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Cytotoxic Effects of Diclofenac and Ibuprofen Zinc (II)-Nicotinamide Ternary Complexes in Breast Cancer Cell Lines

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HIGHLIGHTS

- Diclofenac and ibuprofen-derived complexes decrease viability of breast cancer cell lines
- Complex **1** presented antitumor effect in all breast cancer cell lines tested including on triple negative cell line (MDA-MB-231) (IC50 202µM)
- Complex **1** was selective for both 4T1 and MDA-MB-231 cells

Abstract: Breast cancer is one of the leading types of cancer worldwide, and the search for new treatment options are crucial. Nonsteroidal anti-inflammatory drugs (NSAIDs) —specially ibuprofen and diclofenac—, have shown antitumoral effect against several types of cancer. The synthesis of organometallic compounds has shown significant improvements in pharmacological properties and efficacy of organic molecules. Two zinc II ternary complexes containing the NSAIDs diclofenac and ibuprofen and nicotinamide neutral linker (Nic) were obtained by the two-step solvent metalligand complexation method. The compounds $Zn_2(Diclof)_4(Nic)_2$ (complex **1**) and $Zn_2(Ibup)_4(Nic)_2$ (complex **2**) were tested in breast cancer cell lines (4T1, MCF-7 and MDA-MB-231) to evaluate their cytotoxicity, comparing to ibuprofen and diclofenac as controls. We found that both complex **1** and **2** exerted more than 60% reduction in 4T1 viability at 250µM, and complex

2 decreased cell viability at 250 µM and 137.5 µM in MCF-7 (34.35% and 26.42% reduction, respectively) and in MDA-MB-231 (57.2% and 22.88% reduction, respectively), all compared to controls. Complex **1** was selective only in MCF-7, and complex **2** was selective in both MCF-7 and MDA-MB-231. In summary, our data showed that the cytotoxic effect of complex **1** and **2** is increased comparing to their original NSAID in different breast cancer cell lines, highlighting their potential anti-tumoral activity.

Keywords: NSAIDs; Zinc complexes; cytotoxicity; coordination compounds; cell viability.

INTRODUCTION

Cancer is the second most cause of death worldwide, with 9.6 million deaths estimated in 2018. (1) According to Global Cancer Statistics 2018 (2), for females, breast cancer is the leading type of cancer, with 24.2% of incidence, and leading cause of cancer death, with 15.0% mortality rate. Breast cancer involves an inherited component, with multiple susceptibility genes linked to it, and has a wide variation in tumor morphology and clinical response. Like all cancer types, it implicates in high costs treatments, with several side effects, therefore the search for more specific and efficient compounds against breast cancer is crucial. (2)

Nonsteroidal anti-inflammatory drugs (NSAIDs) are a class of molecules that act on inflammation, vasodilatation, vasoconstriction, and as analgesics and antipyretics. Their mechanism of action is based on the inhibition of COX and LOX enzymes, which are involved in the biosynthesis of prostaglandins (PGs) (3,4). Arachidonic acid (AA) is converted by COX and LOX enzymes into lipid mediators known as eicosanoids, which include prostaglandins, thromboxanes, leukotrienes, lipoxins, resolvins, and eoxins, and are involved in inflammation and cancer (5–7). There have been several studies showing an inverse correlation between the use of NSAIDs and the incidence of several types of cancer, such as breast (8,9), lung (10,11), prostate (12), bladder (13), ovary (14,15), esophagus (16) and stomach (17). These correlations have prompted studies on antitumoral effects of NSAIDs. (18)

Chronic inflammation can lead to the initiation of cancer (19–21), and COX enzymes (specially COX-2 (22)) are overexpressed in many malignant lesions (23), such as colon (24), stomach (25) and breast cancer (26). This enzyme is also involved in cancer development (27), poorer patient prognosis (28) and tumor invasion (29). Thus, NSAIDs like ibuprofen and diclofenac can have an antitumoral effect on cancer, as they inhibit both COX-1 and COX-2 enzymes (30).

Organometallic complexes are formed when a metal coordinates with organic compounds, being considered as an intermediate form between organic and inorganic derivatives. Studies have shown that these complexes can be potential anticancer drugs (31–35), since they exhibit a greater effect than the original organic molecule, and higher kinetic stability, structural diversity, ability to bind biological targets, variable oxidation states and the possibility of rational ligand design to control kinetic properties (36,37). Metallodrugs formed using ibuprofen and diclofenac as chelating agents have shown anticancer activity against cancer cell lines (38–41). Studies using metallodrugs containing ibuprofen showed antiproliferative activity *in vitro*, and compounds using both diclofenac and ibuprofen also led to inhibition of COX and LOX enzymes (38,39).

