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Calpain inhibitor MDL28170 modulates Aβ formation by inhibiting the formation of intermediate $Aβ_{46}$ and protecting Aβ from degradation

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ABSTRACT

The observations that three major cleavages within the transmembrane domain of APP, namely, the γ-cleavage, ε-cleavage, and the newly identified ζ-cleavage, are involved in the generation of secreted $Aβ_{40}$ and $Aβ_{42}$ prompted us to determine how the calpain inhibitor III MDL 28170 influences these three cleavages and Aβ formation. With the use of a cell culture system, our data demonstrate that *1*) at either high concentrations, or at a low range of concentrations, at early time points, MDL 28170 inhibits the formation of secreted $Aβ_{40}$ and $Aβ_{42}$. However, this effect is due to inhibition of the intermediate $Aβ_{46}$ generation by ζ-cleavage and not due to direct inhibition of the γ-cleavage that produces $Aβ_{40/42}$ from $Aβ_{46}$; *2*) at low range of concentrations and at late time points, MDL 28170 causes an increase in secreted $Aβ_{40/42}$ that likely results from inhibition of degradation of both the initial substrate, CTFβ, and the final product, $Aβ_{40/42}$, of γ-secretase. These data strongly suggest that formation of $Aβ_{46}$ is a key step in the γ-secretase mediated generation of $Aβ_{40/42}$ and provide a new target for the development of Aβ inhibitors. These data also suggest that calpain and related proteases, which are sensitive to MDL 28170, play an important role in the accumulation of secreted Aβ.

Key words: amyloid β-peptide • ζ-cleavage

The production and deposition of amyloid β-peptide (Aβ) are believed to be the crucial early events in Alzheimer's disease (AD) pathogenesis (1). Aβ is proteolytically produced from amyloid precursor protein (APP) by the sequential actions of β-secretase and γ-secretase. After β-secretase cleavage, the resulting C-terminal fragment of APP (CTFβ) undergoes further cleavage within its transmembrane domain by γ-secretase to produce the full-length Aβ. Studies have also revealed that the majority of Aβ species released by cultured cells and isolated from biological fluids is $Aβ_{40}$, with the C terminus ending at residue 40 of the Aβ sequence. Only a small fraction (5–10% of the total Aβ) extends to residue 42. However, the

finding that $A\beta_{42}$ is a major constituent of the amyloid plaque cores suggests that it may play an important role in the initiation of Aβ deposition and amyloid plaque formation (2). This idea is supported by the observation that $A\beta_{42}$, which possesses two additional hydrophobic residues at its C terminal, is a more aggregation-prone species (3). This idea is further supported by the observations that AD-linked mutations in the AD genes, APP and presenilins (PS1, PS2), have been shown to increase the production of $A\beta_{42}$ (the Swedish mutation in APP increases both $A\beta_{40}$ and $A\beta_{42}$) (1). Thus, the underlying mechanism of production and secretion of $A\beta_{42}$ and the nature of γ-secretase that generates the C termini of Aβ have become the focus of Alzheimer's disease research.

Calpains are cytosolic calcium-activated cysteine proteases. It has been suggested, based on the finding that some of the calpain inhibitors can modulate the production of Aβ, that these proteases may be involved in the γ-secretase mediated processing of APP (4, 5). Furthermore, based on the observations that both calpain inhibitor III (MDL 28170) (6) and calpain inhibitor I (ALLN) (7) selectively inhibit the formation of $A\beta_{40}$ but have less effect on $A\beta_{42}$ formation, it has been suggested that $A\beta_{40}$ and $A\beta_{42}$ may be generated by distinct protease activities (6, 7). However, the possible mechanism underlying the effects of the calpain inhibitor on the formation of Aβ is not well understood. Moreover, the reported observations of the effects of the calpain inhibitor on the formation of Aβ remain controversial. For example, Figueiredo-Pereira et al. (8) reported that MDL 28170 inhibits the formation of both $A\beta_{40}$ and $A\beta_{42}$ but ALLN has no effect on Aβ production. In contrast, Mathews et al. (9) and Zhang et al. (10) reported that at low concentrations, MDL 28170 and ALLN increase both $A\beta_{40}$ and $A\beta_{42}$ with a relatively stronger enhancing effect on $A\beta_{42}$ formation than that on $A\beta_{40}$.

The intramembrane cleavage of APP, which generates the C termini of $A\beta_{40}$ and $A\beta_{42}$, is now specifically referred to as γ-cleavage (11). During the course of studying the mechanism of γ-secretase processing of APP, a second γ-secretase mediated cleavage, the ε-cleavage was identified at Aβ49. This cleavage produces the N terminus of the CTFε, a major component of the APP intracellular domain (AICD) produced by γ-secretase processing (11–14). Recently, in an effort to determine the missing residues between γ-cleavage at Aβ40/42 and ε-cleavages at Aβ49, we identified a third γ-secretase mediated cleavage site, the ζ-cleavage site at Aβ46, which is between the known γ- and ε-cleavage sites (15). The existence of ζ-cleavage site at Aβ46 is further supported by a recent study showing that $A\beta_{46}$ is the predominant intracellular Aβ species (16). Thus, APP undergoes at least three major cleavages, namely γ-cleavage, ε-cleavage, and the newly identified ζ-cleavage within its transmembrane domain. Identification of the ε- and ζ-cleavage sites prompted us to examine whether and how the calpain inhibitor MDL 28170 influences the three major γ-secretase mediated cleavages and modulates the production of $A\beta_{40}$ and $A\beta_{42}$. To this end, we examined the effect of MDL 28170 on these three cleavages by determining the formation and turnover of CTFε produced by ε-cleavage and the intermediate $A\beta_{46}$ produced by ζ-cleavage. We report that at early time points, MDL 28170 inhibits the formation of secreted $A\beta_{40/42}$ by inhibiting the formation of the intermediate $A\beta_{46}$. At low concentrations and late time points, MDL 28170 increases both $A\beta_{40}$ and $A\beta_{42}$ at rates that are not significantly different from each other and this effect on the increase in $A\beta_{40}$ and $A\beta_{42}$ is likely the result of inhibition of the degradation of Aβ species.

MATERIALS AND METHODS

Reagents

MDL 28170 (carbobenzoxy-valinyl-phenylalaninal also known as calpain inhibitor III), fluorescent calpain substrate Suc-Leu-Leu-Val-Tyr-AMC, and γ-secretase inhibitors (DAPT and compound E) were obtained from Calbiochem and dissolved in dimethyl sulfoxide. $A\beta_{40}$ and $A\beta_{42}$ were purchased from American Peptide. $A\beta_{46}$ is a customized peptide.

Cell culture and treatment

N2a cells, stably expressing wild-type presenilin 1 (PS1wt) and Swedish mutant APP (APPsw), were kindly provided by Drs. Sangram S. Sisodia and Seong-Hun Kim (University of Chicago) and maintained as described previously (17). Twenty four hours after splitting, the old DMEM medium containing 10% FBS was replaced with fresh DMEM medium containing 0.5% FBS, and then the cells were treated, with or without inhibitors, for the time periods indicated.

Cell-free assay

In vitro generation of CTFε and turnover of $A\beta_{46}$ by γ-secretase activity were assayed in a cell-free assay system described previously (18) and following the procedure described previously (13) with minor modifications. Briefly, for determining the effect of MDL 28170 on the in vitro generation of CTFε, N2a cells cultured in the absence of inhibitors were harvested in 9 vol of homogenization buffer (10 mM MOPS, pH 7.0, 10 mM KCl) containing protease inhibitors (Complete, Roche) and homogenized by passing through a 20-gauge needle 30 times. After the removal of unbroken cells and nuclei by centrifugation at 800 *g* at 4°C for 10 min, membranes were pelleted by centrifugation at 20,000 *g* at 4°C for 30 min. The membranes were washed once with homogenization buffer and resuspended in assay buffer (150 mM sodium citrate pH 6.4, protease inhibitor cocktail). Aliquots of equal amounts of membranes were then incubated at either 0°C or 37°C for the indicated time, either in the presence or absence of MDL 28170. The reaction was stopped by the addition of Laemmli SDS sample buffer containing 8 M urea. After being boiled for 5 min, the samples were analyzed by Western blotting. To determine the effect of MDL 28170 on the turnover of $A\beta_{46}$, cells were cultured in the presence of 0.5 μM of DAPT for 4 h and the membrane fraction, which contains the accumulated $A\beta_{46}$, was prepared and the in vitro experiments were performed, as described above, in the absence or presence of appropriate inhibitors.

