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Biochemical Pharmacology 69 (2005) 831–839
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Induction of apoptosis by R-flurbiprofen in human colon carcinoma cells: involvement of p53

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Received 20 September 2004; accepted 25 November 2004

Abstract

R-Flurbiprofen, a non cyclooxygenase inhibiting non-steroidal anti-inflammatory drug (NSAID), has been found to inhibit tumor growth in various animal models. In vitro experiments have shown that this effect is based on the induction of a cell cycle block and apoptosis. Cell cycle inhibition has been explained by activation of the c-Jun–N-terminal kinase (JNK) and downregulation of cyclin D1 expression. However, the molecular mechanism leading to apoptosis is unknown. Here, we show that treatment of the human colon carcinoma cell line HCT116 with different concentrations of R-flurbiprofen leads to an accumulation of p53 protein which is accompanied by an increase in phosphorylated p53 at serine 15. Mutation of serine 15 to alanine by site directed mutagenesis and overexpression of the mutated p53 gene in HCT116 cells, revealed that these cells are significantly less sensitive to apoptosis induced by R-flurbiprofen than pcDNA control cells, as measured by PARP-cleavage and flow cytometry. By contrast, no difference was detected between HCT116p53ser15ala cells and HCT116 pcDNA cells with respect to induction of a cell cycle block after R-flurbiprofen treatment. Moreover, in nude mice HCT116p53ser15ala overexpressing xenografts were significantly less sensitive to R-flurbiprofen than HCT116 pcDNA control xenografts. In conclusion, we were able to show that induction of apoptosis in HCT116 cells after R-flurbiprofen treatment is at least partly dependent on the tumor suppressor gene p53 and that mutation of p53 at serine 15 impairs the apoptotic potency of R-flurbiprofen.

Keywords: G_1-phase block; Apoptosis; p53; Colon cancer; Flurbiprofen

1. Introduction

More than 20 years ago, it has been shown for the first time that non-steroidal anti-inflammatory drugs (NSAIDs) reduce the risk for colon cancer [1,2]. For a long time, this effect of NSAIDs was exclusively ascribed to their COX-inhibiting potency. Over the last years, however, a substantial number of publications pointed towards COX-independent mechanisms for the cancer protective effects of NSAIDs [3–13], with each NSAID having a different mode of action. Nevertheless, the molecular COX-independent mechanisms of the anticarcinogenic properties of NSAIDs are not fully understood. R-Flurbiprofen, a 2-arylpropionic acid, is one of the most interesting NSAIDs because it neither inhibits COX-1 nor COX-2 at therapeutically relevant concentrations and showed antinociceptive as well as antitumorigenic effects in vitro and in vivo in different mouse models [14–19]. Moreover, in men R-flurbiprofen is nearly not inverted into its COX-inhibiting antipode, S-flurbiprofen [16,20]. Thus, R-flurbiprofen is of great interest because it does not have the risk to cause the typically known serious side effects in the gastrointestinal tract or the kidney as the classical COX-1 and/or COX-2 inhibiting drugs [21,22].

Abbreviations: COX-2, cyclooxygenase-2; NSAID, non-steroidal anti-inflammatory drugs; FCS, fetal calf serum; PBS, phosphate buffered saline; FACS, fluorescence-activated cell sorter; EDTA, ethylenediaminetetraacetate; PMSF, phenylmethylsulfonylfluoride; DTT, dithiotreitol; SDS–PAGE, sodiumdodecylsulfate–polyacrylamide gel electrophoresis; RT, reverse transcription; PCR, polymerase chain reaction; AUC, area under the curve; ANOVA, univariate analysis of variance; PARP, poly(ADP)-ribose polymerase

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doi:10.1016/j.bcp.2004.11.026

Recently, we were able to show that the anticarcinogenic effect of R-flurbiprofen is based on an induction of a G_1-phase block and apoptosis in human colon carcinoma cell lines. It turned out that the cell cycle block is partly dependent on activation of the c-Jun-N-terminal-kinase (JNK) accompanied by an increase in DNA-binding activity of the transcription factor AP-1 and downregulation of cyclin D1 expression. By contrast, R-flurbiprofen-induced apoptosis was largely independent of JNK activation [15].

One of the main regulators of cell growth and death is the tumour suppressor protein p53. Normal cells express very low levels of p53 protein but various cellular stress stimuli lead to a rapid increase of the p53 protein level which then accounts either for growth arrest or programmed cell death [23]. The increase of p53 protein is mainly dependent on post-translational mechanisms and only to a minor extent due to changes in the transcription rate or an increase in translation of p53 mRNA [24]. Under normal growth conditions, p53 is in complex with the proto-oncoprotein Murine Double Minute-2 (Mdm2) and inactive c-Jun-N-terminal kinase, which are both responsible for the constitutive instability of p53. In response to stress, both proteins are released from the N-terminal region of the p53 protein and p53 is subsequently phosphorylated at several serine and threonine residues leading to its stabilization and accumulation [23,25]. More than 10 different kinases have been described to be involved in phosphorylation of p53 [25]. Several phosphorylation sites have been associated with either stabilization of the protein, modulation of the DNA-binding activity of p53 or activation/inactivation of the sequence specific transactivation domain, which is the binding site of components of the transcriptional machinery and co-activators of transcription [23,25]. Mutations at different phosphorylation sites within the p53 gene gave insight which amino acid residues are necessary for the apoptotic or cell cycle inhibiting activity of p53 [26–29]. Because of the fundamental function of p53 in the induction of apoptosis and cell cycle block, we wanted to analyse whether or not the apoptotic and by that anticarcinogenic effect of R-flurbiprofen depends on modulation of p53. The human colon carcinoma cell line HCT116, which express wild type p53, has been used as model cell line.

