Supporting Information for

A Fluoro-Acetamidine Based Inactivator of Protein Arginine Deiminase 4 (PAD4): Design, Synthesis, and in vitro and in vivo Evaluation

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Calcium dependent inhibition experiments:

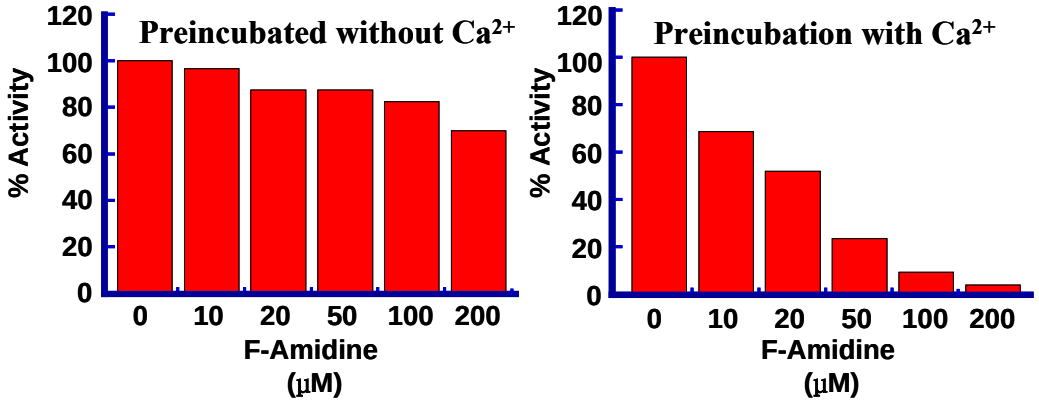


Figure S1. IC_{50} data for F-Amidine, which was preincubated with PAD4 in the absence or presence of 10 mM Ca^{2+}.

Substrate protection experiments:

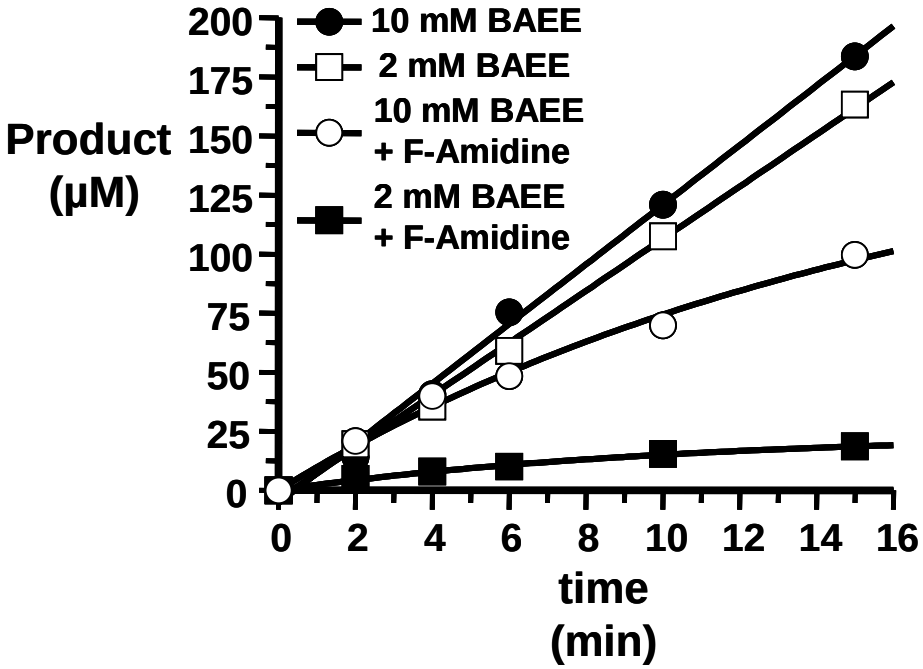
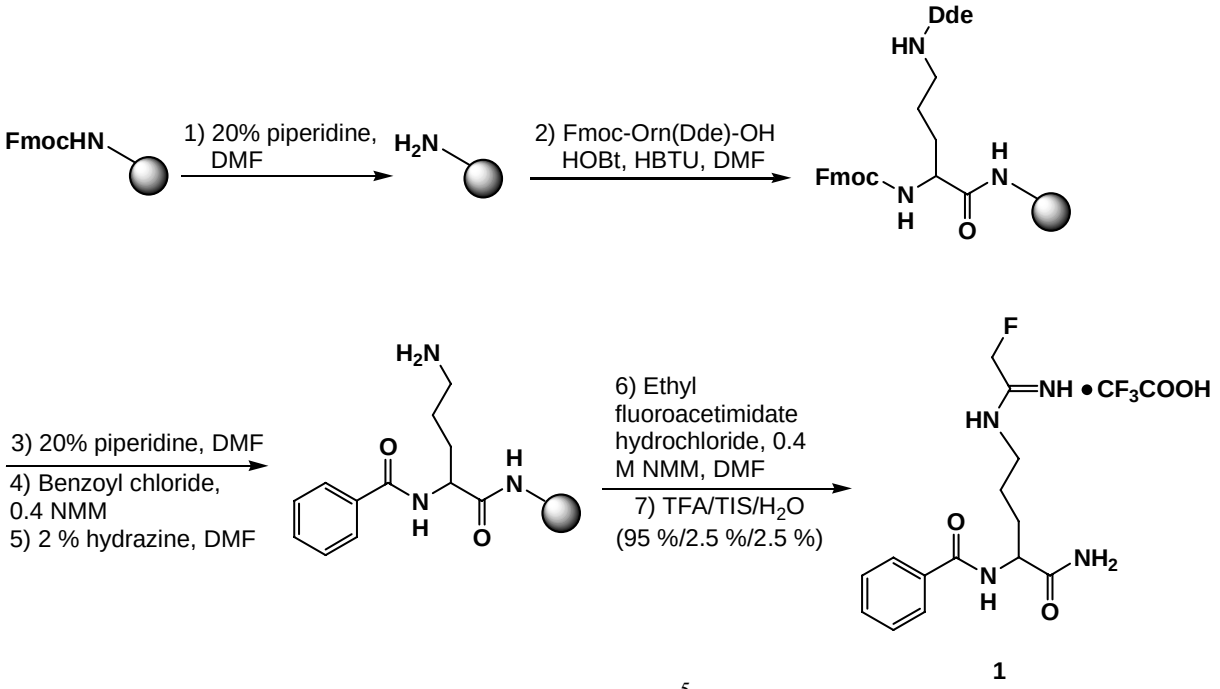


