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RESEARCH ARTICLE

The evaluation of inhibitive effectiveness of the tumour necrosis factor-α converting enzyme selective inhibitors by HPLC

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Abstract

A novel high-performance liquid chromatography (HPLC) method based on the internal standard method was established for assaying the tumour necrosis factor-α converting enzyme (TACE) activity and matrix metalloprotease-9 (MMP-9) activity, and was used to evaluate the inhibitive effectiveness of inhibitors to TACE and MMP-9. In the assay method for TACE and MMP-9, peptides labelled with the ultraviolet group-Dpa were used as substrates. Alanine-Dpa was synthesised and was used as the internal standard for quantitative analysis. After the peptide substrates were hydrolysed by TACE (MMP-9) for 15min (25min) at 37°C, the amount of remaining substrates were determined by reversed-phased HPLC with UV detection at 353nm. The relative peak area of the substrate was linearly dependent on the substrate concentration. This method was then applied to determine the 50% inhibitory concentration (IC_{50}) of GM6001 and inhibitor A for both TACE and MMP-9.

Keywords: High-performance liquid chromatography, internal standard method, tumour necrosis factor-α converting enzyme, inhibitive effectiveness

Introduction

Tumour necrosis factor-α (TNF-α) is implicated in a variety of chronic inflammatory diseases such as rheumatoid arthritis (RA), inflammatory bowel disease (IBD), multiple sclerosis (MS) and chronic obstructive pulmonary disease (COPD). Anti-TNF-α therapy has been shown to be effective in treating these chronic inflammatory diseases [1–4]. The tumour necrosis factor-α converting enzyme (TACE) is a member of the metzincin family and is the primary sheddase for processing pro-TNF-α into its soluble, inflammatory form (5–6]. Thus, as an alternative mediator of TNF- α, small molecule inhibitors of TACE have been widely researched.

TACE has very similar active sites to the matrix metalloproteinases (MMPs). As a result, many of the early TACE inhibitors suffered from a lack of selectivity. Thus, there has been considerable effort directed at finding selective inhibitors for TACE [7].

The methods of assaying TACE and MMPs activity are essential for the evaluation of selective inhibitors of TACE. The main methods for assaying TACE and MMPs *in vitro* include gel electrophoresis zymography [8], enzyme linked immunoassay (ELISA) and high-performance liquid chromatography (HPLC) [9–13]. The HPLC methods are generally faster and more precise than gel electrophoresis and less expensive than ELISA.

In this study, peptides labelled with an ultraviolet group were used as the substrates of TACE and MMP-9. To explore the rules and characteristics of the substrates of TACE and MMP-9, we conducted metrological analysis on data of the substrates of TACE and MMP-9 from medical journal reports in recent years. Here they described that the N-terminal elongation of the recognition sequence by a P4 lysyl residue (Mca-Lys-Pro-Gly-Leu-*f*) creates a novel substrate (FS-6) with markedly improved kinetic properties for hydrolysis by MMPs, specifically for collagenase-1 and -2. They further demonstrate that FS-6 is an excellent substrate for TACE (ADAM-17), a Zn-metalloproteinase that is structurally and functionally closely related to MMPs [14]. Therefore, we use the hard-core (-Lys-Pro-

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Leu-Gly-Leu-) to create a novel substrate of MMP-9 (Dpa-KPLGLAR-NH2) by adding an ultraviolet substance. The fluorescent substrate for the TACE (ADAM-17) assay ((7-methoxycouma-rin-4-yl)-acetyl-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg-Lys-2,4-dinitrophenyl-NH2) was synthesised by Graham Knight, Department of Biochemistry, University of Cambridge. Our previous studies were performed at 27°C in a fluorescence assay buffer (10mM $CaCl_2$, 50mM Tris-HCl, pH 7.5, 0.05% Brij-35, 1% Me_2SO, 0.02% NaN_3) with a Perkin Elmer Life Sciences LS-50B spectrofluorometer equipped with thermostatic cuvette holders as described in our previous studies [15,16]. We used the hard-core (-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg-) to create a novel substrate of TACE (Dpa-SPLAQAVRSSSR-NH2) by adding an ultraviolet substance.