2. Materials and methods

2.1. Cells and reagents

The human colon carcinoma cell line HCT116 was purchased from ATCC (American-type culture collection) and cultured in McCOY's 5A medium with L-glutamin, supplemented with 10% FCS (foetal calf serum) at 37 °C in a 5% CO_2 atmosphere.

R-Flurbiprofen was supplied by PAZ Arzneimittelentwicklungsgesellschaft. The optical purity of this enantiomer was >99% (determined by stereoselective HPLC-analysis). The substance was dissolved in phosphate-buffered saline (PBS).

2.2. Cloning and site directed mutagenesis of wt p53

For cloning wildtype (wt) p53 cDNA total RNA from HCT116 cells was prepared by the method of Chomczynski [30]. RT-PCR was performed with the QIAGEN RT-PCR Kit (QIAGEN). Reverse transcription reaction was incubated at 55 °C for 30 min. PCR amplification with p53 specific primers (FW: 5′-TACGAATTCAGTGGGGAACAAGAAGTGGAG-3′, RV: 5′-CAGTGGGGAACAAGAAGTGGAG-3′) was started with an initial activation step at 95 °C for 15 min. The samples were then denatured at 95 °C for 45 s, annealed at 61 °C for 45 s and extended at 72 °C for 1.5 min in 30 repetitive cycles. The PCR-product was loaded onto a 1% agarose gel stained with ethidium-bromid and extracted with a QIAquick gel extraction kit (QIAGEN). Subsequently, the wt p53-cDNA was used to mutate ser15 to ala by PCR-based oligonucleotide-directed mutagenesis. For PCR, we used the p53ser 15ala forward primer (5′-TACGGATCCATGGAGGAGCCGCAGTCAGATCCTAGCGTCGAGCCCCCTCTGGCTCAGGAAAC-3′) and the wt p53 reverse primer (5′-CAGTGGGGAACAAGAAGTGGAG-3′). The resultant PCR product was cloned into the *Bam*HI and *Eco*RI restriction site of the pcDNA-vector (Invitrogen). All PCR-products were verified by sequencing.

2.3. Stable transfection of HCT116 cells with the p53ser15ala cDNA

HCT116 cells were transfected using FuGene-Transfection Kit (Roche Diagonstics GmbH). Two micrograms of each plasmid (pcDNA or p53ser15ala pcDNA) were mixed with 6 μl FuGene reagent and 4 ml McCOY's 5A medium with L-glutamin, the mixture was left at room temperature for 45 min and then added to the cell culture for at least 6 h. Subsequently, medium was resolved and fresh medium was added containing 10% FCS. After 48 h, incubation time cells were splitted 1:8 in medium containing 0.75 mg/ml G418 and after 10 days incubation time-stable transfected cell clones were analysed for p53 expression.

2.4. Western-blot analysis

5×10^5 cells were seeded per 5 cm dish. After 16 h of incubation, cells were treated with either increasing concentrations of R-flurbiprofen (0, 200, 400, 600, 800, 1000 μM) for 20 h or with 800 μM R-flurbiprofen (this concentration showed significant apoptotic activity) for various time periods, washed with PBS, harvested by scraping them in 1 ml PBS and collected by short centrifugation. For whole cell extracts, cell pellets were resuspended in 1 ml lysis-buffer (20 mM Tris–HCl (pH

8.5), 1 mM EDTA, 1 mM PMSF), sonicated and centrifuged. For Western-blot analysis with phospho-antibodies cell pellets were resuspended in phospho-extraction buffer (20 mM Tris–HCl, pH 7.5, 20 mM *p*-nitrophenylphosphate, 1 mM EGTA, 50 mM sodium fluoride, 50 μM sodium orthovanadate, 5 mM benzamidine, 100 mM NaCl, 5 mM $MgCl_2$, 1 μg/ml protease inhibitors), sonicated and centrifuged. The amount of protein of the supernatants was determined with the Bradford method. Aliquots of 50 μg of total protein extract were electrophorectically separated by 10% or 12% SDS–PAGE and electroblotted onto a nitrocellulose membrane (Amersham Life Science). Equal loading of the gel was checked by ponceau staining (3% trichloroacetic acid, 0.1% ponceau). After overnight incubation of the membrane in blocking buffer (5% nonfat dry milk or 2.5% bovine serum albumin in 0.3% Tween 20 / PBS), the membrane was incubated for 2 h at room temperature with the respective primary antibody directed against p53 and phospho-p53 (Cell Signaling/New England Biolabs GmbH), PARP (1:100), Cyclin A (1:100), Cyclin B (1:100), p21 (1:100), or Erk-2 (1:1000) (all from SANTACRUZ Biotechnology). All antibodies were diluted in blocking buffer. Membranes were washed three times with 0.3% Tween 0 in PBS and then incubated with an IRDye800 or IRDye700 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. Detection of cell cycle distribution and apoptosis by flow cytometry

