

***In Vitro* Antiproliferation in Prostate Cancer Cell Lines with Cytostatics and Combinations with Resistance Modifiers**

OLIVÉR PINTÉR¹, ILONA MUCSI² and JOSEPH MOLNAR²

¹Department of Urology and ²Department of Microbiology,
Medical Faculty, University of Szeged, Szeged, Hungary

Abstract. *The treatment of prostate cancer in an advanced state is still unsatisfactory. In the event of the ineffectiveness of total androgen blockade (TAB) therapy, cytostatic administration may be attempted. In this study, we modelled the drugs used in practice on human prostate cancer cell lines. Studies aimed at decreasing multidrug resistance were performed on PC-3 cells. With the use of various cytostatics, the cell proliferation-inhibiting effects were measured under in vitro conditions on human prostate cancer cell lines LNCaP-FGC and PC-3. Under the given experimental conditions, the examined cytostatics exhibited antiproliferative effects on each of the investigated cell lines. Our results indicate that it is not necessary to wait until the development of a hormone-resistant state. In the studies on the PC-3 cell line, we did not find a multidrug-resistant efflux activity responsible for the resistance of the tumour.*

Prostate cancer is one of the most frequent diseases among men above the age of 50, and occupies a leading position among the causes of death. Hormone therapy has been known since the 1940s. It was reported by Huggins and Hodges that some tumour cells that develop in the prostate gland are sensitive to androgen withdrawal (1). In general, four consecutive stages are distinguished in the progression of the tumour tissue. The local phase is followed by the development of a locally advanced stage, and then by the emergence of advanced and finally hormone-refractory prostate cancer. Certain researchers consider that cells, which earlier were androgen-sensitive, adapt to androgen-ablation treatment (2-4). More or less fixed procedures have been established for the treatment of the individual states.

Correspondence to: Dr Olivér Pintér, Department of Urology, Medical Faculty, University of Szeged, Szeged Kalvaria sgt. 57. H-6722, Hungary. Tel: (+) 36-62-490-590, e-mail: pinter@comser.szote.u-szeged.hu

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In consequence of the well-known screening examinations introduced in the developed countries, the number of patients first seen in an advanced state has declined substantially. The chances of a cure are considerably increased by early recognition and radical surgery or irradiation. However, the literature data reveal that, in spite of surgery, the disease recurs in 19-30% of the cases. As described above, the process progresses in the direction of the hormone-resistant state. In Hungary, almost 80% of prostate cancer patients are first seen in an advanced stage. After 24-36 months, total androgen blockade is ineffective, and a new treatment mode is required. The overall number of patients who are operated on and who undergo medical treatment is considerable, but, following progression, their therapy is not solved. There have been a number of reports on the use of cytostatics. Opinions are divided as to the effectiveness of such treatment. An improvement was found in 10-30% of the cases. These studies, made in the 1980s, remain valid. Chemotherapeutic research on other solid tumours can boast an advantage of close to 20 years relative to the cytostatic treatment of prostate cancer. The 14th International Congress on Anti-Cancer Treatment, held in Paris in 2003, concluded that, despite this 20-year delay, a new attitude, such as that leading to good results with other tumours, should be applied to combat prostate cancer. It is now known that advanced prostate cancer is a dynamic disease, with varying treatment targets. On this basis, we attempted to find new approaches to the therapy (5).

The curing of hormone-resistant prostate cancer remains in dispute, and at present no standard therapeutic protocol is available (6). The contradictory opinions and our own experience led us to seek appropriate treatment forms *via in vitro* model experiments carried out under experimental conditions.

In the human prostate cancer cell lines LNCaP-FGC (containing androgen receptors) and PC-3 (androgen receptor-free), measurements were made of the ability of various cytostatics (already tested in clinical practice) to inhibit cell proliferation under experimental conditions. The individual cytostatics were combined and were

supplemented with the known resistance modifiers, Anafranil and Novantrone.