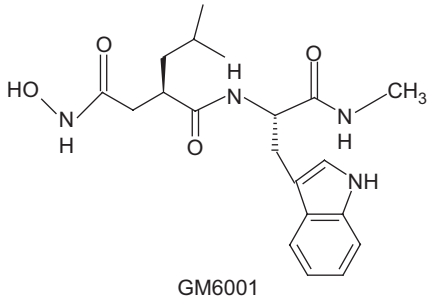
The internal standard method was used instead of the external standard method as a quantitative method. The Ala-Dpa was synthesised and used as the internal standard. The above method was then used to determine the inhibitory effectiveness of GM6001 and inhibitor A on TACE and MMP-9.

Methods

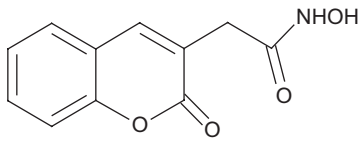
Enzymes and enzyme inhibitor

Recombinant human TACE and Pro-MMP-9 were purchased from R & D Systems (Minneapolis, MN). TACE stock solutions were made in distilled water at 100 μg/mL. Pro-MMP-9 stock solutions were made in 50 mM Tris-HCl buffer (pH7.5, 10mM $CaCl_2$, 150mM NaCl, 0.05%Brij-35) at 100μg/mL.The pro-MMP-9 was activated with *p*-aminophenyl-mercury acetate (APMA) purchased from Sigma Chemical (St Louis, MO). APMA (in DMSO solution) was added to pro-MMP-9 stock solution to give a final APMA concentration of 1mM. Incubation took place at 37°C for 24h [17].

GM6001, one kind of broad spectrum MMPs inhibitor, was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). Inhibitor A was synthesised following the procedure reported by Chun [18]. The structures of these two compounds are shown in below:



GM6001



Inhibitor A

Peptide substrates

TACE peptide substrates (Dpa-SPLAQAVRSSSR-NH_2) and MMP-9 substrates (Dpa-KPLGLAR-NH_2) were custom synthesised by Shanghai Biotech BioScience & Technology (Shanghai, China). Amino acid analysis and mass spectroscopy determined that the purity (as determined using HPLC) was < 95%.

Synthesis of the internal standard

In a typical synthesis, 20 μL 1% 2,4-dinitrofluorobenzene (DNBF) was added to a solution of 100μl 250μM DL-α-Alanine and 100 μl 0.5 M $NaHCO_3$. The solution was mixed well and then put into a 60°C water bath for 1 h. An aliquot of 50 μL 1% trifluoacetic acid (TFA) was added to stop the reaction. After the solution was cooled to room temperature, 1mL phosphate buffer solution (pH 6.86) was added and the solution was stored at –20°C. The internal standard (Ala-Dpa) solution was diluted 1:2 with water before use. The absorption spectra were determined using a Shimadzu UV mini 1240 ultra-visible spectrophotometer (Kyoto, Japan).

Incubation procedure for assaying TACE and MMP-9

The TACE assay was carried out in 25 mM Tris-HCl buffer (pH 9, 2.5 μM $ZnCl_2$ and 0.005% Brij-35). Then, 15ng TACE was added to buffer solution containing 140μM TACE peptide substrate at a final volume of 50 μL. After incubation for 15 min at 37°C, the reaction was stopped with 25 μL of 1% TFA. An aliquot of 25μL of internal standard solution was then added and the solution mixture was analysed by HPLC.

The MMP-9 assay was performed in 50 mM Tris-HCl buffer (pH 7.5, 10mM $CaCl_2$, 150mM NaCl, 0.05%Brij-35). Then, 30ng Active MMP-9 was added to 50 μL of MMP-9 substrate solution (120 μM). After incubation for 25 min at 37°C, the reaction was stopped with 25 μL of 1% TFA. An aliquot of 25 μL internal standard solution was then added and the solution mixture was analysed by HPLC.

