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A Trypsin Inhibitor from Rambutan Seeds with Antitumor, Anti-HIV-1 Reverse Transcriptase, and Nitric Oxide-Inducing Properties

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Abstract *Nephelium lappaceum* L., commonly known as "rambutan," is a typical tropical tree and is well known for its juicy and sweet fruit which has an exotic flavor. Chemical studies on rambutan have led to the identification of various components such as monoterpene lactones and volatile compounds. Here, a 22.5-kDa trypsin inhibitor (*N. lappaceum* trypsin inhibitor (NLTI)) was isolated from fresh rambutan seeds using liquid chromatographical techniques. NLTI reduced the proteolytic activities of both trypsin and α-chymotrypsin. Dithiothreitol reduced the trypsin inhibitory activity of NLTI at a concentration of 1 mM, indicating that an intact disulfide bond is essential to the activity. NLTI inhibited HIV-1 reverse transcriptase with an IC_{50} of 0.73 μM. In addition, NLTI manifested a time- and dose-dependent inhibitory effect on growth in many tumor cells. NLTI is one of the few trypsin inhibitors with nitric oxide-inducing activity and may find application in tumor therapy.

Keywords Rambutan · *Nephelium lappaceum* L. · Nitric oxide · Protease inhibitor · Liquid chromatography · Antitumor

Introduction

Nephelium lappaceum L., commonly known as "rambutan," is a native tropical tree cultivated mainly in Thailand, South Vietnam, Malaysia, Indonesia, the Philippines, and India [1]. The fruit of this tree is well liked for its sweet and juicy sarcocarp. It belongs to the same family Sapindaceae as the subtropical fruits longan (*Dimocarpus longan* Lour.), lychee (*Litchi chinensis* Sonn.), and mamoncillo (*Melicoccus bijugatus* Jacq.). As a multifunctional medicinal plant, the roots show anti-fever effects [2], the bark is an astringent [2], the leaves are used

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as poultices [2], the pericarp can reduce the mortality of infections caused by herpes simplex virus type 1 [3], and the pulp can be used for the treatment of severe dysentery and type 2 diabetes mellitus [4], although it has been reported that it could cause anaphylaxis [5].

Rambutan seeds are soft and crunchy and manifest bitter and narcotic flavors. A limited number of studies have been performed on bioactivity of the seeds [2]. For example, the insecticidal activity of the rambutan seeds was associated with type II cyanolipid components [6]. Ethanol extraction of rambutan seeds can be used to control rice weevils (*Sitophilus oryzae* L.) [7]. Two new diastereomeric monoterpene lactones 1 and 2, purified from the rambutan seeds, exhibit potential antimicrobial characteristics. Only a few studies on rambutan constituents have been reported [8–13]. There are only two reports on proteins from rambutan including glyceraldehyde-3-phosphate dehydrogenase as a major allergen in rambutan-induced anaphylaxis [14] and polyphenol oxidase in rambutan pericarp tissues which play a role in browning [15]. Thus, it is worthwhile to conduct research on the purification of other functional components in the seeds.

Comprising about 2 % of the human genome, proteases are involved in a multitude of essential cellular functions, ranging from regulation of the cell cycle, signal transduction pathways, inflammatory responses to apoptosis, etc. [16]. Two predominant mechanisms on the control of proteases are (1) proprotein/zymogen processing and (2) control of the activated enzymes by protein inhibitors of proteases [17]. Based on their catalytic mechanisms and targeting inhibitors, proteases are divided into five families including cysteine, metallo, aspartic, threonine, and serine proteases [18]. The major groups of serine proteases contain trypsin-like, elastase-like, and chymotrypsin-like proteases, and inhibitors toward such proteases have promising therapeutic uses [19, 20]. Trypsin/chymotrypsin inhibitors from the Leguminosae family are well investigated and have been reported with multifunctional medicinal activities [19–21]. In this study, a trypsin inhibitor was purified from rambutan seeds, and its biochemical and functional activities were investigated.

Experimental

Materials

The red-skinned cultivar of rambutan [*N. lappaceum* L. var. *pallens* (Hiern) Leenh.] was purchased from a local market and identified by Professor Shiu-Ying HU, Honorary Professor of Chinese Medicine, The Chinese University of Hong Kong (CUHK). Bovine pancreatic trypsin, α-chymotrypsin, and soybean trypsin inhibitor were from USB Corporation (Cleveland, USA). All other chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated in the text.

