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Ukrain™, a semisynthetic *Chelidonium majus* alkaloid derivative, acts by inhibition of tubulin polymerization in normal and malignant cell lines

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bstract

Ukrain™ has been described as a semisynthetic *Chelidonium majus* alkaloid derivative, which exhibits selective toxicity wards malignant cells only. Its mechanism of action has hitherto been uncertain. We found that Ukrain™ inhibits tubulin lymerization, leading to impaired microtubule dynamics. This results in activation of the spindle checkpoint and thus a taphase block. The effects of Ukrain™ on the growth, cell cycle progression and morphology of two normal, two transmed and two malignant cell lines did not differ. We could thus find no evidence for the selective cytotoxicity previously orted for Ukrain™. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

ywords: Ukrain; Chelidonium; Alkaloid; Chelidonine; Tubulin polymerization

Introduction

Ukrain™ has been described as a semi-synthetic ophosphoric (triaziridide) derivative of the purified caloid chelidonine isolated from the plant *Chelidonium majus* L. [1]. This drug has previously been orted to be an effective anti-cancer agent with nimal side-effects, because of its selective toxicity wards malignant cells as demonstrated in vitro (see ction 4 for more details). The mechanism of action Ukrain™ is as yet unknown.

The purpose of this study was to determine the echanism of action of Ukrain™ and to confirm its selective toxicity towards malignant cells by examining its effects on the growth, cell cycle progression and morphology of two malignant, two transformed and two normal cell lines.

2. Methods

HeLa (human cervical carcinoma) and Hs27 (human foreskin fibroblast) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA), while Graham 293 (transformed human embryonic kidney) and Vero (transformed African green monkey kidney) cells were obtained from Highveld Biological (Sandringham, SA). The WHCO5 cells, which were originally isolated from a biopsy specimen of a patient with squamous oeso-

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phageal cancer, were a gift of Professor A. Thornley (Department of Zoology, University of the Witwatersrand). The normal monkey kidney cells (isolated from adult vervet monkey kidney) were donated by Mr C. Swanevelder (Department of Virology, University of Pretoria). The cell lines were maintained as monolayer cultures in Eagle's minimum essential medium with Earle's salts and L-glutamine supplemented with 10% heat inactivated fetal calf serum (all obtained from Sigma Chemical Co., St. Louis, MO). No antibiotics were used. Cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Two different batches (lot numbers 544325 and 544324) of Ukrain™ ampoules (1 mg/ml dissolved in H_2O), as well as Ukrain™ powder and *Chelidonium* alkaloid mixture powder were provided by Nowicky Pharmaceuticals (Margaretenstrasse 7, 1040 Vienna, Austria). Chelidonine (lot number 38H0621) was purchased from Sigma Chemicals Co. All chemicals were stored at 4°C until use.

2.1. Growth studies

After Trypan Blue exclusion, cells from stock flasks were seeded at 5×10^3 cells per well of a 96-well plate. After 24 h, 1 ml of medium, containing various concentrations of drug, was added to each well. The alkaloid mixture and Ukrain™ powder were dissolved in water (1 mg/ml). Chelidonine was dissolved in dimethyl sulfoxide (DMSO), and subsequently diluted with medium, so that the final concentration of DMSO in the medium did not exceed 0.05%. Control cells were exposed to ddH_2O or DMSO only, depending on the vehicle of the drug. Growth was terminated after 48 and 120 h, after which the DNA was stained with crystal violet and the chromophore was extracted and spectrophotometrically analyzed according to the method described by [2]. Four to six wells were analyzed for each concentration and time. Data was statistically analyzed for significance using the analysis of variance (ANOVA) single factor model.

2.2. Haematoxylin and eosin (H&E) stains

H&E stains were performed as described previously in [3].

