

A Breast Cancer Stem Cell-Selective, Mammospheres-Potent Osmium(VI) Nitrido Complex

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S Supporting Information

ABSTRACT: The effect of a newly developed osmium(VI) nitrido complex, **1**, on breast cancer stem cells (CSCs) is reported. The complex displays selective toxicity for HMLER breast cancer cells enriched with CD44-positive, CSC-like cells over the same cells having reduced CSC character. Remarkably, **1** also reduces the proportion of CSCs within a heterogeneous breast cancer cell population and irreversibly inhibits the formation of free-floating mammospheres to an extent similar to that of salinomycin, a natural product that targets CSCs. Detailed mechanistic studies reveal that in breast cancer cells **1** induces DNA damage and endoplasmic reticulum stress, the latter being responsible for the CSC selectivity. The anti-CSC properties of **1** provide a strong impetus for the development of new metal-based compounds to target CSCs and to treat chemotherapy-resistant and relapsed tumors.

Cancer relapse is strongly linked to the existence of cancer stem cells (CSCs), a small sub-population of tumor cells that have the ability to self-renew, differentiate, and form secondary or tertiary tumors.¹ Conventional chemotherapy and radiotherapy are ineffective against CSCs.² Although current therapies effectively reduce tumor mass by destroying the bulk of cancer cells, they cannot remove CSCs, which persist and generate new tumors, often of a far more aggressive nature. To improve clinical outcomes, treatments must have the ability to kill the entirety of cancer cells, including CSCs. Compounds capable of selectively killing CSCs and disrupting the micro-environments supporting these cells are currently the subject of intense research.³ Several potential CSC therapeutic targets have been identified, such as cell surface markers⁴ and various deregulated signaling pathways,⁵ but there is still no clinically approved drug that specifically targets CSCs. A recent high-throughput screen of ~16,000 compounds, including commercial libraries and collections of natural extracts, found only four members—salinomycin, abamectin, etoposide, and nigericin—to exhibit prominent CSC specificity.⁶ Thus, there is an urgent need to discover new, selective compounds to add to this limited arsenal of anti-CSC agents.

The quest for new CSC-targeting compounds has been severely hampered by the inability to obtain and sustain CSC-rich cell cultures.⁷ A recent study has shown, however, that enriched populations of CSCs can be achieved by modifying

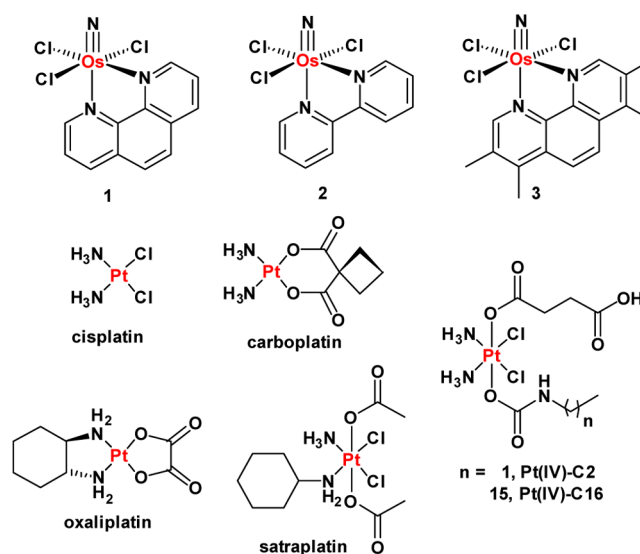


Figure 1. Chemical structures of the platinum(II and IV) and the osmium(VI) nitrido complexes (**1–3**) under investigation.

HMLER breast cancer cells through short hairpin RNA (shRNA)-mediated inhibition of the CDH1 gene, which encodes E-cadherin.⁸ Another study by the same group demonstrated that CSC-enriched cultures could be generated by treating HMLER cells with non-CSC-specific compounds, such as paclitaxel and staurosporine.⁶ The latter approach relies on the ability of the non-CSC-specific agents to kill bulk cancer cells, leaving stem-like cells untouched. Here we sought to use these tools to investigate the CSC-targeting ability of metal-based compounds, including the newly developed osmium(VI) nitrido series, **1–3** (Figure 1). We recently reported their synthesis and anti-proliferative properties.⁹ Encouragingly, **1–3** display selective toxicity for cancer cells over healthy cells and no cross-resistance with cisplatin, a clinically administered anticancer drug. In the current study we examine the anti-CSC activity of **1–3** as well as some well-established Pt(II)- and Pt(IV)-based antineoplastic agents, also illustrated in Figure 1. The Os and Pt compounds were prepared using previously reported methods.^{9,10} Prior to carrying out cellular studies, the stability of **1**, taken as a representative member of the osmium(VI) nitrido series, in

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MEGM cell culture media was established by UV–vis spectroscopy (Figure S1).

