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Induction of apoptosis by hexaosmium carbonyl clusters ☆

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ABSTRACT

A number of tri- and hexaosmium carbonyl cluster derivatives were screened for cytotoxicity against five cancer cell lines, and the hexaosmium carbonyl clusters $Os_6(CO)_{18}$ and $Os_6(CO)_{16}(NCCH_3)_2$ were found to be active against four of these, *viz*., ER+ breast carcinoma (MCF-7), ER-breast carcinoma (MDA-MB-231), metastatic colorectal adenocarcinoma (SW620) and hepatocarcinoma (Hepg2), with IC_{50} values as low as 6 μM. Studies on their mode of action with the MDA-MB-231 cell line pointed to the induction of apoptosis, as has been found earlier for the trinuclear cluster $Os_3(CO)_{10}(NCCH_3)_2$.

1. Introduction

The continuing search for anticancer drugs have led recently to the development of organometallic compounds as potential candidates [1]. These may comprise organometallic modifications of organic compounds known to show therapeutic action, such as ferrocifen [2], and NAMI-A [3], or those which show metal-based anticancer activity in themselves, such as the titanocenes [4], and organometallic clusters [5]. In a recent paper, we reported the screening of six triosmium clusters against five cancer cell lines and one cluster in particular, $Os_3(CO)_{10}(NCCH_3)_2$ (**1a**), was found to be active against four of these, *viz*., ER+ breast carcinoma (MCF-7), ER-breast carcinoma (MDA-MB-231), metastatic colorectal adenocarcinoma (SW620) and hepatocarcinoma (Hepg2) [6]. We have made an initial attempt to study the structure-activity relationship by screening a larger range of osmium clusters, in the course of which we discovered two higher nuclearity osmium clusters which show anticancer activity; the study is reported here.

2. Results and discussion

We have earlier found that besides **1a**, the triosmium clusters $Os_3(CO)_{10}(\mu\text{-}H)_2$ and $Os_3(CO)_{10}(\mu\text{-}OH)(\mu\text{-}H)$ also exhibited cytotoxicity. In contrast, the clusters $Os_3(CO)_{11}(NCMe)$ (**2a**), $Os_3(CO)_{12}$ (**3**), and $Os_3(CO)_{10}(cyclooctene)_2$ exhibited no discernible cytotoxicity up to their maximum soluble concentrations. This indicated that perhaps the molecular requirement for cytotoxicity is the accessibility of two coordination sites on the cluster. To test this hypothesis, we decided to carry out MTS assays on the disubstituted triosmium cluster derivatives $Os_3(CO)_{10}(L)_2$ (**1**, where L = PPh_3 (**b**), $AsPh_3$ (**c**), $SbPh_3$ (**d**), PCy_3 (**e**), $P(OMe)_3$ (**f**) or ^tBuNC (**g**)), the monosubstituted triosmium cluster derivatives $Os_3(CO)_{11}(L)$ (**2**, where L = PPh_3 (**b**), $AsPh_3$ (**c**), or $SbPh_3$ (**d**)), as well as the hexaosmium clusters $Os_6(CO)_{18}$ (**4**) and $Os_6(CO)_{16}(NCMe)_2$ (**5**) (Chart 1).

With the exception of **1a**, **4** and **5**, all the other clusters had maximum solubilities (in DMSO) no higher than 10 μM, and their IC_{50} values clearly exceeded that (Supporting information Table S2, Figs. S1 and S2). The computed growth inhibition (IC_{50}) values for the three active compounds are given in Table 1, and their cytotoxicities are also reflected in the morphological changes of the cells after 24 h incubation with 10 μM solutions (Supporting information Figs. S3–7). A comparison of the cytotoxicities was also made against cisplatin, resveratrol and tamoxifen on both the MCF-7 and MDA-MB-231 cell lines at both 1 and 10 μM concentrations (Fig. 1). The results show that **1a**, **4** and **5** have comparable efficacies, and they are also comparable in efficacy to cisplatin, tamoxifen, and resveratrol. In contrast to **1a**, however, there is no significant difference in the cytotoxicity of **4** and **5** towards cancer vs normal cells. Nevertheless, we sought to confirm that their cytotoxicity is due to the induction of apoptosis with several assays using the MDA-MB-231 cell line.