Immunoprecipitation and Western blot analysis

Immunoprecipitation and Western blot analyses were carried out as described previously (15). Briefly, to determine the intracellular APP derivatives, cells were harvested and lysed in Western blot lysis buffer (50 mM Tris-HCl, pH 6.8, 8 M urea, 5% β-mercaptoethanol, 2% SDS, and protease inhibitors). Secreted Aβ was immunoprecipitated from conditioned media using a monoclonal Aβ-specific antibody 6E10 (Signet Laborotories, Inc.). Both cell lysates and immunoprecipitates were analyzed by 10% Bicine/urea SDS-PAGE, or 10–18% regular SDS-PAGE, and transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore). The membranes were then probed with specific antibodies, and the immunoreactivity bands were visualized using ECL-Plus (Amersham Biosciences).

Measurement of calpain activity

The in vivo calpain activity was measured using the fluorogenic peptide Suc-Leu-Leu-Val-Tyr-AMC (Calbiochem) as a substrate following the procedure described previously (19, 20) with slight modification. Briefly, cells were cultured in 24-well plates in DMEM with 0.5% FBS for 16 h in the absence or presence of different concentrations of MDL 28170 (0, 10, 20, 40, 60, or 100 μM). After being washed twice with phosphate-buffered saline (PBS) containing the same concentrations of MDL 29170 as those in the culture media, Suc-Leu-Leu-Val-Tyr-AMC was added to a final concentration of 80 μM in PBS containing MDL 28170 at the appropriate concentrations. Immediately after addition of Suc-Leu-Leu-Val-Tyr-AMC, fluorescence was recorded at 2 min intervals for 20 min at excitation 360 ± 20 nm and emission 460 ± 20 nm using a Synergy HT Multi-Detection Microplate Reader (BIO-TEK Instruments Inc.). The initial rate of peptidyl-AMC hydrolysis was used as the velocity of enzyme activity. Note that throughout the procedure, MDL 28170 was added to all of the solutions and buffers to maintain the same concentrations of inhibitor as used in the cell culture.

RESULTS

At low concentrations, MDL 28170 causes a slight increase in intracellular accumulation of $A\beta_{46}$

To determine the effect of MDL 28170 (carbobenzoxy-valinyl-phenylalaninal) or calpain inhibitor III on the intramembrane ε-, ζ-, and γ-cleavages, we first examined its effect on the formation and turnover of $A\beta_{46}$ produced by ζ-cleavage. N2a cells stably expressing both wild-type PS1 (PS1wt) and myc-tagged Swedish mutant APP (APPsw), which have been used in previous studies (15, 17, 21), were treated with MDL 28170 at various concentrations for 48 h in DMEM containing 0.5% FBS. Cell lysates were analyzed by regular 10–18% SDS-PAGE (Fig. 1, upper panel) or 10% urea-SDS-PAGE (Fig. 1, lower panel). At low range of concentrations (up to 40 μM), MDL 28170 caused a slight, but dose-dependent increase in the accumulation of intracellular $A\beta_{46}$ (lanes 1–4) as compared with the dramatic increase in $A\beta_{46}$ caused by DAPT (lane 6). At 60 μM of MDL 28170, $A\beta_{46}$ decreased to the basal level. (lane 5).

MDL 28170 causes increase in $A\beta_{46}$ not by inhibiting $A\beta_{40/42}$

In a recent study, we have shown that nontransition state γ-secretase inhibitors, such as DAPT and compound E, cause a dose-dependent increase in the accumulation of intracellular $A\beta_{46}$ and a concomitant decrease in secreted $A\beta_{40/42}$ (15), suggesting a precursor–product relationship between $A\beta_{46}$ and secreted $A\beta_{40/42}$. Calpain inhibitors have also been reported to inhibit the formation of secreted Aβ from CTFβ (4, 5, 8) or, at low concentrations, selectively inhibit the formation of $A\beta_{40}$ (6, 7). We next determined whether the slight increase in $A\beta_{46}$ caused by MDL 28170 is a result of inhibition of the formation of $A\beta_{40/42}$. As shown in Fig. 2*A*, DAPT and compound E cause a dose-dependent increase in $A\beta_{46}$ and a concomitant decrease in $A\beta_{40/42}$, as reported in our previous study (15). However, as shown in Fig. 2*B*, to our surprise, the dose-dependent increase in $A\beta_{46}$ (lanes 2–7) caused by MDL 28170 at a low range of concentrations (up to 40 μM) is not associated with a decrease in $A\beta_{40/42}$. Quite the opposite, significant dose-dependent increases in both $A\beta_{40}$ and $A\beta_{42}$ are observed (lanes 3–7). These observations clearly indicate that the mechanism by which MDL 28170 causes an increase in $A\beta_{46}$ is different from

that by which compound E and DAPT inhibit the formation of $A\beta_{40/42}$. The accumulation of both intracellular $A\beta_{46}$ and secreted $A\beta_{40/42}$ rapidly declined in the presence of high concentrations of MDL 28170 with a concomitant increase in CTFβ, CTFα, and CTFε (lanes 7–9) in a dose-dependent manner. In addition, MDL 28170 affects the formation of $A\beta_{40}$ and $A\beta_{42}$ in a dose-dependent manner at similar rates. Graphs in Fig. 2*C* represent the average of the results of densitometric analysis of at least three repeated Western blots shown in Fig. 2*B*.

MDL 28170 inhibits turnover of CTFβ and the formation of $A\beta_{46}$ and $A\beta_{40/42}$ in a similar time-dependent manner

To further determine the effect of MDL 28170 on the formation of $A\beta_{46}$ and $A\beta_{40/42}$, a time-course experiment was performed. As shown in Fig. 2*B*, in cells treated with MDL 28170 at a concentration of 30 μM, both $A\beta_{46}$ and $A\beta_{40/42}$ reached their peaks at 48 h (lane 6). Accordingly, the time-course experiment was performed by treating cells with 30 μM of MDL 28170. As shown in Fig. 3*A*, as a control, in the absence of inhibitor, secreted $A\beta_{40/42}$ became detectable by Western blotting after 3 h incubation. Graphs in Fig. 3*C* represent the average of the results of densitometric analysis of at least three repeated Western blots shown in Fig. 3*A*. As shown in Fig. 3*C*, the secreted $A\beta_{40/42}$ accumulated in the conditioned medium in a time-dependent manner. The basal level of CTFβ was not changed during the time course, indicating a constant turnover of CTFβ after its formation by β-secretase. The basal level of intermediate $A\beta_{46}$ was barely detectable and was not changed throughout the time course, indicating a quick turnover of $A\beta_{46}$ into $A\beta_{40/42}$, after being formed from CTFβ. In contrast, as shown in Fig. 3*B* and *D*, which graphically represents the average of at least three repeated Western blots shown in Fig. 3*B*, during the first 24 h incubation period MDL 28170 caused time-dependent accumulation of CTFβ and CTFα (Fig. 3*B*, top and third panels; Fig. 3*D*, insert) and neither $A\beta_{46}$ nor $A\beta_{40/42}$ was detectable. Beyond this time point, CTFβ and CTFα began to quickly decline (Fig. 3*B*, top panel; Fig. 3*D*, insert) and as a result the secreted $A\beta_{40/42}$ robustly increased (Fig. 3*B*, bottom panel; Fig. 3*D*). In addition, CTFε was also detected at the same time point at which secreted $A\beta_{40/42}$ became detectable in the conditioned medium (compare Fig. 3*B*, second and bottom panels). At late time points, a small amount of the intracellular $A\beta_{46}$ was also observed (Fig. 3*B*, third panel; Fig. 3*D*).

At low concentrations MDL 28170 has no effect on the turnover of $A\beta_{46}$

The observation that at early time points, MDL 28170 inhibits the formation of $A\beta_{40/42}$ without accumulation of $A\beta_{46}$ suggests two possibilities: *1*) at early time points, MDL 28170 inhibits both of the formation of $A\beta_{46}$ from CTFβ and the formation of $A\beta_{40/42}$ from $A\beta_{46}$, and *2*) MDL 28170 inhibits the formation of $A\beta_{40/42}$ by blocking the formation of the intermediate $A\beta_{46}$. To address this issue, it is necessary to first determine whether MDL 28170 has any inhibitory effect on the turnover of $A\beta_{46}$. To do so, a system that contains preexisting $A\beta_{46}$ is required. Thus, a well-established cell-free system (13, 18) was employed to assay the effect of MDL 28170 on the turnover of $A\beta_{46}$. Cells were cultured in the presence of 0.5 μM of DAPT, which blocks the turnover of $A\beta_{46}$, for 4 h, and then the membrane was prepared as described in Materials and Methods. To determine the effect of MDL 28170 on the turnover of $A\beta_{46}$, the membrane was incubated in the absence or presence of MDL 28170 or DAPT. As shown in Fig. 4, when the membrane was incubated at 0°C for 30 min, the concentration of $A\beta_{46}$ remained unchanged (lane 2). In contrast, when the membrane was incubated at 37°C for 30 min, $A\beta_{46}$ decreased to the basal level (lane 4), indicating a quick turnover of $A\beta_{46}$ in the absence of inhibitors. However,

when the membrane was incubated at 37°C for 30 min in the presence of DAPT, the level of $A\beta_{46}$ remained largely unchanged (lane 3). On the other hand, when the membrane was incubated at 37°C for 30 min in the presence of MDL 28170, it was observed that turnover of $A\beta_{46}$ was not inhibited by MDL 28170 at the low range of concentrations (up to 35 μM, lanes 5–8). In the presence of high concentration of MDL 28170 (100 μM, lane 10), a small amount of residual $A\beta_{46}$ was detected, indicating that at high concentrations, MDL 28170 has a weak inhibitory effect on the turnover of $A\beta_{46}$ and this inhibitory effect may be the result of the inhibition of γ-cleavage of $A\beta_{46}$ at positions 40 or 42. However, it cannot be ruled out that inhibition of random degradation of $A\beta_{46}$ may also contribute to the blockage of the turnover of $A\beta_{46}$ by MDL 28170.

