Combination of Phenylbutyrate and 13-cis Retinoic Acid Inhibits Prostate Tumor Growth and Angiogenesis

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INTRODUCTION

Differentiation therapy represents a novel and alternative therapeutic approach in the treatment of cancer. Among the differentiation agents, retinoids and short-chain fatty acids have shown biological activity as single agents in several preclinical studies of different tumors including PCA.

Aliphatic and aromatic fatty acids such as sodium butyrate, PB, and its metabolite phenylacetate have been reported to induce tumor cell cytostasis, differentiation, and apoptosis in various hematological and solid tumors, including prostate cancer (5–7). HDACs, enzymes that affect eukaryotic chromatin structure, have been shown to be a target of the nuclear hormone receptor superfamily (18–20). These receptors modulate pathways for growth and differentiation, affecting the activity and expression of cell cycle proteins and nuclear receptors (11). Interestingly, inhibitors of HDAC have been shown to dramatically potentiate retinoid-induced differentiation of RA-sensitive human acute promyelocytic leukemia cell lines and to restore retinoid response of RA-resistant human acute promyelocytic leukemia cell lines (12). Aliphatic and aromatic fatty acids have been shown to inhibit HDAC activity (13, 14) and to induce differentiation through G1 cell cycle arrest and p53-independent up-regulation of p21 (15). PB and its metabolite phenylacetate require relatively high concentrations and influence several metabolic pathways. In preliminary clinical trials, PB has been shown to have a low toxicity profile but limited clinical benefit (16, 17).

Retinoids, like the aromatic fatty acids, have been shown to exert their biological activity by promoting cell differentiation, apoptosis, and inhibiting cell proliferation in several human tumor cell lines (prostate, melanoma, neuroblastoma, leukemia, germ cells, and mammary tumor; Ref. 1). Retinoid receptors, RAR and RXR, are members of the nuclear hormone receptor superfamily (18–20). These receptors bind to the RARE as RXR/RAR heterodimer and modulate RA-dependent gene expression. One of the target genes of retinoid receptors is RAR-β, which encodes four transcripts. RAR-β expression is reduced in many malignant tumors, including lung carcinoma, squamous carcinoma of the head and neck, and breast cancer (21, 22). A recent report showed that RAR-β and RXR-β mRNA are selectively and significantly reduced in prostate cancer as well as in adjacent normal prostate tissue, suggesting an association between loss of specific retinoid receptor subtypes and prostate carcinogenesis (23). Interestingly, retinoids, such as short-chain fatty acid, inhibit cell proliferation by inducing G1 cycle arrest and up-regulation of p21 (24).

An extensive series of clinical studies on CRA in patients with advanced established cancers showed no effect (25). However, in primary prevention studies involving heavy smokers (26) and in secondary prevention trials in patients with a history of squamous cell carcinoma of the head and neck, CRA showed a significant clinical benefit (27, 28).

In the past decade, increased interest has been focused on the role of new blood vessel formation in the pathogenesis of tumors since the original observation by Folkman (29, 30). New therapeutic strategies involving angiogenesis inhibitors are actively under development. Differentiation agents, such as retinoids and vitamin D3, have been shown to have a direct inhibitory effect on tumor-induced angiogenesis. Retinoids directly inhibit endothelial cell migration and angiogenesis in vivo (31). Interestingly, retinoids have been shown to down-regulate vascular endothelial growth factor production in normal human keratinocytes (32). Combinations of RA with IFN-α or vitamin D3 have a synergistic antitumor effect by inducing inhibition of both endothelial cell and tumor cell proliferation and reducing tumor growth in vivo and angiogenesis (33, 34). Of interest, both sodium butyrate and retinoids have been shown to up-regulate tissue type plasminogen activator in endothelial cells, suggesting the possible role of increased proteolysis in the modulation of angi-inhibitory cytokines (35, 36).