Based on these studies, our group sought to examine the effect of two organometallic compounds, complex **1** and complex **2**, synthesized and previously described by Moura 2020 (42). The compounds were synthesized using ibuprofen and diclofenac, forming ternary complexes of Zn-Diclof and Zn-Ibup with nicotinamide (Nic) as a nitrogen ligand. Complexes **1** and **2** were tested against three breast cancer (BC) cell lines: MCF-7, a human invasive breast ductal carcinoma, ER^+, $PR^{+/-}$, $HER2^-$ luminal and often chemotherapy responsive; MDA-MB-231, also human invasive breast ductal carcinoma, triple negative, claudin-low and intermediate responsive to chemotherapy; and 4T1, a mouse mammary gland that mimics an animal stage IV human breast cancer (43,44). We aimed to observe the effect of complexes **1** and **2** in inhibiting proliferation of breast cancer cell lines.

MATERIAL AND METHODS

Zinc nitrate, zinc sulphate, nicotinamide, sodium carbonate and ethanol 99% were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Potassium diclofenac 99% and ibuprofen 99% (pharmaceutical grade) were obtained from Sigma-Aldrich. Ultrapure water was obtained from the Milli-Q (Millipore) apparatus. Ethanol and dimethylformamide (DMF) were purchased form Sigma-Aldrich (grade reagent) and used without further purification.

Physical measurements

1H and 13C NMR analysis was performed on a Fourier 300 spectrometer (Bruker) (300.18 MHz from 1H and 75.49 MHz from 13C with 5 mm probe) with 32 scans for 1H and 5000 scans for 13C, using CDCl3 and DMSO-D6 as solvents. Infrared analysis was performed on a Perkin Elmer Spectrum 400 FTIR spectrometer using solid samples by the KBr pellet technique. Spectral data were obtained in the spectral interval from 400 to 4000 cm-1 (wavenumber) with a resolution of 1.0 cm-1 for 32 scans. High resolution mass spectroscopy was performed according to the following conditions: complex solutions (1.0 μg.mL-1 H2O:MeOH 1:1 v/v) were individually infused directly into the ESI source by means of a syringe pump (Harvard Apparatus) at a flow rate of 150 μL.min-1. ESI (+)-MS and tandem ESI (+)-MS/MS were acquired using a hybrid high-resolution and high accuracy (5 μL.min-1) MicroTof (Q-TOF) mass spectrometer (Bruker Scientific) under the following conditions: capillary and cone voltages were set to +3500 V and +40 V, respectively, with a desolvation temperature of 100 °C. For ESI (+)-MS/MS, the energy for the collision-induced dissociations (CIDs) was optimized for each component. For data acquisition and processing in Q-TOF, control data analysis software (Bruker Scientific) was used. The data were collected in the m/z range of 100–2000 at the speed of two scans/s, providing the resolution of 10,000 (FWHM) at m/z 200.

Chemical synthesis

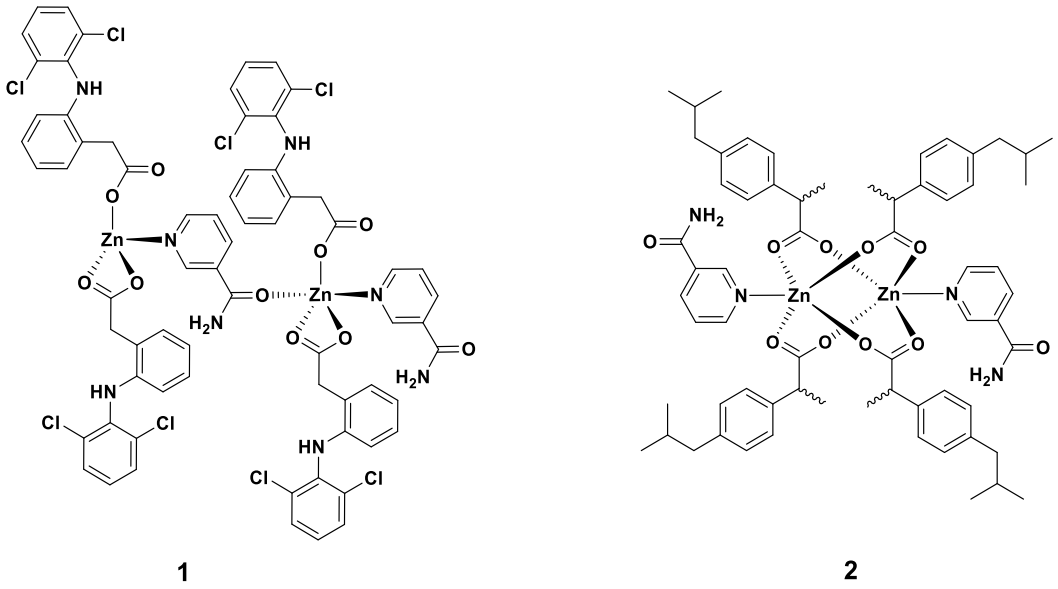

Figure 1. Ternary complexes 1 and 2.

