

## Apoptosis of Human Prostate Androgen-dependent and -independent Carcinoma Cells Induced by an Isopropanolic Extract of Black Cohosh Involves Degradation of Cytokeratin (CK) 18

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**Abstract.** *Background:* The inhibitory effects of black cohosh extracts (*Cimicifuga* syn. *Actaea racemosa* L.) on the proliferation of human breast cancer cells were reported recently. In this study, we turned examined another hormone-dependent, epidemiologically important tumor disease, prostate cancer. The cell growth inhibitory effect of an isopropanolic extract of black cohosh (iCR) on androgen-sensitive LNCaP and androgen-insensitive PC-3 and DU 145 prostate cancer cells was investigated. *Materials and Methods:* The cytotoxic effect of the extract was determined by WST-1 assay. Apoptosis was determined by the appearance of apoptotic morphology, annexin V-FITC adherence and caspase activation. Cytokeratin (CK) 18 degradation was identified with M30 monoclonal antibody. *Results:* Regardless of their hormone sensitivity, the growth of prostate cancer cells was significantly and dose-dependently down-regulated by iCR. The drug concentration producing 50% cell growth inhibition in all cell lines after 72h lay between 37.1 and 62.7 µg/ml. Increases in the level of M30 antigen of approximately 1.8-, 5.9- and 5.3-fold over untreated controls were observed in black cohosh-treated PC-3, DU 145 and LNCaP cells, respectively, with the induction of apoptosis being dose- and time-dependent. *Conclusion:* Black cohosh extract kills human hormone-responsive or-unresponsive prostate cancer cells by induction of apoptosis and activation of caspases. This finding suggests that the cell's hormone responsive status is not an important determinant of the response to the extract and that iCR

extract may represent a novel therapeutic approach for the treatment of prostate cancer.

Prostate carcinoma is the most common male malignancy in many industrialized Western countries and has thus become a major health problem (1). In the United States, prostate cancer is the most common neoplasm and the second leading cause of male death (2). The crucial role of androgens in the development of prostate cancer has long been known and almost all prostate carcinomas are originally androgen-dependent (3). The development of a prostate neoplasm from a localized tumor to a more invasive, metastatic disease is frequently associated with loss of androgen-dependency (4). Therefore, particularly the primary treatment of prostate cancer is currently based on androgen withdrawal therapy, radiation or surgical intervention. For the clinical management of prostate cancer in advanced disease, however, an effective therapeutic regimen is still lacking.

Apoptosis, or programmed cell death, plays a critical role in the multiple steps (transformation, progression and survival of metastases) of tumorigenesis. Potential cytostatic and cytotoxic-based therapies rely on the premise that androgen-insensitive prostate carcinoma cells retain their basic cellular machinery and are therefore able to undergo apoptosis. An increased resistance of tumor cells to apoptosis is one putative underlying molecular mechanism contributing to disease progression and treatment failure (5, 6). Many chemopreventive and therapeutic regimens, aimed at manipulation of the apoptotic process, have been proposed for the clinical treatment of cancer patients (7). The modulation of the apoptotic response of human prostatic cancer cells has provided new hope for therapeutic strategies in this disease (8, 9).

The prostate as an organ has very similar characteristics to the mammary gland, *i.e.* both organs depend on circulating

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*Key Words:* Prostate cancer cells, black cohosh extract, apoptosis, annexin-V-FITC, M30 antibody.

hormones for their growth. As expected, androgens play a paramount role in regulating the growth of normal as well as cancerous prostate epithelial cells (10). As most prostate tumors of human origin comprise a mixture of androgen-dependent and -independent tumor cells, the approach of androgen ablation alone will not permit eradication of the androgen-independent cancer cells. Thus, the therapy of androgen-independent prostate cancer requires treatment protocols combining surgical procedures with the concurrent administration of estrogens or chemotherapeutic agents (11, 12). Experimental drugs, which can induce apoptosis and have only low toxicity, should therefore be of great interest in the research and treatment of cancer.

In recent years, the extracts of the rhizomes of black cohosh (CR) have been recognized as a natural alternative for the treatment and prevention of menopausal disorders (13, 14). Herbal extracts contain a variety of constituents and, therefore, the precise mode of action of CR is not yet known. The plain CR extract does bind weakly to the estrogen receptors ER $\alpha$  and ER $\beta$  (15-18), but contains yet unidentified substances with selective estrogen receptor modulator (SERM) activity like some phytoestrogens (19). In ovariectomized rats, the extract of black cohosh (iCR) is comparable to the synthetic SERM raloxifene in antagonizing hormone deficiency-induced bone resorption (20). Besides direct growth inhibition of ER-positive MCF-7 and ER-negative MDA-MB231 cells, the agonistic activities of estrogen on MCF-7 breast cancer cells were significantly antagonized by iCR, which furthermore enhanced the inhibitory effect of tamoxifen (21-23). Most recently, we reported that the antiproliferative effect of iCR on human breast ER-positive MCF-7 as well as ER-negative MDA-MB231 cells could be related to the induction of apoptosis and that activation of caspases was involved (23).