We investigated the drug sensitivities of these prostate cancer cells, and measured the drug accumulation in the presence and in the absence of the resistance modifiers. The PC-3 cell line was applied in these studies. The multidrug resistance-dependent drug uptake by the tumour cells was followed flow-cytometrically *via* the uptake of Rhodamine 123, a fluorescent dye indicator that is a "cytostatic analogue".

Materials and Methods

Chemicals. The chemotherapeutic agents used in this study were Cisplatin (Platidium inj. Lachema, A.S. Brno, Czech Republic), Mitoxantrone hydrochloride (Novantron inj., Wyeth Medical Ireland, Newbridge, Ireland), Estramustine phosphate (Estracyt inj., Pharmacia and Upjohn AB, Stockholm, Sweden), Gemcitabine hydrochloride (Gemzar inj., Lilly France S.A. Fegersheim, France), Cyproterone acetate (Androcur Depot inj. Schering AG Pharma, Germany), and Clomipramine hydrochloride (Anafranil inj., Novartis, Basel, Switzerland). 3-[4,5-Dimethylthiazol-2-yl]-2-5-diphenyltetrazolium bromide (MTT) and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co, (St. Louis, USA).

Cell cultures. The LNCaP-FGC-10 cell line was isolated by Horoszewicz *et al.* from an aspiration cytological sample taken from a supraclavicular lymph node on the left side in a 50-year-old man, which had proved to be metastatic prostate cancer. The cell line contained androgen and oestrogen receptors (7). The PC-3 cell line was isolated from a bone metastasis caused by prostate cancer in a 62-year-old man; this did not contain either androgen or oestrogen receptors and was suitable for the modelling of a hormone-resistant state (8). The PC-3 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum. The LNCaP-FGC cell line was cultured in RPMI 1640 medium supplemented with 10 mM Hepes, 1.0 mM sodium pyruvate and 10% fetal bovine serum.

Cell proliferation assay. The antiproliferative effects of the chemotherapeutic agents alone and in combination were tested with the MTT assay. The compounds were diluted with culture medium in 96-well flat-bottom culture plates in 100 μ l volume/well, and 50 μ l or 100 μ l cell suspension was then added to the wells with the exception of the medium controls. 1×10^4 PC-3 cells/well and $1.5\text{--}2 \times 10^4$ LNCaP-FGC cells/well were used in these experiments. The final volume per well was 150 or 200 μ l. Cell controls and medium controls were set up in each plate. The culture plates were further incubated at 37°C for 2-3 days. After the incubation period, 20 μ l MTT solution (from 5 mg/ml stock solution), depending on the volume in the wells, was added to each well and the plates were further incubated for 4 h at 37°C. One hundred μ l SDS solution was then added to the wells and the plates were further incubated overnight at 37°C. The inhibitory effect on the cell proliferation was determined by measuring the optical densities (ODs) of the wells. The absorbance was recorded at 540 nm; the reference wavelength was 630 nm; a Multiscan EX reader was used for evaluation. The average values were calculated for parallel wells of each sample and the controls. The

percentage inhibitory effect or cytotoxic activity was determined according to the formula:

$$100 - \frac{OD \text{ sample} - OD \text{ medium control}}{OD \text{ cell control} - OD \text{ medium control}} \times 100$$

Assay of cytotoxicity. Monolayer cultures of the cells were grown in 96-well tissue culture plates for 24 h and were then treated with 100 μ l medium/well containing the compounds at different concentrations for 24 h. The cell viability was tested by means of the MTT assay and the cytotoxicity was evaluated as described above.