HCT116 cells (5×10^5) were starved for 48 h in medium without FCS at 37 °C in 5% CO_2 and then treated for 24 h with medium containing 10% FCS supplemented with increasing concentrations (300, 500, 600, 700, 800, 900, 1000 μM) of R-flurbiprofen. Cells were harvested by trypsinization and washed two times with PBS. Cells were fixed with ethanol for at least 2 h at −20 °C, washed two times with PBS and incubated for 5 min with 0.125% Triton X-100 on ice. After centrifugation cell pellets were stained with propidium iodide in PBS containing 0.2 mg/ml RNAse A. The stained cells were analysed by flow cytometry (Beckton Dickinson). 10,000 cells in the G_1 phase were analysed for each sample. The cell cycle distribution, i.e. the percentage of cells in G_0/G_1, S and G_2/M phase was assessed using WinMDI 2.8. The percentage of apoptotic cells was assessed by calculating the percentage of cells in the sub-G_1 phase.

2.6. Tumor proliferation in nude mice

Seven-week-old NMRI-*nu*/*nu* male mice (Harlan Winkelmann GmbH) were used. HCT116 pcDNA and HCT116p53ser15ala K9 and K17 cells (suspended in PBS) were injected subcutaneously at the right and left dorsal flank (1×10^7 cells per mouse). Mice were treated with 15 mg/kg/day R-flurbiprofen (suspended in PBS) 5 days a week from the day of tumor cell injection up to 3 weeks. This dose has been shown to be effective in previous in vivo tumor-experiments and achieve maximal plasma concentrations of about 192 ± 33 μM R-flurbiprofen [15]. Control mice were left untreated. The substance was administered i.p. Five mice were treated in each group. The tumor volume was assessed three times a week with a calliper rule. 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.

2.7. Statistics

Data are presented as mean ± S.E.M. SPSS 9.01 was used for statistical analyses. To compare the tumor growth in nude mice, the area under the tumor volume versus time curves were calculated using the linear trapezoidal rule. AUCs were submitted to univariate ANOVA. Treatments were then mutually compared using Dunnett *t*-tests. α was set at 0.05.

3. Results

3.1. p53 accumulates after R-flurbiprofen treatment

To ascertain if p53 is a critical factor for the anticarcinogenic effect of R-flurbiprofen, we analysed the expression level of p53 in the human colon carcinoma cell line HCT116 (wt p53 [31]) after treatment with R-flurbiprofen by Western-blotting. R-Flurbiprofen was used at 800 μM, a concentration which has been shown to inhibit cell cycle progression and to induce apoptosis [15]. As shown in Fig. 1, p53 protein level increased 16–28 h after treatment with 800 μM R-flurbiprofen. Accumulation of p53 protein is mainly caused by phosphorylation and subsequent stabilization of p53 protein. Therefore, we investigated the phosphorylation status of p53 using a phospho-p53 antibody sampler kit (Cell Signaling). The kit contains seven different phospho-p53 antibodies directed against different phosphorylation sites of the p53 protein. After treatment with 800 μM R-flurbiprofen phosphorylation of p53 at serine 15 increased 8–28 h in HCT116 cells, according to p53 accumulation (Fig. 1). All other phospho-p53 antibodies (directed against: phospho-p53 (ser 6), (ser 9), (ser 20), (ser 37), (ser 46), (ser 392)) gave no detectable signal (data not shown). ERK-2 expression was used as loading control. Also in two other human colon carcinoma cell lines, Caco-2 and HCT15 (both p53 mutated), p53 and phosphorylation of p53 at serine 15 increased after treatment of these cells

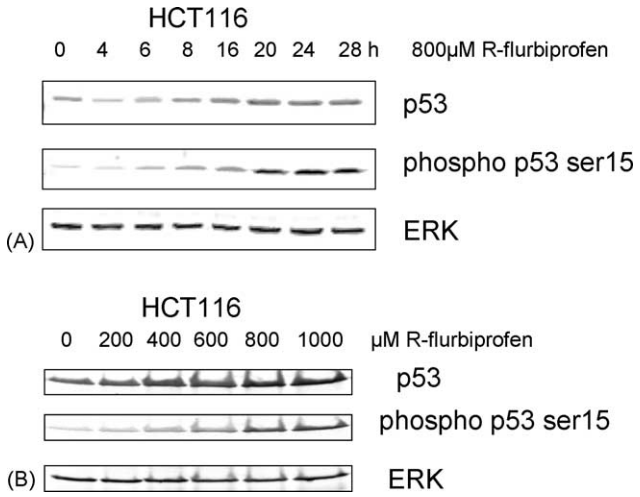

Fig. 1. Phospho-p53 and p53 expression of HCT116 cells after R-flurbiprofen treatment. Cells were treated for the indicated time periods with 800 μM R-flurbiprofen (A) or with increasing concentrations of R-flurbiprofen for 20 h (B). Fifty micrograms of phospho cell extract was loaded per lane onto a 12% SDS–polyacrylamide gel and electro-blotted onto a nitrocellulose membrane. p53 and phospho-p53 serine 15 expression was determined using a specific mouse-monoclonal p53 antibody or a phospho-p53-antibody, respectively. Equal loading of the gel was checked by rehybridisation of the blots with an anti-ERK-2 antibody.

with 800 μM R-flurbiprofen (data not shown), indicating that accumulation and phosphorylation of p53 is a common mechanism appearing in human colon carcinoma cell lines after R-flurbiprofen treatment. Furthermore, we treated HCT116 cells with various concentrations of R-flurbiprofen for 20 h to investigate which is the minimal concentration of R-flurbiprofen to induce p53 and its phosphorylation at serine 15. As Fig. 1B shows, phosphorylation of p53 at serine 15 increased at a concentration of 400 μM R-flurbiprofen already and achieved a maximum at 800 μM which was accompanied by an accumulation of total p53.

