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Research Article

The effects of meloxicam, lornoxicam, ketoprofen, and dexketoprofen on human cervical, colorectal, and mammary carcinoma cell lines

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Received 4 October 2023 • Accepted 12 January 2024 • Published 29 February 2024

Citation: Marinov L, Georgieva A, Toshkova R, Kostadinova I, Mangarov I, Toshkova-Yotova T, Nikolova I (2024) The effects of meloxicam, lornoxicam, ketoprofen, and dexketoprofen on human cervical, colorectal, and mammary carcinoma cell lines. Pharmacia 71: 1–12. https://doi.org/10.3897/pharmacia.71.e113677

Abstract

Targeting the inflammation-related molecules with nonsteroidal anti-inflammatory drugs (NSAIDs) represents a promising approach for cancer prevention/therapy. We evaluated the *in vitro* anticancer effects of meloxicam, lornoxicam, ketoprofen, and dexketoprofen on the proliferation, migration, and apoptosis of human cervical, colorectal, and mammary carcinoma cells. The antiproliferative activity and cytotoxicity of tested NSAIDs on HeLa, HT-29, and MCF-7 cell lines were assessed by the MTT test. The apoptosis-inducing potential was analyzed by fluorescent staining with acridine orange/ethidium bromide and DAPI. Migration activity was assessed by a wound-healing scratch assay. The tested NSAIDs reduced the viability of the used tumor cell lines. The cytomorphological analysis revealed reduced cell density and mitotic activity and the presence of cells with morphological features of early and late apoptosis. Significant inhibition of the migration capacity was established as well. In conclusion, NSAIDs could be candidates for the development of new pharmacological strategies for the treatment and prevention of cancer.

Keywords

anti-proliferative activity, NSAIDs, meloxicam, lornoxicam, ketoprofen, dexketoprofen

Introduction

The association between cancer and inflammation has long been known. However, the possible role of nonsteroidal anti-inflammatory drugs (NSAIDs) in the induction and progression of cancer remains unclear. Some studies revealed a reduced risk of breast, prostate, colorectal, ovarian, and head and neck cancer after NSAIDs therapy, while others demonstrated no association between cancer and NSAID use (Prizment et al. 2010; Wong 2019).

NSAIDs are the most prescribed drugs for the treatment of inflammation, fever, and pain. Their main mechanism of action is the inhibition of cyclooxygenase (COX), an enzyme responsible for the synthesis of prostanoids

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(Ju et al. 2022). In addition to their anti-inflammatory properties, they also have antitumor effects, which include the ability to inhibit cell proliferation and angiogenesis, induce apoptosis, and enhance the cellular immune response (Thunet al. 2002; Hamdani et al. 2015). Meloxicam, lornoxicam, ketoprofen, and dexketoprofen are classified as traditional NSAIDs (Barozzi et al. 2009). Ketoprofen and dexketoprofen are propionic acid derivatives (profens), while meloxicam and lornoxicam are enolic acid derivatives (oxicams). Meloxicam inhibited the growth of colorectal cancer HCA-7 cell lines that express COX-2 but had no effect on the growth of the COX-2 negative HCT-116 cells (Goldman et al. 1998). Meloxicam has also been shown to inhibit the growth and PGE, production of human non-small cell lung cancer cell lines (A549 and PC14), that express COX-2, but not in human small cell lung cancer cell line (H841) that do not express both COX-1 and COX-2 (Tsubouchi et al. 2000). The combination of chemotherapy and meloxicam in prostate cancer cell lines (PC3 and DU-145) demonstrated the growth inhibitory effect of meloxicam and its potential as a possible adjuvant agent to cancer chemotherapy in the treatment of prostate cancer (Montejo et al. 2010). Evidence about the anticancer and antiproliferative effects of lornoxicam is scarce. Its antiproliferative effect may be mediated by a mechanism similar to that of meloxicam.

