

# Antagonistic Interaction Between Bicalutamide<sup>TM</sup> (Casodex<sup>®</sup>) and Radiation in Androgen-Positive Prostate Cancer LNCaP Cells

Laurent Quéro,<sup>1,2,3</sup> Nicole Giocanti,<sup>1,2</sup> Christophe Hennequin,<sup>1,2,3</sup> and Vincent Favaudon<sup>1,2\*</sup>

<sup>1</sup>Institut Curie, Bât. 110-112, Centre Universitaire, Orsay, France

<sup>2</sup>INSERM, U612, Bât. 110-112, Centre Universitaire, Orsay, France

<sup>3</sup>Hôpital Saint Louis, Assistance Publique-Hôpitaux de Paris, Paris, France

**BACKGROUND.** Bicalutamide<sup>TM</sup> (Casodex<sup>®</sup>) reportedly improves high-risk prostate cancer survival as an adjuvant treatment following radiotherapy. However, biological data for the interaction between bicalutamide and ionizing radiation in concomitant association are lacking. **METHODS.** To explore this issue, androgen-dependent (LNCaP) and -independent (DU145) human prostate cancer cells were exposed for 48 hr to 20, 40, or 80  $\mu$ M bicalutamide introduced before (neoadjuvant), during (concomitant), or following (adjuvant) radiation. Growth inhibition and cytotoxicity, cell cycle distribution and expression of the prostate serum antigen (PSA) and androgen receptor (AR), were measured as endpoints.

**RESULTS.** Bicalutamide-induced cytotoxic and cytostatic effects were found to be correlated with a marked G1 phase arrest and S phase depression. The drug down-regulated PSA and AR proteins and *psa* mRNA in LNCaP cells. However, transient up-regulation of the expression of *ar* mRNA was observed in the presence of 40  $\mu$ M drug. DU145 cells did not express PSA and proved to be comparatively resistant to the drug from both cytostatic and cytotoxic effects. Bicalutamide dose-dependently induced a significant decrease of radiation susceptibility among drug survivors in LNCaP cells, whilst the interaction appeared to be additive in DU145 cells.

**CONCLUSIONS.** The antagonistic radiation–drug interaction observed in LNCaP cells is of significance in relation to combined radiotherapy–bicalutamide treatments directed against tumors expressing the AR. The results suggest that bicalutamide is amenable to combined schedule with radiotherapy provided the drug and radiation are not given in close temporal proximity. *Prostate* © 2009 Wiley-Liss, Inc.

**KEY WORDS:** prostate; radiotherapy; bicalutamide; Casodex; association; antagonism

## INTRODUCTION

The androgenic hormones, testosterone and dihydrotestosterone exert their cellular effects by binding with the androgen receptor (AR), a member of the family of intracellular steroid hormone receptors that function as ligand-dependent transcription factors [1]. The AR signaling pathways play a central role in prostate cancer growth and development. Indeed, the majority of newly diagnosed prostate cancers are initially hormone-dependent and respond to androgen deprivation therapies [2]. Based on this observation, and in spite of the fact that advanced prostate carcinomas tend to become resistant to hormone

Abbreviations: AR, androgen receptor; BCLT, bicalutamide; DMSO, dimethyl sulphoxide; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; LH-RH, luteinizing hormone-releasing hormone; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PSA, prostate specific antigen.

Grant sponsor: AstraZeneca; Grant number: CCI419; Grant sponsor: Institut National de la Santé et de la Recherche Médicale; Grant sponsor: Institut Curie.

\*Correspondence to: Vincent Favaudon, Inserm U612, Institut Curie-Recherche, Bat. 110-112, Centre Universitaire, 91405 Orsay, France. E-mail: vincent.favaudon@curie.u-psud.fr

Received 16 April 2009; Accepted 11 September 2009

DOI 10.1002/pros.21074

Published online in Wiley InterScience

(www.interscience.wiley.com).

suppression after 18–24 months [3], androgen deprivation is established as a routine prostate cancer therapy.

Androgen deprivation for advanced or metastatic prostate cancer relies either on (i) surgical castration (bilateral orchidectomy), (ii) chemical castration by synthetic analogs of gonadotropin-releasing hormone (Buserelin acetate, Goserelin acetate, Leuprolin/Leuprorelin acetate, Triptorelin acetate) which act as agonists of luteinizing hormone-releasing hormone (LH–RH), (iii) non-steroidal androgen antagonist monotherapy, or (iv) total androgen blockade by castration or LH–RH agonists plus androgen antagonist treatment.

The combination of radiotherapy with 2–3 years of androgen deprivation by LH–RH agonists is currently the recommended treatment of high risk, locally advanced prostate adenocarcinoma. Four randomized trials have clearly shown a specific survival benefit of such an association compared with radiotherapy alone. (i) The RTOG 85-31 phase III trial compared radiotherapy alone with radiotherapy and Goserelin acetate (Zoladex<sup>®</sup>), a LH–RH agonist. In the combination treatment arm, Goserelin acetate was started during the last week of radiotherapy and administered indefinitely or until the sign of disease progression. This trial demonstrated a decrease of the specific mortality at 10 years from 22% to 16% ( $P=0.0052$ ) in favor of the radiotherapy–hormone therapy combination [4]. (ii) The EORTC 22863 trial demonstrated a 15% absolute specific survival advantage at 5 years in favor of radiotherapy–hormone therapy association compared with radiotherapy alone (94% vs. 79%,  $P=0.0001$ ) [5]. In this trial, androgen deprivation therapy consisted of a total androgen blockade with a steroidal anti-androgen (Cyproterone acetate) and a LH–RH agonist (Goserelin acetate) for 1 month followed by chemical castration by the LH–RH agonist alone (Goserelin acetate) for 3 years. Androgen deprivation therapy started on the first day of radiotherapy in the combination treatment arm. (iii) In the RTOG 86-10 randomized trial, androgen deprivation therapy began 2 months before the start of radiotherapy and continued until the completion of radiotherapy. Androgen deprivation therapy consisted of a total androgen blockade with a non-steroidal androgen antagonist (flutamide) and a LH–RH agonist (Goserelin acetate). In this study, specific mortality also decreased in the combined treatment arm with a 10 years absolute benefit of 8% (77% vs. 64%,  $P=0.01$ ) [6]. In a retrospective analysis, Janoff et al. [7] confirmed that androgen deprivation as the sole therapy for locally advanced prostate cancer, failed to provide as efficient disease control as combined treatments. (iv) This observation was recently confirmed by

the Scandinavian SPCG-7/SFUO-3 phase III trial that showed a benefit in overall survival in favor of radiotherapy–hormone therapy association compared with hormone therapy alone (78.5% vs. 70% at 10 years) [8].

