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Antagonistic Effects of Sodium Butyrate and *N*-(4-Hydroxyphenyl)-retinamide on Prostate Cancer[1]

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Abstract

Butyrates and retinoids are promising antineoplastic agents. Here we analyzed effects of sodium butyrate and *N*-(4-hydroxyphenyl)-retinamide (4-HPR) on prostate cancer cells as monotherapy or in combination *in vitro* and *in vivo*. Sodium butyrate and 4-HPR induced concentration-dependent growth inhibition in prostate cancer cells *in vitro*. The isobologram analysis revealed that sodium butyrate and 4-HPR administered together antagonize effects of each other. For the *in vivo* studies, a water-soluble complex (4-HPR with a cyclodextrin) was created. A single dose of sodium butyrate and 4-HPR showed a peak level in chicken plasma within 30 minutes. Both compounds induced inhibition of proliferation and apoptosis in xenografts of the chicken chorioallantoic membrane. Analysis of the cytotoxic effects of the drugs used in combination demonstrated an antagonistic effect on inhibition of proliferation and on induction of apoptosis. Prolonged jun N-terminal kinase phosphorylation induced by sodium butyrate and 4-HPR was strongly attenuated when both compounds were used in combination. Both compounds induced inhibition of NF-κB. This effect was strongly antagonized in LNCaP cells when the compounds were used in combination. These results indicate that combinational therapies have to be carefully investigated due to potential antagonistic effects in the clinical setting despite promising results of a monotherapy.

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Keywords: Sodium butyrate, *N*-(4-hydroxyphenyl)-retinamide, prostate cancer, *in vivo*, chorioallantoic membrane.

Introduction

Treatment options for early-stage prostate cancer are well defined, and localized prostate cancer can be cured by several strategies. Yet, in advanced prostate cancer disease, the therapeutic outcome is still disappointing [1]. Conventional chemotherapy has not proven to be sufficiently effective and is often limited by toxicity [2]. Recent trials with Docetaxel (Sanofi-Aventis, Bridgewater, NJ) in advanced disease are promising, although the survival benefit is still rather limited [3]. Hence, there is considerable need to identify well-tolerated and easily applicable substances either for chemoprevention of prostate cancer in high-risk patients or for adjuvant chemotherapy in advanced stages.

Agents active against prostate cancer alter cell growth and differentiation by interfering with pathways regulating crucial cell functions. Butyrates, histone deacetylase inhibitors (HDACIs), as well as retinoids, derivates of retinoic acid, are examples of such compounds.

Butyrates are naturally occurring short-chain fatty acids leading to inhibition of tumor cell growth. It has been shown that butyrates can induce cell cycle arrest, differentiation, and apoptosis in many tumor cell types, whereas having a favorable safety profile in humans [4]. We have previously demonstrated that sodium butyrate and tributyrin strongly induce growth inhibition and apoptosis in different human prostate cancer cell lines *in vitro* [5] and on chicken chorioallantoic membrane (CAM) and in nude mice *in vivo* [6].

Naturally occurring *retinoids* possess significant chemopreventive effects in neoplasias such as acute promyelocytic leukemia [7]. Yet the role of retinoids in prostate cancer is still poorly understood. *N*-(4-Hydroxyphenyl)-retinamide (4-HPR) is a synthetic retinoid that has shown antitumor activity in prostate cancer in animal models [8,9]. Compared with the naturally occurring retinoids, 4-HPR is expected to be more effective with fewer side effects, as it may selectively target specific apoptosis-related pathways in prostate cancer [10]. Mitogen-activated

Abbreviations: 4-HPR, *N*-(4-hydroxyphenyl)-retinamide; CAM, chorioallantoic membrane; CD, (2-hydroxypropyl)-β-cyclodextrin; HDACI, histone deacetylase inhibitor; JNK, jun N-terminal kinase; MAPK, mitogen-activated protein kinase

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protein kinase (MAPK) pathways phosphorylate numerous proteins including transcription factors, cytoskeletal proteins, and kinases and thus influence cell proliferation and apoptosis [11]. There is some evidence that jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK)/MAPK pathways might be involved in sodium butyrate– and 4-HPR–mediated cytotoxicity [12–14].

In this study we explored *in vitro* the antiproliferative effects of sodium butyrate and 4-HPR, as single drugs and in combination, on two prostate cancer cell lines. We also developed a drug application system for the highly lipophilic 4-HPR, converting it into a water-soluble complex that could be applied intravenously in a clinical setting. Furthermore, we analyzed the pharmacokinetics of sodium butyrate and 4-HPR *in vivo* in the CAM model. The treatment effects on xenografts were evaluated by immunohistochemistry, using the proliferation marker Ki-67, and by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay.

Materials and Methods

Reagents

All reagents were obtained from Sigma-Aldrich (Munich, Germany). A stock solution of sodium butyrate was prepared in sterile water. 4-HPR was dissolved in DMSO for the *in vitro* experiments or in sterile water as a β-cyclodextrin derivate complex for the *in vivo* studies.

Analysis of Growth Inhibition in Cell Culture

The drug-induced effects were evaluated on the hormone-sensitive LNCaP cells and hormone-independent PC-3 cells (ATCC, Wesel, Germany). The cell lines were cultured in RPMI 1640 (PromoCell, Heidelberg, Germany) and used for the experiments in a growing phase. Cell proliferation was measured by Cell Proliferation Kit II (Roche, Penzberg, Germany) based on the XTT assay. LNCaP (5×10^3) and PC-3 (2×10^3) cells were grown in microtiter plates and treated with the drugs for 72 hours. Drug interaction was analyzed by the isobologram method [15].

