

Supporting Information for:

A Fluopol-ABPP HTS Assay to Identify PAD Inhibitors†‡

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Running Title: Fluopol-ABPP HTS assay for PAD inhibitors

Supplementary Methods

Chemicals. Tris(2-carboxyethyl)phosphine (TCEP), dithiothreitol (DTT), and benzoyl-arginine ethyl ester (BAEE) were acquired from Sigma-Aldrich (St. Louis, MO). The synthesis of RFA has previously been described (12). Recombinant human PAD4 was expressed and purified as previously described (9). NIH compounds (1-10) were purchased from Ryan Scientific (Mount Pleasant, SC).

fluopol-ABPP HTS optimization. The optimized assay time and concentration for the fluopol-ABPP screen were determined by monitoring the Z' value under various conditions. Briefly, 10 μL of PAD4 (0.25, 0.50, 1.0, and 2.0 μM final), PAD4 C645A (2 μM), or no enzyme was incubated in Assay Buffer (50 mM HEPES pH 7.6, 150 mM NaCl, 10 mM CaCl_2 , 0.01% Pluronic F-127, and 1 mM TCEP), and then aliquoted into the wells of a 384 well microplate. The plate was then incubated for 30 min at 37 °C before the addition of RFA (75 nM final). The fluopol signal was then measured at various time points (0, 120, 360, 1440, 2880 min) using an Envision plate reader (Perkin Elmer; Ex 535, Em 595). The Z' value was determined for each concentration of PAD4 at the individual time point. The Z' value was calculated using equation 1,

$$Z' = 1 - [(3 * (\sigma_p + \sigma_n)) / (|\mu_p - \mu_n|)] \quad \text{eq.1,}$$

where σ is the mean, and μ is the standard deviation of the positive (p) and negative (n) controls.

Fluopol-ABPP HTS methods. The PAD4 fluopol-ABPP screen was performed in a 384-well microplate. 10 μL of PAD4 (2 μM final) in Assay Buffer (50 mM HEPES pH 7.6, 150 mM NaCl, 10 mM CaCl_2 , 0.01% Pluronic F-127, and 1 mM TCEP) was added to each of the test and negative control wells. For positive control wells, only 10 μL of assay buffer was added.

Compounds, and DMSO as a control, were added to the wells by pintoole. The plates were then incubated at 37 °C for 30 min, at which point 1.1 µL of RFA (75 nM final) was added to all wells in the plate. The reactions were then allowed to incubate for 5 h at 37 °C. Plates were then read using an Envision plate reader (Perkin Elmer).

ABPP Gel-based Assay. The ABPP gel-based secondary screen was performed similar to previously described methods (10). Briefly, the compounds identified as ‘hits’ using the Fluopole-ABPP HTS were incubated with PAD4 (1 µM) in Assay Buffer (50 mM NaCl, 10 mM CaCl₂, 2 mM DTT, and 100 mM HEPES pH 8.0) for 15 min before the addition of RFA (1 µM). The reaction was allowed to react in the dark for 30 min at 37 °C, quenched with 6X SDS-PAGE loading buffer, boiled, and run on a SDS-PAGE gel. The fluorescence was measured using a Typhoon 9410 (GE Healthcare) and fluorescence intensity was determined using Image Quant.

IC₅₀ studies. IC₅₀ values were determined according to previously established methods that monitor changes in citrulline production (5-6, 12). All measurements were made in duplicate and initial velocity values generally agreed to within 20%. IC₅₀ values were determined by fitting the data to equation 2,

$$\text{Fractional Activity} = 1/(1 + [I]/IC_{50}) \quad \text{eq. 2,}$$

using GraFit version 5.0.11. (15). IC₅₀ is defined as the concentration of inhibitor that corresponds to 50% activity; [I] is the concentration of inhibitor.

PAD Inhibition Assay. NIH compounds 3-9 were tested for % inhibition with PADs 1-3. Each compound (20 µM final) was preincubated with enzyme (PAD1: 0.5 µM, PAD2: 0.5 µM, PAD3:

0.2 μM) for 15 min in a reaction buffer containing, 10 mM CaCl_2 , 2 mM DTT, 50 mM NaCl, and 100 mM Tris pH 7.6. Reactions were initiated with BAEE (10 mM), and allowed to proceed for 15 min. Citrulline formation was measured according to previously established methods (3, 16).