The rationale for combining PB and CRA stems from their distinct

ABSTRACT

Differentiation-inducing agents, such as retinoids and short-chain fatty acids, have an inhibitory effect on tumor cell proliferation and tumor growth in preclinical studies. Clinical trials involving these compounds as single agents have been suboptimal in terms of clinical benefit. Our study evaluated the combination of phenylbutyrate (PB) and 13-cis retinoic acid (CRA) as a differentiation and antiangiogenesis strategy for prostate cancer. On the basis of previous evidence, common signal transduction pathways and possible modulation of retinoid receptors and retinoid response elements by PB could be responsible for such activities. We assessed the effect of the combination of PB and CRA on human and rodent prostate carcinoma cell lines. The combination of PB and CRA inhibited cell proliferation and increased apoptosis in vitro in an additive fashion as compared with single agents (P < 0.014). Prostate tumor cells treated with both PB and CRA revealed an increased expression of a subtype of retinoic acid receptor (retinoic acid receptor-β), suggesting a molecular mechanism for the biological additive effect. The combination of PB and CRA also inhibited prostate tumor growth in vivo (up to 82–92%) as compared with single agents (P < 0.025). Histological examination of tumor xenografts revealed decreased in vivo tumor cell proliferation, an increased apoptosis rate, and a reduced microvessel density in the animals treated with combined drugs, suggesting an antiangiogenesis effect of this combination. Thus, endothelial cell treatment with both PB and CRA resulted in reduced in vitro cell proliferation. In vivo testing using the Matrigel angiogenesis assay showed an additive inhibitory effect in the animals treated with a combination of PB + CRA (P < 0.004 versus single agents). In summary, this study showed an additive inhibitory effect of combination of differentiation agents PB and CRA on prostate tumor growth through a direct effect on both tumor and endothelial cells.

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The abbreviations used are: PCA, prostate carcinoma; PB, phenylbutyrate; CRA, 13-cis retinoic acid; RA, retinoic acid; RXR, retinoic acid receptor; HDAC, histone deacetylase; RXR, retinoid X receptor; RARE, retinoic acid responsive element; PPAR, peroxisome proliferator-activated receptor; FBS, fetal bovine serum; BAEC, bovine aorta endothelial cell; FACS, fluorescence-activated cell sorter; RT-PCR, reverse transcription-PCR; bFGF, basic fibroblast growth factor; MVA, microvessel area; TZZ, troglitazone.

Sodium butyrate and retinoids have been shown to up-regulate tissue type plasminogen activator in endothelial cells, suggesting the possible role of increased proteolysis in the modulation of angi-inhibitory cytokines (35, 36).
DIFFERENTIATION AS ANTITUMOR AND ANTIANGIOGENESIS THERAPY

activity at the nuclear receptor level. Nuclear hormone receptors have recently gained attention as selective targets for antineoplastic drugs (37, 38). The human PPARs are nuclear receptors that act as transcriptional factors. PPARs control the expression of several genes containing PPAR response elements that are involved in the peroxisomal and mitochondrial pathway of lipid metabolism (39). PB and phenylacetate have been shown to activate PPAR-α (40) and PPAR-γ (41). Interestingly, PPARs must form a heterodimer with the retinoid receptor RXR-α to function as a transcriptional factor. Prostate carcinoma cells, similar to other solid tumors such as breast and colon carcinoma, express high levels of PPARs, in particularly the γ subtype (42). Recent preclinical reports showed that TZD, a commonly used antidiabetic drug that acts as a PPARγ agonist, induces differentiation in breast carcinoma (43) and liposarcoma (44) and inhibits prostate carcinoma growth (42).

On the basis of the common signal transduction pathways and possible modulation at the nuclear receptor level, we hypothesized that a combination of PB and CRA may have additive or synergistic inhibitory activity on prostate tumors. In this study, we report on the potent and novel additive inhibitory activity of PB and CRA on several PCA cell line proliferation in vitro and the inhibition of tumor growth in vivo. In addition, we demonstrated that this combination has an additive inhibitory activity on endothelial cell proliferation in vitro and angiogenesis in vivo.

