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The HIV protease inhibitor ritonavir synergizes with butyrate for induction of apoptotic cell death and mediates expression of heme oxygenase-1 in DLD-1 colon carcinoma cells

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1   The protease inhibitor ritonavir is an integral part of current antiretroviral therapy targeting human immunodeficiency virus. Recent studies demonstrate that ritonavir induces apoptotic cell death with high efficiency in lymphoblastoid cell lines. Moreover, ritonavir can suppress activation of the transcription factor nuclear factor-κB and is an inhibitor of interleukin-1β and tumor necrosis factor-α production in peripheral blood mononuclear cells. Thus, ritonavir appears to have anti-inflammatory properties.

2   In the present study, we investigated in DLD-1 colon carcinoma cell effects of ritonavir on apoptotic cell death and expression of heme oxygenase-1 (HO-1), an anti-inflammatory enzyme that may be critically involved in the modulation of colonic inflammation.

3   Compared to unstimulated control, ritonavir resulted in a moderate increase in the rate of apoptotic cell death as observed after 20 h of incubation. Notably, ritonavir potently synergized with the short-chain fatty acid butyrate for induction of caspase-3-dependent apoptosis in DLD-1 cells.

4   Ritonavir enhanced mRNA and protein expression of HO-1 in DLD-1 cells. Ritonavir-induced HO-1 protein was suppressed by SB203580 or SB202190 and preceded by immediate upregulation of cellular c-Fos and c-Jun protein levels. This process was associated with induction of activator protein-1 as detected by electrophoretic mobility shift analysis.

5   The present data suggest that ritonavir has the potential to curb colon carcinogenesis by reducing cell growth *via* mechanisms that include apoptosis and by simultaneously modulating colonic inflammation *via* induction of anti-inflammatory HO-1.

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Introduction

Ritonavir is a potent inhibitor of human immunodeficiency virus (HIV) protease and is successfully used in current highly active antiretroviral therapy. Besides inhibition of HIV protease, cellular proteasome activity is modulated by ritonavir (Andre *et al.*, 1998). Inhibition of the chymotrypsin-like activity of the 20S proteasome with subsequently hindered degradation of inhibitor-κBα likely mediates suppression of nuclear factor-κB (NF-κB) observed in cells exposed to ritonavir (Andre *et al.*, 1998; Pati *et al.*, 2002). Since the transcription factor NF-κB is a pivotal component of the proinflammatory signal transduction machinery (Ardite *et al.*, 1998), these observations concur with the ability of ritonavir to inhibit the release of interleukin (IL)-1β and tumor necrosis factor-α (TNFα) from activated human peripheral blood

mononuclear cells (Pati *et al.*, 2002). In addition, ritonavir mediates antiproliferative effects and induces apoptosis in human lymphoblastoid cell lines. In the study, apoptosis was not observed in nontransformed 3T3 fibroblasts or terminally differentiated bone marrow-derived macrophages, suggesting that proapoptotic effects of ritonavir may primarily affect cancerous cells (Gaedicke *et al.*, 2002).

Heme oxygenase-1 (HO-1), also called heat-shock protein-32, is an enzyme that is critically involved in responses toward diverse types of cellular stress (Immenschuh & Ramadori, 2000). We have recently reported on induction of HO-1 in DLD-1 colon carcinoma cells by nitric oxide and by the activator protein-1 (AP-1)-inducing agent pyrrolidine dithiocarbamate (PDTC) (Hellmuth *et al.*, 2002). Induction of HO-1 *via* this PDTC-inducible pathway has been recently demonstrated *in vivo* in a rat model (Hata *et al.*, 2003). Protective functions of HO-1 are evident in models of diseases that are associated with immunoactivation such as experimental autoimmune encephalomyelitis (Liu *et al.*, 2001) and experi-

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mental colitis (Wang *et al.*, 2001). The beneficial role of HO-1 observed in these disorders may be mediated by anti-inflammatory actions of this enzyme and its reaction products, biliverdin/bilirubin and carbon monoxide. These are illustrated by HO-1-mediated control of TNFα production in macrophages (Otterbein *et al.*, 2000; Lee & Chau, 2002). Interestingly, HO-1 is also inducible by IL-10 (Lee & Chau, 2002) and transforming growth factor-β (Ning *et al.*, 2002), which may contribute to anti-inflammatory properties of these cytokines.

Since anti-inflammatory and proapoptotic functions have been associated with ritonavir and both of these properties potentially affect colon carcinogenesis, cellular actions of this compound on human epithelial-like DLD-1 colon carcinoma cells were investigated.

Methods

Materials

Pure ritonavir (R) was kindly provided by Abbott GmbH (Wiesbaden, Germany). Clasto-Lactacystin, PDTC, SB202190, and SB203580 were purchased by Calbiochem-Novabiochem (Bad Soden, Germany). Sodium butyrate was from Sigma (Deisenhofen, Germany). z-VAD-fmk was purchased from Alexis Biochemicals (Grünberg, Germany).

Cell culture of DLD-1 colon carcinoma cells

Human DLD-1 carcinoma/epithelial cells were obtained from the Centre for Applied Microbiology and Research (Salisbury, U.K.). Cells were maintained in DMEM supplemented with $100 \, U \, ml^{-1}$ penicillin, $100 \, \mu g \, ml^{-1}$ streptomycin, and 10% heat-inactivated FCS (GIBCO-BRL, Eggenstein, Germany). For the experiments, confluent cells grown on polystyrene plates (Greiner, Frickenhausen, Germany) were washed with PBS and incubated with the indicated agents in the aforementioned medium. Unless otherwise indicated, unstimulated control (C) denotes vehicle control that is 0.1% DMSO for ritonavir at $60 \, \mu M$.

Determination of CK18-Asp396 by ELISA

Cell-free culture supernatants were analyzed for cytokeratin-18 neoantigen CK18-Asp396 content by ELISA using a commercial kit (Alexis Biochemicals, Grünberg, Germany) according to the manufacturer's instructions.

