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IN VITRO DETECTION OF (*S*)-NAPROXEN AND IBUPROFEN BINDING TO PLAQUES IN THE ALZHEIMER'S BRAIN USING THE POSITRON EMISSION TOMOGRAPHY MOLECULAR IMAGING PROBE 2-(1-{6-[(2-[^{18}F]FLUOROETHYL)(METHYL)AMINO]-2-NAPHTHYL}ETHYLIDENE) MALONONITRILE

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Abstract—Epidemiological studies have suggested that the chronic use of non-steroidal anti-inflammatory drugs (NSAIDs) reduces the relative risk of Alzheimer's disease (AD). The possible neuroprotection by NSAIDs in AD is generally attributed to anti-inflammatory activity. An additional mode of drug action may involve anti-aggregation of β-amyloid (Aβ) peptides by commonly used NSAIDs. We utilized *in vitro* competition assays, autoradiography, and fluorescence microscopy with AD brain specimens to demonstrate concentration-dependent decreases in the binding of the *in vivo* molecular imaging probe, 2-(1-{6-[(2-[^{18}F]fluoroethyl)(methyl) amino]-2-naphthyl}ethylidene)malononitrile ([^{18}F]FDDNP), against (*S*)-naproxen and (*R*)- and (*S*)-ibuprofen (but not diclofenac) to Aβ fibrils and ex vivo Aβ senile plaques. Conversely, *in vitro* amyloid dyes Congo Red and Thioflavine T were demonstrated in the same experiments not to bind to the FDDNP binding site. FDDNP and the NSAIDs that share the same binding site also exhibit anti-aggregation effects on Aβ peptides, suggesting that the shared binding site on Aβ fibrils and plaques may be a site of anti-aggregation drug action.

Our results indicate for the first time the binding of select NSAIDs to plaques, specifically to the binding site of the molecular imaging probe [^{18}F]FDDNP. Our understanding of the molecular requirements of FDDNP binding may help in the optimization of the Aβ anti-aggregation potency of experimental drugs. [^{18}F]FDDNP has been used to image plaques *in vivo* with positron emission tomography (PET), and investigations into the influence of Aβ anti-aggregation on the risk-reduction effects of NSAIDs on AD could utilize [^{18}F]FDDNP and PET in determining the occupancy rate of NSAIDs and experimental drugs in plaques in the living brain of AD patients.

Key words: anti-aggregation, β-amyloid fibrils, competitive binding, digital autoradiography, fluorescence microscopy, non-steroidal anti-inflammatory drugs.

Epidemiological studies have implicated the chronic use of non-steroidal anti-inflammatory drugs (NSAIDs) in reducing the relative risk (Breitner et al., 1995; Stewart et al., 1997; in t' Veld et al., 2001) or delaying the onset of Alzheimer's disease (AD) (Rogers et al., 1993; Breitner et al., 1995). It is believed that chronic inflammatory response in AD contributes to neurodegeneration (McGeer and McGeer, 1995; Halliday et al., 2000), but it is not clear if the possible neuroprotective effect of NSAIDs in AD is solely due to the suppression of chronic inflammatory response of microglia and astrocytes and reduced neuronal expression of cyclo-oxygenase enzymes (Halliday et al., 2000), or is due to mitigation of other pathological mechanisms in AD (McGeer and McGeer, 1995). Recently, NSAIDs have been suggested to exert an anti-aggregation effect on β-amyloid (Aβ) peptide (Thomas et al., 2001). Aggregates, or fibrils, of Aβ(1–40/42) peptides are major constituents of one of the hallmark lesions in AD, Aβ senile plaques (SPs). These lesions are considered central in the pathogenesis of AD (Selkoe, 1994; Teplow, 1998), suggesting the therapeutic paradigm involving the use of small-molecule drugs to inhibit pathological fibrillogenesis of Aβ peptides (Findeis, 2000). We report herein the previously unrealized *in vitro* NSAID binding to SPs and Aβ fibrils using 2-(1-{6-[(2-[^{18}F]fluoroethyl)(methyl)amino]-2-naphthyl}ethylidene)-malononitrile ([^{18}F]FDDNP; **1**, Scheme I). [^{18}F]FDDNP is a molecular imaging probe developed in our laboratories and previously used in the *in vivo* detection of SPs and neurofibrillary tangles in the living brain of AD patients using positron emission tomography (PET) (Shoghi-Jadid et al., 2002).

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Abbreviations: Aβ, β-amyloid; AD, Alzheimer's disease; ANOVA, analysis of variance; CR, Congo Red; [^{18}F]FDDNP, 2-(1-{6-[(2-[F]fluoroethyl)(methyl)amino]-2-naphthyl}ethylidene)malononitrile; NSAIDs, non-steroidal anti-inflammatory drugs; PBS, phosphate-buffered saline; PET, positron emission tomography; SPs, senile plaques; TT, thioflavine T.

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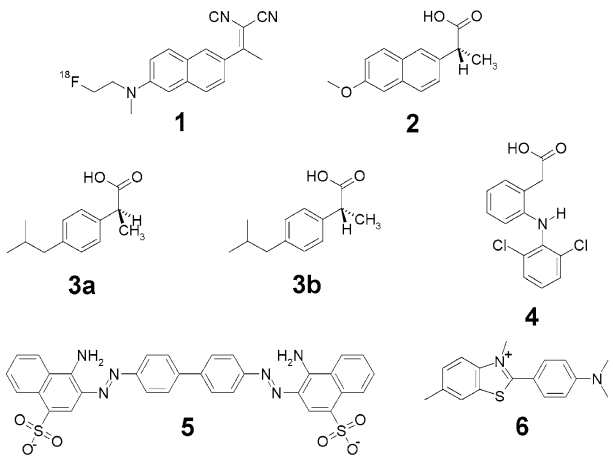

Scheme I. Chemical structures of [^{18}F]FDDNP (1), (*S*)-naproxen (2), (*R*)-ibuprofen (3a), (*S*)-ibuprofen (3b), diclofenac (4), CR (5) and TT (6).

