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# SUPPRESSION OF *IN VITRO* INVASION OF HUMAN FIBROSARCOMA CELLS BY A LEUPEPTIN ANALOGUE INHIBITING THE UROKINASE-PLASMIN SYSTEM

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**SUMMARY**: A leupeptin analogue, pyroglutamyl-Leu-Arg-CHO (Pyr-Leu-Arg-CHO), is an inhibitor of urokinase and plasmin, while leupeptin inhibits only plasmin. Pyr-Leu-Arg-CHO was shown to inhibit *in vitro* invasion of human fibrosarcoma HT1080 cells reducing cellular collagenase activity. Pyr-Leu-Arg-CHO suppressed the invasion of the cells in a Boyden chamber assay with an $IC_{50}$ of 12 μg/ml. Addition of plasminogen to HT1080 cells increased the type IV collagenase activity, and Pyr-Leu-Arg-CHO inhibited this activation of the collagenase with an $IC_{50}$ of 3 μg/ml. Leupeptin inhibited both the invasion and collagenase activation at higher concentrations than that of Pyr-Leu-Arg-CHO. The gelatin zymography of the conditioned medium revealed that a new gelatinolytic band, possibly an activated form of MMP-2, appeared by the addition of plasminogen. The activation of MMP-2 was also inhibited strongly by Pyr-Leu-Arg-CHO. These results indicate that Pyr-Leu-Arg-CHO suppresses the *in vitro* invasion by preventing the activation of type IV collagenase through inhibition of the urokinase-plasmin system. 

Invasion of cancer cells into the extracellular matrix (ECM) and basement membranes, which is accompanied with proteolytic degradation of them, is one of the targets for antimetastatic agents. Basement membranes are composed mainly of type IV collagen, laminin, heparan sulfate proteoglycans, and entactin. Type IV collagenase activity is considered to parallel the metastatic ability of cancer cells (1). Type IV collagenases such as MMP-2 (2) and MMP-9 (3) and their cellular inhibitors such as TIMP-1 (4), TIMP-2 (5) have been identified, and their balance in cancer cells is thought to be related to the invasiveness of the cells. Most MMPs are secreted as inactive precursors (proMMPs). Thus, proteolytic activation of the precursor is required to accomplish the digestion of ECM. Recently, a high amount of urokinase-type plasminogen activator (uPA) and its cellular receptor were found to be coexpressed in highly invasive tumors (6). uPA binds to the receptor, and accelerates production of plasmin from plasminogen on the cell surface (7,8). The produced plasmin may digest ECM components directly or indirectly through activating certain proMMPs (9,10). Thus, invasive cancer cells having the uPA-plasmin system could

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easily degrade ECM. Anti-uPA monoclonal antibodies were shown to suppress cancer metastasis (11,12). Therefore, uPA is considered to play an essential role in the invasion, and to be an attractive target for antiinvasiveness.

Several uPA inhibitors such as amiloride derivatives (13,14) have been reported. However, suppression of cancer metastasis and invasion by uPA inhibitors has not been tested. In the present paper, we describe that a leupeptin analogue that inhibits uPA and plasmin strongly suppresses *in vitro* invasion of human fibrosarcoma cells and type IV collagenase activation more strongly than other protease inhibitors.

## MATERIALS AND METHODS

**Materials**: Pyroglutamyl- and dansyl-Leu-Arg-CHO were synthesized by Dr. T. Saino, Nippon Kayaku Co., Ltd. (15), and other protease inhibitors were kindly supplied by the Institute of Microbial Chemistry, Tokyo. Human urinary urokinase was purchased from Japan Chemical Research. Human plasma plasmin and human plasma plasminogen were obtained from Sigma. Human type IV collagen N-[propionate-2,3-$^3$H]-propionylated (0.14 mCi/mg) was purchased from NEN.

**Enzyme Assays**: For the urokinase assay, urokinase (500 IU/ml) was incubated with Gly-Arg-pNA (0.5 mg/ml) and test chemicals in 50 mM Tris-HCl (pH 7.8) and 0.1 M NaCl at 37°C for 2 h. Then, absorbance at 405 nm was read with a microplate reader. For the plasmin assay, plasmin (1 units/ml) was incubated with 1.0% skim-milk and test chemicals in 50 mM Tris-HCl (pH 7.8) with 0.1 M NaCl at 37°C for 2 h. Then turbidity was measured with a microplate reader.