2.3. Indirect immunofluorescence

Three hundred thousand cells were seeded per heat-sterilized cover slip in a 34.6 mm diameter well. Approximately 24 h later, fresh medium containing 12.5 or 50 μg/ml Ukrain™, 10 μM chelidonine and their respective controls (medium or 0.05% DMSO) were added to separate wells. At 24 h after drug addition, cells were fixed in 10% formalin, 2 mM (ethylenebis(oxononitrilo))tetraacetate (EGTA) in phosphate-buffered saline (PBS) for 10 min and then transferred to 97% methanol, 2 mM EGTA (−20°C) for 10 min. Sequential treatments with anti-tubulin mouse monoclonal antibody (1:100) (Sigma clone TUB 2.1), biotin conjugated anti-mouse IgG (Fab specific) developed in goat (Sigma), diluted 1:100 in FITC conjugate diluent (Diagnostic and Technical Services, Johannesburg, South Africa), ExtrAvidin FITC (Sigma), diluted (1:200) in FITC conjugate diluent, and 4,6-diamino-2-phenylindole (DAPI) (0.1 μg/ml) were performed as described by [4]. Photographs were taken with 1600 ASA film on a Nikon Optiphot microscope equipped with an episcopic-fluorescence attachment and an excitation-emission filter with an average wavelength of 495 nm for FITC and 400 nm for DAPI.

2.4. Flow cytometry

Cells from stock flasks were seeded at 3×10^5 per 25 cm^2 flask after Trypan Blue exclusion and left for 24 h, before administering Ukrain™ containing medium for 24–72 h. Untreated cells served as controls. Growth was terminated by trypsinizing cells in 1 ml of 0.25% trypsin/1 mM ethylene diamine tetra-acetic acid (EDTA), washing with PBS, and then fixing by dropwise addition of ice-cold methanol. Samples were stored at −20°C for 24–72 h, before centrifugation at $250 \times g$ for 5 min and resuspension in 1 ml PBS containing 50 μg/ml of propidium iodide (Sigma). Specimens were examined within 2 h on a Coulter Epics XL Flow Cytometer (system II software), while data was analyzed using MulticycleAV software. At least 10 000 events were counted for each sample.

2.5. Tubulin polymerization assay

Potential antitubulin agents can be evaluated by

determining the concentration of a test compound required to inhibit the extent of glutamate-dependent tubulin polymerization by 50% after 20 min (the IC_{50} value) [5]. Tubulin was previously prepared by purification of bovine brain as described in [6]. Reaction mixtures (final volume of 0.5 ml) contained 0.8 M monosodium glutamate (MSG), 1.0 mg/ml tubulin, 4% DMSO and no Ukrain (control), 10, 20, 30, 40 and 50 μM Ukrain™ in H_2O. Samples were incubated at 30°C for 15 min and then chilled on ice. Subsequently, 10 μl of a 10 mM guanosine triphosphate (GTP) solution was added to each sample to reach a

