INDUCED APOPTOSIS IN HUMAN PROSTATE CANCER
CELL LINE LNCaP BY UKRAIN

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Summary: Exposure of LNCaP prostate cancer cells to Ukrain (NSC-631570), a novel semisynthetic drug from Cheilodendron majus L., results in cell growth inhibition which is concomitant with apoptosis. After 24 h treatment with 3.5 μM of Ukrain as many as 73% cells were found in the G2/M phase. However, at higher drug concentrations (7 μM and 17.5 μM) the changes in cell phase distribution were less dramatic but cell accumulation in the G2/M phase was still evident. The rate of apoptotic cells rose steadily with increased drug concentration in a dose-dependent manner and reached 20% at a dosage of 17.5 μM. To investigate whether the cell cycle control mechanisms are affected in response to Ukrain, we analyzed the expression levels of some cyclins, cyclin-dependent kinases (CDK) and apoptosis-related proteins in drug treated cancer cells. Western blot experiments revealed alterations in levels of CDK1 and CDK2, after treatment. Up-regulation of the CDK inhibitor p27 was observed, which may lead to G2/M cell accumulation, but no substantial changes in expression of Bcl-2 and Bax proteins were found.

Introduction

Prostate carcinoma, the most frequent cancer in men and the second leading cause of male death in many countries, is an escalating health concern because of its age-related onset and the existence of an aging male population (1, 2). However, despite the high incidence of prostate cancer, its causes are not completely known. Multiple genetic and epigenetic factors have been implicated in the oncogenesis and progression of this type of solid tumor (3, 4). Understanding the mechanisms leading to the development and metastasis of prostate cancer and the development of rational strategies for its prevention and treatment are critical health care issues.

Ukrain (NSC-631570), chelidonium thiophosphoric conjugate (C66H72N8O17PS-HCl), an anticancer agent, has been reported to exhibit high cytotoxic activity toward malignant cells (5, 6). It has been found to be an immunostimulating and immunomodulating compound (7) that also possesses antiviral
activity (9). The cytostatic and cytotoxic properties of
Ukrain have been investigated in human malignant
cell lines, nonhuman cell lines and in nude mouse
xenografts (9, 10). The cytotoxicity of Ukrains in vitro
was dose-dependent and estimated from 1 x 10^{-5.5}
to 1 x 10^{-4.2} M (9, 11). In addition, it has recently
been demonstrated to inhibit angiogenic differentiation
of human endothelial cells in vitro (12). While the
mechanism or mechanisms, responsible for its multiple
functions are not clear, fluorometric evaluations sug-
gest that the nucleus is a site of action (13). Ukrains
was screened in vitro by the National Cancer Institute
and found to have cytotoxic effects in 60 human
tumor cell lines representing eight tumor types (9),
although no information is available on the effects of
Ukrain on prostate cancer cells.

In the present study, we examined the cellular and
molecular effects of Ukrains treatment on human
prostate cancer cell line LNCaP, an appropriate
model because the cells most closely resemble typi-
cal human prostate cancer. These cells express
androgen receptor and prostate specific antigen and
retain functional p53 and retinoblastoma protein, all
of which are typical in most prostate cancers (3, 4).
Our data revealed that Ukrains induced cell growth
inhibition and apoptosis and we detected a decrease
in the expression of cyclin-dependent kinases (CDK)
1 and 2 and upregulation of CDK inhibitor p27. We
also observed an accumulation of apoptotic cells
after 24 h of Ukrains treatment at a dose of 3.5 μM,
the lowest concentration used, and the amount of
apoptotic cells rose steadily in a dose-dependent
manner. However, the expression levels of proapop-
totic Bax protein and antiapoptotic protein Bcl-2
were not substantially changed.

Material and methods

The human prostate cancer cell line LNCaP was
purchased from the American Type Culture Collection
(Rockville, MD, USA) and was grown in RPMI medi-
um (Gibco-BRL, Rockville, MD, USA), supplemented
with 10% fetal calf serum. Morphological changes
were monitored in control and treated cells by inver-
ed phase microscopy. Ukrains (1 mg/ml solution in
water) was provided by Dr. J.W. Nowicky (Ukrainian
Anti-Cancer Institute, Vienna, Austria).