MDA-MB-231 cells treated with 10 μM solutions of **1a**, **4** and **5** showed significant changes in chromatin distribution (separate globular structures) compared to the control (rounded, intact nuclei) when analysed under fluorescence microscopy with DAPI staining (Fig. 2). This change is consistent with the induction of apoptosis. Flow cytometry using FITC-conjugated annexin V and

☆ In celebration of Gerard Jaouen's 65th birthday.

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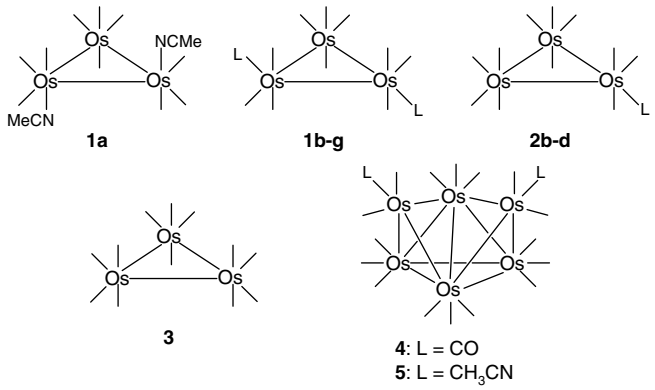

Chart 1.

Table 1
Inhibition of cell growth (IC_{50}, μM) by **1a**, **4** and **5** measured with the six cell lines after 24 h incubation, determined by MTS assay

Compound	MDA-MB-231	MCF-7	HT29	SW620	Hepg2	MCF-10A
1a	4.8 ± 0.7	7.2 ± 1.2	31.3 ± 1.6	8.7 ± 1.0	8.8 ± 0.8	18 ± 2
4	6.0 ± 0.7	6.8 ± 1.5	15.0 ± 1.8	8.2 ± 1.2	6.6 ± 1.2	8.6 ± 1.5
5	6.8 ± 0.9	9.0 ± 1.0	>40	9.8 ± 0.6	27.7 ± 1.5	10 ± 2

propidium iodide (PI) staining (24 h incubation with 10 μM solutions) showed that **1a**, **4** and **5** increased the early apoptosis cell population (FITC stained), reaching ~40–60%, whereas **3** showed a similar result to control (Fig. 3).

Flow cytometric analysis for DNA fragmentation under the same conditions also exhibit cell cycle arrest at the G2 phase; ~35–45% compared to 24.4% and 22.3% for **3** and control, respectively; we did not observe any significant block in other phases (Supporting information Fig. S8). This is in contrast to resveratrol, for example, which is known to show S phase cell cycle arrest in MCF-7 but not in MDA-MB-231 [7]. Finally, proteolytic cleavage of the 116 kDa PARP to an 89 kDa fragment [8], was determined by a Western blot analysis on cells treated with varying concentrations of **1a** and **5** (Fig. 4). The study showed a concentration-dependent increase in the amount of the 89 kDa fragment.

The above assays all point to the induction of apoptosis by **4** and **5**, just as has been observed earlier for **1a** [6]. That **4** showed similar activity to **1a**, in contrast to the low activity shown by **3**, may be attributed to the difference in their substitutional lability. For example, the conversion of **4** to **5** via trimethylamine N-oxide activated substitution can be carried out at low temperatures [9], while a similar conversion of **3** to **1a** required refluxing dichloromethane [10].

