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Breast Cancer Stem Cell Potent Copper(II)–Non-Steroidal Anti-Inflammatory Drug Complexes

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Abstract: *The breast cancer stem cell (CSC) potency of a series of copper(II)–phenanthroline complexes containing the non-steroidal anti-inflammatory drug (NSAID), indomethacin, is reported. The most effective copper(II) complex in this series,* ***4****, selectivity kills breast CSC-enriched HMLER-shEcad cells over breast CSC-depleted HMLER cells. Furthermore,* ***4*** *reduces the formation, size, and viability of mammospheres, to a greater extent than salinomycin, a potassium ionophore known to selectively inhibit CSCs. Mechanistic studies revealed that the CSC-specificity observed for* ***4*** *arises from its ability to generate intracellular reactive oxygen species (ROS) and inhibit cyclooxygenase-2 (COX-2), an enzyme that is overexpressed in breast CSCs. The former induces DNA damage, activates JNK and p38 pathways, and leads to apoptosis.*

Breast cancer recurrence is strongly linked to the existence of breast cancer stem cells (CSCs), a distinct subpopulation of breast cancer cells that resemble stem cells.[1] Basal-, claudin-low-, and Her2-positive-breast tumor are associated with the lowest life expectancy, and display approximately 3–4-fold larger proportions of breast CSCs compared to well differentiated breast tumors.[2] Breast CSCs also play a prominent role in the metastatic progression of breast cancer.[3] Indeed, clinical studies have found much greater proportions of breast CSCs in metastatic tumors compared to the primary site.[3] Conventional chemotherapy and radiotherapy coupled with surgery effectively reduces primary breast tumor size by removing the bulk of breast cancer cells, however, breast CSCs can survive treatment and regenerate secondary tumors with higher metastatic propensity.[4] To improve clinical outcomes treatments must have the ability to eradicate the entire population of breast cancer cells, including CSCs. Although progress has been made in the identification of potential breast CSC therapeutic targets, such as deregulated signaling pathways, certain organelles, cell surface markers, and vulnerable microenvironments,[5] there are still no clinically approved drugs that effectively target breast CSCs. Currently, there are over 60 ongoing or planned clinical trials involving CSC-specific chemical or biological agents.[6] The current batch of small molecules undergoing investigation are typically organic in nature, and target one specific CSC feature.[6] Here, we have sought to shift away from this model by developing copper(II)-containing agents that are able to act across multiple pathways.

Copper is an essential element for many oxygen-dependent organisms, and plays an important catalytic and structural role in many biological pathways.[7] In the last decade, hundreds of copper(I, II) coordination compounds have been prepared and investigated for their anticancer properties.[8] Many of these copper complexes induce their therapeutic effect by cleaving DNA through an oxidative mechanism.[8] Despite the large body of work conducted on copper antineoplastic agents, there is only one active clinical trial (phase I) involving copper for the treatment of cancer, namely the co-administration of disulfiram and copper gluconate for refractory solid malignancies.[9] This treatment regimen relies on the ability of copper, once coordinated to disulfiram, to generate lethal levels of reactive oxygen species (ROS) in cancer cells.

Recent studies have found that, in contrast to bulk breast cancer cells, in which ROS levels are relatively high, breast CSCs maintain low levels of ROS.[10] This is thought to protect them from DNA, protein, and lipid damage and contribute to chemo- and radio-resistance. The fact that some breast CSCs have adapted to survive in ROS-deficient conditions implies that their intracellular redox state is finely controlled and balanced. Therefore, ROS elevation by exogenous chemicals, in combination with CSC-targeted therapies, represents a potentially efficacious strategy to eradicate breast CSCs. Herein, we report the anti-breast CSC properties of a series of copper(II) complexes capable of generating intracellular ROS and delivering indomethacin, a nonsteroidal anti-inflammatory drug (NSAID) that was recently shown to suppress colorectral CSCs.[11] Indomethacin is a potent inhibitor of the cyclooxygenase isoenzymes, COX-1 and COX-2.[12] Cyclooxygenases catalyze the formation of prostaglandins (PG), thromboxanes, and levuloglandins, which are involved in the inflammatory response. The inducible isoform, COX-2 is overexpressed in several mammary carcinomas and linked to breast cancer progression.[13] Evidence suggests that inhibition of this enzyme can attenuate breast tumor dissemination.[14]