3.2. Site directed mutagenesis of wt-p53 at ser15

To gain more insights into the importance of p53 phosphorylation at serine 15 for the anticarcinogenic effect of R-flurbiprofen, we cloned the wildtype p53 cDNA by RT-PCR and introduced point mutations into codon 15 of the p53 cDNA by site directed mutagenesis which led to an amino-acid exchange from serine to alanine. The p53ser15 → ala cDNA was cloned into the eukaryotic expression vector pcDNA3 and transfected into HCT116 cells. Stable neomycine resistant cell clones were isolated and checked for their p53 expression level. Two p53 overexpressing cell clones (HCT116p53ser15ala K9 and K17) as well as a cell clone transfected with the empty pcDNA3 vector were utilised for further experiments.

These clones were treated for up to 28 h with 800 μM R-flurbiprofen and phospho-p53 ser15 and total p53 were analysed by Western-blotting. As shown in Fig. 2, phospho-p53ser15 increased in all three cell clones 8–28 h after R-flurbiprofen treatment, whereby phosphorylation was more pronounced in the pcDNA control clone. The signals of phospho-p53ser15 in HCT116p53ser15ala clone 9 and 17 are due to the phosphorylation of wt p53 protein which is still expressed in these cells. Rehybridisation of these blots with an antibody directed against total p53 revealed that the basal level of p53 in untreated HCT116p53ser15-ala clone 9 and 17 was already increased as compared to the pcDNA control clone (Fig. 2B). Thus, the increase of total p53 8 to 28 h after flurbiprofen treatment in these cells was not conspicuous as in the pcDNA control clone.

3.3. Induction of a cell cycle arrest and apoptosis in p53ser15ala overexpressing cells

Next, we investigated to which extent R-flurbiprofen induced cell cycle arrest and apoptosis was affected by mutation of p53 at ser15 to ala. Therefore, we analysed cell cycle distribution by flow cytometry after treatment of p53ser15ala overexpressing HCT116 cell clones and the pcDNA control clone with various concentrations of R-flurbiprofen. As shown in Fig. 3 and Table 1, treatment of cells with $\geq$500 μM R-flurbiprofen inhibited cell cycle progression in the G_1-phase in all these cell clones. There were no differences in sensitivity between the pcDNA control clone and the p53ser15ala overexpressing cell clones.

In previous works, we have shown that the G_1-phase block after NSAID treatment was accompanied by down-regulation of cyclin A and cyclin B as well as an upregulation of the cell cycle inhibitor protein $p21^{waf1}$ [4,15]. As shown in Fig. 4A–C, expression levels of cyclin A and B decreased, whereas the expression of p21 increased in a time dependent manner after treatment of cells with 800 μM R-flurbiprofen. The increase of p21 protein in HCT116 pcDNA control cells was more pronounced than in p53ser15ala overexpressing cell clones. p21 belongs to the p53-regulated genes [32]. Mutation of p53 at serine 15 to alanine may influence the transcriptional activity of p53.

Additionally, R-flurbiprofen has been shown to induce apoptosis in human colon carcinoma cell lines [15]. To investigate this aspect the $subG_1$ fraction, representing apoptotic cells, was analysed by flow cytometry (Fig. 3 and Table 1). Here, we detected statistically significant differences between the HCT116 pcDNA control clone and the p53ser15ala overexpressing cell clones. The pcDNA control clone was clearly more sensitive against R-flurbiprofen than p53ser15ala overexpressing cells. As a second marker for apoptosis, we analysed poly(ADP)ribose polymerase (PARP) cleavage by means of Western-blot analysis. PARP is a 112 kDa nuclear protein that is specifically cleaved by activated caspase-3 and caspase-6 into 87 and 25 kDa apoptotic fragments. Fig. 5 shows that treatment of HCT116 pcDNA control cells with 800 μM R-flurbiprofen induced PARP cleavage after about 16 h. By contrast in the p53 ser15ala overexpressing cells PAPR cleavage occurred

