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Evidence of COX-2 independent induction of apoptosis and cell cycle block in human colon carcinoma cells after S- or R-ibuprofen treatment

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Abstract

Ibuprofen belongs to the 2-aryl propionic-acid derivatives and consists of two enantiomers, of which S-ibuprofen is a potent cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) inhibitor whereas the R-enantiomer is two to three orders of magnitude less potent to inhibit cyclooxygenases. Beside its positive effects on inflammation and pain several animal studies have shown that ibuprofen also inhibits tumor initiation and proliferation but the molecular mechanisms are not fully understood. To investigate to which extent the antiproliferative effect of ibuprofen depends on COX-inhibition we tested both enantiomers in different human colon carcinoma cell lines (HCA-7 express COX-1, COX-2 and produce high prostaglandin E_2 level; HCT-15 express only COX-1 and produce nearly no prostaglandin E_2). S- and R-ibuprofen reduced concentration dependently cell survival in both cell lines to a similar extent and caused a G_0/G_1 phase block as well as apoptosis. The cell cycle block was accompanied by a down regulation of cyclin A and B and an increase of the cell cycle inhibitory protein $p27^{Kip-1}$. HCA-7 cells were less sensitive against the antiproliferative effects of ibuprofen enantiomers which was probably due to lower ibuprofen concentrations in this cell type. Also in the nude mice model both enantiomers inhibited tumor growth of HCA-7 and HCT-15 xenografts to a similar extent. However, in mice about 54% of R-ibuprofen was unidirectionally inverted to S-ibuprofen, thus the observed antitumorigenic effect of R-ibuprofen in vivo cannot solely be assigned to this enantiomer. In conclusion our data indicate that S- and R-ibuprofen show similar antiproliferative effects in human colon carcinoma cell lines irrespective of its COX-inhibiting potencies.

Keywords: G_1-phase block; Apoptosis; Colon cancer; Ibuprofen

1. Introduction

Colon, prostate and lung cancer are the most common cancers. The American Cancer Society expects that in the year 2005 more than 500,000 Americans will die of cancer, about one 10th of them because of colon cancer (Jemal et al., 2005). Several clinical, laboratory and animal studies have demonstrated that nonsteroidal anti-inflammatory drugs (NSAIDs), which are clinically used in the treatment of pain and inflammation, also have anticarcinogenic effects in colon cancer (Giovannucci et al., 1994; Logan et al., 1993; Peleg et al., 1994, 1996; Thun et al., 1991). For quite a long time the molecular mechanisms of these anticarcinogenic effects have been thought to be exclusively dependent on inhibition of cyclooxygenase-2 (COX-2), which is a key enzyme in the conversion of arachidonic acid to prostaglandins. In line with this theory several studies have demonstrated that prostaglandins are significantly increased in colonic tumor cells (Bennett and Del Tacca, 1975; Bennett et al., 1977; Pugh and Thomas, 1994; Rigas et al., 1993). This is associated with an enhanced expression of COX-2, but not COX-1, in about 80% of all colon carcinoma (Eberhart et al., 1994; Kargman et al., 1995; Sano et al., 1995). In the meantime, several reports have shown that NSAIDs without major COX-inhibiting potency also decrease tumor growth. For example, sulindac sulfon which is a derivative of sulindac and which has no inhibiting effect on prostaglandin synthesis reduced colon cancer growth in rats comparable to the COX inhibitors

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sulindac or piroxicam (Alberts et al., 1995; Piazza et al., 1995). This effect of sulindac sulfon has been related to the induction of apoptosis in cancer cells by activating the Jun-kinase cascade (Soh et al., 2000).

We were able to show that R-flurbiprofen, the so-called inactive enantiomer of flurbiprofen with regard to inhibition of COX, induces apoptosis and a cell cycle block in cell lines with or without COX-2 expression to a similar or even greater extent as the COX-inhibiting isomer S-flurbiprofen. It has been shown that induction of the cell cycle block in the G_1 phase was dependent on activation of the *c*-Jun kinase and the transcription factor activator protein 1 (AP-1) (Grosch et al., 2003), whereas the initiation of apoptosis after R-flurbiprofen treatment was in part dependent on p53 phosphorylation (Grosch et al., 2005). The first hint that NSAIDs act also through COX-independent mechanisms came from Hanif et al., who treated HCT-15 cells which do not produce prostaglandins and HT-29 cells which produce prostaglandins with sulindac sulfide or piroxicam. Both substances inhibited cell proliferation and induced apoptosis in both cell lines, independent of their COX-2 expression and prostaglandin production (Hanif et al., 1996). Treatment of these cells with prostaglandins did not reverse inhibition of proliferation or apoptosis caused by sulindac sulfide and piroxicam, which indicates that these effects are independent of COX inhibition. Moreover, the selective COX-2 inhibitor celecoxib exerts its anticarcinogenic effect by COX-dependent as well as by COX-independent mechanisms (Grosch et al., 2001; Maier et al., 2004).

Also ibuprofen has been shown to inhibit tumor initiation and proliferation in prostate cancer, breast cancer and colon cancer (Andrews et al., 2002; Harris et al., 1999, 2003; Nelson and Harris, 2000; Reddy et al., 1992; Yao et al., 2005). However, little is known about the molecular mechanisms. Ibuprofen exists as two optical isomers that is S-ibuprofen which is the active agent with regard to COX inhibition and R-ibuprofen which inhibits COX at least two orders of magnitude less potently than S-ibuprofen (Boneberg et al., 1996; Neupert et al., 1997). A unique phenomenon of ibuprofen is that in vivo R-ibuprofen is unidirectionally inverted in all species to the COX-inhibiting S-isomer. However, we have shown that after racemate administration inversion of R-ibuprofen provides for only one third of the AUC of S-ibuprofen obtained after administration of the pure S-enantiomer (Geisslinger et al., 1989). Today, beside the ibuprofen racemate also the pure S-enantiomer is commercially available at least in some countries. In the present study, we aimed to investigate the molecular mechanisms of the anticarcinogenic effects of the two ibuprofen enantiomers in colon cancer cell lines in vitro and in vivo. We used two different colon cancer cell lines, a COX-2 expressing cell line (HCA-7) and a COX-2 deficient cell line (HCT-15), in order to distinguish between COX-dependent and COX-independent mechanisms.

