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Original Paper

Neurodegenerative Diseases

Neurodegenerative Dis 2009;6:230–239
DOI: 10.1159/000236902

Received: October 10, 2008
Accepted after revision: June 8, 2009
Published online: September 9, 2009

Destruxin E Decreases Beta-Amyloid Generation by Reducing Colocalization of Beta-Amyloid-Cleaving Enzyme 1 and Beta-Amyloid Protein Precursor

Naohiro Itoh[a, b] Masayasu Okochi[a] Shinji Tagami[a] Kouhei Nishitomi[a, b] Taisuke Nakayama[a] Kanta Yanagida[a] Akio Fukumori[a] Jingwei Jiang[a] Kohji Mori[a] Motoko Hosono[b] Jyunko Kikuchi[c] Yuko Nakano[c] Yoshihiko Takinami[d] Keiji Dohi[e] Atsuko Nishigaki[e] Hiroshi Takemoto[e] Kazuyuki Minagawa[f] Takaaki Katoh[f] Michael Willem[h] Christian Haass[h] Takashi Morihara[a] Toshihisa Tanaka[a] Takashi Kudo[a] Hiroshi Hasegawa[g] Masaki Nishimura[g] Gaku Sakaguchi[b] Akira Kato[b] Masatoshi Takeda[a]

[a]Psychiatry, Department of Integrated Medicine, Division of Internal Medicine, Osaka University Graduate School of Medicine, and [b]Pain and Neurology, [c]Analytical Chemistry, [d]Discovery Technology 1, [e]Discovery Technology 2 and [f]Discovery Chemistry, Shionogi & Co. Ltd., Osaka, and [g]Neurology Unit, Molecular Neuroscience Research Center, Shiga University of Medical Science, Otsu, Japan; [h]Laboratory for Alzheimer's and Parkinson's Disease Research, Department of Biochemistry, Adolf Butenandt Institute, Ludwig Maximilian University, Munich, Germany

Key Words
Alzheimer disease · Amyloid β peptides · β-Amyloid-cleaving enzyme · β-Amyloid protein precursor · Destruxin E · Notch signaling · Presenilin/γ-secretase

Abstract
Alzheimer-disease-associated β-amyloid (Aβ) is produced by sequential endoproteolysis of β-amyloid protein precursor (βAPP): the extracellular portion is shed by cleavage in the juxtamembrane region by β-amyloid-cleaving enzyme (BACE)/β-secretase, after which it is cleaved by presenilin (PS)/γ-secretase near the middle of the transmembrane domain. Thus, inhibition of either of the secretases reduces Aβ generation and is a fundamental strategy for the development of drugs to prevent Alzheimer disease. However, it is not clear how small compounds reduce Aβ production without inhibition of the secretases. Such compounds are expected to avoid some of the side effects of secretase inhibitors. Here, we report that destruxin E (Dx-E), a natural cyclic hexadepsipeptide, reduces Aβ generation without affecting BACE or PS/γ-secretase activity. In agreement with this, Dx-E did not inhibit Notch signaling. We found that Dx-E decreases colocalization of BACE1 and βAPP, which reduces β-cleavage of βAPP. Therefore, the data demonstrate that Dx-E represents a novel Aβ-reducing process which could have fewer side effects than secretase inhibitors.

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Inhibition of β-amyloid (Aβ) generation and enhancement of its degradation are proposed as major strategies for the treatment of Alzheimer disease [1, 2]. A number of compounds have been developed to inhibit β-secretase [i.e. β-amyloid-cleaving enzyme (BACE)]

N.I., M.O. and S.T. contributed equally to this work.

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1660–2854/09/0066–0230$26.00/0

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Masayasu Okochi, MD
Psychiatry, Department of Integrated Medicine, Division of Internal Medicine
Osaka University Graduate School of Medicine, D3, Yamada-oka 2-2, Suita
Osaka 565-0871 (Japan)
Tel. +81 6 6879 3053, Fax +81 6 6879 3059, E-Mail mokochi@psy.med.osaka-u.ac.jp

and γ-secretase [i.e. a complex including presenilin (PS), nicastrin, Aph1 and Pen2] enzymes that are involved in the generation of Aβ. Because these compounds are expected to inhibit the physiological degradation of other secretase substrates, they may have side effects [3–5]. In fact, PS1 knockout mice die perinatally and show the Notch phenotype due to attenuation of NICD (Notch intracellular cytoplasmic domain) generation from Notch receptors [6, 7]. BACE1 knockout mice also show the peripheral nerve hypomyelination phenotype caused by ablation of neuregulin 1 cleavage [5]. It is unclear whether targeting secretases is necessary for inhibiting Aβ generation.

Here, we performed high-throughput screening for inhibitors of Aβ generation and identified destruxin E (Dx-E), a natural compound derived from an entomopathogenic fungus. Interestingly, Dx-E reduced β-cleavage of β-amyloid protein precursor (βAPP) but did not inhibit the secretases. The existence of such a compound suggests that there is a novel mechanism for inhibiting Aβ generation.

Materials and Methods

Antibodies and Reagents

Anti-Aβ antibodies 6E10 and 4G8 were from Signet Laboratories (Dedham, Mass., USA). The anti-sAPPβ Swedish (sw) antibody was from IBL (Takasaki, Japan). The anti-BACE1 antibody was from Chemicon (Temecula, Calif., USA). The anti-Na^+-K^+-ATPase was from Upstate Biotechnology. The anti-calnexin antibody was from Stressgen. The anti-early-endosome-antigen (EEA) and anti-GM130 antibodies were from BD Transduction Laboratories. The γ-secretase inhibitors DAPT and L-685,458 and the BACE inhibitor N-(1S,2R)-1-benzyl-3-(cyclopropylamino)-2-hydroxypropyl-5-[methyl(methylsulfonyl)amino-N′-(1R)-1-phenylethyl]isophthalamide (inhibitor IV) were purchased from Calbiochem (San Diego, Calif., USA). The γ-secretase substrate NMA-GGVVIATVK(DNP)-DRDRDR-NH_2 was purchased from Calbiochem, and the β-secretase substrate MOCAc-SEVNLDAEFRK(Dnp)-RR-NH_2 was from Peptide Institute (Osaka, Japan). Dx-E was purified by Shionogi and Co. Ltd. In the measurement of Aβ40 and Aβ42 at a 384-well plate format, the addition of compounds and seeding of βAPP-overexpressing cells were simultaneously performed and incubated for 24 h. In other experiments, compounds were mixed in culture medium and layered onto adherent cells.