Several metal–NSAID complexes have been reported,[15] yet none have been evaluated for their anti-CSC properties. A large number of copper(II) complexes bearing NSAIDs[16] including indomethacin have been structurally character-

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ized.[17] The di-nuclear copper(II) complex, $[Cu_2(indomethacin)_4(dimethylformamide)_2]$, is used in veterinarian medicine to treat inflammation.[17] The copper(II) complex exhibits higher activity and reduced gastrointestinal toxicity compared to free indomethacin.[18] Recently, the synthesis of copper(II)–indomethacin complexes with polypyridylligands was reported.[19] These complexes displayed strong antioxidant and DNA-binding properties in cell-free systems. Herein, we present the anti-CSC properties of a series copper(II)–indomethacin complexes with tertiary amine bearing phenanthroline ligands.

The compounds investigated in this study are depicted in Figure 1. New phenanthroline-based ligands, **L3–5** were prepared by reacting 5-chloro-1,10-phenanthroline with KOH and the appropriate amino alcohol in dry DMSO. The copper(II) complexes, **1–5**, were synthesized by reacting $CuCl_2{\cdot}2H_2O$ with the appropriate phenanthroline ligand (**L1–5**) and two equivalents of indomethacin in methanol, under basic conditions. The copper(II) complexes were isolated as green solids and were characterized by mass spectrometry, infra-red spectroscopy, and elemental analysis (Supporting Information).

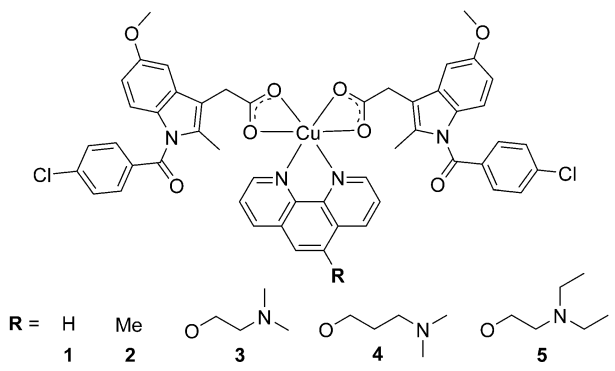

Figure 1. Structures of the copper(II)–nonsteroidal anti-inflammatory drug complexes under investigation.

To investigate the DNA nuclease activity of the copper(II) complexes, **1–5**, gel electrophoresis studies were conducted. Upon incubation of supercoiled plasmid pUC19 DNA (100 ng) with increasing concentrations of **1–5** (0–200 μM for 16 h in the absence of external reducing agents) a clear increase in the amount of nicked circular DNA was observed (Supporting Information, Figures S1, S2), suggestive of DNA cleavage. The tertiary amine-bearing complexes, **3–5**, displayed the highest nuclease activity (complete conversion of supercoiled to nicked circular DNA was observed at 5 μM). DNA cleavage was markedly reduced with $CuCl_2$, dichloro(1,10-phenanthroline)copper(II), and indomethacin (Figure S3). To decipher the oxidative mechanism by which **3–5** induces DNA cleavage, nuclease activity was probed in the presence of ROS scavengers (DMSO, t-BuOH, KI, and NaN_3; Figure S4). KI displayed the highest inhibitory effect for all of the complexes, **3–5**, suggesting that hydrogen peroxide is the major ROS intermediate formed during the DNA cleavage process. We propose that **1–5** undergo reduction to the corresponding copper(I) form by guanine bases in pUC19 DNA. The copper(I) form then reduces molecular oxygen to superoxide, which generates hydrogen peroxide.

