Pathol. Oncol. Res. (2009) 15:335–344
DOI 10.1007/s12253-008-9126-9

ORIGINAL PAPER

Enhancement of 5-Fluorouracil Efficacy on High COX-2 Expressing HCA-7 Cells by Low Dose Indomethacin and NS-398 but not on Low COX-2 Expressing HT-29 Cells

Andrea Réti · Gábor Barna · Éva Pap · Vilmos Adleff · Viktor L. Komlósi · András Jeney · Judit Kralovánszky · Barna Budai

Received: 1 October 2008 / Accepted: 5 November 2008 / Published online: 2 December 2008
© Arányi Lajos Foundation 2008

Abstract The antiproliferative effect of 5-fluorouracil (5-FU) in the presence of low dose non-steroidal anti-inflammatory drugs (NSAIDs) on high cyclooxygenase-2 (COX-2)-expressing HCA-7 and low COX-2-expressing HT-29 colon carcinoma cell lines was investigated. Pharmacogenetic parameters were studied to characterize the 5-FU sensitivity of the two cell lines. Thymidylate synthase (TS) and methylenetetrahydrofolate reductase (MTHFR) polymorphisms were determined by PCR analysis. Cell proliferation was measured by SRB assay, cell cycle distribution and apoptosis by FACS analysis. Cyclooxygenase expression was detected by Western blot and also by fluorescence microscopy. Prostaglandin E_2 (PGE_2) levels were investigated with ELISA kit. The HT-29 cell line was found to be homozygous for TS 2R and 1494ins6 and T homozygous for MTHFR 677 polymorphisms predicting high 5-FU sensitivity (IC_{50}: 10 μM). TS 3R homozygosity, TS 1496del6 and MTHFR 677CT heterozygosity may explain the modest 5-FU sensitivity (IC_{50}: 1.1 mM) of the HCA-7 cell line. Indomethacin and NS-398 (10 μM and 1.77 μM, respectively) reduced the PGE_2 level in HCA-7 cells (>90%). Low concentrations of NSAIDs without antiproliferative potency increased the S-phase arrest and enhanced the cytotoxic action of 5-FU only in HCA-7 cells after 48-hours treatment. The presented data suggested that the enhancement of 5-FU cytotoxicity by indomethacin or NS-398 applied in low dose is related to the potency of NSAIDs to modulate the cell-cycle distribution and the apoptosis; however, it seems that this effect might be dependent on cell phenotype, namely on the COX-2 expression.

Keywords COX-2 · PGE_2 · HT-29 and HCA-7 cells · 5-fluorouracil · Indomethacin · NS-398 · Cell cycle phase

Abbreviations

5-FU	5-fluorouracil
CRC	colorectal cancer
COX-2	cyclooxygenase-2
dUMP	2’-deoxyuridine 5’-monophosphate
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorter
FBS	fetal bovine serum
IC_{50}	50% inhibitory concentration
MTHFR	5,10-methylenetetrahydrofolate
NSAIDs	non-steroidal anti-inflammatory drugs
PBS	phosphate-buffered saline
PGE_2	prostaglandin E_2
SRB	sulphorhodamine B
TBS	Tris-buffered saline
TS	thymidylate synthase
UTP	uridine 5’-triphosphate

Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer death among men and women in Hungary. Epidemiological studies have suggested that non-steroidal anti-inflammatory drugs (NSAIDs) may reduce the risk of colon

A. Réti · É. Pap · V. Adleff · V. L. Komlósi · J. Kralovánszky (✉) · B. Budai
National Institute of Oncology,
Budapest, Hungary
e-mail: kralo@oncol.hu

G. Barna · A. Jeney
1st Institute of Pathology and Experimental Cancer Research,
Semmelweis University,
Budapest, Hungary

cancer and decrease, the number and size of polyps in patients with familial adenomatous polyposis [1] implying that NSAIDs could modulate carcinogenesis and the development of colorectal carcinomas. Several studies have established that COX-2 overexpression is common in a variety of human malignancies, including cancer of the colon and promotes tumor cell growth, angiogenesis, tumor invasion and metastasis [2, 3]. COX-2 tumorigenicity likely involves the production of specific prostaglandins (PGs) and their metabolites, but the role of PGs in various stages of carcinogenesis remains to be elucidated [4]. COX-2 is the molecular target of most NSAIDs. Beside of COX-dependent mechanisms of NSAIDs COX-independent pathways are known to be involved in the anti-proliferative and proapoptotic effects of these drugs, as well [2].

5-fluorouracil (5-FU), a fluorinated pyrimidine analog, which has been used for more than 50 years is still one of the most widely applied drug in the therapy of patients with colorectal cancer. The anticancer effect and toxicity of 5-FU are thought to be caused mainly by its anabolites, 5-fluoro-dUMP and 5-fluoro-UTP. 5-fluoro-dUMP inhibits DNA synthesis by forming a stable ternier complex with thymidylate synthase (TS) and 5,10 methylenetetrahydro-folate (MTHFR) thus blocking the conversion of dUMP to dTMP. 5-fluoro-UTP is incorporated into RNA inducing translational errors [5]. Further on the DNA or RNA directed cytotoxicity and apoptosis induced by 5-FU depends on the status of p53 tumor suppressor gene [6].

In order to improve the effectiveness of 5-FU therapy different drugs and modifiers have been studied. In an experimental study it was demonstrated that antioxidants augmented the apoptosis, induced by 5-FU in colon carcinoma cells [7].

Various cytotoxic agents combined with NSAIDs showed promising results in experimental studies [8]. On the other hand, in clinical trials the cytotoxic agents were combined with the mostly used NSAID, celecoxib, resulting in a better response and longer time to progression of breast cancer patients [9]. Moreover, it has to be mentioned that patients also use NSAIDs for their cancer pains during chemotherapy [10].

In many studies reviewed by Zha et al. high concentrations of NSAIDs - higher than those required to inhibit prostaglandin synthesis [11] - have been shown to induce COX-2-independent effects (Bcl-x_L and NF-κB suppression, PPARδ inhibition, Akt activation block, etc) in colon carcinoma cell lines [3].

COX-2-dependent effects of NSAIDs occur even at low concentrations and would rely on the expression of COX-2 in tumor cells. It has to be emphasized, that low dose NSAIDs usually do not activate the COX-independent signaling pathways. It was found that 10 μM indomethacin is below that required for significant direct PPARγ activation [12]. In myeloma cells Zhang et al. demonstrated that NS-398 even at concentration of 50 μM did not influence the expression of the pro-apoptotic protein, BAX, and the anti-apoptotic proteins, Bcl-xl or Bcl-2 [13]. Aggarwal et al. also found that at least 100 μM indomethacin needed to increased the expression of BAX in SEG-1 esophageal cells, which constitutively expressed COX-2 similarly to HCA-7 cells [14].

