Synergic effects of the cyclin-dependent kinase (CDK) inhibitor olomoucine and androgen-antagonist bicalutamide on prostatic cancer cell lines*

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Currently, mechanisms leading to both apoptosis induction and the development of hormone-independence of prostate carcinoma cells are intensively studied. Attention is also given to the possibility of restoring cell sensitivity to hormone-antagonists. The present study focuses on the effect of the combined synthetic cyclin-dependent kinase [CDK] inhibitor, olomoucine and the antiandrogen bicalutamide on hormone-insensitive (DU-145) and hormone-sensitive (LNCaP) prostate cancer cell lines. In both cell lines reduction in cell viability was significantly higher when olomoucine and bicalutamide were applied in combination when compared to separate application of both these drugs. The setting of optimal concentrations for both substances was important for the final effect on both cell lines. The proliferation arrest was accompanied by a decrease in cyclin D1 expression and the activation of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ pathways in both cell lines. Contrary to the previously described effect of 200 μM olomoucine, weak AR induction after treatment with effective concentrations of olomoucine was not seen in the hormone-insensitive cell line DU-145. The related reaction of DU-145 and LNCaP cell lines to treatment with combined olomoucine and bicalutamide likely provides evidence that the inhibitory effect of bicalutamide may not only be associated with its antiandrogenic properties. The tested substances probably influence different regulatory pathways and these have co-operative impact on the cell cycle outcome. Understanding antitumor and antihormone actions of both agents is essential for the development of novel therapeutic schemes integrating substances with different action. Our results show that the combination of synthetic CDK inhibitors and hormone-antagonists may be one of a number of possible alternatives.

Key words: prostate cancer, bicalutamide, olomoucine, cyclin-dependent kinase, proliferation, apoptosis

Common features of cancer cells are defects in the regulatory processes that govern normal cell proliferation and homeostasis. It is suggested that human tumors of all types arise from several genomic changes affecting the cell physiology and growth. Among characteristics distinguishing cancer cells from normal cells are self-sufficiency or higher sensitivity to growth signals, insensitivity to growth-inhibitory signals and evasion of programmed cell death (apoptosis). During their development, cancer cells also acquire limitless replicative potential, the ability to sustain angiogenesis and the ability to invade surrounding tissue and form metastasis. Detailed understanding of these processes in cancer cells will lead in future to the development of targeted and more efficient anticancer therapies [17].

Prostate cancer is one of the most severe of male neoplasms [31] and the background of its development is not yet fully elucidated [14]. The proliferation and survival of prostatic epithelial cells are dependent on androgens and this dependence is largely maintained even in prostate tumors as well [8, 21, 42]. Initially, the majority of prostate tumors are androgen-dependent and thus patients with advanced inoperable forms of this cancer are usually treated with androgen-deprivation therapy. This lowers the level of circulating androgens or blocks the effect of male sex hormones at the level of androgen receptors (AR). The therapy comprises castration – surgical or chemical (LHRH analogues), or treatment with androgen-antagonists. Usual-

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ly, the combination of all procedures is used in order to achieve maximal androgen blockade [7, 29]. Tumor growth following this therapy is unfortunately only temporarily slowed down and androgen-dependent tumors shift to androgen independence. Androgen-independent tumors are resistant to conventional chemo- and radiotherapy and at present there is no effective treatment available [9, 13, 22].

The molecular basis of the transition from androgen-dependent to androgen-independent status however is largely unknown. Currently, it is not clear whether the androgen-independent phenotype is the result of selection of a subgroup of genetically distinct prostatic tumor cells which are already hormone resistant or a genetic adaptation of prostatic tumour cells to hormone therapy [4, 8]. Molecular mechanisms contributing to androgen-independence include alterations in AR, activation of oncogenes, inactivation of tumour suppressor genes, defects in the genes regulating apoptosis and the cell cycle, or autocrine growth stimulation [5, 21, 34].

Cyclin-dependent kinase [CDK] inhibitors have potential therapeutic importance owing to their ability to regulate the cell cycle and to interact with oncogenes and tumor suppressors. Presently, the most studied topics is the problem of direct inhibition of the catalytic activity of CDKs by their synthetic inhibitors [15, 30]. The first highly specific inhibitors were butyrolactone I, flavopiridol, and olomoucine. Olomoucine (C2, C6, N9-trisubstituted purine) became the precursor for the synthesis of other purines blocking the cell cycle and by modifications of its structure, more effective compounds were obtained, e.g. bohemine and roscovitine [18, 45]. All specific synthetic CDK inhibitors inhibit CDKs reversibly, competitively in the binding pocket for ATP and they are effective at both micro and nanomolar concentrations. All the above mentioned compounds can be found naturally or they are close derivatives of natural substances. Flavopiridol resembles by its effects, inhibitors of the CIP/KIP family. It inhibits both evolutionary related kinases cdk1, cdk2, and cdk5 and their more distant cognates cdk4 and cdk6. In contrast, butyrolactone I and inhibitors from the olomoucine group inhibit the subset of kinases cdk1, cdk2, cdk5, and cdk7 but they do not inhibit kinases cdk4 and cdk6 [30, 39]. Specific synthetic CDK inhibitors universally affect the cell cycle of a wide range of organisms. They block the cell cycle and induce apoptosis of both normal as well as cancer cells. However, normal cells are less sensitive than malignant cells [16]. Apart from cyclin-dependent kinases, as targets for CDK inhibitors, were identified proteins playing important roles in cell functions such as glycolysis, the biosynthesis of proteins, and rearrangement of the cytoskeleton. The cellular effect of these substances is probably not only dependent on the ability to inhibit CDKs. They may be considered to affect additional regulatory pathways as well, and to act in a wide ranging manner [23, 25].

