Supporting Information for

Seeing Citrulline: Development of phenylglyoxal-based probes to visualize protein citrullination

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Abbreviations

High-performance liquid chromatography (HPLC); Dimethylformamide (DMF); Dimethylsulfoxide (DMSO); Trifluoroacetic acid (TFA); Acetonitrile (ACN); O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU); N-Hydroxybenzotriazole (HOBt); Methanol (MeOH); t-Butanol (^{t}BuOH); Tris-(benzyltriazolylmethyl)amine (TBTA); Rhodamine-Phenylglyoxal (Rh-PG); Dithiothreitol (DTT); Trichloroacetic acid (TCA); Ethylenediaminetetraacetic acid (EDTA); triethylamine (Et_3N).

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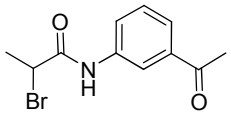
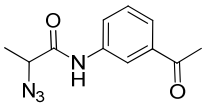
Rh-PG Synthesis and Characterization.

***N*-(3-Acetylphenyl)-2-bromopropionamide:**



To a stirred solution of 3-amino acetophenone (1.0 g, 7.35 mmol) in dimethylformamide (DMF) (10.0 mL) was added triethylamine (Et_3N) (1.11 g, 11.03 mmol) at 0 °C followed by the slow addition of 2-bromopropionyl bromide (1.75 g, 8.08 mmol). The reaction was stirred at rt under N_2 atmosphere for 3 h, diluted with water (50.0 mL) and the precipitated pale brown solid was filtered and dried to give the title product in 80% yield. ^{1}H NMR ($CDCl_3$, 400 MHz): 8.47 (br s, NH), 8.09 – 8.08 (m, 1H), 7.92 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H), 7.73 (dd, J = 8.0 Hz, J = 1.2 Hz, 1H), 7.46 (t, J = 8.0 Hz, 1H), 4.60 (q, J = 6.8 Hz, 1H), 2.62 (s, 3H), 1.96 (d, J = 7.2 Hz, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz): 198.0, 167.8, 137.8, 137.7, 129.5, 124.8, 124.6, 119.5, 44.8, 26.7, 22.7. ESI-MS calcd for $C_{11}H_{12}BrNO_2$ (M + Na)$^+$ 291.99, found 292.0.

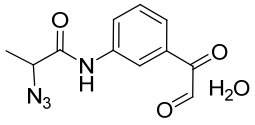
***N*-(3-Acetylphenyl)-2-azidopropionamide:**



To a stirred a solution of *N*-(3-Acetylphenyl)-2-bromopropionamide (800 mg, 2.97 mmol) in DMF (10.0 mL) was added NaN_3 (290 mg, 4.46 mmol). The reaction was stirred at rt

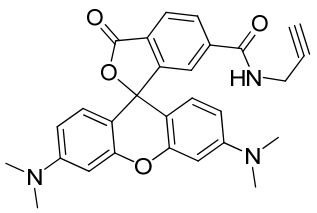
under N_2 atmosphere for 12 h, diluted with water, and the precipitated solid was filtered and dried to give the title product in 50% yield. ^{1}H NMR ($CDCl_3$, 400 MHz): 8.36 (br s, NH), 8.08 – 8.07 (m, 1H), 7.90 – 7.87 (m, 1H), 7.74 – 7.71 (m, 1H), 7.44 (t, J = 8.0 Hz, 1H), 4.25 (q, J = 6.8 Hz, 1H), 2.61 (s, 3H), 1.65 (d, J = 6.8 Hz, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz): 197.7, 168.2, 137.8, 137.5, 129.4, 124.6, 124.5, 119.5, 59.4, 26.7, 17.1. ESI-MS calcd for $C_{11}H_{11}N_4O_2$ (M - H)$^+$ 231.09, found 231.1.

2-Azido propionamido-N-3-phenylglyoxyl hydrate:



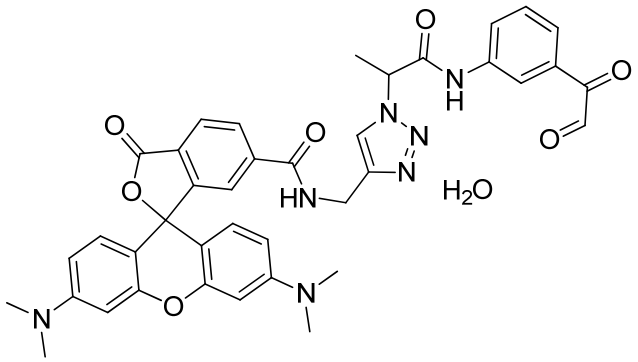
To a stirred solution of azidoacetophenone (200 mg, 0.86 mmol) in dioxane/H_2O (4:1, 10.0 mL) was added SeO_2 (145 mg, 1.29 mmol) and the reaction was refluxed under N_2 atmosphere for 24 h. The reaction mixture was filtered through SiO_2/celite plug, concentrated and purified by reverse phase HPLC using a linear gradient of H_2O/ACN plus 0.05% TFA to give the title product in 40% yield. ^{1}H NMR (CD_3OD, 400 MHz): 8.37 (br s, 1H), 7.92 – 7.84 (m, 2H), 7.52 – 7.47 (m, 1H), 5.54 (s, 1H), 4.09 - 4.06 (m, 1H), 1.57 – 1.52 (m, 3H). ^{13}C NMR (CD_3OD, 100 MHz): 195.3, 171.6, 139.7, 135.7, 130.2, 126.6, 126.5, 122.2, 96.5, 59.8 17.2. ESI-MS calcd for $C_{11}H_{11}N_4O_4$ (M - H)$^+$ 263.08, found 263.1.

***N*-(Prop-2-ynyl)-5(6)-carboxamidotetramethyl rhodamine (rhod-YNE):**



To a stirred solution of 5(6)-carboxytetramethyl rhodamine (50 mg, 0.12 mmol) in DMF (1.0 mL) were added Hunig's base (61 μL, 0.36 mmol, 3.0 eq), propargyl amine (19 mg, 0.36 mmol, 3.0 eq) followed by HBTU (132 mg, 0.36 mmol, 3.0 eq) and HOBt (48 mg, 0.36 mmol, 3.0 eq). The reaction was stirred at rt under N_2 atmosphere for 3 h and then diluted with H_2O/ACN plus 0.05% TFA (3:2), and purified by reverse phase HPLC using a linear gradient of H_2O/ACN plus 0.05% TFA to give the title product in 84% yield. ^{1}H NMR (CD_3OD, 400 MHz, major isomer): 8.81 (d, *J* = 1.6 Hz, 1H), 8.29 (dd, *J* = 1.6 Hz, *J* = 8.0 Hz, 1H), 7.55 (d, *J* = 8.0 Hz, 1 H), 7.18 – 7.16 (m, 2H), 7.08 (dd, *J* = 9.2 Hz, *J* = 2.4 Hz, 2H), 7.01 (d, *J* = 2.4 Hz, 2H), 4.27 (d, *J* = 2.8 Hz, 2H), 3.32-3.33(4 s, 12H, **4**NCH_3), 2.69 (t, *J* = 2.4 Hz, 1H). ^{13}C NMR (CD_3OD, 100 MHz): 167.7, 167.3, 160.5, 159.1, 159.0, 138.4, 137.2, 133.0, 133.4, 131.9, 131.4, 115.6, 114.7, 97.5, 80.5, 72.4, 40.9, 30.2. ESI-MS calcd for $C_{28}H_{26}N_3O_4$ $(M + H)^+$ 468.18, found 468.2.

***N*-(3-Glyoxyl)phenylamino)propionyl)-1*H*-1,2,3-triazol-4-yl)methyl- 5(6)-carboxamidotetramethyl rhodamine hydrate (Rh-PG)**



To a stirred solution of rhod-YNE (6.0 mg, 12.84 mmol) and 2-Azido propionamido-N-3-phenylglyoxyl hydrate (4.2 mg, 16.06 mmol) in DMSO/H_2O (1:1, 1.0 mL) were added sodium ascorbate (9.0 mg, 38.54 mmol) in H_2O (500 μL) followed by TBTA (1.3 mg, 2.57 mmol) in DMSO/H_2O/^{t}BuOH (3:1:1, 500 μL). To this stirred solution $CuSO_4$•$5H_2O$ (0.6 mg in 100 μL H_2O) was added and the reaction was stirred at rt for 1 h then diluted with ACN/H_2O (2:1, containing 0.05% TFA) and purified by reverse phase HPLC to give the title product in 90% yield. ^{1}H NMR (CD_3OD, 400 MHz, mixture): 8.83 (d, J = 1.6 Hz, 1H), 8.35 – 8.30 (m, 2H), 8.23 (s, 1H), 7.88 – 7.84 (m, 2H), 7.54 (d, J = 7.6 Hz, 1H), 7.48 (d, J = 8.0 Hz, 1H), 7.17 – 7.14 (m, 2H), 7.08 (dd, J = 6.4 Hz, J = 3.2 Hz, 2H), 7.0 (d, J = 2.4 Hz, 2H), 5.64 (q, J = 7.2 Hz, 1H), 5.51 (s, 1H), 4.79 (s, 2H), 3.33 (s, 12H), 1.94 (d, J = 6.8 Hz, 3H). ^{13}C NMR (CD_3OD, 100 MHz): 193.7, 167.8, 166.6, 159.1, 157.5, 157.4, 138.2, 134.3, 130.4, 128.7, 120.4, 114.1, 113.2, 95.9, 95.1, 59.6, 39.4, 34.9, 17.0. HRMS calcd for $C_{39}H_{38}N_7O_8$ $(M + H)^+$ 732.2782, found 732.2789.

