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Lack of specific Aβ(1-42) suppression by NSAIDs in young, plaque-free Tg2576 mice and in guinea pig neuronal cultures.

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Running Title:

NSAID effects on Aβ *in vivo* and in primary neurons

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Abbreviations: Aβ, amyloid-β; AD, Alzheimer's disease; APP, amyloid-precursor protein; CSF, cerebrospinal fluid; DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester; ELISA, enzyme-linked immunosorbent assay; E25, embryonic day 25; LY-411575, N^2-[(2S)-2-(3,5-difluorophenyl)-2-hydroxyethanoyl]-N^1-[(7S)-5-methyl-6-oxo-6,7-dihydro-5H-dibenzo[b,d]azepin-7-yl]-L-alaninamide; NSAID, non-steroidal anti-inflammatory drug; PO, oral.

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ABSTRACT

Recent studies indicating that some non-steroidal anti-inflammatory drugs (NSAIDs) selectively modulate γ-secretase cleavage of amyloid precursor protein (APP) while sparing Notch processing have generated interest in discovery of novel γ-secretase modulators with the "NSAID-like" efficacy profile. The objective of the present studies was to compare the efficacy of a subset of NSAIDs with previously reported classical γ-secretase inhibitors, LY-411575 and DAPT, in Tg2576 mice. Flurbiprofen (10 and 25 mg/kg/d) was overtly toxic and elicited significant (but nonselective) reductions in both Aβ(1-40) and Aβ(1-42) in the plasma in one of two studies. Flurbiprofen also produced a small reduction in Aβ(1-40) in the cortex at 25 mg/kg/d but did not affect Aβ levels in hippocampus or CSF. Ibuprofen and sulindac sulfide were neither overtly toxic nor efficacious at doses up to 50 mg/kg/d. The NSAIDs, LY-411575 and DAPT effects were tested in guinea pig embryonic neuronal cultures to determine whether the selective reductions in Aβ(1-42) observed in cell lines over-expressing human mutant APP can be reproduced in a neuronal model of physiological Aβ production and secretion. Flurbiprofen and sulindac non-selectively reduced Aβ(1-40) and Aβ(1-42) at concentrations ≥125 μM, although cytotoxicity was noted at ≥250 μM of sulindac. Ibuprofen had no effect at concentrations up to 500 μM. In contrast, DAPT and LY-411575 potently and completely inhibited Aβ(1-40), Aβ(1-42), and Aβ(1-38) in the absence of cytotoxicity. The divergence of the present data from published reports raises the need to examine the conditions necessary to perceive selective Aβ(1-42) reduction by NSAIDs in neuronal tissue.

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Alzheimer's disease (AD) is characterized by the presence of neurofibrillary tangles, progressive neurodegeneration, amyloid-β protein (Aβ) aggregates in the form of parenchymal and vascular plaques and evidence of several markers of neuroinflammatory processes in the brain. It is perhaps the latter of these pathologies that initially led to the evaluation of a relationship between non-steroidal anti-inflammatory drug (NSAID) use and the risk to develop AD (McGeer et al., 1996). The epidemiological studies published to date indicate that chronic NSAID use reduces prevalence or severity of AD (for review, see Launer, 2003; Pasinetti, 2002). In agreement with the epidemiological findings, preclinical *in vivo* studies have shown that when transgenic mice over-expressing human mutant APP (amyloid-precursor protein) are fed a diet supplemented with ibuprofen for several months, there are reductions not only in inflammatory markers such as activated microglia, but also in amyloid plaque burden and brain Aβ levels (Lim et al., 2000; Jantzen et al., 2002; Yan et al., 2003).

Although the classical mechanism attributed to NSAID pharmacology is inhibition of cyclooxygenase enzyme activity, an increasing number of recent studies demonstrate effects of certain NSAIDs on Aβ(1-42) levels *via* mechanisms independent of their cyclooxygenase-dependent anti-inflammatory properties. For example, some of the NSAIDs have been shown to modulate Aβ levels by affecting the activity of β-secretase and Rho protein in cell cultures (Sastre et al., 2003; Zhou et al., 2003). Additionally, Weggen et al. (2001) demonstrated that a subset of NSAIDs (ibuprofen, indomethacin, sulindac sulfide) preferentially reduced Aβ(1-42) in several culture systems and this effect was not dependent upon inhibition of cyclooxygenase (COX) activity. Furthermore, subacute administration (3 day dosing paradigm) of these NSAIDs

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to young, transgenic mice expressing APPsw (Tg2576 mice) significantly reduced brain Aβ(1-42) levels (Weggen et al., 2001; Eriksen et al., 2003). Using broken cell assays to enrich for γ-secretase activity, several recent studies provide evidence that the above discussed Aβ lowering effects of a selected set of NSAIDs may be attributable to direct modulation of the γ-secretase activity responsible for generation of Aβ(1-42) from APP (Morihara et al., 2002; Takahashi et al., 2003; Weggen et al. 2003). Interestingly, the subset of NSAIDs tested reduced Aβ without inhibiting the γ-secretase cleavage of Notch, which has been implicated in the toxicity of classical γ-secretase inhibitors (Searfoss et al., 2003). These data provided an exciting new possibility of discovering novel γ-secretase modulators with NSAID-like Aβ lowering effects without the side effects associated with disruption of Notch signaling.

Previously others and we have reported dose- and time-dependent reductions in CSF, brain and plasma Aβ levels in the Tg2576 mice by two classical γ-secretase inhibitors, DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester) and LY411575 (N^2-[(2S)-2-(3,5-difluorophenyl)-2-hydroxyethanoyl]-N^1-[(7S)-5-methyl-6-oxo-6,7-dihydro-5H-dibenzo[b,d]azepin-7-yl]-L-alaninamide) (Dovey et al., 2001; Lanz et al., 2003, 2004). The present study sought to examine how NSAID effects may compare to those of DAPT and LY411575 in the central and peripheral compartments. We selected ibuprofen, flurbiprofen and sulindac sulfide for these studies and used a dosing paradigm identical to that reported by Weggen et al., 2001 and Eriksen et al., 2002, since these investigators reported consistent reductions in Aβ(1-42) levels in Tg2576 mice using these drugs and the dosing paradigm. In addition, previous studies suggest differential effects of NSAIDs on secreted Aβ in different cell culture models.

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For example, although NSAIDs selectively reduced Aβ(1-42) in APPsw transfected cell lines derived from peripheral (HEK293, CHO) or glial (H4) lineage (Weggen et al., 2001; Morihara et al., 2002; Eriksen et al., 2003), they had none or only a modest selectivity for reduction in Aβ levels in cells of neuronal origin (N2a cells transfected with APPsw and rat primary cortical neuronal cultures) (Gasparini et al., 2004). Since familial AD cases with APP mutations account for a small percent of the total incidence of AD, we developed a neuronal cell culture model from the guinea pig to assess effects of NSAIDs and γ-secretase inhibitors on endogenous Aβ at the physiological expression level of APP. The guinea pig was chosen to take advantage of the homologous Aβ sequence between the guinea pig and humans (Johnstone et al., 1991), which enabled us to use the same ELISA for Aβ assays in both the *in vivo* and cell culture studies.

Surprisingly, our overall results differ considerably from those of Weggen et al. (2001) and Eriksen et al. (2003). The reasons for the discrepancy between our findings and previous reports by Weggen et al. (2001) and Eriksen et al. (2003) will be discussed.

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MATERIALS AND METHODS

Animals and *in vivo* drug administration

Young female, transgenic mice over-expressing the human *APP* gene with the Swedish double mutation (K670N/M671L) under the transcriptional control of the hamster prion protein promoter (Tg2576 line; Hsiao et al., 1996) were used for the studies (N=15 per group at the start of the experiment for each study). Young, female mice were chosen to facilitate comparison to the *in vivo* studies by Weggen et al. (2001) and Eriksen et al. (2003), who also used female Tg2576 mice. All animal treatment protocols were compliant with the Animal Welfare Act Regulations.

Study 1: 4-month-old mice were dosed orally with vehicle (5% ethanol in corn oil) or solutions of 25, 50, or 100 mg/kg/day of racemic flurbiprofen at 10 ml/kg/d four times daily for three days. The daily doses were divided into 4 equal parts and administered at 6 h intervals. This vehicle was selected for study 1 since it produced a formulation in which flurbiprofen was fully soluble and we have previous experience with this vehicle (Lanz et al., 2003). On day 4, mice were given two additional doses of the vehicle or drug solutions and euthanized 2 h later as described below.

Study 2: This study replicated dosage, dosing frequency and vehicle reported by Weggen et al. (2001) and Eriksen et al. (2003). Thus, ibuprofen (50 mg/kg/day) or racemic flurbiprofen (10 or 25 mg/kg/d) were suspended in Kool-Aid and were dosed orally to 3.3-month-old mice for 3 days. Control mice received an oral gavage of Kool Aid (10 mL/kg). The daily dose was divided into four equal doses and administered every 4 hours, with a 12-hour interval between the last dose at night and the first dose the next

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day. On the fourth day, mice were treated with two additional doses 4 hours apart and euthanized precisely 2 hours after the final dose as described below.

