ORIGINAL ARTICLE

Enhanced 5-fluorouracil cytotoxicity in high cyclooxygenase-2 expressing colorectal cancer cells and xenografts induced by non-steroidal anti-inflammatory drugs via downregulation of dihydropyrimidine dehydrogenase

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Abstract

Purpose To prove that 5-FU cytotoxicity could be increased by combination with low-dose non-steroidal anti-inflammatory drugs (NSAIDs) (indomethacin or NS-398) in high cyclooxygenase-2- (COX-2) expressing cells and xenografts through the modulation of dihydropyrimidine dehydrogenase (DPD) mRNA expression and/or enzyme activity.

Methods HT-29 cells were grown on collagen IV coated plates (HT-29-C). The antiproliferative effect of 5-fluorouracil (5-FU) ± NSAIDs was examined on non-COX-2 expressing HT-29 and COX-2-expressing HT-29-C cells by sulphorhodamine B assay. The COX-2 and DPD expressions were visualized by immunofluorescent staining, and prostaglandin E_2 levels were measured by ELISA kit. The HT-29 xenograft was established in SCID mice and treated with 5-FU ± NSAIDs for 5 days. The tumor volume, enzyme activity, and DPD mRNA expression were investigated by caliper, radioenzymatic method, and real-time RT-PCR, respectively. The drug interaction was calculated for both combinations (5-FU + indomethacin and 5-FU + NS-398).

Results Collagen IV up-regulated significantly the COX-2 and DPD mRNA, and protein expressions, and also their enzyme activities in HT-29 cells. NSAIDs enhanced in a synergistic manner the cytotoxic effect of 5-FU treatment both in vitro and in vivo. Downregulation of DPD was observed after 5-FU monotherapy, but the combined effect of NSAIDs and 5-FU on DPD mRNA expression, and enzyme activity was superior to the effect of 5-FU alone.

Conclusions Since 5-FU + NSAID treatment can alter the DPD enzyme activity resulting in an enhanced cytotoxic effect, further studies in clinical practice are warranted.

Keywords Indomethacin · NS-398 · 5-Fluorouracil · Cyclooxygenase-2 · Dihydropyrimidine dehydrogenase

Introduction

One of the most widely used anticancer agents for treatment of colorectal cancer is 5-FU. The rate-limiting enzyme of 5-FU catabolism is dihydropyrimidine dehydrogenase (DPD). Besides the liver and the peripheral mononuclear cells, tumoral DPD has become of clinical interest because elevated intratumoral DPD can decrease the tumor response to 5-FU therapy since more than 80% of the administered 5-FU is catabolized in vivo by DPD to 5,6-dihydro-5-fluorouracil. The degradation of 5-FU reduces its efficiency and requires extremely high doses and the fluorinated products may cause side-effects in the nervous system [1].

In order to improve the effectiveness of 5-FU, a new subclass of orally administrated fluoropyrimidines, UFT and S-1, containing potent DPD inhibitors, i.e., uracil and 5-chloro-2,4 dihydroxypyridine (CDHP), respectively, was developed [2]. An irreversible inhibitor of DPD, ethynyluracil, was also studied and found to be effective in clinical trials [3]. Kralovánszky et al. [4] proved that 5-ethyl-2′-deoxyuridine enhanced the therapeutic index of 5-FU by reducing its catabolism.

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Cyclooxygenase-2 (COX-2) is a rate-limiting enzyme in the synthesis of prostanoids and is common in human malignancies, including cancer of the colon. The upregulation of COX-2 promotes tumor cell growth, angiogenesis, tumor invasion, and metastasis. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit the COX-2 enzyme activity. Several preclinical and clinical studies have shown that NSAIDs are effective chemopreventive and antitumor agents either alone or in combination with standard cancer therapies [5–7]. Previously it was shown that NSAIDs, indomethacin and NS-398, applied in low dose effectively increased the 5-fluorouracil (5-FU)-sensitivity in high COX-2 protein-expressing HCA-7 colon cancer cells [8]. In a recent report from our laboratory it was also demonstrated that two NSAIDs: indomethacin and NS-398 in parallel with the COX-2 inhibition decreased also the DPD enzyme activity of high COX-2-expressing HCA-7 cells and HT-29 xenografts. It is noteworthy that the HT-29 xenograft was characterized by high COX-2 and DPD enzyme activity after a phenotype change of the implanted low COX-2 and DPD expressing HT-29 cells [9].

