Supporting Information for:

The Development of Benzimidazole-Based Clickable Probes for the Efficient Labeling of Cellular Protein Arginine Deiminases (PADs)

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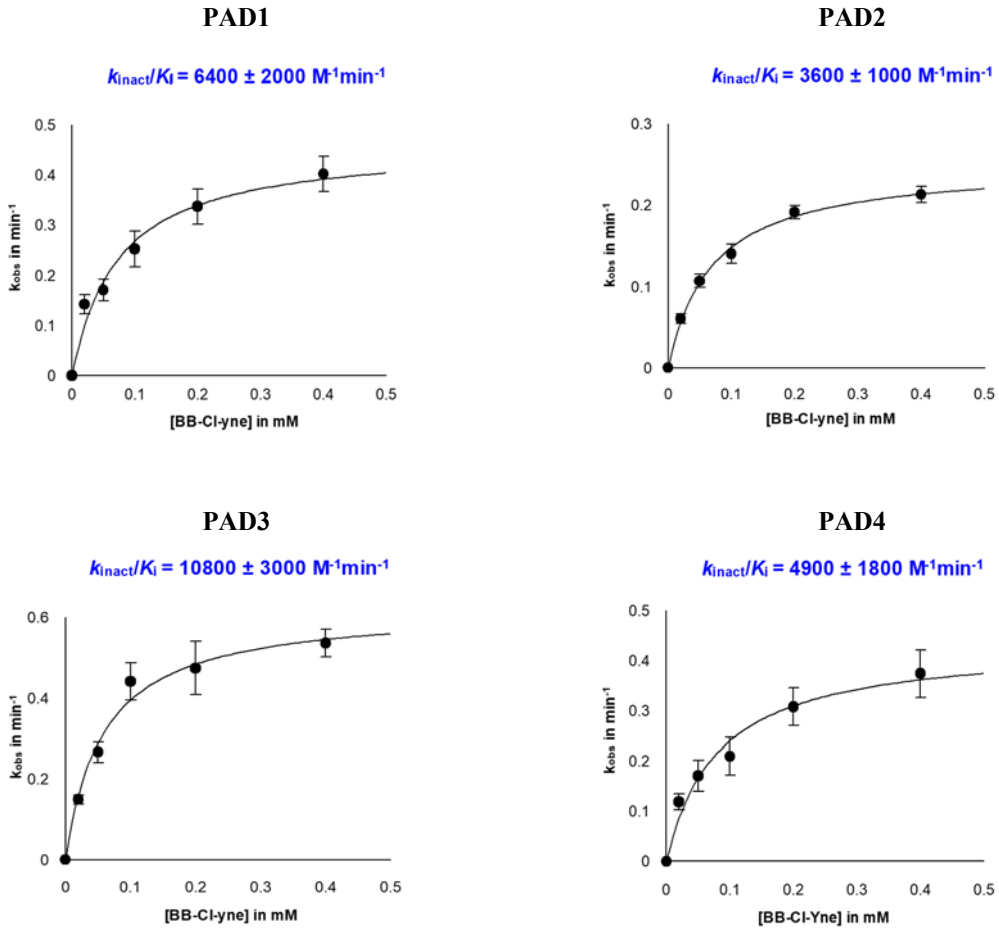
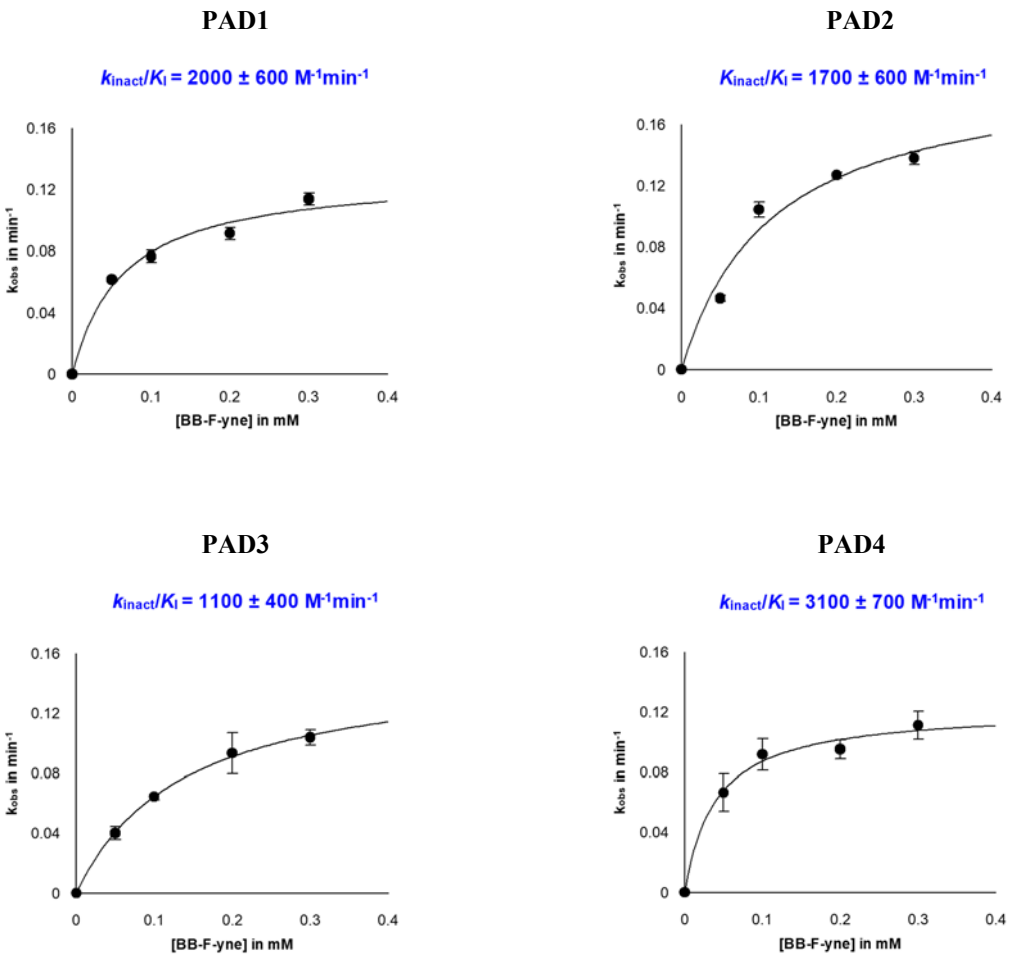
Supporting Figures S1-S8 and Supporting Table S1:



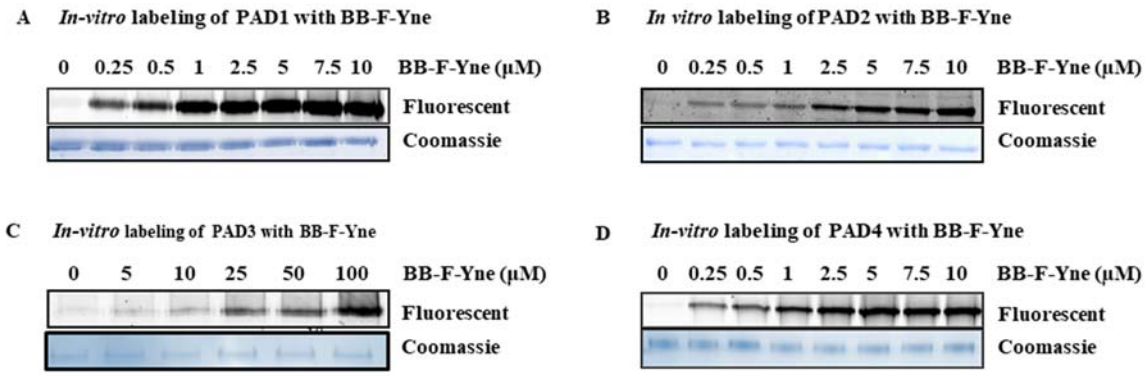
Supplementary Figure S1: Plots showing the fit of k_{obs} versus BB-Cl-Yne concentration for all PAD isoforms. k_{inact} and K_I values are shown above each plot. The data is an average from at least two different replicates.



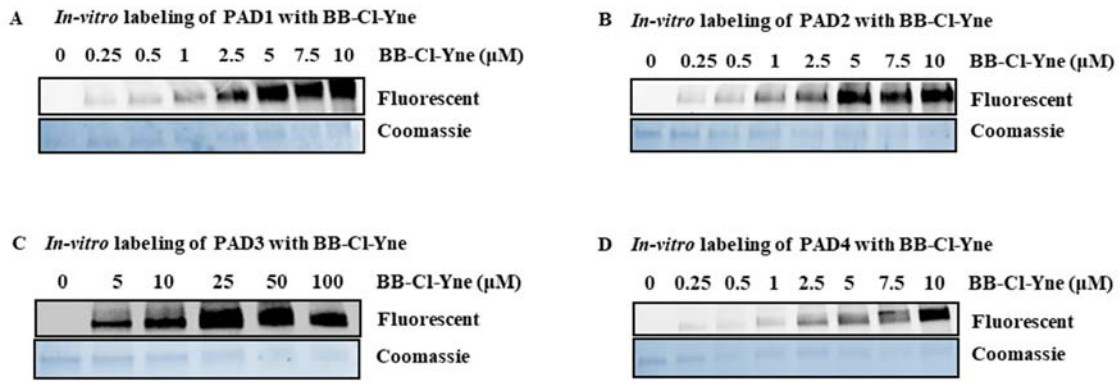
Supplementary Figure S2: Plots showing the fit of k_{obs} versus BB-F-Yne concentration for all PAD isoforms. k_{inact} and K_I values are shown above each plot. The data is an average from at least two different replicates.

Table S1: k_{inact}, K_I and k_{inact}/K_I values for both BB-Cl-Yne and BB-F-Yne against all PAD isoforms

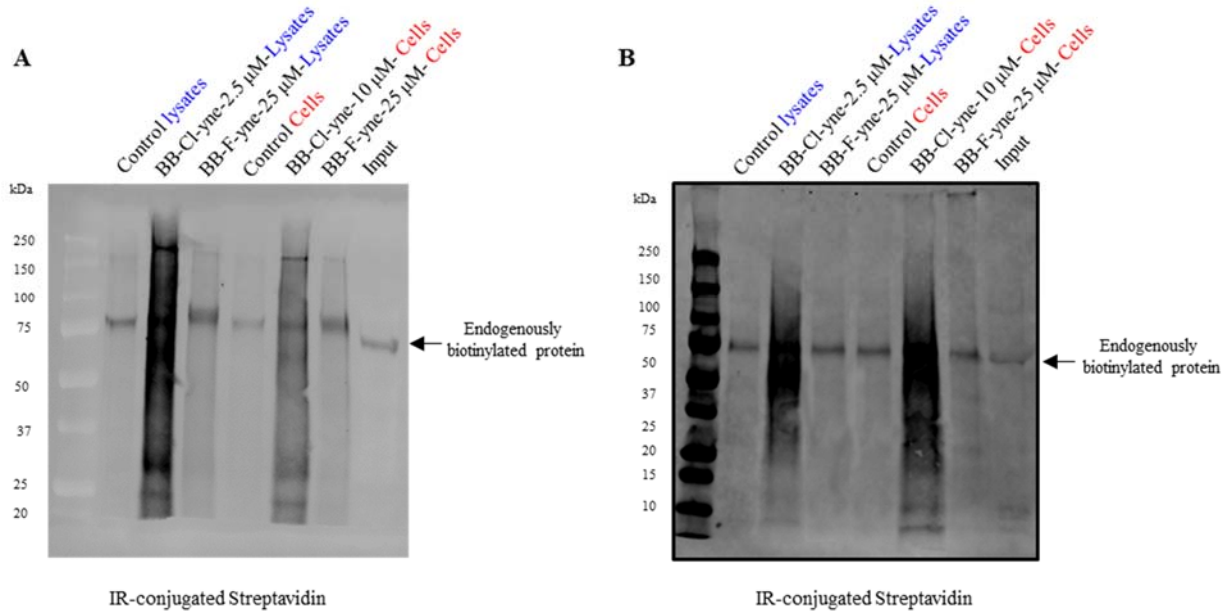
	BB-Cl-Yne			BB-F-Yne		
PAD isozymes	k_{inact} (min^{-1})	K_I (μM)	k_{inact}/K_I (M^{-1} min^{-1})	k_{inact} (min^{-1})	K_I (μM)	k_{inact}/K_I (M^{-1} min^{-1})
PAD1	0.46 ± 0.1	73 ± 18	6400	0.13 ± 0.1	64 ± 19	2050
PAD2	0.25 ± 0.1	69 ± 7	3600	0.19 ± 0.1	115 ± 45	1700
PAD3	0.62 ± 0.1	58 ± 11	10800	0.15 ± 0.1	137 ± 12	1100
PAD4	0.43 ± 0.1	81 ± 21	4900	0.12 ± 0.1	40 ± 10	3100