Preparation of the 4-HPR/β-Cyclodextrin Complex for In Vivo *Testing*

For the solubilization of 4-HPR in water, different cyclodextrins and derivatives thereof were tested. Because of the size of 4-HPR, a (2-hydroxypropyl)-β-cyclodextrin (CD) has been chosen. A 4-HPR/CD complex at a molar ratio of 1:14 was used. The maximum solubility for the 4-HPR/CD at room temperature is 0.2 g/ml water, corresponding to a 10 mM solution of 4-HPR.

Chicken Chorioallantoic Membrane Assay

The xenotransplantations onto CAMs of fertilized chicken eggs were carried out as previously described [16,17]. Briefly, at day 7 of fertilization, a double-silicone ring (6 mm; distance between rings, 3 mm) was placed onto the CAM. The cells (1×10^6) were seeded onto one ring in 20 μl 50% Matrigel (BD Biosciences, Heidelberg, Germany) in serum-free RPMI 1640. Starting on the day after inoculation the drugs were administered onto the second ring three times daily for 4 days. Tumor tissues were sampled, fixed, paraffin-embedded, and serially sectioned (5 μm). Slides were processed for staining and immunohistochemistry for human cytokeratin and Ki-67 [16,17] (antibodies from Dako, Hamburg, Germany). The images were digitally recorded at 50× magnification with an Axiophot microscope (Carl Zeiss, Jena, Germany) and a Sony (Köln, Germany) MC-3249 CCD camera using Visupac 22.1 software (Zeiss). Photomicrographs were analyzed with Optimas 6.51 from Media Cybernetics (Silver Spring, MD).

For the *in situ* detection of apoptotic cells in paraffin-embedded tissue sections, the TUNEL method was used (Roche Diagnostics). The sections were counterstained with hematoxylin.

To determine the drug plasma levels, drugs were applied once and blood was drawn from the chorioallantoic vessels at different time points.

Pharmacokinetic Analysis of Sodium Butyrate and 4-HPR in Plasma

Plasma was purified by PS-OH solid-phase extraction chromatography and analyzed by HPLC using Reprosil-Pur ODS3 column (Dr. Maisch GmbH, Ammerbuch, Germany). The concentrations of compounds were calculated by the dynamic linear calibration method using dilutions of standard compounds [18]. The intra-assay coefficient of variation was 11.7% for sodium butyrate and 3.1% for 4-HPR. The inter-assay coefficients of variation were 9% and 6% for sodium butyrate and 4-HPR, respectively ($n = 3$).

Western Blot Analysis

Cells were treated for 3 hours with individual compounds or in combination. Whole-cell lysates containing equal amounts of protein were separated by SDS-PAGE, transferred to Polyvinylidene diflouride (PVDF) membranes, probed with specific antibodies, and detected as described previously [17]. Antibodies for phosphorylated forms of p38, JNK, ERK1/2, IκBα (all from Cell Signaling, Danvers, MA) were used. Actin (Chemicon, Hampshire, UK) was used as a loading control.

Statistical Analysis

The drug effects were statistically evaluated using Newman-Keuls test. Values are presented as mean ± SEM. *P* values <.05 were considered statistically significant.

Results

Drug-induced Inhibition of Cell Proliferation In Vitro

The PC-3 and LNCaP prostate cancer cell lines were treated with increasing concentrations of sodium butyrate and 4-HPR for 72 hours. A 50% growth inhibition of LNCaP cells was achieved by sodium butyrate at a concentration of 3.0 mM and 0.87 μM 4-HPR (Figure 1, *A* and *B*). The IC_{50} for