Complexes 1 and 2 were prepared and analyzed according to Dos Santos and coauthors (42). For complex 1, 0.655 g (1.0 mmol) of zinc diclofenac salt previously obtained according to Abu Ali and coauthors (45)was dissolved in 20 mL of ethanol 99% at room temperature and vigorous stir. An ethanolic solution of nicotinamide (0.244 g, 2.0 mmol) was then added dropwise over first solution and keep stirring for two h. The white powder was obtained after one week by filtration and vacuum drier. Yield: 0.70 g, (90%); MP: 180° C; IR (cm-1, KBr pellet): 3310, 3183, 3095, 3072, 3039, 2969, 2925, 1679, 1622, 1607, 1592, 1576, 1564 (νa COO-), 1506, 1415 (νs COO-), 1351, 1303, 1283, 1249, 1199, 1164, 1094, 1058, 869, 837, 774, 747, 720, 698, 656; (Δν COO-) 149; 1HNMR (δ-ppm, DMSO-D6): 3.59 (s-4H, 2CH2), 6.28 (d-2H, 2CH, JH-H = 9Hz), 6.82 (dt-2H, 2CH, JH-H = 7Hz), 7.01 (dt-2H, 2CH, JH-H = 7Hz), 7.08 (dd-2H, 2CH, JH-H = 8Hz), 7.15 (dd-2H, 2CH, JH-H = 7Hz), 7.42 (d-4H, 4CH, JH-H = 8Hz), 7.48 (m-1H, CH(Nic), JH-H = 5Hz), 7.63 (s-1H, NH(Nic)), 8.19 (s-1H, NH(Nic)), 8.21 (m-1H, CH, JH-H = 8Hz), 8.30 (s-2H, 2NH(Diclof)), 8.69 (dd-1H, CH(Nic), JH-H = 5Hz), 9.03 (ds-1H(Nic), CH); 13CNMR (δ–ppm, DMSO-D6): 40.73 (2CH2), 116.34 (2CH), 120.82 (2CH), 123.60 (CH(Nic)), 124.59 (2CH), 126.56 (2CH), 126.85 (2C), 128.97 (2CH), 129.12 (4CH), 129.82 (CH(Nic)), 130.61 (2C), 135.46 (CH(Nic)), 137.57 (2C), 142.86 (4CCl), 148.75 (CH(Nic)), 151.92 (CH(Nic)), 166.45 (CON(Nic)), 177.40 (2COO-(Diclof)); HRMS ESI (+) m/z 1253.9743 (C54H42Cl6N7O8Zn2) [M-diclof]+, 1131,9225 (C48H36Cl6N5O7Zn2) [M-diclof-nic]+, 1009.8843 (C42H30Cl6N3O6Zn2) [Zn2(diclof)3]+, 796.9771 (C34H26Cl4N4NaO5Zn) [Zn(diclof)2Nic+Na]+, 774.9993 (C34H27Cl4N4O5Zn) [Zn(diclof)2Nic+H]+, 674.9318 (C28H20Cl4N2NaO4Zn) [Zn(diclof)2+Na]+, 652.9525 (C28H21Cl4N2O4Zn) [Zn(diclof)2+H]+, 602.0314 (C26H22Cl2N5O4Zn) [Zn(diclof)(nic)2]+, 478.9849 (C20H16Cl2N3O3Zn)

[Zn(diclof)(nic)]+, 398.9627 (C14H14Cl2N2NaO2Zn) [Zn(diclof)+NH4+Na]+, 374.9478 (C14H12Cl2NO3Zn) [Zn(diclof)+H2O]+, 357.9379 (C14H10Cl2NO2Zn) [Zn(diclof)]+, 318.0053 (C14H11Cl2NNaO2) [diclof+H+Na]+, 296.0233 (C14H12Cl2NO2) [diclof+2H]+.