The cyclooxygenase (COX-1 and COX-2) inhibitory properties of **3–5**, indomethacin, and aspirin (positive control) were determined using an enzyme immunoassay (EIA). The IC_{50} values, the concentration required to inhibit COX-1- and COX-2-catalyzed conversion of arachidonic acid to PG by 50%, are reported in Table S1. The copper(II) complexes, **3–5**, inhibited COX-1 activity to a lesser degree than indomethacin and aspirin (the fact that **3–5** contain two indomethacin moieties per molecule was taken into account in our comparisons; Figure 2). Interestingly the copper(II) complexes, **3–5**, inhibited COX-2 activity in a concentration-dependent manner, to a similar or better extent than indomethacin and aspirin. Moreover, the data shows that despite coordination of indomethacin to copper in **3–5**, it retains the COX-1 and COX-2 inhibitory properties.

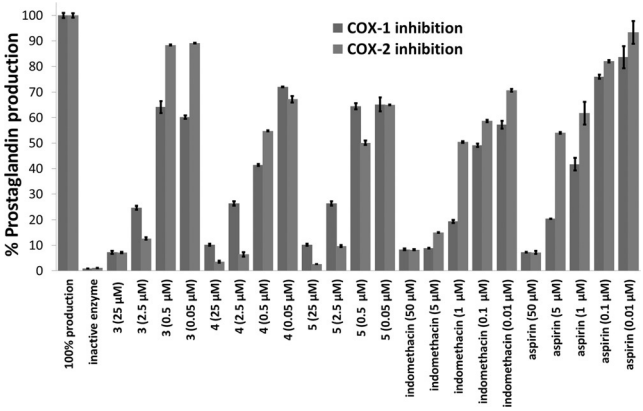

Figure 2. Cyclooxygenase (COX-1 and COX-2) inhibitory properties of **3–5**, indomethacin, and aspirin (positive control).

The lipophilicity of the copper(II) complexes, **1–5**, was determined by measuring the extent to which they partitioned between octanol and water, *P*. The experimentally determined log *P* values for **1–5** varied from 0.37 to 0.94 (Table S2), indicative of hydrophobicity. The hydrophobic character of **1–5** suggests that the complexes will be readily internalized by cells. UV/Vis and high-resolution ESI mass spectroscopy studies were carried out to assess the stability of **4**, taken as a representative member of the copper(II) series. In PBS, **4** is stable over a period of 24 h at 37 °C (Figure S5A), however, in the presence of whole cell lysate (5×10^3 HMLER-Ecad cells), a marked decrease in absorption was observed (Figure S5B). In DMSO, new ion cluster peaks corresponding to $[\mathbf{4} + DMSO + H]^+$ (1140.1880 *m/z*) and $[\mathbf{4} + DMSO + Na]^+$ (1162.1735 *m/z*) appeared in the ESI mass spectrum after 24 h (Figure S5C). This data shows that **4** is able to coordinate to nucleophiles such as DMSO in solution, probably through the displacement of one of the Cu−O(indomethacin) bonds. This could explain the lower stability of **4** in the presence of cell lysates (Figure S5B), which contains several biomolecules that could act as nucleophiles. Overall the results suggest that **4** is reasonably stable under physiological-like conditions.

Before carrying out cellular studies, the stability of **4** in mammary epithelial cell growth medium (MEGM) was established (Figure S5D).

To determine the breast CSC potency and specificity of the copper(II) complexes, two human mammary epithelial cell lines were used; HMLER and HMLER-shEcad cells. HMLER cells exhibit a stable CSC-like population of 5–8%, whereas HMLER-shEcad cells display a significantly larger CSC-like population (approximately 90%).[5a] The anti-proliferative properties of **1**–**5** against HMLER and HMLER-shEcad cells were assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Salinomycin, a breast CSC-specific compound, was used as a positive control.[5a] The anti-proliferative properties of indomethacin and dichloro(1,10-phenanthroline)copper-(II) were also determined. The IC_{50} values, the concentration required to reduce viability by 50%, were determined from dose-response curves (Figures S6–8) and are summarized in Tables 1 and S3.

