Calcium-dependent Hit Identification for PAD4 Inhibitors with FDA-approved and Fragment-based Library Approaches

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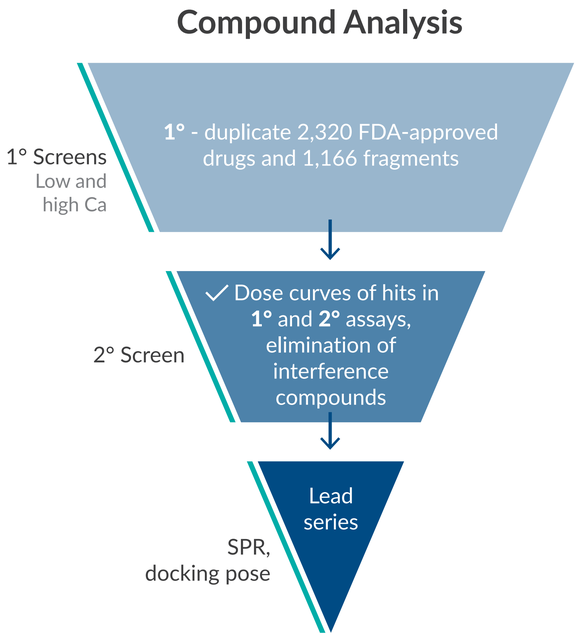
KEY FINDING

Through optimization of assay conditions, both fragment-based and small molecule library screening approaches identified calcium-dependent PAD4 inhibitors for immunomodulator development.

INTRODUCTION

PAD4 catalyzes in a calcium-dependent manner the conversion of arginine to citrulline with the elimination of ammonia. In cells, this generates the post-translational modification of arginine-containing proteins to citrullinated proteins. When the target is histones, the positively charged arginine is replaced with neutral citrulline, which regulates gene expression in response to PAD4 activation. This process is of great importance for immune cell differentiation and neutrophil extracellular trap formation. Increased inflammation associated with citrullinated antigens, such as fibrinogen and collagen, are implicated in autoimmune diseases. PAD4 inhibitors may also be useful in disease states with destructive inflammatory drivers, such as stroke.

In this high-throughput screening (HTS) program, we ran screens under two different conditions in order to assess the calcium sensitivity of PAD4 inhibitor fragments and compounds. The resultant assays maintained requirements for high-throughput screening in a 384-well format (Z' > 0.5) and in comparison allowed for identification of calcium-sensitive and -insensitive compounds for further development.



HIGH-THROUGHPUT PRIMARY SCREENING METHODS AND RESULTS

Our objective was to optimize our workflow for performance of a HTS of 2,320 FDA-approved small molecule drugs and a 1,166 fragment compound library using an assay for PAD4 activity. The primary screen involved chemical detection of ammonia with *o*-phthalaldehyde in both low and high calcium conditions. This allowed for determination of the calcium sensitivity of inhibition for mechanism evaluation and future SAR studies. We developed workflows for both 1° screening at a single drug concentration, to be followed by cherry-picking of hits for dose curves in the 1° and 2° screens to eliminate interference compounds, including reactive primary amines in the ammonia assay.

