Application Note

Molecular Biology and Biochemical

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

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Introduction

Extracellular-Signal Regulated Kinase (ERK) signaling is linked to memory and regulated by environmental stimuli, whereas progressive cognitive impairment is a classic characteristic in Alzheimer's disease. The deficits are believed to be the result of progressive synaptic dysfunction initiated by aggregated β-amyloid peptide 1-42 (Aβ). For example, research shows that Aβ induces disruption of kinases critical for memory.[1] Additionally, in late stage Alzheimer's disease, ERK activation is suppressed relative to early stages and controls.[2] Finally, *in vitro* studies demonstrate that under certain conditions, Aβ or fragments inhibit ERK or the downstream cAMP response element-binding protein (CREB) in neuronal cell models.[3]

This application note evaluates the ability to detect changes in ERK and CREB phosphorylation levels following treatment with Aβ using the SH-SY5Y neuroblastoma cell line. Aβ was oligomerized using a previously published method.[4] A two-step HTRF assay process was incorporated such that cell plating and treatment were carried out in a 96-well clear-bottom imaging plate. Following lysis, aliquots were transferred to separate low-volume, 384-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 immunofluorescence and microscopic analysis. All microplate reading and cellular imaging steps were performed using a novel cell imaging multimode reader. The combination provides an efficient, robust method for testing of new molecules to combat the degenerative effects of the disease.

HTRF phospho- and total protein assay principle

HTRF assays from Cisbio Bioassays combine fluorescence resonance energy transfer (FRET) and time-resolved fluorescence (TRF) to eliminate background interference, and are 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 Eu^{3+} cryptate and an anti-protein-d2 antibody labeled with d2. In the presence of phosphorylated or total protein, and upon excitation of the Eu^{3+} cryptate, energy is transferred to the d2 molecule, and increased emission at 665 nm is seen. In the absence of the protein, no energy is transferred, and virtually no 665 nm signal is detected.

