Differential Effects of Inhibitors on the γ-Secretase Complex

MECHANISTIC IMPLICATIONS*

Received for publication, January 16, 2003, and in revised form, March 13, 2003 Published, JBC Papers in Press, March 18, 2003, DOI 10.1074/jbc.C300019200

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γ-Secretase is a protease complex of four integral membrane proteins, with presenilin (PS) as the apparent catalytic component, and this enzyme processes the transmembrane domains of a variety of substrates, including the amyloid β-protein precursor and the Notch receptor. Here we explore the mechanisms of structurally diverse γ-secretase inhibitors by examining their ability to displace an active site-directed photoprobe from PS heterodimers. Most γ-secretase inhibitors, including a potent inhibitor of the PS-like signal peptide peptidase, blocked the photoprobe from binding to PS1, indicating that these compounds either bind directly to the PS1 site or alter it through an allosteric interaction. Conversely, some reported inhibitors failed to displace this interaction, demonstrating that these compounds do not interfere with the protease by affecting its active site. Differential effects of the inhibitors with respect to photoprobe displacement and in cell-based and cell-free assays suggest that these compounds are important mechanistic tools for deciphering the workings of this intramembrane-cleaving protease complex and its similarity to other polytopic aspartyl proteases.

Cerebral accumulation of the amyloid-β protein (Aβ)† is considered a central event in the pathogenesis of Alzheimer’s disease (AD). Aβ is produced via β- and γ-secretase proteases that have become important therapeutic targets for AD (1). γ-Secretase plays a crucial role in determining the proportion of two forms of Aβ, Aβ40 and Aβ42. The 42-residue Aβ42 is more prone to fibril formation and is disproportionately present in the plaques characteristic of the AD brain. Accumulating evidence (2–4) strongly suggests that γ-secretase is an intramembrane-cleaving aspartyl protease with presenilin (PS) as the catalytic component. Three other multipass membrane proteins, nicastrin, Aph-1, and Pen-2, are genetically linked to γ-secretase activity (5–7), and biochemical isolation has provided evidence that these proteins are indeed necessary members of the protease complex (8–10). Despite the remarkable progress in uncovering the identity of γ-secretase, its mechanism of action remains unclear.

A body of work (11) suggests that γ-secretase cleaves amide bonds within the transmembrane regions of its substrates, a poorly understood process of hydrolysis within a hydrophobic environment. Elucidating the molecular interaction between an inhibitor and its enzyme target can help identify the enzyme and provide insight into the catalytic mechanism. The study of peptidomimetic inhibitors of γ-secretase that contain classic aspartyl protease transition state-mimicking moieties led to the suggestion that γ-secretase is an aspartyl protease and that the conserved aspartates in presenilins are catalytic residues (12). PS is processed into N-terminal (NTF) and C-terminal (CTF) fragments. These fragments are metabolically stable, remain associated, and their formation is tightly regulated, suggesting that together they are the bioactive form of PS (12). The direct binding of transition state analog γ-secretase inhibitors to these fragments strongly suggests that the active site is at the NTF/CTF heterodimeric interface (3, 4), consistent with the fact that each subunit contributes one of the two critical aspartates (2).

The recent discovery of structurally diverse inhibitors suggests that these new compounds may also be important molecular probes for the protease complex. Several non-transition state analog inhibitors are very potent; however, unlike transition state analogs, their site(s) of interaction within the γ-secretase complex is unclear. To probe the mechanism of γ-secretase inhibitor action, we studied the ability of structurally diverse compounds to displace a transition state-based photoactive molecule from its target, the γ-secretase active site at the PS1 NTF/CTF interface. Differential effects of these compounds suggest that they inhibit γ-secretase by distinct mechanisms and are thus important new probes to elucidate the workings of this complex protease. Moreover, the ability of a transition state analog inhibitor of signal peptide peptidase (SPP), a multipass membrane aspartyl protease with presenilin-like motifs (13, 14), to displace the γ-secretase photoprobe suggests that the active site topographies of these two proteases are similar.

