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Butyrate Analogue, Isobutyramide, Inhibits Tumor Growth and Time to Androgen-Independent Progression in the Human Prostate LNCaP Tumor Model

Martin E. Gleave,[1,2]* Naohide Sato,[2] Marianne Sadar,[2] Virginia Yago,[2] Nicholas Bruchovsky,[1,2] and Lorne Sullivan[1]

[1]Department of Surgery, University of British Columbia, Vancouver General Hospital, Vancouver, British Columbia, Canada
[2]Department of Cancer Endocrinology, British Columbia Cancer Agency, Vancouver, British Columbia, Canada

Abstract Progression to androgen independence remains the main obstacle to improving survival and quality of life in patients with advanced prostate cancer. Induction of differentiation may serve as a rational basis for prevention of progression to androgen independence by modulating gene expression activated by castration or upregulated during androgen-independent progression. The objectives of this study were to characterize the in vitro effects of sodium butyrate on human prostate cancer cell growth, PSA gene expression, and differentiation in the LNCaP tumor model and to determine whether tumor progression in vivo is delayed by isobutyramide, an orally bioavailable butyrate analogue with a longer half-life. The effects of isobutyramide on LNCaP tumor growth and serum PSA levels in both intact and castrate male mice were compared to controls. At concentrations >1 mM, butyrate induced dose-dependent changes towards a more differentiated phenotype, G1 cell cycle arrest, and an 80% decrease in LNCaP cell growth rates. PSA gene expression was increased threefold by butyrate, indicative of differentiation-enhanced gene expression. The half-life of isobutyramide in athymic mice was determined by gas chromatography to be 4 h. During a 4 week period in intact-placebo mice, tumor volume and serum PSA increased 4.1- and 6.6-fold, respectively, compared to twofold and 2.7-fold increases in tumor volume and serum PSA in intact-treated mice. During a 7 week period in castrate-placebo mice, tumor volume and serum PSA levels increased 2.4-fold and fourfold, respectively, compared to a 50% reduction in tumor volume and a twofold increase in serum PSA above nadir levels in castrate mice treated with adjuvant isobutyramide. Isobutyramide treatment induced pronouced morphological changes in LNCaP tumor cells, with loss of defined nucleoli and dispersion of chromatin distribution. LNCaP tumor PSA mRNA levels actually increased threefold, indicative of differentiation-enhanced gene expression. This study demonstrates that butyrate causes LNCaP cell cycle arrest and increased PSA gene expression, both indicative of differentiation. The combination of castration and adjuvant isobutyramide was synergistic in delaying tumor progression. Decreased tumor cell proliferation and increased PSA gene expression induced by isobutyramide results in disconcordant changes in serum PSA and tumor volume and reduces the utility of serum PSA as a marker of response to therapy. J. Cell. Biochem. 69:271–281, 1998.

Key words: butyrate; isobutyramide; prostate cancer; LNCaP

No treatment surpasses androgen withdrawal therapy for patients with advanced prostate cancer. However, despite response rates of 80%, progression to androgen independence ultimately occurs and remains the main obstacle to longterm cure [Denis and Murphy, 1993]. Conventional chemotherapy is usually ineffective because of low proliferation rates and is limited by toxicity [Eisenberger et al., 1987]. Future improvements in survival may be possible through development of new therapeutic strategies designed to prevent or delay progression to androgen independence. Induction of differentiation may serve as a rational basis for prevention of progression to androgen independence by modulating gene

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*Correspondence to: Martin E. Gleave, University of British Columbia, Division of Urology, Vancouver General Hospital, D-9, 2733 Heather Street, Vancouver, BC, V5Z 3J5, Canada.

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expression activated by castration or upregulated during androgen-independent progression.

The rationale for differentiation therapy in prostate cancer is supported by several lines of evidence. First, prostatic cancer growth and differentiation can be altered by various differentiating agents, including retinoids [Slawin et al., 1991; Pollard et al., 1991; Dahiya et al., 1995], dimethylsulfoxide (DMSO) [Carvalho et al., 1989; Kaneski et al., 1991], butyrate [Figg et al., 1994], and phenylacetate [Samid et al., 1993]. For example, noncytotoxic doses of DMSO reduce Dunning rat prostate cancer cell growth rates [Carvalho et al., 1989; Kaneski et al., 1991], while fenretinide inhibits carcinogenesis and metastases in Lobund-Wistar rats [Pollard et al., 1991] as well as tumor incidence and growth of *ras*+*myc*-induced carcinomas in the mouse prostate reconstitution model [Slawin et al., 1991]. Second, when androgens are used as differentiation agents and replaced intermittently after castration, repeated cycles of androgen-induced differentiation and androgen withdrawal–induced PSA nadirs are possible, and the time to androgen independence is delayed [Akakura et al., 1993; Sato et al., 1996]. If the adaptive/epigenetic processes involved in progression to androgen independence can be modulated by the differentiation-inducing effects of androgens, these processes may also be prevented by agents capable of inducing cellular differentiation when used in an adjuvant setting with androgen ablation therapy. Indeed, breast cancer in vivo studies found that the combination of fenretinide and the antiestrogen tamoxifen is superior to either agent alone in preventing progression following excision of the first palpable tumor [Ratko et al., 1989]. These studies provide parallel data to support the hypothesis that androgen-independent progression proceeds along a pathway of aberrant cellular differentiation, and it is therefore plausible to assume a similar synergistic effect for differentiation and androgen ablation therapy in the treatment of prostate cancer.

Controlled investigation of the physiologic modifications associated with androgen-independent progression of prostate cancer has proved difficult because no animal model exists that mimics the course of the clinical disease. Of the available human prostate cancer cell lines, only the LNCaP cell line is androgen-responsive, prostate-specific-antigen (PSA)-secreting, and immortalized in vitro [Horoszewicz et al., 1980]. PSA is a secretory glycoprotein produced only by differentiated prostatic epithelial cells and as such may serve as a marker of differentiation [Oesterling, 1991]. As in human prostate cancer, serum PSA levels in the LNCaP tumor model are initially regulated by androgen and are directly proportional to tumor volume, and loss of maintenance of androgen-regulated PSA gene expression is the end point of progression to androgen independence [Gleave et al., 1992]. After castration, serum and tumor-cell PSA levels decrease up to 80% and remain suppressed for 3–4 weeks. Following a prolonged period of growth (>21 days) in castrate hosts, LNCaP tumors regain their ability to produce PSA in amounts similar to their precastrate state, heralding the onset of androgen-independent PSA gene expression [Sato et al., 1996; Gleave et al., 1992]. Loss of androgen dependence of PSA gene regulation involves epigenetic mechanisms via upregulation of alternate transcription factors, which may in turn be suppressed by induction of differentiation [Hsieh et al., 1993].