Study 3: 3-month-old mice were dosed with an optimized formulation vehicle (10% solutol in 0.05M sodium phosphate buffer) or solutions of 50 mg/kg/day ibuprofen or 25 or 50 mg/kg/day sulindac sulfide. The same dosing schedule as that used in Study 2 (i.e., 4X daily for 3 days followed by 2 doses on day 4 and euthanasia 2 h later) was used here.

Two hours after the final dose, mice were anaesthetized with a mixture of ketamine/xylazine (200/5 mg/kg, SC; Butler Company, Columbus, OH). CSF, plasma, cortex and hippocampus were extracted from each mouse as described previously for subsequent Aβ ELISA analysis (Lanz et al., 2003). In Study 3, a separate aliquot of plasma was held aside for drug level analysis by LC/MS (see Lanz et al., 2004 for description of these methods).

Isolation, Culturing and Treatment of Guinea Pig Neurons

A modification of the method reported by Beck et al. (1999) was used. Briefly, Hartley guinea pigs were mated at 3-5 months of age (approximately 500 g) in-house. Pregnancy was confirmed by the vaginal plug and ultrasound examination (Toshiba Power Vision 6000). On the 25th day of gestation, the pregnant female was anaesthetized in a chamber of isoflurane, and then euthanized. The embryos were harvested, and cortex and hippocampus were dissected out and stored in Hibernate E (BrainBits, Springfield, IL) with B27 (Invitrogen, Carlsbad, CA) for 1-2 hours on ice until neurons were isolated according to the procedure of Brewer et al. (1993). Cells were plated at a density of 1.5 x 10^5 cells/cm^2 on Biocoat plates (Becton Dickinson, San Jose, CA) pre-coated with poly-

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D-lysine. Primary neurons were grown under serum-free conditions, and media was changed every 4 days. After 14 days *in vitro*, a complete media change was accompanied by exposure to vehicle (1% DMSO) or drug (DAPT, LY-411575, racemic flurbiprofen, ibuprofen, or sulindac sulfide) at various concentrations in triplicate wells. The neurons were allowed to grow for four days in the presence of the drug or vehicle. The four day incubation was required to be able to measure Aβ(1-42) levels in vehicle-treated cultures. On the last day, half the media was taken and frozen for subsequent Aβ ELISA analysis. Neurons then incubated in the presence of an MTS solution (Promega, Madison, WI) to determine cell viability; media was collected after 2h incubation at 37^{o}C and measured in a plate reader. Each drug was tested in two independent cell cultures.

Aβ ELISA

Brain tissue samples were homogenized in 5M guanidine buffer (5M guanidine HCl in 50 mM Tris-Cl, pH 8.3; Sigma-Aldrich, St. Louis, MO) at 1:10 w/v dilution. Homogenates were agitated at room temperature for 3-4 hours, then stored at -20^{o}C. The day before running the ELISA, the brain homogenates were diluted further 1:10 for a final guanidine concentration of 0.5M. These diluted homogenates were then spun down at 14,000 rpm for 20 min at 4^{o}C and supernatants were used for Aβ determination by ELISA. CSF, plasma, and culture media were diluted into blocking buffer (PBS, 0.05% Tween 20, 1% bovine serum albumin). CSF was diluted 1:20 for Aβ(1-42) and 1:40 for Aβ(1-40); plasma was diluted 1:2.5 for Aβ(1-42) and 1:5 for Aβ(1-40); media was diluted 1:2. Brain extracts were assayed as already diluted in 0.5M guanidine. Standards for each assay were prepared in the appropriate buffer (i.e. 50% blank media in blocking buffer for neurons, 0.5M guanidine buffer for brains, and 100% blocking buffer for CSF

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and plasma). Aβ(1-40) and Aβ(1-42) were assayed using ELISA using 6E10 as the capture antibody and Rb209 and Rb321 as reporter antibodies, respectively, for Aβ(1-40) and (1-42) as detailed in Lanz et al. (2004). Rb341 was used as a reporter antibody for Aβ(1-38); this antibody does not recognize Aβ(1-40) or Aβ(1-42) up to 8000 pg/mL and specificity has been confirmed by western blot (Ellerbrock et al., 2003).

Statistical Analysis and Data Presentation

For each study, one-way analysis of variance was used to detect a significant treatment effect on Aβ (raw values as well as Aβ(1-42)/Aβ(1-40) ratios for each subject) or MTS reduction. Following a significant main effect by ANOVA, individual group differences were analyzed using Dunnett's multiple comparison test; $p<0.05$ was used as a statistically significant level. For these analyses as well as estimation of IC_{50} values for Aβ inhibition in the neuronal culture, Graphpad Prism software was used. To facilitate comparisons between all studies, data are presented as % of the corresponding vehicle control ± standard error of the mean (SEM); absolute values for the vehicle group are included in the figure legend for each study. For neuronal cultures, each drug was tested in two separate cultures (every concentration was tested in triplicate wells in both studies). The results are the mean % vehicle control ± standard error of the mean (SEM) for the two trials.

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RESULTS

In vivo *effects of NSAIDs*

In Study 1 (Fig. 1), racemic flurbiprofen had no significant effect at any doses tested on either Aβ(1-40) or Aβ(1-42) in brain or plasma (CSF was not collected in the first experiment). ANOVA values are as follows: cortex Aβ(1-40) $F_{3,12}$=1.69, p=0.19, Aβ(1-42) $F_{3,12}$=4.56, p<0.01; hippocampus Aβ(1-40) $F_{3,12}$=2.18, p=0.11, Aβ(1-42) $F_{3,12}$=1.03, p=0.39; plasma Aβ(1-40) $F_{3,12}$=2.17, p=0.11, Aβ(1-42) $F_{3,12}$=0.53, p=0.66. While cortical Aβ(1-42) showed a significant treatment effect by ANOVA, no group was significantly different from vehicle in the post-hoc test. The ratio of Aβ(1-42) to Aβ(1-40) in each compartment was generally not reduced by drug treatment (Table 1). A treatment effect was detected in cortex $F_{3,12}$=5.28, p<0.01 due to a significant increase in the 42/40 ratio by the 50 mg/kg/day dose (p<0.01); no significant effect on ratio was observed in hippocampus ($F_{3,12}$=1.59, p=0.22). A treatment effect was observed in plasma ($F_{3,12}$=3.74, p<0.05), however only the 25 mg/kg/day was significantly different from vehicle (p<0.05), and as in cortex this was an increase in the 42/40 ratio. In general, the only significant treatment effect was mortality related to the gastric liability of this drug; 7 of 15 mice died in the 25 mg/kg/day group, and 8 of 15 mice died in each of higher dose groups (50 and 100 mg/kg/day).

Study 2 tested lower doses of flurbiprofen (10 and 25 mg/kg/day) as well as 50 mg/kg/day ibuprofen, each drug administered as a suspension in Kool-Aid. A significant main effect of treatment on Aβ(1-40) was detected in the cortex ($F_{4,11}$=5.427, p<0.01). Post-hoc tests showed that flurbiprofen at 25 mg/kg/day dose elicited a significant reduction in cortical Aβ(1-40) (p<0.01, Figure 2*A*). No significant effect on Aβ(1-42)

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was detected in the cortex ($F_{3,12}$=0.77, p=0.52), and neither Aβ(1-40) nor Aβ(1-42) were significantly altered in hippocampus ($F_{3,12}$=1.95, p=14 for Aβ(1-40) and $F_{3,12}$=1.78, p=0.16 for Aβ(1-42)) (Fig. 2*B*) or CSF ($F_{3,12}$=0.82, p=0.49 for Aβ(1-40) and $F_{3,12}$=1.53, p=0.22 for Aβ(1-42)) (Fig. 2*C*). Ibuprofen had no effect on either Aβ(1-40) or Aβ(1-42) in the brain or CSF. In plasma, however, both Aβ species were reduced by drug treatment ($F_{3,12}$=10.2, p<0.001 for Aβ(1-40) and $F_{3,12}$=23.51, p<0.001 for Aβ(1-42)). Both doses of flurbiprofen significantly reduced Aβ levels when compared to the vehicle (p<0.001) whereas ibuprofen was without a significant effect (Fig. 2*D*). No consistent trend is seen with respect to the Aβ(1-42) to Aβ(1-40) ratio with either drug (Table 1). In cortex, the overall treatment effect on 42/40 ratio was significant ($F_{3,12}$=4.11, p<0.05), but no group was significantly different from vehicle. Hippocampus ($F_{3,12}$=1.36, p=0.27) and CSF ($F_{3,12}$=0.30, p=0.74) did not exhibit a treatment effect on 42/40 ratio. In plasma ($F_{3,12}$=6.41, p<0.01), ibuprofen produced a significantly increased the ratio of Aβ(1-42) to Aβ(1-40) (p<0.01). As with the previous study, flurbiprofen treatment was associated with mortality; in the 10 mg/kg/day group, 2 of 15 mice died, while in the 25 mg/kg/day group, 7 of 15 mice died. No deaths occurred in either the vehicle or ibuprofen groups.