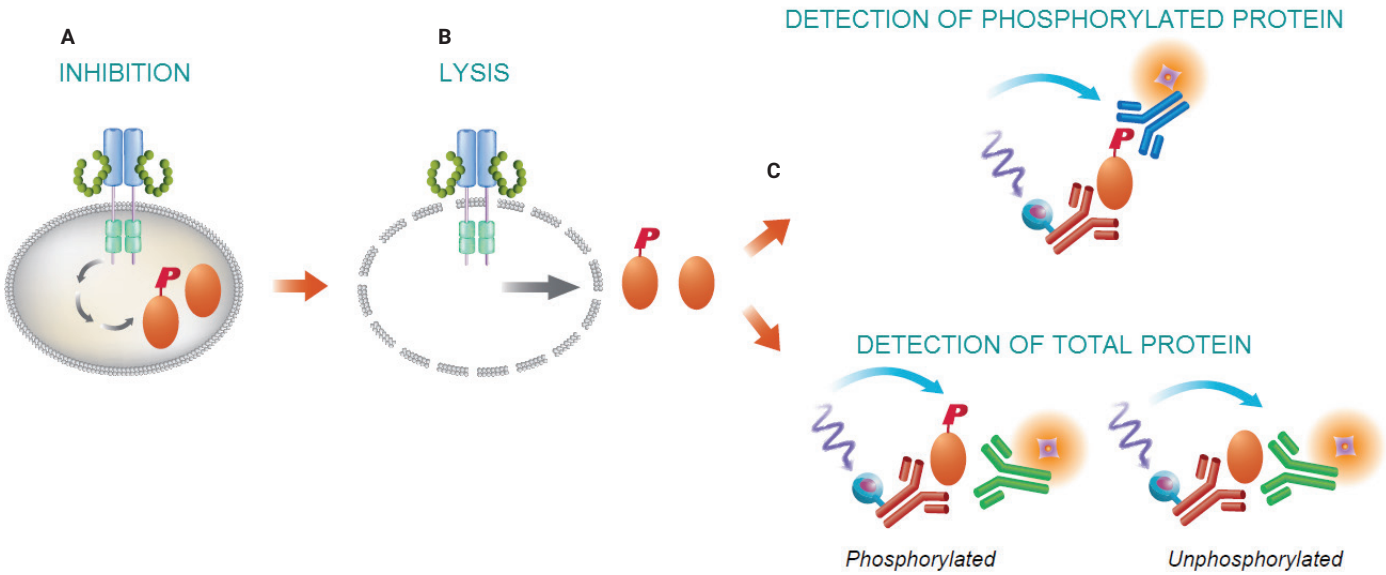

Figure 1. HTRF phospho- and total protein assays. The two-plate HTRF Human ERK and CREB protein assays are run in three steps. (A) Cells are incubated with modulators. (B) Cells are then lysed, releasing phosphorylated and unphosphorylated proteins. (C) Cell lysate is transferred to a second plate, followed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

Materials and methods

Materials

Assay and experimental components

Advanced phospho-ERK1/2 (Thr202/Tyr204) Cellular Assay Kit (part number 64AERPEG), ERK Total Kit (part number 64NRKPEG), CREB phospho-S133 Kit (part number 64CREPEG), and CREB Total Kit (part number 63ADK052PEG) were donated by Cisbio Bioassays, US (Bedford, MA).

Lyophilized β-amyloid (1-42) peptide (part number ALX-151-002-P001), amyloid β A4 (CT, 1-42) monoclonal antibody (8G7) (part number ADI-905-804-100), and Goat anti-mouse IgG1 (ATTO 590 conjugate) (part number ALX-211-204TM-C100) were donated by Enzo Life Sciences (Farmingdale, NY).

Ammonium hydroxide solution (part number 338818) was purchased from Sigma-Aldrich (Saint Louis, MO). SH-SY5Y neuroblastoma cells (part number CRL-2266) were purchased from ATCC (Manassas, VA). Oligomer A11 antibody (part number AHB0052) was purchased from ThermoFisher (Waltham, MA). Purified anti-β-amyloid, 1-16 antibody (part number 803001) was purchased from BioLegend (San Diego, CA).

Agilent BioTek Cytation 5 cell imaging multimode reader

The Cytation 5 cell imaging multimode reader combines a modular multimode microplate reader with automated digital microscopy in one compact unit. 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 performs 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 Agilent BioTek Gen5 data analysis software controls Cytation 5 and was also used for initial data analysis. The imaging module was used to visualize peptide binding effects on the cells, while the filter-based system was used to detect the 665 and 620 nm fluorescent emissions from the HTRF phospho- and total protein assays. The following settings were used: Delay after plate movement: 0 msec; Delay after excitation: 150 µsec; Integration time: 500 µsec; Read height: 7.5 mm.

Other equipment

Hamilton Gastight Syringe, 1700 Series (part number 81175, Hamilton Company, Reno, NV).

Methods

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. A Hamilton Gastight syringe was used to equally divide the volume among three polypropylene vials, which were then sealed and incubated at room temperature for greater than 2 hours to allow monomerization. The solution in each vial was then concentrated at 800 g, room temperature using a vacuum concentrator centrifuge. The vials now containing peptide films were then 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 2.0×10^5 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 to 0 µM. Media was completely removed from the wells and replaced with media containing the peptide, followed by incubation with the cells for 2, 6, 12, or 24 hours.

Inhibitor antibody addition: Media was completely removed from the wells and replaced with A11 or anti-β-amyloid, 1-16 antibodies at 5x concentrations ranging from 1,000 to 0 ng/mL in 20 µL and incubated for 30 minutes at 37 °C. Oligomerized peptide was then added in an 80 µL volume to a final 1x concentration of 10 µM and incubated as previously described.

Imaging: In the initial cell plate, cell fixing and permeabilization was first completed followed by primary amyloid β and secondary ATTO 590 IgG1 antibody incubations. Fluorescent cellular imaging was then carried out with the Cytation 5 using the DAPI Channel to image Hoechst 33342 stained nuclei; GFP Channel for Alexa Fluor 488 phalloidin stained actin; and Texas Red to capture signal from the ATTO 590 goat anti-mouse IgG1 secondary antibody.

