A HIGH-CONTENT DRUG SCREEN IDENTIFIES URSOLIC ACID AS AN INHIBITOR OF AMYLOID β INTERACTIONS WITH ITS RECEPTOR CD36

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Running Title: Ursolic Acid blocks Amyloid Binding to CD36

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Background: Amyloid β binds CD36 and activates microglia to produce cytokines and neurotoxins leading to neurodegeneration.

Results: We developed an assay to find inhibitors of Amyloid β binding to CD36, and identified ursolic acid as such an inhibitor.

Conclusions: ursolic acid blocks CD36-mediated microglial activation by Amyloid β.

Significance: ursolic acid is a potential therapeutic agent for Alzheimer’s disease.

SUMMARY

A pathological hallmark of Alzheimer's disease (AD) is deposition of amyloid β (Aβ) in the brain. Aβ binds to microglia via a receptor complex that includes CD36 leading to production of pro-inflammatory cytokines and neurotoxic reactive oxygen species (ROS), and subsequent neurodegeneration. Interruption of Aβ binding to CD36 is a potential therapeutic strategy for AD. To identify pharmacologic inhibitors of Aβ binding to CD36, we developed a 384-well plate assay for binding of fluorescently labeled Aβ to Chinese hamster ovary cells stably expressing human CD36 (CHO-CD36) and screened an FDA-approved compound library. The assay was optimized based on the cells’ tolerance to DMSO, Aβ concentration, time required for Aβ binding, reproducibility, and signal-to-background ratio. Using this assay, we identified four compounds as potential inhibitors of Aβ binding to CD36. These compounds were ursolic acid, ellipticine, zoxazolamine and homomoschatoline. Of these compounds, only ursolic acid—a naturally occurring pentacyclic triterpenoid—successfully inhibited binding of Aβ to CHO-CD36 cells in a dose dependent manner. The ursolic acid effect reached a plateau at ~20 µM, with a maximal inhibition of 64%. Ursolic acid also blocked binding of Aβ to microglial cells and subsequent ROS production. Our data indicate that cell-based high-content screening of small molecule libraries for their ability to block binding of Aβ to its receptors is a useful tool to identify novel inhibitors of receptors involved in AD pathogenesis. Our data also suggest that ursolic acid is a potential therapeutic agent for AD via its ability to block Aβ-CD36 interactions.

The incidence of Alzheimer’s disease (AD) is projected to triple over the next 40 years(1). This devastating neurodegenerative disorder is believed to be the result of accumulation of neurotoxic peptides 40-43 amino acids in length termed amyloid β (Aβ)(2).
Deposition of Aβ in the brain is associated with a sterile inflammatory response characterized by microglial activation (3). In patients with Aβ plaques present in the brain, CD36 expression is elevated compared with patients whose brains are without Aβ deposition (4). Microglia are intimately associated with sites of Aβ deposits, and interaction of microglia with Aβ in vitro leads to activation of these cells to produce reactive oxygen (ROS) and nitrogen (RNS) species and other neurotoxins (5,6). Several microglial receptors for Aβ have been identified. These include several members of the family of scavenger receptors such as SCARA-1, SCARB-1 and SCARB-2 also known as CD36 (6-9).

CD36 is a class B scavenger receptor expressed on macrophages (10), brain endothelium and microglia (8). We have recently shown that Aβ binding to CD36 leads to formation of a receptor complex composed of CD36 and toll-like receptors (TLR) 4 and 6 (11). Binding of Aβ to this receptor complex is necessary for activation of microglia to produce ROS, RNS, and proinflammatory cytokines and for Aβ-induced neurotoxicity (11). Aβ-induced microglial activation requires all three members of this receptor complex to be present since the absence of CD36, TLR4 or TLR6, significantly reduces Aβ-induced production of cytokines, RNS and neurotoxicity (11). CD36 is an essential component of this signaling complex on microglia. We and others have shown that binding of Aβ to CD36 activates the downstream signaling molecules Src and Fyn and mitogen-activated protein kinase, p44/42. Inhibiting this CD36-mediated signaling abrogates ROS production, chemokine release and subsequent microglial recruitment to amyloid plaques in vivo (12). Furthermore, CD36 signals through p130Cas, pyk2 and paxillin, a complex linked to the actin cytoskeleton that is required for microglial migration. Disruption of this motility complex inhibits migration of microglia to sites of amyloid deposition (13).