2. Material and methods

2.1. Cells and reagents

The human colon carcinoma cell line HCA-7 was purchased from the European Collection of Animal Cell Cultures (ECACC, Wiltshire, UK). HCT-15 cells were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). HCA-7 cells were cultured in Dulbecco's MEM (modified Eagle's medium) supplemented with 10% fetal calf serum, L-glutamine, 110 mg/l sodium pyruvate and 1% of penicillin/streptomycin. HCT-15 cells were cultured in RPMI-1640 medium with glutamax-1 supplemented with 10% fetal calf serum and 1% of penicillin/streptomycin. All cell lines were cultured at 37 °C in an atmosphere containing 5% CO_2. R- and S-ibuprofen were supplied by PAZ Pharma F and E GmbH (Bad Oeynhausen, Germany). The optical purity of the enantiomers was >99% (as determined by stereoselective high-performance liquid chromatography (HPLC) analysis). Both substances were dissolved in PBS (phosphate buffered saline).

2.2. Proliferation assay

Due to the different proliferation rates of HCT-15 and HCA-7 cells we seeded either 1×10^4 (HCT-15) or 5×10^4 (HCA-7) cells in 2 cm dishes in medium containing 10% fetal calf serum and incubated for 24 h at 37 °C in an atmosphere containing 5% CO_2. Then cells were treated with increasing concentrations (0, 200, 400, 600, 700, 800, 900 and 1000 μM) of S- or R-ibuprofen for 8 days. At the end of the incubation period cells were harvested by trypsinisation and stained with trypan blue solution (0.4%, Sigma-Aldrich, Taufkirchen, Germany). The living cells were counted and the cell survival rate was calculated as the percentage of surviving cells.

2.3. Detection of cell cycle arrest and apoptosis using flow cytometry

HCT-15 and HCA-7 cells were seeded in 10 cm dishes at a density of 5×10^5 cells in medium containing 10% fetal calf serum and incubated for 24 h at 37 °C in an atmosphere containing 5% CO_2. Then, cells were synchronised by serum withdrawal for 48 h (HCT-15) or 24 h (HCA-7) and treated with increasing concentrations of ibuprofen enantiomers (0, 200, 400, 600, 800, 900 and 1000 μM) in medium containing 10% fetal calf serum. Measurement of cell cycle distribution occurred after a treatment period of 24 h (HCT-15) or 20 (HCA-7), and apoptosis was measured after 72 h. Therefore, cells were harvested by trypsinisation, washed two times with cold PBS (phosphate buffered saline) and fixed overnight with 80% ethanol at −20 °C. The fixed cells were washed with PBS and incubated for 5 min with 0.125% triton X-100 on ice. After centrifugation cells were resuspended in PBS containing ethidium-bromide and 200 μg/ml RNaseA. Cell cycle distribution and apoptosis were analysed by flow cytometry (Beckton Dickinson FACSCalibur). The percentage of cells in the different cell cycle phases (G_0/G_1, S and G_2/M) was assessed using WinMDI 2.8 and the percentage of apoptotic cells was calculated from the number of cells in subG_1 phase.

2.4. Western blot analysis

5×10^5 HCT-15 and HCA-7 cells were seeded in 10 cm dishes. The cells were treated with 900 μM S- or R-ibuprofen for different

time periods (4 h, 8 h, 16 h, 24 h, 32 h, 48 h and 72 h). We chose this concentration, because 900 μM S- or R-ibuprofen caused a cell cycle block and apoptosis in both cell lines (flow cytometry). The cells were washed with 10 ml PBS, scraped in 1 ml PBS, and centrifuged for 1 min at 11,000 *g*. After resuspending the cells in 1 ml lysis-buffer [10 mM Tris/HCl (pH 7.2), 1 mM EDTA (ethylenediamine tetraacetic acid), 1 mM mercaptoethanol, 5% glycine, 1 mM PMSF (phenyl–methyl–sulfonyl fluoride), 5 mM DTT (dithiothreitol)] the cells were sonicated and centrifuged at 21,000 *g* for 10 min. Protein concentrations of supernatants were determined by Bradford. 30 μg of total protein extract was separated electrophoretically by 10–12% SDS–PAGE (sodium-dodecylsulfate–polyacrylamide gel electrophoresis) and electro-blotted onto a nitrocellulose membrane (Hybond-C, Amersham Life Science, Freiburg). After an overnight incubation of the membranes in blocking buffer (5% nonfat dry milk in 0.3% Tween 20/PBS) or Odyssey blocking-reagent (LI-COR Biosciences, Bad Homburg, Germany) the membranes were incubated for 2 h at room temperature with the respective primary antibody directed against poly (ADP)-ribose polymerase (1:1000, rabbit polyclonal), cyclin A (1:200, rabbit polyclonal), cyclin B (1:200, rabbit polyclonal), cyclin D1 (1:1000, rabbit polyclonal), extracellular signal-regulated kinase 2 (1:2000, mouse monoclonal) or p27 (1:100, rabbit polyclonal) diluted in the respective blocking buffer. The anti-poly (ADP)-ribose polymerase and anti-cyclin D1 antibody were purchased from Cell Signaling (Massachusetts, USA), all other antibodies from Santa Cruz Biotechnology (Heidelberg, Germany).

Membranes were washed three times with 0.3% Tween 20 in PBS for 10 min, and then incubated with an IRDye680 or IRDye800 conjugated secondary antibody (BIOTREND Chemikalien GmbH) in blocking buffer for 1 h. After extensive rinsing in 0.3 % Tween 20 in PBS protein-antibody complexes conjugated with IRDye800/700 were visualised on the Odyssey Infrared Imaging System (LI-COR Biosciences).

2.5. Tumor proliferation in nude mice

For the in vivo experiments 6–8 week old NMRI (nu/nu) male mice (Harlan Winkelmann GMbH, Borchen, Germany) were used. HCT-15 (1×10^7 cells per mouse) and HCA-7 cells (3×10^7 cells per mouse) were injected subcutaneously at the right and left dorsal flank. 3 days after tumor cell injection nude mice were treated five days a week intraperitoneally either with 15 mg/kg/day S-ibuprofen or R-ibuprofen, suspended in PBS (pH 7), up to 4 weeks. Every group consisted of six to eight mice, the control group was treated with the vehicle. The tumor volume was assessed three times a week with a calliper rule in a blinded fashion. After administration of the last dose of S- or R-ibuprofen the mice were killed and blood samples were taken at different time points (5 min, 15 min, 30 min, 45 min, 60 min and 120 min) for determination of S- and R-ibuprofen plasma concentrations. In all experiments the ethics guidelines for investigations in conscious animals were obeyed and the experiments were approved by the local Ethics Committee for Animal Research.