For complex 2, 0.475 g (1.0 mmol) of zinc ibuprofen salt previously obtained according to Abu Ali and coauthors (46) was dissolved in 20 mL of dimethylformamide (DMF) at room temperature and vigorous stir. Nicotinamide (0.244 g, 2.0 mmol) was then added dropwise over first solution and keep stirring for two h. The clear solution was concentered by vacuum evaporation at 20% of start volume and keeped in dark flask at room temperature for 30 days. The clear crystals were then obtained by filtration, washed with cold DMF and dried over freeze drier by 24 h. Yield: 0.42g (70%); MP:148 °C; IR (cm-1, KBr pellet): 3440, 3318, 3283, 3201, 3182, 2959, 2930, 2867, 1689, 1636 (νa COO-), 1604, 1575, 1512, 1458 (νs COO-), 1412, 1372, 1287, 1199, 1056, 793, 699, 601, (Δν COO-) 178; 1HNMR (δ-ppm, CDCl3): 0.82 (d-12H, 4CH3, JH-H = 6Hz), 1.35 (d-6H, 2CH3, JH-H = 7Hz), 1.74 (sep-2H, 2CH, JH-H = 7Hz), 2.27 (d-4H, 2CH2, JH-H = 7Hz), 3.62 (q-2H, 2CH, JH-H = 7), 4,98 (s-1H, 1NH(Nic)), 6.83 (d-4H, 4CH, JH-H = 8Hz), 7,00 (d-4H, 4CH, JH-H = 8Hz), 7.43 (dd-1H, 1CH(Nic), JH-H = 8Hz), 8,26 (s-1H, 1NH(Nic)), 8.43 (dt-2H, 2CH(Nic), JH-H = 8Hz), 8.59 (dd-2H, 2CH(Nic),JH-H = 5Hz), 8.85 (ds-2H, 2CH(Nic), JH-H = 2Hz); 13CNMR (δ-ppm, CDCl3): 19.54 (2CH3), 22.33 (4CH3), 30.09 (2CH), 44.86 (2CH), 46.24 (2CH2), 124.57 (CH(Nic)), 127.09 (4CH), 129.07 (4CH), 130.70 (CH(Nic)), 137.01 (2C), 139.76 (CH(Nic)), 140.06 (2C), 146.83 (CH(Nic)), 150.26 (C(Nic)), 164.39 (CON(Nic))183.07 (2CO2-); HRMS ESI (+): m/z 1093.3807 (C58H74N2NaO9Zn2) [M-Nic+Na]+, 1071.3983 (C58H75N2O9Zn2) [M-Nic+H]+, 987.3219 (C51H63N4O8Zn2) [M-ibup]+, 865.2731 (C45H57N2O7Zn2) [Zn2(ibup)3(Nic)]+, 883.2853 (C45H59N2O8Zn2) [Zn2(ibup)3(Nic)+H2O]+, 619.2110 (C32H40N2NaO5Zn) [Zn(ibup)2(Nic)+Na]+, 597.2296 (C32H41N2O5Zn) [Zn(ibup)2(Nic)+H]+, 513.1469 (C25H29N4O4Zn) [Zn(ibup)(Nic)2]+, 475.1813 (C26H35O4Zn) [Zn(ibup)2+H]+, 328.0889 (C13H23NNaO3Zn) [Zn(ibup)+H2O+NH4+Na]+, 287.0620 (C13H19O3Zn) [Zn(ibup)+H2O]+, 229.1201 (C13H18NaO2) [ibup+Na+H]+.

Chemical synthesis

The compounds were synthesized according to Moura 2020 [42].

Cell lines

The cell lines MCF-7 (human mammary gland adenocarcinoma), 4T1 (Mus musculus mammary gland), MDA-MB-231 (human mammary gland adenocarcinoma) and Vero (Cercopithecus aethiops kidney normal) were purchased from the American Type Culture Collection (ATCC). MCF-7 cells were cultured in DMEM Low glucose, MDA-MB-231 and Vero in DMEM High glucose, and 4T1 in RPMI, all supplemented with 10% Fetal bovine serum (FBS). All cells were incubated in a humidified incubator at 5% CO_2 and 37 °C, and experiments were performed using freshly thawed cells after three passages. Cell lines were tested for Mycoplasma contamination.

Cytotoxicity assay

Cells were seeded in 96-well flat-bottom plates at concentrations of $5x10^3$ cells/well for MCF-7, MDA-MB-231 and 4T1 cells and $2x10^3$ cells/well for Vero cells. After 24 h of incubation, 250 μM, 137.5 μM, 25 μM, 13.75 μM, or 2.5 μM of complex **1** and complex **2** was added and the plates were incubated for an additional 24 h at 37°C in a humidified incubator with 5% CO2. For the treatment curve, 4T1 cells were incubated with 250 μM of compounds and controls for 6h, 12h, 24h and 48h. For the vehicle control, DMSO was added in the highest concentration that was used to dilute the compounds. As an additional negative control (NC), untreated cells were also assayed. Cell viability was assessed using a colorimetric assay based on the reduction of 3-[4,5-dimethylthiazol-2-y1]-2,-diphenyltetrazolium bromide (MTT) by mitochondrial enzymes (Molecular Probes™, Thermo Fisher Scientific, Waltham, MA, USA). Briefly, 100μL of medium was removed and 40μL of MTT reagent (5-mg/mL) was added into each well. Cells were incubated for 4h and the precipitated formazan crystals were dissolved in dimethyl sulfoxide (DMSO). Finally, the optic density (OD) was analyzed at 570/620 nm using a micro-plate reader (EZ Read 400, Biochrom). Assays were performed in triplicate. For cytotoxicity calculation, the OD of the treated cells was multiplied by the percentage equivalent to the cells treated with DMSO, and then divided by the OD of the cells treated with DMSO, as the following:

OD of NC — 100%
OD of DMSO treated cells — x
Viability = (OD of treated cells x % equivalent to DMSO) / (OD of DMSO treated cells)

Data analysis

The half maximal inhibitory concentrations (IC50) of compounds and controls were calculated using linear and polynomial regression analyses using Microsoft Excel 2009, with consequent resolution of the line equation of the graph with Wolfram Alpha. The IC50 values were reported as a mean of two independent experiments. The selectivity index (SI) was calculated according to the following equation:

SI = (IC50 of Vero cells) / (IC50 of tumor cells)

where a SI >1 indicates that the compound is more cytotoxic to tumor cell, and a SI<1 indicates the opposite (45,46).

Statistical analyses were performed with GraphPad Prism 5 (GraphPad Software Inc., CA) using two-way ANOVA followed by Bonferroni correction.

RESULTS

Cytotoxic effects

Cytotoxic effects of complex **1** and complex **2** were tested against breast cancer cell lines MCF-7, MDA-MB-231 and 4T1, and Vero cells were used as non-tumorigenic cell line. Different concentrations were tested (250 µM, 137.5 µM, 25 µM, 13.75 µM and 2.5 µM), and ibuprofen and diclofenac were used in the same concentrations in cell lines as a control.

Both complex **1** and complex **2** exhibited more than 60% reduction in 4T1 cell viability at the concentrations of 250 and 137.5 µM, when compared to controls (Figure 2A and 2E). Complex **1** also decreased viability at 25 µM (22.88% reduction). Only complex **1** decreased cell viability at the concentrations of 250 µM and 137.5 µM in MCF-7 (34.35% and 26.42% reduction, respectively) (Figure 2B) and in MDA-MB-231 (57.2% and 22.88% reduction, respectively) (Figure 2C). Complex **2** showed no significant reduction in MCF-7 and MDA-MB-231, compared to ibuprofen (Figure 2F and 2G). Using non-tumorigenic cell lines (Vero), we observe that both compounds decreased viability at 250 µM (29.96% for complex **1** and 29.97% for complex **2**), and at 137,5 µM, only complex **1** caused reduction (30.58%)**,** showing a cytotoxic and non-selective effect (Figure 2D and 2H).