The finding that tumor growth of prostate cells is dependent on their escape from normal homeostatic control has motivated search for the mechanisms leading to both apoptosis and to the development of hormone-independence. Attention is also given to the possibility of restoring cell sensitivity to hormone-antagonists. Synthetic CDK inhibitors owing to their unique features represent an appropriate tool for studying the mutual relations between factors implicated in proliferation, survival, and cell death signalling. They may contribute to explanations of the mechanisms of hormone-independence development. Expression of some of the above factors may also, in the case of prostate cells, be regulated by steroid hormones and related ligands via nuclear receptor pathways. However, currently, there are few studies dealing with the effect of combined chemotherapeutics and hormone-antagonists. Synthetic CDK inhibitors present themselves as an appropriate part of such combined therapy.

The aim of the present work was to analyse viability and protein expression following application of the combined synthetic CDK inhibitor olomoucine and the antiandrogen bicalutamide in androgen-dependent (LNCaP) and androgen-independent (DU-145) prostate cancer cell lines.

Material and methods

Chemicals. Olomoucine (2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine) was prepared in the Laboratory of Growth Regulators (Palacky University and AS CR, Olomouc, Czech Republic). Stock solution (10 mM) was prepared by dissolving relevant amounts of substance in 10% DMSO and 60 mM HCl and stored in –20 °C. Bicalutamide ((2RS)-4'-cyano-3-(4-fluorophenylsulphonyl)-2-hydroxy-2-methyl-3'-(trifluoromethyl)-propionanilid) was kindly provided by prof. von Angerer from the Pharmacy department (Regensburg University, Germany). Stock solution (100 mM) was prepared by dissolving relevant quantity of substance in DMSO and stored in –20 °C.

Cell lines. Human prostatic cancer cell lines, LNCaP and DU-145, were obtained from the American Type Culture Collection (ATTC, USA). LNCaP cells were cultured in RPMI 1640 medium (Sigma, USA), DU-145 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma, USA). Both media were supplemented with 10% foetal calf serum, L-glutamine (250 mg/l), streptomycin (100 μg/ml), and penicillin (20000 U/ml). Both cell lines were kept in a humidified atmosphere of 5% CO_2 at 37 °C and subcultivated twice a week using 0.1% trypsin-0.02% EDTA (Amresco, USA).

MTT cytotoxicity test. A quantity of 3000 (DU-145) or 4500 (LNCaP) cells per well was seeded in 80 μl of standard media to microtiterplates. Cells were grown 24 h (DU-145) or 48 h (LNCaP) and then equivalent amount of media

containing tested substances in different concentrations was added. After 24, 48, and 72 h of cultivation MTT (3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromi-de) (Lachema, CR, 50 μg/well), was added to each well. MTT was reduced by metabolically active cells to a coloured, water-insoluble formazan salt. After 4 h incubation the crystals formed were solubilized in 10% SDS (100 μl/well, 24 h). Quantification was done by measuring absorbance with an ELISA reader Labsystem Multiscan RC at 540 nm. The amount of formazan salt directly correlated with the number of viable cells [1, 33]. The viability of treated cells was related to the viability of control cells which represented 100%. Each experiment was performed in quadruplet and independently repeated minimally twice. Tested substances were for DU-145: 50 μM and 100 μM (olomoucine), 10 μM, 50 μM and 100 μM (bicalutamide), LNCaP: 25 μM and 50 μM (olomoucine), 10 μM, 50 μM and 100 μM (bicalutamide).

Western blot analysis

Sample preparation. To prepare cell lysates, cells were seeded in standard medium to ∅10 cm cultivation dishes to reach approximately 80% confluence after 24 h (DU-145) or 48 h (LNCaP). The culture medium was then replaced with media containing tested substances in concentrations chosen for a given experiment. After 24, 48 and 72 h of cultivation dishes were washed three times with PBS buffer and cells were lysed with SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 20% glycerol, 2% SDS, 0.025% bromphenol blue). Samples were then immediately boiled (4 min) and stored in –20 °C. Protein concentration was estimated using a modified Lowry method which allowed assessment of samples in SDS buffer [26]. Concentrations used for individual experiments were for DU-145: 100 μM olomoucine and 50 μM bicalutamide, for LNCaP: 50 μM olomoucine and 50 μM bicalutamide.

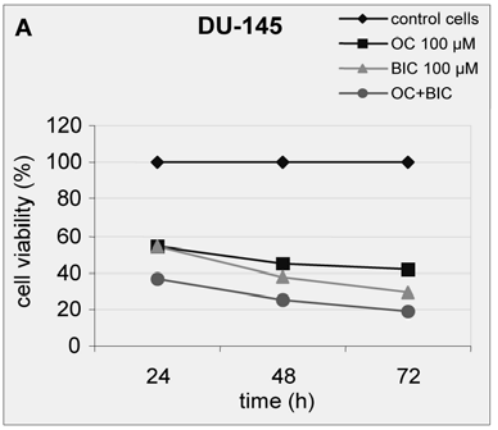
Electrophoretic separation of proteins and western blot. Beta-mercaptoethanol was added to each sample to a final concentration of 5%. Equal aliquots of total protein (15–30 μg/well) were loaded on 8%, 10% or 12% stacked SDS polyacrylamide gels (Mini PROTEAN II Electrophoresis Cell, BIO-RAD, USA). Separated proteins were transferred to nitrocellulose membranes (Amersham, UK) in Mini-Tank™ Electroblotter (Owl Scientific, USA) (2.5 h, 250 mA, blotting buffer: 39 mM glycin, 48 mM Tris, 0.037% SDS, 20% methanol, pH 8.3). Coloured markers (Rainbow™; Amersham, UK) were used as protein molecular weight standards [3].