In Study 3 mice were dosed with a solution of 50 mg/kg/day ibuprofen or 25 or 50 mg/kg/day sulindac sulfide. As in the previous experiment, ibuprofen had no significant effect on either Aβ(1-40) or Aβ(1-42) in any tissue analyzed (Fig. 3). Similarly, sulindac sulfide did not affect Aβ levels in the brain, CSF, or plasma despite achieving high concentrations of the drug in plasma (60.87 μM at the 25 mg/kg/day dose, 94.5 μM at the 50 mg/kg/day dose). ANOVA values are as follows: cortex Aβ(1-40) $F_{3,12}$=0.39, p=0.76, Aβ(1-42) $F_{3,12}$=0.90, p=0.45; hippocampus Aβ(1-40) $F_{3,12}$=1.51, p=0.22, Aβ(1-42)

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$F_{3,12}$=0.97, p=0.41; CSF Aβ(1-40) $F_{3,12}$=0.33, p=0.81, Aβ(1-42) $F_{3,12}$=0.21, p=0.89; plasma Aβ(1-40) $F_{3,12}$=0.22, p=0.89, Aβ(1-42) $F_{3,12}$=1.41, p=0.25. As with the previous experiments, no consistent trend was observed in the Aβ(1-42) to Aβ(1-40) ratio (Table 1). No treatment effect on the 42/40 ratio in cortex ($F_{3,12}$=0.95, p=0.42), hippocampus ($F_{3,12}$=1.22, p=0.31), or CSF ($F_{3,12}$=0.49, p=0.69). A significant effect was seen in plasma ($F_{3,12}$=3.34, p<0.05), as the 50 mg/kg/day group had a significantly higher ratio of Aβ(1-42) to Aβ(1-40). In addition to Aβ(1-40) and Aβ(1-42), all tissues from this experiment were analyzed for Aβ(1-38) levels, and no treatment group showed significantly different Aβ(1-38) levels from the vehicle group (Figure 3). The ANOVA for Aβ(1-38) are as follows: cortex $F_{3,12}$=0.35, p=0.79; hippocampus $F_{3,12}$=0.80, p=0.50; CSF $F_{3,12}$=2.17, p=0.11; plasma $F_{3,12}$=0.45, p=0.72. No treatment-induced deaths occurred in this experiment.

Guinea Pig Primary Neuronal Cultures

Effects of flurbiprofen, ibuprofen, and sulindac sulfide on secreted Aβ were tested in guinea pig neuronal cultures at concentrations of 0.5 to 500 μM. After 4 days of treatment *in vitro*, racemic flurbiprofen elicited non-selective reduction in Aβ(1-40), Aβ(1-42), and Aβ(1-38) starting at 125 μM (Fig. 4*A*). The estimated IC_{50} for Aβ(1-40) was 215.4 μM, and the IC_{50} for Aβ(1-42) was 145.1 μM, and the estimated IC_{50} for Aβ(1-38) was >500 μM. Maximal inhibition obtained with flurbiprofen (at 500 μM) brought the Aβ(1-40) levels to 40.3 ± 14.4% of vehicle, Aβ(1-42) levels to 34.7 ± 2.4% of vehicle, and Aβ(1-38) levels to 61.8 ± 13.2% of vehicle. Ibuprofen did not have any inhibitory effect on Aβ at the concentrations tested (Fig. 4*B*). Sulindac sulfide decreased

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both Aβ(1-40) and Aβ(1-42) at high concentrations (Fig. 4*C*); the IC_{50} for Aβ(1-40) appeared to be 187.1 μM, and 129.9 μM for Aβ(1-42). At the maximal concentration of 500 μM, sulindac sulfide reduced Aβ(1-40) levels to 5.6 ± 1.9% of vehicle and Aβ(1-42) levels of 22.9 ± 1.5% of vehicle. It should be noted, however, that efficacious concentrations were accompanied by cytotoxicity as assessed by the MTS assay (23% cytotoxicity at 250 μM and 61% toxicity at 500 μM of sulindac sulfide). Only these toxic doses reduced Aβ(1-38), causing it to appear to have an IC_{50} value of 255 μM. At the highest nontoxic dose (125 μM), however, Aβ(1-38) was elevated to 129 ± 8.5% of vehicle levels, while Aβ(1-40) levels were 73.0 ± 5.2% of vehicle and Aβ(1-42) levels were 62.1 ± 12.3% of vehicle. No significant cytotoxicity was detected with flurbiprofen or ibuprofen at the concentrations tested. Inter-assay vehicle variability was 5.5% for Aβ(1-40), 8.5% for Aβ(1-42), and 4% for Aβ(1-38). As shown in Table 2, no dose-dependent changes in the ratio of Aβ(1-42) to Aβ(1-40) were observed with any of the NSAIDs tested. With flurbiprofen ($F_{11,13}$=0.52, p=0.86), and ibuprofen treatment ($F_{11,13}$=0.34, p=0.96), no treatment effect on 42/40 ratio was detected. Sulindac sulfide produced a significant treatment effect ($F_{11,13}$=23.9, p<0.001), though only the 500 μM dose was significantly different from vehicle (p<0.01).

The activity of the benchmark γ-secretase inhibitors, DAPT and LY-411575 (for review, see Josien, 2002), were also assayed in neuronal cultures for 4 days. DAPT was tested at 0.1 nM to 10 μM (Fig. 5*A*). The IC_{50} values for Aβ(1-40), Aβ(1-42), and Aβ(1-38) were 144.1 nM, 117.6 nM, and 107 nM, respectively. Cytotoxicity began to be detected when compound reached micromolar concentrations; 30-35% toxicity was detected at 3 and 10 μM. LY-411575, a much more potent γ-secretase inhibitor, elicited

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a steep dose-response when tested from 0.1 pM to 10 nM (Fig. 5*B*). The IC_{50} values for Aβ(1-40), Aβ(1-42), and Aβ(1-38) were 86.9 pM, 60.9 pM, and 66 pM, respectively. No cytotoxicity was observed until the 10 nM dose (20%). Maximal Aβ reduction for both γ-secretase inhibitors reached 100% (i.e., reduction of Aβ levels to below 0.3 pM, the assay's lower limit of detection).

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DISCUSSION

The present studies failed to detect reductions in brain Aβ(1-42) levels following subchronic treatment of young Tg2576 mice with NSAIDs purported to selectively reduce Aβ(1-42) by γ-secretase modulation (Weggen et al., 2001; Eriksen et al., 2003). In a dosing paradigm identical to that reported previously (ibid), ibuprofen and sulindac had no effects on Aβ in the brain, CSF or plasma. Flurbiprofen reduced plasma Aβ(1-40) and Aβ(1-42) and cortical Aβ(1-40), although these effects were not seen consistently, and may be confounded by in vivo toxicity. This is in contrast to the potent, classical γ-secretase inhibitors, DAPT and LY-411575, which dose- and time-dependently reduce Aβ levels in the brain, CSF and plasma of young Tg2576 mice (Dovey et al., 2001; Lanz et al., 2003, 2004). Additionally, using a guinea pig primary embryonic neuronal model to assess the effects of NSAIDs on endogenous, secreted Aβ, we fail to see selective Aβ(1-42) reduction as reported previously by several investigators using cells lines of non-neuronal lineage transfected with APPsw or presenilin-1 constructs (Weggen et al., 2001; Morihara et al., 2002; Eriksen et al., 2003).

The reasons underlying the discordant results between the present *in vivo* studies and those reported previously remain unclear. In the first study, we used a vehicle that fully dissolved flurbiprofen and tested three doses (25, 50 or 100 mg/kg/d), all of which induced lethality but failed to significantly alter Aβ levels. To rule out a formulation effect, we performed the second study in which treatment conditions (vehicle, doses, dose frequency) were identical to those described by Weggen et al. (2001) for ibuprofen (50 mg/kg/d) and flurbiprofen (10 and 25 mg/kg/d). Although no mortality was seen in the ibuprofen group, flurbiprofen again showed dose-dependent lethality, indicating that the

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drug had *in vivo* pharmacological effects. However, Aβ(1-42) levels in the cortex, hippocampus or CSF were not reduced by either ibuprofen or flurbiprofen. Notably, a significant reduction in both Aβ(1-40) and Aβ(1-42) was evident in the plasma of flurbiprofen treated mice. In the last *in vivo* study, we re-tested ibuprofen alongside sulindac sulfide, and observed no changes in Aβ levels nor overt toxicity. Interestingly, the plasma concentration of sulindac was 3-5 times that reported by Eriksen et al. (2003). Thus, the failure to observe *in vivo* Aβ(1-42) inhibition by sulindac is not likely due to lack of adequate drug exposure. Although unlikely, one potential source of this discrepancy could be related to differences in brain regions examined; Eriksen et al. (2003) used mouse hemi-brains whereas the present studies dissected out hippocampus and cortex. Another difference between the studies is the extraction procedure and antibodies used for Aβ ELISAs. The present study used an alkaline guanidine solution to extract brain Aβ, while the aforementioned studies by Eriksen et al. (2003) and Weggen et al. (2001) used 70% formic acid. Since the age of the mice used in all of the experiments precedes the onset of plaque deposition in Tg2576 mice (Kawarabayashi et al., 2001) it is unclear how the extraction method may have effects on the recovery of (presumably soluble) brain Aβ and its recognition by antibodies. A comparison of absolute brain Aβ levels in the present study with those reported in the literature indicate that although our data are in relative agreement with most studies of pre-plaque Tg2576 brains (Table 3), the Aβ(1-40) levels reported here are higher than those reported by Eriksen et al. (2003). It should be noted also that our previous studies of potent γ-secretase inhibitors, DAPT and LY411575, used the guanidine extraction procedures described here and demonstrated dose- and time-dependent reductions in Aβ(1-40) and