**Cell Culture**: Human fibrosarcoma HT1080 cells (CCL 121) were obtained from the Japanese Cancer Research Resources Bank, and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (Bioserum) and 100 μg/ml kanamycin (Sigma) at 37°C in 5% $CO_2$. For the cell growth assay, the cells were inoculated at $1x10^4$ cells/well in 48-well plates (Costar), and incubated at 37°C overnight. Test chemicals were added next, and the cells were further incubated for 2 days; then the cell numbers were counted.

***In Vitro* Invasion Assay**: *In vitro* invasion of HT1080 cells was assayed by the method described previously (16,17). Polycarbonate filters (8 μm pore size, Nucleopore) were coated with 50 μg of Matrigel (Becton Dickinson), and placed in a modified Boyden chamber. The conditioned medium (1.5 ml), obtained by incubation of NIH3T3 cells in serum-free medium for 24 h, was placed in the lower compartment of the Boyden chamber as a chemoattractant. The cells were scraped into $Ca^{2+}$, $Mg^{2+}$-free PBS (PBS[-]) containing 0.5 mM EDTA and resuspended in 10% FBS DMEM at $4x10^5$ cells/ml. The cells ($2x10^5$ cells) and test chemicals were added to the upper chamber and incubated for 6 h at 37°C in 5% $CO_2$. Then, the cells on the upper surface of the filter were completely removed by use of cotton slabs, and the filters were fixed with methanol and stained with Harris' hematoxylin. The cells that had penetrated through the filter were counted in 10 fields under a microscope ( x400).

**Type IV Collagenase Assay**: A 96-well plate (Costar) was precoated with 50 μl 0.01 μCi [$^3$H]type IV collagen in 50 mM Tris-HCl (pH 7.5). After overnight incubation at 37°C, the plate was washed three times with ice-cold PBS[-] and placed on ice until used. HT1080 cells were scraped into PBS[-] containing 0.5 mM EDTA and resuspended in serum-free DMEM at $5x10^5$ cells/ml. An aliquot (100 μl) of the cell suspension was added to the precoated plate. Plasminogen (5 munits/ml) and test chemicals were then added to the plate, and the cells were incubated for 6 h at 37°C in 5% $CO_2$. Then, 50 μl of the medium was taken to measure its radioactivity in a liquid scintillation counter.

**Zymography**: HT1080 cells were inoculated at $2x10^5$ cells/well in a 24-well plate (Costar). After overnight incubation at 37°C in 5% $CO_2$, the cells were washed three times with PBS, and 300 μl of serum-free DMEM was added to the plate. After addition of plasminogen (5 munits/ml) and test chemicals, the cells were further incubated for 6 h at 37°C in 5% $CO_2$. Then the cell-conditioned medium was mixed with SDS-PAGE sample

buffer without a reducing agent, and applied to SDS-PAGE using 0.1% gelatin gel. After electrophoresis, the gel was incubated twice in 2.5% Triton X-100 for 1 h, and incubated overnight in 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 10 mM $CaCl_2$ at 37°C. Then, the gel was stained with Coomassie blue.

## RESULTS AND DISCUSSION

Leupeptin inhibits plasmin but not urokinase or thrombin. Leupeptin analogues, pyroglutamyl-Leu-Arg-CHO (Pyr-Leu-Arg-CHO) and dansyl-Leu-Arg-CHO, were found to inhibit urokinase and thrombin, respectively (15). Effects of protease inhibitors on human uPA and plasmin activities were tested in our assay system and are summarized in Table 1. Pyr-Leu-Arg-CHO inhibited both uPA and plasmin activities most strongly, and its $IC_{50}$ values were 5.8 and 1.3 μg/ml, respectively. None of the protease inhibitors listed in Table 1 affected the growth of HT1080 cells, even at 100 μg/ml, in 2 days (data not shown). First, we examined the effect of these protease inhibitors on *in vitro* invasion of HT1080 cells (Fig. 1A). As a result, at the concentration of 30 μg/ml Pyr-Leu-Arg-CHO was the most effective to inhibit the *in vitro* invasion. While leupeptin suppressed the invasion about 50% at 100 μg/ml, Pyr-Leu-Arg-CHO did dose-dependently with an $IC_{50}$ of 12 μg/ml (Fig. 1B). Dansyl-Leu-Arg-CHO, which inhibits plasmin only weakly, inhibited the invasion; and the other protease inhibitors which do not inhibit plasmin failed to suppress the invasion. These results indicated that the uPA-plasmin system contributes to the invasion.