Fluorescence uptake assay. The effects of different compounds on the fluorescence uptake of PC-3 cells were investigated. The PC-3 cells were propagated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum. For the fluorescence uptake assay, the adherent cell culture was trypsinized and the cells were adjusted to a density of 2×10^6 /ml, resuspended in serum-free culture medium and distributed into 0.5 ml aliquots in Eppendorf centrifuge tubes. The test compounds were added to 0.5 ml cell suspensions in 1-20 μ l stock solution and the samples were incubated for 10 min at room temperature. Ten μ l of the indicator Rhodamine 123 (5.2 μ M final concentration) was then added to the samples and the cells were further incubated for 20 min at 37°C. The cells were next washed twice and resuspended in 0.5 ml phosphate-buffered saline (PBS) for analyses. The fluorescence of each cell population was measured by flow-cytometry using a Beckton Dickinson FACS scan instrument.

Results

Since Novantron (Mitoxantrone) is used as monotherapy or in combination with steroid in clinical practice, we studied its antiproliferative effect. On the LNCaP cell line, the ID₅₀ of Novantron alone was 0.04 μ g/ml. When applied in combination with 12.5 μ g/ml Anafranil, the ID₅₀ of Novantron was 0.02 μ g/ml. Besides the considerable antiproliferative effect of 25 μ g/ml Anafranil, the ID₅₀ of Novantron was in the low range (Figure 1).

On the PC-3 cell line, the ID₅₀ of Novantron was 1.25 μ g/ml. In the checkerboard experiment, a 1:2 dilution series of 5 μ g/ml Novantron was combined with 50, 25, 12.5 or 6.25 μ g/ml Melipramin on the PC-3 cell line. The ID₅₀ of Novantron in the presence of these combinations of Melipramin was <0.035, 0.035, 0.3 and 0.6 μ g/ml, respectively.

The PC-3 cell line was also subjected to combined treatment with Novantron and Depersolon. Depersolon alone and Novantron alone had ID₅₀ values of 20.0 and 1.1 μ g/ml, respectively. The ID₅₀ of Novantron in the combination varied as a function of the concentration of Depersolon: at 125 μ g/ml Depersolon in the combination, the ID₅₀ of Novantron was 0.2 μ g/ml.

It is well known that, in the event of the ineffectiveness of TAB treatment, the drug of first choice is Estracyt. On the LNCaP cell line, the ID₅₀ of Estracyt in a concentration of 100 μ g/ml was not attained.

