Trivarcic Acid, a Potent Depside Human Leukocyte Elastase Inhibitor

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Human leukocyte elastase (HLE) is a serine protease implicated in several inflammatory diseases, and represents a major target for anti-inflammatory drug development. In the present study, nordivarcic acid (1), divarinyl divarate (2), and trivarcic acid (3), three depsides isolated from the culture of a soil derived fungal strain were identified as inhibitors of HLE. Two didepsides 1 and 2 showed low inhibitory activity. In contrast, trivarcic acid, a *para*-tridepside, exhibited highly potent inhibitory activity with an IC₅₀ value of 1.8 μM and a K_i of 0.6 μM. Kinetic investigations with trivarcic acid showed that this inhibition is reversible, competitive pattern. Further studies on the selectivity of three depsides toward serine proteases showed that they did not inhibit chymotrypsin, trypsin and thrombin even at 150 μM.

Key words human leukocyte elastase; depside; trivarcic acid; fungus

Human leukocyte elastase (HLE, EC 3.4.21.37), also known as human neutrophil elastase (HNE) and human granulocyte elastase, is a member of the chymotrypsin superfamily of serine proteases, and highly expressed in the azurophilic granules of polymorphonuclear leukocytes (PMNLs). HLE can very efficiently degrade tissue matrix proteins such as elastin, collagen, fibronectin, laminin and proteoglycan by activating procollagenase, prostromelysin, and progelatinase, and is potentially one of the most destructive enzymes in the body.¹⁾ Under physiological conditions, HLE is released from PMNLs because of inflammatory stimuli and mediators, and is inactivated by a number of endogenous proteinase inhibitors such as α₂-antiplasmin, α₁-antichymotrypsin, antithrombin, and tissue inhibitor of metalloproteinases.^{2,3)} The imbalance between HLE and its endogenous inhibitors leads to abnormal tissue destruction which is associated with a number of inflammatory diseases including pulmonary emphysema,⁴⁾ adult respiratory distress syndrome (ARDS),⁵⁾ chronic bronchitis,⁶⁾ chronic obstructive pulmonary disease (COPD),^{7,8)} rheumatoid arthritis (RA),⁹⁾ and acute lung injury (ALI).¹⁰⁾ In addition, HLE plays an important role in invasion and metastasis of human breast and lung cancer.¹¹⁾ Hence, HLE has been an important therapeutic target.

Research and development of potent small molecular HLE inhibitors has been conducted for over three decades worldwide, and a number of synthetic and natural inhibitors have been discovered and evaluated biologically both *in vitro* and *in vivo*.^{12,13)} The most advanced synthetic inhibitor is ONO-5046 (sivelestat) which has been launched in Japan (2002) as an injectable formulation for the treatment of acute lung injury associated with systemic inflammatory response syndrome, ARDS and ALI.^{14,15)} MR-889 (midesteine), an irreversible HNE inhibitor, is also in pre-registration for the treatment of COPD in Italy.¹⁶⁾

In our searching for novel HLE inhibitors from natural or synthetic compounds, three depsides from the culture of a soil derived fungal strain were identified as inhibitors of HLE.

MATERIALS AND METHODS

Enzymes and Substrates Human leukocyte elastase (EC 3.4.21.37), α-chymotrypsin (EC 3.4.21.1), trypsin (bovine pancreatic type III, EC 3.4.21.4) and thrombin (EC 3.4.21.5), and their substrates *N*-MeO-Suc-Ala-Ala-Pro-Val-*p*NA (M-4765), Suc-Ala-Ala-Pro-Phe-*p*NA (S7388), Bz-Ile-Glu-Gly-Arg-*p*NA (B4875) and *N*-Benzoyl-Phe-Val-Arg-*p*-nitroanilide hydrochloride hydrate (B7632) were all purchased from Sigma.