Figure S2. Substrate protects against F-Amidine inactivation of PAD4. Plots of product formation versus time in the absence or presence of 600 μM of F-Amidine were performed at two different concentrations of BAEE, i.e. 2 and 10 mM BAEE. Linear fits of the data obtained in the absence of F-Amidine are depicted; whereas the data obtained in the presence of F-Amidine were fit to equation 2 (see supplementary methods for details).

Materials and Methods

Synthesis of N-α-benzoyl-N^5-(2-fluoro-1-iminoethyl)-L-ornithine amide trifluoroacetic acid (F-amidine, 1)



Scheme S1. Solid-phase synthesis of N-α-benzoyl-N^5-(2-fluoro-1-iminoethyl)-L-ornithine amide trifluoroacetic acid (F-amidine, **1**).

1) Removal of Fmoc group from resin. Rink Amide AM Resin (300 mg, 0.186 mmol, 1 equiv.) was pre-swelled in 5 ml DMF for 1 h. DMF was then filtered away and the resin was washed twice with DMF (5 ml each time). 5 ml of 20 % piperidine (in DMF) was added to the resin and the suspension was rocked gently at room temperature for 20 min. This treatment was repeated once. Resin beads were washed three times with DMF (5 ml each time).

2) Coupling of protected ornithine to resin. Fmoc-Orn(Dde)-OH (386 mg, 0.744 mmol, 4 equiv.), HOBt (114 mg, 0.744 mmol, 4 equiv.) and HBTU (282 mg, 0.744 mmol, 4 equiv.) were dissolved in 2.3 ml DMF. This mixture was let stand at room temperature for 10 min before N-

methylmorpholine (0.4 M in DMF) (3.7 ml, 1.488 mmol, 8 equiv.) was added to it. The resin was incubated in this new mixture at room temperature with gentle rocking for 3 h. After the solution was filtered away, the resin was washed three times with DMF (5 ml each time).

3) Removal of Fmoc group from resin-bound ornithine. 5 ml of 20 % piperidine (in DMF) was added to the resin and the suspension was rocked gently at room temperature for 20 min. This treatment was repeated once. The resin was then washed three times with DMF (5 ml each time).

4) Functionalization of α-NH_2 of resin-bound ornithine. A mixture of benzoyl chloride (105 mg, 0.09 ml, 0.744 mmol, 4 equiv.) and N-methylmorpholine (0.4 M in DMF) (1.488 mmol, 8 equiv.) was added to the resin. The suspension was rocked gently at room temperature overnight (16 h). After the solution was filtered away, the resin was washed three times with DMF (5 ml each time).

5) Removal of Dde group from resin-bound ornithine. The resin was incubated in 5 ml of hydrazine (2 % in DMF) for 2 h and then washed three times with DMF (5 ml each time), two times with ethanol and two times with methylene chloride. After being dried under vacuum overnight, the resin was used in the next reaction step.

