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Osmium Carbonyl Clusters: A New Class of Apoptosis Inducing Agents

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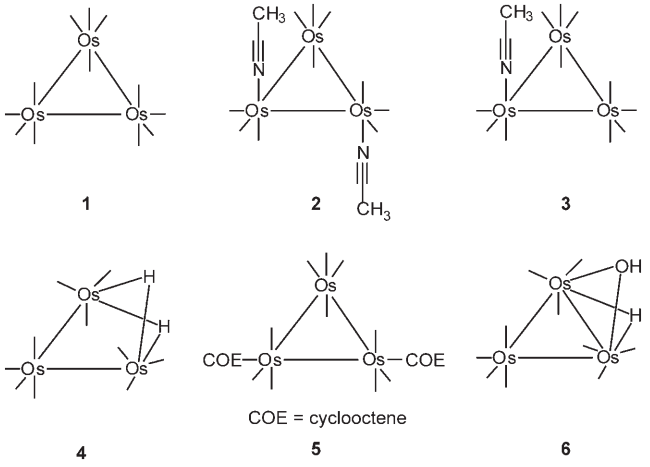
Osmium carbonyl clusters, especially the cluster $[Os_3(CO)_{10}(NCCH_3)_2]$, were found to be active against four cancer cell lines, namely, ER+ breast carcinoma (MCF-7), ER− breast carcinoma (MDA-MB-231), metastatic colorectal adenocarcinoma (SW620), and hepatocarcinoma (Hep G2). The mode of action was studied in MCF-7 and MDA-MB-231 cell lines by a number of morphological and apoptosis assays, all of which pointed to the induction of apoptosis.

Introduction

As a result of the success of cisplatin, as well as its limitations, the search for new inorganic anticancer drugs has continued unabated.[1] One class of inorganic compounds that is currently being actively developed is that of organometallic compounds. These can be divided into those involving inorganic or organometallic modifications of organic compounds known to show therapeutic action, such as ferrocifen and paullones,[2] and those that show metal-based anticancer activity such as NAMI-A[3] and the metallocenes and metallocenyls.[4] More recently there has been a number of reports suggesting that organometallic clusters, compounds which contain metal–metal bonds, may hold promise in pharmacological applications.[5,6] The idea that transition metal carbonyl clusters may have potential applications in cancer therapy was first mooted by Dyson,[6] and his group recently reported that triruthenium–arene clusters exhibited remarkable anticancer activity against ovarian carcinoma.[7]

In a recent paper, we reported the employment of organometallic clusters of osmium for imaging of cells in the mid-infrared range.[8] In the course of that study, we observed that cell viability was affected at higher concentrations, which prompted us to look at the possibility of osmium clusters showing useful cytotoxicity. The cytotoxicity of osmium compounds has been investigated recently[9] as has the telomerase inhibition by triosmium clusters.[10] We would like to report herein our evaluation of the anticancer activity of a series of triosmium clusters against five cancer cell lines.



Results

The cytotoxicity of a series of triosmium carbonyl clusters, $[Os_3(CO)_{12}]$ **1**, $[Os_3(CO)_{10}(NCCH_3)_2]$ **2**, $[Os_3(CO)_{11}(NCCH_3)]$ **3**, $[Os_3(CO)_{10}(\mu\text{-}H)_2]$ **4**, $[Os_3(CO)_{10}(COE)_2]$ **5**, and $[Os_3(CO)_{10}(\mu\text{-}OH)(\mu\text{-}H)]$ **6** was determined by testing for inhibition of cell growth activity against five cancer cell lines, namely, ER+ breast carcinoma (MCF-7), ER− breast carcinoma (MDA-MB-231), colorectal adenocarcinoma (HT29), metastatic colorectal adenocarcinoma (SW620), and hepatocarcinoma (Hep G2), and a normal breast epithelial cell line (MCF-10A). The computed growth inhibition (IC_{50}) values are given in Table 1.

In contrast to **1**, **3**, and **5**, which showed no discernible cytotoxicity up to their maximum soluble concentrations (10 μM) for all the cell lines tested, compounds **2**, **4**, and **6** showed appreciable cytotoxicity for all but the HT29 (colorectal adenocarcinoma) cell line. Whereas compound **4** showed little selectivity, **2** and **6** showed significantly different IC_{50} values for all but one of the cancer cell lines compared with the normal epithelial cell line. Interestingly, **2** was the only one among those tested here which was cytotoxic, albeit at a high treatment

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Table 1. Inhibition of cell growth by the triosmium carbonyl clusters measured with the six cell lines after 24 h incubation, as determined by MTS assay.

