Supporting Information for

D-amino acid based protein arginine deiminase inhibitors: Synthesis, pharmacokinetics, and in cellulo efficacy

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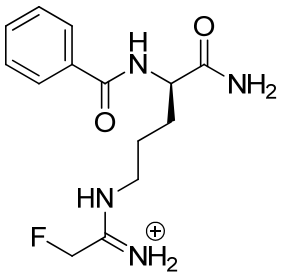
Abbreviations

Trifluoroacetic acid (TFA); Methanol (MeOH); Acetonitrile (ACN); Dimethylformamide (DMF); N-methylmorpholine (NMM); O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU); Triisopropylsilane (TIS); Dithiothreitol (DTT); Benzoyl-L-arginine ethyl ester (BAEE); Protein arginine deiminase (PAD); Color Development Reagent (COLDER); Maximum tolerable dose (MTD).

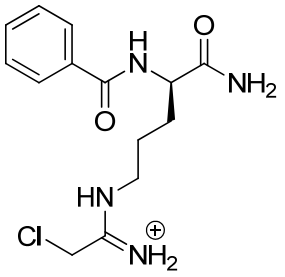
D-F-amidine



D-F-amidine was synthesized analogously to previously described methods.[1] Briefly, N^5-(tert-butoxycarbonyl)-D-ornithine (0.861 mmol) was added to diethyl ether (2.5 mL) and 3.5 M NaOH (0.6 mL) on ice. To this was added benzoyl chloride (100 µL; 0.861 mmol) and 8.5 M NaOH (100 µL) in alternating 20 µL portions over 10 min. The reaction was allowed to warm to 23 ˚C and stirred 18 h. Ether was added and the organic layer removed before acidifying the aqueous layer to pH 4 with HCl. The resulting precipitate of N^1-benzoyl-N^4-Boc-D-ornithine acid was filtered, washed with water, and dried under vacuum. In the next step of the reaction, N^1-benzoyl-N^4-Boc-D-ornithine acid, which was used without purification, was dissolved into dichloromethane (CH_2Cl_2; 10 mL) and cooled on ice. To this was added N-methylmorpholine (142 µL; 1.29 mmol) and isobutylchloroformate (169 µL; 1.29 mmol). After 10 min, ammonium hydroxide (335 µL; 8.61 mmol) was added and stirred on ice 10 min. The reaction was allowed to warm to rt and stirred for 16 h. The resulting precipitate was filtered off and washed with CH_2Cl_2 and dried under vacuum 3 h. The Boc protecting group on the resultant N^1-benzoyl-N^4-Boc-D-ornithine amide, which was used without further purification, was then removed by treating the compound with neat trifluoroacetic acid (TFA; 10 mL) for 1 h at rt. The TFA was bubbled off with N_2 and the product precipitated with cold diethyl ether. The precipitate was collected by centrifugation and dried under vacuum for 1 h. The warhead was then installed by

dissolving the precipitate in methanol (MeOH; 10 mL). To this was added triethylamine (Et_3N; 180 µL; 1.5 eq) and ethyl fluoroacetimidate HCl (193 mg; 1.5 eq) and the reaction was stirred 2 h at rt. Solvent was removed by rotary evaporation and the reaction mixture was purified by reverse phase HPLC with a linear gradient of H_2O/ACN plus 0.05% TFA to give the title product in 40% overall yield. ^{1}H NMR (CD_3OD, 400 MHz) δ (ppm): 7.71 (d, *J* = 7.2 Hz, 2H), 7.57-7.54 (m, 1H), 7.49-7.46 (m, 2H), 5.33 (d, *J* = 45.2 Hz, 2H), 4.63 (m, 1H), 3.49-3.38 (m, 2H), 2.15-1.88 (m, 2H), 1.82 (m, 2H). ^{13}C NMR (CD_3OD, 100 MHz) δ (ppm): 176.8, 170.4, 164.2, 135.0, 133.1, 129.6 (2C), 128.6 (2C), 54.7, 43.0, 30.3, 25.3, 9.2. LRMS [EI^+] calculated for ($C_{14}H_{20}FN_4O_2^+$) 295.15, found 295.20.

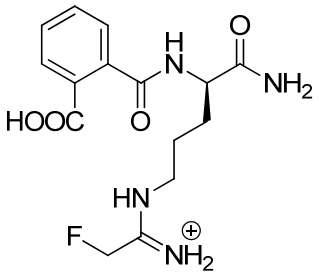
D-Cl-amidine



D-Cl-amidine was generated by first dissolving N^1-benzoyl-D-ornithine amide in methanol (MeOH; 10 mL). To this was added triethylamine (Et_3N; 180 µL; 1.5 eq) and ethyl chloroacetimidate HCl (193 mg; 1.5 eq) and the reaction was stirred 2 h at rt. Solvent was removed by rotary evaporation and the reaction mixture was purified by reverse phase HPLC with a linear gradient of H_2O/ACN plus 0.05% TFA to give the title product in overall 40% yield. Product was >90% pure, as determined by LC-MS. ^{1}H NMR (CD_3OD, 400 MHz) δ (ppm): 7.91 (d, *J* = 7.2 Hz, 2H), 7.58-7.54 (m, 1H), 7.49-7.45 (m, 2H), 4.68 (m, 1H), 4.40 (s,

2H), 3.46-3.41 (m, 2H), 2.15-1.91 (m, 2H), 1.81 (m, 2H). ^{13}C NMR (CD_3OD, 100 MHz) δ (ppm): 176.8, 170.5, 164.6, 135.1, 133.1, 129.7 (2C), 128.6 (2C), 54.7, 43.7, 30.3, 25.2, 9.2. HRMS calculated for ($C_{14}H_{20}ClN_4O_2^+$) 311.1269, found 311.1270.

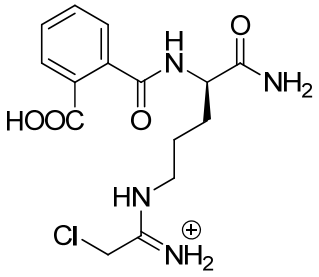
D-o-F-amidine



This compound was synthesized analogously to previously described methods.[2] Briefly, Knorr amide resin (300 mg; 0.75 mmol/g) was swollen in dimethylformamide (DMF) for 30 min, followed by removal of the Fmoc protecting groups in 20% piperidine in DMF for 10 min twice, followed by washing with DMF 3x. Fmoc-D-Orn(Boc)-OH (408.6 mg; 0.9 mmol; 4 eq) was activated with O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU; 341 mg; 0.9 mmol; 4 eq) in 5% N-methylmorpholine (NMM) in DMF for 10 min. This solution was added to the resin and agitated for 1 h at rt. Resin was washed with DMF 3x and the N-terminal Fmoc protecting group removed with 20% piperidine in DMF for 10 min twice, followed by washing with DMF 3x. Phthalic anhydride (133.2 mg; 0.9 mmol; 4 eq) in 5% NMM in DMF was then added and the resin agitated for 16 h at rt, followed by washing with DMF 3x. Resin was then treated with TFA/TIS/H_2O (95/2.5/2.5) for 1 h at rt and the filtrate collected and evaporated by N_2 bubbling. The compound was precipitated with diethyl ether, collected by

