Synthesis and activity evaluation of a new bestatin derivative LYP2 as an aminopeptidase N inhibitor

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As a ubiquitous enzyme overexpressed on the epithelium of the tumor, aminopeptidase N (APN) plays important roles in the angiogenesis and metastasis of the tumor. Bestatin as an effective inhibitor against APN is used in the ancillary treatment of various cancers. In this study, we modified the structure of a bestatin derivative LYP reported in our former study to provide a new bestatin derivative LYP2 with enhanced stability. We also tested the inhibitive activity of LYP2, which retained good efficacy *in vitro* and *in vivo* towards APN. *Anti-Cancer Drugs* 22:99–103 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2011, **22**:99–103

Keywords: aminopeptidase N, A549, bestatin, 2-dimethylaminoethylamine, ES-2, HL60, H7402, K562, PLC

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Received 5 December 2009 **Revised form accepted** 8 April 2010

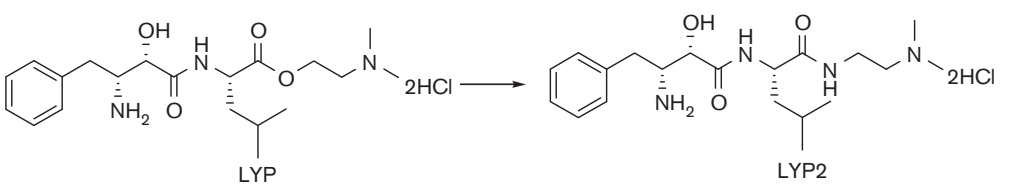
Introduction

Aminopeptidase N (APN), a zinc-dependent metalloprotease, belongs to the M1 aminopeptidase family [1]. APN is a ubiquitous enzyme distributed in many kinds of organs, tissues and cells with the biological function of removing free amino acid from the N terminal of various unsubstituted biologically active peptides. APN possesses extensive substrates such as extracellular matrix, enkephalin, etc. Especially on the epithelial of the tumor, APN is overexpressed and plays key roles in the angiogenesis and metastasis of the tumor [2]. APN can degrade the extracellular matrix to facilitate metastasis, which is the lethal feature of the tumor [3]. On the other hand, neovascularization could supply nutrition and oxygen for tumor survival. In brief, inhibition of APN activity has been a promising avenue for the treatment of cancers.

Bestatin, [(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine, is a well-known inhibitor of APN [4,5], which can be used in leukemia therapy directly or act as an ancillary drug with other anti-cancer agents because of its immunostimulant effect. As the only marketed inhibitor of APN, the pharmacokinetics and biotransformation of bestatin have been well investigated. To improve the water solubility of bestatin, we designed and synthesized a derivative of bestatin (LYP) in our earlier study [6]. However, LYP showed poor stability because of the fragile ester bond in its structure. To overcome this shortcoming, we optimized the structure of LYP by substituting the ester bond with amide linkage to give a new bestatin derivative LYP2 whose stability was well improved. The structures of LYP and LYP2 are showed in Fig. 1.

LYP2 was formed with bestatin and 2-dimethylaminoethylamine through an amide bond because of which LYP2 had an absolutely better stability than that of LYP. Besides, LYP2 also showed good water solubility and efficacy towards APN *in vitro* and *in vivo*, which showed that LYP2 was a more successful reformation of bestatin than LYP.

Fig. 1



The structure of LYP and LYP2.

0959-4973 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e32833ab78a

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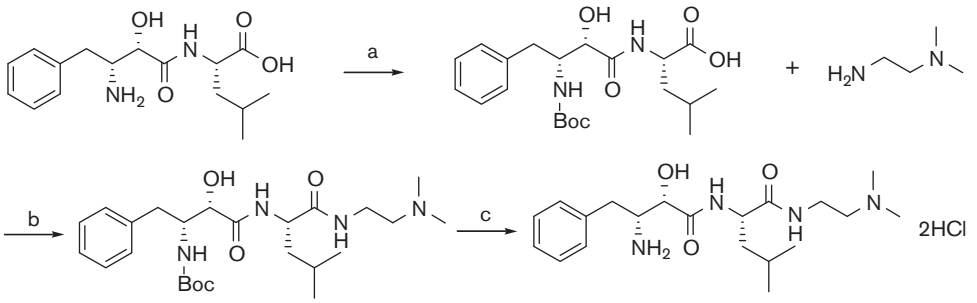
Synthesis