3. Conclusion

In this study, we set out to test the hypothesis that the molecular requirement for the cytotoxicity exhibited by triosmium clusters such as $Os_3(CO)_{10}(NCCH_3)_2$ is the accessibility of two coordination sites on the cluster. The study was limited by the low solubilities of the compounds but the data does suggest that labile ligands are required. More significantly, we have uncovered two hexaosmium clusters $Os_6(CO)_{18}$ and $Os_6(CO)_{16}(NCCH_3)_2$, which exhibit good anticancer potential. These clusters show high cytotoxicity for four of the five types of cancer cells tested, and it has been demonstrated that the cytotoxicity is the result of induction of apoptosis. The contrast in cytotoxicity between $Os_6(CO)_{18}$ and $Os_3(CO)_{12}$ suggests that substitutional lability is an important factor. Interestingly, however, the hexaosmium clusters show comparable cytotoxicity towards normal cells. The underlying reason for this difference in behaviour from the triosmium cluster is not understood and awaits studies into their mode of action.

4. Experimental

4.1. General procedure

All manipulations for chemical synthesis were carried out using standard Schlenk techniques under an argon or nitrogen atmosphere [11]. The osmium carbonyl clusters $Os_3(CO)_{10}(L)_2$ (**1**, L = $NCCH_3$ (**a**) [10], PPh_3 (**b**) [12], $AsPh_3$ (**c**) [13], $P(OMe)_3$ (**f**) [14], and ^tBuNC (**g**) [15]), $Os_3(CO)_{11}L$ (**2**, L = PPh_3 (**b**) [16], $AsPh_3$ (**c**) [17], and $SbPh_3$ (**d**) [18]) and $Os_6(CO)_{16}(L)_2$ (L = CO (**4**) [19], and $NCCH_3$ (**5**) [9]) were prepared according to reported procedures. **1d** and **1e** were prepared from the reaction of $SbPh_3$ and PCy_3, respectively, with **1a** [10]. $Os_3(CO)_{12}$, **3**, was purchased from Oxkem; all other chemicals were purchased from other commercial sources and used as supplied.

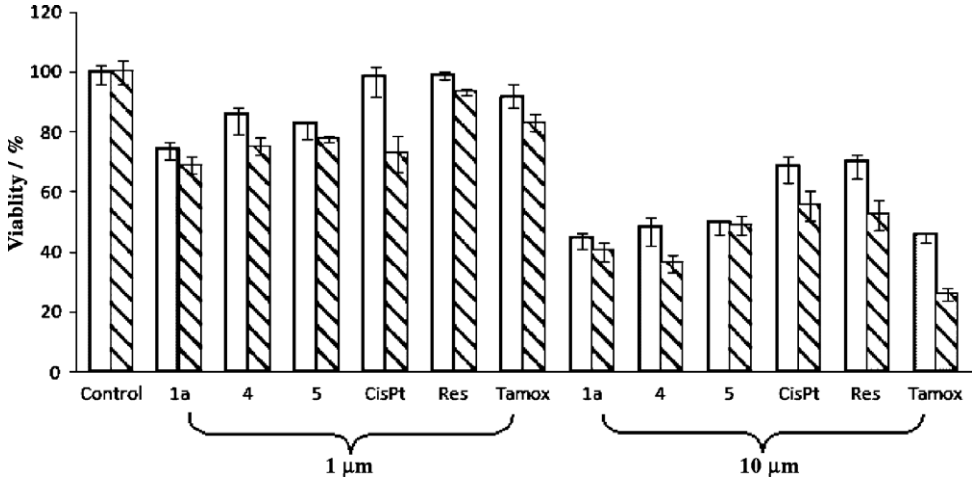

Fig. 1. Comparison of cell viability of MCF-7 (shaded) and MDA-MB-231 (unshaded) after 24 h incubation with 1 and 10 μM concentrations of **1a**, **4**, **5**, cisplatin (CisPt), resveratrol (Res) and tamoxifen (Tamox).