The antiproliferative effect of ketoprofen on the expression of the HE4 gene and viability of the A2780 human ovarian cancer cell line was demonstrated by Yahyaie et al. (2021). Ketoprofen derivatives showed comparable effects on cell viability of colon carcinoma cells HT-29 and Caco-2 expressing high and low levels of COX-2, respectively. These findings suggest that the profens may also act through COX-independent mechanisms (Santos et al. 2012). Fenoprofen and ketoprofen have modest anti-proliferative activity on different human tumor cell lines and normal human fibroblasts in vitro, whereas their amide derivatives show stronger antiproliferative effect, probably because of a greater lipophilicity and/or better cell uptake (Marjanović et al. 2007). The increased activity of Pt (IV) conjugates of ketoprofen and naproxen on a panel of human tumor cell lines (lung A-549, colon HT-29, HCT 116, SW480, ovarian A2780, and biphasic MPM MSTO-211H), have been demonstrated by Ravera et al. (2019). Arginine-glycine-aspartic acid (RGD) and Asparagine-Glycine-Arginine (NGR) conjugates of ketoprofen were evaluated against a group of cancer cell lines and higher cytotoxic activity was established against OVCAR3 and HT-1-80 cell lines, with overexpression of integrin and aminopeptidase N (two receptors that play important roles in angiogenesis) (Shokri et al. 2018). A ketoprofen-RGD conjugate significantly reduced the mammosphere formation rate and the expression of three out of six stemness markers of breast cancer stem-like cells and parental cells. Ketoprofen-RGD conjugate remarkedly decreased viability and induced apoptosis in these cells compared to controls (Noori et al. 2021). Subcutaneous administration of ketoprofen delays Ehrlich solid tumor growth in mice by inhibiting cell proliferation and angiogenesis (Souza et al. 2014). High cytostatic activity against melanoma and colon cancer cell lines has been reported for carborane-containing derivatives of ketoprofen, which have been associated with the potential of ketoprofen to stimulate autophagy and oxidative stress in cells (Buzharevski et al. 2019). There is data that dexketoprofen has a protective effect against breast and colorectal cancers (Sheehan et al. 1999; Khuder and Mutgi 2001). The underlying mechanism can be explained by the inhibition of COX-2 (Sheehan et al. 1999; Dannenberg et al. 2001), but it could also act similarly to ketoprofen and modulate the shedding of L-selectin.

The present study aimed to evaluate *in vitro* anticancer effects of the following NSAIDs: meloxicam, lornoxicam, ketoprofen, and dexketoprofen on cell viability, proliferation, migration, and apoptosis of the human tumor cell lines HeLa, HT-29, and MCF-7. The signal transduction changes were outside the scope of the present study but be the subject of a future study together with the interaction of those NSAIDs with antineoplastic agents.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM) 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). All other chemicals of the highest purity commercially available were purchased from local agents and distributors. All sterile plastic ware was purchased from Orange Scientific (Belgium).

NSAIDs

Meloxicam (Melbek 15 mg/1.5 mL); lornoxicam (Xefo 4 mg/ml; 2.0 mL); ketoprofen (Profenid 100 mg powder and solvent for solution for injection), and dexketoprofen (Dexofen inject – 50 mg/2 mL solution for injection) were used in the experiments. Solutions of NSAIDs were prepared according to the instructions for each preparation and dilutions with a complete cell culture medium were made to obtain the concentrations desired for the different *in vitro* assays.

Cell lines

Permanent human tumor cell lines – HeLa (cervical cancer), HT-29 (colorectal adenocarcinoma), MCF-7 (adenocarcinoma of the mammary gland) and BALB/3T3 (mouse embryonal fibroblasts) purchased from the American Type Culture Collection (Rockville, MD, USA) were used. HeLa, HT-29 and MCF-7 cells were used in our study as models of some of the most common types of oncologic diseases and BALB/3T3 cells were used as a nontumor control. The cells were cultured in DMEM medium, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin, at 37 °C in a humidified 5% CO₂-incubator (Thermo Scientific, HEPA Class 100). Confluent cell monolayers were trypsinized (a mixture of 0.05% trypsin – 0.02% ethylenediaminetetraacetic acid) and cells were used at the exponentially growing phase.

Cell viability assay

The effects of the NSAIDs on cell viability and proliferation were evaluated using a standard colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay (Mossmann 1983). The test method is based on the formation of violet formazan crystals as a result of the reduction of the yellow tetrazolium salt MTT by the living cells. The formation of formazan crystals is proportional to the activity of mitochondrial enzymes and, accordingly, to cell viability.

Cells in a concentration of 10⁴ cells/well were seeded in 100 µL complete culture medium in 96-well flat-bottom culture plates and pre-cultured for a day before the beginning of treatment. The sub-confluent cells were then exposed for 24 and 48 h to eight different concentrations of the NSAIDs ranging from 3.125 to 800 µg/mL. Each concentration was applied in 6 replicates. Cells grown in a medium without any addition of compounds were used as a control. At the end of the incubation periods (24 and 48 h), 100 µL of MTT (5 mg/mL in PBS) was added to each well, and plates were cultivated at 37 °C for 3 h in the dark. The MTT-containing medium was removed and 100 µL DMSO: ethanol (1:1) was added to each well, and the plates were shaken for 5 min. The optical densities (OD) were measured at 570 nm with a reference wavelength of 630 nm using an ELISA spectrophotometer (TECAN, SunriseTM, Grödig/Salzburg, Austria). The percentage of cell viability was calculated as follows:

> Cell viability (%) = OD of treated cells/ OD of untreated cells × 100

Inhibitory concentration IC_{50} , resulting in a 50% reduction in cell viability, as compared to controls was determined. All assays were performed in triplicate.