6) Functionalization of δ-NH_2 of resin-bound ornithine. Ethyl fluoroacetimidate hydrochloride (17 mg, 0.124 mmol, readily synthesized according to literature procedure [1]), dry triethyl amine (13 mg, 0.124 mmol) and resin-bound N-α-benzoyl-ornithine (50 mg, 0.027 mmol) from the previous step were mixed in 1 ml dry DMF. This reaction mixture was stirred at room temperature overnight (16 h) under nitrogen. Then the resin was filtered and washed sequentially with DMF, ethanol and methylene chloride.

7) Cleaving final compound from resin. The resin was incubated with a mixture of TFA/TIS/H_2O (95%/2.5%/2.5%) for 3 h with gentle rocking at room temperature before it was filtered and washed with 95% TFA (in ddH_2O) several times. The filtrate and all the washings were combined. From the combined solution, TFA was blown off with flowing nitrogen. To the remaining residue,

cold ether was added to yield a white precipitate. This precipitate was washed twice with cold ether and then lyophilized. Purification with preparative reverse phase HPLC yielded N-α-benzoyl-N^5-(2-fluoro-1-iminoethyl)-L-ornithine amide trifluoroacetic acid (**1**) as white hydroscopic powder upon lyophilization.

*N-α-benzoyl-N^5-(2-fluoro-1-iminoethyl)-L-ornithine amide trifluoroacetic acid (F-amidine, **1**).* ^{1}HNMR (400 MHz, CD_3OD) δ(ppm): 7.90-7.46 (m, 5H), 5.30-5.19 (d, $^2J_{H-F}$ = 45.3 Hz, 2H), 4.65-4.61 (dd, 1H), 3.47-3.36 (m, 2H), 2.03-1.73 (m, 4H). ^{13}CNMR (400 MHz, CD_3OD) δ(ppm): 176.46, 170.32, 164.44-164.25 ($^2J_{C-F}$ = 20 Hz), 135.02, 133.10, 129.64, 128.52, 79.85-78.07 ($^1J_{C-F}$ = 179 Hz), 54.13, 42.98, 30.37, 25.11. ^{19}FNMR (400 MHz, CD_3OD) δ(ppm): -158.03, -158.15, -158.27 ($^2J_{H-F}$ = 45.2 Hz). MS-ES$^+$: 295. HRMS ($C_{14}H_{20}FN_4O_2^+$): calculated 295.1570, observed 295.1569. Elemental analysis ($C_{16}H_{20}F_4N_4O_4$): calculated C, 47.06 %; H, 4.94 %; found C, 44.72 %; H, 4.86 %. Lyophilization of **1,** after reverse phase HPLC purification, results in the formation of the TFA salt. Yield 36.6 %. F-Amidine is stable at physiological pH (pH 7.0), as evidenced by the fact that negligible decomposition is observed even after a 5-day incubation at room temperature; degradation percentage of F-amidine at pH 8.0 and pH 9.0 after a 5-day incubation is 28.2 % and 70.4 %, respectively. Identical results were obtained for incubations performed in the presence of 10 mM β-mercaptoethanol.

Protein purification

PAD4 used in this work was GST-cleaved full-length PAD4 purified in our laboratory. A recombinant human PAD4 *Escherichia coli* expression system that was obtained from the Yamada group was transformed into *E. coli* Rosetta cells (EMD Biosciences) for the expression of full-length PAD4 with an 8-residue N-terminal linker that is fused in frame to a PreScission protease

cleavable GST tag. The predicted molecular mass of PAD4 including this 8-residue linker is 74879 g/mol. The procedure for purifying this enzyme is described in [2].

IC_{50} Assays

IC_{50} values of F-amidine (**1**) were determined with variable concentrations of this compound in a reaction buffer containing 100 mM HEPES (pH 7.6), 50 mM NaCl, and 0.5 mM TCEP. The aforementioned reaction mixture was pre-incubated with PAD4 (0.2 μM) (in the presence or absence of 10 mM $CaCl_2$) at 37°C for 15 min prior to the addition of BAEE (10 mM final concentration) (and 10 mM $CaCl_2$ if $CaCl_2$ was absent in the pre-incubation) to initiate the reaction. After 15 min the reactions were quenched by flash freezing in liquid nitrogen. For color development, 200 μL of freshly prepared COLDER solution (2.25 M H_3PO_4, 4.5 M H_2SO_4, 1.5 mM $NH_4Fe(SO_4)$, 20 mM diacetyl monoxime, and 1.5 mM thiosemicarbazide) was added to each of the quenched reactions, vortexed to ensure complete mixing, and then incubated at 95° C for 30 minutes2,3. The absorbance at 540 nm was then measured and compared to a Cit standard curve to determine the concentration of Cit produced during the course of the reactions. IC_{50} values were determined by fitting the concentration-response data to Eq. (1) using the Grafit™ version 5.0.11 software package [4].

$$\text{Fractional activity of PAD4} = 1/(1+([\text{F-amidine}]/IC_{50})) \quad \text{(Eq. 1)}$$

The concentration of an inhibitor that corresponds to the midpoint (fractional activity = 0.5) was referred to as the IC_{50}.