Bicalutamide<sup>™</sup> (BCLT), also known as Casodex<sup>®</sup>, as well as Flutamide<sup>™</sup> and Nilutamide<sup>™</sup>, is a non-steroidal competitive androgen receptor antagonist able to block androgen-regulated prostate cell growth. In combination with a LH–RH agonist analog, BCLT has been shown to result in a longer median survival than Flutamide<sup>™</sup>, with better tolerance [9]. BCLT can also be used at low doses in the treatment of metastatic prostate cancer in combination with an LH–RH agonist providing a complete androgen blockade. Compared with castration, BCLT provides better maintenance of physical capacity and sexual interest. BCLT also decreases the risk of fracture by maintaining bone mineral density, which is lost with castration by LH–RH analogs [10–12]. Recently, a phase III study (EPC) demonstrated, after a median follow-up of 7.2 years, an improved overall survival of adjuvant high-dose BCLT following radiotherapy compared to radiotherapy alone (69.6% versus 57.6%,  $P=0.03$ ) in a subgroup of patients with locally advanced prostate cancer [13,14]. The gain in survival observed in this study is comparable to the benefit from androgen deprivation by a LH–RH agonist in association with radiotherapy in the RTOG 85-31 study for high-risk prostate cancer [4]. Whether concomitant treatment with BCLT and radiotherapy results in an enhanced radiosensitivity of tumor cells responding to BCLT, is still open to question. In this article we evaluate the outcome of neoadjuvant, concomitant, and adjuvant treatment with BCLT, and radiation in prostate cancer cells expressing (LNCaP) or not (DU145, PC3) a functional form of the androgen receptor with cell survival, proliferation, cell cycle distribution, and AR or prostate serum antigen (PSA) expression as endpoints.

## MATERIALS AND METHODS

### Cell Lines

LNCaP (human prostate carcinoma, ATCC CRL-1740; wild-type p53), DU145 (human prostate carcinoma, ATCC HTB-81; p53 mutated), PC3 (human prostate carcinoma, ATCC CRL-1435; p53 mutated) and MRC5 cells (normal human fibroblasts, ATCC CCL-171; wild-type p53) were grown as monolayers in RPMI 1640 medium supplemented with 10% fetal bovine serum, 10 mM HEPES, 1 mM sodium pyruvate, 100 IU/ml penicillin, and 100 µg/ml streptomycin, at 37°C in a humidified, 5% CO<sub>2</sub> atmosphere. All products for cell culture were from Invitrogen (Cergy-Pontoise,

France). LNCaP are androgen-sensitive cells; they express endogenous PSA and a mutant, functional form of the AR (missense mutation in the ligand-binding domain). DU 145 and PC3 do not express the AR and are androgen-independent [15,16].

### Treatments With Bicalutamide and/or Radiation

BCLT (Bicalutamide<sup>TM</sup>, Casodex<sup>®</sup>) was kindly provided by AstraZeneca (Macclesfield, Cheshire, UK) as a micronised powder. Aliquots (10 mM) were prepared in pure, sterile dimethyl sulphoxide (DMSO) and stored at  $-20^{\circ}\text{C}$ . Dilutions were made daily in growth medium. The final concentration of DMSO (0.26%) was found not to alter cell growth or radiation responses.

Cells were exposed either to 20, 40, or 80  $\mu\text{M}$  BCLT or to DMSO alone at the same concentration in medium (0.26%), usually for a total of 48 hr. These concentrations are in the same range as those measured in the serum of patients treated for prostate cancer either alone or in combination with LH–RH agonists [11].

Irradiation with or without concomitant exposure to BCLT was performed at room temperature in medium equilibrated with 5%  $\text{CO}_2$  in air, using an IBL-637 ( $^{137}\text{Cs}$ )  $\gamma$ -ray irradiator (CIS-Bio International, Saclay, France) at a dose rate of 1.0 Gy/min. Either one of the following time schedules was used in combined treatment with BCLT. In neoadjuvant assays, cells were incubated with BCLT for 48 hr; the drug was removed immediately before irradiation. In concomitant assays, irradiation was performed 24 hr after the start of drug contact; cells were incubated with drug for a further 24 hr. In adjuvant treatment, the drug was introduced immediately after irradiation and was present for 48 hr.

The cell growth or colony count relative to mock-irradiated cells ( $S$ ) was adjusted for best fit to an exponential (Eq. 1a or 1b) or to the classical linear-quadratic equation (Eq. 2),

$$S = e^{-\alpha D} \quad (1a)$$

$$S = (1 - S_{\infty})e^{-\alpha D} + S_{\infty} \quad (1b)$$

$$S = e^{-\alpha D - \beta D^2} \quad (2)$$

where  $D$  is the radiation dose, and  $\alpha$  and  $\beta$  adjustable parameters characterizing radiation sensitivity.  $S_{\infty}$  (Eq. 1b) is a parameter that takes into account the existence of a plateau at high radiation doses, as frequently observed in cells that undergo radio-induced senescence.

### Growth Inhibition and Clonogenic Assays

Growth inhibition by radiation (up to 8 Gy), BCLT (up to 80  $\mu\text{M}$ ) or a combination of both, was assessed by

manual cell count. Flasks ( $25\text{ cm}^2$ ) were seeded with  $2.5 \times 10^5$  LNCaP,  $1 \times 10^5$  DU 145,  $0.75 \times 10^5$  PC3, or  $2 \times 10^5$  MRC5 cells and incubated for 24 hr prior to neoadjuvant, concomitant, or adjuvant assays.

A clonogenic assay was used for the determination of the cytotoxic response to drug and radiation. Briefly,  $25\text{ cm}^2$  flasks were seeded with  $8 \times 10^2$  (DU145) or  $1.5 \times 10^4$  cells (LNCaP) yielding ca. 200 colonies in untreated controls. Cells were returned to the incubator for 4 (DU145) or 16 hr (LNCaP), then exposed to BCLT or vehicle (DMSO, 0.26%) preceding or following irradiation. At the end of treatments, the flasks were rinsed twice with Hank's balanced salt solution, and cells returned to normal growth medium. For growth assays, cells were incubated for 5 (PC3, MRC5), 6 (DU145), or 8 days (LNCaP) corresponding to three cell generations, harvested with trypsin, collected by centrifugation, resuspended in medium and counted in a Malassez cuvette. For colony formation assays, cells were incubated for 8 (DU145) or 20 days (LNCaP) before ethanol fixation and coloration. Colonies were scored manually under a magnifying lens.

