Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters
ELSEVIER
Bioorganic & Medicinal Chemistry Letters 14 (2004) 1527–1531

KMI-358 and KMI-370, highly potent and small-sized BACE1 inhibitors containing phenylnorstatine

Tooru Kimura,[a] Daisuke Shuto,[a] Soko Kasai,[a] Ping Liu,[a] Koushi Hidaka,[a] Takashi Hamada,[a] Yoshio Hayashi,[a] Chinatsu Hattori,[b] Masashi Asai,[b] Shinobu Kitazume,[c] Takaomi C. Saido,[d] Shoichi Ishiura[b] and Yoshiaki Kiso[a,*]

[a]*Department of Medicinal Chemistry, Center for Frontier Research in Medicinal Science, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607-8412, Japan*
[b]*Department of Life Sciences, Graduate School of Arts and Sciences, University of Tokyo, Meguro-ku, Tokyo 153-8902, Japan*
[c]*Glyco-chain Functions Laboratory, Supra-biomolecular System Group, Frontier Research System, RIKEN, Wako-shi, Saitama 351-0198, Japan*
[d]*Laboratory for Proteolytic Neuroscience, RIKEN Brain Science Institute, Wako-shi, Saitama 351–0198, Japan*

Received 1 December 2003; accepted 26 December 2003

Abstract—Recently, we reported a novel substrate-based octapeptide BACE1 inhibitor KMI-008 containing hydroxymethylcarbonyl (HMC) isostere as a transition-state mimic. Using KMI-008 as a lead compound, a small-sized and highly potent BACE1 inhibitor KMI-370 ($IC_{50}=3.4$ nM) was designed and synthesized.

1. Introduction

Cerebral deposition of amyloid fibrils as senile plaques is a pathological hallmark of Alzheimer's disease (AD).[1] The predominant constituents of amyloid fibrils are 40- and 42-residue amyloid β peptides (Aβ). Aβ is generated from proteolytic processing of a transmembrane protein, β-amyloid precursor protein (APP).[2,3] Two proteases, called β- and γ-secretases, cleave APP to generate the Aβ peptide. β-Secretase which forms the N-terminus of Aβ has been identified as a novel membrane-bound aspartic protease, BACE1 (memapsin 2 or Asp-2).[4–7] BACE1 plays a critical role in the progression of AD since the cleavage of APP by β-secretase is the first step in Aβ formation. Therefore, the development of BACE1 inhibitor is valuable for the elucidation of AD pathology. Several transition-state analogue BACE1 inhibitors modelled on the β-secretase cleavage site have been reported with relatively low IC_{50} values.[8–10] However, the molecular sizes of these inhibitors are too large to be viable drug candidates.

On the basis of knowledge of our HIV-1 protease inhibitor studies,[11,12] we recently reported an octapeptide BACE1 inhibitor **1** (KMI-008) containing an unnatural amino acid, phenylnorstatine [(2*R*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid: Pns], with a hydroxymethylcarbonyl (HMC) isostere as a transition-state mimic (Fig. 1).[13] KMI-008 showed potent inhibition against recombinant enzyme ($IC_{50}=413$ nM) and decreased the secretion of sAPPβ (a soluble form of APP generated by the action of β-secretase) from COS-7 cells co-transfected with both the APP and BACE1. However, the characteristics of KMI-008 were not suitable as a drug due to its large molecular weight, the presence of many natural peptide bonds, and no protection against exopeptidase. In this paper, we describe the design and synthesis of truncated BACE1 inhibitors which are more potent than KMI-008.

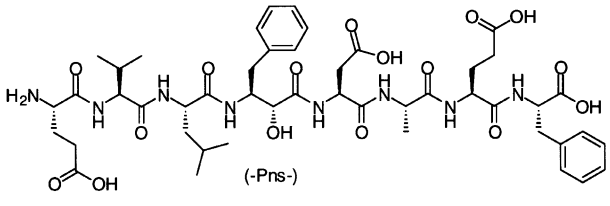
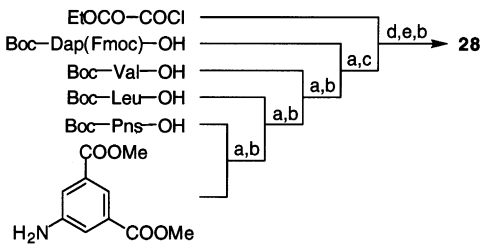


Figure 1. Structure of octapeptide BACE1 inhibitor, **1** (KMI-008).

