Facile Fluorescence-Based Detection of PAD4 Mediated Citrullination

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Background Information:

Objective

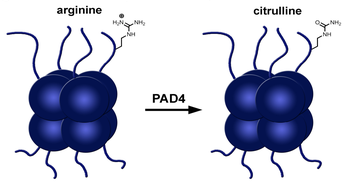
Due to PAD4's overexpression in several types of cancer and autoimmune diseased tissues, there has been a call to develop an effective small molecule inhibitor to use as a potential cancer therapeutic. Research in this field is currently limited by the lack of an effective assay for PAD4 activity. We propose an improved assay which is fast, facile, and fluorescence-based to test for PAD4 activity. By using a small-molecule mimic of PAD4's natural substrate, the assay will then take advantage of the protease trypsin, to monitor PAD4 activity, ultimately leading to the fluorescence output.

Histones

- Assist in DNA condensation and packaging
- A core of eight proteins (H2A, H2B, H3, and H4) provide a scaffold for DNA to wrap around
- Histone core proteins each contain a "tail" domain
    - This domain is heavily post-translationally modified
    - This reversible class of modifications is called epigenetics and is known to play a role in gene expression[1]

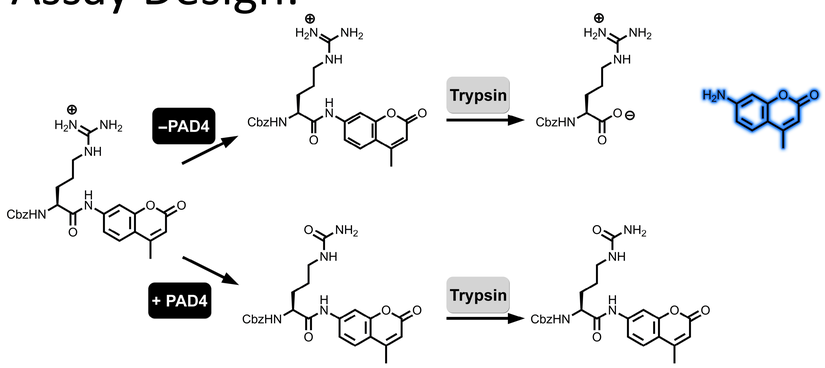
PAD4

- Part of the Protein-Arginine Deiminase enzyme family[2]
- Converts peptidyl arginine to peptidyl citrulline, often on the histone tail[2]



- PAD4 was found to be overexpressed in several malignant tumors, including breast, lung, and bone cancers, but its presence in non-tumor tissue was undetectable[3]
- PAD4 knockdown in tumor cells was sufficient to induce cell death[4]
- Few effective PAD4 inhibitors are known, however, treatment with the most the most potent PAD4 inhibitor available was able to cause up to 70% tumor shrinkage[4]
- Current inhibitor research is limited by the availability of an effective assay that could be utilized for small molecule library screenings; we propose an assay that is simple, fast, requires few reagents, and gives reliable data when preformed in a 384-well plate

Assay Design:



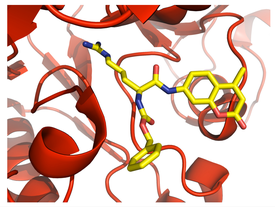
- Incubation of **Z-Arg-AMC** with PAD4 results in conversion to **Z-Cit-AMC**
- Incubation without PAD4, or if PAD4 has been inhibited, results in no conversion (remains **Z-Arg-AMC**)
- The reaction mixture is then incubated with an excess of the protease trypsin
    Trypsin cleaves the peptide amide bond and is selective for arginine residues over citrulline
- When PAD4 has not been added, or when PAD4 has been inactivated by an inhibitor, trypsin is able to cleave the amide bond, subsequently unmasking the fluorophore
- Active PAD4 yields a citrulline product, which is not recognized by trypsin, causing an unchanged fluorescence reading

Results:

Substrate Design:

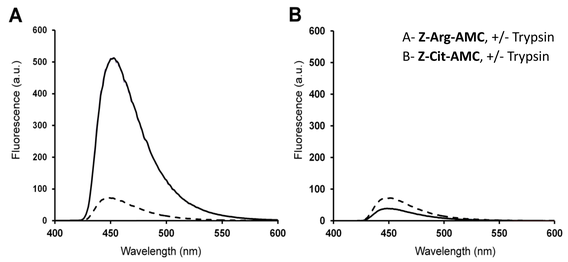
Will the substrate analog designed be accommodated in PAD4's active site?

*The overlay of our substrate analog with an available crystal structure of PAD4 shows that the active site of PAD4 is on the exterior of the protein PAD4 will have adequate room to interact with our designed substrate, **Z-Arg-AMC.***



Fluorescence Emission Verification:

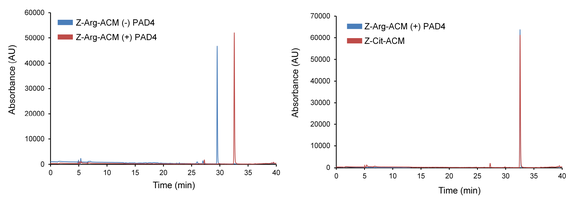
Will trypsin be selective for arginine over citrulline?



*The data from the **Z-Arg-AMC** (A) shows that addition of trypsin increases fluorescence emission over the background signal by ~6.5 fold. Furthermore, data from **Z-Cit-AMC** (B) proves Trypsin's specificity for arginine.*

Substrate Modification Verification:

Will the **Z-Arg-AMC** be a suitable substrate for PAD4? Does PAD4 in fact modify **Z-Arg-AMC** to the anticipated product, **Z-Cit-AMC**?