Inhibitor Bioavailability. MCF-7 cells ($\sim 5 \times 10^5$) were added to each well of a 12-well plate in DMEM phenol red free media containing 10% charcoal stripped FBS. Cells were incubated in the plate at 37 °C, 5% CO_2 . After 48 h, the media was removed and replaced with Locke's solution (0.15 M NaCl, 5 mM KCl, 5 mM HEPES, 2 mM CaCl_2 , 0.1% glucose pH 7.3). Cl-amidine (10 μM) or streptonigrin (1 nM, 10 nM, and 100 nM) were added to the cells and incubated for 15 min before addition of estrogen (0.1 μM) and incubated for an additional 15 min at 37 °C, 5% CO_2 . Cells were rinsed with cold PBS and lysed with SDS lysis buffer (2% SDS, 62.5 mM Tris pH 6.8, and 10% glycerol).

HL-60 cells (1×10^6 ml/cell) were treated with all-trans retinoic acid (1 μM) for 48 h at 37 °C, 5% CO_2 to induce the differentiation of these cells into granulocytes. Cells were split into 12-well plates and treated with 2 mM CaCl_2 and either Cl-amidine (10 μM) or streptonigrin (1 nM, 10 nM, and 100 nM final). After 15 min at 37 °C, 5% CO_2 , the calcium ionophore, A23187 (4 μM final) was added. Cells were harvested after 15 min, rinsed with cold PBS, and lysed with SDS lysis buffer. Proteins were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane for western blot analysis. The levels of histone H3 and citrullinated histone H3 were detected using either a polyclonal anti-Citrulline H3 antibody (Abcam, ab5103) or polyclonal anti-Histone H3 antibody (Abcam, ab1791).

