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Meloxicam and Its Metal Complexes: Cytotoxic Activity and Ability to Induce Autophagy in Human Triple Negative Breast Cancer Cells

Tanya Zhivkova[1], Zdravka Petrova[1*], Lora Dyakova[2], Rossen Spasov[1,3], Chukwuemeka Obinna Ekeh[1,4], Abedulkadir Abudalleh[1], Daniela-Cristina Culita[5], Gabriela Marinescu[5], Radostina Alexanderova[1**]*

[1] *Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria;*
[2] *Institute of Neurobiology, Bulgarian Academy of Sciences, Sofia, Bulgaria;*
[3] *Faculty of Medicine, Sofia University " St. Kliment Ohridski", Sofia, Bulgaria;*
[4] *Faculty of Biology, Sofia University " St. Kliment Ohridski", Sofia, Bulgaria;*
[5] *Institute of Physical Chemistry "Ilie Murgulescu", Romanian Academy, Bucharest, Romania*

* Both authors - T. Zh. and Z. P., contributed equally to this work
** Corresponding author e-mail: rialexandrova@hotmail.com

The study aimed to evaluate the influence of the selective non-steroidal anti-inflammatory agent meloxicam and its metal [Zn(II), Cu(II), Co(II), Ni(II)] complexes on viability and proliferation of cultured MDA-MB-231 human triple negative breast cancer cells as well as the ability of these compounds to induce autophagy. Thiazolyl blue tetrazolium bromide (MTT) test, double staining with acridine orange and propidium iodide (AO/PI) and immunocytochemical analysis were applied. Zn(II) complex of meloxicam (Zn-Mel) was found to be the most pronounced cytotoxic agent among the compounds examined with CC_{50} calculated to be 376.5 μg/ml (470.0 μM) and 298.7 μg/ml (372.9 μM) after 48 and 72 h of treatment, respectively. Apoptosis was observed in cells incubated for 72 h with 250 μg/ml Zn-Mel (late apoptosis) and Co-Mel (early apoptosis) after double staining with AO/PI. Autophagosomes were detected in MDA-MB-231 cells after treatment for 48 h with 100 μg/ml meloxicam, Co-Mel and Ni-Mel, but not in the case of Zn-Mel and Cu-Mel.

Key words: Non-steroidal anti-inflammatory drugs, meloxicam, human triple negative breast cancer cells, cytotoxic activity, autophagy

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are a chemically diverse class of more than 20 medications frequently prescribed in the world because of their anti-inflammatory, analgesic and antipyretic properties. The biological activity of these agents is based on their ability to suppress the enzyme cyclooxygenase (COX) that

converts arachidonic acid to prostaglandine H2 (PGH2) - the key first step in synthesis of biologically active molecules with significant physiological importance such as prostanoids, prostacyclins and thromboxanes. There are two isoforms of COX: COX-1 – constitutively expressed in most tissues playing a crucial role in homeostasis; and COX-2 – induced by inflammation signals (e.g. IL-1β, TNF-α, lipopolysaccharide), mitogenic or oncogenic stimuli (e.g. phybole esters, oncogene v-src) [1].

According to their capacity to inhibit both of COX isoforms or predominantly the COX-2 enzyme, NSAIDs are divided into two groups – nonselective and selective NSAIDs, respectively. In recent years, there has been an increasing interest in the antitumor activity of NSAIDs. COX-2 expression has been documented in various solid tumors including colorectal cancer, cancers of the prostate, mammary gland, pancreatic and lung cancer, which makes this molecule an attractive target for selective antitumor treatment. The antitumor activity of NSAIDs can be explained also by COX-independent mechanisms of action [1, 8, 11, 16].

The so-called triple negative breast cancer (TNBC) is one of the major challenges in modern clinical oncology, as TNBC cells do not express receptors for estrogen, progesterone, and epidermal growth factor (Her2/Neu), and currently available (targeted) therapeutic strategies are limited [11].