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Aβ(1-42) in the brain, CSF, and plasma (Lanz et al., 2003; Lanz et al., 2004). Interestingly, the magnitude of Aβ reduction following DAPT and LY-411575 treatment was greater in the CSF and plasma than in the brain tissue. In view of these data, the complete lack of effect of the three NSAIDs tested here in the CSF is surprising. Similarly, plasma Aβ was generally not affected by NSAID treatment with the exception of the second trial with flurbiprofen, which reduced plasma levels of both Aβ(1-40) and Aβ(1-42). Eriksen et al. (2003) also analyzed plasma Aβ, and reported either no change or a decrease in both Aβ species in 8 of 12 mice treated with flurbiprofen, and 3 of 6 mice treated with ibuprofen. The selective Aβ(1-42) reduction in the brain accompanied by non-selective Aβ reduction in the plasma shown by Eriksen et al. (2003) raises the question about the mechanism underlying molecular interactions between NSAIDs and γ-secretase complex in the brain versus peripheral tissues.

In addition to *in vivo* studies, the three NSAIDs were tested in guinea pig embryonic primary neuronal cultures to assess whether they may alter secreted Aβ in a model of physiological expression of native APP. Flurbiprofen and sulindac partially reduced secreted Aβ at high drug concentrations, but this effect was not selective for Aβ(1-42). These data also contrast those of other groups, including Ellerbrock et al. (2003) who used the same ELISA assays described presently to demonstrate selective Aβ(1-42) reduction and Aβ(1-38) elevation in APPsw transfected cells of non-neuronal origin (e.g. HEK293). However, the present data in the guinea pig primary neurons are in general agreement with those of Gasparini et al. (2004), who demonstrated that flurbiprofen and sulindac, respectively, have none or only a modest selectivity for Aβ inhibition in cells of neuronal origin (N2a cells transfected with APPsw and rat primary

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cortical neuronal cultures). The low *in vitro* potency for Aβ(1-40) and Aβ(1-42) inhibition by flurbiprofen and sulindac are also in agreement with those reported by Gaspirini et al. (2004) and Takahashi et al. (2003) in N2a cells. It should be noted that the concentration range of sulindac that reduced Aβ overlapped at the high end with concentrations deemed toxic by the MTS assay; the switch from induction to inhibition of Aβ(1-38) at 250 μM of sulindac illustrates this point. Thus, although the IC_{50} value for Aβ(1-42) inhibition by sulindac was similar to that reported by Eriksen et al. (2003), the contribution of cell toxicity to Aβ reduction cannot be ruled out. In view of data from neuronal cultures, the lack of *in vivo* efficacy of the NSAIDs on Aβ(1-40) or Aβ(1-42) in the present studies may be a result of low potency of these compounds.

Unlike NSAIDs, the classical γ-secretase inhibitors, DAPT and LY-411575, potently reduced Aβ(1-40), Aβ(1-42), and Aβ(1-38) in a concentration-dependent manner in guinea pig neuronal cultures. The Aβ inhibition IC_{50} estimates for DAPT and LY-411575 in the guinea pig primary neuronal cultures were comparable to the previously reported potency of these compounds in clonal cell lines (Josien, 2002). These data demonstrate that guinea pig primary neurons offer a viable model to study drug effects on endogenous Aβ.

While the Aβ(1-42) modulatory effects of subacute NSAID treatment do not appear to be fully reproducible, several groups have reported beneficial effects of chronic ibuprofen treatment *in vivo*. Dosing APP over-expressing, aged mice with ibuprofen in the chow for at least 3 months reduces plaque load (Lim et al., 2000; Jantzen et al., 2002; Yan et al., 2003) as well as Aβ levels in the soluble and insoluble fractions (Lim et al. 2001, 2002; Yan et al., 2003). It is possible that the anti-inflammatory properties of the

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drug indirectly result in Aβ-lowering and plaque reduction. In support of this contention, chronic ibuprofen treatment has been shown to reduce microglial activation (Lim et al., 2000; Yan et al., 2003) and interleukin-1β expression (Lim et al., 2000, 2001) in APP transgenic mice that harbor increased signs of inflammation with aging (e.g. cytokine induction) and the presence of activated microglia, especially in the vicinity of plaques (Abbas et al., 2002; Frautschy et al., 1998; Gordon et al., 2002). Regardless of its precise interaction in the brain, chronic ibuprofen appears to have beneficial effects on APP over-expressing mice by reducing inflammation, lowering Aβ, slowing plaque development and affecting behavioral phenotypes.

In summary, epidemiological studies clearly implicate protective effects of certain NSAIDs on the incidence and severity of AD. Studies in transgenic mice chronically treated with ibuprofen demonstrate the reduction in plaque load and markers of neuroinflammation and thereby implicate a cyclooxygenase-dependent anti-inflammatory mechanism. However, evidence also exists for several cyclooxygenase-independent mechanisms in modulation of Aβ by a subclass of NSAIDs and includes direct modulation/inhibition of γ-secretase (Morihara et al., 2002; Takahashi et al., 2003; Weggen et al., 2003), β-secretase (Sastre et al., 2003), rho kinase (Zhou et al., 2003), and peroxisome proliferator-activated receptor-γ (PPARγ) (Tegeder et al., 2001) activity. Of these, the modulation of γ-secretase activity to selectively lower Aβ(1-42) while sparing Notch cleavage has received significant attention following reports by Weggen et al. (2001) and Eriksen et al. (2003). The studies presented here question the ability to observe selective Aβ(1-42) reduction in neuronal tissue after NSAID treatment and suggest that many facets of the mechanism of NSAID action need to be investigated in

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neuronal background to aid discovery of novel γ-secretase modulators to reduce brain Aβ while sparing γ-secretase cleavage of other substrates.

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REFERENCES

Abbas N, Bednar I, Mix E, Marie S, Paterson D, Ljungberg A, Morris C, Winblad B, Nordberg A, and Zhu J (2002) Up-regulation of the inflammatory cytokines IFN-gamma and IL-12 and down-regulation of IL-4 in cerebral cortex regions of APP(SWE) transgenic mice. *J Neuroimmunol* **126**: 50-57.

Beck M, Bruckner MK, Holzer M, Kaap S, Pannicke T, Arendt T, and Bigl V (1999) Guinea pig primary cell cultures provide a model to study expression and amyloidgenic processing of endogenous amyloid precursor protein. *Neuroscience* **95**: 243-254.

Brewer GJ, Torricelli EK, Evege EK, and Price PJ (1993) Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J Neurosci Res* **35**: 567-576.

Chang WP, Koelsch G, Wong S, Downs D, Da H, Weerasena V, Gordon B, Devasamudram T, Bilcer G, Ghosh AK, and Tang J (2004) *In vivo* inhibition of Aβ production by memapsin 2 (β-secretase) inhibitors. *J Neurochem* **89**: 1409-1416.

Dovey HF, John V, Anderson JP, Chen LZ, de Saint Andrieu P, Fang LY, Freedman SB, Folmer B, Goldbach E, Holsztynska EJ, Hu KL, Johnson-Wood KL, Kennedy SL, Kholodenko D, Knops JE, Latimer LH, Lee M, Liao Z, Lieberburg IM, Motter RN, Mutter LC, Nietz J, Quinn KP, Sacchi KL, Seubert PA, Shopp GM, Thorsett ED, Tung

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on August 30, 2004 as DOI: 10.1124/jpet.104.073965
This article has not been copyedited and formatted. The final version may differ from this version.

JS, Wu J, Yang S, Yin CT, Schenk DB, May PC, Altstiel LD, Bender MH, Boggs LN, Britton TC, Clemens JC, Czilli DL, Dieckman-McGinty DK, Droste JJ, Fuson KS, Gitter BD, Hyslop PA, Johnstone EM, Li WY, Little SP, Mabry TE, Miller FD, and Audia JE (2001) Functional gamma-secretase inhibitors reduce beta-amyloid peptide levels in brain. *J Neurochem* **76**: 173-181.

Ellerbrock BR, Fleck TJ, Landrum CS, Pauley AM, Carter DB, Peach ML, Laborde AL, Miller DL, Mehta P, Nye JS (2003) NSAID modulators of the gamma secretase show similar potencies at inhibition of Aβ1-42 and induction of Aβ1-38 as measured by a novel neoepitope-specific ELISA. *Soc Neurosci Abstr* 667.10.

Eriksen JL, Sagi SA, Smith TE, Weggen S, Das P, McLendon DC, Ozols VV, Jessing KW, Zavitz KH, Koo EH, and Golde TE (2003) NSAIDs and enantiomers of flurbiprofen target γ-secretase and lower Aβ42 in vivo. *J Clin Invest* **112**: 440-449.