EXPERIMENTAL PROCEDURES

Compound Synthesis—Photoprobe III-63 and analog III-31-C were prepared using methods described previously (8). JLK2 and JLK6 were synthesized as described previously. Boc-Lys(Cbz)-OH (Bachem) instead of Boc-Lys(Cbz)-OH. All D-peptide Boc-D-Val-Gly-Val-epoxide was synthesized using a procedure similar to the one reported before (18), only employing Boc-Lys(Cbz)-OH. The cell-free assay was performed as reported previously (19). The cell-free assay was performed as reported previously (19).

Production—

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TABLE I

Table: Chemical structures, inhibition properties, and the displacement ability of γ-secretase inhibitors used in the study

In cells IC₅₀ values for inhibition of Aβ production were determined using Chinese hamster ovary cells stably expressing human APP. Cell-free IC₅₀ values were measured using solubilized γ-secretase prepared from HeLa cells and a recombinant APP-based (C100FLAG) substrate (see Fig. 1). Displacement indicates the ability of the inhibitor to prevent the transition state-based photolabel from binding to PS1 heterodimer (see Fig. 3).

| Compound       | Structure | In cells | Cell-free | Displacement | Type          |
|----------------|-----------|----------|-----------|--------------|---------------|
| III-31-C       |           | 0.2 µM   | 10 nM     | YES          | Transition state |
| DAPT           |           | 20 nM    | 10 nM     | YES          | Non-transition state |
| Compound E     |           | 0.3 µM   | 3 nM      | YES          | Non-transition state |
| Isocoumarin    |           | 80 µM    | >200 µM   | NO           | Non-γ-secretase  |
| D-Helical peptide 294 | | 3 µM     | 0.1 µM    | NO           | Substrate mimic |
| Epoxide        |           | 20 µM    | 20 µM     | YES          | Irreversible   |
| (Z-LLL)γ-ketone, a SFPI inhibitor | | >100 µM  | 30 µM     | YES          | Apoptotic protease inhibitor |


designed in Table I. In cell-free assays CHAPSO-solubilized HeLa cells stably transfected with human APP were used to evaluate the ability of these inhibitors to displace the photoprobe from its molecular target. All subsequent competition experiments were performed in cell lysates, because this method requires smaller amounts of initial cellular material, but yields similar results. We used competitor concentrations equal to 25 times their IC₅₀ value in vitro. The latter was chosen to compare the degree of displacement with the positive control of displacement by III-31-C at such a concentration (250 nM, 25 times above the 10 nM IC₅₀ value).

Effects of DAPT and Compound E—Several reported inhibitors were identified from small molecule library screening and subsequent optimization, but the means by which they inhibit the protease are unclear. For example, the dipeptide DAPT (Table I) is a non-transition state analog, but nevertheless a very potent inhibitor, that substantially decreased Aβ levels in the brains of APP transgenic mice. In our hands, this compound inhibited Aβ production with an IC₅₀ of 10 nM in the cell-free γ-secretase assay and with an IC₅₀ of 20 nM in APP-transfected cells. After addition of DAPT as a competitor to photolabel III-63, exhibited an in vitro IC₅₀ value similar to the parent compound, indicating that the modifications did not affect the ability of the compound to bind the γ-secretase active site.

