Physiological and Biophysical Factors That Influence Alzheimer’s Disease Amyloid Plaque Targeting of Native and Putrescine Modified Human Amyloid β40

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ABSTRACT

Amyloid β40 (Aβ40) and its derivatives are being developed as probes for the ante-mortem diagnosis of Alzheimer’s disease. Putrescine-Aβ40 (PUT-Aβ40) showed better plaque targeting than the native Aβ40, which was not solely explained by the differences in their blood-brain-barrier (BBB) permeabilities. The objective of this study was to elucidate the physiological and biophysical factors influencing the differential targeting of Aβ40 and PUT-Aβ40. Despite better plaque-targeting ability, 125I-PUT-Aβ40 was more rapidly cleared from the systemic circulation than amyloid β40 labeled with 125I (125I-Aβ40) after i.v. administration in mice. The BBB permeability of both compounds was inhibited by circulating peripheral Aβ40 levels. 125I-Aβ40 but not 125I-PUT-Aβ40 was actively taken up by the mouse brain slices in vitro. Only fluorescein-Aβ40, not fluorescein-PUT-Aβ40, was localized in the brain parenchymal cells in vitro. The metabolism of 125I-PUT-Aβ40 in the brain slices was twice as great as 125I-PUT-Aβ40, 125I-Aβ40 efflux from the brain slices was saturable and found to be 5 times greater than that of 125I-PUT-Aβ40. Thioflavin-T fibrillogenesis assay demonstrated that PUT-Aβ40 has a greater propensity to form insoluble fibrils compared with Aβ40, most likely due to the ability of PUT-Aβ40 to form β sheet structure more readily than Aβ40. These results demonstrate that the inadequate plaque targeting of Aβ40 is due to cellular uptake, metabolism, and efflux from the brain parenchyma. Despite better plaque targeting of PUT-Aβ40, its propensity to form fibrils may render it less suitable for human use and thus allow increased focus on the development of novel derivatives of Aβ with improved characteristics.

Amyloid β (Aβ) protein is a hydrophobic peptide that is thought to be neurotoxic. A consensus is emerging that Aβ proteins, as soluble monomers or polymers, play a critical role in the neurotoxicity and subsequent development of amyloid plaque formation, which contributes to the pathology of Alzheimer’s disease (AD). The extracellular accumulation of Aβ peptides into plaques is one of the pathological hallmarks for the definitive post-mortem diagnosis of AD. A diagnostic imaging technique capable of directly visualizing these amyloid plaques will provide not only a more definitive premortem diagnosis of AD but also a tool for prognostication and evaluation of putative therapies (Poduslo et al., 2002).

Currently, no method exists to image “individual” amyloid plaques in humans for a definitive and early diagnosis of AD. Radiolabeled molecular probes that bind to β-amyloid plaques have recently been demonstrated both in vitro and in animal studies (Skovronsky et al., 2000; Wengenack et al., 2000a; Agdeppa et al., 2001; Baeskai et al., 2001). The most notable of these probes is the Pittsburgh compound-B, which allowed the researchers to visualize amyloid plaques by bulk tissue enhancement after positron emission tomography imaging for the first time in AD patients (Klunk et al., 2004). Although scintigraphic imaging of β-amyloid plaques is...
promising, certain difficulties may be envisioned for clinical application. The most obvious is poor spatial resolution. The spatial resolution of clinically available tomographic scintigraphic techniques (positron emission tomography or single-photon emission computed tomography) is several times poorer than that of standard three-dimensional magnetic resonance imaging (MRI).

The ability of human Aβ40 and putrescine-Aβ40 (PUT-Aβ40) to selectively target amyloid plaques in AD transgenic mouse (APP,PS1) brain has provided an opportunity to use them as molecular probes to image amyloid plaques with MRI (Zlokovic et al., 1993; Maness et al., 1994; Poduslo et al., 1997, 1999; Mackie et al., 2002); however, the plaque-targeting ability of 125I-Aβ40 after i.v. injection in AD transgenic mice was low (Wengenack et al., 2000a). PUT-Aβ40 was shown to have 2-fold higher BBB permeability than unmodified Aβ40 and was able to target and label amyloid plaques in APP,PS1 animals after i.v. administration. When covalently linked to gadolinium diethylenetriaminepentaacetic acid, which provides contrast for MRI imaging, Gd-PUT-Aβ40 was able to provide enough contrast to image amyloid plaques in APP,PS1 mice, whereas Gd-Aβ40 failed to provide the required contrast (Poduslo et al., 2002). The differences in the efficacy of these compounds to label plaques ex vivo cannot be completely explained by the differences in their permeability values at the BBB.

Permeability at the BBB, endothelial transcytosis, as well as diffusion within and efflux from the brain parenchyma are important factors that determine the amount of Aβ-probe available to target plaques. The proportion of Aβ-probe in the extracellular space of the brain parenchyma versus intracellular uptake is another important consideration that will determine the successful targeting of amyloid plaques. Adequate knowledge of the transport kinetics of human Aβ protein and its derivatives at the BBB and in the brain will allow us to design smart molecular probes capable of labeling plaques in Alzheimer’s patients as well as provide further information regarding the dynamics of Aβ transport in the progression of AD.