Cytomorphological studies

Double staining with acridine orange and ethidium bromide

The tumor cells were grown on sterile cover glasses placed on the bottom of 24-well plates (2.0×10^5 cells/well) for 24 h in a CO₂ incubator to form a cell monolayer. The next day, the NSAID solutions with appropriate concentrations, chosen based on the results of the cell viability assays were added. Meloxicam and lornoxicam were applied at concentrations of 400 µg/mL and dexketoprofen

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at concentrations of 800 μ g/mL for all cell lines. Ketoprofen was used in a concentration of 200 μ g/mL for the MCF-7 cells and 800 μ g/mL for the HeLa and HT-29 cells. Tumor cells from the respective cell line, cultured only in medium served as controls. After 24 h of incubation, the coverslips were removed and washed twice with phosphate-buffered saline (PBS). Equal volumes of fluorescent dyes containing AO (10 μ g/mL in PBS) and EtBr (10 μ g/ mL in PBS) were added to the cells. Freshly stained cells were placed on a glass slide and examined immediately under a fluorescence microscope (Leica DM 5000B, Wetzlar, Germany) before the fluorescent color started to fade.

DAPI staining to assess nuclear morphology

The cells were seeded, treated, and cultured as described in the previous paragraph. After incubation, the glass lamellae were washed twice with phosphate-buffered saline (PBS) to remove non-adhered tumor cells. DAPI staining of the cells was performed after fixation with 3% paraformaldehyde according to the instructions of the manufacturer's protocol. Samples of treated and untreated cells were coated with Mowiol, mounted on slides, and stored in the dark until examination with a fluorescence microscope (Leica DM 5000B, Wetzlar, Germany).

Wound-healing assay

To investigate the migration of tumor cells treated with NSAIDs, a wound-healing scratch assay was performed as previously described (Nikolova et al. 2018). The tumor cells $(2 \times 105 \text{ cells}/1\text{mL/per well})$ from the respective line suspended in DMEM medium with 10% FBS and antibiotics were seeded in the 24-well plates and cultured till the formation of an almost confluent (>90%) monolayer. Cell monolayers were scratched in a single stripe using a 200 µl pipette tip, and then rinsed several times with phosphate-buffered saline (PBS) to remove cell debris (detached cells). Cell monolayers were then treated with NSAIDs at concentrations of approximately $\frac{1}{2}$ of the IC₅₀ values determined by the MTT test at 48h. The plates were cultured in a CO₂ thermostat under standard conditions. Monolayers of tumor cells with vertical wounds, cultured only in medium, served as controls. The wound was allowed to heal for 72 h at 37 °C. The average extent of the wound closure was evaluated by measuring the width of the wound. For this purpose, microphotographs of the cell cultures were taken at the beginning of the experiments and after regular intervals (24, 48, and 72 h), using a phase-contrast microscope Olympus, equipped with a digital camera. The surface of the wound area from three independent experiments was measured using ImageG software and the migration activity of the control and treated tumor cells was expressed as a percentage of the wound closure.

Statistical analysis

The data were analyzed by the one-way analysis of variance (ANOVA) using GraphPad PRISM (Version 5) and presented as mean \pm standard deviation (SD). Values of $\rm P < 0.05$ were considered statistically significant. Nonlinear regression (curve fit) analysis (GraphPad Prism) was applied to determine the concentrations inducing 50% inhibition of the cell growth (IC_{50} values).

Results

Effects of the NSAIDs on the viability and proliferation of human cancer cell lines

The NSAIDs meloxicam, lornoxicam, ketoprofen, and dexketoprofen induced statistically significant and concentration-dependent reduction in the viability of the cervical, colorectal, and mammary carcinoma cell lines HeLa, MCF-7, and HT-29, respectively (Fig. 1).

