Sigma-2/PGRMC1 antagonist pharmacodynamic target engagement biomarker discovery for Alzheimer's disease

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C. Rehak, N. Izzo, K. Mozzoni, C. Silky, R. Yurko, H. Safferstein, and S. Catalano
Cognition Therapeutics Inc., Pittsburgh, PA

ABSTRACT

Cognition Therapeutics Inc. (CogRx) discovered CT1812, a novel Abeta oligomer receptor antagonist which is the only drug candidate demonstrated to prevent and displace Abeta oligomer binding to neuronal receptors. By stopping the initiating event in the Abeta oligomer cascade, this first-in-class drug blocks downstream synaptotoxicity and restores memory to normal in transgenic mouse models of Alzheimer's disease (AD). CT1812 displaces receptor-bound oligomers by allosterically antagonizing the sigma-2/PGRMC1 receptor (Izzo et al., 2014a, b). CT1812 is the first disease modifying therapeutic that will test the oligomer hypothesis of AD. Biomarkers in patient biofluids that change following CT1812 engagement with the target receptor enable independent verification of compound activity. Despite prior clinical experience with sigma-2 ligands, biomarkers of sigma-2/PGRMC1 antagonist functional target engagement are not reported in the literature. Sigma-2/PGRMC1 has been demonstrated to regulate expression levels and subcellular localization of several proteins, including EGF receptor (Ahmed et al., 2010), mPRα receptor (Thomas et al., 2014), UNC 40/DCC (Runko et al., 2004), and GLP-1 receptor (Zhang et al 2014). We hypothesize that sigma-2/PGRMC1's role in regulating expression levels and subcellular localization of several proteins may provide an opportunity to measure drug-target engagement, which may manifest as changes in target protein expression or downstream signaling in clinically relevant samples. In 21DIV neurons, sigma-2/PGRMC1 localization was visualized by immunocytochemistry and quantified via image processing. In neurons, GLP-1R was expressed predominantly in the cytoplasm at low levels. Addition of Abeta oligomers caused a significant increase in GLP-1 receptor protein expression in the nucleus. Treatment with sigma-2/PGRMC1 antagonist CT1344 (analog of clinical candidate CT1812) at therapeutic brain concentrations blocked this increase, restoring GLP-1R expression pattern to normal, but did not affect expression in the absence of Abeta oligomers. The magnitude of protein concentration changes associated with the effects of Abeta oligomers on expression levels of GLP-1R was modest (i.e. 20%), but was completely reversed by CogRx sigma-2/PGRMC1 antagonist. Target engagement "fingerprints" (patterns of consistent changes in amounts of several proteins, each modest in magnitude) have been used successfully as pharmacodynamic biomarkers in clinical studies (Paweletz et al. 2009; Tsitoura et al. 2015).

METHODS

Human Clinical Trial: Human clinical trials were done at Nucleus Networks (Melbourne, AU). Single ascending dose (SAD) contained 6 cohorts of CT1812 (n=6, 0.13-14.93 mg/kg) and placebo (n=2) treated participants observed during in-unit confinement for 72 hrs. Multiple ascending dose (MAD) contained 3 cohorts of healthy young participants (18-64 y.o., n=8 treated, n=2 placebo) dosed at 280, 560, and 840 mg once daily for 14 days. One cohort of healthy elderly subjects (65 y.o.) were dosed at 560 mg (n=8 treated, 2 placebo). Clinical chemistry was done at Sydpath (Sydney, AU). Pharmacokinetics were done at CPR Pharma Services (Adelaide, AU).

GLP-1R Translocation: CT1344 was applied to DIV21 cortical/hippocampal cultures from 0.004uM to 0.9uM for 1 hr followed by Abeta at 0.5uM for 1 hr. Neurons were fixed in 3.75% formaldehyde and stained with GLP-1R(Novus), MAP2(Millipore), and 6E10(Biolegend) antibodies. Imaging was preformed on Cellomics VTi automated microscope with a 20X, 0.75 NA objective and analyzed using a compartmental analysis algorithm to measure staining in the nucleus versus cytoplasm.

