Abstract. Background: The active metabolite of vitamin D₃, 1,25(OH)₂D₃, is known to possess anti-proliferative and pro-differentiative activities in prostate cancer (PCa) cells. However, its clinical use is limited because of the risk of hypercalcemia. Concurrent administration of lower doses of 1,25(OH)₂D₃ together with other anticancer drugs may help to overcome this obstacle and lead to an effective and tolerable therapy. In the present in vitro study, we investigated the combined anti-cancer effect of 1,25(OH)₂D₃ and ibuprofen, a well-known non-steroidal anti-inflammatory drug (NSAID) that is also recognized for its ability to reduce prostate cancer development. Materials and Methods: An androgen-sensitive prostate cancer cell line (LNCaP), grown in medium containing androgen (5α-dihydrotestosterone (DHT)) or without it, was treated with 1,25(OH)₂D₃ or ibuprofen alone or with a combination of both drugs. The effects of the treatments on LNCaP cell proliferation, cell cycle and apoptosis were evaluated by the thymidine incorporation method, the propidium iodide method and ELISA, respectively. The unpaired t-test was used for statistical analysis. Results: Simultaneous treatment of LNCaP cells grown without DHT with 10 nM 1,25(OH)₂D₃ and 0.2 mM ibuprofen decreased cell growth by 42% (p<0.001, compared to the control cells). This effect was found to be additive since each single drug reduced cell proliferation by only 24%. On the other hand, highly significant synergistic cell growth inhibition (67%, p<0.001) was achieved by combined treatment of 1,25(OH)₂D₃ and ibuprofen in DHT-stimulated LNCaP cells. This combined treatment was also found to be effective in decreasing the cell transition from G₁- to S-phase (p<0.003) and effective in enhancing apoptosis. Conclusion: Although both 1,25(OH)₂D₃ and ibuprofen demonstrate in vitro anti-carcinogenic activities as a single drug treatment, the present results showed that the combined use of 1,25(OH)₂D₃ with ibuprofen is superior to treatment with a single drug.

In addition to its classic role in regulating calcium and phosphate homeostasis (1), the active metabolite of vitamin D, 1,25-dihydroxyvitamin D₃(1,25(OH)₂D₃) possesses anti-proliferative and pro-differentiative activities in a variety of normal and malignant cells (2-4). Vitamin D deficiency is one of the major factors playing a role in prostate cancer (PCa) development. The hypothesis that vitamin D deficiency increases the risk of PCa was initially put forward by Schwartz and Hulka (5). This deficiency occurs quite frequently in aging men (6), and is a result of reduced efficiency in pre-vitamin D photo-production in advanced age (7, 8). It is established that mortality rates from PCa are inversely proportional to ultraviolet radiation (9, 10), and that patients with reduced serum 1,25(OH)₂D₃ levels have an increased risk of PCa (11, 12). Besides systemic vitamin D deficiency, reduced synthesis of 1,25(OH)₂D₃ by the prostate gland in PCa patients, as well as in human PCa cell lines, resulting from significant reduction in activity of the enzyme 25-hydroxyvitamin D-1-hydroxylase, has been observed (13). Restoration of 1α-hydroxylase activity by transfection of PCa cells (LNCaP) with 1α-hydroxylase cDNA has been found to restore the conversion of 25-OH-D₃ to the hormonal form of vitamin D (1,25(OH)₂D₃) and resulted in an anti-proliferative activity of 25-OH-D₃ in these cells (14, 15). However, the therapeutic use of this hormonal form of vitamin D as an anti-cancer agent is limited because of its hypercalcemic effect (16). Combined treatment of vitamin D with other anti-cancer agents can help to overcome this obstacle. A combination of two drugs may allow the use of lower doses of each drug, thus reducing the risk of adverse reactions. In a recent clinical study, a combination of 1,25(OH)₂D₃, and docetaxel therapy was found to be effective and well tolerated by PCa patients (17).
Materials and Methods

Materials. 1,25(OH)2D3 was kindly provided by Hoffmann La-Roche (Basel, Switzerland). DHT and ibuprofen were purchased from Sigma (St. Louis, MO, USA). [3H]-thymidine (3H-dTTP) was obtained from Basal, Switzerland. DHT and ibuprofen were purchased from Sigma, and nystatin, L-glutamine, HEPES buffer, sodium pyruvate and heat-inactivated fetal calf serum (FCS) was acquired from Beit Haemek, Israel. The ELISA kit for apoptosis detection was purchased from Roche Diagnostics Gmbh (Roche Diagnostics GmbH). Stock solutions of 1,25(OH)2D3 and DHT (1x10-4-1x10-5 M and 2x10-6 M, respectively) were prepared in ethanol and stored at –20°C in the dark. Ibuprofen (2 M) was dissolved in ethanol and stored at room temperature in the dark. The final concentrations of ethanol in the cell culture media did not exceed 0.1%.