Based on these data, blocking the interactions of Aβ with CD36 is a potential therapeutic strategy for AD. For this purpose, we sought to identify pharmacological inhibitors of CD36-Aβ binding, and used a high-content approach to screen a Food and Drug Administration (FDA) approved compound library to identify such inhibitors.

High content imaging is an emerging technology that utilizes an automated microscope to capture cellular images from a large number of wells in a tissue culture plate. The images can subsequently be analyzed by imaging software to provide very detailed information about the cells in question. The technology has been previously used to find small molecule regulators of G-protein coupled receptors (14), develop assays to find inhibitors of metastasis (15) and to find compounds that increase the growth of pancreatic β cells (16). Using this approach we developed an assay to quantify binding of Aβ to CHO cells stably transfected with CD36. Using this assay we screened a library of FDA approved drugs and identified ursolic acid as an inhibitor of Aβ-CD36 interactions. Our data suggest that cell based high-content screening of small molecule libraries is an effective method for discovering novel pharmacologic inhibitors of receptors involved in AD progression and that Ursolic acid has potential as a therapeutic agent for AD by its ability to block interaction of Aβ to CD36.

**EXPERIMENTAL PROCEDURES**

**Reagents-** 488-Hilyte-Fluor human Aβ 1-42 was obtained from Anaspec (Fremont, CA). Soluble β-amyloid was prepared by dissolving in DMSO. DAPI, Calcein, Cell Mask Deep Red and Cell Mask Red were from Invitrogen (Carlsbad, CA). Black sided, and clear bottom 384 well plates were from Becton Dickinson (Bedford, MA). FITC was from Sigma (Saint Louis, MO). Human Aβ 1-42 (unlabeled) was from American Peptide Co (Sunnyvale, CA).

**Preparation of FITC-labeled fibrillar β-amyloid 1-42-Aβ** was made fibrillar as previously described (8). Briefly, 500ug Aβ was incubated with 100ug FITC dissolved in DMSO for 30 minutes with shaking at room temperature. The Aβ was then dialyzed against PBS to remove excess FITC and the protein concentration determined using the BCA protein assay (Pierce chemical Co, Rockford IL).

**Scavenger receptor plasmid construction and stable cell generation-Human CD36 cDNA was obtained from Open Biosystems, 5’ NotI and...**
3’ XbaI ends were added via PCR and cloned into pCDNA3.1+. CHO-CD36 and control vector transfected CHO cells were previously generated in our lab(17). Briefly, 3µg plasmid DNA was transfected into CHO cells with Lipofectamine as per the manufacturer’s instructions. Cells were grown under geneticin selection, and positive colonies were picked, sorted by antibody staining and confirmed stable by QPCR.

**Cell Culture**-CHO-CD36 and control vector transfected CHO cells were maintained in Ham’s F12 media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Pen/Strep), and grown at 5% CO₂ and 37°C. Cells were plated into 384 well tissue culture plates with Multidrop (Thermo-Fisher, Waltham, MA) automated liquid handler.

**IN Cell 488-Hilyte-Fluor β-amyloid Uptake Assay**-CHO and CHO-CD36 were plated at 10,000 cells per well in triplicate in a 384 well plate, the plate was sealed with a gas-permeable plate sealer and cells were allowed to adhere overnight. The next day, the media was changed and replaced with 1µM 488-Hilyte-Fluor β-amyloid 1-42 (HF488 Aβ) in fresh media. Uptake was allowed to proceed for 2hours at 37°C. Cells were washed in PBS using an automated plate washer for a total of four washes. For IN Cell imaging, the cells were fixed in 4% paraformaldehyde for 20 minutes and washed in PBS. The cytoplasm was stained with Cell Mask Deep Red according to manufacturer’s instructions, and nuclei were stained with DAPI.

**IN Cell Imaging and Analysis**-Plates were imaged with a GE IN Cell 1000 automated high content microscope (General Electric Corporation Piscataway, NJ), with 3 fields taken per well. Image analysis was performed with GE IN Cell software, and results were expressed as the average percentage β-amyloid signal per well. T-test statistical analysis was used and a p value of <0.05 was considered significant.