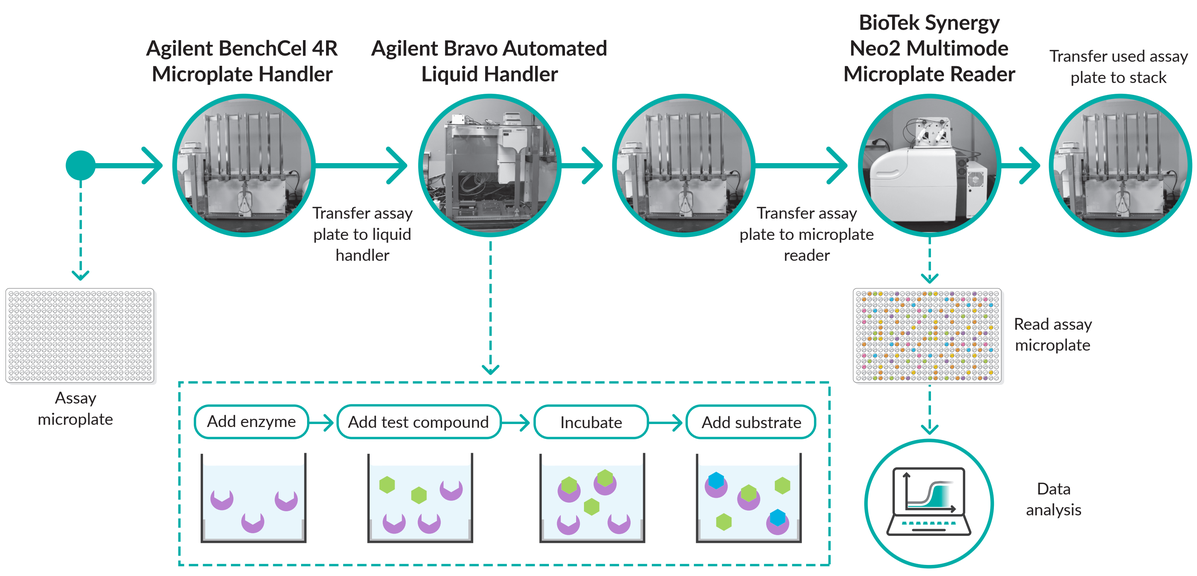


FIGURE 1 – Workflow for PAD4 treatment and readout.

PAD4 was incubated with 2,320 FDA-approved compounds at 10 µM from 8 × 384-well library stock plates in DMSO, or 1,166 fragments at 500 µM from 4 × 384-well library stock plates in DMSO. Substrate with low (0.1 mM) or high (10 mM) calcium treatment was then added. Plate readout was obtained after incubation with either *o*-phthalaldehyde for ammonia 1° assay or 7-amino-4-methylcoumarin (AMC)-substrate digest in 2° assay. CI-Amidine control was used as an irreversible PAD4 inhibitor. Note that CI-amidine reactivity with PAD4 is calcium dependent. GSK484 was used as a potent reversible PAD4 inhibitor control.

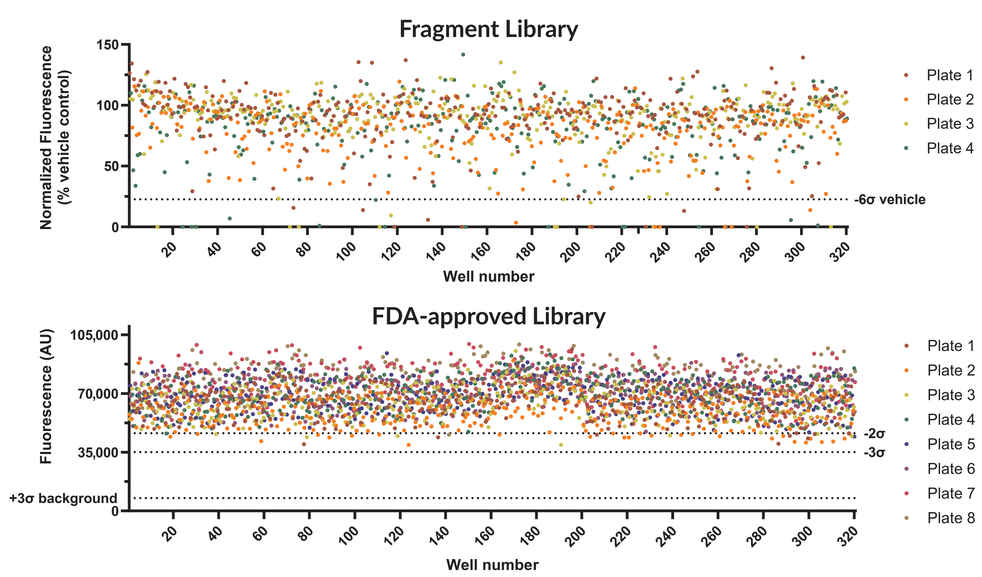


FIGURE 2 – Dot plot of 1° screen hits.