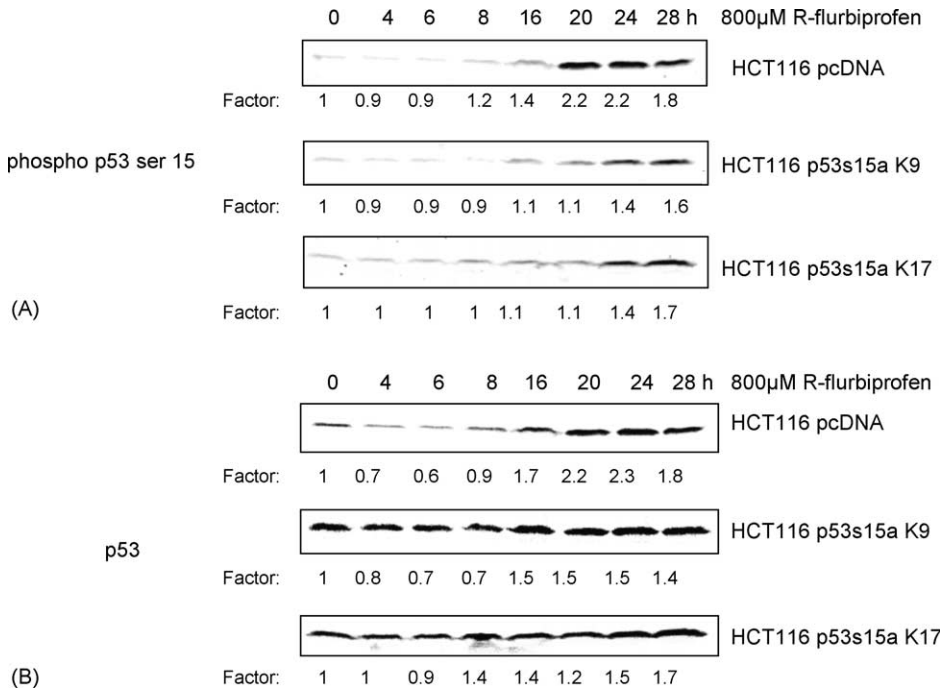

Fig. 2. Phospho-p53 and p53 expression of HCT116 pcDNA control cells and HCT116p53ser15ala clone 9 and 17 after R-flurbiprofen treatment. Cells were treated for the indicated time periods with 800 μM R-flurbiprofen. Fifty micrograms of phospho cell extract was loaded per lane onto a 12% SDS–polyacrylamide gel and electro-blotted onto a nitrocellulose membrane. Equal loading of the gel was checked by staining the membrane with ponceau solution. Phospho-p53 serine 15 expression was determined using a specific phospho antibody (A), p53 was determined by rehybridisation the blots with a mouse monoclonal p53 antibody (B).

only at 20–24 h. These data indicate that the p53ser15ala overexpressing HCT116 cell clones are less sensitive to R-flurbiprofen than control cells.

3.4. Anticarcinogenic effect of R-flurbiprofen in vivo

To analyse if phosphorylation of p53 at ser15 has also relevance for the anticarcinogenic effect of R-flurbiprofen in vivo, we injected HCT116 pcDNA control cells as well as the p53ser15ala overexpressing cells into nude mice and treated them for 3 weeks with 15 mg/kg R-flurbiprofen 5 days a week. Tumour volume was monitored three times a week with a calliper rule. As shown in Fig. 6, xenografts of p53ser15ala overexpressing cells were statistically significantly less sensitive to R-flurbiprofen treatment as compared to the pcDNA control. Thus, it can be concluded that

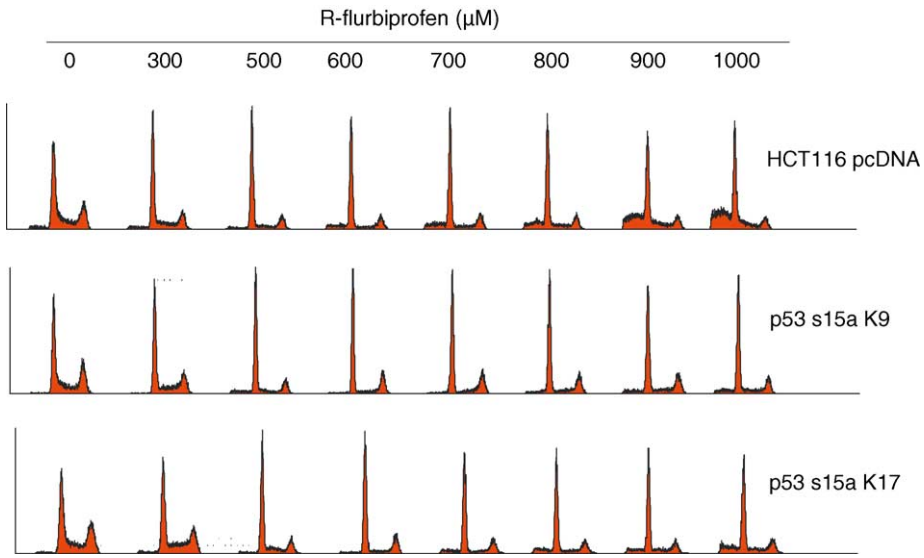

Fig. 3. Cell cycle analysis of HCT116 pcDNA control cells and of HCT116p53ser15ala clone 9 and 17, respectively, after treatment with increasing concentrations of R-flurbiprofen for 21 h. Cells were harvested after trypsinization, fixed with ethanol and the DNA content was assessed using propidium iodide staining and FACS analysis. A representative experiment out of three separate experiments is shown.