Degradation of type IV collagen is essential for cancer cells to penetrate the basement membranes. But collagenolytic activities of uPA and plasmin themselves are very weak. It has been suggested that this uPA-plasmin system could confer invasiveness to the cancer cells by activaiton of type IV collagenase (18,19), so we examined the effect of protease inhibitors on type IV collagenase activity. Whereas HT1080 cells degraded only a basal amount of collagen in serum-free medium, the addition of plasminogen to the cells increased

**Table 1. Effect of protease inhibitors on human uPA and human plasmin activities**

| Inhibitors | $IC_{50}$ (μg/ml)[a] | | Target proteases |
|---|---|---|---|
| | uPA | plasmin | |
| Leupeptin | 42 | 7.4 | plasmin, trypsin, papain, cath. B[b] |
| Pyroglutamyl-Leu-Arg-CHO | 5.8 | 1.3 | + uPA |
| Dansyl-Leu-Arg-CHO | >100 | 15 | + thrombin |
| Antipain | >100 | 92 | papain, trypsin, cath. B |
| Pepstatin | >100 | >100 | pepsin, cath. D, renin |
| Chymostatin | >100 | >100 | chymotrypsin, papain, cath. B |

a) uPA and plasmin activities were assayed at 37°C for 2 h with 0.5 mg/ml Gly-Arg-pNA and 1.0% skim milk, respectively, used as substrates. Values are means of duplicate determinations. The difference was less than 10%.

b) cathepsin B.

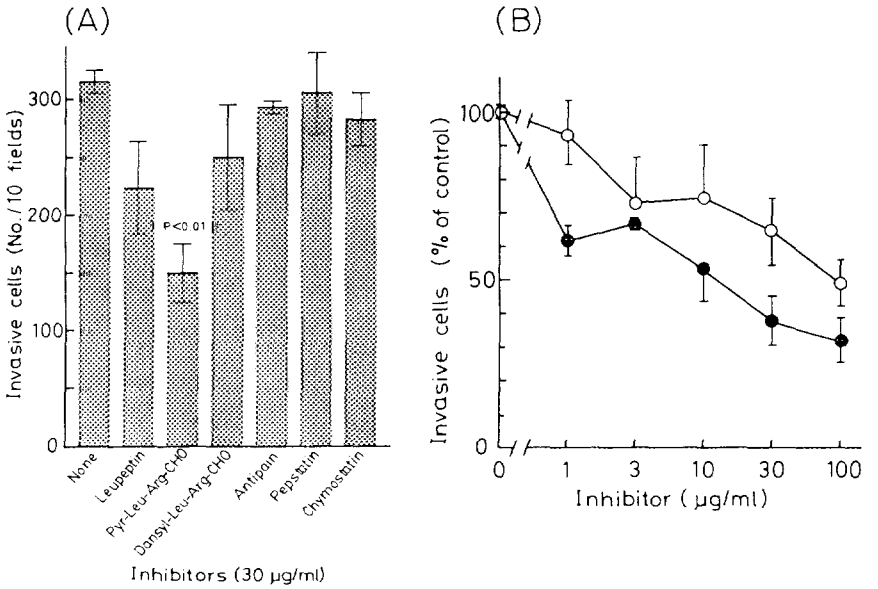


Fig. 1. Inhibition of *in vitro* invasion of HT1080 cells by protease inhibitors. (A) Effect of protease inhibitors at 30 μg/ml on the invasion of the cells in 6 h was examined as described in Materials and Methods. (B) The cells were incubated in Boyden chambers for 6 h with various concentrations of Pyr-Leu-Arg-CHO ( ● ) or leupeptin ( ○ ). Values are means ± SD of triplicate determinations.

the collagenase activity dramatically. When protease inhibitors were added with plasminogen, its activation was inhibited by leupeptin-family inhibitors (Fig. 2A). Among them, Pyr-Leu-Arg-CHO was the most potent, and it inhibited the activation dose-dependently with an $IC_{50}$ of 3 μg/ml. Leupeptin inhibited it at higher concentrations than that of Pyr-Leu-Arg-CHO (Fig. 2B).