Fungal Strain and Fermentation The fungal strain was isolated from a soil sample collected in Yunnan Province, P.R. of China and was cultured on slants of potato dextrose agar (PDA) at 25°C for 10 d. The agar plugs were inoculated in 500 mL Erlenmeyer flasks containing 80 mL of seed media (2.0% starch, 1.0% glucose, 0.6% malt extract, 0.3% yeast extract, 0.2% hot-pressed soybean cake meal, 0.2% NaCl, 0.1% MgSO₄·7H₂O, and 0.2% CaCO₃, final pH 7.0 before sterilization), and the flask cultures were incubated at 27°C on a rotary shaker at 220 rpm for 3 d. Twenty 1000 mL Fernbach flasks, each containing 150 g of solid media (97.5% rice, 2.5% hot-pressed soybean cake meal) were individually inoculated with 1.5 mL of the seed culture and fermented at 27°C under static conditions for 14 d.

Isolation and Determination of Compounds The fermented rice substrate (1.8 kg) was extracted with 90% ethanol (3.6 L), and the organic solvent was evaporated under vacuum to give slurry. The slurry was centrifuged to separate the precipitate. And the precipitate was dissolved into 5 mL methanol and subjected to a Sephadex LH-20 column (2.6×60 cm). The column was eluted with methanol and the active fractions (1.3 g) were collected. Further purification was performed by medium pressure ODS (Merck Co., 30–40 μm, 100 Å) chromatography and three active pure compounds **1** (200.0 mg), **2** (25.0 mg) and **3** (426.0 mg) were obtained. Their ¹H-, ¹³C-NMR spectra were recorded on a Bruker AV-500 NMR spectrometer with tetramethylsilane (TMS) as internal standard. MS spectral data were obtained on a Waters Xevo TQ spectrometer for electrospray ionization (ESI)-MS. Based on ESI-MS, ¹H-, ¹³C- and 2D-NMR spectroscopic data analysis, they were identified to be nordivarcic acid,¹⁷⁾ divarinyl divarate,¹⁸⁾ and

The authors declare no conflict of interest.

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Fig. 1. Chemical Structures of Three Depsides

Nordivarcic acid (1), divarinyl divarate (2), trivarcic acid (3).

trivarcic acid,¹⁹⁾ respectively (Fig. 1). Their assignments of ¹H- and ¹³C-NMR data were given below.

Nordivarcic Acid (1): White amorphous powder. ESI-MS: 373 [M-H]⁻. ¹H-NMR (500 MHz, acetone-*d*₆) δ: 6.80 (1H, d, *J*=2.4 Hz, H-3'), 6.77 (1H, d, *J*=2.4 Hz, H-5'), 6.42 (1H, d, *J*=2.4 Hz, H-5), 6.33 (1H, d, *J*=2.4 Hz, H-3), 3.02 (2H, t, *J*=7.5 Hz, H-7'), 2.94 (2H, t, *J*=7.5 Hz, H-7), 1.71 (2H, m, H-8), 1.68 (2H, m, H-8'), 0.98 (3H, t, *J*=7.4 Hz, H-9'), 0.96 (3H, t, *J*=7.5 Hz, H-9); ¹³C-NMR (125 MHz, acetone-*d*₆) δ: 172.0 (C-10'), 168.8 (C-10), 165.2 (C-2), 163.8 (C-2'), 162.7 (C-4), 153.9 (C-4'), 148.1 (C-6), 147.9 (C-6'), 115.5 (C-5'), 111.2 (C-5), 110.1 (C-1'), 108.2 (C-3'), 103.4 (C-1), 100.8 (C-3), 38.3 (C-7), 37.6 (C-7'), 24.8 (C-8), 24.6 (C-8'), 13.4 (C-9), 13.3 (C-9').