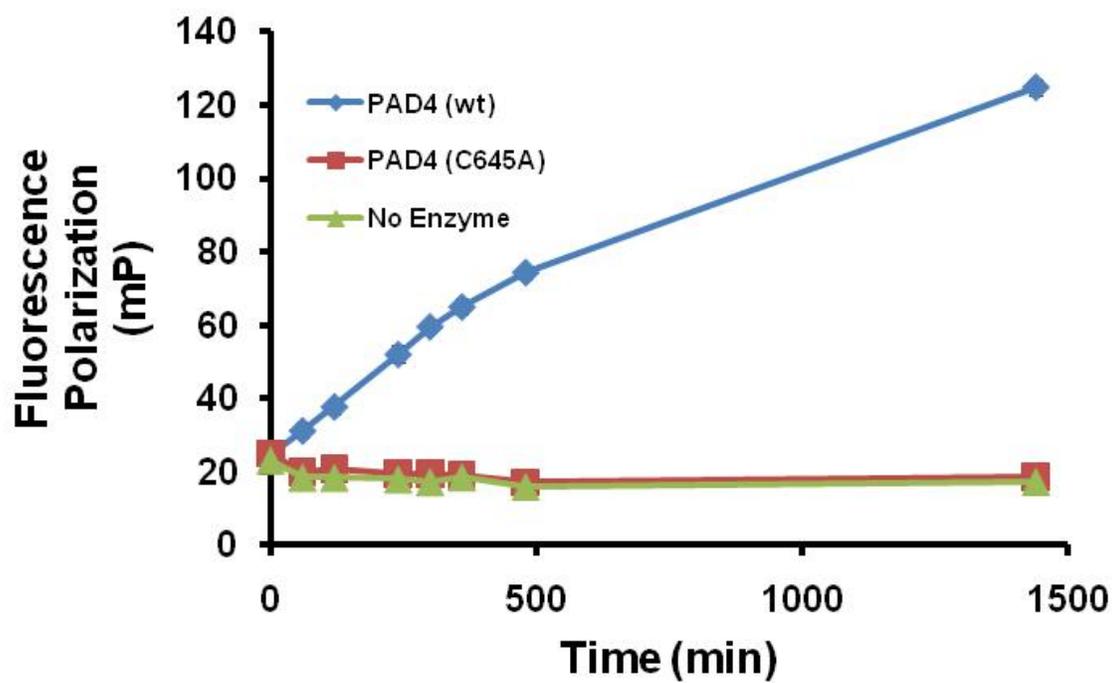
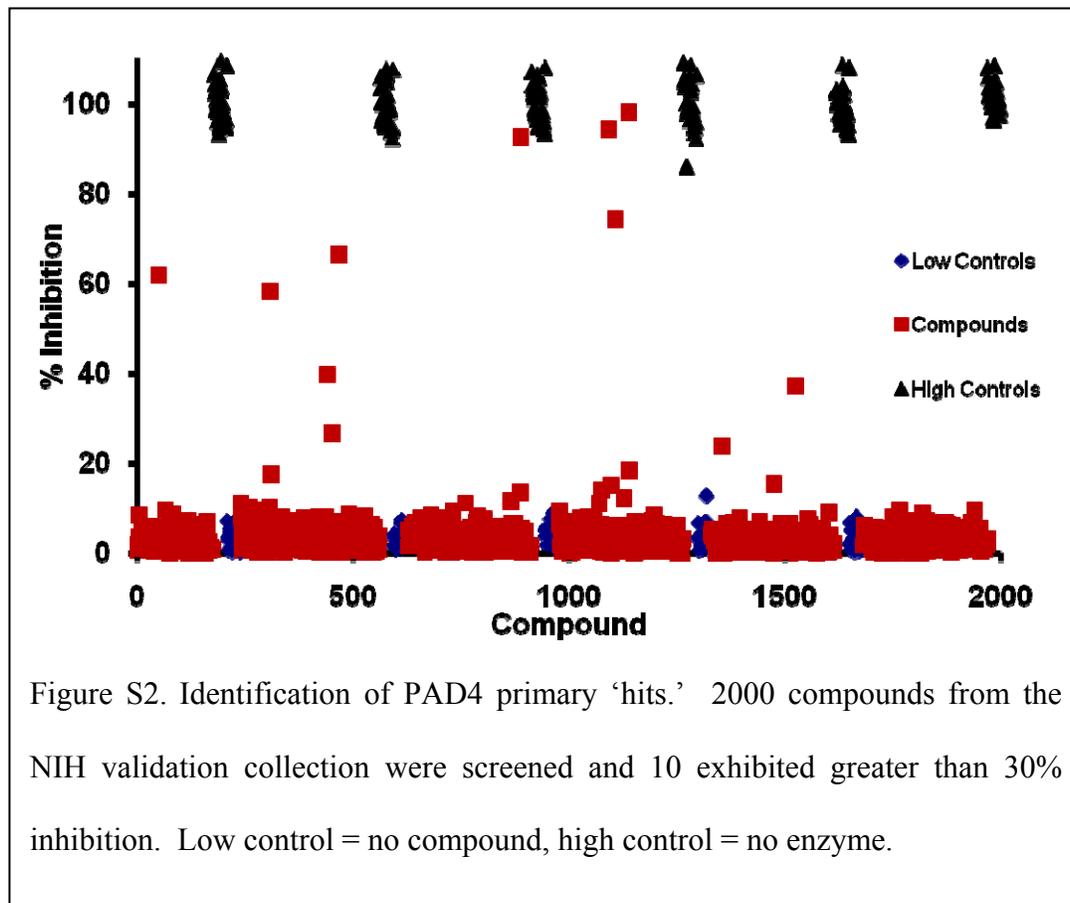


Fig. S1 RFA reacts with PAD4 in a time dependent manner. A strong, time-dependent increase in fluopol was observed at 300 min.



