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IN VITRO EFFECTS OF UKRAIN ON THE ACTIVITY OF TRYPSIN-LIKE PROTEASES

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Summary: *The interactions between Ukrain and peptide hydrolases from the intestinal tracts of* drosophila *were studied. It was established that the active ingredients of Ukrain could stimulate or inhibit the proteolytic activity of these enzymes. The possible importance of these findings is discussed.*

Introduction

A considerable body of evidence indicates that cell-cell interactions, and cell interactions with the exacellular matrix (ECM), are essential organizing principles that help define the nature of the tissue context, and that these interactions play important roles in regulating homeostasis and tissue specificity.

The ECM is a complex structural entity that surrounds and supports cells within mammalian tissues. It is composed of three major classes of biomolecules: structural proteins, such as collagen and elastin; specialized proteins, such as fibrillin, fibronectin and laminin; and proteoglycans—protein cores that are attached to long chains of repeating disaccharide units termed glycosaminoglycans. The ECM has recently received considerable attention because of its importance in cell-cell signaling, wound repair, cell adhesion and tissue function. The status of the ECM is important in the development of cancer diseases: tumor cells produce a number of proteases that degrade the ECM and promote angiogenesis. In general, the proteases involved in the invasive process of many tumors are members of four classes of endopeptidases: matrix metalloproteases, serine proteases, aspartic proteases and cysteine proteases (1).

It is mainly lysosomal cysteine proteinases that are involved in tumor progression. Increased levels of cathepsins B and L have been shown to correlate with the rapid growth and metastasis of tumors and

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transformed cell lines. A strong correlation between overexpression and malignancy has been confirmed for cathepsins B and L, but not for cathepsin H. Among cysteine proteinases, cathepsin S shows tissue-specific expression (lymphoid tissue, lymphocytes) (2).

It has previously been shown that Ukrain normalizes the status of proteinases and their inhibitors after single or repeated administration in rats (3, 4). Ukrain also acts as an antiangiogenic drug (5) that can exert a suppressing effect on new blood vessel formation through the modulation of protease activity. Recently, beneficial results have been demonstrated from the use of protease inhibitor-based antiretroviral therapy in AIDS patients (6). Ukrain can also improve the immune status of patients with AIDS (7).

All these findings formed the background for the present study, which sought to identify that part of the malignotoxic impact of Ukrain that might be the result of protease activity.

The investigations were performed *in vitro*, studying the direct effects of Ukrain on two different enzymes: bovine pancreatic trypsin, and peptide hydrolase from the intestinal tracts of *Drosophila*.

Materials and methods

The investigations used bovine pancreatic trypsin (Sigma, St. Louis, MO, USA), and peptide hydrolase from the intestinal tracts of *Drosophila*. The latter was obtained by extraction from the homogenates of *Drosophila* larvae.

N_{alpha}-benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA) and N_{alpha}-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were used as the substrates. Substrate hydrolysis was performed at pH 9.0 for 60 min at 42 °C using 0.1 M glycine-NaOH buffer prepared by ourselves.

The speed of substrate hydrolysis product formation was recorded at wavelengths of 382.5 nm (p-nitroaniline) and 253 nm (benzoyl-arginine).

Two investigative procedures were followed. In the first, the enzyme solutions (100 μg in 100 μl buffer) together with Ukrain (Nowicky Pharma, Vienna, Austria) (100 μg in 100 μl H_2O) and the substrate solutions (1 mM in 500 μl) were injected into the buffer system (2.3 ml). In this case, direct interaction between the reactive compounds that could cause enzyme inactivation was impossible. In the second procedure, the enzyme was incubated with Ukrain for 10 min at room temperature, and then the buffer and substrate were added to the mixture. The control sample contained only buffer solution (2.5 ml) and a matching substrate solution (500 μl). Enzyme activity was determined by changes in the extinction rate. The data presented characterized the activity of the two enzymes studied. A detailed description of the measurement of proteolytic activity using the above substrates has been presented in previous work (8-10).