Rh-PG Supplementary Methods

Chemoselectivity of Phenylglyoxal – To examine the chemoselectivty of phenylglyoxal, this compounds was reacted with two peptides (H4-13 and H4-13-Cit) under neutral and acidic pH. The H4-13 peptide (Ac-SG**R**GKGGKGLGKG-NH_2) and a derivative, H4-13-Cit (Ac-SG**R**GKGGKGLGKG-NH_2), based off of the 13 N-terminal residues of histone H4, were synthesized using standard Fmoc strategies[2] and purified by reverse-phase HPLC. Three samples of each peptide (50 μM) in 50 mM HEPES (50 μL; pH 7.6) was prepared. One sample of each peptide was treated as follows; 1. untreated; 2. with phenylglyoxal (5 mM) at pH 7.6; and 3. with phenyl glyoxal (5 mM) and 20% trichloroacetic acid (TCA; pH <1). Samples were incubated at 37 °C for 3 h and analyzed by MALDI-TOF using α-cyano-4-hydroxycinamic acid (CHCA) as the matrix.

Amino Acid Selectivity of Rh-PG – Various amino acids (100 mM; i.e., citrulline, Arg, Lys, Asp, Glu, Thr, Ser, Tyr, Gln, Asn, Cys, Met, His, cystine, and homocitrulline) were prepared in 50 mM HEPES (250 μL; pH 7.6). To this was added 20% TCA and 0.1 mM Rh-PG and samples were incubated at 37 °C for 30 min. Samples were analyzed by LC-MS (H_2O:ACN + 0.1% formic acid gradient) and the ratio of unmodified to amino acid modified Rh-PG determined.

Peptide Analysis of Cysteine Cross-Reactivity – To test the hypothesis that the Cys—Rh-PG complex was not stable at neutral pH, a peptide containing both citrulline and Cys (i.e., Ac-AHACitACARA-NH_2) was synthesized using standard Fmoc strategies[2] and purified by

reverse-phase HPLC. Three aliquots of this peptide (0.5 mM) were prepared in 50 mM HEPES (pH 7.6). Samples were treated as follows; 1. untreated; 2. treated with PG (5 mM) and 20% TCA; and 3. treated with PG (5 mM) and 20% TCA. Samples were incubated at 37 °C for 30 min. Samples 1 and 2 were analyzed directly by MALDI-TOF. Sample 3 was first neutralized to a pH of ~7.5 using 3 M NaOH, followed by MALDI-TOF analysis.

Rh-PG Probe Concentration Dependence – To determine the ideal probe concentration, histone H3 (2 μM) was treated with PAD4 (0.2 μM) in reaction buffer (30 μL; 50 mM HEPES; 50 mM NaCl; 10 mM $CaCl_2$, and 2 mM DTT; pH 7.6) at 37 ˚C for 1 h and the reaction was then quenched with 50 mM EDTA. This solution of citrullinated H3 and autodeiminated PAD4 was treated with trichloroacetic acid (TCA) (6.0 μL) and various concentrations of Rh-PG (100, 10, 1, or 0.1 μM Rh-PG). Samples were incubated at 37 ˚C for 30 min. Note that incubations at higher temperatures led to the formation of SDS insoluble aggregates that could not be separated by SDS-PAGE. Excess probe was quenched with 100 mM citrulline before cooling the solutions on ice for 30 min to complete the TCA precipitation. Proteins were isolated by centrifugation at 14,000 rpm for 15 min at 4 ˚C. The supernatant was removed and samples were washed with cold acetone and dried at 100 ˚C for 5 min. Proteins were resuspended in 25 μL of 50 mM HEPES. All samples were then boiled with 6x SDS loading dye and analyzed by SDS-PAGE (15%; 170 V; 50 min) and fluorescent imaging on a Typhoon Imager (Ex. 532 nm; Em. 580 nm).