EXPERIMENTAL PROCEDURES

Synthesis of [^{18}F]FDDNP

Non-radioactive FDDNP was prepared by a modified method developed for the synthesis of DDNP (Jacobson et al., 1996). The Bucherer reaction of 1-(6-hydroxy-2-naphthyl)-1-ethanone with 2-(methylamino)ethanol yielded 1-{6-[(2-hydroxyethyl)(methyl) amino]-2-naphthyl}-1-ethanone. The Knoevenagel reaction of the Bucherer product with malononitrile yielded 2-1-{6-[(2-hydroxyethyl)(methyl)amino]-2-naphthyl}ethylidene malononitrile which upon reaction with *p*-toluenesulfonyl chloride resulted in the tosylated precursor. Reaction of the precursor with KF/Kryptofix 222 (Sigma, St. Louis, MO, USA) in acetonitrile yielded FDDNP. This product and its intermediates described above were fully characterized by ultraviolet, nuclear magnetic resonance (^{1}H, ^{19}F and ^{13}C), mass, and fluorescence spectroscopy. [^{18}F]FDDNP (**1**) was similarly prepared by reaction of the tosylate group with no-carrier-added [^{18}F]KF/Kryptofix 222. After purification by semipreparative high-performance liquid chromatography (Whatman Partisil 10 silica column, Whatman, Clifton, NJ, USA; 50×1 cm; 7:3 dichloromethane:hexane; flow rate: 5.0 ml/min), radiochemically and chemically pure **1** was prepared in approximately 11% radiochemical yield (end-of-synthesis) in a synthesis time of 90 min.

Aβ(1–40) fibril formation

Aβ(1–40) (Biosource, Camarillo, CA, USA) fibrils were prepared according to published methods (Klunk et al., 1999). Briefly, 0.5 mg of Aβ(1–40) was dissolved in 1 ml phosphate-buffered saline (PBS), pH 7.4, and mixed with a magnetic stir bar for 3 days at 37 °C resulting in a visibly cloudy solution. Fibrils were used immediately after their production was confirmed. The production of Aβ fibrils was confirmed by imaging with a Jeol 100CX transmission electron microscope (Jeol, Peabody, MA, USA). A 5-μl drop from a fibril solution was allowed to settle for 30 s on a treated copper grid before being washed away with a drop of 2% uranyl acetate solution. Finally, an additional drop of 2% uranyl acetate was added to the grid to negatively stain the fibrils. Fibrils were used immediately after their production was confirmed. Additional tests for fibril formation using Congo Red (CR, **5**, Sigma) (Klunk et al., 1999) and Thioflavine T (TT, **6**, Sigma) (LeVine, 1993) were performed, as well. The absence of fibrils in the filtrate in the *in vitro* competition assays described below was determined by the same tests using CR.

In vitro competition assays with [^{18}F]FDDNP against NSAIDs and charged amyloid dyes

Solutions of nonradioactive (*S*)-naproxen (**2**, Sigma), (*R*)-ibuprofen (**3a**; BIOMOL, Plymouth Meeting, PA, USA), (*S*)-ibuprofen (**3b**, Sigma), diclofenac (**4**, Sigma), CR (**5**) and TT (**6**) were prepared fresh for each radioactive competition assay. APFF glass fiber filters (0.7-μm particle retention; Millipore, Bedford, MA, USA) were used in a 1225 sampling manifold (Millipore) that was modified with stainless steel support screens (Millipore) and glass sample chambers. Vacuum filtration involved 0.865 μg/ml of *in vitro* fibrils of synthetic Aβ(1–40) and 37 kBq/ml of [^{18}F]FDDNP incubated for 1 h in PBS, pH 7.4 (1% ethanol) with various concentrations of nonradioactive 1–6 with the range of 0.1 pM to 83 μM. Each filter was then washed twice with 3 ml of PBS, pH 7.4. The radioactivity retained by the filters was measured and decay-corrected to a common reference time with a Packard Cobra II Auto-Gamma gamma counter (Packard, Meriden, CT, USA). The binding of radiolabeled **1** to Aβ fibrils without competitor determined 100% specific binding. One hundred percent competition against radioactive **1** occurred with 40 μM of nonradioactive **1**. All competition assays were performed in triplicate. Competition results were analyzed and then K_i values calculated using the Ligand Binding Module for SigmaPlot 2001 (SPSS, Chicago, IL, USA). The comparison of one- and two-site models for binding involved calculating the *F* statistic with a significance level of $P<0.05$.

Preparation of brain tissue

Brain specimens from a 79-year-old female postmortem-diagnosed definite AD patient were obtained from the Department of Pathology and Laboratory Medicine, UCLA School of Medicine, and treated as previously described (Agdeppa et al., 2001a). Briefly, formalin-treated, cryoprotected brain specimens were sectioned 70 μm thick coronally, mounted on gelatin-coated glass slides, allowed to dry, and were defatted for 40 min in xylene (Loopuijt et al., 1987) prior to rinsing of the tissue with ethanol. Finally, lipofuscin autofluorescence in some brain specimens was quenched prior to staining using 10-mM $CuCl_2$ in 50-mM ammonium acetate buffer, pH 5 (Schnell et al., 1999). The quenching determined the origin of lipofuscin fluorescence in brain specimens.

Digital autoradiography

Adjacent postmortem-diagnosed definite AD brain specimens (70 μm thick) were pretreated with either 100 nM of fresh batches of nonradioactive **1** and **2** or 40 μM **3**–**6** in 10% ethanol in PBS, pH 7.4, for 60 min and then the liquid decanted prior to digital autoradiography with [^{18}F]FDDNP. Pretreated and cryosections with no competitor were incubated for 25 min at room temperature with 3.7 MBq of [^{18}F]FDDNP dissolved in 10 ml of 1% ethanol in 0.9% saline (w/v) per cryosection. Following incubation, the sections were optimally washed with water (30 s); 60% 2-methyl-2-butanol (3 min; Sigma) agitated at 40 r.p.m. on a Junior Orbit Shaker (Laboratory-Line Instruments, Melrose Park, IL, USA) for differentiation (Bancroft and Stevens, 1990); and then water (30 s). The sections were dried on a warm hot plate with a steady stream of warm air, exposed to β^{+}-sensitive phosphor plates for 40 min (Fuji Film Medical Systems USA, Stamford, CT, USA), and scanned with a FUJI BAS 5000 Phosphorimager (Fuji Film Medical Systems USA) at a resolution of 25 μm, as described previously (Agdeppa et al., 2001a). Radioactivity in tissue scrapings from the imaged specimens were subsequently measured in a Packard Cobra II Auto-Gamma (Packard), decayed to common reference time, and used as radioactive standards to quantify the amount of specific binding of [^{18}F]FDDNP (radioactivity/area, Bq/

mm^2; Fig. 2Q) in the autoradiograms. Autoradiography was carried out at least in triplicate for each competitor. Statistical analysis of the autoradiograms involved one-way analysis of variance (ANOVA) with Dunnett's post test with a significance level of $P<0.05$ using Prism 3.02 (GraphPad, San Diego, CA, USA) to compare the differences in the ratio of gray-matter to white-matter (Fig. 2Q) radioactivity of [^{18}F]FDDNP in autoradiograms pretreated with nonradioactive **1**–**6** and without pretreatment. Unpaired *t*-tests with a significance level $P<0.05$ were performed to compare the difference of measured [^{18}F]FDDNP radioactivity per area of tissue (Bq/mm^2) in the gray and white matter of each autoradiogram.

