

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

^aPsychiatry, Department of Integrated Medicine, Division of Internal Medicine, Osaka University Graduate School of Medicine, and ^bPain and Neurology, ^cAnalytical Chemistry, ^dDiscovery Technology 1, ^eDiscovery Technology 2 and ^fDiscovery Chemistry, Shionogi & Co. Ltd., Osaka, and ^gNeurology Unit, Molecular Neuroscience Research Center, Shiga University of Medical Science, Otsu, Japan; ^hLaboratory 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 ($A\beta$) is produced by sequential endoproteolysis of β -amyloid protein precursor (β APP): 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 $A\beta$ generation and is a fundamental strategy for the development of drugs to prevent Alzheimer disease. However, it is not clear how small compounds reduce $A\beta$ production without inhibition of the secretases. Such compounds are expected to avoid some of the side effects of secretase inhibi-

tors. Here, we report that destruxin E (Dx-E), a natural cyclic hexadepsipeptide, reduces $A\beta$ 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 β APP, which reduces β -cleavage of β APP. Therefore, the data demonstrate that Dx-E represents a novel $A\beta$ -reducing process which could have fewer side effects than secretase inhibitors.

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Inhibition of β -amyloid ($A\beta$) 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.

and γ -secretase [i.e. a complex including presenilin (PS), nicastrin, Aph1 and Pen2] enzymes that are involved in the generation of A β . 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 A β generation.

Here, we performed high-throughput screening for inhibitors of A β 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 (β APP) but did not inhibit the secretases. The existence of such a compound suggests that there is a novel mechanism for inhibiting A β generation.

Materials and Methods

Antibodies and Reagents

Anti-A β antibodies 6E10 and 4G8 were from Signet Laboratories (Dedham, Mass., USA). The anti-sAPP β 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-N¹-(1R)-1-phenylethyl]isophthalamide (inhibitor IV) were purchased from Calbiochem (San Diego, Calif., USA). The γ -secretase substrate NMA-GGVVIATVK(DNP)-DRDRDR-NH₂ was purchased from Calbiochem, and the β -secretase substrate MOCAC-SEVNLDAEFRK(Dnp)-RR-NH₂ was from Peptide Institute (Osaka, Japan). Dx-E was purified by Shionogi and Co. Ltd. In the measurement of A β 40 and A β 42 at a 384-well plate format, the addition of compounds and seeding of β APP-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 β APP/PS1/Notch derivatives were maintained in DMEM containing 10% FCS and antibiotics (200 μ g/ml G418 for β APP selection, 200 μ g/ml zeocin for PS1 selection and 100 μ g/ml hygromycin for Notch1 selection).

Measurement of A β Secretion

For high-throughput screening, A β 40 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 μ l/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 A β 40 and A β 42 in a 96-well plate format, an A β sandwich enzyme-linked immunosorbent assay (Wako, Japan) was used. The cells were incubated for 16 h with compounds. A β 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-SEVNLDAE-FRHDSGC) 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 μ g/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) \times 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 β APP were metabolically labeled with 450 μ Ci of [³⁵S]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 \times radioimmunoprecipitation assay buffer (1% sodium dodecyl sulfate, 5% deoxycholate, 10% Triton-X 100) and diluted with phosphate-buffered saline containing protease inhibitor cocktail. The β APP 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 β APP 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 β APP (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 β APP [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 A β Generation

A search for inhibitors of A β generation in a library of more than approximately 50,000 compounds by high-throughput screening identified Dx-E, a cyclodepeptide (fig. 1A). To confirm the ability of Dx-E to inhibit A β generation, we labeled SH-SY5Y cells stably expressing wt β APP by a 30-min pulse with [³⁵S]methionine followed by a 2-hour chase. As shown in figure 1B, Dx-E (1 μ M) decreased the level of secreted A β /p3 species (IC₅₀ approx. 230 nM; fig. 1B, upper panel), although it did not significantly change the rate of β APP holoprotein degradation (fig. 1B, middle and lower panels). We also examined whether Dx-E affects the rate of A β degradation in cell culture. Cells were incubated in conditioned medium containing radiolabeled A β and in the absence or presence of Dx-E. As shown in figure 1C, the rate of A β degradation was unchanged by Dx-E treatment. Thus, it appears that Dx-E inhibits A β /p3 generation in cell culture.