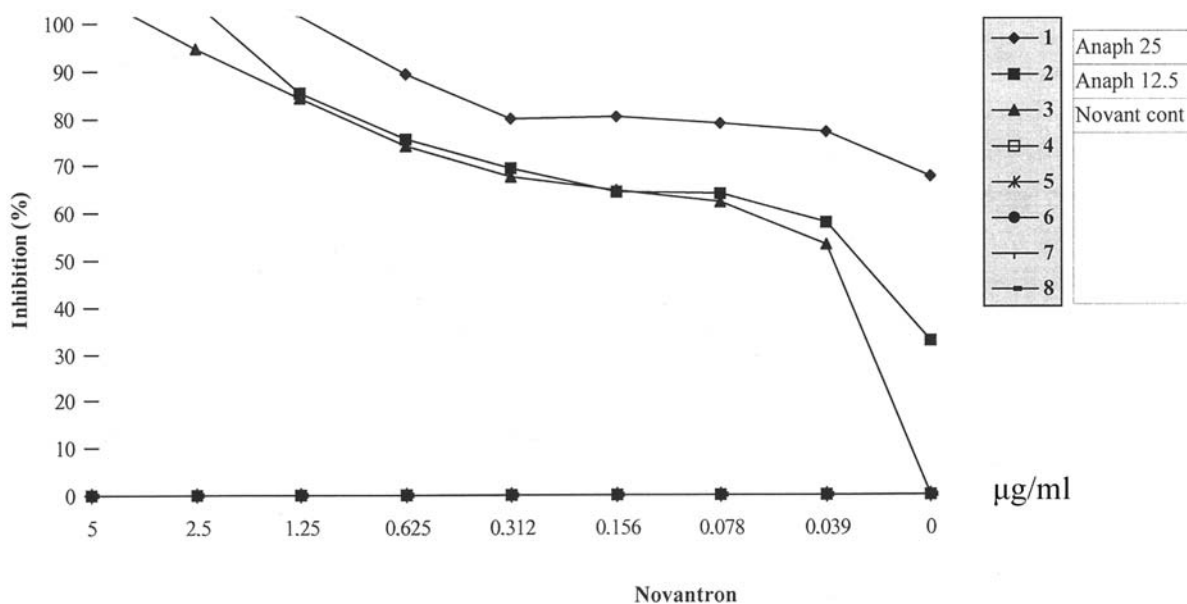


Figure 1. Combined antiproliferative effect of Novantron and Anafranil at different concentrations on the LNCaP cell line.

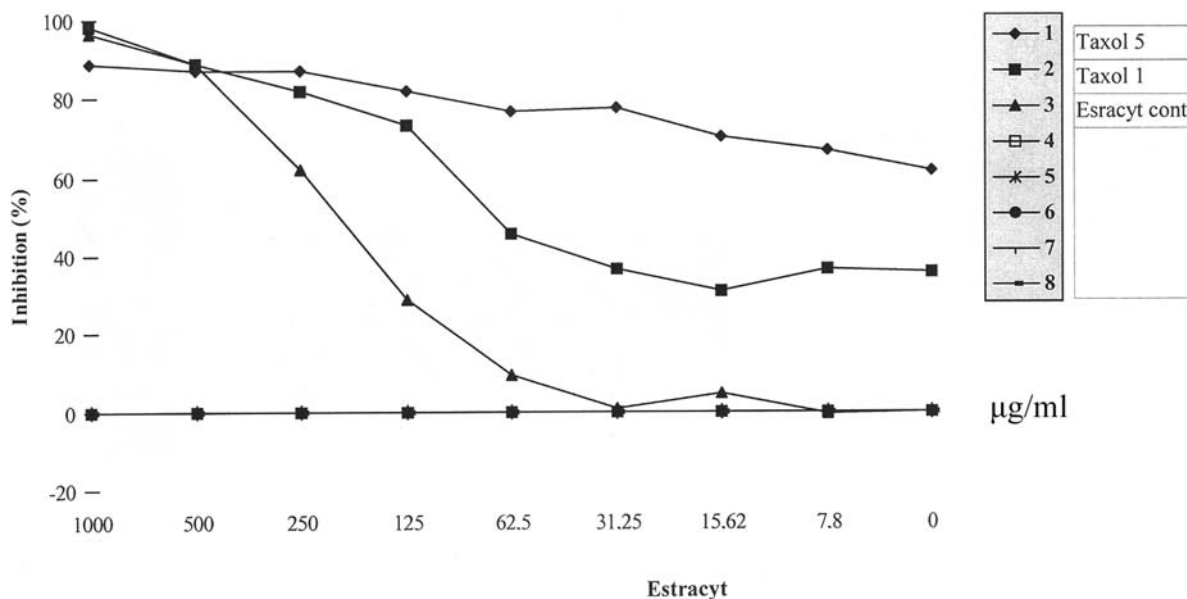


Figure 2. Antiproliferative effect of Estracyt at different concentrations combined with Taxol on the PC-3 cell line.

On the PC-3 cell line, the antiproliferative effect of Estracyt alone was indicated by an ID₅₀ of 185 μg/ml. The ID₅₀ of Estracyt was decreased significantly by its combination with 5 or 1 μg/ml Taxol. At 5 μg/ml Taxol, the ID₅₀ of Estracyt was 50 μg/ml (Figure 2).

When the effectiveness of treatment with Gemzar alone was examined on the LNCaP line, its ID₅₀ proved to be 0.01 μg/ml.

The combination of Estracyt and Gemzar was also studied on the oestrogen and androgen receptor-free PC-3 cell line. The ID₅₀ of Estracyt in combination with 1 μg/ml Gemzar was 140 μg/ml, which fell to 125 μg/ml at 10 μg/ml Gemzar. It remained at around this value independently of the amount of Estracyt, indicating that the effect of Gemzar was manifested (Figure 3).