Fluorescence microscopy

The same brain specimens used for autoradiography were examined using fluorescence microscopy. Tissues were mounted with Vectashield (Vector, Burlingame, CA, USA) and observed with a Nikon Labophot fluorescence microscope (Nikon USA, Melville, NY, USA) with a FITC filter set.

Fluorescence microscopy of tissue previously used for autoradiography with [^{18}F]FDDNP is possible due to the fluorescent properties of FDDNP (Jacobson et al., 1996) and the labeling of SPs by residual nonradioactive FDDNP. The specific activity (activity per unit mass) of non-carrier-added [^{18}F]FDDNP at the end of synthesis was 74–222 GBq/μmol (2000–6000 Ci/mmol), about 10^3 times lower than the maximum theoretical specific activity for ^{18}F (Sorenson and Phelps, 1987). Thus, after ^{18}F decay the residual nonradioactive FDDNP bound to SPs in AD brain specimens may be imaged with fluorescence microscopy.

Anti-aggregation of Aβ(1–40) using NSAIDs and FDDNP

Inhibition of Aβ fibril formation was performed using a modified method previously reported (Thomas et al., 2001). Briefly, inhibition of Aβ fibril aggregation involved >170 μM of Aβ(1–40) peptide (Biosource) and 0 (as a control), 100, 200, or 300 μM of nonradioactive **1**–**4** co-incubated in PBS, pH 7.4, at 37 °C for 3 days with gentle agitation. After incubation, a 37.5-μl aliquot from the Aβ-inhibitor solution and 40-μl of a 1 mg/ml CR solution (10% ethanol (v/v) in water) were added to 2.96 ml of PBS, pH 7.4. The resulting solution was briefly vortexed, equilibrated for 30 min at room temperature, then CR absorption at 540 nm was measured with a DU640 spectrophotometer (Beckman, Fullerton, CA, USA) to determine the extent of Aβ fibril formation. The CR absorption values at 540 nm for the Aβ-inhibitor solutions were corrected for absorption by inhibitor, free CR, PBS, Aβ fibrils and ethanol. Anti-aggregation experiments were performed in triplicate. The inhibition results were represented as a percentage of the corrected control absorption of CR and Aβ fibrils with no inhibitor. Statistical analysis of the extent of inhibition of Aβ fibril formation involved one-way ANOVA with Dunnett's post test with a significance level of $P<0.05$ using Prism 3.02 (GraphPad) to compare the differences in absorption of Aβ-inhibitor solutions with absorption of CR and Aβ with no inhibitor.

Reversal of Aβ(1–40) aggregation experiments were performed in the same manner as described above. Briefly, the Aβ fibrils were formed in the absence of nonradioactive competitors in PBS, pH 7.4, at 37 °C for 3 days with gentle agitation. Afterward, nonradioactive **1**–**4** (at the concentrations described above) were co-incubated with the Aβ fibrils for additional 3 days under the same conditions. After the final incubation, the CR absorption values were determined and analyzed as described above.

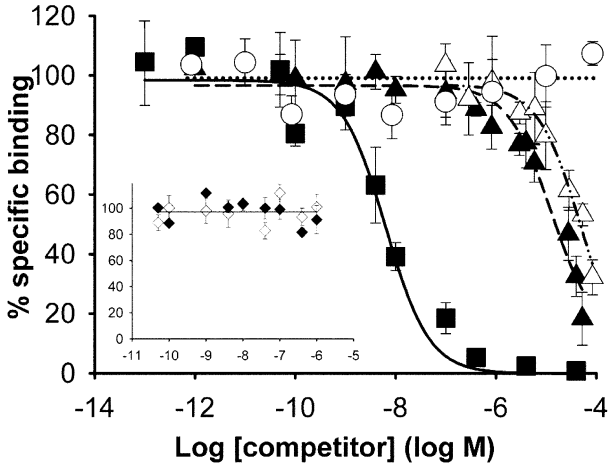

Fig. 1. Competition curves of 2-(1-{6-[(2-[^{18}F]fluoroethyl)(methyl)amino]-2-naphthyl}ethylidene)malononitrile ([^{18}F]FDDNP) (**1**, Scheme I) against non-steroidal anti-inflammatory drugs (NSAIDs) and *in vitro* charged amyloid dyes for β-amyloid (Aβ)(1–40) fibrils. Various concentrations (0.1 pM to 83 μM) nonradioactive (*S*)-naproxen (**2**, black squares), (*R*)-ibuprofen (**3a**, white triangles), (*S*)-ibuprofen (**3b**, black triangles), diclofenac (**4**, white circles), Congo Red (**5**, CR, black diamonds, inset), and Thioflavine T (6, TT, white diamonds, inset) were incubated with 0.865 μg/ml of *in vitro* fibrils of synthetic Aβ(1–40) and 37 kBq/ml of [^{18}F]FDDNP (specific activity >74 GBq/μmol at end-of-synthesis) for 1 h in phosphate-buffered saline, pH 7.4 (1% ethanol). The binding of radiolabeled **1** to Aβ fibrils without competitor determined 100% specific binding. Radioactivity measured by all competition assays were performed in triplicate. (*S*)-naproxen had a K_i=5.70±1.31 nM (±S.D.), (*R*)-ibuprofen K_i=44.4±17.4 μM (±S.D.), and (*S*)-ibuprofen K_i=11.3±5.20 μM (±S.D.). Diclofenac, CR, and TT did not exhibit a dose-dependent decrease in the specific binding of [^{18}F]FDDNP.