Dx-E Does Not Affect γ -Secretase Cleavage of β APP

A β 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-NH₂. 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 μ M) 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 β APP in living cells (fig. 2B). For the experiments, we used K293 cells stably expressing the CTF of β APP (CTF100), a constitutive substrate for PS/ γ -secretase. In contrast to the case of full-length β APP, Dx-E did not inhibit A β generation from CTF100 (fig. 2B). Collectively, these results show that the reduction of A β 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 A β generation at relatively higher concentrations than those causing changes in the relative ratio of A β 42 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 A β 42 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 A β production. Also, as expected, DAPT, a specific γ -secretase inhibitor, reduced both A β generation and Notch signaling (fig. 3B). Therefore, Dx-E does not perturb Notch signaling even though it inhibits A β /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 A β and NICD, we examined whether its effects are substrate specific. Thus, we analyzed secretion of S2/S4 cleavage-derived N β , also known as Notch1 A β -like peptides [8, 15] (fig. 3C). In contrast to specific γ -secretase inhibitors, Dx-E treatment did not inhibit N β secretion from N1CS-

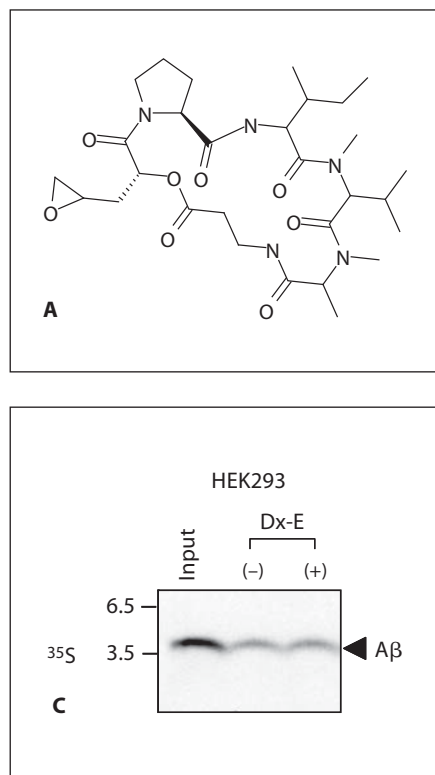
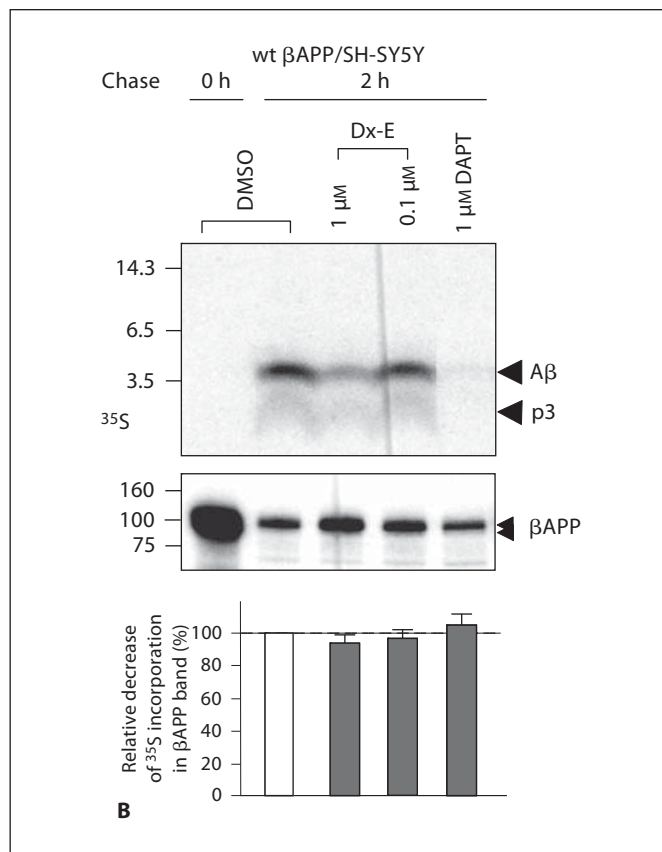


