MOLECULAR MECHANISMS OF POTENTIAL SYNERGISTIC EFFECT OF KETOPROFEN AND MELOXICAM WITH CONVENTIONAL CYTOSTATICS IN HUMAN CERVIX CANCER CELL LINE

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Cyclooxygenases clearly appear to be implicated in carcinogenesis. It has been reported that COX-2 is active throughout the entire process of cancer development and progression. Various molecular mechanisms may be responsible for this. Epidemiological and experimental studies have revealed that nonselective non-steroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors can reduce the risk of cancer. Inhibition of COX provides a plausible explanation of the data on NSAIDs and cancer. However, the molecular pathways of this effect are still unclear, more complex and likely involve multiple COX-2-dependent and independent mechanisms, where pro- and anti-apoptotic Bcl-2 family members may take part.

We examined the effects of ketoprofen (KT), as nonselective COX-1/2, and meloxicam (MK), as selective COX-2 inhibitor, alone and combined with 5-fluorouarcil (FU) and cisplatin (CP), on the proliferation by MTT test and Bcl-2/Bax expression in HeLa cells (human cervical carcinoma cells).

MC alone or combined with conventional anticancer drugs, FU and CP, showed better cytotoxic and antiproliferative effect than KT. The levels of Bcl-2 were decreased while the levels of Bax were increased dose-dependently by KT and MC. A significant increase in the expression of Bax protein in HeLa cells was more pronounced for MC.

The synergy observed in the effects of ketoprofen and meloxicam with cisplatin and 5-fluorouracil on the cervical cancer cell line was generated by an enhancement of apoptosis. Therefore, ketoprofen and meloxicam may represent therapeutic candidates to improve access of cervical cancer chemoprevention and chemotherapy.
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Introduction

Chronic inflammation and overexpression of cyclooxygenase enzymes (COX) take part in the development of epigenetic changes caused by environment/lifestyle factors that contribute to the accumulation of genetic mutations associated with cancer development and progression (1). COX enzymes clearly become dysregulated in cancers and all previous research indicate that these metabolic pathways are involved in carcinogenesis and tumor progression (2). COX-1 is up-regulated in cervical and ovarian cancers (3). On the other hand, COX-2, which is normally undetectable in healthy tissue, is markedly overexpressed in colorectal (4), lung (5), prostate (6), cervical (7), ovarian (8), breast, gastric, pancreatic (9) and certain head and neck squamous cell cancers (10). COX-2 is commonly found in premalignant lesions, carcinoma in situ, invasive cancer, and metastatic disease. It is required throughout the entire evolutionary process of cancer development and progression. Various molecular mechanisms may be responsible for the initiation and promotion of carcinogenesis by COX-2 (11). COX-2 expression in tumors is associated with aggressive tumor growth, increased propensity of tumors to

metastasize, resistance to standard radiotherapy and chemotherapy, and poor prognosis (12).

A large number of epidemiological and experimental studies have revealed that prolonged treatment with non-steroidal anti-inflammatory drugs (NSAIDs), which are COX inhibitors, can reduce the risk of cancer (13). NSAIDs inhibit cell proliferation and induce apoptosis of a number of cancer cells in vitro and in vivo, which is considered to be an important mechanism for the anti-tumor and chemopreventive activity of NSAIDs (14). However, the molecular pathways of this process are still unclear. Selective COX-1/2 and nonselective COX inhibitors modulate the cell cycle machinery at several sites, which may explain some of their antiproliferative/apoptotic effects (15). The proapoptotic effects and the chemopreventive potential of NSAIDs cannot be accounted only by COX inhibition alone. NSAIDs have been shown to inhibit proliferation and induce apoptosis in malignant cell lines which do not express either COX-1 or COX-2 (16). Overexpression of COX enzymes can be associated with changes in expression of members of Bcl-2 family which may influence the apoptosis (17).

The aim of this study was to examine the effects of ketoprofen (KT), as nonselective COX-1/2, and meloxicam (MK), as selective COX-2 inhibitor, alone and combined with 5-fluorouarcil (FU) and cisplatin (CP), on the proliferation and Bcl-2/Bax expression in human cervix cancer cell line (HeLa).