Table 1: IC_{50} values of the copper(II)–nonsteroidal anti-inflammatory drug complexes, **1**–**5**, dichloro(1,10-phenanthroline)copper(II), and salinomycin against HMLER cells, HMLER-shEcad cells, and HMLER-shEcad mammospheres.

Compound	HMLER IC_{50} [μM][a]	HMLER-shEcad IC_{50} [μM][a]	Mammosphere IC_{50} [μM][b]
1	4.4 ± 0.1	4.3 ± 0.1	n.d.
2	2.5 ± 0.2	2.5 ± 0.1	n.d.
3	7.5 ± 1.4	2.7 ± 0.2	16.6 ± 0.6
4	7.4 ± 0.3	2.2 ± 0.5	13.8 ± 0.8
5	6.9 ± 1.4	4.2 ± 0.6	26.3 ± 1.3
dichloro(1,10-phenanthroline) copper(II)	4.9 ± 0.2	7.9 ± 0.3	n.d.
salinomycin	11.4 ± 0.4	4.2 ± 0.3	40.3 ± 2.2

[a] Determined after 72 h incubation (mean of three independent experiments ± SD). [b] Determined after 96 h incubation (mean of three independent experiments ± SD). n.d. = not determined.

The copper(II) complexes, **1**–**5**, and dichloro(1,10-phenanthroline)copper(II) displayed micromolar toxicity towards both cell lines. Strikingly, **3** and **4** exhibited selective potency for HMLER-shEcad cells over HMLER cells (2.8- and 3.3-fold, respectively). The potency and selectivity observed for **4** towards HMLER-shEcad cells was significantly ($p < 0.05$) better than that of salinomycin. Indomethacin was relatively non-toxic towards both cell lines ($IC_{50} >$ 100 μM), which is somewhat surprising considering its ability to eliminate colorectal CSCs at micromolar concentrations.[11] Overall the cytotoxicity data shows that **3** and **4** are able to selectively kill CSC-enriched HMLER-shEcad cells over CSC-depleted HMLER cells.

Due to their stem cell-like character, breast CSCs have the ability to form multicellular three-dimensional structures called mammospheres in anchorage-independent, serum-free cell cultures.[20] The ability of **3**–**5**, dichloro(1,10-phenanthroline)copper(II), indomethacin, and salinomycin (at their IC_{20} values, 96 h) to inhibit the formation of mammospheres from single cell suspensions of CSC-enriched HMLER-shEcad cells was determined using the mammosphere formation assay. Addition of the copper(II) complexes, **3**–**5**, significantly ($p < 0.05$) reduced the number of mammospheres formed relative to the untreated control (Figure 3A). The most effective complex, **4**, reduced mammosphere formation (45%) to a better extent than salinomycin (36%). Microscopy studies revealed that the size of the mammosphere also decreased upon incubation with **3**–**5** (Figure 3B and S10). Dichloro(1,10-phenanthroline)copper-(II)- and indomethacin-treatment did not affect the number of mammospheres formed, however, the size of the mammospheres was moderately reduced (Figure S9,S10). To assess the ability of **3**–**5** to reduce mammosphere viability, the colorimetric resazurin-based reagent TOX8 was used. TOX8 has been previously established as a reliable reagent for determining tumor spheroid viability.[21] The IC_{50} values (concentration required to reduce mammosphere viability by 50%) of **3**–**5** were in the micromolar range (Figure 3C and