The treatment of HeLa and HT-29 tumor cells with meloxicam for 24 h induced significant antiproliferative and cytotoxic effects at all tested concentrations higher than 6.3 μ g/mL. At the highest concentration used in the experiments, the viability of HeLa and HT-29 cells was 79% and 75% compared to the control, respectively. The inhibitory effects enhanced at 48 h of treatment and statistical significance of the differences between the control and treated cells was established at all used concentrations. The cell viability values of the cervical and colorectal carcinoma cells recorded at 48 h of exposure ranged from 83% to 43% and from 87% to 47%, respectively. Meloxi-



Figure 1. Effects of the NSAIDs meloxicam, lornoxicam, ketoprofen, and dexketoprofen on the cell viability and proliferation of the human tumor cell lines HeLa, HT-29, and MCF-7 and the nontumor cell line BALB/3T3.

cam significantly inhibited the proliferation of MCF-7 tumor cells at concentrations higher than 50 µg/mL at 24 h and higher than 100 µg/mL at 48 h. The mean cell viability at 24 h ranged between 84.86 ± 6.65% and 70.14 ± 3.54%, and between 87.35 ± 3.03% and 82.64 ± 2.36% at 48 h.

Lornoxicam induced a statistically significant reduction of the viability of HeLa tumor cells only at concentrations of 200 µg/mL and 400 µg/mL after 24 h and at all tested concentrations after 48 h of exposure. The cell viability values for 24 h were 88.36 \pm 0.30% and 79.95 \pm 0.53%, and at 48 h were in the range between 87.91 \pm 2.12% and 40.69 \pm 2.45%. The antiproliferative effect of lornoxicam in HT-29 cells was observed at concentrations of 100 µg/mL, 200 µg/mL and 400 µg/mL at both time intervals, with corresponding values of 92.58 \pm 2.72%, 82.50 \pm 2.99%, and 70.38 \pm 2.09% at 24 h and 77.24 \pm 4.49%; 52.09 \pm 2.58%, and 38.93 \pm 3.10% at 48 h. Lornoxicam showed no statistically significant effect on the cell viability of MCF-7 cells at all applied concentrations and time intervals.

A statistically significant cytotoxic effect of ketoprofen against HeLa tumor cells was observed at concentrations of 200 μ g/mL and 400 μ g/mL at 24 h (78.15 ± 4.39 % and $49.90 \pm 2.16\%$, respectively), and all concentrations higher than 25 μ g/mL at 48 h (with values from 85.60 \pm 3.12% to $22.73 \pm 0.93\%$). Ketoprofen significantly decreased the viability of HT-29 tumor cells at all concentrations studied. The mean values of the cell viability recorded at 24 h were in the range from $87.03 \pm 1.75\%$ to $55.54 \pm 5.13\%$ and at 48 h – from $82.92 \pm 4.40\%$ to $24.62 \pm 2.24\%$. The effect was strongest at a concentration of 800 µg/ml at 24 and 48 h. In MCF-7 tumor cells, ketoprofen was effective at concentrations higher than 25 µg/mL at 24 h with cell viability values of $84.92 \pm 6.54\%$ to $30.41 \pm 2.53\%$ and at concentrations higher than 50 µg/mL at 48 h with values from $78.12 \pm 5.30\%$ to $20.50 \pm 2.35\%$, with a dose-dependent effect.

Only the concentration of 800 µg/mL of dexketoprofen inhibited the proliferation of HeLa cells at 24 h (75.69 ± 2.82%). At 48 h, concentrations of 400 µg/mL and 800 µg/ mL were effective with values of $85.74 \pm 13.53\%$ and $44.84 \pm 7.74\%$, respectively. High concentrations (of 200 µg/mL, 400 µg/mL, and 800 µg/mL) at 24 h and all concentrations The cytotoxicity of the tested drugs was also assessed on the nontumor cell line BALB/3T3. As evident from Fig. 1, in most of the cases normal cell appeared to be less sensitive to the effects of the tested NSAIDs than the tumor cells.

The half maximum inhibitory concentrations (IC_{50}) (µg/mL) of the tested NSAIDs, calculated by nonlinear regression analysis of the dose-response curves (percentage of living cells relative to the concentration of the corresponding NSAIDs) were determined. The data are presented in Table 1.

The IC₅₀ value is the concentration required to reduce cell viability by 50%; therefore, the lower the IC₅₀ concentration, the higher the cytotoxicity of the tested compound. The lowest IC₅₀ values were observed in MCF-7 cells at 24 h and 48 h after treatment with ketoprofen, and in Hela and HT-29 cells at 48 h after treatment with lornoxicam. The remaining IC₅₀ values were higher than the maximal concentration used in the present study (Table 1).

The blood plasma concentration of ketoprofen in therapeutic doses according to Schulz et al. (2020) is between $1-6 \mu g/ml$. IC₅₀ value of ketoprofen on the 24 h in MCF-7 is 241.2 $\mu g/mL$ which cannot be clinically achived. For the other three NSAIDs the IC₅₀ values are also not clinically achievable (Schultz et al. 2020) (Table 2).