Compd	IC_{50} [μM] MDA-MB-231	MCF-7	HT29	SW620	Hep G2	MCF-10A
1	>10	>10	>10	>10	>10	>10
2	6.2±1.2	8.4±1.6	32±4	8.2±1.1	5.1±1.7	17.3±1.8
3	>10	>10	>10	>10	>10	>10
4	9.7±1.8	9.1±1.1	>10	>10	>10	8.5±1.5
5	>10	>10	>10	>10	>10	>10
6	8.5±0.9	10.8±1.3	>10	9.4±1.7	7.6±1.3	13.6±1.6

concentration, against the HT29 cells. These results were also reflected in the morphological changes of the cells; MDA-MB-231 and MCF-7 cells exhibited significant morphological changes after 24 h incubation with 10 μM solutions of **2**, **4**, and **6**, whereas the SW620 and Hep G2 cells were only affected by **2** and **6**, and the HT29 cells only by **2** (Supporting Information figures S3–7). The efficacy of **2** for both ER+ and ER− breast carcinoma was particularly interesting and hence our subsequent investigations were directed mainly at **2** and with these two cell lines.

The cellular uptake of osmium after 24 h incubation for MDA-MB-231 and MCF-7 cell lines, as determined by ICP, demonstrated that accumulation was <0.1 ppm, below the sensitivity threshold of the method, for **1**, **3**, and **5** (Supporting Information table S1). In contrast, the osmium contents following incubation with **2**, **4**, and **6** for both cell lines were 0.29–0.67 ppm. A similar cellular uptake study for varying concentrations of **2** on MDA-MB-231, MCF-7, and MCF-10A showed that osmium accumulation was significantly higher for the two cancer cell lines, especially for MDA-MB-231; osmium uptake for concentrations from 1 μM and below were not detectable (Figure 1). Although the cell panel investigated here is small, it is evident that the cytotoxicity correlates with the cellular uptake of osmium, pointing to the balance between influx and efflux of triosmium carbonyl clusters as a possible cytotoxicity marker.

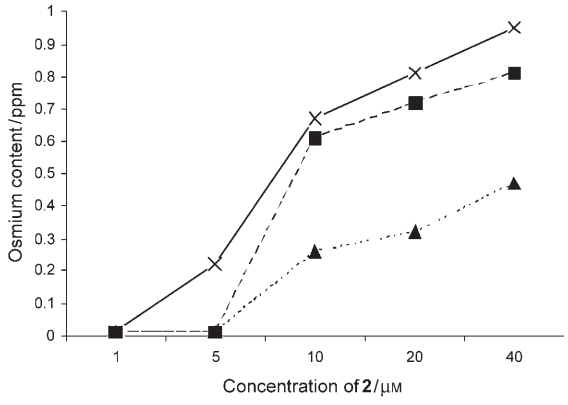

Figure 1. Osmium content (ppm) in cells after 24 h incubation with various concentrations of **2**, determined by ICP. Osmium content for concentrations of **2** at 0.1 and 0.5 μM were <0.1 ppm; values <0.1 ppm are plotted as close to zero. ×: MDA-MB-231, ■: MCF-7, ▲: MCF-10A.

The concentration- and time-dependent decreases in cell proliferation for the MDA-MB-231 and MCF-7 cell lines with **2** are shown in Figure 2. In both cell lines, >50% reduction in cell proliferation was observed at concentrations above 10 μM, and significant reduction in cell proliferation was observed as