CT1812 Displaces Abeta: A. 0.5uM synthetic Abeta oligomers were added to in DIV21 neurons for 1 hr prior to 0-1uM of CT1812. Neurons were stained and imaged as in Figure 2. B. AD patient tissue sections were incubated with CT1812. C. Synapses were measured after addition of 0.5uM Abeta for 1hr and 0-1uM CT1812 then stained with Synaptophysin (Anaspec), 6E10 (Biolegend) and imaged on Cellomics VTi automated microscope with 20X, 0.75NA objective. D. 1.8uM of synthetic oligomers were applied to DIV 21 cultures for 1 hr before CT1812 (1uM-10uM). Tetrazolium salts (3-(4,5-dimethylthiazol-2yl)-2,5diphenyl tetrazolium bromide, Roche Molecular Biochemicals, 5mg/mL in PBS) were added and incubated at 37ºC for 1h and extracted with 1.6% Tween-20 and read at 690nM on a Synergy HT plate reader.

Biomarker Discovery: Figures 1, 2. Thy1-hAPPLond/Swe+ AD mice were treated with CT1812 or vehicle for 8 days (N=9 or 10 animals/group respectively), and terminal CSF and plasma were subject to LC/MSMS analysis at Caprion Biosciences Inc.. After immunodepletion of albumin, IgG, and transferrin with MARS3 columns then trypsinization, samples were either fractionated (plasma only) or left whole and injected into a NanoAcquity UPLC coupled to a Q Exactive Plus MS. Peptide separation was achieved using a nanoAcquity Symmery UPLC Trap column and nanoAcquity UPLC BEH300 analytical column. The 12 most intense peaks per survey scan with charge state >1 were fragmented and scanned with a mass range from 200 to 2000 m/z at a resolution of 17,500. Raw spectromoter data files for each LC-MS run were aligned independently using Elucidator software. The MS/MS spectra were matched to corresponding peptide sequences found in the Uniprot Mus musculus protein database using Mascot software with modifications allowing for up to 2 missed cleavages, a peptide tolerance of 20ppm, and an MS/MS tolerance of 0.05Da. Outlier detection was performed by investigating the average log-intensity of all isotope groups (IG) over injection order for all samples. Samples with an average value lower than 2 standard deviation against the mean were further investigated. Following data transformation and normalization, expression analysis of the identified isotope groups and proteins were performed and the statistical significance of each comparison was assessed via t-test, based on the coefficient of a linear model (LM). Expression analysis was also performed at the peptide and protein levels, which used the same methodology as above, but applied to peptide/protein intensities. Peptide and protein intensities were built by rolling-up the corresponding isotope group intensities. Isotope groups not meeting the Detection Rate (DR) thresholds were not used for the roll-up. All statistical test *p*-values were adjusted for multiple testing by conversion to *q*-value using Storey's method (Storey, 2012; Storey, 2013). Figure 3. Alzheimer's Disease Neuroimaging Initiative (ADNI) and the AD CSF Biomarkers Consortium, identified proteins in CSF that were either up- or down-regulated in AD patients compared to controls. CogRx CSF proteins which normalize AD protein dysregulation are listed. Figure 4. Independent researchers associated with ADNI and/or ABIL identified plasma proteins which were either up- or down-regulated in AD patients compared to healthy controls. CogRx plasma proteins which normalized AD protein dysregulation are listed.

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CT1812 is well tolerated in first-in-man double blind, placebo controlled Phase Ia study in healthy volunteers

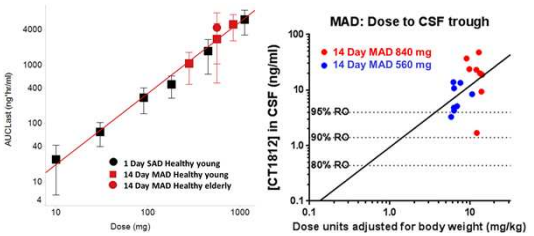

Figure 1. Pharmacokinetics of CT1812 in human plasma (A) and CF (B). A. CT1812 exhibits well behaved pharmacokinetics in plasma with concentrations that were linear across two orders of dosing magnitude [0.13-14.93 mg/kg] with no evidence of accumulation. **B.** CT1812 exhibits dose dependent increases in CSF concentrations that are estimated to reach >90% estimated receptor occupancy at sigma-2/PGRMC1 receptors in brain.

Biomarker Discovery Workflow

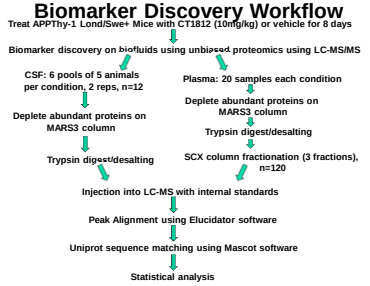

Figure 4. Unbiased LC MS/MS candidate biomarker biofluid analysis. Unbiased MS-based discovery study was performed to identify candidate biomarkers in the plasma and CSF of transgenic (*Thy1-APPSwe/Lond*) mice treated with CT1812 versus vehicle. Each digested plasma sample was fractionated into 3 fractions by SCX chromatography prior to liquid chromatography-tandem mass spectrometry (LC-MS/MS). CSF samples were not fractionated before desalting and LC-MS/MS. Proteins detected with at least one unique peptide mapping to them were selected.