HPLC conditions

HPLC analysis was performed using a Arcus EP-C18 (4.6 × 250 mm, 5 μm) column on a Shimadzu LC-3 HPLC instrument (Kyoto, Japan). The mobile phase consisted of 0.1% TFA (Sigma Chemical, St Louis, MO) and water/methanol, 45/55/ (v/v) for the TACE assay and 40/60 (v/v) for the MMP-9 assay. The flow rate was 0.9 mL/ min and the injection volume was 20μL. The oven temperature was set to 40°C. The effluents were detected by measuring the absorbance at 353 nm. Chromatograms were integrated using the N2000 software (Hangzhou, China).

Calibration and enzyme activity calculation

The standard solutions were prepared at 40, 80, 120, 160 and 200 μM using the TACE peptide substrates and the MMP-9 peptide substrates. Each standard solution (100 μL) contained 25 μL of the internal standard solution. Each concentration level was injected in triplicate and

the calibration curves were constructed by considering the relative peak area (the ratio of substrate peak to IS peak) as a function of substrate concentration.

The amount of enzyme that was required for the catalytic conversion of 1μmol of substrate per minute was defined to be 1 unit. The enzyme activity was calculated according to the following equation:

$$\text{enzyme activity}\,(\text{U}) = \frac{C_0 - C_t}{t} \times V$$

where *V*, is the volume of reaction solution; *t*, time of reaction (min); C_0, initial concentration of substrate; and C_t, concentration of the substrate after the enzymatic reaction. The initial concentration was 140 μM for TACE and 120 μM for MMP-9. The value of C_t was obtained from the calibration curves according to the ratio of the substrate peak to the IS peak in the chromatograms.

Evaluation of inhibitor activity

GM6001 and inhibitor A were evaluated under the standard assay conditions. The 15ng TACE was added to 30 μL of 25mM tris-HCl buffer (pH 9, 2.5 μM $ZnCl_2$, 0.005% Brij-35) containing varying concentrations of GM6001 (0, 20, 80, 120, 200, 300, 600, 1500, 2500 nM) or inhibitor A (0, 15, 45, 90, 120, 165, 300, 600, 1200 nM). After incubation for 10 min at 37°C, 2μL of the 3.5nM TACE substrate was added. The final volume was adjusted to 50 μL and the final concentration of the peptide substrate was 140 μM.

Then 30ng MMP-9 was added to 30 μL of 50mM Tris-HCl buffer (pH 7.5, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% Brij-35) containing varying concentrations of GM6001 (0, 30, 60, 100, 300, 600, 1000, 2000, 4000 pM) or inhibitor A (0, 15, 60, 105, 165, 900, 1500, 1800, 2100 nM). After incubation for 30 min at 37°C, 2 μL of 3 nM MMP-9 substrate was added. The final volume was adjusted to 50 μL and the final concentration of the peptide substrate was 120 μM.

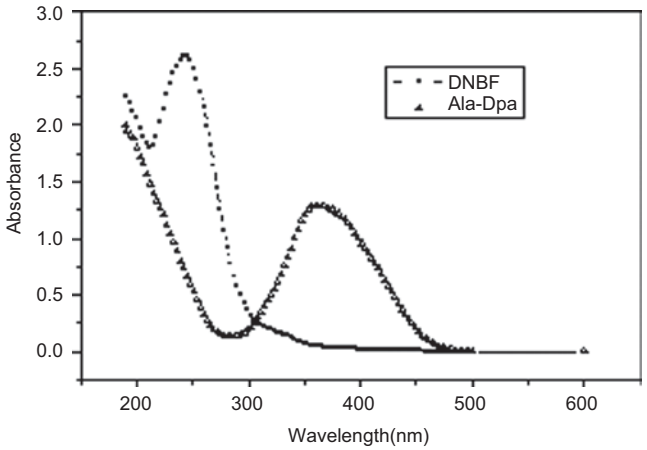


Figure 1. Absorption spectra of DNBF (□) and internal standard Ala-Dpa (△). The absorption maximum of DNBF and Ala-Dpa were 240nm and 360nm, respectively.