This study, therefore, was designed to evaluate the potential apoptotic activity of an isopropanolic extract from black cohosh (iCR) on human prostate cancer cells. Its cytotoxic effects and the mode of cell death (apoptosis) on the androgen-sensitive LNCaP and androgen-insensitive PC-3 and DU145 cell lines were investigated using the adhesion of Annexin V to phosphatidylserine (PS) on the outer leaflet of the cell membrane. The present study also investigated the role of caspases in the iCR-induced apoptosis by the cleavage of the intermediate filament cytokeratin (CK) 18 in epithelial cells recognized by the monoclonal antibody M30.

## Materials and Methods

**Reagents.** An aqueous-isopropanolic (60% v/v) preparation of *Cimicifuga racemosa* extract (B.Nr. 010720, iCR) provided by Schaper & Brümmer GmbH & Co.KG.(Salzgitter, Germany) was tested. The concentration of the extract was 100 mg/ml (iCR) in relation to the dry residue. The amount of the total triterpene glycosides calculated as standard triterpene glycosides (27-deoxyactein) was 26 mg/ml. As

controls, tamoxifen citrate (TAM) and 17 beta-estradiol (E2) from Sigma, (Buchs, Switzerland) and genistein (4', 5, 7-trihydroxyisoflavone; GEN) and staurosporine (STP) from Alexis Corporation (Lausen, Switzerland), were included in the investigations. The cell proliferation reagent WST-1 was purchased from Roche Diagnostica (Rotkreuz, Switzerland).

**Cell lines.** The prostate carcinoma cell lines LNCaP (androgen-sensitive) and DU145 and PC-3 (androgen-insensitive) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). For routine maintenance, the DU145 and LNCaP cells were grown in RPMI 1640 medium and the PC-3 cells in RPMI 1640/Ham's F-12 (1:1) medium supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine and 1% penicillin/streptomycin. All cultures were grown in a fully humidified atmosphere, 5% CO<sub>2</sub> at 37°C.

The test medium in the cytotoxicity and apoptosis assays was RPMI 1640 medium without phenol red, containing 5% charcoal-stripped FCS and 0.2% penicillin/streptomycin. All cell culture reagents were from Sigma. In all experiments, untreated and vehicle-treated cells were included as controls.

**Determination of cell growth and inhibition.** In order to determine the effect of individual compounds on cell growth, human prostate cancer cells (LNCaP, DU145 and PC-3) were each seeded at a density of 3000 cells per well in 96-well plates and treated for 68 h. After a 4-h incubation with a tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate), the cleavage of WST-1 to formazan by metabolically active cells was quantified by scanning the plates at 450 nm and 650 nm reference wavelength in a microtiter plate reader (24). Each treatment, performed in triplicate from three independent sets of experiments, was evaluated. Control cultures were similarly treated with a 0.5% final concentration of the solvent isopropanol, which was the highest concentration of solvent used in the iCR-treated cell media. This concentration did not influence the outcome of the current proliferation and apoptosis experiments. As background control, the test medium alone was used. The growth inhibition rate was calculated as the percentage of parallel untreated controls. The direct reductive potential of all the substances was tested in a cell-free system (25). None of them differed from the blank (medium only). As positive controls, TAM, GEN and E2 in pharmacological concentrations were used. The GI<sub>50</sub> value (concentration which reduces cell growth by 50%) for each individual cell line was evaluated from the dose-response curves using Microsoft-Excel computer software.

### Detection of apoptosis

**Morphological estimation of cell death.** Giemsa-stained cytospin slides (Biotac™, B-D, Mountain View, CA, USA) of 24-h cultured 10x10<sup>3</sup> cells were examined by oil-immersion light microscopy for the occurrence of morphological changes consistent with apoptosis. The scatter characteristics (cell size-forward scatter, cell granularity-right angle scatter) were investigated by flow cytometry in parallel with Annexin V adherence.

**Assessment of phosphatidylserine externalization in prostate cancer cells.** To identify and quantify the apoptotic cells, flow cytometric analysis was performed. Apoptosis was detected in 2x10<sup>4</sup> treated cells by Annexin V-FITC (Roche Diagnostics) binding to the PS on the cell surface in conjunction with a propidium iodide (PI) dye exclusion as

described (26). Five thousand to 10,000 events per sample were collected and analyzed on a FACSCalibur flow cytometer (B-D) using the Cell Quest acquisition and analysis software. Histograms of treated cells were compared to untreated controls. Vehicle-treated cells (0.5% isopropanol) were always included and did not reveal any significant difference to the non-exposed (control) cells apoptosis.

**M30-Apoptosense™ assay.** M30-Apoptosense, an enzyme-linked immunosorbent assay (ELISA Kit, PEVIVA), for the quantitative measurement of the apoptosis-associated M30 neoepitope in tissue culture media, was purchased from Alexis Corporation (Lausen, Switzerland). Treatment of the LNCaP, DU145 and PC-3 cells with the iCR, TAM, GEN and STP at a density of 10,000 per well was initiated after 6 h. After a further 40 h, NP-40 was added to a final concentration of 0.5% and the plates were frozen. By this procedure, it was possible to assay the total M30 antigen produced (cell associated + antigen released into medium). Twenty-five  $\mu$ l of the medium/extract was used for the M30-Apoptosense (sandwich) assay, performed according to the instructions of the manufacturer. The absorbance of samples was measured in a microplate reader at 450 nm. The amount of M30 antigen in the samples was calculated from a standard curve and expressed as Unit per Litre (U/L).