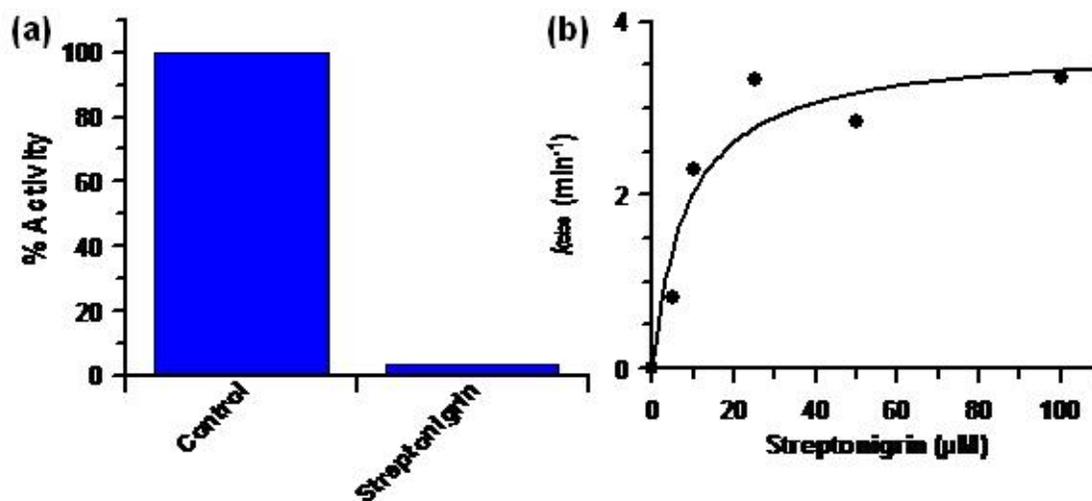


Fig. S3. Streptonigrin is an irreversible PAD4 inactivator. (a) The preformed PAD4•streptonigrin complex was dialyzed for 20 h and the % activity remaining determined. (b) Plot of the pseudo-first order rate constants of inactivation, i.e. k_{obs} , versus streptonigrin concentration.