Table 1
Cell cycle distribution of HCT116 pcDNA control cells and HCT116p53ser15ala clone 9 and 17 cells after treatment with various concentrations of R-flurbiprofen for 21 h

	0	300	500	600	700	800	900	1000	R-Flurbiprofen (μM)
$SubG_1$	3 ± 1	5 ± 0.5	8 ± 1	10 ± 2	14 ± 2	25 ± 6	31 ± 6	38 ± 4	HCT116 pcDNA
G_1	49 ± 2	57 ± 5	70 ± 2	68 ± 2	65 ± 3	54 ± 4	47 ± 2	42 ± 1	
S	21 ± 2	20 ± 2	11 ± 4	10 ± 3	8 ± 1	10 ± 2	11 ± 2	10 ± 1	
G_2/M	27 ± 4	20 ± 5	12 ± 1	13 ± 1	13 ± 2	12 ± 3	11 ± 5	9 ± 2	
$SubG_1$	2 ± 0.5	2 ± 0.5*	2 ± 0.5*	3 ± 1*	4 ± 1*	9 ± 2*	14 ± 5*	17 ± 4*	HCT116p53s15a K9
G_1	48 ± 3	53 ± 5	64 ± 4	71 ± 2	68 ± 2	64 ± 2	58 ± 4	57 ± 4	
S	23 ± 2	22 ± 2	14 ± 2	9 ± 0.5	9 ± 0.5	9 ± 2	10 ± 2	11 ± 1	
G_2/M	28 ± 2	24 ± 5	19 ± 4	18 ± 4	20 ± 3	19 ± 3	18 ± 4	16 ± 3	
$SubG_1$	2 ± 1	3 ± 0.5*	5 ± 1*	6 ± 2*	9 ± 1*	12 ± 1*	17 ± 6*	23 ± 7*	HCT116p53s15a K17
G_1	51 ± 4	50 ± 2	62 ± 4	67 ± 2	67 ± 1	65 ± 2	60 ± 5	53 ± 4	
S	22 ± 1	21 ± 2	16 ± 1	10 ± 2	8 ± 2	8 ± 1	9 ± 3	9 ± 3	
G_2/M	26 ± 4	26 ± 1	16 ± 2	17 ± 3	17 ± 1	16 ± 1	16 ± 1	15 ± 1	

The table shows mean values from three independent experiments.
* Statistically significant differences of the percentage of cells in the $subG_1$ phase between HCT116 pcDNA control cells and HCT116p53ser15ala clones, $p < 0.05$.

phosphorylation of p53 at ser15 plays also a crucial role for the anticarcinogenic effect of R-flurbiprofen in vivo.

4. Discussion

Since in tumor therapy, long term treatment with anticarcinogenic agents is necessary, a reasonable benefit–risk ratio of the substances used is of vital importance. Therefore, R-flurbiprofen, which does not inhibit cyclooxygenases at therapeutic concentrations, represents a promising new anticarcinogenic candidate with a reduced risk causing gastrointestinal side-effects as compared to conventional COX inhibitors [16,33].

In previous experiments, R-flurbiprofen has been shown to induce a cell cycle block and apoptosis in different human colon carcinoma cell lines [15]. Meanwhile, we were able to show that inhibition of the cell cycle is at least partially due to activation of JNK [15]. However, the molecular mechanisms leading to apoptosis are still unknown. Here, we show that induction of apoptosis by R-flurbiprofen is in part dependent on activation of the tumour suppressor p53. Using specific phospho-p53 antibodies, we were able to show that in different human colon carcinoma cell lines 8–28 h after R-flurbiprofen treatment p53 is phosphorylated at serine 15 which is accompanied by an accumulation of total p53 protein. Mutation of serine 15 to alanine by site directed mutagenesis and overexpression of mutated p53 in the human colon carcinoma cell line HCT116 revealed that phosphorylation of p53 at serine 15 is crucial for the apoptotic effects of R-flurbiprofen. Also in vivo in nude mice experiments, HCT116-p53ser15ala overexpressing cells were less sensitive to the antitumorigenic potency of R-flurbiprofen in comparison to HCT116 control cells. Most interestingly, this phosphorylation site had

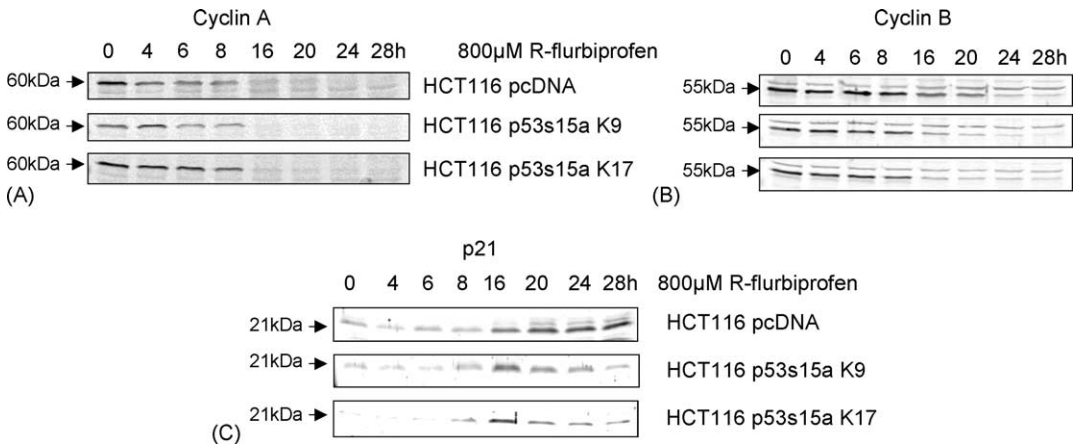

Fig. 4. Western-blot analysis of cell cycle regulating proteins. HCT116 pcDNA control cells and HCT116p53ser15ala clone 9 and 17 cells were treated for different times with 800 μM R-flurbiprofen. For Western-blot analysis, 50 μg of total protein extract were separated onto a 12% SDS–polyacrylamide gel and electro-blotted onto a nitrocellulose membrane. The membrane was then incubated with antibodies directed against cyclin A (A), cyclin B (B) or $p21^{waf\text{-}1}$ (C). Equal loading of the gel was checked by staining the membrane with ponceau solution.