centrifugation, and dried under vacuum 1 h. The precipitate, N^1-(2-carboxy)benzoyl-D-ornithine amide, was dissolved in MeOH and then treated with Et_3N (94 µL; 3 eq) and ethyl fluoroacetimidate HCl (48.3 mg; 1.5 eq) for 1 h at rt. Solvent was removed by rotary evaporation and the reaction mixture was purified by reverse phase HPLC with a linear gradient of H_2O/ACN plus 0.05% TFA to give the title product in 57% yield overall. Product was >90% pure, as determined by LC-MS. ^{1}H NMR (CD_3OD, 400 MHz) δ (ppm): 8.05 (d, *J* = 8.4 Hz, 1H), 7.69-7.65 (m, 1H), 7.61-7.56 (m, 1H), 7.51 (d, *J* = 8.0 Hz, 1H), 5.29 (d, *J* = 45.6 Hz, 2H), 4.57-4.54 (m, 1H), 3.44-3.41 (m, 2H), 2.09-1.87 (m, 2H), 1.80 (m, 2H). ^{13}C NMR (CD_3OD, 100 MHz) δ (ppm): 176.7, 173.2, 169.4, 164.2, 139.9, 133.7, 131.6, 130.9, 129.8, 129.1, 54.3, 43.0, 29.6, 25.2, 9.2. HRMS calculated for ($C_{15}H_{19}FN_4O_4^+$) 339.1463, found 339.1475.

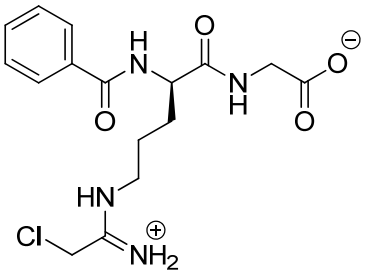
D-o-Cl-amidine



N^1-(2-carboxy)benzoyl-D-ornithine amide, obtained as described above for D-o-F-amidine, was dissolved in MeOH and then treated with Et_3N (94 µL; 3 eq) and ethyl chloroacetimidate HCl (48.3 mg; 1.5 eq) for 1 h at rt. Solvent was removed by rotary evaporation and the reaction mixture was purified by reverse phase HPLC with a linear gradient of H_2O/ACN plus 0.05% TFA to give the title product in 57% yield overall. ^{1}H NMR (CD_3OD, 400 MHz) δ (ppm): 7.94 (d, *J* = 7.6 Hz, 1H), 7.57-7.53 (m, 1H), 7.49-7.45 (m, 1H), 7.37 (d, *J* =

7.6 Hz, 1H), 4.45 (m, 1H), 4.33 (s, 2H), 3.33-3.29 (m, 2H), 2.03-1.83 (m, 2H), 1.73 (m, 2H). ^{13}C NMR (CD_3OD, 100 MHz) δ (ppm): 176.6, 173.1, 169.3, 164.7, 139.9, 133.7, 131.6, 130.8, 129.8, 129.0, 54.3, 43.5, 40.1, 29.5, 25.0. LRMS calculated for ($C_{15}H_{19}ClN_4O_4^+$) 355.11, found 355.10.

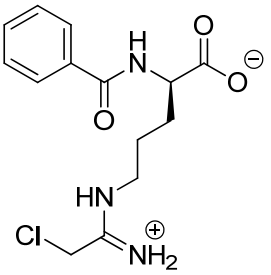
D-Cl-Amidine-Gly



Fmoc-Gly-Wang resin (50 mg; 0.79 mmol/g) was swollen in dimethylformamide (DMF) for 30 min. The Fmoc protecting group was removed with 20% piperidine in DMF for 10 min twice, followed by washing with DMF 3x. Fmoc-D-Orn(DDE)-OH (77.7 mg; 0.15 mmol; 4 eq) was activated with HBTU (57 mg; 0.15 mmol; 4 eq) in 5% N-methylmorpholine (NMM) in DMF for 10 min. This solution was added to the resin and agitated for 1 h at rt. Resin was washed with DMF 3x and the N-terminal Fmoc protecting group removed with 20% piperidine in DMF for 10 min twice, followed by washing with DMF 3x. Benzoic acid (19 mg; 0.15 mmol; 4 eq) was activated with HBTU (57 mg; 0.15 mmol; 4 eq) in 5% NMM in DMF for 10 min, before agitating this solution with the resin for 1 h. After washing 3x with DMF, the DDE protecting groups were then removed by treating with 5% hydrazine in DMF for 1 h at rt, followed by washing with DMF 3x. Resin was then treated with ethyl chloroacetimidate HCl (25 mg; 0.15 mmol; 4 eq) in DMF with Et_3N (44 μL; 8 eq) for 16 h at rt, followed by DMF

washing. Compound was cleaved from the resin with TFA/TIS/H_2O (95/2.5/2.5) for 1 h at rt and the filtrate collected and evaporated by N_2 bubbling. The compound was precipitated by ethyl ether, collected by centrifugation, and dried under vacuum for 1 h. The pellet was resuspended in water plus 0.1% TFA and purified by reverse phase HPLC with a linear gradient of H_2O/ACN plus 0.05% TFA to give the title product in 45.8% overall yield. ^{1}H NMR (CD_3OD, 400 MHz) δ (ppm): 7.91 (d, J = 8.8 Hz, 2H), 7.59-7.56 (m, 1H), 7.51-7.47 (m, 2H), 4.71 (m, 1H), 4.39 (s, 2H), 3.97 (AB_q, $\Delta\delta_{AB}$ = 0.064, J_{AB} = 17.6 Hz), 3.45-3.39 (m, 2H), 2.13-1.92 (m, 2H), 1.87 (m, 2H). ^{13}C NMR (CD_3OD, 100 MHz) δ (ppm): 174.3, 172.9, 170.4, 164.7, 135.0, 133.1, 129.6 (2C), 128.6 (2C), 54.5, 43.6, 41.9, 40.1, 30.3, 24.9. LRMS calculated for ($C_{16}H_{22}ClN_4O_4^+$) 369.13, found 369.20.