Mouse plasma samples were extracted by solid phase extraction and analysed for S- and R-ibuprofen employing a stereoselective high-performance liquid chromatography (HPLC) method as described previously (Menzel-Soglowek et al., 1990). The coefficient of correlation for all measured sequences was at least 0.99. The intra-day and inter-day variability was <10%.

2.6. Detection of intracellular ibuprofen concentrations in human colon cancer cells

1×10^7 HCA-7 or HCT-15 cells, respectively, were seeded in dishes and incubated for 24 h at 37 °C. Because 900 μM ibuprofen was the most effective concentration in our in vitro experiments we used the same concentration for determination of intracellular drug concentrations. The cells were treated with 900 μM S- or R-ibuprofen for various time intervals (4 h, 8 h, 16 h and 24 h), washed four times with 10 ml ice cold PBS, scraped in 1 ml PBS and transferred in Eppendorf-caps. After centrifugation for 1 min at 2000 g the PBS supernatant was removed carefully and the weight of the remaining cell pellet was determined accurately to 0.01 mg using a precise balance (H54AR, Mettler, Giessen, Germany). The cells were then covered exactly with 200 μl PBS and the soluble content of the cells was squeezed out by ultracentrifugation (180 000 *g* for 16 h). The supernatants were transferred into new Eppendorf-caps and ibuprofen was extracted by solid phase extraction and analysed by stereoselective HPLC analysis (Menzel-Soglowek et al., 1990). To estimate the approximate intracellular volume (μl), which was released into the PBS supernatant during centrifugation, the cell weight before and after centrifugation was determined. The total intracellular concentration was estimated using the resulting dilution and the ibuprofen concentrations in the supernatant.

2.7. Statistics

Proliferation and cell cycle data are presented as mean ± S.E.M. The SPSS 9.01 computer software was used for statistical analyses. The percentage of cells in the different cell cycle phases of treatment groups were compared with the control by *t*-tests using a Bonferroni α-correction for multiple comparisons (α was set at 0.05). IC_{50} values were analysed using GraphPad Prism 4. For comparison of tumor growth in nude mice the area under the tumor volume versus time curve was calculated using the linear trapezoidal rule. The AUCs (area under the curves) were submitted to univariate ANOVA (analysis of variance).

3. Results

3.1. Cyclooxygenase expression in tumor cells

For the experiments two different colon carcinoma cell lines (HCA-7 and HCT-15) which differ in their cyclooxygenase expression status and PGE_2 production were used. HCA-7 cells express COX-1 and COX-2 constitutively, whereas HCT-15 cells show only COX-1 expression and no COX-2 protein (Fig. 1A).

The cyclooxygenase converts arachidonic acid to PGH_2 which itself is a substrate for various prostaglandin synthases. PGE_2 is the major product resulting from these reactions. Therefore, we determined the PGE_2 levels in conditioned media of HCA-7 and

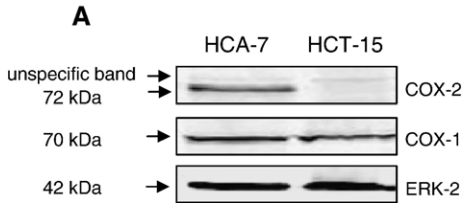

Fig. 1. A) Western blot analysis of cyclooxygenase expression in HCA-7 and HCT-15 colon carcinoma cells. 50 μg total protein extract of the untreated colon carcinoma cells was separated on a 12% SDS-polyacrylamid gel and transferred to a nitrocellulose membrane. COX-1 and COX-2 expression was detected using a rabbit polyclonal anti-COX-1 and anti-COX-2 antibody. A mouse monoclonal extracellular signal-regulated kinase-2 (ERK-2) antibody was used as a loading control.

HCT-15 cells using a PGE_2 EIA™-kit (data not shown). As expected only HCA-7 cells produced high amounts of PGE_2 (>3000 pg/1 × 10^6 cells), whereas the PGE_2 level in medium of HCT-15 cells were below the detection limit (<5 pg/1 × 10^6 cells).

3.2. Cell proliferation assay and determination of intracellular ibuprofen concentrations

As mentioned in the introductory part ibuprofen shows antiproliferative effects in different cancer cells. In order to investigate if these effects depend on COX-inhibition we analysed the survival rate of HCA-7 and HCT-15 cells after treatment with either S-ibuprofen which inhibits COX-1 and COX-2 activity or R-ibuprofen which inhibits COX activity two to three orders of magnitude less potently. As shown in Fig. 2A, S- and R-ibuprofen inhibited survival of HCT-15 and HCA-7 cells measured after treatment for 8 days. Although R-ibuprofen was less effective in both cell lines, comparison of the IC_{50} levels revealed no significant difference for both enantiomers ($IC_{50\ S\text{-ibuprofen HCT-15}}$ = 265.4 μM ± 36.16 and $IC_{50\ R\text{-ibuprofen HCT-15}}$ = 315.8 μM ± 42.65 and $IC_{50\ S\text{-ibuprofen HCA-7}}$ = 576.9 μM ± 45.15 and $IC_{50\ R\text{-ibuprofen HCA-7}}$ = 637.9 μM ± 24.4). However, the COX-2 deficient HCT-15 cells were more susceptible to the proliferation inhibitory effect of S- and R-ibuprofen than the COX-1 and COX-2 expressing cell line HCA-7 (P < 0.01). The data of the proliferation assay indicate that the antiproliferative effect of S- and R-ibuprofen is at least partly independent of COX inhibition because both enantiomers reduced cell viability in COX-2 deficient HCT-15 cells as well as in COX-2 expressing HCA-7 cells nearly to the same extent.