* Corresponding author. Tel.: +81-75-595-4635; fax: +81-75-591-9900; e-mail: kiso@mb.kyoto-phu.ac.jp

0960-894X/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2003.12.088



Scheme 1. Reagents and conditions: (a) EDC·HCl, HOBt·H_2O/DMF; (b) 4 M HCl/dioxane; (c) 20% Et_2NH/DMF; (d) Et_3N/DMF; (e) 4 M KOH/DMF.

2. Synthesis

Compounds containing a C-terminal carboxylic acid were synthesized by the 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase peptide synthesis. For starting resins, *p*-benzyloxybenzyl alcohol resins or 2-chlorotrityl chloride resins coupled with C-terminal Fmoc-amino acid derivatives were employed. Each synthetic cycle consisted of the following steps: (1) deprotection with 20% piperidine in DMF, (2) a coupling with Fmoc-amino acid derivative using diisopropylcarbodiimide (DIPCDI) and 1-hydroxybenzotriazole (HOBt). In the case of the synthesis of **26** and **27**, the P4 ligand was incorporated by sequential coupling of N^{α}-Boc-N^{β}-Fmoc-L-2,3-diaminopropionic acid [Boc-Dap(Fmoc)-OH] and oxalic acid mono-*t*-butyl ester. After the peptide-chain elongation, the peptide was cleaved from the resin by the treatment with TFA in the presence of *m*-cresol and thioanisole as scavengers. The product was finally purified by preparative RP-HPLC and identified by MALDI-TOF MS. The inhibitory activity of the synthetic compounds against rh-BACE1 was examined as described previously.[13]

Compounds **16**, **21** and **28** were synthesized by traditional solution method. For example, the synthetic route to **28** is shown in Scheme 1. The 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl)–HOBt method was employed for each coupling and the N^{α}-Boc group was removed with 4M HCl/dioxane. The P4 ligand was incorporated by two steps of couplings with Boc-Dap(Fmoc)-OH and ethyl oxalyl chloride. After assembly of the peptide-chain, saponification and following 4M HCl/dioxane treatment gave the desired product.

3. Results and discussions

In order to determine the minimum structure having BACE1 inhibitory activity, peptide ladders were designed by the removal of amino acids from KMI-008 (**1**) (Table 1). As shown in Table 1, the compounds lacking P4-P2 positions (**2**–**4**) showed no inhibitory activities at 2 μM. However, the removal of C-terminal residues had mild influence on the activity (**5**–**8**). Especially, compound **7** had slightly weaker activity (61%) than KMI-008 (**1**). These results indicate that the interactions of N-terminal residues were very important while those of the C-terminus were not significant. They also suggested that the inhibitors consisting of P4–P1′ positions were the minimal requirement for the activity.

To confirm this finding, we attempted the Gly-scan of each amino acid consisting of octapeptide inhibitor **1** (Table 1). The compounds having a Gly-substitution at P4-P2 (**9**–**11**) showed no inhibition, but compounds **12**–**15** substituted at P1′–P4′ were active (54–73%). It is clear that the side chains of P4–P2 residues have significant interactions with S4–S2 subsites of BACE1. This is consistent with the previous observation that the side chain of P3′–P4′residues are not involved in any specific interaction in S3′–S4′ subsites.[14]

Next, we attempted to optimize the structures of each position of the inhibitors consisting of P4–P1′. The studies of structure–activity relationship (SAR) focused on P2 or P3 have already been performed on the development of KMI-008 (**1**), and leucine at P2 and valine at P3 were the best among the tested compounds.[13] Hence, SAR at P1′ and P4, corresponding to both the N- and C-terminals, was carried out to increase the potency and stability of the inhibitors under physiological conditions.