Cell Culture

K293 and SH-SY5Y cells stably expressing βAPP/PS1/Notch derivatives were maintained in DMEM containing 10% FCS and antibiotics (200 μg/ml G418 for βAPP selection, 200 μg/ml zeocin for PS1 selection and 100 μg/ml hygromycin for Notch1 selection).

Measurement of Aβ Secretion

For high-throughput screening, Aβ40 secretion was measured with a homogeneous time-resolved fluorescence kit (Nihon Schering, Osaka, Japan) as described in the manufacturer's instructions. Briefly, in a typical 384-well plate assay, 55 ng/ml antibody europium cryptate, 400 ng/ml antibody XL665 and 50 mM phosphate buffer (pH 7.4) containing 0.2% BSA and 0.5 M FK were added into each well. Samples of conditioned cell culture medium or synthetic peptide standards were added to give a total assay volume of 20 μl/well. The reaction mixture was left at 4°C to allow equilibrium binding and then read on an ALVO multilabel counter (Wallac Oy and Perkin Elmer Inc., Turku, Finland). For measurement of Aβ40 and Aβ42 in a 96-well plate format, an Aβ sandwich enzyme-linked immunosorbent assay (Wako, Japan) was used. The cells were incubated for 16 h with compounds. Aβ levels were normalized by cell viability as measured using a WST system (Nacalai Tesque, Japan).

Pulse-Chase Experiments

Pulse-chase experiments were carried out as previously described [8].

Notch Downstream Assays

Notch signal reporter assays were carried out as previously described [9].

In vitro β-Secretase Assay

The peptide substrate for β-secretase (biotin-X-SEVNLDAEFRHDSGC) was labeled with europium cryptate (CIS Bio, Marcoule, France). Recombinant ectodomain human BACE1 (R & D Systems) in reaction buffer (50 mM sodium acetate, pH 5.0, and 0.008% Triton X-100) with various concentrations of inhibitors were mixed with the substrate (18 nM) in 96-well plates and then incubated for 3 h at 30°C. Streptavidin-XL665 (0.4 μg/well) was added, and the reaction was incubated for 1 h at 30°C. The homogeneous time-resolved fluorescence value [(ratio of fluorescence at 665 nm to that at 620 nm) × 10,000] in each well was measured using an ALVO multilabel counter.

In vitro γ-Secretase Assay

Cell-free in vitro γ-secretase assays were carried out as previously described [10, 11].

Cell-Free β-Secretase Assay

Following starvation in methionine- and serum-free minimal essential medium for 40 min, the K293 cells stably expressing sw βAPP were metabolically labeled with 450 μCi of [^{35}S]methionine/cysteine (Redivue Promix; Amersham) for 20 min in methionine- and serum-free minimal essential medium. The crude membrane fraction was extracted, and the cell-free assay was performed as previously described with a minor change [12]. Briefly, samples of the crude membrane fraction were preincubated with each compound for 1 h and then incubated for 40 min at 37°C (cell-free incubation). The reaction was solubilized by the addition of 10× radioimmunoprecipitation assay buffer (1% sodium dodecyl sulfate, 5% deoxycholate, 10% Triton-X 100) and diluted with phosphate-buffered saline containing protease inhibitor cocktail. The βAPP C-terminal fragments (CTF) produced during the cell-free reaction were immunoprecipitated with antiserum 6618 [12]. Immunoprecipitates were separated on

10–20% Tris-tricine gels (Invitrogen, Carlsbad, Calif., USA) and analyzed by autoradiography.

Immunocytochemistry

K293 cells stably expressing sw βAPP and wild-type (wt) PS1 were cultured on poly-L-lysine-coated coverslips. Cells were treated with the reagents at indicated concentrations for 16 h. Cells were fixed in 4% paraformaldehyde, permeabilized by 0.1% Triton-X 100 and blocked with 1% defatted milk, in phosphate-buffered saline. The following primary antibodies were used for immunostaining: Monoclonal antibodies for BACE1 and βAPP (22C11) were from Chemicon. Polyclonal antibody for N-terminal (46–65) BACE1 was from Calbiochem. Polyclonal antibody 6618 was raised against the C terminus of βAPP [12]. Secondary antibodies used were labeled with Alexa 488 or 594 (Invitrogen).

Subcellular Fractionation

Subcellular fractionation was carried out as previously described [12].

Results

Dx-E Inhibits Aβ Generation

A search for inhibitors of Aβ generation in a library of more than approximately 50,000 compounds by high-throughput screening identified Dx-E, a cyclodepepsipeptide (fig. 1A). To confirm the ability of Dx-E to inhibit Aβ generation, we labeled SH-SY5Y cells stably expressing wt βAPP by a 30-min pulse with [^{35}S]methionine followed by a 2-hour chase. As shown in figure 1B, Dx-E (1 μM) decreased the level of secreted Aβ/p3 species (IC_{50} approx. 230 nM; fig. 1B, upper panel), although it did not significantly change the rate of βAPP holoprotein degradation (fig. 1B, middle and lower panels). We also examined whether Dx-E affects the rate of Aβ degradation in cell culture. Cells were incubated in conditioned medium containing radiolabeled Aβ and in the absence or presence of Dx-E. As shown in figure 1C, the rate of Aβ degradation was unchanged by Dx-E treatment. Thus, it appears that Dx-E inhibits Aβ/p3 generation in cell culture.