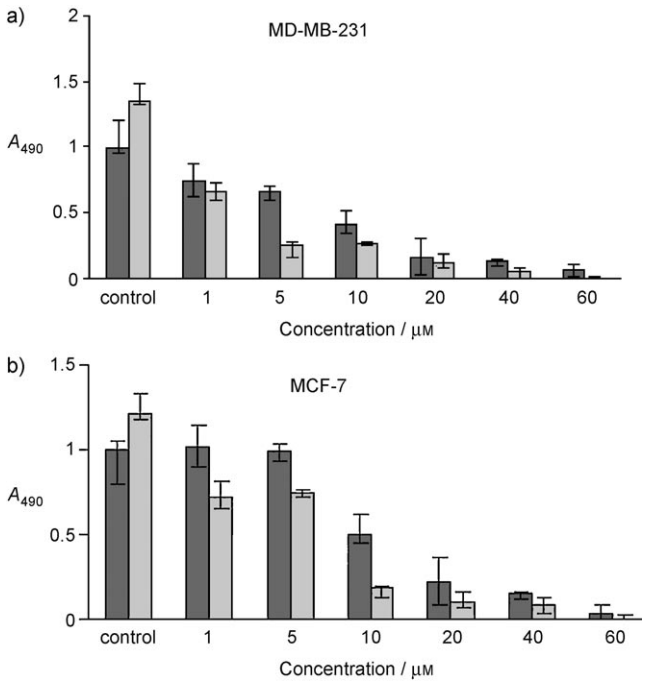

Figure 2. Time- and concentration-dependent effects of **2** on the proliferation of the a) MDA-MB-231 and b) MCF-7 cell lines. Cells were treated with the indicated concentrations of **2** in DMEM containing 1% serum. Dark-grey bars: 24 h incubation, Light-grey bars: 48 h incubation.

early as 24 h. Maximal effects were observed at 48 h treatment, whereby almost all cells were dead. The time-dependent loss in cell viability was also accompanied by morphological changes (Supporting Information figures S8 and S9); cells incubated in medium containing different concentrations of **2** for up to 24 h exhibited characteristic morphological features of apoptosis such as detachment and cell shrinkage. This was further confirmed by the characteristic changes in morphological features of the nucleus stained with DAPI (4′,6-diamidino-2-phenylindole). Figure 3 shows the comparison made of the cytotoxicity of **2** with cisplatin, resveratrol, and tamoxifen, which are commonly used in breast cancer treatment. It is clear that **2** has greater efficacy than cisplatin and resveratrol at both one and 10 μM, and is at least similar to tamoxifen. In particular, both the ER+ and ER− cell lines are sensitive to **2**.

Several assays to confirm apoptosis induction by **2** were carried out. The presence of chromatin condensation in MCF-7

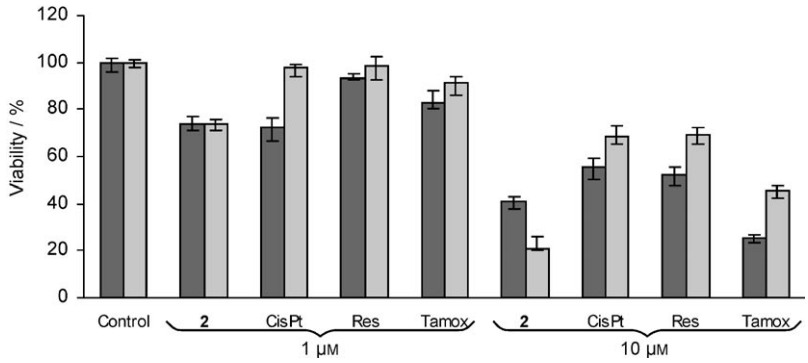

Figure 3. Comparison of cell viability of MCF-7 (dark-grey) and MDA-MB-231 (light-grey) after 24 h incubation with **2** (at 1 and 10 μM), cisplatin (CisPt), resveratrol (Res), and tamoxifen (Tamox) in serum-free DMEM.

cells treated with **2** was analysed by fluorescence microscopy using the DNA-binding fluorescent dye DAPI (Figure 4). In contrast to the control, which presented a homogeneous nuclei distribution (or rounded, intact nuclei), cells treated with 20 μM of **2** showed significant changes in chromatin distribution (separate globular structures). This change is consistent with the induction of apoptosis. Flow cytometry using FITC-conjugated annexin V and propidium iodide (PI) staining were carried out for both the MDA-MB-231 and MCF-7 cells after 24 h incubation with one to 40 μM solutions of **2**; the results for MDA-MB-231 for 10, 20, and 40 μM solutions are shown in Figure 5. The results showed that **2** increased the early apoptosis cell population (FITC stained) in a concentration-dependent manner for both cell lines, reaching 60–70% at 20 μM. At 40 μM, only 30–50% of the cells were in the early apoptotic phase as some of the cells have proceeded to late apoptosis (double stained by FITC and PI).