To elucidate the possible mechanism of this phenotype change in the present study, the HT-29 cells were cultured with extracellular matrix (ECM) components i.e., fibronectin, laminin or collagen IV in order to see if they are able to modify the COX-2 and DPD expression of the cells. Moreover, it was intended to prove whether the 5-FU cytotoxicity could be increased by combination with NSAIDs in the collagen IV-induced HT-29 or in the constitutively high COX-2-expressing HCA-7 cells and in the HT-29 xenografts through the modulation of DPD mRNA expression and/or enzyme activity.

Materials and methods

Cell lines

HT-29 and HCA-7 cells were purchased from the European Collection of Cell Cultures (Salisbury, UK) and were cultured in Dulbecco's modified Eagle-medium and RPMI, respectively, supplemented with 10% (v/v) fetal bovine serum (Sigma–Aldrich, St. Louis, USA).

Coating of plates or glass coverslips

Six- and 96-well plates or glass coverslips coated with 10 μg/ml human collagen type IV, fibronectin or laminin (Sigma–Aldrich, St. Louis, USA) were incubated for 1 h at 37°C then washed with PBS. The HT-29 cells were seeded on the coated plates and incubated for 4 h.

Immunofluorescence staining of COX-2 and DPD proteins

The HT-29 cells were incubated for 4 h on glass coverslips coated with ECM components. Then cells were fixed in methanol at −20°C for 10 min and washed twice with PBS. Monolayers were incubated with COX-2 (Cayman Chemicals, Ann Arbor, MI, USA) or DPD (Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary monoclonal antibodies (1:100 and 1:50, respectively) in Superblock Blocking Buffer in TBS for overnight at 4°C. Following PBS washes monolayers were incubated with the FITC-conjugated secondary antibodies (1:1,000, respectively) (Alexa Fluor 488, Invitrogen, USA) 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 HT-29 cells (1×10^6) in 1 ml culture media were seeded onto non-coated or collagen IV coated wells of six-well plates. After 4 h conditioned media were harvested, and the concentration of prostaglandin E_2 (PGE_2) was measured by ELISA (Assay Designs, Ann Arbor, MI, USA) according to the manufacturer's protocol. Measurements were made in triplicate in three separate experiments.

Determination of cell growth inhibition

The uninduced and collagen-induced HT-29 cells grown in 96-well plates were treated with 10 μM 5-FU [8] alone or combined with non selective COX-2 inhibitor, indomethacin (Sigma–Aldrich, St. Louis, USA) or selective COX-2 inhibitor, NS-398 (Cayman Chemicals, Ann Arbor, USA) (10 and 1.77 μM, respectively) for 8 h. The sulphorhodamine B (SRB) colorimetric assay was used to determine the cell growth inhibitory effect of the drugs in 96-well plates. The detailed SRB assay was described earlier [8]. The experiments were done in six wells of the 96-well plates and repeated three times.

Treatment of collagen-induced HT-29 (HT-29-C) and HCA-7 cells for determination of DPYD mRNA expression and enzyme activity

Collagen-induced HT-29 cells grown in six-well plates were treated for 8 h with 5-FU alone or combined with indomethacin or NS-398 as described above. HCA-7 cells grown in six-well plates were treated with 1 mM 5-FU [8]

alone or combined with indomethacin or NS-398 (10 and 1.77 µM, respectively) for 24 h.

Total RNA extraction and cDNA synthesis

HT-29-C and HCA-7 cells grown in six-well plates were harvested and total RNA was isolated using RNeasy mini prep kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The concentration of RNA was determined by measuring the absorbance at 260 nm with spectrophotometer (BIO-TEK Instruments, VM, USA). Reverse-transcription was performed using RevertAid M-MuLV reverse transcriptase (Fermentas, Glen Burnie, MD, USA). The mRNA templates (2 µg) were transcribed with 0.5 µg oligo (dT) primer (Fermentas) according to the manufacturer's protocol.