Fig. 1. Effects of Dx-E on A β production and degradation. **A** Structure of Dx-E. **B** Effect of Dx-E on β APP metabolism. Pulse-chase analysis of β APP metabolites was performed in SH-SY5Y cells stably expressing wt β APP. Cells were labeled with a 30-min pulse of [³⁵S]methionine followed by a 2-hour chase. The conditioned media were collected and immunoprecipitated with antibody 4G8 (A β ; upper panel). The β APP 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 ³⁵S in-



corporation in β APP holoprotein was analyzed by fluorography using a STORM 820 (Amersham). The ratio of β APP holoprotein degradation during the 2-hour chase period in untreated cells was defined as 100%. **C** Effect of Dx-E on degradation of A β in cell culture. K293 cells stably expressing sw β APP were metabolically labeled with a 30-min pulse of [³⁵S]methionine followed by a 2-hour chase. The medium containing labeled A β 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 β APP but not of Notch1.

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

Dx-E reduces A β generation without affecting γ -cleavage of β APP or Notch signaling/cleavage. Therefore, we examined whether Dx-E affects β -cleavage of β APP by BACE. We examined β -secretase activity in vitro using an intramolecularly quenched fluorogenic peptide probe, MOCAC-SEVNLDAEFRK(Dnp)-RR-NH₂, and recombinant BACE1. Dx-E did not affect BACE1 activity at any concentration of Dx-E tested (0.6 nM to 10 μ M),

although the specific BACE inhibitor, inhibitor IV (IC₅₀ 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 CTF β from β APP holoprotein (fig. 4B). After a 20-min pulse with [³⁵S]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 CTF β , although inhibitor IV decreased the level of CTF β by approximately 70% ($p < 0.001$ (t test); fig. 4B, lower panel). These results indicate that Dx-E does not affect BACE activity.