Each measurement was performed in duplicate (growth inhibition) or triplicate (clonogenic assay). Calculations were made through non-linear least-squares regression taking all data points into account, using Kaleidagraph software (Synergy Software, Reading, Pennsylvania). Statistical analysis was performed using StatEL (AD Science, Paris, France, www.adscience.eu), a software operating on the spreadsheet of Microsoft Excel (Microsoft, Redmond, Washington).

### Cell Cycle Analysis

Cell cycle progression was monitored by dual-parameter flow cytometry using a FACStar PLUS cytofluorometer (Becton-Dickinson Biosciences, Le Pont de Claix, France). Cells were grown for 2 days without or with BCLT at the appropriate concentration; irradiation was eventually performed after 24 hr contact with drug. Twenty minutes before harvest, cells were exposed to 5-bromo-2'-deoxyuridine (10  $\mu\text{M}$ , 10 min) for pulse labeling of S-phase cells. Cells were pooled after trypsinisation, and fixed in 70% ice-cold ethanol. Treatment of fixed cells, data acquisition and processing were performed as described [17]. Cell cycle analysis was performed with CellQuest Pro software (Becton-Dickinson Biosciences).

### Western Blotting

Total extracts were made from ca.  $3 \times 10^6$  cells using M-PER reagent (Pierce, Perbio-Sciences, Brebières, France) containing protease and phosphatase inhibitors (Sigma–Aldrich Chemicals, Saint Quentin

Fallavier, France). Proteins were titrated by the Bradford method using the Bio-Rad protein assay (Bio-Rad, Marnes-la-Coquette, France). Cell extracts were boiled in Laemmli loading buffer and separated on 7.5% (AR) or 12.5% (PSA) sodium dodecyl sulfate polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) and blocked for 1 hr in 5% bovine serum albumin in 10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.5 buffer (TBST) at 37°C. Membranes were subsequently incubated with primary monoclonal antibodies overnight at 4°C in TBST buffer, washed for 1 hr, and incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson Immuno-Research Laboratories, Soham, Cambridgeshire, UK) in TBST buffer and revealed with an enhanced chemiluminescence detection kit (GE Healthcare Life Sciences, Orsay, France). Nitrocellulose membranes were rehybridized with anti- $\alpha$ -tubulin antibody (Sigma-Aldrich Chemicals).

#### Determination of Apoptosis

Apoptosis was determined using an Annexin V assay.  $10^6$  cells were suspended in 1 ml ice-cold binding buffer (10 mM HEPES, 150 mM NaCl, 2.5 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 4% BSA, pH 7.4). Ten microliters of media-binding buffer and 1.25  $\mu\text{l}$  of a 200  $\mu\text{g}/\text{ml}$  solution of Annexin V-FITC (Oncogene Research Products, San Diego, California) were added to 500  $\mu\text{l}$  of the cell suspension for 15 min at room temperature, in the dark. Cells were harvested by centrifugation and resuspended in 0.5 ml ice-cold binding buffer. Propidium iodide was subsequently added to a final concentration of 30  $\mu\text{g}/\text{ml}$  and parallel analysis of both PI and FITC fluorescence was carried out with CellQuest Pro software (Becton-Dickinson Biosciences) as described [18]. Cells that bound Annexin V-FITC without propidium iodide staining were considered as early apoptotic cells; necrotic or apoptotic cells in terminal stages were positive for both annexin V-FITC and propidium iodide.

#### $\beta$ -Galactosidase Staining and Determination of Senescent Cells

For the determination of the amount of senescence induced by treatments, LNCaP or DU145 cells were exposed to 80  $\mu\text{M}$  BCLT (48 hr contact), 3 Gy (LNCaP), or 8 Gy radiation (DU145) or a combination of both treatments. The cultures were subsequently allowed to grow for 5 days in drug-free medium, at which time the medium was removed by aspiration. The flasks were washed with PBS, and cells were fixed sequentially by 20% formaldehyde followed by 2% glutaraldehyde in PBS. After three PBS washes the samples were

incubated (37°C) with 1 mg/ml 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside using a  $\beta$ -galactosidase staining kit (Cell Signaling Technology, Danvers, Massachusetts) according to manufacturer's recommendations.  $\beta$ -galactosidase staining was visualized by microscope examination.

#### Real-Time Quantitative PCR

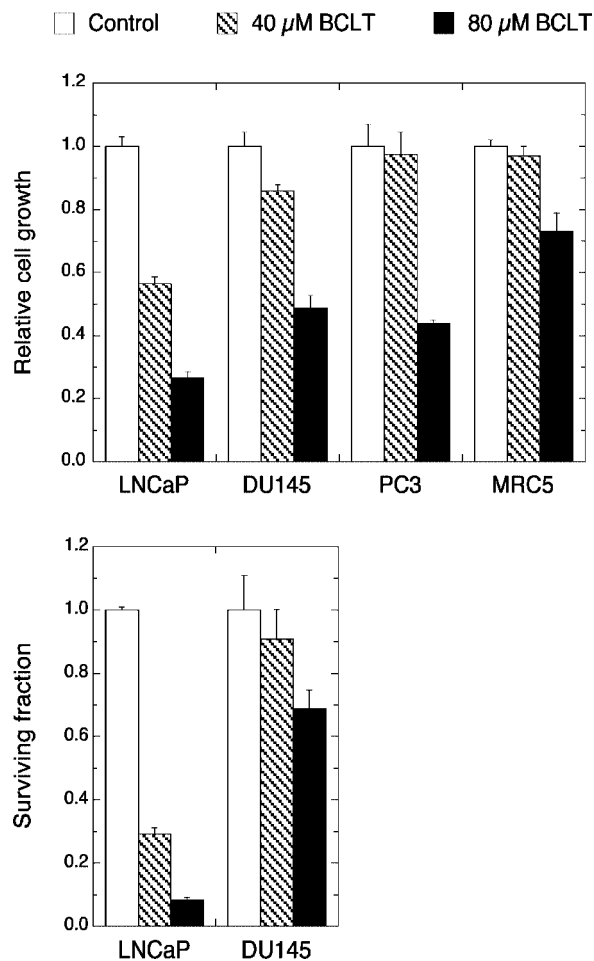
Total RNA was extracted from treated or non-treated cells with NucleoSpin RNA II kit (Macherey-Nagel EURL, Hoerd, France). First-strand cDNA was generated from 1  $\mu\text{g}$  total RNA with i-Script cDNA synthesis kit (Bio-Rad), and real-time PCR was carried out with the i-Cycler iQ System (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad) according to manufacturer's instructions. Twenty nanograms of cDNA product were used as a template in a 25  $\mu\text{l}$  PCR reaction containing 12.5  $\mu\text{l}$  of SYBR mixture (iQ<sup>TM</sup> SYBR Green Supermix, Bio-Rad), 300 nM of each primer, and nuclease-free water. The sequence of AR, PSA, and GAPDH primers were designed with OligoAnalyzer 1.0.2 (Kuopio University, Finland) based on human AR, PSA, and GAPDH mRNA sequences, respectively, as follows: AR forward 5'-GAGACTGCCAGGGACCAT-3' and reverse 5'-CCATAGTGACACCCAGAAGC-3', PSA forward 5'-AGTTTCCCTTCTCCCAGT-3' and reverse 5'-GCCCAGACCATATCTTGAG-3', GAPDH forward 5'-CCTCCCGCTTCGCTCTCT-3' and reverse 5'-CGACCTTACCTTCCCCAT-3'. Two measurements were performed, all in triplicate. Up- and down-regulation of AR and PSA were determined by the  $2^{-\Delta\Delta C_t}$  method [19] and normalized relative to *GAPDH* expression in each sample.