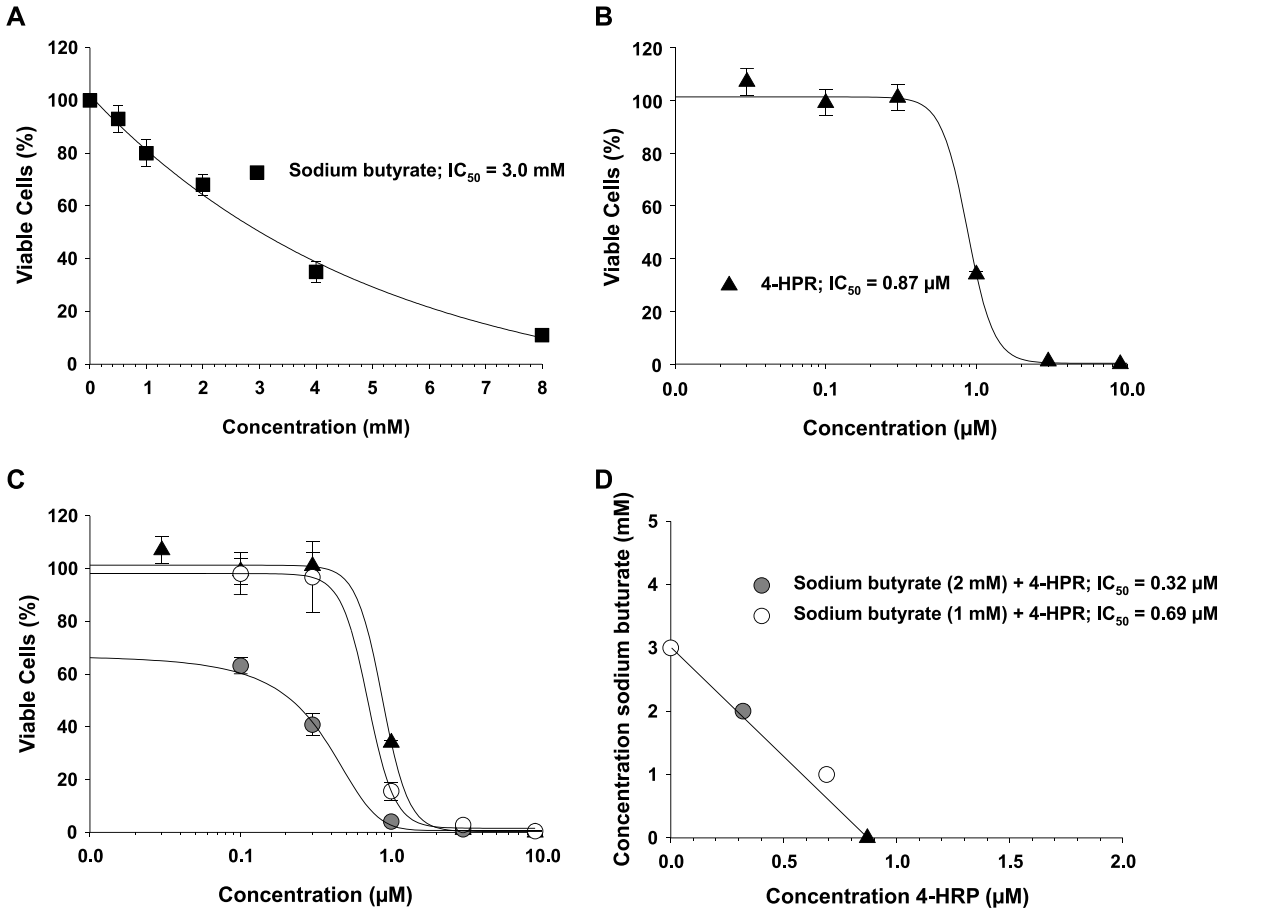

Figure 1. *Inhibitory effect of sodium butyrate and 4-HPR as monotherapy or in combination on proliferation of LNCaP cells. Cells were treated* in vitro *for 72 hours with increasing concentrations of sodium butyrate (A), 4-HPR (B), or 1 to 2 mM sodium butyrate and increasing concentrations of 4-HPR (C). Cell proliferation was analyzed by a modified formazan assay (XTT). The number of viable cells in the control group was set to 100%. Results are presented as mean ± SEM of three experiments. (D) Detection of the antagonistic effects of sodium butyrate and 4-HPR on proliferation of LNCaP by using the isobologram method. The IC_{50} of 4-HPR in the presence of 2 mM sodium butyrate is 0.32 µM and in the presence of 1 mM sodium butyrate is 0.69 µM. The values lie above the line connecting two points representing the IC_{50} of 4-HPR and sodium butyrate used alone indicating the antagonistic effects of drugs.*

4-HPR was 0.32 µM in the presence of 2 mM sodium butyrate, and 0.69 µM with 1 mM sodium butyrate (Figure 1*C*). Drug interactions were analyzed by the isobologram method [15]. The IC_{50} values for each drug used as a monotherapy were placed on the x- and y-axis and connected by a line, which is the locus of points that produce a simple additive effect. In the presence of 1 to 2 mM sodium butyrate the concentrations of 4-HPR that induced 50% inhibition of proliferation of LNCaP cells are located above the line, which clearly indicates antagonistic effects (Figure 1*D*). Similarly, the IC_{50} of sodium butyrate and 4-HPR in PC-3 cells were 2.8 mM and 2.48 µM, respectively (Figure 2, *A* and *B*). In the presence of sodium butyrate the IC_{50} for 4-HPR was slightly reduced to 2.10 and 1.43 µM with 1 and 2 mM sodium butyrate, respectively (Figure 2, *C* and *D*). The data indicate that sodium butyrate and 4-HPR antagonize effects of each other in both cell lines.

Pharmacokinetics of Sodium Butyrate and 4-HPR in the CAM Model

For *in vivo* analysis of cytotoxic effects of the drugs in LNCaP and PC-3 tumors xenografted on the CAM, we created a water-soluble 4-HPR/CD complex (Figure 3*A*). Single doses of sodium butyrate (5 µmol) and 4-HPR/CD (0.6 µmol) were applied on the CAM (Figure 3*B*) and the pharmacokinetics were analyzed (Figure 3, *C* and *D*). The maximum plasma level of sodium butyrate was detected 20 minutes after application, and of 4-HPR, 30 to 40 minutes after application. Under these conditions $t_{1/2}$ was 17 minutes for sodium butyrate and 90 minutes for 4-HPR.