Since the above presented data underline the role of COX-2 in the progression of colon carcinomas our intention was to investigate the effect of COX inhibitors i.e. indomethacin and NS-398 on the efficacy of 5-FU in HCA-7 and HT-29 cells.

Our special aim was to apply low concentrations of these NSAIDs, which according to previous studies have exclusively PGE_2 reducing potency.

Materials and Methods

Reagents and Antibodies

All culture and chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated. NS-398 was purchased from Cayman Chemicals (Ann Arbor, MI, USA) and PGE_2 ELISA kit from Assay Designs (Ann Arbor, MI, USA).

Antibodies to COX-1 and COX-2 were bought from Cayman Chemicals (Ann Arbor, MI, USA) and anti-beta-actin antibody from DAKO (Glostrup, Denmark). Horse-radish peroxidase-conjugated secondary antibodies were obtained from Pierce Biotechnology (Rockford, IL, USA), and Alexa Fluor 488-conjugated secondary antibody from Invitrogen (Parsley, UK).

5-FU (500 mg/10 ml sterile injection) was purchased from Pharmachemie (Haarlem, Hollandia). For higher concentrations 5-FU powder was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell Culture

HCA-7 colony 29 cells and HT-29 cells were obtained from European Collection of Cell Cultures (Salisbury, UK). HCA-7 cells constitutively express high level of COX-2 protein, while HT-29 is a low COX-2 expressing cell line [15]. HCA-7 and HT-29 cells were cultured in Dulbecco's modified Eagle medium or RPMI, respectively, supplemented with 10% (*v/v*) FBS. Viable cell counts were obtained using a Bürker chamber by exclusion of 0.04% (*v/v*) Trypan Blue.

Characterization of HCA-7 and HT-29 Cells for 5-FU Sensitivity

The DNA was isolated from the cells using MasterPure DNA purification kit (Epicentre Technologies, Madison, WI, USA).

The *5'TSER polymorphism* was determined by PCR analysis, respectively, as described earlier [16]. Shortly, the amplicons were analysed by 10% non-denaturing polyacrylamide gel electrophoresis (10% PAGE).

The *3'TSUTR (1494ins/del6bp)* and the *MTHFR C677T* genotypes were assessed by PCR-RFLP. In case of 3'TSUTR and MTHFR the PCR amplicons were digested with *Dra*I and *Hinf*I endonucleases, respectively, and the products were separated by 10% PAGE. The gels were stained with ethidium bromide and visualized by GelDoc2000 image documentation system (Bio-Rad, Hercules, CA, USA) [17].

5-fluorouracil sensitivity was determined by calculating the IC_{50} value from the dose response curve based on the results of SRB assays (see "Cell proliferation assay"). The concentration of 5-FU was increased from 1 nM to 10 M.

Dihydropyrimidine dehydrogenase activity of the cells was investigated by the method described earlier in details [18]. The cell cytosol was incubated with [6-^{14}C] 5-FU as a substrate (Moravek Biochemicals, Brea, CA; 52 mCi/mmol), NADPH, $MgCl_2$ and nicotinamide in sodium phosphate buffer at 37°C for 0 and 20 min. The reaction was stopped by adding ice-cold ethanol. The mixture was filtered in a VectaSpin 0.2 μM microfilter (Whatman, Maidstone, UK) centrifuged and separated on HPLC (Merck-Hitachi, Tokyo, Japan). The radioactivity was measured by BioScan detector (Hidex, Turku, Finland). The activities were expressed as the amount of dihydro-fluorouracil formed in 1 min from 1 mg cytosolic protein (pmol/min/mg protein). Measurements were made in parallels in two independent experiments.

Drug Treatments and Cell Proliferation Assay

In all investigations, except the analysis of dose response curves (see above), HCA-7 and HT-29 cells were treated with 5-FU at their IC_{50} concentration. Indomethacin and NS-398 were made up as a 10 mM stock solution dissolved in dimethyl sulfoxide. The required final concentrations were prepared by diluting the stock solution with cell culture media. The final concentration of the vehicle in all cultures was 0.03%. At these concentration, dimethyl-sulfoxide alone did not affect cell viability (data not shown).

Published IC_{50} values for indomethacin on COX-2 enzymes vary within 0.01–24.6 μM concetration range [19, 20]. At 10 μM concentration indomethacin potently blocked PGE_2 production without changing cell viability [21]. NS-398 has a 50% inhibitory concentration of 0.1–3.8 μM [22]. The IC_{50} concentration of NS-398 (1.77 μM) was determined by radiometric assay [20]. In order to assess the effects of low concentrations of indomethacin and NS-398, 10 and 1.77 μM were applied, respectively.

The quantitative sulphorhodamine B (SRB) colorimetric assay was used to determine the growth inhibitory effect of drugs in HCA-7 and HT-29 cells. Cells were seeded at $1x10^4$ per well in 96-well plates and grown for 24 h. Medium was replaced and cells were treated with the drugs or with their combinations for 24 or 48 h. At the end of the treatment cells were fixed with 10% trichloracetic acid (1 h at 4°C), stained for 15 min at room temperature with 100 μl of a 0.4% *w/v* SRB solution in 1% acetic acid. SRB was then removed and cells were quickly rinsed four times with 1% acetic acid. After air-drying, protein-bound dye was dissolved in 200 μl of 10 mM unbuffered Tris base (pH 10.5) for 5 min on a Heidolph Titramax (gyratory) shaker (Schwabach, Germany). The pink SRB was quantified by measuring the optical density at 540 nm by spectrophotometer (BIO-TEK Instruments, VM, USA). The experiments were done in six wells of the 96-well plates for each time point and repeated three times. The average cell number and the standard deviation were calculated for each treatment.

Cell Cycle Distribution Analysis

To investigate the cell cycle phases and the apoptosis, cells were fixed in 70% ethanol at −20°C, followed by alkaline extraction (200 mM Na_2HPO_4, pH 7.8) and ethidium bromide staining. Measurements were made with a FACScan flow cytometer (Becton-Dickinson, San Diego, CA, USA) and the data were analyzed by Winlist software (Verity Software House, Topshan, ME, USA).

