Rigor and Replication in Alzheimer's Therapeutic Development: A Case Study

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Disclosure: The Authors (independently) have or previously had short positions and/or related hedges in Cassava Sciences stock, and have engaged in paid consulting to discuss Cassava Sciences and other companies.

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Evaluation of Preclinical & Clinical Simufilam Studies: Strange Observations Raise Scientific Doubts

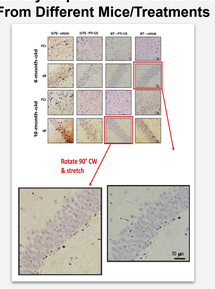
The Simufilam Story

- Simufilam (PTI-125) is an investigational drug in Ph3 trials for AD (NCT04994483, NCT05026177) sponsored by **Cassava Sciences** (Pain Tx)
- First proposed as non-opiod analgesic or modulator of opioid signaling / addictiveness, based on mimicking apocryphal paradoxical effects of "ultra-low-dose Naloxone" **[1, 2]**
- Wang 2008 **[1]** reported Naloxone (**NLX**) bound Filamin-A (**FLNA**) with pM affinity at specific 5mer VAKGL to modulate opioid signaling, and that NLX bound the isolated VAKGL peptide, which competed with FLNA to bind NLX
- Simufilam claimed to have been discovered **[3]** as competitor to NLX binding to VAKGL and FLNA, without opioid antagonism
- Wang 2012 **[4]** claimed Simufilam reduced pathogenesis in a mouse AD model by modulating toxic signaling of Aβ42 via α7nAChR, by binding FLNA & changing protein intx
- Cassava received **>$20M from NIH** for Simufilam development

Reports of Manipulated Images & Data

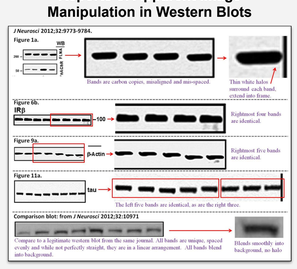
- In 2021, many scientists independently flagged concerns about images & data in **>30** papers authored by **Dr. Hoau-Yan Wang** of City College (CCNY) of **City University of New York** (**CUNY**) [Petitions & Letters to FDA; PubPeer]

Apparently Duplicated / Rotated Photos From Different Mice/Treatments



PTI-125 binds and reverses an altered conformation of filamin A to reduce Alzheimer's disease pathogenesis
Neurobiology of Aging 2017 [PubPeer]

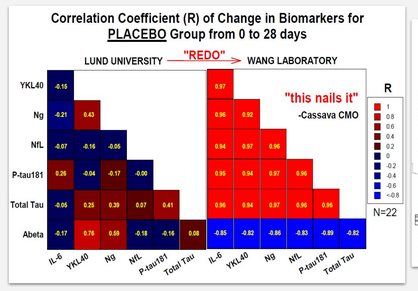
Reports of Apparent Image Manipulation in Western Blots

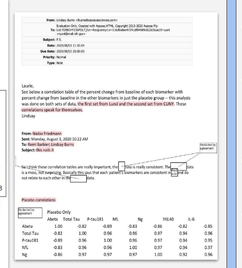


Reducing amyloid-related Alzheimer's disease pathogenesis by a small molecule targeting FLNA
Journal of Neuroscience 2012
[PubPeer; Citizens Petition]

The "Re-Do" & Puzzling Correlations

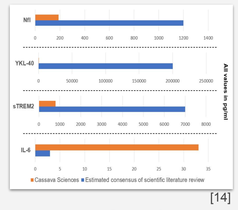
- 28-day P2b **failed** when initially reported (**Lund analysis**)
- Back-up CSF samples sent to **Dr. Wang** at for "re-do"
- Wang reported highly significant CSF biomarker effects
- Use of Wang's data was justified based on ***correlations*** between ***changes of different biomarkers in placebo*** (patient-level; 28d)
- No valid statistical rationale for this justification; in short term, uncorrelated random errors are expected in placebo





Implausible Phase 2 Biomarker Data

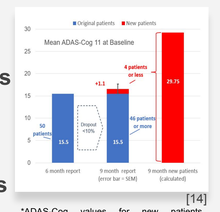
- Wang analyzed **all** P2 CSF samples **7 of 9** CSF biomarkers appeared:
  - Inconsistent with scientific literature
  - Inconsistent with human biology
  - Inconsistent with assay (Luminex v ELISA)