Experimentally transformed HMLER breast cancer cells¹¹ were used to assess the CSC specificity of the metal complexes. Under standard cell culture conditions, HMLER cells contain an inherent CSC population of 5–8%. HMLER CSCs overexpress CD44,^{4a} a cell surface glycoprotein involved in cell signaling, adhesion, and migration,¹² and thus are defined by a CD44^{high} marker profile. Following reported protocols, CSC-enriched HMLER cells were generated by paclitaxel treatment (10 nM for 4 days; Figure S2).⁶ This strategy enabled access to CSC-enriched (>30%), CD44^{high} HMLER cells (hereafter referred to as HMLER^{tax} cells).

The anti-proliferative properties of **1**–**3** against HMLER and HMLER^{tax} cells were assessed using the MTT assay. Identical studies were also performed with compounds known to have CSC-selective potency, such as salinomycin and abamectin. FDA-approved Pt(II) anticancer drugs cisplatin, carboplatin, and oxaliplatin,¹³ and Pt(IV) pro-drugs such as satraplatin¹⁴ and the recently developed fatty-acid mimics Pt(IV)-C2 and Pt(IV)-C16,^{10d} were also investigated. The IC₅₀ values (concentration required to induce 50% inhibition) were derived from dose–response curves (Figures 2A and S3–S12) and are summarized in Table 1. Osmium complexes **1**–**3** displayed micromolar toxicity toward both cell lines. Moreover, the 1,10-phenanthroline-bearing complex **1** exhibited selective toxicity for HMLER^{tax} cells over HMLER cells (2.3-fold). Salinomycin and abamectin also killed HMLER^{tax} cells preferentially over HMLER cells (8.3- and 2.1-fold, respectively). None of the Pt-based agents showed CSC selectivity. In fact, oxaliplatin, satraplatin, and Pt(IV)-C16 exhibited 2–4.5-fold greater toxicity for HMLER cells over HMLER^{tax} cells (Figures S9, S10, and S12). This result is consistent with the tendency of Pt compounds to induce CSC enrichment rather than CSC depletion (vide infra). Overall, the anti-proliferative data suggest that **1** can selectively reduce the viability of CSC-enriched HMLER^{tax} cells over CSC-depleted HMLER cells, in the same order of magnitude as salinomycin and abamectin, two of the most selective CSC-targeting compounds identified to date. Although salinomycin displays better selectivity for CSCs than **1**, **1** exhibits a larger toxicity differential (the concentration difference between the IC₅₀ values for HMLER and HMLER^{tax} cells). These properties are highly desirable for selecting CSC drug candidates in preclinical studies.

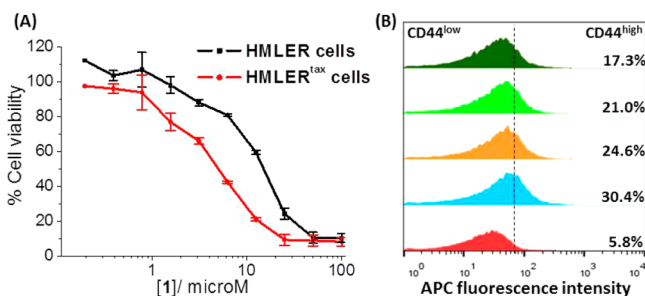


Figure 2. (A) Average dose–response curves for the treatment of HMLER and HMLER^{tax} cells with **1** ($n = 30$ for each point). (B) Representative histograms displaying the red fluorescence emitted by anti-CD44 APC antibody-stained HMLER cells (red), HMLER^{tax} cells (blue), and HMLER^{tax} cells treated with **1** (5 μ M, orange; 10 μ M, light green; 20 μ M, dark green) for 4 days followed by 4 days recovery in compound-free MEGM media.

