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Induced G2/M Arrest and Apoptosis in Human Epidermoid Carcinoma Cell Lines by Semisynthetic Drug Ukrain

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Abstract. *Exposure of ME180 and A431 carcinoma cells to Ukrain (NSC-631570), a novel semisynthetic drug from* Chelidonium majus L, *results in cell growth inhibition which is concomitant with reversible G2/M cell cycle arrest and apoptosis at doses as low as 7 μM. In contrast, the same drug concentrations were not affective towards normal human keratinocytes. In order to investigate whether cell cycle control mechanisms are effected in response to Ukrain, we analyzed cell cycle distribution and levels of cyclins and cyclin-dependent kinases in drug treated carcinoma cells. We found alterations in levels of mitotic cyclins A and B1, and cyclin-dependent kinases CDK1 and CDK2, after treatment. We also observed an upregulation of CDK inhibitor p27 in both cancer cell lines which may lead to the G2/M cells accumulation.*

It has been reported that Ukrain, chelidonine thiophosphoric conjugate ($C_{66}H_{75}N_6O_{18}PS \bullet 6HCl$), the semisynthetic drug consisting of alkaloids isolated from *Chelidonium majus L.* (Papaveraceae), is an anti-cancer agent exhibiting high cytotoxic activity toward malignant cells (1, reviewed in 2). Ukrain has been found to be an immunostimulating and immunomodulating compound (3), also possessing antiviral activity (4). In addition, Ukrain also inhibits angiogenic differentiation of human endothelial cells *in vitro* as demonstrated recently (5). *In vitro* screening of Ukrain by the National Cancer Institute has shown it to have cytotoxic effects on 60 human tumor cell lines representing 8 tumor types. While the mechanism(s) responsible for its multiple functions are not clear, fluorometric evaluations suggest that the nucleus is a site of action (6). In addition, inhibition of DNA, RNA, and protein synthesis were demonstrated in malignant cell lines at relatively high Ukrain concentrations and to a much lesser extent in normal cells (2).

In the present study, we examined the cellular and molecular effects of Ukrain-treatment in normal human keratinocytes and cancer cell lines A431 and ME180. Despite some differences in sensitivity found among cancer cell lines to this compound, our data revealed reversible G2/M arrest and apoptosis for both cancer cell lines in conjunction with decreased levels of cyclins A and B1 and increased levels of the cyclin-dependent kinase inhibitor p27. In contrast, normal keratinocytes remained unaffected by Ukrain treatment.

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Material and Methods

Cell culture and treatment. Primary human keratinocytes were isolated from neonatal skin specimens obtained at the time of circumcision following separation of epidermis from dermis by dispase treatment (15 units/ml at 4°C). Epidermal sheets were trypsinized and single cell suspensions collected by centrifugation. Neonatal keratinocytes (NeoK) were plated on dishes and coverslips in complete serum-free keratinocyte growth medium SFM (Gibco) supplemented with 5mg/L epidermal growth factor (EGF) and 50mg/L bovine pituitary extract (BPE) at low calcium concentrations (0.1mM). Cells were passaged by trypsinization and replated at a 1:3 ratio. Cultures of NeoK were grown to 50% confluence prior to treatment. The human epidermoid cancer cell lines A431 and ME180 were purchased from the American Type Culture Collection (Rockville, MD) and were grown in complete Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal calf serum (FCS). Ukrain (1mg/ml solution in water) was provided by J.W. Nowicky (Ukrainian Anti-Cancer Institute, Vienna, Austria).

Flow cytometric cell cycle analysis. Control and Ukrain-treated cells were trypsinized and fixed in ice cold 70% ethanol. Cells were then incubated in PBS containing propidium iodide (PI) (8 mg/ml), and RNase A (1mg/ml) for 30 min. Analysis was performed on an EPICs Profile flow cytometer (Coulter Counter, Coulter Electronics, Inc., Miami, FL) 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).