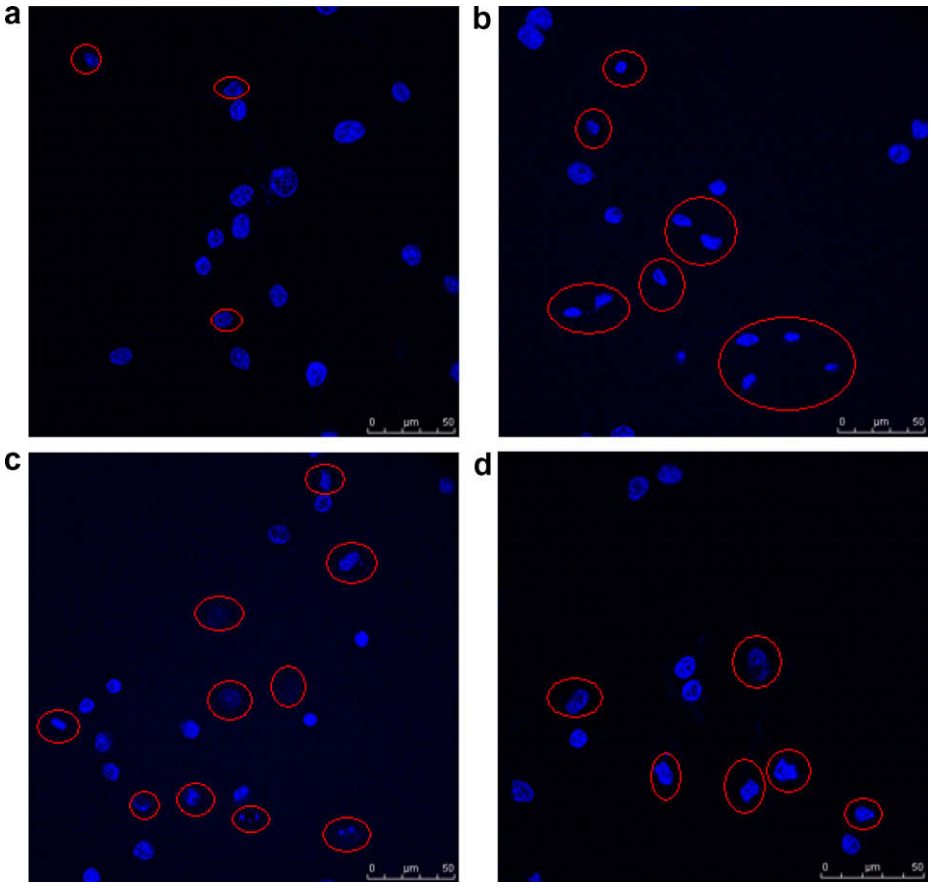

Fig. 2. Fluorescence image (100×) of MDA-MB-231 cells stained with DAPI: (a) control, and after 24 h incubation with 10 μM solution of (b) **1a**, (c) **4**, and (d) **5**. Some cells showing abnormal chromatin structures are circled in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4.2. Cell culture and drug treatment

Experimental cultures of the cell lines MDA-MB-231, MCF-7, MCF-10A, HT29, SW620 and Hepg2 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 osmium carbonyl clusters, cisplatin, tamoxifen and resveratrol were dissolved in dimethylsulfoxide (DMSO) with final concentration used for treatment being 0.1%. The 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 then 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).

4.3. Proliferation assay

The cells were treated with the indicated concentrations of osmium carbonyl clusters in serum-free DMEM condition and incubated for 24 h, after which, 20% of Cell Titer 96® Aq_{ueous} One Cell Proliferation Assay (Promega) was added to each well. This was then left to incubate in a 37 °C incubator with 5% CO_2 over 2 h. The absorbance at 490 nm were then measured and the cell proliferation relative to the control sample was calculated. Each sample was analyzed in triplicates. The IC_{50} are absolute values obtained from plots of percent viability against the logarithm of the dose of compound added, and fitted with a sigmoidal model.