To address the observed significant difference in sensitivity between HCT-15 and HCA-7 cells we determined the intracellular concentrations of S- and R-ibuprofen and ascertained that in HCA-7 cells both S- and R-ibuprofen reached only one third of the intracellular concentrations as compared to HCT-15 cells (Fig. 2B). The intracellular concentrations of ibuprofen in HCT-15 cells after incubation with 900 μM S- or R-ibuprofen for 24 h were 377.5 ± 63.4 μM S-ibuprofen and 431.8 ± 81.9 μM R-ibuprofen, respectively. In contrast, in HCA-7 cells the intracellular concentrations after incubation with 900 μM S- or R-ibuprofen for 24 h were only 43.5 ± 8.1 μM S-ibuprofen and 173.6 ± 25.6 μM R-ibuprofen. These data indicate that the effective ibuprofen concentrations were lower than the total concentrations in the medium. Furthermore, the measurement of the intracellular concentrations revealed that in both cell lines R-ibuprofen has not undergone chiral inversion to S-ibuprofen.

These data let us conclude that the differences in sensitivity of the two cell lines observed in the proliferation assay (HCA-7 cells were significantly less sensitive against S- and R-ibuprofen than HCT-15 cells) might be dependent on the different intracellular ibuprofen concentrations of these cells. Furthermore, this

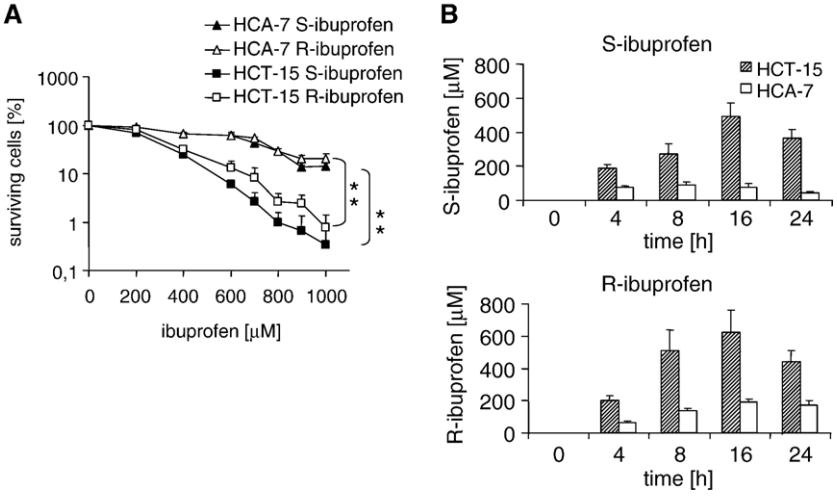

Fig. 2. A) Survival rate of HCA-7 and HCT-15 cells in the proliferation assay. 5 × 10^4 cells (HCA-7) or 1 × 10^4 cells (HCT-15) were seeded in 2 ml dishes, incubated for 16 h and treated with increasing concentrations of S- or R-ibuprofen for 8 days. After the incubation time the cells were harvested by trypsinization, stained with trypan blue solution and counted. The mean number of untreated control cells was set at 100% survival rate. Values are the mean ± S.E.M. of three independent experiments, each performed in duplicate. Statistically significant differences in the proliferation rate of both cell lines treated with S- or R-ibuprofen are indicated with an asterisk. *P < 0.05, **P < 0.01. B) Detection of intracellular ibuprofen concentrations in human colon cancer cells after treatment of HCA-7 and HCT-15 cells with 900 μM S- or R-ibuprofen for different time points (4, 8, 16 and 24 h). The cells were seeded, incubated with S- or R-ibuprofen and after the incubation time, scraped in 10 ml PBS. The cell pellets were resuspended with 200 μM PBS and ultracentrifuged for 16 h. Supernatants were measured by stereoselective HPLC analysis and the ibuprofen concentrations calculated as described.

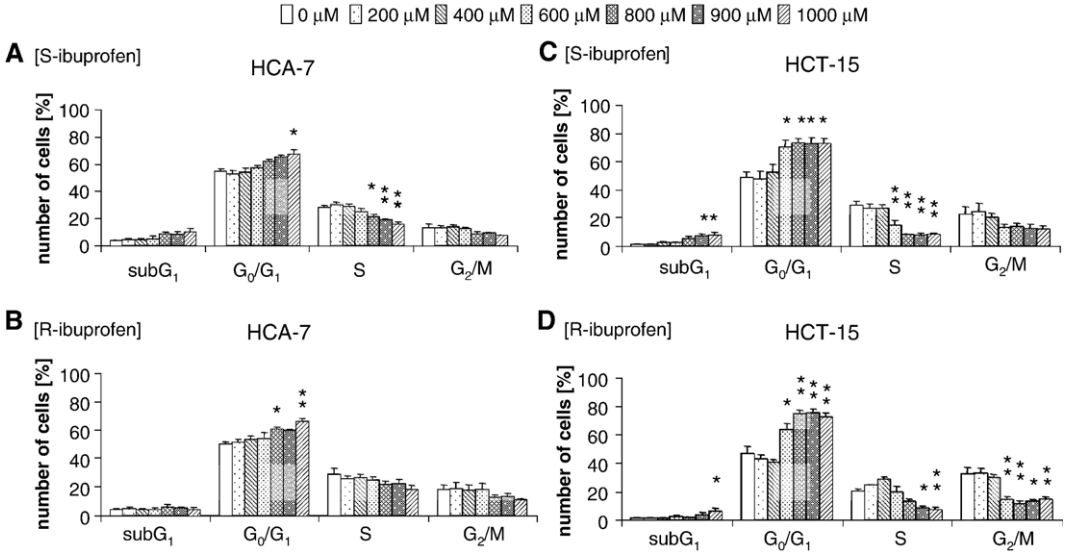

Fig. 3. Cell cycle analysis of HCA-7 and HCT-15 cells after treatment with increasing concentrations of S-ibuprofen (A, C) or R-ibuprofen (B, D). 5×10^5 cells were seeded in 10 ml dishes, synchronized by serum withdrawal and treated with S- or R-ibuprofen (200, 400, 600, 700, 800, 900 and 1000 µM) for 20 h (HCA-7) or 24 h (HCT-15). The cells were harvested by trypsinisation, fixed with 80% ethanol and the DNA content was measured using ethidium-iodide staining and flow cytometric analysis. Data represent the percentage of cells in the different cell cycle phases ($subG_1$, G_1/G_0, S and G_2/M phases) and are the mean±S.E.M. of three independent experiments. Statistical significant differences of cells treated with S- or R-ibuprofen versus untreated cells were indicated with an asterisk. *$P<0.05$, **$P<0.01$.

intracellular concentration of ibuprofen depends on the free drug concentrations in the medium which are about 193.5±75.1 µM for 900 µM S-ibuprofen and 208.3±82.5 µM for 900 µM R-ibuprofen.