Material and methods

Reagens

In this experiment we used commercial preparations of ketoprofen, meloxicam, cisplatin, and 5-fluorouracil, which are used in conventional clinical protocols for the treatment of colon and cervix carcinoma (18,19). Ketoprofen was purchased from Sandoz Pharmaceuticals, Switzerland (Ketonal®, 100 mg/2 mL), meloxicam from Boehringer Ingelheim, Espana S.A. (Movalis®, 15 mg/1.5 mL), 5-fluorouracil from Pharmachemie BV - Netherlands (Fluorouracil-TEVA®, 50 mg/mL) and cisplatin from Ebewe Pharma Austria (Cisplatin Ebewe®, 10 mg/20 mL). DMEM (Dulbecco's Modified Eagle Medium), FBS (Fetal Bovine Serum), antibiotic/antimycotic solution, L-glutamine and Trypsin-EDTA solution were purchased from PAA Laboratories (PAA Laboratories, Austria) and 3-(4,5-dimethylthiazol-2-yl)-2,5–diphenyltetrazolium bromide (MTT) was purchased from Carl Roth (Carl Roth, Germany). Trypan blue stain was purchased from Invitrogen. Primary anti-Bcl-2 and anti-Bax antibodies and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell line

In this study we used HeLa S3 human cervical carcinoma cell line obtained from the Leibniz Institute DSMZ. Cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine and antibiotic/antimycotic solution at 37 ºC in an atmosphere with 5% CO2 and saturated humidity. Replacement of the culture medium was performed every 2 to 3 days.

Treatment of cells

Confluent culture of HeLa cells was harvested using Trypsin-EDTA solution, washed in buffer solution and the total number of cells was determined by Trypan blue dye exclusion test. Cells were seeded in 96-well plates (Greiner Bio-One, Germany) at density 3 x 104 cells per well and cultured for 24 h under standard cell culture conditions. After that, the examined compounds, alone or in combination, were added to the cells. KT, MC, CP, and FU were diluted in DMEM and three concentrations of each of these compounds were tested (group 1 – the lowest concentration, group 2 – middle concentration and group 3 – the highest concentration). Final concentrations of the tested compounds were the following: 2, 20, and 200 µM of KT, 10 µM, 50 µM, and 500 µM of MC, 1.66, 3.32, and 6.64 µM of CP and 10, 100, and 1000 µM of FU. We have also combined KT and MC with CP and FU, respectively. Combining was performed using compounds' concentrations from the same group as following: group 1 with group 1, group 2 with group 2 and group 3 with group 3 in the ratio 1:1 so the effective concentrations of compounds in combinations were twice less than the concentrations of compounds that were applied alone. As control, we used cells that were incubated only with completed cell culture medium, DMEM, without the assayed compounds. Cells were incubated with examined compounds for the next 48 h. After that, cell growth was examined by performing MTT test and the level of Bcl-2 and Bax proteins was also measured.

MTT test

Cell growth was examined by performing MTT test according to the protocol by Damnjanovic et al. [20] Results are presented as: the mean value of absorbance ± standard deviation from four to eight replications for the assayed compounds as well as control.

Measurement of Bcl-2 and Bax protein levels

For determining the levels of Bcl-2 and Bax proteins, the cells were treated as it was described in the section "Treatment of cells". After 48 h of incubation with the assayed compounds, cells were further processed according to the protocol by Kocic et al. [21] Briefly, the cells were washed with phosphate-buffered saline (PBS), fixed by using 70% methanol and permeabilized with 0.1% Triton in PBS. The cells were incubated with the primary anti-Bax and anti-Bcl-2 antibodies, washed three times and incubated with the FITC-conjugated secondary antibodies. The

mean fluorescence intensity (MFI; logarithmic scale) was determined and analyzed on a Victor™ multi-plate reader (Perkin Elmer-Wallace, Wellesley, MA). The presented results were obtained following the subtraction of blank values obtained by the treatment with the secondary antibodies only.

Statistical analysis

The data were analyzed by the commercially available statistics software package (SPSS for Windows®, v. 17.0, Chicago, USA) using the Student's t-test and the ANOVA test. The results were presented as mean ± SD. The statistical significance was set to $p < 0.05$.

