**Apoptotic Effects of Etodolac in Breast Cancer Cell Cultures**

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Additional information is available at the end of the chapter

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**Abstract**

Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used as anti-inflammatory and analgesic agents. This family of drugs suppresses prostaglandin synthesis through inhibition of cyclooxygenase (COX) enzymes. Recent studies displayed that anti-carcinogenic actions of these drugs are mediated by COX-2 enzyme. Currently, there is intense research on COX-2 inhibitors as therapeutic targets. Etodolac is not perfectly selective but shows ‘preferential selectivity’ for COX-2. Here, in this study, we wanted to take gene expression snapshots of several apoptotic proteins under different conditions of drug exposure. The aim, therefore, focused to determine differential effects of etodolac on the regulation of apoptotic genes in hormone-responsive MCF-7 and triple-negative MDA-MB-231 cancer cell lines. Our data suggest that MDA-MB-231 is more responsive to etodolac exposure. Cell proliferation and apoptosis consistently regulated upon drug addiction. Furthermore, COX-2/HER2 was explicitly an up-regulated, phosphorylated form of Bad accumulated and anti-apoptotic proteins SAG and survivin increased in both transcriptional and translational levels. Changes in mitochondrial Bcl-2 family proteins were moderate and pro- and anti-apoptotic proteins showed similar levels of regulation in both cell lines. We believe that these findings would be supportive for future studies targeting etodolac-based therapies, as it reveals apoptotic factors differentially regulated in hormone-responsive and invasive cell lines.

**Keywords:** apoptosis, MCF-7, MDA-MB-231, MTT, Bad, SAG

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1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs), regularly used for their anti-inflammatory and analgesic effects, were shown to have potency for cancer prevention as well [1, 2]. This family of drugs suppresses prostaglandin synthesis through inhibition of cyclooxygenase (COX) enzymes. COX enzymes have two isoforms COX-1 and COX-2, with a recent addition of a splice variant of COX-1, COX-3, which is not functional in humans. COX-1 is commonly expressed in body, showing constitutive activation. COX-2, on the other hand, is hardly detectable in normal conditions but is induced upon stimulation by mitogenic agents, cytokines, growth factors, and so on. Later reports, however, demonstrated that COX-2 is also constitutively expressed in basal levels at several tissues including gastric mucosa, developing brain or kidney [3–5].

COX isoforms catalyse prostaglandin G/H (PGG2/PGH2) synthesis from arachidonic acid, and these prostaglandins are then converted to stable forms like PGD2, PGE2, PGF2, prostacyclin (PGI2) or thromboxane A2 (TXA2) depending on the cell type. Inhibition of COX enzymes, therefore, could result in improper prostaglandin activity, which in turn may cause different side effects. Since COX-1 was mostly expressed in gastrointestinal tract (GI), intestinal side effects of NSAIDs were suggested to decline if selective COX-2 inhibitors were used. A detailed discussion about the role of COX enzymes in GI damage can be found in a review by Lazzaroni et al. [6].

A wide range of COX-2 inhibitors (coxibs) were introduced to the market. Soon after clinical approval and usage, most of these drugs were withdrawn due to the emerging new side effects. Selective COX-2 inhibition partly reduced GI-related pathologies, but they were marked for their adverse effects in cardiovascular (CV) system. Regulation of blood coagulation involves the action of COX enzymes: COX-1 in platelets produces TXA2, and COX-2 in endothelial cells produces PGI2, achieving a balance between thrombotic/anti-thrombotic activities [7]. Drugs targeting one of the enzymes specifically could alter this balance and can cause bleeding or thrombosis. In this context, prevalent COX isoform in vascular endothelium was also a point of debate and a recent report showed that it is the COX-1, rather than COX-2, that is responsible for prostacyclin release in these cells [8]. Altogether, these findings imply necessity of further work on the side effects of COX-2 inhibitors.

Early reports about the role of NSAIDs in cancer were from the studies on colorectal cancer (CRC). Inducible form of COX enzymes, namely COX-2, was found to be elevated in colorectal cancer patients and early polyp formations were preceded by COX-2 induction [9]. Regular use of NSAIDs reportedly reduced CRC occurrence in 30–50%, mostly by COX inhibition, though later studies revealed that COX-2 inhibitors could also act on COX-independent pathways [2, 10]. These studies were further confirmed by many others and in different types of cancers like prostate, breast or lung cancers [11–13].