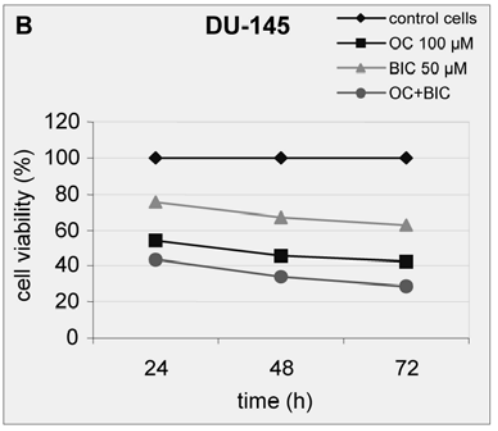
Immunodetection. The blots were blocked with 5% (w/v) non-fat dry milk PBS-T buffer (0.1% Tween-20 in PBS) at least for 2 h at room temperature and incubated overnight (4 °C) in an optimally diluted primary antibody (see below). After washing in PBS-T buffer (30 min), blots were incubated in secondary peroxidase-conjugated anti-mouse antibody (dilution 1:6500) (Santa Cruz Biotechnology, Santa Cruz, USA) for 30 min at room temperature. Protein bands were visualized by the enhanced chemiluminescent detection system, ECL Plus, according to the protocol provided by the manufacturer (Amersham, UK). Equality of protein loading was confirmed by Coomasie Brilliant Blue R-250 staining of gels after electrophoretic separation [35], by Ponceau S red (Sigma, USA) staining of membranes and by immunoblotting for alpha-tubulin (clone DM1A, Sigma, USA). The monoclonal antibodies used were: clone 118 (anti-$p21^{Waf1/Cip1}$, kindly provided by Dr. Vojtěšek, Masaryk Memorial Cancer Institute Brno, CR), clone SX53G8 (anti-$p27^{Kip1}$, Dako, Denmark), clone 100 (anti-Bcl-2, Biogenex, USA), clone DCS-6 (anti-cyclin D1, Novocastra, UK), clone DO-7 (anti-p53, kindly provided by Dr. Vojtěšek, Masaryk Memorial Cancer Institute Brno, CR), clone AR441 (anti-AR, Dako, Denmark), clone PC10 (anti-PCNA, Dako, Denmark).

Statistics. The statistical significance of the effect of tested substances on cell viability was determined using the Student t-test. The levels of significance chosen were $p \leq 0.05$, $p \leq 0.01$.

Results

Effect of combined synthetic CDK inhibitor olomoucine and antiandrogen bicalutamide on cell viability. The results of MTT tests are presented in Figure 1, 2 and Table 1, 2. In DU-145 cells, 10 μM bicalutamide had only low inhibitory effects and these did not significantly influence the effect of 100 μM olomoucine. The most pronounced effect and significant difference between the influence of separately applied substances compared with the influence of their combination was achieved using 100 μM olomoucine (cell viability after treatment with olomoucine compared to control cells was lowered to 42.2%) and 50 μM bicalutamide (cell viability after treatment with bicalutamide compared to control cells was lowered to 62.6%). Cell viability (cell viability decreased to 29.2% compared to control cells) was in this case significantly lower than after treatment with substances used separately and this represented a reduction of ~30% ($p \leq 0.01$) vital cells compared to the action of olomoucine alone and a reduction of ~50% ($p \leq 0.01$) vital cells compared to the action of bicalutamide alone. For Western blot analysis this most effective combination of concentrations was used. After the application of combined 100 μM bicalutamide and 100 μM olomoucine a significant decrease in cell viability compared to the effect of substances applied separately was also achieved. However, the effect was not as marked as it was using a combination of 50 μM bicalutamide a 100 μM olomoucine. None of the above mentioned concentrations of bicalutamide combined with 50 μM olomoucine revealed similar inhibitory effect. 10 μM a 50 μM





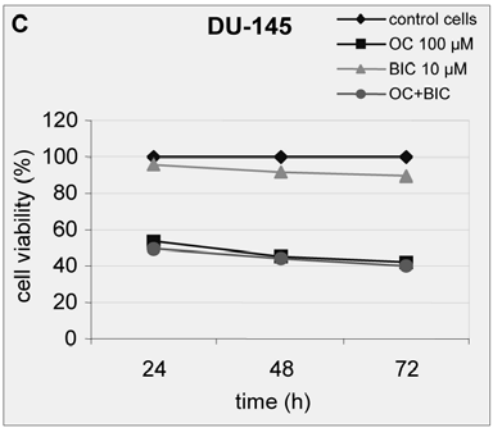
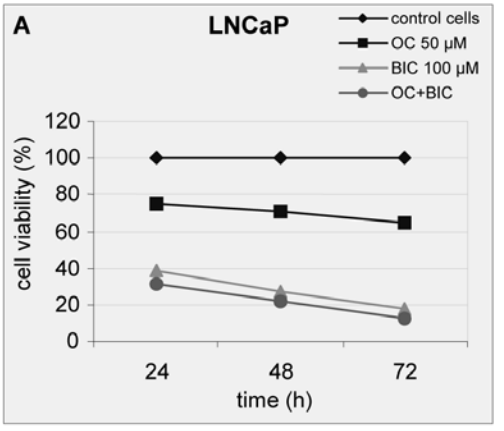
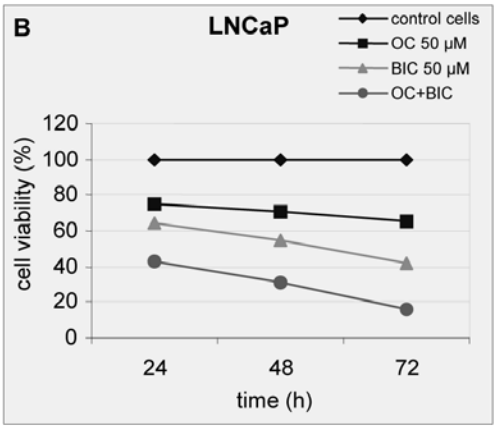


Figure 1. Effect of olomoucine, bicalutamide and their combination on DU-145 cell viability (MTT test) (Graphic illustration of Tab. 1). Values are related to control cells and expressed in %. Used concentrations of bicalutamide were (A) 100 μM, (B) 50 μM a (C) 10 μM and olomoucine 100 μM. Results are means of two independent experiments performed in quadruple.