The objective of the current study was to investigate various factors affecting the in vivo uptake of Aβ40 and PUT-Aβ40 at the BBB, accumulation within and efflux from the brain tissue in vitro, and binding to the amyloid plaques, as a means to explain the better targeting ability of PUT-Aβ40 over native Aβ40.

**Materials and Methods**

### Synthesis of Human Aβ40

Aβ40 was synthesized on an ABI 433 peptide synthesizer (Applied Biosystems, Foster City, CA) using 2-(H-benzotriazol-1-yl)1,1,3,3-tetramethyl urea hexafluorophosphate activation and the manufacturer’s suggested synthesis protocols. The starting resin was Val-Novasyn TGA resin (Calbiochem-Novabiochem, San Diego, CA). The peptides were cleaved from the solid support using 5% crystalline tritylamine for 2 h at room temperature and were purified by reverse-phase high-performance liquid chromatography using a heptad 250-× 21.2-mm C18 Jupiter column (Phenomenex, Torrance, CA). The weights of the peptides were confirmed by electrospray ionization mass spectrometry (Sciex API 165; Applied Biosystems/MDS Sciex, Foster City, CA).

### Synthesis of Fluorescein-Labeled Aβ40 (F-Aβ40)

After the final deprotection of the N-terminal Nα-fluoren-9-yl-methoxycarbonyl group, the peptide resin was washed with 12% diisopropylethylamine/dichloromethane (DCM). N-Hydroxysuccinimide-fluorescein (0.2 mM; Pierce, Rockford, IL) was dissolved in 6 ml of dimethylformamide and added to the resin saturated with 12% diisopropylethylamine/DCM. The resin slurry was mixed overnight at room temperature, followed by several washes with dimethylformamide and DCM. The success of the fluorescein addition was confirmed by a negative ninhydrin reaction.

### Putrescine Modification of Aβ40 and F-Aβ40

Putrescine modification of synthetic human Aβ40 and F-Aβ40 was performed by covalent linkage of the polyamine to carboxylic acid groups using carbodiimide at pH of 6.7 as described previously (Poduslo and Curran, 1996a,b).

### Radiodination of Proteins

Five hundred micrograms of human Aβ40 or PUT-Aβ40 was labeled with carrier-free Na125I, whereas the bovine serum albumin (BSA) (500 μg) was labeled with carrier-free Na131I using the chloramine-T procedure as described previously (Poduslo et al., 2001). Free radioactive iodine was separated from the radioabeled protein by dialysis against 0.01 M phosphate-buffered saline at pH 7.4 (Sigma-Aldrich Co., St. Louis, MO). Purity of the radioabeled proteins was determined by trichloroacetic acid (TCA) precipitation. The radioabeled protein was considered to be acceptable if the precipitable radioactive counts were greater than 95% of the total counts. The specific activity of the proteins thus obtained was determined as 2.0 ± 0.1 μCi/μg. No significant difference was observed between the specific activities of 125I-Aβ40 and 125I-PUT-Aβ40.

### Animals

Wild-type (WT) mice (B6/SJL strain) were obtained from the Jackson Laboratory (Bar Harbor, ME). The mice were housed in a virus-free barrier facility under a 12-h light/dark cycle, with ad libitum access to food and water. All the experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals using protocols approved by the Mayo Institutional Animal Care and Use Committee.

### PUT-Aβ40 Pharmacokinetics Studies

Before the beginning of the experiment, the femoral vein and the femoral artery of each mouse were catheterized under general anesthesia (Poduslo et al., 2001). An i.v. bolus dose of 125I-PUT-Aβ40 (100 μCi in 100 μl) was administered through the femoral vein. Blood samples (20 μl) were obtained from the femoral artery at 0.25-, 1-, 3-, 5-, 10-, and 15-min intervals. At the end of the experiment, an i.v. bolus dose of 131I-BSA (100 μCi in 100 μl) was administered to serve as a measure of residual plasma volume (Vp). One minute after the 131I-BSA injection, the final blood sample was collected, and the animal was sacrificed. The blood samples were diluted to a volume of 100 μl using normal saline, centrifuged, and the supernatant was obtained. After TCA precipitation, the supernatant was assayed for 125I and 131I radioactivity in a two-channel gamma counter (Cobra II; PerkinElmer Life and Analytical Sciences, Boston, MA). The measured activity was corrected for the background and crossover of 131I activity into the 125I channel. At the end of the experiment, the brain of the animal was removed from the cranial cavity, dissected into cortex, caudate putamen, hippocampus, thalamus, brain stem, and cerebellum, and assayed for 125I and 131I radioactivity. The effect of Aβ40 on the 125I-PUT-Aβ40 uptake at the blood brain barrier was
Metabolism of 125I-PUT-\(\text{A}_{\beta 40}\)

