Analysis of the Effect of Aggregated β-Amyloid on Cellular Signaling Pathways Critical for Memory in Alzheimer's Disease

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Introduction

According to literature references, Extracellular-Signal Regulated Kinase (ERK) signaling has been linked to memory and regulated by environmental stimuli. In Alzheimer's disease, progressive cognitive impairment is seen as a classic characteristic. These deficits are believed to be the result of progressive synaptic dysfunction initiated by aggregated β–amyloid peptide 1-42 (Aβ). Zhu *et al.* (2002) showed that Aβ induced disruption of kinases critical for memory. In late stages of Alzheimer's disease, ERK activation is suppressed relative to early stages and controls (Webster *et al.* 2006). *In vitro* studies have also shown that under certain conditions Aβ or fragments inhibit ERK or the downstream cAMP-response element-binding protein (CREB) in neuronal cell models (Daniels *et al.* 2001).

Here we evaluate the ability to detect changes in phosphorylation levels of ERK and CREB following treatment with Aβ using the SH-SY5Y neuroblastoma cell line. Aβ was oligomerized using a method previously described in the literature (FA *et al.* 2010). A two-step HTRF® assay process was incorporated such that cell plating and treatment are carried out in a 96-well clear-bottom imaging plate. Following lysis, aliquots were transferred to separate LV384-well assay plates to perform the phospho and total ERK and CREB assays. A neutralizing antibody was also tested for its capacity to counteract the inhibitory effects of Aβ. Aβ binding and antibody neutralization were detected *via* immunocytochemisty and microscopic analysis. All microplate reading and cellular imaging steps were performed using a novel cell imaging multi-mode reader. The combination provides an efficient, robust method for testing of new molecules to combat the degenerative effects of the disease.

BioTek Instrumentation

Cytation™ 5 Cell Imaging Multi-Mode Reader. Cytation 5 is a modular multi-mode microplate reader combined with automated digital microscopy. Filter- and monochromator-based microplate reading are available, and the microscopy module provides up to 60x magnification in fluorescence, brightfield, color brightfield and phase contrast. The instrument can perform fluorescence imaging in up to four channels in a single step. With special emphasis on live-cell assays, Cytation 5 features temperature control to 65 °C, CO_2/O_2 gas control and dual injectors for kinetic assays. Integrated Gen5™ Data Analysis Software controls Cytation™ 5. The filter-based system was used to detect the 665 and 620 nm fluorescent emissions from the HTRF ERK and CREB assay with the following settings: Delay after plate movement: 0 msec; Delay after excitation: 150 µsec; Integration time: 500 µsec; Read height: 7.5 mm. The imaging module was used to analyze the tumoroid structure, confirm cellular lysis and visualize test compound cytotoxic effects. Gen5™ Data Analysis Software was used for initial data analysis.

HTRF® Total and Phospho Protein Assays

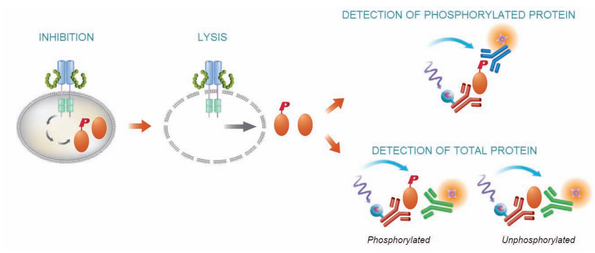


Figure 1. HTRF Phospho and Total Kinase Assays. The two-plate HTRF® Human ERK and CREB protein assays are run in three steps. A) Cells are incubated with modulators. B) Cells are then lysed, releasing the phosphorylated and unphosphorylated proteins. C) Cell lysate is transferred to a second plate, followed by antibody addition and plate reading.