**Statistical analysis.** The experiments were repeated at least three times to confirm similar results. The significance of the differences in mean values between the untreated controls and iCR-treated samples was determined using the Student's two-tailed test. Probability values equal to or less than 0.05 were considered significant.  $GI_{50}$  values were obtained by linear or exponential regression analysis of data using the Microsoft Excel software. The histograms were compared statistically using the Kolmogorov-Smirnov (K-S) two samples test for overlaid histograms.

## Results

**iCR extract inhibits growth of LNCaP, DU145 and PC-3 cells regardless of hormone sensitivity.** We observed the cytotoxic activity of iCR in the human prostate cancer androgen-sensitive LNCaP cells and two androgen-independent DU145 and PC-3 cell lines. All three cell lines were treated with varying concentrations of iCR (0.02 - 200  $\mu$ g/ml), which correspond to the dilutions of  $5 \times 10^{-6}$  -  $5 \times 10^{-2}$ , for 72 h. Treated cells were then subjected to WST-1 assays to determine cytotoxicity. Cell proliferation in this assay was measured indirectly by the cleavage of tetrazolium salt WST-1 to formazan by mitochondrial dehydrogenases in viable cells. This assay revealed that, with increasing concentrations of iCR, its cytotoxicity in all three cells increased, compared to controls, and independent of hormonal sensitivity. The growth of PC-3 and LNCaP cells was significantly inhibited ( $p < 0.01$ ) above 25  $\mu$ g/ml iCR and in DU145 cells above 50  $\mu$ g/ml of iCR (Figure 1). The  $GI_{50}$  of iCR for inhibition of growth was reached at dilutions of approximately 1:1600 - 1900 in PC-3 and DU145 cells, whereas in hormone-responsive LNCaP a  $GI_{50}$  dilution of 1:2700 was required. The corresponding mean concentrations of iCR ( $\mu$ g/ml) from triplicates of three experiments were closely similar at  $62.7 \pm 16.2$  and  $53.2 \pm 9.8$ ,

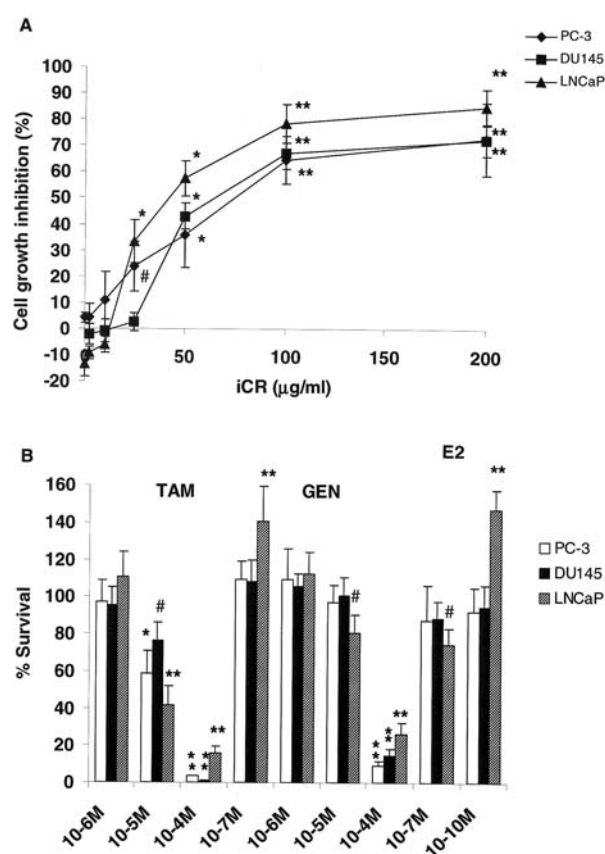


Figure 1. Effect of iCR on the growth of PC-3, DU145 and LNCaP cells (A). Plated cells (3000 per well) were treated for 72 h and stained with WST-1. Average growth inhibition values + SD from triplicates of three independent experiments are shown as dose-response curves of iCR (A). Effect of control substances TAM ( $10^{-6}$  -  $10^{-4}$ M), GEN ( $10^{-7}$  -  $10^{-4}$ M) and E2 ( $10^{-7}$ ;  $10^{-10}$ M) on the survival of cells investigated simultaneously are presented in B. Growth of cells in control medium without iCR was normalized to 100%. In all experiments, the values of vehicle-treated cells (0.5% isopropanol) did not exceed the intra-experimental variance and were less than 10%. (#  $p < 0.05$ ; \*  $p < 0.01$ , \*\*  $p < 0.001$  vs. untreated control; t-test).