## RESULTS

### Cytostatic and Cytotoxic Effects of BCLT

To determine the effect of BCLT on the proliferation of prostate cancer cells, androgen-dependent (LNCaP) and -independent (DU145, PC3, MRC5) cells were exposed to 40 or 80  $\mu\text{M}$  BCLT. The growth of LNCaP cells was reduced by 40% and 75% after treatment with 40 and 80  $\mu\text{M}$  BCLT, respectively (Fig. 1). BCLT (40  $\mu\text{M}$ ) did not affect the growth of DU145 and PC3 cells to a significant extent. However, at the highest drug concentration used (80  $\mu\text{M}$ ) the growth of DU145 and PC3 cells was reduced by 40% and 50%, respectively. MRC5 fibroblasts proved to be even more resistant to the cytostatic effect of BCLT.

Only LNCaP and DU145 cells were investigated in subsequent assays. The cytotoxic potential of BCLT against these cells was determined using a clonogenic assay. BCLT elicited a cytotoxic effect with a concentration-dependent profile similar to that obtained in the



**Fig. 1.** Drug dose-dependence of the loss of the growth or clonogenic potential by BCLT. Cells were seeded at a suitable density (see Materials and Methods Section) and incubated with BCLT (0.26% DMSO in culture medium) for 48 hr. After drug removal cells were allowed either to form colonies (bottom) or to grow for three doubling times (top) before trypsinization and counting in an hemocytometer. Bars, mean deviation (duplicate experiments).

growth assay (Fig. 1). However, the differential response between LNCaP and DU145 cells was more pronounced, and very few LNCaP cells survived 80 μM BCLT.

#### Cytostatic and Cytotoxic Effects in Combined Treatment With BCLT and Radiation

The effect of the neoadjuvant, concomitant, and adjuvant treatment with radiation and 40 or 80 μM BCLT was determined in LNCaP and DU145 cells. The results are shown in Figure 2 and Table I. In LNCaP cells, BCLT dose-dependently induced a major decrease of radiation susceptibility among drug survivors from both growth inhibition and clonogenic survival as endpoints. However, the effect of the drug was less pronounced in the neoadjuvant protocol. The

radiation response of DU145 cells was not significantly affected by BCLT.

#### Apoptosis and Senescence in LNCaP and DU145 Cells After BCLT and Radiation

Apoptosis was determined in LNCaP and DU145 cells using an Annexin V assay, after exposure to BCLT or radiation at doses adjusted to produce the same levels of survival in both cell lines.

In the Annexin assay less than 2% of the bulk cell population was undergoing apoptosis after 3 Gy (LNCaP) or 8 Gy (DU145) irradiation or contact with 80 μM BCLT for 24- or 48-hr. This observation was confirmed by the absence of hypodiploid (Sub-G1) DNA in FACS analysis (data not shown).

Senescence levels in LNCaP and DU145 cells exposed to the same treatments were subsequently determined using a β-galactosidase assay (Fig. 3). The data showed that a substantial fraction of LNCaP cells expressed β-galactosidase following radiation, BCLT or a combination of both. In contrast no β-galactosidase positive cells were found in cultures of DU145 cells.

#### BCLT Alters Cell Cycle Progression in LNCaP and DU145 Cells

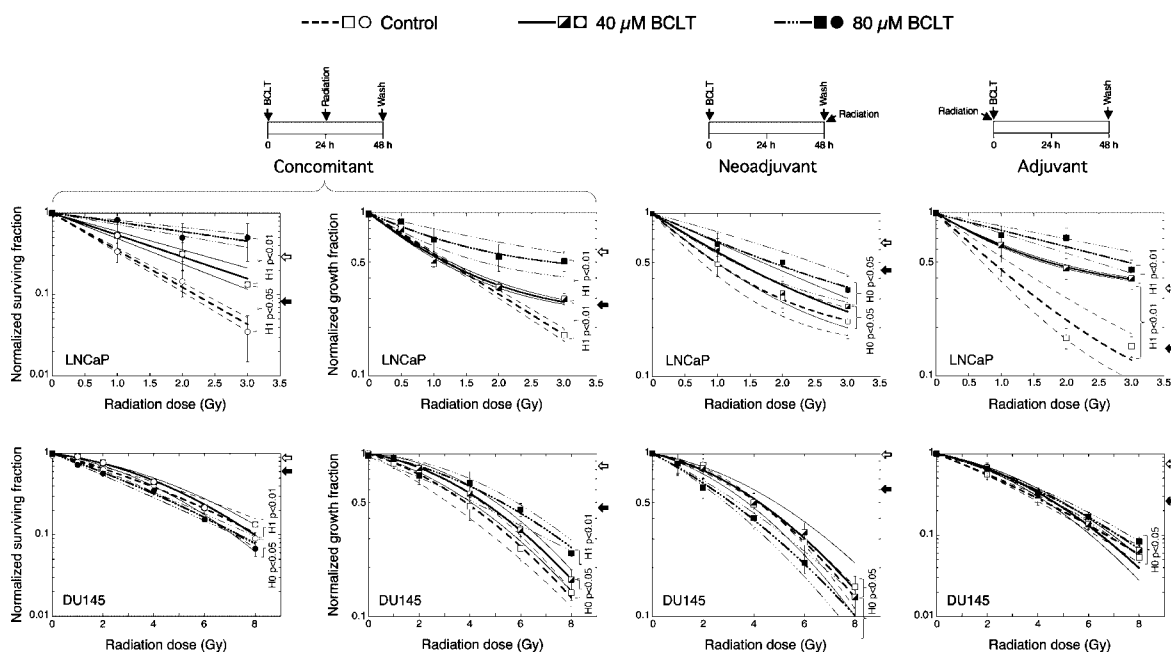
The effect of 80 μM BCLT on cell-cycle phase distribution was investigated in LNCaP and DU145 cells without or with combined exposure to equitoxic doses of radiation in both cell lines. The results are shown in Figure 4. BCLT alone induced an accumulation of cells in G1 with a marked reduction in the proportion of S-phase cells in both cell lines. In combination with radiation, the BCLT-induced G1 block was not reversed, thus preventing the accumulation of cells in G2.