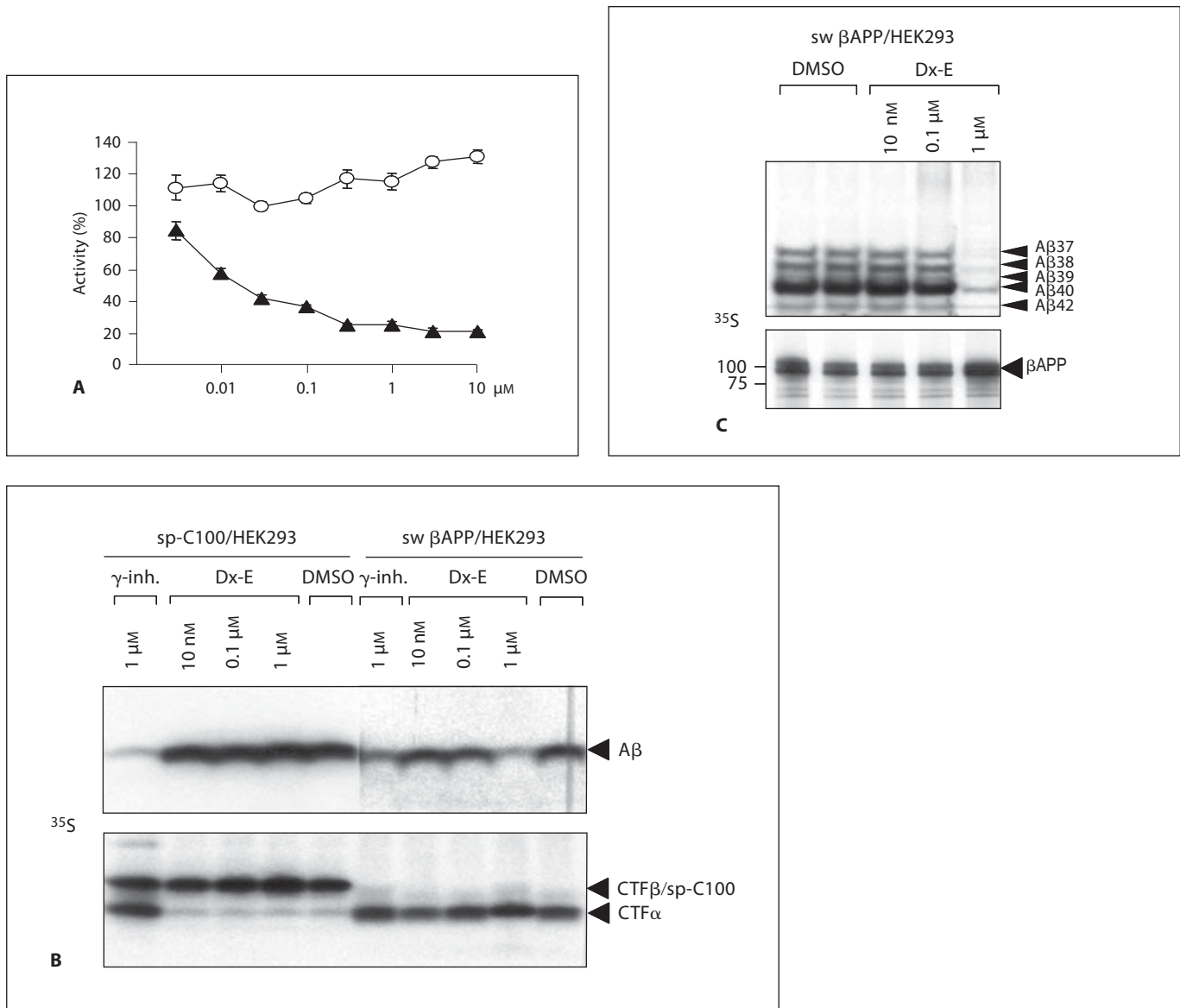


Fig. 2. Effects of Dx-E on γ -secretase/cleavage. **A** In vitro analysis of γ -secretase activity. 1% CHAPSO-solubilized crude membrane fraction was mixed with an intramolecularly quenched fluorogenic peptide probe and various concentrations of Dx-E (open circles) or L-685,458 (filled triangles). **B** Cell-based assay of γ -cleavage. K293 cells stably expressing sp-C100 (β APP-C100 se-

quence is connected to downstream of signal sequence of β APP) were incubated with Dx-E for 16 h; inh. = inhibitor. **C** Cell-based assay of γ -modulation. K293 cells stably expressing sw β APP were used. $A\beta$ in conditioned media was immunoprecipitated with antibody 4G8 and separated by Tris-bicine SDS-PAGE.

Dx-E Inhibits β -Cleavage of β APP 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 β APP were pulse labeled for 10 min and chased for the indicated time. Strikingly, in both cell lines, Dx-E drastically decreased secretion of soluble β APP β , which is released by β -cleavage of β APP,

at the same concentration that reduced $A\beta$ generation (fig. 4C, first panel, and fig. 4D, upper panel). In addition, Dx-E slightly enhanced the secretion of soluble β APP α , which is probably a secondary effect due to decreased β -cleavage of β APP (fig. 4D, lower panel). Dx-E affected neither the expression of BACE1 (fig. 4C, fourth panel) nor the maturation of β APP (fig. 4C, second pan-