Table 1. IC₅₀ Values of Tested Compounds against HMLER and HMLER^{tax} Cells

compound	HMLER IC ₅₀ (μ M)	HMLER ^{tax} IC ₅₀ (μ M)	selectivity for HMLER ^{tax} ^a
1	11.20 \pm 0.48	4.91 \pm 0.86	2.31
2	14.58 \pm 0.20	16.06 \pm 4.12	0.91
3	82.80 \pm 18.43	53.99 \pm 2.45	1.53
salinomycin	0.49 \pm 0.26	0.058 \pm 0.01	8.45
abamectin	1.45 \pm 0.18	0.64 \pm 0.06	2.26
cisplatin	1.95 \pm 0.40	2.06 \pm 0.67	0.95
carboplatin	17.84 \pm 0.58	18.19 \pm 0.80	0.98
oxaliplatin	15.04 \pm 0.41	26.95 \pm 4.42	0.55
satraplatin	1.22 \pm 0.06	2.87 \pm 0.23	0.43
Pt(IV)-C2	39.09 \pm 9.82	40.64 \pm 9.91	0.96
Pt(IV)-C16	0.0254 \pm 0.0016	0.1131 \pm 0.0197	0.22

^aSelectivity = IC₅₀ for HMLER/IC₅₀ for HMLER^{tax}. The values reported are an average of five independent determinations.

To determine the effect of the metal complexes on the heterogeneity of breast cancer cells, flow cytometric studies were carried out. Upon treatment of HMLER^{tax} cells with **1** (5–20 μ M for 4 days), a dose-dependent decrease in the proportion of CD44^{high} cells was observed, indicative of CSC-specific toxicity (Figure 2B). A slight decrease in the CD44^{high} population was also observed upon incubation of HMLER cells with increasing concentrations of **1** (5–40 μ M for 4 days; Figure S13). Taken together, the results demonstrate that **1** can selectively kill CD44^{high} CSC-like cells over bulk cancer cells. HMLER^{tax} cells treated with clinically approved Pt(II) anticancer drugs¹³ cisplatin (1.5 μ M for 4 days), carboplatin (15 μ M for 4 days), and oxaliplatin (15 μ M for 4 days) displayed little change in the fraction of cells with CD44^{high} character compared to the untreated control (Figure S14). Moreover, upon treatment of HMLER cells with the Pt drugs under the same conditions described above, a marked increase in the relative CD44^{high} population was detected (3.2–5.4-fold), consistent with CSC enrichment (Figure S15). These findings highlight the partiality of conventional Pt(II)-based anticancer drugs to kill bulk cancer cells over CSCs. In the same way as the Pt(II) compounds, satraplatin treatment (1.5 μ M for 4 days) did not alter the CSC proportion in HMLER^{tax} cells and propagated CSC enrichment in HMLER cells (Figures S14 and S15), consistent with non-CSC specificity.

Owing to their unlimited self-renewal ability, breast CSCs have the tendency to form de novo tumor-like structures called mammospheres in non-adherent, serum-free cell cultures.¹⁵ The tumor sphere formation assay was used to assess the ability of **1**, salinomycin, paclitaxel, cisplatin, carboplatin, oxaliplatin, and satraplatin (at their respective IC₃₀ values) to inhibit mammosphere formation from HMLER single-cell suspensions. To obtain 3D images of the mammospheres and determine the proportion of CD44-positive cells within a given mammosphere, the cells were stained with Hoechst 33258 dye (7.5 μ M for 30 min) and allophycocyanin (APC)-labeled anti-CD44 antibody (15 μ L, 1:133 dilution for 45 min), respectively, and imaged using a fluorescence microscope (Figure S16). The tumorsphere formation assay showed that treatment with **1** induced a 38% decrease in the number of mammospheres formed relative to the untreated control, providing strong evidence for the inhibition of CSC self-renewal (Figure 3). The microscopy studies revealed that the size of the spheroids decreased by up to 2.4-fold in the presence of **1** (Figure 3). Furthermore, upon incubation with **1**,

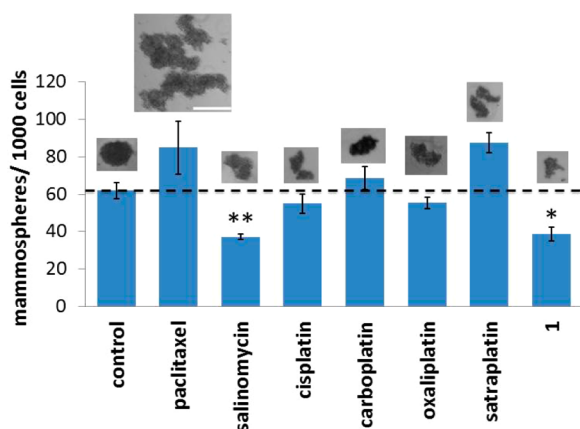


Figure 3. Quantification of mammosphere formation with HMLER cells untreated and treated with the investigated compounds at their respective IC_{30} values for 5 days. Representative bright-field images ($\times 4$) of the mammospheres formed under each condition are presented to scale. Scale bar = 0.3 mm. Student t test, $p < 0.05$ or $p < 0.01$. Error bars represent standard deviations.