RESULTS

Naproxen and ibuprofen share the same binding sites of [^{18}F]FDDNP on Aβ fibrils

In vitro radioactive competition curves carried out using various concentrations of NSAIDs and charged amyloid dyes (2–6) co-incubated with [^{18}F]FDDNP and synthetic Aβ(1–40) fibrils revealed one site-binding competition for (*S*)-naproxen, (*R*)-ibuprofen, and (*S*)-ibuprofen ($P<0.05$; Fig. 1). The concentration-dependent decrease in the binding of [^{18}F]FDDNP versus (*S*)-naproxen, (*R*)-ibuprofen, and (*S*)-ibuprofen yielded K_i values of 5.70±1.31 nM (±S.D.), 44.4±17.4 μM (±S.D.), and 11.3±5.20 μM (±S.D.), respectively, indicating that (*S*)-naproxen binds more tightly to Aβ fibrils (Fig. 1). Diclofenac, CR, and TT did not exhibit a dose-dependent decrease in the specific binding of [^{18}F]FDDNP (Fig. 1).

Autoradiography with naproxen and ibuprofen demonstrate complete blockade of [^{18}F]FDDNP binding sites on *ex vivo* SPs

The gross pattern of radioactivity in the adjacent coronal AD brain specimens with no competitor revealed the specific binding of [^{18}F]FDDNP to areas containing SPs (Fig. 2A). Specific binding of [^{18}F]FDDNP to regions of gray matter with SPs was significantly reduced in AD spec-

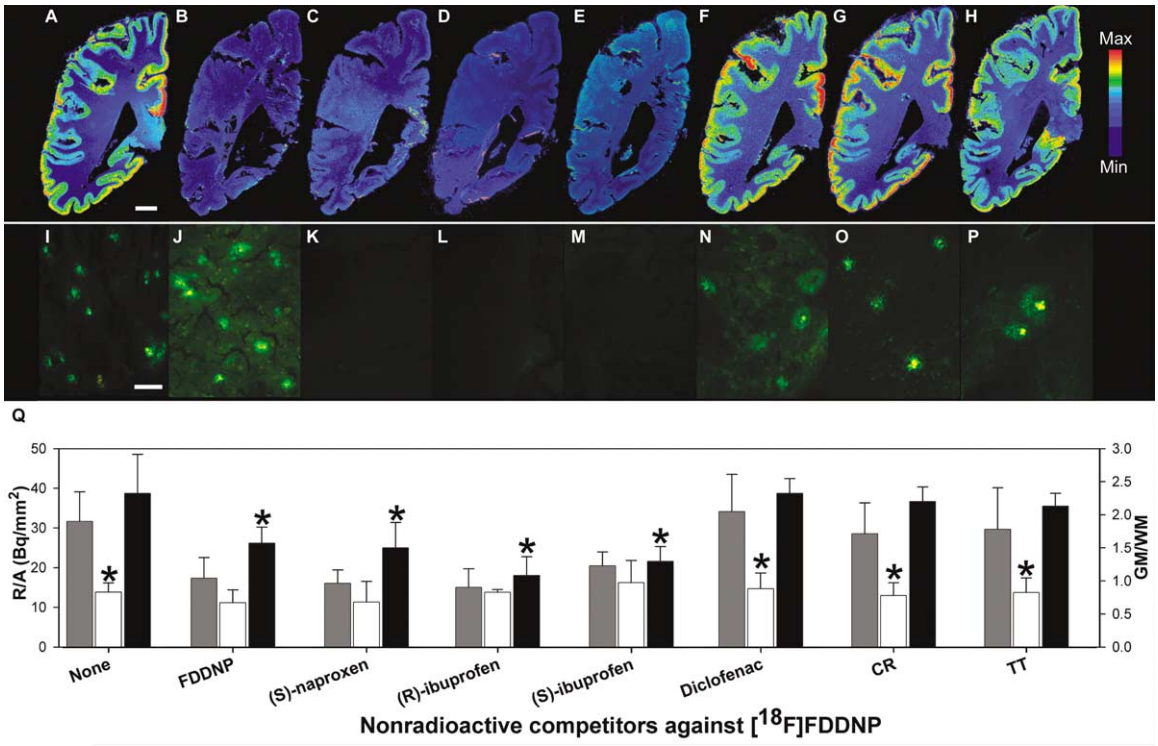

Fig. 2. 2-(1-{6-[(2-[^{18}F]fluoroethyl)(methyl)amino]-2-naphthyl}ethylidene)malononitrile ([^{18}F]FDDNP) digital autoradiography and fluorescence microscopy of Alzheimer's disease (AD) brain specimens treated with non-steroidal anti-inflammatory drugs (NSAIDs) and *in vitro* charged amyloid dyes. AD brain specimens were pretreated with either 100 nM of nonradioactive FDDNP (**1**, B, J) and (*S*)-naproxen (**2**, C, K), or 40 μM of (*R*)-ibuprofen (**3a**, D, L), (*S*)-ibuprofen (**3b**, E, M), diclofenac (**4**, F, N), Congo Red (CR; **5**, G, O) and Thioflavine T (TT; **6**, H, P) for 60 min prior to [^{18}F]FDDNP autoradiography and, after radioactive decay, subsequent fluorescence microscopy. Autoradiography (A) and fluorescence microscopy (I) without pretreatment were also performed. Radioactivity in tissue scrapings from the autoradiography specimens was subsequently measured and used as radioactive standards to quantify the amount of specific binding of [^{18}F]FDDNP (radioactivity/area, R/A, Bq/mm^2, Q) in the senile plaques (SPs)-laden gray-matter areas (gray bars, Q) and white matter (white bars, Q) void of SPs of the autoradiograms. Competition of nonradioactive FDDNP, (*S*)-naproxen, (*R*)-ibuprofen and (*S*)-ibuprofen against [^{18}F]FDDNP exhibited reduced specific binding of [^{18}F]FDDNP to areas with SPs in the autoradiograms (B–E) which was confirmed with fluorescence microscopy (J–M). With these competitors the gray-matter to white-matter (GM/WM) ratios (black bars, Q) of radioactivity in the autoradiograms were significantly lower (*$P<0.05$) in comparison to the ratio for the [^{18}F]FDDNP autoradiograms with no competitor, indicating blockage of specific binding of [^{18}F]FDDNP down to background levels of radioactivity found in the white matter (Q). Diclofenac, CR and TT had minimal effect on specific binding of [^{18}F]FDDNP to SPs as determined by autoradiography (F–H) and fluorescence microscopy (N–P) of the same tissue specimens, and confirmed by quantitative analysis of the significant difference (*$P<0.05$) between gray- and white-matter radioactivity for the autoradiograms pretreated with diclofenac, CR, and TT. A comparison of the ratios of gray-matter to white-matter radioactivity of diclofenac-, CR-, and TT-treated autoradiograms with the ratio in [^{18}F]FDDNP autoradiograms with no competitor indicates the preservation of specific binding of [^{18}F]FDDNP to SPs (Q). Autoradiography was carried out at least in triplicate for each competitor. Scale bar for autoradiograms (A–H)=1 cm. Scale bar for fluorescence micrographs (I–P)=50 μm.

