**EVIDENCE FOR AN ALLOSTERIC MECHANISM**

**Selected Non-steroidal Anti-inflammatory Drugs and Their Derivatives Target γ-Secretase at a Novel Site**

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γ-Secretase is a multi-component enzyme complex that performs an intramembranous cleavage, releasing amyloid-β (Aβ) peptides from processing intermediates of the β-amyloid precursor protein. Because Aβ peptides are thought to be causative for Alzheimer’s disease, inhibiting γ-secretase represents a potential treatment for this neurodegenerative condition. Whereas inhibitors directed at the active center of γ-secretase inhibit the cleavage of all its substrates, certain non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to selectively reduce the production of the more amyloidogenic Aβ(1–42) peptide without inhibiting alternative cleavages. In contrast to the majority of previous studies, however, we demonstrate that in cell-free systems the mode of action of selected NSAIDs and their derivatives, depending on the concentrations used, can either be classified as modulatory or inhibitory. At modulatory concentrations, a selective and, with respect to the substrate, noncompetitive inhibition of Aβ(1–42) production was observed. At inhibitory concentrations, on the other hand, biochemical readouts reminiscent of a non-selective γ-secretase inhibition were obtained. When these compounds were analyzed for their ability to displace a radiolabeled, transition-state analog inhibitor from solubilized enzyme, noncompetitive antagonism was observed. The allosteric nature of radioligand displacement suggests that NSAID-like inhibitors change the conformation of the γ-secretase enzyme complex by binding to a novel site, which is discrete from the binding site for transition-state analogs and therefore distinct from the catalytic center. Consequently, drug discovery efforts aimed at this site may identify novel allosteric inhibitors that could benefit from a wider window for inhibition of γ (42)-cleavage over alternative cleavages in the β-amyloid precursor protein and, more importantly, alternative substrates.

According to the “amyloid cascade hypothesis” an enhanced production or decreased clearance of toxic amyloid-β (Aβ)1 pepti
des is thought to be the cause of Alzheimer’s disease (AD) (1). Aβ peptides are processing products (2) of the type I transmembrane protein β-amyloid precursor protein (βAPP) (3), which has undergone sequential cleavages by β- and γ-secretase enzymes. A common denominator (reviewed by Hardy (4)) for mutations causative of familial AD (FAD) has been revealed, being abnormalities in the metabolism of βAPP that appear to lead to an elevation of the production of the Aβ(1–42) peptide species. This C-terminally elongated Aβ peptide is more prone to aggregation than the shorter and more abundant Aβ(1–40) species. Consequently, the prevention of Aβ production by inhibiting either of the proteases required for processing of βAPP is currently viewed as a promising approach toward a therapy for AD. The membrane-bound aspartyl protease β-site βAPP-cleaving enzyme 1 (5, 6) is the major β-secretase required for the generation of Aβ peptides. β-Site βAPP-cleaving enzyme 1 has been shown to cleave within the βAPP ectodomain to generate membrane-bound βAPP processing intermediates (βAPP C-terminal fragments), a prerequisite for the release of Aβ peptide by γ-secretase as the final processing step. γ-Secretase appears to be an unusual aspartyl protease with loose substrate specificity (7) and cleaves its substrates approxi-
mately in the middle of their transmembrane domains at a specific position relative to the membrane bilayer (8). Intramembranous substrate cleavage at multiple positions leads to the generation of Aβ(1–42) and Aβ(1–40) and various C-terminally truncated Aβ peptides (9, 10). Presenilin 1 and 2 (11, 12) are homologous polytopic membrane proteins that are the most likely candidate polypeptides to form the catalytic center of γ-secretase. Mutations in presenilins account for the majority of cases of familial AD (13); combined with results obtained from presenilin knock-out (14), mutagenesis (15), and photoaffinity labeling studies (16–18), compelling evidence for a catalytic function of presenilins has been generated. Appar-
ently, presenilins alone cannot mediate γ-secretase activity and require co-expression, together with the transmembrane proteins nicastrin (19), anterior pharynx defective 1 (20), and presenilin enhancer 2 (21), to reconstitute γ-secretase activity (22–24).