Effect of Sodium Butyrate and 4-HPR on Prostate Tumor Xenografts on CAM

Tumors were transplanted onto the CAM and treated with 20 µmol sodium butyrate and 0.6 µmol 4-HPR/CD either as monotherapy or in combination three times a day for 4 days. CD was used as control and had no effect either on proliferation or on the apoptosis in xenografts. Both sodium butyrate and 4-HPR inhibited proliferation (number of Ki-67–positive cells) of LNCaP and PC-3 tumors growing on the CAM (Figures 4 and 5). The amount of apoptotic cells of untreated LNCaP cells was already too low to allow statistical analysis. About 22% of PC-3 cells were Ki-67 positive. Treatment with either sodium butyrate or 4-HPR significantly reduced the number of proliferating cells (Figure 5*B*). Interestingly, when both compounds were applied simultaneously, 4-HPR

antagonized the strong inhibitory effect of sodium butyrate on cell proliferation. Similarly, treatment with sodium butyrate significantly increased apoptosis in PC-3 xenografts (Figure 5*C*). 4-HPR in the concentration used had no effect on the apoptosis in PC-3 cells, but reduced the proapoptotic effect of sodium butyrate. Thus, compared with the *in vitro* assay, sodium butyrate and 4-HPR showed strong antagonistic effects in the *in vivo* model.

Effect of Sodium Butyrate and 4-HPR on MAPK Activation

Analysis of MAPK activity by using antibodies against phosphorylated/activated forms of kinases demonstrated that LNCaP and PC-3 cells exhibit constitutively active p38 and ERK, but not JNK (Figure 6).Treatment of LNCaP and PC-3 cells either with sodium butyrate or with 4-HPR triggered a prolonged activation of JNK, which was reduced when the combination of both compounds was used. Sodium butyrate and 4-HPR had no effect on p38 activation in PC-3 cells (Figure 6), but sodium butyrate induced a marked decrease in p38 activation in LNCaP cells, which was abolished in the presence of 4-HPR (Figure 6). Both sodium butyrate and 4-HPR inhibited constitutively active ERK1/2 in prostate cells.

LNCaP and PC-3 cells exhibited significant amounts of phosphorylated IκBα, indicating constitutive activation of the NF-κB transcription factor. Sodium butyrate and 4-HPR inhibited NF-κB in both cell lines. However, when both compounds were added together, the NF-κB inhibition was abolished in LNCaP cells. These data indicate that MAPK as well as NF-κB pathways might be involved in antagonistic effects of sodium butyrate and 4-HPR on prostate cancer.

Discussion

As prostate cancer is one of the leading causes of deaths caused by malignancies worldwide, there is a tremendous need to identify active substances against this disease. Because of the heterogeneous pattern of prostate cancer, one can hypothesize that a combination of different drugs affecting various pathways involved in prostate cancer growth and survival may be advantageous. In the present study we