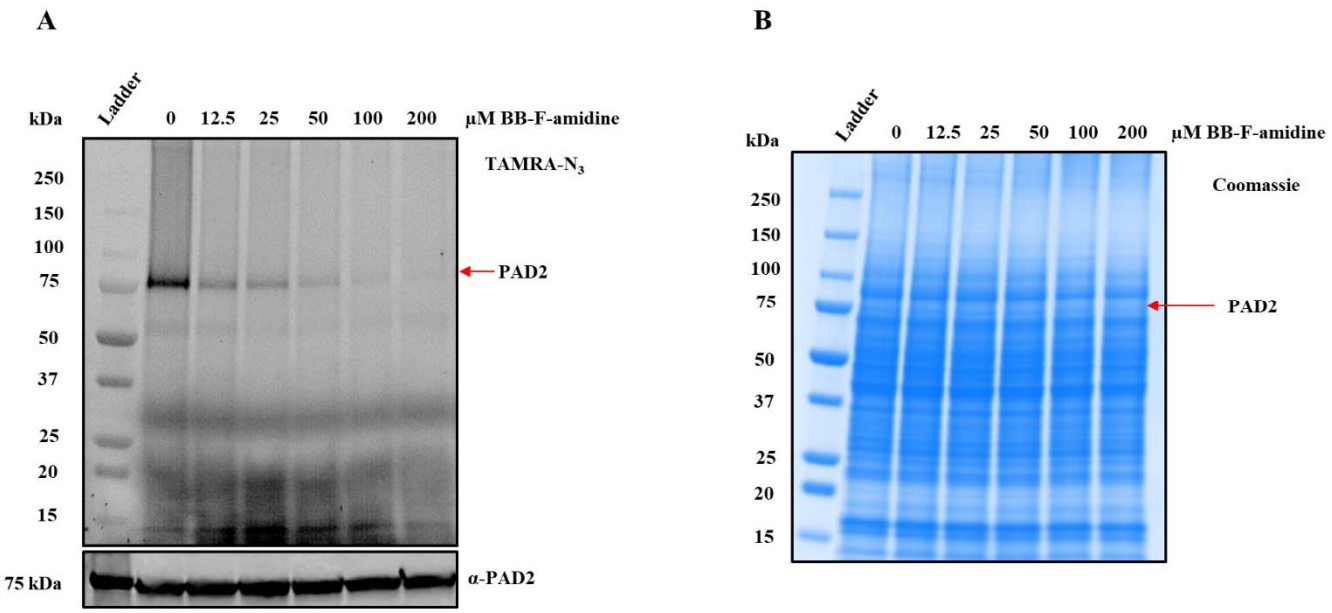
Supplementary Figure S3. *In vitro* labeling of the four active PAD isozymes with BB-F-Yne. For labeling studies, the probes were incubated with PADs for 1 h in the presence of 2 mM $CaCl_2$. (A) Concentration-dependent labeling of recombinant PAD1 with BB-F-Yne (**6**). PAD1 was treated with increasing concentrations of BB-F-Yne (**6**) and then "Clicked" with TAMRA-N_3. (B) Concentration-dependent labeling of recombinant PAD2 with BB-F-Yne (**6**). PAD2 was treated with increasing concentrations of BB-F-Yne (**6**) and then "Clicked" with TAMRA-N_3. (C) Concentration-dependent labeling of recombinant PAD3 with BB-F-Yne (**6**). PAD3 was treated with increasing concentrations of BB-F-Yne (**6**) and then "Clicked" with TAMRA-N_3. (D) Concentration-dependent labeling of recombinant PAD4 with BB-F-Yne (**6**). PAD4 was treated with increasing concentrations of BB-F-Yne (**6**) and then "Clicked" with TAMRA-N_3.



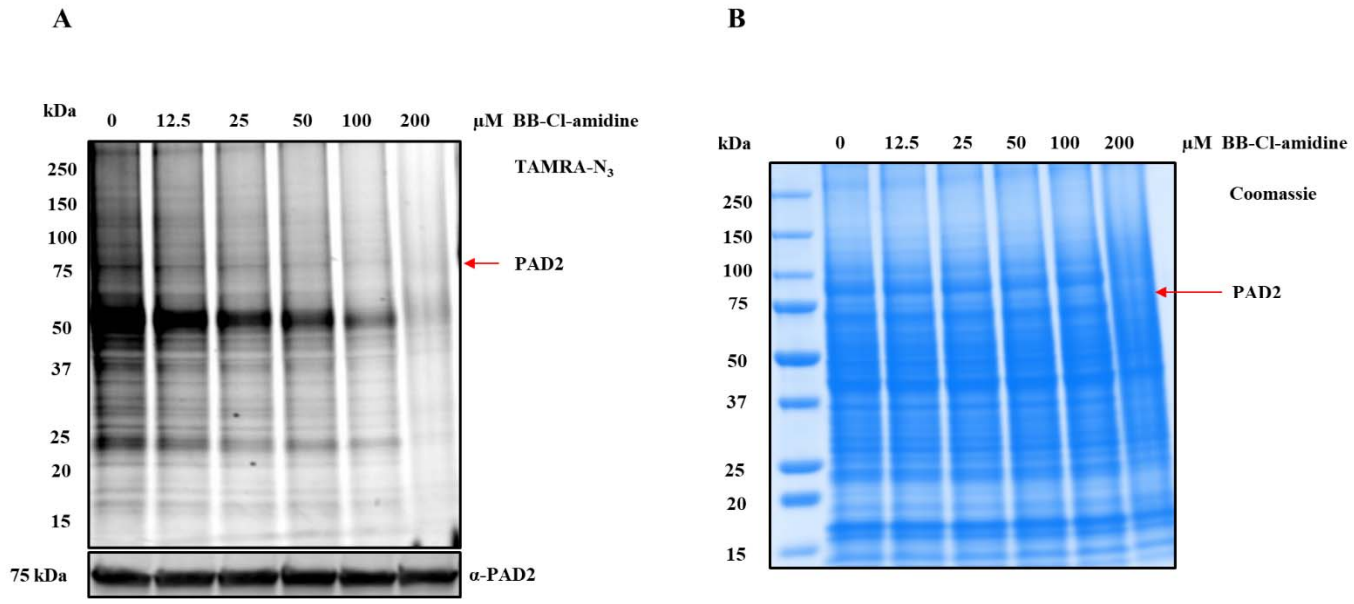
Supplementary Figure S4. In vitro labeling of the four active PAD isozymes with BB-Cl-Yne. For labeling studies, the probes were incubated with PADs for 1 h in the presence of 2 mM $CaCl_2$. (A) Concentration-dependent labeling of recombinant PAD1 with BB-Cl-Yne **(5).** PAD1 was treated with increasing concentrations of BB-Cl-Yne (**5**) and then "Clicked" with TAMRA-N_3. (B) Concentration-dependent labeling of recombinant PAD2 with BB-Cl-Yne (**5**). PAD2 was treated with increasing concentrations of BB-Cl-Yne (**5**) and then "Clicked" with TAMRA-N_3. (C) Concentration-dependent labeling of recombinant PAD3 with BB-Cl-Yne (**5**). PAD3 was treated with increasing concentrations of BB-Cl-Yne (**5**) and then "Clicked" with TAMRA-N_3. (D) Concentration-dependent labeling of recombinant PAD4 with BB-Cl-Yne (**5**). PAD4 was treated with increasing concentrations of BB-Cl-Yne (**5**) and then "Clicked" with TAMRA-N_3.