Investigations and results

The effect of different concentrations of KT and MC alone as well as combined with CP and FU on the growth of HeLa cells

As shown in Figure 1, HeLa cells treated with three different concentrations of CP and FU showed a statistically significant decrease in cell growth compared to the control group. MC reduced the growth of HeLa cells in a dose-dependent manner after 48 h. The combination of KT and CP showed a statistically significant dose-dependent decrease in cell growth in comparison with the control group, until the combination of KT and FU showed a statistically significant effect on cell growth only when middle examined concentration was used, as compared to the control group. The combination of MC and CP showed a statistical significance in the effect on cell growth in dose dependent manner, as compared to the control group. All tested concentrations of MC-FU combination showed a statistical significance in the effect on HeLa cells in comparison with the control group (Figure 1).
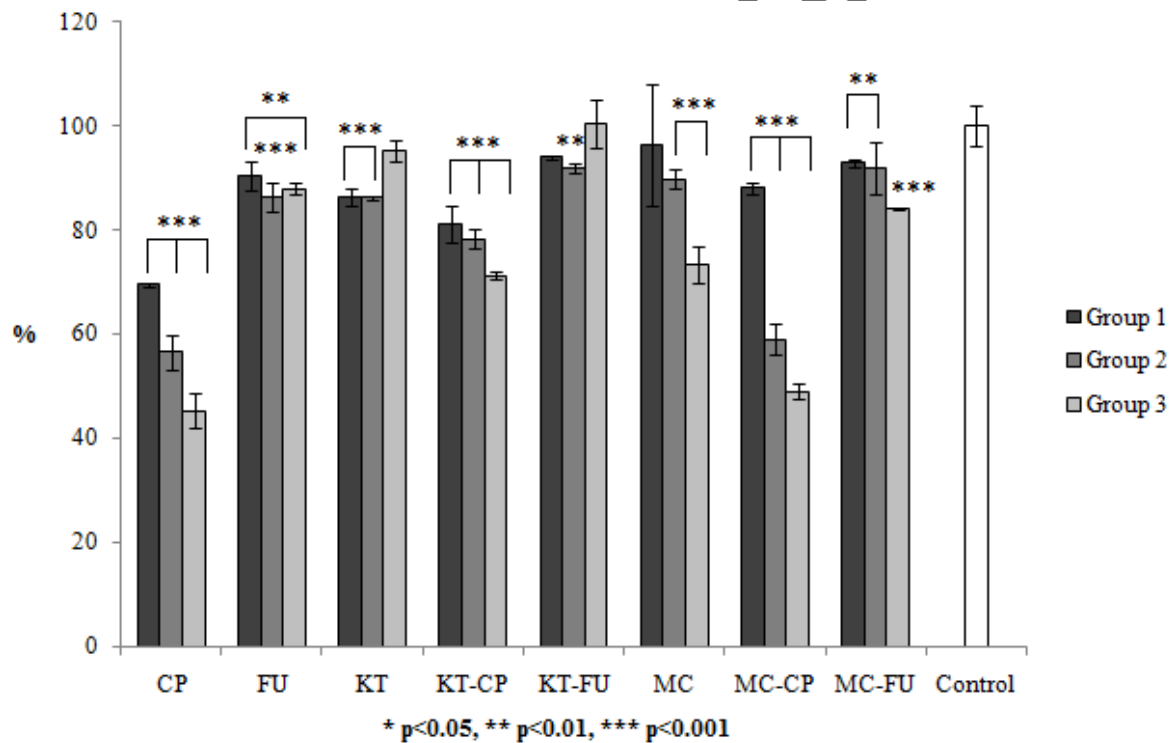

Figure 1: KT, MC alone as well as combined with CP and FU inhibit HeLa cells proliferation.
Final concentrations of the tested compounds were the following:
2, 20, 200 µM of KT; 10 µM, 50 µM, 500 µM of MC;
1.66, 3.32, 6.64 µM of CP; 10, 100, 1000 µM of FU.

The effect of different concentrations of KT and MC alone as well as combined with CP and FU on Bcl-2 expression in HeLa cells

To investigate the mechanism by which apoptosis was induced by combinations of KT or MC with cytostatic drugs (CP and FU), we evaluated the expression levels of Bcl-2 and Bax as apoptotic markers. The treatment of HeLa cells with the middle and the highest concentrations of KT-CP combination significantly decreased the expression of

Bcl-2 (Figure 2a). The expression of Bcl-2 in HeLa cells was significantly decreased after the treatment with the highest concentration of MC. The combination of MC and CP showed a statistically significant decrease in Bcl-2 expression level (Figure 2b).

The effect of different concentrations of KT and MC alone as well as combined with CP and FU on Bax expression in HeLa cells.

