirred for 2 h at 40 °C under argon, and formation of the product was monitored by IR spectroscopy. The mixture was then filtered through silica gel to remove any unreacted Me_3NO, and the solvent was removed in vacuo to give $Os_3(CO)_{11}$(COE) (**2**) as a yellow oil (21.3 mg, 98%): ^{1}H NMR: $\delta = 3.66$–3.69 (m, 2H, vinylic protons), 2.49–2.54 (m, 2H, allylic protons), 1.88–1.97 (m, 2H, allylic protons), 1.68–1.82 (m, 4H, 4H of C_8H_{14}), 1.43–1.53 ppm (m, 4H, 4H of C_8H_{14}); IR (cyclohexane): $\tilde{\nu}_{CO} = 2114$ (w), 2061 (s), 2041 (s), 2025 (vs), 2006 (w), 2000 (m), 1989 (w), 1979 (w), 1974 (w), 1962 cm^{-1} (w).

Synthesis of $Os_3(CO)_{11}(PPh_3)$: Cluster **2** (21 mg, 21 μmol) and PPh_3 (5.6 mg, 21 μmol) were dissolved in CH_2Cl_2 (5 mL). The mixture was then stirred for 1 h at room temperature. Formation of the product was monitored by IR spectroscopy. The CH_2Cl_2 was removed in vacuo, and the crude product was purified by TLC (hexane/CH_2Cl_2, 1.75:1, *v*/*v*) to give $Os_3(CO)_{11}(PPh_3)$ as a yellow solid (15.8 mg, 66%): IR: $\tilde{\nu}_{CO} = 2107$ (m), 2055 (s), 2034 (ms), 2018 (s), 1988 (m), 1978 cm^{-1} (m); Lit. values (cyclohexane):[8] $\tilde{\nu}_{CO} = 2108$ (m), 2055 (s), 2035 (s), 2019 (s), 2000 (ms), 1989 (m), 1978 cm^{-1} (m).

Synthesis of $Os_3(CO)_{11}(NCCH_2CH_2OH)$ (3b) and $Os_3(CO)_{11}(NCC_6H_4OH$-*p*) (3c): An identical procedure to that used for the synthesis of $Os_3(CO)_{11}(PPh_3)$ was followed, with the exception that no further purification was carried out. Removal of the solvent afforded a yellow solid.

3b (18.5 mg, 93%): mp: 121 °C; ^{1}H NMR: $\delta = 3.92$ (s, broad, 2H, CH_2OH), 3.22–3.25 ppm (t, 2H, $J = 6.4$ Hz, $NCCH_2$); IR: $\tilde{\nu}_{CO} = 2106$ (w), 2067 (m), 2053 (vs), 2040 (vs), 2017 (s, sh), 2008 (vs), 1980 cm^{-1} (m); ESI: 951.52 $[M+H]^+$.

3c (18.2 mg, 87%): mp: 143 °C; ^{1}H NMR: $\delta = 7.53$–7.57 (m, 2H, 2H of C_6H_4), 6.90–6.98 (m, 2H, 2H of C_6H_4), 5.61 ppm (OH); IR: $\tilde{\nu}_{CO} =$ 2106 (w), 2068 (m), 2053 (vs), 2040 (vs), 2017 (s, sh), 2007 (vs), 1982 (m) cm^{-1}; ESI: 998.55 $[M+H]^+$.

Biological methods

General: Experimental cultures of MCF-7, MDA-MB-231, and MCF-10A cell lines were obtained from American Type Culture Collection (ATCC) and cultured in tissue culture dishes (Nunc Inc., Naperville, IL, USA). MCF-7 and MDA-MB-231 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine (Gibco Laboratories), and 1% penicillin (Gibco Laboratories) at 37 °C in 5% CO_2 atmosphere. MCF-10A cells were maintained in DMEM:F12 supplemented with 7.5% FBS, 1% L-glutamine, 0.4% gentamicin (Gibco Laboratories), and growth factors EGF (Invitrogen), insulin (Invitrogen), hydrocortisone (Sigma–Aldrich) and cholera toxin (Sigma–Aldrich) at 37 °C in 5% CO_2 atmosphere. Phosphate-buffered saline (PBS) was obtained from Gibco. For all apoptosis assays, the control refers to cells incubated with DMSO (vehicle), and positive control refers to cells incubated with **1**.

Proliferation assay: The osmium carbonyl clusters were first dissolved in sterile DMSO to make 10 mM stock solutions. They were then diluted with serum-free medium, with final concentrations in a range of 0.1–200 μM used for treatment. The cells were then treated with the indicated concentrations of osmium carbonyl clusters and incubated for 24 h at 37 °C in 5% CO_2, after which, Cell Titer 96_Aqueous One Cell Proliferation Assay (20 μL, Promega) was added to each well. This was then left to incubate at 37 °C in 5% CO_2 for 2 h. Absorbance at 490 nm was then measured, and cell proliferation relative to the control sample was calculated.

Curves were then generated using a sigmoidal dose–response (variable slope) equation and GraphPad Prism 5 software.

Annexin V–FITC and PI staining for flow cytometry: MDA-MB-231 cells were plated in six-well plates at the same initial density and were allowed to adhere and grow for 24 h at 37 °C in 5% CO_2. They were then serum-starved for 24 h before treatment with 10 μM of **1** or **4a**. The cells were harvested after the incubation period (15 h) and washed in cold PBS (5 mL). The washed cells were centrifuged to obtain a pellet, the supernatant was discarded, and the pelleted cells were washed with PBS (2×5 mL) before resuspension in 1× annexin-binding buffer (Invitrogen). Annexin V–FITC (5 μL) and PI (1 μL) stains were then added, and the stained cells were analyzed using a BD FACSCalibur flow cytometer.

Nuclear staining with 4,6-diamidino-2-phenylindole (DAPI): MDA-MB-231 cells were plated in single-well plates (Ibidi) at the same initial density and were allowed to adhere and grow for 24 h at 37 °C in 5% CO_2. They were then serum-starved for 24 h before treatment with 5 μM of **4a** for 3 h at 37 °C. After treatment, the cells were fixed with 3.7% formalin (room temperature, 1 h), washed with PBS (2 mL), stained with 4,6-diamidino-2-phenylindole (DAPI, 1 μg mL^{-1} in PBS, 5 min at 37 °C), washed, and then photographed using a confocal microscope equipped with a UV light filter (Nikon D Eclipse C1).

DNA fragmentation using PI: MDA-MB-231 cells were plated in T25 flasks at the same initial density and were allowed to adhere and grow for 24 h at 37 °C in 5% CO_2. They were then serum-starved for 24 h before treatment with 10 μM of **4a** for 15 h. After treatment, the cells were fixed with 70% EtOH (0.7 mL, −20 °C, overnight). They were then centrifuged (200 *g*, 5 min), suspended in PBS (0.8 mL), and centrifuged again (300 *g*, 5 min). The cell pellet was then suspended in PBS (0.05 mL), DNA extraction buffer (0.04 mL) was added, and the mixture was incubated for 5 min at room temperature to facilitate the extraction of low molecular weight DNA. The cells were then centrifuged, PI (0.15 mL) was added, and they were incubated at room temperature in the dark for 30 min. The stained cells were then analyzed using a BD FACSCalibur flow cytometer.

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Keywords: apoptosis · carbonyl clusters · estrogen receptors · osmium · structure–activity relationships

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