Compound III-63 was used for photolabeling of PS1 under in vitro conditions that preserve γ-secretase activity. We used lysates as well as microsomes isolated from HeLa cells by lysing and/or solubilizing with the detergent CHAPSO. The labeled species were precipitated with streptavidin beads and analyzed by Western blot, detecting with anti-PS1 antibodies. The observed biotinylated proteins, a major band at about 21 kDa and a minor band at about 31 kDa, were identified as PS1 CTF and PS1 NTF, respectively (Fig. 2, lane 1). These results strongly suggest that the III-63 photolabel directly binds to the interface of PS1 heterodimers. This observation is essentially the same as seen by Li et al. (4), who used a nearly identical photoprobe and labeled the CTF of PS1 exclusively. Apparently, our closely related compound can also label small amounts of NTF. When the unbiotinylated parent compound III-31-C (250 nM) was used as a competitor, no labeled PS1 NTF and PS1 CTF species were detected (Fig. 2, lane 2). No labeled proteins were detected when irradiation was not applied (data not shown). These observations demonstrated specific labeling of PS1 heterodimers by III-63. This direct photoprobe binding provided a means of assessing the mechanism of structurally diverse γ-secretase inhibitors by testing the ability of these inhibitors to displace the photoprobe from its molecular target. All subsequent competition experiments were performed in cell lysates, because this method requires smaller amounts of initial cellular material, but yields similar results.
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Fig. 2. Covalent labeling of PS1 by photoactivatable active site-directed γ-secretase inhibitor III-63. III-63 (50 nM) was incubated with HeLa lysates in the absence or presence of III-31-C (250 nM), and the samples were irradiated at 350 nm. Biotinylated proteins were precipitated with immobilized streptavidin and detected by SDS-PAGE/immunoblotting using antibodies to PS1-NTF and PS1-CTF. The asterisk denotes a nonspecifically labeled protein.

Fig. 3. Probing the effect of γ-secretase inhibitors on its active site. A, the photoactivatable and biotinylated inhibitor III-63 (50 nM) was incubated with HeLa lysates in the absence or presence of III-31-C (250 nM). DAPT (250 nM and 2 μM), Compound E (75 and 600 nM) and irradiated at 350 nm. Biotinylated proteins were precipitated with immobilized streptavidin and probed by SDS-PAGE/immunoblotting using antibodies to the PS1-CTF. The same experiment was performed in the presence of JLKs (500 μM) and helical peptide 294 (6 μM) (B), epoxide (250 μM, no preincubation and 2 h preincubation at 37°C (C), and (Z)-LL-ketone (200 μM) (D). Each panel shows the results from a single scan of a single blot.

When 2 μM DAPT (200 times the IC50) was used (Fig. 3A). This observation that DAPT prevents labeling with a transition state analog affinity reagent suggests that DAPT either directly binds to the active site between PS1 heterodimers or alters it through an allosteric interaction (20). Interestingly, DAPT does not displace the photoprobe as well as III-31-C does at the same relative concentration (25 times the IC50), despite being essentially equivalent inhibitors in the solubilized γ-secretase assay. This intriguing observation suggests that transition state analog III-31-C and non-transition state analog DAPT act partially, but not completely, overlapping sites. Similar results were obtained with Compound E, a non-transition state analog inhibitor containing a benzodiazepine moiety. In our assays, this compound inhibited Aβ production with an IC50 of 3 nM in microsomes and with an IC50 of 0.3 nM in APP-transfected cells. After addition of Compound E as a competitor to photoprobe, the level of labeled species was reduced somewhat (Fig. 3A) at a competitor concentration of 75 nM (25 times the IC50), and almost no labeled species were detected when 600 nM (200 times the IC50) Compound E was used.

Isocoumarin-based Compounds—Isocoumarin “JLK” compounds (Table I) reported by Petit et al. (15) were also examined in the displacement assay. These compounds were reported to inhibit Aβ production in cells without affecting proteolysis of the Notch receptor. The Notch signaling pathway is involved in cell fate decisions, and presenilin linkers are linked to proteolysis of the transmembrane region of the Notch receptor as a key step in the signaling mechanism (22). As we reported previously (23), we confirmed that JLK compounds lower Aβ production in cell culture with an IC50 of 80 μM; however, the isocoumarins failed to block γ-secretase activity in our cell-free assays. Moreover, we observed no change in the degree of PS1 heterodimers labeling by the photoprobe (Fig. 3B) when the most active analogs, JLK2 and JLK6, were used as the competitors at concentrations as high as 0.5 mM. This observation indicates that JLKs fail to block the interaction of III-63 with its molecular target, suggesting that these compounds are not likely interacting with or otherwise affecting the active site of γ-secretase. Taken together, our evidence implies that the isocoumarins probably work upstream of γ-secretase.