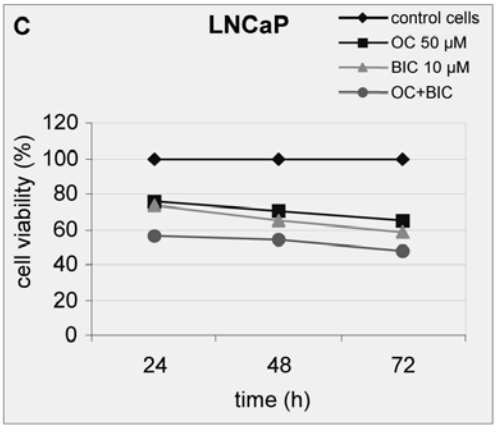


Figure 2. Effect of olomoucine, bicalutamide and their combination on LNCaP cell viability (MTT test) (Graphic illustration of Tab. 2). Values are related to control cells and expressed in %. Used concentrations of bicalutamide were (A) 100 μM, (B) 50 μM a (C) 10 μM and olomoucine 50 μM. Results are means of two independent experiments performed in quadruple.

bicalutamide combined with olomoucine did not show any significantly different effect compared to that of separately applied 50 μM olomoucine, and a combination of 100 μM bicalutamide and 50 μM olomoucine showed no significantly different effect compared to that of bicalutamide alone (data are not shown).

In the LNCaP cell line there was no significant difference between the action of separately applied 10 μM bicalutamide and of separately applied 50 μM olomoucine. Also combined olomoucine and bicalutamide in the above mentioned concentrations showed no more remarkable effect that the action of the substances applied alone. 100 μM

Table 1. Effect of olomoucine, bicalutamide and their combination on DU-145 cell viability (MTT test)

DU-145	24 h		48 h		72 h	
	OC 0 μM	OC 100 μM	OC 0 μM	OC 100 μM	OC 0 μM	OC 100 μM
BIC 0 μM		54.0 $\pm$ 8.7		45.4 $\pm$ 0.9		42.2 $\pm$ 2.7
BIC 10 μM	96.0 $\pm$ 5.2[a]	49.3 $\pm$ 3.3[b]	91.9 $\pm$ 5.5[a]	44.3 $\pm$ 3.9[d]	89.7 $\pm$ 5.1	39.7 $\pm$ 2.0[d]
BIC 50 μM	75.2 $\pm$ 4.5	43.4 $\pm$ 4.0[b]	67.2 $\pm$ 5.8[b]	34.2 $\pm$ 2.8	62.6 $\pm$ 3.0	29.2 $\pm$ 3.1
BIC 100 μM	53.8 $\pm$ 4.8[d]	36.6 $\pm$ 4.7	37.2 $\pm$ 6.1[d]	24.8 $\pm$ 3.6	29.3 $\pm$ 2.4[d]	18.9 $\pm$ 2.7

Values are related to control cells (viability 100%) and expressed in %. The results are means $\pm$ standard deviations of two independent experiments performed in quadruple. Nonmarked results: $p \leq 0.01$ compared to control untreated cells, cells treated with 100 μM olomoucine and bicalutamide in mentioned concentrations. a, b, c: $p \leq 0.05$ compared to control cells (a), cells treated with 100 μM olomoucine (b) or bicalutamide (c). d, e: $p > 0.01$ compared to cells treated with olomoucine (d) or bicalutamide (e).

Table 2. Effect of olomoucine, bicalutamide and their combination on LNCaP cell viability (MTT test)

LNCaP	24 h		48 h		72 h	
	OC 0 μM	OC 50 μM	OC 0 μM	OC 50 μM	OC 0 μM	OC 50 μM
BIC 0 μM		75.3 $\pm$ 0.3		70.5 $\pm$ 0.4		64.9 $\pm$ 1.7
BIC 10 μM	73.5 $\pm$ 2.4[b]	56.4 $\pm$ 3.2	64.5 $\pm$ 0.2[d]	53.6 $\pm$ 1.3[b]	58.6 $\pm$ 4.2[d]	47.4 $\pm$ 5.6[d]
BIC 50 μM	63.9 $\pm$ 0.0[d]	42.8 $\pm$ 3.2	54.1 $\pm$ 5.1[b]	30.8 $\pm$ 0.9	41.6 $\pm$ 0.1	15.9 $\pm$ 6.0
BIC 100 μM	38.9 $\pm$ 1.3	31.3 $\pm$ 7.0[e]	27.0 $\pm$ 4.9	21.9 $\pm$ 0.5[e]	17.8 $\pm$ 2.6	12.8 $\pm$ 3.6[c]

Values are related to control cells (viability 100%) and expressed in %. The results are means $\pm$ standard deviations of two independent experiments performed in quadruple. Nonmarked results: $p \leq 0.01$ compared to control untreated cells, cells treated with 50 μM olomoucine and bicalutamide in mentioned concentrations. a, b, c: $p \leq 0.05$ compared to control cells (a), cells treated with 50 μM olomoucine (b) or bicalutamide (c). d, e: $p > 0.01$ compared to cells treated with olomoucine (d) or bicalutamide (e).