Divarinyl Divarate (2): White amorphous powder. ESI-MS: 329 [M-H]⁻. ¹H-NMR (500 MHz, acetone-*d*₆) δ: 6.66 (1H, t, *J*=1.8 Hz, H-5'), 6.60 (2H, t, *J*=1.8 Hz, H-1', H-3'), 6.40 (1H, d, *J*=2.5 Hz, H-5), 6.32 (1H, d, *J*=2.5 Hz, H-3), 2.95 (2H, m, H-7'), 2.57 (2H, m, H-7), 1.70 (2H, m, H-8, H-8'), 1.64 (2H, m, H-8, H-8'), 0.95 (3H, t, *J*=7.4 Hz, H-9'), 0.94 (3H, t, *J*=7.5 Hz, H-9); ¹³C-NMR (125 MHz, acetone-*d*₆) δ: 170.6 (C-10), 166.2 (C-4), 163.6 (C-2), 158.9 (C-2'), 151.9 (C-4'), 149.1 (C-6), 146.0 (C-6'), 114.0 (C-1'), 113.6 (C-5'), 112.1 (C-5), 107.3 (C-3'), 104.6 (C-1), 101.8 (C-3), 39.4 (C-7), 38.4 (C-7'), 26.0 (C-8), 24.9 (C-8'), 14.0 (C-9), 14.5 (C-9').

Trivarcic Acid (3): White amorphous powder. ESI-MS: 551 [M-H]⁻. ¹H-NMR (500 MHz, acetone-*d*₆) δ: 11.83 (1H, s, 2''-OH), 11.09 (1H, s, 2-OH), 10.37 (1H, s, 2'-OH), 6.86 (1H, d, *J*=2.4 Hz, H-3'), 6.84 (1H, d, *J*=2.4 Hz, H-5'), 6.81 (1H, d, *J*=2.4 Hz, H-3''), 6.78 (1H, d, *J*=2.4 Hz, H-5''), 6.40 (1H, d, *J*=2.4 Hz, H-5), 6.31 (1H, d, *J*=2.4 Hz, H-3), 3.01 (2H, t, *J*=7.7 Hz, H-7''), 2.92 (4H, m, H-7, H-7'), 1.77–1.63 (6H, m, H-8, H-8', H-8''), 0.98 (3H, t, *J*=7.7 Hz, H-9'), 0.97 (3H, t, *J*=7.7 Hz, H-9''), 0.95 (3H, t, *J*=7.7 Hz, H-9); ¹³C-NMR (125 MHz, acetone-*d*₆) δ: 173.2 (C-10''), 170.0 (C-10'), 168.8 (C-10'), 166.4 (C-2), 165.1 (C-2''), 164.0 (C-4), 161.4 (C-2'), 155.2 (C-4''), 154.4 (C-4'), 149.13 (C-6), 149.08 (C-6''), 146.7 (C-6'), 116.4 (C-5''), 116.2 (C-5'), 114.7 (C-1'), 112.3 (C-5), 111.3 (C-1''), 109.2 (C-3''), 109.0 (C-3'), 104.5 (C-1), 102.0 (C-3), 39.4 (C-7), 38.7 (C-7''), 37.8 (C-7'), 25.9 (C-8)*, 25.7 (C-8'')*, 25.5 (C-8')*, 14.5 (C-9)[#], 14.4 (C-9'')[#], 14.3 (C-9')[#].

(*[#]: Assignments can be interchanged.)

Enzyme Activity Assay The activity of human leukocyte elastase was measured according to the previous method,²⁰⁾ with minor modification. Briefly, 50 μL reaction buffer (*N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) pH 6.5, 0.5 M NaCl) containing 0.0015 unit HLE (one unit will release one nanomole of *p*-nitrophenol per second from BOC-L-alanine *p*-nitrophenyl ester at pH 6.5 at 37°C) was incubated with 2 μL test compounds (dissolved in dimethyl sulfoxide (DMSO)) or DMSO (as blank control) in 96-well plate at room temperature for 15 min, the reaction was started by addition of 50 μL substrate solution containing a 300 μM final concentration of *N*-MeO-Suc-Ala-Ala-Pro-Val-*p*NA. After incubation at 37°C for 30 min, the hydrolysis product of *p*-nitroaniline was measured by the optical density (OD) at 405 nm using a microplate reader (Wallac 1420 Victor², Perkin-Elmer). Ulinastatin, a kind of broad-spectrum protease inhibitor, was purchased from Guangdong Biochemistry Medicine Corporation of China as positive control. Its specific activity is 1666 U/mg protein, 1 unit being the amount necessary to inhibit the activity of 2 μg trypsin by 50%.