HeLa cells were exposed to different con centrations of KT and MC alone as well as combined with CP and FU in order to investigate the effects on Bax expression. The expression of Bax was significantly increased after the treatment of HeLa cells only with the highest concentration of KT (Figure 3a). The treatment of HeLa cells with the MC showed a statistical significance in increasing the expression level of Bax in all analyzed groups. The MC-CP and MC-FU combinations, compared to the control group, showed a statistical significance in increasing the expression level of Bax in all analyzed groups (Figure 3b).
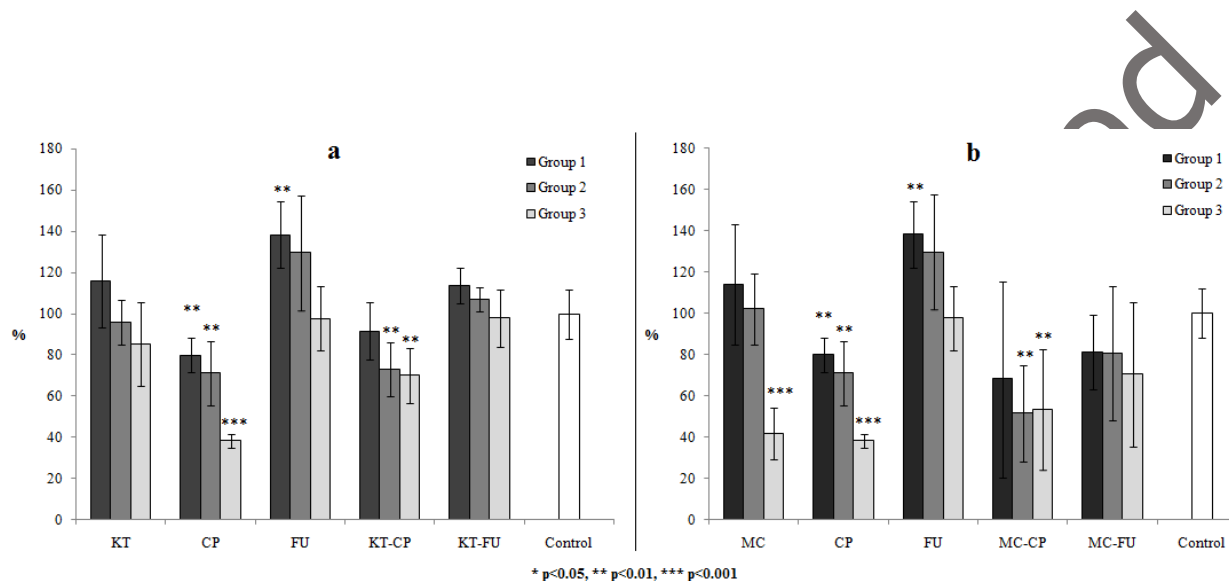

Figure 2: The effect of different concentrations of KT and MC alone as well as combined with CP and FU on Bcl-2 expression in HeLa cells.
Final concentrations of the tested compounds were the following:
2, 20, 200 µM of KT; 10 µM, 50 µM, 500 µM of MC;
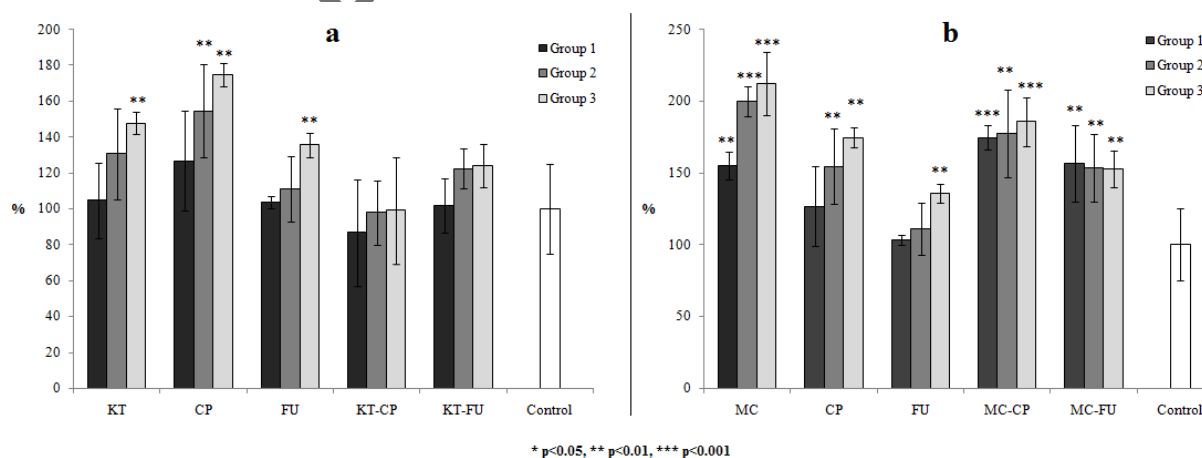1.66, 3.32, 6.64 µM of CP; 10, 100, 1000 µM of FU.