Detection of HO-1, caspase-3, c-Fos, and c-Jun protein by immunoblotting

After stimulation, total cell lysates were prepared using lysis-buffer ($300 \, mM$ NaCl, $50 \, mM$ Tris-HCl (pH 7.6), 0.5% Triton X-100) supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany). Unless otherwise stated, $70 \, \mu g$ of total cellular proteins were separated by 10 or 12% SDS–polyacrylamide gel electrophoresis and transferred onto Immobilon membrane (Millipore, Bedford, U.K.). HO-1 (Stressgene, Hamburg, Germany), caspase-3, c-Fos, and c-Jun (Santa Cruz Biotechnology Inc., Heidelberg, Germany) were detected using rabbit polyclonal antibodies as primary antibodies, horseradish peroxidase labeled secondary antibo-dies (Biorad, Munich, Germany), and a chemiluminescence detection kit (Amersham Pharmacia Biotech, Freiburg, Germany) according to the manufacturer's instructions.

Determination of mRNA for HO-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by reverse transcriptase-PCR (RT–PCR)

Total RNA was isolated using Tri-Reagent according to the manufacturer's instructions (Sigma). RNA ($1 \, \mu g$) was used for RT–PCR (GeneAmp RNA PCR kit using Amplitaq Gold, Perkin-Elmer Corp., Weiterstadt, Germany). The following sequences were performed for PCR: $94°C$ for 10 min (one cycle); $94°C$ for 45 s, $60°C$ (GAPDH) or $58°C$ (HO-1) for 45 s, and $72°C$ for 60 s (numbers of cyles see below); the final extension was at $72°C$ for 10 min. Sequences of the primers, numbers of cycles performed, and length of resulting amplicons were as follows: HO-1 (F): 5'-cagcatgcccaggatttg-3', HO-1 (R): 5'-agctggatgttgagcagga-3', 29 cycles, 616 bp; GAPDH (F): 5'-accacagtccatgccatcac-3', GAPDH (R): 5'-tccaccaccctgttgctgta-3', 23 cycles, 452 bp (Hellmuth *et al.*, 2002). PCR products were run on a 1.5% agarose gel containing $0.5 \, \mu g \, ml^{-1}$ ethidium bromide. The identity of amplicons was confirmed by sequencing (310 Genetic Analyzer, Perkin-Elmer Corp.).

RNase protection assay for analysis of HO-1 and GAPDH mRNA accumulation

RNA was isolated using Trizol-Reagent (GIBCO-BRL) according to the manufacturer's instructions. Total RNAs ($10 \, \mu g$) were used for RNase protection assay, performed as described recently (Hellmuth *et al.*, 2002). Briefly, DNA probes were cloned into the transcription vector pBluescript II KS (+) (Stratagene, Heidelberg, Germany). After linearization, an antisense transcript was synthesized *in vitro* with T3 or T7 RNA polymerase and $[\alpha\text{-}^{32}P]UTP$ ($800 \, Ci \, mmol^{-1}$). RNA samples were hybridized at $42°C$ overnight with 100,000 c.p.m. of the labeled antisense transcript. Hybrids were digested with RNase A and T1 for 1 h at $30°C$. Under these conditions, every single mismatch was recognized by the RNases. Protected fragments were separated on 5% $(w \, v^{-1})$ polyacrylamide/8 M urea gels and analyzed using a PhosphoImager (Fuji, Straubenhardt, Germany). The individual gene expression of HO-1 was evaluated on the basis of the GAPDH housekeeping gene expression. The cDNA probes were cloned by RT–PCR, and correspond to nucleotides (nt) 148–302 (for GAPDH) and nt 706–949 (for HO-1) of the published sequences (Hellmuth *et al.*, 2002).

Electrophoretic mobility shift assay (EMSA)

Preparation of crude extracts from DLD-1 cells was performed as described previously (Hellmuth *et al.*, 2002). Consensus oligonucleotides used in the binding reactions were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Sequences of the double-stranded oligonucleotides used for analysis of specific binding of AP-1 to DNA are as follows: AP-1, wild type 5'-cgc ttg atg act cag ccg gaa-3'; AP-1, mutated 5'-cgc ttg atg act **tg**g ccg gaa-3'. Complementary oligonucleotides were end labeled by T4 polynucleotide kinase (MBI Fermentas, St Leon-Roth, Germany) using $[\gamma\text{-}^{32}P]ATP$

(3000 Ci mmol^{-1}, Amersham Pharmacia Biotech, Braunschweig, Germany). Binding reactions were performed for 2 h on ice with 7.7 µg of protein in 20 µl of binding buffer containing 4% Ficoll, 20 mM HEPES (pH 7.9), 50 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.25 mg ml^{-1} BSA, 2 µg of poly(dI–dC), and 10,000 c.p.m. of ^{32}P-labeled oligonucleotide. For AP-1 super-shift analysis, nuclear proteins were preincubated for 20 min at room temperature with a polyclonal anti-c-Jun antibody (Santa Cruz, CA, U.S.A.) before the binding reaction was performed. DNA–protein complexes were separated from unbound oligonucleotide by electrophoresis through a 4% polyacrylamide gel using 0.5 × TBE buffer. Thereafter, gels were fixed and analyzed by PhosphoImager analysis (Fuji). Competition experiments were performed by coincubation with a 100-fold excess (20 pmol) of unlabeled double-stranded oligonucleotide in the DNA–protein binding reaction.

Detection of apoptotic cell death by analysis of cytosolic oligosome-bound DNA

Cytosolic oligonucleosome-bound DNA was quantified using an ELISA according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). Absorbance values (A$^{405\,nm}$) give a semiquantitative measure of ongoing DNA fragmentation, a common marker for cell death by apoptosis. Data are expressed as an enrichment factor calculated as a ratio of absorbances from treated *versus* untreated cells.

