Abstract. Background: Targeting of the epidermal growth factor receptor (EGFR) pathway is a promising treatment strategy for aggressive androgen-refractory prostate cancer (PCa). The effect of treating the androgen-resistant PCa cell line DU145 with a combination of the anti-EGFR drug cetuximab and 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) was evaluated. Materials and Methods: DU145 cells were treated with 5 nM cetuximab, 100 nM 1,25(OH)₂D₃ or a combination of both. The effect of the treatments on cell growth, cell-cycle and apoptosis was evaluated. Results: Single-drug treatments decreased DU145 cell growth by up to 25% and caused a 1.5- to 1.7-fold increase of apoptosis, but did not affect the cell-cycle distribution. However, dual treatment with a combination of cetuximab and 1,25(OH)₂D₃ inhibited DU145 cell proliferation by 40%, caused considerable cell-cycle arrest in the G₀/G₁-phase, and enhanced apoptosis by 2.5-fold (compared to the control, p<0.0001, p<0.006 and p<0.0001, respectively). Conclusion: A combination of cetuximab and 1,25(OH)₂D₃ efficiently suppresses hormone-resistant PCa cell growth and could provide a basis for its clinical application.

The development of androgen resistance is a common outcome in patients with advanced prostate cancer (PCa), resulting in fewer therapeutic options and a limited median survival of 10-12 months for patients (1). The progression from hormone-dependent to hormone-independent prostate cancer disease is accompanied by activation of the epidermal growth factor receptor (EGFR) family and its cross-talk with androgen receptor pathways (2). The EGFR is a transmembrane tyrosine kinase receptor which is stimulated by growth factors such as EGF or transforming growth factor alpha (TGF-α), both of which bind to the extracellular domain of the receptor. Ligand binding induces receptors to dimerise, and dimer formation leads to activation of the intrinsic receptor tyrosine kinase domain. This tyrosine kinase activation results in autophosphorylation within the C-terminal tail of the EGFR in the cytoplasm. Signaling complexes are then formed in the cytoplasm and activate gene transcription, which, in turn, induces responses such as cell proliferation (3, 4). In addition, the EGFR-EGF complex may be internalized and translocated to the nucleus to act as a nuclear transcription factor. It has been shown that nuclear accumulation of EGFR correlates with increased expression of cyclin D1, inducible nitric oxide synthase (iNOS) and B-Myb (5-7), all of which are associated with increased cell proliferation. The role of EGFR-pathway activation in malignancy is supported by the findings of Fry et al. (8) and Bos et al. (9). These authors demonstrated that targeting the receptor catalytic domain of the EGFR by small molecule tyrosine kinase inhibitors (TKIs) causes an anticancer effect. TKIs inhibit the growth of cancer cells by inducing cell-cycle arrest and/or apoptosis (10). The essential role of the nuclear EGFR-pathway in malignancy was confirmed by findings which demonstrated an anticancer effect of compounds that inhibit nuclear translocation of the EGFR-EGF complex and consequently decrease EGFR function as a transcription factor (11). Based on these evidence, several anti-EGFR strategies that target different components of the EGFR-pathway have been developed (12-14).
The goal of this research was to study the effect of a dual treatment composed of cetuximab (a newly developed anti-EGFR antibody) and 1,25-dihydroxyvitamin D₃; (1,25(OH)₂D₃; the vitamin D active metabolite) on the androgen-resistant prostate cancer cell line DU145. Binding of cetuximab to the EGFR prevents stimulation of the receptor by endogenous ligands and results in inhibition of cell proliferation, enhanced apoptosis, and reduced angiogenesis, invasiveness and metastasis (15). 1,25(OH)₂D₃ in addition to its physiological regulation of calcium and phosphate homeostasis, acts as an antineoplastic agent (16, 17, 18). It has been shown that the inhibition of EGF-stimulated cancer cell growth is one of the mechanisms by which 1,25(OH)₂D₃ exerts its anticancer activity (19). Thus, it was hypothesized that a combination of cetuximab and 1,25(OH)₂D₃ might effectively inhibit the EGFR-pathway and result in a strong suppression of PCA cell growth.

Materials and Methods

**Materials.** Cetuximab was a generous gift of Merck KGaA, Darmstadt, Germany. 1,25(OH)₂D₃ was kindly provided by Hoffmann La-Roche, Basel, Switzerland. Tissue culture media RPMI-1640, 0.25% trypsin, penicillin with streptomycin and nystatin, L-glutamine, HEPES buffer, sodium pyruvate, and heat-inactivated fetal calf serum (FCS) were acquired from Beit Haemek, Israel. The APOPercentage™ apoptosis assay kit was purchased from Biocolor Ltd. (BT36 7LS, Newtonabby, Northern Ireland). Cetuximab solution (2 mg/ml) was kept at 2-6°C. Stock solution of 1,25(OH)₂D₃ (100 µM) in ethanol was stored at −20°C in the dark.