imens pretreated with nonradioactive FDDNP, (*S*)-naproxen, (*R*)-ibuprofen, and (*S*)-ibuprofen (Fig. 2B–E) compared with autoradiography in the absence of those competitors (Fig. 2A). With these competitors the difference in the radioactivity (radioactivity/area, Bq/mm^2; Fig. 2Q) between SP-laden gray-matter areas and white matter void of SPs was not significant ($P>0.05$) and was consistent with the significantly lower ($P<0.05$) gray-matter to white-matter ratio (Fig. 2Q) of radioactivity in comparison to the ratio for the [^{18}F]FDDNP autoradiograms with no competitor. These results indicate blockage of specific binding of [^{18}F]FDDNP down to background levels of radioactivity found in the white matter. All these results were confirmed by fluorescence microscopy with the same brain specimens (Fig. 2I–M). Diclofenac, CR and TT had minimal effect on specific binding of [^{18}F]FDDNP to SPs as determined by autoradiography (Fig. 2F–H) and fluorescence microscopy (Fig. 2N–P) of the same tissue specimens. The difference between gray- and white-matter radioactivity was significant ($P<0.05$) for diclofenac, CR, and TT, as was the case for [^{18}F]FDDNP autoradiography with no competitor (Fig. 2Q). The comparison of the ratios of gray-matter to white-matter radioactivity between diclofenac, CR, and TT with [^{18}F]FDDNP with no competitor indicate the preservation of specific binding of [^{18}F]FDDNP to SPs (Fig. 2Q).

Anti-aggregation of Aβ peptide is demonstrated by FDDNP, naproxen, and ibuprofen

The extent of Aβ(1–40) aggregation in the Aβ solutions co-incubated with increasing concentrations of each nonradioactive compound **1**–**4** was measured using the CR

Table 1. Anti-aggregation effect of FDDNP and select NSAIDs on Aβ(1–40) peptide[a]

[Inhibitor] (μM)	Percentage of absorption of control at 540 nm				
	FDDNP	(*S*)-naproxen	(*R*)-ibuprofen	(*S*)-ibuprofen	Diclofenac
100	80.7±18.3	87.8±1.5	78.4±10.2	101.4±6.1	100.2±1.4
200	69.1±0.6	69.7±6.9	72.6±3.4	92.7±2.3	97.1±0.7
300	43.3±7.1***	63.9±16.0*	64.8±3.5*	79.6±10.3	95.1±1.1

[a] Anti-aggregation results were represented as a percentage of the control absorption measurements of CR and Aβ fibrils with no inhibitor. Data expressed as mean±S.E.M.

* $P<0.05$, *** $P<0.001$, significantly different from absorption measurements with no inhibitor.

FDDNP indicates 2-(1)-{6-[(2-^{18}F]fluoroethyl)(methyl)amino]-2-naphthyl}ethylidene)malononitrile; NSAIDs, non-steroidal anti-inflammatory drugs; and Aβ, β-amyloid.

absorption maximum at 540 nm which is characteristic of CR bound to aggregated Aβ (Klunk et al., 1999; Thomas et al., 2001). Reduced absorption was observed as the concentration of **1**–**3** co-incubated with Aβ(1–40) peptide increased from 100 to 300 μM (Table 1). Substantial differences between control and Aβ-inhibitor absorption readings were obtained with 300 μM of FDDNP ($P<0.001$), (*S*)-naproxen ($P<0.05$), (*R*)-ibuprofen ($P<0.05$), and (*S*)-ibuprofen ($P>0.05$) (Table 1). Minimal inhibition of Aβ aggregation with diclofenac was observed (Table 1). Reversal or dissolution of aggregated Aβ(1–40) fibrils also was observed with 300 μM of the following nonradioactive compounds: FDDNP produced 58.7±0.6% (±S.E.M.) absorption relative to CR-fibrils alone, (*S*)-naproxen 65.7±8.0%, (*R*)-ibuprofen 75.5±4.1%, and (*S*)-ibuprofen 57.6±7.6%.

DISCUSSION

In this work we report concentration-dependent decreases in the *in vitro* binding of [^{18}F]FDDNP to Aβ fibrils and SPs in competition against commonly prescribed NSAIDs, including (*S*)-naproxen, (*R*)-ibuprofen, and (*S*)-ibuprofen (Breitner et al., 1995; Stewart et al., 1997; in t' Veld et al., 2001). Conversely, another epidemiologically examined NSAID, diclofenac (in t' Veld et al., 2001), and commonly used *in vitro* amyloid dyes, Congo Red and Thioflavine T, showed minimal effect on the specific binding of [^{18}F]FDDNP to Aβ aggregates (Fig. 1) and failed to reduce [^{18}F]FDDNP labeling of SPs in AD brain specimens (Fig. 2). The compounds sharing the same binding site on fibrils and SPs, namely FDDNP, (*S*)-naproxen, (*R*)-ibuprofen, and (*S*)-ibuprofen, were also potent inhibitors of Aβ aggregation (Table 1). The *in vitro* binding determinations provide evidence of NSAIDs binding to SPs and may identify a site of anti-aggregation action on Aβ fibrils (Thomas et al., 2001).

The lack of competitive binding of diclofenac to Aβ(1–40) fibrils may suggest other binding sites; however, its minimal anti-aggregation effects on Aβ(1–40) may be consistent with its postulated mode of action of mitigating the neurotoxic inflammatory response in AD (Halliday et al., 2000). Alternatively, a subset of NSAIDs is reported to lower Aβ(1–42) production (Weggen et al., 2001), but it does not include (*S*)-naproxen and other NSAIDs that appear equally protective (Breitner et al., 1995; Anthony et al., 2000). However, the clinical effectiveness of (*S*)-naproxen in AD patients has recently been questioned (Breteler et al., 2002). If other therapeutic mechanisms (e.g. anti-aggregation) may be involved *in vivo*, [^{18}F]FDDNP-PET could be useful to evaluate *in vivo* the efficacy of select NSAIDs as anti-aggregation agents.