Results

Spectral properties of the internal standard

The absorption spectra of DNBF and the internal standard Ala-Dpa is shown in Figure 1. The absorption maximum of DNBF and the internal standard Ala-Dpa were 240nm and 360nm, respectively. This result indicated that the excess DNBF in the internal standard solution did not interfere with the Ala-Dpa signal in the HPLC analysis since the measurement wavelength detector was set at 353nm.

Optimisation of the enzymatic reaction

Reaction time: 0.3mg/L TACE was reacted with 140μM TACE substrate for 5, 10, 15, 20 and 25 min at a concentration of 0.6 mg/L, the MMP-9 was reacted with 120μM MMP-9 substrate for 5, 10, 20, 30 and 40 min. The reacted solutions were analysed with HPLC and the ratios of the product peak area to the internal standard peak area (A_i /A_{is}) were determined. As shown in Figure 2, the ratios of the product peak area to the internal standard peak

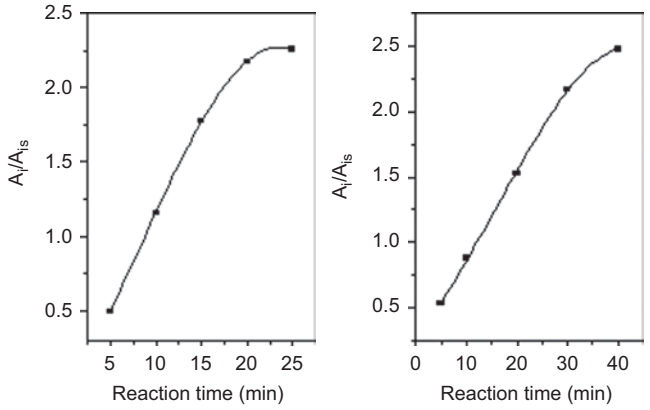


Figure 2. The impact of reaction time on the extent of the enzymatic reaction for TACE (Left) and MMP-9 (Right). The ratios of the product peak area to the internal standard peak area (A_i /A_{is}) as determined by the HPLC method increased linearly with reaction time from 0–15min for TACE and 0–25min for MMP-9.

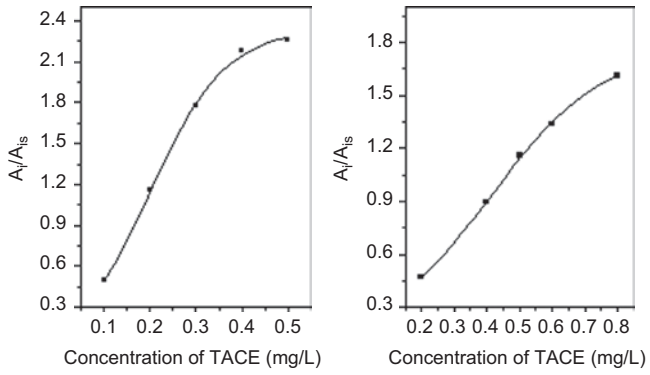


Figure 3. The impact of TACE (Left) and MMP-9 (Right) concentrations on the extent of the enzymatic reaction. The ratios of the product peak area to the internal standard peak area (A_i /A_{is}) as determined by the HPLC method increased linearly with the enzyme concentration from 0.1–0.3 mg/L for TACE and 0.2–0.6 mg/L for MMP-9.

area (A_i /A_{is}) increased linearly with reaction time from 0–15min for TACE and 0–25min for MMP-9. So, the optimal reaction times were 15 min for TACE and 25 min for MMP-9.