MDL 28170 inhibits the formation of $A\beta_{46}$ in living cells

The observation that at low concentration (up to 35 μM), MDL 28170 has no inhibitory effect on the turnover of $A\beta_{46}$ (Fig. 4) suggests that at early time points, MDL 28170 blocked the formation of secreted $A\beta_{40/42}$ by inhibiting the formation of the intermediate $A\beta_{46}$ from CTFβ, rather than by directly inhibiting the formation of secreted $A\beta_{40/42}$ from $A\beta_{46}$. To further determine the inhibitory effect of MDL 28170 on the formation of $A\beta_{46}$, we first determined the time course of $A\beta_{46}$ formation. Since the basal level of $A\beta_{46}$ is very low because of its rapid turnover (Fig. 3*A*), it is necessary to first block the quick turnover of $A\beta_{46}$ to determine the time course of $A\beta_{46}$ formation. Therefore, cells were cultured in the presence of 3 nM of compound E, and the formation of $A\beta_{46}$ was monitored by Western blot analysis of the cell lysates at a series of time points. As shown in Fig. 5*A*, in the presence of compound E, a significant amount of $A\beta_{46}$ was observed after 30 min of treatment (lane 2). The accumulation of $A\beta_{46}$ reached its peak after 3 h of treatment and remained unchanged during the following incubation period of 32 h (lanes 3–7). Note, as shown in Fig. 3*B*, in the presence of MDL 28170, no $A\beta_{46}$ was detected in the first 24 h incubation period (lanes 2–6) and only a small amount of $A\beta_{46}$ was detected at a late time points (36–48 h, lanes 7 and 8). Thus, the delayed appearance of $A\beta_{46}$ and time-dependent accumulation of CTFβ caused by MDL 28170 during the first 24 h incubation (Fig. 3*B*) strongly suggest that MDL 28170 has an inhibitory effect on the formation of $A\beta_{46}$ from CTFβ by γ-secretase activity.

To further substantiate the inhibitory effect of MDL 28170 on the formation of $A\beta_{46}$, the following experiments were performed. Cells were treated with either MDL 28170 or compound E or with a combination of the two. As a control, cells were also cultured in the presence of the vehicle Me_2SO only. As shown in the top panel of Fig. 5*B*, after 24 h incubation, in spite of the accumulation of a significant amount of CTFβ, only a trace amount of $A\beta_{46}$ was detected in cells treated with MDL 28170 (compare lane 2 with lane 1 the control). In contrast, a remarkable amount of $A\beta_{46}$ had accumulated in the cells treated with compound E (lane 3, Fig. 5*B*, top panel). It is notable that when cells were treated with both compound E and MDL 28170, $A\beta_{46}$ dramatically decreased with a concomitant increase in CTFβ in comparison with cells treated with compound E alone (compare lane 4 with lane 3, Fig. 5*B*, top panel). These results clearly indicate that, at early time points, MDL 28170 inhibits the formation of $A\beta_{46}$ by γ-secretase from CTFβ.

It was noticed that the concentration of accumulated CTFβ in the cells treated with the combination of MDL 28170 and compound E is higher than those of cells treated with either

MDL 28170 or compound E individually (compare lane 4 with lanes 2 and 3 at 24 h time point and also compare lane 8 with lanes 6 and 7 at the 48 h time point, Fig. 5*B*, top panel), indicating the additive inhibitory effects of the two inhibitors on the turnover of CTFβ. It was also noticed that as the incubation continues, the concentration of accumulated CTFβ in MDL 28170-treated cells was significantly reduced in a time-dependent manner, while, the concentration of accumulated CTFβ in cells treated with compound E decreased only slightly (compare lane 7 with lane 3, Fig. 5*B*, top panel). These results indicate that the inhibitory effect of MDL 28170 on the turnover of CTFβ by γ-secretase activity is apparently not as strong as that of compound E. That this inhibitory effect of MDL 28170 on the turnover of CTFβ is weaker was also substantiated by the fact that, at the 48 h time point, an increased amount of $A\beta_{46}$ was observed in cells treated with both compound E and MDL 28170 (compare lane 8 with lane 4, Fig. 5*B*, top panel). Moreover, up to 48 h incubation, the concentration of $A\beta_{46}$ in cells treated with compound E decreased only slightly (compare lane 7 with lane 3, Fig. 5*B*, top panel); however, in spite of the decrease in CTFβ, the $A\beta_{46}$ produced from CTFβ in cells treated with MDL 28170 did not increase but rather decreased to a basal level (compare lane 6 with lanes 1 and 2, Fig. 5*B*, top panel). This result also further confirms the results, observed in Figs. 3*B* and 4, that MDL 28170 has a very weak, if any, inhibitory effect on the turnover of $A\beta_{46}$ by the γ-cleavage. It was also noted that CTFε was detected in cells treated with either MDL 28170 alone, or with MDL 28170 in combination with compound E (lanes 2, 4, 6, and 8, Figs. 3*B* and 4, bottom panel) but not in cells treated with compound E alone. This is probably because of the inhibition of the degradation of CTFε by MDL 28170.

At high concentration MDL 28170 inhibits CTFε formation

As shown in Fig. 5*B*, the observation that CTFε was detected in cells treated with either MDL 28170 alone or with MDL 28170 in combination with compound E (lanes 2, 4, 6, and 8, Fig. 5*B*, bottom panel), suggests an inhibition of the degradation of CTFε by MDL 28170. In this regard, as shown in Fig. 2*B*, it was also noted that at high concentrations, MDL 28170 causes a dose-dependent increase in CTFε as well as CTFβ and CTFα with concomitant decrease in $A\beta_{46}$ and $A\beta_{40/42}$ (lanes 7–9, Fig. 2*B*, second panel). Since both CTFε and Aβ ($A\beta_{46}$ and $A\beta_{40/42}$) are produced from CTFβ by γ-secretase, it is impossible that MDL 28170 inhibits the N-terminal product Aβ but not the C-terminal product CTFε of the same γ-secretase-mediated processing of CTFβ. One possibility is that at high concentrations, MDL 28170 may have inhibitory effects on both the cleavage of CTFβ and CTFα catalyzed by γ-secretase and the degradation of CTFε mediated by calpain and other MDL 28170-sensitive proteases. Therefore, the accumulation of CTFε caused by high concentrations of MDL 28170 is possibly a result of inhibition of the degradation of CTFε produced by the residual γ-secretase activity after the addition of MDL 28170, as well as the CTFε produced before the addition of MDL 28170.

To determine whether accumulation of CTFε by MDL 28170 at high concentrations is due to the protection of CTFε from degradation rather than a failure to inhibit CTFε formation, a system that contains no preexisting CTFε is required. For this purpose, the cell-free system was employed to assay the inhibitory effect of MDL 28170 on CTFε formation. Cells were cultured in the absence of any inhibitors and the cell membranes were prepared as described in Materials and Methods. As shown in Fig. 6, since the residual soluble CTFε produced during the culture was washed away during the preparation of the membrane, CTFε was not detected in the control of membrane incubated at 0°C for 2 h (lane 1). When the membrane was incubated at 37°C for 2

h, de novo CTFε generated from both exogenous myc-tagged APP and endogenous APP with a concomitant decreases in CTFβ and CTFα was detected (compare lane 2 with lane 1, Fig. 6). When the membranes were incubated at 37°C in the presence of MDL 28170, a dose-dependent decrease in the de novo generation of CTFε with a concomitant increase in the unprocessed CTFβ and CTFα was observed (lanes 3–8). At 100 μM, MDL 28170 almost completely abolished the formation of CTFε (lane 8). In agreement with a previous report (11), this result clearly indicates that, although it may be less specific, MDL 28170 does have an inhibitory effect on γ-secretase mediated ε-cleavage.

