Inhibitors and Inactivators of Protein Arginine Deiminase 4: Functional and structural characterization[†]

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Running Title: Inhibitors and inactivators of PAD4

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Supplemental Results

Supplemental Figure S1. Time course experiments with 3, 6 and 9.

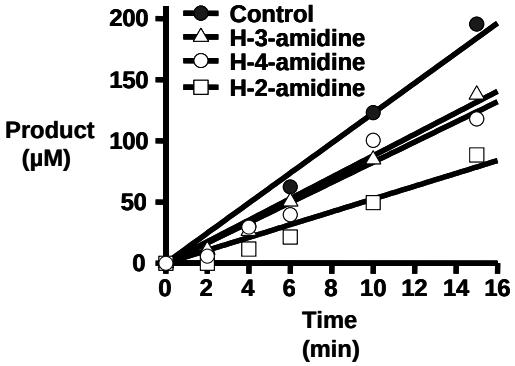


Figure S1. Plots of product formation versus time in presence of 25 mM of H2-amidine, H3-amidine and H4-amidine.

Supplemental Figure S2. Rapid-dilution time course experiments with 4, 5,

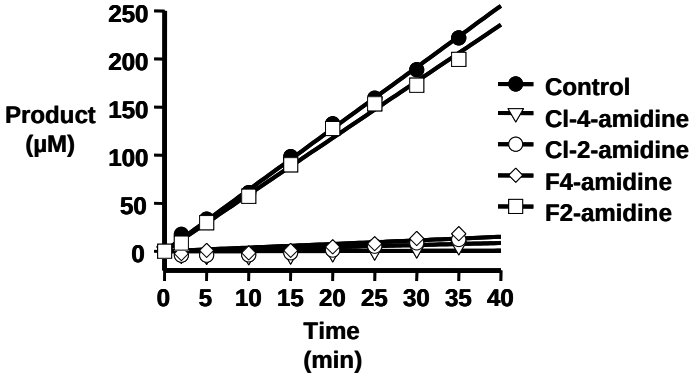


Figure S2. Rapid dilution of a preformed complex of PAD4▪F2-amidine, PAD4▪F4-amidine, PAD4▪Cl2-amidine, or PAD4▪Cl4-amidine into assay buffer containing excess substrate.

7 and 8.

Supplemental Figure S3. Lineweaver-Burk plots for H3-amidine.

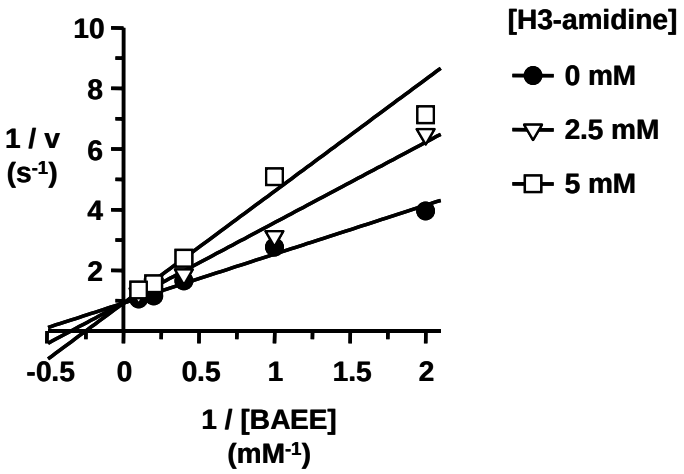
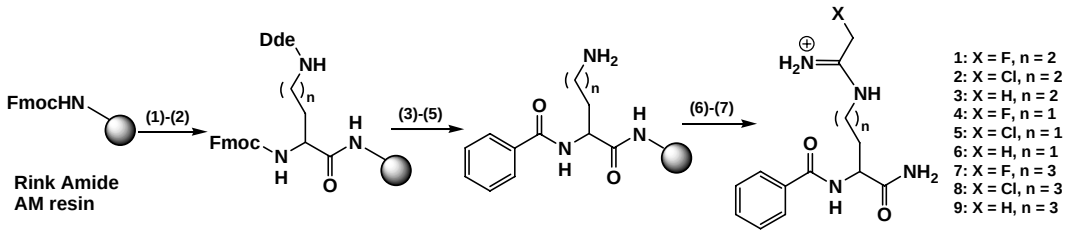


Figure S3. H3-amidine is a competitive inhibitor of PAD4. The Lineweaver-Burk plots (1/vi versus 1/[BAEE]) depicted above are consistent with H3-amidine being a competitive inhibitor. For these studies, the steady state kinetic parameters for the deimination of BAEE were determined in the absence and presence of increasing amounts of H3-amidine.