Quantification of DNA fragmentation by the diphenylamine assay

DNA fragmentation was assayed as described previously (Mühl *et al.*, 1996). Following incubations, cells were centrifuged, resuspended in 250 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA (pH 8.0)), and lysed by adding 250 µl of cold lysis buffer, containing 2 mM EDTA, 0.5% (v v^{-1}) Triton X-100, and 5 mM Tris-HCl (pH 8.0). Samples were allowed to lyse for 30 min on ice prior to centrifugation (15 min, 13,000 r.p.m.) to separate intact chromation (pellet) from DNA fragments (supernatant). Pellets were resuspended in 500 µl TE buffer and the DNA content of pellets and supernatants was determined using the diphenylamine reagent.

Quantification of viable cells via 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reduction

The viability of DLD-1 cells cultured in 96-well plates was quantified by measuring the amount of soluble formazan produced by cellular reduction of MTS according to the manufacturer's instructions. After 20 h of incubation, 20 µl of CellTiter 96@AQ$_{ueus}$ One solution-MTS Reagent (Promega, Mannheim, Germany) were added to each well. After 1 h at 37°C, absorbance was measured at 490 nm. In each experiment, increasing cell numbers were used to ascertain the linearity of the assay.

Statistics

For experiments using DLD-1 cells, data are shown as mean absorbance ± s.d. or as the percentage of control ± s.d. and were analyzed by unpaired Student's *t*-test on raw data using Sigma Plot (Jandel Scientific, Munich, Germany).

Results

Ritonavir mediates apoptotic cell death of DLD-1 colon carcinoma cells

Previous studies revealed that ritonavir induces apoptotic cell death in lymphoblastoid cells (Gaedicke *et al.*, 2002) and in Kaposi sarcoma-derived cell lines (Pati *et al.*, 2002). Therefore, we initially analyzed effects of ritonavir on viability of DLD-1 colon carcinoma cells. As shown in Figure 1a and b, ritonavir alone was able to significantly increase the rate of apoptosis in these cells during a 20 h incubation period. Induction of DNA fragmentation was associated with a reduction of cell viability within the 20 h of incubation to 56.5 ± 8.4% compared to vehicle control (0.1% DMSO) set as 100% ($P < 0.05$; $n = 4$). Ritonavir at 30 µM was the lowest concentration able to induce significant DNA fragmentation in DLD-1 cells (170% for ritonavir *versus* vehicle control set at 100% ($P < 0.05$; $n = 4$).

Butyrate is a short-chain fatty acid that is produced by intestinal bacteria and is supposed to be an important regulator of colonic epithelial cell biology (Wächtershauser & Stein, 2000). Since butyrate is known to sensitize colon carcinoma cells for induction of apoptosis (Bonnotte *et al.*, 1998; Luhrs *et al.*, 2002), DLD-1 cells were treated with the combination ritonavir plus butyrate. These experiments revealed a strong synergism between ritonavir and butyrate with regard to induction of apoptotic cell death (Figure 1a and b). Under these conditions and after a 20 h incubation period, viability was significantly reduced to 31.3 ± 8.0% compared to vehicle control (0.1% DMSO) set at 100% (ritonavir at 60 µM, butyrate at 10 mM; $P < 0.05$; $n = 4$). Detailed dose–response curves demonstrating synergistic induction of apoptosis by ritonavir plus butyrate in DLD-1 cells are shown in Figure 1c.

Induction of apoptosis in DLD-1 cells was associated with processing of procaspase-3 and generation of the large p17 subunit of the active protease (Figure 2a). Moreover, enhanced caspase activity was detected under the influence of ritonavir, particularly in combination with butyrate (Figure 2b). In these experiments, upregulation of caspase activity was quantified by detection of a caspase-specific cytokeratin-18 neoantigen (CK18-Asp396) that is released from apoptotic cells (Biven *et al.*, 2003). Butyrate alone did not enhance caspase activity in DLD-1 cells. By use of the general caspase inhibitor z-VAD-fmk, ritonavir/butyrate-induced apoptosis was determined to be strongly dependent on cellular caspase activity (Figure 3a and b).