3.3. Effect of S- and R-ibuprofen on cell cycle progression

To investigate whether or not the observed antiproliferative effects of S- and R-ibuprofen are mediated by inhibition of cell cycle progression, we determined the percentage of HCA-7 and HCT-15 cells in the different cell-phases after treatment with various concentrations of S-ibuprofen (Fig. 3A and C) or R-ibuprofen (Fig. 3 B and D) for 20 h (HCA-7) and 24 h (HCT-15) using flow cytometry. The time points were used due to the various proliferation rates of the two tumor cell lines. In both cell lines a concentration dependent increase in the number of cells in the G_0/G_1 phase was detected accompanied by a corresponding reduction of the proportion of cells in the S and G_2/M phases after treatment of these cells with S- or R-ibuprofen. In HCT-15 cells a statistically significant increase of cells in the G_0/G_1 phase was observed at concentrations ≥600 µM (S- or R-ibuprofen). However, for HCA-7 cells a significant increase of cells in the G_0/G_1 phase could only be detected at 1000 µM (S-ibuprofen) or at concentrations ≥800 µM (R-ibuprofen) (Fig. 3A and B; *$P<0.05$, **$P<0.01$). Again, there was no clear difference between S- and R-ibuprofen treatment in each cell line. The observed differences in sensitivity between HCT-15 and HCA-7 cells may depend on the different intracellular ibuprofen concentrations in these colon carcinoma cells as already mentioned above.

Cell cycle progression is regulated by the activity of different cyclin-dependent kinases in complex with cyclins and by cell cycle inhibitors such as $p21^{Waf1}$ and $p27^{Kip1}$. To analyse the molecular mechanisms which lead to the observed G_1-phase block after ibuprofen treatment we determined the expression status of different cyclins and cell cycle inhibitors by western blot analysis. Cells were treated with 900 µM S- or R-ibuprofen, because this concentration caused a cell cycle block in both cell lines after 20 h and 24 h, respectively. As shown in Fig. 4A the expression of cyclin A decreased already after an incubation period for 8–16 h with 900 µM S- or R-ibuprofen. In untreated control cells the expression of cyclin A was unchanged over the

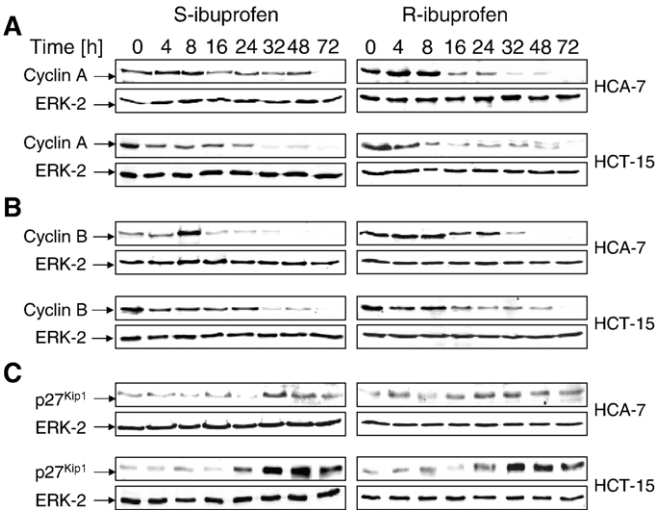

Fig. 4. Western blot analysis of cyclin A (A), cyclin B (B), and $p27^{kip1}$ (C) protein expression in HCA-7 and HCT-15 cells after S- ibuprofen or R-ibuprofen treatment. 5×10^5 cells were seeded in 10 ml dishes and treated with 900 µM S- or R-ibuprofen for 4, 8, 16, 24, 32, 48 and 72 h. 30 µg of total protein extract was loaded per lane on 12% SDS-polyacrylamid gels and blotted onto nitrocellulose membranes. The membranes were incubated with a rabbit polyclonal anti-cyclin A antibody, a polyclonal rabbit antibody against cyclin B, or a polyclonal rabbit antibody against $p27^{kip1}$, respectively, and the respective secondary antibody conjugated with IRDye680 or IRDye800. A mouse monoclonal ERK-2 antibody was used as a loading control. Representative experiments out of three for each antibody are shown.

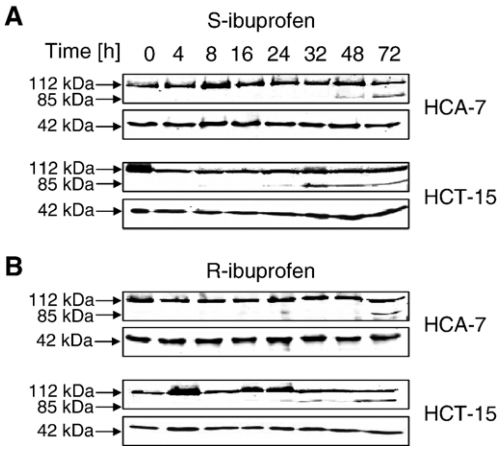

Fig. 5. Western blot analysis of PARP cleavage in HCA-7 and HCT-15 colon carcinoma cells after treatment with 900 μM S-ibuprofen (A) or R-ibuprofen (B) for different time periods. 5×10^5 cells were seeded in 10 ml dishes and treated with S- or R-ibuprofen for 4, 8, 16, 24, 32, 48 and 72 h. 50 μg of total protein extract was loaded per lane on a 12% SDS-polyacrylamid gel and blotted onto a nitrocellulose membrane. PARP (112 kDa) and the apoptotic cleavage product (85 kDa) were detected using a rabbit polyclonal anti-PARP antibody. The membranes were incubated with a mouse monoclonal ERK-2 antibody as a loading control. A representative experiment out of three is shown.

respective time period (data not shown). In addition, the expression of cyclin B time-dependently decreased in both cell lines after treatment with 900 μM S- or R-ibuprofen (Fig. 4B). The cyclin B expression in the control cells remained unchanged (data not shown). There was no clear difference in the sensitivity of the cells towards S- and R-ibuprofen induced decrease in the expression of cyclin A and B. In addition the expression of cyclin D1 was also investigated, however, no changes in expression levels were detected following ibuprofen treatment as compared to untreated control cells (data not shown).