Quantitative real-time RT-PCR

The *DPYD* and *COX-2* mRNA expressions were determined by real-time RT-PCR. The β-actin (*ACTB*) gene was used as reference. The *ACTB* primers were 5′-GTGG GGCGCCCCAGGCACCA and 5′-CTCCTTAATGTCA CGCACGATTTC. The primers used for amplification of *DPYD* were 5′-GAAATGGCCGGATTGAAGTTT and 5′-TCGAAGAGCTTTTGAAGCTGG and that for *COX-2* were 5′-TTCAAATGAGATTGTGGGAAAATTGCT and 5′-AGTTCATCTCTGCCTGAGTATCTT. The amplifications were performed in a mixture of 1 µl cDNA, 0.2 µl of primers, 5 µl IQ SybrGreen Supermix (Bio-Rad, Hercules, CA, USA), and 3.6 µl H_2O on a RotorGene 2000 machine (Corbett Research, Mortlake, Australia). The 3-min denaturation at 95°C was followed by 40 cycles of 20 s denaturation at 95°C, 20 s annealing at 62°C, and 30-s extension at 72°C. The amplicon purity was checked by melting curve analysis. The relative expression levels were calculated as described by Pfaffl [10]. The results are presented as mean of three experiments.

HT-29 xenografts

A suspension of 2×10^6 HT-29 cells in physiological saline (0.1 ml) was injected subcutaneously into the hind leg of a 6-week-old female SCID mice. All animal experiments were performed according to the ethically approved guidelines of the Animal Care Facility of the first Institute of Pathology and Experimental Cancer Research, Semmelweis University, Budapest.

The animal weight (g) and tumor diameters (mm) were measured before the treatment and on the second, fourth, and sixth days with caliper and the tumor volume (mm³) was calculated as follows: $\pi \times (\text{shortest diameter})^2 \times (\text{longest diameter})/6$.

Treatment of xenograft-bearing mice

The HT-29 tumor-bearing mice were randomly separated into seven groups, four mice each: vehicle control, 6 or 30 mg/kg/day 5-FU and 6 or 30 mg/kg/day 5-FU + indomethacin, or NS-398.

The 5-FU (Pharmachemie, Haarlem, Hollandia) was given in two doses: 6 and 30 mg/kg body weight in 0.2 ml saline and was administered by i.p. injection for 5 days. The applied 30 mg/kg × 5 days 5-FU was regarded as the maximal tolerated dose [11].

The dose of indomethacin and NS-398 was 2.5 and 5 mg/kg, respectively, in 0.2 ml saline and given by oral gavage for 5 days. These doses are qualified as relatively low doses [12, 13].

Antitumor activity was evaluated by the change of relative tumor growth (RTG). RTG = (tumor volume on the sixth day − tumor volume before treatment)/tumor volume before treatment.

Measurement of DPD activity

The DPD enzyme activity of the cells and the xenografts was determined by radioenzymatic method as described previously [8, 9]. Measurements were made in parallel in three independent experiments.

Analysis of drug interaction

The effect of the combination of 5-FU and indomethacin or NS-398 was evaluated according to the method of Kern et al. [14]. The expected cell survival (S_{exp}), defined as the product of the survival observed for 5-FU alone and the survival observed for indomethacin or NS-398 alone: $S_{exp} = (S_{5\text{-FU}}) \times (S_{indomethacin\ or\ NS\text{-}398})$ and the observed cell survival (S_{obs}), obtained after combination of 5-FU and indomethacin or NS-398 were used to construct a synergistic ratio $R = S_{exp}/S_{obs}$ (R) defining the drug interaction. The values are $R = 1$ additive effect; >1, synergistic effect; <1, antagonistic effect.

In case of xenografts the percentage ratio between the tumor volume of treated mice and the tumor volume of controls was used for the Kern's index calculation as described above.

Statistical analysis

Statistical analyses were performed using GraphPad PRISM software (San Diego, CA, USA). The significance level was determined by analysis of variance (ANOVA) and Tukey's post hoc test. Differences between the collagen-induced and control cells were tested by using

Student's t test for paired variants. The p values <0.05 were considered as significant.