Enzyme concentration: 140μM TACE substrate was reacted with 0.1, 0.2, 0.3, 0.4 and 0.5 mg/L TACE for 15 min, and 120 μM MMP-9 substrate was reacted with 0.2, 0.4, 0.5, 0.6 and 0.8 mg/L MMP-9 for 25 min. The

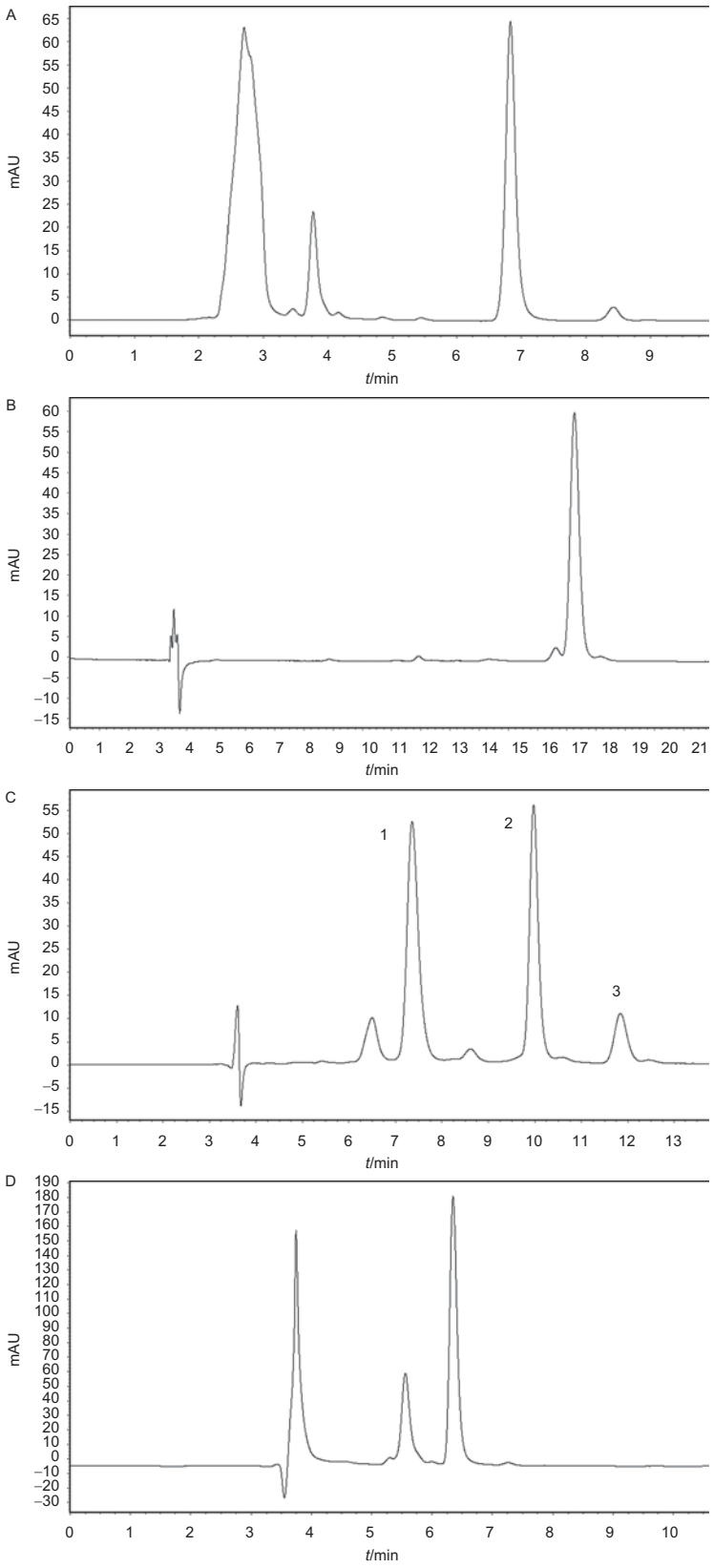


Figure 4. The chromatograms of the eluted reacted substrate using (A) 55% acetonitrile solution, (B) 45% methanol solution, (C) 55% methanol solution, and (D) 70% methanol solution as mobile phases. The peaks in (C) are: (1) Dpa-SPLAQAVRSSSR, (2) Ala-Dpa, and (3) Dpa-SPLAQA.