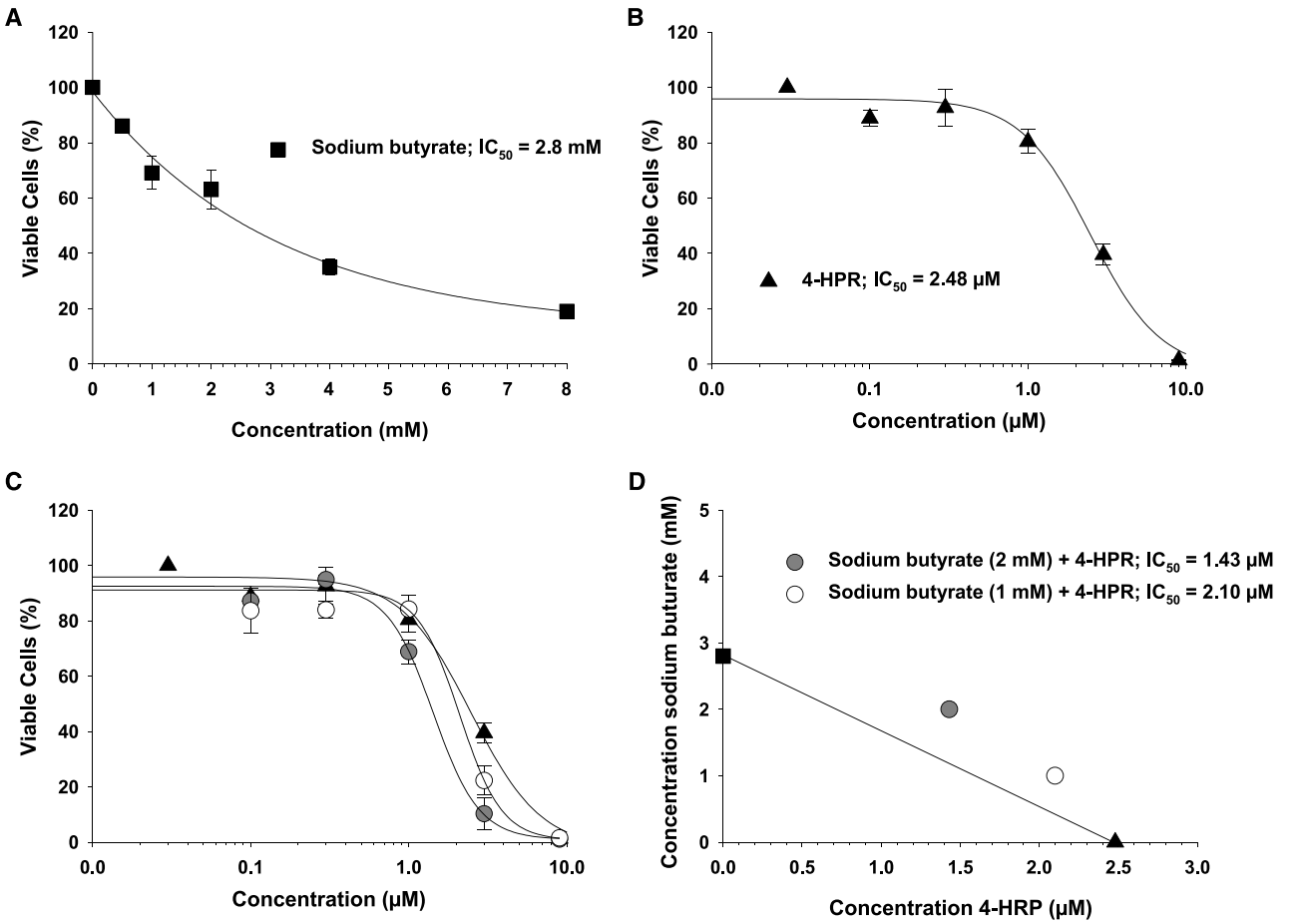

Figure 2. *Inhibitory effect of sodium butyrate and 4-HPR as monotherapy or in combination on proliferation of PC-3 cells. Cells were treated* in vitro *for 72 hours with increasing concentrations of sodium butyrate (A), 4-HPR (B), or 1 to 2 mM sodium butyrate and increasing concentrations of 4-HPR (C). Cell proliferation was analyzed by a modified formazan assay (XTT). The number of viable cells in the control group was set to 100%. Results are presented as mean ± SEM of three experiments. (D) Detection of the antagonistic effects of sodium butyrate and 4-HPR on proliferation of PC-3 by using the isobologram method. The IC_{50} of 4-HPR in the presence of 2 mM sodium butyrate is 1.43 µM and in the presence of 1 mM sodium butyrate is 2.1 µM. The values lie above the line connecting two points representing the IC_{50} of 4-HPR and sodium butyrate used alone indicating the antagonistic effects of drugs.*