Extensive DNA fragmentation is considered as a specific marker for apoptosis. Thus we analysed DNA fragmentation by fractional DNA content analyses for MDA-MB-231 and MCF-7 cells treated with varying concentrations of **2** for 12 and 24 h (Supporting Information figure S12). The flow cytometry profiles revealed an accumulation of cells in the G2-phase. Furthermore, the results also showed a concentration dependence of the cell cycle distribution; accumulation of cells in the G2-phase can be observed after application of a 40 μM solution for 12 h, and >5 μM for 24 h incubation. With the higher concentrations, the characteristic hypodiploid DNA content (sub-G_1) peak can also be seen, again pointing to the induction of apoptosis by **2**.

Caspase activation is another specific marker for apoptosis. We have studied caspase activation by **2** using two assays. In the first, cells were pretreated with the pan-caspase inhibitor Z-VAD-FMK followed by a 24 h treatment with a 20 μM solution of **2**. Examination of the cell morphology showed that the pretreatment with Z-VAD-FMK resulted in almost complete blockage of the cytotoxic effect of **2** on both the MDA-MB-231 and MCF-7 cells (Supporting Information figure S13). The second assay was the detection of poly(ADP-ribose) polymerase (PARP) cleavage; proteolytic cleavage of the 116 kDa PARP to an 85 kDa fragment is another key feature of apoptosis.[11] Cells incubated with **2** for up to 24 h showed that the PARP was cleaved, as determined by immunoblotting using a polyclonal antibody which recognises the intact form and the 85 kDa fragment (Figure 6). The study also demonstrated a concentration-dependent increase in the amount of the 85 kDa fragment.

An initial attempt at elucidating the primary cellular target of the compounds was made through an examination of their

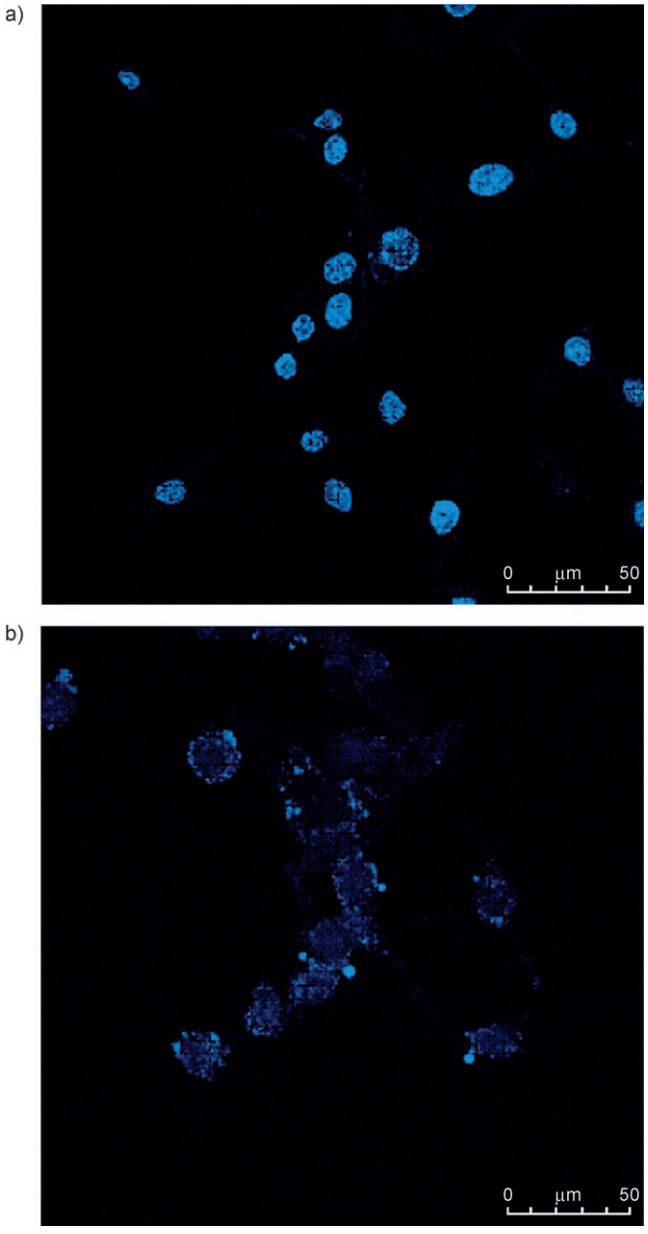

Figure 4. Fluorescence images (100×) of MCF-7 cells stained with DAPI: a) control and b) after 24 h incubation with a solution of **2** at 20 μM.