The metabolism of 125I-PUT-\(\text{A}_{\beta 40}\) in the plasma of WT mice was determined by adding 0.1 \(\mu\)Ci of 125I-PUT-\(\text{A}_{\beta 40}\) to 350 \(\mu\)l of plasma. A 20-\(\mu\)l sample was taken from the mixture at regular intervals up to 60 min, and the amount of intact 125I-PUT-\(\text{A}_{\beta 40}\) in the sample was determined by TCA precipitation. The metabolism of 125I-PUT-\(\text{A}_{\beta 40}\) in the presence of brain, liver, kidney, and spleen slices was determined using the organs obtained from WT mice after perfusion with PBS. The organs were weighed, chopped into 1-mm-thick slices using a tissue slicer (Stoelting Co., Wood Dale, IL), and placed in oxygenated (95\% \( \text{O}_2\) and 5\% \( \text{CO}_2\)) KRB until the start of the experiment. The tissue slices were then transferred to 5 ml of KRB containing 0.1 \(\mu\)Ci of 125I-PUT-\(\text{A}_{\beta 40}\) and incubated at 37\(^\circ\)C for 24 h. The initial rates of efflux (15 min) of these compounds from the slices were calculated as:

\[
\text{Efflux Rate} = \frac{q_{\text{efflux}}}{t_{15\text{min}}}
\]

where \(q_{\text{efflux}}\) is the radioactivity (counts per minute) of the compound present in the tissue at 15 min, and \(t_{15\text{min}}\) is the time taken for the washout. The rates of efflux were normalized by the donor concentrations and plotted against log donor concentrations.

Brain Slices

After sacrificing with an overdose of sodium pentobarbital (200 mg/kg i.p.), the animals were decapitated, and the brains were carefully removed from the cranial cavity. Each brain was cut coronally into 1-mm-thick slices containing cortex and hippocampus. The slices were placed in oxygenated (95\% \( \text{O}_2\) and 5\% \( \text{CO}_2\)) KRB until the start of the experiment.

Brain Slice Uptake of 125I-\(\text{A}_{\beta 40}\) and 125I-PUT-\(\beta 40\)

Effect of Time. After the equilibration in KRB, each brain slice was incubated in 1 ml of donor solution containing 125I-\(\text{A}_{\beta 40}\) (0.8 \(\mu\)Ci/ml KRB) at 37\(^\circ\)C. The brain slices were harvested at various time points: 0, 10, 15, 30, 45, and 60 min, rinsed with KRB, and incubated in 5 ml of KRB (receiving medium). The brain slices were then transferred to 1 ml of donor solution (preincubate medium). The brain slices were harvested at various time points: 0, 10, 15, and 30 min, rinsed with KRB, and incubated in 5 ml of KRB (receiving medium). The brain slices were harvested at various time points: 0, 10, 15, 30, 45, and 60 min, rinsed with KRB, and incubated in 5 ml of KRB (receiving medium). The brain slices were then transferred to 1 ml of donor solution containing 125I-\(\text{A}_{\beta 40}\) (0.8 \(\mu\)Ci/ml KRB). After 15 min, the brain slices were removed from the donor solutions, rinsed with KRB, and assayed for radioactivity using a dual-channel gamma counter.

Effect of Donor Concentration. After the equilibration, each brain slice was incubated at 37\(^\circ\)C in a 1-ml donor solution containing different concentrations of 125I-\(\text{A}_{\beta 40}\) (0.05–28.4 \(\mu\)Ci/ml KRB). After 15 min, the brain slices were removed from the donor solutions, rinsed with KRB, and assayed for radioactivity using a dual-channel gamma counter.

Effects of Metabolic Inhibitors. After the equilibration, brain slices were preincubated with the metabolic inhibitors ouabain (1 \(\mu\)M), androgens (0.1 \(\mu\)M), and actinomycin D (1 \(\mu\)g/ml) to inhibit proteolytic degradation. The brain slices were then transferred to 1 ml of donor solution (preincubate medium). The brain slices were harvested at various time points: 0, 10, 15, and 30 min, rinsed with KRB, and incubated in 5 ml of KRB (receiving medium). The brain slices were then transferred to 1 ml of donor solution containing 125I-\(\text{A}_{\beta 40}\) or PUT-\(\beta 40\) maintained at 37\(^\circ\)C. After 15 min, the brain slices were removed, rinsed with KRB, and assayed for radioactivity in a two-channel gamma counter.

Cellular Accumulation of F-\(\text{A}_{\beta 40}\) and F-PUT-\(\beta 40\) in the Brain Parenchyma. Each brain slice was incubated in 1 ml of donor solution containing F-\(\text{A}_{\beta 40}\) or F-PUT-\(\beta 40\) (40 \(\mu\)g/ml KRB) at 37\(^\circ\)C. After 15 min, the slice was removed from the donor solution, placed on a coverslip moistened with KRB, and imaged under a Zeiss LSM 410 (Carl Zeiss, Thornwood, NY) laser confocal microscope.

125I-\(\text{A}_{\beta 40}\) and 125I-PUT-\(\beta 40\) Efflux from Brain Slices

WT mice brain slices were equilibrated in KRB for 15 min at 37\(^\circ\)C. Each slice was then incubated in a 1-ml donor solution containing various concentrations of 125I-\(\text{A}_{\beta 40}\) or 125I-PUT-\(\beta 40\) at 37\(^\circ\)C for 30 min, washed with KRB, and incubated in 5 ml of KRB (receiving medium). The brain slices were harvested at various time points: 0, 10, 15, and 30 min, rinsed with KRB, and assayed for 125I radioactivity. The initial rates of efflux (<15 min) of these compounds from the brain slices were calculated at various donor concentrations. The rates of efflux were normalized by the donor concentrations and plotted against log donor concentrations.