Ritonavir induces expression of HO-1 in DLD-1 colon carcinoma cells

Since ritonavir exhibits anti-inflammatory properties, we investigated expression of HO-1 in DLD-1 cells exposed to ritonavir. RT–PCR analysis and RNase protection assay (Figure 4a–c) revealed that ritonavir increases steady-state levels of HO-1 mRNA. Ritonavir-induced HO-1 mRNA translated into enhanced expression of HO-1 protein as detected by immunoblot analysis (Figure 5a and b). To investigate whether inhibition of proteasome function may
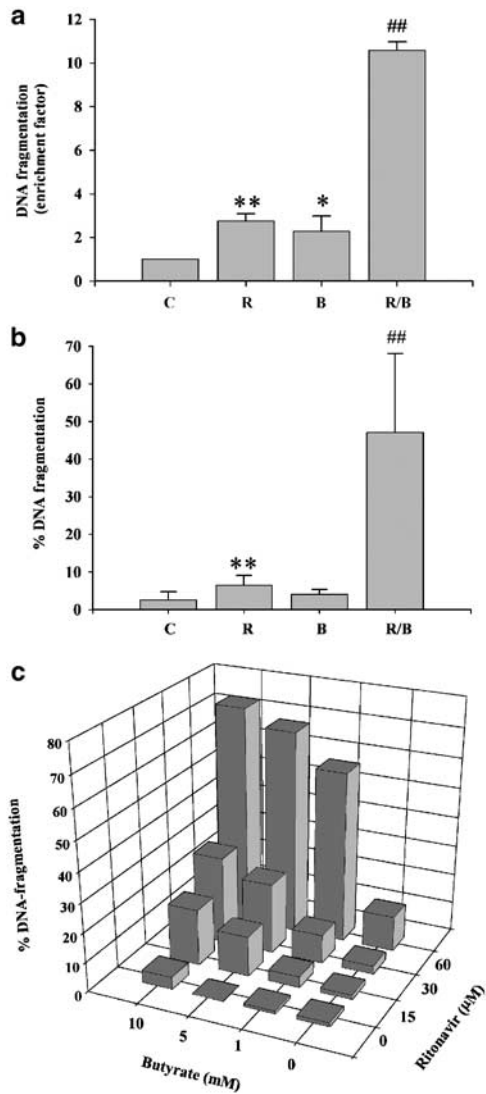
Figure 1 Ritonavir synergizes with butyrate for induction of apoptosis in DLD-1 cells. (a) Analysis of apoptotic DNA fragmentation in DLD-1 cells exposed to ritonavir (R, $60\,\mu$M), butyrate (B, $10\,$mM), or ritonavir plus butyrate (R/B). DLD-1 cells were kept as vehicle control (0.1% DMSO) or were stimulated as indicated. After $20\,$h, cells were harvested and DNA fragmentation was semiquantitively analyzed by an ELISA based on the detection of cytosolic histone/DNA complexes. Data are shown as mean enrichment factor$\pm$s.d. ($n=3$). **$P<0.01$ compared to unstimulated controls; *$P<0.05$ compared to unstimulated controls; \#\#$P<0.01$ compared to ritonavir or butyrate alone. (b) Diphenylamine-based analysis of apoptotic DNA fragmentation in DLD-1 cells exposed to ritonavir (R, $60\,\mu$M), butyrate (B, $5\,$mM), or ritonavir plus butyrate (R/B). DLD-1 cells were kept as vehicle control (0.1% DMSO) or stimulated as indicated. DNA fragmentation after $20\,$h of incubation was quantified using the diphenylamine reaction. Data are shown as the mean percentage of DNA fragmentation$\pm$s.d. ($n=3$). **$P<0.01$ compared to unstimulated control; \#\#$P<0.01$ compared to ritonavir or butyrate alone. (c) Dose–response curves of synergistic induction of apoptosis in DLD-1 cells exposed to combinations of ritonavir plus butyrate. Cells were either kept as vehicle control or exposed to the indicated concentrations of ritonavir and/or butyrate. After $20\,$h of stimulation, DNA fragmentation was quantified using the diphenylamine reaction. Data are shown as the percentage of DNA fragmentation. One representative of two independently performed experimental series is shown.
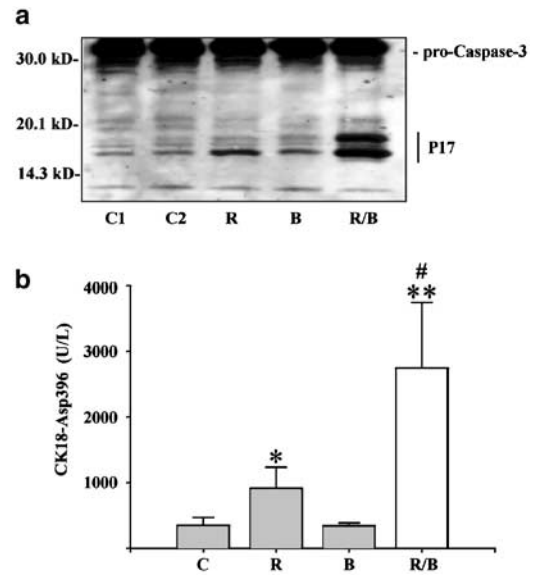

Figure 2 Activation of caspase-3 by ritonavir plus butyrate in DLD-1 cells. (a) DLD-1 cells were kept as unstimulated control (C1), as vehicle control (C2, 0.1% DMSO), or were stimulated with ritonavir (R, $60\,\mu$M), butyrate (B, $5\,$mM), or ritonavir plus butyrate (R/B). After $20\,$h of stimulation, DLD-1 cells were harvested and homogenates were assayed for caspase-3 protein by immunoblot analysis. One representative blot of two independently performed experiments is shown. Total protein ($100\,\mu$g) was applied per lane. (b) After $20\,$h of stimulation, cell-free culture supernatants were assayed for the presence of the cytokeratin-18 neoantigen CK18-Asp396 by ELISA. Data are shown as mean CK18-Asp396 concentrations$\pm$s.d. ($n=4$). Vehicle control (0.1% DMSO). *$P<0.05$ compared to unstimulated control; **$P<0.01$ compared to unstimulated control; \#$P<0.05$ compared to ritonavir alone.

mediate HO-1 expression in DLD-1 cells, these were incubated with clasto-Lactacystin or sodium butyrate. Both agents have been associated with inhibition of proteasome activity (Yin et al., 2001; Almond & Cohen, 2002). As shown in Figure 6, clasto-Lactacystin (Figure 6a) and sodium butyrate (Figure 6b) both mediated HO-1 expression. Combined treatment with ritonavir plus sodium butyrate resulted in efficient induction of apoptotic cell death (Figure 1). Compared to unstimulated control, this condition was likewise associated with enhanced levels of HO-1 protein (Figure 6c). In some experiments, the combination of ritonavir plus butyrate resulted in additive effects with regard to HO-1 expression. However, this observation was not consistent within the set of experiments performed, which likely is due to massive cell death under these conditions (data not shown). Previous data revealed that the p38 mitogen-activated protein (MAP) kinase pathway can mediate expression of HO-1 in response to several stimuli, for example, nitric oxide (Chen & Maines, 2000). In accordance with these reports, we observed that coincubation with the p38 MAP kinase inhibitors SB203580 (Figure 7a) or SB202190 (Figure 7c) suppressed ritonavir-induced HO-1. In contrast, blockage of p38 MAP kinase activity did not significantly affect apoptosis of DLD-1 cells in response to ritonavir or ritonavir plus butyrate (Figure 7b). Moreover, inhibition of apoptosis by coincubation with z-VAD-fmk did not significantly modulate expression of HO-1 in DLD-1 cells exposed to ritonavir plus butyrate ($n=3$; data not shown).
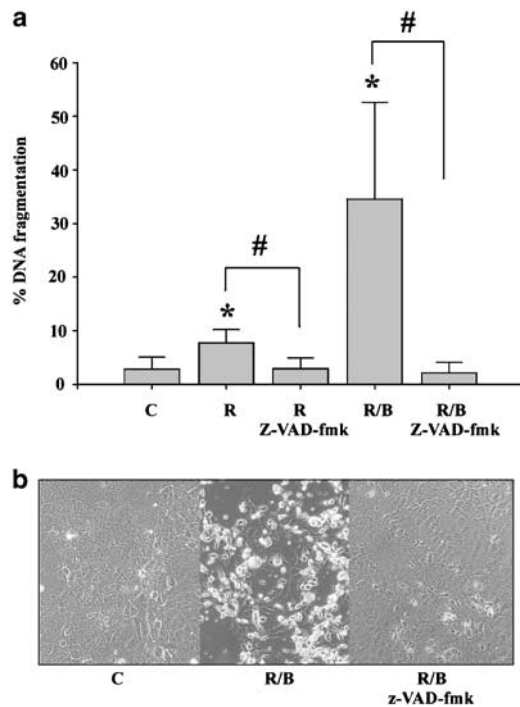
a