**FDA-approved Compound Screen**-1408 FDA-approved compounds, selected for their likelihood to cross the blood-brain-barrier were transferred using a robotic liquid handler into 22 columns a 384 well plate where columns 1-23 previously plated CHO-CD36 and column 24 contained CHO cells. Columns 23 and 24 had DMSO added at a concentration of 0.1%. Compounds were allowed to settle onto the cells for 30 minutes before HF488 Aβ uptake assay, and cell staining was performed as described above. Imaging of each well was done in triplicate by IN Cell, and ‘hits’ were determined as being 6 times below the standard deviation of the positive control column.

**Isolation of primary microglia**- Primary mouse microglia were isolated as previously described (18). Brains from C57/BL6 mice were perfused with PBS/1mM EDTA and chopped with double razor blades in Hibernate containing 2mM EDTA without CaCl₂ (BrainBits, Springfield, IL). Brains were triturated in MACS C tubes (Miltenyi Biotec, Columbus, OH) in between incubations at 37°C. The brain homogenate was passed over a 100µM filter and separated by Percoll gradient. Microglia were isolated by CD11b magnetic bead binding (Miltenyi Biotec, Columbus, OH).

**Flow cytometry staining of CD36 on CHO and primary microglia**-CD36-CHO or primary microglia treated with either ursolic acid or volume equivalent of DMSO were suspended in PBS/0.1% FBS. Anti-human CD36-PE, or isotype control IgM-PE (BD Pharmingen, San Diego, CA) for CHO, or anti-mouse CD36-Alexa 647 or isotype control IgG-Alexa 647 for primary microglia (eBioscience, San Diego, CA) was added at 1:100 for 30 minutes on ice. Cells were washed twice with PBS/0.1% FBS and analysed on an Accuri C6 flow cytometer (Ann Arbor, MI).

**qPCR for CD36**- CD36-CHO or primary microglia treated with either 50µM ursolic acid for CD36-CHO or 100µM ursolic acid for primary microglia or volume equivalent of DMSO and RNA was harvested using Qiagen Micro RNA extraction kit (Valencia, CA). 100ng of RNA was transformed to cDNA using Invitrogen first strand synthesis kit (Carlsbad, CA) and qPCR for human CD36 mouse CD36, mouse or CHO GAPDH (see Supplemental Figure 2 for sequences), was performed on Lightcycler 480 (Roche Applied Science, Indianapolis, IN).

**Assay for uptake of HF488 Aβ by flow cytometry**- Microglia or CHO cells were seeded in triplicate in a 96 well plate and allowed to adhere overnight. The next day, the media was changed and replaced with 1µM HF488 Aβ containing media, either with ursolic acid at 50µM or the volume equivalent of DMSO. Uptake was
allowed to proceed for 2 hours at 37°C. Cells were washed twice with PBS and detached by scraping. To differentiate between internalized and cell surface bound Aβ, we incubated the cells with trypan blue as described (19). Trypan blue quenches extracellular fluorescence but does not affect intracellular fluorescence allowing the quantification of internalized Aβ. Cells were analyzed on Accuri C6 Sampler Flow Cytometer (Ann Arbor, MI).

Western blot for Aβ-1µM HF488 Aβ was electrophoresed on a 4-16% NativePAGE or 10% reducing gel, transferred to PVDF using iBlot (Invitrogen, Calsbad, CA) and probed for Aβ according to the manufacturer’s instructions (Cell Signaling Technologies, Danvers, MA). Densitometry was performed with ImageJ. (NIH, Bethesda, MD)

RESULTS

CD36 is a receptor for soluble and fibrillar β-amyloid—We have previously shown that Bowes melanoma cells stably transfected with CD36 gain the ability to adhere to Aβ-coated surfaces (8). To test whether CHO-CD36 are also capable of binding Aβ in solution we incubated these cells with 1µM soluble HF488 Aβ for 2 hours as described in materials and methods and measured cell-associated Aβ by flow cytometry. As seen in figure 1A CHO-CD36 cells had a threefold higher cell associated Aβ compared to CHO cells (CHO 5530 ±409, CHO-CD36 16515 ±892, p<0.003). Fluorescence microscopy confirmed that HF488 Aβ was indeed cell associated (Figure 1B). These data indicate that similar to its ability to bind fAβ CD36 can also mediate binding to soluble Aβ.