It has been found in our previous investigations that selective non-steroidal anti-inflammatory agent meloxicam and its metal [Zn(II), Cu(II), Co(II), Ni(II)] complexes decrease viability and proliferation of human (cervical carcinoma, glioblatoma multiforme) and animal (chicken hepatoma, rat sarcoma) tumor cells [4, 6]. The aim of the present study was to evaluate the cytotoxic/antitumor effect of these compounds in cultured human triple negative breast cancer cells as well as their ability to induce autophagy.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (D-MEM) and fetal bovine serum (FBS) were purchased from Gibco-Invitrogen (UK). Dimethyl sulfoxide (DMSO) and trypsin were obtained from AppliChem (Germany); thiazolyl blue tetrazolium bromide (MTT) was from Sigma-Aldrich Chemie GmbH (Germany). The antibiotics (penicillin and streptomycin) were from Lonza (Belgium). Immunocytochemistry detection system (Novolink Polymer Detection System) was purchased from Leica Biosystems, UK and Rabbit polyclonal antibody to LC3B protein (abcam 48394) is manufactured by Abcam, UK. The Bio-Mount DPX Medium viscosity covering for microscope slides and nuclear dye (Mayer's Haematoxylin) were delivered from Biognost, Croatia. All other chemicals of the highest purity commercially available were purchased from local agents and distributors. All sterile plastic ware was from Orange Scientific (Belgium).

Compounds

The metal complexes with meloxicam were obtained following the method reported in a previous work [4]. All complexes were characterized by various physicochemical methods. Their molecular formulae have been established by correlating the elemental analysis data with the spectral data (infrared, ultraviolet-visible) and magnetic measurements. All these data were presented and discussed in one of our previous papers [4].

The complexes were denoted Zn-Mel (molecular formula: $[Zn(HMel)_2(H_2O)_2]$), Cu-Mel (molecular formula: $[Cu(HMel)_2(H_2O)_2]$), Co-Mel (molecular formula: $[Co(HMel)_2(H_2O)_2]$), Ni-Mel (molecular formula: $[Ni(HMel)_2(H_2O)_2]\cdot H_2O$).

Cell lines and cultivation

The permanent cell line MDA-MB-231 established from human triple negative breast cancer and proved to express COX-2 was used as a model system [7]. The cells were routinely grown as monolayer cultures in a D-MEM medium, supplemented with 5-10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin. The cultures were maintained at 37°C in a humidified CO_2 incubator (Thermo Scientific, HEPA Class 100). For routine passages the cells were detached using a mixture of 0.05% trypsin – 0.02% ethylendiaminotetraacetic acid.

Cytotoxicity assay

The cells were seeded in 96-well flat-bottomed microplates for cell culturing at a concentration of 1 x 10^4 cells/well. At the 24th hour the culture medium was removed and changed with media containing different concentrations of the compounds examined (10-500 μg/ml). Each concentration was applied in 6 to 8 wells. The concentration of the solvent DMSO in the working solutions containing 500, 250, 200, 100, 50 and 10 μg/ml of the investigated compounds (meloxicam and its metal complexes) was calculated to be 2.00, 1.00, 0.80, 0.40, 0.20 and 0.04%, respectively. Samples of cells grown in non-modified medium (Culture medium control) and in medium containing the corresponding amount of DMSO (DMSO-control) served as controls. The effect of the compounds on cell viability and proliferation was evaluated using thiazolyl blue tetrazolium bromide (MTT) test after 48 h and 72 h of incubation as it was earlier described [6].

Double staining with acridine orange (AO) and propidium iodide (PI)

The cells were grown on sterile cover glasses in 6-well plates (3 – 3.5 x 10^5 cells / well) in the presence of the compound tested. Culture-medium controls as well as DMSO-controls were included in the experiments. After 72h of incubation, the coverslips were removed and washed with phosphate buffered saline (PBS) for 2 min. Equal volumes of fluorescent dyes containing AO (10 μg/mL in PBS) and PI (10 μg/mL in bidistilled water) were added to the cells [6]. Fresh stained cells were placed on a glass slide and examined under fluorescence microscope (Leica DM 5000B, Leica Microsystems, Germany) within 30 min before the fluorescent color started to fade.