We characterized the effects of DMSO, fenretinide, and butyrate in the LNCaP tumor model and found that although all these agents inhibited cell growth in vitro, no in vivo activity was identified [Gleave et al., 1996]. Butyrate was the only agent that caused dose-dependent increases in PSA gene expression concomitant with cell cycle arrest, consistent with a differentiation effect. Butyrate is a naturally occurring short chain fatty acid that changes chromosome structure, gene expression, and differentiation of numerous cell types [Norton et al., 1989; Candido et al., 1978; Nakagawa et al., 1988]. The major known pharmacological action of butyrate in vitro is inhibition of histone deacetylase, which leads to increases in histone acetylation and subsequent changes in chromatin structure [Candido et al., 1978]. Although butyrate is a potent inhibitor of tumor cell proliferation in vitro, its short half-life in vivo limits its use as a therapeutic agent. To circumvent problems with short half-lifes, we began testing a branched-chain butyrate analogue, isobutyramide, that has a longer half-life and is orally bioavailable [Perrine et al., 1994]. The objectives of this study were to characterize the effects of sodium butyrate on LNCaP cell growth, PSA gene expression, and differentiation both in vitro and in vivo and to determine whether the combination of castration and iso-

butyramide is superior to either treatment alone in inhibiting LNCaP tumor growth and progression to androgen independence.

MATERIALS AND METHODS

In Vitro Mitogenic Assays

LNCaP cells were maintained in RPMI 1640 (Terry Fox Laboratory, Vancouver, BC, Canada) with 5% fetal bovine serum (GIBCO, Burlington, ON, Canada) as previously described [Gleave et al., 1992]. The effect of sodium butyrate (Sigma, St. Louis, MO) on LNCaP cell growth rate, morphology, and PSA mRNA expression was assessed. To measure the effect of butyrate on LNCaP cell growth in vitro, we used a 96-well assay based on the uptake and elution of crystal violet dye by the cells in each well [Gleave et al., 1991]. Three thousand LNCaP cells were plated per well in 96-well plates (Falcon, Lincoln Park, NJ) in RPMI with 5% fetal bovine serum, and the cell media was changed to various concentrations of sodium butyrate (100 µM, 1 mM, and 5 mM) 24 h later. The medium was changed every 2 days; 7–10 days later the cells were fixed in 1% glutararaldehyde (Sigma) and stained with 0.5% crystal violet (Sigma). Plates were washed and air-dried, and the dye was eluted with 100 µl Sorensen's solution (9 mg trisodium citrate in 305 ml distilled H_20, 195 ml of 0.1 N HCl, and 500 ml 90% ethanol). Absorbance of each well was measured by a Titertek Multiskan TCC/340 (Flow Laboratories, McLean, VA) at 560 nm. Control experiments demonstrated that absorbance is directly proportional to the number of cells in each well.

Flow Cytometric Evaluation

LNCaP cells (3.5×10^6) were plated on 40 mm plates for 3 days. Subsequently, the media was changed to 1 mM and 5 mM sodium butyrate for periods of 4, 8, 12, 24, 36, and 48 h. The cells were then trypsinzed into a single cell suspension, centrifuged at 1000 rpm for 10 min, and fixed with 0.5% paraformaldehyde for 15 min at 4°C. The cells were again centrifuged and resuspended in 1 ml of 0.1% Triton X-100 for 3 min at 4°C. After centrifugation for 10 min at 1,000 rpm, the supernatent was discarded, and the cells were incubated with RNAse (1 mg/ml) at 37°C for 30 min. After centrifugation the cells were stained with 1 ml of 50 µg/ml propdium iodide, a fluorescent dye that intercalates double-stranded DNA. The DNA profile was then analyzed using a dual laser flow cytometer.

DNA Fragmentation Analysis

DNA fragmentation was analyzed by agarose gel electrophoresis as described by Sellins and Cohen, 1987. Briefly, fragmented genomic DNA was extracted by incubation of cells in Tris extraction buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0,5% Triton X-100) at 4°C for 5 min. The cell suspension was centrifuged for 10 min at 4°C. The resultant supernatent was incubated for 1 h at 37°C in the presence of proteinase K (400 µg/ml) before extracting with phenol/chloroform. The fragmented DNA was precipitated with salt and isopropyl alcohol and then analyzed by agarose gel electrophoresis.

Northern Blot Analysis

Northern analysis was used to determine the effect of the differentiation agents fenretinide, DMSO, dimethylformamide (DMF), and sodium butyrate on PSA mRNA levels in LNCaP cells in vitro and of isobutyramide in vivo. For in vitro Northern analyses, LNCaP cells were weaned gradually from serum using a serum-free defined media supplement (RPMI plus 2% TCM [Celox Co., Minnetonka, MN]). LNCaP cells were plated in 100 mm tissue culture dishes (Falcon) in RPMI, 2% TCM, and 1% fetal bovine serum (FBS). When the cell cultures were 70–80% confluent, the media was changed, cells were washed with phosphate buffered saline (PBS), and media was replaced with serum-free RPMI with 2% TCM and various concentrations of fentretinide, DMSO, or sodium butyrate. After 48 h, the cells were harvested, and their RNA was isolated for Northern analysis. Total cellular RNA was prepared from cultured LNCaP cells or LNCaP tumors using the 4 M guanidinium thiocyanate extraction method. Electrophoresis, blotting, hybridization, and densitometric analyses were carried out as previously reported [Sato et al., in press; Gleave et al., 1992]. The DNA probe for PSA was a 1.4 kb EcoRI fragment of PSA cDNA [Lundwall and Lilja, 1987]. Density of bands for PSA was normalized against that of 18s rRNA.

Pharmacokinetics of Isobutyramide in Athymic Mice

Mice were administered 50 mg of isobutyramide orally in 200 µL water. Blood (100 µL) was

obtained from a tail vein at 2, 4, 8, and 12 h after the single oral dose. Samples were treated by adding 20 µL of a 50% trichloroacetic acid (TCA) solution to 80 µL of the blood to precipitate proteins. The tubes were then centrifuged and the supernatent applied to a gas chromatography column at 220°C. Isobutyramide peaks were found at approximately 4 min and were clear of all plasma peaks.