#### BCLT Down-Regulates AR and PSA Expression in LNCaP Cells

Western blots showed that, in LNCaP cells treatment with BCLT dose-dependently decreased the expression of the AR protein, reaching a 73% reduction after 80 μM BCLT (Fig. 5). BCLT also lowered PSA expression, with a 62% reduction of PSA levels after exposure to 20 μM BCLT and complete suppression at higher drug concentrations. Radiation did not affect PSA and AR expression to a significant extent. DU145 did not express any detectable levels of AR and PSA, as anticipated from reports by other authors [15,16].

#### Altered *ar* and *psa* Transcription by BCLT and Radiation in LNCaP Cells

BCLT alone down-regulated *psa* gene transcription in LNCaP cells, with virtually complete inhibition after



**Fig. 2.** Effect of concomitant treatment with BCLT and radiation on the growth ability and survival (left) of LNCaP and DU145 cells. Triplicate culture flasks were seeded at a suitable density for growth or clonogenic assays (see Materials and Methods Section), and irradiated without or with 40 or 80  $\mu\text{M}$  BCLT. Concomitant, neoadjuvant, and adjuvant assays were performed according to the time schedules indicated. After treatment cells were incubated in drug-free medium for three doubling times, collected by trypsinization and counted, or allowed to form colonies. The curves were drawn for best fit to an exponential model (LNCaP, Eq. 1a or 1b) or to the linear-quadratic equation (DU145, Eq. 2, see Materials and Methods Section). The curves were normalized to take into account the cytostatic or cytotoxic effect of BCLT; the light and solid arrows on the right side of the ordinates indicate the response to 40 or 80  $\mu\text{M}$  drug at null radiation dose, respectively. The bold lines represent the curves drawn for best fit with experimental data (see Table I) taking all data points into account. The light lines represent the upper and lower limits drawn from the standard deviation to the mean curves. To determine whether the drug-induced modification of radiation response was significant or not, a non-parametric Mann–Whitney *U*-test was performed on paired sets of data (Controls vs. 40  $\mu\text{M}$ , and 40  $\mu\text{M}$  vs. 80  $\mu\text{M}$  BCLT) taking into consideration the survival values at the highest radiation dose applied in each cell line. H0 states that there is no significant difference between median values; H1 infers that the medians are statistically distinct. *P* is the calculated error risk estimate relative to the model (H0 or H1) considered.

exposure to 80  $\mu\text{M}$  BCLT (Table II). This observation is in agreement with the full suppression of PSA protein synthesis determined by Western blotting (Fig. 5). Radiation alone resulted in a minor decrease of *psa* transcription and did not potentiate the effect of BCLT in combined treatment.

In contrast with the Western blot analysis showing a minor decrease of the AR protein expression in response to radiation, the transcription of the *ar* gene appeared to be raised by as much as 50% after 2 Gy irradiation in LNCaP cells. BCLT also promoted *ar* transcription in a dose-dependent manner culminating at 60% up-regulation for 40  $\mu\text{M}$  BCLT (Table II). At the highest concentration used (80  $\mu\text{M}$ ), however, BCLT overcame this effect and decreased *ar* transcription by about 60% relative to controls. This effect was unchanged in combination with radiation, consistent with the absence of a synergistic interaction. No significant expression of the *psa* and *ar* genes was found in DU145 cells.

## DISCUSSION

Vicentini et al. [20] previously reported that BCLT dose-dependently inhibits cell proliferation in AR-positive and -negative prostate cancer cells. Our experimental data actually showed a dose-dependent inhibition of cell proliferation by BCLT alone, but this was cell line specific with only LNCaP cells which express a functional yet mutated form of the androgen receptor, demonstrating a high susceptibility to 40  $\mu\text{M}$  BCLT. At this concentration the AR-negative DU145 cells were only marginally affected and 80  $\mu\text{M}$  BCLT was necessary to produce a substantial decrease of the growth potential in these cells as well as in another AR-negative prostate cancer cell line (PC3). Normal fibroblasts (MRC5) were even more resistant. The growth inhibitory effect was correlated with an accumulation of cells in G1 phase, independently of whether cells were irradiated or not. In addition BCLT induced a major cytotoxic effect in LNCaP cells that

