DOMINANCE OF AMYLOID PRECURSOR PROTEIN SEQUENCE OVER HOST CELL SECRETASES IN DETERMINING β-AMYLOID PROFILES

STUDIES OF INTERSPECIES VARIATION AND DRUG ACTION BY INTERNALLY STANDARDIZED IMMUNOPRECIPITATION/MASS SPECTROMETRY.

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Running Title: Aβ Profiles by MALDI-TOF Mass Spectrometry

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List of non-standard abbreviations: Aβ, β-amyloid; AD, Alzheimer’s disease; APP, amyloid precursor protein; APP-CTFβ, amyloid precursor protein C-terminal fragment beta; BACE, β-amyloid converting enzyme, or β-secretase; AICD, amyloid intracellular domain; CSF, cerebrospinal fluid; DEA, diethylamine; MALDI-TOF MS, matrix-assisted laser desorption time of flight mass spectrometry; IP, immunoprecipitation; IP/MS, immunoprecipitation followed by mass spectrometry; Aβ1-40N15, 15N-substituted Aβ 1-40; PS, presenilin; PS1, presenilin-1; PS2, presenilin-2.

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ABSTRACT

β-amyloid peptides, tentatively regarded as the principal neurotoxins responsible for Alzheimer’s Disease, make up a set of products that varies significantly between different biological systems. The full implications of this complexity and its variations have yet to be defined. In this work, Aβ peptide populations were extracted from animal brain tissue or cell-conditioned media, immunoprecipitated with specific antibodies, and analyzed by matrix-assisted laser desorption time-of-flight mass spectrometry. 15N-Substituted Aβ internal standards were added to gauge variations in the profile of captured peptides. Results from a range of species, including guinea pig, dog, rabbit and wild-type and transgenic mice, showed that the Aβ peptide population in each system was mainly determined by the species of origin of the amyloid precursor protein (APP) and not by the host tissue or cell line. The same method was used to gauge the effect on the Aβ peptide profile of an inhibitor of γ-secretase, one of the two proteinases that excises Aβ peptides from the precursor protein with different effects on specific peptides. Overall, the results demonstrate that the species of origin of the APP substrate dictates the outcome of APP processing to a greater extent than the origin of the processing enzymes, an important consideration in rationalizing the properties of different model systems.
INTRODUCTION

Aβ peptides are tentatively regarded as the principal neurotoxins responsible for AD (Watson et al., 2005), and are formed by sequential proteolytic events near the C-terminus of the 770-residue APP. They are also the main components of amyloid plaques that are the classical biomarker of AD in post-mortem brain. In the first step of pathological processing, the membrane-bound aspartic proteinase BACE cleaves APP after Met-671 to generate a C-terminal fragment called APP-CTFβ. Aβ species are liberated from this fragment by the intramembrane proteinase γ-secretase. Both secretases are seen as important drug targets, because the amyloid hypothesis of AD implies that blocking the production of Aβ peptides will retard the disease (Gandy, 2005). The alternative α-secretory pathway does not lead to Aβ peptide production.

BACE is an atypical aspartic proteinase (Haniu et al., 2000), but it clearly belongs to that enzyme family and can be crystallized (Hong et al., 2000). γ-Secretase is a much more enigmatic target. It too is an aspartic-dependent proteinase, but of a specialized type adapted for regulated intramembrane proteolysis (Landman and Kim, 2004). Its proteolytic activity resides in a complex that includes PS and several other proteins (Edbauer et al., 2004; Chyung et al., 2005). γ-Secretase cleaves APP at various sites to produce Aβ peptides of different lengths. Cleavage at the “γ-site” produces Aβ 1-40 and Aβ 1-42, the products widely attributed a major role in AD. These peptides oligomerize, aggregate and ultimately accumulate in plaques (Citron et al., 1996). γ-Site cleavage may be preceded by action of γ-secretase at the “ε site” located after Leu-720 of APP, which releases a 50-residue peptide known as AICD (Gao and Pimplikar, 2001). More recently, formation of a 46-residue fragment of APP has also been attributed to γ-secretase activity and termed the ζ cleavage (Zhao et al., 2004).
Analysis of AD brain tissue and brain tissue from transgenic mice expressing human APP has detected a variety of different Aβ peptides in extracellular amyloid deposits. In addition to Aβ 1-40 and 1-42, N-terminally and C-terminally truncated peptides and C-terminally elongated peptides have been reported. Aβ peptides with an N-terminal Glu-3 or Glu-11 (Naslund et al., 1994), as well as peptides with pyroglutamyl N-termini at positions 3 and 11 (Miravalle et al., 2005; Rufenacht et al., 2005) have been detected in amyloid plaques from human brain. Plaques from transgenic mice expressing both human APP and PS2 as well as plaques from human AD brains have been reported to contain Aβ peptides 1-16 and 17-28 (Rufenacht et al., 2005). C-terminally truncated peptides including Aβ 1-37, 1-38, and 1-39 have also been found in plaques from two different transgenic mouse lines (Lewis et al., 2004) and in CSF of AD patients (Lewczuk et al., 2003). The biological function and disease-relevance of the various Aβ peptides is not understood, and the full complexity of processing, both of the precursor protein and Aβ fragments, has yet to be resolved.