Development of a high content IN CELL based assay for quantifying association of HF488 Aβ with CHO-CD36 cells. To determine the optimum length of time for HF488 Aβ uptake, and to provide the largest signal to background ratio between CHO-CD36 and CHO cells, we incubated these cells with HF488 Aβ and measured cell associated Aβ at various time points using the IN CELL analyzer. As seen in figure 2, the best signal to background binding of HF488 Aβ to CHO-CD36 and CHO cells occurred at 2 hours (p<0.04) CHO-CD36 has threefold ratio over CHO of average % amyloid-β signal per well (CHO 5.34±0.51 CHO-CD36 16.64±1.22). For this reason, all the assays described below were performed for 2 hours. To identify the concentration of HF488 Aβ that gives the highest signal to background ratio we incubated CHO and CHO-CD36 cells with increasing concentrations of HF488 Aβ and measured cell associated Aβ at various time points using the IN CELL analyzer. As seen in figure 3A, 1µM HF488 Aβ showed the highest ratio of CHO-CD36 to CHO cell associated fluorescence. Since both soluble and fibrillar forms of Aβ bind CD36 (8) we tested whether such binding can be measured using IN CELL. For this purpose, we incubated CHO and CHO-CD36 cells adherent to a 384 well plate with 1µM soluble HF488 Aβ or fibrillar FITC-Aβ and measured cell associated fluorescence. As expected, cell association of both soluble and fibrillar Aβ was
quantifiable using IN CELL (HF488 Aβ p<3X10^-7, fibrillar FITC Aβ p<0.01) (Figure 3B). So for all subsequent experiments cells were incubated with 1µM HF488 Aβ for 2 hours and we measured cell associated fluorescence using IN CELL.

The reagents in the compound library to be screened are dissolved in DMSO, with a final concentration of 0.1% DMSO per well. To determine if DMSO affects cell binding to HF488 Aβ, we incubated CHO and CHO-CD36 cells with 1µM HF488 Aβ dissolved in increasing concentrations of DMSO and measured cell associated fluorescence by IN CELL. Concentrations of up to 0.5% DMSO did not significantly affect association of HF488 Aβ with CHO or CHO-CD36 cells (Figure 3C).

To assess intra and inter plate variability in our IN CELL assay, we seeded CHO-CD36 cells in 23 columns of a 384 well plate, and CHO cells were seeded in the last column. We then added HF488 Aβ in 0.1% DMSO, Ham's F12, 10% FBS, 1% pen/strep to the cells using a robotic liquid handler and incubated the cells at 37°C for 2 hours and performed the assay as described in materials and methods. IN Cell analysis of each column of CHO-CD36 cells show a significant ability of these cells to bind HF488 Aβ over CHO cells (p value ≥0.00001 for each column over CHO) (Figure 4), demonstrating good intra and inter plate robustness of the assay. This assay was performed in quadruplicate plates.

FDA-approved compound screen-CHO-CD36 cells were plated as described in experimental procedures and individual FDA-approved compounds (Supplementary table 1) were added to columns 1-22 of a 384 well plate at a concentration of 10µM each. Columns 23-24 were spotted with an equivalent concentration of DMSO using a robotic liquid handler. Each compound plate was performed in duplicate. IN Cell analysis determined several ‘hits’ as being below 6 times the standard deviation of the positive control. These included ursolic acid, ellipticine, zoxazolamine and homomoschatoline. Amongst these compounds of interest was ursolic acid, a pentacyclic triterpenic acid, which has previously been shown to bind to CD36(20).

Ursolic acid does not down-regulate CD36 expression-To determine if the observed effect of ursolic acid was due to down regulation of the expression of CD36 on CD36-CHO and primary microglia, we incubated these cells with ursolic acid, or volume equivalent of DMSO for the duration of the uptake assay (2 hours). We then measured Surface expression and mRNA levels of CD36 by flow cytometry and qPCR respectively. As seen in figure 5A, surface expression of CD36 was not affected by ursolic acid treatment. Similarly, CD36 mRNA levels were also unaffected by ursolic acid treatment (Figure 5B). These data indicate that ursolic acid does not downregulate CD36 expression at the protein or mRNA levels.