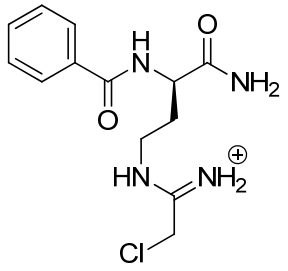
D-Cl-Amidine-OH



N^1-benzoyl-N^4-Boc-D-ornithine acid (75 mg; 0.323 mmol), obtained as described above for the synthesis of D-F-amidine, was treated with neat TFA (10 mL) for 1 h at rt. The TFA was bubbled off with N_2 and the product precipitated with cold diethyl ether. The precipitate was collected by centrifugation and dried under vacuum for 1 h. After dissolving in MeOH (10 mL), Et_3N (180 μL; 1.5 eq) and ethyl chloroacetimidate HCl (77 mg; 1.5 eq) were added and the reaction was stirred for 2 h at rt. Solvent was removed by rotary evaporation and the reaction

mixture was purified by reverse phase HPLC with a linear gradient of H_2O/ACN plus 0.05% TFA to give the title product in 45.1% overall yield. ^{1}H NMR (CD_3OD, 400 MHz) δ (ppm): 7.89 (d, *J* = 7.2 Hz, 2H), 7.59-7.55 (m, 1H), 7.51-7.47 (m, 2H), 4.69 (m, 1H), 4.39 (s, 2H), 3.46-3.40 (m, 2H), 2.17-2.09 (m, 2H), 1.86 (m, 2H). ^{13}C NMR (CD_3OD, 100 MHz) δ (ppm): 175.1, 170.5, 164.7, 135.2, 133.0, 129.7 (2C), 128.6 (2C), 53.7, 43.5, 40.5, 29.8, 25.2. LRMS calculated for ($C_{14}H_{19}ClN_3O_3^+$) 312.10, found 312.10.

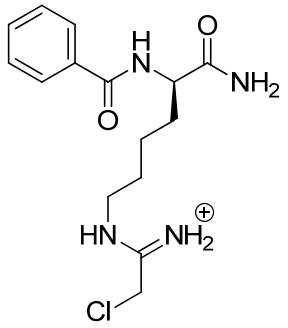
D-Cl2-Amidine



N^5-(tert-butoxycarbonyl)-D-diaminobutanoic acid (0.861 mmol) was added to diethyl ether (2.5 mL) and 3.5 M NaOH (0.6 mL) on ice. To this was added benzoyl chloride (100 µL; 0.916 mmol) and 8.5 M NaOH (100 µL) in alternating 20 µL portions over 10 min. The reaction was allowed to warm to 23 ˚C and stirred 18 h. Ether was added and the organic layer removed before acidifying the aqueous layer to pH 4 with HCl. The resulting precipitate was filtered, washed with water, and dried under vacuum. Without further purification this compound was dissolved into dichloromethane (CH_2Cl_2; 10 mL) and cooled on ice. To this was added N-methylmorpholine (142 µL; 1.29 mmol) and isobutylchloroformate (169 µL; 1.29 mmol). After 10 min, ammonium hydroxide (335 µL; 8.61 mmol) was added and stirred on ice for 10 min. The reaction was allowed to warm to rt and stirred 16 h. The resulting precipitate was filtered

off and washed with CH_2Cl_2 and dried under vacuum 3 h. Without further purification, this compound was treated with neat trifluoroacetic acid (TFA; 10 mL) for 1 h at rt. The TFA was bubbled off with N_2 and the product precipitated with cold diethyl ether. The precipitate was collected by centrifugation and dried under vacuum 1 h. Without further purification, this compound was dissolved into methanol (MeOH; 10 mL). To this was added triethylamine (Et_3N; 180 μL; 1.5 eq) and ethyl chloroacetimidate HCl (193 mg; 1.5 eq) and the reaction was stirred 2 h at rt. Solvent was removed by rotary evaporation and the reaction mixture was purified by reverse phase HPLC with a linear gradient of H_2O/ACN plus 0.05% TFA to give the title product in 25% yield overall. ^{1}H NMR (CD_3OD, 400 MHz) δ (ppm): 7.88 (d, J = 6.8 Hz, 2H), 7.59-7.55 (m, 1H), 7.50-7.46 (m, 2H), 4.71 (m, 1H), 4.47 (s, 2H), 3.61-3.44 (m, 2H), 2.39-2.14 (m, 2H). ^{13}C NMR (CD_3OD, 100 MHz) δ (ppm): 176.1, 170.5, 164.9, 134.9, 133.2, 129.6 (2C), 128.7 (2C), 52.6, 41.2, 40.2, 30.8. LRMS calculated for ($C_{13}H_{18}ClN4O_2^+$) 297.10, found 297.10.

D-Cl4-Amidine



N^5-(tert-butoxycarbonyl)-D-lysine (0.861 mmol) was added to diethyl ether (2.5 mL) and 3.5 M NaOH (0.6 mL) on ice. To this was added benzoyl chloride (100 μL; 0.916 mmol) and 8.5 M NaOH (100 μL) in alternating 20 μL portions over 10 min. The reaction was allowed to warm to

23 ˚C and stirred for 18 h. Ether was added and the organic layer removed before acidifying the aqueous layer to pH 4 with HCl. The resulting precipitate was filtered, washed with water, and dried under vacuum. Without further purification this compound was dissolved into dichloromethane (CH_2Cl_2; 10 mL) and cooled on ice. To this was added N-methylmorpholine (142 μL; 1.29 mmol) and isobutylchloroformate (169 μL; 1.29 mmol). After 10 min, ammonium hydroxide (335 μL; 8.61 mmol) was added and stirred on ice 10 min. The reaction was allowed to warm to rt and stirred 16 h. The resulting precipitate was filtered off and washed with CH_2Cl_2 and dried under vacuum 3 h. Without further purification, this compound was treated with neat trifluoroacetic acid (TFA; 10 mL) for 1 h at rt. The TFA was bubbled off with N_2 and the product precipitated with cold diethyl ether. The precipitate was collected by centrifugation and dried under vacuum 1 h. Without further purification, this compound was dissolved into methanol (MeOH; 10 mL). To this was added triethylamine (Et_3N; 180 μL; 1.5 eq) and ethyl chloroacetimidate HCl (193 mg; 1.5 eq) and the reaction was stirred 2 h at rt. Solvent was removed by rotary evaporation and the reaction mixture was purified by reverse phase HPLC with a linear gradient of H_2O/ACN plus 0.05% TFA to give the title product in 17% yield overall. ^{1}H NMR (CD_3OD, 400 MHz) δ (ppm): 7.89 (d, J = 7.2 Hz, 2H), 7.58-7.54 (m, 1H), 7.50-7.46 (m, 2H), 4.60 (m, 1H), 4.37 (s, 2H), 3.36-3.32 (m, 2H), 2.02-1.86 (m, 2H), 1.73 (m, 2H), 1.57 (m, 2H). ^{13}C NMR (CD_3OD, 100 MHz) δ (ppm): 177.1, 170.5, 164.5, 135.2, 133.0, 129.6 (2C), 128.6 (2C), 54.9, 43.9, 40.1, 32.7, 28.0, 24.3. LRMS calculated for ($C_{15}H_{22}ClN4O_2^+$) 325.14, found 325.10.