Purification

First, 120 g fresh rambutan seeds were homogenized in 400 ml of 0.02 M NH_4HCO_3 buffer (pH 9.4) thoroughly using a blender followed by centrifugation at 20,000×*g* at 4 °C for 40 min. The supernatant was filtered through filter paper and then applied to a Q-Sepharose column (18×5 cm) previously equilibrated with 0.02 M NH_4HCO_3 buffer (pH 9.4). The column was washed with the equilibration buffer to elute the unbound fraction until OD_{280} reached a baseline value, and the bound fraction was then eluted with 1.0 M NaCl in the same buffer. The eluate which exhibited trypsin inhibitory activity was dialyzed exhaustively against deionized water at 4 °C. NH_4HCO_3 buffer (2 M) was added until the NH_4HCO_3 concentration

reached 0.02 M. The fraction was then applied to a Mono Q 5/50 GL column and eluted successively with two linear concentration gradients of NaCl (0~0.2, 0.2~1 M) using an AKTA Explorer 100 FPLC System. Fractions with trypsin inhibitory activity (peak MQ-II) were pooled, dialyzed, lyophilized, and chromatographed on a Superdex 75 10/300 GL column in 0.2 M NH_4HCO_3 buffer (pH 9.4). The purified *N. lappaceum* trypsin inhibitor (hereafter referred to as NLTI) in peak SUP-I was dialyzed extensively and freeze-dried.

Purity and Molecular Weight Determination

The purity of the purified protein was ascertained by molecular exclusion chromatography on a Superdex 200 10/300 GL column (previously equilibrated in 0.2 M NH_4HCO_3 buffer) and then eluted with the same buffer at a flow rate of 0.5 ml/min. For molecular mass determination, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12 % polyacrylamide gel) was carried out under reducing conditions [22]. Furthermore, the molecular weight of the purified protein was also determined by fast protein liquid chromatography (FPLC) gel filtration as described previously [23].

Determination of Trypsin/Chymotrypsin Inhibitory Activity and Its Stability

The assay was conducted by using a spectrophotometric method as reported before [20]. Firstly, NLTI (20 μL) serially diluted fourfold from 1,000 to 1 μM in 50 mM Tris–HCl buffer (pH 7.6) was incubated with 40 μL trypsin (1 mg/mL in 50 mM Tris–HCl containing 0.2 M $CaCl_2$, pH 7.6) for 5 min at 37 °C. Residual trypsin activity was measured by adding 1.44 mL 0.25 mM *N*-α-benzoyl-L-arginine ethyl ester (BAEE) as substrate. After mixing immediately by inversion, the increase in A_{253} was recorded for 5 min. Reaction without addition of NLTI was used as a positive control. Chymotrypsin inhibitory activity was determined by using the same assay but using *N*-benzoyl-L-tyrosine ethyl ester (BTEE) as substrate instead. One chymotrypsin unit hydrolyzes 1.0 μmol of BTEE per minute at pH 7.6 and 25 °C. Furthermore, 1 % casein was used as substrate for the description of a dose-dependent relationship between NLTI and residual trypsin activity as mentioned previously [20]. Soybean trypsin inhibitor from USB Corporation was used as a positive control. The effects of temperature, pH, as well as the reducing agent dithiothreitol (DTT) on stability (marked as residual trypsin inhibitory activity) of NLTI were investigated following the method reported previously [19].

The trypsin inhibitory activity was calculated with the following equation as reported previously [19]: BAEE units/mg trypsin inhibitor=(ΔA_{253}/min positive−ΔA_{253}/min sample tested)/[(0.001) (0.025)−(sample)], where (0.001) = the change in $A_{253/min}$ per unit of trypsin at pH 7.6 and 25 °C in a 1.5-ml reaction mix and (0.025) or (sample) = solid (mg) of trypsin or sample used in the reaction. In addition, 1 unit of trypsin inhibitor activity is the amount of inhibitor which reduces the trypsin activity by 1 BAEE-U. One BAEE-U (1 trypsin unit) is the amount of enzyme which increases the absorbance at 253 nm by 0.001/min with BAEE as substrate at pH 7.6 and 25 °C.

For chymotrypsin inhibitory activity, the same equation was used.

Assay of HIV-1 Reverse Transcriptase (HIV-1 RT) Inhibitory Activity

The assay was performed in view of the report of HIV-1 RT inhibitory activity in trypsin inhibitors [21]. An HIV-1-RT (recombinant) ELISA kit was used following the instructions of the manufacturer (Boehringer Mannheim, Germany) [21]. Pinto bean lectin with anti-HIV-1-RT activity was chosen as a positive control [24].