To confirm that ursolic acid blocks binding of Aβ to CD36, we incubated CHO-CD36 cells with 1µM HF488 Aβ and increasing concentrations of ursolic acid and measured cell associated fluorescence by flow cytometry. Ursolic acid blocked binding of Aβ to CHO-CD36 cells in a dose dependent manner (Figure 6A). The ursolic acid effect started to plateau at 20µM, and reached maximal inhibition (~64%) at a concentration of 100µM (Figure 6A). To determine if ursolic acid blocks binding of both Aβ1-40 and 1-42 to CD36, we incubated CHO-CD36 cells with HF488 Aβ 1-40 and HF488 Aβ 1-42 in the presence of 50µM ursolic acid. At this concentration, ursolic acid blocked CD36-mediated binding of HF488 Aβ 1-40 by ~50% and nearly completely blocked binding of HF488 Aβ 1-42 to CHO-CD36 cells (p<0.05) (Figure 6B) indicating that ursolic acid blocks binding of both Aβ 1-40 and 1-42 to CD36 albeit to different degrees.

To determine if the HF488 Aβ 1-42 used in our experiments was in monomeric, oligomeric or large aggregate forms, we used native and denaturing gel electrophoresis followed by Western blot analysis with anti-Aβ antibodies and analysis by ImageJ densitometry as described in material and methods. Figure 6C shows that >75% of our HF488 Aβ preparations were in oligomeric form between 4-mers and 7-mers. These data indicate that ursolic acid is a competitive inhibitor of binding of neurotoxic forms of Aβ to CD36.

Ursolic acid inhibits HF488 Aβ binding by primary microglia-CD36 is a major microglial receptor for Aβ(6,8,11). To determine if ursolic inhibits binding of Aβ to microglia, we incubated primary mouse microglia with 1µM HF488 Aβ...
and 50µM ursolic acid. Both primary microglia (Figure 7A) and N9 microglia (Figure 7B) bound significantly less HF488 Aβ in the presence of ursolic acid (p<0.05). These data indicate that ursolic acid is a competitive inhibitor of Aβ binding to microglia and suggest that ursolic acid and/or its derivatives have the potential for use as therapeutics agents in AD.

Ursolic acid blocks Aβ-induced ROS production by N9 microglia—Binding of Aβ to CD36 induces production of ROS and cytokines (6) to determine if the concentrations of ursolic acid that block binding of Aβ to N9 microglia also inhibit Aβ-induced microglial activation, we incubated N9 microglia with increasing concentrations of ursolic acid and measured ROS production using the DHR123 assay as described in experimental procedures. At concentrations of 20 µM or more, ursolic acid blocked Aβ induced ROS production by N9 microglia (Figure 7C). These data indicate that at concentrations that block binding of Aβ to microglia, ursolic acid also blocks activation of these cells by Aβ. These data further support a potential therapeutic role for ursolic acid and/or its derivatives in AD.

Ursolic acid does not affect Aβ internalization by N9 microglia—Data shown in Figure 7 demonstrate ursolic acid reduces binding of Aβ to N9 microglia. Since microglia can phagocytose and clear Aβ, and binding is necessary for phagocytosis to occur, inhibiting Aβ uptake and internalization by microglia may prove deleterious during plaque formation in the brain, since it could lead to reduced Aβ clearance and increased accumulation. To test this possibility, we incubated N9 microglia with HF488 Aβ 1-42 and either 50 µM ursolic acid or volume equivalent of DMSO and measured HF488 Aβ 1-42 uptake after 2 hours. To measure the amount of internalized Aβ, extracellular fluorescence was quenched with Trypan blue, and intracellular HF488 Aβ 1-42 was measured by flow cytometry. As shown in Figure 8, internalization of HF488 Aβ 1-42 by N9 microglia is unaffected by ursolic acid. This suggests that the effects of ursolic acid are specific to CD36 and that Aβ binding to other known Aβ receptors such as SCARA-1 and the ability of these receptors to clear Aβ is not affected by ursolic acid.