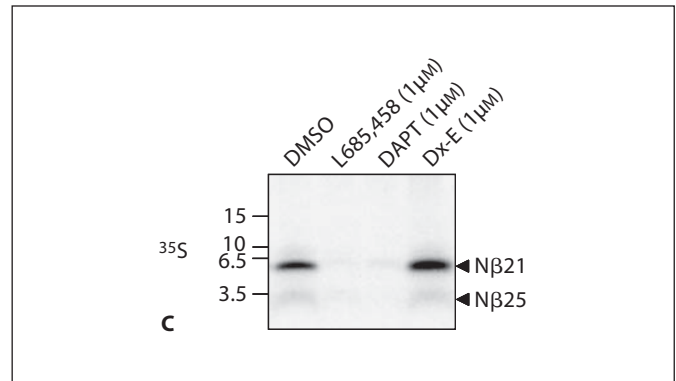
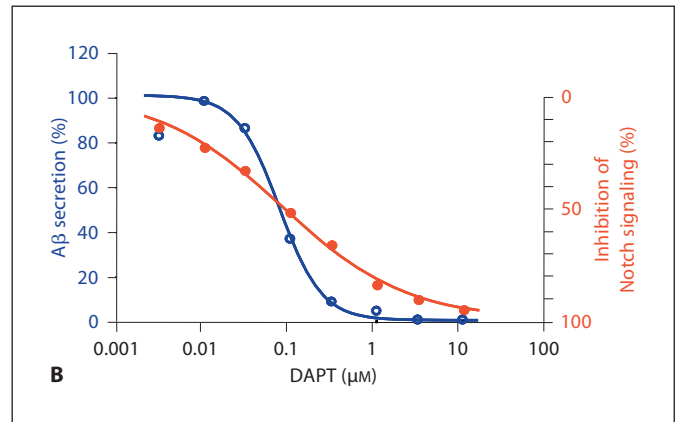
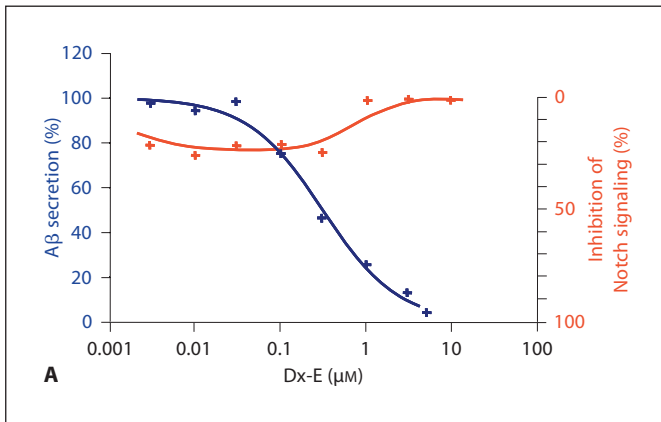


Fig. 3. Effects of Dx-E on Notch signaling/cleavage. **A, B** Notch downstream assay. After transient transfection with *HES-1* and pRL-TK, cells stably expressing N1CS, sw β APP and wt PS1 were treated with Dx-E (**A**) or DAPT (**B**) for 16 h. To analyze Notch signaling, the transcriptional activity of the *HES-1* promoter was assessed in each condition (red). Conditioned media were collected, and the level of A β was measured (blue). **C** N β production assay. N1CS-expressing cells were labeled by pulse-chase with [35 S]methionine. Following treatment with γ -secretase inhibitors or Dx-E, the N β in conditioned media was analyzed by immunoprecipitation/autoradiography. Note that N β consists of two distinct molecular species (N β 21 and N β 25).

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

Dx-E Decreases Colocalization of β APP and BACE1 in Cultured Cells

Our data that Dx-E decreases β -cleavage of β APP in living cells without targeting to BACE enzyme prompted us to study a possibility that it affects relative subcellular locations of BACE and β APP. To address this, we performed immunohistochemical analysis (fig. 5A–J). In untreated cultured cells, immunostaining with anti- β APP 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 β APP.