Frautschy SA, Yang F, Irrizarry M, Hyman B, Saido TC, Hsiao K, and Cole GM (1998) Microglial response to amyloid plaques in APPsw transgenic mice. *Am J Pathol* **152**: 307-317.

Gasparini L, Rusconi L, Xu H, del Soldato P, and Ongini E (2004) Modulation of beta-amyloid metabolism by non-steroidal anti-inflammatory drugs in neuronal cell cultures. *J Neurochem* 88: 337-348.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on August 30, 2004 as DOI: 10.1124/jpet.104.073965
This article has not been copyedited and formatted. The final version may differ from this version.

Gordon MN, Holcomb LA, Jantzen PT, DiCarlo G, Wilcock D, Boyett KW, Connor K, Melachrino J, O'Callaghan JP, and Morgan D (2002) Time course of the development of Alzheimer-like pathology in the doubly transgenic PS1+APP mouse. *Exp Neurol* **173**: 183-195.

Haugabook SJ, Le T, Yager D, Zenk B, Healy BM, Eckman EA, Prada C, Younkin L, Murphy P, Pinnix X, Onstead L, Sambamurti K, Golde TE, Dickson D, Younkin SG, and Eckman CB (2000) Reduction of Aβ accumulation in the Tg2576 animal model of Alzheimer's disease after oral administration of the phosphatidylinositol kinase inhibitor wortmannin. *Faseb J* **15**: 16-18.

Hsiao K, Chapman P, Nilsen S, Eckman C, Harigaya Y, Younkin S, Yang F, and Cole G (1996) Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice. *Science* **274**: 99-102.

Jantzen PT, Connor KE, DiCarlo G, Wenk GL, Wallace GL, Rojiani AM, Coppola D, Morgan D, and Gordon MN (2002) *J Neurosci* **22**: 2246-2254.

Johnstone EM, Chaney MO, Norris FH, Pascual R, and Little SP (1991) Conservation of the sequence of the Alzheimr's disease amyloid peptide in dog, polar bear, and five other mammals by cross-species polymerase chain reaction analysis. *Molec Brain Res* **10**: 299-305.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on August 30, 2004 as DOI: 10.1124/jpet.104.073965
This article has not been copyedited and formatted. The final version may differ from this version.

Josien H (2002) Recent advances in the development of γ-secretase inhibitors. *Curr Opin Drug Discov Dev* **54**: 513-525.

Kawarabayashi T, Younkin LH, Saido TC, Shoji M, Ashe KH, and Younkin SG (2001) Age-dependent changes in brain, CSF, and plasma amyloid (beta) protein in the Tg2576 transgenic mouse model of Alzheimer's disease. *J Neurosci* **21**:372-81.

Lanz TA, Himes CS, Pallante G, Adams L, Yamazaki S, Amore B, and Merchant KM (2003) The γ-secretase inhibitor, DAPT, reduces Aβ levels *in vivo* in plasma and CSF in young (plaque-free) and aged (plaque-bearing) Tg2576 mice. *J Pharmacol Exp Ther* **305**: 864-871.

Lanz TA, Hosley JD, Adams WJ, and Merchant KM (2004) Studies of Abeta pharmacodynamics in the brain, cerebrospinal fluid, and plasma in young (plaque-free) Tg2576 mice using the gamma-secretase inhibitor N2-[(2S)-2-(3,5-difluorophenyl)-2-hydroxyethanoyl]-N1-[(7S)-5-methyl-6-oxo-6,7-dihydro-5H-dibenzo[b,d]azepin-7-yl]-L-alaninamide (LY-411575). *J Pharmacol Exp Ther* **309**: 49-55.

Launer L (2003) Nonsteroidal anti-inflammatory drug use and the risk for Alzheimer's disease: dissecting the epidemiological evidence. *Drugs* **63**: 731-739.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on August 30, 2004 as DOI: 10.1124/jpet.104.073965
This article has not been copyedited and formatted. The final version may differ from this version.

Lim GP, Yang F, Chu T, Chen P, Beech W, Teter B, Tran T, Ubeda O, Hsaio Ashe K, Frautschy SA, and Cole GM (2000) Ibuprofen suppresses plaque pathology and inflammation in a mouse model for Alzheimer's disease. *J Neurosci* **20**: 5709-5714.

Lim GP, Yang F, Chu T, Gahtan E, Ubeda O, Beech W, Overmier JB, Hsiao-Ashec K, Frautschy SA, and Cole GM (2001) Ibuprofen effects on Alzheimer pathology and open field activity in APPsw transgenic mice. *Neurobiol Aging* **22**: 983-991.

McGeer PL, Schulzer M, and McGeer EG (1996) Arthritis and anti-inflammatory agents as possible protective factors for Alzheimer's disease: a review of 17 epidemiologic studies. *Neurol* **47**: 425-432.

Morihara T, Chu T, Ubeda O, Beech W, and Cole GM (2002) Selective inhibition of Aβ42 production by NSAID *R*-enantiomers. *J Neurochem* **83**: 1009-1012.

Pasinetti GM (2002) From epidemiology to therapeutic trials with anti-inflammatory drugs in Alzheimer's disease: the role of NSAIDs and cyclooxygenase in beta-amyloidosis and clinical dementia. *J Alzheimer's Dis* **4**: 435-445.

Sastre M, Dewachter I, Landreth GE, Willson TM, Klockgether T, van Leuven F, and Heneka MT (2003) Nonsteroidal anti-inflammatory drugs and peroxisome proliferator-activated receptor-gamma agonists modulate immunostimulated processing of amyloid precursor protein through regulation of beta-secretase. *J Neurosci* **23**: 9796-9804.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on August 30, 2004 as DOI: 10.1124/jpet.104.073965
This article has not been copyedited and formatted. The final version may differ from this version.

Searfoss GH, Jordan WH, Calligaro DO, Galbreath EJ, Schirtzinger LM, Berridge BR, Gao H, Higgins MA, May PC, and Ryan TP (2003) Adipsin, a biomarker of gastrointestinal toxicity mediated by a functional gamma-secretase inhibitor. *J Biol Chem* **278**: 46107-46116.

Takahashi Y, Hayashi I, Tominari Y, Rikimaru K, Morohashi Y, Kan T, Natsugari H, Fukuyama T, Tomita T, and Iwatsubo T (2003) Sulindac sulfide is a noncompetitive gamma-secretase inhibitor that preferentially reduces Abeta 42 generation. *J Biol Chem* **278**: 18664-18670.

Tegeder I, Pfeilschifter J, and Geisslinger G (2001) Cyclooxygenase-independent actions of cyclooxygenase inhibitors. *FASEB J* **15**: 2057-2072.

Weggen S, Eriksen JL, Das P, Sagi S, Wang R, Pietrzik CU, Findlay KA, Smith TE, Murphy MP, Bulter T, Kang DE, Marquez-Sterling N, Golde TE, and Koo EH (2001) A subset of NSAIDs lower amyloidogenic Aβ42 independently of cyclooxygenase activity. *Nature* **414**: 212-216.

Weggen S, Eriksen JL, Sagi S, Pietrzik CU, Ozols V, Fauq A, Golde TE, and Koo EH (2003) Evidence that nonsteroidal anti-inflammatory drugs decrease Aβ42 production by direct modulation of γ-secretase activity. *J Biol Chem* **278**: 31831-31837.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on August 30, 2004 as DOI: 10.1124/jpet.104.073965
This article has not been copyedited and formatted. The final version may differ from this version.

Yan Q, Zhang J, Liu H, Babu-Khan S, Vassar R, Biere AL, Citron M, and Landreth G (2003) Anti-inflammatory drug therapy alters β-amyloid processing and deposition in an animal model of Alzheimer's disease. *J Neurosci* **23**: 7504-7509.

Zhou Y, Su Y, Li B, Liu F, Ryder JW, Wu X, Gonzalez-DeWhitt PA, Gelfanova V, Hale JE, May PC, Paul SM, and Ni B (2003) Nonsteroidal anti-inflammatory drugs can lower amyloidogenic Abeta42 by inhibiting Rho. *Science* **302**: 1215-1217.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on August 30, 2004 as DOI: 10.1124/jpet.104.073965
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Figures & Tables

Figure 1. Effects of 3-day subchronic administration of 25, 50, or 100 mg/kg/day (mpk) (in 4 divided doses) of flurbiprofen on Aβ(1-40) (black bars) and Aβ(1-42) (stippled bars) on cortex (*A*), hippocampus (*B*), and plasma (*C*) 3 hours after the final dose. Bars represent mean Aβ concentrations shown as % of vehicle control + SEM. Horizontal lines represent mean values for vehicle group; floating error bars on the right of each graph represent the % SEM for the vehicle group (solid line for Aβ(1-40) and dashed line for Aβ(1-42)). The vehicle mean and SEM for Aβ(1-40) concentrations (pmol/g brain or pmol/mL plasma) for each tissue are as follows: cortex, 76.3 ± 4.4; hippocampus, 87.0 ± 4.4; plasma, 4.4 ± 0.19. Vehicle mean and SEM for Aβ(1-42) concentrations (pmol/g brain or pmol/mL plasma) for each tissue are as follows: cortex, 10.6 ± 0.51; hippocampus, 10.0 ± 1.4; plasma, 0.44 ± 0.02.