The production of Aβ 1-42 is favored by mutations of APP or PS linked to early-onset or familial forms of AD (Clark et al., 1995). Because Aβ 1-42 forms fibrils more readily than most other Aβ peptides and is the major component of congophilic plaques in AD brain, the production of Aβ 1-42 is believed to favor progression of the disease. Thus, both the substrate sequence and mutations within the enzyme may influence the final profile of products from γ-secretase-catalyzed cleavage of APP, and subtle differences in these profiles may be highly significant.

These considerations led us to use IP followed by MALDI-TOF MS to observe Aβ profiles from the brains of several preclinical species, and from transgenic mice expressing mutant forms of human APP. Recently, several groups have reported the incorporation of
internal standards into $\beta\beta$ samples subjected to MS, allowing quantitation of specific $\beta\beta$ peptides (Miravalle et al., 2005; Rufenacht et al., 2005). In this work, we incorporated multiple internal standards to monitor changes in several specific $\beta\beta$ peptides. The results indicated that the sequence of APP is the major factor governing which $\beta\beta$ peptides are produced by proteolytic processing, and that the species identity of the host cell or tissue is a less important factor.
METHODS

Immobilization of Capture Antibodies - Biotinylated monoclonal antibodies 6E10 and 4G8 (both from Signet Laboratories, Dedham, MA) were immobilized by incubation with ImmunoPure Immobilized Streptavidin (Pierce) at a ratio of 0.06 mg of antibody for each 0.06 ml of bead slurry, after which unoccupied biotin-binding sites on the beads were blocked with biotin. The choice of antibody depended on the sample. With brain extracts of wild type mouse and rat, 0.06 mg of 4G8 antibody was used for each sample. For brain extracts of dog, rabbit, Tg2576 transgenic mouse or Dutch transgenic mouse, or with conditioned medium of human H4 cells, 0.03 mg each of both 6E10 and 4G8 was used for capture. For brain extracts of guinea pig, either 0.06 mg of 4G8 alone or 0.03 mg each of 6E10 and 4G8 were used. Before use, the antibody beads were washed twice in five volumes of Dulbecco’s phosphate-buffered saline. For control experiments, biotinylated nonspecific murine IgG (Jackson IR Laboratories) was immobilized in the same fashion.

Brain Extracts - Methods for handling animals were approved by the Animal Care and Use Committee. Animals were euthanized according to animal care guidelines, and the brains were removed and frozen in liquid N2. Brains were homogenized in a Polytron using a nine-fold milliliters-to-grams ratio of ice cold 0.2% DEA/0.05 M NaCl (Wong et al., 2004). The homogenate was incubated at 0 ℃ for 3 h, then centrifuged at 135,000 x g for 1 h. Supernatants were collected and stored frozen at –80 ℃.

Immunoprecipitation from Brain Extract – Brain extract was adjusted to pH 8.5 by adding a 1:100 dilution of 2 M Tris-HCl, pH 8.5, after which the sample was treated with three-quarters of a tablet of Complete EDTA-Free Protease Inhibitor Cocktail (Roche Applied Science). Proteins that bound nonspecifically to beads were removed by treating the sample with 0.8 ml of a slurry
of ImmunoPure Immobilized Protein A/G beads (Pierce) and incubating it overnight at 4 °C with end-over-end rotation, after which it was centrifuged to remove the beads. Next, the supernatant was treated with isotopically-substituted Aβ standards (rPeptide, Bogart, GA), chosen to allow quantification of the relevant peptides. In specific experiments with animal models expressing human sequence Aβ one or two or all of the following 15N-substituted human standards were used, in the amounts: 7 ng Aβ1-40N15, 3.5 ng Aβ1-42N15 and 3.5 ng Aβ1-43N15. In specific experiments with animal models expressing murine/rat sequence, 7 ng Aβ1-40N15 substituted murine/rat standard was added. Beads loaded with Aβ-specific antibodies were then added to the sample (see above), and the samples were incubated with rotation for 24 h, after which the beads were separated from the rest of the sample by centrifugation. The beads were washed three times for 300 s each with 50 ml of ice cold 0.2% DEA, 0.02 M Tris HCl, 0.05 M NaCl, pH 8.5 and recovered from each wash by centrifugation at 1530 x gs. They were then transferred to a 1.5 ml microfuge tube, and washed twice with 1.5 ml of ice cold 0.02 M Tris HCl, 0.05 M NaCl, pH 8.0. Finally, the captured peptides were recovered by eluting them from the antibody beads with 50% CH3CN/0.1% TFA, and the eluate was then neutralized with NH4OH.