MATERIALS AND METHODS

Cell Lines and Reagents. Human prostate carcinoma cell lines LNCaP, PC3, TSU, DU-145 (American Type Culture Collection repository), and Dunning rat prostate carcinoma cell lines R-3327 (G-Tumor; a generous gift from Dr. John Isaacs, Johns Hopkins Oncology Center) were maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FBS (Sigma Chemical CO) and 2 mm L-glutamine (Life Technologies, Inc.). BAECs (American Type Culture Collection) and human umbilical vein endothelial cells (Clonetics) were maintained in DMEM supplemented with 10% FBS and endothelium-specific medium (EGM; Clonetics), supplemented with 2% FBS, respectively. Stock solutions of 10⁻³ M CRA (Sigma Chemical Co.) and 250 mM PB (Triple Crown America) were prepared in DMSO and PBS, respectively, and stored at 4°C.

Proliferation Assay. Briefly, tumor cell lines and BAECs were plated in 24-well plates (5–10× 10⁵/well), and after overnight incubation in complete medium, cells were treated with 2.5 mM PB or 10 μM CRA or a combination in complete medium containing <0.1% DMSO. These concentrations were chosen based on preliminary in vitro studies (data not shown), reports in the literature, and achievable peak blood concentrations. At different time points, cells were trypsinized, and each condition was counted in triplicates on a Coulter Z1 cell counter (Coulter Electronics). Results are expressed as mean cell counts/well ± SE. The experiments were repeated twice.

Colony Formation Assay. Colony formation assay was performed as published previously by other investigators (45). Tumor cells and BAECs were harvested and replated in six-well plates (5 × 10⁵ cell/well) in absence of drugs and in complete medium. After 48 h, to allow cell attachment, cells were treated with 2.5 mM PB or/and 10 μM CRA for 72 h as in the proliferation assay. Then, cells were washed, and drug-free complete medium was added. As the colonies became visible (10 days), cells were fixed with methanol, stained with crystal violet, and counted at the inverted light microscope (Zeiss). Results are expressed as a mean of total colonies/well ± SE. The experiments were repeated twice.

Cell Cycle and Apoptosis Assays. The FlowTACS Apoptosis Detection kit (R&D Systems), which is designed specifically for in situ detection of apoptotic cells by flow cytometry, was used according to the manufacturer’s recommendations. Briefly, tumor cells or BAECs were plated in T75 flasks and treated with the 2.5 mM PB or/and 10 μM CRA for 72 h. Cells were harvested, and their membranes were permeabilized with CytoPore reagent. Next, terminal deoxynucleotidyl transferase (R&D Systems) added biotinylated nucleotides to the 3′-ends of the DNA fragments. Streptavidin-conjugated fluorescein (FITC) specifically bound to the biotinylated DNA fragments and was detected by flow cytometry (FACS). The cell cycle analysis was performed under the same experimental conditions as described above. Cells were harvested and resuspended in citrate buffer and stored at −20°C. Prior to analysis, cells were incubated for 30 min with staining solution containing 0.1 mg/ml propidium iodide (Sigma Chemical Co.). The solution was then passed through a nylon mesh filter and analyzed on a Becton Dickinson FACSScan. In a parallel experiment, BAEC nuclei were stained with 4,6-diamidino-2-phenylindole (Sigma Chemical Co.) and analyzed at the fluorescence microscope (Zeiss).