Supplementary Methods

Inactivation Kinetics – Inactivation kinetic parameters were determined by incubating PAD1, 2, or 4 (2.0 μM) or PAD3 (5.0 μM) in a pre-warmed (10 min; 37 °C) inactivation mixture (50 mM HEPES; 10 mM $CaCl_2$, and 2 mM DTT; pH 7.6) containing various concentrations of inhibitor. Aliquots were removed at various time points (0, 2, 6, 10, 15, 20, and 30 min) and added to a pre-warmed (10 min 37 °C) reaction mixture (50 mM HEPES, 50 mM NaCl, 10 mM $CaCl_2$, 2 mM DTT, and 10 mM BAEE; pH 7.6). After 15 min, reactions were quenched in liquid nitrogen and citrulline production quantified using the COLDER assay.[3] Data were plotted as a function of time and fit to **eq 1**,

$$v = v_o e^{-kt} \qquad \textbf{eq 1,}$$

using GraFit version 5.0.11, where v is velocity, v_o is initial velocity, k (or k_{obs}) is the pseudo-first order rate constant of inactivation, and t is time. When saturation was reached upon plotting k_{obs} versus inactivator concentration, the data were fit to **eq 2**,

$$k_{obs} = k_{inact}[\mathrm{I}]/(K_I + [\mathrm{I}]) \qquad \textbf{eq 2,}$$

using GraFit version 5.0.11, where k_{inact} corresponds to the maximal rate of inactivation and K_I is the concentration of inhibitor that gives half-maximal inactivation. If the plot of k_{obs} versus [I] was linear and did not saturate, then the value for k_{inact}/K_I equaled the slope of the line.

For plots where cooperativity was observed, data were fit to **eq 3**,

$$k_{obs} = k_{inact}[\mathrm{I}]^n/(K_I^n + [\mathrm{I}]^n) \qquad \textbf{eq 3,}$$

using GraFit version 5.0.11, where k_{inact} corresponds to the maximal rate of inactivation, K_I is the concentration of inhibitor that gives half-maximal inactivation, and n is the cooperativity coefficient.

Competitive ABPP – To show that D-series compounds bind to the active site of PAD1, we performed a gel based competitive ABPP assay using our L-amino acid based fluorescent probe, rhodamine-Cl-amidine (RCA).[4] Briefly, a mixture of reaction buffer (50 mM HEPES, 50 mM NaCl, 10 mM $CaCl_2$, 2 mM DTT) containing RCA (10 μM) and various concentrations of D-Cl-amidine or D-o-F-amidine (i.e., 0, 15, 25, 50, 100, 250, and 500 μM) was incubated at 37 ºC 10 min. Subsequently, PAD1 (0.2 μM) was added and the mixture incubated at 37 ºC for 30 min prior to quenching with 50 mM EDTA. All samples were then boiled with 6x SDS loading dye and analyzed by SDS-PAGE (12%; 170 V; 50 min) and fluorescent imaging on a Typhoon Imager (Ex. 532 nm; Em. 580 nm).

Inhibitor Reversibility – To examine the reversibility of the two most potent D-series inhibitors, PAD1 (2 μM) was incubated with either no inhibitor, D-Cl-amidine (1 mM), or D-o-F-amidine (1 mM) in reaction buffer (100 μL; 50 mM HEPES, 50 mM NaCl, 10 mM $CaCl_2$, and 2 mM DTT; pH 7.6) at 37 °C for 1 h. Samples were transferred to a 3.5 kDa dialysis cassette and dialyzed against PAD long term storage buffer (500 mL; 20 mM Tris, 500 mM NaCl, 1 mM EDTA, 0.5 mM TCEP, 10% glycerol; pH 7.6) at 4 °C for 16 h (5000-fold dilution). Samples were collected from the cassettes and enzyme activity measured by adding 6 μL of each sample to a mixture containing 10 mM BAEE in reaction buffer. After incubating 15 min at 37 °C,

samples were frozen in liquid nitrogen and analyzed by COLDER analysis, reading the absorbance at 540 nm.[3]

Maximum Tolerable Dose Studies – MTD (maximum tolerated dose) for Cl-Amidine, D-Cl-amidine, and D-o-F-amidine administered orally (p.o.) was investigated in healthy male and female C57BL/6J mice (8-10 weeks of age). The MTD was defined as the highest dose that allows 100% of the animals to survive without clinical signs of toxicity, such as blood in the stool, stool consistency; general signs, including shaking, anxiousness, hunching and breathing patterns, as well as maintaining body weight. 5 male and 5 female mice from each group received a single dose of 0 mg/kg (group 1), 5 mg/kg (groups 2), 25 mg/kg (group 3), 50 mg/kg (group 4), 75 mg/kg (group 5), 100 mg/kg (group 6), or 150 mg/kg (group 7) inhibitor, followed by observation at 1 h, 2 h, 4 h., 8 h, 24 h, 48 h, 72 h, and 96 h. Variation in body weight, stool consistency, and blood in the stool were recorded at 0 h, 24 h, 48 h, 72 h, and 96 h. Animals showing weight loss exceeding 20% were euthanized, as changes of this magnitude often indicate lethal toxicity.[5]

Western Blotting of PAD1 – MDA-MB-231 or MCF-7 cells were lysed in RIPA buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1% NP-40, 0.1% SDS, 0.5% deoxycholate, and 0.2 mM PMSF and 1X general protease inhibitor. Protein concentration in lysates was determined by Bradford Assay prior to gel loading to ensure equal protein loading. 6X sample buffer (300 mM Tris-HCl, pH 6.8, 60% glycerol, 30 mM DTT, 6% SDS) was added to yield a final concentration of 1X and lysates were boiled for 5 min. Samples were subjected

to SDS polyacrylamide gel electrophoresis on a 8% gel (acrylamide:bis-acrylamide ratio of 29:1) and electro-blotted to Immobilin PVDF membranes (Millipore). Membrane was blocked in 1X casein diluted in Tris buffered saline (TBS). Anti-PAD1 1:1000 (Sigma) was incubated overnight at 4^{o}C. The blot was washed and then incubated with a 1:10,000 dilution of anti-rabbit HRP (Jackson ImmunoResearch Labs, West Grove, PA) for 2 h at room temperature. The blot was washed for 30 min (3 x 10 min) with TBS-Tween and then visualized by chemiluminescence using Millipore Immobilon Western. To confirm equal protein loading, the membrane was stripped and re-probed with anti-GAPDH 1:6000 (Chemicon).