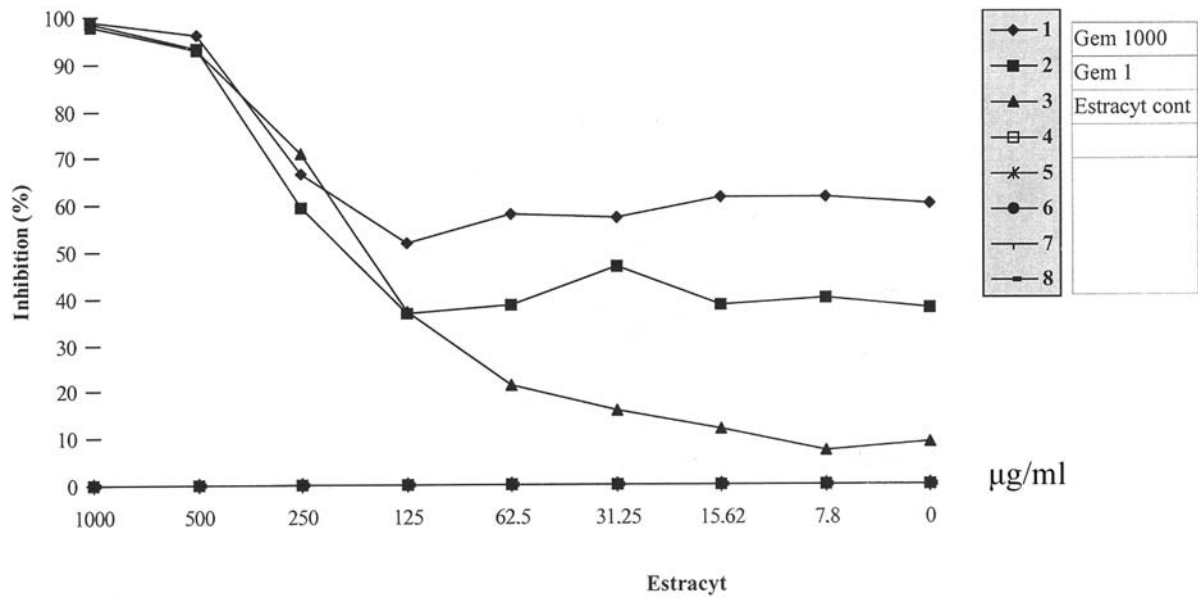


Figure 3. Antiproliferative effect of Estracyt at different concentrations combined with Gemcitabine on the PC-3 cell line.

The effect of Farmorubicin on the PC-3 line was investigated too. When the starting concentration of 100 µg/ml was varied in a dilution series of 1:2, the ID₅₀ was found to be 1.5 µg/ml. Examination of the antiproliferative effect of Farmorubicin on the LNCaP cell line, using a starting concentration of 10 µg/ml and 1:2 dilution series, revealed an ID₅₀ of 0.03 µg/ml.

The ID₅₀ of Anafranil alone as resistance modifier on the LNCaP cell line proved to lie in the range 25-50 µg/ml. The variation in the effect of Farmorubicin on the LNCaP line was investigated in the presence of this resistance modifier. On the addition of 12.5 µg/ml Anafranil, the ID₅₀ of Farmorubicin was 0.015 µg/ml; the ID₅₀ remained high when 25 µg/ml Anafranil was added (Figure 4).

Taxol as monotherapy proved effective on the PC-3 cell line. Its ID₅₀ was 3.23 µg/ml. At concentrations higher than this, the extent of inhibition was 90-100%.

The antiproliferative effect of Cisplatin too was examined on the PC-3 cell line. It may be stated that many of the preparations employed in combination in practice are effective. The ID₅₀ was found to be 6.13 µg/ml (Figure 5).