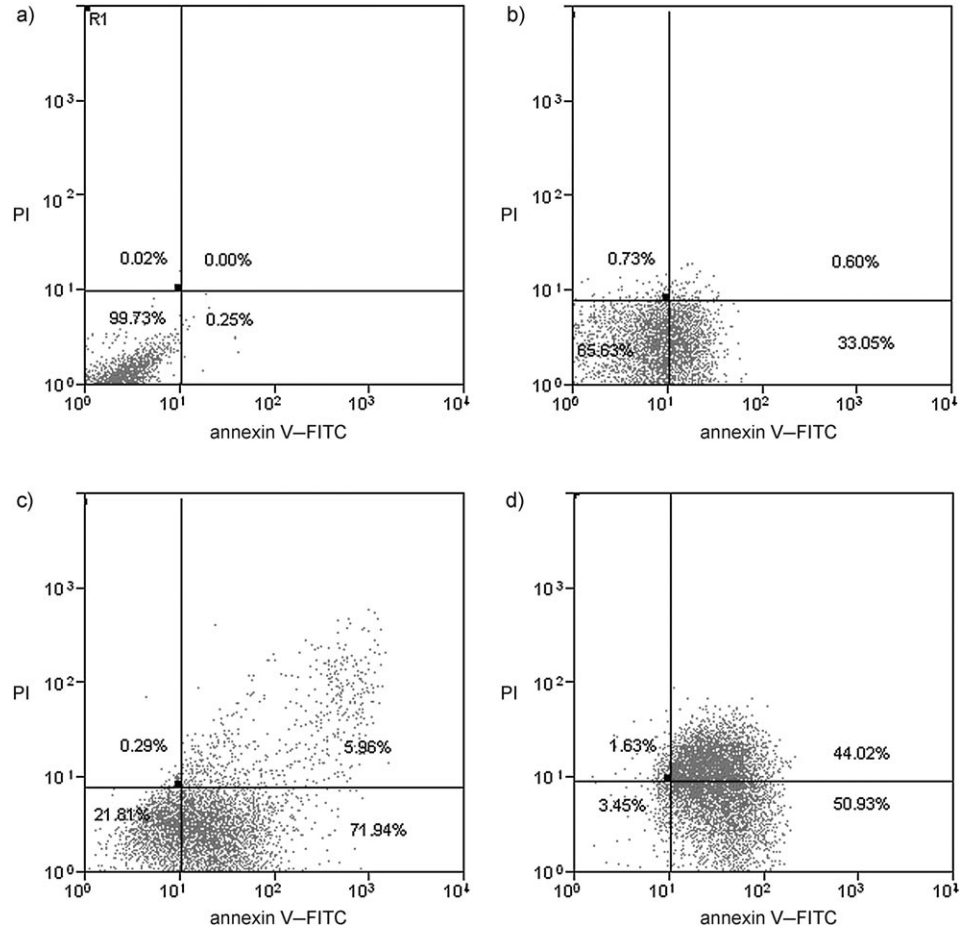

Figure 5. Detection of early and late apoptotic MDA-MB-231 cells after staining with annexin V–FITC and PI. Cells were incubated for 24 h with solutions of **2** at concentrations of: a) 0, b) 10, c) 20, or d) 40 μM before staining.

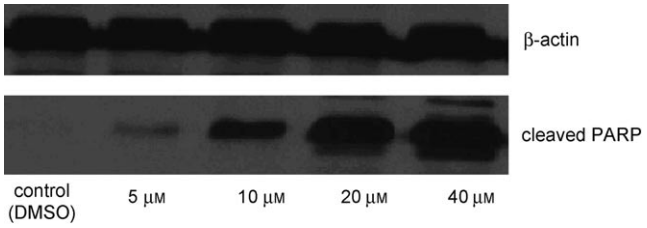

Figure 6. Gel electrophoretogram of MDA-MB-231 cells treated for 24 h with various indicated concentrations of **2**.