Cellular analysis: Two separate cellular analyses were carried out. The first was performed to determine the number of cells per 20x image. Using the signal emanating from the Hoechst 33342 stained nuclei, captured with the DAPI channel, primary and advanced cellular parameters were optimized in Gen5 to automatically place object masks around each nuclei in the well. A second analysis was then conducted to quantify the number of oligomerized β-amyloid peptide binding sites in the same image. Here fluorescence from the ATTO 590 secondary antibody, captured using the Texas Red channel, was used. Cellular analysis parameters were again optimized to accurately identify the smaller, pinpoint areas of binding. Finally, the total number of binding sites identified per image was divided by the number of nuclei to normalize the amount of β-Amyloid peptide binding per treatment condition.

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 at room temperature. The remaining assay procedure was performed as previously described in Figure 1.

Results and discussion

β-amyloid binding to SH-SY5Y cells

First, the oligomerized β-amyloid (1-42) peptide's ability to bind to the SH-SY5Y neuronal cell model using the aforementioned procedure was validated. Immunofluorescence was then performed as previously described followed by imaging using a 20x or 40x objective.

Per Figure 2 images, oligomerized β-amyloid (1-42) peptide binds to SH-SY5Y cells and can be detected by the included primary and secondary antibodies. Binding occurs in both a dose and time dependent manner, as demonstrated by the graph in Figure 3, where cellular analysis was performed to determine the average number of amyloid binding sites per cell in each test well.

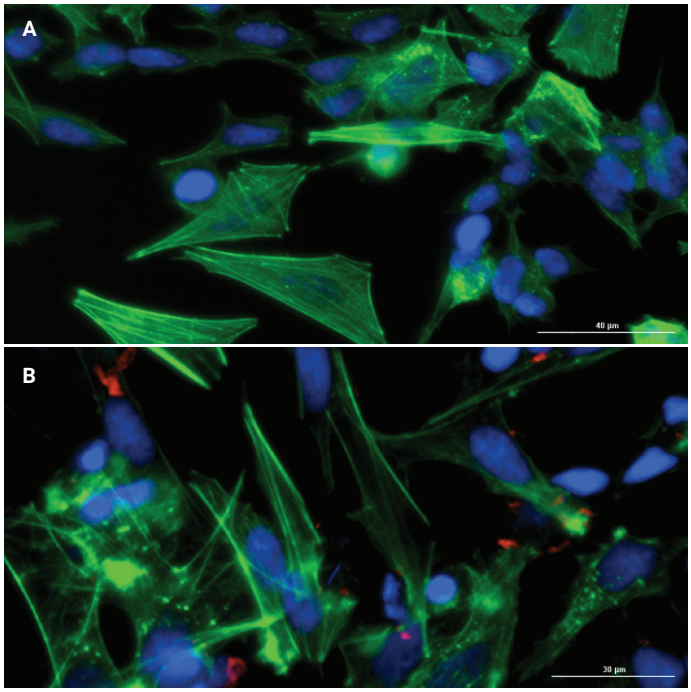

Figure 2. β-amyloid peptide binding. Overlaid images showing β-amyloid peptide binding to SH-SY5Y cells following a 24-hour incubation. 40x images showing incubation with (A) 0 µM; or (B) 10 µM peptide. DAPI Channel: Hoechst 33342 stained nuclei; GFP Channel: Alexa Fluor 488 phalloidin; Texas Red: ATTO 590 goat anti-mouse IgG1.