Figure 2. Effects of 3-day subchronic dosing of 50 mg/kg/day (mpk) ibuprofen or 10 or 25 mg/kg/day flurbiprofen on Aβ(1-40) (black bars) and Aβ(1-42) (stippled bars) on cortex (*A*), hippocampus (*B*), CSF (*C*), and plasma (*D*) 3 hours after the final dose. Bars represent mean Aβ concentrations shown as % of vehicle control + SEM. Horizontal lines represent mean values for vehicle group; floating error bars on the right of each graph represent the % SEM for the vehicle group (solid line for Aβ(1-40) and dashed line for Aβ(1-42)). The vehicle mean and SEM for Aβ(1-40) concentrations (pmol/g brain or pmol/mL CSF and plasma) for each tissue are as follows: cortex, 104 ± 7.3; hippocampus, 70.9 ± 2.4; CSF, 19.3 ± 2.76; plasma, 3.11 ± 0.15. The vehicle mean and

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SEM for Aβ(1-42) concentrations (pmol/g brain or pmol/mL CSF and plasma) for each tissue are as follows: cortex, 13.6 ± 0.90; hippocampus, 13.9 ± 0.96; CSF, 2.78 ± 0.26; plasma, 0.30 ± 0.01. **p<0.01 versus vehicle.

Figure 3. Effects of 3-day subchronic dosing of 50 mg/kg/day (mpk) ibuprofen or 25 or 50 mg/kg/day sulindac sulfide on Aβ(1-40) (black bars), Aβ(1-42) (stippled bars) and Aβ(1-38) (open bars) on cortex (*A*), hippocampus (*B*), CSF (*C*), and plasma (*D*) 3 hours after the final dose. Bars represent mean Aβ concentrations shown as % of vehicle control + SEM. Horizontal lines represent mean values for vehicle group; floating error bars on the right of each graph represent the % SEM for the vehicle group (Aβ(1-40), Aβ(1-42), and Aβ(1-38) are denoted numerically). The vehicle mean and SEM for Aβ(1-40) concentrations (pmol/g brain or pmol/mL CSF and plasma) for each tissue are as follows: cortex, 155 ± 12.3; hippocampus, 108 ± 11.9; CSF, 38.2 ± 4.08; plasma, 7.62 ± 0.29. The vehicle mean and SEM for Aβ(1-42) concentrations (pmol/g brain or pmol/mL CSF and plasma) for each tissue are as follows: cortex, 10.2 ± 0.41; hippocampus, 12.2 ± 1.07; CSF, 1.17 ± 0.26; plasma, 0.69 ± 0.14. The vehicle mean and SEM for Aβ(1-38) concentrations (pmol/g brain or pmol/mL CSF and plasma) for each tissue are as follows: cortex, 5.24 ± 0.28; hippocampus, 9.38 ± 0.56; CSF, 2.10 ± 0.15; plasma, 0.16 ± 0.02 **p<0.01 versus vehicle.

Figure 4. Effects of flurbiprofen, ibuprofen and sulindac sulfide on Aβ secretion in guinea pig embryonic neuronal cultures. E25 guinea pig neurons (plated at 1.5 x 10^5 cells/cm^2) were incubated for 4 days in the presence of varying concentrations of the

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NSAIDs or vehicle (1% DMSO in media). Each point represents mean Aβ concentrations plotted as % of respective vehicle control for flurbiprofen (*A*), ibuprofen (*B*), and sulindac sulfide (*C*). Open circles on dashed curves represent Aβ(1-40), filled triangles on solid curves represent Aβ(1-42), and asterisks represent Aβ(1-38). The mean Aβ(1-40) concentrations (pmol/mL) for the vehicle groups are as follows: flurbiprofen, 1.76 ± 0.23; ibuprofen, 1.60 ± 0.65; sulindac sulfide, 1.81 ± 0.33. The mean Aβ(1-42) concentrations (pmol/mL) for the vehicle groups are as follows: flurbiprofen, 0.35 ± 0.02; ibuprofen, 0.32 ± 0.03; sulindac sulfide, 0.04 ± 0.02. The mean Aβ(1-38) concentrations (pmol/mL) for the vehicle groups are as follows: flurbiprofen, 0.141 ± 0.002; ibuprofen, 0.127 ± 0.001; sulindac sulfide, 0.139 ± 0.004.

Figure 5. Effects of DAPT and LY-411575 on Aβ secretion in guinea pig embryonic neuronal cultures. E25 guinea pig neurons (plated at 1.5 x 10^5 cells/cm^2) were incubated for 4 days in the presence of varying concentrations of classical γ-secretase inhibitors or vehicle (1% DMSO in media). Each point represents mean Aβ concentrations plotted as % of respective vehicle control for DAPT (*A*) and LY-411575 (*B*). Open circles on dashed curves represent Aβ(1-40), filled triangles on solid curves represent Aβ(1-42), asterisks represent Aβ(1-38). The mean Aβ(1-40) concentrations (pmol/mL) for the vehicle groups are as follows: DAPT, 1.06 ± 0.04; LY-411575, 1.15 ± 0.03. The mean Aβ(1-42) concentrations (pmol/mL) for the vehicle groups are as follows: DAPT, 0.067 ± 0.004; LY-411575, 0.079 ± 0.004. The mean Aβ(1-38) concentrations (pmol/mL) for the vehicle groups are as follows: DAPT, 0.069 ± 0.001; LY-411575, 0.079 ± 0.002.

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Table 1. Ratio of mean Aβ(1-42) to Aβ(1-40) levels are given for each group in all tissues from the experiments in Tg2576 mice.

*p<0.05, **p<0.01 versus vehicle

Ratio of Aβ(1-42) to Aβ(1-40): *in vivo*				
Study 1	Cortex	Hippocampus		Plasma
Vehicle	0.140	0.131		0.100
25 mg/kg/d Flurbiprofen	0.138	0.125		0.148*
50 mg/kg/d Flurbiprofen	0.169**	0.148		0.107
100 mg/kg/d Flurbiprofen	0.157	0.113		0.085
Study 2	Cortex	Hippocampus	CSF	Plasma
Vehicle	0.130	0.196	0.144	0.096
50 mg/kg/d Ibuprofen	0.110	0.234	0.112	0.124**
10 mg/kg/d Flurbiprofen	0.120	0.202	0.081	0.112
25 mg/kg/d Flurbiprofen	0.172	0.170	0.128	0.105
Study 3	Cortex	Hippocampus	CSF	Plasma
Vehicle	0.066	0.112	0.031	0.091
50 mg/kg/d Ibuprofen	0.063	0.113	0.023	0.092
25 mg/kg/d Sulindac	0.067	0.101	0.031	0.097
50 mg/kg/d Sulindac	0.054	0.080	0.034	0.106*

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Table 2. Ratio of mean Aβ(1-42) to Aβ(1-40) levels are given for each concentration of flurbiprofen, ibuprofen, and sulindac sulfide as assessed in guinea pig primary neuronal culture media.

*p<0.05, **p<0.01 versus vehicle

Ratio of Aβ(1-42) to Aβ(1-40) in guinea pig primary neuronal culture			
Drug Concentration	Flurbiprofen	Ibuprofen	Sulindac Sulfide
0	0.020	0.020	0.021
500 μM	0.019	0.018	0.088**
250 μM	0.016	0.021	0.021
125 μM	0.020	0.018	0.017
62.5 μM	0.022	0.023	0.018
31.3 μM	0.023	0.021	0.023
15.6 μM	0.020	0.021	0.019
7.81 μM	0.019	0.021	0.019
3.90 μM	0.020	0.028	0.019
1.95 μM	0.021	0.019	0.019
0.98 μM	0.023	0.022	0.019
0.49 μM	0.021	0.022	0.025

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Table 3. Vehicle levels of Aβ(1-40) and Aβ(1-42) from 3- to 5-month-old Tg2576 mice from various published studies are presented. Despite the use of different antibody cassettes and homogenization techniques, there is only modest variation in Aβ levels from study to study.

Reference	Aβ(1-40) (pmol/g)	Aβ(1-42) (pmol/g)	Extraction Buffer
Present dataset	71 – 155	10 - 14	5M guanidine
Eriksen et al., 2003	18.5	7.7	70% formic acid
Hsiao et al., 1996	32 - 71	2 - 21	70% formic acid
Haugabook et al., 2000	46	16	70% formic acid
Kawarabayashi et al., 2001	33.1	9.8	70% formic acid
Chang et al., 2004	75		5M guanidine

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Figure 1.