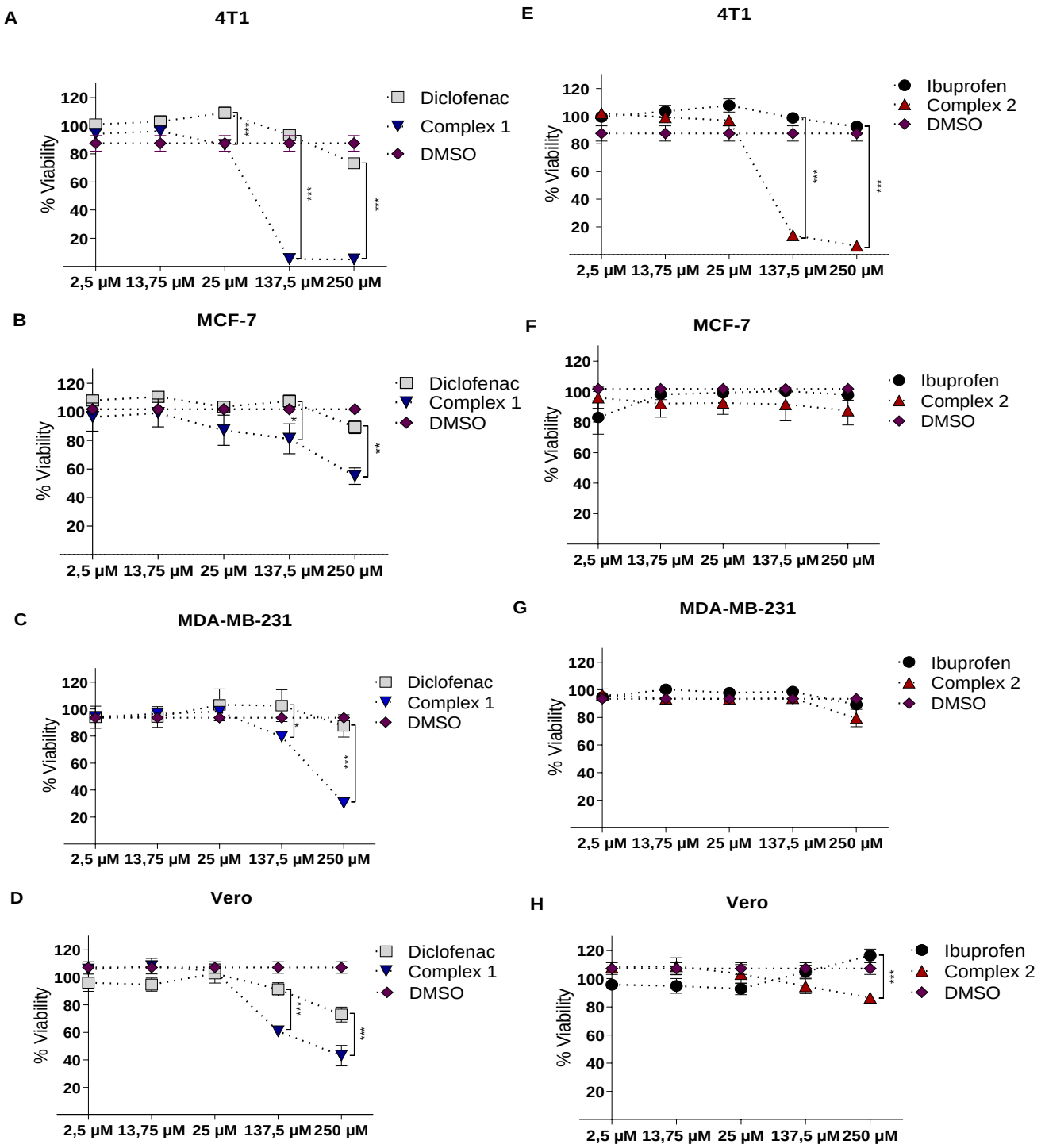

Figure 2. Cytotoxicity of complex 1, complex 2 and controls (ibuprofen and diclofenac). (A) Viability of 4T1 cells treated with complex 1 and diclofenac. (B) Viability of MCF-7 cells treated with complex 1 and diclofenac. (C) Viability of MDA-MB-231 cells treated with complex 1 and diclofenac. (D) Viability of Vero cells treated with complex 1 and diclofenac. (E) Viability of 4T1 cells treated with complex 2 and ibuprofen. (F) Viability of MCF-7 cells treated with complex 2 and ibuprofen. (G) Viability of MDA-MB-231 cells treated with complex 2 and ibuprofen. (H) Viability of Vero cells treated with complex 2 and ibuprofen. Cell viability was measured by MTT assay. Dose response curves represent viability 24h after treatment with 2.5 – 250 µM of each compound performed in triplicates. DMSO was used as a vehicle control. Values represent the mean and standard deviation of two independent experiments. * indicates p-values lower than 0.05, ** indicates p-values lower than 0.01 and *** indicates p-values lower than 0.001.

IC50 values

The IC50 values (the concentration of a drug that is required for 50% inhibition *in vitro)* were calculated for each complex and controls (Table 1). We found that for all cell lines tested, complex **1** exhibited the lowest IC50 compared to controls and complex **2** in all cell lines, indicating that it had the greatest cytotoxic effects.

Although complex **1** was effective at inhibiting tumor cell lines growth, it also inhibited Vero cells. Complex **2** presented lower IC50 value for 4T1 cells comparing to the other cell lines, so 4T1 was the cell line that was more affected by the compounds' activity. When controls were tested, diclofenac showed lower IC50 values than ibuprofen, because it is more cytotoxic.

Table 1. Cytotoxic activity (IC50 μM) of compounds (complex **1** and complex **2**) and controls (ibuprofen and diclofenac).

Cell line	Complex 1	Complex 2	Diclofenac	Ibuprofen
4T1	103,3	117,23	459,77	1252,08
MDA-MB-231	202,56	838,98	1017,02	1737,15
MCF-7	298,05	1770,56	1788,79	5061,17
Vero	251,32	787,14	729,92	972,15

Selectivity index

Selectivity index (SI) (Table 2) is a ratio that measures the window between cytotoxic and antitumoral activity. We used the IC50 found in Vero cells and divided by IC50 of tumoral cells. For both 4T1 and MDA-MB-231 cells, complex **1** was selective, as its SI was >1. Complex **2** presented selective effects only on 4T1 cells. For MCF-7, neither the compounds nor the controls exhibited a SI >1, showing that they are all not selective for this cell line. Diclofenac had a SI >1 for 4T1, and ibuprofen showed a SI <1 for all cell lines tested, meaning that it is not selective.