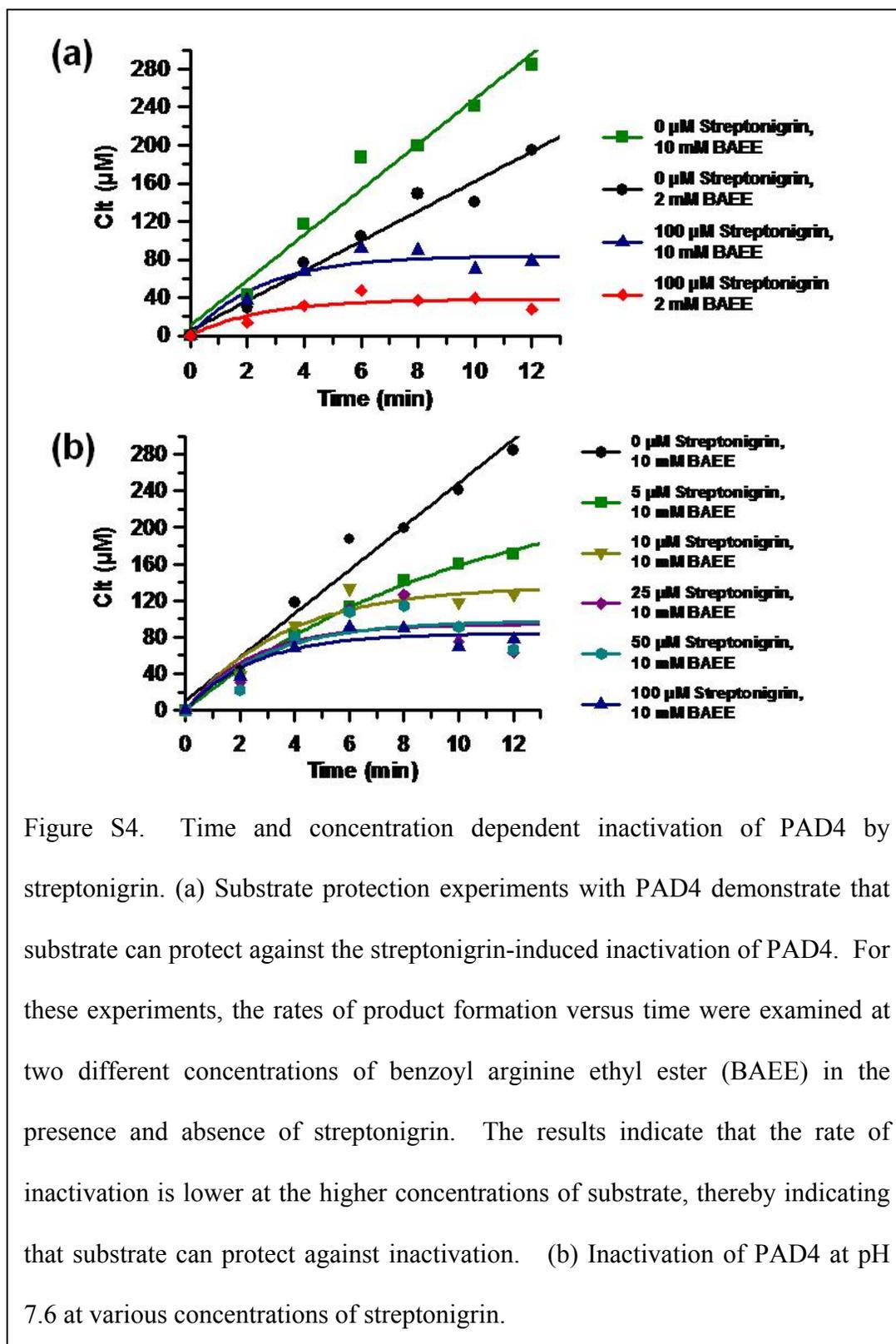


Figure S4. Time and concentration dependent inactivation of PAD4 by streptonigrin. (a) Substrate protection experiments with PAD4 demonstrate that substrate can protect against the streptonigrin-induced inactivation of PAD4. For these experiments, the rates of product formation versus time were examined at two different concentrations of benzoyl arginine ethyl ester (BAEE) in the presence and absence of streptonigrin. The results indicate that the rate of inactivation is lower at the higher concentrations of substrate, thereby indicating that substrate can protect against inactivation. (b) Inactivation of PAD4 at pH 7.6 at various concentrations of streptonigrin.

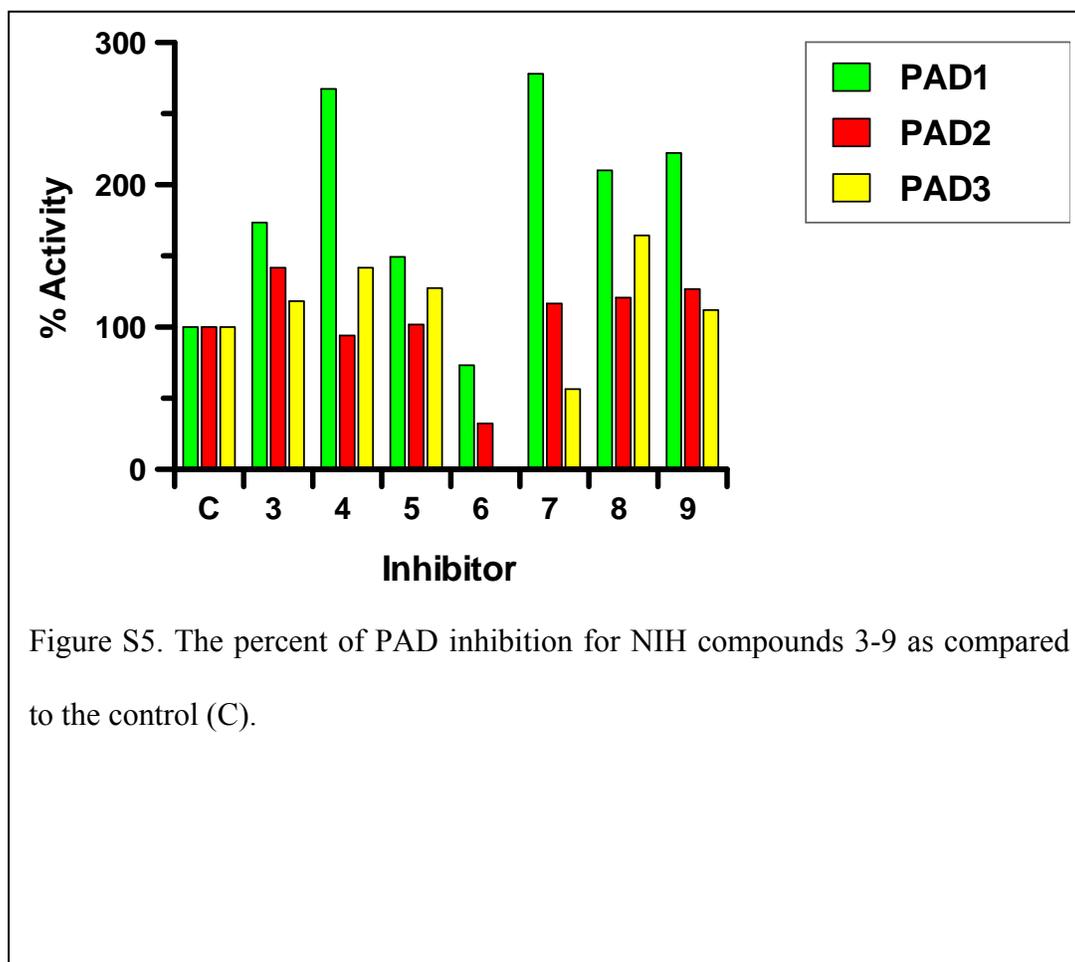


Figure S5. The percent of PAD inhibition for NIH compounds 3-9 as compared to the control (C).