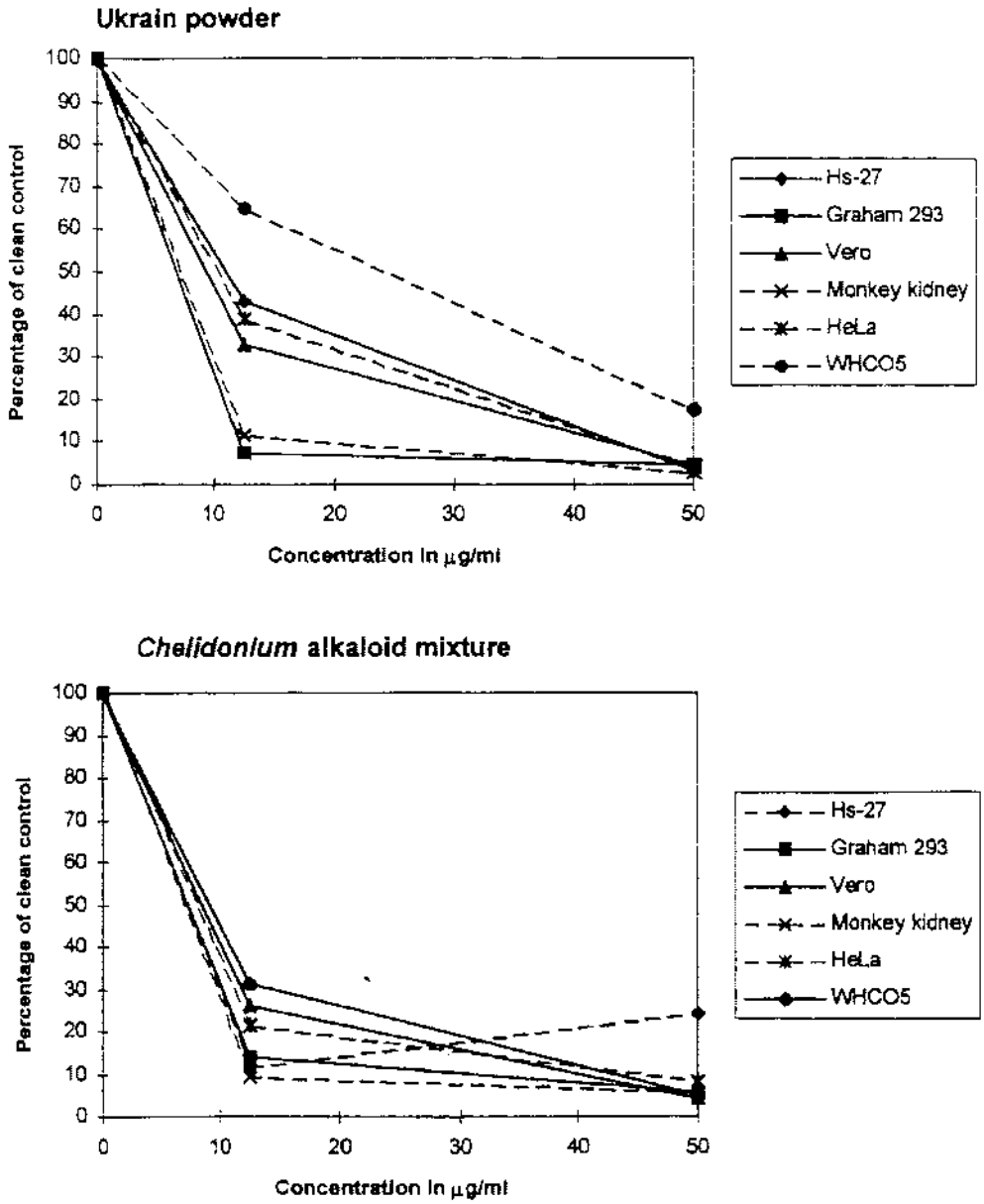


Fig. 1. The effects of Ukrain™ and the *Chelidonium* alkaloid mixture on cell growth at 48 h. Results are expressed as a percentage of untreated controls.

final concentration of 0.4 mM. Samples were then transferred to a Gilford 250 recording spectrophotometer equipped with electronic temperature controllers. Baselines were established with the cuvettes held at 0°C, and the reaction was initiated by a 75 s jump to 30°C. Polymerization was followed for 20 min at 30°C. Results of the net absorbance at 350 nm were measured [5,7].

3. Results

3.1. Growth studies

In initial studies we could find no evidence of selective toxicity of Ukrain™ for malignant cell lines, since the Hs27 and primary monkey kidney cells were as sensitive to Ukrain™ (in powder form; Fig. 1, top panel) or in solution (two separate batches, data not shown) as the transformed or tumor cell lines. In multiple experiments when any of the six cell types were exposed to 50 μg/ml Ukrain™ for 48 or 120 h, there was significant growth inhibition relative to controls ($P < 0.01$). Furthermore, the effects of Ukrain™ seemed to be similar to those of the *Chelidonium* alkaloid mixture (prior to reaction with thiophosphamide; Fig. 1, lower panel). Maximum inhibitory effects with chelidonine were obtained at the lowest concentration tested (10 μM, data not shown). Growth inhibition with chelidonine was greater than 50% in only three cell lines (HeLa, monkey kidney and Vero cells). Growth inhibition found after 120 h exposure to the above drugs was similar to the results demonstrated after 48 h (data not shown).

