ANTICANCER RESEARCH *25*: 4277-4280 (2005)

4-OH Tamoxifen does not Interfere with Bicalutamide Inhibitory Effects on Human Prostatic Cancer Cells *In Vitro*

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Abstract. *Background: The present study was aimed at investigating the effects of the co-administration of 4-OH Tamoxifen (4HT) and bicalutamide (BIC) on the human prostate cancer cell line LNCaP in vitro. Materials and Methods: The LNCaP FGC prostate cancer cell line was grown in vitro within a three-dimensional matrix formed by collagen gel under dihydrotestosterone (DHT) (0.1 nM) stimulation. Cells were incubated in the presence either of BIC at escalating concentrations (1 nM; 100 nM; 10 μM) or 4HT Tamoxifen (10 nM; 100 nM) or both compounds at the different concentrations studied. The cells were incubated for 144 hours, and growth was evaluated in non trypsinised cells by crystal violet vital dye staining assay. Results: BIC appeared to exert a dose-dependent inhibitory action, with the maximum inhibitory effect achieved by the 10 μM concentration. A comparable inhibitory effect was also observed after exposure to 4HT at both doses tested. No statistically significant interference was observed when BIC was combined with 4HT. Conclusion: 4HT, even at the higher concentration employed here, showed no major interference with the inhibitory effects of BIC.*

Monotherapy with pure anti-androgens is a valuable treatment option for advanced prostate cancer, namely for those patients who wish to avoid the adverse effects of castration (1-3), and is also being investigated in the treatment of early prostate cancer, following radical prostatectomy or radiotherapy, with promising results (4, 5). Anti-androgen monotherapy is usually well tolerated and offers a number of putative advantages over androgen deprivation (3, 6). However gynecomastia and breast pain occur in most patients under anti-androgenic treatment and is the cause of treatment discontinuation in a significant proportion of them (1-5). In order to prevent gynecomastia and breast pain, the combination of pure anti-androgens with tamoxifen, an anti-oestrogen commonly used in the treatment of breast cancer, has been suggested. Two studies have recently demonstrated that the use of tamoxifen is safe and highly effective in preventing the development of gynecomastia in men receiving bicalutamide for their prostate cancer (7, 8).

In particular, we conducted a double-blinded, placebo-controlled trial where 114 patients, assigned to bicalutamide (BIC) monotherapy for localised, locally advanced or biochemically recurrent prostate cancer, were randomly allocated to receive concurrent treatment with tamoxifen or anastrozole, an aromatase inhibitor (7). Our study demonstrated that tamoxifen, at the dose and schedule usually employed for the management of breast cancer (*i.e.* 20 mg daily), was highly effective in preventing both gynecomastia and breast pain and was much more effective than anastrozole. Gynecomastia developed in 73% of the men assigned to BIC and placebo but in only 10% of those receiving concurrent treatment with tamoxifen (TAM) (the incidence rate of gynecomastia in the patients assigned to BIC and anastrozole was 54%) (7). These results were achieved without any increase in adverse events or worsening of quality of life or sexual functioning. Moreover, no major interference on PSA control during BIC monotherapy was attributable to the co-administration of TAM.

In parallel with the clinical evaluations, pharmacodynamic and pharmacokinetic studies were performed to study the possible pharmacological interference between these drugs. These studies failed to show any interference of TAM on BIC pharmacokinetics (9). The present study was performed to investigate whether the co-administration of 4-OH tamoxifen (4HT), the active metabolite of TAM, would interfere with the antiproliferative activity of BIC on the dihydrotestosterone (DHT)-induced growth of the androgen-sensitive human prostate cancer cell line LNCaP *in vitro*.

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Key Words: LNCaP cells, bicalutamide, 4-OH tamoxifen.

Materials and Methods

Cell culture and propagation. The LNCaP FGC prostate cancer cell line was purchased from ATCC/LGC Promochem s.r.l. Milan, Italy. The cells were thawed at 37˚C in a water bath and immediately added to RPMI 1640 routine culture medium at room temperature. They were centrifuged at 200 x g in order to eliminate storage medium, plated in 25-cm^2 culture flasks at 37˚C in an atmosphere of 5% CO_2 in air and left to stand for 48 h. The routine culture medium was supplemented with 15% foetal calf serum (FCS) and L-glutamine 2 mM plus antibiotics (pen/strep 100 IU / 100 μg/ml). The medium was changed every second day.

When the flasks reached a confluence of about 85%, the cells were digested with trypsin/EDTA solution (1x) in phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}. Briefly, the flasks were washed with buffered saline to remove traces of routine medium; trypsin working solution was added and left for 3 min at 37˚C to detach cells from the plastic support; at the end of this period 10 ml of fresh medium were added to stop the digestion by dilution. The cells were gently suspended with a pipette and set in a tube and centrifuged for 5 min at 200 x g. At the end of centrifugation, the supernatant was discarded and the cell pellet resuspended in 10 ml of fresh medium; an aliquot of 100 μl was collected from the suspension and counted with trypan blue solution (1:1 v:v) in a Bürker haemacytometer chamber. Original concentrations were obtained and the cells seeded into new 75-cm^2 flasks with a splitting ratio (dilution) of 1:3-1:4 in order to maintain the cells in log phase.

