DRUGS EXPTL. CLIN. RES. **XXVI**(5/6) 141-147 (2000)

INDUCED APOPTOSIS IN HUMAN PROSTATE CANCER CELL LINE LNCaP BY UKRAIN

ROUBLEVSKAIA I.N.,[1] HAAKE A.R.,[1,2] LUDLOW J.W.,[2,3] POLEVODA B.V.[3]

1) Department of Dermatology, University of Rochester School of Medicine and Dentistry, Rochester, USA.
2) University of Rochester Cancer Center, Rochester, USA.
3) Department of Biochemistry and Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, USA.

Summary: *Exposure of LNCaP prostate cancer cells to Ukrain (NSC-631570), a novel semisynthetic drug from* Chelidonium 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 G_2/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 G_2/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 G_2/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), chelidonine thiophosphoric conjugate ($C_{66}H_{75}N_6O_{18}PS{\cdot}6HCl$), 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

Address for correspondence: Bogdan V. Polevoda, Department of Biochemistry and Biophysics, University of Rochester Medical Center, P.O. Box 712, 601 Elmwood Avenue, Rochester, NY 14642, USA.
Tel: 716-275-3329 Fax: 716-271-2683
E-mail: Bogdan_Polevoda@urmc.rochester.edu

0378-6501/2000/5/6 00141+7 $02.50/0

© 2000 Bioscience Ediprint Inc.

activity (8). 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 Ukrain *in vitro* was dose-dependent and estimated from $1 \times 10^{-5.5}$ to $1 \times 10^{-4.2}$ 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 suggest that the nucleus is a site of action (13). Ukrain 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 Ukrain treatment on human prostate cancer cell line LNCaP, an appropriate model because the cells most closely resemble typical 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 Ukrain 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 Ukrain 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 proapoptotic 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 medium (Gibco-BRL, Rockville, MD, USA), supplemented with 10% fetal calf serum. Morphological changes were monitored in control and treated cells by inverted phase microscopy. Ukrain (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 experiments, control and Ukrain-treated cells were collected after the indicated time, trypsinized, then washed in phosphate buffer saline (PBS) and fixed in ice-cold 70% ethanol. Cells were incubated in PBS containing propidium iodide (8 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 histogram analysis was performed using the Cell Cycle Profile Multicycle Software package (Phoenix Flow Systems, Inc., San Diego, CA, USA).

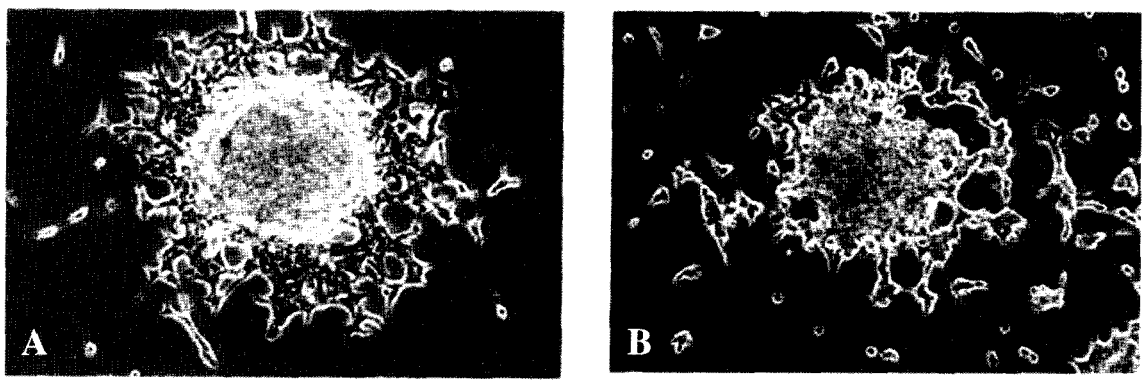
Western immunoblot analysis. Control and treated cells were lysed with ice-cold buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 0.5% Tween 20, 10 mg/ml each of chymostatin, leupeptin and pepstatin and scraped from the dish. Following centrifugation at 14,000 x g for 20 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 G_0/G_1 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 G_2/M cells occurred. Following treatment, approximately 75% of cells were found in G_2/M compared with 10%