Table 1. Ladders of BACE 1 inhibitor and Gly-scan

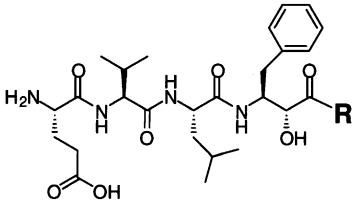
Compd (KMI No)		P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'		BACE1 inhibition (%)[a]
1 (−008)	H–	Glu	–Val	–Leu	–Pns	–Asp	–Ala	–Glu	–Phe	–OH	>90
2 (−070)		H	–Val	–Leu	–Pns	–Asp	–Ala	–Glu	–Phe	–OH	<20
3 (−196)			H	–Leu	–Pns	–Asp	–Ala	–Glu	–Phe	–OH	<20
4 (−251)				H	–Pns	–Asp	–Ala	–Glu	–Phe	–OH	<20
5 (−069)	H–	Glu	–Val	–Leu	–Pns	–Asp	–Ala	–Glu	–OH		60
6 (−098)	H–	Glu	–Val	–Leu	–Pns	–Asp	–Ala	–OH			46
7 (−122)	H–	Glu	–Val	–Leu	–Pns	–Asp	–OH				61
8 (−204)	H–	Glu	–Val	–Leu	–Pns	–OH					34
9 (−193)	H–	Gly	–Val	–Leu	–Pns	–Asp	–Ala	–Glu	–Phe	–OH	<20
10 (−194)	H–	Glu	–**Gly**	–Leu	–Pns	–Asp	–Ala	–Glu	–Phe	–OH	<20
11 (−195)	H–	Glu	–Val	–**Gly**	–Pns	–Asp	–Ala	–Glu	–Phe	–OH	<20
12 (−249)	H–	Glu	–Val	–Leu	–Pns	–**Gly**	–Ala	–Glu	–Phe	–OH	54
13 (−250)	H–	Glu	–Val	–Leu	–Pns	–Asp	–**Gly**	–Glu	–Phe	–OH	73
14 (−252)	H–	Glu	–Val	–Leu	–Pns	–Asp	–Ala	–**Gly**	–Phe	–OH	56
15 (−253)	H–	Glu	–Val	–Leu	–Pns	–Asp	–Ala	–Glu	–**Gly**	–OH	65

[a] At 2 μM.

To study the SAR at the P1′ position, we selected compound **7** as a lead compound and prepared compounds containing different moieties at P1′ (Table 2). As shown in Table 2, compound **16** exhibited weak activity while compounds **17**–**20**, containing benzoic acid analogues, were moderately active (52–82% inhibition at 2 μM). These data revealed that the carboxyl group at the P1′ position gave a suitable interaction with the S1′ pocket of BACE1. Interestingly, the phenylamide analogues **19** and **20** were more potent than the benzylamide analogues **17** and **18**. Thus, the rigid conformation of the phenyl ring at the P1′ position may be preferred for binding to the enzyme. Furthermore, compound **21** bearing two carboxyl groups at the P1′ position exhibited more potent inhibitory activity (IC_{50} = 55 nM) than KMI-008 (**1**). It was supposed that the remarkable increase was caused by the interaction of the P1′ moiety with both active site and flap region of BACE1.

The SAR study focused on P4 was carried out using heptapeptide inhibitor **5** as a lead compound, which had moderate potency (Table 3). The inhibitory activity of **5**, **22** and **23** indicated that the amino and carboxyl groups at the P4 position significantly affected the BACE1 binding. In addition, the length of the side chain at the P4 position also has an important effect on the activity (**5** versus **24** and **25**). The aspartic acid analogue **24** showed no activity against BACE1, but homoglutamic acid analogue **25** had slightly decreased inhibitory activity. Therefore, we designed analogue **26**, which has two carbonyl groups at the side chain corresponding to the glutamic acid and homoglutamic acid. Surprisingly,

Table 2. BACE1 inhibitory activity (P1′ position)