The starting material is bestatin. After the protection of the primary amine group with di-*tert*-butyl dicarbonate (Boc group), coupling and removal of the Boc group, the targeted compound LYP2 was obtained with a reasonable overall yield as shown in the following scheme.



a. $(Boc)_2O$, NaOH; b. HOBt/DCC, dry THF; c. dry HCl in EtOAc

Water solubility assay

Solubility limits were estimated as the concentrations (mmol/l) obtained in saturated solutions. Large amounts of bestatin and LYP2 were stirred at room temperature for 1 day in 1 ml of PBS. The resulting two saturated solutions were clarified twice by centrifugation, and the concentrations were measured spectrophotometrically after a 200-fold dilution in PBS using the standard curve of absorbance as a function of concentration according to the Lambert–Beer law.

Biological activity assay

Enzyme inhibition assay

APN inhibition assay

The IC_{50} value against APN was determined by using L-leucine-*p*-nitroanilide as the substrate and microsomal amino-peptidase from Porcine Kidney Microsomes (Sigma, St. Louis, Missouri, USA) as the enzyme in 50 mmol/l PBS, pH 7.2 at 37°C. The hydrolysis of the substrate was monitored by following the change in the absorbance measured at 405 nm with a UV–visible spectrophotometer Pharmacia LKB, Biochrom 4060. All the solutions of the inhibitors were prepared in the assay buffer (PBS), and the pH was adjusted to 7.2 by the addition of 0.1 mol/l HCl or 0.1 mol/l NaOH. All the inhibitors were preincubated with APN for 30 min at room temperature. The assay mixture, which contained the inhibitor solution (six concentrations), substrate, enzyme solution (4 μg/ml final concentration) and buffer solution, was finally adjusted to 200 μl.

MMP-2 inhibition assay

Our laboratory also devoted efforts to find new inhibitors of MMP-2, which was also a zinc-dependent metalloproteinase. Thus, the inhibition activity towards MMP-2 was measured simultaneously to test the selectivity of LYP2. MMP-2 and TNBS were purchased from Sigma. The substrate was synthesized as described by Vijaykumar *et al.* The gelatinase, substance and inhibitor were dissolved in sodium borate (pH8.5, 50 mmol/l) and incubated for 30 min at 37°C, and then 0.03% TNBS was added and incubated for another 20 min; the resulting solution was detected under 450-nm wavelength to measure absorption.

Cell assay

The tumor cell lines we used were HL60, ES-2, K562, A549, H7402 and PLC.

Tumor cells seeded in a 96-well microplate and incubated at 37°C with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 were exposed to different concentration of LYP2 and bestatin 4 h later with a solvent plate as the blank group. After 48 h, 10 μl 0.5% MTT was added in each well. The supernatant was separated by centrifugation (2500 rpm, 20 min) and discarded. After adding 100 μl DMSO, the supernatant was subjected to fluorometric determination (excitation, 570 nm; emission, 630 nm). The activity was expressed as the inhibition rate. Triplicate experiments with triplicate samples were performed.

Anti-metastasis assay in vivo

In vivo, the efficacy of LYP2 was evaluated in H22 xenografts mice that were supplied by the Drug Analysis Center of Shandong Academy of Sciences.

Each mouse was injected with 0.2 ml suspended solvent of tumor cell (6×10^6) under sterile conditions. After 24 h, the mice were randomly divided into three groups, including the blank group, bestatin group and LYP2 group.

Both the compounds were dissolved in 0.5% sodium carboxymethycellulose solution with a concentration of 2 mg/ml and administrated intragastrically for 12 days.

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The mice were killed and the metastases on the lungs were counted. The result was shown through the inhibition rate:

$$\text{inhibition rate} = \frac{C - T}{C} \times 100\%$$

C = control group, T = experimental group.

Results

Chemistry

3.08 g (10 mmol) of bestatin was stirred in 10 ml 1 mol/l NaOH solution under an ice bath. 2.34 g (10.74 mmol) di-*tert*-butyl dicarbonate dissolved in 5 ml of THF was then added into the solution. After 2 h, the ice bath was removed. The mixture was stirred for another 24 h. After removing THF under vacuum, the solution was acidified to pH 3 with saturated citric acid. The solution was washed with 30 ml acetic ether three times. The organic layer was collected and dried over anhydrous sodium sulfate overnight. After evaporating the solvent, the residue was dried in vacuum to obtain 3.93 g of white solid (Boc-bestatin) with a yield of 99.1%.

