Application of non-steroidal anti-inflammatory drugs to enhance 5-fluorouracil efficacy on experimental systems

Ph.D. thesis

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Introduction

Colorectal cancer (CRC) is one of the most serious, unresolved problems of healthcare, worlwide diagnoses of bowel cancer approximate an estimated one million cases per year. In the development of colorectal cancer, both genetic and environmental factors play a role. Approximately 88-94% of colorectal cancer cases are sporadic and the remaining are familial disease.

Although, the availability of new drugs and new regimens have been introduced in the therapy, 5-fluorouracil (5-FU) is still the most important of the basic drugs for more than 40 years. The anticancer effect and toxicity of 5-FU are thought to be caused mainly by its anabolites inhibiting the thymidilate synthase and incorporating into the DNA and RNA.

The bioavailability of 5-FU is greatly limited by its rapid catabolism. Dihydropyrimidine dehydrogenase (DPD) is the first and rate-limiting enzyme in the catabolic pathway of 5-FU. Determination of tumoral DPD has become of clinical interest because the elevated intratumoral DPD can decrease the tumor response to 5-FU therapy as a result of decreased drug level. In order to improve the effectiveness of 5-FU therapy different drugs and modifiers of 5-FU have been studied.

Several studies have established that COX-2 overexpression is common in a variety of human malignancies, including cancer of the

breast, lung, oesophagus and colon. In colorectal cancer COX-2 overexpression was associated with increased tumor size, deeper invasion and numbers of positive lymph nodes The unfavorable prognostic significance of the elevated COX-2 expression was proven in several studies. Overexpression of COX-2 is related to the accumulation of prostanoids especially prostaglandin E2 (PGE_2) PGE_2 has been shown to affect the carcinogenesis and tumor progression processes, including cell proliferation, motility and angiogenesis. A number of epidemiological studies have indicated that long term usage of non-steroidal anti-inflammatrory drugs (NSAID) is associated with 30-50% reduction in the risk of colorectal cancer or adenomatous polyps or death from colorectal cancer. The chemopreventive properties of NSAIDs have been attributed to COX-2 inhibition and a reduction in prostaglandin levels. COX enzymes are the best defined targets of NSAIDs.

Several studies showed that NSAIDs can increase the efficacy of traditionally used chemotherapeutic drugs. It has to be mentioned, however, that the majority of these studies high concentrations of NSAIDs - higher than those required to inhibit prostaglandin synthesis - were used, thus NSAIDs could activate COX-independent signaling pathways as well. However, there are only few investigations presenting the effect of low dose NSAIDs combined with 5-FU treatments on cancer cells.

Aim of the study

The purpose of the study was to investigate the effect of COX-2 inhibitors on the efficacy of 5-FU on experimental systems. The aim was to ascertain the following problems:

1.) If there is any association between the COX-2 expression and cytotoxic and antitumor effects of 5-FU on high and low COX-2 expressing HCA-7 and HT-29 human colon adenocarcinoma cell lines, respectively and also on xenografts derived from HT-29 cells.

2.) If the non-selective COX-2 inhibitor indomethacin and the selective COX-2 inhibitor NS-398 could enhance the cytotoxic and antitumor effects of 5-FU on the above mentioned cell lines and xenograft.

3.) To understand the mechanism(s) by which NSAIDs could enhance the cytotoxic effect of 5-FU

To answer the above questions the following investigations were carried out:

1)

a) Characterization of the HCA-7 and HT-29 cell lines and HT-29 derived xenografts cencerning their pharmacogenetic and pharmacobiochemical parameters, (thymidylate synthase (TS), (MTHFR) *polymorphisms* and *DPD activity*) - which might influence the sensitivity of these cell lines against 5-FU

b) Determination the COX-2 epression and 5-FU sensitivity of HCA-7 and HT-29 cells and HT-29 xenografts. (*IHC, Western blot and immunofluorescent staining*)

2)

a) Investigation of the effect of NSAIDs i.e. indomethacin and NS-398 on the cytotoxic and antitumor effects of 5-FU on cells and xenografts. (*IC_{50} values, SRB, tumor volume measurement)*

b) Evaluation of the drug interactions between 5-FU and NSAIDs.

3)

a) Cell-cycle analysis and apoptosis determination after 5-FU, NSAID and 5-FU + NSAID treatments. (*FACS analysis*)

b) Study of the effect of 5-FU, NSAIDs and 5-FU + NSAID treatments on the COX-2 expression and its enzyme activity on the cell lines and xenografts. *(IHC, WB, ELISA)*

c) Influence of the 5-FU, NSAIDs and 5-FU + NSAID treatments on the alterations of COX- and DPD enzyme activities and mRNA expressions (*ELISA, DPD activity, RT-PCR)*

Materials and Methods

Pharmacogenetic characterization of 5-FU sensitivity

To determine the 5-FU sensitivity of HCA-7 és HT-29 cell lines and xenografts the *5'-TSER* és *3'TSUTR* polymorphisms were determined by PCR analysis, the MTHFR *C677T* polymorphism was assessed by PCR-RFLP. The IC_{50} value of 5-FU was determined by calculating its value from the dose response curve, based on the results of SRB assays

Immunofluorescent staining, immunohistochemistry and Western blott analysis

Investigations were made to determine the COX-2 expressions. The DPD protein expression was investigated by immunofluorescent staining as well.