**TABLE I. Parameters Characterizing Radiation Response of LNCaP and DU145 Cells Without or With BCLT**

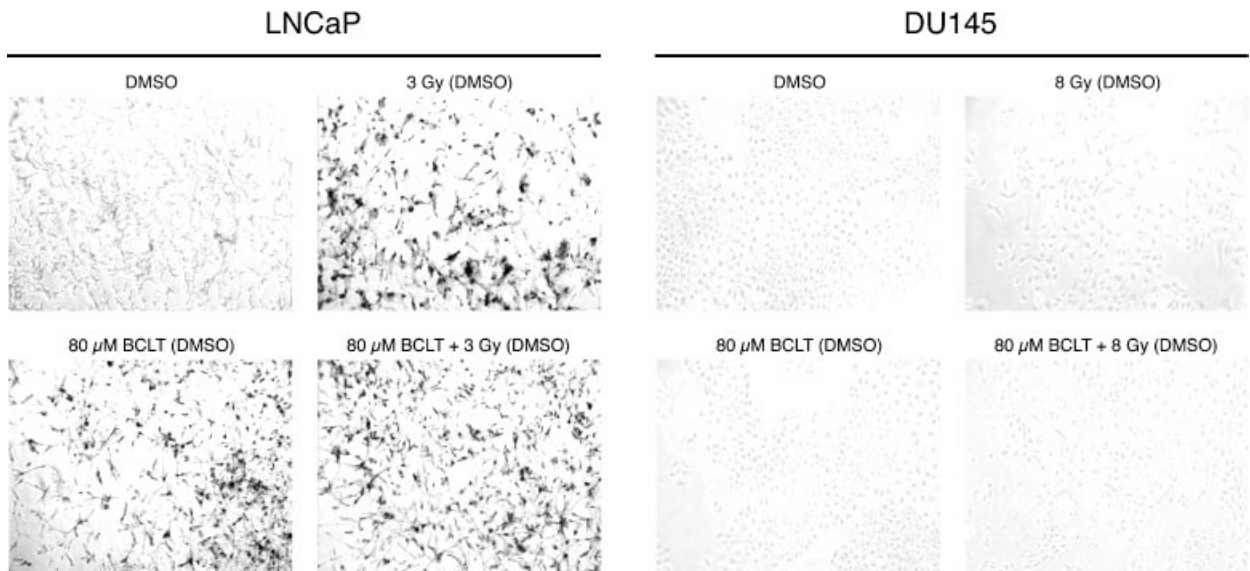
	LNCaP		DU145			
	Exponential equation (Eq. 1a or 1b)		Linear-quadratic equation (Eq. 2)			
	Growth assay	Clonogenic assay	Growth assay		Clonogenic assay	
	$\alpha$ [ $S_{\infty}$ ]	$\alpha$ [ $S_{\infty}$ ]	$\alpha$	$\beta$	$\alpha$	$\beta$
C						
Control	0.573 ± 0.030	1.048 ± 0.065	0.111 ± 0.008	0.0179 ± 0.0019	0.193 ± 0.019	0.0111 ± 0.0040
40 μM BCLT	0.892 ± 0.166 [0.231 ± 0.056]	0.618 ± 0.099	0.0588 ± 0.0085	0.0204 ± 0.0017	0.0939 ± 0.0213	0.0244 ± 0.0046
80 μM BCLT	0.658 ± 0.217 [0.407 ± 0.095]	0.261 ± 0.060	0.0639 ± 0.0254	0.0127 ± 0.0046	0.253 ± 0.038	0.00816 ± 0.00830
N						
Control	0.948 ± 0.029 [0.173 ± 0.009]		0.0679 ± 0.0135	0.0233 ± 0.0028		
40 μM BCLT	0.653 ± 0.157 [0.127 ± 0.096]		0.0773 ± 0.0175	0.0203 ± 0.0036		
80 μM BCLT	0.493 ± 0.121 [0.162 ± 0.113]		0.180 ± 0.025	0.0132 ± 0.0057		
A						
Control	0.873 ± 0.197 [0.057 ± 0.079]		0.277 ± 0.011	0.0099 ± 0.0024		
40 μM BCLT	0.803 ± 0.045 [0.333 ± 0.015]		0.143 ± 0.028	0.0328 ± 0.0073		
80 μM BCLT	0.237 ± 0.030		0.186 ± 0.018	0.0187 ± 0.0043		

C, concomitant; N, neoadjuvant; A, adjuvant.

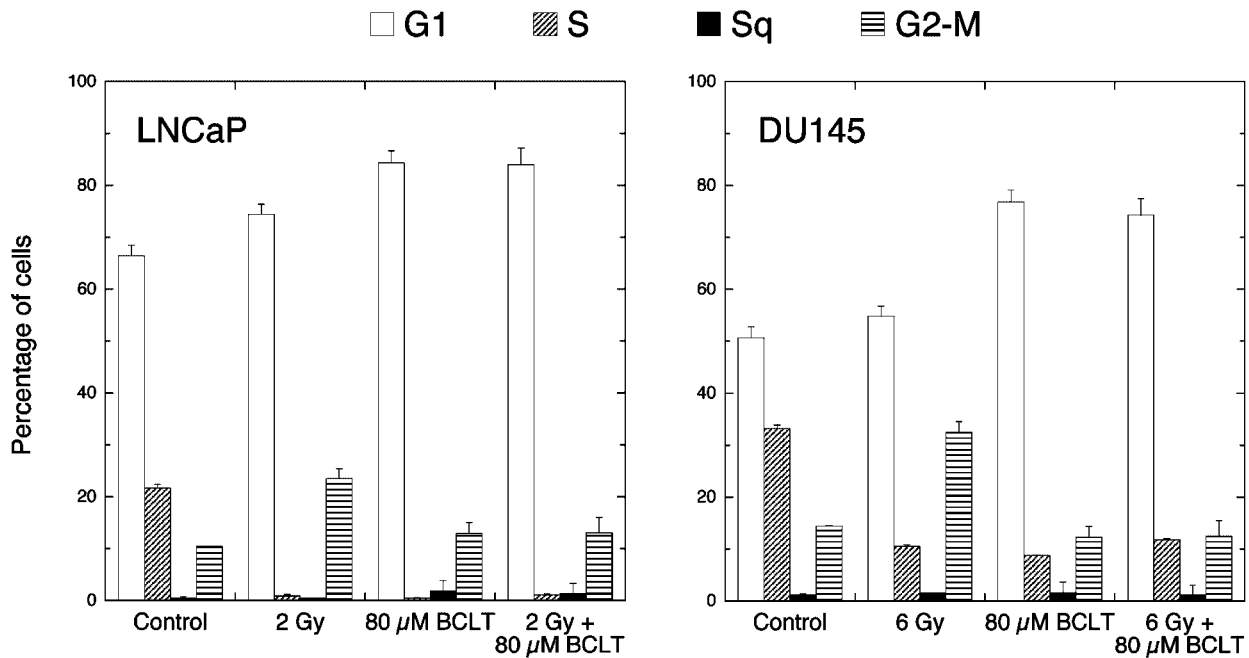
The  $\alpha$  ( $Gy^{-1}$ ),  $\beta$  ( $Gy^{-2}$ ) and  $S_{\infty}$ , parameters (Eqs. 1a, 1b, and 2, see Materials and Methods Section) were calculated for best fit with the experimental data as described under legend to Figure 2.