Time Course Inactivation Assays

Time course experiments were performed in assay buffers containing 2 mM BAEE, 100 mM HEPES (pH 7.6), 50 mM NaCl, 500 μM TCEP and 10 mM $CaCl_2$ in the presence of various

concentrations (0, 150, 300, 600 and 900 μM) of F-amidine. Additional time course experiments were performed in the presence of 10 mM BAEE and 0, 250, 500, and 1000 μM F-Amidine. The assay buffers were pre-incubated at 37°C for 10 min. Reactions were initiated by addition of PAD4 to a final concentration of 0.2 μM. At different time points (0, 2, 4, 6, 10, and 15 min), 60 μl of the reactions was withdrawn and quenched by flash freezing in liquid nitrogen. Color development and absorbance measurement at 540 nm of the samples were done as described in IC_{50} assays. The data obtained were fit to Eq. (2) using the Grafit™ version 5.0.11 software package [4].

$$[P] = v_i [1-\exp(-k_{obs} t)]/k_{obs} \quad \text{(Eq. 2)}$$

where v_i is the initial velocity, k_{obs} is the apparent pseudo-first-order rate constant, and [P] refers to the concentration of citrulline produced during the reaction process. In order to obtain k_{inact}, K_I and k_{inact}/K_I, the apparent k_{obs}'s were multiplied by the transformation $(1 + [S]/K_m)$ to obtain the pseudo-first-order rate constant, k_{obs} [5], and these values were plotted versus F-amidine concentrations and fit to Eq. (3) using the Grafit™ version 5.0.11 software package [4].

$$k_{obs} = k_{inact}[\text{F-amidine}]/(K_I+[\text{F-amidine}]) \quad \text{(Eq. 3)}$$

Rapid Dilution Time Course Inhibition Assays

In order to determine the reversibility of inhibition, rapid dilution time course experiments were performed for F-amidine (**1**) by measuring the recovery of PAD4 activity over time after a rapid 95-fold dilution of a PAD4-F-amidine complex. PAD4-F-amidine complex was pre-formed by incubating PAD4 (9.5 μM) with F-amidine (167 μM) at 37°C for 30 min. The reaction was initiated by the addition of 6.3 μL of the preformed PAD4-F-amidine complex to a reaction buffer containing 10 mM BAEE, 100 mM HEPES (pH 7.6), 50 mM NaCl, 500 μM TCEP and 10 mM $CaCl_2$ (final volume 600 μL). At various time points (0, 3, 6, 10, 15, 20, 25, and 30 min), 60 μl of

the reaction was withdrawn and quenched by flash freezing in liquid nitrogen. Color development and absorbance measurement at 540 nm of the samples were done as described for the IC_{50} assays.

Dialysis experiments

To further assess the irreversibility of inactivation, control and F-Amidine treated PAD4 were dialyzed against 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 500 mM NaCl, 1 mM DTT, and 10% glycerol for 20 hours, and the residual activity quantified using our assay for Cit production. The results indicated that there was no recovery of activity.

Transient Transfection and Mammalian Two Hybrid Assay

CV-1 cells were maintained in DMEM with 10% FBS. Transfection and luciferase assays were performed in 12-well dishes as described (6). Targefect (Targeting Systems, Santee, CA) was used for transfection according to manufacturer's protocol. Plasmids encoding the Gal4 DBD fused to the p300 GBD (GRIP1 binding domain) (250 ng each) were transfected into CV-1 cells with 125 ng of GK1 reporter plasmid and plasmids for GRIP1 AD1 (p300 binding domain) fused to VP16 AD, and PAD4 WT or mutant (250 ng each). DNA and transfection mixtures were incubated with cells for three hours and replaced with DMEM (with 10% FBS) including 0~200 μM of F-Amidine. Luciferase activities of the transfected cell extracts were measured after 40 hours

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(8) IC_{50} for N-a-benzoyl-N^5-(2-fluoro-1-ketoethyl)-L-ornithine amide is >> 500 mM.

(9) Because the line representing the F-Amidine rapid dilutions experiments fits well to a linear fit, the small amount of product formation observed after extended incubations is most likely due to the presence of a small amount of active PAD4 (< 5 % -- based on comparing the slopes of the two lines) that was not inactivated during the preincubation of PAD4 with Ca^{2+} and F-Amidine.

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