Cell Culture and Assay for Cell Viability

The assay was performed in view of the report of antiproliferative activity in trypsin inhibitors [21]. The human nasopharyngeal carcinoma (NPC) cell lines CNE-1 (well-differentiated), CNE-2 (poorly differentiated), HNE-2, and SUME-α were bought from Sun Yat-sen University of Medical Sciences, Guangzhou, China. The human hepatocellular carcinoma Hep G2 and human breast tumor MCF-7 cell lines were purchased from American Type Culture Collection. All cell lines were maintained in RPMI 1640 medium containing 10 % fetal bovine serum and 1 % penicillin and streptomycin. The MTT test was used to monitor inhibition of cell growth with details reported elsewhere [20].

Assay of Nitric Oxide-Inducing Activity

The Griess assay was applied to detect the concentration of nitrite and nitrate, as the surrogate marker for nitric oxide (NO), in BALB/c mouse peritoneal macrophages [25].

Statistical Analysis

The IC_{50} values were calculated by Sigmaplot software. SPSS 11.0 (SPSS, Chicago) was applied for data analysis. All the data are shown in mean±standard deviation (SD) from at least two independent experiments. The two-tailed Student's *t* test was chosen for between-group comparisons, and a p value of <0.05 was considered significant.

Results

Purification of NITI

The crude extract of rambutan seeds was first loaded on a Q-Sepharose column. The adsorbed fraction containing trypsin inhibitory activity, eluted by 1 M NaCl, was subsequently applied to a Mono Q 5/50 GL column with an FPLC system. Fraction MQ-II was finally loaded on a Superdex 75 10/300 GL column. As Fig. 1 illustrates, fraction MQ-II (Fig. 1a), which retained trypsin inhibitory activity, was then applied to a Superdex 75 column and the purified NLTI within SUP-I (Fig. 1b) was harvested. The yields of successive chromatographic purification steps are summarized in Table 1. The final yield of NLTI was about 200 mg from 120 g fresh rambutan seeds with a 23.6 purification fold compared to the crude extract. SDS-PAGE in Fig. 1c shows the purification achieved after each chromatographic step. After gel filtration on Superdex 75, a single band of NLTI with a molecular weight of about 22.5 kDa was acquired. The molecular weight was in agreement with results of gel filtration chromatography on Superdex 75 10/300 (Fig. 1d). N-terminal sequence of the purified protein did not yield positive results indicating the protein had a blocked N-terminus.

Biological Characterization and Its Thermal, pH, and Chemical Stability

NLT exhibited trypsin inhibitory activity of 20,425 BAEE units/mg and α-chymotrypsin inhibitory activity of 46 BTEE units/mg, respectively. The linear dose (log_{10} concentration)–residual trypsin/α-chymotrypsin activity relationships are shown in Fig. 2a, b. Results of the positive control were similar to our previous report [21].