HTRF® assays from Cisbio Bioassays combine fluorescence resonance energy transfer and time-resolved fluorescence to eliminate background interference. The assay is based on a sandwich immunoassay principle (Figure 1). After cell membrane lysis, phosphorylated and total protein may be detected upon addition of two monoclonal antibodies: an anti-phospho protein-K (phosphorylated assay) or anti-protein-K (total assay) antibody labeled with Eu3+-cryptate and an anti-protein-d2 antibody labeled with d2. In the presence of phosphorylated or total protein, and upon excitation of the Eu3+-cryptate, energy is transferred to the d2 molecule, and an increase in emission at 665 nm is seen. In the absence of the protein, no energy is transferred, and virtually no 665 nm signal is detected.

Materials and Methods

Assay and Experimental Components: Advanced ERK phospho-T202/Y204 Kit (Catalog No. 64AERPEG), ERK Total Kit(Catalog No. 64NRKPEG), CREB phospho-S133 Kit (Catalog No. 64CREPEG), and CREB Total Kit (Catalog No. 63ADK052PEG) were donated by Cisbio ,US (Bedford, MA).

β-Amyloid (1-42) Peptide (Catalog No. ALX-151-002-P001), Amyloid β A4 (CT, 1-42) monoclonal antibody (8G7) (Catalog No. ADI-905-804-100), and Goat anti-mouse IgG1 (ATTO 590 conjugate) (Catalog No. ALX-211-204TM-C100) were donated by Enzo Life Sciences (Farmingdale, NY).

Ammonium hydroxide solution (Catalog No. 338818) was purchased from Sigma-Aldrich (Saint Louis, MO). SH-SY5Y neuroblastoma cells (Catalog No. CRL-2266) were purchased from ATCC (Manassas, VA). Oligomer A11 antibody (Catalog No. AHB0052) was purchased from ThermoFisher (Waltham, MA). Purified anti-β-amyloid, 1-16 antibody (Catalog No. 803001) was purchased from BioLegend (San Diego, CA).

Peptide Oligomerization Procedure: Lyophilized β-amyloid (1-42) peptide was resuspended in 1% ammonium hydroxide at a concentration of 1 mg/mL and sonicated for 1 minute. The volume was divided into three equal aliquots using a GasTight Hamilton syringe into polypropylene vials, sealed, and incubated for greater than 2 hours at room temperature to allow monomerization. The solution was then concentrated using a SpeedVac centrifuge (800g/RT) and the vials containing peptide films stored at -80 °C. Films were then resuspended in 100% DMSO at 1 mM, sonicated for 10 minutes, and stored at -20 °C. To complete oligomerization, vials were removed from storage, diluted to 500 µM in sterile phosphate buffer, and incubated at +4 °C for 24 hours.

Assay Procedure: Cell Plating: SH-SY5Y cells were added to a 96-well imaging plate at a concentration of $2.0x10^5$ cells/mL in a volume of 100 µL and incubated at 37 °C/5% CO_2 until reaching 80% confluency. Media exchanges were performed every two days during this period. Oligomerized Peptide Treatment: Oligomerized β-amyloid (1-42) peptide was diluted in serum-free DMEM/F12 media to 1X concentrations ranging from 10-0 µM and incubated with cells for 2, 6, 12, or 24 hours. Inhibitor Antibody Addition: A11 or anti-β-amyloid, 1-16 antibodies were added to cells at 5X concentrations ranging from 1000-0 ng/mL in 20 µL and incubated for 30 minutes. Oligomerized peptide was then added in an 80 µL volume to a final 1X concentration of 10 µM and incubated as previously described. HTRF Assay Performance: Media containing peptide or peptide and antibody was removed, replaced with 80 µL of lysis buffer, and the plate was shaken for 60 minutes. The remaining assay procedure was performed as previously described. Imaging: In a separate plate, cell fixing and permeabilization was first completed followed by primary Amyloid β and secondary ATTO 590 IgG1 antibody incubations using Enzo antibodies. Cellular imaging using the Cytation 5 was then performed.