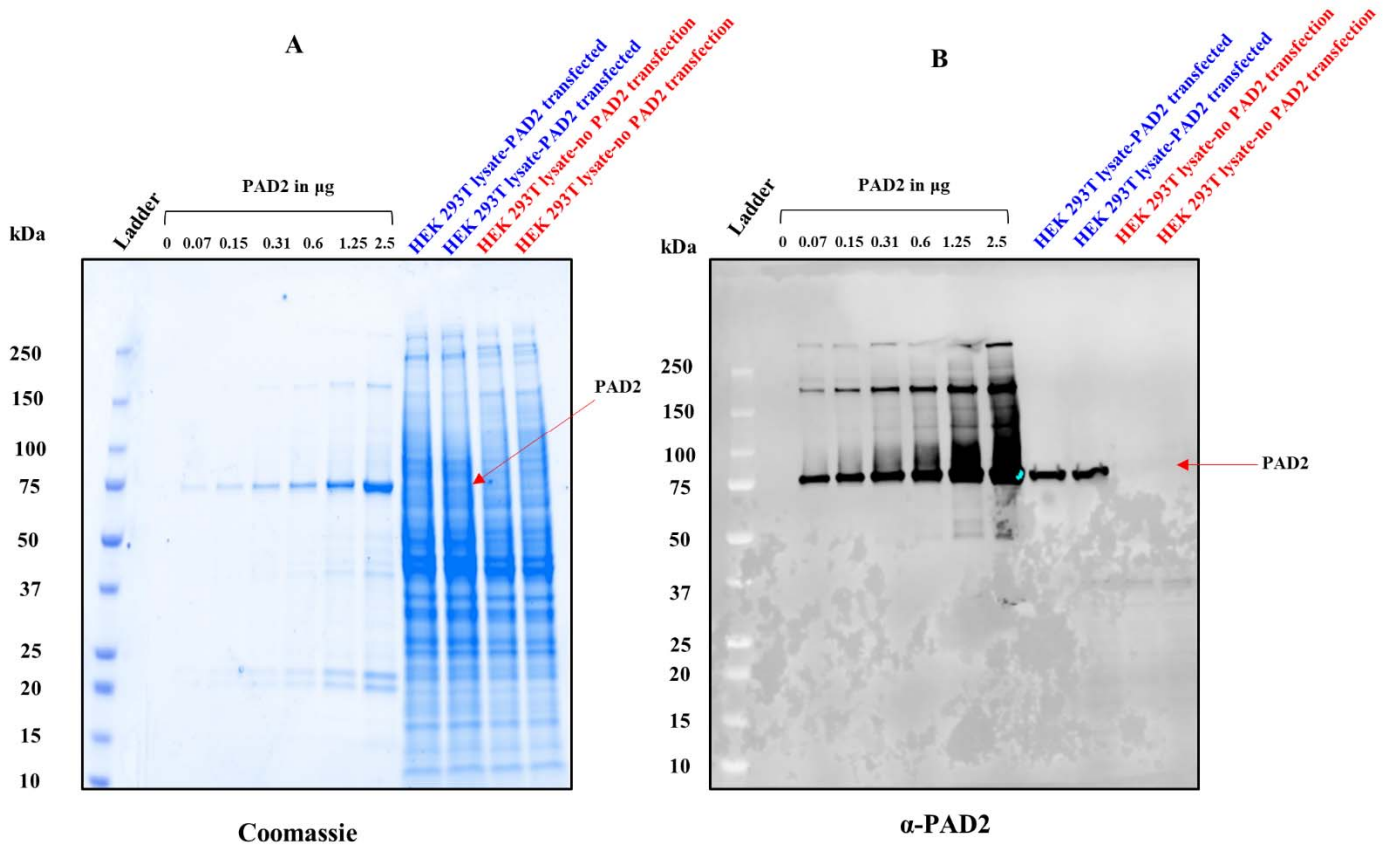
Supplementary Figure S5. (**A**) HEK293T/PAD2 cells were treated with BB-Cl-Yne (2.5 µM), BB-F-Yne (25 µM), and DMSO, or (**B)** BB-Cl-Yne (10 µM), BB-F-Yne (25 µM), and DMSO and incubated at 37 °C for 0.5 h. 5 µM ionomycin and 1 mM $CaCl_2$ were added and incubated at 37 °C for another 1h. The cells were sonicated and the probe labeled proteins were tagged with Biotin-N_3. Biotin-tagged proteins were incubated with streptavidin agarose beads and the unbound proteins were washed off. The Streptavidin beads were heated with 1.2% SDS 95 °C and the supernatants subjected to SDS PAGE and western blotting. Biotinylated proteins were detected by IR-conjugated streptavidin.