The specific binding of [^{18}F]FDDNP in the absence of competitor (Fig. 2A, I) is entirely consistent with previous reports of *in vitro* [^{18}F]FDDNP binding in AD brain tissue (Agdeppa et al., 2001a). These results correlated with *in vivo* binding of [^{18}F]FDDNP in the brain of AD patients using PET (Agdeppa et al., 2001b). Therefore, the *in vitro* competition results with tissue pretreated with nonradioactive FDDNP, (*S*)-naproxen, (*R*)-ibuprofen, and (*S*)-ibuprofen point to the exciting possibility of using [^{18}F]FDDNP with PET (Shoghi-Jadid et al., 2002) in the *in vivo* determination of occupancy rates of SP sites by NSAIDs, and other therapeutic candidates, in the living brain of AD or at-risk AD patients who are medicated. This can be correlated to the drug-mediated anti-aggregation effects on Aβ fibrils in SPs. In turn, [^{18}F]FDDNP-PET may provide an *in vivo* tool to help separate the apparent risk-reduction effects of different NSAIDs on AD in terms of anti-aggregation and anti-inflammatory effects, which may provide explanations for the apparent efficacy of NSAIDs for prevention (Stewart et al., 1997; in t' Veld et al., 2001; Zandi et al., 2002) but not for treatment (Scharf et al., 1999; Aisen et al., 2000; Van Gool et al., 2001). Although we focused our investigation on commonly used NSAIDs that show possible neuroprotective effects in prospective studies, we recently found that (*R*)-naproxen, which lacks the anti-inflammatory activity of (*S*)-naproxen (Adams et al., 1976), competes with [^{18}F]FDDNP for the same binding site on Aβ(1–40) fibrils (K_i value=2.76±0.95 nM (±S.D.) perhaps providing an example of using [^{18}F]FDDNP-PET to separate the anti-aggregation and anti-inflammation effects. Many factors need to be considered for these future *in vivo* investigations, including the effect of the unidirectional chiral inversion of *R*-aryl propionic acid NSAIDs to *S*-isomers in the body (Caldwell et al., 1988; Mayer, 1990). Thus, longitudinal determinations in treated patients would provide direct evidence of the therapeutic efficacy and mode of action of these drugs.

The promise of extending the *in vitro* results to *in vivo* molecular imaging depends on the bioavailability of these

NSAIDs to the brain. An extrapolation of dose and plasma or brain concentrations of (*S*)-naproxen and racemic ibuprofen in rats to humans suggests the possibility of the *in vivo* competition of [^{18}F]FDDNP at clinically relevant doses of the drugs. Many patients in epidemiological studies, who showed reduced risk of AD, were consuming NSAIDs for treatment of arthritis (Breitner et al., 1995; Halliday et al., 2000). For rheumatoid arthritis and osteoarthritis, the recommended dose of (*S*)-naproxen would produce brain concentrations of (*S*)-naproxen well above those needed ($<$0.1 μM) for complete blockade of [^{18}F]FDDNP binding (Fig. 1). Specifically, plasma concentrations of 130–390 μM of (*S*)-naproxen are achieved by recommended (*S*)-naproxen doses of 250–500 mg twice daily for 2 weeks (McEvoy, 2002). By comparison, a single dose of (*S*)-naproxen (143 mg/kg) produces approximately 29–42 μM of the drug in the rat brain at 0.5 h after dosing (Micheli et al., 1993). This brain concentration is approximately 10^3 times higher than the one needed ($<$0.1 μM) for complete blockade of SP sites in the AD brain. Thus, it appears likely that this concentration in the AD brain would be easily achieved with the recommended clinical doses of (*S*)-naproxen (McEvoy, 2002).

Recommended anti-inflammatory doses of racemic ibuprofen, and possibly chronic lower doses, are also expected to produce blockade of SP sites. A racemic ibuprofen dose of 40 mg/kg/day to rats produced approximately 4 μM of the drug in plasma and approximately 2 μM in the brain (Adams et al., 1969), whereas a clinical dose of 2400 mg/day (34 mg/kg/day) to humans results in approximately 54 μM of ibuprofen in plasma (Bradley et al., 1992; McEvoy, 2002). Chronic use of racemic ibuprofen, even at lower doses (Broe et al., 2000), may reach higher than expected brain concentrations considering the cerebrospinal fluid half-life of ibuprofen is approximately two times longer than the plasma half-life (Bannwarth et al., 1995). Overall, extrapolation of interspecies pharmacokinetics would be favorable for humans (Barrio et al., 1989) because of faster drug metabolism and elimination in rats [(*S*)-naproxen plasma $t_{1/2}$=5.1 h; racemic ibuprofen plasma $t_{1/2}$=2.13 h] than in humans [(*S*)-naproxen plasma $t_{1/2}$=13.9 h; racemic ibuprofen plasma $t_{1/2}$=2–4 h] (Runkel et al., 1972; Parrott and Christensen, 1984; McEvoy, 2002). Ultimately, future [^{18}F]FDDNP-PET studies of AD patients who chronically use NSAIDs are necessary to test these assumptions. Until then, care should be taken to note the medication, including NSAIDs, taken by AD research subjects scanned with [^{18}F]FDDNP and other potential amyloid probes (Klunk et al., 2001; Kung et al., 2001; Engler et al., 2002).