**Cell culture and treatment.** The DU145 androgen refractory prostate cancer cell line (American Tissue Culture Collection, Manassas, VA, USA) was cultured in culture flasks (Corning) in RPMI-1640 medium containing phenol red supplemented with 10% FCS, 1% penicillin/streptomycin/nystatin, L-glutamine, HEPES buffer, sodium pyruvate, and heat-inactivated fetal calf serum (FCS) were acquired from Beit Haemek, Israel. The APOPercentage™ apoptosis assay kit was purchased from Biocolor Ltd. (BT36 7LS, Newtonabby, Northern Ireland). Cetuximab solution (2 mg/ml) was kept at 2-6°C. Stock solution of 1,25(OH)₂D₃ (100 µM) in ethanol was stored at −20°C in the dark.

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**Apoptosis assay.** Thirty thousand cells (1 ml) per well were seeded in 24-well plates. The medium was replaced the following day with 1 ml of the medium containing the test compound. At the end of the treatment period, the APOPercentage dye was added to the cells and incubated for 1 h at 37°C (21). Unabsorbed dye was removed by two consecutive gentle washes of the cells with PBS. The dye absorbed by the cells which underwent apoptosis was released by adding 300 µl of APOPercentage dye release reagent; 270 µl of the released dye were transferred to 96-well plates and the OD was measured at 550 nm using a microplate reader. To normalize the OD measured in the apoptosis test to the cell number, the OD of the assay of apoptosis was divided by the OD of the growth test also performed in 24-well plates. The results of the apoptosis assay on the treated cells were expressed as the ratio of the values of the control cells. The APOPercentage apoptosis assay used in the present study is based on the ability of the cells undergoing apoptosis to uptake APOPercentage dye.

**Statistical analysis.** Results were evaluated using the unpaired t-test. Differences were considered statistically significant when p<0.05. Results are expressed as mean±SEM.

**Results**

The cell growth experiments showed that 100 nM 1,25(OH)₂D₃ and 5 nM cetuximab applied as single drugs suppressed DU145 cell growth by approximately 25% as compared to untreated cells (p<0.0001) (Figure 1). However, a combination of both drugs was much more effective and inhibited prostate cancer cell proliferation by 40%. This inhibition was found to be highly significant compared to both the control and the single treatments (p<0.0001).
In contrast to the cell growth experiments, cell-cycle analysis did not reveal any effect of 1,25(OH)\textsubscript{2}D\textsubscript{3} or cetuximab applied alone on the cell-cycle distribution of DU145 cells (Table I). However, the combination of the two drugs notably changed the cell-cycle distribution pattern (Figure 2) as evidenced by a considerable accumulation of DU145 cells in the G\textsubscript{0}/G\textsubscript{1} stage and the decreased percentage of these cells in the S-phase of the cell-cycle (\(p<0.03\) and \(p<0.02\), compared to control, respectively) (Table I).

The APOPercentage apoptosis assay demonstrated enhanced apoptosis of DU145 cells in response to treatment with either 1,25(OH)\textsubscript{2}D\textsubscript{3} or cetuximab alone (Figure 3). Treatment with 1,25(OH)\textsubscript{2}D\textsubscript{3} increased apoptosis 1.7-fold, while cetuximab increased apoptosis 1.5-fold (\(p<0.0001\) and \(p<0.0002\), compared to the control, respectively). The combined treatment was especially effective, bringing about a 2.5-fold increase in DU145 cell apoptosis (\(p<0.0001\) compared to the control and single drug treatments).

![Figure 1. Effects of 1,25(OH)\textsubscript{2}D\textsubscript{3}, cetuximab and their combination on DU145 cell growth. a: \(p<0.0001\) compared to control; b: \(p<0.0001\) compared to 1,25(OH)\textsubscript{2}D\textsubscript{3} and cetuximab.]

![Table I. Cell-cycle distribution of DU145 cells treated with 1,25(OH)\textsubscript{2}D\textsubscript{3}, cetuximab or their combination (n=5, mean±SEM).](attachment:Table_I.jpg)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>G\textsubscript{0}/G\textsubscript{1}-phase (%)</th>
<th>S-phase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>47.4±3.1</td>
<td>27.0±2.3</td>
</tr>
<tr>
<td>1,25(OH)\textsubscript{2}D\textsubscript{3}</td>
<td>48.6±4.3</td>
<td>24.7±3.1</td>
</tr>
<tr>
<td>cetuximab</td>
<td>49.4±2.0</td>
<td>24.5±1.2</td>
</tr>
<tr>
<td>1,25(OH)\textsubscript{2}D\textsubscript{3} and cetuximab</td>
<td>58.4±2.5\textsuperscript{a}</td>
<td>19.1±1.4\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\(\textsuperscript{a}p<0.03\) compared to control; \(\textsuperscript{b}p<0.02\) compared to control.