Three grams (7.35 mmol) of Boc-bestatin was dissolved in 100 ml dry THF. 1.07 g (7.93 mmol) HOBt was added into the solution under an ice bath. 1.67 g (8.03 mmol) DCC dissolved in 50 ml dry THF was added dropwise into the mixture. The solution was stirred for 8 h at room temperature. 1.96 g (22 mmol) 2-dimethylaminoethylamine dissolved in 20 ml dry THF were added into the solution directly. The mixture was stirred for 24 h. After filtration of DCU and removal of THF, the residue was dissolved in 150 ml ethyl acetate followed by washing with saturated sodium bicarbonate solution and brine twice, respectively. The organic layer was collected and dried over anhydrous sodium sulfate overnight. Removal of the solvent followed by purification on column chromatography by ethyl acetate/methanol (50 : 1) solution gave a colorless oil (Boc-LYP2) 2.1 g with a yield of 56.7%. 0.48 g (1 mmol) Boc-LYP2 was dissolved in 10 ml saturated solution of hydrogen chloride in ethyl acetate. The solution was allowed to stand at 0°C overnight. 0.37 g white solid (LYP2) was obtained after removing the solvent under vacuum and recrystallization (ethyl acetate and ether) with a yield of 96%. Melting point = 140–142°C. MS-ESI: 190.5 (M/2 + 1), 379.6 (M + 1). ^{1}HNMR (D_2O): δ 0.72 (d, J = 4.2 Hz, 6H, CH $(CH_3)_2$); δ 1.44 (m, 2H, $CH_2CH(CH_3)_2$); δ 1.52 (m, 1H, $CH_2CH(CH_3)_2$); δ 2.70 (s, 6H, N $(CH_3)_2$); δ 2.75–2.96 (m, 2H, $NHCH_2$); δ 3.10 (t, 2H, $CH_2N(CH_3)_2$); δ 3.34–3.47 (m, 2H, $PhCH_2CH$); δ 3.67 (m, 1H, NHCHC = O); δ 4.12 (m, 1H, CH_2CHNH_2); δ 4.14 (m, 1H, OCH); δ 7.14–7.25 (m, 5H, Ph).

Docking result

The program SYBYL-FlexX was performed here. APN is a zinc-dependent metalloprotease. The zinc ion is located at the catalytic site. In the three-dimensional structure of APN, there are three hydrophobic pockets S1, S1′, and S2′. S1 and S1′ are small pockets, whereas S2′ is a bigger one. The benzene ring and isobutyl group of bestatin can occupy the S1 and S1′ pockets, respectively, leaving the S2′ pocket empty. However, in the structure of LYP2, the *N,N*-dimethyl ethanolamine fragment can effectively occupy the S2′ pocket. The docking result is showed in the Figs 2 and 3 below.

Water solubility

Bestatin has poor water solubility that limits its usage, whereas LYP2 is a double hydrochloride salt that has better water solubility. To determine and compare the water solubility of LYP2 and bestatin, we made standard lines of absorbance to the concentration of LYP2 and bestatin, respectively. The results are shown in Table 1 below.

From the table, we can see that the water solubility of LYP2 was nearly 13-fold more than that of bestatin, which made LYP2 suitable for a higher number of dosage forms.

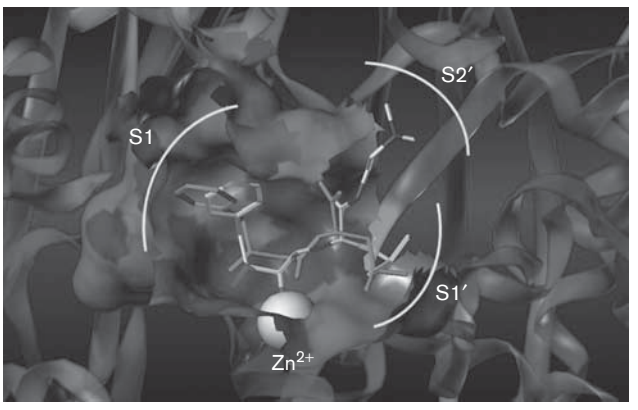
Enzyme inhibition assay

The IC_{50} (μmol/l) towards APN and MMP-2 is shown in Table 2.

Cell assay

APN is overexpressed in various tumor cells playing important roles in the angiogenesis and metastasis of the tumor. Accordingly, we used six tumor cell lines to test the inhibitory activity of LYP2 towards APN. From the results, LYP2 exhibited good inhibitive activity towards the six tumor cell lines we had selected. The IC_{50} of LYP2 is lower than that of bestatin. The results are shown in Table 3.