Dx-E Does Not Affect γ-Secretase Cleavage of βAPP

Aβ generation requires two independent proteolytic steps, namely β- and γ-cleavages. We first evaluated the possibility that Dx-E reduces γ-secretase activity. We performed an in vitro γ-secretase assay [10] using an intramolecularly quenched fluorogenic peptide probe, NMA-GGVVIATVK(DNP)-DRDRDR-NH_2. This probe was mixed with the 1% CHAPSO-soluble membrane fraction of cultured cells (containing active PS/γ-secretase complex) and incubated for 16 h with Dx-E or L685,458, a specific γ-secretase inhibitor. As shown in figure 2A, Dx-E (0.003–10 μM) did not affect γ-secretase activity in vitro, although L-685,458 inhibited the activity in a dose-dependent manner. Next, we analyzed whether Dx-E inhibits γ-cleavage of βAPP in living cells (fig. 2B). For the experiments, we used K293 cells stably expressing the CTF of βAPP (CTF100), a constitutive substrate for PS/γ-secretase. In contrast to the case of full-length βAPP, Dx-E did not inhibit Aβ generation from CTF100 (fig. 2B). Collectively, these results show that the reduction of Aβ generation by Dx-E is not due to inhibition of γ-secretase/cleavage.

Modulators of γ-secretase, such as a subset of nonsteroidal anti-inflammatory drugs, can inhibit Aβ generation at relatively higher concentrations than those causing changes in the relative ratio of Aβ42 generation [13]. We therefore investigated whether Dx-E is a γ-secretase modulator. Pulse-chase experiments revealed that Dx-E does not change the relative ratio of Aβ42 species at lower concentrations, indicating that it is not a γ-secretase modulator.

Dx-E Does Not Affect Notch1 Degradation or Notch Signaling

The major side effects of γ-secretase-inhibiting compounds are due to inhibition of Notch signaling. If Dx-E does not affect the activity of PS/γ-secretase, it should have no influence on Notch signaling. To confirm this, we examined whether Dx-E affects Notch signaling in cell culture (fig. 3A). We used cells stably expressing the N1CS construct, a derivative of the Notch1 receptor [14]. The N1CS polypeptide constitutively undergoes sequential endoproteolysis in the absence of ligand binding [14]. The extent of Notch signaling was determined using a luciferase reporter assay in cells expressing *HES-Y* [9]. As shown in figure 3A, Dx-E treatment did not affect the rate of Notch signal transmission, although it dose-dependently reduced Aβ production. Also, as expected, DAPT, a specific γ-secretase inhibitor, reduced both Aβ generation and Notch signaling (fig. 3B). Therefore, Dx-E does not perturb Notch signaling even though it inhibits Aβ/p3 generation.

The sequential endoproteolysis of Notch1 consists of S2/S3/S4 cleavages. NICD is generated by the S3 cleavage. Because Dx-E has distinct effects on the generation of Aβ and NICD, we examined whether its effects are substrate specific. Thus, we analyzed secretion of S2/S4 cleavage-derived Nβ, also known as Notch1 Aβ-like peptides [8, 15] (fig. 3C). In contrast to specific γ-secretase inhibitors, Dx-E treatment did not inhibit Nβ secretion from N1CS-