bicalutamide combined with 50 μM olomoucine was not significantly more effective than 100 μM bicalutamide alone. The effect of 50 μM bicalutamide in combination with 50 μM olomoucine was the most marked (cell viability after combination of these substances decreased to 15.9% compared to control cells). The viability of treated cells was significantly lower than after the action of single substances. Cell viability was lowered to 64.9% after treatment by olomoucine only and to 41.6% after treatment by bicalutamide only compared to control cells. This represented a reduction of ~75% ($p \leq 0.01$) viable cells compared to the action of olomoucine alone and a reduction of ~60% ($p \leq 0.01$) viable cells compared to the action of bicalutamide alone. This combination of concentrations was chosen for the Western blot analysis. Similar results were achieved using 25 μM olomoucine in combination with the above mentioned concentrations of bicalutamide. However, the total inhibition was lower owing to the weaker inhibitory ability of olomoucine at this concentration (data are not shown).

Cell morphology after treatment with combined olomoucine and bicalutamide. Figure 3 and 4 show the morphological changes in DU-145 cells and LNCaP cells after 72 h treatment with olomoucine, bicalutamide and a combination of substances. Concentrations used are given in the legends to figures. In both cell lines there was lowered confluence of cells, loss of adhesion, rounding of cells and the occurrence of cell debris from disintegrated cells. These manifestations were visible both in cells treated with single substances only and, more distinct, in cells treated with a combination of compounds.

Effect of combined olomoucine and bicalutamide on protein expression. Changes in protein expression after treatment with synthetic CDK inhibitor olomoucine, antiandrogen bicalutamide and after their combination are shown in Figure 5. In DU-145 cell line, Bcl-2 protein was expressed on the threshold of the detection limit. Conversely, in LNCaP cell line, Bcl-2 expression was high. At both time points after olomoucine treatment compared to control cells, there was no change in the level of Bcl-2 expression. After bicalutamide and combined bicalutamide and olomoucine treatment the expression was increased only nonsignificantly. In DU-145 cell line, the level of CDK inhibitor protein $p21^{Waf1/Cip1}$ was increased after 24 h treatment with bicalutamide and there was even more significant increase after olomoucine and combined olomoucine and bicalutamide treatment. After 72 h treatment with olomoucine and combined olomoucine and bicalutamide the level of $p21^{Waf1/Cip1}$ expression was further increased. However, after treatment with bicalutamide alone compared to control cells, there was a slight decrease. In LNCaP cell line, after 24 h treatment compared to control cells the level of $p21^{Waf1/Cip1}$ was considerably increased and expression rose in the order: olomoucine < bicalutamide < olomoucine + bicalutamide. After 72 h in all treated cells the level of $p21^{Waf1/Cip1}$ decreased. However, it remained above the

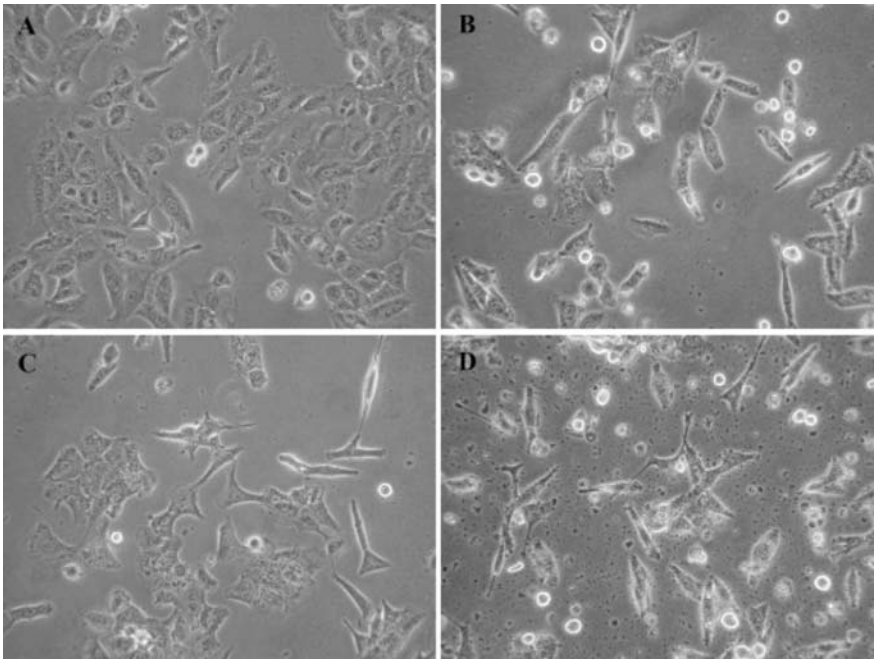


Figure 3. Morphology of DU-145 cells after 72 h treatment with (B) 100 μM olomoucine, (C) 50 μM bicalutamide and (D) their combination documenting an increase in number of detached nonvital cells. (A) Control cells. (200x)