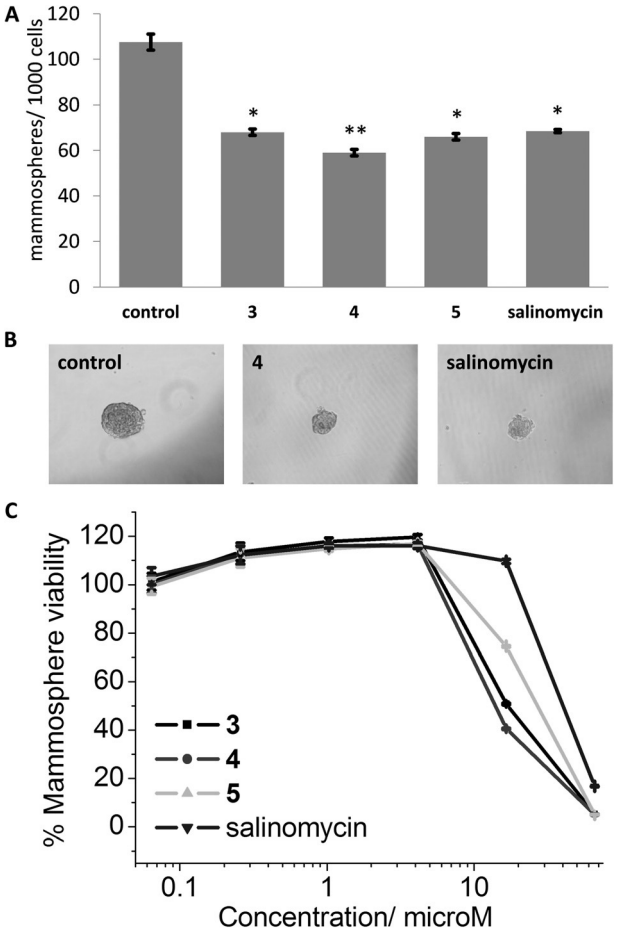

Figure 3. A) Quantification of mammosphere formation with HMLER-shEcad cells untreated and treated with **3**–**5**, and salinomycin at their respective IC_{20} values for 96 h. Error bars = SD and Student *t-test*, ** = $p < 0.01$ and * = $p < 0.05$. B) Representative bright-field images (×10) of the mammospheres in the absence and presence of **4** or salinomycin, at their respective IC_{20} values. C) Representative dose-response curves for the treatment of HMLER-shEcad mammospheres with **3**–**5** and salinomycin. Error bars = SD.

Table 1). Notably, **4** displayed 3-fold better mammosphere-potency than salinomycin. Collectively, these studies show that the copper complexes, in particular **4**, are able to markedly reduce mammosphere formation, size, and viability.

Breast CSCs express high levels of CD44, a type I transmembrane glycoprotein that facilitates cell–cell and cell–matrix interactions through its affinity for hyaluronic acid.[22] Therefore, by monitoring the change in the CD44-positive cell population upon treatment with a given compound, its CSC-specificity can be determined. Flow cytometric studies were carried out to determine the effect of **3**–**5** on the proportion of CSC-like, CD44-positive cells within a heterogeneous population of HMLER-shEcad cells. Upon incubation of HMLER-shEcad cells with **4** (5 μM for 72 h), the fraction of CD44-positive cells decreased markedly (Figure S11). This provides further evidence that **4** selectively kills CSC-like cells over bulk cancer cells. The addition of **3** and **5** (5 μM for 72 h) reduced the proportion of CD44-positive cells to a lesser extent than **4** (Figure S11). As expected, salinomycin-treatment (5 μM for 72 h) also reduced the number of CD44-positive cells (Figure S11).