At the range of concentrations used throughout the study, MDL 28170 strongly inhibits calpain activity in living cells

Next, we determined the inhibitory effect of MDL 28170 on calpain activity in the N2a cells used in this study. To determine the in vivo calpain activity, a procedure using a fluorogenic Suc-Leu-Leu-Val-Tyr-AMC peptide as substrate (19) was employed. Suc-Leu-Leu-Val-Tyr-AMC is a calpain protease substrate (22). Since the proteolytic hydrolysis of the peptidyl-7-amino bond liberates a highly fluorescent 7-amino-4-methylcoumarin (AMC) moiety and especially since Suc-Leu-Leu-Val-Tyr-AMC has been found to be membrane permeant (23, 24), this fluorogenic peptide has been successfully used to quantitatively determine the calpain activity in living cells in previous studies (19, 20). As described under Materials and Methods, the inhibitory effect of MDL 28170 on calpain was determined in cells after culturing in DMEM with 0.5% FBS for 16 h in the presence of different concentrations of MDL 28170 (0, 10, 20, 40, 60, or 100 μM). At this time point, MDL 28170 caused accumulation of CTFβ and blocked the formation of Aβ (Fig. 3*B* and *D*), indicating a strong inhibitory effect of MDL 28170. As shown in Fig. 7, calpain activity, determined by the initial rate of the peptidyl-AMC hydrolysis, decreased in a dose-dependent manner. The calpain activity decreased by 93% in cells cultured in the presence of 60 μM MDL 28170. This result clearly indicates that, at the range of concentrations used throughout this study, MDL 28170 strongly and dose dependently inhibits calpain activity in living cells. In the presence of MDL 28170 up to 60 μM, cells remained adherent over the time course (including both the culture period and the enzymatic assay time course) with no loss of viability as determined by the lack of increase in lactase dehydrogenase (LDH) release (data not shown). When the cells were treated with 100 μM of MDL 28170, no significant decease in calpain activity was observed (from 93 to 95%). However, at this high concentration (100 μM), MDL 28170 caused loss of the viability of cells (over 25%) during late time course (later than 12 h of the culture period and specifically during the enzymatic assay time course) as determined by the release of LDH and changes in morphology (data not shown).

DISCUSSION

In this study, by examining its effect on the formation and turnover of $A\beta_{46}$ and CTFε, we have attempted to determine the effect of the calpain inhibitor MDL 28170 on the three major APP intramembrane cleavages, namely ε-cleavage, γ-cleavage, and the newly identified ζ-cleavage. First, our results clearly indicate that ζ-cleavage, which produces the intermediate $A\beta_{46}$, plays an important role in the generation of secreted $A\beta_{40/42}$ by the γ-secretase mediated processing of APP. We found that, at early time points (up to 24 h), and at a concentration of 30 μM, MDL 28170 strongly inhibited the formation of secreted $A\beta_{40/42}$ and caused a concomitant increase in the initial substrate CTFβ, without an accumulation of the intermediate $A\beta_{46}$ (Fig. 3*B*). These

observations indicate that the effect of MDL 28170 on the formation of $A\beta_{46}$ and $A\beta_{40/42}$ is different from those of the nontransition state γ-secretase inhibitors, such as compound E and DAPT, both of which inhibit the formation of $A\beta_{40/42}$ by blocking the turnover of $A\beta_{46}$ (15). Furthermore, we found that, at low concentrations, MDL 28170 had no effect on the quick turnover of $A\beta_{46}$ and, at high concentrations, MDL 28170 had only a very weak, if any, inhibitory effect on the turnover of $A\beta_{46}$ (Fig. 4). These observations suggest that MDL 28170 inhibits the formation of $A\beta_{40/42}$, not by blocking the turnover of $A\beta_{46}$, but rather by inhibiting the formation of the intermediate $A\beta_{46}$. In support of this hypothesis, our data clearly demonstrate that MDL 28170 strongly inhibits the formation of $A\beta_{46}$ (Fig. 5). Thus, these observations clearly indicate that the formation of the intermediate $A\beta_{46}$, or in other words, the ζ-cleavage, which produces $A\beta_{46}$, plays an important role in the formation of secreted $A\beta_{40/42}$. It is notable that the ζ-cleavage site at $A\beta_{46}$ is the APP717 mutation site, which is one of the well-characterized AD-linked mutations in APP and the pathogenic role of this mutation has been attributed to its effect on the selective increase in the production of long amyloidogenic $A\beta_{42}$ (25). The fact that the APP717 mutation site happens to be the ζ-cleavage site suggests a possibility that this mutation may cause abnormal Aβ production by altering the ζ-cleavage. Interestingly, a recent study showed that Aβ peptides with C termini ending at Aβ43, Aβ44, Aβ45, and Aβ46 were detectable in the senile plaques of APP(V717F) FAD individuals (26). This observation provides strong support for the idea that the APP717 mutations may affect ζ-cleavage, resulting in the formation of longer Aβ species.

Second, our observation that the effect of MDL 28170 on the formation of Aβ is completely dose and time dependent may provide an answer to the controversial results reported by previous studies regarding the effect of MDL 28170 on the formation of Aβ. Some of the previous studies have shown that MDL 28170 inhibited the formation of both $A\beta_{40}$ and $A\beta_{42}$ (5, 8); another showed that MDL 28170 selectively inhibited the formation of $A\beta_{40}$ and had less effect on formation of $A\beta_{42}$ (6) and a further study showed that, at low concentrations, MDL 28170 increased both $A\beta_{40}$ and $A\beta_{42}$ and had a stronger enhancing effect on the formation of $A\beta_{42}$ (9). In this current work, we have performed comprehensive dose-curve and time-course studies. Our data clearly demonstrate that, at early time points, MDL 28170 inhibits the formation of all of the γ-secretase processing products, including secreted $A\beta_{40/42}$, intermediate $A\beta_{46}$, and CTFε, but causes a concomitant accumulation of CTFβ and CTFα, the initial substrates of γ-secretase (Fig. 3*B*). These results strongly suggest that at early time points, MDL 28170 inhibits γ-secretase mediated APP processing. We also found that, at late time points, the secreted $A\beta_{40/42}$ robustly increased, and this increase was associated with a dramatic decrease in CTFβ and CTFα. It was noted that at the time point of 48 h incubation, both the substrates (CTFβ and CTFα) and the products (the final products $A\beta_{40/42}$ and CTFε and the intermediate product $A\beta_{46}$) were all detectable (lane 8, Fig. 3*B*). Based on these observations, dose-curve experiments were performed at this 48 h time point. Our data clearly demonstrate that, at a low range of concentrations (up to 40 μM), MDL 28170 caused a remarkable increase in both secreted $A\beta_{40}$ and $A\beta_{42}$ and at rates that are not significantly different from each other (Fig. 2*C*). At high concentrations, MDL 28170 caused a rapid decline in both $A\beta_{40}$ and $A\beta_{42}$, again, at similar rates (Fig. 2*C*). Thus, our data clearly demonstrate that the effect, either an enhancing or an inhibiting effect, of MDL 28170 on the formation of secreted $A\beta_{40/42}$, is totally dependent on the dose of the inhibitor used and the duration of treatment. Thus, the conflicting results reported by previous studies may be due to differences in the dose of inhibitor used or differences in duration of treatment of cells with the inhibitor.

Third and more importantly, our data strongly suggest that MDL 28170 inhibits the degradation of Aβ species and other APP derivatives produced by γ-secretase, and this inhibition of the degradation of Aβ is the major factor contributing to the increase in secreted $Aβ_{40/42}$ caused by MDL 28170. Regarding the mechanism underlying the effect of MDL 28170 on the formation of secreted $Aβ_{40/42}$, a previous study has suggested that this effect may be a result of redistribution of APP to the cell surface caused by this inhibitor (9). However, the redistribution of APP is unlikely to be the key factor that leads to the increase in secreted $Aβ_{40/42}$ caused by MDL 28170 in our study. First, our time-course data clearly demonstrate that treatment with MDL 28170 evidently delays the formation of both secreted $Aβ_{40/42}$ and the intermediate $Aβ_{46}$ (Fig. 3). This result indicates that MDL 28170 does not facilitate the formation of $Aβ_{40/42}$, a finding that is in contrast to the model in which MDL 28170 causes redistribution of APP to the cell surface, thus enhancing the production of CTFβ, which, in turn, facilitates the generation of $Aβ_{40/42}$ (9). Specifically, the fact that within the period of the first 24 h culture, a significant amount of $Aβ_{40/42}$ was detected in control cells (Fig. 3*A* and *C*), but $Aβ_{40/42}$ was not detected in cells treated with MDL 28170 (Fig. 3*B* and *D*), strongly indicates that at early time points, the time-dependent increase in CTFβ in cells treated with MDL 28170 is due to the inhibition of γ-secretase mediated processing of CTFβ, which produces $Aβ_{40/42}$. Beyond the 24 h time point, CTFβ starts to rapidly decrease with a concomitant increase in secreted $Aβ_{40/42}$ (Fig. 3*B* and *D*), and this is possibly due to the decay of the inhibitor itself. Second, in agreement with the observation reported in the previous study (9), our comprehensive dose-curve experiment clearly demonstrates that MDL 28170 causes a dose-dependent increase in CTFβ (Fig. 2*B* and *C*). Specifically, at high concentrations (>50 μM), MDL 28170 caused a rapid decline in $Aβ_{40/42}$ and a concomitant increase in CTFβ, strongly indicating that at high concentration, MDL 28170 modulates the production of $Aβ_{40/42}$ by directly affecting the γ-secretase mediated processing of CTFβ.