[14]

Open-Label Cognitive Baseline Shifts

- Reported ADAS-Cog **improvement** of **1.6pts at 6mo** and **3pts at 9mo**
- "Improvement" likely due to 4 (est.) patients dropped-in w/ **baseline scores 2x as severe** as the original cohort*
- Absolute scores **did not change** significantly 6mo → 9mo: 13.6 vs.13.9
- After this was noticed, **baseline values** were not reported in 12mo read-out (Δ-1.5pts)



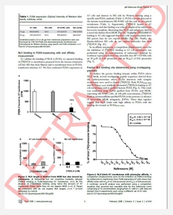
[14]

*ADAS-Cog values for new patients calculated based on the assumption that the mean score for drop-outs didn't differ from that of other patients

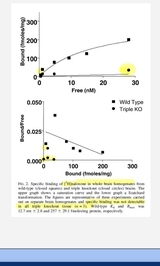
A Foundational Question: Does Simufilam Really Bind Its Purported Molecular Target, VAKGL peptide in Filamin-A?

Claim 1: A New Naloxone Target
Naloxone Binds Filamin-A?

- The only paper **[1]** to report Naloxone binding FLNA is by Wang and Burns and was retracted by PLoS editors



- There is no reported non-opioid specific binding site for Naloxone **[9]**; Naloxone does not distribute to tissues with high FLNA expression **[10]** (50yrs of study)



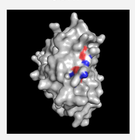
European Journal of Neuroscience, Vol. 16, pp. 1705–1712, 2002 © Federation of European Neuroscience Societies

Autoradiography of opioid and ORL1 ligands in opioid receptor triple knockout mice

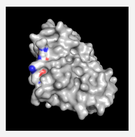
Claim 2: A Specific New Naloxone Binding Site
Naloxone Binds VAKGL Peptide?

- No reported analogous small molecule ligands for any other pentapeptides
- No binding pocket near VAKGL in structure of the FLNA dimerization domain [3CNK]
- No model ever proposed for how binding to VAKGL causes conformational change, pI shift, or allosteric modulation of FLNA protein interactions or function
- VAKGL occurs in dozens of other human proteins; if Naloxone bound VAKGL (both as a pentapeptide and in native FLNA), unclear why it would not bind other proteins with VAKGL

3CNK



3CNK



Claim 3: A Novel Molecule Mimicking Naloxone
Simufilam Binds VAKGL and Filamin-A?

- Simufilam reportedly discovered with *in vitro* screen against biotinylated VAKGL competing with FITC-tagged NLX **[3]**
- Simufilam claimed to bind AD brain tissue in displacement assay vs [^{3}H]NLX **[5]**
- Simufilam claimed to induce pI shift of FLNA from AD mice and AD patients in ***lymphocytes*** [**5, 6**]
- VAKGL claimed to compete vs FLNA for Simu & block effects on FLNA intx in synaptosome preps

Reported Simufilam Effects on FLNA from AD Patient Lymphocytes

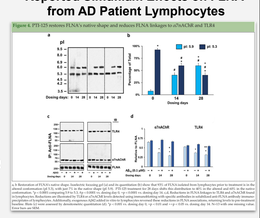


Fig. 4 from "PTI-125 Reduces Biomarkers of Alzheimer's Diseas in Patients" JPAD **[6]**

Claim 4: High Affinity & Two FLNA Conformations
Simufilam binds altered FLNA; fM IC50?

Reported Radiolabeled Simufilam Binding Assay

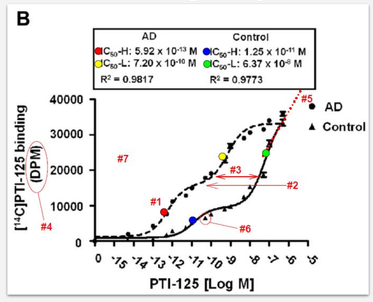


Fig. 1 from "PTI-125 binds and reverses an altered conformation of filamin A to reduce Alzheimer's disease pathogenesis" **[5]**
Neurobiology of Aging 2017
[Expression of Concern][PubPeer]

1. 4 Affinities
2. Wrong Ratio
3. Thermodynamic Paradox
4. Physically Impossible Radiochemistry
5. No Asymptote
6. n=6 but no error bars
7. Time to equilibrium

Experiment & Results: No Evidence for Naloxone or Simufilam Binding VAKGL By Isothermal Titration Calorimetry

What is ITC? How does it work?