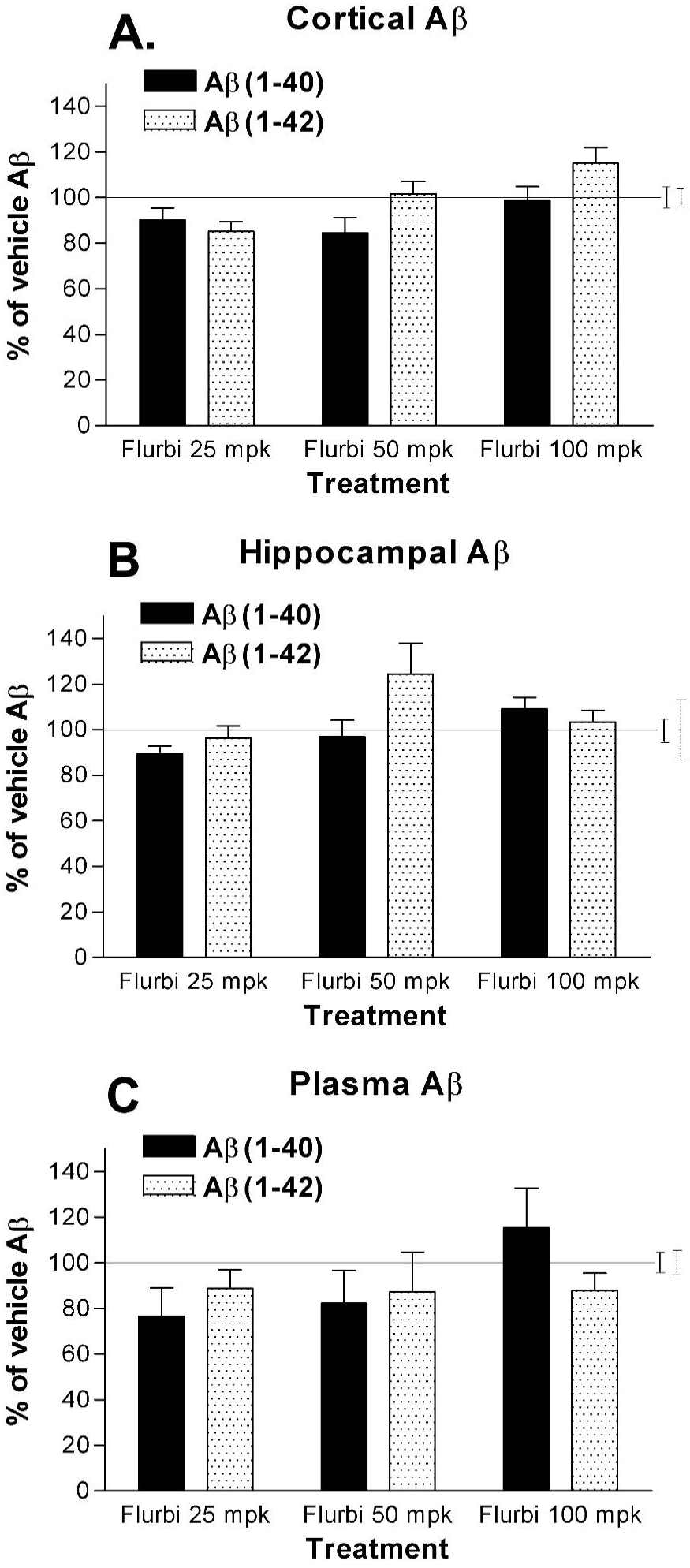

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Figure 2.

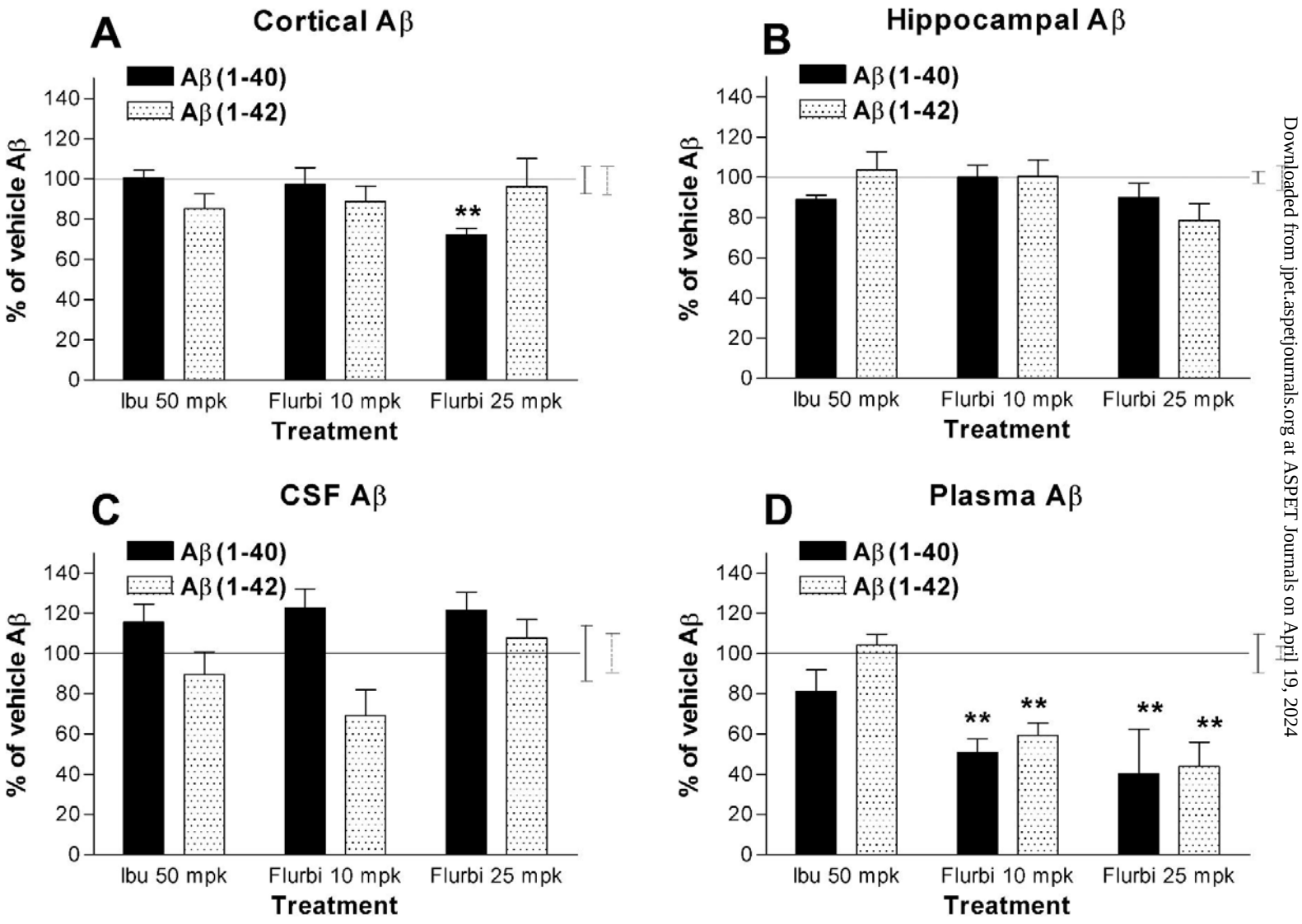

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Figure 3.