To further confirm that Dx-E decreases the colocalization of β APP 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 β APP/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 β APP 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

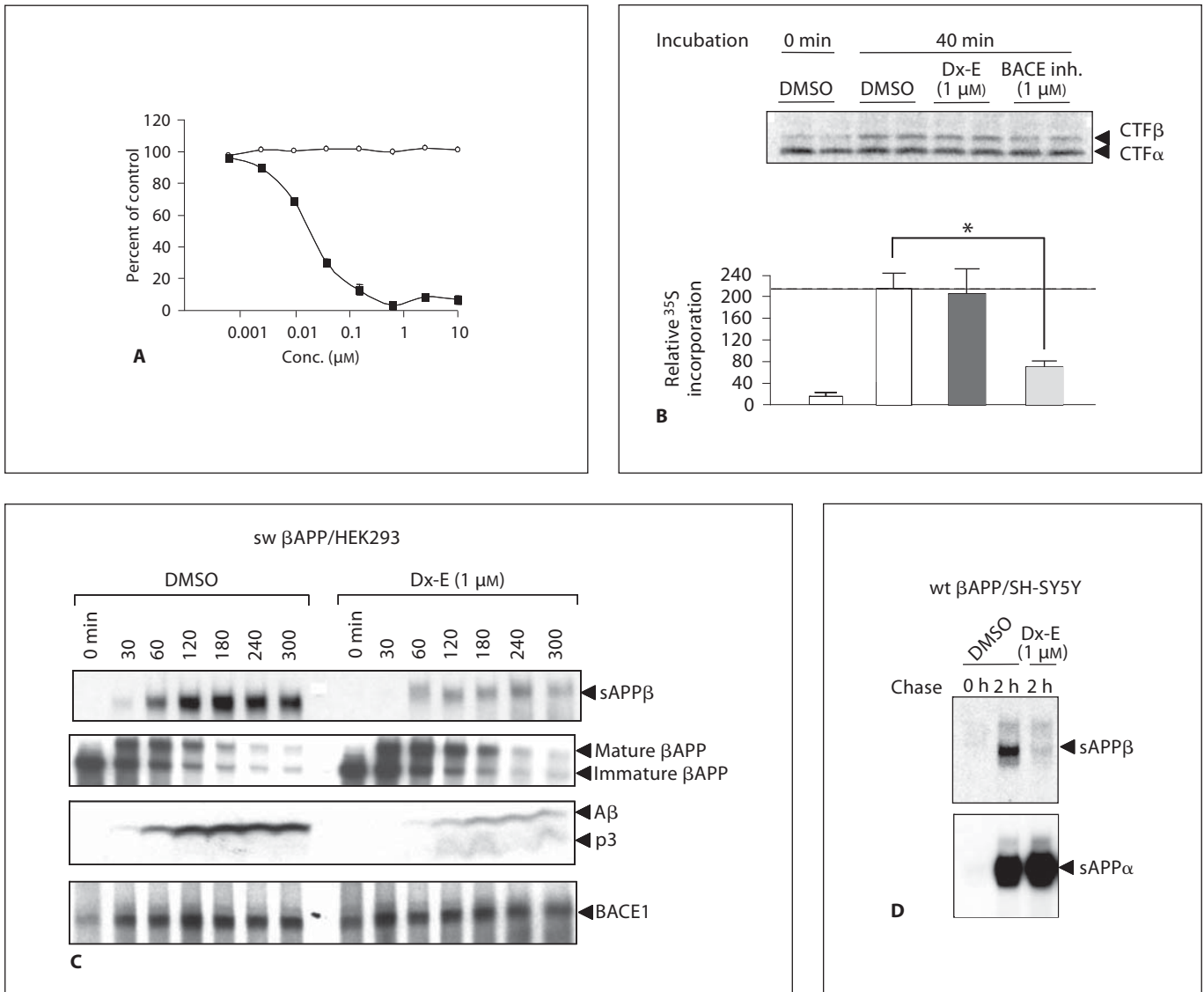


Fig. 4. Effects of Dx-E on β -secretase activity and β -cleavage of βAPP in cell culture. **A** Recombinant BACE1 was mixed with substrates and various concentrations of Dx-E (open circles) or inhibitor IV (filled squares). **B** The crude membrane fraction from [^{35}S]methionine-labeled K293 cells stably expressing sw βAPP was prepared. The cell-free β -secretase activity was assessed by measuring the de novo production of CTF β ; 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 (A β) or anti-sw-sAPP β antibody. The levels of βAPP holoprotein and BACE1 in the cell lysates were also analyzed. **D** Production of sAPP β and sAPP α 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 A β 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 sub-cellular location of BACE1 and β APP. Cells were treated with dimethylsulfoxide (DMSO, **A, B**) or 1 μ M Dx-E (**C, D**) for 16 h. Then, β APP (**E, H**) and BACE1 (**F, I**) were immunostained, and cell nuclei were counter-stained with diamidophenylindole (**G, J**).

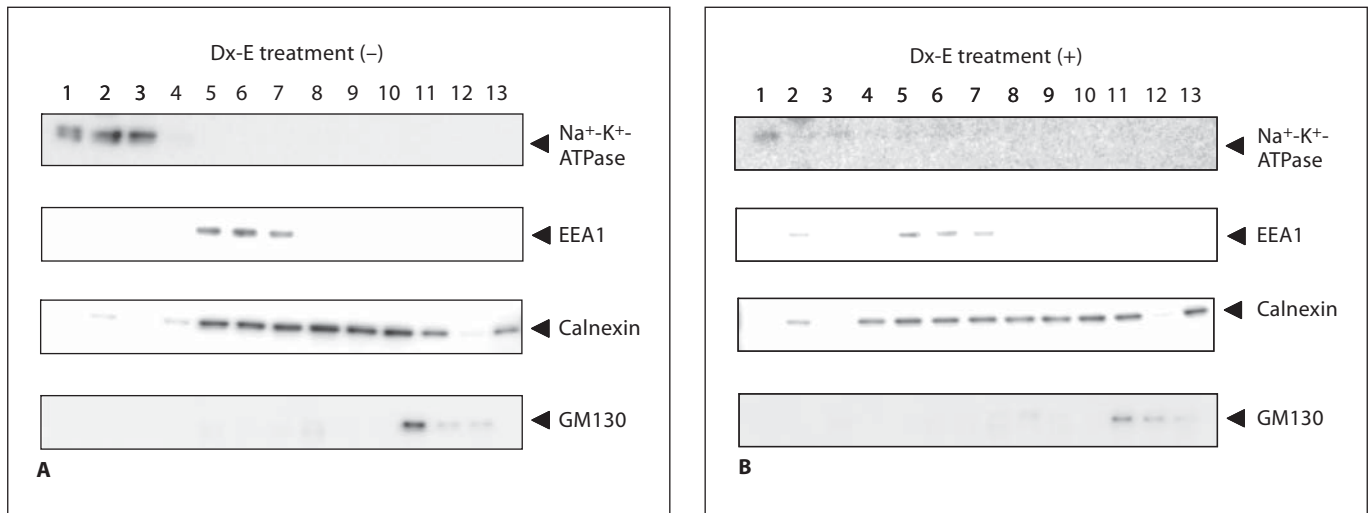
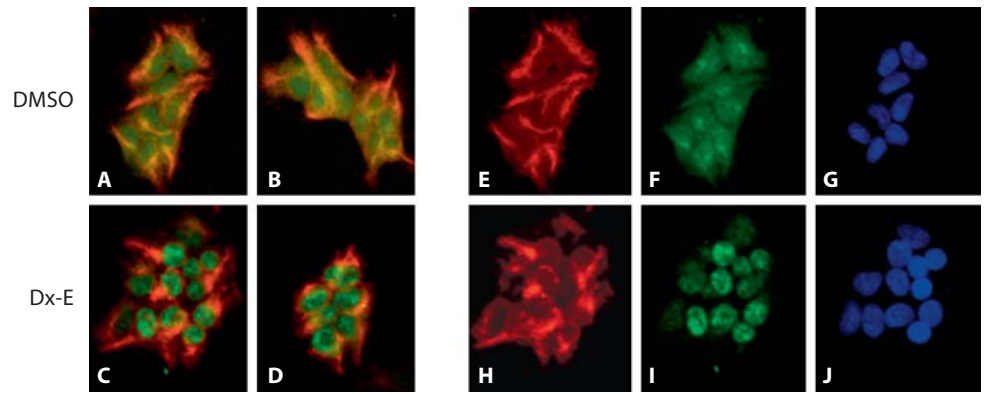
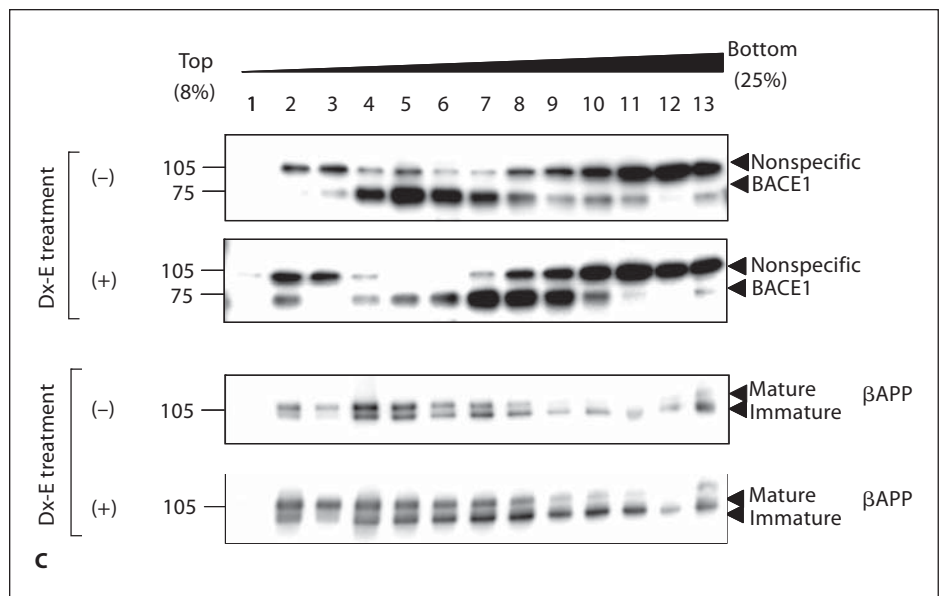