RNA Preparation and RT-PCR. Prostate tumor cells were plated in T75 flasks, grown until 60–70% confluent, and then treated with 2.5 mM PB or/and 10 μM CRA for 72 h. Cells were harvested, and extraction of total RNA was performed using Trizol (Life Technologies, Inc.) according to the manufacturer’s instructions. Total RNA samples also were prepared from human breast carcinoma cell lines Hs578t (positive expression of RAR-β and MDA-MB-231 (negative expression of RAR-β) as positive and negative controls, respectively. The Superscript One-Step RT-PCR System (Life Technologies, Inc.) was used to amplify 50 ng of DNA-treated total RNA. Samples were processed in a Perkin-Elmer 9600 GeneAmp thermocycling system under the following conditions: 30 min at 50°C for the reverse transcriptase reaction; 2-min denaturation step at 94°C; followed by 35 amplification cycles (15 s at 94°C for denaturation, 30 s at 55°C for primer annealing, and 1 min at 72°C for primer extension), and final extension at 72°C for 10 min. PCR products were analyzed on 1.5% agarose gels. Amplification of RAR-β promoter (256 kb) was performed by using published primer sequences: exon 5 (sense strand 5′-GAC TGT ATG GAT GTT CGT TCA G-3′) and exon 6 (antisense strand 5′-ATT TGT CCT GGC AGA CGA ACA AGC A-3′; Ref. 46). RT-PCR with primers encoding for 36B4 (human acidic ribosomal phosphoprotein, 150 kb; sense strand 5′-GAT TGG CTA CCC AAC TGT TGC A-3′ and antisense strand 5′-CAT GAG GAG CTC CCA AAA AGG C-3′; Ref. 47) was used as an internal RNA control. Primers were a generous gift from Drs. Smitha Subramanyan and Nicoletta Sacchi (48), Johns Hopkins Oncology Center.

Tumor Growth in Vivo. Male athymic nude mice (Taconic), 4–6 weeks of age, were kept in a temperature-controlled room on a 12/12-h light/dark schedule with food and water ad libitum. Animals were injected s.c. in the flank regions bilaterally with 2 × 10⁶ prostate carcinoma cells resuspended in serum-free medium (Life Technologies, Inc.) and mixed with Matrigel (1:1; Collaborative Biomedical Products) in a final volume of 0.2 ml. Twenty animals for each tumor were randomly placed in four groups (five animals/group): control, PB, CRA, and PB + CRA. Animals in the control group were treated with daily administration of vehicle (polyethylene glycol; Fisher) by 20-gauge gavage needle and with i.p. injections of PBS. The in vivo doses and schedules of PB and CRA were chosen based on preliminary studies (data not shown) and reports in the literature (6, 7, 49, 50). PB (600 mg/kg/day) was administered concomitantly by i.p. injections (300 mg/kg/day, 9 a.m. and 5 p.m.) and by 2-week osmotic pumps (300 mg/kg/day; Alza Corp). The osmotic pumps were replaced once. CRA was administered by gavage needle (30 mg/kg/day, 9 a.m.). Daily treatment (6 days/week) with PB or/and CRA was initiated 2–5 days after implantation. Tumor volume was measured with a caliper twice a week and calculated according to the formula: length × width × height × 0.5236 and reported as mean mm³ ± SE. The animals were treated for ~4 weeks and then were euthanized; tumors were harvested for histological studies. All of the in vivo experiments were repeated once. Animals treated with CRA and CRA + PB sometimes showed a transient weight loss during the second week of treatment with spontaneous recovery.

Immunohistochemistry Study. Formalin-fixed, paraffin-embedded tissue was generated from G-Tumor xenotransplants. Sections were pretreated with Pronase for 20 min at 37°C and a rabbit antihuman factor VIII antibody (Dako, Carpinteria, CA; 1:1000) was used on all sections as a specific marker for endothelial cells. Then, sections were incubated with a secondary biotin-conjugated goat antirabbit IgG antibody (1:100 × 30 min at room temperature. Avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA) was prepared as per the manufacturer’s instructions and allowed to incubate on the sections. Next, sections were incubated in diaminobenzidine solution, washed, and counterstained in methyl green. The Image-Pro analysis system was used to quantify the area occupied by factor VIII-stained vessels. The mean area/field from 10 to 20 fields/section/plug (×200 = ×20 objective lens and ×10 ocular lens; Zeiss Axio-skop) was calculated. The same sections were also
processed in a similar fashion and stained with monoclonal antibody anti Ki-67/MIB-5 (Beckam Coulter Immunotech) as proliferation marker and with Terminal Transferase (terminal deoxynucleotidyltransferase-mediated nick end labeling technique; Boehringer Mannheim) for apoptotic cell detection. Each section was also stained with H&E.