Autophagy detection

The intracellular expression of the autophagy marker - the LC3B protein – was identified by immunocytochemical method using a rabbit polyclonal antibody.

The cells were seeded in 6-well plates on sterile cover glasses, 2 x 10^5 cells / well. After culturing for 24 hours, negative control (untreated cells), positive control (cells treated with known autophagy inducer - Rapamycin), as well as cells treated with studied compounds – meloxicam and its Zn(II), Cu(II), Co(II) and Ni(II) complexes were incubated for 48 h under the same conditions. The cover glasses were then washed PBS and fixed with 10% neutral buffered formalin (10 minutes), washed three times with PBS and processed by immunocytochemical detection protocol. It includes blocking endogenous peroxidase (5 minutes), protein block (5 minutes), incubation with primary antibody against LC3B protein (60 minutes), incubation with secondary antibody (30 minutes), incubation with enzyme-conjugating polymer (30 minutes). DAB (3,3′-diaminobenzidine) - 50μl plus 1 ml Substrate buffer (5 minutes) was used as the chromogen. After each step, the slides were washed three times with PBS. As a result of the antigen site, brown colored precipitates were obtained. After staining, the

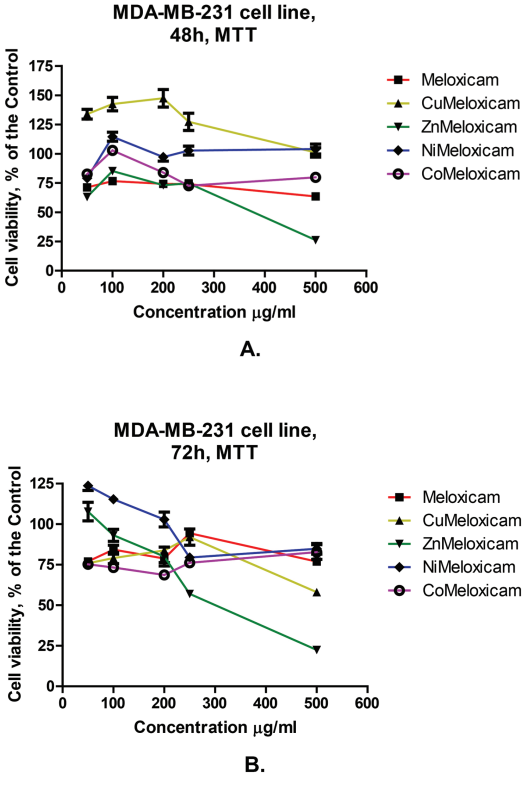
preparations were washed several times with distilled water and counterstained with hematoxylin for 3 minutes, then dehydrated with an ascending series of alcohols and incorporated onto slides using Bio-Mount DPX synthetic resin. The procedure was carried out at room temperature and the slides were incubated in a humidified chamber.

The prepared immunocytochemical samples were microscoped with a Leica DM 5000B, Leica Microsystems, Germany and documented with a Leica DFC 420 C camera, Leica Suite 3.1.0 software.

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett post-hoc test and Origin 6.1™.

Results and Discussion

Information about cytotoxic activity of meloxicam and its Zn(II), Cu(II), Co(II) and Ni(II) complexes in cultured MDA-MB-231 human triple negative breast cancer cells is obtained by MTT assay – the gold standard for such type of investigations. Concentration response curves based on experimental data are presented in **Fig. 1**. Cytotoxic concentration 50 (CC_{50}) that reduces the amount of viable cells by 50% as compared to the DMSO control has been determined (where possible) from these curves. Zn(II) complex of meloxicam (Zn-Mel) has been found to be the most pronounced cytotoxic agent among the compounds examined with CC_{50} calculated to be 376.5 μg/ml (470.0 μM) and 298.7 μg/ml (372.9 μM) after 48 and 72 h of treatment, respectively. In the case of meloxicam and its Cu(II), Co(II) and Ni(II) complexes the viability of the treated cells has been determined to be >50% as compared to the DMSO control.