3.2. Flow cytometric and morphological studies

Ukrain™ and chelidonine both lead to a dose-dependent G2M arrest in all cell types studied. WHCO5 cells, which were exposed to Ukrain™ for 24 h, are shown as an example (Fig. 2). Ukrain™ treated WHCO5 cells were also examined following

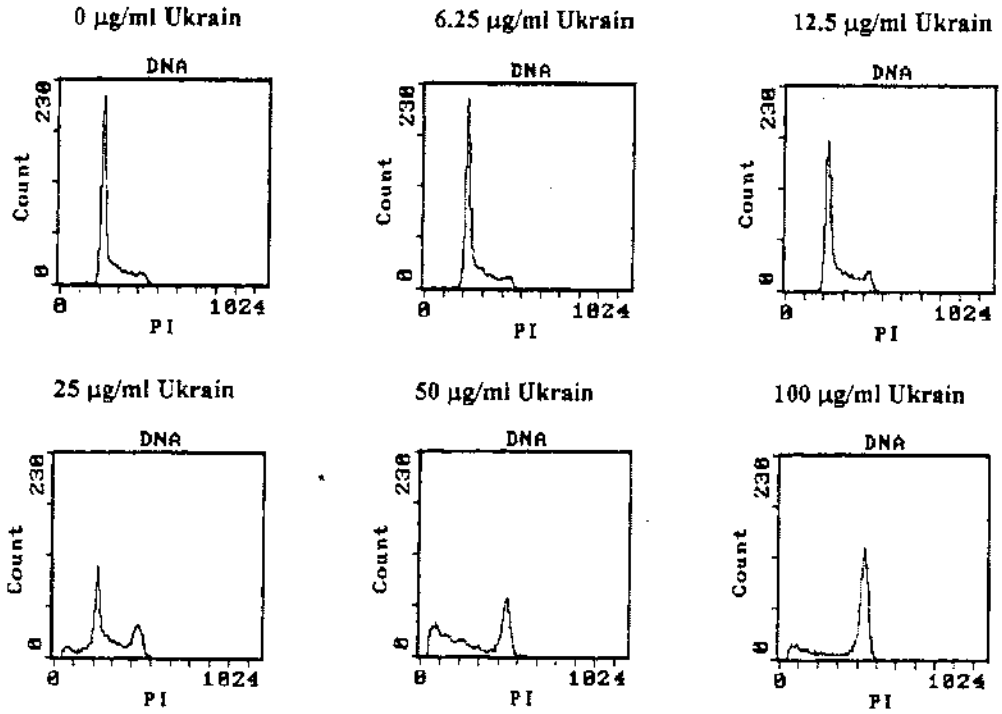


Fig. 2. DNA histograms of WHCO5 cells exposed to 0, 6.25, 12.5, 25, 50 and 100 μg/ml Ukrain™ for 24 h. Propidium iodide staining of DNA is plotted on the *x*-axis, while the *y*-axis indicates total cell count.