DNA extraction and gel electrophoretic analysis of DNA. Cultured cells were washed with phosphate buffered saline (PBS) and scraped from the dishes in 1ml of lysis buffer (10mM Tris pH8.0, 1mM EDTA, 150 mM NaCl, 0.5% sarcosyl). After Proteinase K treatment, DNA was collected by ethanol precipitation and re-suspended in Tris-EDTA buffer. RNase

Table I. *Cell cycle progression in Ukrain treated cells as detected by flow cytometry.*

Time (hours)	Apoptotic cells (%)		G0/G1 cells (%)		S phase cells (%)		G2/M cells (%)	
	A431	ME180	A431	ME180	A431	ME180	A431	ME180
0 h	0	0	45.1±5.7	50.3±5.5	36.2±4.3	32.2±6.8	18.7±4.1	17.4±5.8
24h	5.3±2.4	0	13.2±5.1	2.7± 2.5	7.0±4.8	4.3± 2.1	74.5±5.9	92.1±3.8
48h	11.4±4.0	7.2±4.6	20.8±4.1	8.2 ±3.7	20.3±3.5	14.8±2.8	46.8±4.8	69.2±4.2
72h	15.2±4.7	13.5±3.1	7.4±3.3	8.8±3.5	32.8±4.2	1.5±1.4	44.1±5.1	76.8±4.4

Cells were treated with 7 μM Ukrain for 0, 24, 48, and 78 hours. Values shown are the means of three separate samples (10,000 cells were counted for each sample).

A digested DNA samples were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide following visualization on a UV transilluminator.

Western blot analysis. Control and treated cells were lysed with ice cold buffer (20mM Tris, pH7.5, 100mM NaCl, 1mM EDTA, 0.5% Tween 20, 10mg/ml each of chymostatin, leupeptin and pepstatin) and scraped from the dish. Following centrifugation at 14,000 xg for 20 min at 4°C, the supernatant was collected. Protein concentrations were determined using the Bio-Rad Protein Assay Kit. Equal amounts of total protein (15 μg) from control and treated cells were separated by SDS-PAGE and transferred to ECL nitrocellulose membrane (Amersham Life Sciences, Inc.). Cyclin A, cyclin B1, cyclin E, CDK1, CDK2 and p27 (C19) antibodies were purchased from Santa Cruz (Santa Cruz, CA). Immunoreactive proteins were visualized using ECL Western Blotting Detection System (Amersham Life Sciences, Inc.).

Results

Ukrain inhibits cell cycle progression in a dose-dependent manner. In order to determine if Ukrain can induce growth arrest, cells were treated with various concentration of drug for 24 h and analyzed by flow cytometry. As shown in Figure 1, following treatment with 3.5 μM Ukrain, approximately 50% of ME180 cells can be found in G2/M. A431 cells were found to be less sensitive to this drug concentration exhibiting only a slight increase (~10%) in G2/M cells. At 7.0 μM Ukrain, a significant increase in G2/M was detected for both cell lines (90% and 75% for ME180 and A431, respectively), with only a subtle change at higher doses. In contrast, NeoK cells remained unaffected by 3.5 and 7 μM concentrations. There was an increase in G2/M for the NeoK cells at higher concentrations of Ukrain, but not so dramatic as in cancer cells.

Ukrain-treatment affects cell cycle distribution and induces apoptosis in A431 and ME180 cells. In order to address this apparent selective mechanism of cell cycle inhibition more thoroughly, subsequent experiments were performed at the 7.0 μM Ukrain when most of cancer cells were found accumulated in G2/M. Following a 24 h treatment, the population of ME180 cells in G0/G1 decreased from 50% to 3%, and remained stable at approximately 9% by 72 h (Table I). The amount of S-phase cells first decreased to ~4% at 24 h, then increased to ~15% by 48 h, but at 72 h of treatment this population was reduced to ~2%. The percentage of G2/M-cells after a sharp increase at 24 h did not significantly decline at 48 h of treatment and was ~80% until the experiment ended at 72 h. While there was no detectable apoptotic cell population for the first 24 h of Ukrain treatment, we observed an increase of apoptotic cells at 48 h and at 72 h to ~14%. In summary, Ukrain treatment of ME180 cells during 72 h led to a substantial increase in the number of cells in G2/M phase and apoptotic cells, while the number of G0/G1 cells was decreased and S-phase cells were almost eliminated.

Somewhat different results in cell cycle phase distribution were observed for A431 cells following Ukrain treatment (Table I). The number of cells remaining in the G2/M phase after 48 and 72 h of treatment was approximately 30% lower compared to the 24 h time-point. This suggests that some cell cycle progression through G2/M is taking place. However, the amount of G0/G1 cells reduced from 50% to less then 10% by 72 h. The percentage of cells in S-phase after an initial 5-fold drop at 24 h was restored to control level by 72 h. Unlike the ME180 cell line, a notable amount of A431 apoptotic cells were apparent by 24 h. The number of apoptotic cells rose steadily until 72 h, reaching 15%.