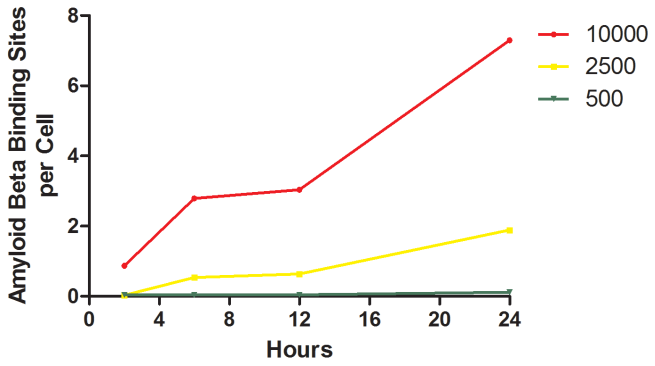

Figure 3. Time and dose dependent β-amyloid peptide binding to SH-SY5Y cells. Binding sites identified following treatment with β-amyloid peptide concentrations of 10,000, 2500, and 500 nM for 2, 6, 12, or 24 hours.

β-amyloid interruption of ERK-CREB signaling

Following validation of β-amyloid peptide binding, it was determined whether or not binding would interfere with signaling pathways leading to ERK and CREB phosphorylation, as previously reported.[3] A second plate was treated with oligomerized β-amyloid (1-42) peptide in a similar manner as previously described. Following peptide and lysis buffer incubations, four sets of liquid transfers were made to separate wells of a low-volume, 384-well plate, each containing four 16 µL aliquots, with peptide concentrations ranging from 10,000 to 50 nM. One aliquot set each was used to perform either the HTRF advanced phospho-ERK1/2 (Thr202/Tyr204) (pERK) assay, ERK total protein assay, CREB phospho-S133 (pCREB) assay, or CREB total protein assay. Total protein concentrations compared to untreated cells, in addition to percent phosphorylated protein, compared to total protein, was calculated for the concentrations of β-amyloid (1-42) peptide tested at each time point (Figure 4).

While total protein levels were unaltered by oligomerized β-amyloid (1-42) peptide treatment, phosphorylated protein decrease proportionately in response to higher β-amyloid peptide concentrations and increased incubation times with pERK and pCREB assays (Figure 4).

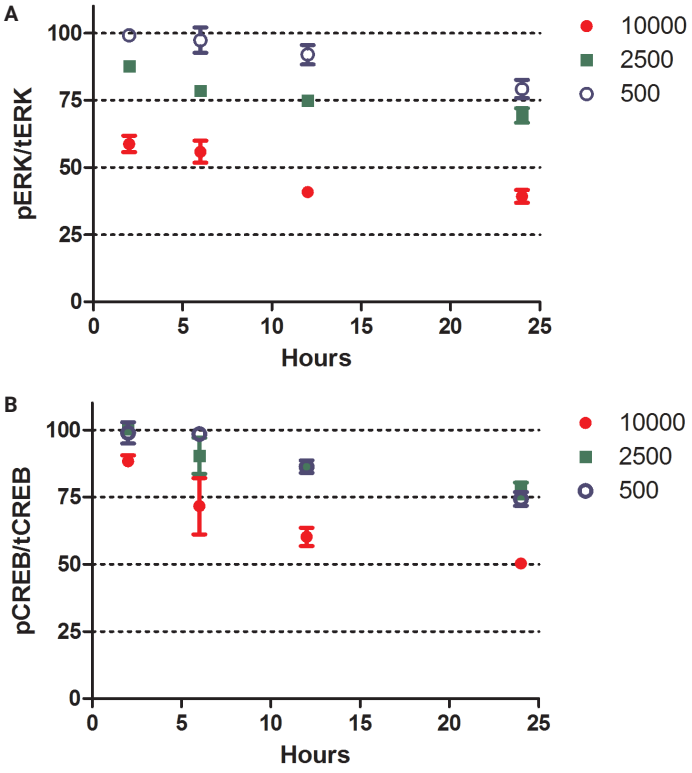

Figure 4. Time-dependent percent phosphorylated protein levels. Phosphorylated protein expressed as a percentage of total protein levels per sample following peptide treatments of 10,000, 2500, and 500 nM incubated with SH-SY5Y cells for 2, 6, 12, or 24 hours. Results reported for (A) pERK; and (B) pCREB assays.