Supplemental Methods



Scheme S1. Solid-phase synthesis of haloacetamidine-based PAD4 inactivators/inhibitors. (1) 20 % piperidine in DMF; (2) Fmoc-AA(Dde)-OH, HOBt, HBTU, NMM, DMF; (3) 20 % piperidine, DMF; (4) benzoyl chloride, NMM, DMF; (5) 2 % hydrazine, DMF; (6) $XCH_2C(=NH)OEt\cdot HCl$, Et_3N, DMF; (7) 95 % TFA, 2.5 % TIS, 2.5 % H_2O. (X= F, Cl, or H; n=1, AA=Dab; n=2, AA=Orn; n=3, AA=Lys; NMM: N-methyl morpholine; DMF: N, N-dimethylformamide; TFA: trifluoroacetic acid; TIS: triisopropylsilane)

Inhibitor Synthesis

(Halo)acetamidine-based PAD4 inhibitors/inactivators were readily synthesized using adaptations of previously described methodologies (*1*) (Scheme S1). Briefly, the Fmoc group on the resin was first deprotected with 20% piperidine to generate a free amine. A diamino acid (Dab, Orn or Lys), with both amino groups protected, was attached to Rink Amide AM resin via standard uronium based coupling methods, i.e. the α-carboxylic acid group of the diamino acids were activated with HBTU and HOBt. After removal of the Fmoc protective group, the α-amino group was benzolated with benzoyl chloride. The Dde group was then removed with 2 % hydrazine and the free side chain amine reacted with either ethyl-, ethyl fluoro-, or ethyl-chloro-acetimidate hydrochloride to form the (halo)acetamidine, i.e. for the synthesis of compounds **2**-**9.** The warheads in compounds **10** and **11** were generated by reacting the free amine with either fluoroacetic acid or chloroacetic acid and DIC (1,3-

diisopropylcarbodiimide). The ethyl-, ethyl fluoro, or ethyl-chloro-acetimidates were derived from the corresponding acetonitrile derivatives by reacting these compounds with ethanol in acidified ether. (*2*) Final compounds were cleaved from resin by a mixture of 95 % TFA, 2.5 % TIS and 2.5 % H_2O, and purified on a reverse phase HPLC. Purity was assessed by ^{1}H-NMR, ^{13}C-NMR, and reverse phase HPLC.

Structural Characterization of (Halo)acetamidine-Based Inactivators

1. N-α-benzoyl-N^5-(2-chloro-1-iminoethyl)-L-Orn amide (**2, Cl3-amidine**)

2 was synthesized similarly to **1**; see above and ref. (*1*). ^{1}HNMR (400 MHz, CD_3OD) δ(ppm): 7.79-7.37 (m, 5H), 4.56-4.52 (dd, 1H), 4.26 (s, 2H), 3.34-3.20 (m, 2H), 1.98-1.64 (m, 4H). ^{13}CNMR (400 MHz, CD_3OD) δ(ppm): 176.46, 170.31, 164.68, 135.03, 133.08, 129.63, 128.53, 54.17, 43.53, 40.11, 30.37, 25.00. MS-ES^+: 311 $(M+1)^+$. HRMS ($C_{14}H_{20}ClN_4O_2^+$): calculated 311.1275, observed 311.1266.

2. N-α-benzoyl-N^5-(1-iminoethyl)-L-Orn amide (**3, H3-amidine**)

3 was synthesized similarly to **1**; see above and ref. (*1*). ^{1}HNMR (400 MHz, D_2O) δ(ppm): 7.71-7.42 (m, 5H), 4.43-4.39 (dd, 1H), 3.23-3.20 (t, 2H), 2.09 (s, 3H), 1.96-1.63 (m, 4H). ^{13}CNMR (400 MHz, CD_3OD) δ(ppm): 176.68, 170.46, 166.33, 135.21, 133.20, 129.77, 128.68, 54.39, 43.21, 30.57, 25.35, 19.02. MS-ES^+: 277 $(M+1)^+$. HRMS ($C_{14}H_{21}N_4O_2^+$): calculated 277.1665 observed 277.1660.