Rh-PG Labeling Time Dependence – Citrullinated H3 and autodeiminated PAD4, prepared as above, were incubated with TCA and 0.1 mM Rh-PG at 37 ºC. Aliquots (30 μL) were removed

at various times (i.e., 0, 0.5, 1, 2, 3, and 4 h) and quenched with 100 mM citrulline for 30 min. Samples were then cooled on ice for 30 min, and centrifuged at 14,000 rpm for 15 min at 4 ˚C. The supernatant was removed and samples were washed with cold acetone and dried at 100 ˚C for 5 min. Proteins were resuspended in 25 μL of 50 mM HEPES, boiled with 6x SDS loading dye and analyzed by SDS-PAGE (15%; 170 V; 50 min) and imaged on a Typhoon Imager (Ex. 532 nm; Em. 580 nm).

Rh-PG Limit of Detection – Citrullinated H3 and autodeiminated PAD4, prepared as described above, were diluted into 50 mM HEPES pH 7.6 to final concentrations of (2.2 μM, 0.22 μM, 22 nM, 2.2 nM, and 0.22 nM H3) and (0.16 μM, 16 nM, 1.6 nM, 0.16 nM, and 0.016 nM PAD4). These samples were treated TCA and 0.1 mM Rh-PG at 37 ˚C for 30 min. Solutions were quenched with 100 mM citrulline, cooled on ice for 30 min, and centrifuged at 14,000 rpm for 15 min at 4 ˚C. The supernatant was removed and samples were washed with cold acetone and dried at 100 ˚C for 5 min. Proteins were resuspended in 25 μL of 50 mM HEPES, boiled with 6x SDS loading dye and various amounts of protein (i.e., 1000, 100, 10, 1, 0.1, and 0 ng citrullinated H3 and 150, 15, 1.5, 0.15, 0.015, and 0 ng autodeiminated PAD4) loaded onto a 15% SDS-PAGE gel. Gel electrophoresis was performed at 170 V for 50 min and the gels analyzed on a Typhoon Imager (Ex. 532 nm; Em. 580 nm).

Temperature Dependence of TCA Precipitations – To determine the effect of temperature during trichloroacetic acid (TCA) precipitation on protein loading onto a gel, TCA precipitations were carried out at five different temperatures for three different proteins. For this, protein samples

(i.e., PAD4 (4.44 μM); H3 (22.2 μM); and H4 (27.9 μM)) were prepared in 50 mM HEPES. To this was added 20% TCA and samples were incubated at various temperatures (i.e., 0, 23, 37, 42, and 55 ºC) for 30 min. Samples were cooled on ice 30 min, centrifuged at 14000 rpm at 4 ºC for 15 min, and the supernatant was removed. After washing with cold acetone, samples were again centrifuged for 5 min, the supernatant was removed, and samples were dried at 100 ºC for 5 min. Proteins were resuspended in 50 mM HEPES, boiled in 6x SDS loading dye for 10 min, and separated by SDS-PAGE (15%; 210 V; 50 min). Gels were coomassie stained and analyzed by Adobe Photoshop 7.0.

Autodeimination Time Course Experiments – For these experiments, PAD4 (0.2 μM) was incubated in reaction buffer at 37 ˚C and 50 μL aliquots were removed and quenched with 50 mM EDTA at various times (i.e., 0, 2, 4, 6, 10, 15, 30, and 60 min). Each aliquot was then divided to give two replicates of the time series. One replicate was incubated at 37 ˚C for 30 min in 20% TCA with 0.1 mM Rh-PG. Solutions were quenched with citrulline, cooled on ice for 30 min, and centrifuged at 14,000 rpm for 15 min at 4 ˚C. The supernatant was removed and samples were washed with cold acetone and dried at 100 ˚C for 5 min. Proteins were resuspended in 25 μL of 50 mM HEPES, boiled with 6x SDS loading dye, and analyzed by gel electrophoresis (12%; 170 V; 50 min) and fluorescent imaging (Ex. 532 nm; Em. 580 nm).