reacted solutions were analysed with HPLC and the ratios of the product peak area to the internal standard peak area (A_i /A_{is}) were determined. The relationship between A_i /A_{is} and enzyme concentration are shown in Figure 3. A linear relationship between the initial reaction rates and the enzyme concentration was obtained from 0.1–0.3 mg/L for TACE and 0.2–0.6 mg/L for MMP-9. So, 0.3mg/L and 0.6mg/L were considered the

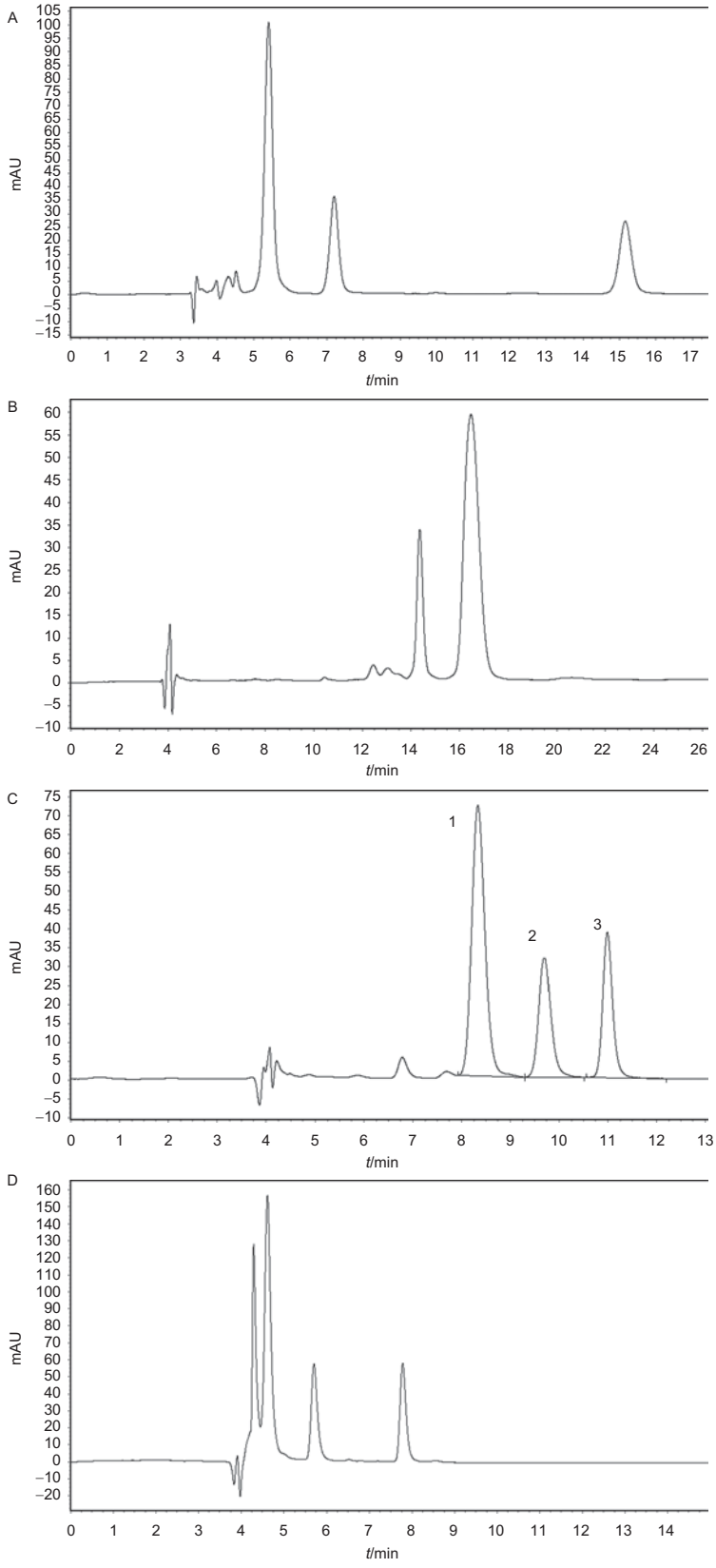


Figure 5. The chromatograms of the eluted reacted substrate using (A) 40% acetonitrile solution, (B) 50% methanol solution, (C) 55% methanol solution, and (D) 70% methanol solution as mobile phases. The peaks in (C) are: (1) Dpa- KPLGLAR, (2) Ala-Dpa, and (3) Dpa- KPLG.

optimal enzyme concentrations for TACE and MMP-9, respectively.