Results

Expression of COX-2 and DPD in HT-29 cells induced by collagen

In order to verify if the ECM components may induce the COX-2 and DPD expressions, the HT-29 cells were cultured in the presence of collagen IV, fibronectin or laminin. The 4-h incubation of HT-29 cells on collagen IV caused significant increase of COX-2 and DPD protein expressions (Fig. 1), but the fibronectin and laminin treatments were ineffective (data not shown). At the same time the PGE_2 level and DPD activities were significantly upregulated as well. *DPYD* and *COX-2* mRNA expressions also showed a 287- and 750-fold increase, respectively, compared to the control cells (Table 1).

Influence of indomethacin or NS-398 on 5-FU cytotoxicity in HT-29, HT-29-C cells

In the uninduced HT-29 cells after 8-h treatment, 5-FU alone caused a very moderate cell growth inhibition which could not be potentiated with NSAIDs.

In HT-29-C cells 5-FU was almost ineffective in growth inhibition; however, the combination of 5-FU with indomethacin or NS-398 resulted in a remarkable growth inhibition (37 and 41% inhibition vs. control,
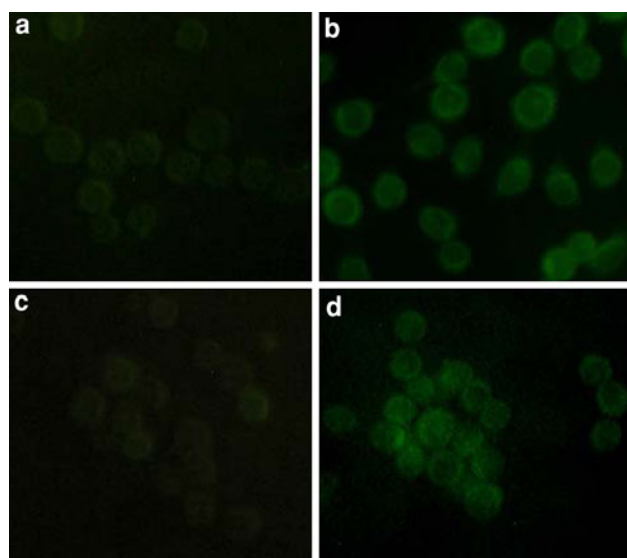
Fig. 1 Representative immunofluorescent staining of COX-2 in HT-29 (**a**) and collagen-induced HT-29-C cells (**b**), and DPD proteins in HT-29 (**c**) and collagen-induced HT-29-C cells (**d**) (×250)

Table 1 Comparison of cyclooxygenase-2 and dihydropyrimidine dehydrogenase status of uninduced HT-29 and collagen-induced HT-29-C cells

	HT-29	HT-29-C
DPD enzyme activity (pmol/min/mg protein)	9 ± 2.7	$17.44 \pm 2.87^{\#}$
PGE_2 level (pg/ml)	ND	$50.85 \pm 12.3^{\#}$
Relative *DPYD* mRNA expression[a]	$8.09 \pm 0.4 \times 10^{-3}$	$2328 \pm 464 \times 10^{-3\#}$
Relative *COX-2* mRNA expression[a]	$4.8 \pm 1.4 \times 10^{-6}$	$3624 \pm 1221 \times 10^{-6\#}$

ND non-detectable

[#] $p < 0.05$ versus uninduced cells

[a] Related to β-actin mRNA

respectively) (Fig. 2). The potentiation of 5-FU action by NSAIDs was similar to our earlier results obtained in HCA-7 cells, which demonstrated that indomethacin or NS-398 significantly enhanced the 5-FU cytotoxicity after 48-h treatment (35 and 38% vs. 5-FU, respectively) [8]. It is noteworthy, however, that in HT-29-C cells, characterized by enhanced COX-2 and DPD expressions, the potentiation of 5-FU action could be achieved already after 8-h treatment compared to the constitutively high COX-2 and DPD expressing HCA-7 cells where 48 h were necessary to obtain a significant 5-FU cytotoxic effect.

Influence of indomethacin or NS-398 on DPD mRNA expression and enzyme activity in HT-29-C and HCA-7 cells

We have examined whether treatment of HT-29-C and HCA-7 cells with 5-FU and its combination with NSAIDs regulates the DPYD mRNA expression and enzyme activity, one of the most important determinants of 5-FU action. In HT-29-C cells the *DPYD* mRNA expression was significantly decreased by 5-FU treatment ($p < 0.01$). This effect was potentiated by combining 5-FU with NSAIDs ($p < 0.05$ vs. 5-FU).