Protein Extraction and Western Blotting

The attached HCA-7 and HT-29 cells were lysed with ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μg/ml pepstatin and 1 mM phenylmethylsulfonylfluoride). The cell lysate was centrifuged for 10 min. at 10,000 g at 4°C. The protein concentration in the samples was measured using Bio-Rad protein assay (Hercules, CA, USA) according to the manufacturer's instructions. Samples extracted from the control or from the treated cells containing 60 μg of protein were subjected to SDS-PAGE using 12% denaturing polyacrylamide gels. The proteins were transferred electrophoretically to a PolyScreen PVDF membrane (PerkinElmer, Boston, MA, USA) at 25 V for overnight at 4°C. Membranes were blocked for non-specific binding by incubating in Superblock Blocking Buffer in TBS (Pierce, Rockford, IL, USA) for 6 h. Membranes were incubated overnight with the primary antibody (1:1,000) followed by the secondary antibody for 3 h (1:200,000). After the incubation membranes were washed three times in TBS containing 0.05% Tween 20. Finally, the membranes were incubated in SuperSignal West Femto Maximum Sensitivity Substrate solution (Pierce,

Rockford, IL, USA) for 5 min. and exposed to X-ray film. The bands were analysed with GelDoc2000 image documentation system (Bio-Rad, Hercules, CA, USA). Human recombinant COX-2 protein was used as positive control (Alpha Diagnostic, San Antonio, TX, USA).

Immunofluorescence Microscopy

HCA-7 and HT-29 cells were grown on glass coverslips and treated under culture conditions as described above. Cells were then fixed and permeabilized in methanol at −20°C for 10 min. and washed twice in PBS. Monolayers were incubated with primary antibody (1:100) in Superblock Blocking Buffer in TBS for overnight at 4°C. Following PBS washes monolayers were incubated with the secondary antibody (1:1.000) in Superblock Blocking Buffer in TBS for 2 h at 4°C. After further PBS washes the fluorescence was detected by Olympus CKX41 fluorescence microscope (Tokyo, Japan) and documented with Olympus C-5060 widezoom camera (Tokyo, Japan).

Prostaglandin E_2 Assay

The concentration of prostaglandin E_2 (PGE_2), the major product of arachidonic acid metabolism, was measured by ELISA (Assay Designs, Ann Arbor, MI, USA) from cell culture medium, according to the protocol of the manufacturer. Measurements were made in triplicate in three separate experiments.

Statistical Analysis

Data were expressed as mean ± standard deviation (SD). Statistical significance was determined by analysis of variance (ANOVA) and Tukey's post hoc test. IC_{50} values were calculated with GraphPad PRISM software (San Diego, CA, USA). The p values <0.05 were considered as significant.

Results

Characterization of 5-FU Sensitivity of HCA-7 and HT-29 Cells

To investigate the modulating effect of COX inhibitors on 5-FU cytotoxicity in HCA-7 and HT-29 cells, the pharmacogenetic and pharmacobiochemical characteristics, which might influence the sensitivity of this cell lines against 5-FU, were studied. Genetic polymorphisms of pyrimidine enzymes i.e. TS, the most important molecular target of 5-FU and that of MTHFR, producing folate cofactor for 5-FU action as potential predictors of 5-FU responsiveness were determined. In HCA-7 cells in the 5'untranslated region of the TS gene (5'TSER) a homozygous triple repeat of a 28 bp long nucleotide sequence (3R/3R), while in HT-29 cells a homozygous double repeat (2R/2R) was found. In the 3' untranslated region of the gene (3'TSUTR) a 6 bp long insertion resulting in 6 bp/0 bp heterozygosity in HCA-7 cells and 6 bp/6 bp homozygosity in HT-29 cells was present. For the C677T polymorphism of the MTHFR gene HCA-7 cells were heterozygous, and HT-29 cells were homozygous mutant.

The dihydropyrimidine dehydrogenase enzyme activity was found to be 101±15 pmol/min/mg protein in HCA-7 cells, and 9±3 pmol/min/mg protein in HT-29 cells. Based on these findings in agreement with our earlier studies [16, 18] HT-29 could be characterized as 5-FU sensitive and on the contrary HCA-7 as 5-FU insensitive cell lines.

The 48-hour 5-FU treatment inhibited HCA-7 and HT-29 cell growth in a dose-dependent manner. The IC_{50} values - calculated from the 5-FU dose response curve - were 1.1 mM for HCA-7 cells and 10 μM for HT-29 cells, respectively, showing a good relationship with the results of pharmacogenetic and pharmacobiochemical studies (Fig. 1). For the further experiments the applied 5-FU concentration was 1 mM for HCA-7 and 10 μM for HT-29 cell lines, respectively.

Growth Inhibition of HCA-7 by 5-FU ± NSAIDs

To evaluate the dose dependent effect of 5-FU ± NSAIDs on the proliferation rate cells were incubated for 48 h with different concentrations of 5-FU±10 μM indomethacin or 1.77 μM NS-398.

Indomethacin or NS-398 applied for 48 h potentiated significantly the growth inhibitory effect of 5-FU in HCA-7 cells. The IC_{50} value of 5-FU + indomethacin treatment was 0.44 mM ($p<0.001$ vs 5-FU), and that of 5-FU + NS-398 treatment was 0.28 mM ($p<0.001$ vs 5-FU). In HT-29 cells the IC_{50} value of 5-FU + indomethacin treatment was 11.62 μM, and that of 5-FU + NS-398 treatment was 12.32 μM, which were non-significant changes compared to the IC_{50} value of 5-FU treatment (Fig. 1).

In HCA-7 cells the 24-hours treatment with 1 mM 5-FU + NSAIDs decreased the cell proliferation, but the difference compared to 5-FU alone did not reach the level of significance. After 48 h 5-FU alone produced 42% cell growth inhibition compared to the control and this effect was significantly enhanced by indomethacin (62% inhibition vs control), or NS-398 (64% inhibition vs control). Compared to the 5-FU treatment 5-FU + indomethacin resulted in a 20% ($p<0.01$) and 5-FU + NS-398 in a 22% ($p<0.001$) decrease in the cell number.

In case of HT-29 cells both the 24- and 48-hours treatments with 10 μM 5-FU caused a significant reduction of the cell number ($p<0.0001$ vs. control). The combined treatment with NSAIDs did not enhance the cytotoxic effect of 5-FU.