Assessment of In Vivo Tumor Growth

LNCaP cells (1×10^6) were inoculated s.c. with 0.1 ml of Matrigel (Becton Dickinson Labware, Bedford, MA) in the flank region of 6- to 8-week-old male athymic nude mice (BALB/c strain; Charles River Laboratory, Montreal, PQ, Canada) via a 27 gauge needle under methoxyfluorane anesthesia. Tumors were thereafter measured twice weekly, and their volumes were calculated by the formula $L \times W \times H \times 0.5236$ [Gleave et al., 1991]. The effect of isobutyramide was tested in intact and castrated mice. Changes in tumor growth rate and serum PSA levels were compared between intact-placebo (200 µL water three times daily) and intact-treated (50 mg of isobutyramide in 200 µL water three times daily) mice as well as between castrate-placebo and castrate-treated mice. Mice bearing tumors between 100 and 200 mm^3 in volume were assigned to either intact or castrate groups and randomly selected for placebo vs. treatment with isobutyramide. Data points were expressed as average tumor volumes ± standard errors of the mean based on five determinations.

Determination of Serum PSA Levels

Blood samples were obtained with tail vein incisions of mice before treatment and then once weekly after starting treatment with isobutyramide or a placebo in both intact and castrate groups. Serum PSA levels were determined by an enzymatic immunoassay kit with a lower limit of sensitivity of 0.2 µg/L (Abbott IMX, Montreal, PQ, Canada) according to the manufacturer's protocol. PSA velocity is defined as the rate of change of serum PSA over time, while PSA doubling time is defined as the number of doubings of serum PSA over the treatment period. Time to androgen-independent PSA regulation was defined as the duration of time required after castration for serum PSA levels to return to or increase above precastrate levels. Data points were expressed as average tumor volumes ± standard errors of the mean based on five determinations.

RESULTS

Butyrate-Induced Changes in LNCaP Cell Morphology In Vitro and In Vivo

LNCaP cell morphology changes to a more differentiated epithelioid phenotype within 48 h after exposure to 1 and 5 mM sodium butyrate, concomitant with decreased growth rates (Fig. 1A,B). No evidence of apoptosis or necrosis was observed with concentrations of 1 mM or less, but at concentrations of 5 mM cell detachment began after 48 h and was associated with DNA laddering on agarose gel electrophoresis, consistent with apoptosis (Fig. 2). Changes in LNCaP tumor cell morphology were also apparent in vivo following isobutyramide treatment. After 1 week of isobutyramide therapy in mice bearing LNCaP tumors, nuclei in LNCaP tumor cells become larger with dispersion of chromatin and loss of nucleoli. Increases in apoptosis of necrosis were not apparent (Fig 1C,D).

Butyrate Treatment Results in Cell Cycle Arrest in LNCaP Cells In Vitro

The growth rate of both the LNCaP cell line is reduced in a dose-dependent manner by sodium butyrate at concentrations above 1 mM (Fig. 3). Flow cytometry revealed that sodium butyrate results in G1 cell cycle arrest over a 48 h period (Fig. 4), thereby reducing the fraction of cells in S phase by 99%. The absence of hypodiploid and DNA fragmentation suggests that no obvious apoptosis or necrosis, respectively, occurs at 1 mM butyrate.

Isobutyramide Is Orally Bioavailable and Has a Longer Half-Life In Vivo

To determine the pharmacokinetic parameter determinations of isobutyramide in athymic mice, we measured plasma concentrations using gas chromatography in five mice. After a single oral dose of 50 mg of isobutyramide, gas chromatography of mouse sera measured peak levels above 1 mg/ml with a half-life of 4 (±0.28) h. Isobutyramide pharmacokinetics follow a one compartment elimination model.

Isobutyramide Treatment Inhibits LNCaP Tumor Growth and Serum PSA Doubling Time in Intact and Castrate Male Mice Compared to Controls

To evaluate the effect of isobutyramide treatment in androgen-dependent LNCaP tumors,