On the other hand, it was also noticed that the increase in CTFβ caused by MDL 28170 at low range of concentrations (up to 40 μM) was not associated with a decrease but rather an increase in $Aβ_{40/42}$ (Fig. 2*B* and *C*), indicating that inhibition of γ-secretase activity is not the only mechanism by which MDL 28170 increases CTFβ. The other possibility is that, at a low range of concentrations, the MDL 28170-caused increase in CTFβ is the result of the inhibition of the degradation of CTFβ. This inhibition of the degradation of CTFβ, the initial precursor of $Aβ_{40/42}$, may also in turn contribute to the increase in $Aβ_{40/42}$ by increasing the concentration of CTFβ, the substrate of γ-secretase, as suggested by a previous study (10). Nevertheless, as shown in Figs. 2*B* and 3*A*, since a significant amount of CTFβ was detected in the absence of any inhibitors and under the same condition where only a trace amount of $Aβ_{46}$ was detected, it is unlikely that the formation of CTFβ is the rate-limiting step in the formation of secreted Aβ. Therefore, inhibiting the degradation of CTFβ, the initial precursor of $Aβ_{40/42}$, may not be the major means by which, at low concentrations, MDL 28170 causes an increase in secreted $Aβ_{40/42}$. The other possibility, and a more likely one, is that MDL 28170 may also cause an increase in secreted $Aβ_{40/42}$ by directly inhibiting its degradation. In this regard, it is notable that our data suggest that MDL 28170 inhibits the degradation of all APP derivatives produced during proteolytic processing. For example, at concentrations >40 μM, MDL 28170 indeed inhibits the formation of CTFε from CTFβ and CTFα (lanes 7 and 8, Fig. 6) in a cell free system, strongly suggesting that the increase in CTFε caused by MDL 28170 at these concentrations in living cells (Fig. 2*B*) is not due to the inability of MDL 28170 to inhibit the formation of CTFε. Rather, and more likely, the increase in CTFε is due to the ability of MDL 28170 to inhibit the degradation of CTFε produced by the

residual γ-secretase activity after the addition of MDL 28170, as well as the CTFε produced before the addition of MDL 28170. This possibility is also supported by the fact that in the absence of inhibitors, only a very small amount of CTFε can be detected in living cells (lane 1 of Figs. 2*B* and 5*B*), indicating a quick turnover of CTFε under normal conditions.

In addition, in contrast to the two- to threefold increase in $A\beta_{42}$ and <1.5 fold increase in $A\beta_{40}$ reported in the previous study (9), our data from this study reveal that MDL 28170 can dramatically increase $A\beta_{40}$ formation by 18-fold and increase $A\beta_{42}$ by 20-fold (Fig. 2*C*). One possibility is that these inconsistencies are a result of using different cell lines and conditions of culture and treatment of cells. We cultured cells in a medium containing 0.5% FBS. As discussed above, our results strongly suggest that the MDL 28170-caused increase in Aβ is most likely due to its inhibiting the degradation of Aβ and other APP derivatives. When cells are cultured in media containing high concentration of FBS, which contains many kinds of protease inhibitors, Aβ may also be protected from degradation by these protease inhibitors contained in the FBS. Thus, the inhibitory effect of MDL 28170 on the degradation of Aβ may be shadowed by that of the protease inhibitors contained in the FBS and this may lead to the underestimation of the extent of the increase in Aβ by MDL 28170.

In summary, our data presented in this study clearly revealed that, at early time points and high concentrations, MDL 28170 inhibits the formation of secreted $A\beta_{40/42}$ by inhibiting the γ-secretase-mediated CTFβ processing, specifically, by inhibiting the formation of the intermediate $A\beta_{46}$ produced by ζ-cleavage. This observation strongly suggests that $A\beta_{46}$ is the rate-limiting proteolytic intermediate in Aβ generation. Our data also strongly suggest that, at late time points and low range of concentrations, MDL 28170 causes an increase in secreted $A\beta_{40/42}$, mainly by inhibiting its degradation. In support of this hypothesis, it was found that at the range of concentrations used in this study, MDL 28170 strongly inhibits calpain activity (Fig. 7), suggesting that calpain protease may be involved in the degradation or clearance of Aβ in living cells. Thus, the knowledge obtained from this study would contribute to a better understanding of the mechanism by which γ-secretase produces Aβ from APP and the mechanism underlying the effect of calpain inhibitor MDL 28170 on the formation of secreted $A\beta_{40/42}$. The information obtained from this study would also be important to the study of γ-secretase inhibitor development and Aβ clearance.

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Fig. 1

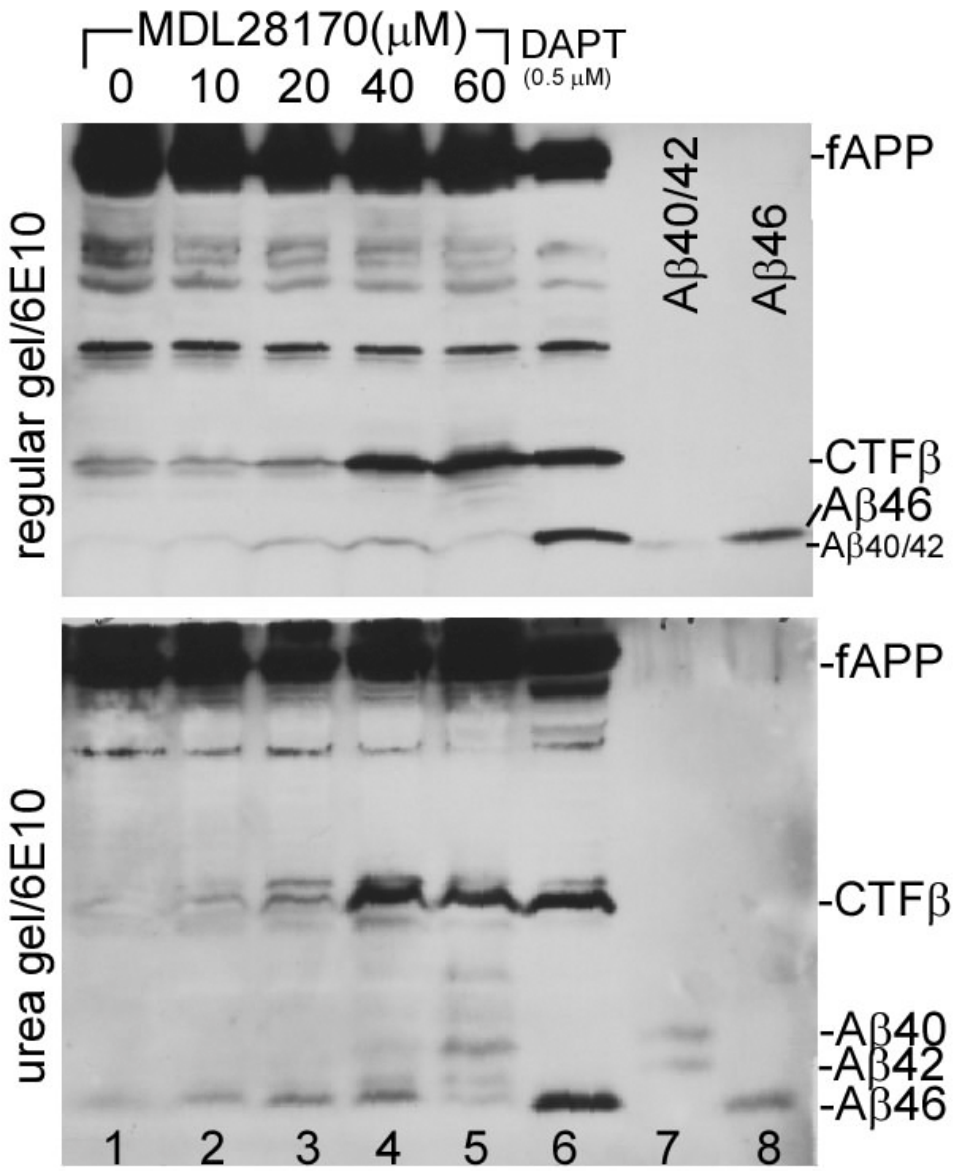

Figure 1. At low concentrations, MDL 28170 causes a slight increase in intracellular accumulation of $A\beta_{46}$. Lane 1: control cells treated with vehicle Me_2SO. Lanes 2–5: cells treated with MDL 28170 at indicated concentrations. Lane 6: positive control of cells treated with DAPT. Lane 7: mix of synthetic $A\beta_{40}$ and $A\beta_{42}$; Lane 8, synthetic $A\beta_{46}$. Aβ species and CTFβ were probed using 6E10, a monoclonal antibody that recognizes residues 1-17 of human Aβ (27). ***Upper panel***) 10–18% SDS-PAGE. ***Bottom panel***) 10% urea-SDS-PAGE. As shown in ***bottom panel***, $A\beta_{40/42}$ can be clearly separated from $A\beta_{46}$ by a 10% urea-SDS-PAGE system, which separates Aβ peptides based on their hydrophobicity. The longer and more hydrophobic Aβ peptides run at a faster migration rate than shorter Aβ peptides do (28).