respectively, for hormone-independent PC-3 and DU145 prostate cells. The hormone-dependent LNCaP cells were more sensitive to the cytotoxicity exerted by iCR as reflected in the  $GI_{50}$  value of  $37.1 \pm 8.8$   $\mu$ g/ml. However, the differences in the  $GI_{50}$  values of iCR in DU145 and PC-3 in comparison to that of LNCaP cells were not significant ( $p = 0.177$  and  $p = 0.168$ ). In addition, TAM ( $10^{-7}$  -  $10^{-4}$ M), an ER antagonist and apoptosis inducer (27), two estrogens, E2 ( $10^{-10}$  -  $10^{-7}$ M), that increases the speed of the cell cycle and acts as an antiapoptotic factor (28) and GEN ( $10^{-7}$  -  $10^{-4}$ M), a phytoestrogen with estrogenic and antiestrogenic activity (29), were tested in parallel. TAM ( $10^{-4}$  M; 56.4  $\mu$ g/ml) inhibited the growth of all three cell lines almost completely, while the isoflavone GEN ( $10^{-4}$  M, 27  $\mu$ g/ml) caused cells inhibition between 65-85%. There was a dual effect of E2 and GEN on

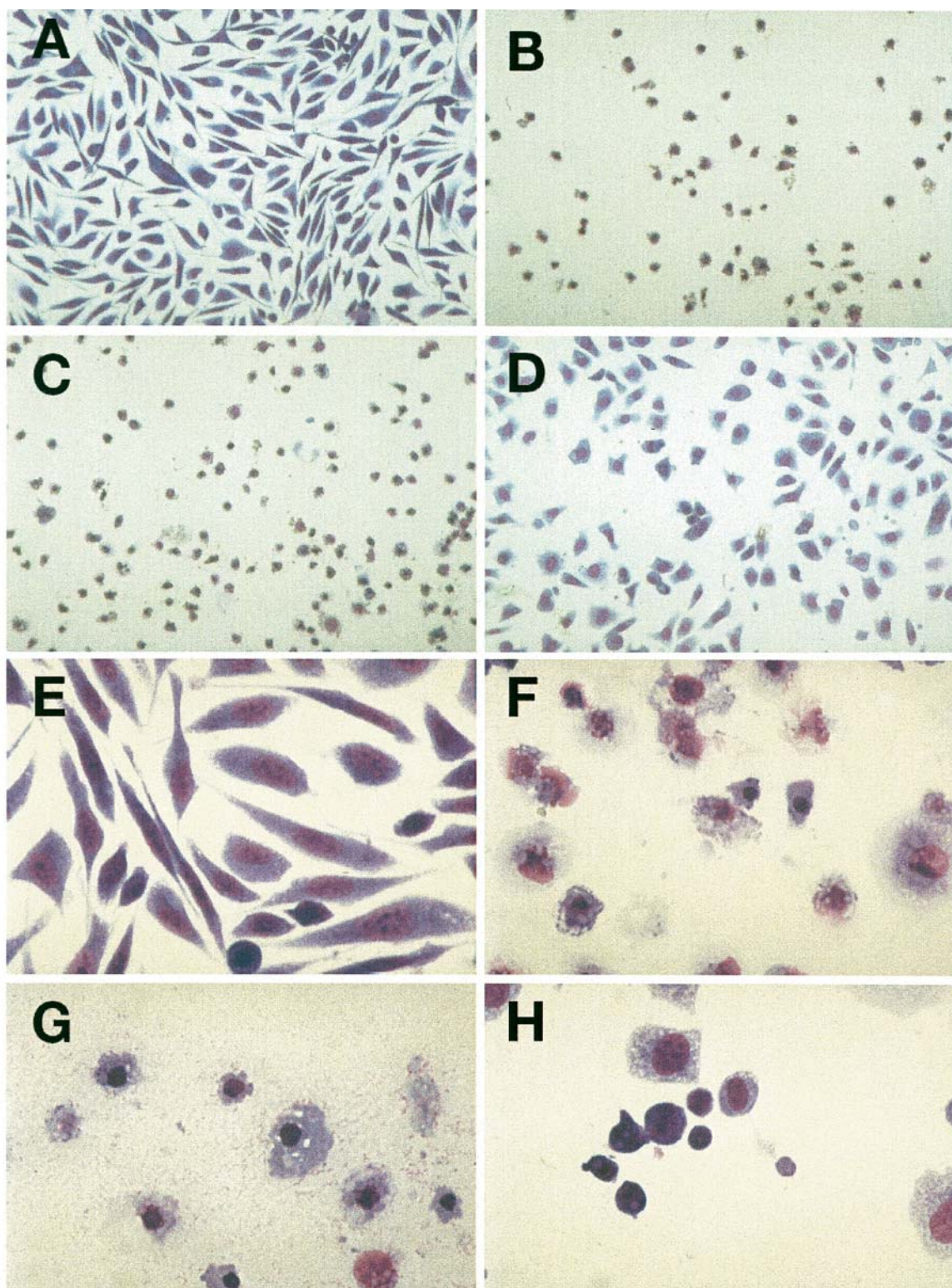


Figure 2. Effect of *iCR* on cellular and nuclear morphology of androgen-insensitive prostate PC-3 cells. In association with the significant decrease in cell count (A-D), a dramatic change in cellular and nuclear morphology (E-H) suggestive of cell death (apoptosis, necrosis) appeared after 24-h treatment with *iCR* (C, G-200  $\mu\text{g/ml}$ ; D, H-40  $\mu\text{g/ml}$ ) in comparison to untreated controls (A, E). As positive control, 10  $\mu\text{M}$  TAM (B, F) was used. Morphological changes of PC-3 cells detected by microscopy are shown on photomicrographs at original magnifications  $\times 100$  (A-D) or  $\times 400$  (E-H). Note that different stages of the apoptotic process can be recognized.