Circular Dichroism Spectroscopy

The secondary structures of \(\text{A}_{\beta 40}\) and PUT-\(\beta 40\) were determined using circular dichroism (CD) spectroscopy. A 20 \(\mu\)M solution of each protein was prepared in PBS, pH 7.2, filtered using a 0.22-\(\mu\)m syringe filter, and transferred to a 0.2-cm path length quartz cuvette. The CD spectra of \(\text{A}_{\beta 40}\) and PUT-\(\beta 40\) were obtained from 260 to 200 nm at 4°C, scanning every 1 nm with an averaging time of 5 s, on an AVIV CD spectrometer model 215 (Aviv Instruments, Newington, NH).

Data Analysis

The 125I-PUT-\(\beta 40\) plasma concentration profile after the single i.v. bolus dose of 125I-\(\beta 40\) was best described by a biexponential disposition function \(C(t) = A e^{-\lambda t} + B e^{-\beta t}\), where \(C(t) = 125I-\beta 40\) per milliliter of plasma, A and B are the intercepts and \(\alpha\) and \(\beta\) are the slopes of the biexponential curve. Pharmacokinetic parameters were estimated by nonlinear curve fitting using Gauss-Newton (Levenberg and Hartley) algorithm and iterative reweighting (WinNonlin Professional, version 4.1; Pharsight, Mountain View, CA). Secondary parameters such as \(C_{\text{max}}\) (maximum plasma concentration), the first \(t_{1/2\alpha}\) and the second \(t_{1/2\beta}\) phase half-lives, the plasma clearance (Cl), the steady-state volume of distribution \(V_p\), and area under the plasma concentration curve (AUC) were also calculated using WinNonlin. The mean values of controls and treatments were compared by Student’s \(t\) test using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA).

Cerebrovascular permeability coefficient-surface area product (PS) and \(V_p\) measurements were calculated as described previously by Poduslo (1993). The residual brain region \(V_p\) (microliters per gram) is calculated as:

\[
V_p = q_p \times \frac{C}{C \times WR}
\]

where \(q_p\) is the radioactivity (counts per minute) of 131I-BSA present in the tissue. \(C_s\) is the 131I-BSA concentration (counts per minute per milliliter) in plasma, \(W\) is the dry weight (grams) of the brain region, and \(R\) is the wet weight/dry weight ratio for mice of a defined age group. From the total 125I-\(\text{A}_{\beta 40}\) content \(q_{\text{total}}\) (counts per minute) of the brain region, the amount of 125I-\(\text{A}_{\beta 40}\) that enters the brain region extravascular space \(q\) (counts per minute per gram) is calculated as:

\[
q = q_{\text{total}} - V_p C_s \times 10^3
\]

where \(C_s\) is the final 125I-\(\text{A}_{\beta 40}\) concentration (counts per minute per milliliter) in plasma. The PS (milliliters per gram per second) of the BBB is calculated as:

\[
V_p = q_p \times \frac{C}{C \times WR}
\]
The initial rates of efflux of $^{125}$I-AB40 and $^{125}$I-PUT-A$\beta$40 from the WT brain slices were normalized by the donor concentrations and plotted against log donor concentrations (log C). The IC$_{50}$ of $^{125}$I-AB40 efflux from the brain slices in vitro was determined by fitting the following equation to the data using GraphPad Prism version 3.03 (GraphPad Software).

$$\text{NE} = \frac{(\text{NE}_{\text{max}} - \text{NE}_{\text{min}})}{1 + 10^{\text{logC} - \text{logC}_{50}}}$$

where NE$_{\text{max}}$ is the maximum normalized efflux value, which was obtained at the lowest $^{125}$I-AB40 concentration, NE$_{\text{min}}$ is the minimum NE value obtained at the highest $^{125}$I-AB40 concentration.

**Results**

The in vivo targeting abilities of molecular probes may be compromised because of rapid elimination from the systemic circulation, competitive inhibition by high circulating A$\beta$ levels in APP,PS1 mice, proteolytic degradation in the brain parenchyma, uptake and degradation by neurons, and efflux from the brain. In this study, we systematically studied and compared the influence of each of the above factors on the amyloid plaque targeting of A$\beta$40 and PUT-A$\beta$40.

$^{125}$I-PUT-A$\beta$40 Plasma Pharmacokinetics

Upon i.v. administration, the $^{125}$I-PUT-A$\beta$40 concentrations in the plasma of WT mice declined rapidly, exhibiting a biexponential disposition with short $\alpha$ and $\beta$ half-lives ($t_{1/2\alpha}$ and $t_{1/2\beta}$, respectively) (Fig. 1; Table 1). The $C_{\text{max}}$, $t_{1/2\alpha}$, and AUC were significantly lower, whereas the Cl and Vss were significantly higher metabolism of $^{125}$I-PUT-A$\beta$40 in the peripheral tissues compared with that of $^{125}$I-AB40.