b

Figure 3 Induction of apoptosis by ritonavir plus butyrate in DLD-1 cells is potently suppressed by coincubation with z-VAD-fmk. (a) DLD-1 cells were either kept as unstimulated control (C, 0.1% DMSO), or were stimulated with ritonavir (R, 60μM), with ritonavir plus z-VAD-fmk (30μM), with ritonavir/butyrate (B, 5 mM), or with with ritonavir/butyrate plus z-VAD-fmk. After 20 h, DNA fragmentation was quantified using the diphenylamine reaction. Data are shown as the mean percentage of DNA fragmentation $\pm$ s.d. ($n = 4$). *$P < 0.05$ compared to unstimulated control; [#]$P < 0.05$ compared to ritonavir or ritonavir/butyrate in the absence of z-VAD-fmk. (b) Protective effects of z-VAD-fmk in the same experimental setting as shown by light microscopy.

Ritonavir activates the AP-1 signaling pathway in DLD-1 colon carcinoma cells

Since AP-1 is a key regulator of HO-1 transcription (Alam & Den, 1992), and basal expression of c-Fos and c-Jun can be augmented by appropriate stimuli in colon carcinoma cells (Wilson *et al.*, 1999; Yao & O'Dwyer, 2003), we investigated the effects of ritonavir on the AP-1 system. Immunoblot analysis of total cell lysates revealed that within 2 h of incubation expression of both c-Jun and c-Fos is upregulated in DLD-1 cells exposed to ritonavir (Figure 8a and b). Activation of the AP-1 pathway was furthermore investigated by EMSA analysis. Compared to background AP-1 binding activity in unstimulated cells, we observed enhanced specific binding of AP-1 to the respective wild-type oligonucleotides when nuclear extracts of DLD-1 cells were analyzed that had been exposed to ritonavir (Figure 8c). Appearance of the shifted band was prevented by coincubation with an anti-c-Jun antibody. In contrast, a constitutive band, which was apparent on the EMSA gel was not diminished when the anti-c-Jun antibody was added to nuclear extracts derived from ritonavir-activated DLD-1 cells (lane 2 *versus* lane 3). The band that was induced by ritonavir comigrated with a signal that was inducible by PDTC, a known activator of AP-1 in various cell types, among them DLD-1 cells (Hellmuth *et al.*, 2002).
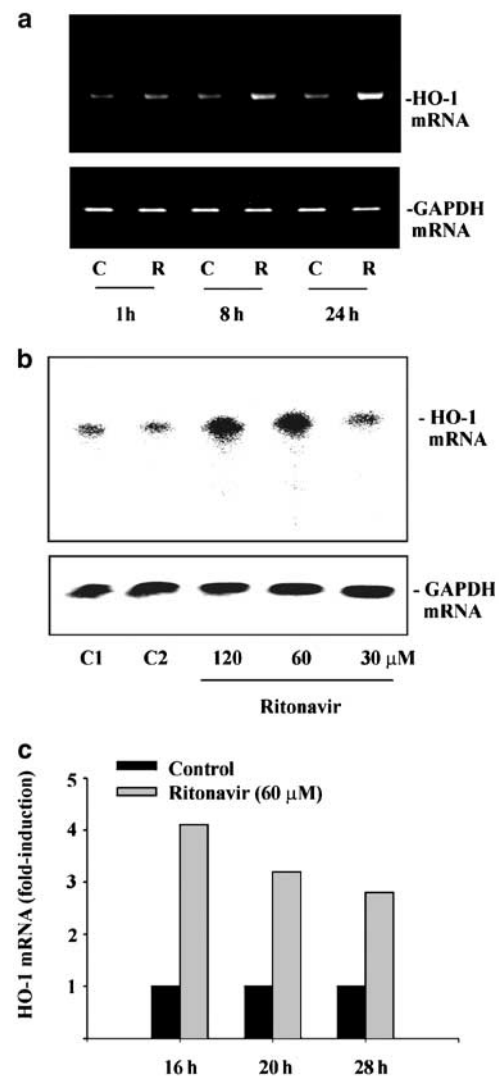
a

b

c

Figure 4 Ritonavir induces HO-1 mRNA in DLD-1 cells. (a) DLD-1 cells were either kept as unstimulated control (C, 0.1% DMSO) or were stimulated with ritonavir at 60μM (R). After the indicated time periods, cells were harvested, total RNA was isolated, and mRNA expression of HO-1 and GAPDH was determined by semiquantitative RT–PCR analysis. One representative of three independent experiments evaluating HO-1 expression by RT–PCR is shown. (b and c) Ritonavir-induced HO-1 mRNA induction was assessed by RNase protection assay. DLD-1 cells were stimulated in three independent experiments with the indicated concentrations of ritonavir for either 16, 20, or 28 h. One representative of these RNase protection assays is shown in (b); cells were stimulated for 20 h. (c) The degree of HO-1 mRNA induction by ritonavir (60μM) relative to GAPDH mRNA was quantified by PhosphoImager (Fuji) analysis of the radiolabeled gels. C1 (0.1% DMSO) and C2 (0.2% DMSO) represent vehicle control for ritonavir at 60 and 120μM, respectively.