the growth of hormone-dependent LNCaP cells. Their growth was inhibited by  $10^{-7}$  M E2 and  $10^{-5}$  M GEN, whereas the growth of LNCaP cells was stimulated by  $10^{-10}$ M E2 and  $10^{-7}$ M GEN. No stimulatory effect comparable to that elicited by E2 and GEN was observed with iCR, except an increase of up to 13% in LNCaP cells with the lowest concentration of iCR (Figure 1). The survival of vehicle-treated cells was  $101.1 \pm 0.9\%$ ,  $97.4 \pm 4.8\%$  and  $89.6 \pm 5.3\%$  for DU145, PC-3 and LNCaP prostate cancer cells, respectively.

*iCR induces apoptosis in prostate cancer cells.* In this phase of the study, the type of iCR-induced cell death in LNCaP, PC-3 and DU145 cells was investigated. Apoptosis-induced morphological changes manifested by detachment of some cells from adjacent cells and the rounding of detached cells, nuclear condensation, cell shrinkage and evidence of pyknosis (indicative of necrotic death) were visualized by oil-immersion light microscopy on Giemsa-stained slides after 24-h treatment with concentrations of iCR between 20-200  $\mu\text{g/ml}$ . The morphological changes of PC-3 cells resulting from iCR treatment are shown in Figure 2, being also typical of iCR-induced apoptosis and secondary necrosis in DU145 and LNCaP. The occurrence of morphological changes consistent with apoptosis was confirmed by flow cytometric assessment of the light scatter properties of cells. Morphological changes for the cells in the early phase of apoptosis were well reflected by reduced ability to scatter light in the forward direction and an increase, or no change, in the  $90^\circ$  light scatter (data not shown). In addition, the changes occurring in the early stages of apoptosis, the breakdown of asymmetrically distributed plasma membrane phospholipids, were assessed by binding Annexin V to PS exposed on the outer layer of the cell membrane. As secondary necrotic cells bind both Annexin V and PI, PI staining indicates final necrosis but does not exclude cell death through apoptosis. Thus, we established the kinetics of cell death induction by iCR. The extent of apoptosis induction was directly proportional to the drug concentration and treatment time and varied between individual cell lines, as shown in Figure 3. After 24-h incubation with 40  $\mu\text{g/ml}$  iCR, the percentage of PS-expressing DU145 and PC3 cells did not differ from untreated controls, being between 6-15%. After 24-h incubation, only TAM (10  $\mu\text{M}$ ) caused 65% apoptosis in both androgen-unresponsive prostate cells, whereas GEN (50  $\mu\text{M}$ ) exerted an apoptotic effect only after 72-h treatment. With increasing concentrations of iCR and treatment time, the percentage of cells in advanced stages of apoptosis/or necrotic morphology increased in all the cell lines, but the response varied amongst them (Figure 3). After 72-h, iCR at 40  $\mu\text{g/ml}$  exhibited 46.8% and 61.9% apoptosis in DU145 and PC-3 cells, an effect which was achieved after only 24-h incubation with a higher concentration (200  $\mu\text{g/ml}$ ) of iCR. The effect of iCR was comparable to that of TAM (10  $\mu\text{M}$ ) after 48 h and superior