Neutralization of β-amyloid binding

Antibodies, such as the Oligomer A11 antibody, demonstrate the ability to immunoneutralize β-amyloid peptide oligomers, and thus restore signaling pathway activity. This phenomenon, and the ability to track subsequent cellular activity changes, were tested by treating SH-SY5Y cells with the test A11 antibody, in addition to the negative control anti-β-amyloid, 1-16 antibody which is known to exhibit no neutralization effect. Following a 30-minute incubation at 37 °C with the antibodies, cells were treated with 10 µM peptide for 2, 6, 12, or 24 hours. Immunofluorescence was once again performed, followed by fluorescent imaging using a 20x or 60x objective (Figure 5).

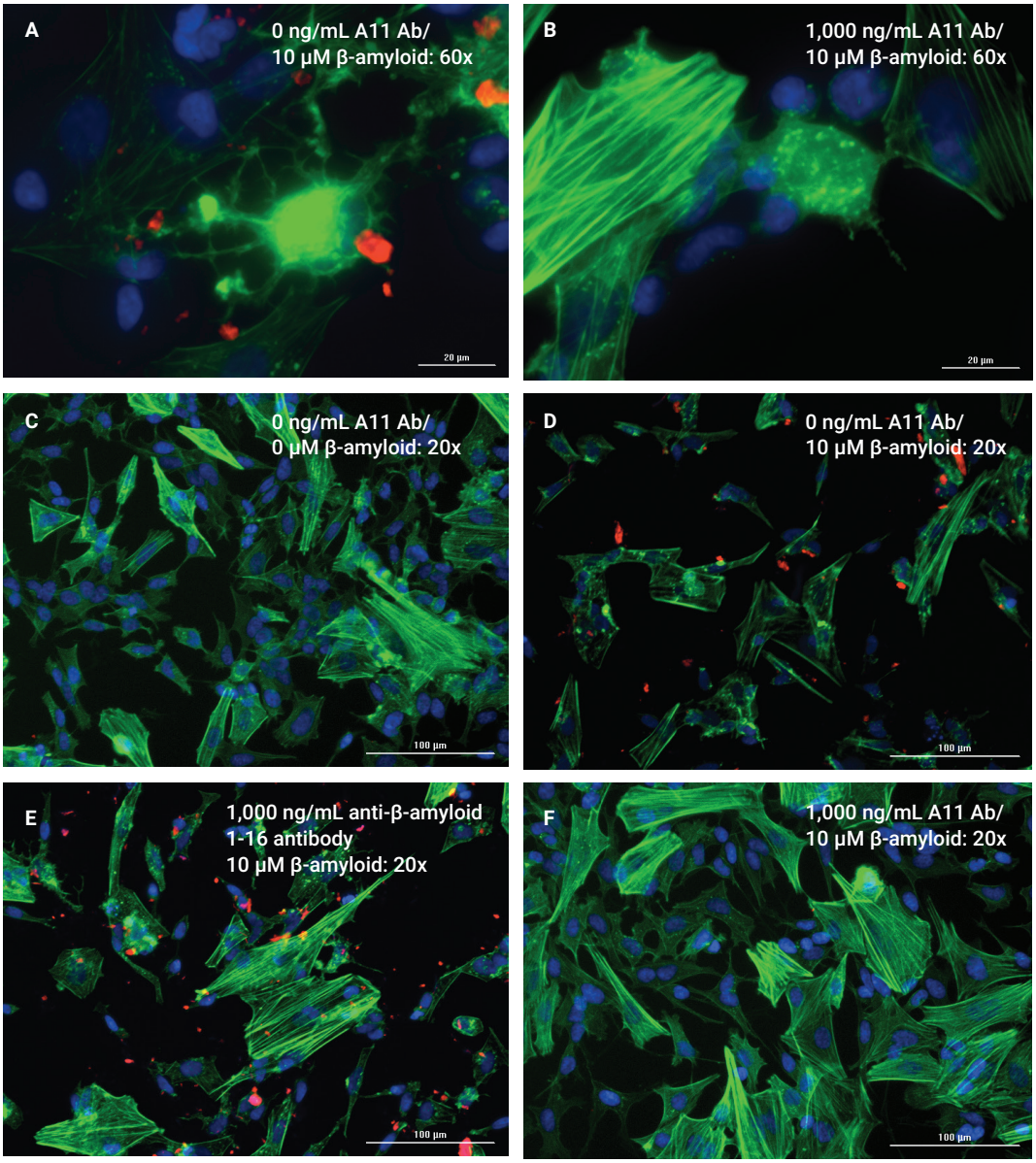

Figure 5. Neutralization of β-amyloid peptide binding. Overlaid images showing antibody treatment effects on β-amyloid peptide binding to SH-SY5Y cells following a 24-hour incubation with 10 µM β-amyloid peptide. 60x images showing initial incubation with (A) 0 ng/mL, or (B) 1,000 ng/mL A11 antibody. 