The other time series replicates were separated by gel electrophoresis (12% 200 V; 45 min) and electrotransferred to polyvinylidene difluoride (PVDF; tris-glycine buffer; 80 V; 70 min). Autodeiminated PAD4 was then detected using the Anti-Citrulline (Modified) Detection Kit. For this analysis, the PVDF membrane was washed with water twice. Citrulline

modification solution was then prepared by combining 5 mL of Reagent A (0.025% $FeCl_3$ in water/98% H_2SO_4/85% H_3PO_4 (55%/25%/20%); prepared in house) with 5 mL of Reagent B (0.5% 2,3-butanedione monoxime, 0.25% antipyrine, and 0.5 M acetic acid; provided). This solution was added to the blot and incubated without agitation overnight at 37 ˚C. The blot was then washed with water (5x 5 min) and blocked with 5% milk in TBST for 1 h at rt with constant agitation. After blocking, the blot was incubated with the primary anti-citrulline (modified) antibody at a dilution of 1:1000 in 5% milk in TBST for 1 h at rt. This solution was removed and the blot washed with TBST (3x 5 min). The blot was then incubated with HRP-conjugated goat anti-rabbit IgG at a dilution of 1:4000 in 5% milk in TBST for 1 h at rt. After washing the blot with TBST (5x 5 min) citrullinated proteins were visualized by enhanced chemoluminescence (ECL). These experiments were performed in duplicate on different days.

Histone H3 Deimination Time Course Experiments – For these experiments, histone H3 (1.0 μM) was incubated with PAD4 (0.2 μM) in reaction buffer at 37 ˚C and 75 μL aliquots were removed and quenched with 50 mM EDTA at various times (i.e., 0, 2, 4, 6, 10, 15, 30, and 45 min). Each aliquot was then divided to give two replicates of the time series. One replicate was incubated at 37 ˚C for 30 min in 20% TCA with 0.1 mM Rh-PG. Solutions were quenched with citrulline, cooled on ice for 30 min, and centrifuged at 14,000 rpm for 15 min at 4 ˚C. The supernatant was removed and samples were washed with cold acetone and dried at 100 ˚C for 5 min. Proteins were resuspended in 25 μL of 50 mM HEPES, boiled with 6x SDS loading dye, and analyzed by gel electrophoresis (12%; 170 V; 50 min) and fluorescent imaging (Ex. 532 nm; Em. 580 nm).

The second replicate was analyzed by the Anti-Citrulline (Modified) Detection Kit as described above. The third replicate was probed first with anti-H3 (Cit) antibody (catalogue #ab5103; Abcam, Cambridge, MA) and an anti-H3 antibody (catalogue #ab1791, Abcam, Cambridge, MA). For this analysis, samples were separated by gel electrophoresis (12% 200 V) and electrotransferred to PVDF (tris-glycine buffer; 80 V; 70 min). The blot was blocked with 5% milk in TBST for 1 h at rt before treating with anti-H3 (Cit) polyclonal antibody (2 μL; 1:5000; catalogue #ab5103; Abcam, Cambridge, MA) in 2.5% milk in TBST for 1 h at rt. After washing with TBST, the blot was then incubated with HRP-conjugated goat anti-rabbit IgG (2 μL; 1:5000) in 5% milk in TBST for 1 h at rt. After washing the blot with TBST (5x 5 min) citrullinated proteins were visualized by enhanced chemoluminescence (ECL). To confirm equal loading amounts of histone H3, this same blot was stripped and probed with an anti-histone H3 antibody. Briefly, the blot was treated 2x with acidic stripping buffer (0.2 M glycine, 10 mM Tween-20, 0.1% SDS, pH 2.2) (10 min), washed 2x with PBS (10 min), and 2x with TBST (5 min). The blot was subsequently blocked with 5% milk in TBST for 1 h at rt and treated with anti-histone H3 polyclonal antibody (2 μL; 1:5000; catalogue #ab1791, Abcam, Cambridge, MA) in 2.5% milk in TBST for 1 h at rt. After washing 3x with TBST (5 min), the blot was treated with goat anti-rabbit (HRP) secondary antibody (2 μL; 1:5,000) in 2.5% milk in TBST for 1 h at rt. The blot was then washed 3x with TBST (5 min) and visualized by ECL. These experiments were performed in duplicate on different days.

Kinetic Assays – For kinetic measurements, a citrulline standard was first generated to convert fluorescent intensity to citrulline concentration. For this histone H3 (100 μM) was treated with PAD4 (0.2 μM) in reaction buffer at 37 ºC for 1 h, followed by quenching with 50 mM EDTA. A portion of this was then analyzed by COLDER in duplicate at two different dilutions to

determine the concentration of citrulline in the solution. This value was determined to be 25.6 μM citrulline.