Appearance of this PDTC-inducible complex was likewise prevented by cotreatment with the anti-c-Jun antibody (lane 4 *versus* lane 5). The specificity of the ritonavir-induced complex was furthermore characterized by the observation that the signal could be quenched by excess unlabeled wild-type oligonucleotides but not by excess unlabeled mutated oligonucleotides (lane 2 *versus* lanes 6 and 7). In contrast, the constitutive band was not modulated by coincubation with excess unlabeled wild-type oligonucleotides (lane 2 *versus* lane 6).
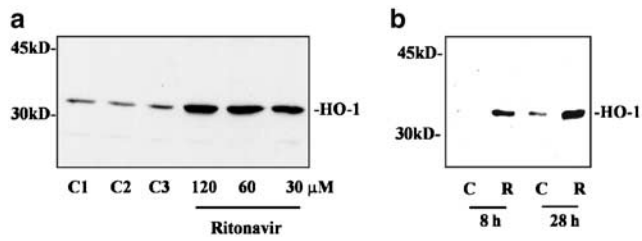
Figure 5 Ritonavir induces expression of HO-1 protein. (a) Dose–response curve of ritonavir-induced HO-1 protein. DLD-1 cells were kept as unstimulated control or were stimulated for 16 h with the indicated concentrations of ritonavir. Thereafter, cells were harvested and homogenates were assayed for HO-1 protein expression by immunoblot analysis. C1 denotes medium alone; C2 (0.1% DMSO) and C3 (0.2% DMSO) represent vehicle control for ritonavir at 60 and 120 μM, respectively. One representative of four independently performed experiments is shown. (b) Kinetic analysis of ritonavir-induced HO-1 protein. DLD-1 cells were stimulated with ritonavir (R, 60 μM) for the indicated periods of time. Thereafter, cells were harvested and homogenates were assayed for HO-1 protein expression by immunoblot analysis. One representative of three independently performed kinetics is shown.
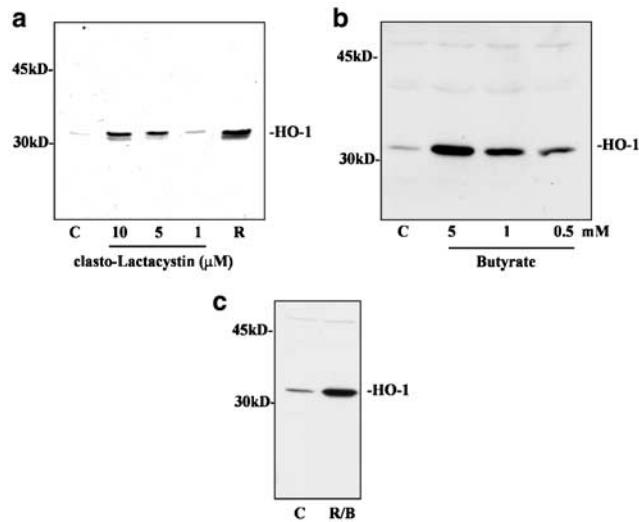

Figure 6 Induction of HO-1 by clasto-Lactacystin, butyrate, and ritonavir plus butyrate. (a) DLD-1 cells were kept as unstimulated vehicle control (C, 0.1% DMSO) or were stimulated with the indicated concentrations of clasto-Lactacystin or with ritonavir (R) at 60 μM. After 24 h, cells were harvested and homogenates were assayed for HO-1 protein expression by immunoblot analysis. (b) DLD-1 cells were kept as unstimulated control (C, without DMSO) or were stimulated with the indicated concentrations of sodium butyrate (B). After 24 h, cells were harvested and homogenates were assayed for HO-1 protein expression by immunoblot analysis. One representative of four independent experiments is shown. (c) DLD-1 cells were kept as unstimulated control (C, 0.1% DMSO) or were stimulated with sodium butyrate (B, 10 mM) plus ritonavir (R, 60 μM). After 20 h, cells were harvested and homogenates were assayed for HO-1 protein expression by immunoblot analysis. One representative of five independent experiments is shown.

Discussion

HO-1 is an anti-inflammatory enzyme, the deficiency of which is associated with inflammation in mice and humans (Poss & Tonegawa, 1997; Yachie et al., 1999). Upregulation of HO-1 in
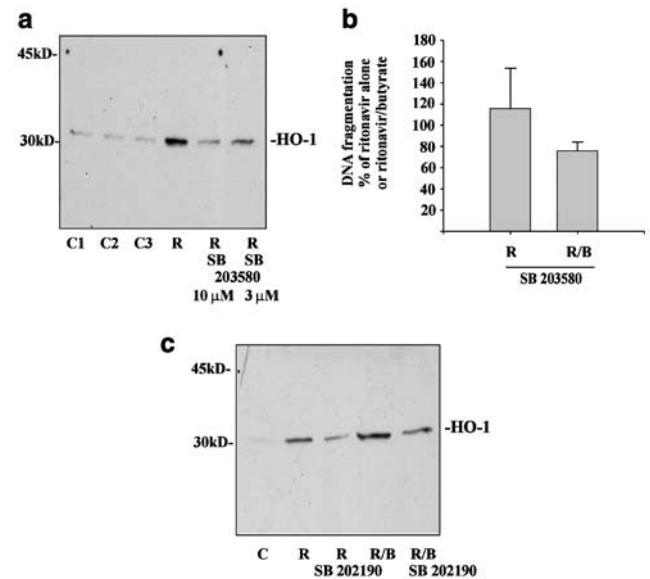