DNA flow cytometry. For flow cytometry experi-
ments, control and Ukrains-treated cells were collect-
ed after the indicated time, trypsinized, then washed
in phosphate buffer saline (PBS) and fixed in ice-cold
70% ethanol. Cells were incubated in PBS contain-
ing propidium iodide (6 mg/ml) and RNase A (1
mg/ml) for 30 min. Analysis was performed on an
EPICS Profile flow cytometer (Coulter Counter,
Coulter Electronics, Inc., Miami, FL, USA) at the
University of Rochester Cancer Center. DNA his-
togram analysis was performed using the Cell Cycle
Profile Multicycle Software package (Phoenix Flow
Systems, Inc., San Diego, CA, USA).

Western immunoblot analysis. Control and treated
cells were lysed with ice-cold buffer (20 mM Tris, pH
7.5, 100 mM NaCl, 1 mM ethylenediamine tetra-
acetic acid, 0.5% Tween 20, 10 mg/ml each of chy-
mostatin, leupeptin and pepstatin and scraped from
the dish. Following centrifugation at 14,000 x g for 20
min at 4 °C, the supernatant was collected. Protein
concentrations were determined using the Bio-Rad
Protein Assay Kit (Gercules, CA, USA). Equal
amounts of total protein (15 μg) from control and
treated cells were separated by 10-15% SDS-PAGE
and transferred to ECL nitrocellulose membrane
(Amersham Life Sciences, Inc., Arlington Heights, IL,
USA). Cyclin A, cyclin E, CDK1, CDK2, p27 and Bax
antibodies were purchased from Santa Cruz (Santa
Cruz, CA, USA). Bcl-2 antibody from Oncogene
(Cambridge, MA, USA), p21 antibody from Calbiochem (La Jolla, CA, USA) and p53 antibody
from Novocastra (Newcastle, UK). Immunoreactive
proteins were visualized using the ECL Western Blotting Detection System (Amersham Life Sciences).

Results

The effect of Ukrain on LNCaP cancer cells was first evident from the morphological changes, as observed with inverted phase microscopy. Control, nontreated cells remained in colonies (Fig. 1A), while cancer cells exhibited scattering of colonies and irregular cell morphology. Cells became elongated and spindle-shaped (Fig. 1B).

**Effects of Ukrain-treatment on cell cycle distribution and apoptosis in LNCaP cells.** To determine whether Ukrain can affect cell cycle progression, LNCaP cells were treated with 3.5 μM, 7 μM and 17.5 μM of the drug for 24 h and analyzed by flow cytometry. As shown in Table I, following 24 h treatment with 3.5 μM of Ukrain, the cell population in the G0/G1 phase dropped from 73% in control cells to as low as 2% in treated cells. The number of S-phase cells remained almost the same as in control cells. Also, dramatic changes in the percentage of G2/M cells occurred. Following treatment, approximately 75% of cells were found in G2/M compared with 10%

![Control](image1.png) ![Ukraine treated](image2.png)

**Fig. 1** Micrographs of the morphology exhibited by control and Ukrain-treated cells. (A) Control LNCaP cells; (B) LNCaP cells treated with 17.5 μM of Ukrain for 48 h. Photographs were taken with an Olympus inverted microscope with 30 x objective.

<table>
<thead>
<tr>
<th>Drug concentration (μM)</th>
<th>Apoptotic cells (%)</th>
<th>G0/G1 cells (%)</th>
<th>S-phase cells (%)</th>
<th>G2/M cells (%)</th>
</tr>
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<tr>
<td>0</td>
<td>0</td>
<td>73.2 ± 5.4</td>
<td>17 ± 6.2</td>
<td>9.8 ± 5.1</td>
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<td>3.5</td>
<td>10.1 ± 2.4</td>
<td>1.7 ± 1.5</td>
<td>15.1 ± 3.1</td>
<td>73.1 ± 2.5</td>
</tr>
<tr>
<td>7</td>
<td>15.8 ± 5.3</td>
<td>42.3 ± 4.9</td>
<td>8.4 ± 6.2</td>
<td>33.5 ± 5.7</td>
</tr>
<tr>
<td>17.5</td>
<td>19.7 ± 3.5</td>
<td>34.2 ± 2.6</td>
<td>24.7 ± 3.2</td>
<td>21.4 ± 3.1</td>
</tr>
</tbody>
</table>

Table I Cell cycle progression in Ukrain-treated LNCaP cells analyzed for DNA content by flow cytometry.