The activities of α-chymotrypsin, trypsin and thrombin were assayed with the same procedures as that of HLE, except for using 200 μM Suc-Ala-Ala-Pro-Phe-*p*NA, 300 μM Bz-Ile-Glu-Gly-Arg-*p*NA, and 300 μM *N*-Benzoyl-Phe-Val-Arg-*p*-nitroanilide hydrochloride hydrate as substrates, respectively, and the buffer used was 0.05 M Tris-HCl containing 0.15 M NaCl, pH 7.2.

IC₅₀ value, the half maximal inhibitory concentration, was determined from the logarithmic regression line of a dose-response curve. All experiments were undertaken in triplicate and repeated at least three times. Data are presented as average values.

Kinetics Study Kinetic experiments were conducted as described.²¹⁾ Reversible or irreversible inhibition mode is distinguished by the first-order enzyme-catalyzed reactions, which were performed at 300 μM *N*-MeO-Suc-Ala-Ala-Pro-Val-*p*NA substrate, three different concentration inhibitor, and HLE with increasing concentration. Lineweaver-Burk double reciprocal plot of 1/*v* versus 1/[*S*] was made by the velocities of enzyme reactions that were carried out at a variety of

Fig. 2. Inhibitory Curves of Depsides against HLE and Inhibitory Kinetics of Trivarcic Acid on HLE

(A) Inhibitory curves of nordivarcic acid (1) (▲), divarinyll divarate (2) (●), and trivarcic acid (3) (■) against HLE. (B) A plot of OD versus concentrations of HLE in presence of three different trivarcic acid concentrations of 0.0 μM (▲), 3.0 μM (●), or 10 μM (■). (C) Lineweaver-Burk plot of $1/v$ versus $1/[S]$ for inhibition of HLE by 0.0 μM (▲), 1.0 μM (●) or 3.0 μM (■) trivarcic acid. (D) Dixon plot of $1/v$ versus $[I]$ for HLE inhibitor trivarcic acid at two substrate concentrations of 0.3 mM (▲) and 1.0 mM (●). Data are mean values of triplicate experiments.

substrate concentrations (0.1–1.0 mM) and 0.0015 unit HLE in the presence or absence of the inhibitor, where the Michaelis constant K_m (concentration of substrate that produces half-maximal velocity) and apparent K_m (K_{mapp} , K_m in the presence of an inhibitor) were obtained from the curve. Dixon plot of $1/v$ versus $[I]$ (concentration of inhibitor) was made by the linearisations of the rates of enzyme reactions performed at a dilution serial of concentrations of inhibitor and three different substrate concentrations (0.0, 0.3, 1.0 mM).

RESULTS

Inhibitory Activity against HLE Inhibitory effects of the depsides against HLE were tested with *N*-MeO-Suc-Ala-Ala-Pro-Val-*p*NA as the substrate. The dipeptides, nordivarcic acid (1) and divarinyll divarate (2) could weakly inhibit HLE with the IC_{50} values of 45.1 μM and 92.6 μM , respectively. Trivarcic acid (3), one tripeptide, exhibited high inhibitory activity toward HLE with IC_{50} value of 1.8 μM (Fig. 2A), its inhibitory potency toward HLE is approximately 25–50-folds over that of two dipeptides. Ulinastatin, a positive control in the assay, exhibited an IC_{50} value of 1.1 $\mu\text{g/mL}$ (1.8 U/mL).