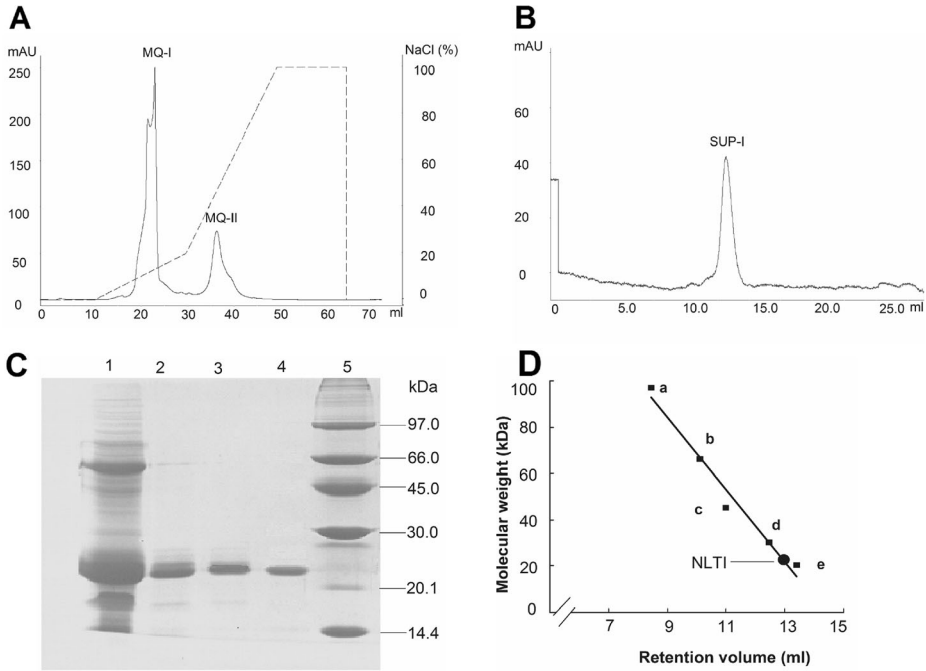


Fig. 1 Purification and molecular weight determination of NLTI. **a** The crude extract of rambutan seeds was applied to a Q-Sepharose column, and the bound fraction eluted by 1 M NaCl (not shown) was then applied to a Mono Q 5/50 GL column. Only peak MQ-II contained trypsin inhibitory activity. The *dashed lines* represented the concentration of NaCl employed (100 % NaCl equals 1 M NaCl). **b** Fraction MQ-II was subsequently loaded on a Superdex 75 10/300 GL column to yield purified trypsin inhibitor (SUP-I). **c** SDS-PAGE (reducing conditions) showing purity and molecular weight of NLTI. *Lane 1*, crude extract; *lane 2*, fraction bound on Q-Sepharose; *lane 3*, fraction MQ-II bound on Mono Q column; *lane 4*, peak SUP-I from Superdex 75 column; *lane 5*, molecular weight marker. **d** Calibration curve of Superdex 75 column eluted with 0.2 M NH_4HCO_3 buffer at the flow rate of 0.5 ml/min. The markers included **a** phosphorylase b (97.0 kDa), **b** albumin (66.0 kDa), **c** ovalbumin (45 kDa), **d** carbonic anhydrase (30 kDa), and **e** trypsin inhibitor (20 kDa). Elution volume of NLTI was 12.95 ml corresponding to a molecular weight of 22.5 kDa

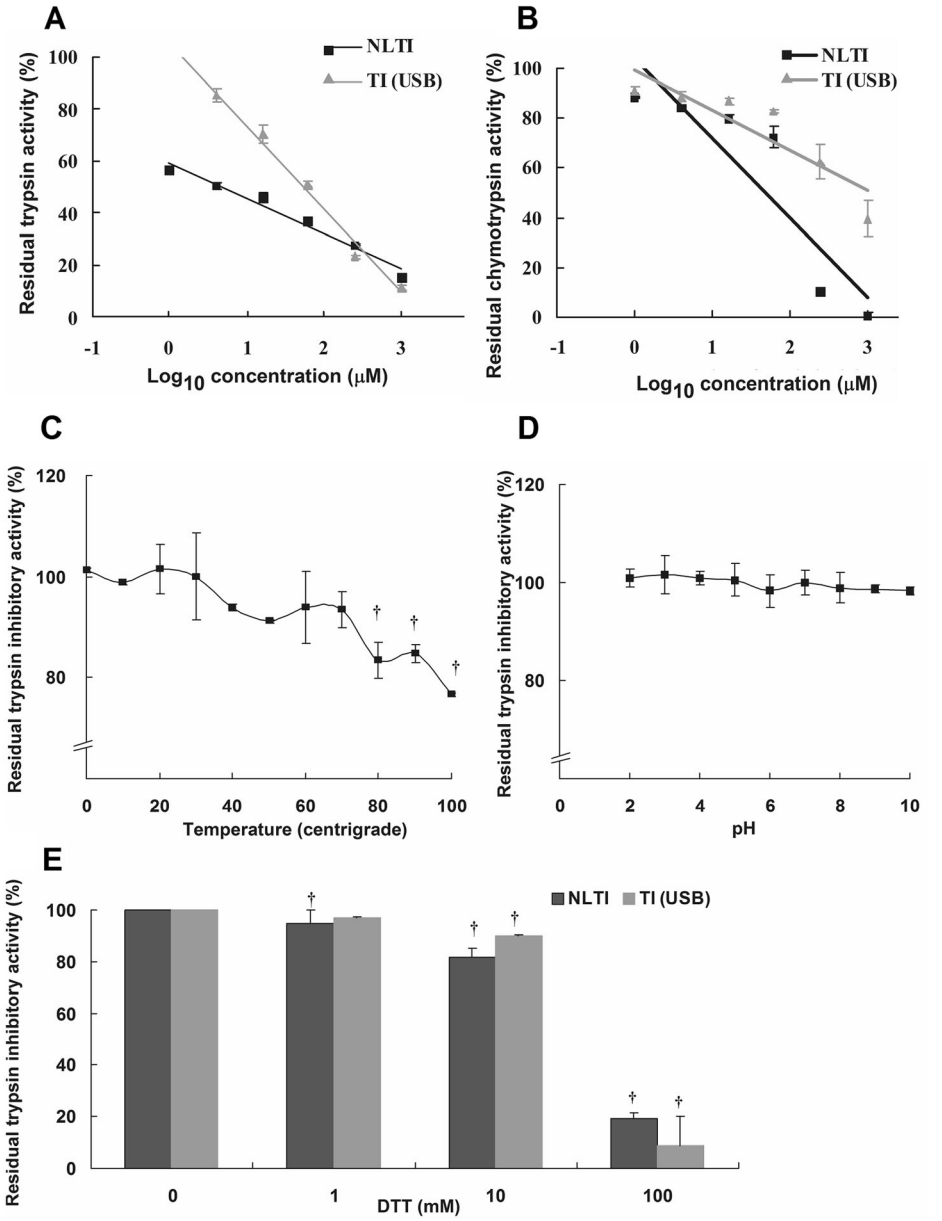
The trypsin inhibitory activity was stable throughout the temperature range 0–70 °C (Fig. 2c). At 80 °C, activity dropped to 83.42 % of the value at 30 °C ($p<0.01$). After