In Cellulo Efficacy in MDA-MB-231 Cells – MDA-MB-231 cells were seeded into 25 cm^2 tissue culture flasks at 1.5 x 10^6 viable cells /flask in triplicate for each sample. Cells were allowed to settle and attach overnight at 37 ^{0}C in 5% CO_2. The next morning growth medium (DMEM, Lonza Cat #12-741F, with 10% FBS, and antibiotics) was replaced with fresh medium containing 0, 50, 100, 200, or 400 μM drug. Flasks were harvested after 4 days incubation as above. On day 2 of incubation, an additional 3 flasks were treated with tunicamycin (10 μg/mL) as a positive control for apoptosis, or with tunicamycin (30 μg/mL) as a positive control for Ki-67 levels. These flasks were harvested at 48 h with the drug treated flasks.

To harvest the cells, the flask supernatant, washes and cells from each flask were placed in the same tube on ice so that a total cell count for each flask (viable and dead) could be made. Cells were detached from the flask with Accutase (Innovative Cell Technologies, Inc., Cat # AT104) which is superior to trypsin-EDTA for producing single cell suspensions for flow cytometry. Cell suspensions were centrifuged at 450xg for 5 min, the supernatant discarded and

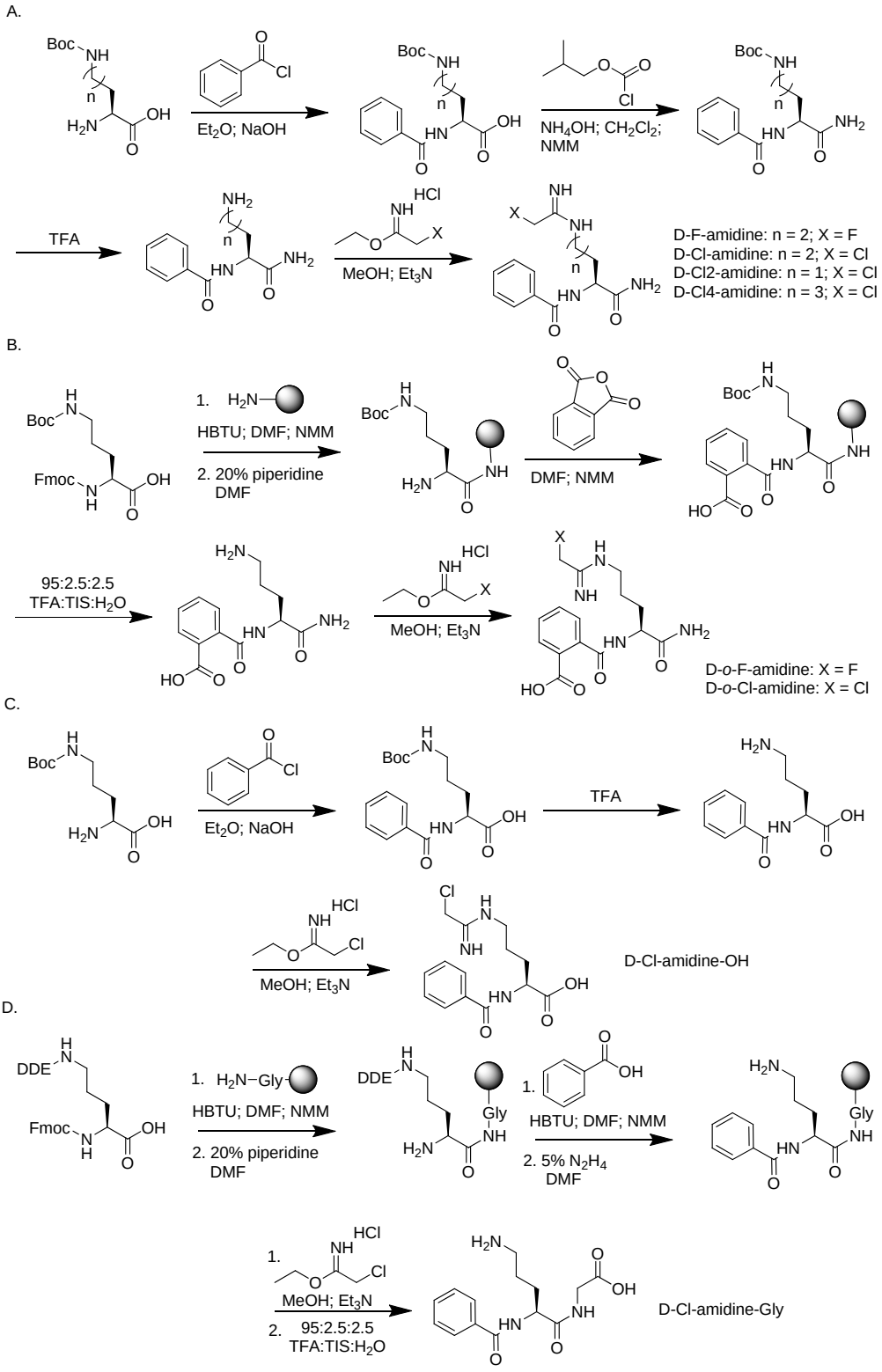
the pellet made up in 1 mL of ice cold HBSS (Gibco, Cat # 14170). A small aliquot of cells was mixed 1:1 in 0.4% trypan blue and total cell counts and viability determined using a BioRad TC-10 Automated Cell Counter.

Remaining cells were fixed in 2% paraformaldehyde in 0.1 M Dulbecco's phosphate buffered saline (PBS) for 15 min at room temperature, washed with PBS and blocked in permeabilization buffer (PB) (PBS containing 0.1% Triton X-100, 1% BSA, 0.02% sodium azide) with 10% normal goat serum for 1 h at rt. After centrifugation as above, cell pellets were mixed with 100 µL of diluted (in PB) antibodies (rabbit anti-cleaved caspase 3, Cell Signaling Cat#D175, and mouse anti-Ki67, BD Biosciences Cat# 556003) and incubated at rt for 1 h. Cells were washed 3 times with PB without Triton and gently mixed with 100 µL of diluted secondary antibodies (goat anti-rabbit conjugated to Alexa 488 and goat anti-mouse conjugated to Alexa 647, Invitrogen, Cat # A11034 and A21236, respectively) and incubated as for primaries. Cells were washed 3 times as described. Pellets were reconstituted to 200 µL with PB without Triton containing 1 µg/mL DAPI. Cell populations were analyzed on a Gallios flow cytometer (Beckman Coulter) and population data determined with Kaluza (Beckman-Coulter) and Flow-Jo (TreeStar) software. Data was reported as means of the three replicates of percent activated caspase positive (as a marker for apoptosis), and % Ki67 positive (as a marker for proliferating cells). The DAPI staining was used to determine percent of cells in G_0/G_1, S and G_2/M cell cycle phases.