Figure 3: The effect of different concentrations of KT and MC alone as well as combined with CP and FU on Bax expression in HeLa cells.
Final concentrations of the tested compounds were the following:
2, 20, 200 µM of KT; 10 µM, 50 µM, 500 µM of MC;
1.66, 3.32, 6.64 µM of CP; 10, 100, 1000 µM of FU.

Discussion

The idea that NSAIDs could have a variety of molecular targets, not only provides a much-needed explanation of apparently disparate observations, it also underscores the opportunity which these targets represent for cancer chemoprevention and for improving therapeutic efficacy in cancer therapy (15).

Our results show that KT and MC, in examined concentrations, exert some cytotoxic and antiproliferative effects on HeLa cells. The treatment of cells with examined NSAIDs alone or in combination with conventional anticancer drugs, FU and CP, showed better cytotoxic and antiproliferative effect in groups treated with MC than in groups treated with KT, especially when the combination of these concentrations compounds at the highest tested were applied (Figure 1). So the obtained results could potentially indicate the synergistic cytotoxic effect of MC and conventional cytostatics (Figure 1).

Currently, there is an evidence that nonselective NSAIDs and selective COX-2 inhibitors inhibit proliferation of different cancer cell types such as a highly invasive mouse CRC cell model-MC-26 (22), osteosarcoma MG-63 cells (23), prostate cancer models (24), HT-29 human colon cancer and HCT 15 cells (25). Our results are consistent with the data of other authors, who reported that various nonselective NSAIDs and highly selective COX-2 inhibitors decreased cancer cell proliferation (26, 27).

A body of evidence indicates a role of inflammation in the development and modulation of different steps of cancer progression (28). Amongst different mediators of inflammation, the cyclooxygenases clearly appear to be implicated in cancer development (29). COX-1, which is an isoform constitutively expressed in many tissues, is believed to function as a housekeeping enzyme (30). Conversely, COX-2 is a pro-inflammatory factor that shows rapid up-regulation in response to stimuli such as mitogens, cytokines, growth factors, and tumor promoters. Inhibition of COX is the best known biochemical effect of NSAIDs provided a plausible explanation of the epidemiological data on NSAIDs and cancer (15). NSAIDs and COX-2-specific inhibitors exert their effect in carcinogenesis by inhibiting COXs, especially COX-2, the isoform overexpressed in cancer (31). Our results suggest that KT and MC have distinct actions on cellular apoptosis and the growth of cervical cancer cells. Obtained results can be explained by different COX isoenzyme expression in various cancer cells (29), the physico-chemical properties of KT and MC (32) as well as their affinity for inhibiting COX isoenzyme (16).

It is important to say that the COX-independent effects of NSAIDs come from several lines of evidence. NSAIDs have antiproliferative and/or proapoptotic effects in cell lines that do not express either COX-1 or COX-2 (33). The cancer chemopreventive properties of NSAIDs are much more complex and likely involve multiple COX-2-independent effects where mitochondria and mitochondrial markers of apoptosis are key players (34).

In the mitochondrial pathway, apoptosis could be regulated in part by changes in the expression levels of various pro - and anti-apoptotic members of the Bcl-2 family. Among them, Bcl-2, Bcl-xL and Bcl-w effectively inhibit cellular apoptosis, while Bax, Bcl-xs and Bak promote it (35). Apoptosis deregulation in cancer cells appears to primarily affect the signaling pathways upstream of Bax/Bak and mitochondria, leaving the downstream core apoptotic machinery mostly intact (36). This presents a great opportunity for restoring apoptosis in cancer cells by manipulating the balance between the pro - and anti-apoptotic Bcl-2 family members (36).

Results of our research showed that the levels of anti-apoptotic marker Bcl-2 were decreased while the levels of pro-apoptotic marker Bax were increased dose-dependently by KT and MC in HeLa cells (Figure 2). The synergistic effect of the KT and CP in the culture of HeLa cells was observed. Decrease of Bcl-2 protein was more pronounced in HeLa cells treated with MC alone or combined with conventional cytotoxic drugs (CP and FU). Similar results were obtained in the study conducted by Gao et al (37).

In our study Bax levels were upregulated in the treatment with KT and MC, alone or in combination with CP and FU. KT, alone or combined with cytostatics, lead to a significant increase in the expression of pro-apoptotic Bax protein in HeLa cells, but the effect of MC was more pronounced (Figure 3). It should be noted that MC and FU showed the synergistic effect on Bax expression in HeLa cells.