To shed light on the mechanism of action of the copper(II) complexes, detailed mechanistic studies were carried out. Complexes **3**–**5** were subjected to a predictive functional genetic assay based on RNAi.[23] This approach uses cancer cells that are partially infected with eight distinct short hairpin RNAs (shRNAs). Depending on the survival advantage or disadvantage conferred by a given shRNA, the shRNA-bearing cells will either enrich or deplete relative to the uninfected population upon treatment with the test compound. The pattern of resistance and sensitivity of each shRNA for the test compound is then compared to a reference set of compounds with known mechanisms of action through a K-nearest neighbors based algorithm. This reference set includes all classes of clinically used cytotoxic agents, as well as some targeted therapeutics. If the resulting p-value is below 0.05, then one can confidently conclude that the mechanism of action of the compound of interest is shared by that set of compounds in the reference set. If the p-value is above 0.05, then one can conclude that the mechanism of action of the new compound is unlike any in the reference set. The signatures obtained for **3**–**5** were completely distinct from any of the established mechanisms of action represented in the reference set (Figure 4A). However, when comparing **3**–**5** to our database of previously assayed compounds that are not included in the reference set, dichloro(1,10-phenanthroline)copper(II) ranked in the top 3 for being most similar. For **3**–**5**, the most similar compound was a rhenium(V)–oxo complex previously characterized as a ROS-inducing, necroptotic agent (Figure S12).[24] To compare similarities among **3**–**5** relative to other compounds, the intra- and inter-compound distances were calculated by averaging the sum of the absolute Euclidian distance between the compounds in question (Table S4). As the average intra-compound distance between **3**–**5** is 0.5, and the average distance from the rhenium(V)–oxo complex and dichloro(1,10-phenanthroline)copper(II) is 1.9 and 2.2, respectively, **3**–**5** are most likely to have related, but distinct, mechanisms of action.

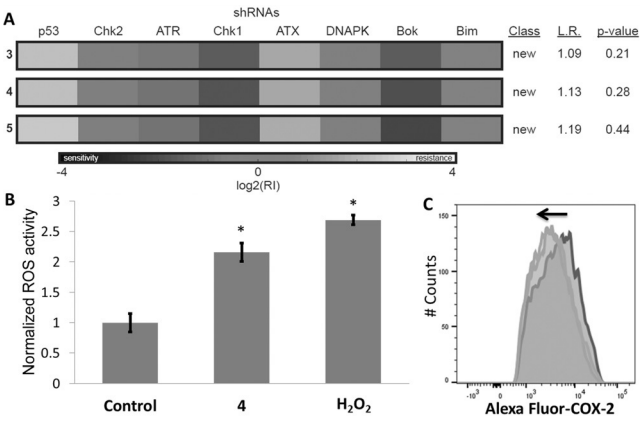

Figure 4. A) RNAi signatures derived from the treatment of Eμ-Myc-$p^{19arf-/-}$ lymphoma cells with **3**–**5** at the LD80-90 concentration for each compound. B) Normalized ROS activity in untreated HMLER-shEcad cells (control) and HMLER-shEcad cells treated with **4** (3 μM for 48 h) and H_2O_2 (6 μM for 48 h). C) Representative histograms displaying the green fluorescence emitted by anti-COX-2 Alexa Fluor 488 nm antibody-stained HMLER-shEcad cells treated with LPS (1 μM) for 24 h (bold black line) followed by 48 h in media containing **4** (5 μM, grey line) and indomethacin (10 μM, light grey line).

A recent study showed that co-administration of copper(II) chloride and disulfiram induced ROS-mediated apoptosis in aldehyde dehydrogenase (ALDH)-positive glioblastoma CSCs.[25] Given this finding, we investigated the role of ROS in the breast CSC-specificity observed for **4**. Intracellular ROS production was quantified using 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), a well-established ROS indicator. Upon incubation of CSC-rich HMLER-shEcad cells with **4** (3 μM for 48 h) a marked increase in ROS levels was observed compared to the untreated control cells (Figure 4B). Addition of H_2O_2 (6 μM for 48 h) also enhanced ROS levels relative to the untreated cells. As expected, **4**- and H_2O_2-mediated ROS production was attenuated in the presence of N-acetylcysteine (3 mM), a ROS scavenger (Figure S13). Remarkably, the intracellular ROS concentration in CSC-deficient HMLER cells in the absence and presence of **4** (3 μM for 48 h) was statistically similar ($p > 0.05$; Figure S14). Thus, **4** generates ROS more readily in CSCs than in non-CSCs. This may partly explain the CSC-selective killing profile observed for **4**.