Cytomorphological studies

Double staining with acridine orange and ethidium bromide

Cytomorphological changes at the cell and nucleus level that occurred after treatment with NSAIDs visualized by live/dead AO/EtBr staining are presented in Fig. 2.

Control HeLa tumor cells were slightly elongated in shape, monolayered, uniformly green in color, with one or more bright green nuclei, and perinuclear orange-colored granules (Fig. 2a). After treatment with meloxicam, impaired monolayer growth and morphological changes characteristic of early and late apoptosis were observed – round cells, cell membrane blebbing, chromatin condensation, nucleus fragmentation and apoptotic body formation (Fig. 2d). Lornoxicam also caused a change in the growth and morphology of HeLa cells, but cells with early

Table 1. Inhibitory concentrations IC_{50} (µg/mL) of the NSAIDs meloxicam, lornoxicam, ketoprofen, and dexketoprofen, established by MTT test after exposure of human tumor cells and nontumorigenic cells for 24 and 48 h.

Cell lines	Lornoxicam		Meloxicam		Ketoproten		Dexketoprofen	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
HeLa	>400 (1.1 mM)	235.6 (0.6 mM)	>400 (1.4 mM)	529.3 (1.5 mM)	852.5 (3.1 mM)	455.9 (1.8 mM)	>800 (3.1 mM)	744.6 (2.9 mM)
HT-29	>400 (1.1 mM)	258.8 (0.7 mM)	>400 (1.4 mM)	973.9 (2.8 mM)	>800 (3.1 mM)	489.4 (1.9 mM)	>800 (3.1 mM)	541.6 (2.1 mM)
MCF-7	>400 (1.1 mM)	>400 (1.4 mM)	>400 (1.4 mM)	>400 (1.4 mM)	241.2 (0.9 mM)	360.9 (1.4 mM)	818.7 (3.2 mM)	917.0 (3.6 mM)
BALB/3T3	>400 (1.1 mM)	>400 (1.4 mM)	>400 (1.4 mM)	>400 (1.4 mM)	>800 (3.1 mM)	>800 (3.1 mM)	>800 (3.1 mM)	>800 (3.1 mM)

at 48 h of dexketoprofen reduced the viability of HT-29 and MCF-7 cells. The lowest values of HT-29 cell viability were observed at a dose of 800 μ g/mL – 79.39 \pm 2.66% and 32.82 \pm 3.87% at 24 and 48 h, respectively. For MCF-7 cells, the values were 46.32 \pm 4.55% and 45.98 \pm 3.87% at 24 and 48 h).

Table 2. Therapeutic blood-plasma concentration (µg/mL).

Substance	Blood-plasma concentration (µg/mL)
Lornoxicam	0.1-0.8
Meloxicam	0.4-2
Ketoprofen	1-6
Dexketoprofen	appr. 3.7



Figure 2. Fluorescence microscopy of human tumor cells HeLa, HT-29, and MCF-7 treated with the NSAIDs meloxicam, lornoxicam, ketoprofen, and dexketoprofen. Control (**a**-**c**); HeLa cells (**a**, **d**, **g**, **j**, **m**); HT-29 cells (**b**, **e**, **h**, **k**, **n**); MCF-7 cells (**c**, **f**, **i**, **l**, **o**); Meloxicam (**d**-**f**); Lornoxicam (**g**-**i**); Ketoprofen (**j**-**l**); Dexketoprofen (**m**-**o**); Lens 40X; AO/EtBr staining.

apoptotic changes predominated and single cells with late apoptotic characteristics were seen (Fig. 2g). Ketoprofen and dexketoprofen caused identical morphological changes in HeLa tumor cells similar to those described for meloxicam – rounded cells with typical signs of early and late apoptosis (Fig. 2j, m).

Control-untreated HT-29 tumor cells were round, with monolayer and three-dimensional growth (glandular-like

structures), uniformly green with one or more light green nuclei and light orange granules in the cytoplasm (Fig. 2b). Treatment with meloxicam and lornoxicam led to changes in cell growth and morphology – there was a reduction in cell number, impaired monolayer and three-dimensional growth, the presence of single (when exposed to lornoxicam) and a significant number (when exposed to meloxicam) of cells with optically empty (round) spaces, cells with morphological features of early and late apoptosis (Fig. 2e, h). Changes in growth and morphology (reduced cell number, single cells with optically empty circular spaces in the cytoplasm, cells with signs of early and late apoptosis) of HT-29 cells after treatment with ketoprofen and dexketoprofen were similar (Fig. 2k, n).