Of the resistance modifiers, the effects of Pipolphen and Anafranil on the PC-3 line were studied. The ID₅₀ of Pipolphen was 50 µg/ml, while that of Anafranil was 25-50 µg/ml.

The mechanism of action of the GnRH analogues is known: they exhaust the receptors of the tumour tissue according to a cascade principle. Their antiproliferative effects on cells are not known. Hence, we studied their

possible antiproliferative action on PC-3 cells. Our investigations revealed that both the Decapeptyl depot and Lucrin depot were without effect.

Of the antiandrogens, the antiproliferative action of Androcur on the LNCaP line was studied. This agent did not have any essential effect on cell proliferation.

The cells of the PC-3 line exhibited a significant Rhodamine 123 accumulation. Depending on their concentrations, Novantron and Estracyt slightly depressed the Rhodamine 123 uptake by the cells. A study was made of the drug interactions in the Novantron-Depersolon and Novantron-Estracyt combinations, which caused further decreases in the Rhodamine 123 uptake by the tumour cells. The labelling was investigated in the presence of various resistance modifiers (Verapamil, Melipramin and Pipolphen). It emerged that the Rhodamine 123 accumulation decreased in response to the customary resistance modifiers, but the fluorescence of the cytosol barely changed; they were ineffective on this cell line.

Discussion

Novantron as monotherapy was effective on both cell lines. When Novantron was combined with Anafranil treatment on the LNCaP cell line, the ID₅₀ decreased considerably, since Anafranil itself has an antiproliferative effect on both cell lines. This observation can be utilized in clinical practice. Anafranil reduces pain and appreciably improves the condition of the patient as an antidepressant. If

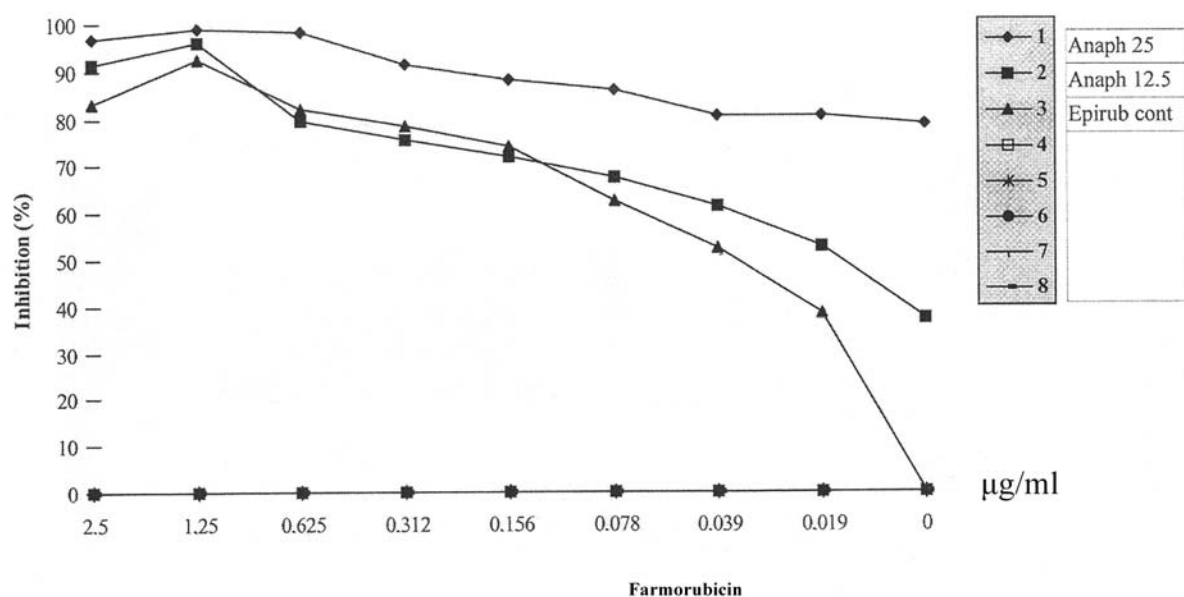


Figure 4. Combined antiproliferative effect of Farmorubicin and Anafranil at different concentrations on the LNCaP cell line.