Cells were treated with 0, 3.5 μM, 7 μM and 17.5 μM of Ukrain for 24 h. Values shown are the means of three separate samples (10,000 cells were counted for each sample).
of control cells. In addition, approximately 10% cells underwent apoptosis. After a sharp increase at 24 h, the percentage of G2/M-cells significantly declined at 60 h of treatment and was approximately 12% (data not shown). This suggests that cell cycle progression through G2/M takes place at low Ukrain concentrations and cells can overcome G2/M arrest after a period of adaptation.

After LNCaP cell treatment with 7.0 μM of Ukrain, the percentage of cells in G2/G1 phase was reduced to 42% and the number of S-phase cells (10%) was not significantly reduced compared with control levels. Even so, a significant accumulation of LNCaP cells was found in G2/M, which was 40%, or four times higher than in control cells. After treatment with 17.5 μM of Ukrain, the number of cells remaining in the G2/M phase was approximately 21%, 52% lower than with the 3.5 μM drug concentration (G2/G1 cells 34% and S-phase cells 25%). However, a substantial number of apoptotic cells was apparent at 7 μM Ukrain, 15%, and the number rose steadily, reaching 20% at 17.5 μM of Ukrain. After prolonged incubation of the cells in 7 μM of drug-containing medium for 60 h and 76 h, the rate of apoptotic cells increased even more, to 21.8% and 29.4%, respectively, while the percentage of G2/M cells was 12.9% and 14.6% (data not shown).

Taken together, our data show that Ukrain treatment of LNCaP prostate cancer cells led to substantial changes in cell cycle distribution, concomitant with apoptosis. The major difference between the lower (3.5 μM) and the higher (17.5 μM) drug concentrations was that at the lower concentration, LNCaP cells initially preserved a significant proportion of G2/M cells during treatment. With time, however, the number of apoptotic cells increased steadily and in a dose-dependent manner.

Expression of the CDK and CDK inhibitor p27 changes after Ukrain treatment. We next investigated the possibility that changes in the expression of cyclins, CDKs, and CDK inhibitors occurred in Ukrain-treated cells. The cell cycle is regulated by the coordinated action of CDKs in association with their specific regulatory cyclin proteins. Cyclin A and B are required to modulate the activities of CDK1 and CDK2, which are necessary for mitotic progression, while cyclins D and E are required for regulation of CDKs 4, 6 and 2, respectively, for proper G1 progression (14, 15). The kinase activity of cyclin E/CDK2, cyclin D/CDK4/6 and other cyclins/kinase binary complexes is suppressed by CDK inhibitors including p27 (16, 17). p27 has also been suggested to be a tumor suppressor and possible promoter of apoptosis (18).

Following Ukrain treatment for 48 h at a concentration of 17.5 μM, LNCaP cells expressed less CDK1 and CDK2 as detected by Western blot analysis, whereas expression of cyclin E was very high and remained little changed (Fig. 2). In contrast, p27

![Fig. 2](image-url)
protein expression was slightly increased. The observed diminished expression of CDK1 and CDK2 in LNCaP cells and the elevated expression of p27 after Ukrain treatment is in agreement with the detected cell growth inhibition and G_{2}/M cell accumulation.

Expression of antiapoptotic Bcl-2 and proapoptotic Bax proteins. Additionally, we investigated the expression of apoptotic regulatory proteins Bcl-2 and Bax in Ukrain-treated cells. Susceptibility to apoptosis is partly modulated by the Bcl-2 protein family and the tumor suppressor gene p53 (19). Apoptosis is inhibited by Bcl-2 and Bcl-x, and promoted by proapoptotic Bax, Bak and Bcl-X_{S} proteins. Moreover, p53 is a direct transcriptional activator of Bax expression (20) and a suppressor of Bcl-2 and normally an increase in Bax expression is accompanied by a decrease in Bcl-2 levels. We did not observe significant changes in cellular levels of these two proteins by Western immunoblotting (Fig. 2). However, expression of Bcl-2 protein in LNCaP cells is very low (21, 22), as is expression of p53 protein (22). LNCaP cells that were derived from human lymph node metastasis have normal, wild type p53 (23). We found that wild type p53 was not elevated in response to Ukrain treatment (data not shown) although proliferation was significantly inhibited by this drug.