Control untreated MCF-7 tumor cells were elongated, uniformly green in color with several light green nuclei and light orange granules in the cytoplasm, and with monolayer growth (Fig. 2c). Typical apoptotic changes (round cells, cell membrane blebbing, condensed chromatin in the form of bright green luminous dense areas, nucleus fragmentation, and apoptotic bodies) were characteristic of almost all MCF-7 cells treated with meloxicam and lornoxicam (Fig. 2f, i). The presence of entoses was also observed after treatment with meloxicam (Fig. 2f). Cultivation of MCF-7 tumor cells with ketoprofen led to less pronounced morphological changes, but typical of cell death by apoptosis - cell membrane blebbing, condensed chromatin, nuclear fragmentation, and apoptotic bodies (Fig. 2l). Dexketoprofen caused extremely severe morphological changes in MCF-7 tumor cells indicative of apoptosis. Most treated cells had cell membrane blebbing, condensed chromatin, nuclear fragmentation, and apoptotic bodies. Single necrotic cells were also observed (Fig. 2o).

DAPI staining

To assess the nuclear morphology of NSAID-treated and untreated (control) tumor cells, fluorescent dye staining with 4',6-diamino-2-phenylindole (DAPI) was applied. The DAPI molecule can cross the intact cytoplasmic membrane, making it a suitable agent for studying the nuclear morphology of both living and fixed cells. The results are presented in Fig. 3.

The nuclei of control untreated HeLa tumor cells were intact, approximately uniform in shape and size, with smooth edges and homogeneously distributed chromatin (Fig. 3a). As a result of treatment with meloxicam, changes occurred in the nuclei of HeLa cells, expressed in different in shape and size nuclei with inhomogeneously distributed chromatin with granular structure, fragmentation of the nuclei, and formation of apoptotic bodies (Fig. 3d). The nuclei of HeLa tumor cells treated with lornoxicam had apoptotic morphology, they were pale blue, with condensed granular chromatin and fragmentation (Fig. 3g). Similar apoptotic changes in the nuclei, such as chromatin condensation, nucleus fragmentation, and apoptotic bodies, were observed after treatment of HeLa tumor cells with ketoprofen and dexketoprofen, with the severity of the changes being more obvious with dexketoprofen treatment (Fig. 3j, m).

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Control HT-29 tumor cells had intact nuclei, uniform in shape and size, with smooth edges, and evenly distributed nuclear chromatin (Fig. 3b). The nuclei of HT-29 cells treated with meloxicam and lornoxicam were different in size, with uneven edges, unevenly colored, with highly condensed and aggregated chromatin, some were fragmented with the presence of apoptotic bodies (Fig. 3e, h). The nuclei of HT-29 cells cultured in the presence of ketoprofen and dexketoprofen have clear signs of apoptosis – uneven edges, condensed, unevenly distributed chromatin, single fragmented nuclei, and apoptotic bodies (Fig. 3k, n).

Control MCF-7 tumor cells had intact nuclei, uniform in shape and size, with smooth edges, and uniformly distributed nuclear chromatin, with one to several nucleoli in the nucleus. There were nuclei of cells in mitosis (Fig. 3c). Treatment with meloxicam and lornoxicam led to condensation and margination of chromatin in the nuclei of MCF-7 cells, fragmentation, and formation of apoptotic bodies (Fig. 3f, i). The apoptotic changes in the nuclei of MCF-7 cells treated with ketoprofen and dexketoprofen were identical to the described, but more pronounced – enlarged nuclei with uneven edges, highly condensed chromatin, fragmented nuclei, and apoptotic bodies. Cell nuclei after entosis process were seen (Fig. 3o).

Wound-healing assay

The migration of human tumor cells treated with NSAIDs was studied using an in vitro wound healing method in which scrape wounds were induced in monolayer cell culture. The results are presented in Fig. 4.

The migration of HeLa tumor cells cultivated in the presence of NSAIDs was inhibited with the best effect for meloxicam and dexketoprofen at 24 h ($39.59 \pm 3.37\%$ and $45.17 \pm 2.41\%$ respectively). At the same time $88.92\pm1.35\%$ of the wound area was filled in control cultures. At 48 and 72 h the highest inhibitory effect was observed for dexketoprofen ($65.26 \pm 2.09\%$ and $81.04 \pm 1.94\%$ respectively). The decrease in the HeLa cell migration capacity in the presence of lornoxicam was much weaker. At 24 h, the wound was filled to $82.18 \pm 1.93\%$. Ketoprofen did not affect the migration of HeLa cells and the values were the same as in the control (100% wound healing).