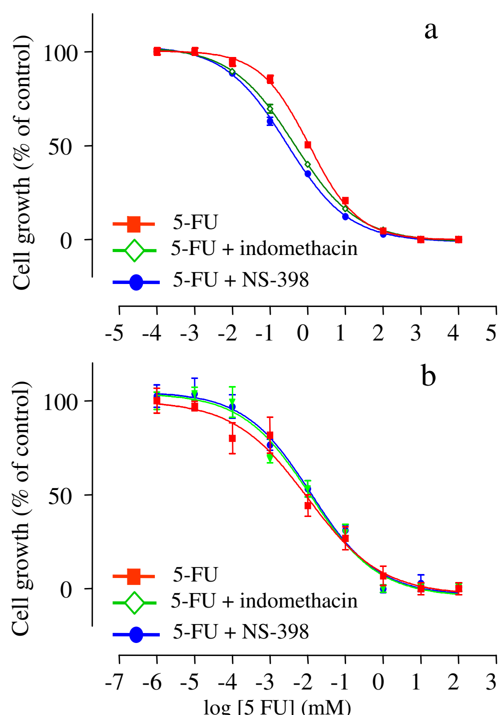

Fig. 1 Dose dependent effect of 5-fluorouracil ± indomethacin or NS-398 treatment on HCA-7 and HT-29 cell growth. Cells were treated with different concentrations of 5-FU ± indomethacin (10 μM) or NS-398 (1.77 μM). After 48 h the cell proliferation was measured by sulphorhodamine B assay and the results were plotted as % of control (cells without exposure to drugs). The data represent the mean ± SD of two independent experiments performed in quadruplicates. **a**. The IC_{50} values in HCA-7 cells were: 5-FU=1.1 mM, 5-FU + indomethacin= 0.44 mM ($p<0.001$ vs 5-FU) and 5-FU + NS-398=0.28 mM ($p<0.001$ vs 5-FU) **b**. In HT-29 cells the IC_{50} values were: 5-FU=10 μM, 5-FU + indomethacin=11.62 μM and 5-FU + NS-398=12.32 μM

Neither indomethacin, nor NS-398 alone influenced the proliferation of HCA-7 or HT-29 cells after 24 and 48 h (Fig. 2).

Cell Cycle Analysis and Apoptosis Determination

After 48-hour treatment with 5-FU or 5-FU + NSAIDs the FACS analysis showed a significant accumulation of the cells in S-phase and in parallel a decrease of G2/M fraction compared to the control in both cell lines (Table 1). The amount of cells in S-phase after combined treatment seems to be dependent on the extent of S-phase arrest induced by 5-FU and NSAID treatments, separately. A significant increase (~5–10%) of S-phase after 5-FU + NSAID treatment compared to 5-FU alone was seen only in case of HCA-7 cells, where 5-FU and NSAIDs alone enhanced the S-phase arrest by 27% and 8–15%, respectively. In contrast, in case of HT-29 5-FU alone induced a more extended S-phase arrest (~66% increase), whereas, NSAIDs alone decreased the rate of S-phase by 10–12%.

Compared to the untreated cells the apoptosis rate after 48-hour 5-FU treatment was nearly doubled in both cell lines, while NSAIDs alone did not induce apoptosis. All the same, the combined treatment resulted in a significant increase of apoptotic rate by 30–40% (compared to the 5-FU treatment) only in case of HCA-7 cells (Fig. 3).

The Effect of 5-FU ± NSAIDs on PGE_2 Production

The PGE_2 synthesis was evaluated in HCA-7 and HT-29 cells as a measure of COX-2 activity. In HT-29 cells the PGE_2 production was under the detection limit. In HCA-7 cells the treatment with 10 μM indomethacin or 1.77 μM NS-398 resulted in more than a 90% inhibition of PGE_2 production. Combined treatments (5-FU + NSAID) also resulted in the same inhibitory effect of COX-2 enzyme activity (Table 2).

COX-2 Protein Expression Determined by Western Blotting and Immunofluorescence Microscopy

The two isoforms of COX enzyme: COX-1 and COX-2 were investigated in HCA-7 and HT-29 cells by Western

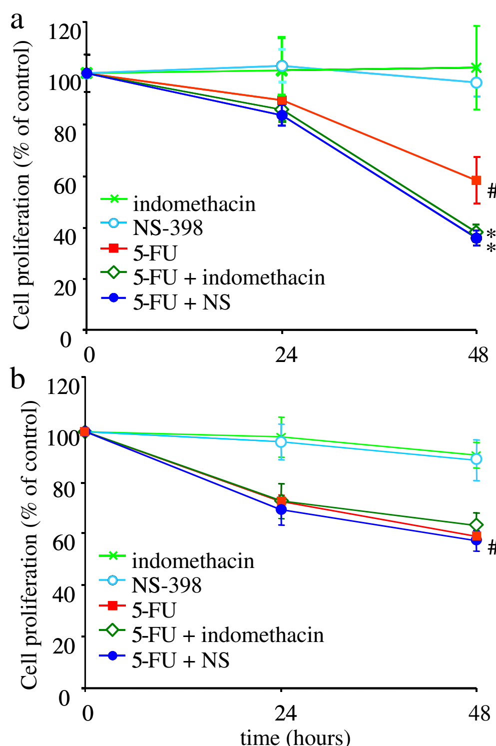

Fig. 2 Effect of 5-FU, indomethacin, NS-398 and their combinations on HCA-7 and HT-29 cell proliferation measured by sulphorhodamine B assay. Each point represents the mean ± SD in % of control of six independent experiments. **a**. HCA-7 cells were treated with 10 μM indomethacin, 1.77 μM NS-398 and 1 mM 5-FU **b**. HT-29 cells were incubated with 10 μM indomethacin, 1.77 μM NS-398 and 10 μM 5-FU. # $p<0.001$ vs control; * $p<0.01$ vs 5-FU

Table 1 Cell cycle phase distribution of HCA-7 and HT-29 cells after 24- and 48-hour treatment with 5-FU (F), indomethacin (I), NS-398 (N) and their combination[1]

Cell cycle Phase	Treatment type: 24 hours						48 hours				
	C[+]	F	I	N	F+I	F+N	F	I	N	F+I	F+N
HCA-7											
G1	48±4	39±3**	47±5	44±11	36±3**	38±3**	39±7**	44±3	45±4	34±2**	36±6**
S	48±2	61±3**	48±1	51±11	64±3**	62±3**	61±1**	55±1**	52±2**	66±1**,*	64±1**,*
G2/M	4±3	0±0**	5±2	5±1	0±0**	0±0**	0±0**	1±2	3±1	0±0**	0±0**
HT-29											
G1	57±7	45±12	59±16	60±15	44±17	47±16	32±9**	62±7	61±3	32±9**	36±8**
S	41±5	55±12	37±16	37±16	56±17	53±16	68±18**	37±13	36±4	68±19**	64±16**
G2/M	2±1	0±0**	4±1**	3±1	0±0**	0±0**	0±0**	1±1	3±1	0±0**	0±0**