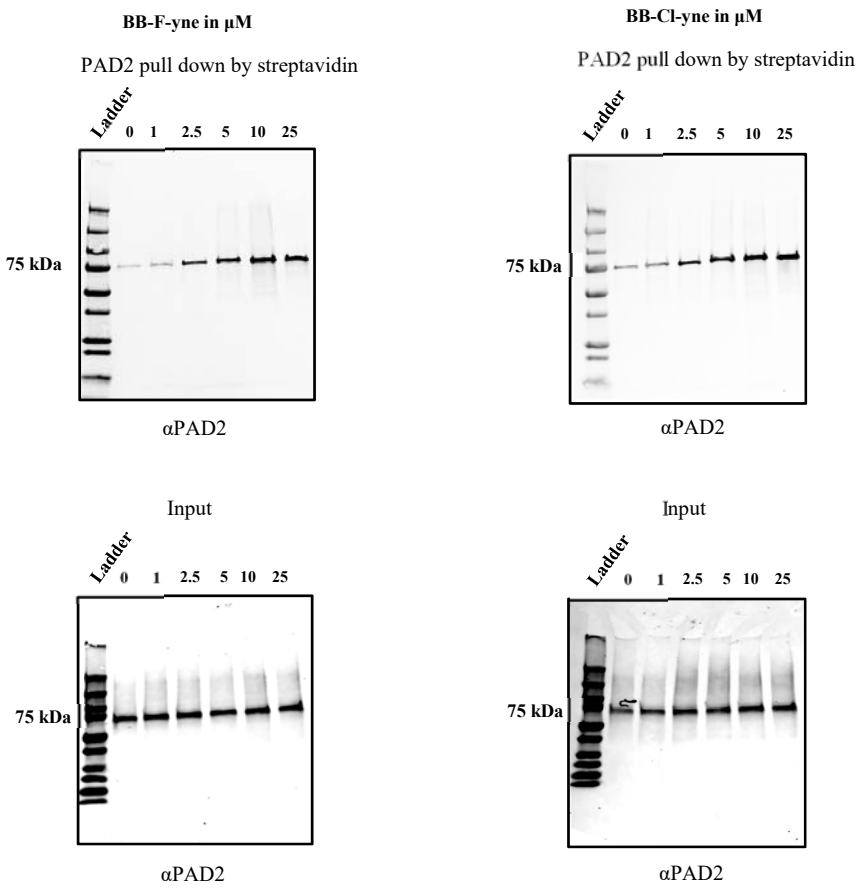
Supplementary Figure S6. Competitive ABPP experiments with BB-F-amidine. (**A**) HEK 293T cell lysates were incubated with increasing concentrations of BB-F-amidine in the presence of 1 mM $CaCl_2$ for 15 min at 37 °C. BB-F-Yne (25 µM) was added and the mixture was further incubated for 1 h at 37 °C. The probe labelled proteins were tagged with TAMRA-azide to facilitate fluorescent visualization after SDS page. The visualized gel was transferred to a PVDF membrane and anti-PAD2 antibody was used to visualize PAD2 by western blot (shown in the bottom). (**B**) Coomassie staining of the SDS page gel showing equal protein loading.



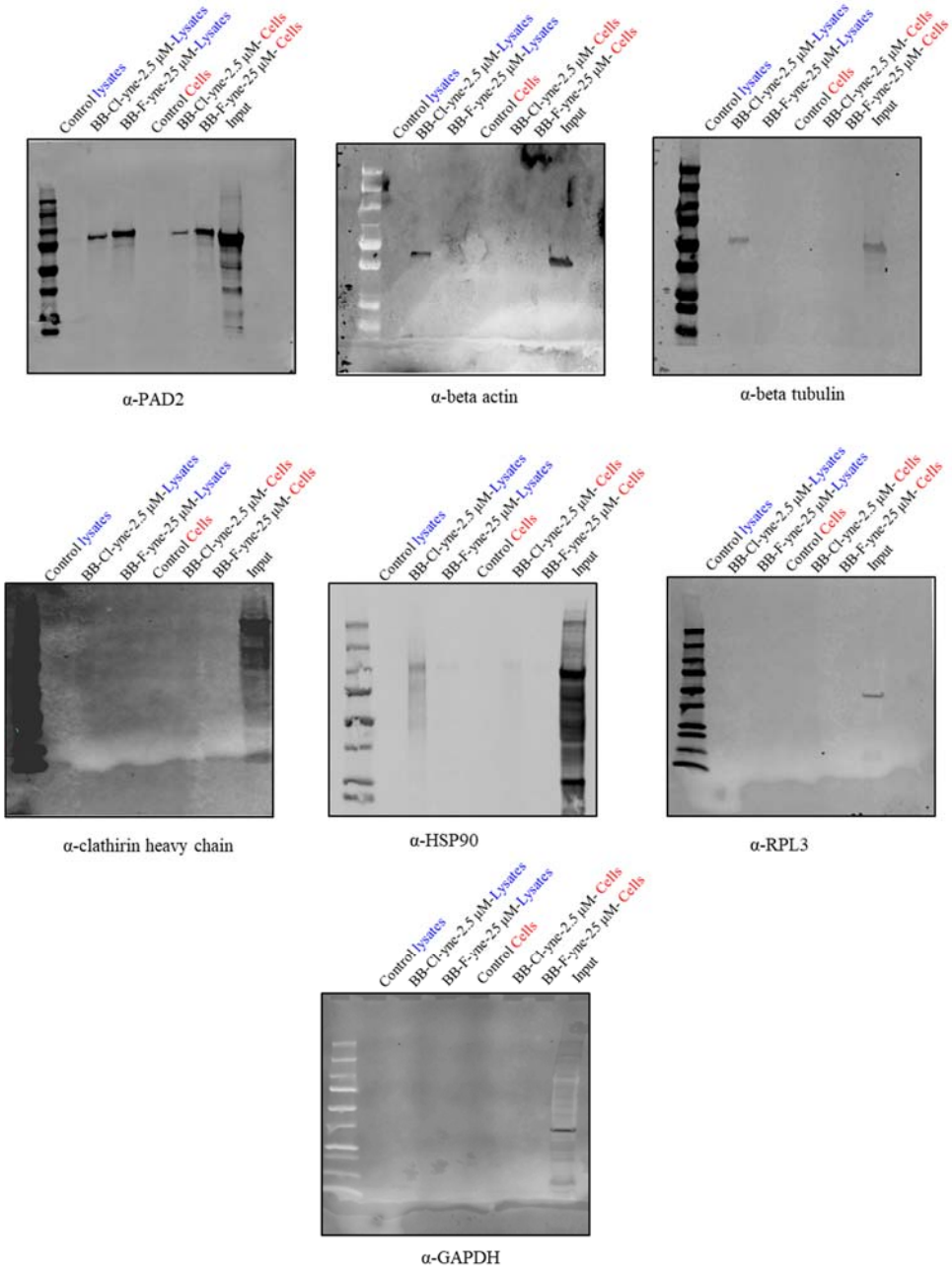
Supplementary Figure S7. Competitive ABPP experiment with BB-Cl-amdine. (**A**) HEK 293T cell lysates were incubated with increasing concentrations of BB-Cl-amidine in the presence of 1 mM $CaCl_2$ for 15 min at 37 °C. BB-Cl-Yne (25 µM) was added and the mixture was further incubated for 1 h at 37 °C. The probe labelled proteins were tagged with TAMRA azide to facilitate fluorescent visualization after SDS page. The visualized gel was transferred to a PVDF membrane and anti-PAD2 antibody was used to visualize PAD2 by western blot (shown in the bottom). (**B**) Coomassie staining of the SDS page gel showing equal protein loading.