Figure 7 Ritonavir-induced expression of HO-1 is inhibited by SB203580 (a) or SB202190 (c). (a) DLD-1 cells were kept as unstimulated control or were stimulated with ritonavir (R, 60 μM) alone or in combination with the indicated concentrations of SB203580. After 20 h, cells were harvested and homogenates were assayed for HO-1 protein expression by immunoblot analysis. C1 denotes medium alone; C2 (0.1% DMSO) and C3 (0.2% DMSO) representing vehicle control for ritonavir and ritonavir plus SB203580 (10 μM), respectively. One representative of four independent experiments is shown. (b) DLD-1 cells were stimulated with ritonavir (R, 60 μM) or with ritonavir plus butyrate (B, 5 mM) in the presence or absence of SB203580 (10 μM). After 20 h of stimulation, DNA fragmentation was quantified using the diphenylamine reaction. Data are shown as mean DNA fragmentation (percentage of ritonavir alone or ritonavir/butyrate alone) $\pm$ s.d. ($n = 3$). (c) DLD-1 cells were kept as unstimulated vehicle control or stimulated with ritonavir (R, 60 μM), or ritonavir/butyrate (B, 5 mM) in the presence or absence of SB202190 (20 μM). After 20 h of stimulation, cells were harvested and homogenates were assayed for HO-1 protein expression by immunoblot analysis. One representative of four independently performed experiments is shown.

intestinal tissues is associated with reduction of proinflammatory parameters such as TNFα and inducible nitric oxide synthase and is protective in models of experimental colitis (Wang et al., 2001; Fujii et al., 2003). Accordingly, augmentation of HO-1 expression by pharmacological means is likely a promising therapeutic strategy for the treatment of inflammatory bowel diseases. Since colonic inflammation can be regarded a precancerogenic condition, HO-1 induction may have the potential to interfere with the process of colon carcinogenesis at an early stage. Here, we report that exposure to ritonavir mediates expression of HO-1 in DLD-1 colon carcinoma cells. Induction by ritonavir was apparent at concentrations of 30 μM or higher. Common treatment regimes for ritonavir in HIV therapy consist of 600 mg orally twice a day and peak concentrations in sera of patients can reach up to 46 μM (Gaedicke et al., 2002). Thus, it is reasonable to assume that local concentrations of ritonavir, particularly at the colonic epithelium, may actually reach higher levels. Upregulation of HO-1 in DLD-1 cells by clasto-Lactacystin suggests that the action of ritonavir described herein may be related to modulation of cellular proteasome activity. Similarly, HO-1 was inducible by butyrate, which also has been shown to
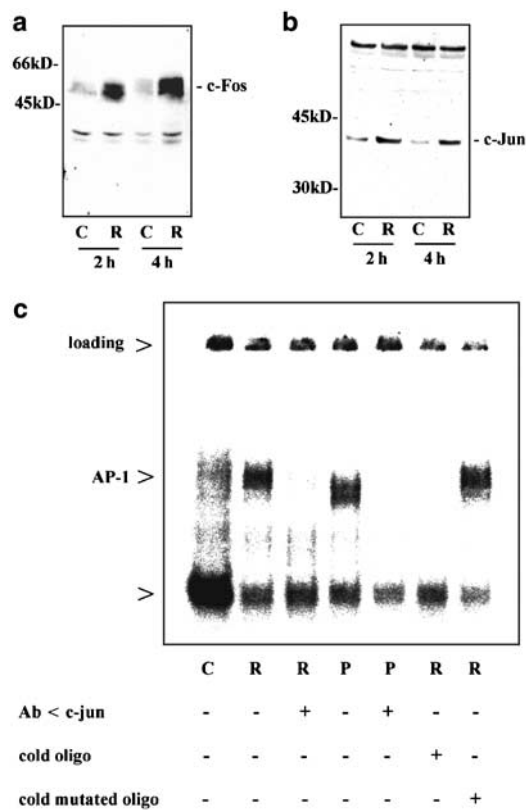
Figure 8 Ritonavir activates the AP-1 signaling pathway in DLD-1 cells. (a and b) ritonavir induces expression of c-Fos and c-Jun in DLD-1 cells. DLD-1 cells were kept as unstimulated control (C, 0.1% DMSO) or were stimulated with ritonavir (R, 60 μM). After the indicated time periods, cells were harvested and homogenates (140 μg total protein/lane) were assayed for c-Fos (a) or c-Jun (b) protein expression by immunoblot analysis. One representative of three independent experiments performed analyzing ritonavir-induced c-Fos and c-Jun expression is shown. (c) Detection of ritonavir-induced AP-1 DNA binding activity in DLD-1 cells by EMSA analysis. DLD-1 cells were incubated as unstimulated control (C, 0.1% DMSO), or stimulated with ritonavir (R, 60 μM), or with PDTC (PDTC, 200 μM) for 2 h. Thereafter, nuclear extracts were prepared and EMSA analysis was performed using ^{32}P-labeled AP-1 consensus oligonucleotide. As indicated, experiments were performed in the presence or absence of an anti-c-Jun antibody, or of excess cold wild-type or mutated oligonucleotide.

diminish proteasome activity in colon carcinoma cells (Yin *et al.*, 2001). In accordance with these observations, it has recently been described that the proteasome inhibitor MG132 mediates gene induction of HO-1 in rat cardiomyocytes (Lüss *et al.*, 2002). Suppression of HO-1 induction by coincubation with SB203580 or SB202190 suggests that activation of the p38 MAP kinase is an essential component of the signal transduction pathway leading to ritonavir-induced HO-1. Interestingly, MG132-induced HO-1 in cardiomyocytes is similarly dependent on activation of p38 MAP kinase. Indeed, inhibition of proteasome function can mediate activation of this kinase (Lüss *et al.*, 2002), again implying that modulation of proteasomal activity may be related to ritonavir-induced HO-1.