**125I-PUT-A$\beta$40 Metabolism in the Peripheral Tissues**

To investigate the contribution of peripheral tissue metabolism on the rapid elimination of $^{125}$I-PUT-A$\beta$40 from the systemic circulation, in vitro degradation of $^{125}$I-PUT-A$\beta$40 in the presence of WT mice tissue slices (liver, kidney, and spleen) as well as plasma was studied. Although $^{125}$I-PUT-A$\beta$40 degradation in the WT plasma was slightly higher than the degradation in Dulbecco’s modified Eagle’s medium, not more than 10% of the initial amount of $^{125}$I-PUT-A$\beta$40 was degraded in 60 min (Fig. 2). The degradation of $^{125}$I-PUT-A$\beta$40 in the presence of liver slices was similar to that of $^{125}$I-AB40, with substantial degradation in 60 min. The extent of $^{125}$I-PUT-A$\beta$40 degradation in the kidney and spleen was significantly lower compared with that of $^{125}$I-PUT-A$\beta$40 (Fig. 2). These results demonstrate that despite rapid clearance from the peripheral circulation compared with $^{125}$I-AB40, the metabolism of $^{125}$I-PUT-A$\beta$40 in the peripheral organs of elimination was significantly lower than that of $^{125}$I-AB40.

$^{125}$I-PUT-A$\beta$40 Brain Uptake

It has been established in our earlier studies that the PS value, which is a measure of BBB permeability of a protein, of $^{125}$I-PUT-A$\beta$40 is significantly higher than that of $^{125}$I-AB40 in APP,PS1 mice (Wengenack et al., 2000a). These transgenic mice develop distinct plaques at the age of 3 months with substantial amyloid burden by 6 months, mostly in the cortex and hippocampus (Wengenack et al., 2000) and carry significantly higher levels of A$\beta$40 (13.9 pmol/ml) in the peripheral circulation compared with WT mice (2.9 pmol/ml) at 6 months (Poduslo et al., 2001). It was shown that the plaque burden in APP,PS1 animals increases linearly with age (Wengenack et al., 2000b), and the endogenous A$\beta$40 levels in the peripheral circulation are expected to follow a similar trend. The efficacy of PUT-A$\beta$40 to target plaques in older APP,PS1 animals with high endogenous levels of A$\beta$40 may be compromised if A$\beta$40 competes with PUT-A$\beta$40 for the BBB uptake. Hence, it is important to determine whether A$\beta$40 can competitively inhibit the PUT-A$\beta$40 absorption at the BBB. It was observed that when 1 mg of A$\beta$40 was coadministered with 100 $\mu$Ci of $^{125}$I-PUT-A$\beta$40, the PS values of $^{125}$I-PUT-A$\beta$40 decreased significantly in various brain regions (Fig. 3). The $V_p$ values, however, did not change significantly because of the coadministration of unlabeled A$\beta$40 (Fig. 3). To verify that the reduction in the PS values was not due to change in the pharmacokinetics of $^{125}$I-PUT-A$\beta$40 resulting from a possible interaction with unlabeled A$\beta$40 in the syringe before injection, the PS values were determined by first injecting 1 mg of unlabeled A$\beta$40 immediately followed by 100 $\mu$Ci of $^{125}$I-PUT-A$\beta$40. The PS values

![Fig. 1. Plasma pharmacokinetics of $^{125}$I-AB40 ($n = 12$; from Kandimalla et al. (2005)] and $^{125}$I-PUT-A$\beta$40 in 24-week-old WT mice ($n = 3$). Data are mean ± S.D.; lines indicate the fit of the two-compartment pharmacokinetic model to the plasma concentration-time data.

**TABLE 1**

Comparison of the plasma pharmacokinetic parameters of $^{125}$I-AB40 and $^{125}$I-PUT-A$\beta$40 in 24-week-old WT mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>$^{125}$I-AB40 $^a$</th>
<th>$^{125}$I-PUT-A$\beta$40</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$ (µCi/ml)</td>
<td>17.4 ± 2.7</td>
<td>8.6 ± 3.4</td>
<td>*</td>
</tr>
<tr>
<td>$t_{1/2\alpha}$ (min)</td>
<td>0.9 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>**</td>
</tr>
<tr>
<td>$t_{1/2\beta}$ (min)</td>
<td>9.2 ± 2.3</td>
<td>8.9 ± 3.6</td>
<td>N.S.</td>
</tr>
<tr>
<td>Clearance (ml/min/g)</td>
<td>0.09 ± 0.02</td>
<td>0.23 ± 0.08</td>
<td>*</td>
</tr>
<tr>
<td>Vss (ml/g)</td>
<td>0.9 ± 0.17</td>
<td>2.2 ± 0.7</td>
<td>*</td>
</tr>
<tr>
<td>AUC (µCi/ml/min)</td>
<td>50.5 ± 7.3</td>
<td>18.5 ± 7.0</td>
<td>**</td>
</tr>
</tbody>
</table>

$^a$ From Kandimalla et al. (2005).
of $^{125}$I-PUT-Aβ40 obtained from this experiment were not significantly different from the PS values obtained when $^{125}$I-PUT-Aβ40 was coadministered with Aβ40 (data not shown). These results demonstrate that the endogenous Aβ40 levels in the peripheral circulation can reduce the permeability of $^{125}$I-PUT-Aβ40 as well as $^{125}$I-Aβ40 (Kandimalla et al., 2005) at the BBB.