Considerable effort has been put into the development of potent γ-secretase inhibitors for the treatment of AD because they have the potential to block the generation of all amyloidogenic peptides from βAPP-derived substrates (10). This class of compound suffers from the disadvantage that it does not

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4 The abbreviations used are: Aβ, amyloid-β; AD, Alzheimer’s disease; βAPP, β-amyloid precursor protein; NSAID, non-steroidal anti-inflammatory drug; CHAPS, 3-(3-cholamidopropyl)dimethyl-
ammonio)-1-propanesulfonate; AICD, βAPP intracellular domain.
discriminate between the cleavage of alternative substrates for γ-secretase, such as the Notch receptor (25, 26). Consequently, it has been demonstrated that γ-secretase inhibitors interfere with the Notch signaling pathway, which can among other responses lead to a repression of thymocyte development (27, 28). In contrast, a distinct group of non-steroidal anti-inflammatory drugs (NSAIDs) has been shown to selectively reduce the production of the Aβ[1–42] peptide (29). Although the production of the potentially less amyloidogenic Aβ[1–35] peptide is comparatively increased, claims have been made that neither Aβ[1–40] nor Aβ[1–42] peptide production nor the Notch receptor cleavage are adversely affected (29, 30). To determine the underlying mechanism of this selective inhibition, we have profiled representative NSAID-like compounds in biochemical assays monitoring various aspects of γ-secretase enzyme function. Taken together, our results indicate that NSAID-like compounds can act as allosteric inhibitors by directly targeting the presenilin-dependent γ-secretase complex at a novel site discreet from the binding site for transition-state analog γ-secretase inhibitors.

**EXPERIMENTAL PROCEDURES**

**Materials**—Sulindac sulfide, sulindac suflate (Calbiochem), and R-flurbiprofen (Sigma Aldrich) were obtained from commercial sources. The synthesis of Merck A (31, 32) has been described, and the tritiated version [3H]-Merck A was prepared as follows. A solution of FMOC-4,5-dehydro-Leu-0H (50.5 g), N,N-diethylalaninamide, 1,3-dimethylamino- propyl)-3-ethylcarbodiimide hydrochloride (0.32 g), and 1-hydroxybenzotriazole hydrate (0.23 g) in N,N-dimethylformamide (5 ml) was stirred at room temperature for 2 h. The reaction mixture was diluted with ethyl acetate, washed with aqueous citric acid, aqueous sodium bicarbonate and brine, dried (MgSO₄), filtered, and evaporated in vacuo. The resulting solid was treated with 50% piperidine in N,N-dimethylformamide solution, stirred for 10 min, and then evaporated in vacuo. A portion of the resulting amine (253 mg) was treated with 2R-benzyl-SS-tet-t-butoxycarbonylamino-4R-(tert-butylmethylsilanyloxy)-6-phenyl-hexanoic acid (33) (485 mg), 1,3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (191 mg), 1-hydroxybenzotriazole hydrate (136 mg), and N,N-dimethylformamide (5 ml) and stirred for 2 h. The reaction mixture was diluted with ethyl acetate, washed with aqueous citric acid, aqueous sodium bicarbonate and brine, dried (MgSO₄), filtered, and evaporated in vacuo. Purification by column chromatography yielded 550 mg (76%) of the amide as a white solid. A portion of the foregoing amide (250 mg) was treated with tetrabutylammonium fluoride (25 ml, 4 ml) and stirred overnight. The reaction mixture was diluted with ether, water, and citric acid solution. The resulting precipitate was collected by filtration, washed with water, ether, and dried to give the alkene (200 mg, 90%). A portion of this compound (3 mg, 4.84 × 10⁻³ mmol) was dissolved in N,N-dimethylformamide (1 ml) and mixed with 10% palladium on carbon (5 mg). The reaction mixture was degassed thoroughly, cooled to 78 °C, and then stirred with carrier-free tritium gas at room temperature for 2 h. Upon the completion of the reaction, the unreacted tritium gas was removed, and the catalyst was filtered off using a Whatman auto-vial syringeless filter. Any labile tritium in the filtrate was removed by the removal of 50 mM 2-[14C]-mercaptoethanesulfonic acid, pH 6.5, 0.15 mM NaCl, 5 mM MgCl₂, 5% Me₂SO, 5% Me₂SO, and 0.5% CHAPSO while shaking for 90 min at 37 °C. Nonspecific binding was determined for all experiments by adding 600 nM unlabeled Merck A to the reaction, and serial dilutions of the tritiated ligand [3H]-Merck A (50-6 nM final concentration) were used to obtain saturation binding isotherms. Bound ligand was separated from free ligand as described (35) by adding an excess complex to polyethyleneimine-coated glass fiber filter plates (UniFilter-96, GF/B, Packard) and rapid filtration in a Packard Filtermate 196 cell harvester, followed by washing with ice-cold 5 mM Tris-HCl, pH 7.4. After drying of the plates overnight, Microscint 0 scintillant was added, and the plates were read on a Packard Topcount Microplate Scintillation counter. Subsequent data analyses were performed by nonlinear regression analysis using GraphPad Prism (GraphPad Software Inc.). Equilibrium dissociation constants (Kᵣ) for competition of [3H]-Merck A binding were determined by incubating serial dilutions of NSAID derivatives in the presence of 1 nM [3H]-Merck A. Kᵣ values were calculated using the Cheng and Prusoff equation (36): 