Moreover, we determined the expression level of the cell cycle inhibitor protein $p27^{Kip1}$ which increased 16 h after treatment with 900 μM S- or R-ibuprofen in both cell lines (Fig. 4C). The increase in HCT-15 cell was more pronounced than in HCA-7 cells both after S- and R-ibuprofen treatment, which is in line with the flow cytometry data from cell cycle analysis.

3.4. Induction of apoptosis

To evaluate whether or not the reduction in cell number in the cell proliferation assay was not only caused by cell cycle arrest but also by induction of apoptosis, we assessed different apoptosis marker. First, we detected poly (ADP-ribose) polymerase (PARP) cleavage using western blot analysis. PARP is a 112 kDa nuclear protein which is specifically cleaved by activated caspase-3 and caspase-7 during apoptosis into 85 kDa and 29 kDa fragments. PARP can thus be used as an early apoptosis marker. For apoptosis detection we treated both cell lines with either 900 μM S-ibuprofen (Fig. 5A) or R-ibuprofen (Fig. 5B) for up to 72 h. PARP cleavage occurred in HCT-15 cells already after 32 h for S- and R-ibuprofen, whereas in HCA-7 cells it was first detected after 48–72 h for S- and R-ibuprofen, respectively.

As a second apoptotic marker we determined the percentage of cells in the $subG_1$ phase by flow cytometry. Therefore, we treated HCA-7 and HCT-15 cells with increasing concentrations of S- and R-ibuprofen for 72 h, a time point were PARP cleavage was detected in both cell lines. As shown in Fig. 6A–D the percentage of cells in the $subG_1$ phase increased in a concentration dependent

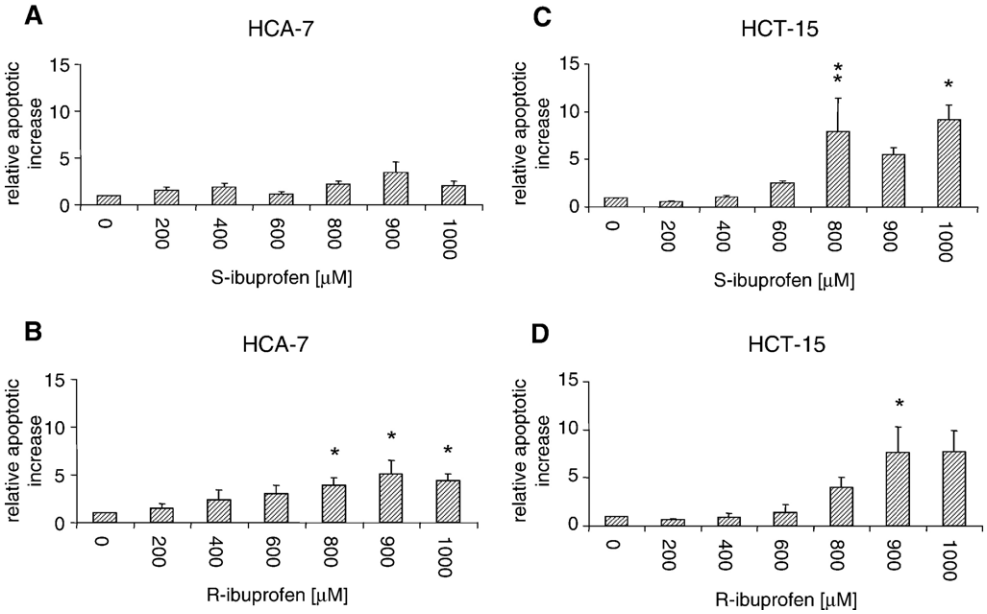

Fig. 6. Analysis of the number of apoptotic cells in the G_1-phase after treating colon carcinoma cells (HCA-7 and HCT-15) with 900 μM S-ibuprofen (A, C) or R-ibuprofen (B, D). 5×10^5 cells were seeded in 10 ml dishes, synchronized by serum withdrawal and treated with S- or R-ibuprofen (200, 400, 600, 700, 800, 900 and 1000 μM) for 72 h. The cells were harvested by trypsinisation, fixed with 80% ethanol and the DNA content was measured using propidium iodide staining and FACS analysis. Data are mean ± S.E.M. of the relative number of apoptotic cells in the $subG_1$ phase versus untreated control cells. The asterisks indicate statistical significant increase in the number of apoptotic cells after treatment with S- or R-ibuprofen in comparison to untreated control cells. *$P<0.05$, **$P<0.01$.

manner. A significant increase was observed after treatment of HCA-7 cells with concentrations ≥800 μM R-ibuprofen and in HCT-15 cells after treatment with concentrations of ≥800 μM S-ibuprofen or 900 μM R-ibuprofen. The increase of apoptotic cells in the HCA-7 cell line after S-ibuprofen treatment was not statistically significant (Fig. 6A).

3.5. Tumor growth in nude mice

HCA-7 and HCT-15 cells were used for tumor growth assays in nude mice in order to examine the antiproliferative effects of S- and R-ibuprofen not only in cell culture assays but also in vivo. HCA-7 (3×10^7 cells per mouse) and HCT-15 (1×10^7 cells per mouse) cells were injected subcutaneously at the right and left dorsal flank. 3 days after tumor cell injection the mice were treated with S- or R-ibuprofen (15 mg/kg/day) for 3 weeks. As shown in Fig. 7 both S- and R-ibuprofen treatment reduced significantly the growth of HCA-7 (A) and HCT-15 (B) colon cancer xenografts in comparison to untreated control mice. These data indicate that the antiproliferative effects of S- and R-ibuprofen could not only be observed in vitro but also in vivo.