Cell culture and treatment. LNCaP cells were cultured in culture flasks (Corning) in RPMI-1640 medium with phenol red, supplemented with 10% FCS, 1% penicillin/streptomycin/nystatin, HEPES buffer, sodium pyruvate and heat-inactivated fetal calf serum (FCS) was acquired from Beit Haemek, Israel. The ELISA kit for apoptosis detection was purchased from Roche Diagnostics GmbH. Stock solutions of 1,25(OH)2D3 and DHT (1x10-4-1x10-5 M and 2x10-6 M, respectively), subsequently were prepared in ethanol and stored at –20°C in the dark. Ibuprofen (2 M) was dissolved in ethanol and stored at room temperature in the dark. The final concentrations of ethanol in the cell culture media did not exceed 0.1%.

Cell growth. Six thousand LNCaP cells suspended in 100 μL of medium, supplemented with 3% FCS, were seeded in each well of a 96-well plate (Corning). The outer border wells in each plate were filled with medium only. After 2 days of incubation, 70 μL of cell medium was replaced by the same volume of medium containing 3% FCS and different compounds: i) 0.1% ethanol (control), ii) 1,25(OH)2D3 (10 nM), iii) ibuprofen (0.2 mM), iv) combination of 1,25(OH)2D3 (10 nM) and ibuprofen (0.2 mM). In a parallel series of experiments, the same treatments were tested in DHT (1nM)-stimulated cells. The treated cells were incubated for an extra 48 h. Each treatment was tested in 10 repetitions for each experiment.

Thymidine incorporation and cell harvesting. Following the incubation period, 50 μL of medium containing 5 μCi 3H-thymidine were added to each well. The cells were incubated for 3 h under the same conditions. The cells' DNA was harvested on a glass micro-filter (934-AH, Tamar Equipment, Israel) by an Inotech cell harvester. Unincorporated 3H-thymidine was removed by 6 repeated washes of the filter with water. The dried filter with the radioactive-labelled DNA was exposed against a radioactive sensitive image plate (Fuji) and locked in the dark in a sealed box for two days. The image plate was detected by a Fuji BAS-1000 scanner and the results quantified using BAS-1000 software on a Macintosh Quadra 840 av.

Cell cycle and apoptosis. Aliquots of 5 ml LNCaP cell suspension (500,000 cells) were seeded in 25-cm² flasks (Corning). After incubation for 2 days, the medium was replaced by the same volume of medium containing 3% FCS and the specific tested compound. Treatments were as in the proliferation experiments. LNCaP cell cycle was assessed by the propidium iodide (PI) method. Briefly, cells were detached from the flask by light trypsinization, washed and centrifuged in phosphate-buffered saline (PBS), pH 7.4. One million washed cells were fixed in ice-cold 70% ethanol at 4°C for 30 min and stored at –20°C until analysis. Prior to analysis, the fixed cells were washed twice with PBS and resuspended in 1.0 mL of PBS containing 0.1% Triton X-100, 100 μg Dnase free Rnase A and 15 μg of PI (Sigma-Aldrich). Then the cells were incubated in dark at room temperature for 30 min before being analyzed on a FACScan flow cytometer (Becton Dickinson) for DNA content. The percentage of cells in each phase of the cell cycle was determined by a ModFit LT computer program (Verity Software, House). The one-step sandwich ELISA (Roche Diagnostics GmbH) method was used for apoptosis detection. Histone-complexed DNA fragments were measured in cell lysates as follows: 3x104 LNCaP cells from control and from each treatment were resuspended in 200 μL of lysis buffer. The suspension was incubated for 30 min and centrifuged at 200 x g for 10 min. Aliquots of 20 μL of each supernatant were applied for analysis. ELISA determinations of apoptosis were performed in accordance with the manufacturer's instructions.

Statistical analysis. The results were evaluated by the unpaired t-test. Differences were considered statistically significant when p ≤ 0.05. The results are expressed as mean±SEM.