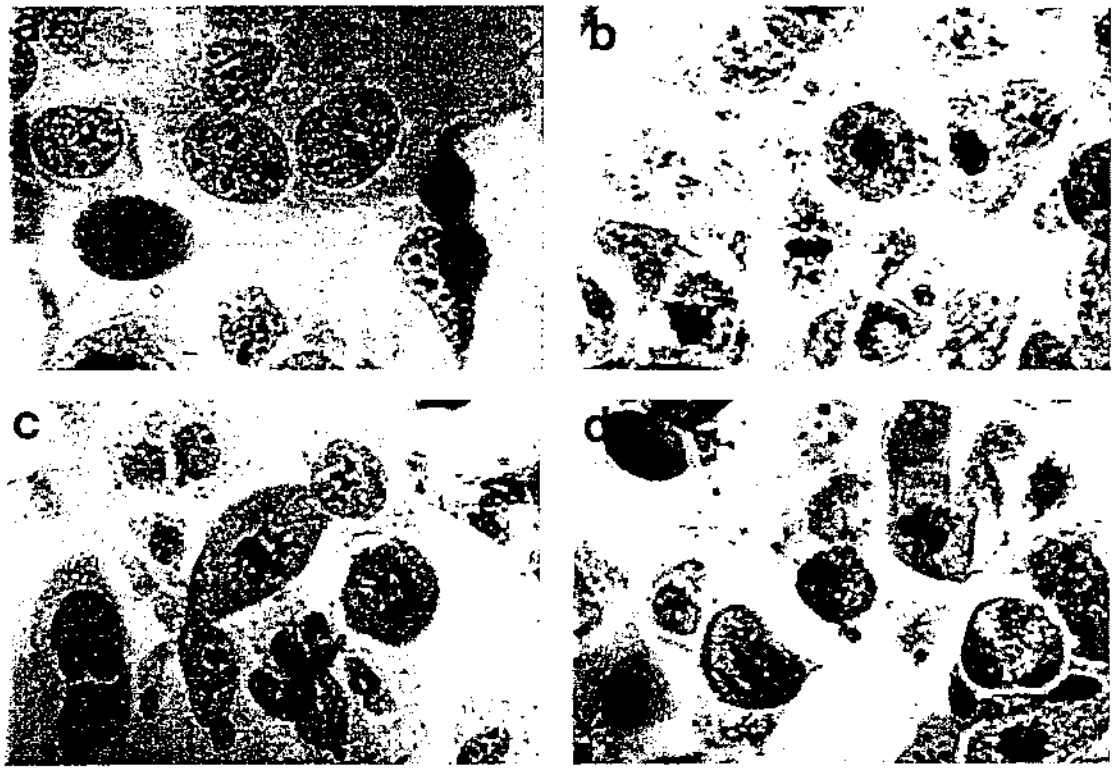


Fig. 3. H&E stains of WHCO5 cells which were exposed to 0 (a), 12.5 (b), 25 (c) and 50 μg/ml Ukrain™ (d) for 24 h.

ation and H&E staining (Fig. 3). An increase in etaphase cells with abnormal morphology, e.g. here not all chromosomes are aligned on the meta-ase plate, is evident.

3. *Indirect immunofluorescence*

Abnormal metaphase spindles were present in all ll types studied. Hs27 and Graham 293 cells are own as examples in Figs. 4 and 5, respectively. ote the normal metaphase and anaphase cells in e control Hs27 cells. Abnormal metaphase spindles e evident in the 12.5 μg/ml Ukrain™ exposed Hs27 lls: an abnormal spindle, in which chromosomes m equal masses on either side of the metaphase ate is seen on the left, while a spindle which appears onopolar and is enclosed by a ball-shaped mass of romosomes, is evident on the right. In the untreated raham 293 cells (Fig. 5), a normal metaphase and ophase cell can be seen. Ukrain™ treatment leads to the formation of abnormal spindle figures. In the Graham 293 cells, nearly complete disappearance of microtubules in interphase cells is also evident.

3.4. *Tubulin polymerization*

For the purposes of this experiment the molar mass of Ukrain™ was taken as 1470. Potential antitubulin agents can be evaluated by determining the concentration of a test compound required to inhibit the extent of glutamate-dependent tubulin polymerization by 50% after 20 min (the IC_{50} value) [7]. In this system, in which 10 μM tubulin is used, the most potent antitubulin agents yield IC_{50} values of about 1.0 μM. Such agents typically arrest cells in mitosis at media concentrations of 1–10 nM. Both Ukrain™ and chelidonine had weak activity in this system, yielding IC_{50} values of 23 ± 2 ($n = 3$) and 24 ± 2 ($n = 3$) μM.