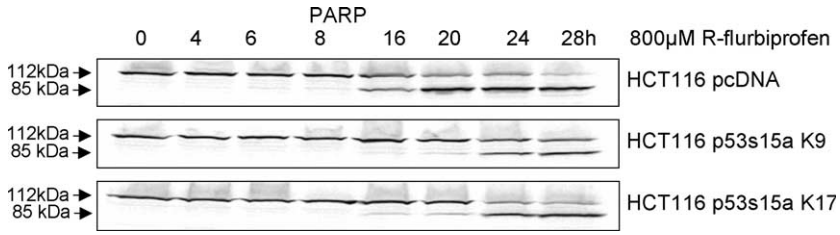

Fig. 5. Detection of PARP-cleavage in HCT116 pcDNA control cells and in HCT116p53ser15ala clone 9 and clone 17 cells by Western-blot analysis. Cells were treated for the indicated times with 800 μM R-flurbiprofen. Fifty micrograms of total protein extract was separated on a 10% SDS–polyacrylamide gel and electro-blotted onto a nitrocellulose membrane. PARP (112 kDa) and the apoptotic cleavage product of PARP (85 kDa) were detected using a rabbit polyclonal anti-PARP antibody.

no influence on induction of a cell cycle block after R-flurbiprofen treatment (measured by flow cytometry).

The p53 tumor suppressor protein is involved in diverse cellular processes, including the regulation of the cell cycle, apoptosis, senescence, DNA-repair, cell differentiation and angiogenesis. These activities are mediated by p53 through different mechanisms for example by transcriptional activation or transrepression of a large set of target genes [23]. Induction of the p53 response upon stress occurs largely through modifications in the p53 protein. One mechanism leading to p53 activation is phosphorylation of p53 at several sites which occurs after different cellular stress stimuli such as DNA-damage, hypoxia, heat shock, and exposure to nitric oxide [24]. Phosphorylation of p53 at serine 15 has been shown to occur in response to different DNA-damaging agents such as ionizing radiation or UV radiation [34], exposure to cisplatin [35], and cadmium [36] and has been linked to induction of apoptosis [28,37] and to inhibition of cell cycle progression [26]. This phosphorylation site is adjacent to the Mdm2