The experimental data obtained by double staining with acridine orange and propidium iodide are presented in **Fig. 2**. While untreated MDA-MB-231 cells (control) form dense and vital monolayer (**Fig. 2a**), MDA-MB-231 cells treated with meloxicam and its metal complexes show different (in type and degree) cytopathological changes. Cells treated with 500 μg / mL meloxicam for 72 hours have a 30% cell population reduction, as well as visible cellular and nuclear polymorphism (**Fig. 2b**). The cytoplasm is swollen and filled with vacuoles, the chromatin is roughly dispersed. Cells treated with 250 μg / mL Zn-Mel are non-vital, with signs of late apoptosis – highly reduced cytoplasm and apoptotic patches on the cell sur-

Fig. 1. Effect of meloxicam and its metal [Zn(II), Cu(II), Co(II), Ni(II)] complexes on viability / proliferation of MDA-MB-231 human triple negative breast cancer cells evaluated by MTT test after 48 (**A**) and 72 h (**B**) treatment periods.

face (**Fig. 2c**), while treated with 250 µg / mL Cu-Mel cells are non-vital, but their cytoplasm is highly inflated and the nuclei are stacked (**Fig. 2d**). Vital cells in early apoptosis were observed after 72 h treatment with 500 µg / mL Co-Mel (**Fig. 2e**). In the treated cells for 72 h with 500 µg / mL Ni-Mel, a pronounced cellular polymorphism - spindle-shaped vital cells were observed, along with rounded non-vital cells with vacuolated and strongly enlarged cytoplasmic volume (**Fig. 2f**).