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Kᵣ = \frac{IC₅₀ \times [1 + (IC₅₀)^{-1}]}{IC₅₀}
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with IC₅₀ being the concentration of competitor that competes for half of the specific binding, (L) is the free ligand concentration, and Kᵣ is the equilibrium dissociation constant of the radioligand. To determine the antagonist competitiveness of NSAID-like compounds, [3H]-Merck A dose-response curves were analyzed in the presence of fixed concentrations of these compounds.

**In Vitro Generation of AICD and Aβ Peptides in Membranes**—Membranes from SH-SYSY neuroblastoma P2 cells expressing the γ-secretase substrate precursor SPA4CT (37) (SP-LEC99) were prepared according to Beher et al. (32). For in vitro generation of βAPP intracellular domain (AICD) and Aβ peptides, 240 μg of membrane protein was incubated for 2 h at 37 °C with increasing amounts of NSAID-like compounds as indicated in the figure legends, in 120 μl of 0.5% CHAPSO, 20 mM HEPES, pH 7.3, 2 mM EDTA, and 1 mM EDTA-free protease inhibitor mixture (Roche Molecular Biochemicals). Me₂SO vehicle (0.5% v/v) or 10 μM Merck A was used as a control, and for quantitation of de novo production of Aβ[1–40] and Aβ[1–42] peptide, 0.5 μl and 5 μl aliquots of the corresponding reactions, respectively, were analyzed by an electrochemiluminescence assay as described above. The solubilized enzyme was incubated in the presence of [3H]-Merck A in 0.5% CHAPSO, 20 mM HEPES, pH 7.3 (10 ml final volume). After centrifugation for 10 min at 4000 × g, the supernatant was incubated overnight at 4 °C with the antibody-coupled surface-enhanced laser desorption ionization protein chip (Ciphergen Biosystems, Fremont, CA), which was processed for surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (10).

**Surface-enhanced Laser Desorption Ionization Time-of-Flight Mass Spectrometry**—Immunocapture of Aβ peptides generated in the solubilized enzyme assay from recombinant C100Flag substrate was performed analogous to methods described previously using monoclonal antibodies 6E10 or W0-2 (10). Briefly, after incubation for 90 min at 37 °C, 2.5 ml of the in vitro γ-secretase reaction was adjusted to 0.5% (v/v) Triton X-100, 25 mM HEPES, pH 7.3 (10 ml final volume). After centrifugation for 10 min at 4000 × g, the supernatant was incubated overnight at 4 °C with the antibody-coupled surface-enhanced laser desorption ionization protein chip (Ciphergen Biosystems, Fremont, CA), which was processed for surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (10).
RESULTS

Selected NSAID-like Compounds Act as Noncompetitive Inhibitors of Aβ Peptide Formation—To profile the NSAID sulindac sulfide and the NSAID-like derivative R-flurbiprofen for their inhibitory potential toward γ-secretase enzyme activity, we evaluated these compounds in an exogenous substrate enzyme assay (16). Because the assay uses CHAPSO-solubilized membrane preparations from human neuroblastoma cells as a source of enzyme and a recombinant C100Flag substrate, it allows a direct examination of the effects of compounds on enzyme catalysis. Furthermore, the cell-free nature of the assay allowed the use of these compounds at higher concentrations (100 to 300 μM), where normally decreased cell viability would be observed. Sulindac analogs were chosen because sulindac sulfide, but not its close analog sulindac sulfone (serving as a less potent control; data not shown), has been described to selectively lower Aβ(1–42) production in cells (29). R-Flurbiprofen, the cyclooxygenase-inactive enantiomer of the NSAID S-flurbiprofen, was chosen as a structurally distinct agent described to selectively lower Aβ(1–42) production (38, 39).