β-Amyloid Binding to SH-SY5Y Cells

The first test was performed to validate the ability of the oligomerized β-amyloid (1-42) peptide to bind to the SH-SY5Y neuronal cell model. Concentrated peptide was diluted in DMEM/F12 media to 1X concentrations ranging from 0-10 µM. Complete media was removed from cells and replaced with media containing peptide and incubated for 2, 6, 12, or 24 hours. Immunocytochemistry was then performed as previously described followed by imaging using a 20x or 40x objective.

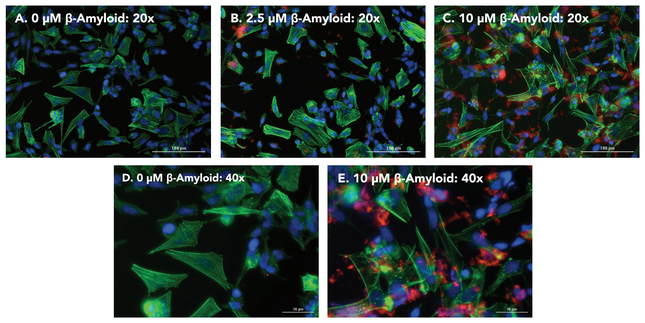
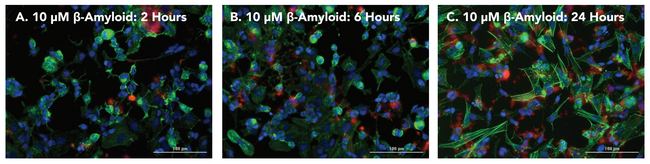


Figure 2. Dose dependent β-amyloid peptide binding. Overlaid images showing β-amyloid peptide binding to SH-SY5Y cells following a 24 hour incubation. 20x images showing incubation with **(A)** 0; **(B)** 2.5; or **(C)** 10 µM peptide. 40x images showing incubation with **(D)** 0; or **(E)** 10 µM peptide. DAPI Channel: Hoechst 333423 stained nuclei; GFP Channel: Alexa Fluor 488 phalloidin; Texas Red: ATTO 590 goat anti-mouse IgG1.



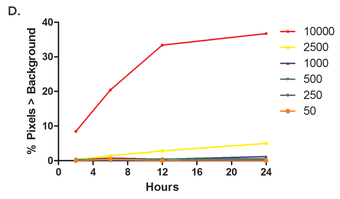


Figure 3. Time dependent β-amyloid peptide binding. Overlaid 20x images showing β-amyloid peptide binding to SH-SY5Y cells following a **(A)** 2; **(B)** 6; or **(C)** 24 hour incubation with 10 µM peptide. **(D)** Cellular analysis of 20x images demonstrating image pixel percentage above background in the Texas Red channel. Peptide treatment concentrations ranging from 10000-50 nM. DAPI Channel: Hoechst 333423 stained nuclei; GFP Channel:Alexa Fluor 488 phalloidin; Texas Red: ATTO 590 goat anti-mouse IgG1.

The images in Figure 2 and 3 visually confirm that oligomerized β-amyloid (1-42) peptide binds to SH-SY5Y cells in a time and dose dependent manner, and can be detected by the included primary and secondary antibodies. This was validated by the results in Figure 3D where analysis was performed to determine the pixel percentage demonstrating fluorescent signal in the Texas Red channel above background levels. Increasing percentages indicate higher levels of fluorescent signal emanating from the well, and therefore greater peptide binding.

β-Amyloid Interruption of ERK-CREB Signaling

Following confirmation of β-amyloid peptide binding it was then determined whether binding would interfere with signaling pathways leading to ERK and CREB phosphorylation, as reported in the literature. A second plate was treated with oligomerized β-amyloid (1-42) peptide in a similar manner as previously explained. Following peptide and lysis buffer incubations, four 16 µL aliquots were transferred to separate wells of a low volume 384-well plate where either the HTRF advanced ERK phospho-T202/Y204, ERK total protein, CREB phospho-S133, or CREB total protein assays were performed.