3. N-α-benzoyl-N^4-(2-fluoro-1-iminoethyl)-L-Dab amide (**4, F2-amidine**)

4 was synthesized similarly to **1**; see above and ref. (*1*). ^{1}HNMR (400 MHz, CD_3OD) δ(ppm): 7.93-7.47 (m, 5H), 5.35-5.24 (d, $^{2}J_{H\text{-}F}$=45.4 Hz, 2H), 4.71-4.67 (dd, 1H), 3.57-3.41 (m, 2H), 2.39-2.10 (m, 2H). ^{13}CNMR (400 MHz, CD_3OD) δ(ppm): 176.13, 170.56, 164.87, 164.68 (($^{2}J_{C\text{-}F}$ = 19.7 Hz), 135.00, 133.28, 129.76, 128.82, 80.04, 78.26 ($^{1}J_{C\text{-}F}$ = 179.4 Hz), 52.50, 40.76, 31.12. ^{19}FNMR (400 MHz, CD_3OD) δ(ppm): -158.09, -158.21, -158.33 ($^{2}J_{H\text{-}F}$=45.8 Hz). MS-ES^{+}: 281 $(M+1)^{+}$. HRMS ($C_{13}H_{18}FN_4O_2^{+}$): calculated 281.1414, observed 281.1404.

4. N-α-benzoyl-N^{4}-(2-chloro-1-iminoethyl)-L-Dab amide (**5, Cl2-amidine**)

5 was synthesized similarly to **1**; see above and ref. (*1*). ^{1}HNMR (400 MHz, CD_3OD) δ(ppm): 7.92-7.46 (m, 5H), 4.70-4.67 (dd, 1H), 4.41 (s, 2H), 3.57-3.40 (m, 2H), 2.39-2.10 (m, 2H). ^{13}CNMR (400 MHz, CD_3OD) δ(ppm): 176.09, 170.56, 165.05, 135.00, 133.27, 129.76, 128.83, 52.55, 41.37, 40.30, 30.95. MS-ES^{+}: 297 $(M+1)^{+}$. HRMS ($C_{13}H_{18}ClN_4O_2^{+}$): calculated 297.1118, observed 297.1119.

5. N-α-benzoyl-N^{4}-(1-iminoethyl)-L-Dab amide (**6, H2-amidine**)

6 was synthesized similarly to **1**; see above and ref. (*1*). ^{1}HNMR (400 MHz, CD_3OD) δ(ppm): 7.92-7.46 (m, 5H), 4.70-4.67 (dd, 1H), 3.48-3.33 (m, 2H), 2.39-2.07 (m, 2H), 2.23 (s, 3H). ^{13}CNMR (400 MHz, CD_3OD) δ(ppm): 176.21, 170.54, 166.61, 135.04, 133.24, 129.75, 128.81, 52.61, 40.80, 31.10, 19.15. MS-ES^{+}: 263 $(M+1)^{+}$. HRMS ($C_{13}H_{19}N_4O_2^{+}$): calculated 263.1508, observed 263.1496.

6. N-α-benzoyl-N^{6}-(2-fluoro-1-iminoethyl)-L-Lys amide (**7, F4-amidine**)

7 was synthesized similarly to **1**; see above and ref. (*1*). ^{1}HNMR (400 MHz, CD_3OD) δ(ppm): 7.88-7.45(m, 5H), 5.28-5.17 (d, $^{2}J_{H\text{-}F}$ = 45.4 Hz, 2H), 4.60-4.57 (dd, 1H), 3.35-3.32 (t, 2H), 2.02-1.80 (m, 2H), 1.78-1.65 (m, 2H), 1.61-1.43 (m, 2H). ^{13}CNMR (400 MHz, CD_3OD) δ(ppm): 177.12, 170.39, 164.39, 164.20 ($^{2}J_{C\text{-}F}$=19 Hz), 135.30, 133.14, 129.76, 128.67, 79.98, 78.21 ($^{1}J_{C\text{-}F}$=179 Hz), 54.81, 43.48, 32.81, 28.23, 24.40. ^{19}FNMR (400 MHz, CD_3OD) δ(ppm): -157.90, -158.03, -158.15 ($^{2}J_{H\text{-}F}$ = 45.8 Hz). MS-ES^{+}: 309 $(M+1)^{+}$. HRMS ($C_{15}H_{22}FN_4O_2^{+}$): calculated 309.1727, observed 309.1727.