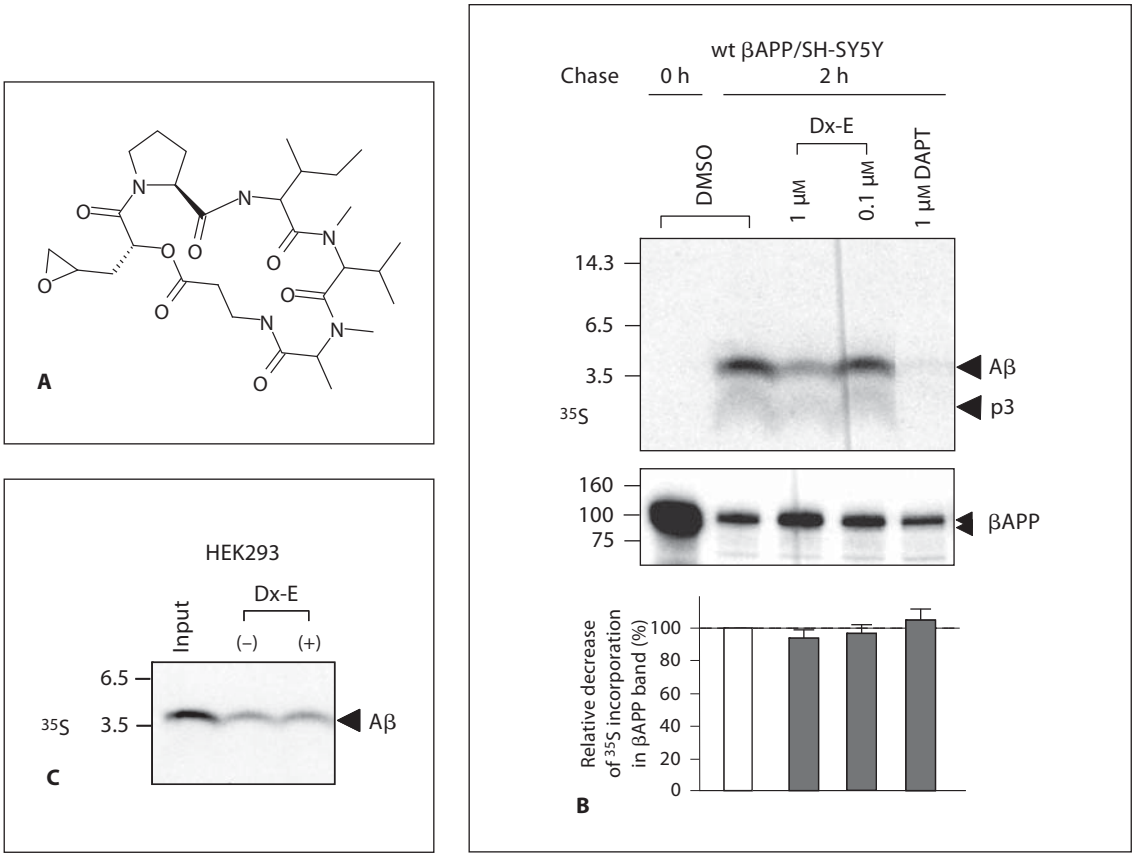


Fig. 1. Effects of Dx-E on Aβ production and degradation. **A** Structure of Dx-E. **B** Effect of Dx-E on βAPP metabolism. Pulse-chase analysis of βAPP metabolites was performed in SH-SY5Y cells stably expressing wt βAPP. Cells were labeled with a 30-min pulse of [^{35}S]methionine followed by a 2-hour chase. The conditioned media were collected and immunoprecipitated with antibody 4G8 (Aβ; upper panel). The βAPP holoprotein was immunoprecipitated from cell lysate using antiserum 6618 [12] (middle panel). Immunoprecipitates were separated by SDS-PAGE and analyzed by autoradiography, and the relative decrease in ^{35}S incorporation in βAPP holoprotein was analyzed by fluorography using a STORM 820 (Amersham). The ratio of βAPP holoprotein degradation during the 2-hour chase period in untreated cells was defined as 100%. **C** Effect of Dx-E on degradation of Aβ in cell culture. K293 cells stably expressing sw βAPP were metabolically labeled with a 30-min pulse of [^{35}S]methionine followed by a 2-hour chase. The medium containing labeled Aβ was transferred to unlabeled cells. The cells were then cultured in the presence or absence of Dx-E for 3 h. The labeled conditioned media were collected and immunoprecipitated with 4G8.

expressing cells (fig. 3C). Therefore, it appears that Dx-E inhibits sequential endoproteolysis of βAPP but not of Notch1.

Dx-E Does Not Affect β-Cleavage of βAPP in vitro

Dx-E reduces Aβ generation without affecting γ-cleavage of βAPP or Notch signaling/cleavage. Therefore, we examined whether Dx-E affects β-cleavage of βAPP by BACE. We examined β-secretase activity in vitro using an intramolecularly quenched fluorogenic peptide probe, MOCAc-SEVNLDAEFRK(Dnp)-RR-NH_2, and recombinant BACE1. Dx-E did not affect BACE1 activity at any concentration of Dx-E tested (0.6 nM to 10 μM), although the specific BACE inhibitor, inhibitor IV (IC_{50} approx. 20 nM), affected the activity (fig. 4A). We also used a newly established cell-free β-secretase assay that can detect de novo generation of CTFβ from βAPP holoprotein (fig. 4B). After a 20-min pulse with [^{35}S]methionine, we extracted the crude membrane fraction from cultured cells and incubated it for 40 min in vitro. Notably, addition of Dx-E to the membrane fraction did not decrease the generation of CTFβ, although inhibitor IV decreased the level of CTFβ by approximately 70% ($p < 0.001$ (t test); fig. 4B, lower panel). These results indicate that Dx-E does not affect BACE activity.