Table 1 Summary of purification of NLTI from 120 g fresh rambutan seeds

Column	Fraction with trypsin inhibitory activity	Yield (mg)	Specific activity (BAEE units/mg)[a]	Purification fold[b]
–	Crude extract	2,540	864±12	1.0
Q-Sepharose	Q bound fraction	860	4,240±231	4.9
Mono Q	MQ-II	280	20,250±1,025	23.5
Superdex 75	SUP-I	200	20,425±890	23.6

[a] Mean±SD for three independent experiments are shown

[b] Purification fold equals value of specific activity of the chromatographic fraction divided by value of specific activity of crude extract



incubation at 100 °C for 10 min, there was 76.63 % activity remaining ($p<0.01$). The trypsin inhibitory activity was stable after incubation in buffers at pH 2–10 for 30 min (Fig. 2d). The activity was reduced by DTT at a minimum concentration of 1 mM ($p<0.05$) (Fig. 2e). Following exposure to 100 mM DTT for 30 min at room temperature, there was only 19.16 % residual trypsin inhibitory activity. This indicated the importance of disulfide bond(s) in NLTI linked to the activity.

◀ **Fig. 2** Trypsin and α-chymotrypsin inhibitory activities of NLTI and its stability. After incubation with different concentrations of NLTI (fourfold dilution of 1 to 1,000 μM, including 1,000, 250, 62.5, 15.6, 3.9, and 0.98 μM) for 30 min, the remaining activity of **a** bovine pancreatic trypsin or **b** α-chymotrypsin was measured using 1 % casein as substrate. Means±SD of three independent experiments are shown. Correlation coefficient (*r*) values: −0.83 (NLTI to trypsin); −0.91 (TI to trypsin); −0.32 (NLTI to chymotrypsin); and −0.29 (TI to chymotrypsin). Values of *r* were from three independent experiments. **c** Thermal stability was tested after NLTI (50 μM in Tris–HCl buffer) had been exposed to 0 to 100 °C for 30 min. **d** pH stability of NLTI was measured after dissolving the lyophilized protein in buffers of different values to a final concentration of 50 μM followed by incubation for 0.5 h at 37 °C. Residual trypsin inhibitory activity in both **c** and **d** was measured by using BAEE as substrate. **e** NLTI was incubated with DTT at different final concentrations (0, 1, 10, and 100 mM) for 30 min at 37 °C. Twice the amount of iodoacetamide was used to stop the reaction, and the residual trypsin inhibitory activity was measured by using BAEE as substrate. Soybean trypsin inhibitor was used as a positive control. Means±SD of three independent experiments are shown, and the Student's *t* test was applied for statistical analysis where appropriate. In **c**, $p<0.05$ (*dagger*) versus the residual activity at 30 °C. In **d**, no statistical difference was found between the value at pH 7 and the other conditions. In **e**, $p<0.05$ (*dagger*) versus control, respectively

Anti-HIV-1-RT Activity

NLTI exhibited significant HIV-1-RT inhibitory activity with an IC_{50} of 0.73 μM (Fig. 3). The 62-kDa pinto bean lectin, used as a positive control in this assay, revealed an IC_{50} value of 2.93 μM which was commensurate with previously reported results [21, 24].