COX-2 is the main form participating in PGE2 production. When its activity is suppressed, protein expression is up-regulated [14]. Since there is strong evidence that COX-2 expression-related PGE2 increase acts on tumourigenesis and possible side effects could be reduced
by targeting molecular downstream effectors taking part in this pathway such as cell cycle regulation, inhibiting proliferation and inducing apoptosis.

Presented study summarizes current knowledge available on various coxib agents in molecular level. Besides, it focuses on the molecular effects of etodolac in cancer cells. As a part of our study, we are testing various etodolac derivatives synthesized by our collaborators. Etodolac has different structural characteristic than other coxibs, in that it has no sulphonyl, sulphonamide or sulphone groups to facilitate COX-2 binding. Its toxicity and relative selectivity for COX-2 are low, and although there are numerous studies about its anti-cancerogenic activity, dose-response relationship on various cancer cell lines and molecular downstream effectors was not well documented. Here, we present our preliminary results on the expression changes of various apoptotic proteins between hormone responsive and nonresponsive breast cancer cell lines under different doses and points.

2. COX-2 inhibitors and their biological effects

2.1. COX-2 inhibitors (coxibs) in common use

Specific COX-2 inhibitors rofecoxib and valdecoxib were withdrawn from the market due to their cardiovascular side effects [15]. Celecoxib, brand name Celebrex, presented to the market by Pfizer, is still in use, with precaution for possible cardiovascular thrombotic events. In addition to its anti-inflammatory and analgesic properties, celecoxib is also known to reduce premalignant adenomatous polyps and affects signalling pathways involved in malignant transformation in tumours, but not in normal tissues [16]. Celecoxib showed COX-1/COX-2 ratio of 30 in IC50 values, meaning that it has 30 times more potency at inhibiting COX-2 with respect to COX-1. Rofecoxib, was introduced to the market at the same time, has nearly 272-fold potency in COX-2 selectivity. This higher selectivity on COX-2 inhibition resulted in more severe adverse effects of the drug, and its use was banned by Food and Drug Administration (FDA).

Etoricoxib is developed by Merck & Co. It is approved in many countries worldwide, with the exception of US. Selectivity of this drug for COX-2 is nearly 100-fold more than COX-1 [17]. Parecoxib is another COX-2 inhibitor drug introduced to the market. Since parecoxib is a pro-drug of valdecoxib, it has similar pharmacodynamic properties as valdecoxib, which have a COX-1/COX-2 IC50 ratio of around 60, 2–2.5-fold higher than that of celecoxib, and it has no anti-thrombotic activity. Both drugs were not approved by the Food and Drug Administration (FDA) yet, but they are available in Europe and many other countries. Lumiracoxib (Novartis AG) is one of the most selective COX-2 inhibitors with significant reduction in gastrointestinal side effects [18]. Though it is being approved in more than 50 countries, it was not approved by FDA.

Several randomized clinical studies suggest that the novel coxibs have comparable efficacy to nonselective NSAIDs in the treatment of osteoarthritis, rheumatoid arthritis and acute pain, but they share similar renal side effects. The apparent dose dependence of renal toxicity may
limit the use of higher doses of the novel coxibs for improved efficacy. Large-size randomized clinical trials are ongoing to define the gastrointestinal and cardiovascular safety of the novel coxibs.

Etodolac is one of the first NSAIDs approved by FDA. It has been grouped in family showing ‘preferential selectivity’ for COX-2 together with meloxicam and nimesulide [19]. It has approximately threefold higher selectivity for COX-2, but full dose could be inhibitory for COX-1, too.

2.2. COX-2/PGE2 signalling pathway

To prevent adverse side effects of NSAIDs and to better describe their anti-carcinogenic properties, it is important to clarify COX-2-related signalling pathways, especially on proliferation, cell cycle and apoptosis. Since most of their anti-carcinogenic effects are produced by COX-2/PGE2 regulation, direct intervention with the downstream players could be a promising approach to reduce unwanted side effects.