Three-dimensional substrate. Lyophilised acid soluble type I collagen (100mg) was used for setting up the three-dimensional substrate for cell culturing. Prior to utilisation, collagen was sterilised overnight in absolute ethanol at 4˚C. Excess ethanol was then removed in sterile conditions under a laminar flow hood until complete dryness was achieved. The desiccated powder was resuspended in 40 ml sterile solution of distilled water/acetic acid 0.1% (v/v) overnight at 4˚C with continuous mixing. The resulting solution was centrifuged at 4000 x g for 45 min, and the supernatant collected and stored at 4˚C (stock solution).

When necessary, the working solution was prepared from collagen to which a neutralising solution (NaOH 0.34 M: RPMI 1640 10x 2:1 v/v) was added in the proportion of 4:1 (v/v). The final mixture was kept cold on ice to avoid collagen polymerisation.

Experimental. In our incubation experiments, LNCaP cells were cultured in monolayer using white RPMI (no phenol red) medium supplemented with 15% charcoal-treated FCS (ctFCS), L-glutamine 2 mM plus antibiotics (pen/strep 100 IU / 100 μg/ml) in the presence of DHT 0.1 nM (10^{-10} M).

Log-phase growing cells were harvested from 75-cm^2 flasks by trypsinisation and centrifugation, as previously described, and adjusted to a final density of $1x10^6$ cells/ml. The final concentrated cells, *i.e.* $1x10^6$ cells, were spun again at 200 x g for 5 min and the supernatant decanted; 1 ml of ice-cold gel was added to the pelleted cells and thoroughly, but gently, mixed in order to obtain a homogeneous cell/collagen gel suspension. A triplicate for each treatment point was set.

Suspension aliquots (6 x 100 μl) ($1x10^5$ cell/well final density) were loaded onto a 6-multiwell plate by pipetting and avoiding bubble formation within the gel. The plates were set into a 5% CO_2 humidified incubator at 37˚C for 1 h to allow the gel to polymerise.

At the end of the polymerisation, 3 ml of fresh white medium, containing DHT 0.1 nM only, were added and left to pre-incubate for 72 h at 37˚C in the CO_2 incubator. The cells were subsequently incubated for a further 72 h (total incubation time: 144 h), either with BIC (1 nM - 100 nM - 10 μM) or 4HT (10 nM - 100 nM), or the combination of the two drugs, still in the presence of 0.1 nM DHT. A separate set of gels was pre-incubated for 72 h in fresh medium without DHT and left to grow for another 72 h (total growth time: 144 h) and served as the external control. All experiments were carried out in triplicate, and results were expressed as the mean (SE) of all results.

Cell growth was evaluated in non-trypsinised cells by the crystal violet vital dye staining assay. Briefly, the gel lysate was spun at 200 x g for 5 min to obtain a cell pellet. Saline-diluted crystal violet stock staining solution (500 μl) in RPMI (4:1 v:v) was added to each tube with pellet and incubated for 20 min at room temperature. At the end of the incubation time, dyed cells were centrifuged at 2000 x g for 10 min to remove the dye. The cells were subsequently rinsed three times with tap water to remove traces of dye solution and centrifuged at 2000 x g for 10 min plus a final wash with distilled water. After removing the supernatant, the tubes with pelleted dyed cells were dried and left to stand. Before reading at 595 nm, in the UV/Vis spectrophotometer, the dye was eluted with 1 ml of eluent solution (absolute ethanol/distilled water/glacial acetic acid 50%/49.9%/0.1% v/v).

Comparisons between the groups were performed by the Student's *t*-test.

Results

Results are summarised in Figure 1. The number of cells in the control gel at 144 h was about 3-fold the number of seeded cells, but was about a half of the cells exposed to DHT ($p=0.0004$). When added to DHT, BIC appeared to exert a dose-dependent inhibitory action, with the maximum inhibitory effect achieved by the 10 μM concentration ($p=0.02$ *vs.* DHT alone). 4HT also appeared to exert an inhibitory effect on DHT-induced cell growth. Though the effect was comparable for the two concentrations tested, it was significantly different only for the 10 nM concentration ($p=0.01$ *vs.* DHT alone).

4HT did not appear to interfere with the effect of BIC when this anti-androgen was employed at the lower concentration, which was not very effective in inhibiting DHT-stimulated cell growth. However, some form of interference was evident when the anti-oestrogen was added to BIC at the concentrations that were more effective in inhibiting DHT-stimulated cell growth, though in no case was a statistically significant difference recorded.