Discussion

Prostate cancer is the most commonly diagnosed neoplasm and one of the leading causes of male death. Multiple factors have been identified that are involved in its oncogenesis and progression (1, 2, 24). However, the molecular mechanisms underlying the disease are largely still unknown. Androgen withdrawal therapy remains the only treatment for advanced prostate cancer (24). Therapeutic options include radiotherapy and chemotherapy, mostly with limited antitumor activity and low response rate. Some studies implicate dietary and nutritional factors in the onset and progression of prostate cancer, particularly vitamin D (2). Among many approaches to the treatment of hormone-resistant prostate cancer, the Bcl-2/Bax ratio has recently been reported to be a possible predictive marker for therapeutic response to radiotherapy (25). Another approach to the treatment of prostate cancer is the use of new chemotherapeutic drugs or natural/synthetic chemicals. Most drugs used in chemotherapy induce apoptosis or mediate cytotoxicity preferentially in proliferating cancer cells. However, human prostate cancer cells demonstrate very slow growth kinetics and thus do not respond well to currently available drugs. The angiogenesis inhibitor, llinomide (26), and a plant product, β-lapachone (27), have been shown to have promising apoptosis-inducing effects on prostate cancer cells in a proliferation-independent manner. Thus, modulation of the apoptotic response represents a novel mechanism-based approach for the development of new therapeutic regimens for the treatment of prostate cancer (24).

We have tested the effects of the anticancer drug Ukrain on human prostate cancer cell line LNCaP and found that Ukrain-treatment at concentrations of 3.5 μM-17.5 μM resulted in cell growth arrest and apoptosis of cancer cells. Our data revealed that at lower concentrations and short exposure Ukrain induced G_{2}/M cell arrest. Also, we observed the appearance of apoptotic cells even when the lowest drug concentration was applied and the amount of apoptotic cells rose steadily in a dose-dependent manner reaching 28% of cells after 72 h incubation with 17.5 μM Ukrain. Accumulation of cancer cells in the G_{2}/M phase suggests that Ukrain belongs to the class of antimitotic, antitubulin drugs. In support of this, the extensive polyploidy of DNA and “blister cell death” observed in the K562 human leukemia cell line in response to high concentrations of Ukrain is
believed to be mediated by prevention of microtubule formation (11). Similar Ukrain effects were obtained after treatment of two human epidermoid carcinoma cell lines, ME180 and A431, with striking G2/M cell arrest, but the proportion of apoptotic cells was lower (28). G2/M cell arrest was reversible at Ukrain concentrations as high as 30 μM. The drug’s reversible effects were also recently found for two other human carcinoma cell lines and one transformed monkey cell line (29).

In agreement with cell cycle arrest, the significant decrease in the expression of CDK1 and CDK2 and up-regulation of the CDK inhibitor p27 were detected. These results are consistent with the findings that induction of apoptosis and blocking of cell cycle at G2/M are important determinants of the sensitivity of cancer cells to Ukrain. The role of cyclin and CDK activity for an orderly progression through the cell cycle is well established (14, 15). Also, low expression of p27 has been observed in many types of human tumors and correlates strongly with tumor aggressiveness (17). However, the expression of proapoptotic Bax protein, antiapoptotic protein Bcl-2 and global transcription activator p53 was not substantially changed. It is likely that Ukrain-promoted apoptosis of cancer cells is p53/Bcl-2/Bax independent. Indirect evidence in support of this is the observation of apparent apoptosis in Ukrain treated ME180 and A431 cells where p53 is mutated or not functional, respectively (28). Further studies outside the scope of this report should be performed to address this issue.

Our findings extend those of other studies supporting the development of Ukrain as a possible therapeutic agent and provide new insight into at least some of the possible mechanism of Ukrain action at the molecular cellular levels. Understanding, as well as possible modulation, of this mechanism involved in cancer cell death could be used as a model for providing safer and effective new cancer therapies. It is also evident that more studies are needed to estimate the optimal concentration of this drug for use in the treatment of prostate cancer cells that would still be safe for normal cells and the whole organism as well as to identify the drug’s primary target or targets in the cell.

Acknowledgments

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References

Ukrain-induced apoptosis in LNCaP cell line


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