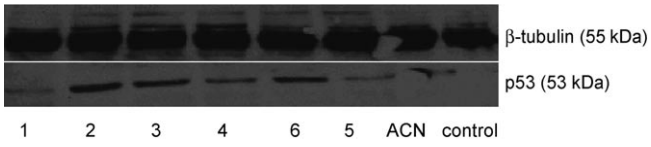

Figure 7. Gel electrophoretogram of MDA-MB-231 cells treated for 24 h with indicated compounds at 10 μM; ACN = acetonitrile.

effect on p53 activity in MDA-MB-231 cells. Treatment with 10 μM solutions showed that p53 levels were increased for **2**, **3**, **4**, and **6** (Figure 7).

Discussion

The triosmium carbonyl clusters which we have chosen for evaluation here are readily obtainable and, except for the parent cluster **1**, are known to undergo reactions readily. Not only are they different chemically from other mononuclear metal-based drugs such as cisplatin, but they are also different from other clusters which show anticancer activity via their ligands. Several of these compounds at least matched cisplatin in their efficiency in cell growth inhibition. They thus represent a potentially new class of anticancer agents. Of particular interest was the high activity and selectivity of **2**, for both ER+ and ER− breast cancer cells, as well as for hepatocarcinoma.

Through a number of assays with **2**, that is, morphological assay (differential staining), phosphatidylserine inversion and membrane integrity analysis (annexin V–FITC and PI staining), DNA fragmentation (DNA content with fractional DNA content), caspase inhibition, and caspase activation (PARP cleavage), we have established that cell growth inhibition was via the induction of apoptosis. Cleaved PARP and caspases are at the heart of the apoptotic machinery[12] and several caspases have been shown to be the key executioners of apoptosis mediated by various inducers, including antitumour agents.[13] However, although the appearance of cell death apparently requires caspase activation,[14] we cannot at this point exclude the involvement of other pathways, in particular the signalling pathway involving Fas and caspase 8, as has been described for cisplatin.[15]

Cell cycle checkpoints can be targeted for cancer therapy either by activating checkpoint-mediated apoptosis or by exploiting chemical sensitivity because of loss of checkpoint function.[16] Anticancer drugs generally inhibit growth by blocking the cells at various phases of the cell cycle, depending on the drugs and the cell type. For example, resveratrol caused S phase cell cycle arrest in MCF-7, whereas MDA-MB-231 did not show any cell cycle arrest at S phase.[17] In contrast, **2** showed remarkable effect on cell cycle arrest at the G2 phase for both

MDA-MB-231 and MCF-7; we did not observe any significant block in other phases, although other cell lines may be differently affected. In particular, we have observed increased levels of p53; this has also been reported for several other anticancer drugs,[18] and points to DNA damage as the likely drug action.

Several of the compounds were limited by their solubility, although an IC_{50} value above 10 μM is not too desirable. Nevertheless, it is interesting to note that with most of the cell lines, the cytotoxicities of **2**, **3**, **4**, and **6** are similar throughout the concentration ranges tested (Supporting Information figure S2). These compounds all have labile ligands. The exception is **5** which, although it possessed ligands as labile as those in **2**, showed lower cytotoxicity. A possible reason may be that **5** is too unstable to survive in any useful form in the medium or under physiological conditions. This is alluded to by the uptake study, which shows that the intracellular accumulation of **2** is much higher than of **5**; drug uptake has been demonstrated to be highly correlated to its efficacy.[19] However, the fact that the cytotoxicity of metal-based drugs is determined by the ligand characteristics is perhaps not too surprising. For example, modifications of the arene ligand on ruthenium anticancer drugs has been shown to affect its efficacy.[20] However, the possibility of the ligand (CH_3CN) being directly responsible for the observed cytotoxicity has been ruled out (Figure 7 and Supporting Information figure S2).

We are currently carrying out further investigations towards the development of **2** and similar derivatives for their potential use in cancer treatments. This includes in vivo studies as well as investigations into the molecular mechanism involved. Our findings for the latter will be reported separately.

Conclusions

We have screened six osmium carbonyl clusters against five cancer cell lines and a normal cell line for anticancer activity. Two of the compounds showed good activity against four of the cell lines, including one metastatic cell line, and did not affect normal cells. We have also shown by a number of assays that the mode of action is the induction of apoptosis. This thus represents the first report of osmium carbonyl clusters as a new class of apoptosis-inducing agents, with anticancer therapeutic potential.