Control **Ukrain treated**

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

Drug concentration (μM)	Apoptotic cells (%)	G_0/G_1 cells (%)	S-phase cells (%)	G_2/M cells (%)
0	0	73.2 ± 5.4	17 ± 6.2	9.8 ± 5.1
3.5	10.1 ± 2.4	1.7 ± 1.5	15.1 ± 3.1	73.1 ± 2.5
7	15.8 ± 5.3	42.3 ± 4.9	8.4 ± 6.2	33.5 ± 5.7
17.5	19.7 ± 3.5	34.2 ± 2.6	24.7 ± 3.2	21.4 ± 3.1

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 G_2/M-cells significantly declined at 60 h of treatment and was approximately 12% (data not shown). This suggests that cell cycle progression through G_2/M takes place at low Ukrain concentrations and cells can overcome G_2/M arrest after a period of adaptation.

After LNCaP cell treatment with 7.0 μM of Ukrain, the percentage of cells in G_0/G_1 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 G_2/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 G_2/M phase was approximately 21%, 52% lower than with the 3.5 μM drug concentration (G_0/G_1 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 G_2/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 G_2/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 G_1 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

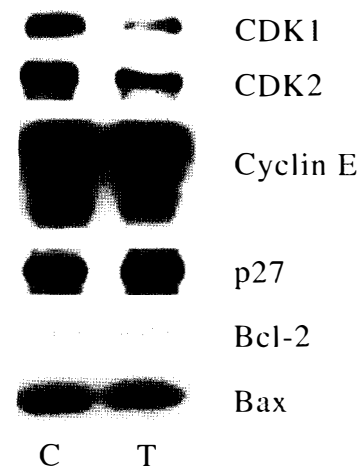


Fig. 2 Expression of the mitotic cyclins, cyclin-dependent kinases (CDK), CDK inhibitor p27 and apoptotic proteins Bcl-2 and Bax in LNCaP cells. Western blots were carried out as described in Materials and methods and probed with the antibodies indicated to the right of each panel. C = control cells; T = cells treated for 48 h with 17.5 μM of Ukrain.

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 proapototic 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 supressor gene p53 (19). Apoptosis is inhibited by Bcl-2 and Bcl-x_L and promoted by proapoptotic Bax, Bak and Bcl-X_S proteins. Moreover, p53 is a direct transcriptional activator of Bax expression (20) and a supressor 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, linomide (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 G_2/M cell arrest, but the proportion of apoptotic cells was lower (28). G_2/M cell arrest was reversible at Ukrain concentrations as high as 35 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 G_2/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 aggression (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 safe 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

We thank Dr. Peter Keng, Regina Harley and Brenda Goodfriend for help in the operation of the flow cytometer and Dr. Ray Konger for providing p27 antibody.

References

(1) Denmeade S.R., Isaacs J.T. *Prostate cancer: Where we are and where are we going?* Br. J. Urol., **79** (suppl 1), 2, 1997.

(2) Kamat A.M., Lamm D.L. *Chemoprevention of urological cancer.* J. Urol., **161**, 1748, 1999.

(3) Sciavolino P.J., Abate-Shen C. *Molecular biology of prostate development and prostate cancer.* Ann. Med., **30**, 357, 1998.

(4) Hegarty N.J., Fitzpatrick J.M., Richie J.P., et al. *Future prospects in prostate cancer.* Prostate, **40**, 261, 1998.

(5) Nowicky J.W., Manolakis G., Meijer D., Vatanasapt V., Brzosko W.J. *Ukrain both as an anticancer and immunoregulatory agent.* Drugs Exptl. Clin. Res., **18** (Suppl), 51, 1992.

(6) Jagiello-Woitowicz E., Kleinrok Z., Urbanska E.M. *Ukrain (NSC-631570) in experimental and clinical studies: A review.* Drugs Exptl. Clin. Res., **24,** 213, 1998.

(7) Liepins A., Nowicky J.W. *Activation of spleen cell lytic activity by the alkaloid thiophosphoric acid derivative: Ukrain.* Int. J. Immunopharmacol., **14,** 1437, 1992.

(8) Potopalsky A.I., Nowicky J.W. *Semisynthetic antitumor alkaloid derivatives as antiviral and potential anti-HIV preparations* [Abstract]. Antivir. Res., **20** (Suppl 1), 57, 1993.

(9) Nowicky J.W., Nowicky W., Liepins A. *Cytostatic and cytotoxic effects of Ukrain on malignant cells.* J. Chemother., **5** (Suppl 1), 797, 1993.