4.4. Morphological analysis

Morphological features of cells were assessed by scoring control and treated cultures (24 h incubation with 10 μM solutions of compounds) 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 to identify cells with an inverted Diavert fluorescence microscope (Leica, Wetzlar, Germany).

4.5. Analysis of nuclear staining with DAPI

Nuclear fragmentation of cells was assessed by scoring control and 24 h incubation with 10 μM of **1a**, **4** and **5** treated cultures on 6-well flat bottom plates. 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) [20].

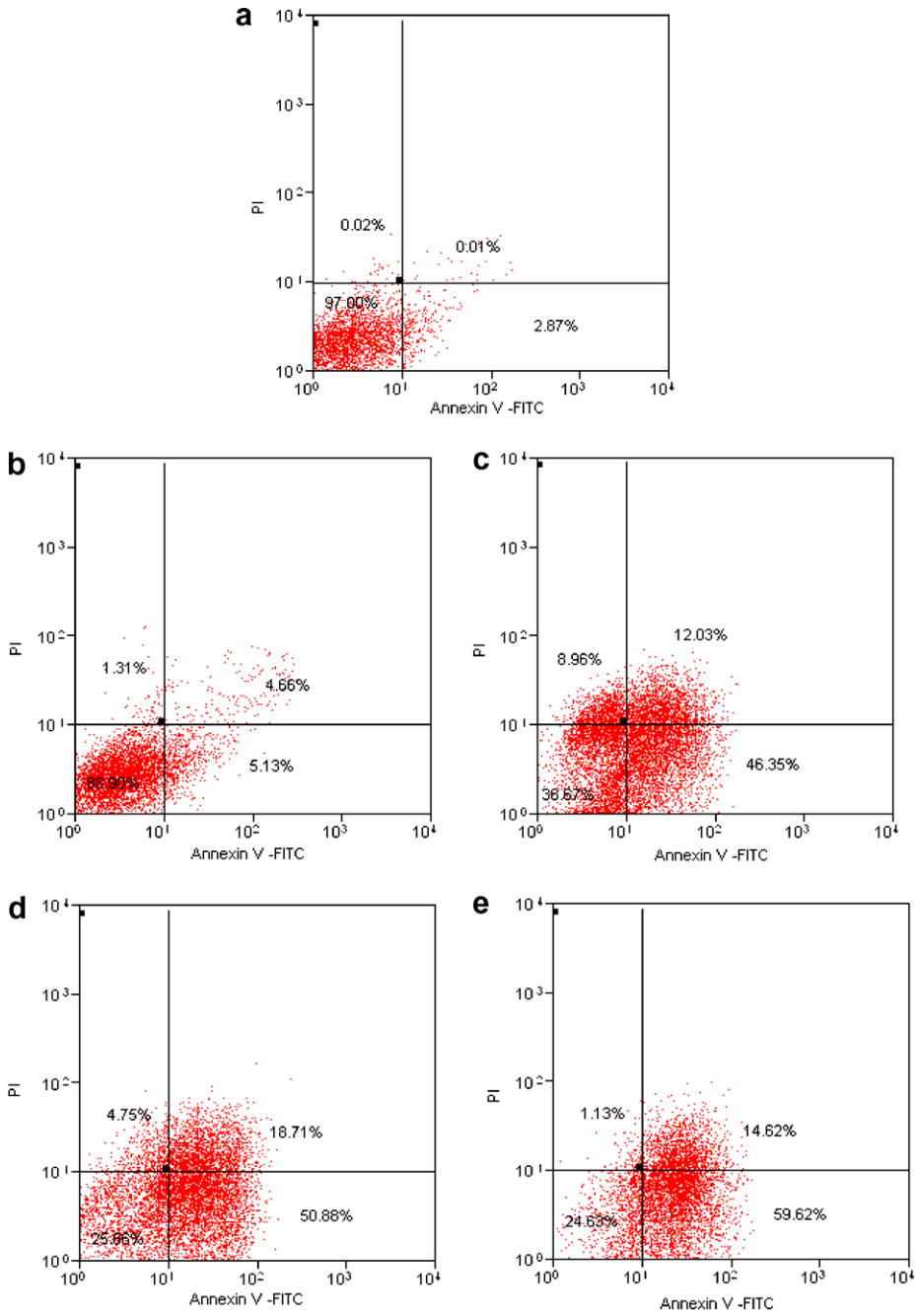

Fig. 3. Detection of early and late apoptotic MDA-MB-231 cells after staining with annexin V-FITC and PI. (a) control, and 24 h incubation with 10 μM solution of (b) **3**, (c) **1a**, (d) **4**, and (e) **5**.

4.6. Annexin V and PI staining for flow cytometry

The percentage of cells actively undergoing apoptosis was determined using annexin V-PE-based immunofluorescence, as described previously [21]. Briefly, MDA-MB-231 cells were plated in 6-well flat bottom plates to yield 80% confluence within 24 h. They were then treated with **1a**, **4**, and **5** (10 μM) and incubated for 24 h, after which the cells were harvested and then double-labelled with annexin V-FITC and PI, as described by the manufacturer (BD Biosciences). The cells were analyzed using a FACScan instrument equipped with FACStation running Cell Quest software (Becton Dickinson, San Jose, CA). All experiments were performed in duplicate.

4.7. Cell cycle by flow cytometry