Downstream targets of PGE2 are four different G-protein-coupled receptors (EP1, EP2, EP3 and EP4) on the membrane, each initiating different signalling systems. The EP1 receptor is coupled to the Gaq protein subunit that activates phosphoinositide signalling through phospholipase C (PLC). PLC hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP2) to generate the secondary messengers inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). This signalling pathway regulates intracellular calcium through PLC/IP3 and activates protein kinase C (PKC) through DAG. Clinical observations do not support any strict correlation between EP1 and cancer. However, since the activation of PLC ultimately leads to the activation of PKC, gene transcription would be effected due to mitogen-activated protein (MAP) kinase, nuclear factor-kappaB (NFκB) or Bcl2/Bad pro-apoptotic pathways [20–22]. EP1 was also shown to contribute to the development of UVB- or chemically induced skin cancers. UVB-induced squamous cell carcinomas display higher levels of EP1 expression than uninvolved skin [23]. Consistent with these conclusions is that the topical application of a selective EP1 antagonist protects against UVB-induced tumours [24].

While EP1 seems to have a secondary role in tumourigenesis, EP2-EP4 receptors obviously effect major cancer-signalling pathways. Secondary messenger systems like Gαs-cAMP-ERK signalling activated by EP2 or Ras/MAPK/ERK signalling activated by EP4 could affect cellular functions like differentiation, cell survival, cell growth or proliferation and apoptosis. EP2 also works through PI3K/Akt system together with axin and APC, leading to the accumulation of unphosphorylated form of β-catenin in the cytoplasm. The result would be a series of events initiated by transcription factors to yield cell proliferation, survival or angiogenesis [20].

A recent study reported interesting relation between PGE2 and insulin-like growth factor (IGF-1)/Akt/mTORC1-signalling pathway in recovering effects of obesity in pancreatic cancer cells. The study reveals that PGE2-stimulated mTORC1 activation occurs not through Akt but rather through cooperative action of EP4/cAMP/PKA and EP1/Ca2+ pathways [25].
EP2 positively and EP3 negatively regulate adenylate cyclase mediated by heterotrimeric G-proteins G-alphas or G-alphai, respectively. Resultant activation/deactivation of protein kinase A changes transcription factor activities of CREB and ERK1/2 through phosphorylation, which are correlated with phosphorylation of Bad or activation of cyclin D1, COX-2 and VEGF, respectively.

EP4 leads to the activation of adenylate cyclase, cAMP formation, activation of MAPK signalling, with an end point of CREB activation. This, in turn, causes the rise in Bcl-2 levels and inhibition of p53-induced apoptosis [26, 27].

2.3. Molecular pathway studies on coxibs

Etodolac-induced apoptosis was studied in Burkitt’s lymphoma cells, and it was shown that the induction is higher with respect to meloxicam, a drug classified in the ‘preferential selective’ COX-2 inhibitors, such as etodolac [10]. In this study, the treatment of cell with 100 μM etodolac was sufficient to reduce Bcl-2, Bcl-xL, cIAP-1 and survivin, and cleaved Procaspase-9, -3 and PARP in a dose-dependent manner. Since these cells do not express COX-2 enzyme, observed effects might be following a COX-2-independent pathway. Down-regulation of Bcl-2 was also reported in prostate cancer cell lines. In accordance with Kobayashi’s report, there was no change in COX-2 levels after etodolac treatment and growth inhibition was correlated with hormone sensitivity. On the other hand, induction of apoptosis by celecoxib did not show any hormone dependency and progressed through Akt regulation, instead of Bcl-2 [11, 28].

Bcl-2, an antiapoptotic protein, prevents induction of apoptosis by sequestering BH-3 only proteins like Bim, Bid, NOXA, PUMA, phosphorylated Bad or BNIP. These BH3-only proteins can either activate (directly or indirectly) Bax or Bak proteins, which are located at the mitochondrial outer membrane and change permeabilization or they inactivate anti-apoptotic Bcl-2 family members [29]. When there is an apoptotic stimulus, BH3-only proteins are up-regulated and they can directly act on Bax and Bak, initiating cytochrome c release through VDAC (voltage-dependent anion channel). In addition to direct activator BH-3-only proteins, sensitizer BH-3 group, such as Bad, NOXA or BNIP3, can release activators from anti-apoptotic BH1-4 proteins and initiate apoptosis through an indirect pathway [30, 31]. PUMA, an activator of BH3 protein, and NOXA, a sensitizer, both are found to be expressed in a p53-dependent manner.