In case of HCA-7 cells, however, the *DPYD* mRNA expression was reduced in a non-significant manner, but when 5-FU was combined with indomethacin or NS-398 the decrease was significant compared to 5-FU treatment ($p < 0.05$ vs. 5-FU). No differences between the enhancing effect of indomethacin and NS-398 could be found (Table 2). The decrease of DPD enzyme activity was non-significant in both cell lines after 5-FU treatment, while at the same time indomethacin or NS-398 potentiated significantly the enzyme inhibitory effect of 5-FU (Table 2).
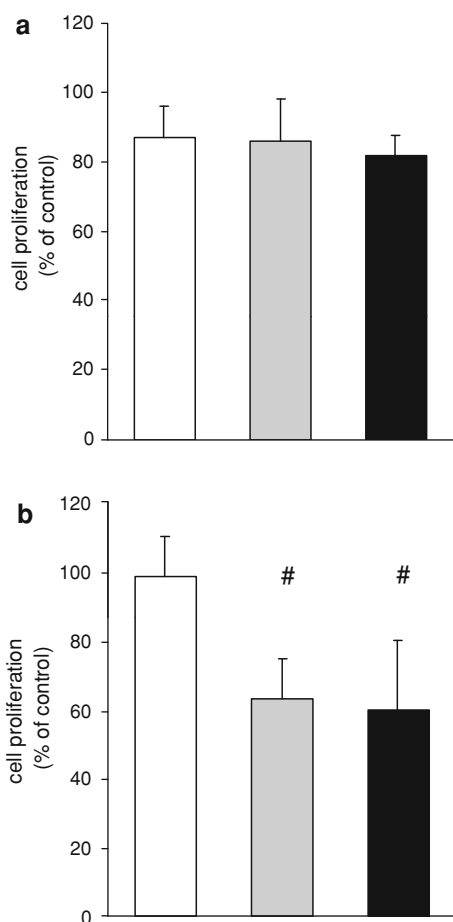
Fig. 2 Cell growth inhibition of HT-29 (**a**), collagen-induced HT-29 (HT-29-C) (**b**) cells after treatment with 10 μM 5-FU (*open bars*), 10 μM 5-FU + 10 μM indomethacin (*gray bars*) and 10 μM 5-FU + 1.77 μM NS-398 (*black bars*) measured by sulphorhodamine B assay. Each bar represents the mean + SD of six independent experiments in percent of control. #$p < 0.05$ versus 5-FU

Influence of indomethacin or NS-398 on the tumor growth inhibitory effect of 5-FU in HT-29 xenografts

The mice tolerated well the administration of 6 and 30 mg/kg/day 5-FU or their combinations with 2.5 mg/kg/day indomethacin or 5 mg/kg/day NS-398, as well.

In HT-29 xenografts 6 mg/kg 5-FU did not affect the tumor growth compared to the control, but the simultaneous treatment with indomethacin or NS-398 potentiated significantly the tumor growth inhibitory effect of 5-FU (33.3 and 42% decrease vs. 5-FU). The tumor growth inhibitory potential of 6 mg/kg 5-FU + NSAIDs was similar to that obtained by 30 mg/kg 5-FU monotherapy, which decreased the relative tumor growth by ∼50% ($p < 0.001$) compared to the control, but it is noteworthy that NSAIDs could not further enhance the cytotoxic effect of 30 mg/kg 5-FU (Fig. 3). Neither indomethacin, nor NS-398 alone influenced significantly the tumor growth of HT-29 xenografts after 5-day treatment (data not shown).

Effect of 5-FU ± NSAIDs on DPD enzyme activity in HT-29 xenografts

5-fluorouracil (6 mg/kg/day) caused a significant decrease in DPD enzyme activity compared to the control, and this enzyme inhibitory effect was potentiated significantly by INDO or NS-398 ($p < 0.05$ and $p < 0.01$, respectively) compared to 5-FU treatment. Noteworthy, that the decrease in DPD activity caused by the 6 mg/kg 5-FU + NSAIDs was similar to that obtained after treatment with 30 mg/kg 5-FU alone (Fig. 4). Similar to the in vitro results the DPD suppressing effect of indomethacin or NS-398 was about the same.