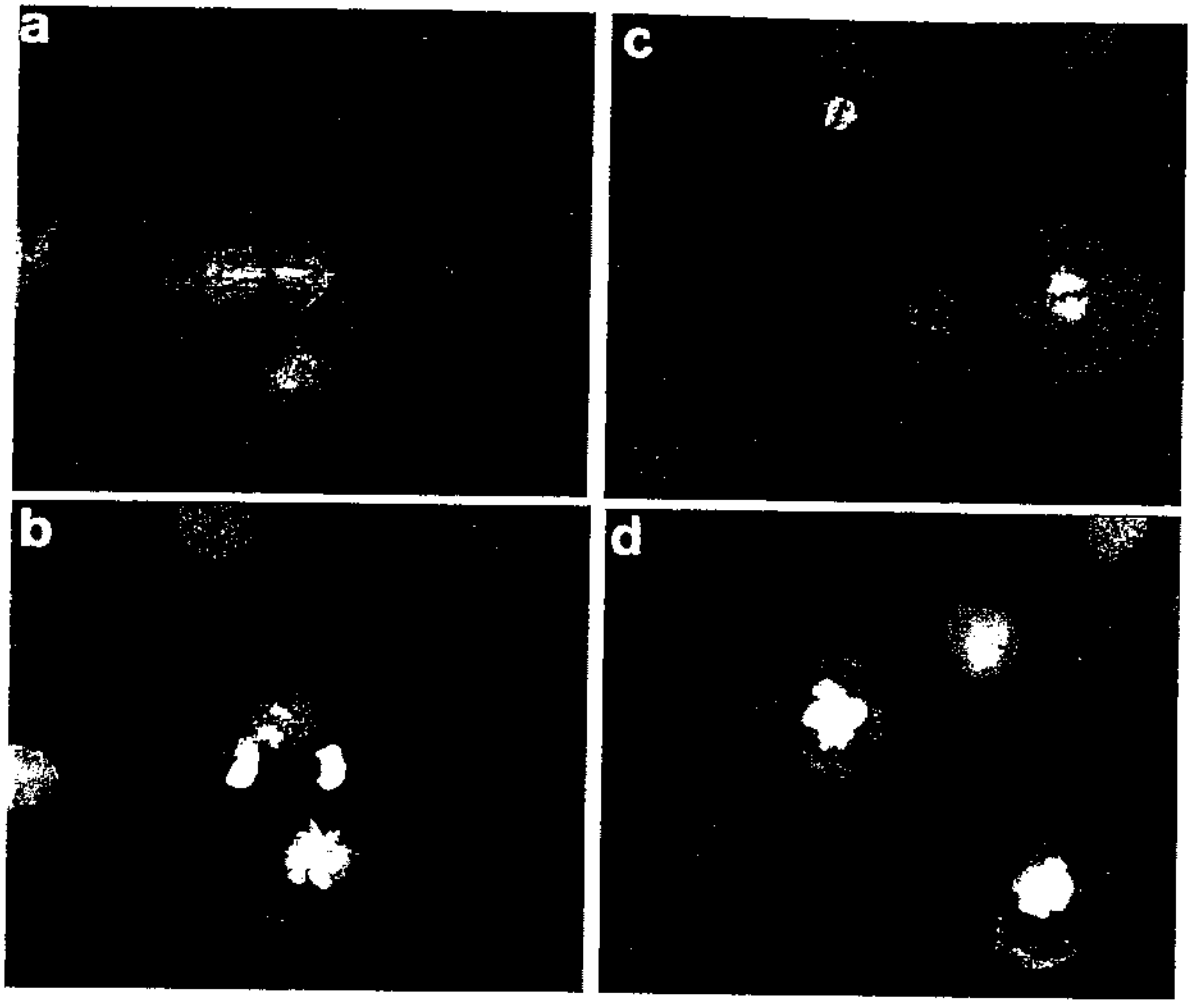


Fig. 4. Indirect immunofluorescence for β-tubulin (to stain the microtubules) and DAPI (to visualize chromosomes) in Hs27 cells exposed to 0 (control) (a,b) or 12.5 μg/ml Ukrain™ (c,d) for 24 h.

4. Discussion

The greater celandine (*C. majus* L.) is a member of the Papaveraceae family and is a common weed in Europe and Western Asia [8]. This plant has been used in the therapy of warts, skin cancers, liver- and gallbladder diseases for many years [9]. Ukrain™ is a semi-synthetic thiophosphoric acid (triaziridide) derivative of the purified alkaloid chelidonine isolated from *C. majus* L. [10].