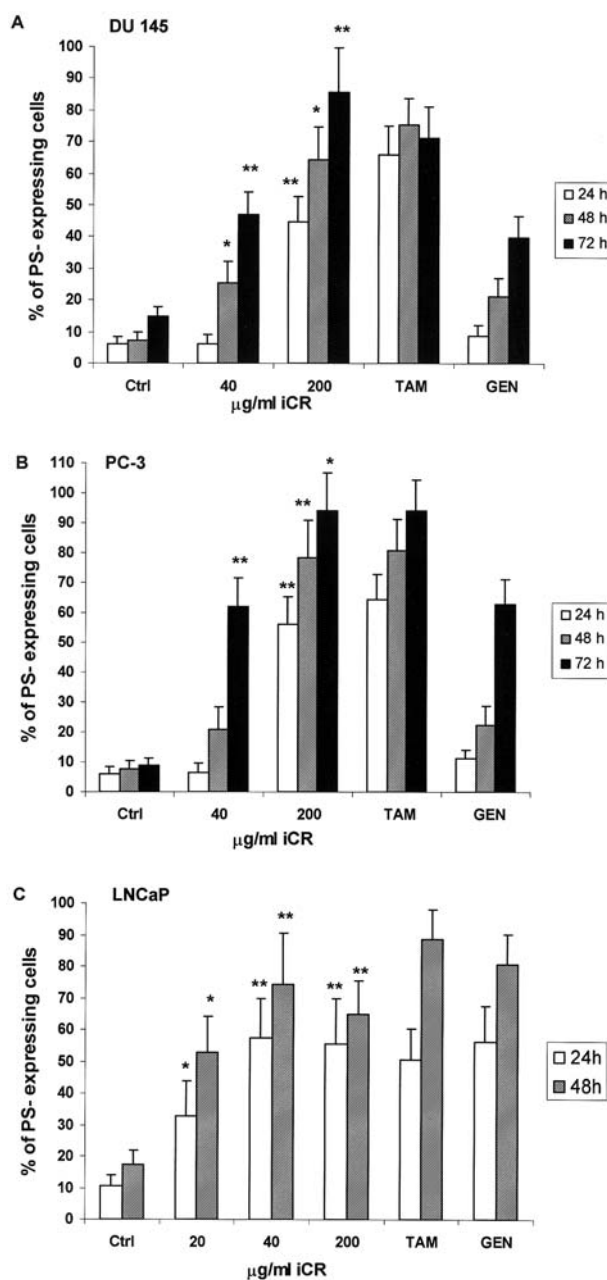


Figure 3. Apoptosis-inducing effect of iCR on DU145 (A), PC-3 (B) and LNCaP (C) prostate cancer cells expressed as percentage of PS-expressing (Annexin V-positive) cells after 24-h, 48-h and 72-h incubation. Cells treated with 10  $\mu\text{M}$  TAM and 50  $\mu\text{M}$  GEN were used as controls. Results are the means  $\pm$  SD of three replicate experiments. (\*  $p < 0.05$ , \*\*  $p < 0.01$ ; t-test).

to that of GEN (50  $\mu\text{M}$ ). PI-positive staining occurred in 10% of DU 145 (72h) and 22% of PC-3 (72h). The response to iCR was very similar in DU145 and PC-3 cells. The highest susceptibility to apoptotic cell death was, however, evident in LNCaP. After 24-h incubation, 40  $\mu\text{g/ml}$  iCR induced 57.5% apoptotic cells, which was comparable to those cultured with

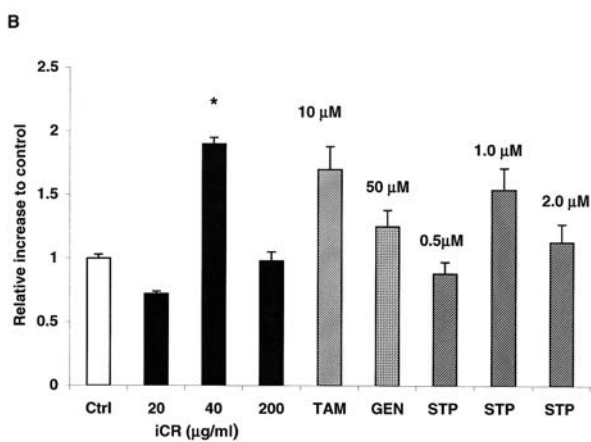
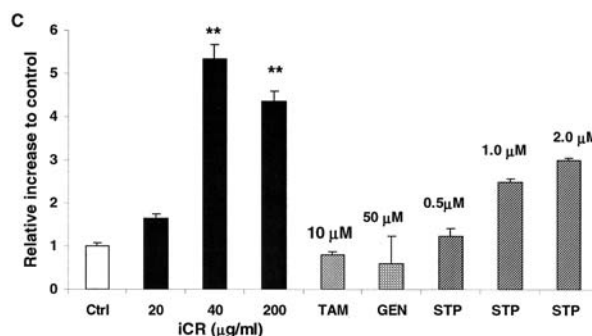
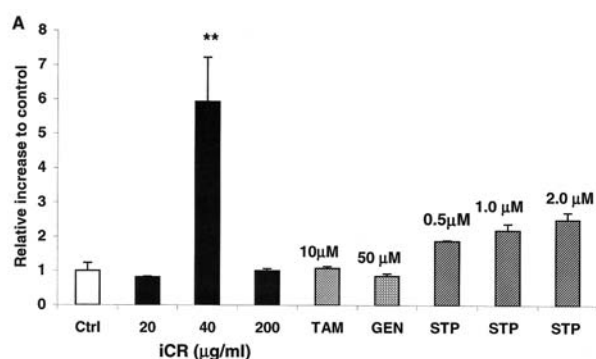


Figure 4. Changes in the level of total CK18 cleavage in cell extracts and tissue culture supernatants in DU145 (A), PC-3 (B) and LNCaP(C) cells measured with M30 antibody. Cells ( $10 \times 10^3$ ) were treated for 40 h with the indicated concentrations of iCR, TAM (10  $\mu$ M), GEN(50  $\mu$ M) and STP (0.1, 1.0 and 2.0  $\mu$ M). M30 activity was measured with ELISA and presented as relative values, where activity of un-induced cells were regarded as 100%. The M30 level was 395, 70 and 241U/L of untreated DU145, PC-3 and LNCaP cells. Bars represents means  $\pm$ SD from two experiments performed in duplicates. (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; t-test).

TAM and GEN. The apoptotic response of LNCaP cells at a low concentration of iCR (40  $\mu$ g/ml) was significantly ( $p < 0.001$ ) higher than in DU145 and PC-3. With an increasing concentration of iCR, the number of necrotic LNCaP cells increased. The spontaneous apoptosis of LNCaP cells was about 10% (24 h) and 24% (48 h).