Statistically significant inhibition of HT-29 cell migration was observed after treatment with lornoxicam, ketoprofen, and dexketoprofen. In the control cell cultures, the migration percentages for the three-time intervals were $14.05 \pm 0.66\%$, $25.81 \pm 0.90\%$, and $27.43 \pm 0.50\%$. For lornoxicam, the migration percentages were $12.34 \pm 9.57\%$, $18.28 \pm 0.99\%$, and $18.61 \pm 0.62\%$ at 24, 48 and 72 h, respectively. The effect of ketoprofen and dexketoprofen was well expressed in all three time intervals with corresponding values of $5.87 \pm 0.7\%$; $6.10 \pm 1.67\%$; $9.65 \pm 0.95\%$ and $7.50 \pm 1.76\%$; $15.60 \pm 1.90\%$; $17.98 \pm 2.9\%$.

All four NSAIDs used showed statistically significant and time-dependent inhibition of MCF-7 cell migration at all time intervals studied. The migration percentages of the untreated cell cultures were $50.75 \pm 0.65\%$, $86.49 \pm 0.65\%$,



Figure 3. Fluorescence microscopy of human tumor cells HeLa, HT-29, and MCF-7 treated with the NSAIDs meloxicam, lornoxicam, ketoprofen, and dexketoprofen. Control (**a**–**c**); HeLa cells (**a**, **d**, **g**, **j**, **m**); HT-29 cells (**b**, **e**, **h**, **k**, **n**); MCF-7 cells (**c**, **f**, **i**, **l**, **o**); Meloxicam (**d**–**f**); Lornoxicam (**g**–**i**); Ketoprofen (**j**–**l**); Dexketoprofen (**m**–**o**); Lens 40X; DAPI staining.

and 100% for 24 h, 48 h, and 72 h. The highest inhibition was reported after treatment with lornoxicam (the migration percentages for the three time intervals were 3.85 \pm 1.77%, 7.77 \pm 1.49%, and 9.06 \pm 1.59%, respectively). Ap-

proximately 5–6-fold lower inhibition of MCF-7 cell migration was observed for meloxicam (15.03 \pm 0.54%; 39.75 \pm 2.47%; 58.85 \pm 2.98%, at the same time intervals). After treatment with dexketoprofen and ketoprofen, the highest



Figure 4. Effects of the NSAIDs meloxicam, lornoxicam, ketoprofen, and dexketoprofen on the migration capacity of the human tumor cell lines HeLa, HT-29, MCF-7.

inhibition of migration was established at 24 h – 20.21 \pm 2.687 and 40.42 \pm 1.61, respectively.

Meloxicam, lornoxicam, ketoprofen, and dexketoprofen inhibited the migration of the tumor cells. According to the results of the wound healing assay, the most sensitive to the NSAID treatment was MCF-7, followed by HeLa and HT-29 cells.

Discussion

The results of the study showed that NSAIDs altered the migration of treated tumor cells. MCF-7 cells were the most sensitive to the NSAIDs, followed by HeLa and HT-29 cells. Dexketoprofen inhibited the migration of HeLa, MCF-7, and HT-29 tumor cell lines at 24, 48, and 72 h with the strongest effect in HeLa carcinoma cells. Lornoxicam decreased cell migration of the three lines, but at different time intervals (in HeLa at 24h and 48h; in HT-29 at 48 and 72 h) and it was the most active for MCF-7 tumor cells. Meloxicam inhibited the migration of HeLa and MCF-7 tumor cells and did not affect the migration of HT-29 cells. Ketoprofen was ineffective in HeLa cells but affected the migration of MCF-7 and HT-29 cells in the three-time intervals. NSAID-induced inhibition of tumor cell migration was the most pronounced in mammary carcinoma cells. The oxicams, meloxicam, and lornoxicam, induced stronger inhibition of MCF-7 tumor cell migration than the profens, ketoprofen, and dexketoprofen. In the present study, the cell viability after treatment with NSAIDs showed different degrees of reduction. Ketoprofen and dexketoprofen decreased the viability of the three tumor cell lines with the strongest effect on MCF-7. Lornoxicam and meloxicam had a more pronounced inhibitory effect on HT-29 and HeLa cells and had a little (meloxicam) and no effect (lornoxicam) on MCF-7. The lowest IC₅₀ values were observed in MCF-7 cells at 24 and 48 h after treatment with ketoprofen and in Hela and HT-29 cells at 48 h after treatment with lornoxicam. Based on these findings, it could be concluded that breast cancer cells were more sensitive to the cytotoxic effects of profens, while the viability of cervical and colon carcinoma cells was more significantly affected by the oxicams. The IC_{50} values of the tested NSAIDs in the three tumor lines were higher than those obtained with diclofenac treatment in our previous studies and comparable to those found for metamizole in the same human tumor lines (Nikolova et al. 2018; Marinov et al. 2021). Our results were supported by data from Jongthawin et al. 2012; Li et al. 2006; Montejo et al. 2010; and Goldman et al. 1998, in which meloxicam showed greater anticancer activities in various cell lines like a human colorectal, hepatocellular, cholangiocarcinoma, osteosarcoma, non-small cell lung cancer, prostate cancer cell lines. The proliferation of M139 and M214 human cholangiocarcinoma cell lines was inhibited compared with untreated control cells, after exposure to 400 and 600 μ M of meloxicam for 24, 48, and 72 h (p < 0.0001), while concentrations equal to or lower than 100 µM had