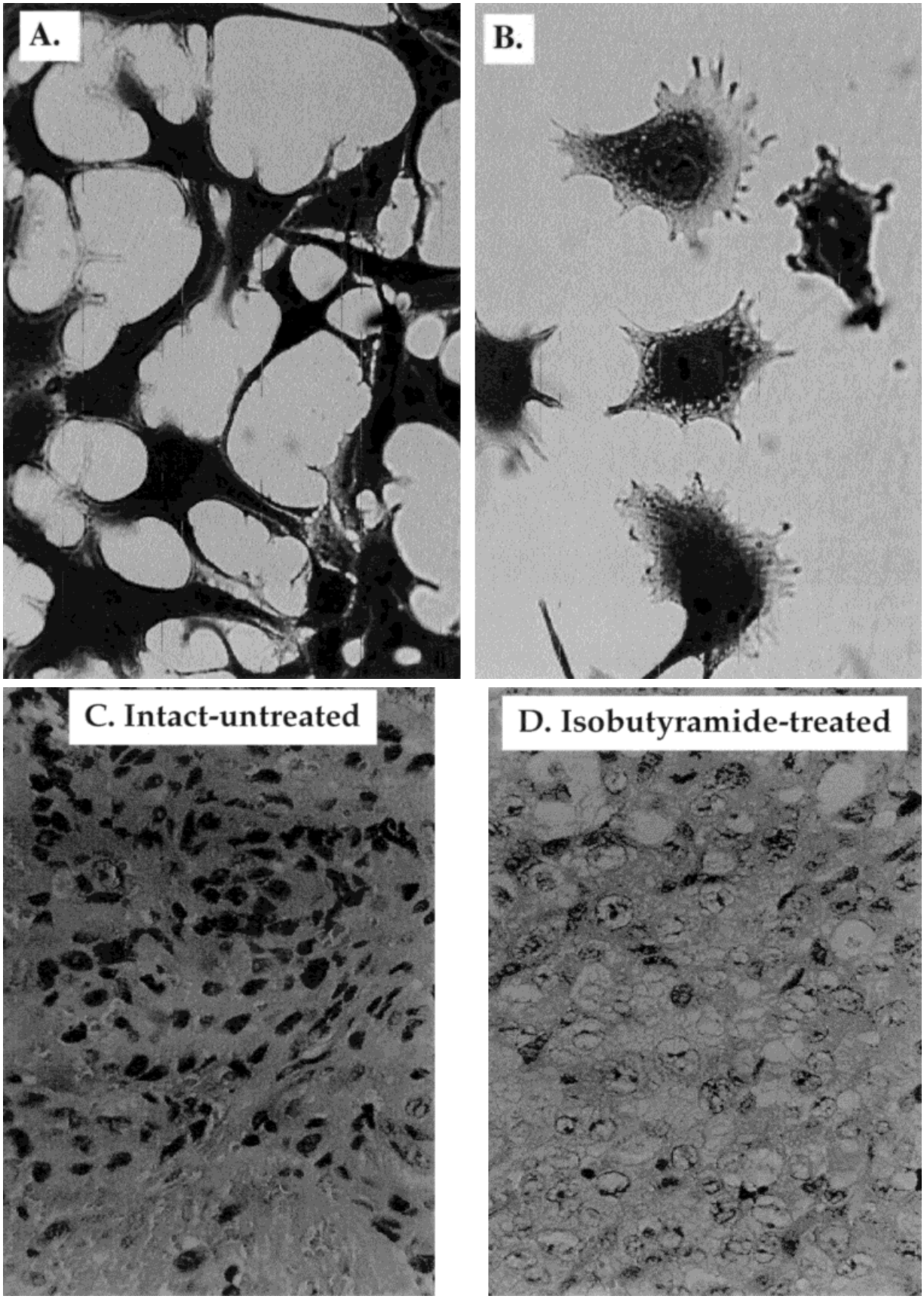

Fig. 1. Changes in LNCaP cell morphology following treatment with butyrate in vitro and isobutyramide in vivo. In vitro control (**A**) and in vitro LNCaP (**B**) cells after 48 h sodium butyrate (1 mM). **C:** Intact-untreated LNCaP tumor. **D:** Intact-treated LNCaP tumor after 1 week isobutyramide treatment. Reduced from $\times 250$.

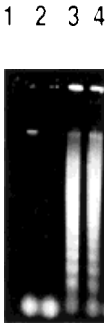

Fig. 2. Agarose gel electrophoresis of fragmented DNA from LNCaP cells after 48 h with RPMI media (**lane 1**), 10 nM R1881 **(lane 2**), 5 mM butyrate (**lane 3**), or both R1881 and butyrate (**lane 4**). M, molecular weight markers (λ and [00d8]X DNA). A total of 10 µg DNA was added to each lane prior to electrophoresis in a 1% agarose gel and staining with ethidium bromide.

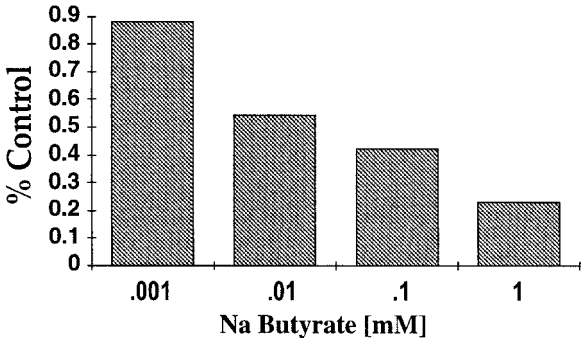

Fig. 3. LNCaP cell growth rates are reduced in a dose-dependent manner by sodium butyrate at concentrations above 1 mM, with a 80% reduction at 5 mM relative to controls.

we randomly selected intact mice bearing tumors for placebo vs. isobutyramide treatment. During a 4 week period in intact-placebo mice (n = 5), tumor doubling time averaged 14 days (range 8–30 days), with a serum PSA doubling time of 8 days (range 6–14). Tumor volume and serum PSA increased 4.1- and 6.6-fold, respectively, in intact-placebo mice compared to twofold and 2.7-fold increases in tumor volume and serum PSA in intact-treated mice (Fig. 5).

For evaluatation of the ability of isobutyramide treatment to delay the time to androgen independence, mice bearing tumors between 100 and 200 mm^3 involume were castrated and randomly selected for placebo vs. adjuvant treatment with isobutyramide. In castrate-controls (n = 5), tumor volume increased an average of 2.4-fold, and serum PSA increased an average of fourfold above nadir levels over a 7 week observation period. In castrate-treated mice (n = 5), tumor volume decreased by 50%, and serum PSA levels increased by twofold above

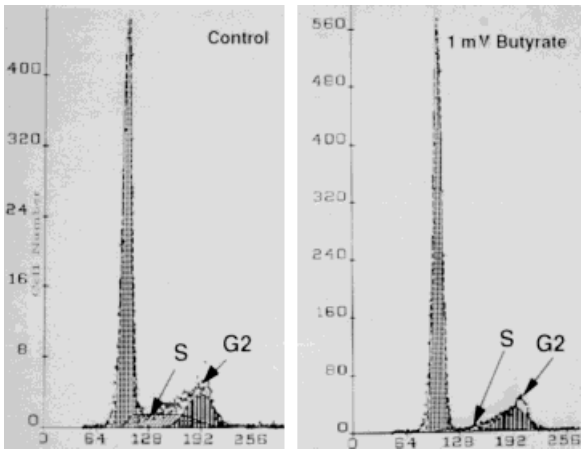

Fig. 4. DNA profile of LNCaP cells was analyzed after treatment with 1 mM sodium butyrate for the times indicated. Flow cytometry indicates G1 cell cycle arrest over a 48 h period, resulting in a 99% decrease in the fraction of cells in S phase.

nadir levels over a 7 week observation period (Fig. 6). After 3 weeks of therapy, however, mice develop reversible central nervous system (CNS) toxicity with quadraparesis that resolved spontaneously after interruption of therapy. In both groups, discontinuation of therapy is associated with tumor progression.

Changes in PSA Gene Expression in LNCaP Cells After Treatment With Butyrate In Vitro and Isobutyramide In Vivo

LNCaP cells were treated for 48 h with either fenretinide (4-HPR), DMSO, DMF, or sodium butyrate (Na-BT). Butyrate increases PSA mRNA levels fourfold in a concentration-dependent manner indicative of differentiation-enhanced expression of this differentiation marker (Fig. 7A). In contrast, DMSO and DMF decrease PSA expression in a concentration-dependent manner. Fenretinide does not significantly affect PSA gene expression.