Results

As shown in Figure 1, incubation of LNCaP cells with 10 nM 1,25(OH)2D3 resulted in 24% inhibition of cell proliferation. This inhibition was found to be highly significant (p<0.001). Similar results were obtained following incubation with 0.2 mM ibuprofen. However, incubation of LNCaP cells with both agents, as a combined treatment, resulted in an additive inhibitory effect of 42% of cell growth inhibition (p<0.001). Incubation of LNCaP cells with DHT...
augmented the proliferation of these cells by 83%, as compared to untreated cells \((p<0.001)\). Treatment of DHT-stimulated LNCaP cells with 1,25(OH)₂D₃ (10 nM) increased the rate of cell growth inhibition to 30.5% (Figure 2). On the other hand, addition of DHT to the medium did not modify the potency of ibuprofen to inhibit cell growth compared to the experiment without DHT (Figures 1 and 2). However, combined treatment of these two agents in DHT-stimulated LNCaP cells resulted in a synergistic and highly effective (67%) suppression of LNCaP cell proliferation (Figure 2).

The effects of 1,25(OH)₂D₃ (10 nM) and of ibuprofen (0.2 mM) on the cell cycle were analyzed alone or in combination in DHT-stimulated and non-stimulated LNCaP cells. As summarized in Table I, incubation of unstimulated LNCaP cells with 1,25(OH)₂D₃ did not affect the ratio between non-proliferating cells (G₁-phase) and the proliferating cells (S- and G₂-M-phases). However, treatment of these cells with ibuprofen significantly decreased the percentage of cells in the proliferative state from 28.2% to 20.8±1.0% \((p<0.006)\). Combined treatment with both compounds did not change the cell cycle (Table I). It seems that 1,25(OH)₂D₃ moderated the inhibitory effect of ibuprofen on the cell cycle. On the other hand, addition of androgen (DHT) to the medium changed the effect of these drugs on the cell cycle (Table II), resulting in the accumulation of LNCaP cells in the non-proliferating G₁-phase and decreasing the percentage of proliferating cells (S- and G₂-M-phases). This effect was found to be significant when cells were treated by ibuprofen, and developed into a synergistic effect when the cells were treated by both agents simultaneously.

The effects on apoptosis of DHT-stimulated LNCaP cells were tested by the Cell Death Detection ELISA PLUS kit. The results, expressed by the enrichment factor (Absorbance U of sample/Absorbance U of control), showed that, compared to the control cells, 1,25(OH)₂D₃ increased apoptosis by 1.7-fold, and ibuprofen by 2.2-fold. The combined treatment of both agents caused a 4.9-fold synergistic enhancement of apoptosis.

**Discussion**

Although an anticancer effect of vitamin D has already been demonstrated (5, 11, 12), this is the first study to investigate the combined effect of 1,25(OH)₂D₃ and a NSAID (ibuprofen) on PCa cells. The results demonstrated that concurrent treatment of LNCaP cells with 1,25(OH)₂D₃ and ibuprofen resulted in an additive inhibition of cell growth (Figure 1). A similar additive effect, but on HL-60 promyelocytic leukemia cells, was described by Sokoloski and Sartorelli (23), who studied differentiation of these cells treated by a combination of NSAIDs with low levels of vitamin D3. We and others (24, 26) have shown that the addition of
optimal concentrations of DHT to the cell medium stimulates the growth of androgen-responsive PCA cells. Supplementation of 1 nM DHT to 3% FCS medium increased the growth inhibitory effect of vitamin D on LNCaP cells from 24% to 30.5%, but did not alter the effect of ibuprofen. However, combined treatment of these two agents in DHT-stimulated LNCaP cells resulted in a synergistic and highly effective (67%) suppression of LNCaP cell proliferation (Figure 2), while in the absence of androgen, combined treatment resulted in a suppression of cell proliferation by only 42% (Figure 1). The role of DHT in the anti-proliferative action of vitamin D demonstrated in the present study is supported by the results of Bao et al. (27), who studied the effect of vitamin D on androgen-sensitive LNCaP and CWR22R cell lines. These investigators found a reduction in vitamin D-mediated growth inhibition when androgen receptors were blocked by anti-androgens. This cross-talk between vitamin D and androgens was also demonstrated by Lou et al. (28), who showed a significant inhibition of 1,25-(OH)2D3-induced expression of 24-hydroxylase in LNCaP cells by DHT, which resulted in decreased catabolism of 1,25(OH)2D3. Our results, with DHT-24-hydroxylase in LNCaP cells by DHT, which resulted in transition from G1- to S-phase (Table II), and by increased combination (Figure 2) can be explained by decreased proliferation by 1,25(OH)2D3, or ibuprofen, or their combination (Table I). These findings are in contradiction with the results of the cell proliferation test that was performed under the same conditions (Figure 1). Zhuang and Brunstein (29) and Moffatt et al. (30) also did not find an effect of 1,25(OH)2D3 on the cell cycle of a PCA cell line, ALVA 31, despite significant cell growth suppression. Moffatt et al. (30) have suggested that 1,25(OH)2D3 may decrease cell proliferation by slowing the rate of cell progression through the S- and G2-M-phases. This retardation of cell cycle progression could not be measured by standard flow cytometric cell cycle analysis (30). Based on this explanation and on our results, it is possible that 1,25(OH)2D3 suppresses androgen non-stimulated LNCaP cell growth by decreasing the transition rate through the S-phase and G2-M-phases, but does not affect the transition from G1- to S-phase. On the other hand, treatment with 1,25(OH)2D3 of DHT-stimulated LNCaP cell resulted in a slow transition from G1- to S-phase and, consequently, to an accumulation of cells in G1-phase. We also showed that increased apoptosis is another explanation for LNCaP cell proliferation retardation. We demonstrated a 1.7-fold increase in apoptosis of DHT-stimulated LNCaP cells following treatment with 1,25(OH)2D3. While a similar increase in apoptosis (2.2-fold) was obtained by ibuprofen treatment, the combined treatment with both agents yielded a 4.9-fold increase in apoptosis. Hence, it is suggested that the mode of action by which 1,25(OH)2D3 and ibuprofen inhibit the proliferation of the prostate cancer cell line LNCaP is by increasing apoptosis and inhibiting cell cycle transition.