Antitumor and NO-Inducing Activities

After 48-h incubation, NLTI manifested a time- and dose-dependent growth inhibitory effect on MCF-7 (IC_{50}=130.7 μM), Hep G2 (IC_{50}=215.3 μM), CNE-1 (IC_{50}=277.0 μM), and CNE-2 (IC_{50}=30.0 μM) tumor cell lines, whereas no significant effect was observed on HEN-2 and SUME-α cells when tested up to 1 mM was observed (Table 2). Since NO is an antitumor molecule [26], we then investigated the NO-inducing activity of NLTI. Figure 4a illustrates that NLTI could dose-dependently stimulate the production of NO in mouse macrophages. The same activity was shown by lipopolysaccharide (LPS) which served as a

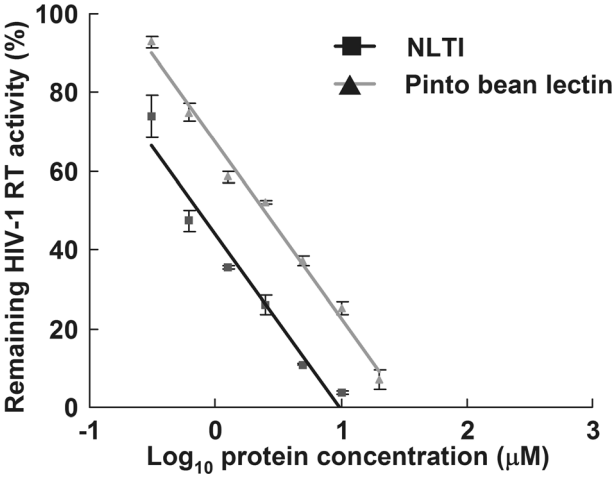


Fig. 3 HIV-1 reverse transcriptase inhibitory activity of NLTI. A serial dilution of NLTI (0.31~10 μM) was used, and anti-HIV-1-RT activity was assayed by using a HIV-1 reverse transcriptase ELISA kit. Pinto bean lectin was used as a positive control (0.31~20 μM). Means±SD of three independent experiments are shown

Table 2 Antiproliferative activity of NLTI on different tumor cells

Tumor cell line	IC_{50} (μM)	
	24 h	48 h
MCF-7	266.2±13.4	130.7±9.0*
Hep G2	337.7±50.2	215.3±7.5*
HNE-2	–	–
CNE-1	–	277.0±31.2
CNE-2	215.6±13.2	30.0±5.9*
SUME-α	–	–

Values of IC_{50} represent mean±SD for n=3 determinations with the MTT assay. Statistical differences were determined by paired T test between 24 and 48 h within each cell line

– means antiproliferative activity was undetectable or value above 1 mM

*p<0.01 compared with IC_{50} (24 h) of the same cell line as determined by one-way ANOVA

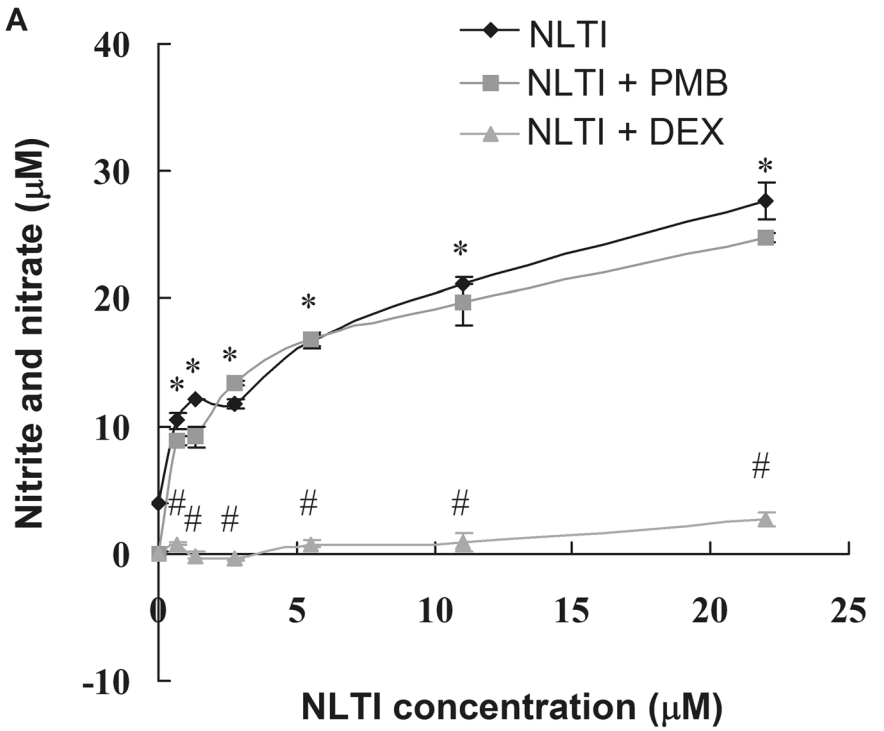
positive control. Furthermore, coadministration of polymyxin B sulfate (PMB), a specific LPS inhibitor, did not attenuate its NO-inducing activity, indicating no detectable LPS contamination in NLTI. On the other hand, addition of dexamethasone (DEX), a specific inhibitor of inducible NO synthase (iNOS), resulted in significant downregulation of NO. It unveiled a complementary role of iNOS in NLTI-induced NO production. Results of the positive control, LPS, were the same as previously reported (Fig. 4b) [26].