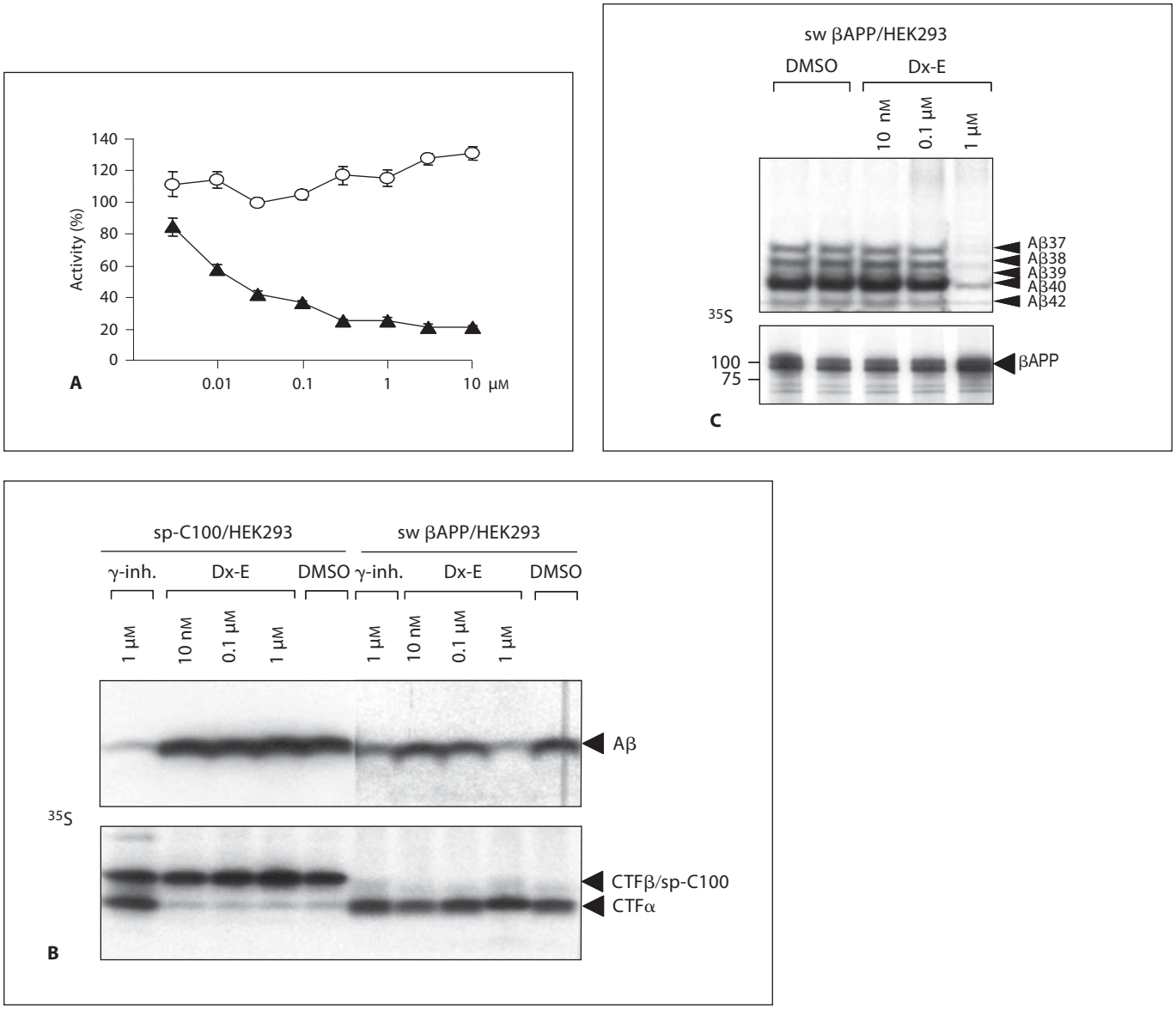
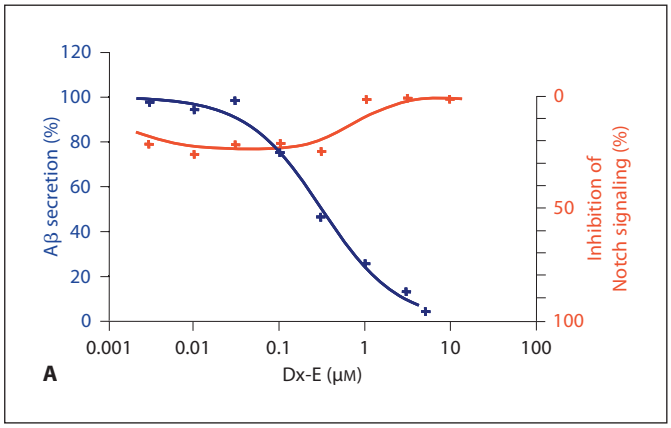
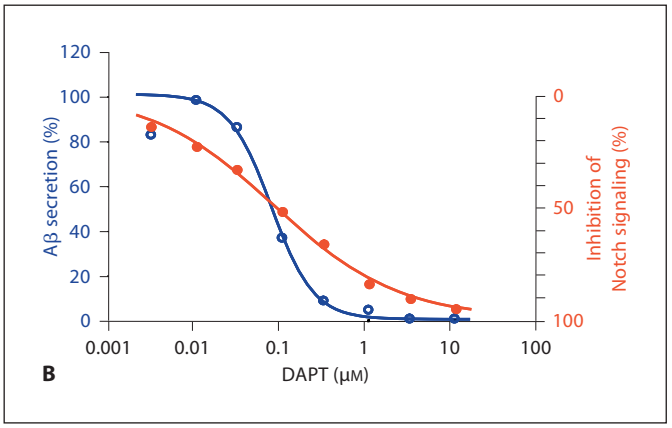


Fig. 2. Effects of Dx-E on γ-secretase/cleavage. **A** In vitro analysis of γ-secretase activity. 1% CHAPSO-solubilized crude membrane fraction was mixed with an intramolecularly quenched fluorogenic peptide probe and various concentrations of Dx-E (open circles) or L-685,458 (filled triangles). **B** Cell-based assay of γ-cleavage. K293 cells stably expressing sp-C100 (βAPP-C100 sequence is connected to downstream of signal sequence of βAPP) were incubated with Dx-E for 16 h; inh. = inhibitor. **C** Cell-based assay of γ-modulation. K293 cells stably expressing sw βAPP were used. Aβ in conditioned media was immunoprecipitated with antibody 4G8 and separated by Tris-bicine SDS-PAGE.

Dx-E Inhibits β-Cleavage of βAPP in Living Cells

We further examined whether Dx-E affects β-cleavage by BACE in living cells. K293 (fig. 4C) and SH-SY5Y (fig. 4D) cells expressing βAPP were pulse labeled for 10 min and chased for the indicated time. Strikingly, in both cell lines, Dx-E drastically decreased secretion of soluble βAPPβ, which is released by β-cleavage of βAPP, at the same concentration that reduced Aβ generation (fig. 4C, first panel, and fig. 4D, upper panel). In addition, Dx-E slightly enhanced the secretion of soluble βAPPα, which is probably a secondary effect due to decreased β-cleavage of βAPP (fig. 4D, lower panel). Dx-E affected neither the expression of BACE1 (fig. 4C, fourth panel) nor the maturation of βAPP (fig. 4C, second pan-