Aib-containing Peptides—We also examined APP-based peptides designed to assume a helical conformation and mimic the APP transmembrane domain upon initial interaction with γ-secretase. Thus, these compounds were designed to inhibit the protease by a different mechanism than transition-state analogs do. Specifically, these peptides are based on the sequence of the APP transmembrane domain, modified with the helix promoting residue Aib. One of the most potent helical peptides, n-294 (Table I), displays IC50 values of 3 μM in cells and 100 nM in the in vitro assay (Fig. 1). When this peptide was used as a competitor to the photoprobe at concentrations as high as 6 μM (60 times IC50), it did not affect the photoprobe binding to PS1 heterodimers (Fig. 3B). When n-294 was used at 100 μM (1000 times IC50), little or no change in the degree of labeling was observed (data not shown). Therefore, despite the fact that this helical peptide directly inhibits γ-secretase activity, it does not interact with or otherwise affect the active site. These observations are consistent with the helical peptide inhibitor competing with the substrate for a separate initial docking site (8).

Epoxide Peptidomimetic—Another class of γ-secretase inhibitor with a distinct structure are epoxide-containing molecules. A number of small epoxide molecules have been identified as irreversible inhibitors of aspartyl proteases that act by alkylating the catalytic aspartates (24). One such molecule (18) was reported by Golde et al. to be an inhibitor of γ-secretase, an unconventional aspartyl protease. We synthesized a similar epoxide (Table I) and analyzed its inhibitory properties toward γ-secretase. This epoxide inhibited γ-secretase activity in the cell-based assay (IC50 of 20 μM) and in the cell-free assay (IC50 of 20 μM) (Fig. 1). When tested in the displacement assay under standard conditions, it did not prevent the photoprobe from binding to the active site (Fig. 3C). However, when the epoxide was pre-incubated with lysate for 2 h before the addition of photoprobe and irradiation, much less photolabeling was observed. Such time-dependent displacement was not observed with compound III-31-C. This result suggests that the epoxide is a mechanism-based inhibitor, inactivating γ-secretase by irreversible binding to the active site.

SPP Inhibitor—Finally, we tested (Z)-LL-ketone (Table I), an aspartyl protease transition state analog inhibitor of SPP, which is a recently discovered presenilin-like aspartyl protease (13). We found this compound to be only moderately active in the γ-secretase cell-free assay, with an IC50 value of 30 μM (Fig. 1). The (Z)-LL-ketone similarly inhibited the formation of the other (FLAG-tagged) proteolytic product generated in this assay (data not shown). When tested in the displacement assay, 200 μM (Z)-LL-ketone (only seven times IC50) completely pre-
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We also found that an SPP inhibitor displays pharmacological crossovers, blocking γ-secretase activity as well. Sequence homology between PS and an entire family of so-called “PS homologs,” which includes SPP, is found primarily at the transmembrane motifs YD and LGLGD that contain the critical and conserved aspartates (14). (Z)-1-(2)-ketone is a transition state analog that directly interacts with SPP (15). By showing that this compound can inhibit γ-secretase and compete for the active site on PS, we provide evidence that γ-secretase and SPP have similar active site analogs and likely share the same proteolytic mechanism. Other than the short, conserved aspartate-containing motifs, SPP and PS share very little homology, suggesting that the two proteins arrived at their aspartyl protease mechanisms via independent evolutionary paths.

Finally, analyzing an epoxide inhibitor in our displacement assay revealed that this molecule affects the active site of γ-secretase in a time-dependent manner. The epoxide may inactivate γ-secretase by irreversible binding to the active site aspartates due to its chemical properties. Further work will focus on studying this interaction in detail, as epoxides might be very good probes for the active site of γ-secretase or other unknown intramembrane aspartate proteases awaiting a discovery.

Acknowledgments—We thank S. Gandy for AB14 antibody; W. T. Kimberly, W. P. Edel, and M. LaVoie for helpful discussions; and I. Buldyrev and T. S. Diehl for expert technical assistance.

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J. Biol. Chem. 2003, 278:16470-16473.
doi: 10.1074/jbc.C300019200 originally published online March 18, 2003

Access the most updated version of this article at doi: 10.1074/jbc.C300019200

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