Discussion

Major inhibitors of serine proteases include Kunitz type inhibitors, Bowman-Birk type inhibitors, potato type I and II inhibitors, and Kazal type inhibitors [27, 28]. The Kunitz type trypsin inhibitors demonstrate a molecular weight of ~20 kDa and two disulfide bonds [20, 21, 28]. Though most Kunitz type trypsin inhibitors have only one reactive site, a few that display two reactive sites are termed double-headed inhibitors, e.g., arrowhead protease inhibitors A and B whose tertiary structures have been published recently [29]. Among them, Kunitz type trypsin inhibitors have been extensively studied and multiple activities/functions have been disclosed, such as significant inhibition of proliferation and metastasis of tumors [20, 30], and even utilization in assessing geographical variation and studying the evolutionary process [31]. As judged by the molecular weight, NLTI is likely a Kunitz trypsin inhibitor. Its biological and functional activities are reported.

The 22.5-kDa NLTI was isolated by simple liquid chromatographic methods. It manifested significant trypsin inhibitory activity (20,425 BAEE units/mg) but a mild inhibition toward α-

Fig. 4 NO production by mouse peritoneal macrophages after different treatments. Macrophages (0.2 million cells/ well) were treated with **a** NLTI (final concentrations from 0.6875 to 22 μM) or NLTI + PMB (polymyxin B sulfate, final concentration 10 U/mL) or NLTI + DEX (dexamethasone, final concentration 10 μM) and **b** LPS (final concentrations from 7.8 to 250 μg/ml) or LPS + PMB (final concentration 10 U/mL) or LPS + DEX (final concentration 10 μM). Supernatants of cultures were evaluated for the concentrations of nitrite and nitrate, as the surrogate marker for NO, using Griess assay. Values represent mean±SD of three independent tests. *Asterisk*: p<0.05, NLTI (or LPS) group compared with negative control (no treatment). *Number sign*: p<0.05, NLTI (or LPS) + DEX compared with NLTI (or LPS) group at the same dose point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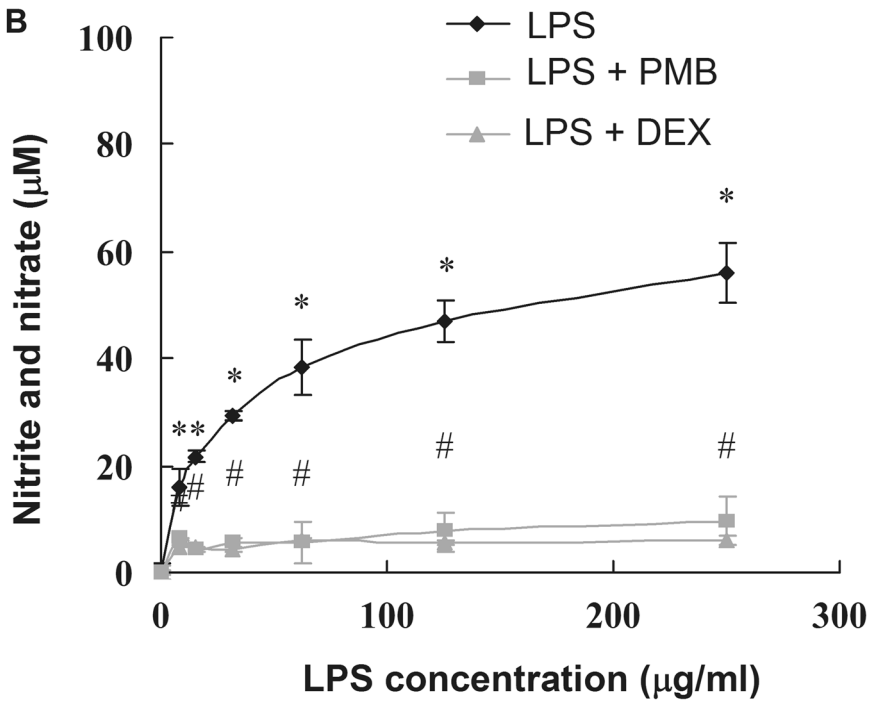
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chymotrypsin (46 BTEE units/mg) (Fig. 2). Its chemical, thermal, and pH stabilities were further tested. Our results showed that NLTI was sensitive to DTT at a final concentration of 1 mM (Fig. 2e). This sheds light on the importance of disulfide bonds in NLTI to its trypsin/α-chymotrypsin inhibitory activity as reported previously [18]. Furthermore, NLTI is stable from 0 to 70 °C and from pH 2 to 10. This may facilitate its potential commercial development as a novel trypsin/chymotrypsin inhibitor.