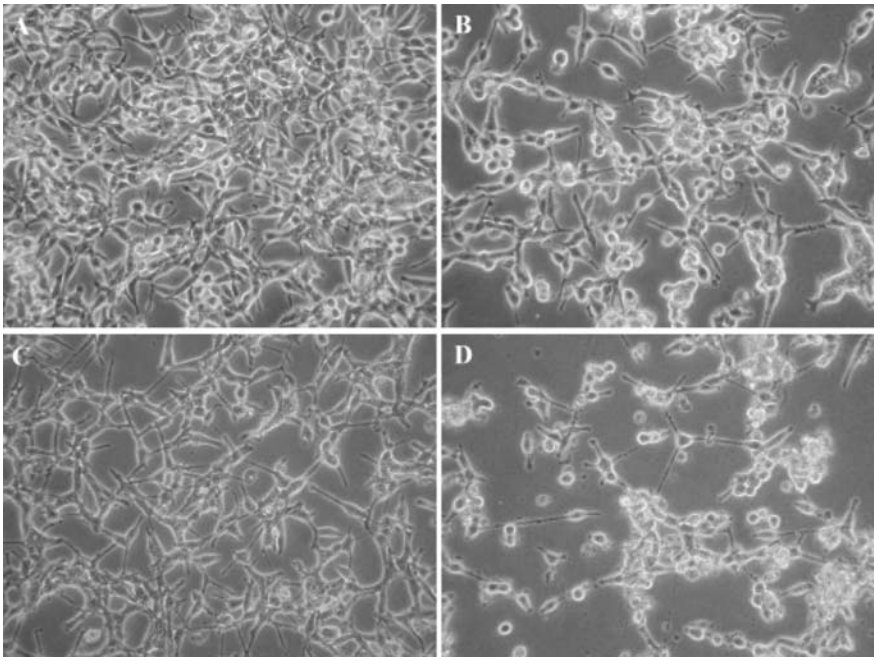


Figure 4. Morphology of LNCaP cells after 72 h treatment with (B) 50 μM olomoucine, (C) 50 μM bicalutamide and (D) their combination documenting an increase in number of detached nonvital cells. (A) Control cells. (200x)

level of expression in control cells. In both cell lines after 24 h and 72 h treatment with bicalutamide and combined olomoucine and bicalutamide $p27^{Kip1}$ protein expression was increased. After treatment with olomoucine in LNCaP cell line there were no changes in $p27^{Kip1}$ expression. In DU-145 cells, the level of $p27^{Kip1}$ was slightly increased after 24 h of treatment and after 72 h the increase in $p27^{Kip1}$ protein expression was already significant. In both cell lines, at both time points, compared to control cells, expression of PCNA remained at the same level. A considerable drop in LNCaP cell line after 72 h of bicalutamide and combined bicalutamide and olomoucine treatment was the only exception. In both cell lines there was the same image of cyclin D1 expression. At both time points after olomoucine treatment cyclin D1 expression was at the level of expression of control cells. A slight decrease was visible after bicalutamide treatment and considerable decrease was visible after combined olomoucine and bicalutamide. In the LNCaP cell line, p53 protein expression was unchanged. In DU-145 cells, after 24 h treatment with separately applied substances or with their combination compared to control cells, a slight increase in p53 protein expression was seen. After 72 h of bicalutamide treatment p53 level remained elevated. However, after olomoucine treatment it decreased and this was not influenced by combined substances. In DU-145 cells, AR expression was on the threshold of the detection limit. In LNCaP cells, at both time points, AR expression was high and this was not influenced by olomoucine treatment. After bicalutamide treatment considerable decrease in AR level was seen and this was more intense after olomoucine and bicalutamide combined.

Discussion

The cell lines used in this study are the most frequently studied prostatic cancer cell lines. Their morphology, cytogenetics, and biochemistry have been well characterized in several studies [6, 10, 19, 38, 41].

Androgen-sensitive cell line LNCaP was derived from a prostatic lymph nodes cancer metastasis and it maintains many characteristics of prostatic epithelial cells. LNCaP