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) 1,000 ng/mL anti-β-amyloid, 1-16 antibody and 10 µM peptide; and (F) 1,000 ng/mL A11 antibody and 10 µM peptide. DAPI Channel: Hoechst 33342 stained nuclei; GFP Channel: Alexa Fluor 488 phalloidin; Texas Red: ATTO 590 goat anti-mouse IgG1.

When comparing the positive control image in Figure 5C to Figure 5B (60x) and Figure 5F (20x), it was confirmed that antibody treatment causes immunoneutralization of oligomerized β-amyloid peptide binding to SH-SY5Y cells. The specific A11 antibody effect was confirmed as no neutralization was seen following treatment with the anti-β-amyloid, 1-16 antibody (Figure 5E). This is specifically evidenced when viewing the 60x images in Figure 5. High levels of peptide binding can be seen in the absence of antibody treatment, which is almost completely neutralized upon incubation with the A11 antibody. The neutralization effect is then shown to be both time- and dose-dependent, as evidenced by the cellular analysis of the 20x images to calculate beta-amyloid binding sites per cell (Figure 6).

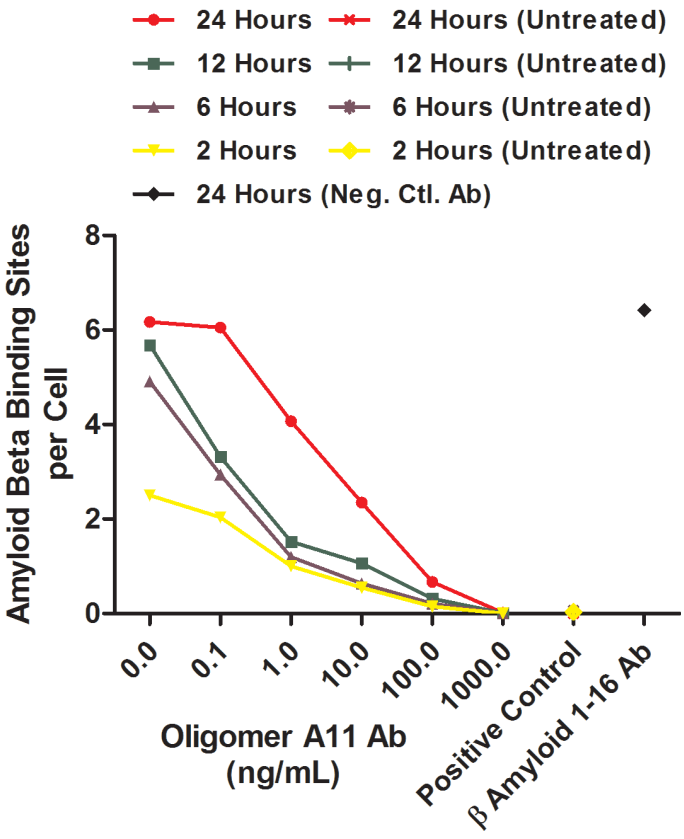

Figure 6. Cellular analysis of 20x images demonstrating β-amyloid peptide binding to SH-SY5Y cells following A11 or anti-β-amyloid 1-16 antibodies.