Similar to butyrate-induced upregulation of PSA mRNA in vitro, isobutyamide increases PSA mRNA levels in LNCaP tumours in vivo two- to threefold (Fig. 7B). Isobutyramide increased levels of PSA mRNA in LNCaP tumors from intact male mice as well as in LNCaP tumors in castrated mice. The increased PSA mRNA expression following isobutyramide treatment likely increases circulating serum PSA levels, which may dissociate treatment-related changes in tumor volume and serum PSA levels.

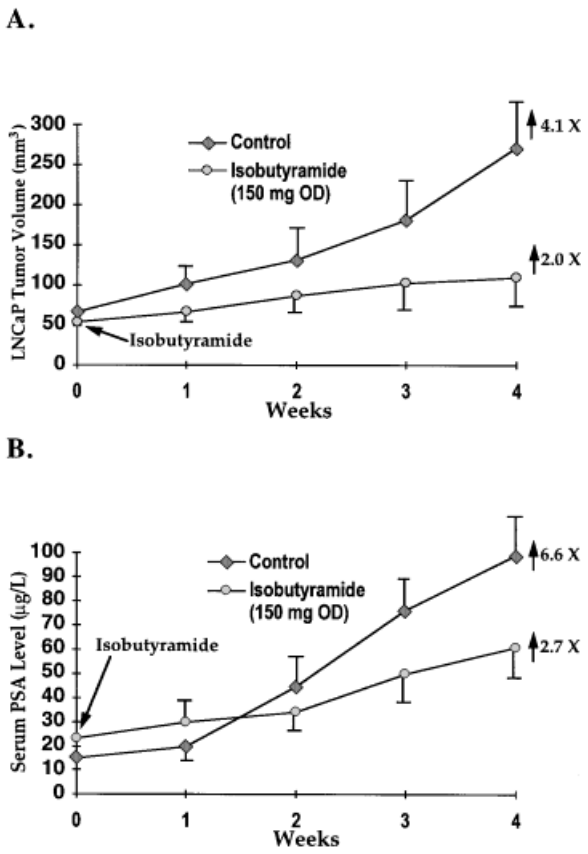

Fig. 5. Isobutyramide delays LNCaP tumor growth and PSA velocity in intact mice. Tumor volume and serum PSA increased 4.1- and 6.6-fold, respectively, in intact-placebo mice compared to twofold and 2.7-fold increases in tumor volume and serum PSA in intact-treated mice. Data points expressed as average tumor volumes ± SEM based on five determinations.

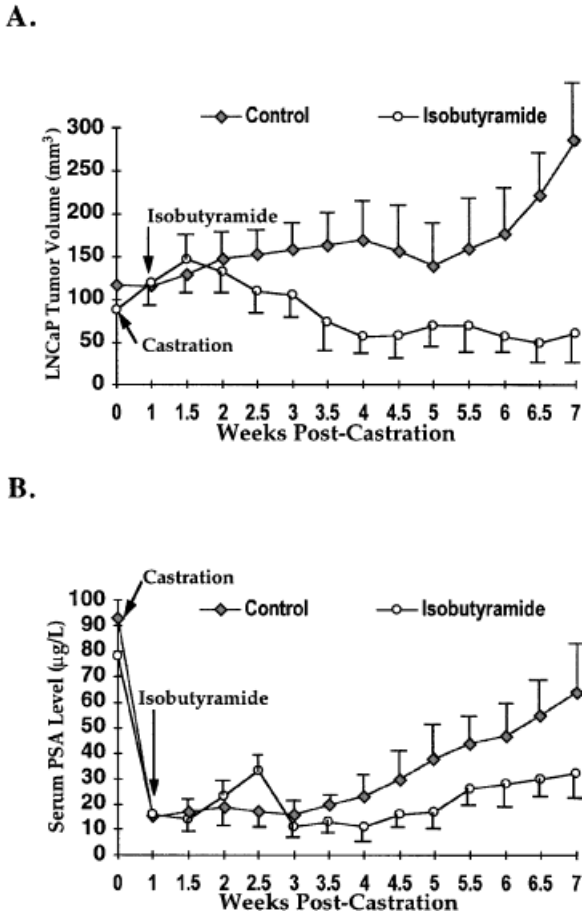

Fig. 6. Isobutyramide decreases LNCaP tumor growth and PSA velocity in castrate mice. Tumor volume and serum PSA levels increased 2.4-fold and fourfold above nadir levels in castrate-controls, while tumor volume decreased by 50% in castrate-treated mice, and serum PSA levels increased by twofold above nadir levels. Data points expressed as average tumor volumes ± standard errors of the mean based on five determinations.

DISCUSSION

Simply stated, most cancers can be considered disorders of differentiation with uncontrolled self-renewal of undifferentiated cells resulting in an imbalance between cell proliferation and programmed cell death. While normal cells are committed to terminal differentiation, neoplastic cells either do not differentiate or the link to terminal differentiation is blocked and programmed cell death does not occur [Waxman et al., 1986]. The induction of differentiation may serve as a basis for cancer chemoprevention and treatment and can be achieved with various agents, including DMSO, sodium butyrate, and retinoids. Broadly speaking, these agents alter cell growth and differentiation by modifying expression of genes or gene products that regulate cell shape, function, adhesion, communication, and growth. For example, DMSO modulates differentiation via various enzyme systems and cell surface and extracellular matrix protein synthesis [Higgins and O'Donnell, 1992; Dairkee and Glaser, 1982]. Retinoids are potent analogues of vitamin A that regulate the synthesis of cytokeratins, collagen, and glycoproteins [Dairkee and Glaser, 1982; Fuchs and Green, 1981]. Butyrate, a naturally occurring short chain fatty acid, changes chromosome structure, gene expression, and differentiation of numerous cell types [Norton et al., 1989; Candido et al., 1978; Nakagawa ete al., 1988]. Additionally, the expression of certain growth factors (e.g., transfroming growth factor-α [TGFα]), growth factor receptors (e.g., EGFR), and oncogenes (e.g., *myc*) that regulate malignant cell growth and behavior can be altered by differentiation agents [Watkins et al., 1988; Westin et al., 1982].