Both sulindac sulfide and R-flurbiprofen inhibit γ(42)-secretase activity (Fig. 1, A and B) with IC_{50} values of 34 and 307 μM, respectively. At higher concentrations, inhibition of γ(40) ac-
tivity was also observed, however, which suggests that these compounds produce a full γ-secretase inhibition. That appears to be distinct mechanistically from the selective modulation of Aβ(1–42) production observed at lower concentrations.

To investigate the mode of inhibitor action, enzyme kinetic analyses were performed with R-flurbiprofen (Fig. 1, C–E). The data from these studies revealed that the inhibition of both γ(42) and γ(40) activities cannot be overcome by increasing the substrate concentration in the assay. The V_max for both γ(42)- and γ(40)-γ-secretase activities were considerably decreased (the latter one at the highest concentration used; Fig. 1C) in the presence of increasing concentrations of R-flurbiprofen (Fig. 1, C and D). This is confirmed by Lineweaver-Burk transformation of the R-flurbiprofen Aβ(1–42) inhibition curve (Fig. 1E), which reveals a change in V_max without affecting the K_m. In accordance with Takahashi and colleagues (40), similar results were obtained for sulindac sulfide (supplementary information).

Such a mode of noncompetitive inhibition with respect to substrate has been observed previously for structurally diverse γ-secretase inhibitors resembling aspartyl protease transition state analogs or other small molecules (41). Combined, this suggests that both NSAID-like inhibitors and prototypical γ-secretase inhibitors, such as the transition state analog inhibitor L-685,458, target a site independent of the substrate binding site of the enzyme.

**Inhibitor Profile of NSAID-like Compounds Mimics That of γ-Secretase Inhibition**—γ-Secretase cleavage of βAPP-derived substrates results in the production of various C-terminally truncated Aβ peptides (9, 10). To investigate whether the inhibition of Aβ(1–40) production observed in the cell-free enzyme assay (Fig. 1, A and B) is accompanied by an inhibition of the production of all Aβ peptide species, we performed mass spectrometric analyses of the recombinant substrate enzyme reactions (Fig. 2). R-Flurbiprofen was chosen because this compound is less selective compared with sulindac sulfide (Fig. 1, C and B). The spectra obtained after treatment with 1 mM R-flurbiprofen were similar to the samples incubated in the presence of 10 μM of the potent γ-secretase inhibitor Merck A. For both compounds, a decrease of the production of all C-terminally truncated Aβ peptides was observed, with Merck A being more potent as expected. This included the Aβ(1–38) peptide that was shown previously to be up-regulated when NSAID-like compounds are used at concentrations where selective inhibition of Aβ(1–42) peptide production occurs (29). In good accordance with this, a reduction of Aβ(1–42) in the presence of 0.3 mM R-flurbiprofen was accompanied by an enhanced production of C-terminally truncated Aβ peptides such as Aβ(1–38) and Aβ(1–37) (Fig. 2; average peak intensities relative to the MeSO control spectra obtained from W0-2 and 6E10 captures were: Aβ(1–42), −15.7%; Aβ(1–40), +0.5%; Aβ(1–38), +25.5%; and Aβ(1–37), −108.5%).

Conflicting reports have been published on sulindac sulfide regarding the inhibition of βAPP intracellular domain (AICD) generation, which is a direct measure of e-cleavage of βAPP-derived processing products in proximity to the cytosolic surface of the membrane. Whereas Takahashi and colleagues (40) observed an inhibition of AICD generation after an initial elevation at low concentrations, Weggen and colleagues (30) reported that sulindac sulfide was not able to inhibit AICD generation. To address this issue, we modified our cell-free γ-secretase assay (31) to establish an AICD assay that allows the parallel measurement of AICD generation with Aβ(1–40) and Aβ(1–42) peptide production from the same sample. The results indicate that both R-flurbiprofen (Fig. 3, A and B) and sulindac sulfide (Fig. 3, C and D) can inhibit the e-cleavage of βAPP, causing a dose-dependent reduction in the production of AICD at concentrations...
concentrations of H9252 generation by infrared imaging and production of Aβ(1–40) and Aβ(1–42) peptides measured by an electrochemiluminescence assay in the same samples as shown in the left panels. Note that for determination of de novo Aβ peptide production in B and D, the values obtained in the presence of 10 μM of the inhibitor Merck A were subtracted from the total values for each reaction. Because this assay uses membranes from cells overexpressing a direct γ-secretase substrate, these membranes contain considerable amounts of preexisting intracellular Aβ. Shown are representative blots, and the data in B and D are the average of three independent experiments, with error bars indicating the standard error of the mean. CTF, C-terminal fragment.

