CANCE
CONGRES
Rio de Janeiro, Bra.
24-28 August 19

Ukrain (NSC-631570) inhibits angiogenic differentiation of human endothelial cells *in vitro*

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SUMMARY

Ukrain, a semi-synthetic drug derived from alkaloids of Chelidonium majus L. conjugated to thiophosphoric acid, has been reported to have therapeutic efficacy in treatment of patients with advanced primary and metastatic malignancies. This drug has unique properties such as direct stimulation of cytolytic activity of immune effector cells and tumor-specific cytotoxic activity. In this study we demonstrated that Ukrain also acts as a potent inhibitor of *in vitro* angiogenesis.

INTRODUCTION

UKRAIN (NSC-631570) represents a semi-synthetic compound formed by alkaloids of Chelidonium majus L. with thiophosphoric acid derivatives, triethylenethiophosphoric acid triamide (Thio-TEPA). The final product contains at least 90% Chelidonium majus alkaloid acid derivative and a maximum of 10% of free Chelidonium majus alkaloids, while Thio-TEPA of free aziridine components can not be detected (Nowicky, 1983). Ukrain has been reported to have therapeutic efficacy in treatment of patients with advanced primary and metastatic malignancies (Musianowycz et. al, 1992, Lohninger et. al, 1993).

It has been shown in several immunological target-effector cell systems that at relatively low concentrations (1μM) Ukrain acts as an effective biological response modifier, directly enhancing the cytotoxic activity of macrophages, lymphocytes and NK cells towards different tumor cells (Liepins et. al, 1996). At higher concentration (10-50μM) Ukrain develops cytotoxic activity via a dose - dependent inhibition of DNA-, RNA- and protein synthesis and induces apoptosis in tumor cells, but not in non-malignant cells (Nowicky et. al, 1996). Antineoplastic effect of Ukrain was analyzed on 60 different human cancer cell lines of the 8 most common types of solid human tumors. Almost all cell lines demonstrated a growth inhibition between 50% and 100% that, at higher concentrations, turned into cytolytic effect (Nowicky et. al, 1993). However, the mechanisms of therapeutic effect of Ukrain remain to be fully elucidated.
The aim of present study was to investigate whether Ukrain could also act as an anti-angiogenic factor. For this purpose we used an *in vitro* angiogenesis model (Kubota et. Al, 1988) based on the ability of human endothelial cells to form networks of capillary tubes on basement membrane extracts from murine Engelbreth-Holm-Swarm tumor (Matrigel).

MATERIALS AND METHODS

Human umbilical vein endothelial cells (HUVECs) were isolated by mild collagenase treatment and grown in gelatine coated plastic flasks using M199 medium containing 20% supplemented calf serum (SCS), 50mg/ml endothelial growth supplement (ECGS) and 5U/ml heparin. The cells were confirmed as endothelial by their "cobblestone" morphology, positive staining with von Willebrand factor antibodies and uptake of immunofluorescent-labeled acetylated low-density lipoprotein.
Capillary tubes formation was analyzed using commercially available Matrigel (Becton Dickinson) diluted 1:1 with M199 medium containing 5% SCS. HUVECs (30.000 cells/well) were plated on polymerized Matrigel and analyzed after 24 h. For quantification of tube-like structures, three randomly selected fields in 16-mm wells of duplicated samples were photographed at a single level beneath the surface monolayer using phase-contrast microscopy. Digitalized images were analyzed with NIH-Image software to calculate the total additive length of skeletonized tube-like structures.
Proliferation and cytotoxicity assay was performed using non-radioactive EZ4U kit (Biomedica) based on the finding that living cells are capable to reduce uncolored tetrazolium salts into intensively colored formalin derivatives. This reduction process requires functional mitochondria, which are inactivated within a few minutes after cell death.

RESULTS AND CONCLUSIONS

Figure 1A demonstrates that treatment of confluent HUVECs cultured on gelatinized plastic with Ukrain (10-50 μM) does not induce significant changes in cell morphology even at concentrations of drug described to be cytotoxic for many different tumor cells (Nowicky et. al, 1993). Proliferation and cytotoxicity assay showed that Ukrain inhibits HUVECs growth in dose-dependent manner (10-50μM) to approximately 50% of control at highest concentration, but has no cytotoxic effect (Fig. 2).