*Caspase induction in the iCR-mediated prostate cell apoptosis.* To determine whether caspase activation is involved in iCR-induced cell death in human prostate cancer, the induction in the M30 antibody level was investigated. Effector caspases cleave CK18 generating the M30 antigen. The increased activity of the M30 antibody in epithelial cells correlates with the caspases activation during apoptosis (30). All cells were treated for 40 h and STP (0.5, 1.0 and 2.0  $\mu$ M) was used as a positive control. The modulation in M30 activity in the treated cells is presented in Figure 4. A comparative study showed differences in M30 antibody levels of the three cell lines in response to iCR. The greatest increases in the total M30 antigen activity (cell associated + antigen released into medium) compared to the uninduced controls were detected at concentrations of 40  $\mu$ g/ml iCR extract in all cell lines. The sensitivity of androgen-dependent LNCaP and hormone-refractive DU145 cells was approximately equal. The level of M30 increased 5.3-(LNCaP)

and 5.9-fold (DU145) compared to un-induced controls with a basic level of 241 and 395 U/L M30, respectively. With increasing iCR concentration, the M30 level decreased. STP showed lower potency in both the aforementioned prostate cancer cell lines, with 3.0 -fold (LNCaP) and 2.5-fold (DU145) induction of the M30 level. In experiments performed in parallel with TAM (10  $\mu$ M) and GEN (50  $\mu$ M), no modulation of M30 activity was found. This observation most probably results from the different kinetics in apoptosis induction. The remaining androgen refractory prostate cancer cells PC-3 showed a low response regarding the M30 level to iCR (1.8 - fold) and also to STP (1.7 - fold) increase, which significantly ( $p < 0.01$ ) differed from that of LNCaP and DU145 cells. The M30 value in the un-induced PC-3 cells was 70 U/L.

**Discussion**

Due to the recently described antiproliferative and apoptotic effect of iCR on ER-positive (MCF-7) and ER-negative (MDA MB231) breast cancer cells (23), we turned our attention to prostate cancer, another hormone-dependent, epidemiologically large-scale, tumor disease. Since prostate cancer is known to be intrinsically heterogeneous and represents a mixture of androgen-responsive and unresponsive cells at the

time of clinical diagnosis, we investigated the apoptotic response to iCR using prostate cancer cells that represent the entire spectrum of stages as well as their hormone's sensitivity. We used the metastasis-derived prostate carcinoma cell lines DU 145 (obtained from brain: androgen-unresponsive), PC-3 (obtained from bone: androgen-unresponsive) and LNCaP (obtained from supraclavicular lymph node: androgen-responsive) (31). The first two are negative for the expression of androgen receptor (AR) and prostate-specific antigen (PSA), whereas LNCaP expresses the AR gene and releases PSA (32). The growth of all three cell lines was down-regulated by iCR. We found no association between the androgen sensitivity of male prostate cancer cells and their responsiveness to the antiproliferative action of iCR. Whereas LNCaP exhibited the highest sensitivity to growth inhibition by iCR, as evident by the lowest  $GI_{50}$ , the difference in  $GI_{50}$  values of either DU145 or PC-3 cells was not significant. Therefore, taking into consideration that androgen signaling is shut down in PC-3 and DU145 cells due to the absence of androgen receptor (AR) expression, but is still functionally intact in LNCaP cells through mutated AR, it is likely that at least part of iCR's antiproliferative signaling is independent of the androgen receptor status. In addition to androgens, other steroid hormones may be involved in the tumorigenesis of prostate cancer. The exact role of estrogens, whose action is mediated by specific nuclear ERs, namely  $ER\alpha$  and  $ER\beta$ , in the development of prostate cancer has remained unclear. It was reported that estrogen exposure during prostate development may initiate cellular processes resulting in neoplasia later in life (33). On the other hand, epidemiological studies suggested that elevated plasma phytoestrogen levels may protect from prostate cancer (34). The estrogen receptor status of prostate cells has been examined by others (35). The prostate tumor cells PC-3 and LNCaP express very low levels, while DU145 cells do not express  $ER\alpha$ . All three prostate cancer cells exhibit equal levels of  $ER\beta$  which are, however, lower than in breast cancer cell lines. As we previously found that iCR inhibited the growth of  $ER^+$  MCF-7 as well as  $ER^-$  MDA-MB231 breast carcinoma cells independently of their estrogen receptor status (23), we suspect that the antiproliferative action of iCR on prostate cancer cells is evoked by a non-ER-mediated mechanism as well. The biphasic effect of TAM and GEN on the proliferation of MCF-7  $ER^+$  cells, namely stimulating at low concentrations and inhibiting at high concentrations (27, 29), was absent for any concentrations of iCR on hormone-dependent LNCaP cells.

Subsequently, we identified the mode of cell death induced by iCR as apoptosis. Apoptosis, or insensitivity towards its induction, is closely involved in the initiation, progression and metastasis of human prostate cancer. To find novel drugs and/or develop new therapeutic regimens which modulate the apoptotic response would be a promising weapon against prostate cancer (36). Apoptosis was assessed in three ways.