Optimisation of the mobile phase

In the TACE assay, 55% acetonitrile aqueous solution, 45% methanol aqueous solution, 55% methanol aqueous solution and 70% aqueous methanol solution (all containing 0.1% TFA) were tested as possible mobile phases. As shown in Figure 4, the best separation effect was obtained using 55% methanol aqueous solution.

For the MMP-9 assay, 40% acetonitrile aqueous solution, 50% methanol aqueous solution, 60% methanol aqueous solution and 70% aqueous methanol solution (all containing 0.1%TFA) were tested as prospective mobile phases. As shown in Figure 5, the best separation effect was achieved using a 60% methanol aqueous solution.

Calibration curves and precision

The fitted calibration equation for the TACE substrates is $y = 0.0172C - 0.0026$. Where, y, relative peak areas (the ratio of substrate peak to IS peak), C, substrate concentration (μM). The fitted calibration equation for the MMP-9 substrates is $y = 0.0148C - 0.0746$. Both the TACE and MMP-9 substrate calibration curves achieved the high R^2 values of 0.9984 and 0.9999, respectively.

The precision tests were performed by using the TACE substrate at concentrations of 50μM, 150μM, and 300μM. The results are shown in Table 1. The relative standard deviations at all concentration levels were less than 4% within day, and less than 5% between days.

Evaluation of inhibitor

The inhibitive effectiveness of GM6001 and inhibitor A on TACE and MMP-9 were evaluated using the enzyme assay methods described above. Figure 6 displays the inhibition activity curve of GM6001 on TACE and MMP-9.

Figure 7 shows the response curve of logarithmic dose of GM6001 to TACE and MMP-9. There is a sigmoidal relationship between the inhibition ratio and the logarithmic concentration of the inhibitor used. The 50% inhibition concentration (IC_{50}) of the GM6001 and inhibitor A for TACE and MMP-9 are shown in Table 2. The GM6001 shows strong inhibitive effectiveness on MMP-9 and poor inhibitive effectiveness on TACE. Inhibitor A synthesised in our laboratory showed poor inhibitive effectiveness on both TACE and MMP-9.

Table 1. The relative standard deviation (RSD) for the stability of the reacted TACE substrates at varying concentrations (n = 6).

Concentration of substrate	50 μM	150 μM	300 μM
within day RSD			
RSD of A_i	7.48%	5.33%	6.32%
RSD of A_i/A_s	3.27%	3.60%	2.00%
between day RSD			
RSD of A_i	7.57%	6.84%	5.89%
RSD of A_i/A_s	4.77%	3.18%	3.99%

Discussion

The HLPC method has often been used to screening TACE inhibitor *in vitro* (9–10). In this paper, the method was improved in the following aspects: the substrate labelling with two groups (-Mca and –Dpa) were replaced by

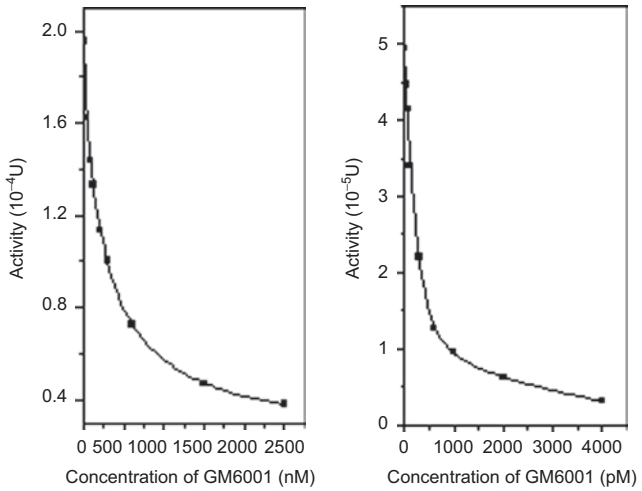


Figure 6. The inhibitive curve of GM6001 on TACE (left) and MMP-9 (right). The activity of TACE and MMP-9 as determined by HPLC method was decreased by the different concentrations of GM6001.