The horrific propagation of acquired immunodeficiency syndrome (AIDS) and its drug resistance perplex scientists. During the past years, many strategies have been used to develop anti-HIV drugs including targeting HIV-RT or HIV protease inhibitors and extracting components from plants and other sources. We used an HIV-1-RT (recombinant) ELISA kit to test the anti-HIV-1-RT activity of NLTI, and the IC_{50} was 0.73 μM (Fig. 3). This value is smaller than some of the previously purified anti-HIV-1-RT proteins, such as a 60-kDa *Phaseolus vulgaris* lectin (IC_{50}=1.8 μM) [26] and the 62-kDa pinto bean lectin (IC_{50}=2.93 μM) [24], but whether it shows efficacy as an anti-HIV adjuvant needs further in-depth studies. The mechanism of the inhibitory action of NLTI on HIV-1-RT activity probably involves protein–protein interaction, in a way similar to some other protease inhibitors of credited clinical effects [32].

Our further in vitro studies focused on the antiproliferative activity of NLTI against cancer cells including NPC cells. The incidence and mortality of NPC remain high in endemic regions such as northern Africa, Alaska, and especially in South China. During the period 2000~2004, the average age-standardized incidence rate of this cancer was 11.8 per 100,000 people during the 5-year period in Hong Kong [33]. Natural components isolated from traditional herbs and plants provide an exciting complementary way of cancer therapy [30]. Four NPC cell lines were used in this study, and NLTI showed antiproliferative activity on the two classical CNE-1 (IC_{50}=277.0 μM) and CNE-2 (IC_{50}=30.0 μM) cell lines but no significant effect on HEN-2 and SUME-α cells up to concentrations of 1 mM. The results disclosed selective antitumor potential of NLTI towards different NPC subtypes. In addition, NLTI demonstrated antiproliferative activity toward other cancer cells including breast cancer MCF-7 cells and hepatoma Hep G2 cells.

NO manifests a wide variety of medicinal activities. It is involved in blood pressure control, neural signal transduction, platelet function, and also has defensive functions (including antimicrobial, anti-antibiotic, and antitumor activities) [26]. Most trypsin inhibitors were reported as suppressors of NO production in different tissues. For instance, a Bowman-Birk trypsin inhibitor from soybean inhibits NO production [34]. A 25-kDa trypsin inhibitor from sweet potato storage roots is a potential NO and peroxynitrite scavenger [35]. Endogenous urinary trypsin inhibitor is protective against LPS-induced iNOS elevation [36]. Nafamostat mesilate, a serine protease inhibitor, suppresses LPS-induced NO synthesis in cultured human trophoblasts [37]. Aprotinin (Kunitz inhibitor) is the first competitive protein inhibitor of NOS activity [38]. However, our results indicated that NLTI manifested good NO-inducing activity, with the possibility of contamination with LPS, a contaminant which may be present in certain reported studies, excluded [26]. Up to now, ancordin, a 23-kDa trypsin inhibitor which is the major rhizome protein of madeira-vine, is the only trypsin inhibitor shown to have stimulatory activity on NO production [39]. To the best of our knowledge, the present report is the second that shows NO-inducing activity of a trypsin inhibitor.

In conclusion, by using a combination of chromatographic techniques, a 22.5-kDa trypsin inhibitor (NLTI) with trypsin and α-chymotrypsin inhibitory activities was purified from

N. lappaceum seeds. NLTI possesses a good medicinal potential in terms of (a) good thermal and pH stability, (b) potent inhibitory activity against HIV-1 RT, and (c) selective toxicity to some tumor cell lines. NLTI is among the few trypsin inhibitors with NO-inducing activity.

Conflict of Interest The authors have declared no conflicts of interest.

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