Angiogenesis Assay in Vivo. The effect of PB and CRA on new blood vessel formation was assessed in the Matrigel assay, performed as described previously (51). Briefly, C57/BL6 mice (The Jackson Laboratory) were randomly divided in four groups (five animals/group): control, PB, CRA, and PB + CRA. Animals received injections s.c. with 0.75 ml of Matrigel supplemented with 150 ng/ml bFGF (R&D Systems). PB and CRA were administered (same doses and schedules as above) starting 5 days prior to the Matrigel injection and until the end of the experiment. Control animals received vehicle only. Mice were sacrificed 10 days after the Matrigel injection. Then, the gels were recovered by dissection and fixed in PBS-buffered 10% formalin containing 0.25% glutaraldehyde, prior to staining the slides with Masson’s Trichrome. The Image-Pro analysis system was used to quantify the area occupied by vessels in the histological sections. The mean area/field from 10 to 20 fields/section/plug (×200) was calculated and expressed as mean percentage area occupied by blood vessels ± SE. The experiment was repeated twice.

Statistical Analysis. Differences between means of unpaired samples were evaluated by Student’s t test using the SigmaPlot program (SPSS, Inc.). P < 0.05 was taken to indicate statistical significance.

RESULTS

The Combination of PB and CRA Has an Additive Inhibitory Effect on PCA Proliferation and Induces Cell Cycle Arrest and Apoptosis in Vitro. To determine the effect of PB and CRA on PCA proliferation, hormone-sensitive (LNCaP and G-Tumor) and hormone-independent (TSU, DU-145, and PC3) cell lines were exposed to 2.5 mM and/or 10 μM concentrations of PB and CRA, respectively. By 72 h, the number of cells in the PB and CRA groups was significantly less than the number of cells in the control groups (67–74% (PB) and 11–45% (CRA) growth inhibition as compared with control; Fig. 1). However, when tumor cells were treated with both PB and CRA, an additive inhibitory effect (76–83% inhibition as compared with control) was observed (P < 0.014 versus single agents). These proliferation data were confirmed also by colony formation assay. Treatment of DU-145 cells with PB + CRA resulted in a 77–80% inhibition of colony formation as compared with control, whereas single agents produced a 48–50% inhibition (P < 0.002 PB + CRA versus control, PB and CRA alone). Similar results were obtained with the other cell lines (data not shown). Treatment of prostate carcinoma cells with PB and PB + CRA resulted in distinct changes in cell morphology. The cells grew slowly with enlarged cytoplasm and pyknotic nuclei. Flow cytometry analysis was performed to identify drug-induced changes in the tumor cell cycle. Treatment of PC3 cells with PB and PB + CRA for 72 h resulted in increased G1 arrest (94%) and 10-fold decrease in S-phase (3.1%), as compared with control (54 and 30%, respectively; Fig. 2A). No significant variation with CRA alone or additive effect with combined drugs was observed. Similar results were observed with the other cell lines (data not shown). Next, induction of apoptosis by PB and CRA was assessed. PCA treated with PB or CRA had an increased apoptotic rate as compared with controls (PB: 7-fold increment, P < 0.024 versus control; CRA: 1.5 fold increment; Fig. 2B). Interestingly, the combination of PB and CRA had an additive effect on apoptosis as compared with single agents (P < 0.006), with 15-fold increments as compared with baseline controls (P < 0.001).