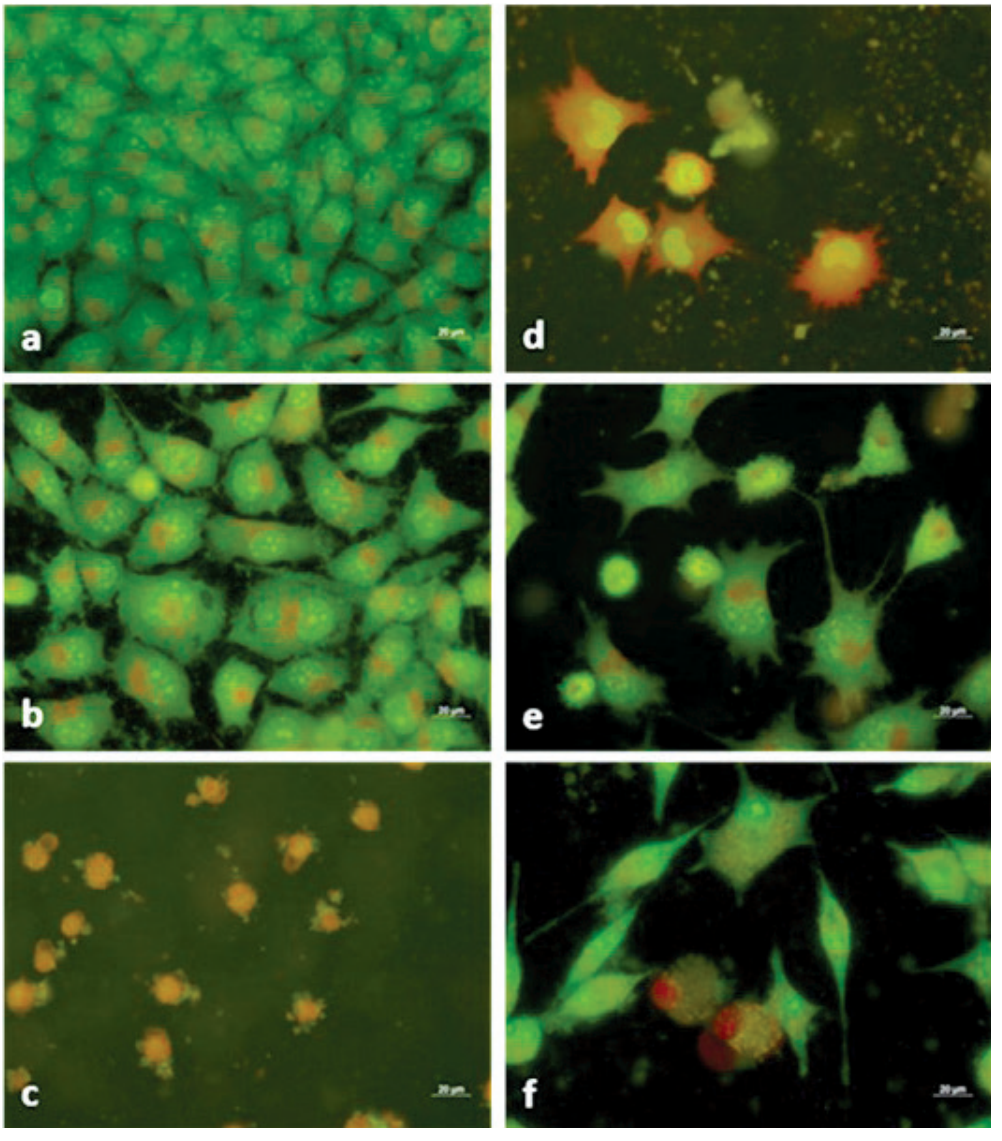


Fig. 2. Non-treated human triple negative breast cancer MDA-MB-231 cells (culture medium control – **a**) and MDA-MB-231 cells after 72 h treatment with 500 µg/mL meloxicam (**b**), 250 µg/mL Zn-Mel (**c**), 250 µg/mL Cu-Mel (**d**), 500 µg/mL Co-Mel (**e**), 500 µg/mL Ni-Mel (**f**). Double staining with acridine orange and propidium iodide. Bar = 20 µm (Leica DM 5000B, Leica Microsystems, Germany, 40x)

Immunocytochemical detection of autophagy in cell cultures and paraffin-embedded tissues has long been established as a reliable method for demonstrating this process [13]. The expression of autophagy marker - LC3B protein, was studied in MDA-MB-231 cells cultured in the presence of Rapamycin, known as autophagy inducer, as well as meloxicam and its metal [Zn(II), Cu(II), Co(II), Ni(II)] complexes. The results obtained are presented in **Fig. 3** and **4**. Untreated control MDA-MB-231 cells demonstrate diffuse cytoplasmic labeling of LC3B, the cells are monomorphic and formed a solid monolayer. In the presence of meloxicam (100 µg /ml, 48h) autophagosomes are observed only in individual cells. The cells treated with Cu-Mel and Zn-Mel (100 µg /ml, 48 h) show an intense cytoplasmic response without autophagosome formation with a well-pronounced cytotoxic effect. In cells cultured in