Fig. 2

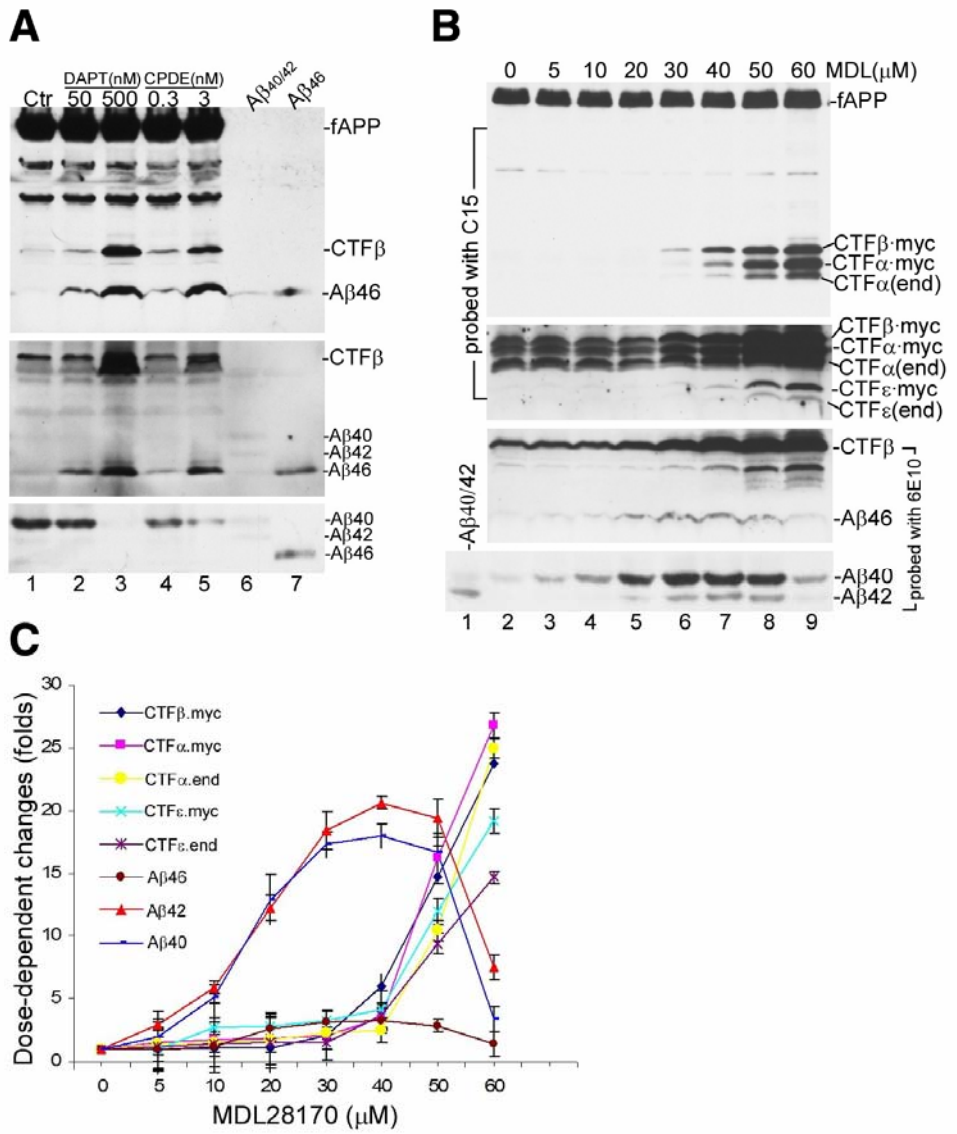

Figure 2. MDL 28170 causes accumulation of intracellular $A\beta_{46}$ by a mechanism different from that of DAPT and compound E. ***A)*** DAPT and compound E cause accumulation of intracellular $A\beta_{46}$ by inhibiting formation of secreted $A\beta_{40/42}$. Lane 1: control cells treated with vehicle Me_2SO. Lanes 2 and 3: cells treated with DAPT for 12 h at 50 and 500 nM, respectively. Lanes 4 and 5: cells treated with compound E (CPDE) for 12 h at 0.3 and 3 nM, respectively. Lane 6: mix of synthetic $A\beta_{40}$ and $A\beta_{42}$. Lane 7: synthetic $A\beta_{46}$. ***Top panel*)** Cell lysates analyzed by 10-18% SDS-PAGE. ***Middle panel*)** Cell lysates analyzed by 10% urea-SDS-PAGE. ***Bottom panel*)** Secreted $A\beta_{40/42}$ immunoprecipitated from conditioned media analyzed by 10% urea-SDS-PAGE. All blots in ***A*** were probed with 6E10. As shown in lanes 6 and 7, $A\beta_{40/42}$ can be clearly separated from $A\beta_{46}$ by 10% urea gel. ***B)*** MDL 28170 causes a slight increase in intracellular accumulation of $A\beta_{46}$ without inhibition of formation of secreted $A\beta_{40/42}$. Cells were treated with MDL 28170 for 48 h at different concentrations. Lane 1: mix of synthetic $A\beta_{40}$ and $A\beta_{42}$. Lane 2: control cells treated with vehicle Me_2SO. Lanes 3–9: cells treated with MDL 28170 at different concentrations as indicated. ***Top panel*)** Cell lysates analyzed by 10–18% SDS-PAGE and probed with APP-C-terminal specific antibody C15. ***Second panel*)** Prolonged exposure of same blot in ***top panel***. ***Third panel*)** Cell lysates analyzed by 10–18% SDS-PAGE and probed with 6E10. ***Bottom panels*)** Secreted $A\beta_{40/42}$ immunoprecipitated from conditioned media analyzed by 10% urea-SDS-PAGE and probed with 6E10. Note, CTFs generated from exogenous APP, which is expressed with a myc tag fused to its C-terminal, were designated as CTFβ•myc, CTFα•myc, and CTFε•myc respectively; CTFs generated from endogenous APP were designated as CTFβ(end), CTFα(end), and CTFε(end), respectively, as described in previous study (21). ***C)*** Data graphically represent average of results of at least 3 repeated experiments shown in ***B***. Dose-dependent effects of MDL 28170 on formation of APP derivatives generated during APP processing are presented by folds of changes in comparison with control of untreated cells.