The identification and characterization of a site of anti-aggregation action on Aβ fibrils is suggested by the observations that the same set of compounds [i.e. FDDNP, (*S*)-naproxen, (*R*)-ibuprofen, and (*S*)-ibuprofen] not only share a common binding site on Aβ(1–40) aggregates and SPs but also are anti-aggregation agents. It is tempting to speculate that SP binding sites of anti-aggregation NSAIDs are Aβ amino acid sequences critical for β-sheet formation in fibrils (Thomas et al., 2001), but supportive high-resolution atomic X-ray crystallographic data for Aβ fibrils are lacking. Tertiary and quaternary structures of Aβ fibrils have not been successfully determined due to the inability of insoluble Aβ fibrils to form single crystals, despite repeated attempts by conventional X-ray crystallography (Lansbury, 1996). In the absence of detailed atomic structural requirements for Aβ fibril binding, the nature of the binding site can be inferred from a congeneric series of molecules (Dean, 1987). The family of probes based on FDDNP (Agdeppa et al., 2000, 2001a, 2002) is well suited for this purpose considering FDDNP requires the macromolecular conformation of the insoluble fibrils for binding (Agdeppa et al., 2001a). Further characterization of the binding site also may involve the drugs that are structurally related to and compete with [^{18}F]FDDNP [e.g. (*S*)-naproxen and (*R*)- and (*S*)-ibuprofen]. Compounds that do not compete with [^{18}F]FDDNP, such as diclofenac, Thioflavine T, and Congo Red, still may provide exclusion criteria for binding (e.g. size of the binding site). Although binding constants for FDDNP and its analogs as well as a general description of the local environment of their binding sites have been reported elsewhere (Agdeppa et al., 2001a), the development of an inferred topographical map of particular intermolecular forces for the binding site of FDDNP, its congeners, and competing drugs would allow for further optimization of the binding of molecular imaging probes for SPs. Most importantly, it would contribute to the characterization of the site of anti-aggregation action in SPs, and provide a tool for the design and evaluation of the therapeutic efficacy of experimental AD drugs.

The results presented herein reveal a previously unrecognized binding of NSAIDs in SPs in AD, may identify the binding site of anti-aggregation agents, may support the rationale of using NSAIDs as anti-aggregation agents on Aβ peptide, and suggest the added value of *in vivo* PET molecular imaging probes as useful tools in the determination of the therapeutic effects of experimental Aβ-anti-aggregation drugs beyond their use as *in vivo* diagnostic probes.

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REFERENCES

Adams SS, Bough RG, Cliffe EE, Lessel B, Mills RFN (1969) Absorption, distribution and toxicity of ibuprofen. Toxicol Appl Pharmacol 15:310–330.

Adams SS, Bresloff P, Mason CG (1976) Pharmacological differences between the optical isomers of ibuprofen: Evidence for metabolic inversion of the (−)-isomer. J Pharm Pharmacol 28:256–257.

Agdeppa ED, Kepe V, Kiziloglu ZN, Petriĉ A, Zabjek A, Satyamurthy N, Cole GM, Vinters HV, Small GW, Barrio JR (2000) FDDNP analogs as probes for plaques and tangles in Alzheimer's disease. J Nucl Med 41 suppl:25P.

Agdeppa ED, Kepe V, Liu J, Flores-Torres S, Satyamurthy N, Petriĉ A, Cole GM, Small GW, Huang S-C, Barrio JR (2001a) Binding characteristics of radiofluorinated 6-dialkylamino-2-naphthylethylidene derivatives as positron emission tomography imaging probes for

β-amyloid plaques in Alzheimer's disease. J Neurosci 21:1–5 RC189.

Agdeppa ED, Kepe V, Petriĉ A, Liu J, Satyamurthy N, Shoghi-Jadid K, Small GW, Huang S-C, Barrio JR (2002) Torsionally modified FDDNP analogs have picomolar binding affinity to plaques in Alzheimer's disease. J Nucl Med 43 (suppl):166P.

Agdeppa ED, Kepe V, Shoghi-Jadid K, Satyamurthy N, Small GW, Petriĉ A, Vinters HV, Huang S-C, Barrio JR (2001b) *In vivo* and *in vitro* labeling of plaques and tangles in the brain of an Alzheimer's disease patient: a case study. J Nucl Med 42 (suppl):65P.

Aisen PS, Davis KL, Berg JD, Schafer K, Campbell K, Thomas RG, Weiner MF, Farlow MR, Sano M, Grundman M, Thal LJ (2000) A randomized controlled trial of prednisone in Alzheimer's disease. Alzheimer's Dis Cooperative Study Neurol 54:588–593.

Anthony JC, Breitner JC, Zandi PP, Meyer MR, Jurasova I, Norton MC, Stone SV (2000) Reduced prevalence of AD in users of NSAIDs and H2 receptor antagonists: the Cache County Study. Neurology 54:2066–2071.

Bancroft JD, Stevens A (eds) (1990) Theory and practice of histological techniques. New York: Churchill Livingstone.

Bannwarth B, Lapicque F, Pehourcq F, Gillet P, Schaeverbeke T, Laborde C, Dehais J, Gaucher A, Netter P (1995) Stereoselective disposition of ibuprofen enantiomers in human cerebrospinal fluid. Br J Clin Pharmacol 40:266–269.

Barrio JR, Satyamurthy N, Huang S-C, Keen RE, Nissenson CH, Hoffman JM, Ackermann RF, Bahn MM, Mazziotta JC, Phelps ME (1989) 3-(2'-[^{18}F]fluoroethyl)spiperone: *in vivo* biochemical and kinetic characterization in rodents, nonhuman primates, and humans. J Cereb Blood Flow Metab 9:830–839.

Bradley JD, Rudy AC, Katz BP, Ryan SI, Kalasinski LA, Brater DC, Hall SD, Brandt KD (1992) Correlation of serum concentrations of ibuprofen stereoisomers with clinical response in the treatment of hip and knee osteoarthritis. J Rheumatol 19:130–134.

Breitner JCS, Welsh KA, Helms MJ, Gaskell PC, Gau BA, Roses AD, Pericak-Vance MA, Saunders AM (1995) Delayed onset of Alzheimer's disease with nonsteroidal anti-inflammatory and histamine H2 blocking drugs. Neurobiol Aging 16:523–530.

Breteler MMB, in t' Veld B, Hofman A, Stricker BHC (2002) Aβ-42 peptide lowering NSAIDs and Alzheimer's disease. Neurobiol Aging 23 (suppl):S286.

Broe GA, Grayson DA, Creasey HM, Waite LM, Casey BJ, Bennett HP, Brooks WS, Halliday GM (2000) Anti-inflammatory drugs protect against Alzheimer disease at low doses. Arch Neurol 57:1586–1591.

Caldwell J, Winter SM, Hutt AJ (1988) The pharmacological and toxicological significance of the stereochemistry of drug disposition. Xenobiotica 18 (suppl):59–70.

Dean PM (1987) Molecular foundations of drug-receptor interaction. Cambridge University Press: Cambridge.