Prostaglandin E_2 assay

The concentration of PGE_2 was measured by ELISA from cell culture medium and from tumor cytosol

Cell proliferation assay

The quantitative sulphorhodamine B (SRB) colorimetric assay was used to determine the growth inhibitory effect of 5-FU, NSAIDs, and 5-FU + NSAID treatments.

Cell culture with collagen IV

Six- and 96-well plates or glass coverslips were coated with collagen IV. The HT-29 cells were seeded on the coated plates and incubated for 4 hours. The collagen-induced HT-29 cells were named as HT-29-C cells.

Measurement of DPD enzyme activity

The DPD activity of the cells and the xenografts was determined by radioenzymatic method. The cytosol was incubated with [6-^{14}C] 5-FU as a substrate. The 5-FU and the generated H_2FU were separated on HPLC. The radioactivity was measured by BioScan detector. The activities were expressed as the amount of H_2FU formed in 1 minute from 1 mg cytosolic protein (pmol/min/mg protein).

Drug treatments

In all investigations HCA-7 and HT-29 cells were treated with 5-FU at their IC_{50} concentration. Our special aim was to apply NSAIDs on COX-2 enzyme inhibitory concentration. Based on published data in the *in vitro* experiments the applied concentration of non-selective COX-2 inhibitor indomethacin was 10 μM, and that of NS-398 (selective COX-2 inhibitor) was 1.77 μM.

Cell cycle distribution and apoptosis analysis

Measurements were made on a FACScan flow cytometer to investigate the effect of 5-FU, NSAIDs and their combination on the cell cycle phases distribution and on the apoptosis.

Quantitative real-time RT-PCR

The *DPYD* and *COX-2* mRNA expressions were determined by real time RT-PCR.

Treatment of HT-29 xenograft-bearing mice

A suspension of 2×10^6 HT-29 cells in physiological saline was injected subcutaneously into the 6-week-old female SCID mice. The mice were randomly separated into 2 groups for the following experiments: 1.) To investigate the antitumor effect of 5-FU, 5-FU + indomethacin and 5-FU + NS-398 treatments Drugs were administered for 5 days. The 5-FU was given intraperitonally in two doses: 6 and 30 mg/kg body weight. The dose of indomethacin and NS-398 was 2.5 and 5 mg/kg body weight, respectively and were given by oral gavage . Antitumor activity was evaluated by the change of relative tumor growth. 2.) Determination of the effect of NSAIDs on COX-2 and DPD enzyme activities. The NSAID treatments were given for 5 days/week for 3 consecutive weeks.

Analysis of drug interaction between 5-FU and NSAIDs

The effect of the combination of 5-FU and indomethacin or NS-398 was evaluated according to the method of Kern et al. (1988).

Results

1. Characterization of the 5-FU sensitivity and COX-2 exppression of HCA-7 and HT-29 cells and xenografts

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) was in good correlation with their pharmacobiological characteristics. The 5'TSER 3R homozygosity and the MTHFR C677T heterozygous genotype and on the other hand the high DPD enzyme activity contributed to the relative insensitiveness of HCA-7 cells against 5-FU. In case of HT-29 cells on the 5'TSER region a homozygous double repeat (2R/2R) and in the 3'UTR region a 6bp/6bp homozygosity were found. For the C677T polymorphism of the MTHFR gene HT-29 cells were homozygous mutant. These pharmacogenetic characteristics together with the demonstrated low DPD activity could be the predictors of the higher 5-FU sensitivity. In HCA-7 cells strong and in HT-29 cells weak COX-2 expression was present. In case of HT-29 xenografts the COX-2 expression was higher in the xenograft than in the cell line. The PGE_2 concentration was evaluated as a measure of COX-2 activity. It was comparable with the COX-2 protein expression both in cells and xenografts.

2. Cytotoxic and antitumor effect of 5-FU + NSAID treatments on cell lines and xenografts

Indomethacin or NS-398 applied for 48 hours potentiated significantly the growth inhibitory effect of 5-FU in the high COX-2 expressing HCA-7 cells. In case of low COX-2 expressing HT-29 cells the combined treatment with NSAIDs did not enhance the cytotoxic effect of 5-FU.

In the HT-29 xenografts the indomethacin or NS-398 treatment potentiated significantly the tumor growth inhibitory effect of 6 mg/kg 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, but the NSAIDs was not able enhance further the cytotoxic effect of 30 mg/kg 5-FU.