the proportion of CD44-positive cells within a given mammosphere was markedly diminished, indicative of CSC-specific toxicity (Figure S16). Collectively, these data show that **1** inhibits the clonogenic growth of HMLER mammospheres by eliminating CD44-positive, CSC-like cells. As expected, a reduction in both mammosphere number and size (up to 1.4-fold) was observed for salinomycin treatment (positive control, Figure 3). In contrast, paclitaxel, carboplatin, and satraplatin treatment led to an increase in the number of mammospheres formed (10–40%), suggestive of CSC enrichment (Figure 3). This result was corroborated by a 2–4-fold increase in the number of CD44-positive cells within the mammospheres (Figure S16). The number of mammospheres formed was marginally reduced by cisplatin and oxaliplatin treatment, but the size of the spheroids remained largely unaltered (Figure 3).

To determine whether **1** could induce a durable mammosphere inhibitory response, **1**-treated primary mammospheres were dissociated into single-cell suspensions, and their propensity to form secondary mammospheres was assessed (Figure S17). Control studies were also conducted with salinomycin- and paclitaxel-treated primary mammospheres. The secondary mammospheres formed from cells isolated from **1**-treated primary mammospheres were 5-fold fewer than those from the untreated control. This result shows that **1** inhibits the self-renewal of HMLER mammospheres and that this effect is maintained upon serial passage. Cells extracted from salinomycin-treated primary mammospheres displayed non-clonogenic properties, similar to those observed for **1**-treated cells. Single-cell suspensions of paclitaxel-treated primary mammospheres, on the other hand, produced slightly more secondary mammospheres compared to the untreated control.

To account for the CSC-specificity and mammosphere potency observed for **1**, detailed mechanistic studies were conducted. The Os complex **1** was previously characterized via an RNAi signature approach¹⁶ capable of discerning drug mechanism of action.⁹ Although **1** did not resemble any category of drug mechanism present in the reference set, the pleiotropic mechanism of **1**-induced cell death appeared to involve DNA damage. Given the nature of other Os compounds in eliciting endoplasmic reticulum (ER) stress,⁹ we added known ER stress inducers tunicamycin and thapsigargin to the RNAi signature

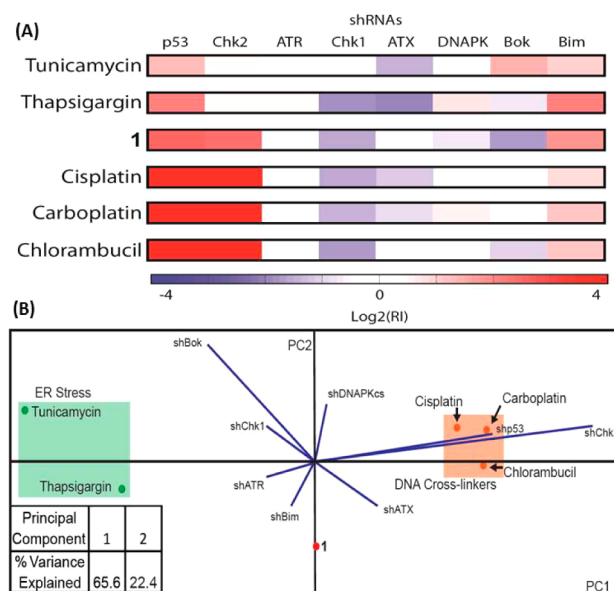


Figure 4. (A) RNAi signatures derived from the treatment of $E\mu$ -Myc^{p19arf-/-} lymphoma cells with **1**, ER stress inducers tunicamycin and thapsigargin, and DNA-cross-linking agents cisplatin, carboplatin, and chlorambucil at the LD_{80-90} concentration for each compound. (B) Principal components analysis plot of the RNAi signatures.

training set (Figure 4A). Upon re-analysis of the RNAi signatures, we found that **1** still did not belong to any training set category. Thus, we next examined the signatures of **1**, ER stress inducers tunicamycin and thapsigargin, and representative DNA-cross-linkers cisplatin, carboplatin, and chlorambucil via principal components analysis (PCA). PCA represents the variance of a multi-dimensional data set in successive principal components where each component represents a larger portion of the data set variance than the next. By plotting the aforementioned compounds via PCA, we found that **1** lies roughly equidistant between the canonical ER stress inducers and DNA-cross-linkers (Figure 4B). This result indicated that **1** can induce cell death via both mechanisms.