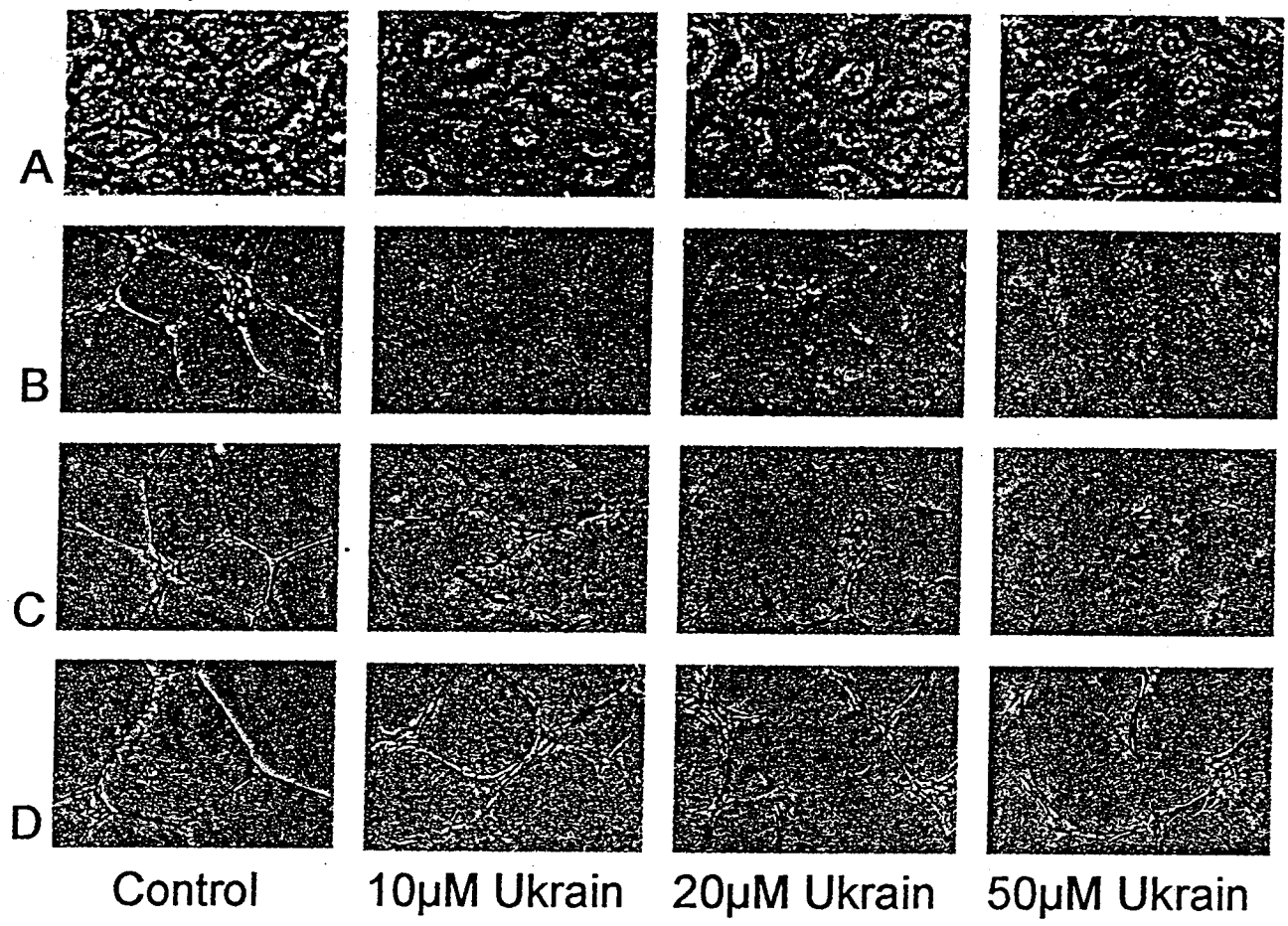

Figure 1. Influence of Ukrain on morphology of HUVECs growing on gelatin and Matrigel. A – HUVECs were grown on gelatin-coated plastic to confluency and incubated overnight with indicated concentration of Ukrain. B – HUVECs were preincubated for 4 h with indicated concentrations of Ukrain and plated on Matrigel. C – HUVECs were plated on Matrigel containing indicated concentrations of Ukrain. D - HUVECs were preincubated for 4 h with indicated concentrations of Ukrain, incubated for 2 h in fresh medium without Ukrain and plated on Matrigel.