Experimental Section

All manipulations for chemical synthesis were carried out using standard Schlenk techniques under an argon or nitrogen atmosphere.[21] The triosmium carbonyl clusters $[Os_3(CO)_{10}(NCCH_3)_2]$ **2**,[22] $[Os_3(CO)_{11}(NCCH_3)]$ **3**,[22] $[Os_3(CO)_{10}(\mu\text{-}H)_2]$ **4**,[23] $[Os_3(CO)_{10}(C_8H_{14})_2]$ **5**,[24] and $[Os_3(CO)_{10}(\mu\text{-}H)(\mu\text{-}OH)]$ **6**,[25] were prepared according to reported procedures. $[Os_3(CO)_{12}]$ **1**, was purchased from Oxkem; all other chemicals were purchased from other commercial sources and used as supplied.

Cell culture and drug treatment: Experimental cultures of the cell lines MDA-MB-231, MCF7, HT29, SW620, and Hep G2 were obtained from the American Type Culture Collection (ATCC) and cultured in tissue culture dishes (Nunc Inc., Naperville, IL, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamate (GIBCO Laboratories), and 1% penicillin/streptomycin (GIBCO Laboratories) at 37 °C in 5% CO_2 atmosphere. Cell cultures were maintained in an antibiotic-free condition during cell growth and experiments. Phosphate-buffered saline (PBS) was obtained from 1st BASE.

The triosmium carbonyl clusters, cisplatin, tamoxifen, resveratrol, and caspase inhibitor Z-VAD-FMK (Calbiochem; La Jolla, CA, USA) were dissolved in dimethyl sulfoxide (DMSO) with final concentration used for treatment being 0.1%. For treatment with the triosmium carbonyl clusters, cisplatin, tamoxifen, and resveratrol, cells were seeded in growth medium at the same initial density, and allowed to adhere and grow for 24 h before treatment (80% confluence). They were washed once with serum-free DMEM and then serum-starved for 6 h before treatment with the indicated concentrations of compounds in DMEM. Control cells were treated with vehicle (0.1% DMSO).

Proliferation assay: Cells were plated in wells within a 96-well plate and allowed to adhere for approximately 4 h. To each well, 20% of Cell Titer 96 Aq_{ueous} One Cell Proliferation Assay (Promega) was added and then left to incubate in a 37 °C incubator with 5% CO_2 over 2 h. The absorbance intensities at 490 nm were then measured and the cell proliferation relative to the control sample was calculated. Each sample was analysed in triplicates. The IC_{50} value was obtained from plots of percent viability against the dose of the compound added. For the time- and concentration-dependence studies of **2** on the proliferation of the MDA-MB-231 and MCF-7 cell lines, 10000 cells were seeded per well in a 96-well plate and allowed to adhere. They were then treated with the indicated concentrations of **2** (1–60 μM) in DMEM containing 1% serum, and cell viability was determined as above for both 24 h and 48 h incubation.

Determination of intracellular osmium level: Cells (1×10^6) were incubated with the compound solution for 24 h, after which they were washed three times with sterile PBS, harvested by trypsinisation, concentrated by centrifugation at 1200 *g*, and then diluted to 1 mL with sterile PBS. Thereafter, mineralisation of cells was carried out with 65% HNO_3 at 120 °C, and the dried digested material was redissolved in 2% HNO_3. The osmium content was then determined by ICP with a PerkinElmer ELAN 6100.

Morphological analysis: Morphological features of cells were assessed by scoring control and triosmium carbonyl cluster-treated cultures (concentration range 0.1–40 μM) seeded in 6-well flat bottom plates. Cultures were fixed in 70% cold ethanol and then stored overnight at −20 °C. Samples were then examined with an inverted Diavert fluorescence microscope (Leica, Wetzlar, Germany).