TSU Cell Treatment with PB Induces Expression of RAR-β in the Presence of CRA. On the basis of previous evidence of induction of RAR-β by phenylacetate in neuroblastoma cells (52), we hypothesized that the additive effect of the combination of PB + CRA might be attributable to tumor cell sensitization to CRA by induction of the retinoid receptor. TSU cells were treated with PB and/or CRA and at different time points total RNA was extracted and analyzed for RAR-β expression by RT-PCR. After 24 h exposure, there was a weak expression of RAR-β in control, PB-, and CRA-treated cells (Fig. 3, Lanes 3–5, respectively). However, in the presence of CRA, there was 4–6-fold induction of RAR-β expression by PB (Fig. 3, Lane 6). RAR-β up-regulation by PB + CRA persisted at 48 and 72 h (Fig. 3, Lanes 10 and 14, respectively).

The Combination of PB and CRA Shows an Additive Inhibitory Effect on Prostate Tumor Growth in Vivo. To determine the effect of PB and CRA on PCA growth in vivo, human (LNCaP, DU-145) and rat (G-Tumor) prostate tumors were established by giving injections to athymic nude mice s.c. with 2 × 106 cells mixed with Matrigel. Single agents PB or CRA resulted in a modest tumor growth inhibition that was generally not statistically significant as compared with controls (Fig. 4). However, the combination of PB and CRA resulted in a significant additive inhibitory effect (up to 90% growth inhibition, as compared with controls; P < 0.025 versus single agents). The tumor volume data were confirmed by tumor weight measurements in separate experiments as shown in Table 1. The
combination of PB and CRA resulted in a 70% inhibition of G-Tumor and DU-145 tumor weights ($P_{0.02}$) versus single agents).

The Combination of PB and CRA Is Cytostatic in Vivo. Next, we asked whether the “dormant” prostate tumors were able to grow after discontinuation of the treatment. In a separate experiment, G-Tumor-bearing animals were treated with the combination PB + CRA. Tumor growth was inhibited during administration of the drugs, but upon discontinuation the tumors resumed growth at the same rate as controls (Fig. 5). After a 3-week interval, drug treatment was resumed, and tumor growth slowed and reached a plateau. Histological examination of the tumor during drug administration showed the presence of small cellular aggregates in the original Matrigel (Fig. 5B) as compared with control (Fig. 5A). These data show the primary cytostatic nature of this treatment and infer the need for continuous administration to achieve the antitumor effect.

The Combination of PB and CRA Inhibits Cell Proliferation, Induces Apoptosis, and Reduces Tumor Microvessel Density in Vivo. The LNCaP and G-Tumor xenografts in the PB + CRA-treated animals did not grow and remained “dormant,” still embedded as nests of cellular aggregates in the Matrigel (Fig. 6B). Tumor samples from G-Tumor xenotransplants underwent histological examination. Immunohistochemistry studies showed a decreased proliferation signal and

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**Table 1** Effect of combination of PB + CRA on prostate tumor weights

<table>
<thead>
<tr>
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<th>Control</th>
<th>PB</th>
<th>CRA</th>
<th>PB+CRA</th>
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<tr>
<td>G-Tumor</td>
<td>2.57 ± 0.40*</td>
<td>2.52 ± 0.34</td>
<td>1.05 ± 0.01b</td>
<td>0.79 ± 0.01c</td>
</tr>
<tr>
<td>DU-145</td>
<td>0.99 ± 0.12</td>
<td>0.72 ± 0.07</td>
<td>0.57 ± 0.03b</td>
<td>0.30 ± 0.08c</td>
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*a* Mean g/tumor ± SE.

*b* $P < 0.02$.

*c* $P < 0.015$ versus control; $P < 0.02$ versus PB and CRA.
Fig. 5. The combination of PB and CRA shows cytostatic activity in vivo. G-Tumor cells (2 × 10^5) were injected in nude mice, and within 1 week, treatment with PB + CRA was initiated. After 40 days, the drugs were discontinued, and one animal from the control group (Fig. 5A) and one from the experimental group (Fig. 5B) were sacrificed for tumor histology. After a 3-week interval, the experimental group was restarted with PB and CRA for an additional 2 weeks, and then treatment was discontinued. Tumors were measured twice a week.