Brain Slice Uptake Studies

After permeating the BBB, $^{125}$I-Aβ40 or $^{125}$I-PUT-Aβ40 must diffuse across the brain parenchyma to reach the plaque sites. The relative diffusivities of $^{125}$I-Aβ40 and $^{125}$I-PUT-Aβ40 across the brain parenchyma may be directly related to their plaque-targeting capabilities. Hence, the uptake of $^{125}$I-Aβ40 and $^{125}$I-PUT-Aβ40 into WT mouse brain slices was determined in vitro.

Effect of Time. The uptake of $^{125}$I-Aβ40 into WT mouse brain slices was linear till 15 min and reached a plateau from 15 to 60 min (Fig. 4).

Effect of Donor Concentration. Uptake of $^{125}$I-Aβ40 across the WT mouse brain slices was nonlinearly dependent upon the donor concentration (Fig. 5). Upon fitting a Michaelis-Menten type expression to the data, $K_m$ and $V_{max}$ were estimated to be 18.4 μCi/ml and 2622 nCi/g wet weight, respectively, whereas 24.8 nCi/g wet weight was estimated to be nonspecifically bound to the tissue.

Localization of F-Aβ40 and PUT-Aβ40 (F-PUT-Aβ40) in the Brain Parenchyma. A laser confocal micrograph of WT brain slice incubated with F-Aβ40 demonstrated intense fluorescence accumulated in the cells, most likely neurons, of cortex with faint background fluorescence (Fig. 6). Laser confocal micrograph of F-PUT-Aβ40 demonstrated numerous dark spots amid intensely fluorescent background in the cortex. These results suggest that F-PUT-Aβ40 was not taken up by the cells and remained in the extracellular space of the brain parenchyma (Fig. 6).

Effects of Metabolic Inhibitors. The uptake of $^{125}$I-Aβ40 in the WT brain slices treated with metabolic inhibitors such as ouabain (ATPase inhibitor) or 2,4-DNP (uncoupler of oxidative phosphorylation) was reduced significantly compared with the untreated brain slices (Fig. 7), suggesting that the brain slice uptake of $^{125}$I-Aβ40 is energy-dependent and carrier-mediated. The uptake of $^{125}$I-PUT-Aβ40 in the WT brain slices was not significantly affected in the presence of a metabolic inhibitor such as 2,4-DNP (Fig. 7). Hence, the uptake of $^{125}$I-PUT-Aβ40 in the brain slices is more likely by passive diffusion.

$^{125}$I-Aβ40 and $^{125}$I-PUT-Aβ40 Efflux from Brain Slices

As indicated above and from earlier studies conducted in our lab, Aβ40 efflux from WT brain slices could be inhibited by 2,4-DNP, which strongly suggested that it is a carrier-mediated process (Kandimalla et al., 2005). In the next experiment, we evaluated the saturability of $^{125}$I-Aβ40 efflux process and compared it with that of $^{125}$I-PUT-Aβ40 (Fig. 8). The initial rates of $^{125}$I-Aβ40 efflux from WT brain slices,
normalized with the donor concentration, decreased with an increase in log donor concentration. In contrast, the normalized initial rates of $^{125}$I-PUT-A$\beta40$ efflux from WT brain slices did not change appreciably with log donor concentration. These results demonstrated that the efflux of $^{125}$I-PUT-A$\beta40$ from WT brain slices is carrier mediated, whereas the efflux of $^{125}$I-A$\beta40$ is predominantly by passive diffusion.

Biophysical Characterization of A$\beta40$ and PUT-A$\beta40$

**CD Spectra.** The CD spectra of A$\beta40$ and PUT-A$\beta40$ illustrated in Fig. 9 demonstrate that A$\beta40$ has a random coil structure, whereas PUT-A$\beta40$ assumes a $\beta$-sheet structure in phosphate-buffered saline, pH 7.2, at 4°C.

**Kinetics of Fibril Formation.** It is believed that high concentration of soluble A$\beta$ accumulates over time in the brain extracellular space, polymerizes into insoluble fibrils, and eventually forms amyloid plaques. The ease with which a molecule can form fibrils is considered a direct measure of its likelihood to provide a seed for plaque formation. The ideal diagnostic probe would not form fibrils after reaching the brain parenchyma but only bind to pre-existing plaques. We studied the fibril formation of A$\beta40$ and PUT-A$\beta40$ using THT assay (Levine, 1993). THT binds to the $\beta$-sheet structure of fibrils and emits fluorescence. The intensity of the fluorescence provides a direct measure of the extent of fibril formation. The intensity of THT fluorescence plotted against time in Fig. 10 describes the kinetics of A$\beta40$ and PUT-A$\beta40$ fibril formation. A$\beta40$ forms fibrils with a lag time of 2 h, whereas PUT-A$\beta40$ forms fibrils readily. Moreover, the extent of fibril formation with PUT-A$\beta40$ was substantially higher than A$\beta40$ reflected in the larger fluorescence intensity. At the end of the experiment, the fibril formation was verified by electron microscopy (data not shown).