DISCUSSION
AD is a chronic degenerative disorder of the brain associated with a sterile inflammatory response. This response is believed to be caused by Aβ deposition and microglial activation, ultimately leading to neuronal degeneration and decreased cognitive abilities and dementia (3). CD36, a scavenger receptor that binds Aβ (8) has been implicated in promoting the progression of AD through activation of local inflammatory intracellular signaling pathways (3,6,21). The increase in local inflammation causes ROS and neurotoxin production and cytokine release. In this study, we sought to develop a method to identify inhibitors of Aβ-CD36 interactions and successfully used a high-content screening (HCS) approach for this purpose.

HCS uses a powerful automated fluorescent microscope to capture a large number of images per field, allowing thousands of cells to be imaged in one experiment(22). The resulting data can be resolved into single cell analyses allowing a detailed examination of the effects of treatments on the cells. The advantage of this method over high throughput screening (HTS) includes the ability to review a series of images and reanalyze them without having to repeat large data sets. Our results presented here, support the benefits of this approach to identify inhibitors of Aβ binding to CHO-CD36 cells.

The Aβ binding/uptake assay was successfully miniaturized to a 384-well plate format, and tested for reproducibility and sensitivity. As proof of concept, we tested the feasibility of such HCS approach using an FDA-approved library of compounds specially selected for their likelihood of penetrating the blood-brain-barrier. From the compound library we identified ursolic acid as an inhibitor of CD36-Aβ interactions. We validated our findings using flow cytometry. We found that at concentrations ≤ 100µM ursolic acid successfully blocked the binding of Aβ to CHO-CD36 cells and to primary microglia. This inhibition of Aβ binding was not due to down-regulation of surface CD36 expression, nor was the mRNA level of CD36 affected. The ability of the microglia to bind to Aβ was decreased upon treatment with ursolic acid, however this treatment did not eliminate uptake of Aβ by the cell, as observed in Figure 8,
indicating that ursolic acid blocks CD36-mediated Aβ signaling and ROS production without affecting the ability of other scavenger receptors such as SCARA-1, also expressed on the surface of microglia to bind and mediate uptake and clearance of Aβ. Ursolic acid is a pentacyclic triterpenoid found occurring naturally in a variety of edible and medicinal plants such as rosemary(23). Ursolic acid has long been known to have anti-inflammatory properties, as previous studies have shown inhibition of inflammation in arthritis and dermatitis models with ursolic acid treatment (24,25). While our results need to be validated in vivo using a mouse model of AD, to our knowledge this is the first report that describes a role for ursolic acid in blocking the interaction of Aβ with microglia and to suggest a potential use for this compound and/or its derivatives for treatment of AD. This is also the first development and optimization of a HCS to investigate small molecule inhibitors of uptake/binding of Aβ to microglial receptors. Because no effective treatments exist to delay or stop progression of AD and since ursolic acid is a prototypic member of a large family of naturally occurring compounds, our data provide a molecular basis for exploring the potential use of ursolic acid and/or its derivatives for therapy of this devastating disorder.

REFERENCES
Ursolic Acid blocks Amyloid Binding to CD36


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FIGURE LEGENDS

Figure 1. CD36 expressed on CHO cells is a receptor for Aβ 1-42. Stably transfected CHO-CD36 and control vector transfected CHO cells were incubated with 1µM Hilyte-Fluor 488 Aβ 1-42 (HF488 Aβ 1-42) for 2 hours and uptake was measured by flow cytometry (A) and visualized by fluorescence microscopy (B). Only CHO-CD36 cells showed cells association with HF488 Aβ 1-42 by flow cytometry and showed evidence of intracellular Aβ by fluorescent microscopy. HF488 Aβ 1-42 is shown in green, nuclei were stained with DAPI (blue) [Original magnification 40X]. Data represent mean ±SEM n=3 p<0.003