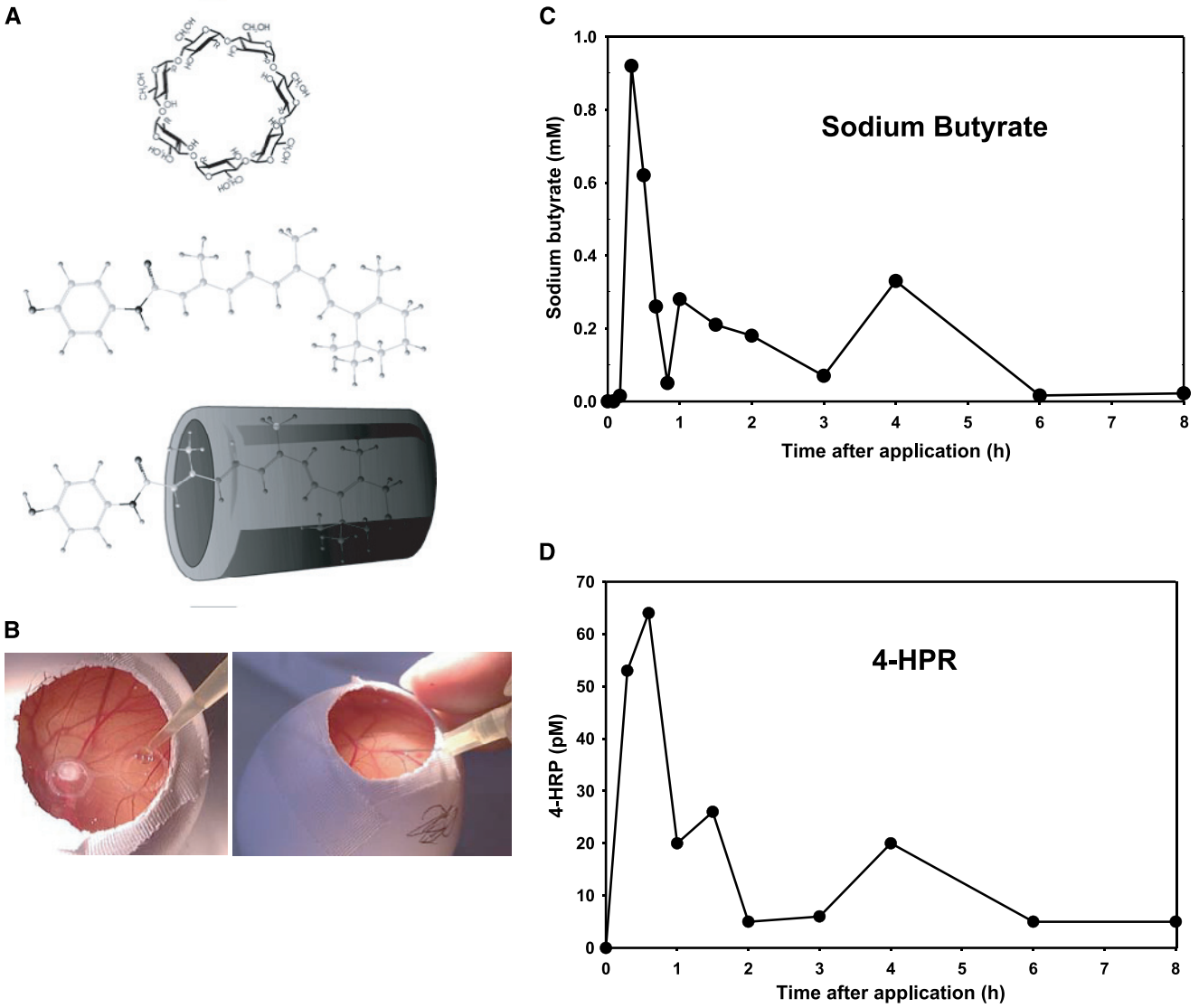

Figure 3. *Analysis of plasma pharmacokinetics of sodium butyrate and water-soluble 4-HPR/CD complex. (A) Top: structure of the (2-hydroxypropyl)-β-cyclodextrin (CD) molecule consisting of seven glucopyranosides. Middle: structure of 4-HPR. Bottom: graphical presentation of 4-HPR within the CD molecule. (B) Principle of CAM assay. For xenotransplantation of tumor cells onto CAM, cells were seeded in 20 μl medium/Matrigel (1:1) in a 6-mm silicone ring placed on CAM of fertilized chicken. Next day after seeding, drugs or the cyclodextrin vehicle were put onto the second ring. (C) For analysis of the plasma-level kinetics, 5 μmol of sodium butyrate in 20 μl was applied topically on the CAM. Blood was collected from chorioallantoic veins at different time points, anticoagulated with 5 mM EDTA, and used for plasma preparation. For each time point, blood from three eggs was analyzed. (D) Pharmacokinetic analysis of plasma levels of 4-HPR was performed essentially as for sodium butyrate; 0.6 μmol of 4-HPR in 15 μl was applied topically on the CAM.*

investigated an effect of a combination of an HDACI, sodium butyrate, with a synthetic retinoid, 4-HPR, on the proliferation and induction of apoptosis in prostate cell lines.

Malignant cells express increased histone deacetylase activity. It is believed that inhibitors could normalize their transcriptional activity. In fact, successful preclinical studies have resulted in the evaluation of HDACIs in the clinical setting [19]. Meanwhile new inhibitors such as suberoylanilide hydroxamic acid (SAHA) [20,21], PXD-101 (TopoTarget, Copenhagen, Denmark) and LAQ-824 (Novartis AG, Basel, Switzerland) were developed.

Sodium butyrate exhibits growth inhibition in LNCaP and PC-3 cells. These effects were apparently independent of the p53 status because PC-3 cells, which are less differentiated, carry mutated p53 [22]. Both cell lines, hormone-sensitive LNCaP and hormone-independent PC-3, were equally sensitive to sodium butyrate treatment.

4-HPR, a synthetic derivative of all-*trans*-retinoic acid, is more effective against cancer cells compared with the naturally occurring retinoic acids especially in hormone-dependent diseases [23]. It exhibits antitumor activity in PC-3 cells *in vitro* [24] and in prostate cancer xenografts *in vivo* [8,25]. Clinical pilot trials with 4-HPR as monotherapy were less successful [26]. However, 4-HPR is expected to act synergistically with other chemotherapeutic drugs [27].

Similar to sodium butyrate, 4-HPR inhibited proliferation of LNCaP and PC-3 cells independently of p53 status of cells. When both substances were applied together, they antagonized effects of each other, demonstrating potential interference within signaling pathways.

