Potent Homophthalimide-Type Inhibitors of B16F10/L5 Mouse Melanoma Cell Invasion

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Received June 21, 1999; accepted August 18, 1999

Recently, we developed a series of novel and potent aminopeptidase inhibitors with a homophthalimide skeleton. Among them, *N*-(2,6-diethylphenyl)homophthalimide (PIQ-22) possesses a specific aminopeptidase-inhibiting activity more potent than that of bestatin or actinonin, as assayed in terms of hydrolysis of L-alanine 4-methylcoumaryl-7-amide (Ala-AMC) by human acute lymphoblastic leukemia MOLT-4 cells. We show here that PIQ-22 and its 2,6-dimethylphenyl derivative (PIQ-11) are more potent inhibitors of tumor cell invasion than bestatin and actinonin in a Matrigel assay using mouse melanoma B16F10/L5 cells.

Key words tumor cell invasion; homophthalimide; aminopeptidase

The malignant metastatic cascade includes a tumor cell invasion step, *i.e.*, passage of tumor cells through connective tissue barriers which consist of various adhesive molecules, including fibronectin, laminin, and other glycoproteins and proteoglycans.^{1–3} Proteases, which degrade tissue barriers and regulate cell adhesion and mobility, are thought to play a critical role in this step, and many different types of proteases have been shown to be involved.^{2–5} Among them, the role of aminopeptidase N (APN; EC 3.4.11.2, also referred to as aminopeptidase M, alanyl aminopeptidase, arylamidase, and acyl-peptide hydrolase), which is identical to the myeloid differentiation antigen CD13, has been emphasized.⁵

In the course of our structural development studies of thalidomide,^{6–8} we recently reported the preparation and structure–activity relationships of novel small-molecular nonpeptide aminopeptidase inhibitors with a cyclic imide skeleton.^{9–12} Among them, *N*-(2,6-diethylphenyl)homophthalimide (PIQ-22; Fig. 1) shows a specific aminopeptidase-inhibiting activity more potent than that of bestatin or actinonin in the assay system used (*vide infra*).^{9,11,12} We had regarded the compound as a specific inhibitor of APN, based on data obtained with a well-established assay system for APN activity, *i.e.*, measuring 7-amino-4-methylcoumarin (AMC) liberated from L-alanine 4-methylcoumaryl-7-amide (Ala-AMC) using intact human acute lymphoblastic leukemia MOLT-4 cells.^{5,9–12} However, our recent studies revealed that PIQ-22 does not inhibit the activity of a standard sample of APN, and that the true target enzyme of PIQ-22 is an enzyme other than APN. This enzyme possesses a

similar substrate selectivity to APN, *i.e.*, it hydrolyzes Ala-AMC efficiently, but it does not hydrolyze glycyl-L-proline 4-methylcoumaryl-7-amide, which is a typical substrate of dipeptidyl peptidase IV (EC 3.4.14.5).^{9–12} The character (substrate specificity, molecular weight, distribution, *etc.*) of the APN-type enzyme which is directly inhibited by PIQ-22 is more similar to puromycin-sensitive aminopeptidase (PSA) than to APN (details to be published elsewhere).^{13,14} Therefore PIQ-22 should be regarded as a specific and potent inhibitor of APN/PSA-type enzyme. This unique and specific high potency of PIQ-22 and its low toxicity^{9–12} led us to evaluate the effect of the compound and its 2,6-dimethylphenyl derivative (PIQ-11) on tumor cell invasion into reconstituted basement membrane (Matrigel).^{5,15}

Both PIQ-11 and PIQ-22 were prepared as described previously,⁹ and gave appropriate analytical values. Tumor cell invasion was assessed by counting mouse metastatic tumor cells (B16F10/L5 mouse melanoma cells)¹⁶ that invaded into Matrigel-coated filters.^{5,15} Briefly, the lower surface and the upper surface of the filter in an invasion chamber (Chemotaxicell, pore size 8 μ m, Kurabo Ltd.) were coated with human plasma fibronectin (the reverse side; 5 μ g/filter, GIBCO BRL) and Matrigel (the surface; 22.5 μ g/filter, Becton Dickinson), respectively, in a 24-well plate (Falcon 3047) containing 650 μ l of Swiss 3T3 cell-conditioned medium. B16F10/L5 cells (3.0×10^4 cells in 200 μ l of DMEM containing 0.1% BSA) were added to the upper part of the Chemotaxicell. The cells were incubated for 3.5 h at 37 $^{\circ}$ C under a 5% CO₂ atmosphere in the presence or absence of a test compound (PIQ-11, PIQ-22, bestatin, or actinonin). After incubation, cells present in the lower part of the cell chamber and on the lower surface of the filter (stained with Crystal violet after careful removal of the cells on the upper surface of the filter by wiping with cotton swabs) were counted manually under a microscope. Each sample was tested in a quadruplicate, and the mean values are shown in Fig. 2, together

