A High Content Drug Screen Identifies Ursolic Acid as an Inhibitor of Amyloid β Protein Interactions with Its Receptor 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 disease.

A pathological hallmark of Alzheimer 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 proinflammatory cytokines and neurotoxic reactive oxygen species 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 dimethyl sulfoxide, 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 homoschotamine. 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 disease (AD)3 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 species (ROS) and reactive nitrogen species (RNS) 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 because 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

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3 The abbreviations used are: AD, Alzheimer disease; Aβ, amyloid β; DHR, dihydorhodamine; DMSO, dimethyl sulfoxide; FDA, Food and Drug Administration; HCS, high content screening; H4488, Hilyte-Fluor 488; qPCR, quantitative PCR; RNS, reactive nitrogen species; ROS, reactive oxygen species; TLR, Toll-like receptor.
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 used previously 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 ursoic acid as an inhibitor of Aβ-CD36 interactions. Our data suggest that cell-based high content screening (HCS) of small molecule libraries is an effective method for discovering novel pharmacologic inhibitors of receptors involved in AD progression and that ursoic acid has potential as a therapeutic agent for AD by its ability to block interaction of Aβ to CD36.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Hilyte-Fluor 488 (HF488) human Aβ 1–42 was obtained from Anaspec (Fremont, CA). Soluble Aβ was prepared by dissolving in DMSO. DAPI, calcein, Cell Mask Deep Red, and Cell Mask Red were from Invitrogen. Black-sided, and clear-bottom 384-well plates were from Becton Dickinson. FITC was from Sigma. 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 described previously (8). Briefly, 500 μg of Aβ was incubated with 100 μg of FITC dissolved in DMSO for 30 min 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).

Scavenger Receptor Plasmid Construction and Stable Cell Generation—Human CD36 cDNA was obtained from Open Biosystems, and 5’ NotI and 3’ XbaI ends were added via PCR and cloned into pCDNA3.1. CHO-CD36 and control vector-transfected CHO cells were generated previously in our laboratory (17). Briefly, 3 μg of plasmid DNA was transfected into CHO cells with Lipofectamine according to 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 medium 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) automated liquid handler.

In-Cell HF488 β-Amyloid Uptake Assay—CHO and CHO-CD36 were plated at 10,000 cells/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 medium was changed and replaced with 1 μM HF488 β-amyloid 1–42 (HF488 Aβ) in fresh medium. Uptake was allowed to proceed for 2 h 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 min and washed in PBS. The cytoplasm was stained with Cell Mask Deep Red according to the manufacturer’s instructions, and nuclei were stained with DAPI.

In-Cell Imaging and Analysis—Plates were imaged with an In-Cell 1000 automated high content microscope (General Electric), with three fields taken per well. Image analysis was performed with In-Cell software (General Electric), and results were expressed as the average percentage of β-amyloid signal/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 min 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 six times below the S.D. of the positive control column.

**Isolation of Primary Microglia**—Primary mouse microglia were isolated as described previously (18). Brains from C57/BL6 mice were perfused with PBS/1 mM EDTA and chopped with double razor blades in Hibernate containing 2 mM EDTA without CaCl₂. Brain bits, Springfield, IL). Brains were triturated in MACS C tubes (Miltenyi Biotec, Columbus, OH) between incubations at 37°C. The brain homogenate was passed over a 100 micron 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 ursoic acid or volume equivalent of DMSO were resuspended in PBS/0.1% FBS. Anti-human CD36-PE, or isotype control IgM-PE (BD Pharmingen) 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 min on ice. Cells were washed twice with PBS/0.1% FBS and analyzed on a C6 flow cytometer (Accuri, Ann Arbor, MI).

qPCR for CD36—CD36-CHO or primary microglia treated with either 50 μM ursoic acid for CD36-CHO or 100 μM ursoic acid for primary microglia or volume equivalent of DMSO and
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RNA was harvested using a Micro RNA extraction kit (Qiagen, Valencia, CA). 100 ng of RNA was transformed to cDNA using an Invitrogen first strand synthesis kit and qPCR for human CD36, mouse CD36, mouse or CHO GAPDH (see supplemental Fig. 1 for sequences), was performed on Lightcycler 480 (Roche Applied Science).

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 medium was changed and replaced with 1 μM HF488 Aβ-containing medium, either with ursolic acid at 50 μM or the volume equivalent of DMSO. Uptake was allowed to proceed for 2 h 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 a C6 sampler flow cytometer.

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

Microscopy of CHO-CD36 and HF488 Aβ Uptake—Cells were seeded at 0.1 × 10⁶/well of an 8-well chamber slide and allowed to adhere for 48 h. 1 μM HF488 Aβ was incubated with the cells overnight. Cells were washed with PBS, fixed in 4% formaldehyde, and the nuclei were stained with DAPI. Cells were imaged by epifluorescence microscopy at a magnification of ×40 using a Nikon Eclipse ME600 (Melville).