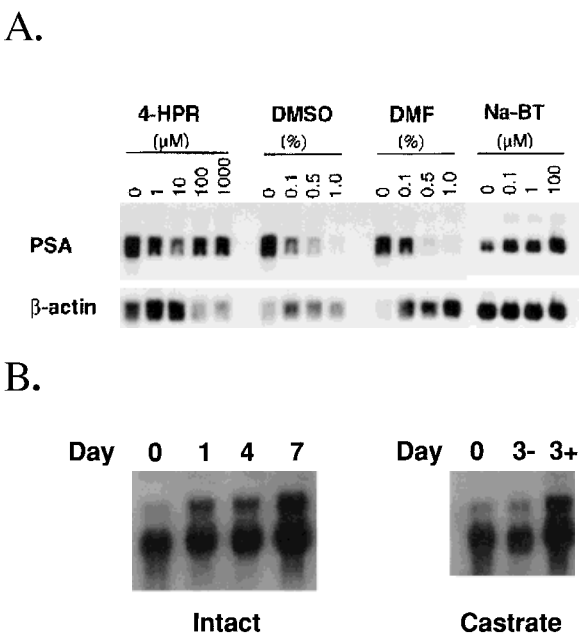

Fig. 7. **A:** Northern analysis was used to determine the effect of fenretinide, DMSO, dimethylformamide (DMF), and sodium butyrate on PSA mRNA levels in LNCaP cells in vitro. After 48 h, butyrate increases PSA mRNA levels fourfold in a concentration-dependent manner, compared to a 90% concentration-dependent reduction in PSA expression by DMSO and DMF. **B:** LNCaP tumors were harvested for Northern analysis after isobutyramide treatment from intact (1, 4, and 7 days posttreatment) and castrate (3+) mice and compared to untreated controls (0, 3-).

Although androgen ablation is the most effective therapy for patients with advanced prostate cancer, progression to androgen independence ultimately occurs and remains the main obstacle to improved survival and quality of life [Denis and Murphy, 1993]. Progression to androgen independence is a complex process involving both clonal selection and adaptive mechanisms occurring in heterogenous tumors composed of subpopulations of cells that respond differently to androgen ablation [Gleave, 1997]. Although clonal expansion cannot be prevented because of resistance to traditional cytotoxic chemotherapies, it may be possible to modulate the adaptive changes in gene expression precipitated by androgen ablation through the adjuvant use of differentiation agents. This hypothesis is indirectly supported by several lines of evidence. In general, tumor cells comprising androgen-independent prostatic tumors are phenotypically and behaviorally less differentiated compared to those in the original tumor prior to castration [Eisenberger et al., 1987]. Analysis of the DNA content of parent and recurrent Shionogi tumors demonstrated that androgen-independent tumors are composed of more poorly differentiated aneuploid cells [Bruchovsky et al., 1990]. Also, markers characteristic of differentiated prostatic cell function (PSA, acid phosphatase, androgen receptor) are less frequently expressed by androgen-independent recurrences compared to untreated prostate cancer [Svanholm and Hrder, 1988; Trachtenberg and Walsh, 1982]. Furthermore, BCL-2 is expressed in the less differentiated basal cell layer of prostatic acini but not in the differentiated luminal cell layer or androgen-dependent prostate cancer cells [McDonnell et al., 1992]. During progression to androgen independence, BCL-2 is upregulated and may play an active role in resistance to apoptosis. Finally, studies using intermittent androgen suppression suggest that modulation of gene expression with intermittent reexposure of tumor cells to testosterone helps maintain androgen dependence [Akakura et al., 1993; Sato et al., 1996], while progression of breast cancer models is delayed when antiestrogens were combined with fenretinide compared to either agent alone [Ratko et al., 1989]. A similar synergistic effect between androgen ablation and other active differentiation agents may exist in the treatment of prostate cancer.

The objectives of this study were to characterize the effects of the differentiation agent butyrate in LNCaP cells in vitro and to determine if tumor growth and androgen-independent progression of LNCaP tumors in vivo could be delayed by its orally bioavailable analogue, isobutyramide. Butyrate was used in these experiments because preliminary experiments identified it as the most potent agent affecting LNCaP cell growth in vitro [Gleave et al., 1996]. The major known pharmacological action of butyrate in vitro is inhibition of histone deacetylase, which leads to histone hyperacetylation and subsequent changes in chromatin structure [Candido et al., 1978]. However, a direct relationship between inhibition of histone deacetylase and induction of differentiation did not exist among a number of butyrate analogues [Lea and Tulsyan, 1995], suggesting that other mechanisms, including nonspecific effects on other enzymes, cytoskeleton, or cell membranes, are involved. The use of more specific inhibitors of histone deacetylase, however, confirmed the critical role of histone acetylation control on cell cycle progression, differentiation, and oncogene-mediated transformation

[Kijima et al., 1993]. Although butyrate is a potent inhibitor of tumor cell proliferation in vitro, its short half-life in vivo limits its use as a therapeutic agent. Other butyrate analogues have been evaluated to circumvent this problem, including phenylbutyrate [Figg et al., 1994; Dover et al., 1992] and isobutyramide. Isobutyramide is a branched-chain butyrate analogue investigated for its ability to induce differentiated hemoglobin expression in sickle cell hemoglobinopathies and is tolerated at millimolar concentrations in human phase I studies [Perrine et al., 1994].

Although PSA levels and LNCaP tumor growth rates decrease after castration, the effects are temporary, and progression to androgen-independent growth and PSA gene expression occurs after 4 weeks. In this study, tumor growth and PSA doubling time were faster in LNCaP tumors in intact compared to castrated mice. Tumor regression rarely occurred following castration, but tumor growth rates were slower in castrate-untreated compared to intact-treated mice. Tumor growth and PSA velocity in intact mice were reduced by isobutyramide, but tumors continued to grow slowly. Only tumors in mice treated by both castration and isobutyramide consistently regressed and decreased in size over the treatment period, suggesting a synergistic effect between androgen ablation and isobutyramide.