(10) Nowicky J.W., Hiesmayer W., Nowicky W., Liepins A. *Influence of Ukrain on human xenographs in vitro.* Drugs Exptl. Clin. Res., **22**, 93, 1996.

(11) Liepins A., Nowicky J.W., Bustamante J.O., Lam E. *Induction of bimodal programmed cell death in malignant cells by the derivative Ukrain (NSC-831570).* Drugs Exptl. Clin. Res., **22**, 73, 1996.

(12) Koshelnick Y., Moskvina E., Binder B.R., Nowicky J.W. *Ukrain (NSC-631570) inhibits angiogenic differentiation of human endothelial cells in vitro.* In: 17th Int. Cancer Cong. Rio de Janeiro, Aug. 24-28, 1998, pp. 91-95

(13) Hohenwarter O., Strutzenberg K., Katinger H., Liepins A., Nowicky J.W. *Selective inhibition of in vitro cell growth by anti-tumor drug Ukrain.* Drugs Exptl. Clin. Res., **18** (Suppl), 1, 1992.

(14) Morgan D.O. *Cyclin-dependent kinases. Engines, clocks, and microprocessor.* Annu. Rev. Cell. Dev. Biol., **13**, 261, 1997.

(15) Sherr C.J., Roberts J.M. *CDK inhibitors: Positive and negative regulators of G_1-phase progression.* Gene Dev., **13,** 1501, 1999.

(16) Polyak K., Lee M-H., Erdjument-Bromage H., et al. *Cloning of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals.* Cell, **78,** 59, 1994.

(17) Lloyd R.V., Erickson L.A., Jin L., et al. *p27kip1: A multifunctional cyclin-dependent kinase inhibitor with prognostic significance in human cancers.* Am. J. Pathol., **154,** 313, 1999.

(18) Katayose Y., Kim M., Rakkar A.N.S., Li Z., Cowan K.H., Seth P. *Promoting apoptosis: A novel activity associated with the cyclin-dependent kinase inhibitor p27.* Cancer Res., **57**, 5441, 1997.

(19) Reed J.C. *Bcl-2 and the regulation of programmed cell death.* J. Cell Biol., **124**, 1, 1994.

(20) Miyashita T., Reed J.C. *Tumor supressor p53 is a direct transcriptional activator of the human bax gene.* Cell, **80**, 293, 1995.

(21) Liu X-H., Yao S., Kirschenbaum A., Levine A.C. *NS398, a selective cyclooxygenase-2 inhibitor, induces apoptosis and down-regulates bcl-2 expression in LNCaP cells.* Cancer Res., **58**, 4245, 1998.

(22) Rokhlin O.W., Bishop G.A., Hostager B.S., et al. *Fas-mediated apoptosis in human prostatic carcinoma cell lines.* Cancer Res., **57**, 1758, 1997.

(23) Carrol A.G., Voeller H.J., Sugars L., Gelmann E.P. *p53 oncogene mutations in three human prostate cancer cell lines.* Prostate, **23**, 123, 1993.

(24) Tang D.G., Porter A.T. *Target to apoptosis: A hopeful weapon for prostate cancer.* Prostate, **32**, 284, 1997.

(25) Mackey T.J., Borkowsky A., Amin P., Jacobs S.C., Kuprianou N. *bcl-2/bax ration as a predictive marker for therapeutic response to radiotherapy inpatients with prostate cancer.* Urology, **52**, 1085, 1998.

(26) Vukanovic J., Isaacs J.T. *Linomide inhibits angiogenesis, growth, metastasis, and macrophage infiltration within rat prostatic cancers.* Cancer Res., **55**, 1499, 1995.

(27) Li C.J., Wang C., Pardee A.B. *Induction of apoptosis by lapachone in human prostate cancer cells.* Cancer Res., **55**, 3712, 1995.

(28) Roublevskaia, I.N., Polevoda, B.V., Ludlow, J.W., Haake, A.R. *Induced G_2/M arrest and apoptosis in human epidermoid carcinoma cell lines by semisynthetic drug Ukrian.* Anticancer Res., **20**, 3163, 2000.

(29) Panzer A., Jouubert A.M., Bianchi P.C., Seegers J.C. *The antimitotic effects of Ukrain, a Chelidonium majus alkaloid derivative, are reversible in vitro.* Cancer Lett., **150**, 85, 2000.