Evidence for apoptotic events were also provided by hematoxylin/eosin staining of both ME180 and A431 cells; nuclei appeared to be fragmented and condensed after staining of Ukrain-treated cells (data not shown). Also, detection of apoptosis was confirmed by DNA fragmentation analysis. After Ukrain treatment, cells display a ladder of DNA fragments with an interval of about 200 bp, characteristic of intranucleosomal cleavage (Figure 2). Taken together, after Ukrain treatment both cell lines appear to undergo substantial changes in their cell cycle distribution followed by detection of apoptotic cells. The major difference between the ME180 and A431 cell lines is that the latter preserved a significant proportion of S-phase cells during

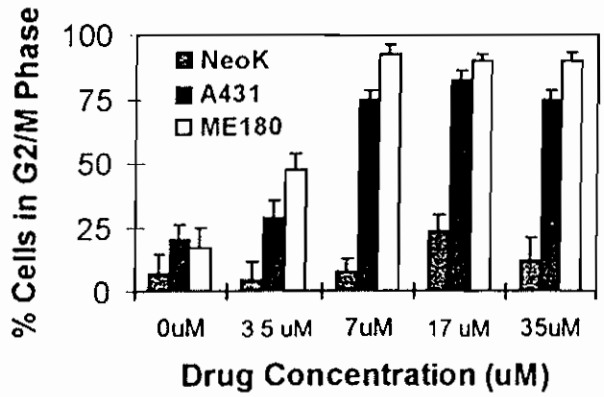

Figure 1. *Effect of different Ukrain concentrations on G2/M cell population. Cells were treated with the indicated concentrations (0; 3.5; 7.0; 17.5; and 35.0 μM) for 24 h and analyzed for DNA content by flow cytometry. The data presented are the percentage of NeoK. A431 and ME180 cell populations in G2/M. Results shown are representative of three independent experiments.*

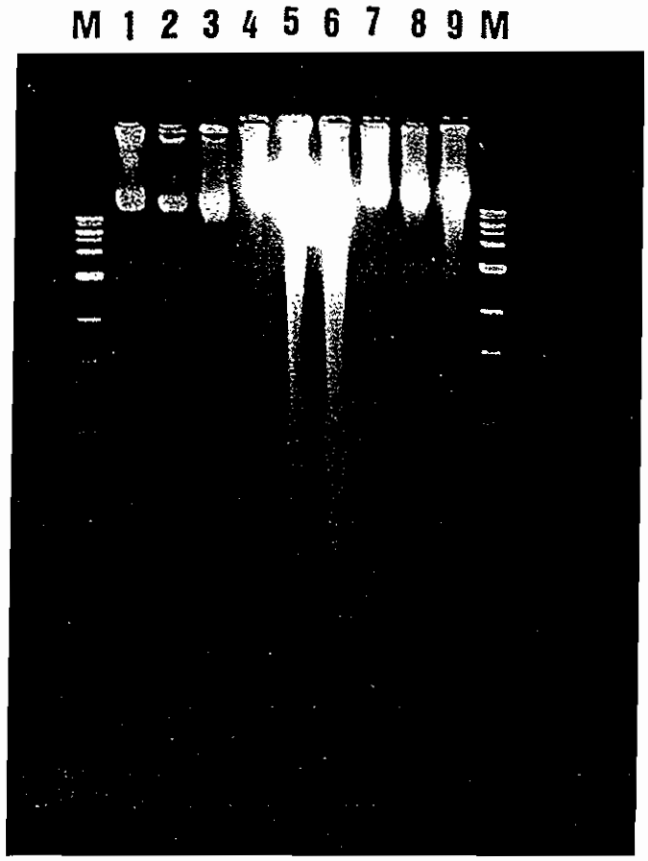

Figure 2. *Progressive DNA degradation with increasing time of Ukrain treatment. Lanes 1-9 contain 10 μg of DNA isolated from cells treated with 7.0 mM Ukrain for 0 h, 24 h, and 48 h, respectively. Normal keratinocytes (lanes 1-3), cell line A431 (lanes 4-6) and cell line ME180 (lanes 7-9). M, markers of DNA fragments, 1 kb ladder (Gibco BRL, Rockville, MD).*

treatment while ME180 accumulated more G2/M cells. This difference may be related to a delayed apoptotic response of ME180 relative to A431 and to the fact that ME180 cells are more differentiated compared to A431, as was judged by analysis of expression of select cellular proteins including keratinocyte transglutaminase (7).

