# Review of: "The orphan drug dichloroacetate reduces amyloid beta-peptide production whilst promoting non-amyloidogenic proteolysis of the amyloid precursor protein"

Darrell Mousseau[1]

1 University of Saskatchewan

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This manuscript reports on the results of study designed to examine the relation, if any, between dichloroacetate (DCA), an inhibitor of pyruvate dehydrogenase kinase 1 (PDK1) that is most often associated with cancer studies and Amyloid Protein Precursor (APP) processing. A quick search for similar articles online brings up a MSc thesis with a very similar title by the second author. The authors propose that pilot data showing changes in Jagged1 cleavage following DCA treatment suggested the possibility that DCA could be affecting all substrates that undergo ADAM10 or BACE-1-mediated shedding and then processing to some active fragment. This would include APP, with relevance to Alzheimer disease.
The authors primarily used viability assays and western blotting to test their hypothesis. The conclusions are that DCA appears to inhibit the BACE1-mediated cleavage of the APP that leads to the cytotoxic Aβ peptide in favour of presumed cleavage via the non-amyloidogenic route (e.g. via ADAM10). This is very interesting and could have significant impact on treatment management.
While I have no concerns that the detection of bands on Western blotting change (or do not, depending on the experiment and target protein), I do have questions about the interpretation of the data. Any concern is more a reflection of the discussion being focused on canonical mechanisms and trying to shoehorn the data into those mechanisms. I think there may be other options that could be considered. The comments below are addressed to the second author, whose thesis work forms the basis of this manuscript.
Comments:
• Abstract (line 26): there is no lead-in to this statement; the question I am left with is why use DCA?
• Abstract (line 37): “did not significantly affect the expression of α-, β- or γ-secretases” is misleading; you measured ADAM10, BACE1 and PS-1 mRNA transcript levels using qPCR and not the actual functional unit, i.e. the protein/secretase.
• If this were my manuscript, I would start the introduction with the paragraph that discusses DCA (e.g. current line 59). I would then move the current first paragraph after ‘amyloid protein precursor’ (current line 83). I would include a discussion of “Amyloid Beta Resistance in Nerve Cell Lines Is Mediated by the Warburg Effect (2011) by J.T. Newington et al in PlosONE, which looks at the association between DCA and amyloid toxicity. I would alos reference Newtington's 2021 JBC paper as well as the Zhao et al (2019)

paper in Front Pharmacol that shows effects of DCA in a rat model of dementia (different molecular targets, but supports the use of DCA in AD/dementia). I think these articles should be acknowledged, They do not take away from the novelty and importance of your findings and actually corroborate some of your results.

• The part of the introduction that begins currently on Line 84-102 is just a detailed summary of your findings and should not really be in the introduction (which is normally a recap of the literature and the knowledge gaps therein). I would expand a bit more of the role of DCA at the mitochondria, how it might affect ER-mitochondrial coupling (which is important for BACE1/secretase processing of APP) and even include a bit on Ca2+ flux/dynamics, which have been implicated in PDK1 function, and even cancer and AD (APP processing). I would finish with a very, very high-level summary of your findings (one-two sentences)…but that's just my preference.

• Materials, Line 111: what is the epitope for the anti-sAPPβ antibody?

• Cell culture, Line 121: you describe SW480 and SH-Sy5y cells, but not HEK293.

• Treatment of cells, Line 133: number of cells plated? also, is this the same number of cells plated for your experiments with cells stably expressing APP or BACE1 (below)?

• Treatment of cells, Line 134: why use the two culture conditions? This needs to be clearly explained as you get drastically different outcomes depending on the plating density and as there could be vast differences in energetics (given that there is likely a bit of contact inhibition and signalling change with the confluence state). For example, the following articles monitor changes in Notch signalling (one of your arguments) and how this relates to cell confluence: Giulia Ferrari-Toninelli, et al (2010) Targeting Notch pathway induces growth inhibition and differentiation of neuroblastoma cells. Neuro Oncol. 12(12): 1231–1243 as well as Sestan N, et al (1999) Contact-dependent inhibition of cortical neurite growth mediated by Notch signaling. Science 286(5440): 741–746. This could represent a confound for the 'viability' assays.

• Peptide quantification, Line 173: just a comment; I do not understand how an antibody (e.g. 6E10) directed at the N-term 16 amino acids of the Aβ peptide can differentiate between two Aβ peptides that differ by two amino acids at their C-term end. I presume this is explained by the manufacturer, but I am having difficulty imagining this concept.

• qPCR primers, Line 190: separate by codon; doesn't change the information, just allows for wrapping of text from one line to the next; tidier.

• Results, Line 210: again, why use full confluence? This is your 'control' condition, so it is important to know why you chose this rather than testing cells in log phase.

• Results, Line 232 (and many others): "Multiple […] immunoblots were quantified"…this is repeated at least 12 times throughout the text and figure legends. Why not just give the 'n=' with each of your P values?

• P values in text and in EVERY figure legend…the P value should be expressed as 'less than' [$P < 0.05$] not 'greater than' [$P > 0.5$ etc].

• This I simply do not understand: Figure legend 1 (and all other instances throughout the manuscript); you

state that the results were normalized to trypan blue data/number of cells'. sAPPα is a secreted fragment, so why do you believe that it should be normalized to the 'number of cells'? Or did you include both blue and non-blue cells in your 'number of cells'. In panel (B), FL-APP would be membrane-bound regardless of membrane-integrity, so probably a better indication of 'number of cells'. Therefore, wouldn't it be more sound to 'normalize' your sAPPα levels to those of FL-APP? In fact, you would have had a completely different outcome if you would have 'normalized' your data to MTS values rather than to the TB values...so, why choose the one over the other as MTS and TB would BOTH be giving an indication of health/number of cells? This comes back to my comment above about trying to shoehorn data into an existing paradigm rather than interpreting what the cells are actually telling you.