Evaluation of drug interaction between 5-FU and NSAIDs

To investigate the therapeutic advantage of the potentiation of 5-FU cytotoxicity by NSAIDs the drug interaction analysis was performed. The type of interaction between 5-FU and indomethacin or NS-398 in HT-29-C cells was synergistic (R: 1.57 and 1.71, respectively) compared to HT-29 cells in which it was only additive. A remarkable synergy was achieved also in case of HT-29 xenografts after treatment with 6 mg/kg 5-FU combined with the two NSAIDs (R: 1.48 and 1.47, respectively) (Table 3).

Discussion

Cyclooxygenase-2 expression was described in several tumor types including colorectal, gastric, esophageal, hepatocellular, breast, etc. cancers and its overexpression is often associated with a shorter time to progression or overall survival [5]. The cyclooxygenase enzymes are the best characterized targets of NSAIDs, which inhibit their activity. Recently, there has been much interest in using COX-2 inhibitors along with conventional anticancer therapy [15]. Previously, it was demonstrated that in high COX-2 expressing cell lines NSAIDs in addition to COX-2 inhibition also reduced the DPD enzyme activity [9].

In the present study, high COX-2-expressing colorectal cancer cell lines and xenograft were used to demonstrate the enhancement of 5-FU cytotoxicity induced by the NSAIDs-related inhibition of DPD mRNA level and enzyme activity.

The results of the present study regarding the induction of COX-2 by collagen IV are in accordance with the findings demonstrating that various ECM proteins induced COX-2 expression in HUVEC cells [16]. Collagen I and IV mediated expression of COX-2 was also described in epithelial cells [17]. Until now, direct association between DPD expression and signals induced by ECM components

Table 2 Change of dihydropyrimidine dehydrogenase status after the treatment with 5-FU ± indomethacin or NS-398 in collagen-induced HT-29 (HT-29-C) and HCA-7 cells

	HT-29-C cells		HCA-7 cells	
	mRNA expression[a]	Enzyme activity (pmol/min/mg protein)	mRNA expression[a]	Enzyme activity (pmol/min/mg protein)
Control	2.3 ± 0.5	17.4 ± 2.9	10.4 ± 3.7	99.6 ± 13.1
5-FU	$0.9 \pm 0.08^*$	14.6 ± 3.6	9.6 ± 1.4	90.2 ± 1.7
5-FU + indomethacin	$0.06 \pm 0.04^{\#}$	$5.1 \pm 1.6^{\#}$	$1.2 \pm 0.4^{\#}$	$54.3 \pm 7.6^{\#}$
5-FU + NS-398	$0.005 \pm 0.004^{\#}$	$5.9 \pm 1.1^{\#}$	$0.6 \pm 0.07^{\#}$	$55.0 \pm 6.3^{\#}$

HT-29 cells were incubated with 10 μM 5-FU alone or combined with either 10 μM indomethacin or 1.77 μM NS-398; HCA-7 cells were incubated with 1 mM 5-FU alone or combined with either 10 μM indomethacin or 1.77 μM NS-398
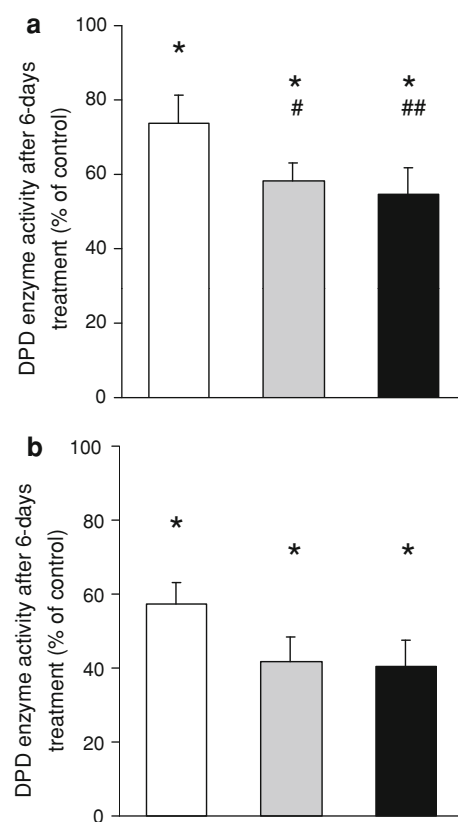
* $p < 0.01$ versus control