Conceptually, differentiation agents should inhibit prostate tumor cell proliferation and enhance PSA gene expression. Indeed, our evidence suggests that butyrate and isobutyramide exhibit this dual functionality. Induction of differentiation was marked by morphological changes towards a more differentiated phenotype, loss of defined nucleoli and dispersion of nuclear chromatin, decreased growth rates, and G1 cell cycle arrest. Furthermore, the dose-dependent increases in PSA gene expression induced by butyrate is consistent with a differentiation effect. Because PSA is a species-specific, a prostate-specific, and a secretory glycoprotein, it may serve as a differentiation marker. In man, the amount of PSA expressed in a prostate cell is a function of the cell's state of differentiation, most abundantly expressed in normal or benign acinar cells, intermediate in well-differentiated cancers, and lower in poorly differentiated cancers [Oesterling, 1991]. In contrast, serum PSA levels in patients with advanced poorly differentiated cancers are usually higher because circulating serum PSA levels are determined by both tumor volume and PSA gene expression. Indeed, PSA is the most useful and versatile tumor marker in all oncology. The LNCaP tumor model mimics the clinical disease in that serum PSA levels are directly proportional to tumor volume and increase as tumor volume increases [Gleave et al., 1992]. However, after isobutyramide treatment this direct relationship is lost in both intact and castrated mice because serum PSA levels continue to increase, albeit at a slower rate than controls, despite stabilized or decreased tumor growth rates. Isobutyramide-induced decreases in tumor cell proliferation and increases in PSA gene expression result in disconcordant changes in serum PSA and tumor volume, and therefore impair the utility of serum PSA as a marker of response to therapy. Taken together, interpretation of response to treatment with isobutyramide must be done using both serum protein and tumor mRNA levels in addition to changes in tumor volume.

Androgens and butyrate are the only compounds identified thus far that increase PSA gene expression. In contrast to androgen, however, butyrate results in cell cycle arrest and decreased cell proliferation. Androgens are both potent differentiation agents and mitogens for prostate cells. Without androgens, the prostate gland does not form in the embryo and does not function in the adult. Furthermore, the ability of benign or malignant prostate cells to undergo apoptosis and express PSA is acquired as a feature of differentiation of prostatic cells under the influence of androgens. Androgens function as differentiation factors that modulate gene expression through binding to intracellular androgen receptors (AR) that function as transacting transcription factors. Expression of PSA is androgen-regulated via androgen response elements (ARE) in its 5× regulatory region [Riegman et al., 1991]. The regulation of PSA and many other key genes are altered during the course of prostate cancer progression to androgen independence, no longer requiring the presence of androgens for expression. It is not clear if this is a direct consequence of changes in androgen receptor function, but it is likely that key changes in transacting factors precipitates the activation of this promoter during tumor progression.

Interestingly, differentiation agents vary in their effect on PSA expression. We found that

PSA mRNA expression is downregulated fivefold by DMSO and DMF but is upregulated by butyrate in vitro and by isobutyramide in vivo. A similar pattern of DMSO-induced suppression and butyrate-induced enhancement has been reported in fetal globin gene expression in erythroid progenitor cells, where butyrate alters gene expression through specific regulatory DNA sequences [Glauber et al., 1991]. Butyrate may alter PSA gene expression either through direct binding to its 5× upstream region, via upregulation of other transciption factors like AP-1 [Karin, 1995], or by protein-protein interactions with AR or other transcription factors. More in-depth mechanistic studies, such as genomic footprinting of the PSA promoter and regulatory analysis, are necessary to study the complex effects of these pleiotropic agents.

In summary, the results from this study suggest that the differentiation agent isobutyramide can modify the biology of human prostate cancer at noncytotoxic doses. An important goal of future therapy for metastatic prostate cancer is stabilization or reduction of tumor volume using treatments that inhibit tumor progression after androgen ablation, which was accomplished in this study using the LNCaP model. In humans, isobutyramide is tolerated at mM concentrations without significant side effects, where its half-life is more than twice that measured in mice [Perrine et al., 1994]. The toxicity that was observed in this murine model was reversible and likely developed because of higher and more frequent dosing necessary to maintain plasma levels of 1 mM. Discontinuation of therapy is associated with tumor progression, and therefore continuous or combination therapy is likely necessary to prevent tumor progression. Due to the heterogenous and slowly progressive nature of prostate cancer, it is likely that development and utilization of multiple therapeutic modalities will be necessary to impact disease mortality.

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REFERENCES

Akakura K, Bruchovsky N, Goldenberg SL, Rennie PS, Buckley AR, Sullivan LD (1993): Effects of intermittent androgen suppression on androgen-dependent tumours: Apoptosis and serum prostate specific antigen. Cancer 71:2782–2790.

Bruchovsky N, Rennie PS, Coldman AJ, Goldenberg SL, To M, Lawson D (1990): Effects of androgen withdrawal on the stem cell composition of the Shionogi carcinoma. Cancer Res 50:2275–2282.

Candido EPM, Reeves R, Davie JR (1978) Sodium butyrate inhibits histone acetylation in cultured cells. Cell 14:105–113.

Carvalho L, Foulkes K, Mickey DD (1989): Effect of DMSO and DFMO on rat prostate tumor growth. Prostate 15: 123–133.

Dahiya R, Zhang DY, Ho RJ, PC Haughney, Hayward SW, Cunha GR, Narayan P (1995) Regression of LNCaP human prostate tumr xenografts in athymic nude mice by 13-cis-retinoic acid and androgen ablation. Biochem Mol Biol Int 35:487–498.

Dairkee SH, Glaser DA (1982): Dimethyl sulfoxide affects colony morphology on agar and alters distribution of glycosaminoglycans and fibronectin. Proc Natl Acad Sci U S A 79:6927–6931.

Denis L, Murphy GP (1993): Overview of phase III trials on combined androgen treatment in patients with metastatic prostate cancer. Cancer 72(Suppl):3888–3895.

Dover GJ, Brusilow S, Samid D (1992): Increased fetal hemoglobin in patients receiving sodium 4-phenylbutyrate. N Engl J Med 327:569–570.

Eisenberger MA, Bezerdjian L, Kalash S (1987): A critical assessment of the role of chemotherapy for endocrine-resistant prostatic carcinoma. Urol. Clin. North Am. 14: 695–706.

Figg WD, Walls RG, Cooper MR, Thibault A, Sartor O, McCall NA, Myers CE, Samid D (1994): In vitro antitumor effect of hydroxyurea on hormone-refractory prostate cancer cells and its potentiation by phenylybutyrate. Anticancer Drugs 5:336–342.

Fuchs E, Green H (1981): Regulation of terminal differentiation of cultured human keratinocytes by vitamin A. Cell 25:617–625.

Glauber JG, Wandersee NJ, Little JA, Ginder GD (1991): 5× flanking sequences mediate butyrate stimulation of embryonic globin gene expression in adult erythroid cells. Mol Cell Biol 11:4690–4697.