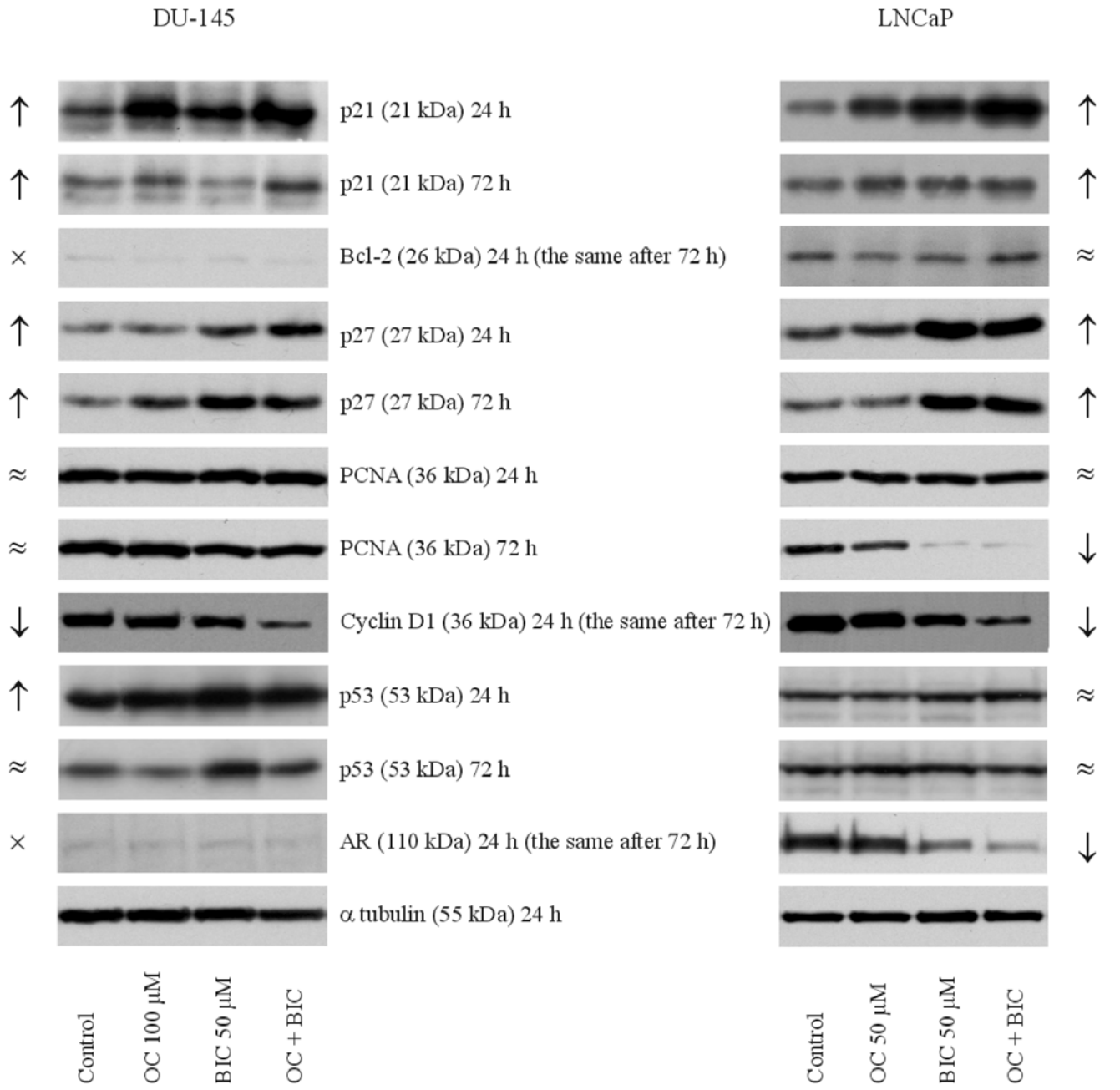


Figure 5. Effect of combined olomoucine and bicalutamide on protein expression in DU-145 and LNCaP cancer cell lines. Protein expression after 24 h and 72 h treatment with studied substances. Concentrations of substances for DU-145 cell line: 100 μM olomoucine, 50 μM bicalutamide and their combination, for LNCaP cell line: 50 μM olomoucine, 50 μM bicalutamide and their combination. Results were compared with protein expression in untreated control cells. Symbols schematically show changes in protein levels in cells treated with combined olomoucine and bicalutamide compared to control cells: ≈ no marked change in expression, ↑ increased expression, ↓ decreased expression, × protein expression on the threshold of the detection limit, – significant changes uninterpretable.

cells respond to androgen stimulation by transcriptional activation of genes coding for prostate specific glandular kallikreins (PSA and hGK2), subsequently by its secretion and increase in cell proliferation. These cells also produce acid phosphatase. The doubling time for this cell line is about 40 hours. HOROSZEWICS at al [19] found it was up to 60 hours. Thus the cells are relatively slow growing. LNCaP cell line expresses functional androgen receptors, which carry a point mutation in the hormone binding domain. These receptors can be detected both in the cytoplasm and nu-

cleus. The cells contain wild-type tumour suppressor gene p53 [6, 19, 32]. This cell line has been used as a model for androgen-sensitive prostate cancer with low metastatic potential.

The DU-145 cell line is derived from brain metastasis of primary prostatic adenocarcinoma and in cell culture, it maintains its original aggressive behaviour. DU-145 cells show only weak acid phosphatase production. Its doubling time is 34 hours. The cell line is neither androgen-sensitive nor androgen-dependent. Expression of wild type AR is either negative or very weak. DU-145 cells contain mutated p53 gene and deletion in Rb gene [6, 10, 38]. This cell line has been used as a model for poorly differentiated, aggressive and strongly metastasizing types of prostate cancer.

Our previous results [28] indicated the ability of higher doses of the synthetic CDK inhibitor olomoucine to induce slightly the AR and to reduce $p21^{Waf1/Cip}$ expression in hormone-independent DU-145 cell line. This finding led us to the idea of combining this ability of olomoucine with the hormonal antagonist bicalutamide. The hypothesis was that induction of AR may lead to the restoration or increase in hormone responsiveness and in this way to enhance the effect of the combined substances. We also intended to check whether the same or an even stronger effect could be achieved by using both substances in lower concentrations and thus decrease possible negative side effects.

In the LNCaP cell line, compared to the effect of bicalutamide alone, there was no significant effect of substances applied in combination when the combination was composed of high (100 μM) or conversely low (10 μM) concentration of bicalutamide combined with olomoucine (50 μM). The most marked cell viability decrease, compared to control cells, was achieved with combined 50 μM olomoucine and 50 μM bicalutamide. In the DU-145 cell line, a low (10 μM) concentration of bicalutamide showed only very weak inhibitory effects. This was not increased even in combination with olomoucine (100 μM) and in this case the inhibitory effect of olomoucine was asserted alone. The combination of 100 μM bicalutamide and 100 μM olomoucine was more effective. The most profound cell viability decrease, compared to control cells, was achieved by combination of 100 μM olomoucine and 50 μM bicalutamide. In general, in both cell lines the effect of combined olomoucine and bicalutamide on the cell viability decrease was more pronounced when compared with the effect of olomoucine alone or bicalutamide alone. Optimally balanced concentrations of both substances were important for the final effect in both cell lines.

The highest concentration of bicalutamide used in our *in vitro* experiments is in agreement with real *in vivo* plasmatic concentrations achieved after peroral administration of bicalutamide in daily doses 10–200 mg [43].

The reasons for the lower sensitivity of arrested cells to the effects of bicalutamide may be cell cycle arrest and changes in protein expression induced by olomoucine. Our results, despite the fact that in the present study we used a lower concentration of olomoucine, than in a previous study [28] (since a higher concentrations totaly masked the effect of bicalutamide), did not confirm this hypothesis, because pre-treatment of both cell types with olomoucine and subsequent application of bicalutamide did not influence the final effect of the combination of both substances (data are not shown). This result is consistent with that of Berchem et al. [2]. In the LNCaP cell line, these authors reported lowered cytotoxicity of etoposide in the presence of androgens. Androgens increase expression of the anti-apoptotic protein Bcl-2 and thus they cause relative resistance to apoptosis induction by cytostatics. The authors assume that apoptosis inducers may be more effective under conditions of androgen deprivation.