The downregulation of Bcl-2 and upregulation of Bax induced by KT, MC and combinations with CP and FU in HeLa cells were also dose-dependent. Up to date there have been only a few studies to compare our results, especially when we want to analyze the effects of MC and KT on the proliferation and expression of Bcl-2 and Bax protein in HeLa cell culture. Zhou et al. showed that overexpression of Bax is closely involved in apoptosis induced by aspirin and indomethacin, without altering Bcl-2 and Bcl-xL expression (38). Study obtained by Liu et al. showed that celecoxib also triggered apoptosis in osteosarcoma MG-63 cells through downregulation of Bcl-2 (23), whereas Naruse et al. suggested that meloxicam at the concentration of 100 mM upregulated Bax in MG-63 cells, but had no significant effect on Bcl-2 expression (39). Similarly, Dong et al. found that treatment of HepG2 cells with meloxicam upregulated the expression of Bax, in a time-dependent manner, but had no effect on the expression of Bcl-xL and Bcl-2 (40). In the view of aforementioned facts, our results suggest that both KT and MC have distinct actions on cellular apoptosis and the growth of cervical cancer cells through a combination of COX-dependent and COX-independent pathways.

In conclusion, we propose that the synergy observed in the effects of ketoprofen and meloxicam with cisplatin and 5-fluorouracil on the growth of cervical cancer HeLa cell line was generated by an enhancement of apoptosis. The results of this study further emphasize the complexity of the role of the dysregulated expression of Bcl-2 family members in successful cancer therapy. According to our results,

ketoprofen and meloxicam may be classified as therapeutic candidates to improve access of cervical cancer chemoprevention and chemotherapy.

Acknowledgement

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Originalni rad

MOLEKULARNI MEHANIZMI POTENCIJALNO SINERGISTIČKOG EFEKTA KETOPROFENA I MELOKSIKAMA SA KONVENCIONALNIM CITOSTATICIMA U ĆELIJSKOJ LINIJI HUMANOG KARCINOMA GRLIĆA MATERICE

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Ciklooksigenaze su jasno uključene u proces karcinogeneze. Zabeleženo je da je COX-2 eksprimirana tokom celog evolutivnog procesa razvoja i napredovanja karcinoma. Različiti molekularni mehanizmi mogu biti odgovorni za te procese. Epidemiološke i eksperimentalne studije su pokazale da nesteroidni antiinflamatorni lekovi (NSAIL) i selektivni COX-2 inhibitori mogu smanjiti rizik za nastanak karcinoma. Inhibicija ciklooksigenaza jedno je od prihvatljivih objašnjenja uloge NSAIL i prevencije karcinoma. Međutim, molekularni putevi ovih efekata još uvek nisu u potpunosti poznati, dok njihova kompleksnost verovatno uključuje COX zavisne i nezavisne mehanizme, proapoptotične i antiapoptotične članove Bcl-2 familije.

U sprovedenom istraživanju ispitivan je efekat ketoprofena (KT), kao neselektivnog COX-1/2 i meloksikama (MC) kao selektivnog COX-2 inhibitora. Efekat KT i MC je ispitivan samostalno ili u kombinaciji sa 5-fluorouarcilom (FU) i cisplatinom (CP) na proliferaciju (MTT test) i ekspresiju Bcl-2/Bax u ćelijskoj liniji humanog karcinoma grlića materice (HeLa ćelije).

MC, sam ili u kombinaciji sa konvencijalnim citostaticima FU i CP, pokazuje bolji citotoksični i antiproliferativni efekat u odnosu na KT. MC i KT dovode do dozno-zavisnog smanjenja ekspresije Bcl-2 i povećanja ekspresije Bax proteina. Značajnije povećanje ekspresije Bax proteina zabeleženo je u ispitivanim grupama HeLa ćelija tretiranim sa MC.

Primećena sinergija u efektima ketoprofena i meloxicama sa cisplatinom i 5-fluorouracilom u liniji humanog karcinoma grlića materice može biti posledica indukcije apoptoze u ćelijama. Stoga, ketoprofen i meloxicam mogu predstavljati nove terapeutske kandidate u cilju poboljšanja hemoprevencije i terapije karcinoma grlića materice.
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Ključne reči: molekularni mehanizmi, sinergistički efekat, ketoprofen, meloksikam, HeLa ćelije