Finally, the percent of phosphorylated protein compared to total protein was once again calculated following A11 and anti-β-amyloid, 1-16 antibody treatment. 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 (Figures 7A and 7B). The effect is also confirmed as specific to the A11 antibody due to the lack of improved phosphorylation levels following treatment with the anti-β-amyloid 1-16 antibody.

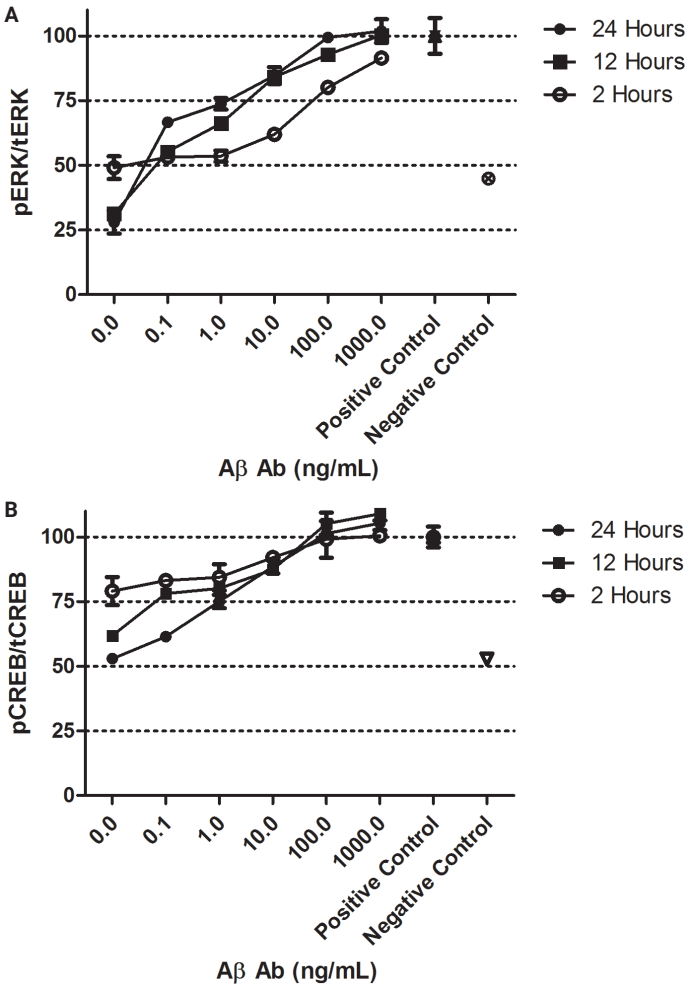

Figure 7. Percent phosphorylated versus total protein levels following antibody and β-amyloid peptide treatment. Percent phosphorylated protein compared to total protein values reported for A11 antibody concentrations ranging from 1,000 to 0 ng/mL, and incubated for 2, 6, 12, or 24 hours. Values also reported for wells treated with 1,000 ng/mL anti-β-amyloid, 1-16 antibody and 10 µM peptide in addition to positive control untreated wells. Results reported for (A) pERK/total ERK; and (B) pCREB/total CREB.

Conclusion

The β-amyloid (1-42) peptide, amyloid β A4 (CT, 1-42) monoclonal antibody, and Goat anti-mouse IgG1 (ATTO 590 conjugate) secondary antibody from Enzo Life Sciences represent sensitive tools to reproduce and monitor β-amyloid peptide binding in an *in vitro* setting. Cisbio Bioassay's HTRF phospho- and total protein assays allow easy determination of peptide binding effects for specific targets. Multiple targets may be assessed from the same well, thereby reducing plate to plate variability. Additionally, the combination of filter-based microplate reading, fluorescent imaging, and image analysis, found in Agilent BioTek Cytation 5 with integrated Agilent BioTek Gen5 data analysis software, allows sample processing and calculations to be performed in a single instrument. Finally, the combination of microplate reading and imaging, along with assay methodologies, 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.

References

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