Molecular studies to understand COX-2 inhibitors’ action mostly concentrated on two specific coxibs, celecoxib and rofecoxib. In HT-29 cells, celecoxib was found to reduce p38 and p55 MAPK phosphorylation, together with a reduction in adhesion molecules ICAM-1 and VCAM-1 [32]. Data indicated that there is an induction of pro-apoptotic response (Bax and Bid) in a dose-time-dependent manner. Global transcription profiling in colon cancers implied modulations on the genes related to cell cycle and apoptosis, but these changes were mostly observed in both COX-2 (+) and (-) cell lines [33]. Celecoxib induces cell cycle arrest at G1-phase, together with decreases in the inhibition of various cyclin expressions. Celecoxib can
inhibit protein kinase B (PKB/Akt) or its upstream kinase phosphoinositide-dependent kinase 1 (PDK-1) [34, 35]. Partial inhibition of PKB/Akt results in a relative activation of cell cycle inhibitors p21 and p27, which can cause the partial inactivation of cyclin-CDK complexes. However, a detailed mechanism has not been elucidated, since there was no change in the expressions of p21 and p27, cyclins or in the phosphorylation of CDK complexes [36].

Cell cycle gene profiling was conducted in normal breast epithelial cells, where 96 genes in p53 pathway were studied under two different doses of etodolac (0.5 and 2 mM) for 48 h [37]. Prominent regulation was observed in ATM, CCND2 (Cyclin D2), CCNF (Cyclin F), CDC20 (p55cdc), CDKN1A (p21) and RAD50. Apoptotic protein BAX was found to be down-regulated only after 2-mM application.

3. Methods

3.1. Cell viability assay

Cell viability was determined using Cell Proliferation Kit I (MTT) (Roche) according to the manufacturer's instructions. Briefly, cells were seeded into 96-well plates (10^4 cells/well) and incubated at 37°C in CO_2 incubator for 24 h. The next day, appropriate doses of drug were added and cells were further incubated for 24 or 48 h. MTT of 10 μL was added to each well for an additional 4 h. The precipitated formazan was dissolved in 100 μL of 10% SDS, and the absorbance was taken at 570 nm [38].

3.2. Assays for apoptosis

Tali™ Image-Based Apoptosis Kit utilizing Annexin V/propidium iodide (PI) binding was used to assess apoptotic cells (green fluorescence), dead cells (red and yellow fluorescence) and live cells (no fluorescence). The Tali™ Image-Based Cytometer has two in-built fluorescence channels: (1) green channel to measure V-Alexa Fluor®488, using 458-nm excitation and 525/20-nm emission filters and (2) red channel to measure propidium iodide, using 530-nm excitation and 585-nm longpass emission filters. The alterations in permeability of mitochondrial membrane were studied using JC-1 Mitochondrial Membrane Potential Kit (Abnova). All assays were performed according to the manufacturer’s protocols. Spectral readout was done using Synergy H1 Multi-Mode Microplate Reader (BioTek).

3.3. Real-time PCR analysis

Total RNA was purified using the RNeasy Plus Mini Kit (Qiagen) containing g-eliminator columns. Purity and quantification of products were tested through absorbance measurements and gel imaging. RNAs of 1 μg were reverse transcribed to cDNAs using Transcriptor High Fidelity cDNA Synthesis Kit (Roche) according to the manufacturer’s guidelines. Real-time PCR was applied using LightCycler 480 SYBR Green I Master Kit (Roche). Custom
plate involving primers for 16 genes of interest was designed and produced by Qiagen. Fold changes were evaluated through on-site web application of the same company.

3.4. Western blot

Cells were lysed with 1× RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP40, 0.5% Na-deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Complete, EDTA-free Protease Inhibitor Cocktail Tablets, Roche) and 10 mM phosphatase inhibitor sodium fluoride (Santa Cruz, SC-24988B). Proteins of 40 μg were resolved by SDS-PAGE and transferred to PVDF membranes. Membranes were incubated with primary and secondary antibodies after optimization. RNF7/SAG (Novus Biologicals, NBP1-85594), survivin (Novus Biologicals, NB500-201H) and phosphorylated Bad (pSer112) (Novus Biologicals, NB100-81807) were polyclonal clones produced in rabbit. Beta-actin (Novus Biologicals, NB600-501) was monoclonal antibody produced in mouse. Detection was performed using chemiluminescent substrates for HRP (Western Bright ECL-Advansta, K-12045-050) and Celvin S Chemiluminescence Imaging System (BioStep).