Fig. 2



The three-dimensional docking result between aminopeptidase N (APN) and LYP2. The grey compound is bestatin whereas the other one is LYP2.

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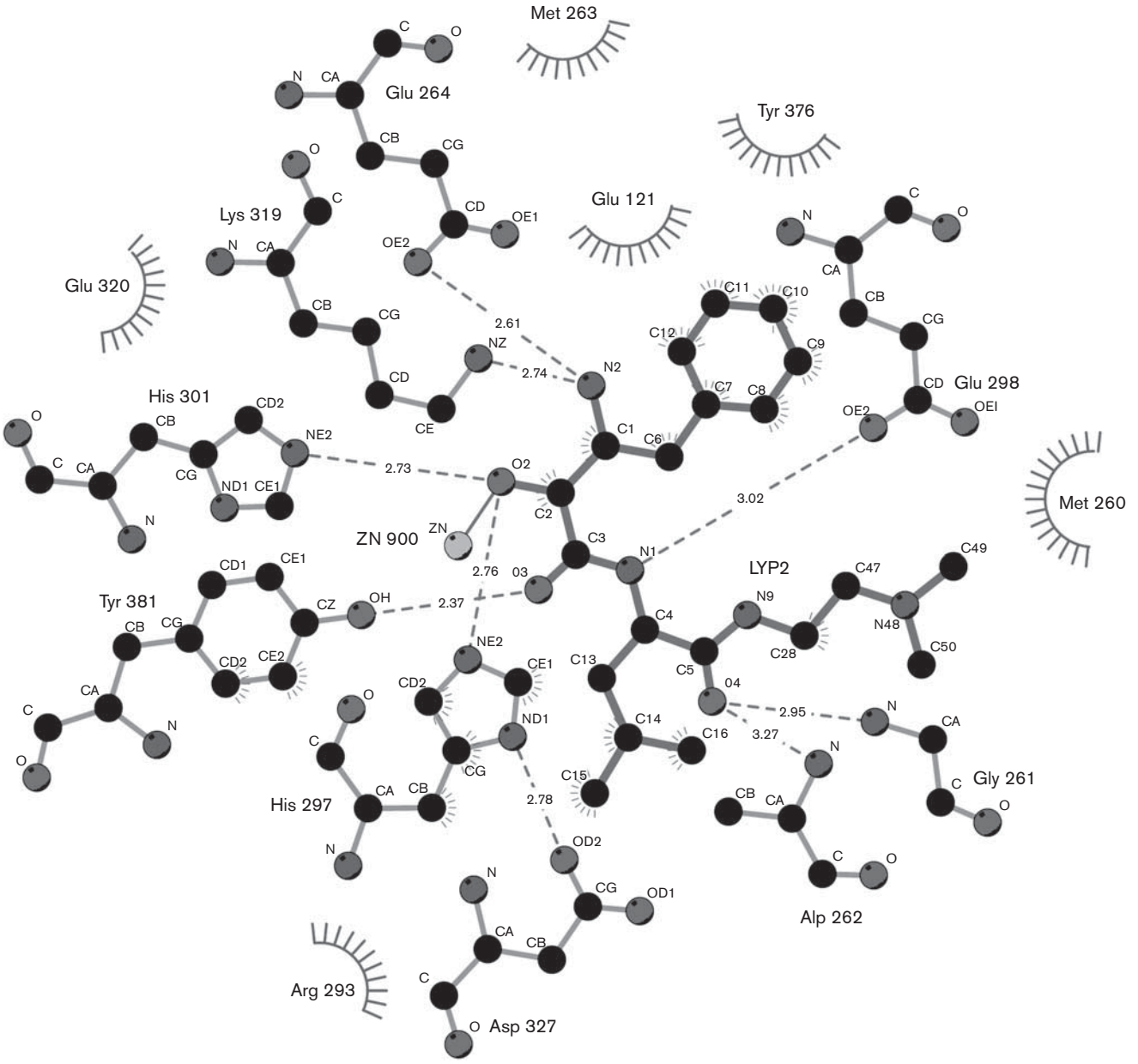
Fig. 3



The two-dimensional view of the binding model between LYP2 and aminopeptidase N (APN).