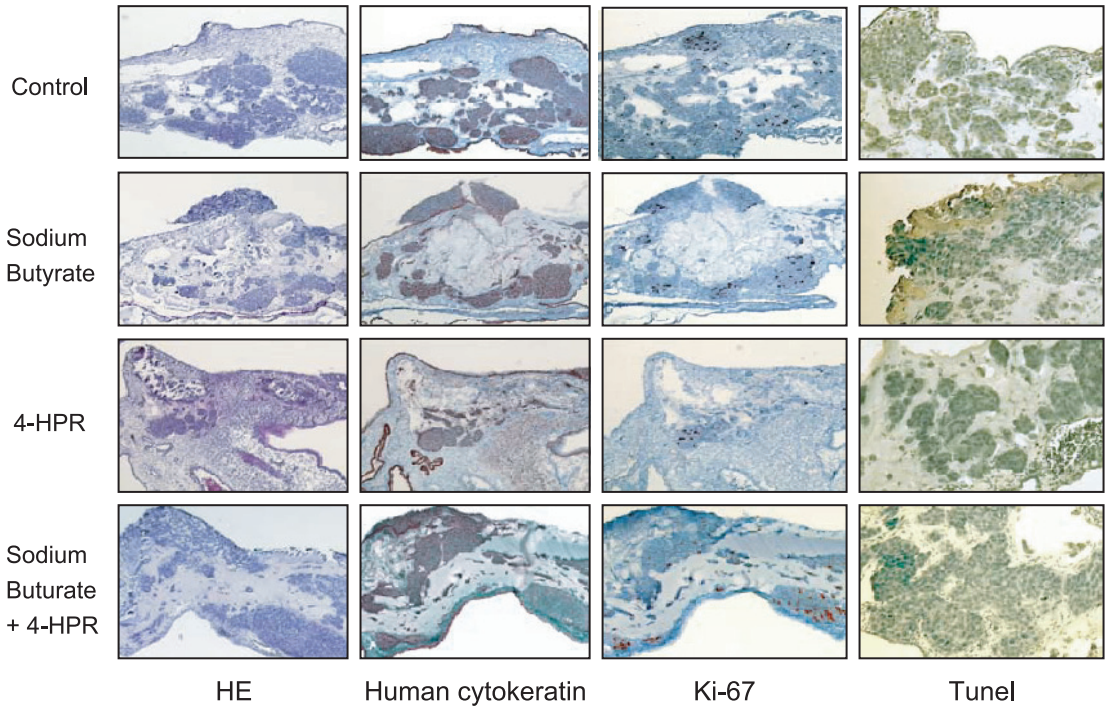

Figure 4. *Inhibitory effect of sodium butyrate and 4-HPR as monotherapy or in combination on proliferation of LNCaP cells* in vivo *in CAM assay. Histological and TUNEL staining of representative center tissue sections of the LNCaP xenografts. Six days after fertilization, 1 × 10^6 cells were grafted onto the CAM of chicken eggs. Next day, the CAMs were topically treated with either sodium butyrate (20 μmol/20 μl), 4-HPR/CD (0.6 μmol/15 μl), or both substances three times a day for 4 days. Sections were stained with hematoxylin-eosin (HE), human cytokeratin, and Ki-67 proliferation antigen and photomicrographed at 50× magnification. Apoptosis was analyzed by TUNEL technique, counterstained with hematoxylin, and photomicrographed at 100× magnification. Data shown are representative of 4 to 6 eggs each.*

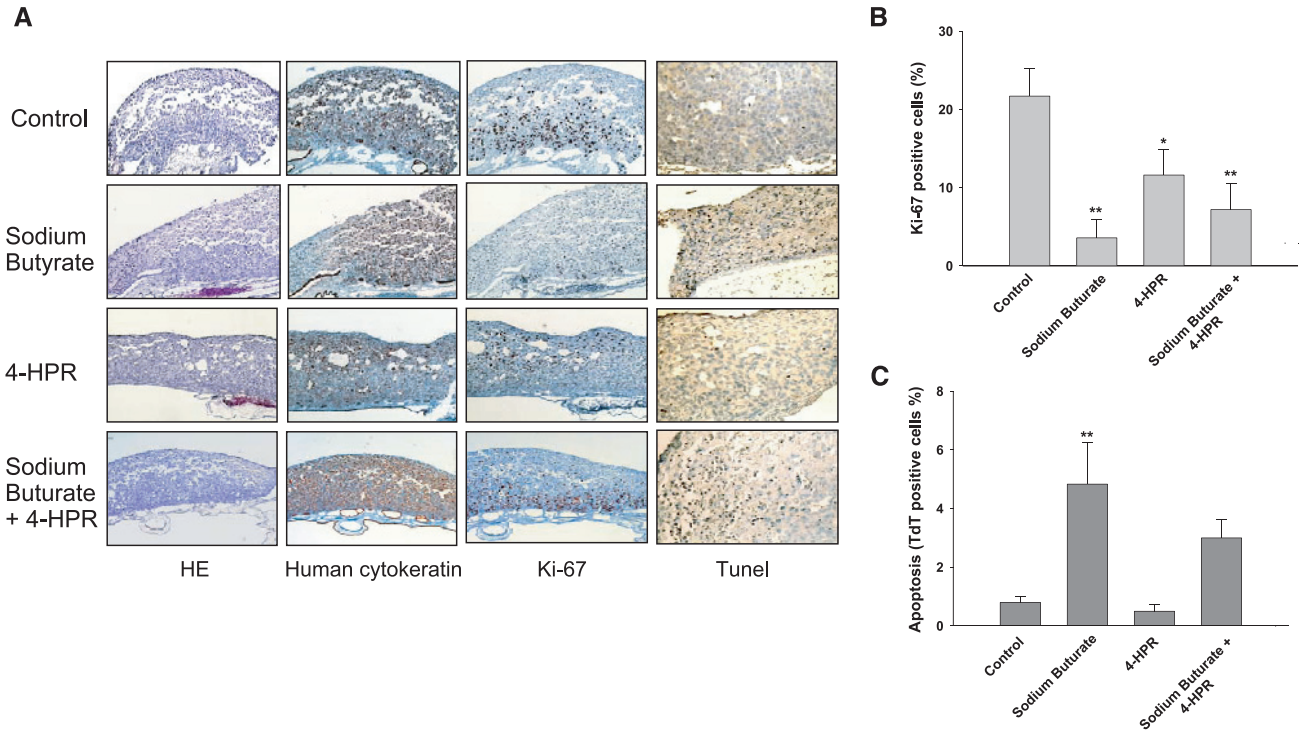

Figure 5. *Inhibitory effect of sodium butyrate and 4-HPR as monotherapy or in combination on proliferation of PC-3 cells* in vivo *in CAM assay. (A) Histological and TUNEL staining of representative center tissue sections of the PC-3 xenografts. Six days after fertilization, 1 × 10^6 cells were grafted onto the CAMs of chicken eggs. Next day, the CAMs were topically treated either with sodium butyrate (20 μmol/20 μl), 4-HPR/CD (0.6 μmol/15 μl), or both substances three times a day for 4 days. Sections were stained with hematoxylin-eosin (HE), human cytokeratin, and Ki-67 proliferation antigen and photomicrographed at 50× magnification. Apoptosis was analyzed by TUNEL technique, counterstained with hematoxylin, and photomicrographed at 100× magnification. Data shown are representative of 4 to 6 eggs each. (B) Histomorphometric analysis of the proliferation antigen Ki-67. For histomorphometry we used digitalized color photomicrographs of serial 5-μm sections 100 μm apart from each other (n = 4–6 eggs in each group). All treatment groups expressed significantly less Ki-67 antigen than the cyclodextrin control group. Results are mean ± SEM of 4 to 6 eggs in each group. *P < .05, **P < .01 compared with control. (C) Histomorphometric analysis of apoptosis. Results presented as percent of terminal deoxynucleotidyl transferase (TdT)–positive cells. **P < .01.*