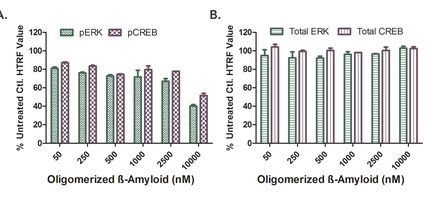


Figure 4. Phosphorylated and total protein levels following 24 hour β-amyloid peptide binding. Percent of untreated control well HTRF values reported for peptide concentrations ranging from 10000-50 nM run with **(A)** phospho-ERK and -CREB; or **(B)** total ERK and CREB assays.

A time course study was again performed to ascertain the level of signaling pathway interruption following 2, 6, 12, or 24 hour peptide incubations.

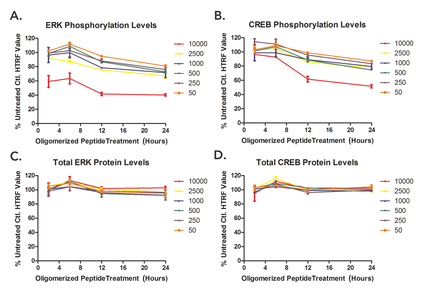
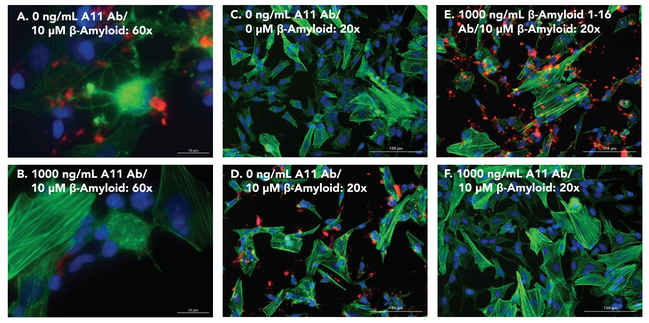


Figure 5. Time dependent phosphorylated and total protein levels. Percent of untreated control well HTRF values reported for peptide concentrations ranging from 10000-50 nM, and incubated for 2, 6, 12, or 24 hours. Lysate aliquots run with **(A)** phospho-ERK; **(B)** phospho-CREB; (C) total ERK; or **(D)** total CREB assays.

The results in Figure 4 and 5 demonstrate that HTRF ratios decrease proportionately in response to higher concentrations of oligomerized β-amyloid (1-42) peptide and increased incubation times with phospho-ERK and –CREB assays. The same phenomenon is not seen with total protein assays where protein levels remain consistent across all tested peptide concentrations and incubation times. The combined outcomes confirm that binding of oligomerized β-amyloid (1-42) peptide to SH-SY5Y cells does indeed interrupt the ERK-CREB signaling pathway.

Neutralization of β-Amyloid Binding

Antibodies, such as the Oligomer A11 antibody, have been developed which demonstrate the ability to immunoneutralize β-amyloid peptide oligomers, and thus restore signaling pathway activity. This phenomenon, and the ability to track subsequent changes to cellular activity were tested by treating SH-SY5Y cells with the test A11 antibody, in addition to a negative control anti-β-amyloid, 1-16 antibody which exhibits no neutralization effect. Following a 30 minute incubation with the antibodies, cells were treated with 10 µM peptide for 2, 6, 12, or 24 hours. Immunocytochemistry was once again performed, followed by imaging using a 20x or 60x objective.