Table 1 The UV absorption characteristic and saturated solution concentration of LYP2 and bestatin in PBS

	LYP2	Bestatin
Function of the line	A=166.58c−0.0093	A=181.52c−0.005
r^2	0.9997	0.9999
Solubility in PBS	479.17±0.98 mmol/l	35.70±0.86 mmol/l

A, absorbance; c, concentration.
$P<0.01$, $n=3$.

Table 2 IC_{50} of LYP2 and bestatin

	LYP2	Bestatin
Molecular weight	451.43	308.37
IC_{50} (APN, μmol/l)	47.4±2.6	23.8±1.2
IC_{50} (MMP-2, μmol/l)	>1000	169.9±6.1

APN, aminopeptidase N.
$P<0.01$, $n=3$.

The result *in vitro* showed that LYP2 had a better IC_{50} as compared with bestatin towards the six tumor cell lines we had selected. From the data, we could also see that LYP2 had a better IC_{50} towards tumor cells that showed high expression of APN such as HL60, ES-2, A549 and PLC compared with those that showed low expression of

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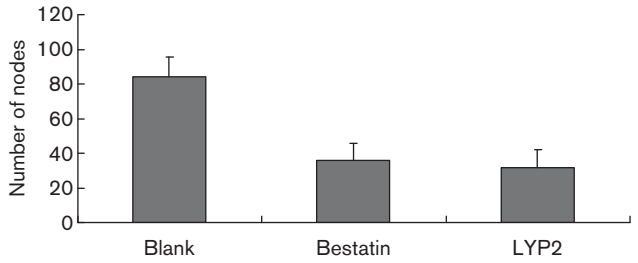
Table 3 IC_{50} **of LYP2 and bestatin (mmol/ml)**

	LYP2	Bestatin
HL60	0.11 ± 0.09	0.28 ± 0.11
ES-2	0.19 ± 0.06	0.87 ± 0.26
K562	1.42 ± 0.22	NA
A549	0.3 ± 0.08	NA
H7402	0.62 ± 0.12	NA
PLC	0.56 ± 0.16	NA

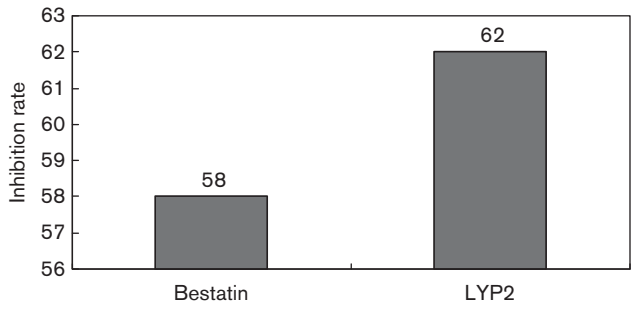
NA, no activity.
P=0.005, n=3.

Fig. 4



The graph shows the number of nodes in the lungs of the LYP2 and bestatin groups.

Fig. 5



The graph shows the inhibition rate of the LYP2 and bestatin groups.

APN such as K562 and H7402, which exhibited the selectivity of LYP2 towards APN.

Anti-metastasis assay *in vivo*

In this assay, the Kunming mice transplanted with H22 liver tumor cells were used. The inhibition of metastasis from the liver to the lungs by bestatin and LYP2 was tested. Bestatin and LYP2 were administered intragastrically. The concentration of bestatin and LYP2 was 2 mg/ml, respectively. The result was shown by the number of nodes in the lung and the inhibition rate as shown in Figs 4 and 5.

LYP2 had a little better anti-metastasis effect than that of bestatin without severe side effects towards important organs such as the liver, kidney, spleen, which were all in good condition after the assembly.

Discussion

In this study, we designed and synthesized a new derivative of bestatin for enhancing its stability. From the *in vitro* and *in vivo* results, the efficacy of LYP2 is better than that of bestatin in the parallel assay condition. Considering that LYP2 is a dihydrochloride salt, its good hydrophilicity can make it suitable for use in a higher number of dosage forms. All of these interesting results showed that LYP2 is a successful optimization of the structure of bestatin.

Acknowledgements

This study was supported by the National Nature Science Foundation of China (Grant No. 30772654 and 36072541), the PhD Programs Foundation of Ministry of Education of PR China (No. 20060422029), 863 National Program Foundation of Ministry of Technology of China (No. 2007AA02Z314), Nature Science Foundation of Important Research Project of China (Grant No. 90713041) and New Drug Research and Discovery Foundation of National Ministry of Technology of China (No. 2009ZX09103-118).

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