**Discussion**

$^{125}$I-A$\beta40$ and $^{125}$I-PUT-A$\beta40$ have demonstrated a differential ability to target amyloid plaques in APP,PS1 transgenic mice after i.v. injection. As a result, they are used in this study as model compounds to investigate various physiological factors (plasma clearance, BBB permeability, parenchymal diffusion and metabolism, and efflux from the central nervous system) and biophysical factors (effect of charge, ability to form fibrils, and affinity to plaques) that affect plaque targeting.

It is generally believed that lower plasma clearance result-
ing in high sustained plasma levels of the probe will enhance its BBB permeability. Our earlier studies demonstrated that \(^{125}\text{I}-\text{A} \beta 40\) is rapidly eliminated from the systemic circulation as a result of significant renal clearance and hepatic metabolism (Kandimalla et al., 2005). Despite better plaque-targeting ability, \(^{125}\text{I}-\text{PUT-A} \beta 40\) is more rapidly cleared from the systemic circulation than \(^{125}\text{I}-\text{A} \beta 40\) after i.v. administration. Unlike \(^{125}\text{I}-\text{A} \beta 40\), the rapid clearance of \(^{125}\text{I}-\text{PUT-A} \beta 40\) did not correlate with its metabolism in the peripheral tissues such as liver, kidney, and spleen. Although the metabolism of \(^{125}\text{I}-\text{PUT-A} \beta 40\) in the liver slices was only slightly lower compared with \(^{125}\text{I}-\text{A} \beta 40\), its metabolism in kidney slices was significantly lower than \(^{125}\text{I}-\text{A} \beta 40\). Because the molecular size of \(^{125}\text{I}-\text{PUT-A} \beta 40\) or \(^{125}\text{I}-\text{A} \beta 40\) is small, these molecules can be eliminated by the kidney without the need for initial catabolism in the liver. Moreover, the microvascular permeability of cationized proteins like \(^{125}\text{I}-\text{PUT-A} \beta 40\) was found to be substantially higher than the native proteins (Dellian et

Fig. 6. Accumulation of fluorescein-labeled A\beta40 and fluorescein-labeled PUT-A\beta40 in the neurons and extracellular space of WT mouse brain slices in vitro, respectively (20×).
al., 2000), resulting in their increased renal clearance. Although higher microvascular permeability can increase the distribution of 125I-PUT-Aβ40 to brain (Poduslo and Curran, 1996b), increase in the renal clearance and rapid distribution to peripheral tissues could diminish the net effect. Hence, it is unlikely that the better plaque-targeting ability of 125I-PUT-Aβ40 compared with 125I-Aβ40 is due to favorable plasma pharmacokinetics.

Like the BBB permeability of 125I-Aβ40, which decreased significantly in the presence of unlabeled Aβ40 (0.125–2 mg) in a dose-dependent manner (Kandimalla et al., 2005), the BBB permeability of 125I-PUT-Aβ40 decreased when coadministered with 1 mg of Aβ40. Even though the PS value of 125I-PUT-Aβ40 is higher than that of 125I-Aβ40 in WT mice, the PS value of 125I-PUT-Aβ40 coadministered with 1 mg of Aβ40 was similar to the PS value of 125I-Aβ40 coadministered with 1 mg of Aβ40. These results indicate that the BBB permeability of 125I-PUT-Aβ40 is also saturable. Therefore, endogenous Aβ40 levels in the peripheral circulation of APP,PS1 animals, which were reported to increase linearly with age, could affect the BBB permeability of 125I-PUT-Aβ40 to a similar extent as 125I-Aβ40. Based on this information, it is likely that a modest 1.5- to 2.0-fold higher PS value of 125I-PUT-Aβ40 compared with 125I-Aβ40 (Wengenack et al., 2000a) may, at the best, have a limited contribution to the differences in their plaque-targeting abilities.

Our earlier work demonstrated that the accumulation of 125I-Aβ40 in the extracellular space of APP,PS1 mouse brain is primarily influenced by the reduced clearance of Aβ40, mediated by metabolism and/or efflux, from the brain parenchyma (Kandimalla et al., 2005). Mouse brain slices containing cortex and hippocampus were used in this study as in vitro model to investigate the role of parenchymal metabolism and uptake on the differential plaque-targeting ability of 125I-Aβ40 and 125I-PUT-Aβ40. This in vitro model has been used by several researchers to study the metabolism (Newman et al., 1990) and diffusion of ions (Newman et al., 1995) and small (Gredell et al., 2004) and large molecules (Patlak et al., 1998) in the brain parenchyma. Uptake of 125I-Aβ40 into WT mouse brain slices followed Michaelis-Menten kinetics, which suggested that the uptake is saturable. The inhibition of 125I-Aβ40 uptake in WT mouse brain slices by metabolic inhibitors such as 2,4-DNP and ouabain suggested that it is energy-dependent and most likely carrier-mediated. Preferential localization of F-AP40 in the brain parenchymal cells, most likely neurons, after a short incubation time (5 min) further confirms the presence of carrier-mediated transport of AP40.