Cutoffs for PAD4 inhibitors were set relative to the inhibitor control (180 µM CI-amidine). The sample values for the fragment library are normalized to the average vehicle signal across the full dataset. The fragment library produced verified hits only with low calcium, whereas the larger molecules in the FDA-approved library identified a true hit in both calcium formats.

HIT CONFIRMATION AND CHARACTERIZATION OF COMPOUNDS

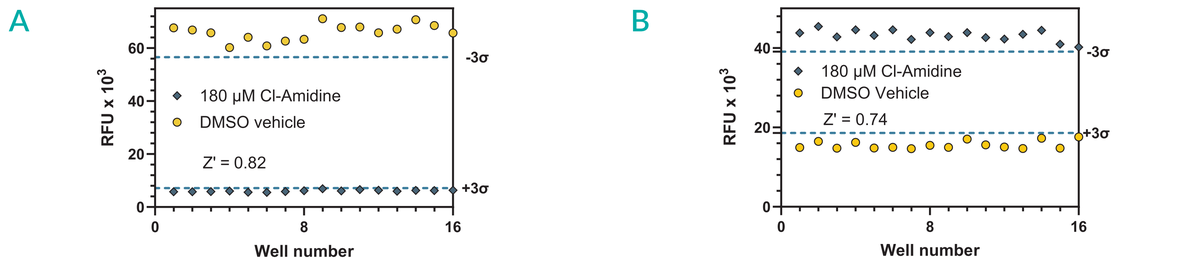


FIGURE 3 – Assay performance for 1° and 2° screening.

To confidently screen and produce dose curves with the 1° and 2° assays in a 384-well format, we verified that assay controls achieved Z' values greater than 0.5. **A)** Example control values from the 1° screen with high calcium. **B)** Example control values from the 2° screen with high calcium.

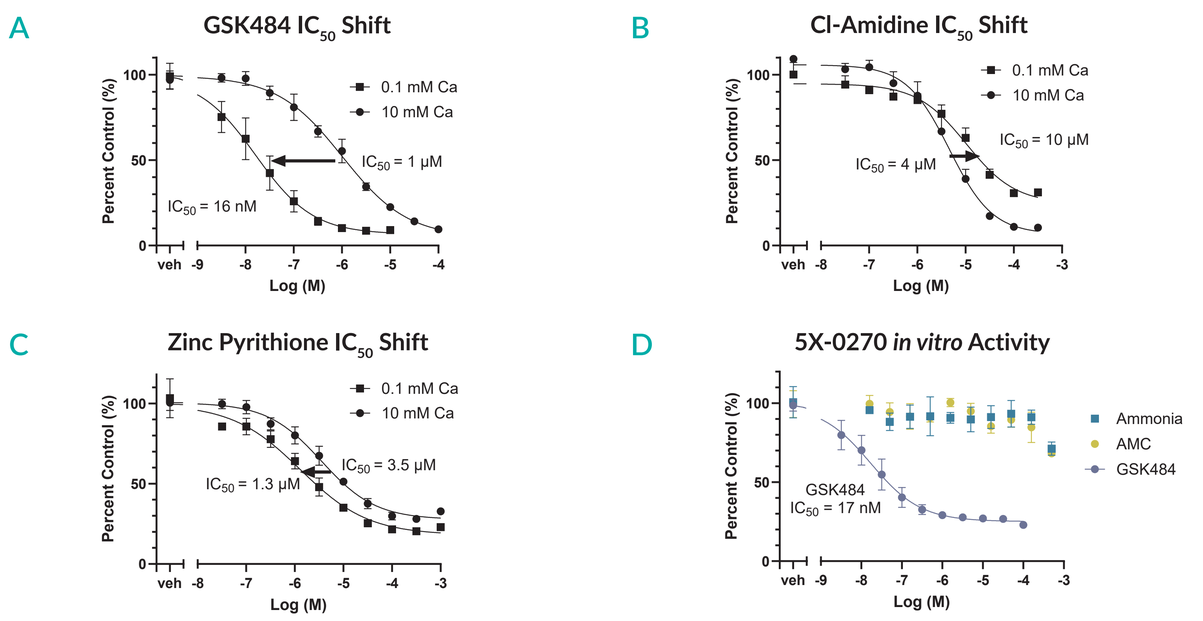


FIGURE 4 – Dose curves of verified hit compounds and example fragment.