Operational Scale of Immunoprecipitation with Different Aβ Sources – The intention of different scaling was to obtain about the same amount of Aβ for mass spectrometry from each IP experiment. With 2 week old male Hartley guinea pigs (Charles River), 30 ml of brain extract was subjected to the preclear step, and 25 ml of the supernatant was taken for IP; with 8 week old male CD/Sprague Dawley rats (Charles River), 25 ml was taken for preclear and 20 ml for IP; with 12 week old Tg2576 transgenic mice, 1.5 ml for preclear and 1.0 ml for IP; with 15
week old male New Zealand White rabbits, 20 ml for preclear and 17.5 ml for IP; and with 8 week old male CD-1 wild-type mice, 20 ml for preclear and 17.5 ml for IP.

H4 human neuroglioma cells (ATTC HTB-148) stably transfected with human Swedish APP were incubated for 16 h with medium at 37 °C, after which the medium was collected and mixed 1:1 with 0.4% DEA/0.1 M NaCl before being stored frozen at –80 °C.

**MALDI-TOF MS Analysis** – For each sample, the eluate containing Aβ peptides was desalted and concentrated with a C18 ZipTip (Millipore) and then mixed with the MALDI matrix α-cyano-4-hydroxycinnamic acid (Applied Biosystems). The mixture was spotted to a 192-well MALDI plate (Applied Biosystems) and analyzed using an Applied Biosystems 4700 Proteomics Analyzer, a MALDI-TOF/TOF instrument, in the linear positive ion mode. The mass range was set at 2000-6000 Da with the focus mass at 4300 Da. The data were collected using automatic data acquisition and a random search pattern. Each MS spectrum was an average of 10,000 laser shots with fixed laser intensity. External calibration was performed using the 4700 Cal Mix (Applied Biosystems). As appropriate for the sample, human or mouse Aβ1-40N15 added prior to IP was also used as an internal standard for peak intensity and mass calibration (human peptide [M+H]+ = 4383.86; mouse peptide [M+H]+ = 4285.77). Direct mass measurements of the standard peptides with the instrument in reflectron mode confirmed these values. Identifications for tissue or cell-derived peptides were based on agreement between experimental masses and theoretical values (see Results).

To provide a basis for quantitation of changes in the relative abundance of Aβ species, known amounts of 15N-substituted human or rat/mouse Aβ peptides 1-40, 1-42 and 1-43 were spiked into each brain extract. The ratio of peak height of each peptide to the corresponding internal standard was calculated, and the changes caused by drug treatment were estimated from
the change in the ratio between the peak for each tissue-derived peptide and the most relevant internal standard.

**Dosing Protocol for LY-411575** – Two week old male Hartley guinea pigs (Charles River) were dosed subcutaneously with 3.2 mg/kg LY-411575 (Wong et al., 2004) using a dosage volume of 1 ml/kg. The compound was solubilized in a vehicle of 20%DMSO/20%EtOH/60%PEG MW400. Guinea pigs were euthanized 3 h after dosing and tissues were collected. There were six animals per dosing group.

**Fluorescence Polarization** – The experiment was performed with a Beacon 2000 instrument, using fluorescein-\(\beta\) as the ligand to 6E10 antibody in phosphate buffer, pH 7.4.

**Surface Plasmon Resonance** - SPR experiments were performed on a Biacore 3000 instrument (Biacore, Inc.). Biotinylated antibody was immobilized to 4000 resonance units on a Biacore SA chip. Experiments were carried out at 25° C in buffer consisting of 20 mM Tris HCl (pH 8.5), 50 mM NaCl. \(\beta\) 1-40 peptide was injected over the antibody surface and an unmodified streptavidin surface at a flow rate of 10 µl/min. Binding data were reference against the streptavidin surface and buffer injections. Dissociation data were fit to a simple exponential using BIAeval software (Biacore Inc.).
RESULTS

The monoclonal antibodies used in this work were 4G8, for which the cognate epitope is residues 17-24 of human or mouse/rat Aβ, and 6E10, which recognizes residues 4-8 of human Aβ. Conditions were sought that gave maximum depletion of the Aβ population in a single step, with capture efficiency assessed by subjecting immunodepleted samples to a second round of IP.

In a preliminary study, either 60 µg of 4G8 (Fig. 1A) or a combination of 30 µg each of 6E10 and 4G8 (Fig. 1B) efficiently captured both endogenous Aβ and the 15N-substituted human Aβ 1-40 internal standard from 25 ml samples of guinea pig brain extract, but 6E10 used alone (Fig. 1C) failed to capture an N-terminally truncated form of Aβ. We elected to use a combination of the two antibodies for IP when analyzing human-like Aβ, as this method seemed most likely at the outset of the work to yield a complete Aβ profile.