Intracellular ROS production often triggers genomic DNA damage and activates Jun-amino-terminal kinase (JNK) and/or p38 MAP kinase (MAPK) pathways.[26] Therefore, we conducted immunoblotting studies to monitor changes in the expression of biomarkers related to these pathways. HMLER-shEcad cells incubated with **4** (2.5–5 μM for 72 h) displayed a marked increase in the expression of the phosphorylated form of H2AX (γH2AX), indicative of genomic DNA damage (Figure S15). This is consistent with the high nuclease activity observed for **4** in cell-free systems (Figures S1, S2). HMLER-shEcad cells dosed with **4** (2.5–5 μM for 72 h) also exhibited enhanced phosphorylation of JNK and p38 and their respective downstream effectors, c-Jun and MAP kinase-activated protein kinase 2 (MAPKAPK-2) (Figure S15). Persistent activation of JNK and p38 pathways

can induce apoptosis.[27] HMLER-shEcad cells exposed to **4** (2.5–5 μM for 72 h) expressed higher levels of cleaved caspase 3 and poly-ADP ribose polymerase (PARP) relative to untreated cells (Figure S15), suggestive of caspase-dependent apoptosis. Collectively, the immunoblotting studies showed that **4**-induced ROS production promotes DNA damage, JNK and p38 pathway activation, and apoptosis.

COX-2 expression in breast cancer cells is heavily implicated in invasiveness and epithelial-to-mesenchymal transition (EMT).[14,28] Given that epithelial breast cancer cells that have undergone EMT are functionally indistinguishable from breast CSCs[29] and the COX-2 inhibitory properties of **4** in cell-free systems (Figure 2), we investigated whether the CSC-selective toxicity observed for **4** was, in part, due to its ability to inhibit COX-2. Incubation of HMLER-shEcad cells with **4** (5 μM for 48 h) and indomethacin (positive control, 10 μM for 48 h), induced a marginal reduction in COX-2 levels, as determined by flow cytometry (Figure S16). A more appreciable decrease in COX-2 expression was observed in HMLER-shEcad cells pretreated with lipopolysaccharide (LPS; 1 μM for 24 h; Figure 4C). LPS was applied to increase basal COX-2 levels (Figure S16). CSC-rich HMLER-shEcad cells express significantly higher levels of COX-2 than CSC-depleted HMLER cells (Figure S16), therefore the COX-2 inhibitory activity of **4** (in addition to its ability to induce ROS, see above) may contribute to its ability to selectively kill CSCs. To confirm this, cytotoxicity studies were conducted with and without prostaglandin E2 (PGE2), the functional product of COX-2. Co-treatment of HMLER and HMLER-shEcad with **4** and PGE2 (20 μM, 72 h), significantly reduced the potency of **4** toward CSC-rich HMLER-shEcad ($p < 0.05$), but not towards CSC-poor HMLER cells (Figure S17). This result confirms that the selective CSC-inhibitory effect of **4** is related to its ability to induce cell death through a COX-2-dependent mechanism.

In summary, we presented a family of copper(II) complexes capable of selectively killing breast CSC-like cells over bulk breast cancer cells. The most promising copper(II) complex in this series, **4**, induces its cytotoxic effect by elevating intracellular ROS levels and inhibiting COX-2 activity. As well as highlighting the largely unstudied potential of metal-containing compounds to effectively kill breast CSCs, this study also showed that a dual-threat approach can be used (within a single molecule) to reduce mammosphere formation, size, and viability. These findings could open the door for the development of other redox-modulating, metal-containing, NSAIDs complexes for CSC-directed chemotherapy.

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Keywords: bioinorganic chemistry · cancer · COX inhibition · nonsteroidal anti-inflammatory drugs · reactive oxygen species

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