[1] Data represent the mean ± standard deviation of four independent experiments expressed in %, [+] The cell cycle phase distribution of the control (C) remained unchanged during the treatment period, ** $p<0.05$ vs control, * $p<0.05$ vs 48-hour 5-FU treatment

blot analysis. In HCA-7 cells strong and in HT-29 cells weak COX-2 expression was present. The COX-1 expression was somewhat higher (~1.5x) in HT-29 cells compared to that of HCA-7 cells. In both cell lines the expression of COX-1 protein remained unaltered by either forms of the treatment (data not shown).

COX-2 protein expression increased in HCA-7 cells after treatment with 5-FU ± NSAIDs, while it was not affected by either form of the treatments in case of HT-29 cells (Fig. 4). The results of immunofluorescence staining - a relatively rapid and easy method to determine the changes in the expression of COX-2 protein, which is localized in the cytoplasm of the cells, correlated well with the results of the Western blot analysis (Fig. 5a,b)

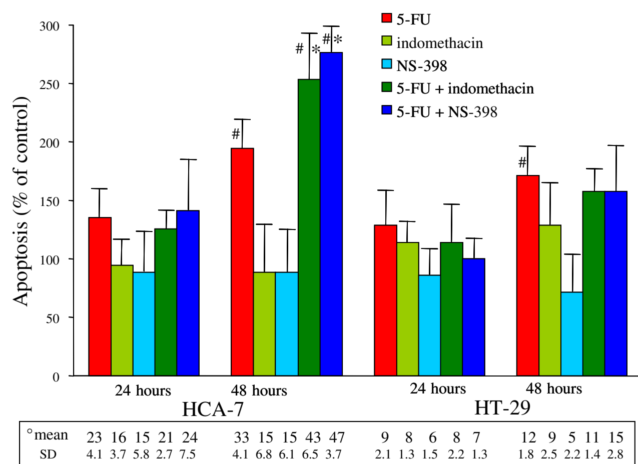

Fig. 3 Apoptotic rate of HCA-7 and HT-29 cells after 24- and 48-hour treatment with 5-fluorouracil, indomethacin, NS-398 and their combination. Each bar represents the average value + SD of four independent experiments in % of control. # $p<0.05$ vs control; * $p<0.05$ vs 48-hour 5-FU treatment. ° apoptotic cells in percentage of all cells. The apoptotic rate of the control cells remained unchanged during the 48-hour period (HCA-7 cells: 17±1.2%; HT-29 cells: 7±2.2%)

Discussion

5-fluorouracil is an effective cytotoxic agent in the management of colorectal cancer, which after inhibiting the pyrimidine metabolism induces apoptosis of cancer cells. Combination of 5-FU with various modulators potentiated its antitumor effects [23] and enhanced the rate of apoptosis [7].

It is well known that the malignant tumors including colorectal cancers overexpress COX-2 resulting in an increased tumorigenic potential [1]. Non-steroidal anti-inflammatory drugs by inhibiting the activity of COX

Table 2 Effect of 5-flourouracil ± indomethacin or NS-398 on prostaglandin E_2 production (PGE_2) of HCA-7 and HT-29 cells after 48 h measured by enzyme immunoassay[1]

Treatment	PGE_2 production of cells (ng/ml/10^6 cells): HCA-7	HT-29
Control	20.4±0.60	Nd
Indomethacin	0.4±0.04**	Nd
NS-398	0.2±0.02**	Nd
5-FU	16.7±1.30	Nd
5-FU + indomethacin	0.2±0.12*	Nd
5-FU + NS-398	0.2±0.05*	Nd

[1] Data represent the mean ± standard deviation of triplicate experiments, *Nd* non-detectable, ** $p<0.001$ vs control, * $p<0.001$ vs 5-FU treatment

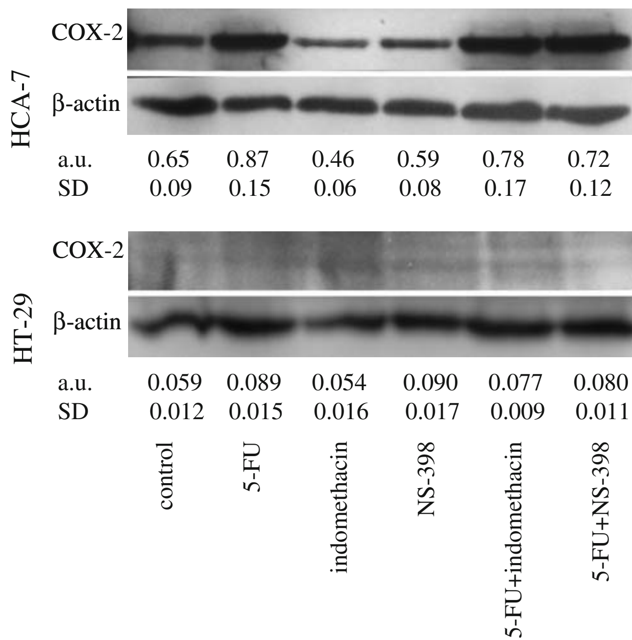

Fig. 4 Western blot analysis of cyclooxygenase-2 and β-actin expressions in HCA-7 and HT-29 cells after 48-hour treatment with 5-FU, indomethacin, NS-398 and their combination. a.u. arbitrary unit represents the optical density ratio of COX-2/β-actin

enzymes have been applied as chemopreventive agents [2] moreover, high concentration of NSAIDs combined with anticancer agents resulted in a higher cytotoxic effect [9].

In the present study, before investigating the NSAID-modulated cytotoxic activity of 5-FU, the characteristics of HCA-7 and HT-29 cell lines regarding their 5-FU sensitivity were examined. In our earlier study polymorphisms in the gene encoding TS have been shown to influence the responsiveness to the 5-FU treatment [16]. Our present results confirm the moderate sensitivity of HCA-7 cells against 5-FU (IC_{50}:1.1 mM) compared to the HT-29 cells (IC_{50}:10 μM), which was in good correlation with their pharmacobiological characteristics. The TS 3R homozygosity and on the other hand the high dihydropyrimidine dehydrogenase enzyme activity contributed to the relative insensitiveness of HCA-7 cells against 5-FU [24]. Etienne et al. reported earlier that the TS genotype 3R/3R have a higher TS activity and a poorer response to 5-FU therapy than those being homozygous for 2R [25].