Using 30 µg of 4G8 antibody in 25 ml of brain extract (the largest volume used) gave a 4G8 concentration of 8 nM (based on a mass of 150 kDa). The bivalent character of IgG means that its effective concentration as a capture agent is twice its molar concentration, i.e. 2*[4G8]. If we consider the binding of 4G8 to Aβ as a simple equilibrium for which $K_d = 2*[4G8][A\beta]/[A\beta-4G8]$, with antibody so greatly in excess over Aβ that its free concentration [4G8] is effectively unchanged by binding all the Aβ to antibody, then an endpoint at which $\geq 90\%$ of Aβ is bound to antibody requires that $[4G8] = 5 \times K_d$ (because $[A\beta-4G8]/[A\beta] = 2*[4G8]/K_d$). $K_d$ for the interaction of 4G8 with Aβ was reported to be 0.8 nM (Hughes et al., 1998), and the selected concentration of 4G8 at 8 nM (10-fold $K_d$) was therefore sufficient on its own according to theory as well as appearing effective by experiment. Additional Aβ binding
capacity was present in the form of an equal concentration of 6E10 antibody, for which a $K_d$ of 1.5 nM from $A\beta$ was measured using a fluorescence polarization assay (data not shown).

After capture, the $A\beta$-antibody complexes were subjected to multiple wash steps to remove nonspecifically bound components. To evaluate the risk of losing $A\beta$ while washing, the off-rate for $A\beta$ bound to immobilized biotinyl-6E10 was measured on a Biacore biosensor system (not shown). The first-order rate constant for dissociation of $A\beta$ into the pH 8.5 wash buffer at 25 °C was $3.3 \times 10^{-4}$ s$^{-1}$, which would result in a 10% loss in a 5 min wash. Including centrifugation, wash steps in our protocol required about 5 min each, but they were performed using ice-cold buffer to retard dissociation. The available data suggested that losses by dissociation of antibody-$A\beta$ complexes would not have had a major effect on the IP/MS profiles obtained.

**Immunoprecipitation and MALDI-MS Analysis** - To check the specificity of $A\beta$ capture, guinea pig brain extract was subjected in turn to the IP protocol using (i) bead-linked nonspecific IgG, followed by (ii) bead-linked anti-$A\beta$ antibodies. Before the first IP, $A\beta1-40N15$ was spiked into the sample as a marker for appropriately specific capture of a desired target (i.e., it should escape IP by nonspecific IgG but be captured by the anti-$A\beta$ antibodies). $A\beta1-42N15$ was added to the captured sample from each round as a positive control for the MALDI-MS step.

No brain-derived $A\beta$ or $A\beta1-40N15$ were captured by nonspecific IgG, because the only peak in the mass spectrum came from $A\beta1-42N15$ added after IP but before MS (Fig. 2A). Subsequent IP from the same extract using $A\beta$-specific antibodies produced a complex spectrum (Fig. 2B) in which the masses of fourteen peaks matched theoretical values for $A\beta$ peptides of the guinea pig (Table I). Assignments of peak identity were based on experimental masses that
agreed to within 0.05% of theory with theoretical masses for individual Aβ species. The spectrometer was operated in linear positive ion mode to maximize its sensitivity.

As the guinea pig Aβ sequence is the same as that for human, dog and rabbit, this series of identifications applies to all four species. Table I also shows theoretical and experimental mass values for Aβ of the mouse and rat and for Dutch variant human peptides, which appear later in Results.

Aβ peptide profiles from model species – Despite the sequence-identity of guinea pig, dog and rabbit Aβ, there is no certainty that the α-, β- and γ-secretase enzymes in these species will cleave APP at similar rates or with similar relative specificities for different cleavage sites. Differences in degradation or clearance of Aβ could also cause interspecies variation in Aβ populations. In Fig. 3, MALDI-TOF MS profiles of Aβ are given for brain extracts of the guinea pig, dog and rabbit (each equivalent to 2.7 g of brain tissue). The three samples gave profiles in which the principal similarities are the prominence of Aβ species 11-34, 11-40, 1-33, 1-34, 1-37, 1-38, 1-40 and 1-42. Aβ 1-43 was also detected in all three extracts. Differences were comparatively minor, and included the peak identified as Aβ 7-39 in the dog sample. The guinea pig has been recommended as an animal model for human APP/Aβ processing because of its human-identical Aβ, while the dog is favored for drug safety evaluation.

Wild-type and transgenic mice - We next examined Aβ populations from three mouse models containing different APP substrates: wild type mouse with its endogenous APP, as well as transgenic mice expressing either the Swedish or Dutch mutant forms of human APP (Fig. 4). Because the wild type mouse has three differences in Aβ peptide sequence compared to human Aβ (see Table I), the human Aβ-specific antibody 6E10 does not capture murine Aβ, and so IP
from the extract of wild type mouse brain was performed with 4G8 alone. The transgenic mouse samples were probed with both 4G8 and 6E10 antibodies. Due to the different levels of total Aβ in these mouse models, the amount of brain extract used for IP was adjusted accordingly. For mouse brain, 1.8 g was subjected to IP compared with 1.17 g of Dutch-mutant mouse brain and 0.11 g of Tg2576 mouse brain. In the Swedish mutant, a two-residue sequence change from wild-type immediately N-terminal to the β-secretase site enhanced the rate of Aβ cleavage causing an elevated ratio of Aβ production to cleavage at the α-secretase site (Citron et al., 1992). In the Dutch variant, alteration of Glu-22 to Gln-22 increases the propensity of the Aβ population to form fibrils (Watson et al., 1999).