correlates with  $\beta$ -galactosidase expression, suggesting the occurrence of premature senescence. The level of  $\beta$ -galactosidase positive cells was below detection in cultures of DU145 cells, and the level of apoptosis was less than 2% of LNCaP and DU145 cells. Absence of significant apoptosis after radiation in LNCaP and DU145 cells was also reported by other authors using

terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) and FACS analysis [21]. Consistent with this observation is that BCLT, even at higher doses than those used in this study, did not elicit changes in mitochondria depolarization ( $\Delta\psi_m$ ) or Bcl-2 status [22]. This is in agreement with Lehmann et al. [23] who showed that p53-dependent cellular

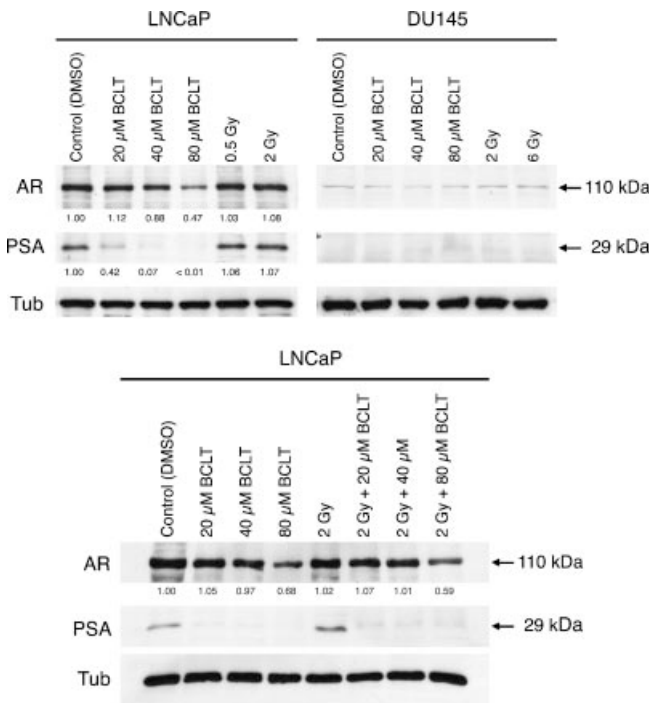


**Fig. 3.** Expression of  $\beta$ -galactosidase after radiation, BCLT, and combined treatment. Cells were irradiated or not at the doses indicated with or without BCLT (80  $\mu$ M) in the presence of 0.26% DMSO. BCLT was introduced 24 hr prior to radiation and was present for 48 hr. Cells were probed for the presence of  $\beta$ -galactosidase after 5 days of post-treatment incubation. Images were recorded using a camera-coupled microscope operated at constant contrast. Blue areas revealing evidence of  $\beta$ -galactosidase positive cells were found in LNCaP cells only and appear as dense spots in the figure.



**Fig. 4.** Altered cell cycle progression of LNCaP and DU145 cells by BCLT and radiation. Mid-log growing cells in duplicate flasks were irradiated or not without or with 80  $\mu$ M BCLT. BCLT was introduced 24 hr prior to irradiation at the doses indicated and was present for a total of 48 hr. Cells were incubated with 10  $\mu$ M 5-bromo-2'-deoxyuridine before harvest and processed for FACS analysis as described under Materials and Methods Section. Sq stands for S-phase arrested (non-synthesizing) cells. For the sake of clarity the minor sub-G1 fraction has not been shown on the diagram. Bars, mean deviation (duplicate experiments).

senescence, not apoptosis is a major mode of radio-induced cell death in LNCaP cells. Cell death in DU145 cells reportedly occurs through mitotic catastrophe with characteristic release of micronuclei [23,24].



BCLT has been reported to be a competitive antagonist of AR and an inhibitor of AR activation by androgens. In this study, we observed that BCLT dose-dependently lowered the expression of the AR protein in the AR-positive LNCaP cells. Low (20  $\mu$ M) and intermediate doses (40  $\mu$ M) of BCLT, either alone or in association with radiation, paradoxically increased *ar* mRNA expression. This phenomenon was reversed at the highest concentration of BCLT used (80  $\mu$ M). As the expression of the *psa* gene is regulated by AR via androgen response elements and other transcription factors, such as the GATA family and the prostate-derived Ets factor [25,26], we also sought to determine

**Fig. 5.** Western blot determination of the expression of PSA and AR following BCLT without or with radiation. Cells were exposed or not to BCLT (48 hr) and irradiated or not after 24 hr contact with drug. Whole cell extracts from treated LNCaP or DU145 cells (20–35  $\mu$ g per lane) were separated by electrophoresis on 7.5% (AR) or 12.5% (PSA) gels. Proteins were transferred to nitrocellulose membranes and probed as described under Materials and Methods Section. The antibodies used were a mouse mAb directed against PSA (clone ER-PR8, Abcam, Cambridge, UK) and a rabbit polyclonal antibody directed against AR (Cell Signaling Technology). The densitometric analysis was performed using ImageQuant software (GE Healthcare Life Sciences). After subtraction of the baseline in each lane, integral values were normalized relative to controls and corrected for the integral of tubulin bands in the same run.

**TABLE II. Altered Transcription of the *psa* and *ar* Genes in Response to Bicalutamide, Radiation, or a Combination of Both**

	LNCaP		DU145	
	PSA	AR	PSA	AR
Control	2.0 (1.6–2.5)	4.0 (3.3–4.8)	0.00 (0.00–0.00)	0.01 (0.00–0.02)
20 $\mu$ M BCLT	0.6 (0.6–0.7)	6.0 (5.4–6.7)	0.00 (0.00–0.01)	0.01 (0.00–0.04)
40 $\mu$ M BCLT	0.6 (0.4–0.7)	6.3 (5.0–8.0)	0.00 (0.00–0.00)	0.01 (0.00–0.02)
80 $\mu$ M BCLT	0.05 (0.03–0.06)	1.6 (1.3–2.0)	0.00 (0.00–0.01)	0.01 (0.00–0.02)
2 Gy	1.6 (0.9–2.8)	5.8 (5.0–6.7)	0.00 (0.00–0.01)	0.01 (0.00–0.01)
20 $\mu$ M BCLT + 2 Gy	0.9 (0.6–1.4)	8.7 (7.0–10.8)	0.00 (0.00–0.01)	0.01 (0.00–0.01)
40 $\mu$ M BCLT + 2 Gy	0.5 (0.5–0.56)	9.0 (5.2–15.6)	0.00 (0.00–0.01)	0.01 (0.00–0.01)
80 $\mu$ M BCLT + 2 Gy	0.01 (0.01–0.02)	3.7 (3.1–4.5)	0.00 (0.00–0.01)	0.02 (0.01–0.04)