Discussion

LNCaP FGC was originally isolated and established by Horoszewicz *et al.* from a lymph node metastasis of a patient with prostate cancer (10) and is one of the most often used experimental models. These cells express high levels of androgen receptor (AR) mRNA and protein and are

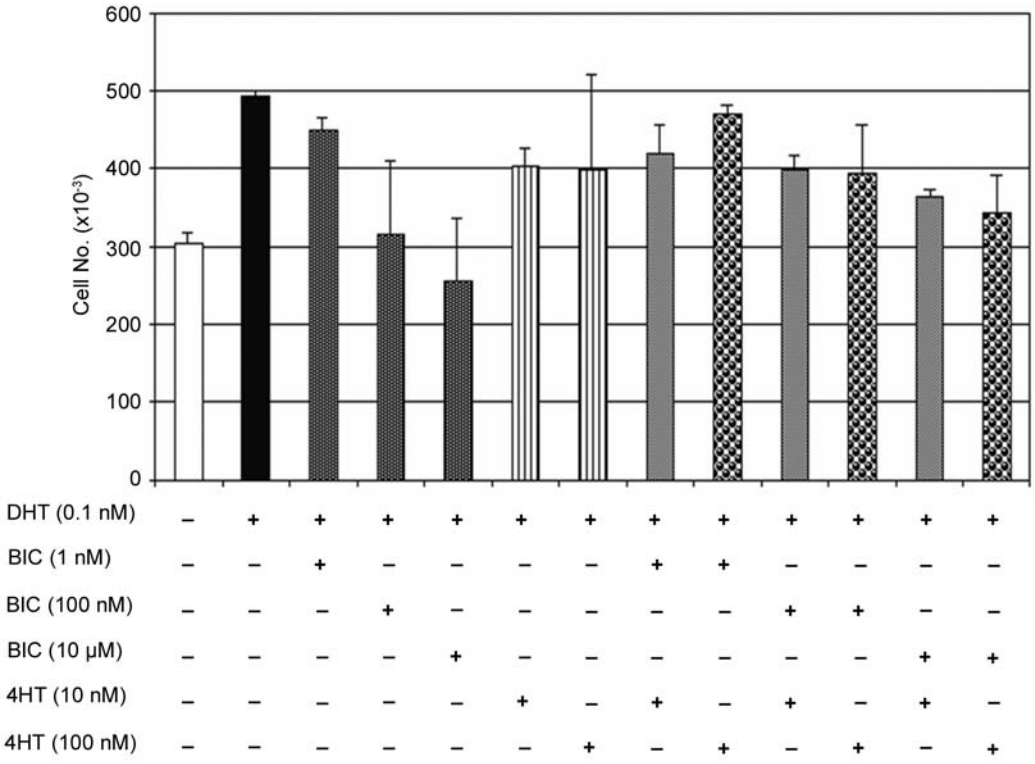

Figure 1. *Inhibitory effects of BIC and 4-OH TAM, either alone, or in combination, on LNCaP cell growth. Cells (1x10^5 cells/well) were seeded in a 3-D collagen gel and preincubated with DHT 0.1 nM for 72 h, then the cells were incubated with different treatments, as reported in the figure, for an additional 72 h. A set of gels used as external control was treated with the fresh medium without DHT. The experiments were repeated in triplicate and results expressed as mean (SE); p values are detailed in the text.*

androgen sensitive. DHT modulates cell growth and stimulates PSA production (11). In order to mimic *in vivo* growth condition, we chose to grow cells within the three-dimensional matrix formed by collagen gel (12, 13). When grown in monolayer, we have observed that LNCaP cells tend to grow in clusters loosely attached to the support (data not reported). Beyond growing in response to androgenic stimuli, LNCaP cells are also capable of growing in response to oestrogenic and progestagenic steroids as well as to non-steroidal anti-androgens, such as hydroxyflutamide and nilutamide (14, 15). Though some investigators have documented the presence of oestrogen receptors (16), it is likely that the growth response to oestrogens and progestagens is mediated by the AR. Indeed, it has been documented that the AR expressed by this cell line is able to bind natural steroids (including oestradiol or progesterone) as well as synthetic steroids, such as R1881 and non-steroidal anti-androgens, with a higher affinity than the wild-type AR (15,16). Unlike hydroxy-flutamide and nilutamide, BIC behaves as a pure anti-androgen and inhibits androgen-stimulated cell growth (17).

Our experiments confirm that the growth of LNCaP cells is sustained by DHT and is inhibited by BIC in a dose-dependent manner, exactly as occurs *in vivo*. Therefore, the chosen model appears to be adequate for study purposes. In this model, 4HT exerted a weak anti-androgenic effect, especially at the lower concentration employed, which was comparable to the effect exerted by BIC at the lowest dose employed. Since the type of steroid receptors expressed by this cell line was not determined, we cannot say whether the weak anti-androgenic effect exerted by the anti-oestrogen was mediated by its binding to the AR or to the oestrogen receptor. Independently of the mechanism involved, it is reassuring that no statistically significant interference with the inhibitory effects of BIC were observed following the co-administration of 4HT. We should, however, be very cautious in extrapolating the results achieved *in vitro* to the clinic, and our findings, though reassuring, do not rule out that some form of interference on cell growth inhibition might be observed after a longer growth period.

Acknowledgements

Casodex™ (bicalutamide - BIC) and 4-hydroxytamoxifen (4HT) were a generous gift from AstraZeneca Italia, Basiglio (MI), Italy.

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Received May 3, 2005
Accepted July 6, 2005