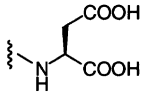
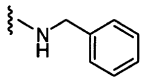
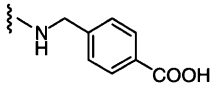
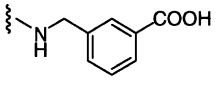
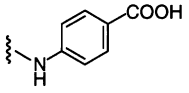
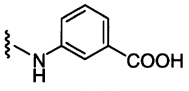
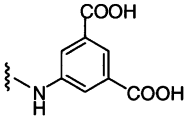
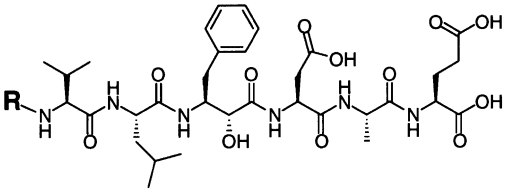
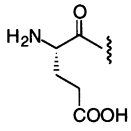
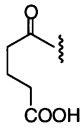
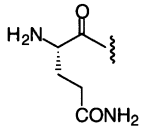
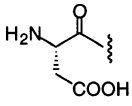
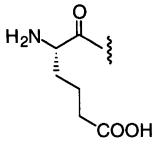
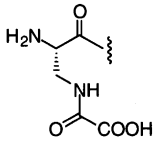
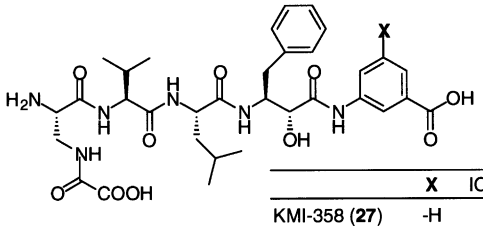
Compd (KMI No)	R	BACE1 inhibition (%) at 2 μM
7 (–122)		61
16 (–205)		30
17 (–158)		52
18 (–293)		64
19 (–287)		79
20 (–260)		82
21 (–360)		>90 (IC_{50} = 55 nM)

Table 3. BACE1 inhibitory activity (P4 position)



Compd (KMI No)	R	BACE1 inhibition (%) at 2 μM
5 (–069)		60
22 (–103)		<20
23 (–124)		<20
24 (–262)		<20
25 (–322)		43
26 (–300)		>90 (IC_{50} = 84 nM)



	X	IC_{50} (nM)
KMI-358 (**27**)	-H	16
KMI-370 (**28**)	-COOH	3.4

Figure 2. Structures and BACE1 inhibitory activities of KMI-358 (**27**) and KMI-370 (**28**).

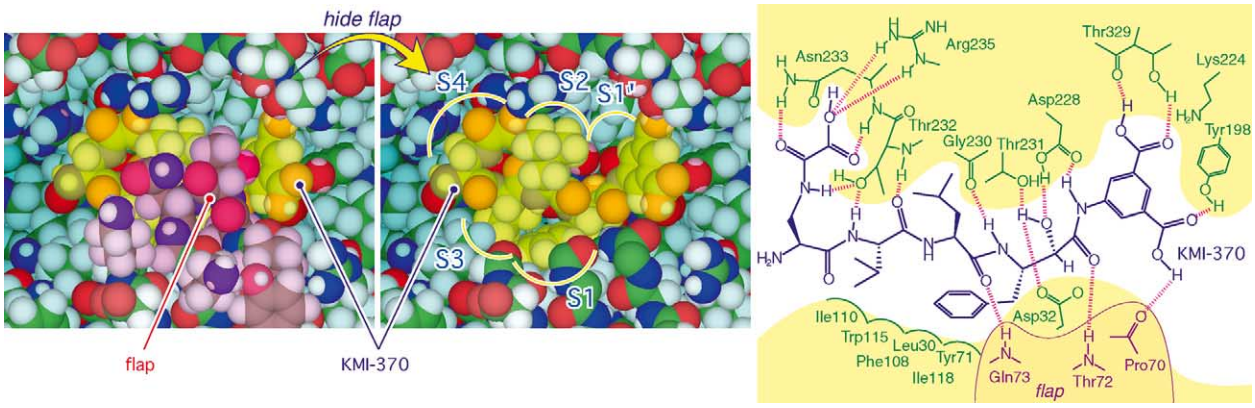


Figure 3. Modelled structure of KMI-370 bound to BACE1 and possible hydrogen bonding pattern. The model was constructed using the crystal structure of BACE1 bound to OM99-2.[14]

compound **26**, which contains N^{β}-oxalyl-L-2,3-diaminopropionic acid, resulted in a further 2–3-fold increase in potency ($IC_{50} = 84$ nM). This result may be due to the increase of affinity for the hydrophilic S4 subsite of enzyme by the two carbonyl groups.