Gleave ME, Hsieh JT, Gao C, von Eschenbach AC, Chung LWK (1991): Acceleration of human prostate carcinoma growth in vivo by factors produced by prostate and bone fibroblasts. Cancer Res 51:3753–3761.

Gleave ME, Hsieh JT, Wu H-C, Chung LWK (1992): Serum PSA levels in mice bearing human prostate LNCaP tumors are determined by tumor volume and endocrine and growth factors. Cancer Res 52:1598–1605.

Gleave ME, Sato N, Rennie P, Bruchovsky N (1996): Prevention of LNCaP prostate tumour growth and progression by isobutyramide, and orally available butyrate analogue. J Urol 155:515A.

Gleave ME (1997): Experimental animal models for prostate cancer. In Lange P, Scher H, Ragavahn O (eds): "Genitourinary Oncology." New York: Lippencott, pp. 367–378.

Higgins PJ, O'Donnell PV (1982): Dimethylsulfoxide-induced alterations in the growth properties and protein composition of in vitro–propagated murine hepatoma cells. Oncology 39:325–330.

Horoszewicz J Leong S, Chu T, Wajsman Z, Freedman M, Papsidero L, Kim U, Chai LS, Kakati S, Arya SK, Sandberg AA (1980): The LNCaP cell line—a new model for studies on human prostatic carcinoma. Prog Clin Biol Res 37:115–132.

Hsieh JT, Wu H-C, Gleave ME, von Eschenbach AC, Chung LWK (1993): Autocrine regulation of prostate-specific antigen gene expression in human prostate cells. Cancer Res 53:2852–2857.

Kaneski CR, Constantopoulos G, Brady RO (1991): Effect of DMSO on the proliferation and glycosaminoglycan synthesis of rat prostate adenocarcinoma cells (PAIII) in vitro: solation and characterization of DMSO-resistant cells. Prostate 18:47–58.

Karin M (1995): The regulation of AP-1 activity by mitogen-activated protein kinases. J Biol Chem 270:16483–16486.

Kijima M, Yoshida M, Sugita K, Horinouchi S, Beppu T (1993): Trapoxin, an antitumor cyclic tetrapeptide, is an irreversible inhibitor of mammalian histone deacetylase. J Biol Chem 268:22429–22435.

Lea MA, Tulsyan N (1995): Disconcordant effects of butyrate analogues on erythroleukemia cell proliferation, differentiation, and histone deacetylase. Anticancer Res 15:879–884.

Lundwall Å, Lilja H (1987): Molecular cloning of human prostate specific antigen cDNA. FEBS Lett. 214:317–322.

McDonnell T, Troncoso P, Brisbay M, Logothetis C, Chung L, Hsieh JT, Tu SM, Campbell ML (1992): Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. Cancer Res 52:6940–6944.

Nakagawa T, Nelkin BD, Baylin SB, deBustros A (1988): Transcriptional and posttransciptional modulation of calcitonin gene expression by sodium *n*-butyrate in cultured human medullary thyroid carcinoma. Cancer Res 48: 2096–2100.

Norton VG, Imai BS, Yau P, Bradbury EM (1989): Histone acetylation reduces nucleosome core particle linking number change. Cell 57:449–457.

Oesterling JE (1991): Prostate specific antigen: A critical assessment of the most useful tumor marker for adenocarcinoma of the prostate. J Urol 145:907–923.

Perrine SP, Dover GH, Daftari P, Walsh CT, Jin Y, Mays A, Faller DV (1994): Isobutyramide, an orally bioavailable butyrate analogue, stimulates fetal globin bene expression in vitro and in vivo. Br J Hematol 88:555–561.

Pollard M, Luckert PH, Sporn MB (1991): Prevention of primary prostate cancer in Lobund-Wistar rats by N-(4-hydroxyphenyl) retinamide. Cancer Res 51:3610–3611.

Ratko TA, Detrisac CJ, Dinger NM, Thomas CF, Kelloff GJ, Moon RC (1989): Chemopreventive efficacy of combined retinoid and tamoxifen treatment following surgical excision of a primary mammary cancer in female rats. Cancer Res 49:4472–4476.

Riegman PHJ, Vlietstra RJ, van der Korput JAGM, Brinkmann AO, Trapman J (1991): The promoter of the prostate-specific antigen gene contains a functional androgen responsive element. Mol Endocrinol 5:1921–1930.

Samid D, Shack S, Myers CE (1993): Selective growth arrest and phenotypic reversion of prostate cancer cells in vitro by nontoxic pharmacologic concentrations of phenylacetate. J Clin Invest 91:2288–2295.

Sato N, Gleave ME, Goldenberg SL, Bruchovsky N, Rennie P, Sullivan LD (1996): Intermittent androgen suppression delays time to androgen-indepenent progression in the LNCaP prostate tumour model. J. Steroid Biochem Mol Biol 58:139–146.

Sellins K, Cohen J (1997): Gene induction by gamma-irradiation leads to DNA fragmentation in lymphocytes. J Immunol 39:3199–3206.

Slawin K, Kadmon D, Park SH, Scardino PT, Anzano M, Sporn MB, Thompson TC (1991): Dietary fenretinide, a syntheitic retinoid, decreases the tumor incidence and the tumor mass of ras+myc-induced carcinomas in the mouse prostate reconstitution model system. Cancer Res 53:4461–4465.

Svanholm H, Hrder M (1988): Clinical application of prostatic markers: I. Classification of prostatic tumors using immunohistochemical techniques. Scand. J. Urol. Nephrol. 107(Suppl):65–70.

Trachtenberg J, Walsh PC (1982): Correlation of prostatic nuclear androgen receptor content with duration of response and survival following hormonal therapy in prostatic cancer. J Urol 127:466–471.

Watkins LF, Brattain MG, Levine AE (1988): Modulation of a high molecular weight form of transforming growth factor-alpha in human colon carcinoma cell lines. Cancer Lett 40:59–70.

Waxman S, Scher W, Scher BM (1986): Basic principles for utilizing combination differentiation agents. Cancer Detect Prev 9:395–407.

Westin EH, Wong-Staal F, Gelmann EP, Dalla Favera R, Papas TS, Lautenberger JA, Eva A, Reddy EP, Tronick SR, Aaronson SA, Gallo RC (1982): Expression of cellular homologues of retroviral oncogenes in human hematopoietic cells. Proc Natl Acad Sci U S A 79:2490–2494.