4. Results

4.1. Effects of etodolac on the proliferation of breast cancer cell cultures

The role of COX-2 inhibitors in tumourigenesis was a focus of interest in recent years. The most commonly used, FDA-approved, COX-2 inhibitor in market is celecoxib. A recent study presented full effects of celecoxib both in vivo and in vitro on breast cancer cells [39]. Growth inhibitory effects of celecoxib were clearly observable between ranges 10 and 40 μM, especially after 72- and 96-h incubations.

Etodolac is another approved COX-2 inhibitor being used generally as adjuvant to chemotherapeutic applications. Early studies on etodolac were performed in colorectal cancers, and later it was tested in various other types such as liver, lung or prostate. In our study, cytotoxicity of etodolac was determined using MTT assay in two breast cancer cell lines, one of which is known for its good prognosis (MCF-7) and the other for its malignant, invasive properties (MDA-MB-231). Proliferative effects of etodolac were insignificant at low concentrations (0–100 μM) in both cell lines. When concentrations were raised to 0.5 or 1 mM, cell viability in both cell lines was considerably decreased (Figure 1A). Etodolac was less effective in MCF-7, but there was a regular dose-response relation in MDA-MB-231 cells at the end of 48 h (Figure 1B). A good correlation was observed with the study where regular HT-29 was compared with the invasive-type colon cancer cell line HT-29/Inv3 by Chen et al., where invasive type was found to be more susceptible to the effect of etodolac with a relative IC50 values of 0.5 versus 1.88 mM for other cell lines [40]. In our case, IC50 value for MDA-MB-231 was found to be 0.69 mM, while no approximation was available for MCF-7.
4.2. Determination of apoptosis

There was no detectable apoptotic effect of etodolac at low doses, in accordance with our observations from MTT assays. When concentrations were raised to mM range, apoptosis was detectable through changes in mitochondrial membrane potential (Figure 2A). Apoptotic effect of etodolac was more prominent in MDA-MB-231 cells at the end of 48 h.

Apoptosis was further confirmed with Annexin V/PI staining using Tali™ Apoptosis Kit (Thermo Fisher Scientific) (Figure 2B).

4.3. Transcriptional profiling of apoptotic proteins of interest

To understand molecular mechanisms underlying observed apoptotic effects of etodolac and to clarify COX-2 dependency of anti-carcinogenic responses, regulatory changes in apoptotic pathways were investigated at molecular level. To achieve this, $10^5$–$10^6$ cells were collected for

![Figure 1. Cell viability effect of etodolac on breast cancer cell lines. (A) Decrease in cell viability upon 0.5 and 1 mM etodolac addition. Each bar represents the mean ± SD of three independent experiments. Two-way ANOVA was applied for each pair. *$P < 0.05$, **$P < 0.001$. (B) Dose-response curve for MCF-7 and MDA-MB-231 cells treated with increasing concentration of etodolac (100, 250, 500, 750 and 1000 μM) for 48 h.](image)

![Figure 2. Effect of etodolac on apoptosis in MCF-7 and MDA-MB-231 cell lines. (A) The ratio of apoptotic to health cells at the end of 48-h incubation period was quantified by JC-1 staining with or without etodolac. (B) Apoptotic/dead cells were counted following Annexin V/PI staining using Tali™ Apoptosis Kit as described in Methods. Two-way ANOVA was applied for each pair. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.](image)
real-time and Western blot analyses as described in Section 3. Cell pellets were stored at –80°C to the day of experiments. Custom-designed 96-well-plate-containing primers for the genes of interest were purchased from Qiagen-SAB Bioscience. Fold changes were calculated from Ct values, using delta-delta Ct method. All values were normalized to GADPH expression and calculated with respect to non-drugged controls (Table 1).