Results

Firstly, it was established that Ukrain has the ability to hydrolyze the synthetic substrate BAPNA. This pseudopeptide can be hydrolyzed only by trypsin or trypsin-like enzymes, such as acrosine and carboxy peptidase, and thus may be used to identify these enzymes. It is very resistant to nonenzymatic hydrolysis at pH 3-10 and may only be cleaved enzymatically. Therefore, according to the experimental data, we can assume that Ukrain or its alkaloids (chelidonine, cheleritrine, berberine, *etc*.) can interact with the chromogenic substrate BAPNA, causing its degradation with p-nitroaniline formation. At 15 min from the start of incubation of Ukrain with trypsin, the enzyme-activating effect was observed. Ukrain displays weak peptide hydrolase activity, but this developed at 60 min from start of incubation. It is possible that the

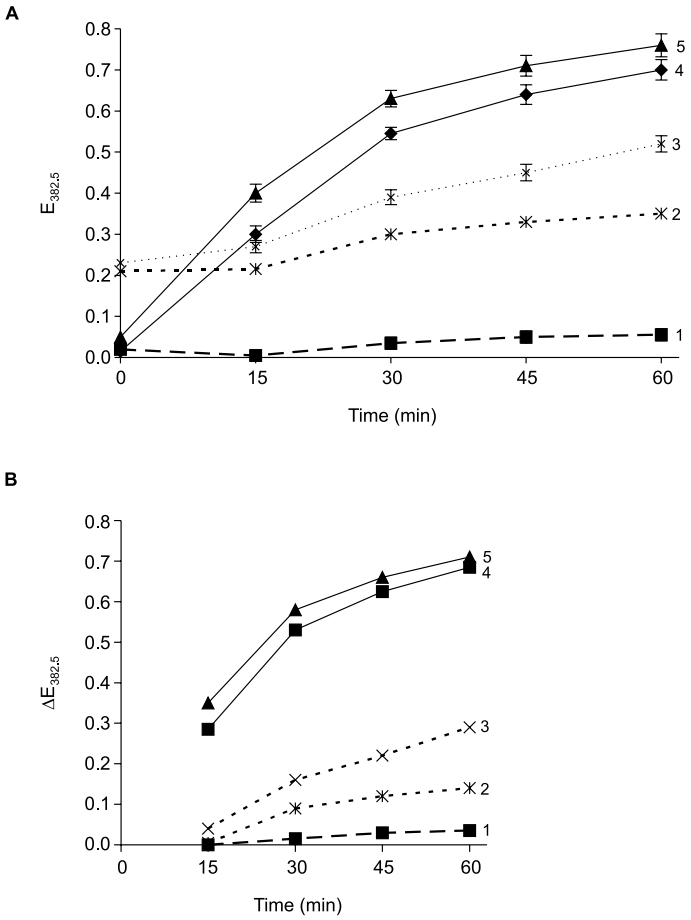

Fig. 1 Effect of Ukrain on the kinetics of BAPNA hydrolysis by trypsin and *Drosophila* intestinal peptide hydrolase: procedure 1. A: with "zero time" extinction; B: without "zero time" extinction. The substrate was added to the reaction system, and then enzyme and preparation were added to the buffer solution. Results are expressed as mean from $n = 6$ experiments. 1 = Ukrain; 2 = peptide hydrolase + Ukrain; 3 = peptide hydrolase; 4 = trypsin; 5 = trypsin + Ukrain.

preparation interacted with the substrate molecules and activated their hydrolysis by trypsin. However, under the same experimental conditions, no hydrolysis of the synthetic substrate BAEE by Ukrain was recorded. The results of these experiments are presented in Figures 1-4.

Investigating the interactions of Ukrain with trypsin *in vitro*, we observed a significant activating effect in the case of BAPNA cleavage and an inhibition of BAEE degradation (Figs. 1B and 3B). *Drosophila* intestinal peptide hydrolase activity under the same conditions was strongly inhibited by Ukrain in the