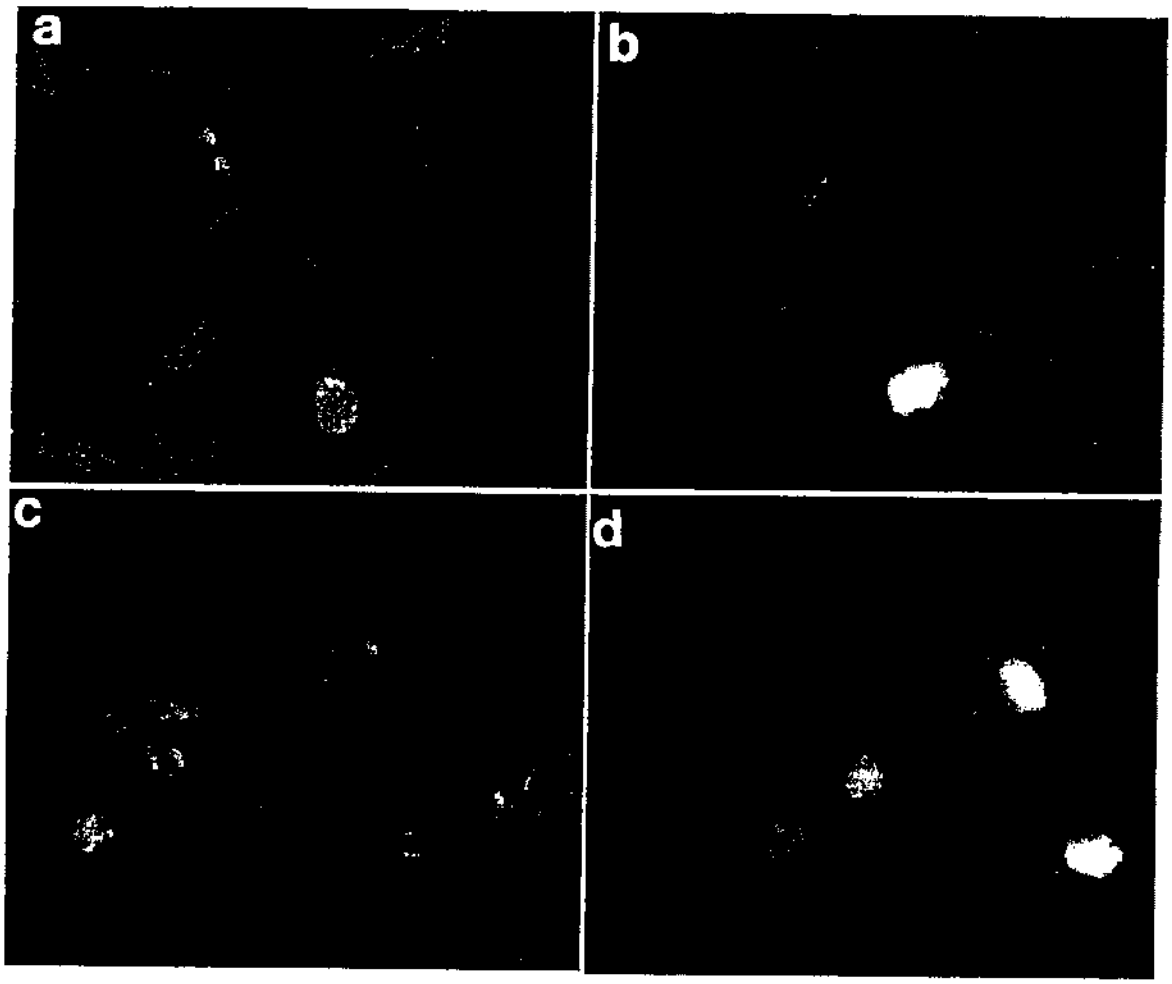
Ukrain™ has been described as causing regression of primary tumors and metastases in as many as 400 cancer patients with a wide variety of tumor types [11–17]. Moreover, there are reports that pretreatment with Ukrain™ can considerably facilitate surgery by reduction in tumor mass [14,18]. Minimal side-effects have been described with Ukrain™ treatment by [10,19], and this has been attributed to the agent's selective toxicity toward malignant cells [20–22].

Ukrain™ was evaluated by the National Cancer Institute (USA) in its drug screening program as NSC 631570 (http://dtp.nci.nih.gov). Results for compounds examined in this screen are expressed as the concentrations of drug that produce 50% inhibition of cell growth (GI_{50}), total inhibition of cell growth (TGI) and 50% reduction of cell biomass

LC_{50}). The highest concentration of Ukrain™ tested was 3.8×10^{-4} M (559 μg/ml). After 48 h the mean values, (i.e. averages for all cell lines successfully tested) obtained were 2.8×10^{-6} M (4.1 μg/ml) for the GI_{50}, 1.6×10^{-5} M (23.5 μg/ml) for the TGI, and 6.7×10^{-5} M (98.5 μg/ml) for the LC_{50}. The highest concentration of Ukrain™ tested was insufficient to reach TGI in three cell lines and LC_{50} in 21 cell lines. Thus, the mean TGI and LC_{50} values are actually underestimates, since the highest concentration tested is taken as the value obtained in the computer generation of mean values.