We performed dose curves in triplicate for compounds with verified activity in the 1° and 2° screens. Calcium-dependent IC_{50}s are shown in the ammonia assay for: **A)** GSK484, which shows over 50-fold decrease in IC_{50}. **B)** CI-Amidine irreversible control compound. Note that calcium dependence is reversed for CI-amidine, due to the inactivation mechanism being dependent on calcium.[1] **C)** Zinc pyrithione with a minor reduction in IC_{50}. **D)** 5X-0270 fragment in ammonia and AMC assay with low calcium. Fragment compound inhibition became detectible in low calcium assays in the high µM range.

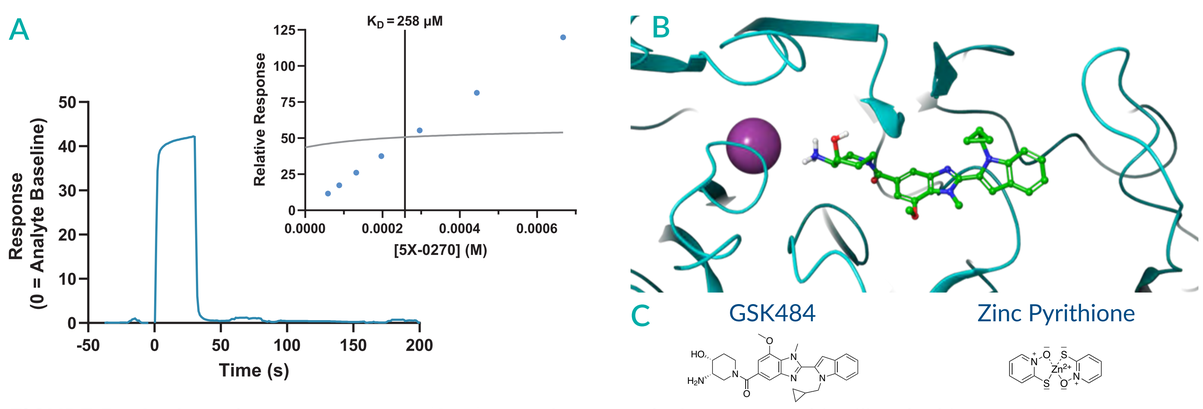


FIGURE 5 – Surface plasmon resonance (SPR) trace for example fragment and *in silico* docking to PAD4.

Binding levels from the affinity screen of the fragment library against PAD4 identified shared compounds with the PAD4 enzyme activity screen. **A)** Example fragment 5X-0270 SPR binding trace to PAD4 with affinity plot inset, demonstrating high µM binding. **B)** GSK484 docked to the calcium-bound form of PAD4.[2] **C)** GSK484 and zinc pyrithione compound structures show core structural similarity.

CONCLUSION

- PAD4 activity assays can be optimized for HTS across a wide range of calcium concentrations.
- The use of low and high calcium screens allowed for segregation of activity for validation of mechanism of action for small molecule inhibitor development and to promote sensitivity for the fragment library screen.
- Future *in vivo* analysis will allow for evaluation of calcium-dependent and -independent inhibitors for selection of appropriate tools for disease studies.

References

1. Lewis, H.D., Liddle, J., Coote, J.E., *et al.* Inhibition of PAD4 activity is sufficient to disrupt mouse and human NET formation. *Nat. Chem. Biol.* **11(3)**, 189-191 (2015).
2. Lewis, H.D., Bax, B.D., Chung, C.-W., *et al.* Crystal structure of human peptidylarginine deiminase type4 (PAD4) in complex with GSK147. *Worldwide Protein Data Bank.* (2015).

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