Fig. 3

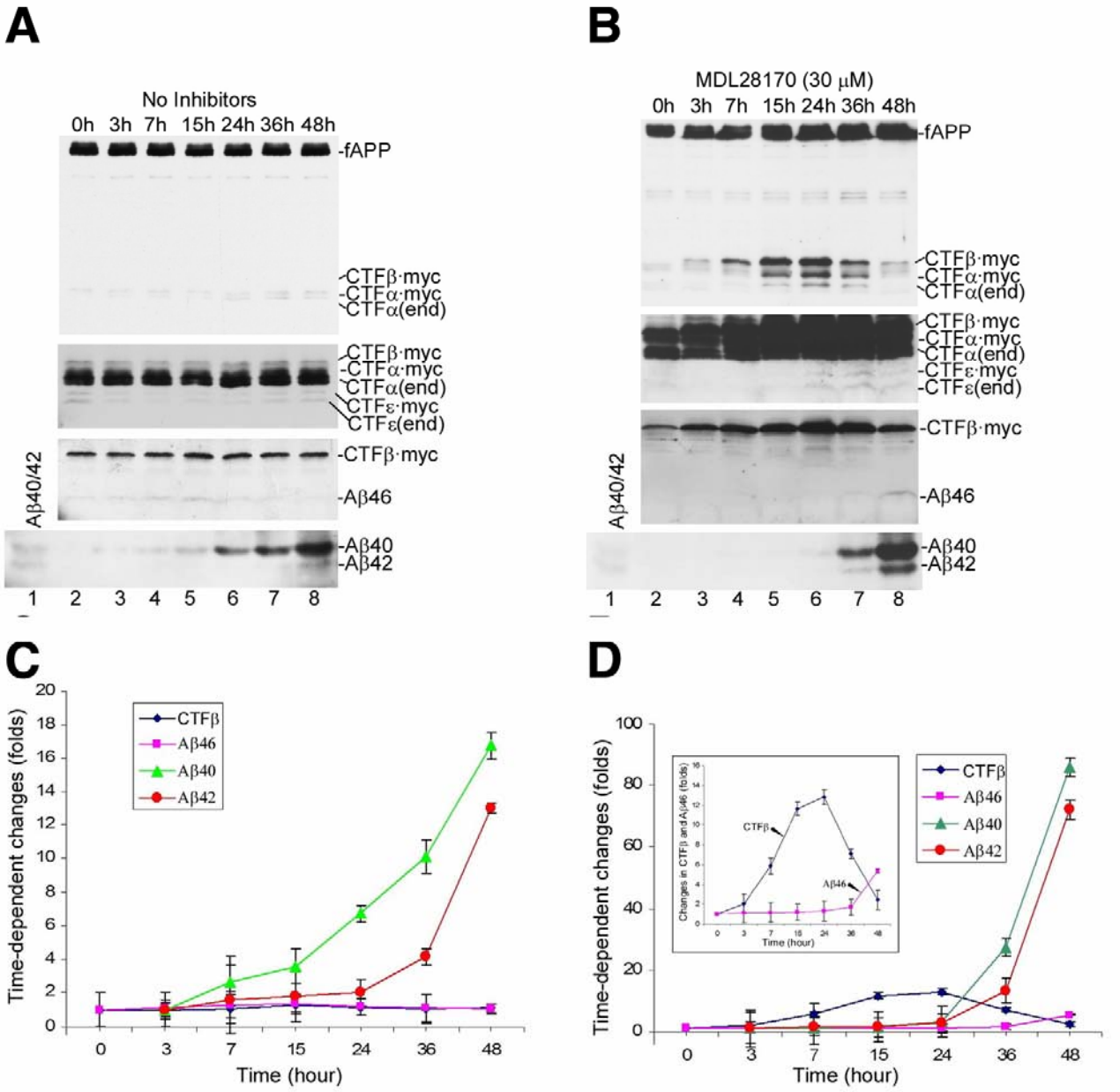

Figure 3. Time course of formation of $A\beta_{46}$ and $A\beta_{40/42}$ in absence or presence of MDL 28170. ***A)*** Time course of formation of $A\beta_{46}$ and $A\beta_{40/42}$ in control cells treated with the vehicle Me_2SO only. Lane 1: mix of synthetic $A\beta_{40}$ and $A\beta_{42}$. ***Top panel*)** Cell lysates analyzed by 10–18% SDS-PAGE and probed with C15. ***Second panel*)** Prolonged exposure of ***top panel***. ***Third panel*)** Cell lysates analyzed by 10–18% SDS-PAGE and probed with 6E10. ***Bottom panels*)** Secreted $A\beta_{40/42}$ immunoprecipitated from conditioned media analyzed by 10% urea-SDS-PAGE and probed with 6E10. ***B)*** Time course of formation of $A\beta_{46}$ and $A\beta_{40/42}$ in cells treated with MDL 28170. Lane 1: mix of synthetic $A\beta_{40}$ and $A\beta_{42}$. Lanes 2–9: cells treated with MDL 28170 and incubated for different time length. ***Top panel*)** Cell lysates analyzed by 10–18% SDS-PAGE and probed with C15. ***Second panel*)** Prolonged exposure of ***top panel***. ***Third panel*)** Cell lysates analyzed by 10–18% SDS-PAGE and probed with 6E10. ***Bottom panel*)** Secreted $A\beta_{40/42}$ immunoprecipitated from conditioned media analyzed by 10% urea-SDS-PAGE and probed with 6E10. ***C)*** Data graphically represent average of results of at least 3 repeated experiments shown in ***A***. ***D)*** Data graphically represent average of results of at least 3 repeated experiments shown in ***B***. Time-dependent effects of MDL 28170 on formation of intracellular CTFβ and $A\beta_{46}$ are presented by folds of changes in comparison with that at initiating time (0). Time-dependent effects of MDL 28170 on formation of $A\beta_{40}$, and $A\beta_{42}$ are presented by folds of changes in comparison with that at 3 h time point at which point secreted Aβ becomes detectable by Western blotting. Insert in ***D*** is replot of changes in CTFβ and $A\beta_{46}$ in an appropriate scale.

Fig. 4

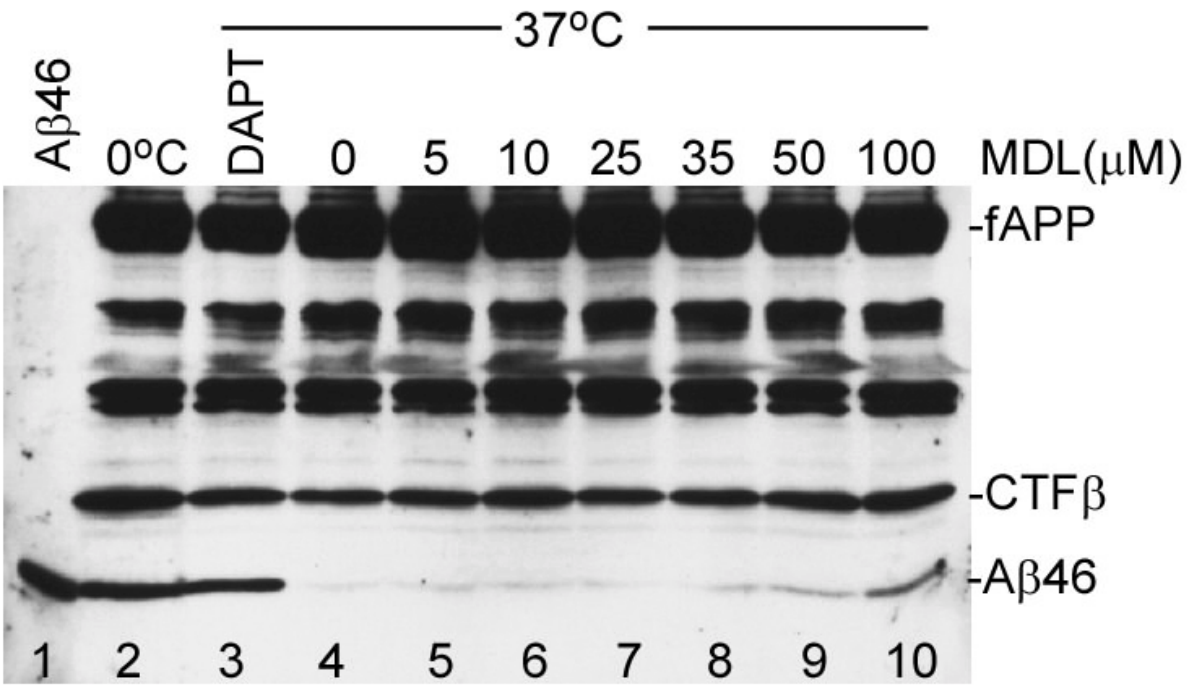

Figure 4. At a low range of concentrations, MDL 28170 has no effect on turnover of $A\beta_{46}$. Lane 1: synthetic $A\beta_{46}$. Lane 2: control membrane incubated at 0°C. Lane 3: membrane incubated at 37°C in the presence of DAPT. Lanes 4–10: cells treated with MDL 28170 at different concentrations as indicated. Samples were analyzed by 10–18% SDS-PAGE and probed with 6E10.