Brain extract from wild type mouse gave an Aβ pattern (Fig. 4A) distinct from that seen in guinea pig, rabbit and dog. Its major feature was the prevalence of Aβ peptides with N-terminal Glu-11 (Gouras et al., 1998). In contrast to the profiles shown in Fig. 3, Aβ peptides 11-34, 11-37, 11-38 and 11-40 yielded prominent peaks and peaks for Aβ 1-40 and Aβ 1-42 were comparatively less abundant. This was not the case for spectra from brains of transgenic mice expressing Swedish- (Fig. 4B) and Dutch-variant (Fig. 4C) human APP, which resembled that of wild-type guinea pig (Fig. 3A) much more than that of wild-type mouse (Fig. 4A). Both transgenic profiles were dominated by peaks of C-terminally truncated Aβ, including Aβ 1-37, 1-38, 1-40 and 1-42, with relatively few Aβ 11-x components. The result clearly indicates how heavily minor differences in the Aβ sequence can alter the product profile. Aβ 1-43 was not detected in any mouse models, including wild-type or human transgene expressing models. This could be due to either a low abundance or lack of cleavage of this fragment in these models.

Transgene-derived human Aβ dominated the profile from Tg2576 mouse (Fig. 4B), with no detection of Aβ sourced from mouse APP. The Tg2576 model has a six-fold higher
expression of the human APP protein relative to the endogenous mouse APP, and this level of expression of the Swedish-mutation variant of APP results in the production of a fivefold increase in the amount of Aβ 1-40 and a fourteen-fold higher amount of Aβ 1-42 relative to endogenous murine Aβ (Hsiao et al., 1996). The failure to detect murine Aβ can be accounted for by recalling that sixteen-fold less brain tissue was used from the Tg2576 mouse than from wild-type to compensate for the robust overexpression of human Aβ in this model. In contrast to the profile from the Tg2576 mice, endogenous mouse Aβ peptides were clearly represented in the spectrum from the transgenic mouse expressing Dutch-variant human APP (Fig. 4C), a line that has approximately a five-fold increase in APP expression, and about a four-fold excess of human Aβ relative to murine (Fig. 4C). The quantity of brain tissue extracted for the Dutch-variant transgenic was 69% of that taken for wild-type mouse. The peak profile of endogenous mouse Aβ from Dutch-mutant mouse (Fig. 4C) was consistent with the profile from wild type mouse (Fig. 3A), with prominent Aβ 11-x peaks (mouse Aβ 11-34, 11-38 and 11-40). The striking feature of the results from Dutch-variant mouse is coexistence within a single brain of different processing patterns for the mouse and human APP’s. On a technical point, the murine Aβ profile obtained from mice carrying the Dutch variant APP transgene by IP using both monoclonals generally matched the profile captured from wild type mice by 4G8 alone. This mitigates any concern that use of 4G8 alone might skew the profile in favor of Aβ 11-X components.

Aβ secreted from human cells - Stably transfected cell lines that produce Aβ are also used in AD research. Fig. 5 shows MALDI-TOF MS profiles for Aβ secreted from H4 human neuroglioma cells stably transfected with either wild type human APP (Fig. 5A) or Swedish-variant APP (Fig 5B). In each case, Aβ was immunoprecipitated from cell-conditioned medium with 6E10 and
4G8 antibodies. A notable difference between the profiles is the relative loss of Aβ11-x peptides in the H4Sw/APP-conditioned medium (Fig. 5B), which can be attributed to the previously detected influence of the Swedish mutation on the relative rates of cleavage before Asp-1 and Glu-11 of Aβ. The spectra in Fig. 5, together with those from the Tg2576 mouse brain (Fig. 4B), support the view that the Swedish mutation favors production of Aβ1-x relative to other potential events (e.g. α-secretase-catalyzed cleavage, or production of Aβ 11-x) (Citron et al., 1994), and also tend to imply that BACE may cleave before either Asp-1 or Glu-11, but does not function sequentially at both sites.

Comparison of wild-type rodent profiles - Fig. 6 compares profiles from rodents commonly used to study Aβ production. These include the guinea pig (Fig. 6A) which produces human-identical Aβ peptides, the wild-type mouse (Fig. 6B) and the wild-type rat (Fig. 6C). Mouse and rat generate almost identical Aβ products that differ from the human sequence at three positions. The result of the comparison is clear. The species that produce human-identical Aβ species give profiles in which Aβ 1-34 and Aβ 1-40 are the largest peaks, while the mouse and rat profiles are dominated by Aβ peptides beginning at Glu-11 of the Aβ 1-40 sequence.

Quantitative analysis of drug effects on Aβ peptide production in guinea pig brain - The ability to detect and identify individual Aβ peptides in a complex pool should allow the evaluation of drug effects on individual peptides in model species. Drug action is quantified by noting how peaks from tissue-derived Aβ change in comparison to peaks for internal standards added at constant levels to all brain extracts. Multiple internal standards allowed more precise quantitation of specific Aβ peptides. The initial selection of internal standards Aβ1-40N15 and Aβ1-42N15 to calibrate the corresponding endogenous Aβ1-40 and 1-42 in guinea pig brain.
extracts was based on their known importance and disease-relevance, and the greater variability that might be expected for longer and more hydrophobic Aβ products. The Aβ1-40N15 peptide also served as internal standard for shorter Aβ peptides, as it was not feasible to have an independent standard for every Aβ in the pool.