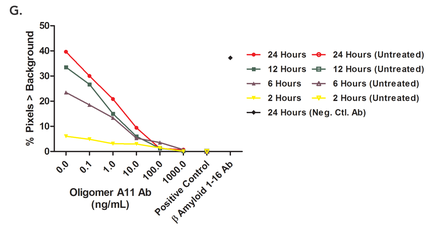


Figure 6. Neutralization of β-amyloid peptide binding. Overlaid images showing antibody treatment effects on β-amyloid peptide binding to SH-SY5Y cells following a 24 hour incubation with 10 µM β-amyloid peptide. 60x images showing initial incubation with **(A)** 1000; or **(B)** 0 ng/mL A11 antibody. 20x images showing initial incubation with **(C)** 0 ng/mL A11 antibody and 0 µM peptide (positive control); **(D)** 0 ng/mL A11 antibody and 10 µM peptide; **(E)** 1000 ng/mL anti-β-amyloid, 1-16 antibody and 10 µM peptide; and **(F)** 1000 ng/mL A11 antibody and 10 µM peptide. **(G)** Cellular analysis of 20x images demonstrating image pixel percentage above background in the Texas Red channel. DAPI Channel: Hoechst 333423 stained nuclei; GFP Channel: Alexa Fluor 488 phalloidin; Texas Red: ATTO 590 goat anti-mouse IgG1.

Upon comparison to the image in Figure 6C, the 60x and 20x images in Figures 6B and 6F, respectively, confirm that antibody treatment causes immunoneutralization of oligomerized β-amyloid peptide binding to SH-SY5Y cells. The specific effect of the A11 antibody was confirmed by the fact that no neutralization was seen following treatment with the anti-β-amyloid, 1-16 antibody (Figure 6E). The effect is both time and dose dependent as evidenced by the image analysis performed with the 20x image collected in the Texas Red channel.

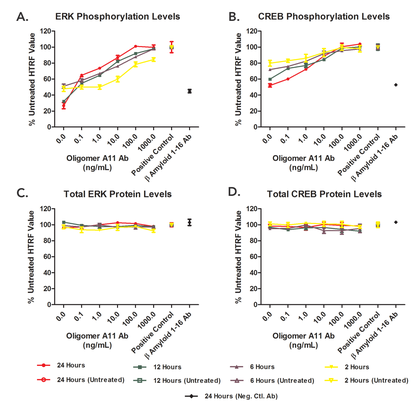


Figure 7. Phosphorylated and total protein levels following antibody and β-amyloid peptide treatment. Percent of untreated control well HTRF values reported for A11 antibody concentrations ranging from 1000-0 ng/mL, and incubated for 2, 6, 12, or 24 hours. Values also reported for wells treated with 1000 ng/mL anti-β-amyloid, 1-16 antibody and 10 µM peptide in addition to positive control untreated wells. Lysate aliquots run with **(A)** phospho-ERK; **(B)** phospho-CREB; **(C)** total ERK; or **(D)** total CREB assays.

The increase in ERK and CREB phosphorylation to levels seen in wells containing untreated cells confirms that immunoneutralization restores signaling pathways impaired by oligomerized β-amyloid peptide binding (Figure 7A and B). Antibody treatment does not change protein concentrations (Figure 7C and D). The effect is also confirmed to be specific to the A11 antibody as witnessed by the lack of improvement in phosphorylation levels following treatment with the anti- β-amyloid 1-16 antibody.

Conclusions

1. The β-Amyloid (1-42) Peptide, Amyloid β A4 (CT, 1-42) monoclonal antibody, and Goat anti-mouse ATTO 590 conjugated IgG1 secondary antibody from Enzo Life Sciences represent a sensitive method to reproduce and monitor β–amyloid peptide binding in an *in vitro* setting.

2. Cisbio HTRF phospho and total protein assays allow the effect of peptide binding to be easily determined for specific targets. Multiple targets can be assessed from the same well, reducing plate to plate variability.

3. The combined filter-based microplate reading, fluorescent imaging, and image analysis capabilities of the Cytation 5 and Gen5 Data Analysis Software, allow sample processing and calculations to be performed by a single instrument

4. The combination of microplate reading and imaging based assay methodologies and instrumentation create robust *in vitro* methods to make phenotypic and target-based determinations that can further the understanding of Alzheimer's disease and how its effects can be counteracted.