To investigate the basis for the competitiveness of sulindac sulfide and R-flurbiprofen, dose-responses for [3H]-Merck A binding were analyzed in the presence of various fixed concentrations of these compounds and the less active control sulindac sulfone (Fig. 4, D–F). A decrease in the maximum binding (Bmax) of the transition state analog inhibitor to solubilized enzyme was observed that was insurmountable and thus characteristic of noncompetitive antagonism. As expected, the rank of order reflected the K, values of the NSAID derivatives and was observed with as little as 50 μM sulindac sulfide (Fig. 4D).

**DISCUSSION**

γ-Secretase is a prime target for a pharmacological intervention in the disease progression of AD. Whereas classical γ-secretase inhibitors, which neither discriminate between the cleavages of alternative substrates for γ-secretase nor the actual cleavage position in βAPP-derived substrates, have been shown conclusively to target presenilins (16–18), substantial evidence for such an interaction of NSAID-like inhibitors with the presenilin-dependent γ-secretase complex has just begun to evolve (40, 45). The noncompetitive mode of inhibition observed in solubilized membrane preparations with either R-flurbiprofen or sulindac sulfide demonstrates that recent findings that were restricted to the latter (40) can be extended to structurally diverse NSAID-like inhibitors. In this respect, it appears that NSAID-like inhibitors share similarities with classical nonselective inhibitors (41) by interacting with the enzyme at a site distinct from the initial substrate binding site. In contrast with nonselective inhibitors, NSAID derivatives are characterized by a distinct window of modulation, where a selective inhibition of Aβ(1–42) generation is observed. When these compounds are used at higher concentrations, however, a classical γ-secretase inhibition occurred, as seen by an inhibition of Aβ(1–40) generation. This finding is in good accordance with a previous study (40) but does not confirm claims that these compounds...
are truly selective inhibitors of γ-secretase (29, 39). In particular, R-flurbiprofen has been a useful compound in our studies because it has a relatively modest window for inhibition of Aβ(1-42) over Aβ(1-40). Accordingly, the mass spectrometric characterization of the recombinant substrate cleavage unequivocally demonstrated that when used at 1 mM, treatment with R-flurbiprofen resulted in a full inhibition of the production of all Aβ peptides, including various C-terminally truncated species, again similar to the profile obtained with a prototypical γ-secretase inhibitor. Consistent with these observations, ε-cleavage of βAPP, which leads to the generation of AICD, was also inhibited by sulindac sulfide and R-flurbiprofen. This inhibition occurred at concentrations where the selectivity for inhibition of Aβ(1-42) over Aβ(1-40) is lost, which indicates that an inhibition of Aβ(1-40) production in this assay reflects a complete γ-secretase inhibition. It is noteworthy that the cell-free AICD/γ-secretase assay simultaneously detects various γ-secretase cleavage products generated in the same sample under identical conditions. Thereby, interassay variations in inhibitor potencies that are commonly observed, even for potent γ-secretase inhibitors, can be avoided. It is likely that these variations, in addition to the different compound concentration ranges chosen for the actual assays, might explain to some extent the conflicting evidence described in the literature (30, 40).

Although the cell-free enzyme assay data suggested a direct interaction of NSAID-like inhibitors with the γ-secretase complex, the radioligand competition data provide, for the first time, direct evidence for such a mechanism. In this paradigm, the transition state analog inhibitor served as a probe for the integrity of the active center because it binds to the catalytic residues in γ-secretase (44). It is noteworthy that this type of competition can only be detected in the radioligand displacement assay because NSAID derivatives do not appear to compete for photolabeling of presenilin 1 and rather increase the capture efficiency in affinity precipitation studies.3 Nevertheless, the noncompetitive antagonist behavior of sulindac sulfide

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3 D. Beher, unpublished observations.
and R-flurbiprofen in the radioligand binding paradigm suggests that these compounds introduce conformational changes into the complex that affect the binding of the radioligand itself or less likely the retention of the enzyme complex on the charged filters. This allosteric modulation of γ-secretase by NSAID derivatives could result either in a direct change of enzyme conformation or, for example, the oligomerization state of the complex. The latter is conceivable because γ-secretase has been proposed recently to exist as a dimer (46). How this can potentially explain this unconventional pharmacological activity is limited. Only certain classes of NSAIDs and NSAID derivatives could result either in a direct change of enzyme conformation or, for example, the oligomerization state of the complex. The latter is conceivable because γ-secretase has been proposed recently to exist as a dimer (46). How this can potentially explain this unconventional pharmacological activity is limited. 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