Fig. 6. Effects of Dx-E on subcellular location of BACE1 and β APP. Fractions from a 8.0–25% linear iodixanol gradient examined by immunoblotting with the indicated antibodies. Cells were treated with dimethylsulfoxide (**A** and **C**, first and third panels) or 1 μ M Dx-E (**B** and **C**, second and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



β APP was reduced by Dx-E. Our results suggest that Dx-E represents a novel and advantageous means of reducing A β 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 A β production might be related to these effects. In addition, Dx-E is not the only member of the destruxin family that can inhibit A β production; we found that destruxin C is a weak inhibitor of A β production [Itoh N., unpubl. observations]. Like Dx-E, bafilomycin and concanamycin inhibit the vacuolar-type H⁺-ATPase and reduce A β secretion from sw- β APP-expressing cells [17, 18]. However, their mechanism of inhibiting β -cleavage is not well understood.

To reduce A β generation, inhibitors of either β -cleavage or γ -cleavage of β APP have been developed. Since typical BACE or PS/ γ -secretase inhibitors inhibit the enzymatic activities, they inevitably perturb cleavages of substrates other than β APP. 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 A β 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 A β in a cell-free assay [19]. How other compounds such as JLK (nonpeptidic inhibitors of γ -secretase), glycogen synthase kinase 3 α inhibitors, cerebrosin and berberine inhibit A β generation is unclear [20–22].

Dx-E, which is neither a BACE nor PS/ γ -secretase inhibitor, can reduce A β generation. Dx-E may decrease A β by reducing colocalization of β APP 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 β APP, 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.

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