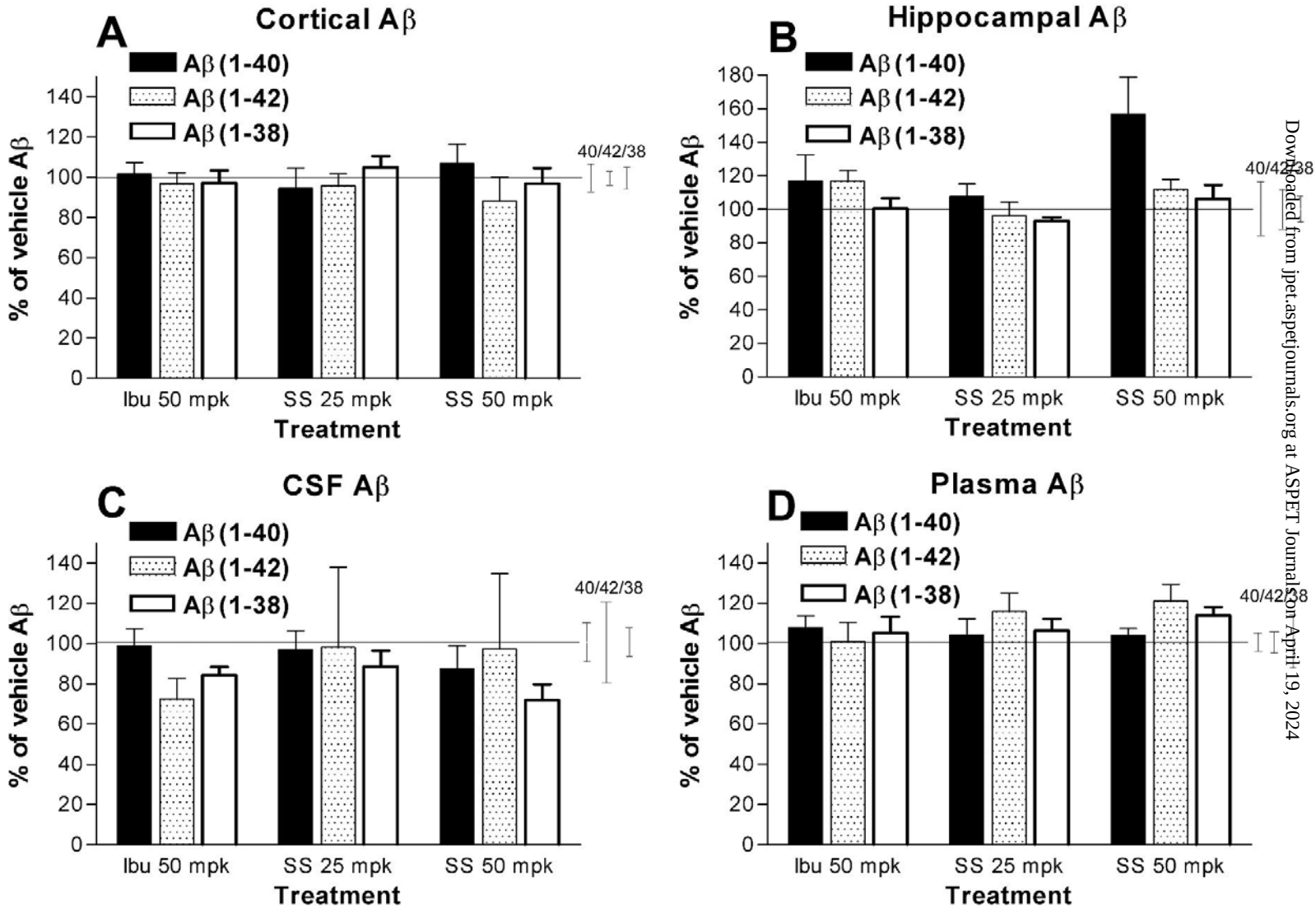

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Figure 4.

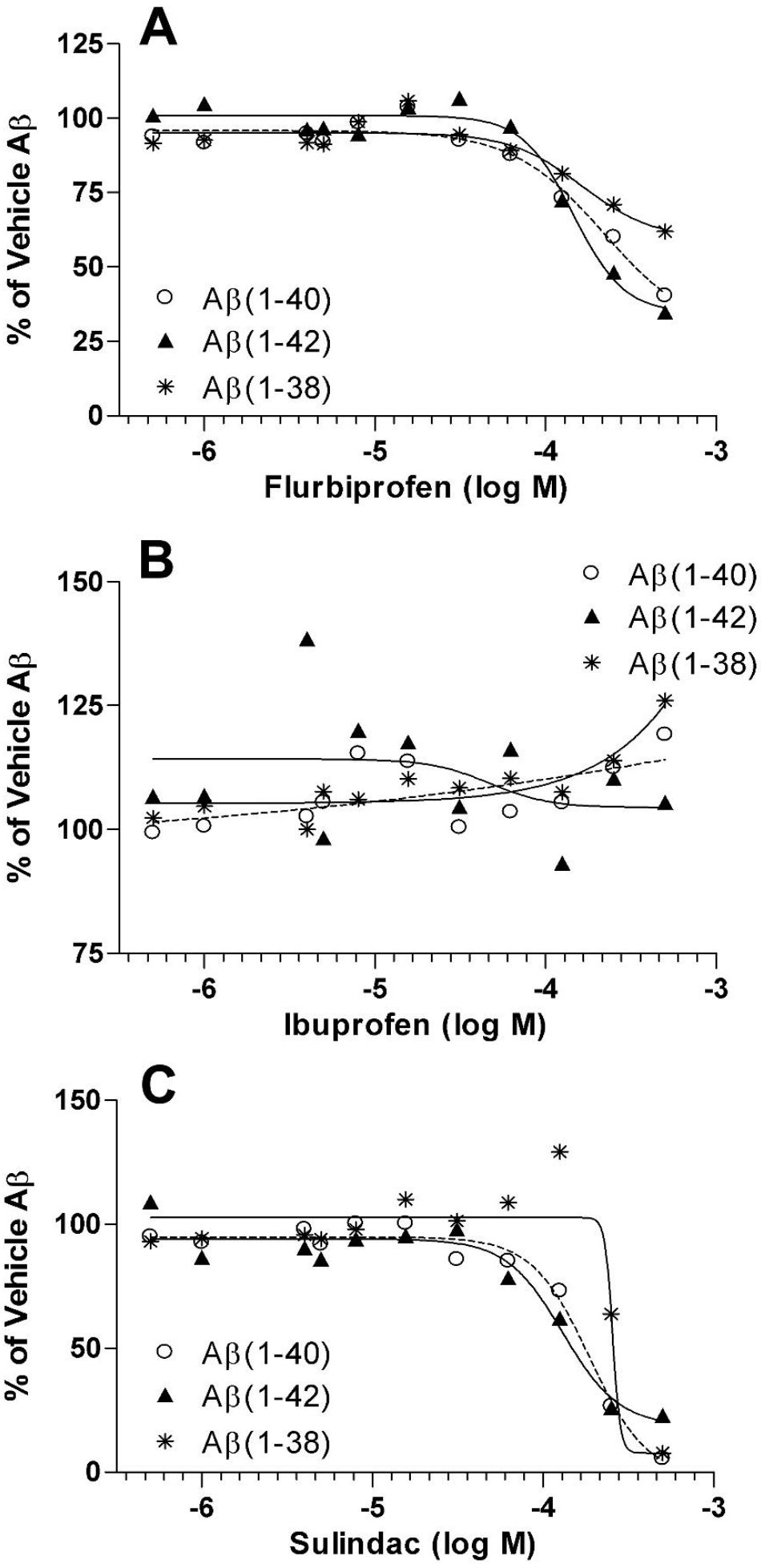

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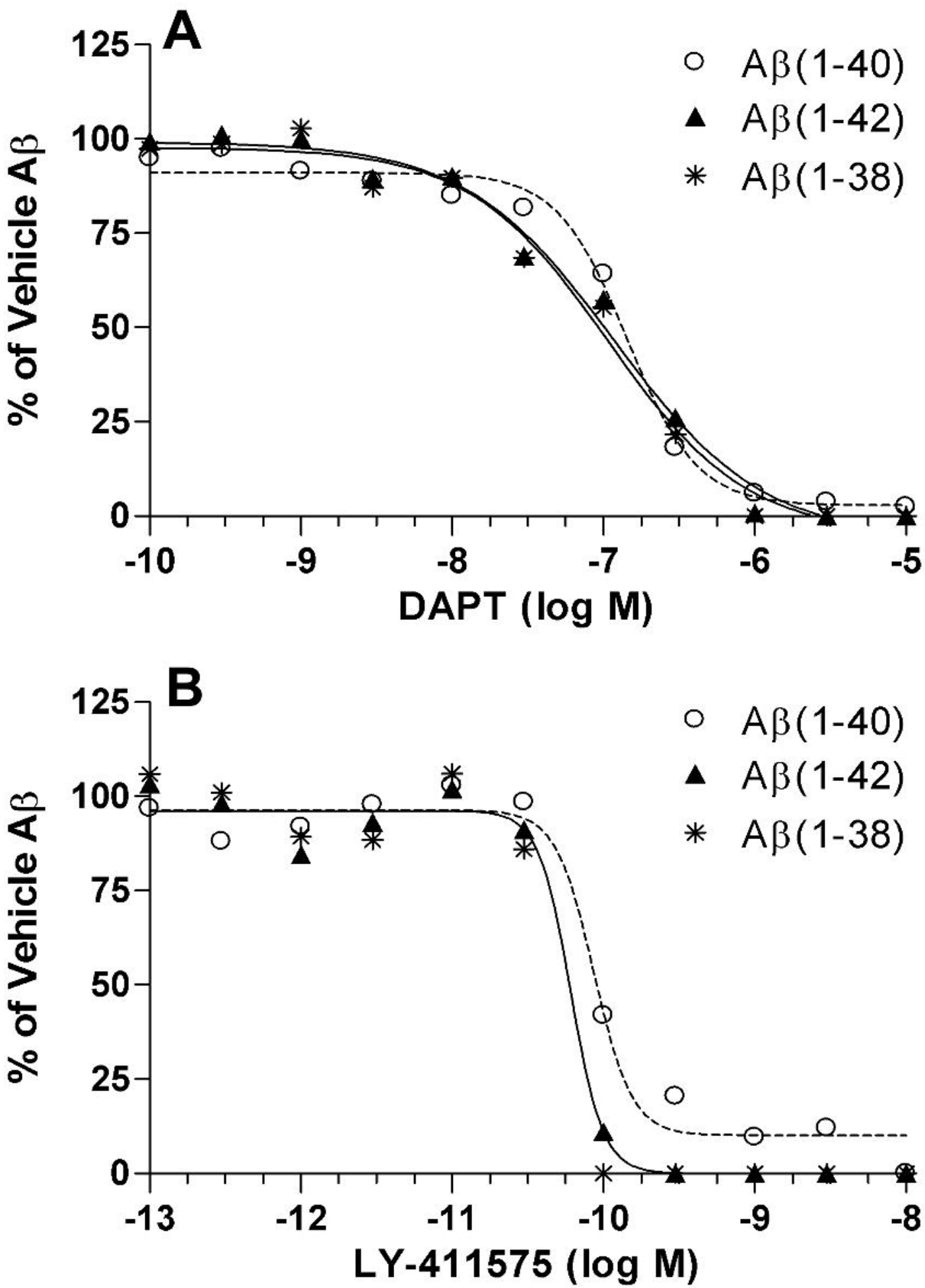
Figure 5.



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