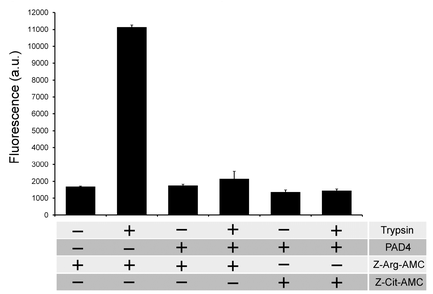


*The HPLC data shows that the **Z-Arg-AMC** is modified to a less polar product after incubation with PAD4, consistent with a conversion from arginine to citrulline (left). Furthermore, we see that the substrate is fully converted into the product and the retention time of the product exactly matches that of our anticipated product, **Z-Cit-AMC** (left). This indicates that **Z-Arg-AMC** is in fact a suitable substrate for PAD4.*

PAD4 Activity Assay:

Does the assay to report the activity of PAD4 in a 96-well plate format?

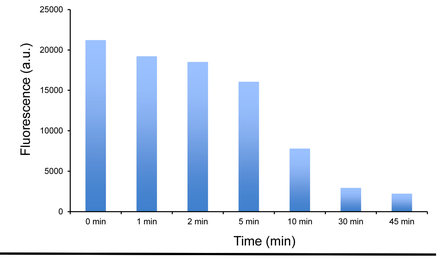
*All previous tests were done in test tubes, The data shows that the assay could be completed in a 96-well plate, using a plate reader to measure fluorescence emission. Furthermore, this test assay showed a high signal to noise ratio, as well as providing the expected results with the **Z-Arg-AMC** vs. **Z-Cit-AMC** substrates. Finally, the data was highly reproducible.*



Time Course Experiment:

Can PAD4 reaction be completed within a reasonable time frame?

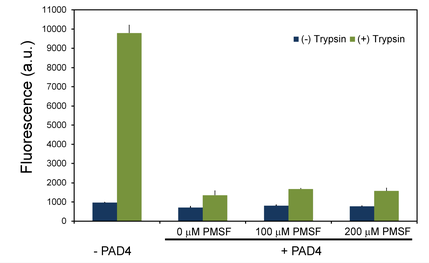
The results of the time course data shows that the PAD4 reaction is nearly completed after 30 minutes; additional incubation time does not significantly decrease the signal.



Effects of a Trypsin Inhibitor:

Will incubation with a trypsin inhibitor result in a false negative signal?

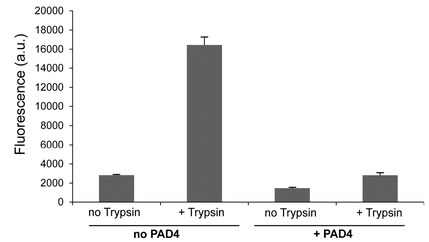
One concern when using an assay dependent on a secondary enzyme is false negatives, where the small molecule inhibits trypsin. We used a known trypsin inhibitor (PMSF) to show that the amount of trypsin being used in the system is enough to still allow the assay to work.



Assay Miniaturization:

Can the assay be miniaturized for use in a 384-well plate?

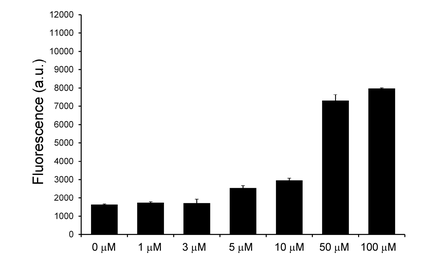
Most small library screening are robotic and use a 384-well plate rather than a 96-well plate. We ensured that the assay could be miniaturized to be used for its intended application.



Testing a known PAD4 Inhibitor:

Does the assay properly report on the introduction of a known PAD4 inhibitor?

The assay was tested by pre-incubating PAD4 with various concentrations of a known PAD4 inhibitor, Cl-Amidine[5].With the addition of 50 μM Cl-Amidine, fluorescence emission signals had significantly risen, indicating that the assay was successful.



Conclusion:

We have developed a novel rapid and facile fluorescence-based assay of PAD4 which relies on the re-design of an arginine substrate, whose fluorescence is controlled by the reaction of the substrate with PAD4. The citrullination reaction by PAD4 leads to a change in the level of blue fluorescence, yielding a reliable read-out of PAD4 activity. We plan to apply our novel assay format for the screening of large small molecule libraries for potent and specific PAD4 inhibitors. Additionally, we will explore the use of our trypsin-linked assay in the context of a histone-mimetic peptide in an attempt to develop isozyme-specific probes. Finally, we plan to develop a live cell assay for PAD4 by implementing similar elements to the assay described here.

References:

(1) Kouzarides, T. Cell 2007, 128, 693.
(2) Vossenaar, E. R.; Zendman, A. J.; van Venrooij, W. J.; Pruijn, G. J. *Bioessays* **2003**, *25*, 1106.
(3) Mohanan, S.; Cherrington, B. D.; Horibata, S.; McElwee, J. L.; Thompson, P. R.; Coonrod, S. A. *Biochem. Res. Int.* **2012**, *2012*, 895343.
(4) Wang, Y.; Li, P.; Wang, S.; Hu, J.; Chen, X. A.; Wu, J.; Fisher, M.; Oshaben, K.; Zhao, N.; Gu, Y.; Wang, D.; Chen, G. *J. Biol. Chem.* **2012**, *287*, 25941.
(5) Luo, Y.; Arita, K.; Bhatia, M.; Knuckley, B.; Lee, Y. H.; Stallcup, M. R.; Sato, M.; Thompson, P. R. *Biochemistry* **2006**, *45*, 11727.