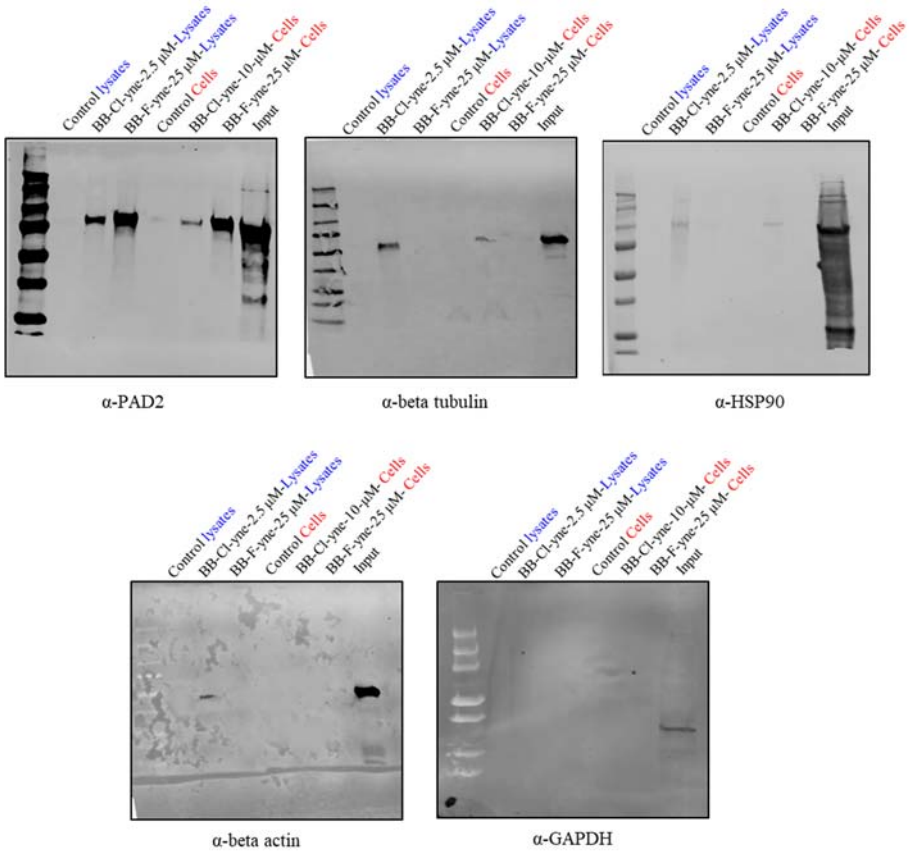
Supplementary Figure S8. Coomassie staining of HEK cell lysates with and without PAD2 transfected. (**A**) Coomassie stained gel of recombinantly expressed PAD2 (0, 0.078, 0.156, 0.312, 0.625, 1.25 and 2.5 µg in each lane) and 25 µg of HEK cell lysates with and without transfected PAD2. Concentration of PAD2 in HEK cell lysates was calculated from a standard curve obtained using different amounts of recombinant PAD2. The amount of PAD2 in the HEK cell lysates transfected with PAD2 was 206 ng, which amounts to about ~0.8% of the total protein concentration. (**B**) Western blot of the HEK cell lysates shows the presence of PAD2 in transfected HEK 293 cell line, whereas the non-transfected HEK cell line does not express detectable amounts of PAD2.



Supplementary Figure S9: HEK293T/PAD2 cells were treated with increasing concentrations of BB-F-Yne (**A**) or BB-Cl-Yne (**B**) for 0.5 h. 5 µM ionomycin and 1 mM $CaCl_2$ were added and incubated at 37 °C for another 1 h. The cells were sonicated and the probe-labeled proteins were tagged with Biotin-N_3. Biotin tagged proteins were then isolated on streptavidin agarose and the eluted proteins were probed for PAD2 by western blot.



Supplementary Figure S10: HEK293T/PAD2 cells were treated with BB-Cl-Yne (2.5 μM), BB-F-Yne (25 μM) or DMSO, followed by 5 μM ionomycin and 1 mM $CaCl_2$ and incubated at 37 °C for 1h. Cells were sonicated and the probe labeled proteins were tagged with Biotin-N_3. Biotin tagged proteins were then isolated on streptavidin agarose and the eluted proteins were probed for various proteins with the indicated antibodies.



Supplementary Figure S11: HEK293T/PAD2 cells were treated with BB-Cl-Yne (10 μM), BB-F-Yne (25 μM) or DMSO, followed by 5 μM ionomycin and 1 mM $CaCl_2$ and incubated at 37 °C for 1h. Cells were sonicated and the probe labeled proteins were tagged with Biotin-N_3. Biotin-tagged proteins were then isolated on streptavidin agarose and the eluted proteins were probed for the isolation of various proteins using the indicated antibodies.