R-ibuprofen is known to be unidirectionally inverted to S-ibuprofen in various species including mice and humans. We, therefore, analysed the plasma concentrations of S- and R-ibuprofen after administration of the last dose. Blood samples were collected at time points from 5 min to 120 min and the plasma concentrations of S- and R-ibuprofen were determined by

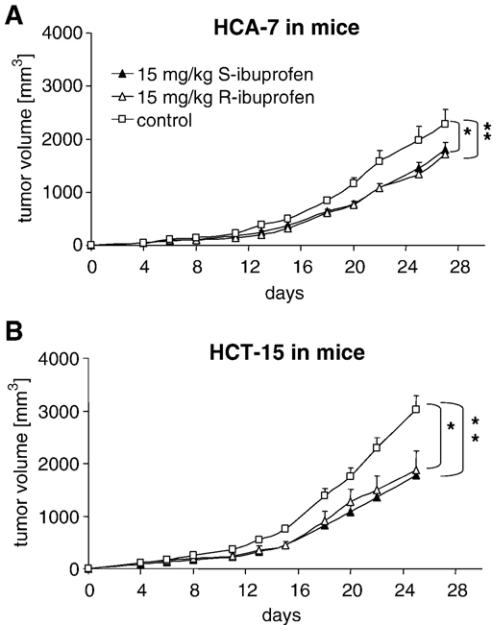

Fig. 7. Tumor growth of HCA-7 (A) and HCT-15 (B) cells in nude mice. 3×10^7 (HCA-7) or 1×10^7 (HCT-15) cells were injected subcutaneously at the dorsal flank. The nude mice were treated 5 days a week with 15 mg/kg/day S- or R-ibuprofen for three weeks, the control group was left untreated. Each group consisted of 5 to 8 animals. The tumor volume was measured every 2nd day and the volumes of the treated groups were compared with the tumor volumes of the control animals. Data are the mean ± S.E.M. of the tumor volumes. The statistical comparisons of the areas under the "tumor volume" versus "time curves" have shown that tumor growth in treated mice was significantly reduced, compared to control mice. *$P<0.05$, **$P<0.01$.

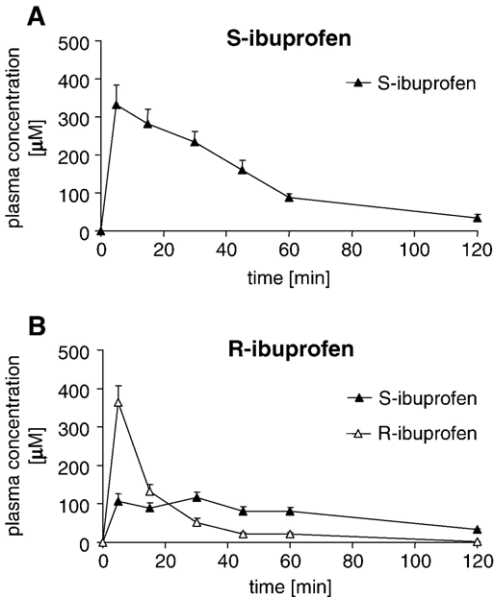

Fig. 8. Plasma concentration of ibuprofen in nude mice after treatment with S- or R-ibuprofen for 3–4 weeks. After the last administration of 15 mg/kg/day S-ibuprofen (A) or R-ibuprofen (B) blood samples were collected at different time points (5, 15, 30, 45, 60 and 120 min) and the concentrations of S- and R-ibuprofen in every plasma sample were measured by stereoselective HPLC analysis. Data are the mean ± S.E.M., for every time point 5 to 8 animals are included.

stereoselective HPLC. For every time point 5 to 8 blood samples were taken. In mice treated with 15 mg/kg/day S-ibuprofen the plasma concentration after 5 min following the last S-ibuprofen dose was 332 ± 51 μM and it decreased after 2 h to a concentration of 34 ± 9 μM S-ibuprofen (Fig. 8A). 5 min after administration of the last dose of R-ibuprofen we detected a plasma concentration of 364 ± 44 μM R-ibuprofen and 106 ± 20 μM S-ibuprofen in these mice. After 2 h it decreased to a concentration of 34 ± 2 μM S-ibuprofen and just a very small amount of R-ibuprofen could be detected (Fig. 8B). Data are the mean ± S.E.M. We determined the area under the plasma concentrations versus time curve (AUC) of S- and R-ibuprofen after administration of R-ibuprofen and calculated the inversion of R- to S-ibuprofen. In mice about 54% of R-ibuprofen was unidirectionally inverted to S-ibuprofen, thus the antitumorigenic effect of R-ibuprofen cannot solely be assigned to this enantiomer.

Moreover, these data indicate that the concentrations of S- and R-ibuprofen which we used in vitro are only 2–3 fold higher than the concentrations we obtained in vivo in the mouse. In comparison to the intracellular ibuprofen concentrations measured after incubation of cells with 900 μM S- or R-ibuprofen the plasma concentrations were similar.

4. Discussion

Since the approval of ibuprofen in 1969 the view about the usage of NSAIDs has dramatically changed. NSAIDs were developed and approved for the treatment of inflammation and pain but a lot of prospective — as well as case-control studies have demonstrated that NSAIDs reduces also the risk of cancer.

For example, Nelson and Harris (2000) have shown that ibuprofen reduces the risk of prostate cancer and Harris et al. have demonstrated in a prospective study that the regular use of ibuprofen for several years was accompanied by a decrease of risk for breast cancer (Harris et al., 1999, 2003). So far, however, the molecular mechanisms of the anticarcinogenic effects of NSAIDs are not clear.

Thus in this study, we investigated the anticarcinogenic effects of S- and R-ibuprofen in vitro by the use of two human colon carcinoma cell lines as well as in vivo in the nude mouse model. Traditionally, anticarcinogenic effects of NSAIDs have been explained by inhibition of cyclooxygenases. But only S-ibuprofen is a potent COX-1 and COX-2 inhibitor whereas the R-enantiomer is two to three orders of magnitude less potent to inhibit COX. Thus, by using ibuprofen enantiomers and two different cell lines as a tool, we put special interest to clarify the questions: 1) to which extent the antiproliferative effect of the ibuprofen-isomers depends on COX-inhibition and 2) are there differences in terms of their anticarcinogenic potency.

For our experiments we used two human colon carcinoma cell lines HCT-15 (produce no PGE_2) and HCA-7 (produce high levels of PGE_2) and treated them in vitro and in vivo after injection into nude mice with different concentrations of S- and R-ibuprofen. Interestingly, the COX-1 and COX-2 expressing cell line HCA-7 was less sensitive against both the cell cycle inhibiting as well as apoptosis inducing potency of S- and R-ibuprofen in comparison to the COX-2 deficient human colon carcinoma cell line HCT-15. In accordance with the observed cell cycle block, which also was more pronounced in HCT-15 cells, western blot analysis of cell cycle regulating proteins revealed that induction of the cell cycle inhibitor $p27^{Kip1}$ was stronger in HCT-15 cells than in HCA-7 cells. This difference was also obvious in vivo in the nude mice model, where the growth of HCA-7 xenografts was slightly less, but still significantly inhibited by S- and R-ibuprofen in comparison to HCT-15 xenografts.