Several studies presented the ability of NSAIDs to increase the efficacy of chemotherapeutic drugs. Specifically, combining flurbiprofen, sulindac or indomethacin with methotrexate, cyclophosphamide, melphalan, vincristine, doxorubicin and 5-FU resulted in an enhancement of cytotoxicity [26–29].

Indeed, NSAIDs might inhibit cell growth and induce apoptosis, however, in a significantly higher concentrations than those required for the inhibition of prostaglandin production. In our experiments low concentrations of NSAIDs had no effect on cell proliferation.

Indomethacin and NS-398 decreased the PGE_2 level in HCA-7 cells. Totzke et al also found that PGE_2 levels in COX-2 expressing HeLa H21 cells treated with low dose NS-398 (1 μM) was significantly reduced after 3–12 h [30]. The PGE_2 level of HT-29 cells was under the detection limit. Data regarding the COX-2 expression of HT-29 cells are controversial [15, 31].

Unfortunately, there are only few investigations presenting the effect of 5-FU treatments on cancer cells combined with low dose NSAIDs. Applying low dose indomethacin (2.8 μM) after 72 h a significant (~60%) IC_{50} reduction was reported after 5-FU treatment on murine Colon 26 cells [28], which constitutively express COX-2 protein [32]. Similar results were found on SKG-2 and HKUS human cervical cancer cells after treatment with indomethacin (0.3 μM), which significantly increased the cytotoxicity of

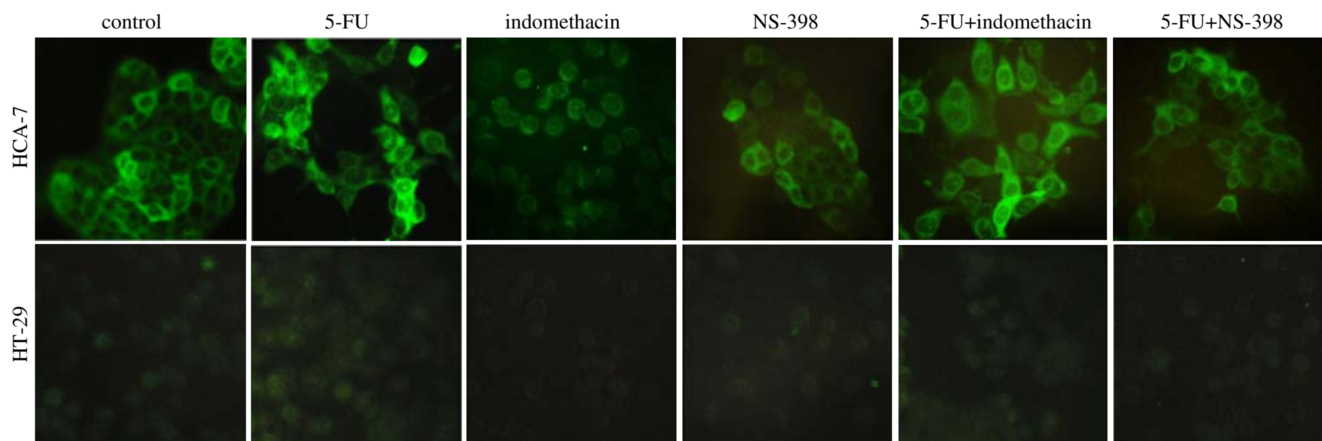

Fig. 5 Immunofluorescent staining of cyclooxygenase-2 protein in HCA-7 and HT-29 cells after 48-hour treatment with 5- fluorouracil ± indomethacin or NS-398. **a**. HCA-7 cells were treated with indomethacin (10 μM); NS-398 (1.77 μM); 5-FU (1 mM) **b** HT-29 cells were incubated with indomethacin (10 μM); NS-398 (1.77 μM); 5-FU (10 μM)

5-FU after 5 h resulting in a 72 and 63% reduction in IC_{50} values, respectively [29]. However, the COX-2 expression level of these cells are unknown.

On the other hand, it was demonstrated on human lung adenocarcinoma cell lines (DLKP, A549, COR L23P, COR L23R, HL60/ADR, 76-2 and 77-4) that indomethacin at low doses (5.6–7 μM) was unable to potentiate the cytotoxicity of 5-FU (3–15.4 μM) after 3–5-day treatment [26, 33, 34]. To the best of our knowledge, the COX-2 expression status of these cell lines was not yet investigated, except for A549 cells, which express COX-2 after stimulation [33].

Interestingly, NS-398 at low dose (1 μM) diminished the cytotoxic action of cisplatin, paclitaxel and also 5-FU on HeLa cervical carcinoma cells after 48 h [35]. It worths to mention that HeLa in that study was also a COX-2 non-expressing cell line.

Mizutani et al found that the selective COX-2 inhibitor (JTE-522) synergistically enhanced - independently of the p53 status - the cytotoxic effect of 5-FU against bladder cancer cells (T24 and HT1197) [36], which both are COX-2 expressing cell lines [37].

The cell cycle distribution of HCA-7 and HT-29 cells after 5-FU + indomethacin or NS-398 treatment presented marked reduction of G2/M and an increase in S-phase fraction. Petak et al. demonstrated that 5-FU treatment of cells with mutant p53 result in an accumulation of cells in S-phase and absence of acute apoptosis [6]. HT-29 cells possess a homozygous mutant p53, which causes an inactive protein (R273H) [38] and consequently, a marked S-phase arrest. Absence of acute apoptosis was observed in our experiments as well.

On the contrary, HCA-7 cells have both wild-type and mutant p53 [39], which is in accordance with our results showing that 5-FU treatment caused a relatively modest S-phase arrest (27% increase vs control compared to the 66% in HT-29 cells after 48 h) and more increased ($p<0.05$) apoptosis compared to HT-29.

Several studies demonstrated that NSAIDs i.e: indomethacin or NS-398 caused a G0/G1 arrest, reducing the ratio of cells in S and G2/M phase leading to apoptosis of the cells. These effects were seen only at high NSAID doses [40]. Interestingly, when HCA-7 cells were treated with low dose indomethacin or NS-398 a significant increase of cells in S-phase was seen compared to control. The hypothetical question if higher doses of NSAIDs would induce a more extended S-phase arrest and apoptosis is pointless, because other already mentioned, different signal pathways could be activated modifying the cell cycle distribution.