Morphological changes consistent with apoptosis were identified by oil-immersion light microscopy of Giemsa-stained cytospin slides (Figure 2). This was confirmed by flow cytometry assessment of the light scatter properties of the cells, cell size according to forward (FSC) and granularity to side (SSC) scatter and by using Annexin V/PI stain. The flow cytometric assay using cells stained with Annexin V-FITC/PI is an interesting and powerful method for measuring apoptosis, especially for early events (26). The induction of apoptosis was dose- and time-dependent and varied among individual cell lines, as shown in Figure 3. LNCaP proved to be the most sensitive cell line. Whereas 57.5% apoptosis was induced by treatment with 40  $\mu$ g/ml extract in LNCaP as early as 24 h, DU145 and PC-3 required an incubation period of 72 h under the same conditions to achieve comparable results. The sensitivity of individual cells to iCR as obtained in apoptosis assays correlated well with their growth inhibition responses. The apoptosis induced by iCR was preceded by a secondary necrosis in prostate cells.

The activation of caspases is essential for the acquisition of apoptotic morphology. During apoptosis, caspases cleave various cellular proteins. In epithelial cells, one of those proteins is the intermediate filament CK18 (37). An increased M30 activity after induction of apoptosis suggests a caspase involvement in drug-induced cell apoptosis (30). In LNCaP and DU145 cells, iCR elicited a 1.8- to 2.4-fold more potent up-regulation of M30 antigen than the apoptosis inducer STP. In spite of the fact that genistein inhibits the growth of both LNCaP and DU145 cells *via* apoptosis mainly and induces activation and expression of caspase-3 in both target cells (38), we, in our test system, after 40-h incubation found no modulation of the M30 antigen on both target cells. This is most probably due to the fact that for GEN a prolonged treatment time would be necessary, whereas the detection of caspases activation by TAM would require a shorter treatment time (27, 29). In contrast to LNCaP and DU145 cells, where iCR activates caspases with the subsequent induction of apoptosis regardless of androgen sensitivity, the hormone refractory PC-3 cells behaved differently. In spite of an iCR-induced PS expression level that was comparable to DU14, PC-3 cells showed only a low response in M30 activity. This, together with the early apoptotic cell morphology after 24-h iCR-treatment, suggests that the chosen time point for the M30 investigation was not optimal for PC-3 cells. Another reason for the lower cleavage of CK18 associated with apoptosis induction could be the constitutive expression of the p53 gene. The p53 protein is an important modulator of the cell cycle, triggering processes leading to apoptosis. The prostate cancer cells were characterized by others (39) on their p53 status and it was found that LNCaP cells carry wild-type p53 whereas DU145 cells carry mutant p53 and PC-3 cells possess a functional null mutation for the p53 gene. In addition, the antiapoptotic regulator Bcl-xL from the Bcl-2

gene family is overexpressed on PC-3 cells and is responsible, for example, for the resistance to STP-induced apoptosis in PC-3 cells (40). Indeed, the peculiarities of both critical proteins implicated in apoptosis, lack of p53 and overexpression of Bcl-xL, could block the caspases activation in PC-3 cells and thus suggest the possibility of the activation of a caspase-independent apoptotic pathway by iCR. On the other hand, in the highly sensitive iCR-responsive LNCaP, Bcl-2 overexpression was possibly overcome and the subsequent caspase-dependent pathway was activated. Inter-cell differences in the level of caspases activation, measured with M30 antibody, were most probably due to the loss of the p53 gene accompanied with overexpressed Bcl-xL protein in PC-3 cells in comparison to LNCaP and DU145 cells.

Adjuvant strategies aimed at up-regulating the protein expression of the caspases in order to prime prostate carcinoma cells for apoptosis are likely to play an important role in the future treatment of patients with this disease (38, 41). In this study, the growth-inhibitory and apoptotic potential of iCR was observed in a variety of prostate carcinoma cells representing different developmental stages and androgen responsiveness. A vast majority of the chemotherapeutic agents currently used in cancer therapy are highly toxic and produce severe damage to normal cells. Therefore, the concept of drug-mediated apoptosis with low toxicity towards normal cells is gaining increasing attention. One such approach could be a combination of non-toxic effective phytochemicals with chemotherapeutic agents, which could enhance the efficacy together with reducing toxicity to normal tissues. Extracts of black cohosh contain several components with diverse biological activity, which could be attributed to the two main groups of constituents, namely triterpene glycosides (e.g. actein, 27-deoxyactein, cimicifugoside, cimircemosides) and phenolic compounds, such as cinnamic acid esters, caffeic acid derivatives (e.g. caffeic acid, fukinolic acid, ferulic acid, cimicifugic acids A, B, E and F) (42, 43). Recently, it was shown that several cycloartane glycosides (actein, 27-deoxyactein, cimircemoside G) from black cohosh inhibited the growth of human oral squamous cell carcinoma cells (44). Further, triterpene glycosides fraction and its compounds actein, 26-deoxyactein and cimircemoside A induced cell cycle arrest at G1 on human breast MCF-7 cancer cells (45). Recently, Sakurai *et al.* described the antitumor-promoting effects of cimigenol (46) and identified actein as an anti-HIV principle of the extract from the rhizome of black cohosh (47). However, further studies are required (are in progress) to determine the active fractions or related components and mechanism(s) of action of iCR.

### Acknowledgements

We acknowledge Dr. Joyce B. Baumann for her kind help in linguistically improving the manuscript.

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Received May 25, 2004

Revised August 23, 2004

Accepted October 4, 2004