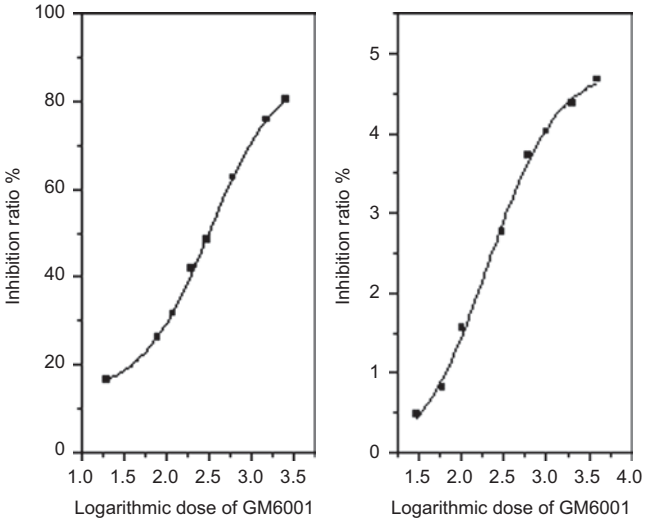


Figure 7. The response curve of the logarithmic dose of GM6001 to TACE (right) and MMP-9 (left). There is a sigmoidal relationship between the inhibition ratio and the logarithmic concentration of GM6001. The value of IC_{50} of GM6001 for TACE and MMP-9 are 317 nM and 0.26 nM (260 pM), respectively.

Table 2. The IC_{50} values of GM6001 and inhibitor A to TACE and MMP-9.

	GM6001	Inhibitor A
TACE	317 nM	175 nM
MMP-9	0.26 nM	537 nM

substrates labelled with a single group (-Dpa); the detection of fluorescence intensity was replaced by detection of absorbance; the external standard method was replaced by an internal standard method. A UV-labelled amino acid (Ala-Dpa) was synthesised and used as an internal standard in the HPLC analysis for the first time. By using this concise and accurate method, the inhibitive effectiveness of GM6001 and inhibitor A on TACE and MMP-9 were measured successfully.

Conclusions

In summary, we have developed a novel, concise, accurate HPLC method for assaying TACE and MMP-9 activity. This method can be used for TACE selective inhibitor screening. Compared with the fluorogenic method, the advantage of our method is: UV-HPLC made it convenient to define the enzyme activity using the International System of Units. The fluorogenic peptide substrates with the fluorophore/quencher-capped ends have found extensive use in monitoring protease activity in the screening of small molecule libraries for protease inhibitors. This substrate is an n-amino-acid peptide capped with an o-aminobenzoyl group on the N-terminal end and with a 3-(2,4-dinitrophenyl)-L-2, 3-diaminopropionic amide group on the C-terminal end. The fluorogen of the substrate was quenched which couldn't be detected with any precision and it was hard to quantitate its concentration after the enzymatic reaction (C_t). The fluorometric detection couldn't define the enzyme activity using the International System of Units. This UV-HPLC method was used to evaluate the activity of MMP-9 and TACE using peptides tagged with the Dpa label. The amount of enzyme required for the catalytic conversion of 1 μmol of substrate per minute was defined to be 1 unit. The enzyme activity was calculated according to the following equation: enzyme activity $(U) = (C_0 - C_t)/t \times V$. The substrate had a UV asorbance, so it was possible to easily calculate its concentration after the enzymatic reaction (C_t). Using UV-HPLC made it possible to define the enzyme activity by International System of Units. Accordingly, there was a broad consistency in determination of results by the different detectors.

Declaration of interest

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