\# $p < 0.05$ versus 5-FU

[a] Related to β-actin mRNA
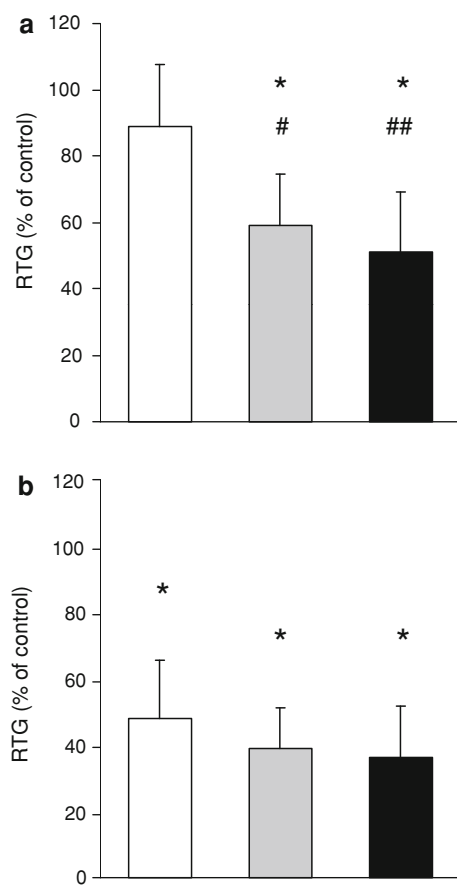
Fig. 3 Inhibitory effect of 5-FU (*open bar*), 5-FU + indomethacin (*gray bar*) or 5-FU + NS-398 (*black bar*) treatment on the tumor growth of HT-29 xenografts determined by the change of relative tumor volume growth (RTG). **a** 6 mg/kg/day and **b** 30 mg/kg/day 5-FU; *p < 0.001 versus control, #*p* < 0.05 versus 5-FU, ##*p* < 0.001 versus 5-FU

Fig. 4 Inhibitory effect 5-FU (*open bar*) 5-FU + indomethacin (*gray bar*) and 5-FU + NS-398 (*black bar*) treatment on the DPD enzyme activity (pmol/min/mg protein) of HT-29 xenografts. **a** 6 mg/kg/day and **b** 30 mg/kg/day 5-FU; *p < 0.001 versus control, #*p* < 0.05 versus 5-FU, ##*p* < 0.01 versus 5-FU

could not be found in the literature. In 3D collagen I gel cultures the Sp1 transcription factor was increased in HSC cells [18]. Zhang et al. [19] demonstrated that the Sp1 binds to the *DPYD* promoter and acts as a potent transactivator. Based on these data, one can speculate that collagen could affect the DPD expression via Sp1. On the other hand, NSAIDs activate proteosome-dependent degradation of Sp1 [20].

Table 3 Drug interaction between 5-FU and indomethacin or NS-398

	R^a_{INDO}	R^a_{NS-398}
HT-29 cells	1.02	1.09
HT-29-C cells	1.57	1.71
HT-29 xenograft 6 mg/kg 5-FU	1.48	1.47
HT-29 xenograft 30 mg/kg 5-FU	1.19	1.13

The synergistic ratio R, was calculated as: $R = S_{exp}/S_{obs}$

[a] Type of interaction $R = 1$, additive effect; >1, synergistic effect; <1, antagonistic effect

In this study NSAIDs enhanced the cytotoxicity effect of 5-FU in vitro and in vivo, as well. Many studies proved that NSAIDs potentiated the effect of conventional chemotherapeutic drugs such as 5-FU, cisplatin, oxaliplatin, S-1, etc [21–23]. However, there are only few investigations presenting the effect of 5-FU combined with low-dose NSAIDs on cancer cells or xenografts. Low-dose indomethacin significantly reduced the IC_{50} of 5-FU treatment on COX-2-expressing murine Colon 26 cells [21]. Similarly, SKG-2 and HKUS human cervical cancer cells after treatment with indomethacin showed significantly increased 5-FU cytotoxicity [24]. An impressive but interestingly non-significant tumor volume decrease (40%) after 28-day low-dose ibuprofen + 5-FU treatment in a COX-2-expressing mouse colorectal tumor model was described [25].