7. N-α-benzoyl-N^{6}-(2-chloro-1-iminoethyl)-L-Lys amide (**8, Cl4-amidine**)

8 was synthesized similarly to **1**; see above and ref. (*1*). ^{1}HNMR (400 MHz, D_2O) δ(ppm): 7.71-7.42 (m, 5H), 4.42-4.38 (dd, 1H), 4.24 (s, 2H), 3.27-3.24 (t, 2H), 1.92-1.70 (m, 2H), 1.69-1.55 (m, 2H), 1.51-1.34 (m, 2H). ^{13}CNMR (400 MHz, CD_3OD) δ(ppm): 177.08, 170.38, 164.71, 135.29, 133.14, 129.76, 128.68, 54.78, 44.00, 40.24, 32.82, 28.11, 24.41. MS-ES^{+}: 325 $(M+1)^{+}$. HRMS ($C_{15}H_{22}ClN_4O_2^{+}$): calculated 325.1431, observed 325.1432.

8. N-α-benzoyl-N^{6}-(1-iminoethyl)-L-Lys amide (**9, H4-amidine**)

9 was synthesized similarly to **1**; see above and ref. (*1*). ^{1}HNMR (400 MHz, CD_3OD) δ(ppm): 7.88-7.45 (m, 5H), 4.60-4.57 (dd, 1H), 3.25-3.22 (t, 2H), 2.17 (s, 3H), 2.00-1.76 (m, 2H), 1.74-1.64 (m, 2H), 1.60-1.44 (m, 2H). ^{13}CNMR (400 MHz, CD_3OD) δ(ppm): 176.96, 170.22, 166.04, 135.17, 132.99, 129.61, 128.51, 54.65,

43.39, 32.68, 28.13, 24.31, 18.82. MS-ES$^+$: 291 $(M+1)^+$. HRMS ($C_{15}H_{23}N_4O_2^+$): calculated 291.1821, observed 291.1812.

9. N-α-benzoyl-N^5-(2-fluoroacetyl)-L-Orn amide (**10**)

10 was synthesized similarly to **1**; see above and ref. (*1*). ^{1}HNMR (400 MHz, CD_3OD) δ(ppm): 7.80-7.30 (m, 5H), 4.76-4.62 (d, 2H, $^2J_{H-F}$ = 47.0 Hz), 4.48-4.43 (dd, 1H), 3.27-3.16 (m, 2H), 1.89-1.68 (m, 2H), 1.62-1.50 (m, 2H). ^{13}CNMR (400 MHz, CD_3OD) δ(ppm): 177.01, 170.70, 170.52, 170.34, 135.24, 132.90, 129.57, 128.55, 81.94, 80.12, 54.67, 39.41, 30.52, 27.15. ^{19}FNMR (400 MHz, CD_3OD) δ(ppm): -53.62, -53.74, -53.87 ($^2J_{H-F}$ = 47.0 Hz). MS-ES$^+$: 296 $(M+1)^+$.

10. N-α-benzoyl-N^5-(2-chloroacetyl)-L-Orn amide (**11**)

11 was synthesized similarly to **1**; see above and ref. (*1*). ^{1}HNMR (300 MHz, CD_3OD) δ(ppm): 7.86-7.44 (m, 5H), 4.61-4.56 (dd, 1H), 4.02 (s, 2H), 3.35-3.21 (m, 2H), 2.01-1.76 (m, 2H), 1.73-1.62 (m, 2H). ^{13}CNMR (400 MHz, CD_3OD) δ(ppm): 176.95, 170.31, 169.45, 135.26, 132.88, 129.55, 128.54, 54.63, 43.18, 40.28, 30.52, 27.02. MS-ES$^+$: 312 $(M+1)^+$.

References

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