In contrast, the metabolic inhibitors such as 2,4-DNP did not affect the uptake of 125I-PUT-Aβ40 significantly, thereby suggesting that the uptake of 125I-PUT-Aβ40 in WT mouse brain slices is most likely by passive diffusion. Localization of F-PUT-Aβ40 in the extracellular space of the brain parenchyma but not in the cells provides further evidence that there is no receptor-mediated uptake of PUT-Aβ40. The cellular uptake of Aβ40 in the brain parenchyma makes it unavailable for targeting the plaques located in the extracellular space. In contrast, PUT-Aβ40 is not taken up by the cells, remains in the extracellular space, and is available for binding to plaques, a desirable feature for imaging amyloid plaques.

In vitro fibril binding studies demonstrated that both 125I-Aβ40 and 125I-PUT-Aβ40 bind to Aβ40 fibrils with similar affinity (data not shown). However, the differences in the extracellular concentrations of the probe can alter plaque binding significantly. The extracellular concentration of 125I-Aβ40 is not only reduced because of cellular uptake but also by the efflux and parenchymal metabolism. The degradation of 125I-PUT-Aβ40 in the WT mouse brain slices was significantly lower than the previously reported degradation of 125I-Aβ40 (Kandimalla et al., 2005). The efflux of 125I-Aβ40 across WT mouse brain slices was found to be saturable and inhibited by 2,4-DNP (Kandimalla et al., 2005), thereby suggesting that it is energy-dependent and carrier-mediated. The efflux of 125I-PUT-Aβ40, on the other hand, was much slower compared with that of 125I-Aβ40 and was not dependent upon the donor concentration, suggesting that it occurs via passive diffusion.

Despite its success in providing contrast enhancement of plaques after i.v. injection during MRI of APP,PS1 mouse brains ex vivo, the utility of PUT-Gd-Aβ40 for diagnostic use in animal models and patients is limited because carbodiimide-mediated modification of Aβ40 with putrescine is associated with problems inherent with the protein itself, such as crosslinking, aggregate and/or fibril formation, and insolubility. In addition, PUT-Aβ40 forms fibrils more readily than Aβ40. The ease with which a molecule can form fibrils is considered as a direct measure of its likelihood to provide a seed for plaque formation. Hence, a new probe, Gd[N-4ab/Q-4ab]Aβ30, was produced as a putative MRI contrast enhancement agent by first synthesizing a glutamyl-4-aminobutane or asparagyl-4-aminobutane, which were then incorporated into the synthesis of the protein using standard solid-phase methods (Poduslo et al., 2004). The complete chemical synthesis of this probe eliminates peptide crosslinking, aggregate and fibril formation, and insolubility that affected the carbodiimide-mediated modification of Aβ40 with putrescine. Apart from having the chemical purity, this probe is devoid of the neurotoxic domain found in Aβ40 and is not amyloidogenic like Aβ40. In addition, Gd[N-4ab/Q-4ab]Aβ30 has good BBB permeability and labels neuritic plaques in vitro with affinity comparable with PUT-Aβ40. However, attempts to image amyloid plaques in APP,PS1 animals in vivo using this agent yielded only modest results. Studies are being conducted to enhance

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**Fig. 10.** Kinetics of 125I-Aβ40 and 125I-PUT-Aβ40 fibril formation. THT binds to the β-sheet structure of the fibrils and emits fluorescence. The intensity of the fluorescence provides a direct measure of the extent of fibril formation.
the in vivo efficacy of this probe through appropriate structural modifications to influence various physiologic factors, parenchymal metabolism and neuronal uptake, that were determined in the present study to adversely affect the plaque-targeting ability.

In summary, these studies demonstrated that both 125I-Aβ40 and 125I-PUT-Aβ40 are rapidly eliminated from the systemic circulation after i.v. administration in WT animals. However, the peripheral pharmacokinetics of the probes did not significantly influence their plaque-targeting capabilities. The higher concentration of endogenous Aβ40 in the peripheral circulation decreases the permeability of both compounds at the BBB. The metabolism of 125I-PUT-Aβ40 is substantially lower in the brain slices compared with that of 125I-Aβ40. This indicates that 125I-PUT-Aβ40 is metabolically stable and may remain intact in the brain parenchyma with higher concentrations being available for plaque targeting than 125I-Aβ40. In addition, the rate of 125I-PUT-Aβ40 efflux in WT brain slices was substantially lower compared with that of 125I-Aβ40. Hence, 125I-PUT-Aβ40 tends to remain in the brain parenchyma longer than 125I-Aβ40. This study also demonstrates that the fluorescein-labeled Aβ40, but not 125I-PUT-Aβ40, is taken up by cells, which makes the former unavailable for targeting the plaques located in the extracellular space. By comparing the distribution and metabolism of 125I-Aβ40 and 125I-PUT-Aβ40 after i.v. administration, it can be concluded that the inadequate targeting of 125I-Aβ40 to amyloid plaques despite its carrier-mediated uptake at the BBB could be due to cellular uptake, metabolism, and efflux of Aβ40 in the brain parenchyma. The knowledge gained from these studies will be very useful in the development of new Aβ derivatives with improved BBB permeability and plaque targeting.

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References