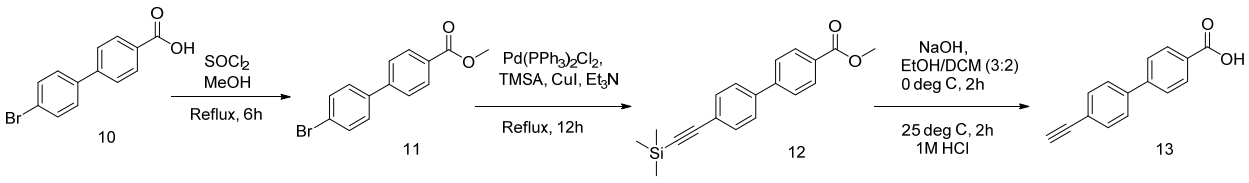
Supporting Information – Detailed Methods

Chemicals and Proteins

Chemistry. ^{1}H NMR were recorded at 400 MHz using a Bruker DRX-400 with a H/C/P/F QNP gradient probe spectrometer and ^{13}C NMR spectra were recorded at 100 MHz. Chemical shifts are reported in δ (ppm) relative to internal chloroform-*d* ($CDCl_3$, 7.26 ppm) or methanol-*d* (CD_3OD, 3.31 ppm). ESI-HRMS signals were recorded with a Micromass Q-TOF I. The purity of all compounds was determined to be >95% purity as determined by ^{1}H NMR and ^{13}C NMR spectra, unless otherwise noted. TLC was performed on glass backed silica gel plates (Uniplate) with spots visualized by UV light. All solvents were reagent grade and, when necessary, were purified and dried by standard methods. Concentrations of solutions after reactions and extractions involved the use of a rotary evaporator operating at reduced pressure.

Synthetic Procedures.

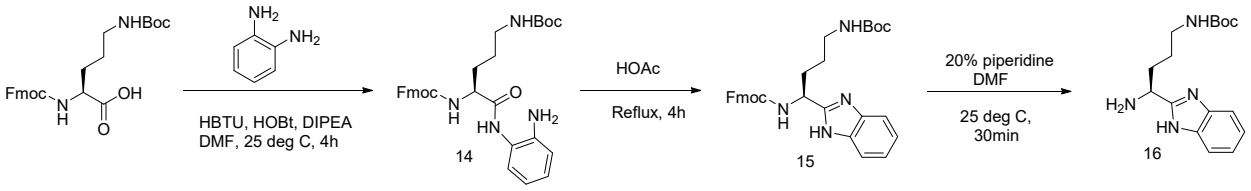
Synthesis of compound 13.



Thionyl chloride (5.3 mL) was added dropwise to a suspension of **10** (2 g, 7.2 mmol) in methanol at 0 °C and the mixture was refluxed for 6 h. The resultant suspension was evaporated *in vacuo* to remove methanol and excess thionyl chloride. Compound **11** was obtained as a white powder (1.8 g, crude yield: 86%) and was used for the subsequent steps without further purification. ^{1}H NMR (d_6-DMSO) δ (ppm): 8.05 (d, J = 8 Hz, 2H), 7.71 (d, J = 8.5 Hz, 2H), 7.71 (d, J = 2 Hz, 4H),

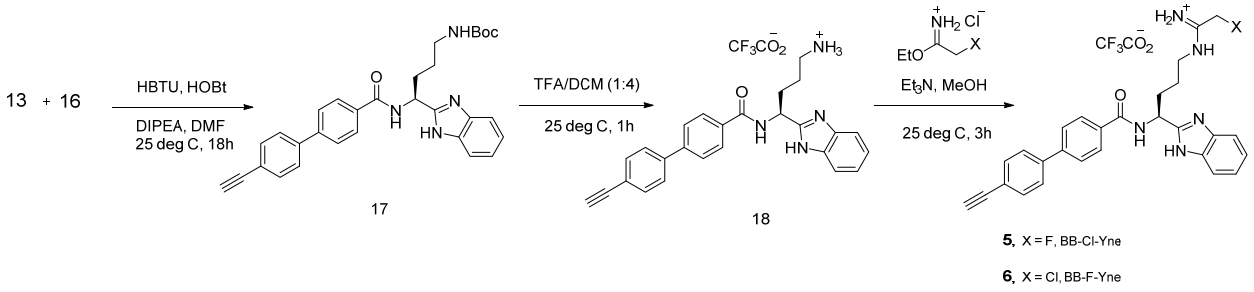
3.88 (s, 3H). Compound **11** was coupled with trimethylsilyl acetylene (TMSA) by following a reported procedure[1] with minor modifications. Briefly, **11** (1g, 3.4 mmol), $Pd(PPh_3)_2Cl_2$ (241 mg, 0.3 mmol), copper (I) iodide (66 mg, 0.3 mmol) and TMSA (1.9 mL, 13.74 mmol) were taken in a two-necked round-bottomed flask and deoxygenated triethylamine (30 mL) was added to the mixture. The resultant mixture was refluxed for 12 h under nitrogen atmosphere. The dark brown-colored suspension was evaporated *in vacuo* to remove the excess triethylamine and was re-suspended in water. Then the mixture was extracted two times with dichloromethane and the combined organic extracts were washed with water, dried over anhydrous sodium sulphate and concentrated *in vacuo*. The crude product was then purified by column chromatography using hexane/ethyl acetate (9:1) as the mobile phase to afford compound **12** as a white solid (0.8 g, yield: 75%). ^{1}H NMR ($CDCl_3$) δ (ppm): 7.99 (d, J = 8 Hz, 2H), 7.52 (d, J = 8 Hz, 2H), 7.45 (s, 4H), 3.83 (s, 3H), 0.18 (s, 9H). Compound **12** (1 g, 3.3 mmol) was dissolved in a mixture of ethanol and dichloromethane (3:2) and 1 M sodium hydroxide (13 mL) was slowly added to the solution from a dropping funnel at 0 °C. The solution was further stirred at 0 °C for 2 h before warming it up to 25 °C over 2 h. The reaction mixture was then diluted with water, washed with diethyl ether and the aqueous layer was acidified with 1 M HCl. The resultant suspension was extracted two times with diethyl ether and the combined organic extracts were washed with water, dried over anhydrous sodium sulfate and concentrated *in vacuo* to afford **13** as an off-white solid (400 mg, yield: 55%). ^{1}H NMR ($CDCl_3$) δ (ppm): 8.12 (d, J = 8 Hz, 2H), 7.63 (d, J = 8.5 Hz, 2H), 7.55 (s, 4H), 3.11 (s, 1H).