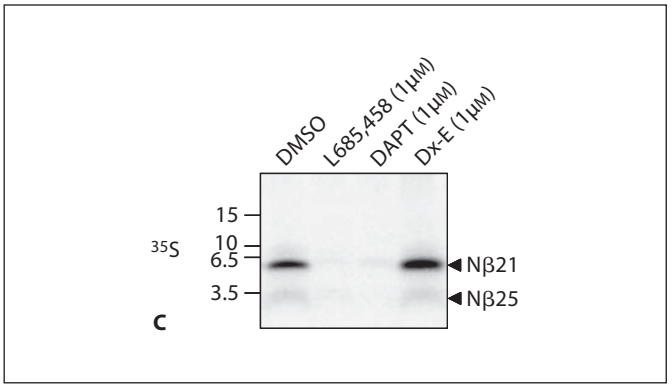


Fig. 3. Effects of Dx-E on Notch signaling/cleavage. **A**, **B** Notch downstream assay. After transient transfection with *HES-Y* and pRL-TK, cells stably expressing N1CS, sw βAPP and wt PS1 were treated with Dx-E (**A**) or DAPT (**B**) for 16 h. To analyze Notch signaling, the transcriptional activity of the HES-1 promoter was assessed in each condition (red). Conditioned media were collected, and the level of Aβ was measured (blue). **C** Nβ production assay. N1CS-expressing cells were labeled by pulse-chase with [^{35}S]methionine. Following treatment with γ-secretase inhibitors or Dx-E, the Nβ in conditioned media was analyzed by immunoprecipitation/autoradiography. Note that Nβ consists of two distinct molecular species (Nβ21 and Nβ25).

el). Therefore, Dx-E decreases β-cleavage of βAPP in living cells.

Dx-E Decreases Colocalization of βAPP and BACE1 in Cultured Cells

Our data that Dx-E decreases β-cleavage of βAPP in living cells without targeting to BACE enzyme prompted us to study a possibility that it affects relative subcellular locations of BACE and βAPP. To address this, we performed immunohistochemical analysis (fig. 5A–J). In untreated cultured cells, immunostaining with anti-βAPP extracellular domain antibody (22C11, red) and BACE1 N-terminal domain antibody (Calbiochem 195101, green) yielded substantial amounts of yellow colors which implies colocalization of the enzyme and the substrate (fig. 5A, B). However, upon Dx-E treatment, a portion of the yellow staining drastically decreased, while red/green stainings reciprocally increased (fig. 5C, D), indicating a marked decrease in the colocalization. In addition, we compared the distribution of each green or red staining with or without Dx-E treatment (fig. 5E–G, H–J), and found that the change of BACE1 distribution looked greater than that of βAPP.

To further confirm that Dx-E decreases the colocalization of βAPP and BACE1, we performed subcellular fractionation of cultured cells and observed the colocalization in each organelle. We immunoblotted each fraction with antibodies to the Na^+-K^+-ATPase α_1-subunit (for plasma membrane marker), EEA1 (for endosome marker), calnexin (for endoplasmic reticulum marker) and GM130 (for Golgi marker), which confirmed successful separation of each organelle (fig. 6A, B). Continuously, we tried to observe changes in subcellular distribution of βAPP/BACE1 with or without the treatment of Dx-E (fig. 6C). Strikingly, the major distribution of BACE1 moved from the relatively light fractions (approx. fractions 4–7) to heavier fractions (approx. fractions 7–9; fig. 6C, first and second panels). However, distribution of βAPP holoprotein and those of the organelle marker proteins did not change so much (fig. 6C, third to fourth panels, and fig. 6A, B). Both the biochemical and immunohistochemical data indicate that Dx-E induces drastic