The results of the evaluation of drug interaction between 5-FU and NSAIDs proved, that the type of interaction between 5-FU and NSAIDs in the high COX-2 expressing HCA-7 cells and HT-29 xenografts was synergistic compared to the low COX-2 expressing HT-29 cells in which it was only additive.

3. The studied mechanism(s) by which the NSAIDs could enhance the cytotoxic effect of 5-FU.

1. The effect of the 5-FU, the NSAIDs and their combinations on the cell-cycle distribution and apoptosis

On HCA-7 cells after 48-hour treatment with 5-FU + NSAIDs the FACS analysis showed a significant accumulation of the cells in S-phase and resulted in a significant increase of apoptotic rate compared to 5-FU treatment alone. In case of HT-29 cells 5-FU alone induced a more extended S-phase arrest whereas, 5-FU + NSAID treatment did not affect the cell-cycle phase distribution and the apoptotic rate compared to 5-FU monotherapy.

2. Influence of the 5-FU, the NSAIDs and their combinations on the COX-2 protein expression

It was found, that on HCA-7 and HT-29 xenografts NSAIDs enhanced the 5-FU cytotoxicity but not through the modification of COX-2 protein expression.

3. Changes of COX-2 and DPD enzyme activities after treatments with NSAIDs

Our intention was to prove, that besides their PGE_2-reducing effect, NSAIDs may also modify the DPD enzyme activity. DPD is the first and rate-limiting enzyme in the catabolic pathway of 5-FU. On high COX-2-expressing HCA-7 cells and HT-29 xenografts NSAID

treatments resulted in a simultaneous decrease of the COX-2 and DPD activities and mRNA expression compared to the control.

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

To investigate the coexistence of COX-2 and DPD enzymes HT-29 cells were cultured in the presence of collagen IV (HT-29-C cells). The 4-hour incubation of HT-29 cells on collagen IV, caused a significant increase both of COX-2 and DPD protein expressions, enzyme activities and mRNA expressions compared to the control cells.

HT-29-C cells were less sensitive to 5-FU than uninduced cells, however, the combination of 5-FU with indomethacin or NS-398 resulted in a remarkable growth inhibition. The type of interaction between 5-FU and indomethacin or NS-398 in HT-29-C cells was synergistic

5. Influence of the 5-FU + NSAID treatment on the DPD mRNA expression and enzyme activity in HT-29-C and HCA-7 cells and in HT-29 xenografts

The treatment of the HT-29-C and HCA-7 cells and the HT-29 xenografts with 5-FU + NSAIDs decreased significantly the DPYD mRNA expression and enzyme activity, compared to 5-FU monotherapy which is attributable to the simultaneous decrease of COX-2 and DPD enzyme activities by the NSAIDs.

Conclusions, *new findings*

On the HCA-7 és HT-29 cells and on theHT-29 xenografts an inverse correlation between the COX-2 expression and 5-FU efficacy could be demonstrated. Consequently the determination of the COX-2 status of the tumors at the design of chemotherapy could be suggested

Indomethacin and NS-398 significantly enhanced 5-FU sensitivity and cytotoxicity on high COX-2 expressing cell lines and xenografts.

Our study is the first, presenting the coexistence of COX-2 and DPD enzymes on cell lines and xenografts. We also proved that on HT-29 cells collagen IV induced simultaneous enhancement of COX-2 and DPD expressions and activities.

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

It is possible to conclude that 5-FU efficacy is 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.

Scientific publications

Publications closely related to the thesis

Réti A, Barna G, Pap E, Adleff V, L Komlósi V, Jeney A, Kralovánszky J, Budai B. 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. Pathol Oncol Res. 2009 Sep;15(3):335-44. **IF:1,260**

Réti A, Pap E, Zalatnai A, Jeney A, Kralovánszky J, Budai B. Co-inhibition of cyclooxygenase-2 and dihydropyrimidine dehydrogenase by non-steroidal anti-inflammatory drugs in tumor cells and xenografts. Anticancer Res. 2009 Aug;29(8):3095-101. **IF:1,390**

Réti A, Pap E, Adleff V, Jeney A, Kralovánszky J, Budai B. 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. Cancer Chemother Pharmacol. 2009 Oct 15. DOI: 10.1007/s00280-009-1149-8 **IF:2,740**

Publications not related to the thesis

Kralovánszky J, Adleff V, Hitre E, Pap E, **Réti A**, Komlósi V, Budai B. [Pharmacogenetic studies on the prediction of efficacy and toxicity of fluoropyrimidine-based adjuvant therapy in colorectal cancer] Magy Onkol. 2007;51(2):113-25.

Szoboszlai N, **Réti A**, Budai B, Szabó Zs, Kralovánszky J, Záray Gy. Direct elemental analysis of cancer cell lines by total reflection X-ray fluorescence Spectrochimica Acta Part B: Atomic Spectroscopy. 2008 December 63(12):1480-1484. **IF:2,853**

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