The LNCaP cell line expresses a high concentration of AR protein and AR mRNA. In this cell line the AR gene carries point mutation leading to the substitution of threonin in the position of alanin in codon 877 corresponding to hormone binding domain [19, 41, 44]. The DU-145 cell line is androgen-insensitive having AR expression either negative or very weak [9, 22]. The absence of AR expression is caused by inhibition on the transcriptional or translational level because the wild type of AR gene exists in this cell line. In both cell lines all 8 exons are presented and deletions or rearrangements of AR gene were not proven [41]. In our DU-145 cell line, we found low, but detectable levels of AR, using two monoclonal antibodies (Biogenex, Dako) recognising the N-terminal transactivation domain of AR [28].

Both cell lines also differ in status of the tumor suppressor gene p53 [20]. LNCaP cells carry nonmutated p53. Conversely, in DU-145 cells the p53 gene carries one mutation in codon 274 and the second one in codon 223. Functionally one or both mutations stabilize mutated protein and lead to strong immunohistochemical p53 positivity. p53 mutation in the LNCaP line has not been confirmed, even though CARROLL et al [6] described mutation in codon 273 after long term *in vitro* cultivation.

The literary data concerning the expression of the Bcl-2 family members in prostatic cancer cell lines are quite controversial. TANG et al [40] described Bcl-2 (26 kDa) protein expression in the LNCaP line. However, in the DU-145 line they detected Bcl-2 in molecular weight 26 kDa only after isolation of a large amount of total proteins and after long exposure time. In contrast to LNCaP cells using the same antibody, in DU-145, they also detected 30 kDa protein. The same authors asserted that DU-145 cell line do not express protein Bax. DORAI et al [11] describe only weak Bcl-2 expression in the LNCaP line and in the DU-145 line they failed to find this protein. FAN et al [12] showed both Bcl-2 and Bax expression in the DU-145 cell line. Bax expression in DU-145 line is also described by SHIRAHAMA et al [37]. Conversely ROKHLIN et al [33] detected neither

Bcl-2 nor Bax. Our LNCaP cell line expressed both Bcl-2 and Bax proteins. In the DU-145 cell line in agreement with FAN et al [12] and SHIRAHAMA et al [37] we detected protein Bax. Bcl-2 levels (analyzed by anti-Bcl-2 antibody clone 100, Biogenex) were very low, almost on the threshold of detection limit. We asssume that these differences might be caused by the different sensitivity of methods used in individual laboratories.

In the LNCaP cell line, after 24 h and 72 h treatment with bicalutamide alone and combined bicalutamide and olomoucine a decrease in AR expression accompanied by drop in cyclin D1 levels was seen. Expression of proliferation marker PCNA also decreased. However, this did not occur until 72 h of treatment. Olomoucine applied separately in effective concentration did not induce significant changes in expression of the above mentioned proteins. However, a combination of olomoucine and bicalutamide led to greater decrease in protein levels than was seen after application of bicalutamide alone. Our results are in agreement with results of PERRY and TINDALL [32]. These authors showed that in the LNCaP cell line expression of proliferation marker PCNA is posttranscriptionaly regulated by androgens and mediated by AR. Also in the DU-145 cell line, unchanged PCNA expression together with AR expression represent indirect evidence for a relation between proliferative activity and expression of AR. The cell cycle arrest in DU-145 cells was accompanied, as in LNCaP, by a decrease in cyclin D1 expression. The results support the fact that androgens influence the entry of cells to the S phase of the cell cycle and that androgen ablation leads to cell cycle arrest in the G1 phase. This arrest is characterized by a reduction in activity of relevant cyclin-dependent kinases and by changes in Rb phosphorylation accompanied by reduction of G1 phase cyclins and alterations of CDK inhibitors [24]. In addition, in both cell lines activation of the CDK inhibitor $p27^{Kip1}$ and stimulation of $p21^{Waf1/Cip}$ protein expression is in agreement with the above mentioned data. Levels of both proteins were considerably more increased after treatment with combination of olomoucine and bicalutamide. Increased expression of tumor suppressor protein p53 after treatment with substances applied separately and after their combination in the DU-145 line shows that transactivation of $p21^{Waf1/Cip}$ is probably p53 independent in these cases.

As described in the literature, low concentrations of androgens induce an upregulation of cdk2 and cdk4 in androgen-dependent prostatic cancer cell lines [27]. From this point of view the bicalutamide could inhibit activity of cdks in LNCaP cells like olomoucine. We can not exlude that this mechanism might in part contribute to the final inhibitory effect but this participation cannot be significant since bicalutamide has the same effect on androgen-independent cells DU-145 as well.

We found that both hormone-sensitive and hormone-insensitive prostate cancer cell lines react to combined olomoucine and bicalutamide treatment in a similar way. This fact provides presumably evidence that the inhibitory effect of bicalutamide may not be associated only with its antiandrogenic properties. Both tested substances probably influence more different regulatory pathways and these have cooperative impact on the outcome of the cell cycle. Knowledge of antitumor substances and antihormone action on the cell cycle is crucial for the development of novel therapeutic schemes integrating substances with different proapoptotic and endocrine effects. Our results show that the combination of the synthetic CDK inhibitor olomoucine and hormone-antagonist bicalutamide may be one possible alternative. Detailed determination of mechanisms responsible for these effects however requires additional research.

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