- Molecules interact due to thermodynamic driving forces. Major contributors to non-covalent interactions are hydrogen bonding and van der Waals forces, hydrophobic interactions, & entropy. **ΔG = ΔH − TΔS**
- Isothermal Titration Calorimetry directly measures the heat (enthalpy; ΔH) of a molecular interaction **[11]** as small volumes of a solution containing the ligand are sequentially injected into solution containing target
- As molecules bind (if they bind), heat is released & measured, until all target binding sites are saturated
- Advantages: Easy; automated; does not require labeling molecules; very sensitive; quantitative

Experimental Design & Methods

- Automated calorimetry: MicroCal Auto iTC 200; 25°C
- No +ve control exists for a small mol binding any pentapeptide; high-affinity Carbonic Anhydrase II (CAII) inhibitors eg. Acetazolamide (K_d ~20nM) often used to benchmark detection of binding intx
- VAKGL(target) and VAAGL (-ve control) peptides synthesized (GenScript)
- Simufilam HCl (MedchemExpress), Naloxone (Selleckchem), Acetazolamide (Fisher) ligands were dissolved in DMSO
- Peptides,CAII dissolved in dH_20 (VAKGL) or PBS
- ITC binding assay in PBS;matched [DMSO] <5%
- Integration & baseline correction using NITPIC **[12]**

ITC Results: Does It Bind???

ITC Thermograms

Water + Water
No Signal, as Expected

Acetazolamide + CAII
Clear Signal of Binding
Binding is saturable

Naloxone + VAKGL peptide
No Signal of Binding!

Simufilam + VAKGL
Simufilam + VAAGL (-ve ctrl)
No Signal of Binding!

Interpretation & Limitations

- **Analysis of published claims together with our experiments suggests that Simufilam does not bind its reported target.**
- We believe Simufilam couldn't have been discovered as patents claim, since NLX doesn't seem to bind FLNA
- Proving a negative result is hard; a single experiment is not determinative. ITC & other expts should be repeated by others
- If Simufilam or NLX bound FLNA, it should be easy to determine structure of bound complex by crystallography, cryo-EM, or NMR. No such structures were ever reported.
- Simufilam authors have failed to offer *any* new experimental data to address concerns for over a year.
- **All pre-clinical & clinical claims about Simufilam [3,4,5,6,7,8] are in doubt if they rely biologically or logically upon retracted, invalidated, fabricated, or falsified scientific claims.**

Conclusion: In Our Opinion, a Drug that Doesn't Bind Its Purported Target Has No MoA, Nor Possible Clinical Utility

Ethics & Law of Trials in Humans

- Human experimentation must be "justified on the basis of a favorable risk/benefit assessment" **[16]**
- Can informed consent be obtained if veracity of science used to justify clinical trials is in question?
- Is offering hope, denying access to alternative trials, & doing lumbar punctures justified if prospect of any clinical benefit is in question?
- Can clinicians & IRB properly evaluate a trial if Investigator Brochure cites dubious papers & science?
- When should FDA take enforcement action to ensure compliance with 21 CFR 312?

Correcting the Scientific Record & Investigating Possible Misconduct

- 7 retractions to date
- Some journals refused to investigate, take further action, or address errors
- Initial 2021 CUNY inquiry determined that an investigation was required under Research Misconduct Policy, yet CUNY has provided no public updates for over a year
- Multiple ongoing federal investigations have been reported [WSJ; New York Times; Reuters]

Simufilam Ph2a Paper References

PTI-125 Reduces Biomarkers of Alzheimer's Disease in Patients [6]

Beyond the Simufilam Case Study

- Many other drugs in development deserve scrutiny; peer review is an ongoing process
- Questionable research practices all too common
- **Mechanism of action matters**, especially for drugs from rational, target-directed discovery
- Large unmet needs & long development timelines create perverse incentives to exaggerate claims
- **Potential conflicts of interest must be considered, but are independent of truth & validity of observations and arguments**
- **Institutions of science must encourage debate, investigate concerns, & protect skeptics & whistleblowers – not attack them [15]**

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Disclosures: The Authors (independently) have or previously had short positions and/or related hedges in Cassava Sciences stock, and have engaged in paid consulting to discuss Cassava Sciences and other companies. The Authors are defendants in a federal defamation lawsuit, Cassava Sciences v. Bredt **[13]**

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