MDA-MB-231 cells were plated in 6-well flat bottom plates to yield 80% confluence after 24 h. They were then incubated with the compounds (10 μM) for 24 h, trypsinized, washed twice with cold PBS, resuspended in 70% ethanol, and 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 samples analyzed by flow cytometry within 1 h.

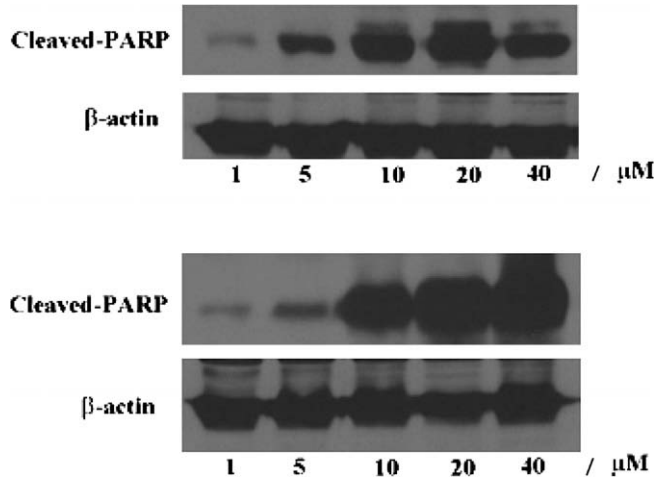

Fig. 4. Gel electrophoretogram of MDA-MB-231 cells treated for 24 h with various concentrations of (a) **1a** and (b) **5**.

4.8. 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 an hour over a period of 2 h, and was kept on ice at all times. The lysate was pre-cleared by centrifugation at 13 000*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 (Bio-Rad) [22].

4.9. SDS gel electrophoresis and Western immunoblot analysis

Equal amounts of proteins 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) non fat 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 and β-actin) were used at a final concentration of 1 μg mL^{-1}. 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).

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Appendix A. Supplementary material

Compound numbering scheme, table of IC_{50} values for all compounds, graphs of cell viability versus concentration for all compounds on all the cell lines tested, optical images of all the cell lines after incubation with the compounds, and cellular DNA content analysis of MDA-MB-231 cells treated with **1a** and **3–5**. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jorganchem.2008.07.039.

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