Synthesis of compound 16.



Fmoc-Orn(Boc)-OH (1 g, 2.2 mmol) and 1,2-phenylenediamine (238 mg, 2.2 mmol) was dissolved in DMF and diisopropyl ethylamine (DIPEA) (1.2 mL, 6.6 mmol), HBTU (1.3 g, 3.3 mmol) and HOBt (297 mg, 2.2 mmol) were added sequentially to the solution. The reaction mixture was stirred under nitrogen atmosphere for 4 h at 25 °C and then poured into water to precipitate compound **14**, which was recovered by vacuum filtration, washed with water and dried *in vacuo*. Crude compound **14** was dissolved in glacial acetic acid (50 mL) and the mixture was refluxed for 4 h. Then the reaction mixture was cooled to room temperature and poured into water. Excess acetic acid was neutralized with saturated sodium bicarbonate solution and the mixture was extracted with excess dichloromethane. The organic extract was then washed extensively with water, brine, dried over anhydrous sodium sulphate and concentrated *in vacuo* to afford compound **15** as a gummy oil. Compound **15** was then treated with 20% piperidine in dimethylformamide (v/v) for 30 min and the reaction mixture was vigorously stirred with excess hexane. The hexane layer was decanted off and this procedure was repeated three times to afford **16** as an orange colored oil. Compound **16** was used for the successive steps without further purification.

Synthesis of BB-F-yne (6) and BB-Cl-yne (5).



DIPEA (0.6 mL, 3.6 mmol), HBTU (918 mg, 2.4 mmol) and HOBt (327 mg, 2.4 mmol) were added sequentially to a mixture of compound **13** (270 mg, 1.2 mmol) and **16** (369 mg, 1.2 mmol) in DMF (10 mL). The reaction mixture was stirred for 18 h under nitrogen atmosphere at room temperature. Then the mixture was poured into water and the precipitate was recovered by vacuum filtration, washed thoroughly with water and dried *in vacuo* to afford compound **17** as an off-white solid (560 mg, crude yield: 90%). Compound **17** (560 mg, 1.1 mmol) was treated with trifluoroacetic acid/dichloromethane (1:4) (10 mL) for 1 h at room temperature and excess trifluoroacetic acid was evaporated under reduced pressure to afford compound **18** as a viscous liquid (563 mg, crude yield: 98%). Triethylamine (0.9 mL, 6.1 mmol) was added to a solution of compound **18** (0.8 g, 1.5 mmol) in methanol (20 mL). Then ethyl-2-choloroacetimidate hydrochloride (483 mg, 3.1 mmol) (for BB-Cl-yne) or ethyl-2-fluoroacetimidate hydrochloride (434 mg, 3.1 mmol) (for BB-F-yne) was added to the reaction mixture and the solution was stirred for 3 h at room temperature. Excess triethylamine was evaporated under vacuum and the resultant solid was dissolved in methanol. The crude product was then purified by reverse phase HPLC using a semi-preparative C18 column and water/acetonitrile gradient supplemented with 0.05% trifluoroacetic acid to afford BB-Cl-yne (**5**) or BB-F-yne (**6**) as off-white solids (yield: 102 mg, 11% for BB-Cl-yne and 105 mg, 12% for BB-F-yne). BB-Cl-yne: ^{1}H NMR (CD_3OD) δ (ppm):

7.96 (d, *J* = 8.5 Hz, 2H), 7.7 (d, *J* = 8.5 Hz, 2H), 7.64-7.66 (m, 2H), 7.59 (d, *J* = 10 Hz, 2H), 7.48 (d, *J* = 10 Hz, 2H), 7.43-7.44 (m, 2H), 5.53-5.56 (m, 1H), 4.28 (s, 1H), 3.5 (s, 1H), 3.34-3.41 (m, 2H), 2.20-2.31 (m, 2H), 1.86-1.93 (m, 1H), 1.74-1.81 (m, 1H); ^{13}C NMR (CD_3OD) δ (ppm): 168.5, 163.4, 153.8, 144.0, 139.8, 132.9, 132.3, 131.9, 128.1, 126.8, 126.7, 125.4, 122.4, 113.9, 82.6, 78.5, 42.0, 38.7, 29.2, 23.6, 22.4; ESI-MS (m/z) calculated for $C_{28}H_{26}Cl_1N_5O_1$ $[M + H]^+$: 484.1899, found 484.2. BB-F-yne: ^{1}H NMR (CD_3OD) δ (ppm): 7.95 (d, *J* = 10 Hz, 2H), 7.7 (d, *J* = 5 Hz, 2H), 7.58-7.63 (m, 4H), 7.48 (d, *J* = 10 Hz, 2H), 7.38-7.40 (m, 2H), 5.51-5.54 (m, 1H), 5.22 (s, 1H), 5.13 (s, 1H), 3.49 (s, 1H), 3.36-3.42 (m, 2H), 2.17-2.31 (m, 2H), 1.86-1.89 (m, 1H), 1.74-1.79 (m, 1H); ^{13}C NMR (CD_3OD) δ (ppm): 168.5, 161.7, 161.4, 153.9, 143.9, 139.8, 132.3, 132.0, 128.1, 126.8, 126.7, 124.9, 122.3, 114.0, 82.6, 78.5, 78.3, 76.9, 41.5, 29.3, 23.7; ESI-MS (m/z) calculated for $C_{28}H_{26}F_1N_5O_1$ $[M + H]^+$: 468.2194, found 468.2.

References.

1. Xiang, Y.; Wang, Q.; Wang, G.; Li, X.; Zhang, D.; Jin, W., Synthesis and coordination of star-shaped electron-deficient hexaheteroarylbenzene derivatives containing three pyrimidylbenzene derivatives. *Tetrahedron* **2016,** *72* (20), 2574-2580.