| Genes over-expressed in | Genes under-expressed in |
|-------------------------|--------------------------|
| Sample name             | Gene symbol (mM)         | Fold regulation | Sample name | Gene symbol (mM) | Fold regulation |
| 24 h                    |                          |                | MCF-7       |                |                |
| BAD1                    | 0.5                      | ++             | BCL2L11     | 0.5            | –              |
| 1                       | ++                       |                | 1           |                | –              |
| BAK1                    | 0.5                      | +              | PMAIP1      | 0.5            | –              |
| 1                       | +                        |                |             |                |                |
| BAX                     | 0.5                      | ++             | HIF1A       | 0.5            | –              |
| 1                       | +                        |                |             |                |                |
| BID                     | 0.5                      | +              | TP53        | 0.5            | –              |
| 1                       | +                        |                |             |                | –              |
| MB231                   |                          |                |             |                |                |
| COX2                    | 0.5                      | ++             |             |                |                |
| 1                       | +                        |                |             |                |                |
| ERBB2                   | 0.5                      | ++             |             |                |                |
| 1                       | +                        |                |             |                |                |
| BAX                     | 0.5                      | ++             |             |                |                |
| 1                       | +                        |                |             |                |                |
| BID                     | 0.5                      | +              |             |                |                |
| 1                       | +                        |                |             |                |                |
| HIF1A                   | 0.5                      | +              |             |                |                |
| TP53                    | 0.5                      | +              |             |                |                |
| 1                       | +                        |                |             |                |                |
| 48 h                    |                          |                | MCF-7       |                |                |
| ERBB2                   | 0.5                      | +              | BAD1        | 0.5            | –              |
|                         |                          |                | BAK1        | 0.5            | –              |
| MB231                   |                          |                | BCL2L11     | 0.5            | –              |
| COX2                    | 0.5                      | ++             | BAX         | 1              | –              |
All analyses were made through Qiagen website facility provided for data analysis.

Etodolac induced an early increase in pro-apoptotic proteins Bad1, Bak1, Bax and Bid gene expression, accompanied by Bcl-2 down-regulation for both concentrations in MCF-7 cells. These changes were seen to decline as period was extended to 48 h. Another prompt response was the up-regulation of HER-2 and COX-2 in MDA-MB-231 cells. There was a remarkable up-regulation of anti-apoptotic SAG protein after 48-h incubation in MDA-MB-231 cells, parallel to BCL2L11 (Figure 3A and B).

4.4. Etodolac promoted anti-apoptotic pathways in MDA-MB-231 cells

In translational level, phosphorylated form of Bad has slightly increased upon etodolac addition in MDA-MB-231 cells. SAG and survivin have also increased similarly in this cell line, in a dose- and time-dependent manner (Figure 4A–C).

Low drug concentrations were also examined in long-term culturing to see differences in relation to various dose-time applications. Etodolac was added into cultures at 100 and 200 μM concentrations. Neither cell proliferation nor cell deaths were significantly affected under these concentrations (data not shown). Real-time PCR analysis implicated that protein expressions were only moderately regulated in transcriptional level for all, except a consistent increase in anti-apoptotic proteins, including SAG, in invasive MDA-MB-231 cells (Figure 5A and B).

Table 1. Fold changes after 24- and 48-h incubation periods upon addition of 0.5 and 1 mM etodolac into cultures.

| Sample name | Gene symbol (mM) | Fold regulation | Sample name | Gene symbol (mM) | Fold regulation |
|-------------|------------------|-----------------|-------------|------------------|----------------|
| ERBB2       | 0.5              | ++              | SAG         | 0.5              | –              |
| BCL2L11     | 0.5              | ++              | Survivin    | 0.5              | –              |
| SAG         | 0.5              | ++              | TP53        | 0.5              | –              |

* + and – signs were used for up- and down-regulations, respectively. Fold regulations >10 were signified using two marks.

Figure 3. Differential expression of 12 genes chosen in relation to COX-2/apoptotic pathway. Fold changes were relative to control cells treated with DMSO after (A) 24 h and (B) 48 h. All expressions were normalized to GAPDH expression of corresponding cell line.
Figure 4. Western blot analysis of anti-apoptotic proteins (A) SAG at 24, 48 and 72 h, and (B) SAG and survivin after 48 h, together with (C) inactivated phosphorylated form of Bad. The data are representative of two independent experiments. Whole cell lysate was loaded as 40 μg proteins in each lane. β-actin was used as house-keeping control.
Figure 5. Effect of 100 μM etodolac addition on gene expressions of 6 genes chosen in relation to COX-2/apoptotic pathway after 72 and 96 h incubations. (A) Scattered plot analysis created by the software. The data points between the lines represent non-regulated proteins with insignificant fold-changes, where dots above the diagonals correspond to up- and dots below the diagonals correspond to down-regulations respectively. (B) Bar graph of fold changes observed in MDA-MB-231 cells. Regulations in MCF-7 cell line were minor, except negative regulation of BCL2L11, which correlated with positive regulations in Bak and Bax genes (data not shown).
5. Discussion