Next, to study what species of collagenase was activated, we conducted gelatin-substrate zymography of the conditioned medium. HT1080 cells are reported to secrete MMP-2 and MMP-9 (20). However, in our assay system they secreted mostly MMP-2, and the production of MMP-9 was very weak. The addition of plasminogen to the cells led to the appearance of a new gelatinolytic band. Although its molecular weight was slightly lower than that of a minophenylmercuric acetate-activated MMP-2, its geltinolytic activity was inhibited by EDTA (data not shown). Thus, this band is considered to be a proteolytic-activated form of MMP-2. When protease inhibitors were added with plasminogen, the activation was inhibited by leupeptin and Pyr-Leu-Arg-CHO. Especially, Pyr-Leu-Arg-CHO inhibited the activation of MMP-2 completely at 30 μg/ml (Fig. 3). Other protease inhibitors did not affect the activation. Although MMP-2 is not activated directly by plasmin or trypsin (21), this result is apparently correlated with that of type IV collagen degradation (Fig. 2). Therefore, the uPA-plasmin system is considered to be linked with the activation of MMP-2 *in situ*.

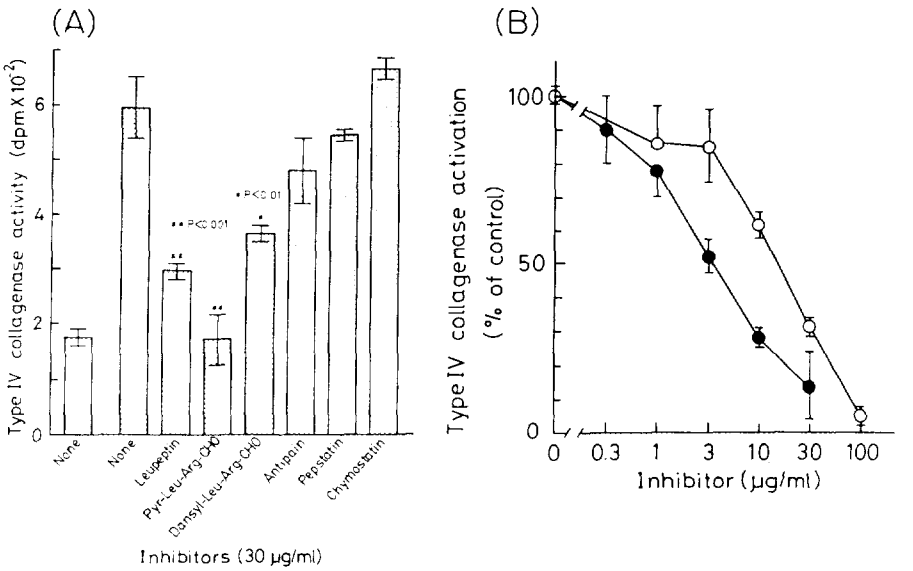


Fig. 2. Inhibition of type IV collagenase activity in HT1080 cells by leupeptin analogues. (A) The cells were incubated in [$^3$H]type IV collagen-precoated well, and incubated in serum-free medium without (white) or with (dotted) plasminogen and protease inhibitors (30 μg/ml) for 6 h. Then the radioactivity in the supernatant was counted. (B) The cells were incubated with various concentrations of Pyr-Leu-Arg-CHO ( ● ) or leupeptin ( ○ ) in the presence of plasminogen. Basal level of collagenolytic activity in the absence of plasminogen was subtracted from all data in B. Values are means ± SD of triplicate determinations.

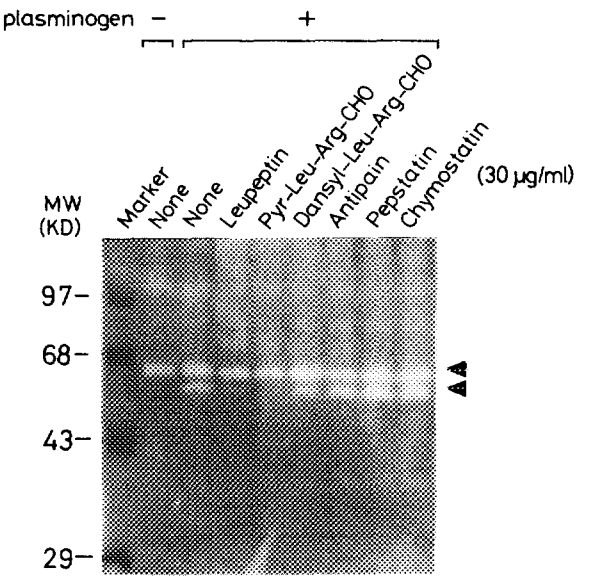


Fig. 3. Effect of protease inhibitors on the secretion of gelatinase by HT1080 cells. HT1080 cells were incubated in serum-free medium with or without plasminogen and protease inhibitors (30 μg/ml) for 6 h. Thus prepared conditioned medium was analyzed by gelatin-substrate zymography. Arrowheads indicate latent MMP-2 (above) and activated MMP-2 (below).