Proliferation arrest of A431 and ME180 cells in G2/M phase by Ukrain is reversible. Cells were treated with 35.0 μM Ukrain for 24 hours then washed with PBS, and incubated for another 24 and 48 h in fresh DMEM. Changes in the DNA distribution were monitored by flow cytometry. As shown in Figure 3A, recovery of ME180 cells to control levels of G0/G1, S, and G2/M was achieved by 48 h. In contrast, recovery from G2/M arrest for A431 cells was close to complete by 24 h, except that approximately 14% cells were in apoptosis and this apoptotic fraction persisted through the end of the experiment at 48 h (Figure 3B).

Expression of the cyclins, CDKs and CDK inhibitor p27 changes after treatment with Ukrain. We next investigated the possibility that changes in the expression of cyclins, CDKs, and CDK inhibitors were occurring 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 cyclin 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 CDK's 4, 6, and 2, respectively, for proper G1 progression (8, 9). The kinase activity of cyclin E/CDK2, cyclin D/CDK4/6 and other cyclins/kinase binary complexes is suppressed by CDK inhibitors including p27 (10, 11). p27 has also been suggested to be a tumor suppressor and possible promoter of apoptosis (12, 13).

Following Ukrain treatment for 24 h at both 3.5 and 7 μM concentrations, ME180 cells expressed less cyclin A and B1, and CDK1, whereas CDK2 expression remained unchanged. In contrast, p27 protein expression was significantly increased, at least several fold (Figure 4, panel A). Changes in expression of these proteins in treated A431 cells were less dramatic than in ME180 cells, however p27 expression was clearly increased and CDK1 and CDK2 slightly increased at 3.5 μM Ukrain (Figure 4, panel B). Diminished expression of cyclin A and B1 in ME180 cells after Ukrain treatment is in agreement with the detected G2/M cell arrest.

Discussion

We have tested the effects of the anticancer drug Ukrain on human epidermoid cancer cell lines as well as primary normal human keratinocytes and found a striking difference in their response. At Ukrain concentrations of 7 μM, cancer cells, but not normal keratinocytes, accumulated in G2/M over a 24 h period. In addition, apoptosis was detected following 48 h

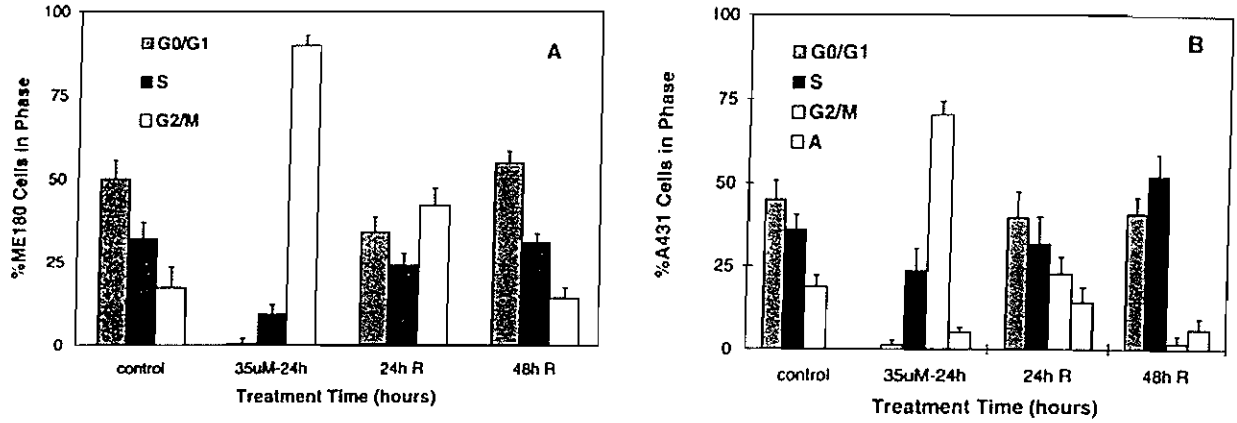

Figure 3. *Recovery from Ukrain treatment of ME180 (A) and A431 (B) cells after exposure for 24 h to 35.0 μM of compound. Following Ukrain removal, the cells were grown in drug-free medium. The summary data are from four samples from two independent experiments.*