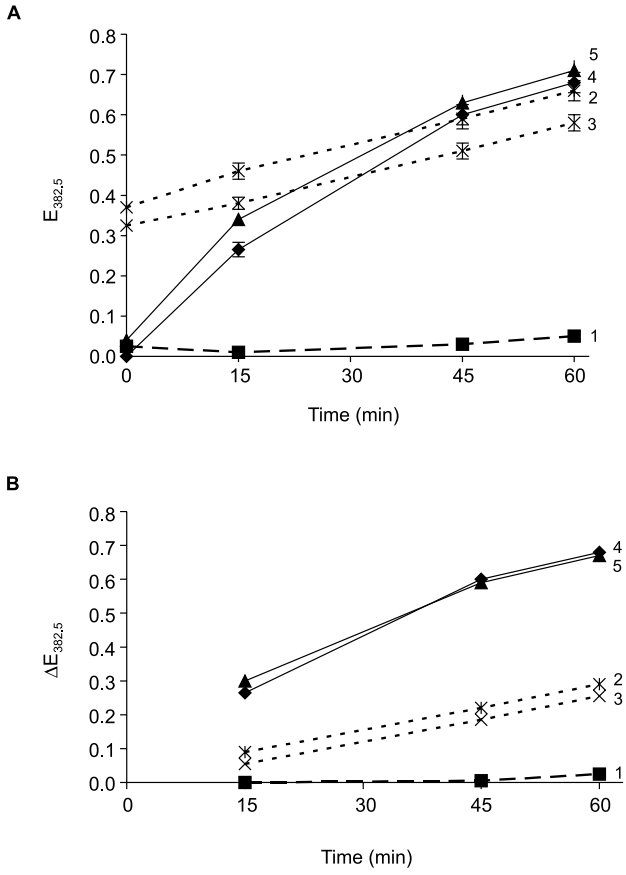

Fig. 2 Effect of Ukrain on the kinetics of BAPNA hydrolysis by trypsin and *Drosophila* intestinal peptide hydrolase: procedure 2. A: with "zero time" extinction; B: without "zero time" extinction. Enzyme and preparation were incubated first, and then buffer and substrate added. Results are expressed as mean from $n = 6$ experiments. 1 = Ukrain; 2 = peptide hydrolase + Ukrain; 3 = peptide hydrolase; 4 = trypsin; 5 = trypsin + Ukrain.

cleavage of both substrates. The inhibition curves are presented in Figures 1 and 3.

In the other test series, the enzymes were first mixed with Ukrain, and then the buffer and substrate were added to this reaction system. The aim of these experiments was to reveal a protease-activating or protease-inhibiting effect of Ukrain in conditions of direct interaction in the incubation system, with regard to the relatively low Ukrain concentration of 1 mg/ml of active substance in the injection solution. In the case of BAPNA hydrolysis, trypsin was activated in the first 15 min, and by 45 and 60 min the action of Ukrain was complete (Fig. 2B). Under the same conditions it was shown that BAEE hydrolysis by trypsin was inhibited after its interaction with Ukrain (Fig. 4B).

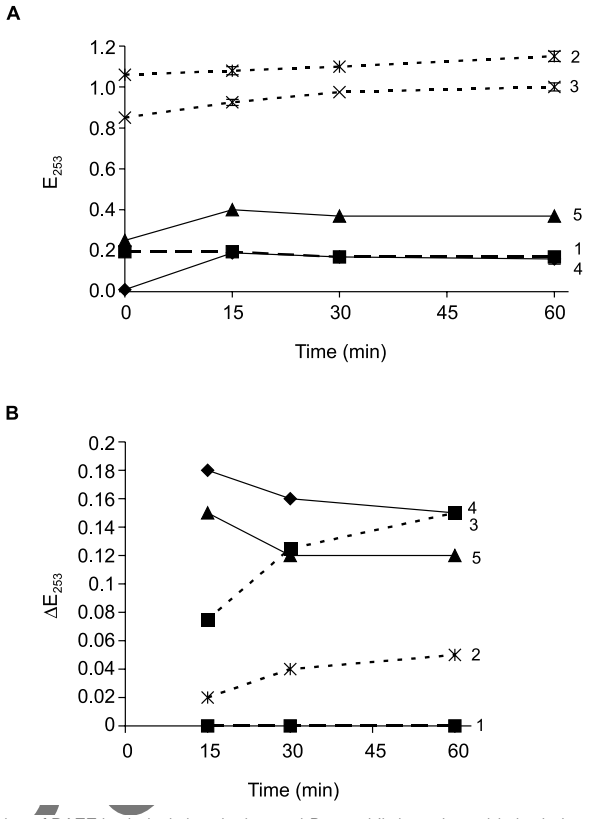

Fig. 3 Effect of Ukrain on the kinetics of BAEE hydrolysis by tripsine and *Drosophila* bowel peptide hydrolase: procedure 1. A: with "zero time" extinction; B: without "zero time" extinction. The substrate was added to the reaction system, and then enzyme and preparation were added to the buffer solution. Results are expressed as mean from $n = 6$ experiments. 1 = Ukrain; 2 = peptide hydrolase + Ukrain; 3 = peptide hydrolase; 4 = trypsin; 5 = trypsin + Ukrain.

The *Drosophila* intestinal hydrolase was more sensitive to the direct action of Ukrain, resulting in its activation (Figs. 2A and 2B).

Discussion

Currently, various aspects of the anticancer action of Ukrain are under discussion. In certain experiments, Ukrain has shown a high accumulation of treated cells in the G2/M phase, whereas the rate of apoptosis of peripheral mononuclear cells did not display any differences between treated and untreated cells; mitogen-stimulated lymphocytes even showed an increased blastogenic response (11).