To assure that rate measurements for k_{cat}/K_m were obtained during the linear phase of histone citrullination, a time course assay was performed. For this assay, histones H3 or H4 (50 μM) in reaction buffer were preincubated at 37 ºC for 10 min before treatment with PAD4 (0.2 μM). Aliquots (10 μL) were removed at time 0, 2, 4, 6, 8, 10, and 15 min and quenched with EDTA (50 mM) and placed on ice. An aliquot of each sample was then diluted 60 fold in 50 mM HEPES (0.5 μL in 29.5 μL buffer) and treated with 20% TCA and 0.1 mM Rh-PG probe at 37 ºC for 30 min. All samples were quenched with citrulline, cooled, centrifuged, washed and dried, as described above. After resuspending in 50 mM HEPES, samples were separated by SDS-PAGE (15%; 170 V; 50 min) and imaged on a Typhoon Imager (Ex. 532 nm; Em. 580 nm). These experiments were performed in duplicate.

For the kinetic assay, varying concentrations of both histone H3 and histone H4 (i.e., 0, 10 23, 50, 75, 100, and 125 μM) were preincubated at 37 ºC for 10 min before treatment with and without PAD4 (0.2 μM) in reaction buffer (30 μL) at 37 ºC for 6 min. Reactions were quenched with 50 mM EDTA and placed on ice. An aliquot of each sample was then diluted 60 fold in 50 mM HEPES (0.5 μL sample in 29.5 μL buffer) and treated with 20% TCA and 0.1 mM Rh-PG probe at 37 ºC for 30 min. Also, a dilution series of citrullinated histone H3 in 50 mM HEPES (25.6 μM stock; 0, 0.25, 0.5, 1.0, and 1.5 μM final) was treated in parallel with 20% TCA and 0.1 mM Rh-PG probe at 37 ºC for 30 min to serve as a citrulline standard. All samples were quenched with citrulline, cooled, centrifuged, washed and dried, as described above. After resuspending in 50 mM HEPES, samples were separated by SDS-PAGE (15%; 170 V; 50 min) and imaged on a Typhoon Imager (Ex. 532 nm; Em. 580 nm). Images were analyzed using

ImageQuant 5.2 and the initial rates fit to eq 1,

$$v = V_{max}[S]/(K_m+[S]) \quad (1),$$

using Grafit 5.0.1.1. All experiments were done in duplicate on different days.

Rh-PG Analysis of Murine Ulcerative Colitis Serum Samples – Serum samples from a previously published study[3] of Cl-amidine efficacy in a mouse model of ulcerative colitis were obtained and analyzed by the Rh-PG method. Briefly, the protein concentration of serum samples was first obtained using a standard Lowry assay. A sample (2 mg/mL; 30 μL) of each serum sample (i.e., 14 total; 7 DSS; 7 DSS + Cl-amidine) was prepared in 50 mM HEPES and treated with 20% TCA and 0.1 mM Rh-PG for 30 min at 37 ºC. Samples were then quenched with citrulline, cooled, centrifuged, washed, and dried as described earlier. After resuspending in 50 mM HEPES, samples were separated by SDS-PAGE (12%; 170 V; 50 min) and imaged on a Typhoon Imager (Ex. 532 nm; Em. 580 nm). Images were analyzed using ImageQuant 5.2 and correlation coefficients were determined using Microsoft Excel. These experiments were run in duplicate on different days. Subsequent statistical analysis was done using STATISTICA, utilizing two-tailed, independent student's t-tests to determine significance (i.e., P-value) between diseased and Cl-amidine treated samples. Correlation coefficients were calculated in Microsoft Excel, utilizing a table of critical values of Pearson's r with a degree of freedom value of 12 (n-2) to determine the significance (i.e., P-value) of the correlation between the fluorescence of a particular protein and a disease score.