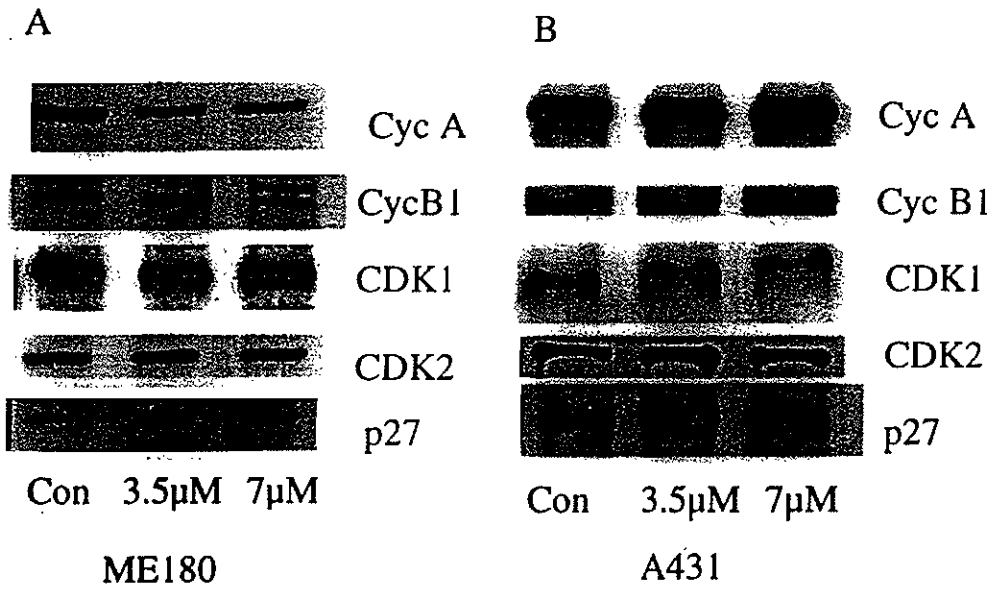

Figure 4. *Expression of the mitotic cyclins, cyclin-dependent kinases, and CDK inhibitor p27 in ME180 (A) and A431 (B) cells. Western blots were probed for cyclin A, cyclin B1, CDK1, CDK2, and p27 as indicated to the left of each panel.*

cancer cell treatment. Accumulation of cancer cells in G2/M phase may suggest that Ukrain belongs to the class of anti-tubulin drugs. This is an agreement with previous studies on the mechanism(s) of action of Ukrain. 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 (14). However, the possibility that Ukrain acts by alternate mechanism cannot be excluded at this time. Perhaps Ukrain damages DNA or affects DNA replication/repair mechanisms. It is well established that cell cycle arrest occurs after cell exposure to ionizing radiation or other DNA-damaging agents. Ukrain-induced G2/M cell cycle arrest and apoptosis, most likely, occurs via a p53-independent pathway since in A431 cells p53 is mutated (15), and in ME180 cells p53 expression is very low (16). Further studies outside the scope of this report will be needed to rigorously address this issue.

Ukrain treatment facilitates an elevation of CDK inhibitor p27 in both cancer cell lines. Elevation of p27 was accompa-

nied by lowering in abundance of cyclins A and B1 in ME180. but not in A431 cells. In contrast, levels of CDK1 and CDK2 were slightly up-regulated in A431 without significant changes in ME180 cells. These results are consistent with the findings that induction of apoptosis and block of cell cycle in G2/M are important determinants of the sensitivity of cancer cells to Ukrain. These data also suggest that alterations in the levels of cyclins/CDKs together with elevation of CDK inhibitor p27 may play an important role in G2/M phase arrest and apoptosis. The role of cyclin and cyclin-dependent kinase activity for an orderly progression through the cell cycle is well established (8). If the cyclin regulatory partner of the kinase is in short supply, it is reasonable to predict that decreased cyclin-dependent kinase activity will occur. Taken together with an increased abundance of the p27 CDK inhibitor, many cyclin/CDK complexes that are formed may have an increased probability of being inactivated due to increased p27 binding (9). Although not directly addressed in this study, this proposed mechanism appears to be valid as reported by us elsewhere (17, 18). Also, low expression of p27 is observed in many types of human tumors and correlates strongly with tumor aggression (19). Given that p27 is a suppressor of tumorogenesis (12) and that Ukrain possesses an intrinsic anti-cancer activity, it would not be surprising to detect elevated p27 level after carcinoma cell treatment by this agent. Our findings extend those of others supporting the development of Ukrain as a possible therapeutic agent and provide new insight into at least one possible mechanism of Ukrain action at the molecular level. Understanding, as well as possible modulation, of this mechanism involved in selective cancer cell death could be used as a model for providing safe and efficacious new therapies for cancers.

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