Table 2. Selectivity index (SI) of compounds (complex **1** and complex **2**) and controls (ibuprofen and diclofenac).

Cell line	Complex 1	Complex 2	Diclofenac	Ibuprofen
4T1	2,43	6,71	1,58	0,77
MDA-MB-231	1,24	0,93	0,71	0,55
MCF-7	0,84	0,44	0,40	0,19

Since the compounds exhibited the greatest effects on tumor cell growth of 4T1 cell line, we sought to examine the effect of the complexes at viability through different time points (6h, 12h, 24h and 48h) in order to observe when the cytotoxic effect starts (Figure 3). Both complex **1** and **2** had a time-dependent effect on cell viability, after 6h of the treatment we found a significant reduction on viability comparing to controls (Figure 3A and 3B). Diclofenac decreased around 30% cell viability after 48h, and in contrast to ibuprofen that did not affect cell viability.

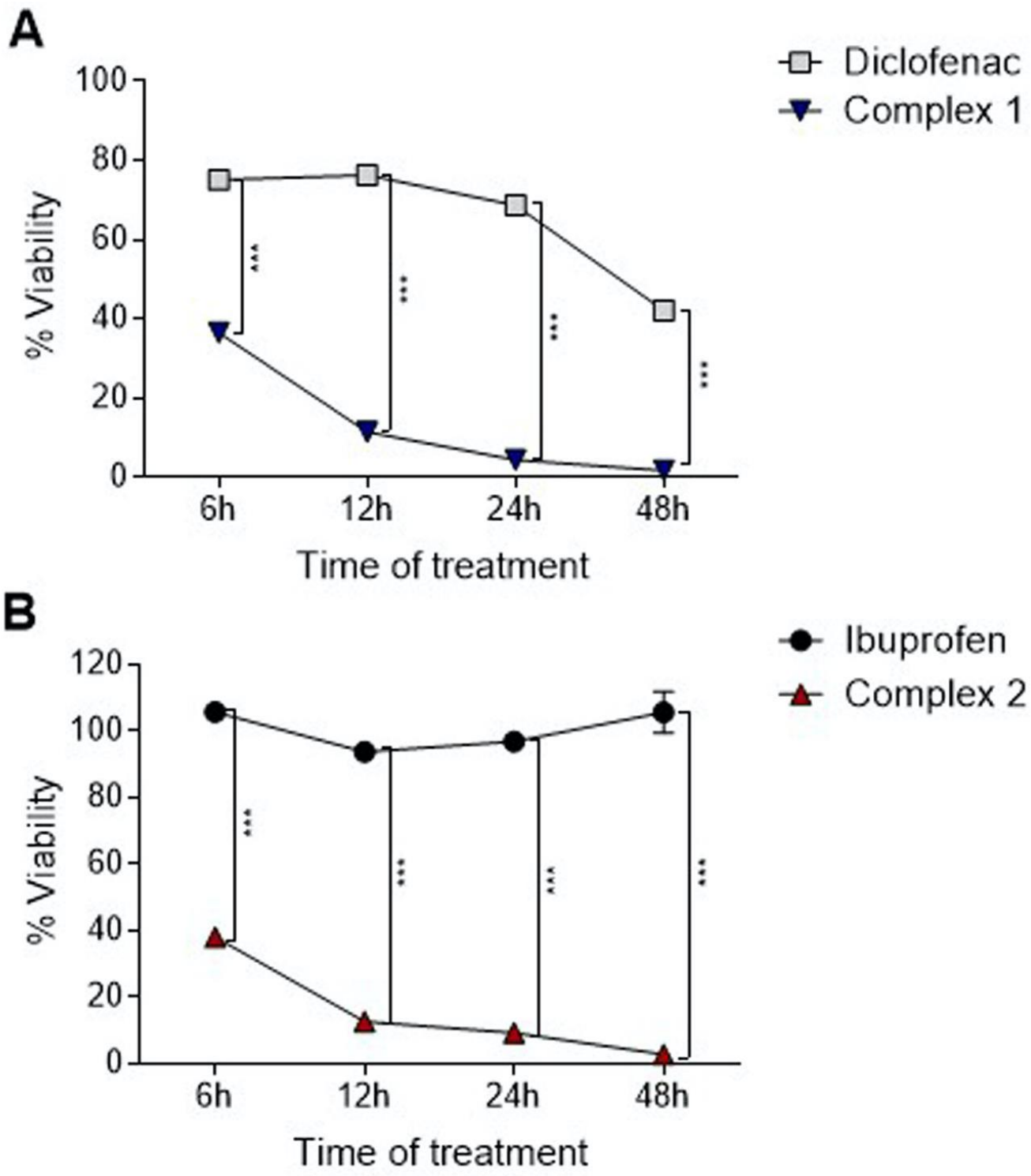

Figure 3. Cytotoxicity of 4T1 cells after 6h, 12h, 24h and 48h of treatment with compounds and controls. (A) 4T1 cells treated with complex 1 and diclofenac. (B) Cells treated with complex 2 and ibuprofen. Cell viability was measured by MTT assay. Cells were treated with 250 µM of compounds and controls. Values represent the mean and standard deviation of three replicates of one experiment. * indicates p-values lower than 0.05, ** indicates p-values lower than 0.01 and *** indicates p-values lower than 0.001.

DISCUSSION

Breast cancer is one of the leading causes of death in the developed world, especially among women, implying in high costs treatments, which sometimes causes major side effects. For this reason, several studies have been performed to better understand the mechanisms of compounds candidates to be used as treatment of cancer.

Our study and others showed that organometallic compounds derived from ibuprofen and diclofenac presented inhibition of tumor cell growth in vitro (38,39,41). We found that complex **1**, derived from diclofenac, and complex **2**, derived from ibuprofen, decreased cell viability in breast cancer cell lines when compared to controls, especially after 48h of treatment, which may be associated with their high stability (42). Since the addition of metals to an organic molecule can augment its pharmacological properties, possibly the coordination to Zn^{+} caused this effect on these complexes. When compounds were tested against 4T1 cells in different time points, we observed that cytotoxicity effects started at 6h and increased over time, suggesting that the compounds might affect cell death. Furthermore, the presence of nicotinamide increases the solubility of compounds (42), which may be related to the cytotoxicity of both complexes (38,47,48). It was previously demonstrated that the complexed form of the Zn II NSAIDs and the Nic binder produces molecules of low toxicity, no DNA cleavage activity (instead of its ability to interact with the molecule) and low chemical lability in polar solvent (42).