The expression of COX-1 and COX-2 in breast cell line cultures was studied in an early report by Liu et al. [41]. The data revealed that COX-2 is one of the markers showing differential expression between metastatic (MDA-MB-231) and non-metastatic hormone-responsive cell lines (MCF-7). Metastatic cell line had clearly high constitutive expression of COX-2 and a correlated increase in PGE2 levels. PGE2 production was firmly determined by phospholipase A2 availability, which is known to be high in metastatic MDA-MB-231 cell line, and COX2 activity, that is also shown to be higher in this cell line. These findings were further confirmed by a later study comparing COX-2 activity between colon cancer cell line HT-29 and its metastatic variant HT-29/Inv3 [40]. In prostate cancer cell lines, dose-response relation was found to be weaker for etodolac compared to NS-398, another selective COX-2 inhibitor [11]. Strange finding was that COX-2 expression did not necessarily correlated with its activity and etodolac was able to suppress PGE2 and tumour invasiveness without effecting protein COX-2 levels [14, 39]. Similarly, COX-2 expression and apoptosis were examined in HT-29 colon cancer cell lines, and high expression of COX-2 was detected in HT-29 cells with mutant APC. When full-length wild-type APC was expressed in the same line, the cell growth was declined and apoptosis was induced parallel to COX-2 down-regulation. However, activity tests showed that even though COX-2 expression exists, it is catalytically inactive in these cells [9].

Following these reports, the role of COX-2/PGE2 signalling in tumourigenesis, angiogenesis or suppression of apoptosis was further documented by many other studies and these fostered new therapeutic approaches based on various selective coxib derivatives. Clinical applications, however, demonstrated that the usage of NSAIDs or in particular coxibs as promoting anti-cancer agents has several drawbacks. Besides their serious side effects in gastrointestinal and cardiovascular systems, problems such as COX-2-independent anti-carcinogenic effects, or interferences with other eicosanoid pathways, signifies that great caution should be taken in the clinical use of these drugs [42]. Therefore, assessment of molecular changes in detail under different conditions could provide valuable foresight for future applications.

Although quite complex, carcinogenesis involves several main routes to follow in cell transformation. Limitless replicative capacity of cells could be a result of uncontrolled responses to growth signals, due to a constitutive receptor/ligand activity or insensitivity to growth signal inhibitors, cell cycle checkpoint defects, interfering with programmed cell death pathways, and sustained angiogenesis causing tumour invasion and metastasis.

Many signal transduction pathways cross talk and further complicate this picture. Studies focusing on cellular mechanisms of coxibs, in particular of etodolac and celecoxib, also reflect similar multi-facet picture.

In light of these observations, proteins playing substantial role in apoptotic fate were investigated to better understand the relations between COX-2/PGE2 and carcinogenesis. HER-2 (ERBB-2) and COX-2 were utilized to verify the already-identified correlation between high expression and invasiveness of the cells. As expected, MDA-MB-231 cells displayed higher expression levels of these genes and expressions were further up-regulated upon etodolac addition. Even though etodolac is a known Cox-2 inhibitor, as discussed above, its anti-proliferative effects may be
Cox-2 independent. A clinical study on breast cancers recently reported a significant increase in COX-2 gene expression levels upon etodolac addition, correlated with cyclin D1 reduction [43].

Etodolac induced an early increase in Bad gene expression, accompanied by Bax up-regulation and Bcl-2 down-regulation in MCF-7 cells, similar to the results previously reported for chemotherapeutic agents Taxol and Thiotepa [44]. Bad is able to regulate apoptosis by binding to the anti-apoptotic Bcl-2 family members Bcl-2 or Bcl-xL. This regulation proceeds post-translationally through modifications by kinases or phosphatases rather than transcriptional processes. Since only the unphosphorylated form of Bad is able to bind Bcl-xL or Bcl-2 to drive apoptotic process, we tested post-translational modifications through Western blot analyses. Phosphorylated form of the protein was enriched not in MCF-7 but in MDA-MB-231 cells. Phosphorylated form of Bad is inactive and explicitly induces growth and cancer development. The relation between protein levels and development or progression of different cancer types, including breast cancer, was recently examined both in cancer cell lines and on large-scale clinical data collected from The Moffitt Cancer Center Total Cancer Care repository [45]. Our results, in agreement with these findings, confirm the role of Bad in breast cancer cells and exhibit antagonistic action of etodolac in two cell lines with benign and malign characteristics. Bad phosphorylation would be one effective point in metastatic behaviour of MDA-MB-231 cell line. In addition to that, SAG and survivin were also clearly up-regulated in MDA-MB-231. Strangely, SAG increment was prominent in cells treated with 100 μM etodolac for 72 h.