Earlier it has been shown, that in addition to COX-2 inhibition NSAIDs increase the level of arachidonic acid (AA) [41]. The accumulation of AA caused an increase of S-phase and induced cell proliferation in embryonic stem cells via cyclin D/E and CDK2/4 [42].

Indeed, in our study on HCA-7 cells low dose NSAIDs alone caused S-phase accumulation and a slight but not significant increase in cell number. The aboved mentioned assumption regarding the effect of AA is feasible to understand our results, because indomethacin and NS-398 decreased PGE_2 level already after 24 h although there was no change in S-phase rate, while after 48 h no further decrease in PGE_2 level was demonstrated yet, a significant accumulation in S-phase appeared.

Informations regarding the molecular pathways influenced by various NSAIDs at different concentrations are controversial at the present time. Recent studies have identified a series of new molecular targets of NSAIDs that are mainly involved in signaling pathways, i.e. activation of MAPKs and AKT [12] inhibition of Delta/Notch1 [43], etc, but in these cases the applied NSAID concentrations, which are required to influence these pathways were more than tenfold higher than those used in our investigations.

As a consequence, other mechanisms should be considered in the explanation why low dose NSAIDs enhance the cytotoxicity of 5-FU. Ogino and Hanazono demonstrated that indomethacin-modified the 5-FU effect by increasing the intracellular uptake of 5-FU. This finding might be in connection with the changes of fatty acid composition of tumor cells influencing the membrane fluidity and permeability caused by indomethacin, affecting the entry of 5-FU into tumor cells [28].

An other mechanism, which may influence the intracellular content of 5-FU was raised by Oguri et al demonstrated that multidrug-resistance-associated protein, MRP8/ABCC11 is an efflux pump for the nucleotide analogues and 5-fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP), as well, and elevated MRP8 level confers 5-FU resistance [44].

Moreover, it was found by Chen et al. that 10 μM indomethacin can decrease the level of MRP8 mediated transport by approximately 30%. They presented that low dose NSAIDs increase the intracellular 5-FU content and consequently its cytotoxicity by suppressing the MRP8-mediated 5-FU efflux [45], which might help to interpret our findings.

In conclusion, our present results suggest that 5-FU-sensitivity of HCA-7 colon cancer cells, which express high level of COX-2, can be increased effectively by applying COX inhibitors indomethacin or NS-398 in low dose. The exact mechanism by which low dose NSAIDs enhances 5-FU cytotoxicity requires further studies. Because of their later potential in clinical implications our findings need to be confirmed on other cell lines and in vivo, as well.

Acknowledgements Authors thank Dr. E. Hitre for critical reading of this manuscript and Cs. Polényi Makácsné, A. Nagy, J. Kútvölgyi, A. Éber Mousáné for their technical assistance. This study was supported by the Jedlik Ányos Grant (NKFP1-00024/2005).