Supplementary Figures

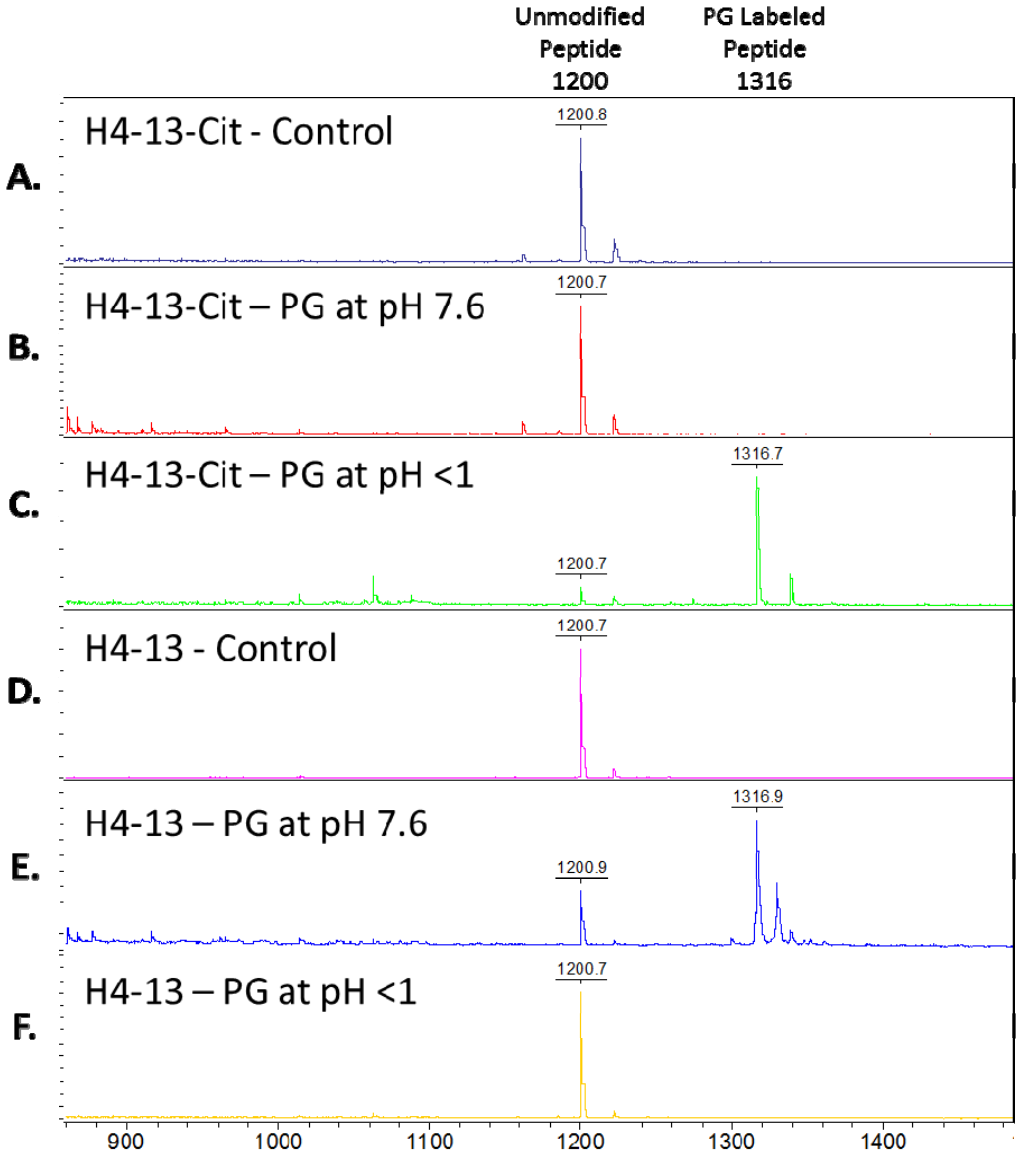


Figure S1. – MALDI spectra of peptides H4-13-Cit (Ac-SG**Cit**GKGGKGLGKG-NH_2) (**A-C**) and H4-13 (Ac-SG**R**GKGGKGLGKG-NH_2) (**D-F**) treated with various conditions at 37 °C for 3 h. Peptides were either untreated (**A** and **D**), treated with PG at pH 7.6 (**B** and **E**), or treated with PG in 20% TCA (**C** and **F**). Data indicate that arginine is selectively labeled under basic conditions and citrulline is selectively labeled under acidic conditions.