CT1812 displaces Abeta binding from neurons in vitro

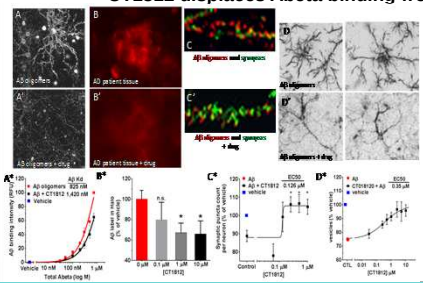

Figure 3. CogRx drug CT1812 displaces Aβ oligomers. CT1812 displaces binding sites on neurons in vitro **A**, **A'**, **A*** and in human AD patient brain sections **B**, **B'**, **B***, and as a result restores synapses **C**, **C'**, **C*** and downstream membrane trafficking to normal **D**, **D'**, **D***. Aβ oligomer-treated neurons (21DIV) in vitro are shown in the upper row of photomicrographs **A**,**C**,**D**, Aβ oligomer + CT1812 treated neurons are shown in the middle row **A'**,**C'**,**D'**. Human AD patient brain section treated with saline **B** or CT1812 **B'** shows displacement of oligomers from plaques in neocortex. The differences in conditions are quantified in the graphs underneath **A*.B*.C*.D***.

Conclusions:

1. **CT1812 is safe and well tolerated in Phase 1 clinical trials in healthy young and elderly volunteers, exhibits well behaved pharmacokinetics in plasma, and CSF concentrations reaching an estimated >90% receptor occupancy.**
2. **Clinically relevant target engagement biomarkers for CT1812 were identified via target-directed and unbiased proteomics methods.**
3. **GLP1 receptor (known to directly bind to CT1812 target sigma-2/PGRMC1 receptor complex) translocates to the nucleus following Abeta oligomer addition to DIV21 neuronal cultures. GLP-1R translocation is prevented with CT1812 analog CT1344.**
4. **Concentrations of several proteins expressed in plasma and CSF of Tg mice change significantly following treatment with CT1812.**
5. **The majority of CSF and plasma proteins previously reported to distinguish AD patients from age-matched cognitively normal controls are changed in Tg mice in a direction that is consistent with a disease-modifying AD therapeutic.**
6. **Significant reversal of AD-related changes in CSF and plasma protein concentrations has not been reported previously for any therapeutic.**

Abeta-induced GLP-1R nuclear translocation normalized with CT1812

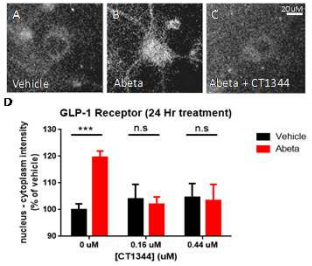

Figure 2. CT1812 normalizes Abeta oligomer-induced receptor signaling in primary neurons. A. DIV 21 neurons express low levels of GLP-1 receptor in the cytoplasm, but not over the nucleus. **B.** Abeta oligomer treatment for 24 hours causes a significant increase in GLP-1R expression over the nucleus vs. vehicle control treatment (***p=0.0068, two way ANOVA). **C.** Treatment with sigma-2/PGRMC1 antagonist CT1344 (0.16 uM) restores GLP-1R expression localization pattern to normal. Bar=20uM **D.** CT1344 blocks the significant Abeta oligomer-induced increase in GLP-1 receptor expression (red bars), without affecting levels of expression in neurons in the absence of Abeta oligomers (black bars). N= >5000 neurons/data point. Scale bar = 20 uM.