In conclusion, the present results point out the role of the active metabolite of vitamin D in inhibiting PCA cell proliferation. The combined treatment of 1,25(OH)2D3 with the NSAID drug ibuprofen was found to be superior in comparison to treatment with each drug alone. However, this finding should be further tested in in vivo models. The present results also clearly suggest that treatment and prevention of PCA by NSAIDs is more effective in patients with higher blood levels of 1,25(OH)2D3. The combined therapeutic use of 1,25(OH)2D3 with a NSAID in PCA patients will allow administration of lower doses of

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### Table I. Cell cycle distribution of LNCaP cells treated with 1,25(OH)2D3 and ibuprofen (n=4, mean±SEM).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>G1-phase</th>
<th>S+G2-M-phases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>71.8±1.4</td>
<td>28.2±1.4</td>
</tr>
<tr>
<td>1,25(OH)2D3 10 nM</td>
<td>71.9±0.9</td>
<td>28.1±0.9</td>
</tr>
<tr>
<td>Ibuprofen 0.2 mM</td>
<td>79.2±1.0</td>
<td>20.8±1.0</td>
</tr>
<tr>
<td>1,25(OH)2D3 10 nM + Ibuprofen 0.2 mM</td>
<td>74.8±1.4</td>
<td>25.2±1.4</td>
</tr>
</tbody>
</table>

* a = p<0.006 compared to control

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### Table II. Cell cycle distribution of DHT stimulated LNCaP cells treated with 1,25(OH)2D3 and ibuprofen (n=4, mean±SEM).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>G1-phase</th>
<th>S+G2-M-phases</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHT 1nm</td>
<td>73.4±1.7</td>
<td>26.6±1.6</td>
</tr>
<tr>
<td>DHT 1nm + 1,25(OH)2D3 10 nM</td>
<td>76.0±1.2</td>
<td>24.0±1.3</td>
</tr>
<tr>
<td>DHT 1nm + Ibuprofen 0.2 mM</td>
<td>79.8±0.7</td>
<td>20.2±0.8</td>
</tr>
<tr>
<td>DHT 1nm + 1,25(OH)2D3 10 nM + Ibuprofen 0.2 mM</td>
<td>83.5±1.1</td>
<td>16.5±1.2</td>
</tr>
</tbody>
</table>

* a = p<0.013 compared to control, b = p<0.003 compared to control
1,25(OH)2D3 without the risk of hypercalcemia. Moreover, as some of the less calcemic vitamin D analogs are as equipotent as 1,25(OH)2D3 in inhibiting cancer cell growth (4), it is likely that their combination with a NSAID will be as efficient and will have the advantage of being a safer therapeutic procedure for the treatment of PCa.

References