Figure 2. Time course for binding/uptake of Hilyte-Fluor 488 Aβ 1-42 to CHO and CHO-CD36 cells. CHO and CHO-CD36 cells were incubated with 1µM HF488 Aβ 1-42. At various time points the cells were washed, fixed and analyzed by GE IN Cell 1000 microscope. The data show that a 2-hour incubation provides the best signal-to-background measurement (Data represent mean ±SEM, n= 4, p<0.04).
Figure 3. Characterization of binding/uptake of CHO-CD36 cells to Aβ: (A) CHO CD36 cells bind soluble and fibrillar Aβ. CHO and CHO-CD36 were incubated with either 1µM soluble or fibrillar HF488 Aβ for 2 hours and cell associated Aβ was measured by IN CELL, n=, p<3X10^-7 (for soluble Aβ) and n=15, p<0.01 for fibrillar Aβ. (B) CHO-CD36 cells bind Aβ in a dose-dependent manner. CHO and CHO-CD36 were incubated with various concentrations of HF488 Aβ for 2 hours and the cells were analyzed by IN Cell. n=6 p<3X10^-7 for 1µM HF488 Aβ. (C) CHO-CD36 cells bind Hilyte-Fluor 488 Aβ in the presence of DMSO concentrations up to 1%. CHO and CHO-CD36 were incubated with 1µM HF488 Aβ for 2 hours with increasing concentrations of DMSO. Data represent mean ±SEM n=15 (0% DMSO p<0.001, 0.1% DMSO, p<0.0009, 0.25% DMSO p<0.0024, 0.5% DMSO p<0.0003, 1% DMSO p<0.01 and 2% DMSO p<0.05).

Figure 4. Binding/uptake of CHO-CD36 cells to Aβ is miniaturized to a 384 well plate. CHO-CD36 cells were seeded in columns 1-23 and CHO cells in column 24 of a 384 well plate. All columns were incubated with 0.1% DMSO and 1µM HF488 Aβ for 2 hours. Cells were analyzed by IN Cell and each column was averaged. Data represent mean ±SEM n=4 (p<0.00001).

Figure 5. Ursolic acid does not affect expression of CD36 on CD36-CHO or primary microglia. CD36-CHO and primary microglia were incubated with either ursolic acid (50µM) or volume equivalent of DMSO. (A) Surface antibody staining for CD36 revealed no difference between ursolic acid or DMSO treatment (black-unstained, red-isotype control, blue-DMSO, yellow-ursolic acid) (B) qPCR for CD36 after either ursolic acid or DMSO treatment also showed no difference in CD36 expression.

Figure 6. Ursolic acid competitively blocks binding of Hilyte-Fluor 488 Aβ 1-42 and 1-40 species to CHO-CD36 cells. CHO-CD36 cells were incubated with 1µM HF488 Aβ 1-42 (A) for 2 hours in the presence of increasing concentrations of ursolic acid DMSO concentrations remain constant in each triplicate. (B) Cell associated Aβ was assessed by flow cytometry for HF488 Aβ 1-40 and HF488 Aβ 1-42. (C) Native Western blot for Aβ and densitometry analysis shows the species present in the Aβ preparation are mostly 4-mers and 7-mers. Denaturing Western blotting for Aβ shows HF488 Aβ is approximately 4.5kDa. For comparison, molecular weight markers were run on the same gels, their corresponding sizes are shown in kDa). (Data for A and B represent the mean±SEM n=3) (p<0.05).

Figure 7. Ursolic acid partially blocks Aβ binding and Aβ-induced activation of microglia to produce ROS. (A) Freshly isolated primary microglia or (B) N9 microglia incubated with 1µM HF488 Aβ for 2 hours with 50µM ursolic acid or volume equivalent of DMSO. Cell associated Aβ was measured by flow cytometry (p<0.05). (C) N9 microglia pre-incubated with ursolic acid for 30 minutes and then stimulated with 1µM amyloid-β for an additional 30 minutes and ROS production measured using the DHR123 assay. Data represent mean ±SEM, each data point is the mean of 3 separate experiments each done in triplicate n=3 (20µM p<0.034, 50µM, p<0.0005, 100µM p<0.0005).

Figure 8. Ursolic acid does not affect internalization of HF488 Aβ by N9 microglia. N9 microglia incubated with 1µM HF488 Aβ for 2 hours in the presence of 50µM ursolic acid or volume equivalent of DMSO followed by Trypan blue quenching of external fluorescence. Internalized HF488 Aβ was measured by flow cytometry. Data represent mean ±SEM, each data point is the mean of 3 separate experiments each done in triplicate n=3.
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