Data points: mean tumor volume of five mice/group; bars, SE. Lines: days of treatment. *, P < 0.004 versus control.

The Combined Effect of PB and CRA on Tumor Growth

PB and CRA alone reduced tumor volume by 62% (P < 0.0001 versus control; Fig. 6H), whereas combined drugs demonstrated a 70% inhibition (P < 0.001 versus control, PB and CRA alone). BAECs, similar to tumor cells, also showed active apoptosis upon treatment with PB + CRA (Fig. 7D), as compared with control (Fig. 7C).

The Combination of PB and CRA Inhibits Angiogenesis in Vivo

We assessed the effect of PB and CRA on new blood vessel formation in vivo. Matrigel mixed with 150 ng/ml bFGF was injected s.c. into C57BL6 mice. Matrigel plugs retrieved from animals treated daily with PB + CRA had an extensive vascularization 10 days after implantation. In contrast, plugs retrieved from animals treated daily with PB + CRA had markedly reduced vascularization (Fig. 8A). Quantitative analysis of the MVA showed an inhibitory effect on bFGF-induced neovascularization upon treatment with either PB or CRA as single agents (39% inhibition; Fig. 8B). However, an additive inhibitory effect was achieved when these drugs were combined (64% inhibition, P < 0.004 PB + CRA versus PB and CRA).

DISCUSSION

These results demonstrate that the combination of two differentiation agents, PB and CRA, inhibits PCA cell growth in vitro and in vivo and inhibits angiogenesis. Previous preclinical studies have shown that these individual agents have activity on PCA, but this is the first time that these two differentiation agents are tested together and found to have additive inhibitory effect on tumor growth in vivo. This combination is effective in both hormone-sensitive and hormone-independent PCA cell lines.

On the basis of the common signal transduction pathways shared by PB and CRA, we expected an increased inhibition of cell proliferation and apoptosis in tumor cells treated with combined drugs. However, this combination of differentiation-inducing agents revealed primarily a cytostatic activity, as shown in our in vivo studies. No tumor regression was observed, as expected, and tumors were kept “dormant” as long the drugs were administered, with resumption of normal growth rate upon discontinuation of the treatment.

The additive inhibitory activity of PB and CRA may be explained by the fact that aromatic fatty acids and retinoids likely act through similar mechanisms to induce tumor cytostasis. Several molecular mechanisms may underlie these common biological effects between these two classes of nuclear receptor activators: (a) the signaling pathways effected by PB and CRA are likely to converge through the formation of heterodimers between their respective receptors, PPARs and RXR; (b) phenylacetate/butyrate up-regulate the expression of RAR-β and, thus, may enhance retinoid-specific activity (52); (c) evidence exists that histone acetylation may modulate RA activity. Trichostatin, a specific inhibitor of HDAC potentiates RA-induced neuronal differentiation by enhancing RXR/RAR heterodimer binding to RARE (53). Inhibitors of HDAC potentiate or restore retinoid-induced differentiation in leukemic cells by removing the corepressor and allowing the transcription of RAREs (12); and (d) PPARγ and RXR-specific ligands may have an additive effect in inducing differentiation, such as in liposarcoma cell lines where the adipogenic activity through activation of the PPARγ/RXRα heterodimer is maximal when both receptors are bound by their respective ligands (44, 54).

The identification of the thiazolidinedione drugs as agonist-ligands for the PPARγ receptor has recently provided an attractive therapeutic opportunity (55, 56). A recent report describes the successful induction of differentiation of human liposarcoma in patients treated with TZD, suggesting the clinical development of receptor-targeted therapeutic interventions to induce differentiation as an antitumor strategy (57).