Pharmacokinetics of D-Series Compounds – C57Bl6 mice, weighing approximately 25 g, were purchased from The Jackson Laboratories and used for all pharmacokinetic studies. All studies

were performed as previously described,[6] using procedures approved by the Institutional Animal Care and Use Committee (IACUC). A total of three mice were used for each experiment. Cl-amidine was administered to each mouse by intraperitoneal injection (IP; 10 mg/kg dose) or intravenous injection (IV; 10 mg/kg) in saline. D-Cl-amidine was administered to each mouse by IP (2.5 mg/kg dose) or IV (10 mg/kg) in saline. Note that a lower dose of D-Cl-amidine was administered by IP during these studies to accommodate the simultaneous analysis of other compounds unrelated to this study. Approximately 20 μL of blood was collected into an EDTA-K_2 tube at times 1, 2, 4, and 8 h. Plasma was isolated from whole blood by standard centrifugation techniques. Plasma samples were mixed 1:5 with ACN containing an internal standard, filtered and, subsequently analyzed by LC-MS/MS (API Sciex 4000).

Supplementary Figures



Scheme S1. Synthesis of D-series compounds.

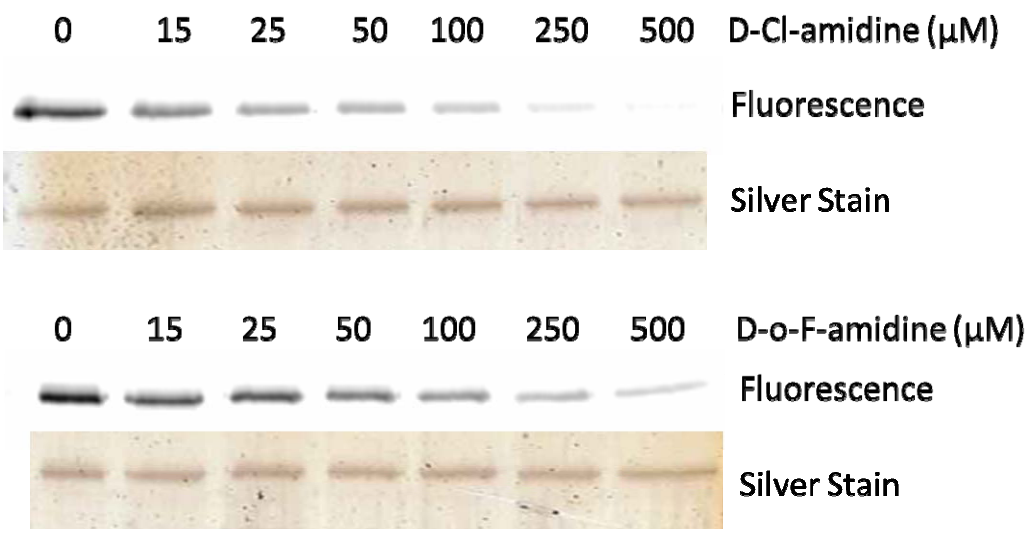


Figure S1. Competitive ABPP experiment between D-Cl-amidine or D-o-F-amidine and the fluorescent probe RCA, indicating that both D-compounds compete for binding to the active site of PAD1.

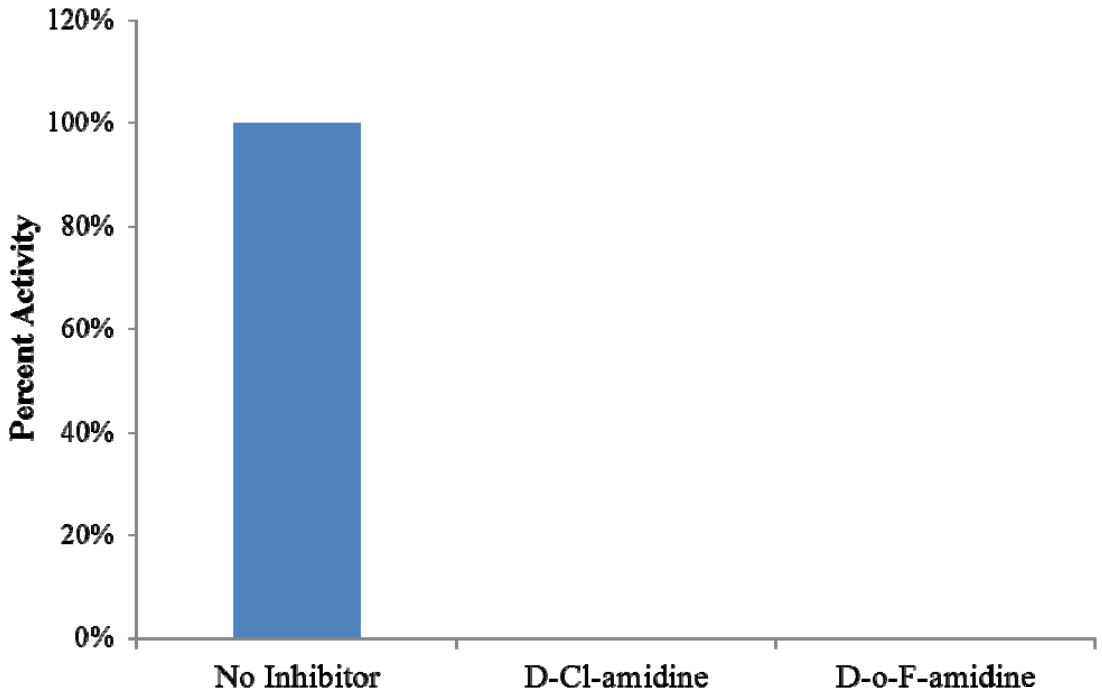


Figure S2. Dialysis experiments indicate that like L-Cl-amidine, D-Cl-amidine, and D-o-F-amidine are irreversible with respect to PAD1.

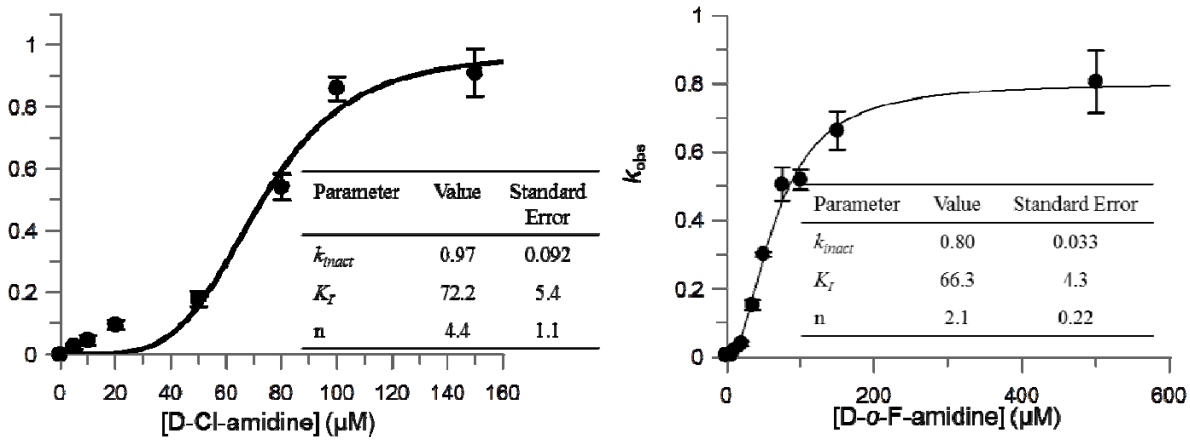


Figure S3. Plot of k_{obs} vs. concentration of inhibitor, indicating that these inhibitors show cooperative inhibition of PAD1.