In another experiment using cancer cell lines A431 and ME180 with normal human keratinocytes as control, it was demonstrated that at a Ukrain concentra-

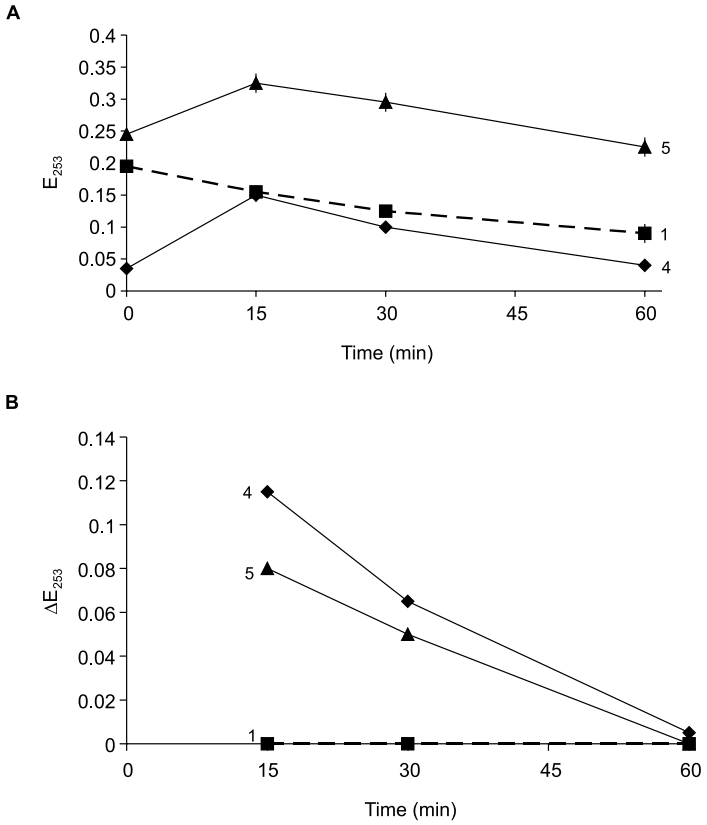

Fig. 4 The effect of Ukrain on the kinetics of BAEE hydrolysis by trypsin: procedure 2. A: with "zero time" extinction; B: without "zero time" extinction. Enzyme and preparation were incubated first, and then buffer and substrate added. Results are expressed as mean from $n = 6$ experiments. 1 = Ukrain; 4 = trypsin; 5 = trypsin + Ukrain.

tion of 7 μM, cancer cells accumulated in the G2/M phase over a 24-h period, but human keratinocytes did not. In addition, apoptosis was detected following 48 h of treatment (12).

Tumor tissues from human breast cancer treated before surgery with Ukrain at a dose of 5 mg i.v. every second day for 20 days and followed by surgery 7-10 days later, showed a number of striking changes compared to the untreated tumors of control patients (13). Histopathological examination demonstrated that the tumor was surrounded by connective tissue (encapsulated) with massive infiltration by mononuclear cells, mostly lymphocytes and plasma cells. Many neoplastic cells surrounded by inflammatory infiltrates were degenerated, enlarged with vacuolated cytoplasm, undergoing necrosis or already necrotic.

Ukrain also seems to reversibly inhibit angiogenesis at relatively low concentrations of 10 to 50 μM, approximately 15 to 75 μg/ml (5).

It is well known that the ECM plays the key role in cell cycle regulation. How does Ukrain act in this context? Ukrain can decrease the number of monocytes in peripheral blood, stimulating macrophage migration into tumor tissue (**3**). Cathepsin D and cysteine proteinases are products of macrophage secretion and can lead to the observed result, tumor cell lysis.

Other authors have hypothesized that the positive antitumor effect of Ukrain is possibly mediated through increased secretion of alpha-1-proteinase inhibitor by macrophages.

Our study demonstrated that Ukrain had different effects on the activity of certain proteolytic enzymes depending on the *in vitro* experimental conditions. We have also shown that Ukrain may inhibit or activate the nonenzymatic hydrolysis of BAPNA, a specific synthetic substrate of trypsin-like enzymes. This is of note because under the same conditions BAEE was not hydrolyzed. The results of the first experimental series showed the strong activation of trypsin by Ukrain, as well as a marked decrease in *Drosophila* intestinal peptide hydrolase activity. In the second experimental series, the effect of Ukrain-dependent trypsin activation was complete at 45 min of incubation, but the interaction of *Drosophila* intestinal hydrolase with Ukrain resulted in enzyme activation, compared with the control reaction system, throughout the period of incubation. In the case of BAEE hydrolysis, Ukrain always inhibits protease activity. This was most clearly observable with the *Drosophila* intestinal protease. Ukrain is a significant modulator of trypsin and trypsin-like enzymes *in vivo*. Interesting data might be obtained by investigating the interactions of Ukrain with proteolytic systems *in vivo*. Further experiments on this theme are warranted, and we suggest the use of *Drosophila* in these investigations.

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