SAG/ROC/Rbx/Hrt, also known as RNF7, is a member of zinc RING finger gene family, first characterized by Sun et al. [46]. This protein is a part of SCF E3 ubiquitin ligase complex and promotes polyubiquitination of various proteins involved in cell metabolism, signal transduction, cell cycle progression and apoptosis. Its reactive oxygen species (ROS)-scavenging activity protects cells from apoptosis induced by mitogenic factors such as ROS, hypoxia, stress, radiation, and so on and promotes cell survival. SAG levels were found to be higher in malignant cells and indicated as a potential prognostic marker at several cancer types [47, 48]. We found a reverse correlation of SAG, Bcl-xL and p53 expressions and overall survival of the advanced stage cervical carcinoma patients, as well as rectal cancer patients [49]. Apoptotic effects of SAG silencing were investigated in different cancer cell lines. Among the major pro-apoptotic proteins (Bax, Bak, Puma, Bim, Bad and NOXA) and anti-apoptotic proteins (Bcl-2, Mcl-1, survivin, XIAP, Bcl-xL and cIAP2), only NOXA was substantially regulated [50]. Therefore, in addition to known apoptotic markers, such as Bcl-2, Bax, Bak and Bad, we also wanted to examine if a potential association exists between SAG/NOXA- and COX-2/PGE2-related mechanisms leading to cell proliferation or apoptosis. As stated above, etodolac addition induced SAG up-regulation in both high-dose-short-time and low-dose-long-time applications in invasive cell line MDA-MB-231. NOXA expression was in basal levels, but there was no detectable down-regulation correlated with SAG levels.

In cancer, uncontrolled proliferation of cells leads to insufficient blood supply to the tissue, through the generation of aberrant microvessels. Lack of oxygen supply induces hypoxia-induced factor (HIF-1), which in turn activates SAG and SAG drives HIF-1α degradation through a feedback mechanism [51]. The role of hypoxia in the regulation of COX-2, on the other hand, was found to be an up-regulation of Cox-2 protein levels and correlated with hypoxia-inducible factor (HIF)-1α induction. A feedback loop, similar to SAG turnover, was reported in COX-2 pathway, in which COX-2/PGE2 up-regulation due to hypoxia enhances
HIF-1α transcriptional activity and this reinforces COX-2 up-regulation through a feedback mechanism [52, 53]. BNIP3 was another protein we tested in our panel. BNIP3 is known to be activated by hypoxia, and this activation results in enhanced mitochondrial membrane permeability and apoptosis [54, 55]. There was no definite change in neither HIF-1α nor BNIP3 levels (data not shown). In brief, among the sensitizers we tested (Bad, NOXA and BNIP3), only Bad was effectively regulated and etodolac does not show any straight regulation on hypoxia-related proteins. Our study marks the fact that in breast cancer, only the triple-negative invasive cell line was responsive to the effects of etodolac. MDA-MB-231 cell line promptly induced COX-2/HER2 expressions upon etodolac addition. HIF-1, BNIP3 and TP53 up-regulations in MDA-MB-231 were weak and these changes were reversing (down-regulation) for MCF-7 cells, but in similar levels. Up-regulation of anti-apoptotic proteins (SAG, survivin and Bcl-2) in MDA-MB-231 cell line was discernible in short times and became more evident when incubation time was extended to 3–4 days.

6. Conclusions

In this study, anti-proliferative and apoptotic effects of etodolac were investigated in breast cancer cell lines, MCF-7 and MDA-MB-231. Anti-proliferative and apoptotic changes were found to be pronounced only after high concentrations. Cox-2/HER2 over-expression was confirmed in invasive cell line MDA-MB-231. Regulation of mitochondrial Bcl-2 family proteins was moderate and pro- and anti-apoptotic proteins showed similar but reverse distributions. However, there was a prompt transcriptional up-regulation of Bad in MCF-7 and a slower response in MDA-MB-231 cells as the accumulation of phosphorylated form of Bad, suggesting a prominent role for Bad-mediated apoptotic pathway. In addition, SAG and survivin proteins increased in MDA-MB-231 cells in a dose-time-dependent manner. We believe that these findings would be supportive for future studies targeting etodolac-based therapies, as it reveals that apoptotic factors are differentially regulated in hormone-responsive and invasive cell lines.

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