On the other hand, NS-398 at low dose protected HeLa cells against the cytotoxic action of cisplatin, paclitaxel, and 5-FU [26]. It is worth mentioning that Shestopal et al. [27] demonstrated that HeLa cells showed the lowest DPD mRNA level among five investigated cell lines.

The selective COX-2 inhibitor, JTE-522, synergistically enhanced the cytotoxic effect of 5-FU against COX-2 expressing bladder cancer cells, T24 and HT1197 [28]. Triptolide, which among others showed COX-2-inhibitory effect too, at low concentration presented synergistic effect with 5-FU on the growth of HT-29 colon carcinoma in vitro and in vivo [29]. Interestingly, in COX-2 expressing LM-H3 colon cancer cells the effect of 5-FU was enhanced by the DPD inhibitor, 5-chloro-2,4-dihydroxypyridine (CDHP). Etodolac and rofecoxib did not increase the cytotoxic effect of 5-FU; however, the combination of S-1 + NSAIDs inhibited the liver metastasis formation compared to S-1 after tumor cell inoculation in the mice spleen [22]. Low dose of nitric oxide-donating NSAID, NCX 4040, enhanced 5-FU cytotoxicity on colon adenocarcinoma cell lines, but not xenografts [23]. Their findings on cell lines are in accordance with the results of the present study, while the discrepant results regarding the xenografts may be due to the different treatment schedules and applied 5-FU doses.

Combination of 5-FU with various modulators, e.g., DPD inhibitors, resulted in an increased antitumor effect and enhanced rate of apoptosis [3, 4, 30].

The present study is one of the first, which demonstrates that the NSAIDs caused decrease of DPD activity enhanced the 5-FU cytotoxicity. Although, the DPD enzyme activity in connection with 5-FU action has been in limelight, up to this day, there are few data about the DPD regulation, although, growing evidence has been accumulated that not only the well-known DPD inhibitors are able to modulate the DPD enzyme activity, e.g., TRAIL, IFN [31, 32]. A very recent article described synergistic effect of 5-FU and a bioactive flavonoid, oroxylin A in vitro and in vivo. The combined treatment significantly decreased the *DPYD* mRNA levels compared to 5-FU alone. In addition, oroxylin A among other effects inhibits the lipopolysaccharide-induced COX-2 expression [33].

DPD activity has previously been reported to correlate with its protein level in human lymphocytes [34] and with mRNA levels in colorectal tumors, and liver tissues [35]. In the present study a similar correlation was found in vitro between DPD enzyme activity and the mRNA expression. On the other hand, others did not find any correlation between DPD enzyme activity, protein level, and mRNA expression [36]. The discrepancy might be due to the non-comparable methods used in different studies.

It is worth mentioning that in our study 5-FU alone caused a decrease in DPD enzyme activity both in vitro and in vivo. Literature data concerning the effect of 5-FU exposure on DPD levels are not conclusive [37–39]; thus, further investigations are needed to clarify in details these findings.

Although it is well known that 5-FU resistance is multifactorial the DPD status is among the most important predictors of 5-FU efficacy in vitro, in vivo, and in clinical practice, as well. The sensitivity of four gastric and three colorectal cancer cell lines to 5-FU were in inverse correlation with DPD protein or mRNA levels [40]. A significant correlation was found between *DPYD* DNA copy number and mRNA or enzyme activity in 58 human cancer cell lines and 31 human cancer xenografts. In cell lines the 5-FU sensitivity was significantly correlated with *DPYD* copy number, which also differed significantly between drug-resistant and drug-sensitive xenografts [41]. The prognostic value of DPD enzyme activity [42] or mRNA level [43] was demonstrated in patients with colorectal cancer treated with 5-FU-based chemotherapy.

In the present study the indomethacin and NS-398 caused downregulation of *DPYD* mRNA expression, and consequently the reduction of the enzyme activity in experimental systems was in good relationship with the enhancement of the antiproliferative potency of 5-FU.

Finally, based on our results it is possible to conclude that 5-FU efficacy could be limited by the COX-2 associated high DPD expression and activity in patients with colorectal cancers as well; therefore, further clinical studies are warranted to decide if NSAIDs in the therapeutic protocol might improve the antitumor potency of 5-FU.

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