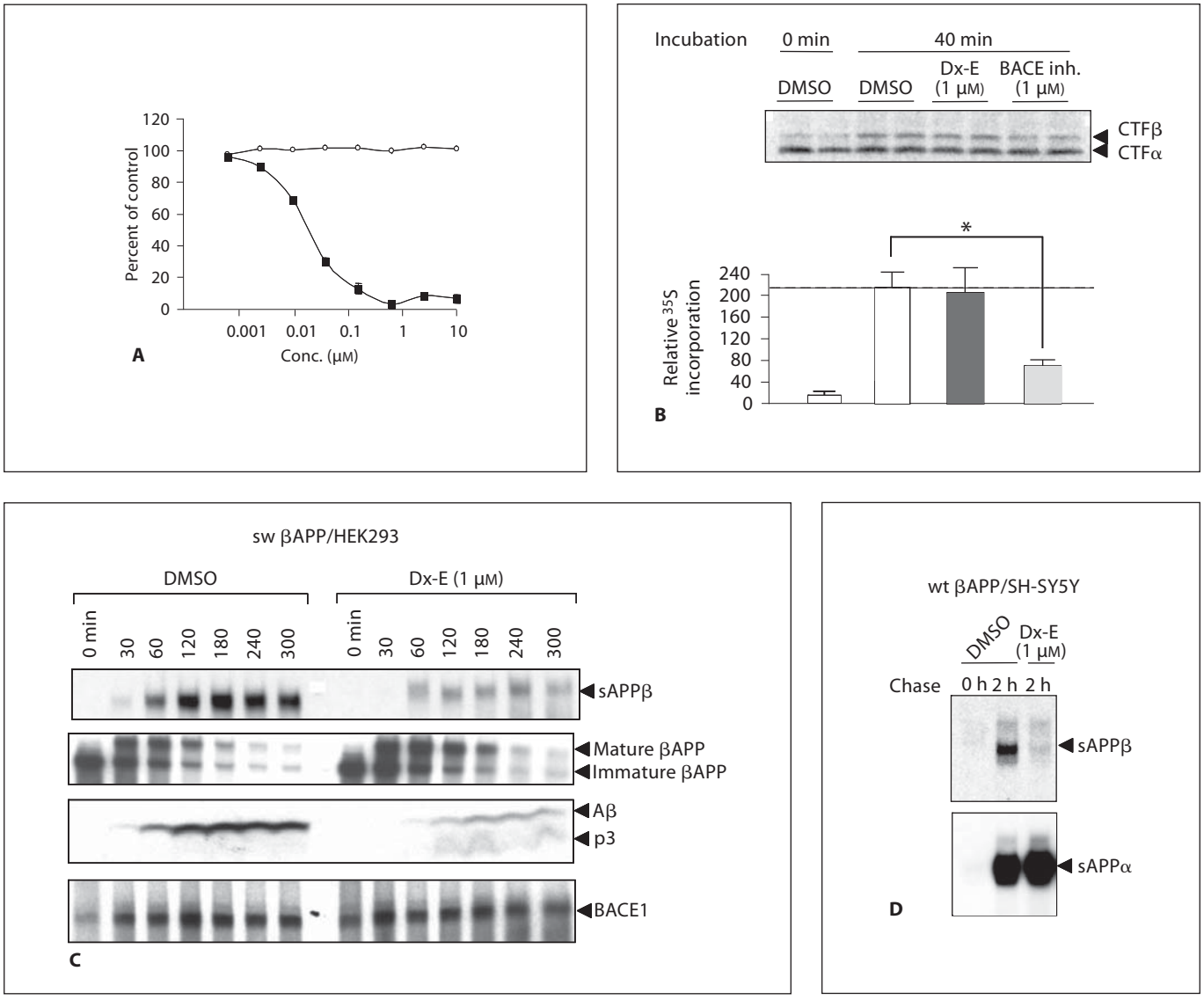


Fig. 4. Effects of Dx-E on β-secretase activity and β-cleavage of βAPP in cell culture. **A** Recombinant BACE1 was mixed with substrates and various concentrations of Dx-E (open circles) or inhibitor IV (filled squares). **B** The crude membrane fraction from [^{35}S]methionine-labeled K293 cells stably expressing sw βAPP was prepared. The cell-free β-secretase activity was assessed by measuring the de novo production of CTFβ; inh. = inhibitor; * $p < 0.001$. **C** Immunoprecipitation/autoradiography analysis of βAPP metabolites from K293 cells stably expressing sw βAPP and wt PS1. After 10 min of metabolic labeling with [^{35}S]methionine, the cells were chased at the indicated time points. The conditioned media were collected and immunoprecipitated with 4G8 (Aβ) or anti-sw-sAPPβ antibody. The levels of βAPP holoprotein and BACE1 in the cell lysates were also analyzed. **D** Production of sAPPβ and sAPPα by SH-SY5Y cells stably expressing wt βAPP.

changes in the subcellular locations of BACE1. Collectively, we suggest that Dx-E induces decreased colocalization of matured βAPP holoprotein and BACE1 enzyme, which may reduce β-cleavage.

Discussion

In this study, we demonstrated that Dx-E reduces Aβ generation in a secretase-independent manner. Dx-E inhibits β-cleavage of βAPP, but it does not affect β- and γ-secretases. Curiously, colocalization of BACE1 and

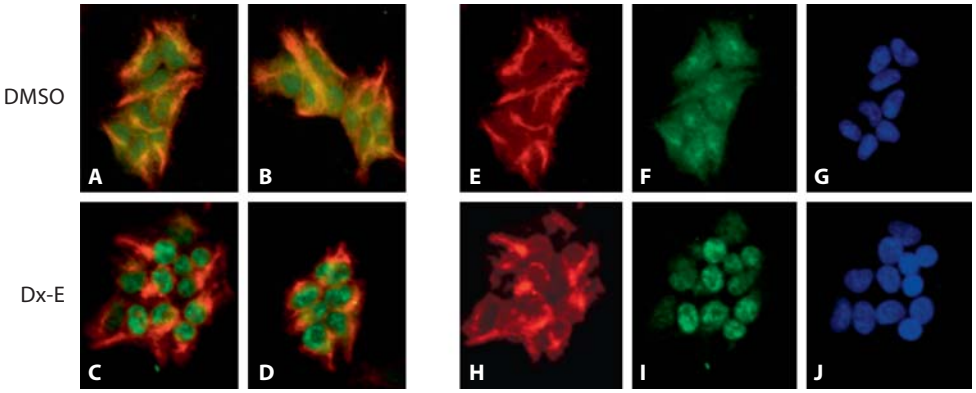
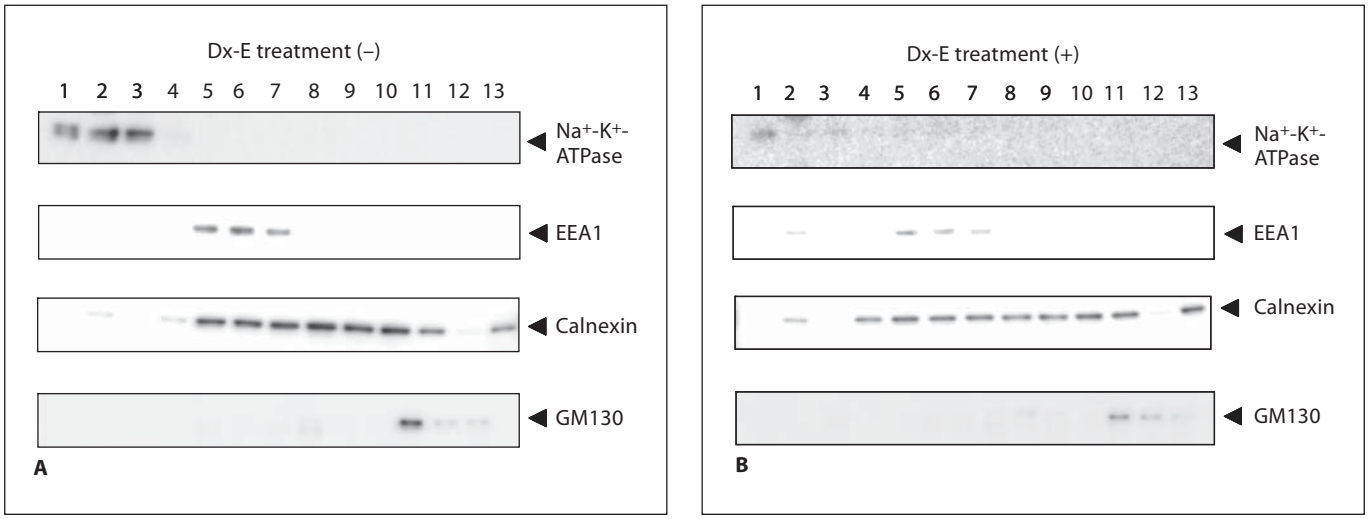


Fig. 5. Effects of Dx-E on the subcellular location of BACE1 and βAPP. Cells were treated with dimethylsulfoxide (DMSO, **A**, **B**) or 1 μM Dx-E (**C**, **D**) for 16 h. Then, βAPP (**E**, **H**) and BACE1 (**F**, **I**) were immunostained, and cell nuclei were counter-stained with diamidinophenylindole (**G**, **J**).