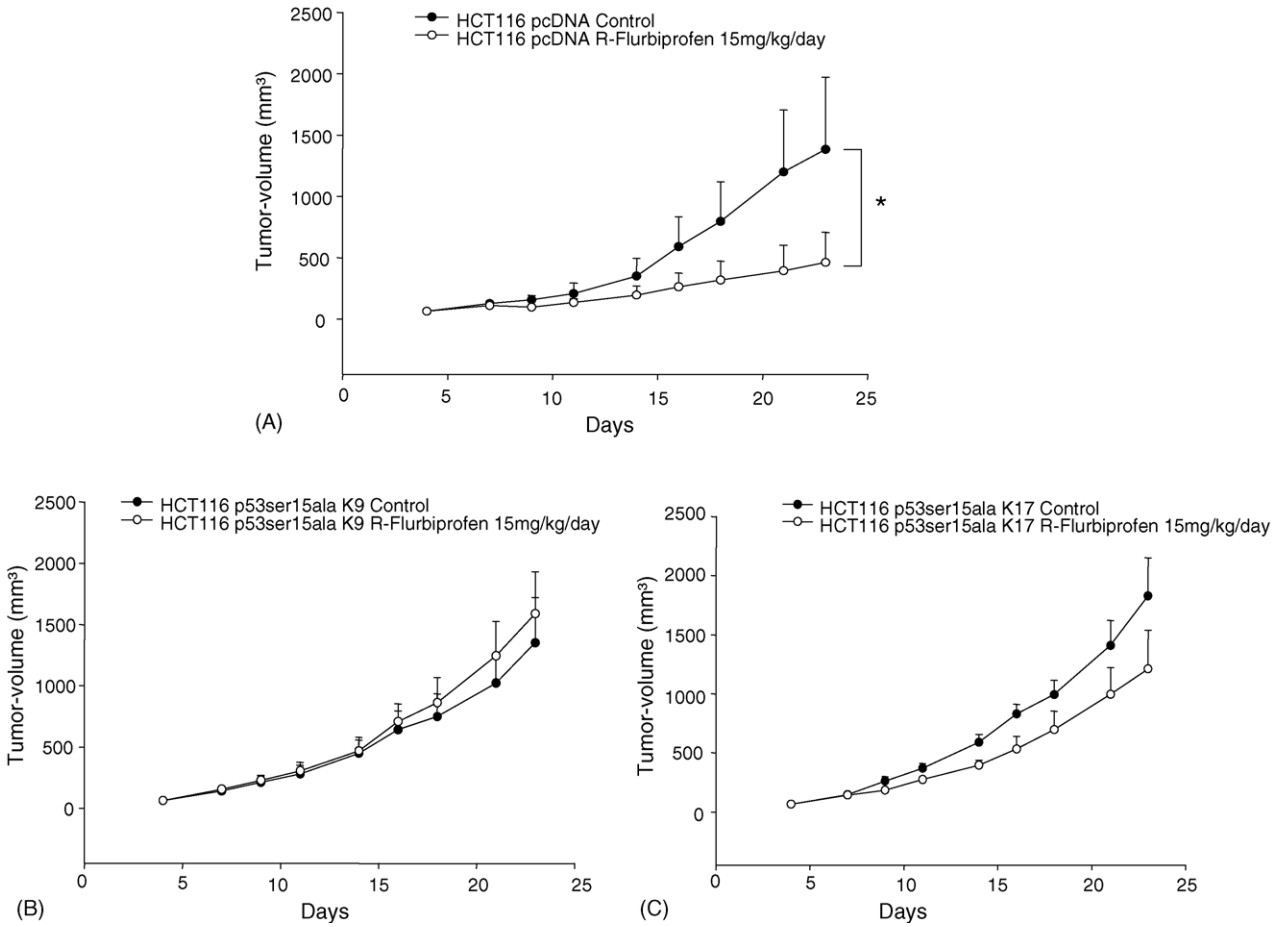

Fig. 6. Tumor growth of HCT116 pcDNA control cells and HCT116p53ser15ala clone 9 and clone 17 cells in nude mice. Nude mice were subcutaneously injected with 1×10^7 HCT116 pcDNA control cells (A) or HCT116p53ser15ala clone 9 (B) or clone 17 (C) cells at the dorsal flank and were treated once daily with 15 mg/kg/day R-flurbiprofen (○) or were left untreated (●). Each group consisted of five animals. Data are presented as mean ± S.E.M. Statistical comparisons of the areas under the "tumor volume" vs. "time curves" revealed that R-flurbiprofen significantly reduced growth of HCT116 pcDNA control xenografts only, indicated with an asterisk ($p < 0.05$).

binding site, and its phosphorylation impairs the binding of Mdm2 to p53 which is necessary for p53 degradation leading to accumulation of the p53 protein [38,39].

It has been shown that mutation of serine 15 to alanine reduces the ability of p53 to inhibit the cell cycle progression [26]. In Fig. 4C, we show that the expression level of the p53 regulated cell cycle inhibiting protein p21 increases to a lesser extent in p53ser15ala clone 9 and 17 than in HCT116 pcDNA control cells after R-flurbiprofen treatment. But the induction of a G_1-phase block (measured by FACS-analysis, Fig. 3 and Table 1) in p53ser15ala overexpressing cells was not significant different from HCT116 control cells, suggesting that beside the induction of p21 also other mechanisms play a pivotal role in the cell cycle block after flurbiprofen treatment [15].

Interestingly, we were able to demonstrate that the p53ser15ala overexpressing HCT116 cells were less sensitive to the apoptotic potency of R-flurbiprofen than HCT116 control cells. Also in vivo in the nude mice, the growth of p53ser15ala overexpressing xenografts was less inhibited after R-flurbiprofen treatment than HCT116 control xenografts. These findings are in line with data from Unger et al., who demonstrated that mutation of serine 15 to alanine impairs also the apoptotic activity of p53 [28]. p53 can induce apoptosis either by the sequence-specific transactivation (SST) function or by SST-independent pathways (reviewed in [40–42]). Proteins which are directly transcriptional activated by p53 and promote apoptosis are: Bax, Puma, Noxa, Bid and Apaf-1. All these proteins are involved in the intrinsic apoptotic pathway. But also the extrinsic apoptotic pathway is regulated by p53. For example Fas/CD95, DR5 and Fas ligand are direct p53 targets [41]. Furthermore, p53 can induce apoptosis independently of its transcription activity, for example by repressing the transcription factor E2F-1 [23,43] or by direct interaction with apoptosis inducing proteins such as the helicases XPB and XPD [44]. Which of these pathways, however, are crucial for the apoptotic effects of R-flurbiprofen remains to be elucidated.

Phosphorylation of serine 15 is mediated by different kinases such as the DNA-PK (DNA-dependent protein kinase), the ATM (ataxia-telangiectasia, mutated)/ATR (ATM and Rad3 related) or by p38/ERKs (extracellular signal-regulated kinase) (reviewed in [25]). All these kinases are activated through genotoxic stress and are either involved in ‘‘sensing’’ or ‘‘detection’’ of DNA damage [45]. This let us suppose that treatment of cells with R-flurbiprofen leads to DNA-damage either directly or in response to cellular events initiated by R-flurbiprofen. Ongoing experiments will probably verify this hypothesis.

In summary, we were able to show that the anticarcinogenic effects of R-flurbiprofen in vitro and in particular in vivo depend on the tumor suppressor gene p53 and that mutation of p53 at serine 15 impairs the apoptotic potency of R-flurbiprofen.

Acknowledgment

The work was supported by the Deutsche Forschungsgemeinschaft (DFG GR2011/1-1) and the European Graduate School ‘‘Roles of Eicosanoids in Biology and Medicine’’ (DFG GRK 757/1).

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