The conclusion that Ukrain™ is non-toxic to normal cells is based on minimal data [20–23]. For example, in one study three normal cell lines were monitored and visual inspection provided no evidence of apoptosis [22]. In another, synthesis of macromolecules in normal tonsil and hepatocyte cell lines, compared to malignant cell lines, was less inhibited by Ukrain™ [24].

The explanation given for this apparent selective toxicity is that different levels of Ukrain™ uptake occur in normal and tumor cells [20]. The mechanism of action of Ukrain™ at the cellular level is thought to involve effects on oxygen consumption [25,26]. Other proposed mechanisms of action are inhibition of DNA, RNA and protein synthesis [24] and induction of apoptosis through an unspecified pathway [27].



5. Indirect immunofluorescence for β-tubulin (to stain the microtubules) and DAPI (to visualize chromosomes) in Graham 293 cells exposed to 0 (control) (a,b) or 12.5 μg/ml Ukrain™ (c,d) for 24 h.

Alternatively, Ukrain™ could have the same mechanism of action as chelidonine, the compound from which it is prepared [1,28]. Chelidonine inhibits microtubule polymerization and causes a mitotic block [29]. A further indication of mechanism of action can be gleaned by using the COMPARE algorithm of the NCI1, in which patterns of cytotoxicity against the 60 cell lines obtained with a test drug are matched with all the other agents in the database [30]. The algorithm calculates the Pearson correlation coefficient (PCC) of the degree of similarity between the patterns obtained with two agents [30]. If the test drug causes a 50% growth inhibition of HL-60 (TB) leukemia cells at a concentration of 1 μM or less, and has a PCC of at least 0.6 with at least one known antimitotic drug, it has a high likelihood of interacting with tubulin [30]. Chelidonine hydrochloride (NSC 406034) has a GI_{50} of $10^{-5.8}$ M (1.58 μM) in the HL-60 (TB) cells, and exhibits a similar cytotoxicity pattern to halichondrin B (PCC 0.776), podophyllotoxin (PCC 0.733), and nocodazole (PCC 0.667), which are all known microtubule inhibiting drugs. Ukrain™ (NSC 631570) was shown to cause a 50% growth inhibition of the HL-60 (TB) cells at $10^{-5.99}$ M (1.023 μM). By using the COMPARE algorithm, Ukrain™ was found to have a similar cytotoxicity pattern to colchicide HCl (PCC 0.715), vinblastine sulfate (PCC 0.655) and maytansine (PCC 0.649), indicating that its mechanism of action may involve interaction with tubulin.

The studies presented here were undertaken, first, to confirm the selective toxicity of Ukrain™ towards malignant cells by examining its effects on the growth, cell cycle progression and morphology of two malignant, two transformed and two normal cell lines and, secondly, to determine whether Ukrain™ had a tubulin-based mechanism of action.

We were unable to confirm earlier reports that Ukrain™ had minimal effects on the growth of non-malignant cells in tissue culture, for we found little difference in its inhibitory effects on the growth of six cell lines (two derived from malignant tumors, two transformed cell lines, and two non-malignant cell lines). The mixture of *Chelidonium* alkaloids from which Ukrain™ is prepared, showed a similar nonselective cytotoxicity pattern (see Fig. 1). Moreover, in all cell lines Ukrain™ caused cells to accumulate at the G2M phase of the cell cycles, and morphological studies demonstrated that these cells were arrested at metaphase with malformed mitotic spindles. This initially unanticipated finding [24–26] led us to explore the possibility that the specific mechanism of action for Ukrain™ was a relatively weak interaction with the spindle protein tubulin. Two lines of evidence support this conclusion. First, the COMPARE algorithm on NCI cell screen data yielded PCC's >0.6 when Ukrain™ was analyzed versus several well-described anti-tubulin drugs. Secondly, we demonstrated that relatively high concentrations of Ukrain™ inhibited the polymerization of purified bovine brain tubulin.

We conclude that Ukrain™ acts by inhibition of tubulin polymerization in cells in a non-selective manner.

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