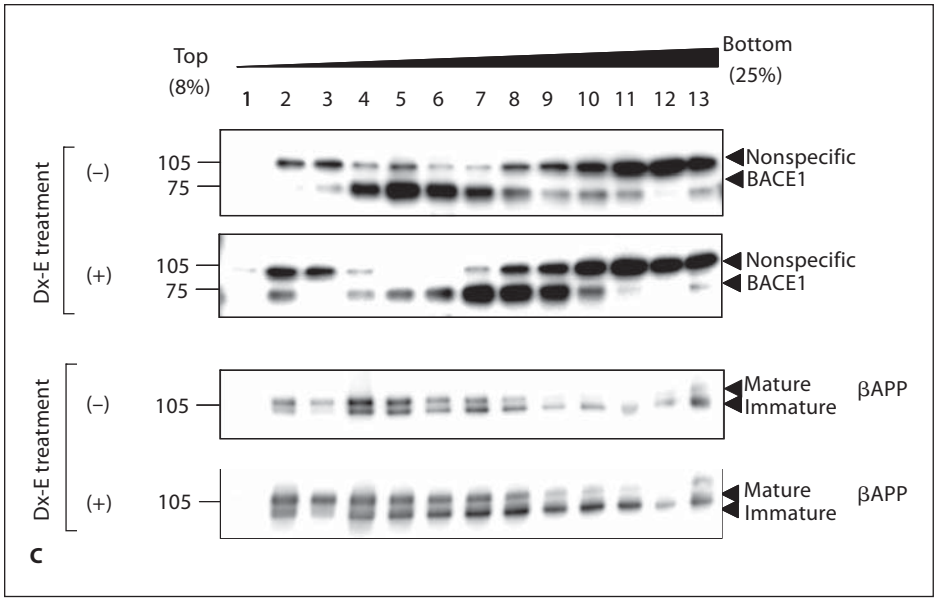


Fig. 6. Effects of Dx-E on subcellular location of BACE1 and βAPP. Fractions from a 8.0–25% linear iodixanol gradient examined by immunoblotting with the indicated antibodies. Cells were treated with dimethylsulfoxide (**A** and **C**, first and third panels) or 1 μM Dx-E (**B** and **C**, second and fourth panels) for 16 h and homogenated followed by subcellular fractionation.

βAPP was reduced by Dx-E. Our results suggest that Dx-E represents a novel and advantageous means of reducing Aβ production in Alzheimer disease.

Dx-E, a member of the destruxin family, is a cyclic hexadepsipeptide containing an α-hydroxy acid and 5 amino acid residues [16]. This peptide is reported to have several biological activities, including inhibition of vacuolar-type H^+-ATPase, cell cycle arrest and inhibition of bone resorption [16]. The ability of Dx-E to reduce Aβ production might be related to these effects. In addition, Dx-E is not the only member of the destruxin family that can inhibit Aβ production; we found that destruxin C is a weak inhibitor of Aβ production [Itoh N., unpubl. observations]. Like Dx-E, bafilomycin and concanamycin inhibit the vacuolar-type H^+-ATPase and reduce Aβ secretion from sw-βAPP-expressing cells [17, 18]. However, their mechanism of inhibiting β-cleavage is not well understood.

To reduce Aβ generation, inhibitors of either β-cleavage or γ-cleavage of βAPP have been developed. Since typical BACE or PS/γ-secretase inhibitors inhibit the enzymatic activities, they inevitably perturb cleavages of substrates other than βAPP. Inhibiting the enzymatic activities may cause unfavorable side effects because these enzymes are deeply involved in neuregulin/Notch signaling and play important roles in vivo. Thus, atypical compounds have been of note which inhibit Aβ generation without disturbing cleavages of other substrates. Especially, inhibitors that do not affect Notch signaling have been sought. For example, Gleevec® is thought to target the PS/γ-secretase because it inhibits the de novo generation of Aβ in a cell-free assay [19]. How other compounds such as JLK (nonpeptidic inhibitors of γ-secretase), glycogen synthase kinase 3α inhibitors, cerebrolysin and berberine inhibit Aβ generation is unclear [20–22].

Dx-E, which is neither a BACE nor PS/γ-secretase inhibitor, can reduce Aβ generation. Dx-E may decrease Aβ by reducing colocalization of βAPP and BACE1. The reduction of the colocalization is probably induced by drastic changes in the subcellular locations of BACE1. Since Dx-E does not change subcellular locations of other proteins such as βAPP, Na^+-K^+-ATPase α_1-subunit, EEA1, calnexin and GM130, the effect of Dx-E may be specific to BACE1. Dx-E may specifically affect trafficking of BACE1. Further study will be needed to elucidate how it is achieved. In future, we expect research and development of small-molecule inhibitors with processes similar to Dx-E.

Acknowledgements

We are grateful to the Program for the Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (05-26) (to M.O., S.T. and M.T.) and KAKENHI from the Ministry of Education, Culture, Sports and Science of Japan (to M.O. and S.T.).

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