Engler H, Blomqvist G, Bergstrom M, Estrada S, Sandell J, Antoni G, Langstrom B, Barletta J, Klunk W, Debnath M, Holt D, Wang Y, Huang G-F, Mathis C (2002) First human study with a benzothiazole amyloid-imaging agent in Alzheimer's disease and control subjects. Neurobiol Aging 23(suppl):S429.

Findeis MA (2000) Approaches to discovery and characterization of inhibitors of amyloid β-peptide polymerization. Biochim Biophys Acta 1502:76–84.

Halliday G, Robinson SR, Shepherd C, Kril J (2000) Alzheimer's disease and inflammation: a review of cellular and therapeutic mechanisms. Clin Exp Pharmacol Physiol 27:1–8.

in t' Veld BA, Ruitenberg A, Hofman A, Launer LJ, van Duijn CM, Stijnen T, Breteler MM, Stricker BH (2001) Nonsteroidal antiinflammatory drugs and the risk of Alzheimer's disease. N Engl J Med 345:1515–1521.

Jacobson A, Petriĉ A, Hogenkamp D, Sinur A, Barrio JR (1996) 1,1-dicyano-2-(6-dimethylaminonaphthalen-2-yl)propene (DDNP): a solvent polarity and viscosity sensitive fluorophore for fluorescence microscopy. J Am Chem Soc 118:5572–5579.

Klunk WE, Jacob RF, Mason RP (1999) Quantifying amyloid β-peptide (Aβ) aggregation using the Congo red-Aβ (CR-Aβ) spectrophotometric assay. Anal Biochem 266:66–76.

Klunk WE, Wang Y, Huang G-f, Debnath ML, Holt DP, Mathis CA (2001) Uncharged thioflavin-T derivatives bind to amyloid-beta protein with high affinity and readily enter the brain. Life Sci 69:1471–1484.

Kung HF, Lee C-W, Zhuang Z-P, Kung M-P, Hou C, Plossl K (2001) Novel stilbenes as probes for amyloid plaques. J Am Chem Soc 123:12740–12741.

Lansbury PT (1996) A reductionist view of Alzheimer's disease. Acc Chem Res 29:317–321.

LeVine H (1993) Thioflavine T interaction with synthetic Alzheimer's disease β-amyloid peptides: detection of amyloid aggregation in solution. Protein Sci 2:404–410.

Loopuijt LD, Sebens JB, Korf J (1987) *In vivo* labeling of dopamine receptors: light microscopic localization at the cellular level by means of dipping autoradiography with the agonist (^{3}H)*N*-n-propylnorapomorphine. J Neural Transm 68:15–23.

Mayer JM (1990) Stereoselective metabolism of anti-inflammatory 2-arylpropionates. Acta Pharm Nord 2:197–216.

McEvoy GK (ed) (2002) AHFS drug information. Bethesda: Authority of the Board of Directors of the American Society of Hospital Pharmacists.

McGeer PL, McGeer EG (1995) The inflammatory response system of brain: implications for therapy of Alzheimer and other neurodegenerative diseases. Brain Res Brain Res Rev 21:195–218.

Micheli L, Fiaschi AI, Cerretani D, Giorgi G (1993) Non-steroidal anti-inflammatory drugs (NSAIDs) affect the glutathione (GSH) levels in the brain. Ann Ist Super Sanita 29:387–390.

Parrott KA, Christensen JM (1984) Influence of cimetidine on the disposition of ibuprofen in the rat. Res Commun Chem Pathol Pharmacol 43:369–380.

Rogers J, Kirby LC, Hempelman SR, Berry DL, McGeer PL, Kaszniak AW, Zalinski J, Cofield M, Mansukhani L, Willson P, Kogan F (1993) Clinical trial of indomethacin in Alzheimer's disease. Neurology 43:1609–1611.

Runkel R, Chaplin M, Boost G, Segre E, Forchielli E (1972) Absorption, distribution, metabolism, and excretion of naproxen in various laboratory animals and human subjects. J Pharm Sci 61:703–708.

Scharf S, Mander A, Ugoni A, Vajda F, Christophidis N (1999) A double-blind, placebo-controlled trial of diclofenac/misoprostol in Alzheimer's disease. Neurology 53:197–201.

Schnell SA, Staines WA, Wessendorf MW (1999) Reduction of lipofuscin-like autofluorescence in fluorescently labeled tissue. J Histochem Cytochem 47:719–730.

Selkoe DJ (1994) Cell biology of the amyloid β-protein precursor and the mechanism of Alzheimer's disease. Annu Rev Cell Biol 10:373–403.

Shoghi-Jadid K, Small GW, Agdeppa ED, Kepe V, Ercoli LM, Siddarth P, Read S, Satyamurthy N, Petriĉ A, Huang SC, Barrio JR (2002) Localization of neurofibrillary tangles and beta-amyloid plaques in the brains of living patients with Alzheimer's disease. Am J Geriatr Psychiatry 10:24–35.

Sorenson JA, Phelps ME (1987) Physics in nuclear medicine. Grune and Stratton: Orlando.

Stewart WF, Kawas C, Corrada M, Metter EJ (1997) Risk of Alzheimer's disease and duration of NSAID use. Neurology 48:626–632.

Teplow DB (1998) Structural and kinetic features of amyloid β-protein fibrillogenesis. Amyloid 5:121–142.

Thomas T, Nadackal TG, Thomas K (2001) Aspirin and non-steroidal anti-inflammatory drugs inhibit amyloid-β aggregation. Neuroreport 12:3263–3267.

Van Gool WA, Weinstein HC, Scheltens P, Walstra GJ, Scheltens PK(2001) Effect of hydroxychloroquine on progression of dementia in early Alzheimer's disease: an 18-month randomised, double-blind, placebo-controlled study. Lancet 358:455–460.

Weggen S, Eriksen JL, Das P, Sagi SA, Wang R, Pietrzik CU, Findlay

KA, Smith TE, Murphy MP, Bulter T, Kang DE, Marquez-Sterling N, Golde TE, Koo EH (2001) A subset of NSAIDs lower amyloidogenic Aβ42 independently of cyclooxygenase activity. Nature 414:212–216.

Zandi PP, Anthony JC, Hayden KM, Mehta K, Mayer L, Breitner JC (2002) Reduced incidence of AD with NSAID but not H_2 receptor antagonists: the Cache County Study. Neurology 59:880–886.

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