Inhibitory Specificity To determine the specificity of these depsides on serine protease, their inhibitory effects toward α -chymotrypsin, trypsin and thrombin was also investigated. Studies showed that they did not exhibit inhibitory

activity against these serine proteases here mentioned even at 150 μM . Trivarcic acid (3) displayed a more than 83-fold selectivity for HLE over some serine protease, suggesting trivarcic acid is a potent and selective HLE inhibitor.

Inhibitory Kinetics on HLE To further elucidate the action mechanism of trivarcic acid (3) toward HLE, the inhibition kinetics was further investigated. Firstly, the reversible or irreversible inhibition was distinguished by a plot of OD versus $[E]$, which was obtained from enzyme reactions carried out with a dilution series of HLE enzyme (0.005, 0.0035, 0.00245, 0.0017, 0.00125, 0.00086 U per well) and three different concentrations of trivarcic acid (10.0, 3.0, 0.0 μM). As shown in Fig. 2B, the velocity of enzyme reaction was increased with increasing concentration of enzyme. In presence of trivarcic acid, the reaction rate was decreased with the concentration of trivarcic acid, and remained a linear relationship with enzyme concentration, indicating the inhibition mode of trivarcic acid is reversible.

Next, a Lineweaver-Burk double reciprocal plot of $1/v$ versus $1/[S]$ was performed by the enzyme reactions which were carried out at a serial dilution of *N*-MeO-Suc-Ala-Ala-Pro-Val-*p*NA substrate concentrations in the range of 0.1–1.0 mM in the presence of three different concentrations of trivarcic acid (0.0, 1.0, 3.0 μM). As the plot (Fig. 2C) shown, the K_m of HLE is about 125 μM , the K_{mapp} were about 371 μM and 769 μM at the presence of 1.0 μM or 3.0 μM trivarcic acid, respectively.

The K_{mapp} is increased with increasing triventric acid concentration, whereas the maximum velocity (V_{max}) of the enzyme reaction apparently remained unchanged, indicating that the inhibition mode is typical competitive. By the equation of $K_{\text{mapp}} = K_m(1 + [I]/K_i)^{21}$ the inhibition constant (K_i) value of triventric acid is calculated to be about $0.55 \mu\text{M}$, which is consistent with the K_i value of $0.68 \mu\text{M}$ obtained from the Dixon plot of $1/v$ versus $[I]$ (Fig. 2D) which was made at a dilution serial of concentrations of inhibitor and two substrate concentrations (0.3 , 1.0 mM).

DISCUSSION

Nature is also the most valuable source of potential lead structures for drug development. So far, a significant number of natural products have been identified as HNE inhibitors with micromolar ranges of activity.^{12,13} Melzig *et al.* reported various phenolic compounds as HLE inhibitors, including flavones, cinnamic acid depside derivatives and caffeic acid derivatives.²⁰ Löser *et al.* reported that fukinolic acid, one of depside derivatives from *Cimicifuga racemosa*, could significantly inhibit HNE with an IC_{50} of $0.23 \mu\text{M}$.²² Depside is a type of polyphenolic compounds composed of two or more monocyclic aromatic units linked by an ester bond. Depsides are most often found in lichens,^{23,24} but have also been isolated from high plants^{25,26} and marine or endophytic fungi.^{27,28} Nordiventric acid (**1**), divaric acid (**2**), and triventric acid (**3**) were all originally isolated from lichens.^{17–19} In the present study, the three depsides, for the first time, are isolated from a soil derived fungal strain.

Depsides have been reported to have a variety of pharmacological activities such as antibiotic, anti-human immunodeficiency virus (HIV), antioxidative.^{29–31} However, there is little bioactivity information reported on nordiventric acid (**1**), divaric acid (**2**), and triventric acid (**3**). In the present study, the three phenolic depsides were discovered as inhibitors of HLE, especially tridepside triventric acid (**3**) was demonstrated to be a high potent, selective, and reversible competitive HLE inhibitor.

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