Fig. 7 shows the effect of a 3.2 mg/kg dose of a γ-secretase inhibitor, LY-411575 (Eli Lilly Inc.), on the Aβ profile from guinea pig brain (see Experimental section for details). LY-411575 generally inhibited production of Aβ in guinea pig brain, with most fragments declining as gauged by their peak intensity relative to the internal standards. An exception was the elevation of Aβ 1-43, which appeared to be an increase of several-fold by reference to the both the Aβ 1-40 N15 and Aβ 1-42 N15 internal standards. Although a corresponding Aβ 1-43 internal standard was not included in this experiment, the robust increase in endogenous Aβ 1-43 is readily quantified with the two internal standards present, and subsequent experiments prove, that employing all three internal standards would not change the outcome of this experiment. Effects of this kind would be difficult to detect without the capacity of mass spectrometry to survey the Aβ population.
DISCUSSION

Animal models are indispensable in AD research, but applications of IP/MS have largely been confined to characterizing Aβ in human brain (Huse et al., 2002), in CSF of AD patients (Lewczuk et al., 2003; Portelius et al., 2006), or in conditioned media from cells transfected with genes encoding human APP (Wang et al., 1996; Vandermeeren et al., 2001). There have been fewer studies of Aβ populations in animal brains (Terai et al., 2001; Lewis et al., 2004; Rufenacht et al., 2005), despite the potential value of such data in interpreting the properties of important models and the actions of drug candidates.

Because different Aβ peptides contribute differently to neurotoxic insult and plaque formation (Parvathy et al., 2001), understanding interspecies differences in Aβ peptide production should help to rationalize differences between different in vivo models. Also, the enzymology of γ-secretase includes subtle responses to pharmacologic intervention, such as uneven shifts in the relative quantities of particular Aβ peptides (Ikeuchi et al., 2003; Beher et al., 2004). As a profiling method, IP/MS offers an excellent approach to both these subjects.

The most favored animal models are transgenic mice that overexpress mutant or wild-type human APP and exhibit amyloid deposits. They express both human and mouse APP at different ratios in the presence of either endogenous murine PS1 (the Tg2576 mouse (Hsiao et al., 1996)) or transgenic human PS1 (the PSAPP mouse (Takeuchi et al., 2000)). The three differences in sequence between human and mouse Aβ cause profound differences between their respective biophysical properties and propensities to form oligomers and plaques (De Strooper et al., 1995). IP/MS was used here to analyze whether Aβ profiles from transgenic mice expressing
different forms of human APP had more resemblance to those from animals that make human-
identical Aβ or to those from animals that make wild-type rat/mouse Aβ.

The low level of Aβ in brains of animals lacking an APP transgene provided a technical
challenge. Our method was designed to capture and detect even minor Aβ species from
relatively large amounts of brain extract for the purpose of thorough characterization of Aβ in
animal brains, including both extracellular and intracellular forms. It clearly detected the
fragments already implicated in AD as well as recognizing several Aβ fragments not reported
elsewhere (to our knowledge). As a critical step, components that bound nonspecifically to IgG
were removed before specific Aβ capture, minimizing interference with capture and mass
analysis.

Aβ profiles from brain extracts of guinea pig, dog and rabbit, three species that make
human-identical Aβ (Johnstone et al., 1991), were similar (Fig. 3) except for a small peak of Aβ
7-39 from dog brain. The longer Aβ forms identified were Aβ 1-40, 1-42 and 1-43. Another
major peak was Aβ 1-34; cleavage of -Leu34-Met35- in Aβ is attributed to BACE (Shi et al.,
2003). The peptide profile from untreated guinea pig was consistent across all experiments
(Figures 3, 6 and 7), demonstrating the reproducibility of the pattern of Aβ peptides obtained
with this methodology.

To gauge how different APP sequences affected Aβ profiles, we next analyzed brain Aβ
from wild-type mice and two transgenic lines expressing mutant human APP (Fig. 4). The
transgenics were (i) Tg2576 mice, expressing Swedish-variant human APP, and (ii) mice
expressing Dutch-variant human APP (in which Glu-22 of Aβ becomes Gln-22). All three lines
produce Aβ by activity of endogenous mouse secretases. IP/MS of wild-type mouse brain
showed the preponderance of Aβ 11-x species reported earlier (Gouras et al., 1998); cleavage of
-Phe\textsuperscript{10}-Glu\textsuperscript{11}- in mouse/rat A\textbeta is attributed to BACE1 (Vassar et al., 1999). Each transgenic model produced a distinctive profile. That from Tg2576 brain was dominated by human-sequence A\textbeta and resembled the guinea pig, dog and rabbit profiles. Dutch-variant APP mouse gave a complex profile containing human and murine A\textbeta’s, but peaks from each APP were easily assigned to their respective A\textbeta sequences. The A\textbeta profile derived from murine APP matched the one from wild-type mouse, and the A\textbeta profile from the Dutch-type human APP was generally similar to the one from animals containing human-identical A\textbeta.