Determination of intracellular drug concentrations by stereoselective HPLC pointed out that after treatment of HCT-15 and HCA-7 cells with 900 μM S- or R-ibuprofen the intracellular concentration of both enantiomers were about three times higher in HCT-15 cells as compared to HCA-7 cells. These differences in intracellular drug concentrations might explain the higher sensitivity of HCT-15 cells against the antiproliferative effects of S- and R-ibuprofen. The molecular mechanisms responsible for these differences in intracellular drug concentrations are not clear. Possibly, the cells express different levels of the organic anion transporter (OAT) or differ in their activity. These transporters are expressed in many tissues and also in human colon adenocarcinoma cells (Hirohashi et al., 2000) and it has been shown that the human OAT1 and OAT3 are involved in the transport of ibuprofen and other NSAIDs (Khamdang et al., 2002). In addition, differences in the expression or activity of metabolizing enzymes may also contribute to the observed variations in intracellular drug concentrations of S- and R-ibuprofen. Which of these mechanisms are responsible for the differences in intracellular drug concentrations in our cell system is not clear and have still to be investigated. Furthermore, determination of free drug concentrations of S- or R-ibuprofen in medium after administration of 900 μM of each enantiomer revealed, that only about 10–30% of each enantiomer were protein-unbound (data not shown). So free medium drug concentrations are comparable with the intracellular concentrations of S- and R-ibuprofen and with mean plasma concentrations measured after treatment of mice with 15 mg/kg S- or R-ibuprofen.

Moreover, the plasma concentrations of ibuprofen after oral administration of 400–600 mg ibuprofen racemate in humans are in the range between 150–300 μM (product information of Contraneural®600 (Pfleger) and Ibuprofen STADA®400 mg (STADA)). These data indicate that the ibuprofen concentrations which we used in vitro for induction of a cell cycle block or apoptosis were comparable to those reachable in vivo in mice and humans. Thus, we can conclude that the molecular mechanisms observed in our in vitro experiments may in principle explain the observed anticarcinogenic effects of ibuprofen in vivo.

In this study we demonstrated that the anticarcinogenic effects of S- and R-ibuprofen occurred in COX-2 expressing as well as in COX-2 deficient colon carcinoma cell lines both in vitro and in vivo in the nude-mice model and that there exists no dramatic difference in the anticarcinogenic potency of both enantiomers. Furthermore, ibuprofen concentrations used to inhibit cell growth are one to two orders of magnitude higher than concentrations are needed for inhibition of COX-1 or COX-2 (Neupert et al., 1997). These data suggest that the observed antiproliferative effects of ibuprofen enantiomers are in part independent of COX-inhibition. Last, but not least several COX-independent targets of ibuprofen have been proposed. Scheuren et al. reported that R-ibuprofen inhibits the translocation of transcription factor nuclear factor-κB (NF-κB) into the nucleus by stabilizing the NF-κB/ IκBα complex in the cytoplasm of the cell (Scheuren et al., 1998). Inhibition of nuclear factor-κB activity has been identified as one possible mechanism contributing to the anticarcinogenic effects of NSAIDs, probably by suppression of nuclear factor-κB regulated protein expression, such as COX-2 and cyclin D_1 (Takada et al., 2004). Another COX-independent target of ibuprofen is the *N*-acetyltransferase (NAT) which is inhibited by ibuprofen in a concentration dependent manner (Chung et al., 1999). This enzyme is expressed in various animals as well as in humans and is involved in chemical carcinogenesis. In prostate cancer cells ibuprofen reduced the expression of the hypoxia-inducible factors HIF-1α and HIF-2α both in COX-2 expressing and COX-2 deficient cells. These proteins regulate the expression of genes involved in angiogenesis and cell survival (Palayoor et al., 2003). Last, but not least it has been shown that ibuprofen induced the expression of the $p75^{NTR}$ neurotrophin receptor in bladder tumor cells, which is a tumor and metastasis suppressor (Khwaja et al., 2004). These results together with our in vitro data corroborate that S- and R-ibuprofen inhibits tumor growth at least partly through COX-independent mechanisms. In our in vivo experiments both enantiomers significantly inhibited the growth of the COX-2 deficient HCT-15 and the COX-2 expressing HCA-7 colon xenografts in comparison with the untreated control group. It is known that R-ibuprofen can be inverted in vivo, including mice and humans (Caldwell et al., 1988; Reichel et al., 1997; Williams, 1990), and it has been shown that already the thioester, R-ibuprofenoyl-CoA which is a prerequisite for inversion, inhibits

COX-2 activity within the same concentration range as S-ibuprofen (Neupert et al., 1997). Thus, for the in vivo situation, we cannot exclude that COX-inhibition plays a major role for the antitumorigenic effect of ibuprofen. However, in a recent study Yao et al. also examined the effects of racemic ibuprofen on mouse and human colon cancer cells in vitro and in animal models. They showed that ibuprofen exerts its anticarcinogenic effect in part by inhibition of angiogenesis which was associated with a reduction of vascular endothelial growth factor (VEGF) expression. Interestingly, this effect was not reversible by exogenous addition of PGE_2 (10 μM) (Yao et al., 2005) indicating that the antitumorigenic effect of ibuprofen is in part independent of COX inhibition also in the in vivo situation.

In summary, our study suggest that the anticarcinogenic effect of ibuprofen is only in part mediated through COX inhibition indicating that also COX-independent mechanisms play a decisive role, which until now are not fully understood. No clear difference was observed between the anticarcinogenic effects of S- and R-ibuprofen, suggesting that in the case of tumor inhibition both ibuprofen enantiomers are more or less equally effective. Interestingly, measurement of intracellular ibuprofen concentrations revealed that there exist clear differences in intracellular drug uptake by different cells which might have an impact on the efficacy of ibuprofen in these cells. Thus, further studies are needed, to evaluate the molecular mechanisms responsible for the different intracellular drug concentrations of both ibuprofen enantiomers.

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