From the results of the above SAR studies, we designed pentapeptide BACE1 inhibitors **27** (KMI-358) and **28** (KMI-370) consisting of P4–P1′ moieties (Fig. 2). They had N^{β}-oxalyl-L-2,3-diaminopropionic acid at P4 and 3-aminobenzoic acid or 5-aminoisophthalic acid at the P1′ position. Of the tested compounds, KMI-370 (**28**) was the most potent inhibitor against BACE1 with an IC_{50} value of 3.4 nM. In our assay, the IC_{50} value of KMI-370 is approximately 20- and 10-fold smaller than those of P_{10}–$P_{4'}$statV[10] ($IC_{50} = 65$ nM) and OM99-2[9] ($IC_{50} = 28$ nM), respectively. The modelled structure of KMI-370 bound to BACE1 (Fig. 3) was prepared from 1FKN (pdb data).[14] KMI-370 could fill the active site-pockets of BACE1 tightly. The carboxylic acids at the P4 and P1′ positions could provide several hydrogen bonds which contribute to the binding affinity. Through our SAR study, we found that the pentapeptide inhibitor KMI-370, which had a relatively low molecular weight (Mw = 728), was a very potent inhibitor. Since both terminals of KNI-370 consist of unnatural amino acids, it was expected that KMI-370 would be more stable to the decomposition by exopeptidase in vivo than KMI-008 with natural amino acids.

Finally, we examined the effects of KMI-358/-370 on β-secretase activity in HEK293 cells transfected with BACE1 (BACE1-HEK293), which are stably expressed both full length-BACE1 and native APP.[15] The secretion of sAPPβ in the culture medium was detected using anti-sAPPβ antibody (Fig. 4). KMI-358 and KMI-370 dose-dependently inhibited the secretion of sAPPβ from BACE1-HEK293 cells. The IC_{50} values of KMI-358 and KMI-370 were 0.65 and 0.20 μM, respectively. These results demonstrate the potential of our inhibitors containing HMC isostere as drugs to attenuate Aβ-formation in vivo.

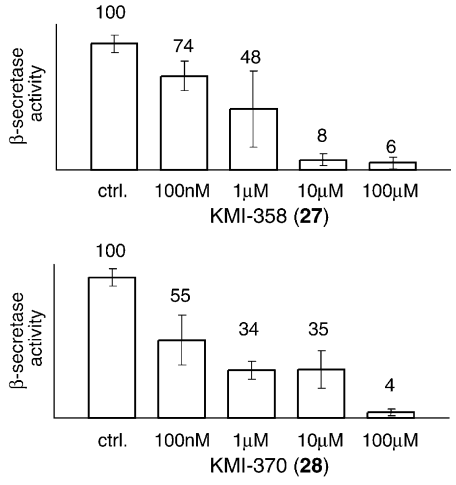


Figure 4. Effects of KMI-358/-370 on β-secretase activity in BACE1-transfected HEK293 cells ($n = 3$).

4. Conclusion

In conclusion, we have established that a pentapeptide containing phenylnorstatine is the minimal molecular size for BACE1 inhibition. Moreover, we have found a highly potent inhibitor, KMI-370, with an IC_{50} value of 3.4 nM, which can reduce the secretion of sAPPβ from BACE1-HEK293 cells dose-dependently.

Acknowledgements

This research was supported in part by the Frontier Research Program of the Ministry of Education, Science and Culture of Japan, and grants from the Ministry of Education, Science and Culture of Japan. The authors thank Dr. Y. Hashimoto (RIKEN) and Ms. S. Shibakawa for their help in the inhibition assay.

References and notes

1. Selkoe, D. J. *Neuron* **1991**, *6*, 487.
2. Selkoe, D. J. *Nature* **1999**, *399*, A23.
3. Sinha, S.; Lieberburg, I. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 11049.
4. Vassar, R.; Bennett, B. D.; Babu-Khan, S.; Kahn, S.; Mendiaz, E. A.; Denis, P.; Teplow, D. B.; Ross, S.;

Amarante, P.; Loeloff, R.; Luo, Y.; Fisher, S.; Fuller, J.; Edenson, S.; Lile, J.; Jarosinski, M. A.; Biere, A. L.; Curran, E.; Burgess, T.; Louis, J. C.; Collins, F.; Treanor, J.; Rogers, G.; Citron, M. *Science* **1999**, *286*, 735.