Comparing profiles from the three mouse models again suggested that it was the APP substrate (presumably its sequence in the A\textbeta and pre-A\textbeta regions) rather than host secretase and A\textbeta-degrading enzymes that determined the A\textbeta profile. Further support came from comparing the native A\textbeta profiles of three different rodent species (Fig. 6). The similar A\textbeta profiles of mouse (Fig. 6B) and rat (Fig. 6C) were dominated by peaks of rat/mouse A\textbeta 11-34 and 11-40, and were distinct from the guinea pig profile (Fig. 6A) with major peaks of A\textbeta 1-40 and 1-34.

The A\textbeta population secreted from H4 cells expressing wild-type human APP included a strong peak of A\textbeta 11-40 (Fig. 5A), but A\textbeta 11-40 was barely detected in medium conditioned by cells expressing Swedish variant (Fig. 5B). This presumably reflected the enhanced kinetics of cleavage at the A\textbeta N-terminus with Swedish-variant APP as substrate. Tg2576 mice, which express the Swedish human variant, as well as H4 cells overexpressing Swedish-variant APP, produced a profile dominated by A\textbeta 1-40, with almost no A\textbeta cleaved after Tyr-10. This was consistent with the report that skin fibroblasts from persons of the Swedish pedigree secreted elevated levels of A\textbeta, with peptides starting at A\textbeta 1 predominant (Citron et al., 1994).
BACE and γ-secretase are drug targets in AD, and IP/MS profiling could apply to either approach. Surprisingly, the more complex γ-secretase has yielded more quickly to medicinal chemistry in terms of getting trial agents to the clinic. To us and others (Beck et al., 2003), the guinea pig has attractions as a preclinical model that include its human-like brain Aβ profile (e.g. compare Figs. 5A and 6A). This made it interesting to test the effects of a γ-secretase inhibitor on the Aβ profile from guinea pig brain.

IP/MS is unique in distinguishing so many different Aβ fragments, and the use of 15N-substituted Aβ standards allows quantitative assessment of drug effects on all brain Aβ peptides simultaneously. A γ-secretase inhibitor, LY-411575, was shown to have induced acute changes in the guinea pig brain Aβ profile 3 h after the animal received a subcutaneous dose of 3.2 mg/kg (Fig. 7). Aβ1-40 and 1-42 were both diminished in abundance relative to their specific internal standards. The other Aβ fragments were also diminished, including Aβ 1-34, a product of two BACE-catalyzed cleavages. This phenomenon was interpreted elsewhere as signifying that γ-secretase must cleave the CT99 fragment of APP before BACE cleaves after Leu-34 (Shi et al., 2003). It was also observed that the γ-secretase inhibitor diminished some fragments more than others, which adds further complexity to the process of secretase inhibition. Of particular interest, the Aβ1-43 fragment was increased more than 2-fold over vehicle levels in drug-dosed animals. We have replicated this result in a second experiment (data not shown). Similar results were observed with cellular systems (Ikeuchi et al., 2003; Qi-Takahara et al., 2005) but have not (to our knowledge) been reported before in animal brain. The importance of Aβ1-43 is not fully understood, but its strongly amyloidogenic nature presumably makes it undesirable (Burdick et al., 1992; Jarrett et al., 1993). Consistent with this, Aβ x-42 and x-43 were shown to be
associated with early AD progression in human patients (Parvathy et al., 2001). This result underlines the complexity of the task of pharmacologically inhibiting Aβ processing through secretase inhibition, reinforcing the importance of thoroughly studying the Aβ profile in animal models. As we detected Aβ1-43 only in animal models endogenously expressing the human Aβ sequence (and flanking sequence), the proper selection of animal models and detailed Aβ profiling may promote understanding the effects of secretase inhibitors and their potential impact in human brain.

Judging by the Aβ profiles presented here for in vivo models and in vitro models, the APP sequence is the principal factor governing the Aβ profile. According to the amyloid hypothesis, the production and toxic effects of Aβ peptides are the primary cause of neurotoxicity and consequent cell death in the brain. The impossibility of direct biochemical analyses on brain tissue from living patients makes it immensely important that animal models of the disease be chosen on the basis of maximum information. A thorough understanding of species differences in Aβ peptide production will enhance our understanding of in vivo models and thereby promote efforts to defeat AD. The IP/MS described above revealed the complexity of APP processing in different species. The unexpected observation of enhanced production of Aβ 1-43 caused by a γ-secretase inhibitor further emphasized the importance of thoroughly assessing the response to drug treatment at a high level of detail. That this result is obtained in an animal model that closely resembles Aβ processing the normal human brain underlines the importance of attempting to gauge the medical potential of emerging pharmaceutical Aβ modifiers in a human-like biological context.
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REFERENCES


FOOTNOTES

Ping Du and Kathleen M. Wood contributed equally to this research. The authors were employees of Pfizer Inc., which supported the work.