![Figure 2. Effect of combined treatment with cetuximab and 1,25(OH)\textsubscript{2}D\textsubscript{3} on the DU145 cell-cycle distribution.]

![Figure 3. Effect of cetuximab, 1,25(OH)\textsubscript{2}D\textsubscript{3} and of their combination on DU145 cell apoptosis. a: \(p<0.0001\), b: \(p<0.0002\) compared to control; c: \(p<0.0001\) compared to 1,25(OH)\textsubscript{2}D\textsubscript{3} and cetuximab.]

Discussion

The present study showed that the combined treatment of androgen-resistant PCa cells was much more effective than treatment with cetuximab or 1,25(OH)2D3 alone.

The combined treatment suppressed DU145 cell growth by 40% while single-drug treatment decreased cancer cell growth by only 25%. The high efficiency of the combined treatment was also demonstrated by more significant apoptosis of PCa cells ($p<0.0001$) compared to single-drug treatments. Moreover, the combined treatment notably delayed progression through the cell-cycle and induced a significant accumulation ($p<0.03$) of cancer cells in the G0/G1 stage, while single-drug treatments did not change the cell-cycle distribution pattern despite their ability to inhibit DU145 cell proliferation. This discrepancy between the ability of a drug to suppress cancer cell growth but not to affect cell-cycle distribution was also observed by Zhuang and Burnstein (22) and Moffatt et al. (23). Neither studies found any effect of 1,25(OH)2D3 on the cell-cycle of the PCa cell line ALVA 31, although cell growth was suppressed significantly. Moffatt et al. (23) suggested that 1,25(OH)2D3 may decrease cell proliferation by slowing down the rate of cell progression through the cell-cycle without necessarily causing a measurable arrest in any particular phase. Our findings can also be interpreted with this explanation.

The increased anticancer effect of the combined treatment obtained in the present study may, to a certain

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extent, be a result of the further enhancement by 1,25(OH)₂D₃ of the EGFR-pathway suppression induced by cetuximab. The EGFR pathways and the relevant effects of cetuximab and 1,25(OH)₂D₃ are presented in Figure 4. This suggestion is in accordance with the results of McGaffin et al. (24) and Cordero et al. (11) who showed that 1,25(OH)₂D₃ induces reduction of EGFR gene transcription as a consequence of vitamin D receptor (VDR) binding to the VDR response element (VDRE) within the EGFR promoter (24). This binding decreases EGFR mRNA and protein expression, which is followed by reduced EGF and TGF-α-dependent phosphorylation of EGFR and decreased EGF-induced nuclear translocation of EGFR (11). Reduction of nuclear EGFR is followed by decreased interaction with STAT3 and consequent sub-regulation of iNOS (6) resulting in suppressed tumor growth. Decreased nuclear EGFR content also results in reduced cooperation of EGFR and E2F1, subsequent decreased activity of the B-Myb gene and consequent slow progression through the G1/S-phase of the cell-cycle (7). In addition, down-regulation of the EGFR reduces binding of EGF and TGF-α resulting in decreased dimerization of EGFR and low activity of the downstream kinase cascades (25, 26).

1,25(OH)₂D₃ exerts its anticancer effects through other mechanisms as well. For example, 1,25(OH)₂D₃ induces synthesis of insulin-like growth factor binding protein-3 (IGFBP-3), which increases the levels of the cell cycle inhibitor p21 resulting in suppression of cancer cell growth (27, 28). Peng et al. (29) demonstrated that this induction of IGFBP-3 production by 1,25(OH)₂D₃ is performed through a genomic mechanism. These researchers identified a functional VDRE in the distal region of the IGFBP-3 promoter and showed that VDR binding to the VDRE of the IGFBP-3 promoter induced IGFBP-3 production in the presence of 1,25(OH)₂D₃.

Conclusion

The results of the present study support our hypothesis that a combination of cetuximab and vitamin D might be effective in combating PCa and could provide the basis for clinical application in the treatment of hormone-refractory PCa. Furthermore, this combination of drugs could be used as a neoadjuvant therapy for DNA-damaging drugs, since inhibition of the EGFR pathway results in decreased DNA-dependent protein kinase activity and consequently reduces DNA repair (30).

Acknowledgements

This study was partially supported by Roche Pharmaceuticals (Israel) Ltd.

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Received November 28, 2006
Accepted January 5, 2007