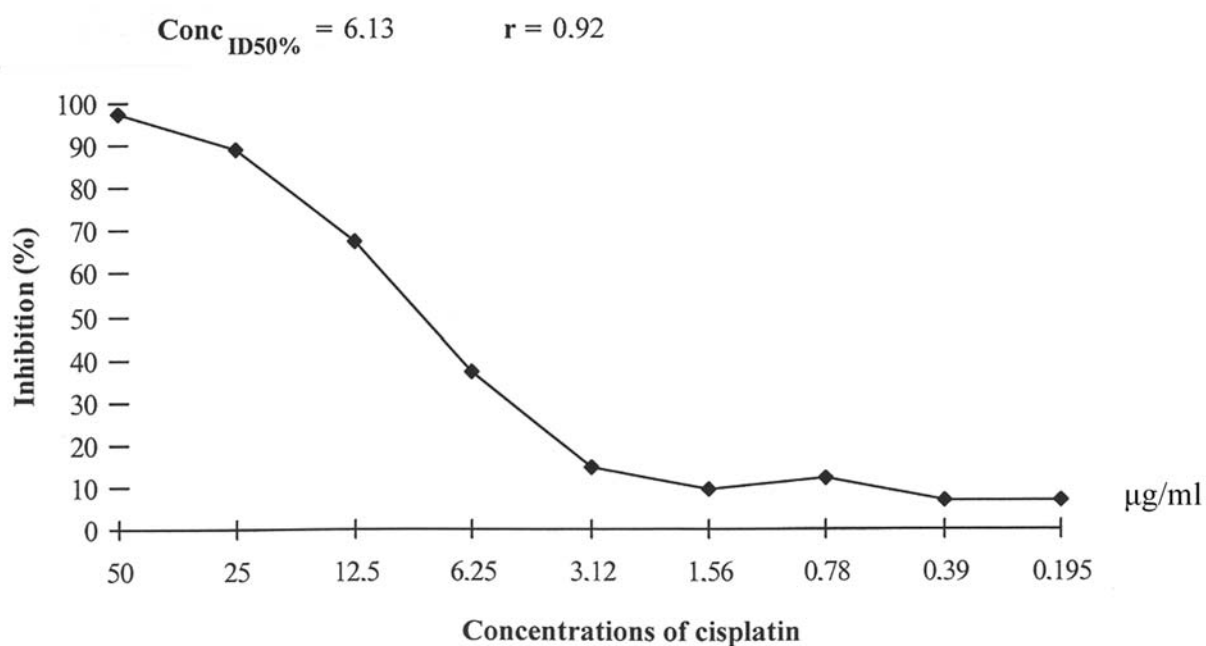


Figure 5. Antiproliferative effect of Cisplatin at different concentrations on the PC-3 cell line.

Anafranil enhances the action of Novantron, a combination of the two drugs may be effective *in vivo*.

The ID₅₀ of Novantron was substantially higher on the PC-3 cell line than on the LNCaP cell line. This may possibly indicate that the effect may be achieved at a higher dose in hormone-resistant states.

The effect of Melipramin monotherapy (similar to that of Apomorphine) on the PC-3 cell line revealed that it did inhibit cell proliferation, but less markedly than did Anafranil.

On the PC-3 cell line, combined treatment with Novantron and Melipramin considerably modified the ID₅₀

of Novantron. As an antidepressant, Melipramin may be administered as supplementary treatment in clinical practice, and it cannot be excluded that it improves the effect of Novantron.