• Line 261 regarding the sAPPβ data; would require some information on this antibody; how would it differentiate between FL-APP, sAPPα and sAPPβ? For example, your sAPPβ bands in Figure 2 appear to migrate very close in size to FL-APP in Figure 1; same for Figure 3 and figure 5 (they look exactly the same). In our experience, sAPPβ migrates at 95 kDa or below, and certainly far below FL-APP. With these higher molecular weight fragments, the difference in kDa is not simply a reflection of the loss of the CTF, but also significant differences in post-translational modification(s), all of which would be important for processing through BACE1, ADAM10 etc..

• Line 289: Ab levels don't match sAPPβ changes in the corresponding figure...also, did you check Aβ levels within cell lysates? Is it possible that DCA inhibits the transport/secretion of Aβ peptides out of the cell and into the medium? If it does, this would give the same pattern (i.e. a decrease in Aβ detection with increasing concentrations of DCA), but would lead to an entirely different conclusion/mechanism.

• Figure 3, Line 315: no sAPPβ/sAPPβ at 695 (or is it 751/770)? Wouldn't this be an important observation to make/address? In fact, from what I can see, you still have FL-APP695 and sAPPβ695 (just above the marker as in Fig 1), but no 751 in either of these; however, you have a load of sAPPβ751, but very little sAPPβ695...what is this telling you?

• Line 328, 'fold-change': I would strongly suggest that you use 'percentage increase' as that is consistent with all of your previous quantifications. Also, there is some debate as to what 'fold change' actually means as many folks suggest a fold-change is exponential (as in folding a piece of paper...you end up with 2, 4, 8, 16 squares with 1, 2, 3, 4 folds, respectively. Better to avoid any confusion and stick with '%'.

• Line 333: a 500% increase in sAPPα (line 328) corresponds to a 79% decrease in sAPPβ and a 34% decrease in Aβ peptides? How do you reconcile this huge discrepancy in proportional change(s)?

• Section title, Line 349: 'APP', is it human? wildtype? Also in title (and subsequent text), 'ablation' usually refers to removal of tissue and in animal experiments it is the destruction of neuronal pathways etc...I would suggest 'reverses' or 'rescues' or 'mitigates' (all fine, and overused, alternatives).

• Line 363: the increases in CTFs with DCA would suggest inhibition of PS-1/γ-secretase...

• Line 399-401: The comment on BACE1 is unclear. It is a rate-limiting enzyme on APP processing, but I would expect to see a bit more processing of APP in an overexpressed model. Relevant ref: J H Stockley, C O'Neill (2008) Understanding BACE1: essential protease for amyloid-beta production in Alzheimer's

disease. Cell Mol Life Sci 65(20): 3265-89.

• Line 403, Stable expression of BACE1: why choose to overexpress BACE1? did you show in any previous lysates that endogenous BACE1 expression was altered? Also, Findlay etal (2015) have shown that Pyruvate dehydrogenase activity (PDH) is reduced in BACE1 overexpressing cells (they also used SH-Sy5ycells)...wouldn't that be a confound?

• Line 437: there were no CTFs, even with detectable FL-APP and overexpressed BACE1? Is this not a bit unexpected? Also, there is significant sAPPα, but no C83-CTF???

• Discussion: The discussion was (primarily) a statement of the results. Some lines (541-543) actually present data(!). I think the manuscript would benefit greatly by a bit more discussion on mechanism. For example, again, if BACE1 was the main event, then its overexpression would rescue/alter the phenotype, which is not the case...therefore, what other mechanistic options are available? inhibition of Aβ transport? BACE1 cleaving at Aβ peptide at residue 11 (which would result in C89-CTF)? Inhibition of PS-1/GS?

• Also, why not discuss how mitochondrial metabolism could be tied into DCA-mediated phenotypes and then relate to the Warburg effect (that has been tied in with cancer, but more and more with AD).

• I think the issue here is that the Western blots do not conclusively identify the various fragments. In many cases, e.g. CTFs, it would benefit to immunoprecipitate CTFs with a C-terminal-directed APP antibody and probe for 6E10 (for C99) or 4G8 (for C89/C83). The same argument applies to the larger fragments. sAPPα and sAPPα can be definitively differentiated by immunoprecipitation protocols; just looking at where a band is on Western blot does not tell you which ‘fragment’ it is as it could be another fragment that has distinct PTMs. Also, why 'demonstrated' lack of changes in ADAM10, BACE1, PS1 using qPCR, which only speaks to transcription and not translation to the functional unit, e.g. the protein. Why didn't you do WB for BACE1, ADAM10, and PS-1 in your lysates? It would have been more meaningful and more informative.

• There are some very clear patterns that emerge from this study, but I am concerned that the interpretation of these patterns has been driven by a need to fit the data into canonical amyloidogenic and non-amyloidogenic APP processing scenarios, when the data could have easily been interpreted based on Aβ transport or γ-secretase inhibition (in some/many instances).

• The conclusion is weak. After all of this work and the interesting observations made, to simply re-cap the title does not do the work justice. Also, I am not sure how your experimental data support the very last statement. You did not measure energetics; rather you measured APP fragments. The conclusion should be focused on APP processing and perhaps expand a bit more on clinical relevance, utility, in comparison to current practice/diagnosis/marker(s)?