In conclusion, Pyr-Leu-Arg-CHO suppressed the invasion of HT1080 cells, possibly by preventing the activation of cellular type IV collagenase through inhibition of the uPA-plasmin system.

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## REFERENCES

1) Liotta, L. A., Tryggvason, K., Garbisa, S., Hart, I., Foltz, C. M., and Shafie, S. (1980) Nature 284, 67-68
2) Collier, I. E., Wilhelm, S. M., Eisen, A. Z., Marmer, B. L., Grant, G. A., Seltzer, J. L., Kronberger, A., He, C., Bauer, E. A., and Goldberg, G. I. (1988) J. Biol. Chem. 263, 6579-6587
3) Wilhelm, S. M., Collier, I. E., Marmer, B. L., Eisen, A. Z., Grant, G. A., and Goldberg, G. I. (1989) J. Biol. Chem. 264, 17213-17221
4) Stricklin, G. P., and Welgus, H. G. (1983) J. Biol. Chem. 258, 12252-12258
5) Stetler-Stevenson, W. G., Krutzsch, H. C., and Liotta, L. A. (1989) J. Biol. Chem. 264, 17374-17378
6) Vecchio, S. D., Stoppelli, M. P., Carriero, M. V., Fonti, R., Massa, O., Li, P. Y., Botti, G., Cerra, M., D'Aiuto, G., Esposito, G., and Salvatore, M. (1993) Cancer Res. 53, 3198-3206
7) Ellis, V., Scully, M. F., and Kakkar, V. V. (1989) J. Biol. Chem. 264, 2185-2188
8) Barnathan, E. S., Kuo, A., Rosenfeld, L., Kariko, K., Leski, M., Robbiati, F., Nolli, M. L., Henkin, J., and Cines, D. B. (1990) J. Biol. Chem. 265, 2865-2872
9) Mackay, A. R., Corbitt, R. H., Hartzler, J. L., and Thorgeirsson, U. P. (1990) Cancer Res. 50, 5997-6001
10) He, C., Wilhelm, S. M., Pentland, A. P., Marmer, B. L., Grant, G. A., Eisen, A. Z., and Goldberg, G. I. (1989) Proc. Natl. Acad. Sci. USA 86, 2632-2636
11) Hearing, V. J., Law, L. W., Corti, Al., Appella, E., and Blasi, F. (1989) Cancer Res. 48, 1270-1278
12) Ossowski, L., Russo-Payne, H., and Wilson, E. L. (1991) Cancer Res. 51, 274-281
13) Vassalli, J.-D., and Belin, D. (1987) FEBS Lett. 214, 187-191
14) Towle, M. J., Lee, A., Maduakor, E. C., Schwartz, C. E., Bridges, A. J., and Littlefield, B. A. (1993) Cancer Res. 53, 2553-2559
15) Saino, T., Someno, T., Ishii, S., Aoyagi, T., and Umezawa, H. (1988) J. Antibiot. 41, 220-225
16) Terranova, V. P., Hujanen, E. S., Loeb, D. M., Martin, G. R., Thornburg, L., and Glushko, V. (1986) Proc. Natl. Acad. Sci. USA 83, 465-469
17) Albini, A., Iwamoto, Y., Kleinman, H. K., Martin, G. R., Aaronson, S. A., Kozlowski, J. M., and McEwan, R. N. A. (1987) Cancer Res. 47, 3239-3245
18) Mignatti, P., Robbins, E., and Rifkin, D. B. (1986) Cell 47, 487-498
19) Reich, R., Thompson, E. W., Iwamoto, Y., Martin, G. R., Deason, J. R., Fuller, G. C., and Miskin, R. (1988) Cancer Res. 48, 3307-3312
20) Kubota, S., Fridman, R., and Yamada, Y. (1991) Biochem. Biophys. Res, Commun. 176, 129-136
21) Okada, Y., Morodomi, T., Enghild, J. J., Suzuki, K., Yasui, A., Nakanishi, I., Salvesen, G., and Nagase, H. (1990) Eur. J. Biochem. 194, 721-730