Cell Viability Assay—Cells were seeded at 0.5 × 10⁶/well in a 48-well tissue culture plate and allowed to adhere overnight. Ursolic acid or the volume equivalent of DMSO was added to the wells in a dose-dependent manner and incubated for 2 h. The cells were washed with PBS, and 2 μM calcein, and 1 μg/ml DAPI were added to the cells for 30 min. Cells were washed and imaged at ×20 with appropriate filters, as described previously.

RESULTS

CD36 Is a Receptor for Soluble and Fibrillar Aβ—We have shown previously 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 1 μM soluble HF488 Aβ for 2 h as described under “Experimental Procedures” and measured cell-associated Aβ by flow cytometry. As seen in Fig. 1A, CHO-CD36 cells had a 3-fold higher cell-associated Aβ compared with CHO cells (CHO, 5530 ± 409; CHO-CD36, 16,515 ± 892, p < 0.003). Fluorescence microscopy confirmed that HF488 Aβ was indeed cell-associated (Fig. 1B). These data indicate that similar to its ability to bind fibrillar Aβ, 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 Fig. 2, the best cell-associated fluorescence. Concen-

![Graph](image)

**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 1 µM soluble or fibrillar HF488 Aβ for 2 h, and cell-associated Aβ was measured by In-Cell, n = 6, p < 3 × 10⁻⁷ 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 h, and the cells were analyzed by In-Cell, n = 6, p < 3 × 10⁻⁷ for 1 µM HF488 Aβ. C, CHO-CD36 cells bind HF488 Aβ in the presence of DMSO concentrations up to 1%. CHO and CHO-CD36 were incubated with 1 µM HF488 Aβ for 2 h with increasing concentrations of DMSO. Data represent mean ± S.E. (error bars). n = 15. 0% DMSO, p < 0.001; 0.1% DMSO, p < 0.0005; 0.25% DMSO, p < 0.0024; 0.5% DMSO, p < 0.0003; 1% DMSO, p < 0.01; and 2% DMSO, p < 0.05.

Ursolic Acid Blocks Amyloid Binding to CD36

![Graph](image)

**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 1 µM HF488 Aβ for 2 h. Cells were analyzed by In-Cell, and each column was averaged. Data represent mean ± S.E. (error bars), n = 4. p < 0.00001.

- To determine whether 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.
acid, or volume equivalent of DMSO for the duration of the uptake assay (2 h). We then measured surface expression and mRNA levels of CD36 by flow cytometry and qPCR, respectively. As seen in Fig. 5A, surface expression of CD36 was not affected by ursolic acid treatment. Similarly, CD36 mRNA levels were also unaffected by ursolic acid treatment (Fig. 5B). These data indicate that ursolic acid does not down-regulate CD36 expression at the protein or mRNA level.

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

To determine whether 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 under “Experimental Procedures.” Fig. 6C shows that >75% of our HF488 Aβ

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 (50 μM) or a 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.
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/H9252 to CD36.

Ursolic Acid Inhibits HF488 Aβ Binding by Primary Microglia—CD36 is a major microglial receptor for Aβ (6, 8, 11). To determine whether ursolic inhibits binding of Aβ to microglia, we incubated primary mouse microglia with 1 μM HF488 Aβ 1–42 and 50 μM ursolic acid. Both primary microglia (Fig. 7A) and N9 microglia (Fig. 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.

**FIGURE 6.** Ursolic acid competitively blocks binding of HF488 Aβ 1–42 and 1–40 species to CHO-CD36 cells. A, CHO-CD36 cells were incubated with 1 μM HF488 Aβ 1–42 for 2 h 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 blotting 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 that HF488 Aβ is ~4.5kDa. For comparison, molecular mass markers were run on the same gels, their corresponding sizes are shown in kDa). Data for A and B represent the mean ± S.E. (error bars). n = 3, p < 0.05.

Ursolic Acid Blocks Aβ-induced ROS Production by N9 Microglia—Binding of Aβ to CD36 induces production of ROS and cytokines (6) to determine whether the concentrations of ursolic 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 DHR 123 assay as described under “Experimental Procedures.” At concentrations of 20 μM or more, ursolic acid blocked Aβ-induced ROS production by N9 microglia (Fig. 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.
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Ursolic Acid Does Not Affect Aβ Internalization by N9 Microglia—Data shown in Fig. 7 demonstrate ursolic acid reduces binding of Aβ to N9 microglia. Because 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 because 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 the volume equivalent of DMSO and measured HF488 Aβ 1−42 uptake after 2 h. 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 Fig. 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 are 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, 13). 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 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 includes the ability to review a series of images and reanalyze them without having to repeat large datasets. 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 an 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 ≤100 μM, 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 Fig. 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 (21, 24). Although 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 because 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.

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