Fig. 1. Structures of Novel Nonpeptide Small-Molecular Aminoprotease Inhibitors (PIQ-11 and PIQ-22), Bestatin, and Actinonin

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Fig. 2. Effects of Aminopeptidase Inhibitors on the Invasion of B16F10/L5 Cells into Matrigel

Cell invasion assay was performed as described in the text. Vertical scale: Number (%) of invading cells based on the control cell number as 100%. * $p < 0.05$, ** $p < 0.01$ (Shirley-Williams test).

with experimental error bars. Cell viability was evaluated by testing the succinate-tetrazolium reductase system using the WST-1 method.¹⁷⁾ None of the test compounds showed toxicity in the concentration range used in our experiments (data not shown).

As shown in Fig. 2, the APN inhibitors bestatin and actinonin inhibited the tumor cell invasion in our assay system, in accordance with the results reported by other researchers.¹⁸⁾ Both PIQ-11 and PIQ-22 inhibited the invasion of B16F10/L5 cells into Matrigel-coated filters in a concentration-dependent manner, with the latter being more potent. PIQ-22 inhibited the invasion almost completely (98.9% inhibition) at 100 μM , and was more potent than bestatin or actinonin (57.9% and 42.1% inhibition, respectively, at 100 μM). PIQ-22 inhibited the invasion with an efficacy of 68.5% even at the concentration of 10 μM , at which concentration bestatin showed no inhibiting activity. This potent activity of PIQ-22 matches its potent inhibiting activity toward APN/PSA-type enzyme, *i.e.*, the IC_{50} value for amino-peptidase of PIQ-22 determined in our assay system (hydrolysis of L-ala-AMC by MOLT-4 cells) was 0.12 $\mu\text{g}/\text{ml}$, while that of bestatin and actinonin was 0.81 and 0.32 $\mu\text{g}/\text{ml}$, respectively.^{9–12)}

Though all of the test compounds used in our experiments possess inhibiting activity toward APN-type enzyme, their target molecule(s) is not the same. While it has been established that bestatin and actinonin act directly on APN, PIQ-11 and PIQ-22 are inactive toward APN itself, but they inhibit another APN/PSA-type enzyme (*vide supra*, details to

Fig. 3. Morphology of B16F10/L5 Cells Treated with or without Amino-peptidase Inhibitors

Cells were treated as described in the text, and photographed under a microscope ($\times 200$).

be published elsewhere). This might suggest that the mechanisms of the tumor cell invasion-inhibiting activity elicited by PIQ-11/PIQ-22 and bestatin/actinonin are different. Moreover, although PIQ-11 possesses inhibiting activity toward APN/PSA-type enzyme which is weaker (IC_{50} value of 8.7 $\mu\text{g}/\text{ml}$ in our assay system) than that of bestatin or actinonin, it showed more potent inhibiting activity toward tumor cell invasion than did bestatin or actinonin (Fig. 2). Therefore the APN/PSA-type enzyme which is the target of PIQ-11/PIQ-22 might be a superior molecular target for novel inhibitors of tumor cell invasion.

To investigate the difference between the actions of PIQ-11/PIQ-22 and bestatin, morphological changes in B16F10/L5 cells caused by the compounds were analyzed (Fig. 3). The cells were treated with the compounds for 2 h under the same conditions as for cell invasion assay, and the morphology was analyzed. PIQ-22 and bestatin did not affect primary cell adhesion, but PIQ-22 appeared to inhibit cell extension, as shown in the photograph in Fig. 3. This might suggest that PIQ-22 possesses inhibitory activity toward cell mobility, which may account for the potent inhibiting activity on tumor cell invasion. PIQ-11 caused similar morphological changes.

In conclusion, we showed that novel nonpeptide small-molecular amino-peptidase inhibitors with an *N*-phenylhomophthalimide skeleton, PIQ-11 and PIQ-22, possess potent inhibiting activity toward metastatic tumor cell invasion. These compounds have no apparent cytotoxicity, and should be superior lead compounds for the development of novel tumor cell invasion inhibitors. Characterization of the amino-peptidase which is the direct target enzyme of PIQ-11/PIQ-22, as a novel molecular target of tumor cell invasion inhibitors, seems to be the next step.

Acknowledgment The work described in this paper was partially supported by funds for the Promotion of Fundamental Studies in Health Science from the Organization for Pharmaceutical Safety Research.

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