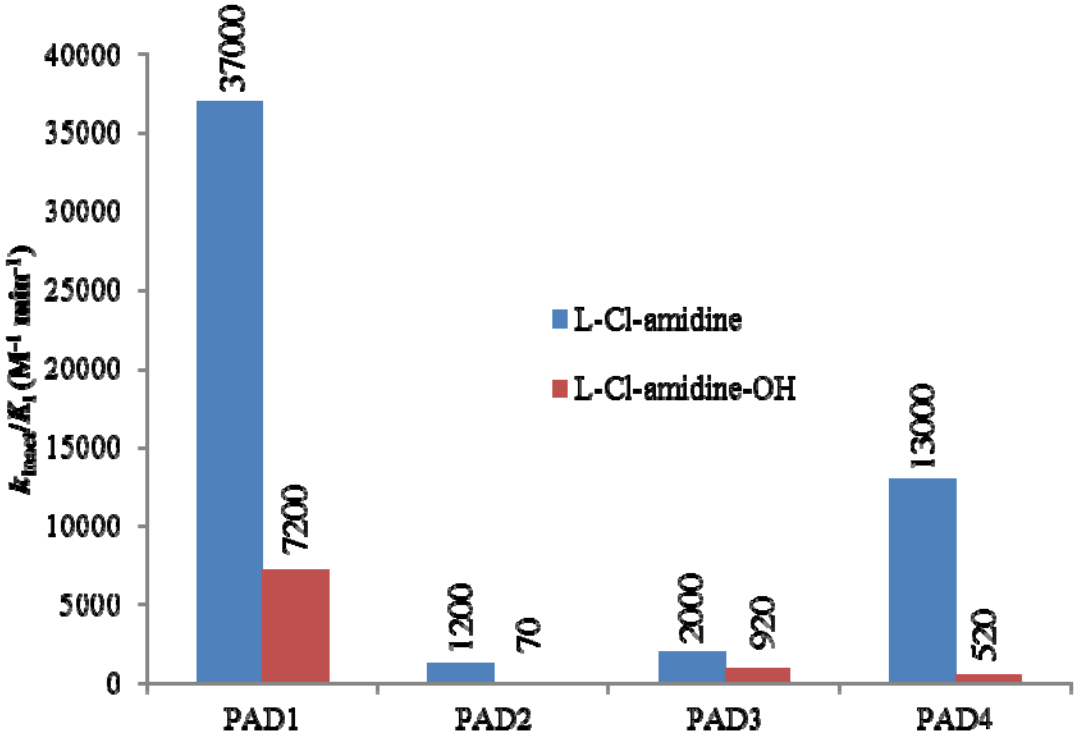


Figure S4. Comparison of L-Cl-amidine and L-Cl-amidine-OH, a possible metabolite of L-Cl-amidine, indicating that hydrolysis of the C-terminal amide would decrease *in vivo* potency.

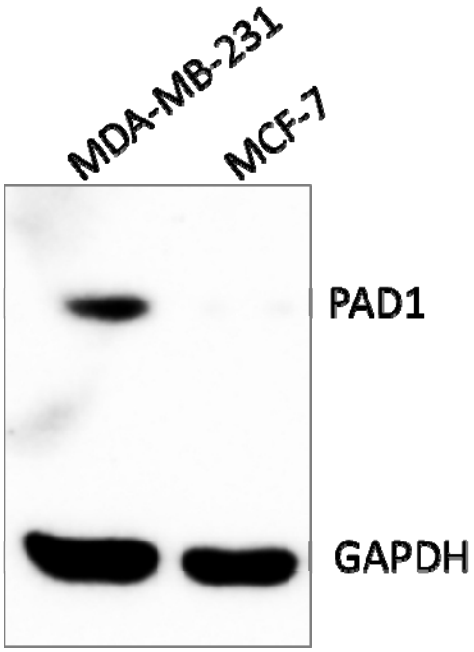


Figure S5. Triple negative breast cancer cells, MDA-MB-231, overexpress PAD1 in comparison to MCF-7 cells.

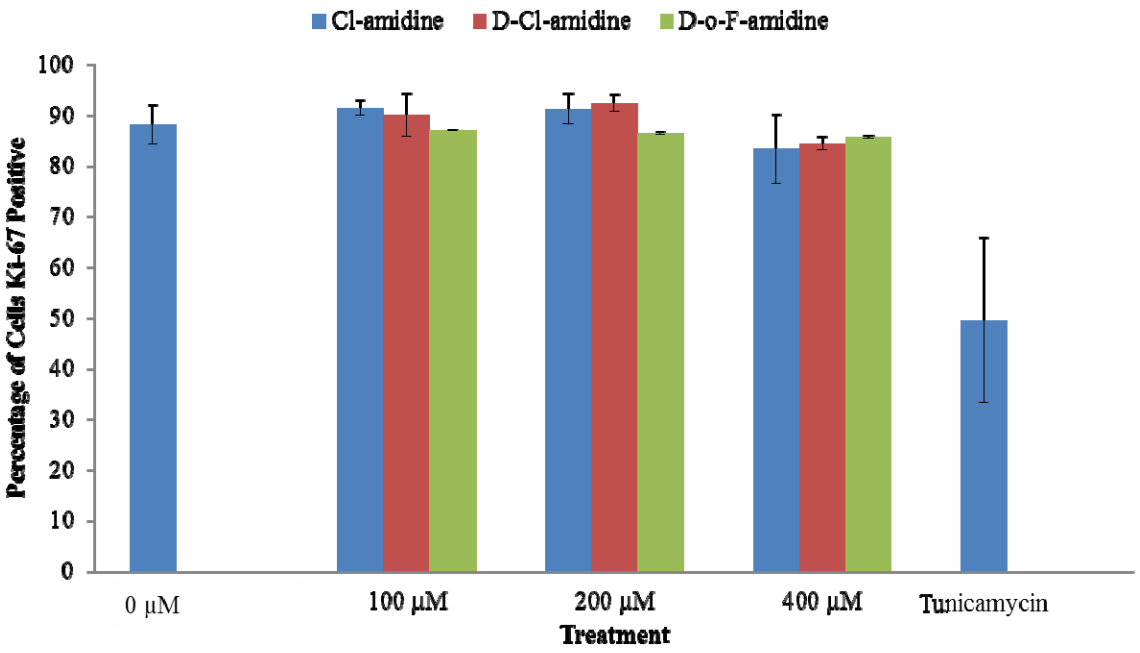


Figure S6. Analysis of Ki-67, a marker of cellular proliferation, in MDA-MB-231 cells in response to varying concentrations of Cl-amidine, D-Cl-amidine, and D-o-F-amidine. No significant decrease in protein levels is observed. Tunicamycin is used as a positive control.