The transcription factor AP-1 is a major trigger for gene expression of HO-1 (Alam & Den, 1992). Therefore, the effects of ritonavir on the AP-1 signaling system were investigated. Here, we demonstrate that ritonavir can efficiently augment cellular levels of c-Fos and c-Jun protein. Furthermore, EMSA analysis revealed upregulation of AP-1-specific binding to the corresponding oligonucleotides, indicative of activation of the AP-1 signal transduction machinery under the influence of ritonavir. In the present study, we did not further investigate molecular mechanisms of HO-1 induction by butyrate. However, it is of interest to note that a previous report demonstrates activation of AP-1-dependent gene transcription in colon carcinoma cells exposed to this short-chain fatty acid (Glinghammar *et al.*, 1999). Altogether, present and previous data suggest that ritonavir, like butyrate, has the capacity to inhibit cellular proteasome function and to activate AP-1. Expression of HO-1 appears to be a consequence of this profile of activation and may mediate protective functions (Liu *et al.*, 2001) of ritonavir in chronic inflammation as seen in experimental autoimmune encephalomyelitis (Hosseini *et al.*, 2001).

Induction of apoptotic cell death in cancerous cells is a fundamental principle in anticancer pharmacotherapy. In fact, colon carcinogenesis is associated with reduction of tumor cell sensitivity toward apoptosis (Bedi *et al.*, 1995). Here, we report that exposure of DLD-1 cells to ritonavir resulted in augmented cell death and apoptotic DNA fragmentation. Induction of apoptosis by ritonavir has been described before in Kaposi sarcoma-derived cells, lymphoblastoid cells, and multiple myeloma cells (Gaedicke *et al.*, 2002; Pati *et al.*, 2002; Ikezoe *et al.*, 2004) and may likewise be related to modulation of proteasome activity (Almond & Cohen, 2002). To further evaluate its potential as an inducer of cell death, DLD-1 cells were coincubated with the combination ritonavir plus sodium butyrate. Butyrate is a short-chain fatty acid, which is primarily provided by intestinal bacteria during fermentation of dietary fiber. Cellular actions of butyrate are diverse and include inhibition of histone deacetylase (Archer & Hodin, 1999; Ishihara *et al.*, 2004), inhibition of proteasome activity (Yin *et al.*, 2001), suppression of hypoxia-inducible factor-1α bioactivity (Zgouras *et al.*, 2003), and activation of AP-1 (Glinghammar *et al.*, 1999). In the context of colorectal cancer, it is of particular interest that butyrate is able to enhance the rate of apoptosis in colon carcinoma cells exposed to the Fas ligand (Bonnotte *et al.*, 1998) or to TNFα (Luhrs *et al.*, 2002). Moreover, local bioactivity of the supposedly tumor suppressive cytokine IL-18 may be augmented by butyrate (Paulukat *et al.*, 2001; Kalina *et al.*, 2002). A low-fiber diet has been considered a risk factor for colorectal cancer (Burkitt, 1971). Actually, dietary supplementation of fiber or application of butyrate enemas can suppress colon carcinogenesis in rat models of disease (D'Argenio *et al.*, 1996). Here, we demonstrate that butyrate ($\geqslant 1$ mM) potently enhanced ritonavir-induced cell death. Notably, peak concentrations of butyrate in the human colon can reach up to 20 mM (Wächtershauser & Stein, 2000). Cell death was associated with activation of caspase-3 and with accumulation of the caspase-dependent neoantigen CK18-Asp396. Furthermore, cell death was potently suppressed by the general caspase inhibitor z-VAD-fmk. These observations further confirm that apoptosis is the prime mode of cell death that is responsible for loss of viability in DLD-1 cell cultures exposed to ritonavir or ritonavir plus butyrate. In accordance with the present data, it has been shown that butyrate synergizes with the proteasome inhibitors MG132 (Giuliano *et al.*, 1999) or bortezomib (Pei *et al.*, 2004) for induction of apoptosis in human Y79

retinoblastoma cells or multiple myeloma cells, respectively. Experiments with z-VAD-fmk also revealed that apoptotic cell death is not the driving force behind HO-1 expression in response to ritonavir plus butyrate. Taking into account our aforementioned data concerning the effects of p38 MAP kinase inhibitors on HO-1 expression and apoptosis, we propose that HO-1 induction and apoptosis are parallel but independent cellular responses toward ritonavir or ritonavir plus butyrate. Altogether, the present observations suggest that increasing the colonic butyrate content by high-fiber diet or by butyrate enemas may dramatically potentiate proapoptotic actions of ritonavir on carcinoma cells in patients with colorectal cancer.

Animal models demonstrate that anti-inflammatory properties of HO-1 can suppress leukocytic infiltration into immunologically active tissues (Ishikawa *et al.*, 1997; Poss and Tonegawa, 1997; Busssolati *et al.*, 2004). Leukocytic infiltration into tumor tissues consists mainly of macrophages and can make up to 50% of the tumor biomass. Since angiogenesis and poor prognosis correlate with the degree of macrophage infiltration in several human malignancies (Bingle *et al.*, 2002) including colorectal cancer (Etoh *et al.*, 2000), induction of HO-1 by ritonavir appears to be of advantage and agrees with tumor suppressive properties of this drug. Moreover, HO-1 activity has been shown to reduce production of proinflammatory factors associated with growth and progression of colon cancer, such as IL-8 (Fox *et al.*, 1998; Brew *et al.*, 2000; Bulger *et al.*, 2003) and NO (Cavicchi *et al.*, 2000; Lagares-Garcia *et al.*, 2001; Wang *et al.*, 2001). Current data suggest an anticancer potential of ritonavir. In fact, ritonavir showed protective effects in a murine model of Kaposi sarcoma (Pati *et al.*, 2002) and in a thymoma model (Gaedicke *et al.*, 2002). The potent synergism between ritonavir and butyrate with regard to induction of colon carcinoma cell death by apoptosis may indicate the potential of ritonavir for the treatment of colorectal cancer.

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