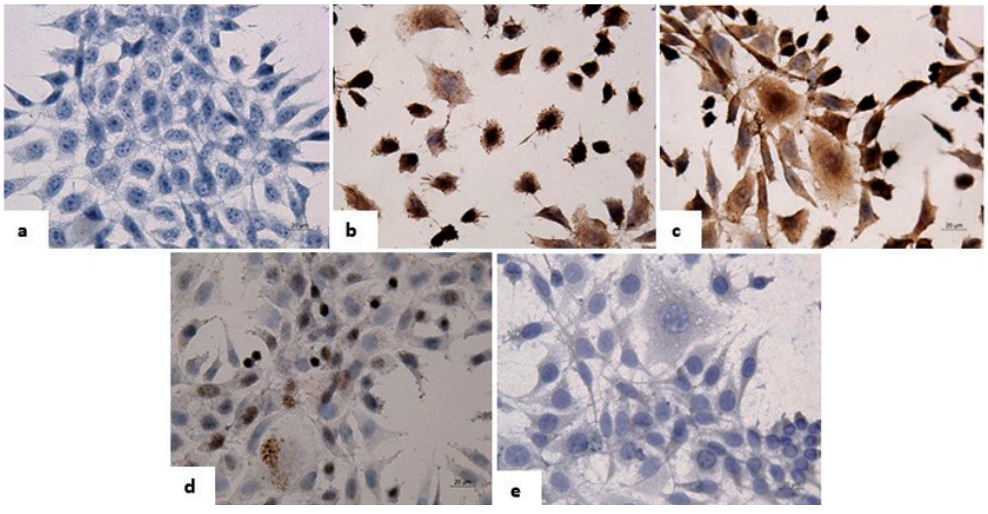


Fig. 3. Human triple negative breast cancer MDA-MB-231 cells. Negativ control – (**a**) and treated with an autophagy inducer Rapamycin applied at a concentyration of 500 nM for various time intervals: 3 hours (**b**), 6 hours (**c**), 24 hours (**d**) and 48hours (**e**). Immunocytochemical reaction with polyclonal antibody against LC3B protein, imaging with chromogen DAB (Diaminobenzidine), Leica DM 5000B, Leica Microsystems, Germany, x40.

the presence of Co-Mel and Ni-Mel (100 µg /ml, 48h) the cytoplasm is greatly increased in volume and a number of autophagosomes are visible near the nucleus, representing vesicular structures positively labeled by the LC3B protein. In the enlightenment of some of the autophagosomes, cellular organelles, which are integrated for the purpose of degradation, are seen (**Fig. 4**).

Because of their remarkable ability to treat pain, inflammation and fever, NSAIDs are among the most often prescribed medications all over the world. Increasing evidence suggests that these preparations also possess potential antitumor properties that are not surprising because of at least two reasons: i) chronic inflammation is well known to be involved in cancer development and progression by inducing proliferation, neoangiogenesis and metastasis as well as facilitating the escape from the immune system and drug resistance of tumor cells; ii) COX-2 is expressed in many types of cancer, including TNBC [1, 8, 11, 16]. Triple negative breast cancer affects younger

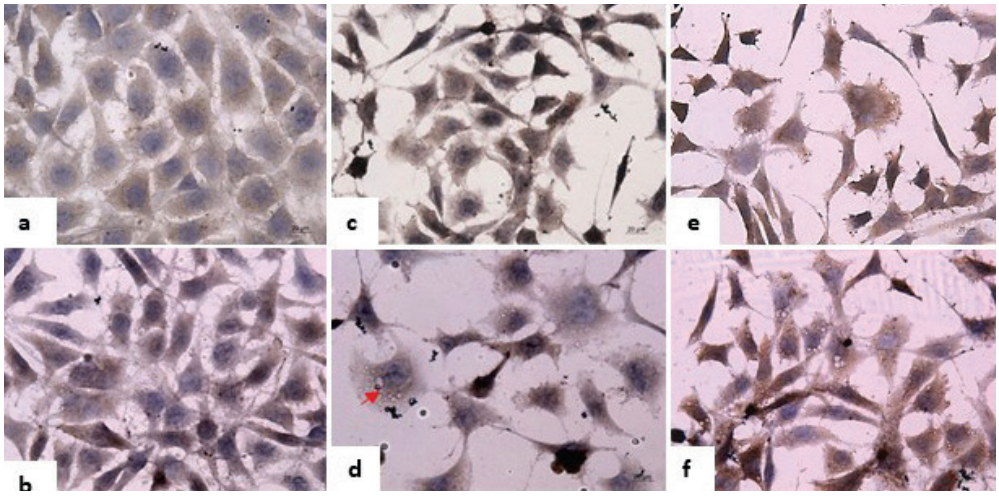


Fig. 4. Non-treated humah triple negative breast cancer MDA-MB-231 cells (culture medium control – **a**) and MDA-MB-231 cells after 48 h treatment with meloxicam **(b)** and its Cu(II) – **(c)**, Co(II) – **(d)**, Zn(II) – **(e)** and Ni(II) – **(f)** complexes at a concentration of 100 μg/ml for 48 hours. Immunocytochemical reaction with polyclonal antibody against LC3B protein, imaging with chromogen DAB (Diaminobenzidine), Leica DM 5000B, Leica Microsystems, Germany, x40. Autophagosomes with integrated cell organelles (CoMel, 100 μg/ml, arrow)

women, usually has an aggressive behavior and is associated with poor prognosis. The treatment of this tumor remains a problem in clinical oncology, as TNBC cells do not express estrogen and progesterone receptors and HER2/neu and therefore do not benefit from the available targeted therapies for breast cancer [11].