In a recent study, the PPARγ agonist TZD inhibited human PCA growth in vitro and in vivo (42). The investigators found that human PCA cell lines and human PCA samples express PPARγ, whereas
normal prostate tissue has very low expression. Dose-response clonogenic assays of PC3 cell line treated with TZD showed an antiproliferative effect. The authors tested the hypothesis of whether the combination of PPAR\(\gamma\) and RXR or RAR agonists may have additive or synergistic effect when combined together. The RXR and RAR ligands tested were effective against LNCaP, PC3, and DU-145, whereas TZD inhibited \textit{in vitro} proliferation of PC3 only. Interestingly, minimal additional inhibition occurred when TZD was combined with a RXR ligand and no additional effect with an RAR agonist. The \textit{in vivo} experiments showed a mild inhibitory effect of TZD on PC3 cell xenograft growth and greater growth inhibition with all-trans RA, but no evidence of an additive inhibitory effect with the combination of these two drugs.

When we treated the PCA cells with ciglitizone, a pure PPAR\(\gamma\) agonist, in the presence of CRA, we did not achieve any additive inhibitory effect \textit{in vitro} (data not shown). These results suggest that the PPAR\(\gamma\) activity of PB is not responsible for the additive biological effect in the presence of CRA.

Recently, reports support the hypothesis that chromatin-remodeling drugs, such as HDAC inhibitors and demethylating agents, might provide a strategy to restore RAR-\(\beta\) activity, and help to overcome the hurdle of RA resistance in tumor cells (i.e., breast cancer; Ref. 48). Our study on RAR-\(\beta\) expression in TSU cells confirms that a HDAC inhibitor, such as PB, is able to activate specific response elements within 24 h of PB exposure. In our case, PB might derepress the RAREs present in the RAR-\(\beta_2\) promoter region and induce the ex-
expression of this receptor in the presence of the ligand. Thus, it would be reasonable to speculate that the additive effect of PB and CRA on PCA shown in our study may be attributable to the HDAC inhibitor activity of PB, which sensitizes tumor cells to RA.

These data are of particular interest. Not only might it explain the molecular mechanism underlying the additive effect of this combination, but also it would suggest a role of this or similar combination in the chemoprevention of prostate cancer. In fact, a growing body of evidence supports the hypothesis that RAR-β is a tumor suppression gene, and its expression is “silenced” in breast and prostate cancer (22, 23). Additional molecular studies are ongoing in our lab to confirm and expand these data.

Our study also suggests that the combination of PB and CRA have a direct effect on endothelial cells and inhibits angiogenesis in vivo. A possible role for PB in inhibiting angiogenesis might be related to its properties as a PPARγ agonist. Tumor-associated macrophages play a key role in tumor angiogenesis by secreting a repertoire of angiogenic products (58). PPARγ nuclear receptor has been identified in monocytes and a wide range of ligands including TZD, 15-deoxy-D12,14-prostaglandin J2, and certain nonsteroidal anti-inflammatory drugs, strongly inhibited macrophage/monocyte cytokine production and reduced nitric oxide, as well as the secretion of other inflammatory products such as gelatinase B (59, 60). A recent report also showed that PPAR activators induce macrophage apoptosis by negatively interfering with the antiapoptotic nuclear factor-κB signaling pathway (61).

Recently, two compelling reports showed that endothelial cells express PPARγ receptor, and that the bio-active prostanooid 15-deoxy-D12,14-prostaglandin J2, which has PPARγ agonist properties, is able to inhibit endothelial cell proliferation in vitro and angiogenesis in vivo (62, 63). Additional molecular studies are in progress in our laboratory to elucidate the molecular mechanism responsible for the biological effect of PB and CRA on endothelial cells.

In conclusion, our results suggest that the combination of PB and CRA may have therapeutic potential in the treatment of PCA. We hypothesize that PB and CRA likely act through similar signal transduction pathways. The additive effect of combination of PB and CRA may attributable to the induction of RAR-β by PB and thus tumor cell sensitization to CRA. Additional molecular studies are necessary to elucidate the mechanisms of this interaction. The completion of these further mechanistic studies will be important so that combination of newer and more selective agents (i.e., new synthetic retinoids, PPARγ agonists, and HDAC inhibitors) can be assessed in preclinical studies and tested in the treatment of prostate cancer.
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