5. Yan, R.; Bienkowski, M. J.; Shuck, M. E.; Miao, H.; Tory, M. C.; Pauley, A. M.; Brashier, J. R.; Stratman, N. C.; Mathews, W. R.; Buhl, A. E.; Carter, D. B.; Tomasselli, A. G.; Parodi, L. A.; Heinrikson, R. L.; Gurney, M. E. *Nature* **1999**, *402*, 533.
6. Sinha, S.; Anderson, J. P.; Barbour, R.; Basi, G. S.; Caccavello, R.; Davis, D.; Doan, M.; Dovey, H. F.; Frigon, N.; Hong, J.; Jacobson-Croak, K.; Jewett, N.; Keim, P.; Knops, J.; Lieberburg, I.; Power, M.; Tan, H.; Tatsuno, G.; Tung, J.; Schenk, D.; Seubert, P.; Suomensaari, S. M.; Wang, S.; Walker, D.; Zhao, J.; McConlogue, L.; John, V. *Nature* **1999**, *402*, 537.
7. Hussain, I.; Powell, D.; Howlett, D. R.; Tew, D. G.; Meek, T. D.; Chapman, C.; Gloger, I. S.; Murphy, K. E.; Southan, C. D.; Ryan, D. M.; Smith, T. S.; Simmons, D. L.; Walsh, F. S.; Dingwall, C.; Christie, G. *Mol. Cell. Neurosci.* **1999**, *14*, 419.
8. (a) Ghosh, A. K.; Shin, D.; Downs, D.; Koelsch, G.; Lin, X.; Ermolieff, J.; Tang, J. *J. Am. Chem. Soc.* **2000**, *122*, 3522. (b) Ghosh, A. K.; Bilcer, G.; Harwood, C.; Kawahara, R.; Shin, D.; Hussain, K. A.; Hong, L.; Loy, J. A.; Nguyen, C.; Koelsch, G.; Ermolieff, J.; Tang, J. *J. Med. Chem.* **2001**, *44*, 2865.
9. Tung, J. S.; Davis, D. L.; Anderson, J. P.; Walker, D. E.; Mamo, S.; Jewett, N.; Hom, R. K.; Sinha, S.; Thorsett, E. D.; John, V. *J. Med. Chem.* **2002**, *45*, 259.
10. Tamamura, H.; Kato, T.; Otaka, A.; Fujii, N. *Org. Biomol. Chem.* **2003**, *1*, 2468.
11. Mimoto, T.; Imai, J.; Tanaka, S.; Hattori, N.; Takahashi, O.; Kisanuki, S.; Nagano, Y.; Shintani, M.; Hayashi, H.; Akaji, K.; Kiso, Y. *Chem. Pharm. Bull.* **1991**, *39*, 2465.
12. Mimoto, T.; Imai, J.; Tanaka, S.; Hattori, N.; Kisanuki, S.; Akaji, K.; Kiso, Y. *Chem. Pharm. Bull.* **1991**, *39*, 3088.
13. Shuto, D.; Kasai, S.; Kimura, T.; Liu, P.; Hidaka, K.; Hamada, T.; Shibakawa, S.; Hayashi, Y.; Hattori, C.; Szabo, B.; Ishiura, S.; Kiso, Y. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 4273.
14. Hong, L.; Koelsch, G.; Lin, X.; Wu, S.; Terzyan, S.; Ghosh, A. K.; Zhang, X. C.; Tang, J. *Science* **2000**, *290*, 150.
15. BACE1-HEK293 cells (80–100% confluent) were replaced with new serum-free medium (DMEM containing 1 mg/mL G418) with 100 μM/10 μM/1 μM/100 nM of KMI-358 or KMI-370, followed by cell incubation for 6 h. The sAPPβ in the culture was concentrated, ran on 7.5% SDS-PAGE and western blotted. The blots were proved with anti-sAPPβ antibody.