The mechanism used by the compounds to reduce cell viability remains unknown, however we hypothesize that it is related to COX inhibition, as other groups found with other NSAIDs-derived compounds (30,49). Both ibuprofen and diclofenac are non-selective COX inhibitors, meaning they inhibit both COX-1 and 2, although diclofenac has a higher selectivity for COX-2 (50,51). MDA-MB-231 and 4T1 were previously shown to have high expression of COX-2 and MCF-7 showed low expression of COX-2 (52). Thus, we hypothesize that complex **1** had more cytotoxic effect on MDA-MB-231 and 4T1 cells because since it is derived from diclofenac, it preferably inhibits COX-2. Specially for breast cancer, COX-2 was sufficient to promote tumorigenesis in transgenic mice (53) and was also related to higher histological grade, tumors with larger sizes, high Ki-67 and p53 expression, negative hormone receptor status, HER-2 amplification and presence of axillary lymph node metastases (18).

Both compounds did not show greater cytotoxic effects in MCF-7 cells — which may be due to low COX-2 expression— and presented more cytotoxic effects against 4T1 cell lines. Interestingly the complex **1** presented a cytotoxic effect on MDA-MB-231. The distinct effects of compounds on BC cell lines are probably due to different phenotype and origin of the cells, as 4T1 cells are originated from mice, and MCF-7 and MDA-MB-231 from human invasive breast ductal carcinoma. Additionally, molecular subtypes of each cell are individual, as MCF-7 is classified as luminal (less aggressive, better prognosis) and MDA-MB-231 as claudin-low (more aggressive, worse prognosis) (44,54). Other study presented alternative pathways that show how NSAIDs, including ibuprofen and diclofenac, can act on MDA-MB-231 cell lines, through inhibiting the sirtuin 1 (SIRT1) deacetylase activity, increasing acetylation and activity of the tumor suppressor p53 and the expression of the antiproliferative gene p21 (55). Moreover, studies with diclofenac showed COX-independent mechanisms, such as modulation of MYC expression and glucose metabolism, resulting in impaired carcinoma cell line proliferation in murine melanoma cell line B16F10 (56).

In summary, our results demonstrated that complexes **1** and **2** are more effective than the NSAIDs ibuprofen and diclofenac in reducing cellular growth in three different breast cancer cell lines, highlighting the potential antitumoral activity of Zinc-NSAIDs and Nic complexes. In this context, future studies should be conducted to better understand the mechanism by which the synthesized compounds, complex **1** and complex **2**, act on cell viability.

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