Immunoblotting studies were conducted to monitor changes in expression of biomarkers related to the DNA damage and ER stress pathways. HMLER cells incubated with **1** (5–20 μ M for 72 h) displayed a marked increase in the expression of the phosphorylated forms of H2AX and CHK2, indicative of DNA damage (Figure S18).¹⁷ DNA damage is usually accompanied by p53 accumulation and upregulation of downstream effectors related to cell cycle arrest, DNA repair, and apoptosis.¹⁸ Owing to the inactivation of p53 in HMLER cells, however, the expression of p21, a p53 effector, remained unchanged upon treatment with **1** (5–20 μ M for 72 h; Figure S18). The inability of p53 to coordinate cellular response following DNA damage in HMLER cells suggests that **1**-induced DNA damage may not be a major determinant of cell death. HMLER cells dosed with **1** (5–20 μ M for 72 h) exhibited an increase in the expression of proteins related to the unfolded protein response, such as phosphorylated eukaryotic initiation factor 2 α (eIF2 α) and C/EBP homologous protein, suggestive of ER stress (Figure S18).¹⁹ Immunofluorescence studies showed that, upon incubation of HMLER cells with **1** (25 μ M for 24 h), the expression of the phosphorylated RNA-dependent protein kinase-like endoplasmic reticulum kinase (phospho-PERK) increased. This finding provided further evidence of ER stress

(Figure S19).¹⁹ A similar result was also observed for HMLER cells treated with thapsigargin (0.25 μ M for 24 h; Figure S19). HMLER cells treated with **1** (5–20 μ M for 72 h) displayed an increase in the expression of cleaved caspase 3 and 7, and poly-ADP ribose polymerase (Figure S18). Thus, the mode of **1**-induced cell death is most likely to be caspase-dependent apoptosis. Taken together, the immunoblotting and immunofluorescence data reveal that in breast cancer cells **1** initiates both DNA damage and ER stress, culminating in apoptotic cell death.

A recent study identified the vulnerability of HMLER CSC-like cells to agents that can induce ER stress through the PERK-eIF2 α axis.²⁰ In light of this report and our mechanistic data, we propose that the CSC specificity observed for **1** could be attributed to the ability of the complex to induce ER stress. To investigate this hypothesis, the toxicity of **1** against HMLER and HMLER^{tax} cells in the absence and presence of a known ER stress inhibitor, salubrinal,²¹ was determined. Co-administration of HMLER and HMLER^{tax} cells with **1** and salubrinal (10 μ M) significantly reduced the cytotoxicity of **1** in CSC-enriched HMLER^{tax} cells (*t* test, *p* < 0.05) but not in CSC-depleted HMLER cells (*t* test, *p* = 0.30; Figure S20). Thus, **1** induces ER stress-mediated cell death in CSCs more readily than in non-CSCs. Overall, our mechanistic studies show that the CSC specificity observed for **1** likely results from the ability of **1** to induce ER stress via the PERK-eIF2 α pathway and the sensitivity of HMLER CSCs to ER stress inducers.

In summary, we present the anti-CSC properties of **1**. To our knowledge, **1** is the first osmium-based compound to exhibit selective toxicity for breast CSC-enriched cell populations. Encouragingly, the CSC-specific potency of **1** challenges some of the most CSC-selective compounds identified to date. Additionally, **1** inhibits the formation of mammospheres by specifically targeting CD44-positive, CSC-like cells. Given our findings and the urgent medical need for CSC-specific chemotherapies to overcome cancer relapse and metastases formation in the clinic, the anti-CSC properties of **1** are pre-clinically very appealing. Overall, this study highlights the great, largely unexplored potential of metal-based complexes for CSC-directed chemotherapy and provides hints about the mechanism and targets of systemic Os toxicity.

■ ASSOCIATED CONTENT

■ Supporting Information

Experimental techniques and data concerning all biophysical and cellular studies. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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