Normalization of AD Biomarkers with CT1812

CSF

Table 1. CSF: Thy1-hAPPLond/Swe: CT1812 vs vehicle

Function	AD biomarker	% change	P-value	Q-value
Inflammation	Y	-77.7	0.0005	0.198
Inflammation	Y	-83.4	0.0001	0.044
Ion channel regulation	Y	+74.5	0.0187	0.957
Growth factor	N	-28.3	0.021	0.775
Heat shock protein	N	-42.2	0.023	0.775
Neuroendocrine system	N	-51.4	0.037	0.775
Neuroendocrine system	N	+46.3	0.0491	0.957
Drug metabolism	N	-52.1	0.044	0.775
Actin binding	N	-32.5	0.045	0.775
Inflammation	N	-97.9	0.046	0.775
Actin binding	N	-41.9	0.049	0.775
Epithelial structure	N	+>100.0	0.0176	0.957
Epithelial structure	N	+>100.0	0.0215	0.957
Epithelial structure	N	+>100.0	0.0225	0.957
Epithelial structure	N	+ >100.0	0.0305	0.957
Epithelial structure	N	+ >100.0	0.0482	0.957
Cell cycle	N	+>100.0	0.0251	0.957
Extracellular matrix	N	+ 41	0.0262	0.957
Extracellular matrix	N	+ 64.5	0.0408	0.957
Inflammation	N	+ 90.7	0.0471	0.957

Plasma

Table 2. Plasma Thy1-hAPPLond/Swe: CT1812 vs vehicle

Function	AD biomarker	% change	P-value	Q-value
Inflammation	Y	-34.8	0.033	0.957
Epithelial structure	Y	-25.4	0.008	0.957
Lipid transport	Y	+97.5	0.028	0.957
Muscle structure	Y	+ 92.9	0.030	0.957
Protease inhibition	N	-27.2	0.005	0.957
Growth factor	N	-25.9	0.039	0.957
Neuroendocrine system	N	+55.8	0.003	0.957
Hemoprotein	N	+87.7	0.027	0.957
Hemoprotein	N	+ 29	0.035	0.957
Heat shock protein	N	+ >100.0	0.029	0.957
Protein folding	N	+ >100.0	0.032	0.957
Transcription factor	N	+ >100.0	0.039	0.957

Table 3. Comparison of mouse results with human study of CSF biomarkers of AD

Function	Literature Change in Human AD CSF	CSF Thy1-hAPPLond/Swe: CT1812 vs veh
Synaptic function	↓	↑
Synaptic function	↓	↑
Synaptic function	↓	↑
Synaptic function	↓	↑
Synaptic function	↓	↑
Ion channel regulation	↓	↑
Differentiation	↓	↑
Neurite growth	↓	↑
Neuronal protein processing	↓	↑
Neuroendocrine system	↓	↑
Lipid metabolism	↓	↑
Methylation	↓	↓
Astrocyte structure	↑	↓
Synaptic function	↓	No Change
Lipid transport	↑	No change
Inflammation	↑	No change

CSF and Plasma proteins respond to drug treatment.

Tables 1 and 2. Twenty (20) CSF and twelve (12) plasma proteins were found to change more than 25% with drug treatment in transgenic AD mice (p <0.05). Proteins reported in literature to be AD biomarkers in mice are indicated. **Table 3.** Orthologs of 16 AD CSF biomarker proteins were identified by LC-MS/MS discovery in mouse CSF. 13 of 16 biomarkers were normalized by treatment with CT1812 - 12 of 13 proteins down-regulated in human AD were increased and 1 had no change with CT1812 treatment. 1 of 3 proteins up-regulated in human AD were decreased and 2 showed no change with CT1812. **Table 4:** 12 of 15 AD plasma biomarkers were normalized by CT1812. 5 of 6 proteins down-regulated proteins were increased and 1 was unchanged. 6 of 9 up-regulated proteins were normalized and 3 were unchanged with CT1812.

Table 4. Comparison of mouse results with human study of Plasma biomarkers of AD

Function	Literature Change in Human AD Plasma	Plasma Thy1-hAPPLond/Swe: CT1812 vs veh
Lipid Transport	↓	↑
Lipid transport	↓	↑
Inflammation*	↓	↑
Inflammation	↓	↑
Inflammation	↓	↑
Synaptic plasticity	↑	↓
Insulin regulation	↑	↓
Lipid transport	↑	↓
Insulin regulation	↑	↓
Steroid transport	↑	↓
Protease inhibitor	↑	↓
Signal transduction	↑	No change
Tissue remodeling	↑	No change
Lipid Transport	↑	No change
Lipid transport	↓	No change

Publications:

1. Izzo NJ, et al. Alzheimer's therapeutics targeting Amyloid beta 1-42 oligomers I: Abeta 42 oligomer binding to specific neuronal receptors is displaced by drug candidates that improve cognitive deficits. PLoS ONE 10: e0111898, 2014.
2. Izzo NJ, et al. Alzheimer's therapeutics targeting Amyloid beta 1-42 oligomers II: Sigma-2/PGRMC1 receptors mediate Abeta 42 oligomer binding and synaptototoxicity. PLoS ONE 10: e0111899, 2014.