Androgen-dependent (LNCaP) and -independent (DU145) prostate cancer cells were exposed to bicalutamide alone, ionizing radiation alone, or a combination of both. Contact with BCLT before harvest was for 48 hr. In combined treatment, BCLT was introduced 24 hr prior to irradiation. For  $\gamma$ -ray treatment alone, cells were collected 24 hr after irradiation. DMSO (0.26%) was constant throughout. The expression of the mRNAs was quantified using real-time reverse transcription-polymerase reaction. The results are expressed as quantity values relative to GAPDH mRNA expression in the same sample.

the changes of PSA protein and mRNA expression in response to BCLT and radiation. PSA was found not to be expressed in the AR-negative DU145 cells, as expected, and both PSA and *psa* expression were down-regulated by BCLT in LNCaP cells in a dose-dependent fashion. This observation is in agreement with Masiello et al. [27] who showed that growth inhibition of LNCaP cells correlates with down-regulation of PSA expression. Concomitant exposure to radiation did not modify this pattern. Taken together, the available data indicate that expression of a functional AR is necessary to mediate cytostatic and cytotoxic responses to low or intermediate concentrations of BCLT, whilst high doses of BCLT are required to induce efficient growth arrest and cell killing in AR-negative cells, thus suggesting that another, AR-independent pathway may operate to compromise survival and depress cell cycle progression in the high range of drug concentration.

BCLT unexpectedly elicited an antagonistic interaction with radiation in LNCaP cells, from both cell growth and clonogenic survival endpoints and independently of whether the drug was present during or after irradiation (Fig. 2). The strength of the effect increased with the concentration of BCLT, whilst the drug did not alter the radiation response of DU145 cells to a significant extent. Interestingly, this effect is similar to that reported for tamoxifen, an estrogen receptor antagonist in breast cancer cells expressing the estrogen receptor (ER). Breast and prostate cancers show parallels in so much that they are both hormone sensitive cancers whose growth depends on estrogens and androgens, respectively. Indeed, 80% of prostate cancers respond to hormone therapy [28] and 75% of breast cancers express the estrogen and/or progesterone receptor [29]. Consistently tamoxifen is used

as adjuvant treatment in hormone sensitive breast cancer [30]. Like BCLT, tamoxifen binds to an intracytoplasmic steroid receptor and then inhibits receptor activation [27,31] and receptor-dependent gene transcription [32] and cellular proliferation [33]. Furthermore, tamoxifen demonstrates a radioprotecting effect in combination with radiation in ER-positive breast cancer cells [34,35], in a similar manner to BCLT (Fig. 2) in the AR-positive LNCaP cells. Also, tamoxifen reportedly induces an accumulation of cells in G1 [36] similar to that observed in our experiments using BCLT (Fig. 4). Induced G1 arrest or quiescence of LNCaP cells by BCLT was also reported by Bai et al. [37]. We would tentatively propose that such a G1 arrest might allow cells to increase the efficiency of potentially lethal damage repair, a subset of DNA damage whose repair is more efficient in quiescent-arrested cells [38,39], thus providing a common mechanism for the increased resistance to radiation in the presence of ER (breast) or AR inhibitors (prostate).

Previous studies involving external beam radiotherapy and permanent implants of  $^{125}\text{I}$  [40,41] suggested that hypofractionated radiotherapy ( $\geq 2.5$  Gy per fraction) may provide a better therapeutic index than conventional fractionation ( $\leq 2.0$  Gy per fraction) in the treatment of prostate carcinoma. In keeping with this hypothesis, Kupelian et al. [42] in a prospective cohort of 770 patients recently showed that hypofractionated radiotherapy provides a good disease control with acceptable toxicities. However, this does not mean that hypofractionation may be used to advantage in association with androgen-suppressing treatment. As a matter of fact, Pollack et al. [43] reported a lack of prostate cancer radiosensitization by androgen deprivation. Moreover, Martinez et al. [44] showed that a short course of androgen deprivation associated with

hypofractionated radiotherapy, did not confer a therapeutic advantage and might even be detrimental, with increased rates of metastasis and cancer mortality. Our data add support to this observation, as we show here that BCLT antagonizes the cell killing effect of radiation in AR-positive LNCaP cells (Fig. 2). However, the data suggest that BCLT can be used in a combined treatment protocol with radiotherapy provided drug and radiation are not given in close temporal proximity. The time sequences of drug and radiation used in the various clinical radiotherapy–hormonotherapy trials that have been published, appear to be consistent with this requirement. In the EORTC 22863 trial Goserelin acetate, a LH–RH agonist, was administered on the first day of irradiation and then every 4 weeks for as long as 3 years [5]. In the RTOG 85-31 trial, Goserelin acetate was administered during the last week of irradiation and given until relapse [4]. In the RTOG 92-02 trial, Goserelin acetate was associated with an anti-androgen treatment beginning 2 months before radiotherapy and prolonged for 2 years [45]. In the SPCG-7/SFUO-3 trial hormone therapy consisted of total androgen blockade with Leuprorelin acetate, another LH–RH agonist for 3 months in association with 250 mg of the oral androgen antagonist Flutamide<sup>TM</sup> three times a day. After 3 months of total androgen blockade, patients started radiotherapy in association with Flutamide<sup>TM</sup>. Patients continued using Flutamide<sup>TM</sup> until progression or death [8]. In the EPC trial, patients received oral BCLT 150 mg once daily following radiotherapy. The planned duration of adjuvant hormonal treatment was 2 years in North America (or until disease progression if earlier), and until disease progression elsewhere with a maximum of 5 years recommended for adjuvant therapy [13]. This notwithstanding, the mechanisms that give rise to additive tumor cure seen with androgen deprivation and normofractionated radiotherapy in the clinic [4,5] remain unclear in the light of experimental evidence in favor of an antagonistic interaction between radiation and bicalutamide in vitro.

### ACKNOWLEDGMENTS

The authors wish to thank Dr. Janet Hall for kind support and helpful discussion. This work was supported by AstraZeneca (grant No. CCIAZ19) and by financial aid from the *Institut National de la Santé et de la Recherche Médicale* and *Institut Curie*.

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