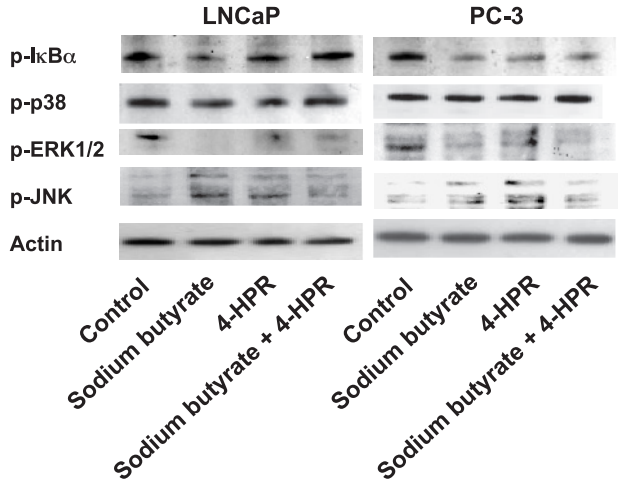

Figure 6. *Effects of sodium butyrate and 4-HPR on NF-κB and MAPK activation in LNCaP and PC-3. Western blotting analysis of phosphorylated IκBα, p38, ERK1/2, and JNK MAPK in the cells treated for 3 hours either with sodium butyrate (2 mM), with 4-HPR (1 μM LNCaP, 3 μM PC-3), or both. Actin served as a loading control. One of three independent experiments is shown.*

We further analyzed if the antagonistic effects of the drugs also occurred *in vivo*. Sodium butyrate induced pronounced growth inhibition and induced apoptosis in PC-3 xenografts. 4-HPR as a monotherapy reduced cell proliferation but had no effect on the level of apoptosis. Application of both drugs in combination reduced antiproliferative and proapoptotic effects seen with sodium butyrate as monotherapy. Thus, both substances act antagonistically *in vitro* and *in vivo*. Limited information exists concerning interactions of retinoids with other antineoplastic agents. Our results, showing the antagonistic effects between 4-HPR and sodium butyrate, prompted us to analyze the possible molecular mechanisms of this antagonism.

Antagonistic effects of 4-HPR on sodium butyrate–induced apoptosis might be a result of interference with the pro- and antiapoptotic signaling pathways, such as MAPK. MAPKs, which include ERK1/2, JNKs, and p38, are activated by phosphorylation [11]. ERK1/2 is constitutively activated in various tumors and associated with cell proliferation [11]. Inactivation of ERK might be involved in HDACI-mediated lethality [13,14]. Treatment of LNCaP and PC-3 with either sodium butyrate or 4-HPR drastically reduced ERK activity, which might contribute to the inhibitory effects on the proliferation of prostate tumor cells.

Members of the JNK family regulate cellular responses to various stresses. Transient JNK activation is associated with gene expression and cell proliferation, whereas prolonged JNK activation promotes cell death [28]. Histone deacetylase inhibitors have been shown to use JNK for induction of apoptosis [14]. In contrast, retinoids, in particular 4-HPR, strongly suppress c-jun (which is a JNK substrate) activity, and thus, activator protein 1 activity in ovarian cancer [29]. In prostate cancer cells, 4-HPR induced JNK activation leading to the induction of apoptosis [12]. We have found that treatment of prostate cells with either sodium butyrate or 4-HPR triggers prolonged JNK activation; coadministration of both drugs resulted in downregulation of JNK phosphorylation and, consequently, inhibition of apoptosis in prostate cells.

The prolonged activation of JNK induced by sodium butyrate and 4-HPR might be a result of concomitant downregulation of NF-κB activity, which triggers increased reactive oxygen species (ROS) [30] NF-κB is a family of transcription factors regulating expression of antiapoptotic genes [28]. Consequently, inhibition of NF-κB activity promotes apoptosis in prostate cancer cells [17]. Sodium butyrate and 4-HPR suppressed phosphorylation of the inhibitor IκBα, resulting in the inhibition of NF-κB activation and downregulation of expression of antiapoptotic genes. This effect was abolished in LNCaP when both compounds were used in combination, rending cells less susceptible to apoptosis.

Together our data show that sodium butyrate and 4-HPR inhibit ERK1/2 and NF-κB, activate JNK, and thus possess inhibitory effects on prostate cancer cells *in vitro* and *in vivo*. Applied together, sodium butyrate and 4-HPR, however, antagonize effects of each other. Studies on the molecular mechanism of action of antineoplastic drugs are the key to allow the identification of highly effective agents and the development of efficacious combinations for targeted therapy of the heterogeneously growing prostate cancer.

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