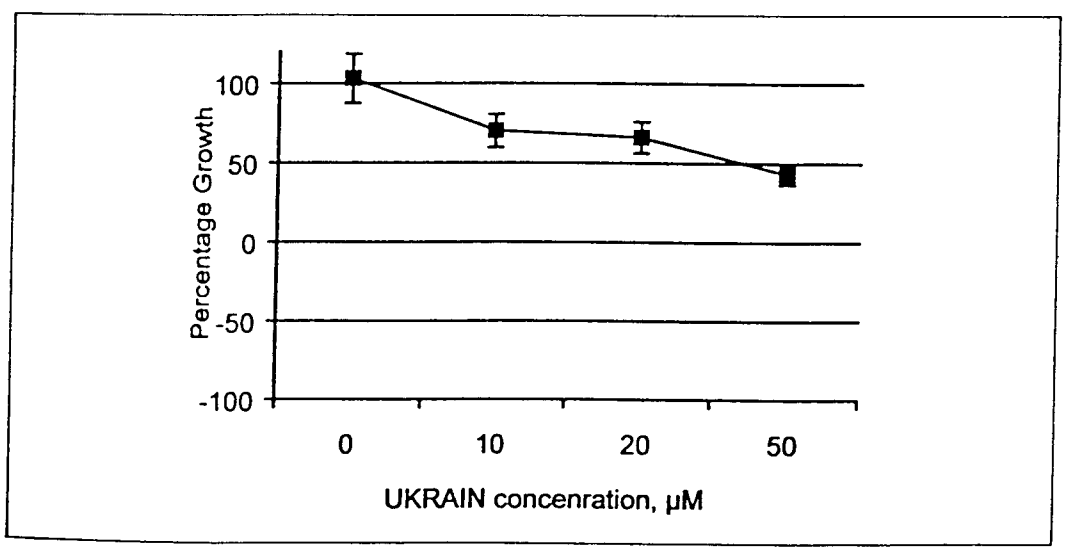

Figure 2. Proliferation assay. HUVECS were grown to 50 % of confluency in 96 well plates, than indicated concentrations of Ukrain were added and cells were grown for additional 72 h. Afterwards, cells were incubated for 3 h with EZ4U substrate, and extinction was measured using microplate reader (at 492 nm filter and 620 nm reference wavelength).

We found that Ukrain (10-50μM) efficiently blocked capillary tube formation of HUVECS plated on Matrigel. This inhibitory effect was observed when Ukrain was added to confluent cultures of HUVECS 4 h before or immediately after plating on Matrigel (Fig. 1B,C). When HUVECs were pretreated with 10-50μM Ukrain for 4 h followed by 2 h incubation in medium without Ukrain, tube forming potential was almost completely restored (Fig.1D, Fig.3). Quantitative data show that inhibitory effect of Ukraine on tube formation is more pronounced when cells are pre-treated with Ukrain (38 % at 10μM, 72% at 20μM and 91% at 50μM). Simultaneous addition of Ukrain to the incubation medium during plating of HUVECs on Matrigel leads to 39%, 52% and 88% inhibition of tube formation, respectively (Fig 1B,C, Fig.3).

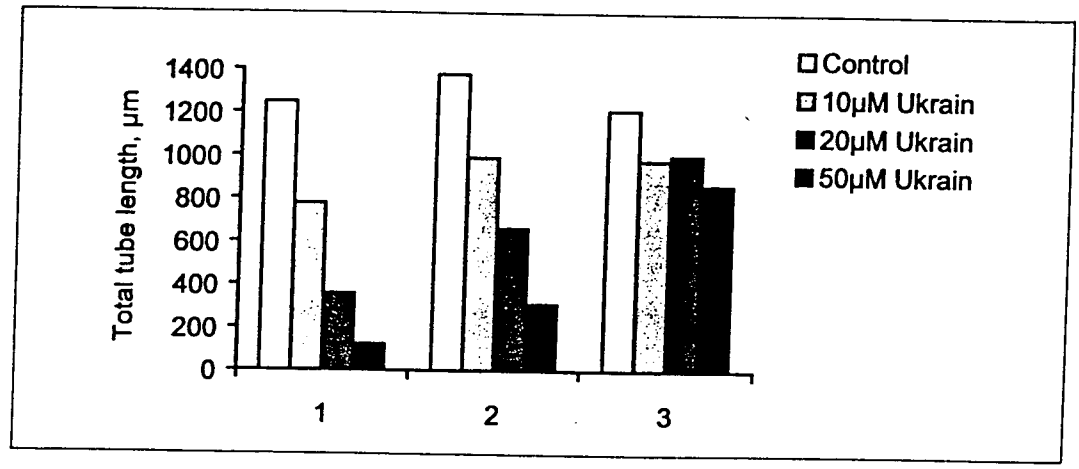

Figure 3. Quantification of capillary tubes length. 1- HUVECs were preincubated for 4 h with indicated concentrations of Ukrain. 2 – HUVECs were plated on Matrigel containing indicated concentrations of Ukrain. 3 – HUVECs were preincubated for 4 h with indicated concentrations of Ukrain and incubated for 2 h in fresh medium without Ukrain.

The major conclusions regarding our findings are:
1. Ukrain demonstrates a strong inhibitory effect on formation of capillary-like tube structures of human endothelial cells *in vitro*. 2. This inhibitory effect is reversible - removal of Ukrain by incubation of cells in fresh medium could rescue the tube-forming potential of endothelial cells. 3. The highest concentration of Ukrain (50μM) almost completely (>90%) inhibits angiogenic tube formation, but is not toxic for endothelial cell.

Whether this effect reflects a direct Ukrain influence on the capillary tube forming capacity of endothelial cells or is mediated indirectly via induction of anti-angiogenic factors has still to be elucidated. Also further investigations of anti-angiogenic activity of Ukrain *in vivo* have to be performed.

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