Novantron and Depersolon treatment proved effective on the PC-3 cell line, and this is a mode of therapy applied clinically. The steroid treatment results in a temporary substantial improvement, but, unfortunately, the condition of the patient deteriorates within a short time, and death generally occurs within 6 months.

Estracyt is the drug of choice after primary TAB treatment. This drug was originally intended for the treatment of breast cancer, but, interestingly, it acquired an important role in the therapy of prostate cancer (9). Because of its oestrogen content, this product decreases the serum testosterone level, while its mustard nitrogen content allows it to act as an alkylating agent, inhibiting the proliferation of tumour cells by blocking the microtubules (10). It exerts an antiproliferative effect on both the PC-3 and the LNCaP cell lines.

The combination of Estracyt and Taxol in clinical practice is known from the literature (11). We found that 5 µg/ml Taxol decreased the ID₅₀ of Estracyt to nearly one-third in the LNCaP cell line.

Gemzar, as monotherapy, was effective even in very low amounts on the LNCaP cell line, and it is known from the literature that it has a strong antiproliferative effect on both cell lines examined here (12).

A study was made of the co-administration of Estracyt and Gemzar to the cells of the LNCaP line, which contains oestrogen and androgen receptors. Ten µg/ml Gemzar decreased the ID₅₀ of Estracyt considerably. Then, independently of the quantity of Estracyt, the level of inhibition remained at about the same level in consequence of the effect of Gemzar.

We have applied Farmorubicin most frequently in clinical practice. This drug was effective on both cell lines. On the PC-3 and LNCaP cell lines, the ID₅₀ was 1.5 µg/ml and 0.03 µg/ml, respectively. Accordingly, the latter cells responded more sensitively to this monotherapy.

When the effect of Farmorubicin was investigated on the LNCaP cell line in the presence of the resistance modifier Anafranil, it was observed that 12.5 µg/ml Anafranil considerably reduced the ID₅₀ of Farmorubicin, from 0.03 µg/ml to 0.015 µg/ml.

On the basis of their mechanisms of action, the GnRH analogues, Decapeptyl depot and Lucrin depot, were not expected to exhibit antiproliferative effects, and they were indeed found to be ineffective.

The products of multidrug resistance genes are able to pump certain chemotherapeutic agents out of cells. One of the causes of the ineffectiveness of many drugs may be their intracellularly decreased concentration. The best known member of the transporter family is MDR1; this gives rise

to the product P-glycoprotein (Pgp), an ATP-binding transmembrane transport protein. Operating as an efflux pump, this reduces the intracellular concentrations of antitumour drugs to below the therapeutic level. Pgp170, coded by the MDR1 gene, is a glycolyl transmembrane protein. Its efflux pump activity was first described by Gottesmann *et al.* (13, 14). A number of agents are known which inhibit the pump mechanism *in vitro*. In our experiments, we applied the resistance modifiers Verapamil, Melipramin and Pipolphen. These compounds did not alter the extent of drug uptake by the cells. It could, therefore, be stated that the efflux pump was not operating. The reason for this may have been that the examined cell line did not contain Pgp.

Conclusion

Under experimental conditions, known cytostatics were demonstrated to be effective on two different prostate cancer cell lines. Therapy with Taxol and Gemzar is currently undergoing clinical testing in cases of advanced prostate cancer. These drugs have not yet been officially approved for such treatment in Hungary, though their application would represent a new possibility in therapy.

It is noteworthy that Farmorubicin, Estracyt and their combination exhibited low ID₅₀ values on the LNCaP cell line, which contains oestrogen and androgen receptors. Consequently, it is perhaps not necessary to wait until the development of a hormone-resistant state before cytostatic administration. Systematic following of the PSA is of good prognostic value: if the post-TAB treatment PSA level increases 2-fold, it may be recommended to change the treatment mode to mono- or combined chemotherapy. Multidrug resistance efflux activity responsible for tumour resistance was definitely not operative in the prostate cancer cell line investigated. It follows from this that the hormone resistance that developed here requires further clarification.

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