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LEGENDS FOR FIGURES

FIG. 1. **Immunoprecipitation with different antibodies.** MALDI-TOF MS spectra of Aβ peptides captured from guinea pig brain extract using (A) 60 µg of 4G8; (B) 30 µg each of 4G8 and 6E10; (C) 60 µg of 6E10. The standard peptide $^{15}$N-substituted human Aβ 1-40 was added to each extract before the immunoprecipitation. See Methods for other details.

FIG. 2. **Specificity of immunoprecipitation.** MALDI-TOF MS spectra of Aβ peptides captured from guinea pig brain extract by (A) nonspecific immunoglobulin G, and (B) a combination of anti-Aβ monoclonal antibodies 6E10 and 4G8. The extract was probed firstly with the nonspecific antibodies and secondly with the anti-Aβ antibodies. $^{15}$N-substituted human Aβ 1-40 standard peptide was added to the extract before the immunoprecipitation with nonspecific IgG, and $^{15}$N-substituted human Aβ 1-42 standard peptide was added to each captured sample before mass spectrometry.

FIG. 3. **MALDI-TOF MS analysis of Aβ peptides from three sources of human-identical Aβ peptides.** (A) guinea pig brain with $^{15}$N-substituted human internal standards for Aβ 1-40, Aβ 1-42 and Aβ 1-43; (B) dog brain with $^{15}$N-substituted human internal standards for Aβ 1-40 and Aβ 1-42; (C) rabbit brain with $^{15}$N-substituted human internal standards for Aβ 1-40, Aβ 1-42 and Aβ 1-43. $^{15}$N-Substituted internal standards were added to the extracts before immunoprecipitation.
FIG. 4. MALDI-TOF MS analysis of Aβ peptides immunoprecipitated from brain extracts of three mouse strains. (A) Endogenous Aβ of wild-type mouse with ^15^N-substituted murine/rat internal standards for Aβ 1-40. Mouse Aβ species are designated m1-40, etc., to distinguish them from the human-identical forms. (B) Aβ peptides from transgenic mouse Tg2576, which expresses the Swedish mutant form of human APP; the Aβ species captured are predominantly human, including the ^15^N-substituted human internal standards for Aβ 1-40, Aβ 1-42 and Aβ 1-43; (C) Aβ peptides from transgenic mouse expressing Dutch mutant APP, in which Glu-22 of Aβ 1-40 is altered to Gln-22, causing a 1 Da mass decrease, with ^15^N-substituted human internal standards for Aβ 1-40 and Aβ 1-42. Both human and mouse Aβ species were detected. Dutch mutant human peptides are designated D1-40, etc.

FIG. 5. MALDI-TOF MS analysis of Aβ peptides immunoprecipitated from conditioned medium of human cells. (A) H4 human cells expressing wild-type human APP with ^15^N-substituted human internal standards for Aβ 1-40; (B) H4 human cells expressing Swedish-mutant variant human APP with ^15^N-substituted human internal standards for Aβ 1-40, Aβ 1-42 and Aβ 1-43.

FIG. 6. MALDI-TOF MS analysis of Aβ peptides immunoprecipitated from brain extracts of three rodent species. (A) guinea pig with ^15^N-substituted human internal standards for Aβ 1-40, Aβ 1-42 and Aβ 1-43; (B) mouse (wild-type) with ^15^N-substituted
murine/rat internal standards for Aβ 1-40; (C) rat with $^{15}$N-substituted murine/rat internal standards for Aβ 1-40.

FIG. 7. **Effect of the γ-secretase inhibitor LY-411575 on the Aβ peptide population recovered from guinea pig brain 3 h after dosing.** Guinea pigs were dosed subcutaneously with (A) vehicle, (B) LY-411575 at 3.2 mg/kg. Extraction, addition of $^{15}$N-substituted human internal standards for Aβ 1-40 and Aβ 1-42 and immunoprecipitation were performed as described in Methods.
TABLE I

Theoretical and observed values of \([M+H]^+\) used to identify A\(\beta\) peptides

Observed mass values were compared with theoretical average mass values for A\(\beta\) peptides.

A. Human/guinea pig/rabbit/dog A\(\beta\)1-43 sequence

(DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIAT)

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This article has not been copyedited and formatted. The final version may differ from this version.
B. mouse/rat $\alpha\beta$ 1-43 sequence (differences from human $\alpha\beta$ are underlined):

\[
(\text{DAEFG}H\text{DSGFEVRH}QKL\text{VFFAEDVGSNK}GAI\text{IGLMV}G\text{VV}IAT)
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C. Human Dutch mutant $\alpha\beta$ 1-43 sequence (the residue altered by the mutation is underlined): (DAEFRHDSGYEVHHQKLVFFAQDVGSNKGAIIGLMVVGVVIAT)
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*ND: not detected
Fig. 5