Fig. 5

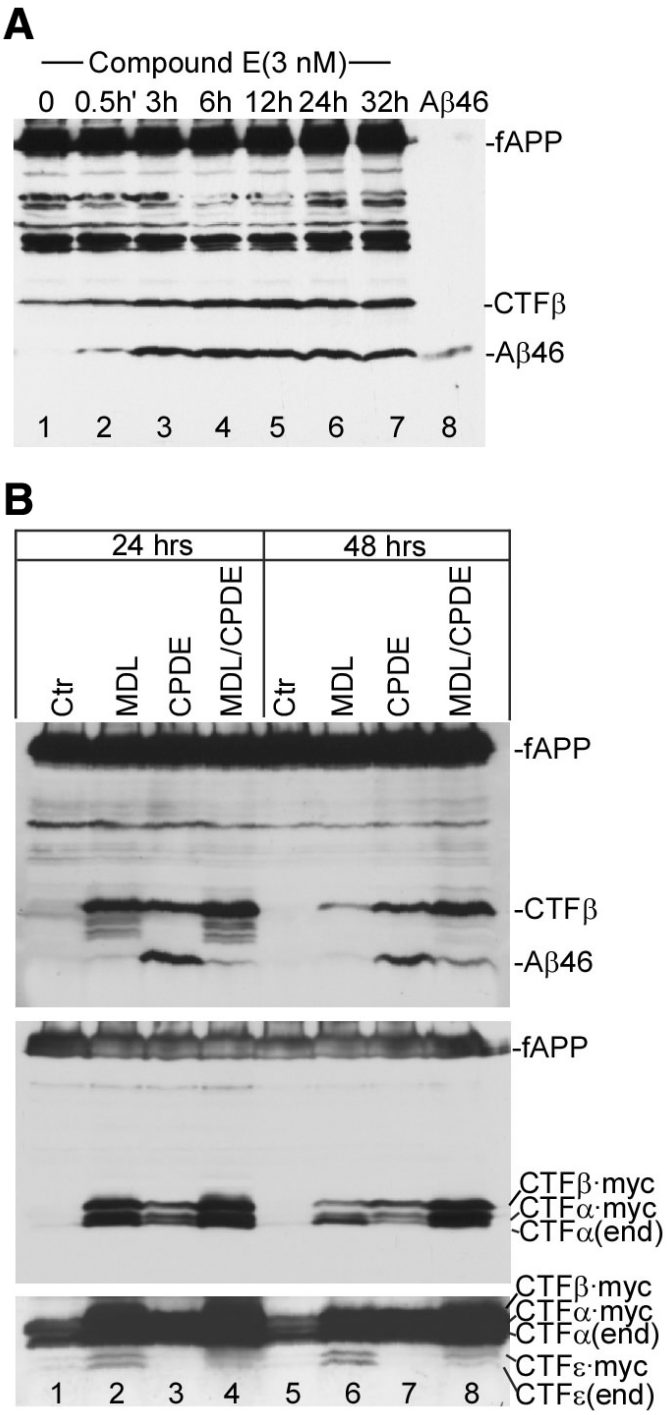

Figure 5. MDL 28170 inhibits formation of $A\beta_{46}$. ***A*)** Time course of formation of $A\beta_{46}$. Lane 1: control cells treated with vehicle Me_2SO. Lanes 2–7: cells treated with 3 nM compound E, which prevents turnover of $A\beta_{46}$, for different time lengths. Cell lysates were analyzed by 10–18% SDS-PAGE and probed with 6E10. Lane 8: synthetic $A\beta_{46}$. ***B*)** MDL 28170 inhibits formation of $A\beta_{46}$ from CTFβ. Lanes 1 and 5: control cells treated with vehicle Me_2SO. Lanes 2 and 6: cells treated with MDL 28170. Lanes 3 and 7: cells treated with compound E (3 nM, CPDE). Lanes 4 and 8: cells treated with both compound E (3 nM) and MDL 28170 (30 μM). ***Top panel*)** Cell lysates analyzed by 10–18% SDS-PAGE and probed with 6E10. ***Middle panel*)** Cell lysates analyzed by 10–18% SDS-PAGE and probed with C15. ***Bottom panel*)** Prolonged exposure of ***middle panel***.

Fig. 6

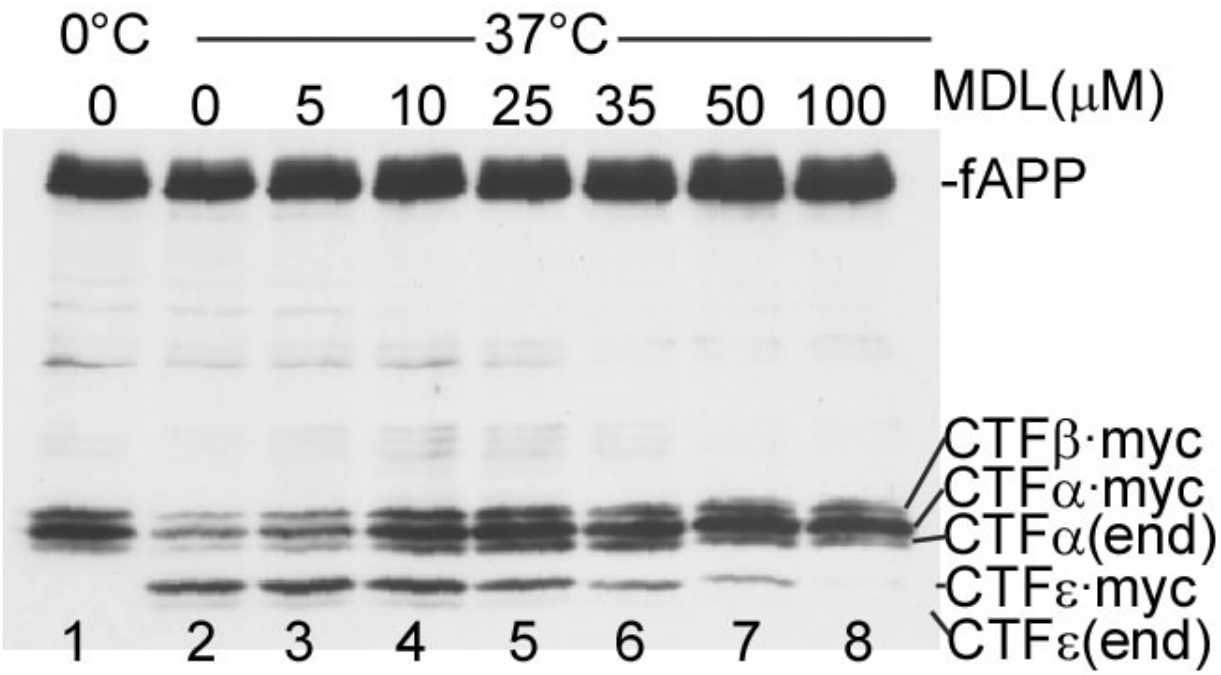

Figure 6. MDL 28170 inhibits de novo generation of CTFε in a cell-free system. Lane 1: control membrane incubated at 0°C. Lanes 2–8: membranes incubated at 37°C in the presence of different concentrations of MDL 28170 as indicated. Samples were analyzed by 10–18% SDS-PAGE and probed with C15.

Fig. 7

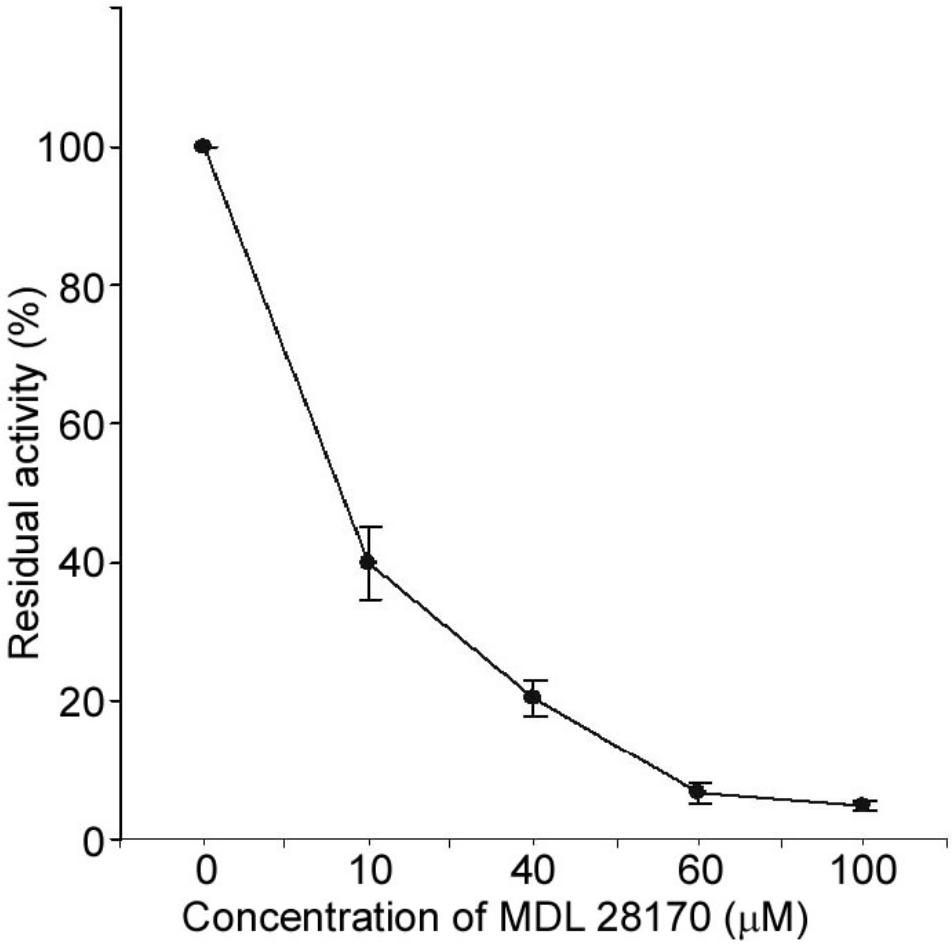

Figure 7. MDL 28170 inhibits calpain activity in living cells. Effect of MDL 28170 on in vivo calpain activity was measured using the fluorescent calpain substrate Suc-Leu-Leu-Val-Tyr-AMC. Inhibitory effects of MDL 28170 on calpain activity are expressed as percentage of control (cells cultured in the absence of inhibitor), considering control as 100%. Data are average of results of 3 repeated experiments.