no effect (Jongthawin et al. 2012). A lower concentration of meloxicam (50 μ M) inhibited cell growth of the human HepG2 hepatocellular carcinoma cells (Li et al. 2006).

The obtained results for ketoprofen are also supported by Yahyaie et al. (2021) who show the displayed anticancer effect through reduction in the expression of the HE4 gene and the survival of ovarian cancer cells A2780S. The percentage of living cells decreased significantly from 82.21 \pm 2.5 (at a concentration of 100 μ M) to 43.43 \pm 5.15 (at a concentration of 1000 μ M). Yahyaie et al. (2021) reported IC₅₀=583.7 μ M for ketoprofen at 24 h, in comparison to IC₅₀=241.2 μ g/mL at 24 h in MCF-7 cells, followed by HT-29 IC₅₀=489.4 μ g/mL at 48 h and HeLa IC₅₀=852.5 μ g/mL at 24 h, respectively in our experiment.

 IC_{50} =241.2 µg/mL at 24 h; IC_{50} =360.9 µg/mL at 48 h, followed by HT-29 IC_{50} =489.4 µg/mL at 48 h and HeLa – IC_{50} =852.5 µg/mL and IC_{50} =455.9 µg/mL at 24 and 48 h respectively.

A reduction of the tumor's weight in ketoprofen-treated Wistar rats with ovarian cancer was shown and this effect was probably associated with an increase of apoptosis, inhibition of angiogenesis, reduction of cell proliferation, and biological modification of the tumor by inhibiting COX (Keramati et al. 2012). The inhibition of COX-2 by ketoprofen is an important step in the treatment and prevention of colon and cervix cancer (Damnjanovic et al. 2015). Ketoprofen-loaded nanocapsules (Keto-NCs) reduced the survival of the glioma cell line while causing no toxicity to astrocytes (da Silveira et al. 2013). The mechanism underlying these biological changes is unclear because various factors are involved in how COX affects living organisms. The anticancer activity data for dexketoprofen and lornoxicam is scarce and our research may provide impetus for new studies.

The *in vitro* antitumor effects of COX inhibitors may differ significantly between cancer cell lines, even when possessing similar COX-2 selectivity (Waskewich et al. 2002). The observed difference in the action of the studied NSAIDs can be explained on the one hand by their different chemical groups and structures, and on the other hand by the different expression of COX enzymes in tumor cells.

Conclusion

The NSAIDs meloxicam, lornoxicam, ketoprofen, and dexketoprofen exerted significant anticancer effects in *in vitro* models of some of the most common types of human epithelial neoplasms, expressed by decreased cell viability, inhibition of tumor cell proliferation and migration, and induction of apoptotic cell death. The different tumor cell lines showed different sensitivity towards the tested NSAIDs. Based on our results, it could be concluded that breast cancer cells were more sensitive to the cytotoxic effects of propionic NSAIDs, while the viability of cervical and colon carcinoma cells was more significantly affected by the oxicams. NSAID-induced inhibition of tumor cell migration was most pronounced in mammary carcinoma

cells. The oxicams meloxicam and lornoxicam induced stronger inhibition of MCF-7 tumor cell migration than the propionic derivatives ketoprofen and dexketoprofen. The results of performed fluorescent microscopy studies indicated that the apoptotic morphological alterations

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were most clearly expressed in NSAID-treated breast cancer cells. The present results indicate that NSAIDs have anticancer potential and could be used together with conventional chemotherapeutics to develop new pharmacological strategies for the treatment and prevention of cancer.

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