Table S1. Average weight changes in mice consuming water *ad libitum* 96 hours after Cl-amidine treatment *per os*.

Group	Dose (mg/kg)	Females (g)	Males (g)	Overall (g)	Females (%)	Males (%)	Overall (%)
1	0	0.44±0.08	0.08±0.14	0.30±0.1	2.07	0.32	1.2
2	25	0.44±0.08	0.10±0.13	0.27±0.09	2.07	0.39	1.23
3	50	0.10±0.08	0.08±0.07	0.09±0.05	0.46	0.3	0.38
4	75	-0.08±0.17	-0.02±0.11	-0.05±0.09	-0.36	-0.08	-0.22
5*	100	-0.14±0.02	-0.28±0.12	-0.21±0.12	-0.63	-1.04	-0.84***
6**	150	-0.18±0.08	-0.22±0.02	-0.14±0.05	-0.81	-0.82	-0.82***

*, indicates at 24 h, all 10 mice in this group developed diarrhea or soft stool that lasted 2-3 days.

**, indicates all 10 mice in this group were notably shaking, anxiousness, hunching and breathing heavily from 1-4 h after treatment. Thereafter, such signs and symptoms dissipated. At 24 h, all mice developed diarrhea or soft stool that lasted 2-3 days.

***, indicates a significant decrease in weight (P-value < 0.05).

Table S2. Average weight changes in mice consuming water *ad libitum* 96 hours after D-Cl-amidine treatment *per os*.

Group	Dose (mg/kg)	Females (g)	Males (g)	Overall (g)	Females (%)	Males (%)	Overall (%)
1	0	0.52±0.23	0.68±0.22	0.56±0.25	2.8	2.86	2.64
2	25	1.20±0.62	0.70±0.38	1.02±0.53	6	2.87	4.57
3	50	0.36±0.65	0.18±0.57	0.27±0.58	1.75	0.84	1.29
4*	75	0.62±0.54	0.00±0.35	0.31±0.54	3.02	0	1.44
5**	100	0.24±0.38	0.74±0.67	0.49±0.58	1.12	3.38	2.67
6***	150	0.04±0.54	0.72±0.42	0.38±0.60	0.19	2.9	1.66

*, indicates at day 2, female mice in this group developed bloody stool.

**, indicates at day 2, female mice in this group developed bloody stool; at day 3, female mice in this group developed soft stool; at day 4, both males and females in this group developed bloody stool.

***, indicates at day 3, female mice in this group developed soft stool; at day 4, females developed blood in stool, male mice developed soft stool.

For 100 mg/kg group, at 2 h, one male mouse was slow moving that lasted for more than 8 h, then recovered at 24 h,

For 150 mg/kg group, 2 female mice were noticeably slow moving that lasted for 24 h. 2 male mice were noticeably slow moving, one of them recovered in 2 h, the other recovered in 8 h.

Table S3. Average weight changes in mice consuming water *ad libitum* 96 hours after D-o-F-amidine treatment *per os*.

Group	Dose (mg/kg)	Females (g)	Males (g)	Overall (g)	Females (%)	Males (%)	Overall (%)
1	0	0.54±0.30	0.26±0.27	0.40±0.31	2.92	1.09	1.89
2	25	0.14±0.47	0.50±0.37	0.32±0.44	0.78	2.04	1.51
3*	50	0.16±0.30	0.60±0.39	0.38±0.40	0.89	2.47	1.79
4*	75	0.28±0.53	0.32±0.44	0.30±0.46	1.45	1.46	1.46
5**	100	-0.02±0.30	0.17±0.28	0.07±0.29	-0.13	0.72	0.35
6***	150	0.44±0.30	0.48±0.27	0.46±0.27	2.35	1.94	2.12

*, indicates at day 2, mice in this group developed soft stool.

**, indicates female mice in this group developed soft stool, male mice in this group developed bloody stool at day 1.

***, indicates female and male mice in this group developed bloody stool at day 1.

Table S4. Student t-test P-values for inhibitor significance versus no treatment in MDA-MB-231 cells.

Compound	Cell Number	% Viability	Caspase 3 Activity
100 μM Cl-amidine	0.362	0.508	0.524
200 μM Cl-amidine	**0.0324**	**0.00490**	0.103
400 μM Cl-amidine	**0.0165**	**0.00134**	**0.00709**
100 μM D-Cl-amidine	0.946	0.627	0.3714
200 μM D-Cl-amidine	0.819	**0.00179**	0.153
400 μM D-Cl-amidine	**0.0235**	**0.00604**	**0.000143**
100 μM D-o-F-amidine	0.978	0.879	0.00659
200 μM D-o-F-amidine	0.0570	0.301	0.00511
400 μM D-o-F-amidine	**0.0387**	0.310	0.00694
Tunicamycin 10 μg/ml			**0.00929**

References

[1] C. P. Causey, P. R. Thompson, *Tetrahedron Lett.* **2008**, *49*, 4383-4385.

[2] C. P. Causey, J. E. Jones, J. L. Slack, D. Kamei, L. E. Jones, V. Subramanian, B. Knuckley, P. Ebrahimi, A. A. Chumanevich, Y. Luo, H. Hashimoto, M. Sato, L. J. Hofseth, P. R. Thompson, *Journal of Medicinal Chemistry* **2011**, *54*, 6919-6935.

[3] P. L. Kearney, M. Bhatia, N. G. Jones, L. Yuan, M. C. Glascock, K. L. Catchings, M. Yamada, P. R. Thompson, *Biochemistry* **2005**, *44*, 10570-10582.

[4] Y. Luo, B. Knuckley, M. Bhatia, P. J. Pellechia, P. R. Thompson, *Journal of the American Chemical Society* **2006**, *128*, 14468-14469.

[5] E. J. Freireich, E. A. Gehan, D. P. Rall, L. H. Schmidt, H. E. Skipper, *Cancer Chemother. Rep.* **1966**, *50*, 219-244.

[6] S. P. Brothers, S. A. Saldanha, T. P. Spicer, M. Cameron, B. A. Mercer, P. Chase, P. McDonald, C. Wahlestedt, P. S. Hodder, *Molecular Pharmacology* **2010**, *77*, 46-57.