The selective non-steroidal anti-inflammatory agent meloxicam has been reported to exhibit antitumor activity alone or in combination with other therapeutic drugs and/or radiation using *in vitro* and *in vivo* experimental models of various human cancers such as osteosarcoma, ovarian cancer, hepatocellular carcinoma [2, 12, 14, 18]. Cardioprotective effect of meloxicam against doxorubicin has been demonstrated in a mouse model of breast cancer [9]. Meloxicam and its Zn(II), Cu(II), Co(II)) and Ni(II) complexes have been found to decrease viability and suppress 2D/3D growth of retrovirus-transformed avian hepatoma (expressing v-myc gene) and rat sarcoma (expressing v-src gene) cells as well as in cell lines established from human cervical carcinoma and glioblastoma multiforme [4, 6]. In this study we report for the first time data about cytotoxic activity of these compounds in MDA-MB-231 human triple negative breast cancer cells and their capacity to induce autophagy. The results obtained indicate that after exposure for 48 hours, the complexes of Co(II) and Ni(II) with meloxicam exhibit pro-autophagic activity in MDA-MB-231 cells, while the cell viability determined by the MTT test is relatively high (≥ 73% for Ni(II)Mel and ≥56% in the case of Co(II) complex with meloxicam), even when the compounds were applied at the maximal examined concentration – 500 μg / ml). Autophagy is not detected in cells incubated in the presence of the compound with the most pronounced cytotoxic activity – Zn-Mel.

The antitumor activity of meloxicam has been associated with its ability to affect apoptotic (i.e. by upregulating Bak and Fas-L, and downregulating survivin and Mcl- 1) and autophagic (PI3K/Akt/mTOR, MAPK/ERK1/2, P53/DRAM, etc.) pathways through COX-dependent and COX-independents mechanisms [5, 15, 18]. It

has been demonstrated that inhibition of autophagy can help to overcome the resistance to meloxicam-induced apoptosis in hepatocellular carcinoma cells [5]. There are data indicating that climacostol, natural compound produced by the ciliated protozoan Climacostomum virens induces apoptosis and impairs autophagy in human and murine melanoma and glioma cells [17].

Autophagy is an evolutionary conservative catabolic process that contributes to maintaining homeostasis in the cell, breaking down damaged and unnecessary macromolecules and organelles, as well as pathogens. Dysregulated autophagy is implicated in various pathological conditions including neoplastic diseases. That is why targeting autophagy is an attractive strategy for cancer treatment. At the same time this approach is very challenging because of the controversial role of autophagy in carcinogenesis – initially suppressing it but subsequently stimulating tumor progression. On one hand, autophagy is essential for a wide range of key biological processes including normal functioning of the immune system and stress response and can inhibit tumorigenesis and progression, therefore induction of autophagy may decrease viability, proliferation, invasiveness and dissemination of tumor cells. On the other hand, the cytoprotective function of autophagy may facilitate the survival and drug resistance of tumor cells [3, 10]. The "situation" becomes more complicated because of interactions between autophagy and apoptosis that are found to share many signaling molecules and pathways but their cross talk is not fully clarified yet.

Conclusion

The anticancer potential of NSAIDs is very attractive because these medications have been used for many years in clinical practice and their pharmacokinetics and pharmacodynamics as well as safety and side effects profiles are well understood. In this study we present for the first time data about cytotoxic activity of selective NSAID meloxicam and its metal [Zn(II), Cu(II), Co(II), Ni(II)] complexes as well as their ability to induce autophagy in MDA-MB-231 cells established from human triple negative breast cancer – an oncological disease whose successful treatment remains a challenge for modern biomedicine. The obtained results suggest that meloxicam and its Zn(II), Cu(II), Co(II) and Ni(II) can be recognized as promising candidates for further evaluation of their potential antitumor, including anti TNBC, properties. The complex role of autophagy in carcinogenesis as well as the relationship between apoptosis and autophagy indicate the need to investigate both the ability to induce apoptosis and autophagy of the potential new antitumor agents.

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