References

1. Prescott SM, Fitzpatrick FA (2000) Cyclooxygenase-2 and carcinogenesis. Biochim Biophys Acta 1470:69–78
2. Kawai N, Tsujii M, Tsuji S (2002) Cyclooxygenases and colon cancer. Prostaglandins Other Lipid Mediat 68–69:187–196
3. Zha S, Yegnasubramanian V, Nelson WG et al (2004) Cyclooxygenases in cancer: progress and perspective. Cancer Lett 215: 1–20
4. Rishikesh MK, Sadhana SS (2003) Prostaglandins and cyclooxygenase: Their probable role in cancer. Indian J Pharmacol 35:3–12
5. Weckbecker G (1991) Biochemical pharmacology and analysis of fluoropyrimidines alone and in combination with modulators. Pharmac Ther 50:367–424
6. Petak I, Tillman DM, Houghton JA (2000) p53 dependence of Fas induction and acute apoptosis in response to 5-fluorouracil-leucovorin in human colon carcinoma cell lines. Clin Cancer Res 6:4432–4441
7. Adeyemo D, Imtiaz F, Toffa S et al (2001) Antioxidants enhance the susceptibility of colon carcinoma cells to 5-fluorouracil by augmenting the induction of the bax protein. Cancer Lett 164: 77–84
8. Ponthan F, Wickström M, Gleissman H et al (2007) Celecoxib prevents neuroblastoma tumor development and potentiates the effect of chemotherapeutic drugs in vitro and in vivo. Clin Cancer Res 13:1036–1044
9. Mazhar D, Ang R, Waxman J (2006) COX inhibitors and breast cancer. Br J Cancer 94:346–350
10. Mercadante S (2001) The use of anti-inflammatory drugs in cancer pain. Cancer Treat Rev 27:51–61
11. Minter HA, Eveson JW, Huntley S et al (2003) The cyclooxygenase 2-selective inhibitor NS398 inhibits proliferation of oral carcinoma cell lines by mechanisms dependent and independent of reduced prostaglandin E2 synthesis. Clin Cancer Res 9:1885–1897
12. Ou YC, Yang CR, Cheng CL et al (2007) Indomethacin induces apoptosis in 786-O renal cell carcinoma cells by activating mitogen-activated protein kinases and AKT. Eur J Pharmacol 563:49–60
13. Zhang M, Abe Y, Matsushima T et al (2005) Selective cyclooxygenase 2 inhibitor NS-398 induces apoptosis in myeloma cells via a Bcl-2 independent pathway. Leuk Lymphoma 46:425–433
14. Aggarwal S, Taneja N, Lin L et al (2000) Indomethacin-induced apoptosis in esophageal adenocarcinoma cells involves upregulation of Bax and translocation of mitochondrial cytochrome C independent of COX-2 expression. Neoplasia 2:346–356
15. Shao J, Sheng H, Inoue H et al (2000) Regulation of constitutive cyclooxygenase-2 expression in colon carcinoma cells. J Biol Chem 275:33951–33956
16. Hitre E, Budai B, Adleff V et al (2005) Influence of thymidylate synthase gene polymorphisms on the survival of colorectal cancer patients receiving adjuvant 5-fluorouracil. Pharmacogenet Genomics 15:723–730
17. Frosst P, Blom HJ, Milos R et al (1995) A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 10:111–113
18. Katona C, Tímár F, Jeney A et al (1999) Modulation of 5-fluorouracil by 5-ethyl-2'-deoxyuridine on cell lines expressing different dihydropyrimidine dehydrogenase activities. Anticancer Drugs 10:561–567
19. Stubbs VE, Schratl P, Hartnell A et al (2002) Indomethacin causes prostaglandin D(2)-like and eotaxin-like selective responses in eosinophils and basophils. J Biol Chem 277:26012–26020
20. Barnett J, Chow J, Ives D (1994) Purification, characterization and selective inhibition of human prostaglandin G/H synthase 1 and 2 expressed in the baculovirus system. Biochim Biophys Acta 1209:130–139
21. Sánchez-Alcázar JA, Bradbury DA, Pang L, Knox AJ (2003) Cyclooxygenase (COX) inhibitors induce apoptosis in non-small cell lung cancer through cyclooxygenase independent pathways. Lung Cancer 40:33–44
22. Han JH, Roh MS, Park CH et al (2004) Selective COX-2 inhibitor, NS-398, inhibits the replicative senescence of cultured dermal fibroblasts. Mech Ageing Dev 125:359–366
23. Kralovánszky J, Katona C, Jeney A et al (1999) 5-ethyl-2'-deoxyuridine, a modulator of both antitumour action and pharmacokinetics of 5-fluorouracil. J Cancer Res Clin Oncol 125:675–684
24. Beck A, Etienne MC, Chéradame S et al (1994) A role for dihydropyrimidine dehydrogenase and thymidylate synthase in tumor sensitivity to fluorouracil. Eur J Cancer 30A:1517–1522
25. Etienne MC, Ilc K, Formento JL et al (2004) Thymidylate synthase and methylenetetrahydrofolate reductase gene polymorphisms: relationships with 5-fluorouracil sensitivity. Br J Cancer 90:526–534
26. Duffy CP, Elliott CJ, O'Connor RA et al (1998) Enhancement of chemotherapeutic drug toxicity to human tumour cells in vitro by a subset of non-steroidal anti-inflammatory drugs (NSAIDs). Eur J Cancer 34:1250–1259
27. Bennett A, Gaffen JD, Melhuish PB, Stamford IF (1987) Studies on the mechanism by which indomethacin increases the anticancer effect of methotrexate. Br J Pharmacol 91:229–235
28. Ogino M, Hanazono M (1999) Indomethacin preferentially augments 5-fluorouracil cytotoxicity in Colon 26 tumors by increasing the intracellular inflow of 5-fluorouracil. Int J Clin Oncol 4:22–25
29. Ogino M, Minoura S (2001) Indomethacin increases the cytotoxicity of cis-platinum and 5-fluorouracil in the human uterine cervical cancer cell lines SKG-2 and HKUS by increasing the intracellular uptake of the agents. Int J Clin Oncol 6:84–89
30. Totzke G, Schulze-Osthoff K, Jänicke U (2003) Cyclooxygenase-2 (COX-2) inhibitors sensitize tumor cells specifically to death receptor-induced appoptosis independently of COX-2 inhibition. Oncogene 22:8021–8030
31. O'Callaghan G, Kelly J, Shanahan F, Houston A (2008) Prostaglandin E2 stimulates Fas ligand expression via the EP1 receptor in colon cancer cells. Br J Cancer 99(3):502–512
32. Yasumaru M, Tsuji S, Tsujii M et al (2003) Inhibition of angiotensin II activity enhanced the antitumor effect of cyclooxygenase-2 inhibitors via insulin-like growth factor I receptor pathway. Cancer Res 63(20):6726–6734
33. Touhey S, O'Connor R, Plunkett S et al (2002) Structure-activity relationship of indomethacin analogues for MRP-1, COX-1 and COX-2 inhibition identification of novel chemotherapeutic drug resistance modulators. Eur J Cancer 38:1661–1670
34. Kobayashi S, Okada S, Yoshida H, Fujimura S (1997) Indomethacin enhances the cytotoxicity of VCR and ADR in human pulmonary adenocarcinoma cells. Tohoku J Exp Med 181:361–370
35. Eichele K, Ramer R, Hinz B (2008) Decisive role of cyclooxygenase-2 and lipocalin-type prostaglandin D synthase in chemotherapeutics-induced apoptosis of human cervical carcinoma cells. Oncogene 27:3032–3044
36. Mizutani Y, Kamoi K, Ukimura O et al (2002) Synergistic cytotoxicity and apoptosis of JTE-522, a selective cyclooxygenase-2 inhibitor, and 5-fluorouracil against bladder cancer. J Urol 168: 2650–2654

37. Smakman N, Schaap N, Snijckers CMJT et al (2005) NS-398, a selective cyclooxygenase-2 inhibitor, reduces experimental bladder carcinoma outgrowth by inhibiting tumor cell proliferation. Urology 66:434–440
38. Rodrigues NR, Rowan A, Smith ME et al (1990) p53 mutations in colorectal cancer. Proc Natl Acad Sci USA 87:7555–7559
39. Liu Y, Bodmer WF (2006) Analysis of P53 mutations and their expression in 56 colorectal cancer cell lines. Proc Natl Acad Sci USA 103:976–981
40. Yip-Schneider MT, Sweeney CJ, Jung SH et al (2001) Cell cycle effects of nonsteroidal anti-inflammatory drugs and enhanced growth inhibition in combination with gemcitabine in pancreatic carcinoma cells. J Pharmacol Exp Ther 298:976–985
41. Meade EA, Smith WL, DeWitt DL (1993) Differential inhibition of prostaglandin endoperoxide synthase (cyclooxygenase) isozymes by aspirin and other non-steroidal anti-inflammatory drugs. J Biol Chem 268:6610–6614
42. Lee SH, Lee MY, Han HJ (2008) Short-period hypoxia increases mouse embryonic stem cell proliferation through cooperation of arachidonic acid and PI3K/Akt signalling pathways. Cell Prolif 41:230–247
43. Zhang H, Ye Y, Bai Z, Wang S (2008) The COX-2 selective inhibitor-independent COX-2 effect on colon carcinoma cells is associated with the Delta1/Notch1 pathway. Dig Dis Sci 53:2195–2203
44. Oguri T, Bessho Y, Achiwa H et al (2007) MRP8/ABCC11 directly confers resistance to 5-fluorouracil. Mol Cancer Ther 6:122–127
45. Chen ZS, Guo Y, Belinsky MG et al (2005) Transport of bile acids, sulfated steroids, estradiol 17-beta-D-glucuronide, and leukotriene C4 by human multidrug resistance protein 8 (ABCC11). Mol Pharmacol 67:545–557