Analysis of nuclear staining with DAPI: Cells were seeded in 6-well plates at a density of 2×10^5 cells/well in growth medium and grew for 24 h. They were then treated with the indicated concentration of the compound for 24 h. After treatment, the cells were fixed with 3.7% formalin (RT, 1 h), washed with PBS, and stained with 4,6-diamidino-2-phenylindole (DAPI, 1 mg mL^{-1} in methanol, 5 min at 37 °C), washed, and then examined and photographed using a Leica SP 5 microscope equipped with a UV light filter. Apoptotic cells were defined on the basis of chromatin condensation and nuclear formation (bead-like formation).[26]

Annexin V and PI staining for flow cytometry: The percentage of cells actively undergoing apoptosis was determined using annex-

in V–PE-based immunofluorescence, as described previously.[27] Briefly, cells were plated in 10 cm culture dishes at concentrations determined to yield 80% confluence within 24 h. They were then treated with **2** (10–40 μM). After 24 h of treatment, the cells were harvested and then double-labelled with annexin V–FITC and PI, as described by the manufacturer. The cells were analysed using a FACScan instrument equipped with FACStation running Cell Quest software (Becton Dickinson, San Jose, CA). All experiments were performed in duplicate and yielded similar results.

Cell cycle analysis by flow cytometry: Cells were plated in 6-well flat bottom plates with 80% confluence after 24 h. They were then incubated with **2** for 12 or 24 h, trypsinised, washed twice with cold PBS, resuspended in 70% ethanol, and then incubated at RT for 30 min. After this, they were spun down at 2000 rpm (400 *g*) for 5 min, washed once, and resuspended again with 1×PBS containing 1% FBS (500 μL). This was then incubated (37 °C for 15 min) after the addition of 1×RNase (20 μL), after which 1×propidium iodide (PI) (50 μL) was added, and the sample analysed by flow cytometry within 1 h.

Cellular protein extraction: Harvested cells were washed with cold 1×PBS (500 μL) and immediately placed in a −20 °C bath for 5 min to allow rapid cooling. The cells were then scraped in the presence of 200 μL (per 3×10^6 cells) of lysis buffer (10 mM HEPES, pH 7.9) (Sigma), 10 mM KCl (Merck), 0.1 mM EDTA, 10% NP40 supplemented with 1×protease inhibitor (Pierce Biotechnology), and phosphatase inhibitors: 50 μM okadaic acid (Sigma) and 200 mM sodium vanadate (Sigma). The mixture was vortexed for 1 min every half hour over a period of 2 h, and was kept on ice at all times. The lysate was pre-cleared by centrifugation at 13000 *g* at 4 °C, and subsequently stored at −20 °C until evaluation by SDS-PAGE. The supernatant was collected and protein concentration was determined by the method of Bradford using a protein assay kit (BioRad).[28]

SDS gel electrophoresis and Western immunoblot analysis: Equal amounts of protein of each sample were subjected to 8% and 15% SDS-PAGE electrophoresis and then electrophoretically transferred to nitrocellulose membranes (Bio-Rad Laboratories) using a wet transfer apparatus (Bio-Rad Laboratories). Precision Plus Protein Dual Color Standards (Bio-Rad Laboratories) were used as a molecular mass standard. After transfer, the membranes were washes in distilled water to remove traces of transfer buffer, and then air dried for a few hours prior to blotting. Membranes were blotted with 1×Tris -buffered saline with Tween-20 (TBST) containing 5% (*w/v*) nonfat milk (10 mL) overnight at 4 °C. The membranes were then washed with TBST before overnight exposure to the appropriate primary antibodies at 4 °C. All primary antibodies (cleaved PARP, β-tubulin, β-actin, and p53) were used at a final concentration of 1 $\mu g\,mL^{-1}$ with overnight exposure at 4 °C. All blots were incubated with mouse antibodies β-actin to confirm equal protein loading. Washes with TBST were carried out again before incubation (RT, 1 h) with the appropriate horseradish peroxidase-labelled secondary antibodies. After secondary body incubation, membranes were washed again with TBST. The antibody-reactive bands were revealed by chemiluminescence-based detection using West Pico Substrate (Pierce).

Supporting Information: Solution IR spectra of compounds **1**–**6**, table of osmium content in cells after incubation with the compounds as determined by ICP, graphs of cell viability versus concentration for **1**–**6** on all the cell lines tested, optical images of the cells after incubation with the compounds, annexin V–FITC and PI staining for MCF-7 incubated with **2**, cellular DNA content analysis of MDA-MB-231 and MCF-7 cells treated with **2**, and optical images of cells after treatment with Z-VAD-FMK followed by **2**.

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Keywords: antitumor agents · apoptosis · cancer · cluster compounds · osmium

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