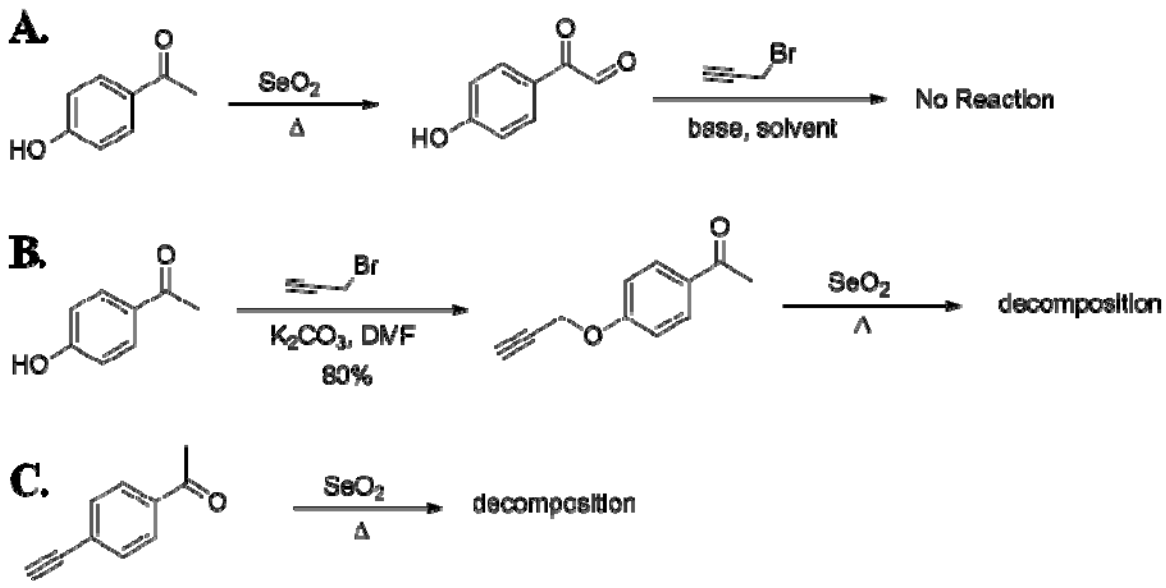


Figure S2. Several attempts were made to synthesize alkyne-containing PG probes linked to reporter tags via the *para* position, however, none of these synthetic routes resulted in the desired compounds.

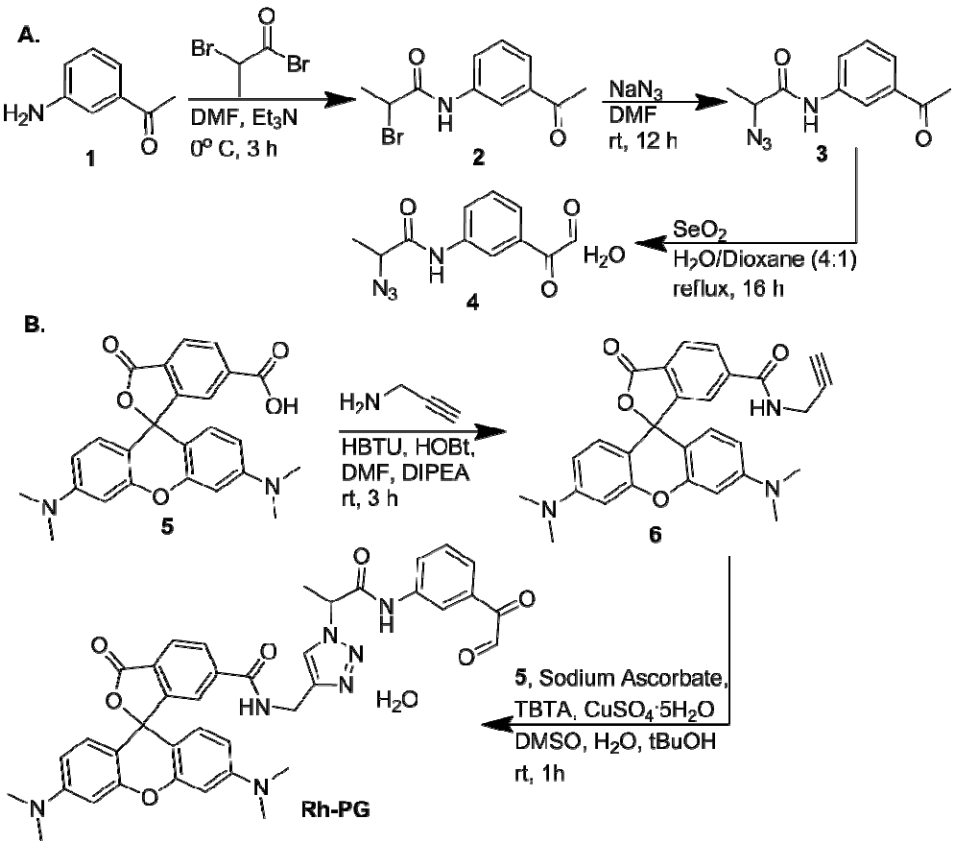


Figure S3. Synthesis of rhodamine phenylglyoxal (Rh-PG).

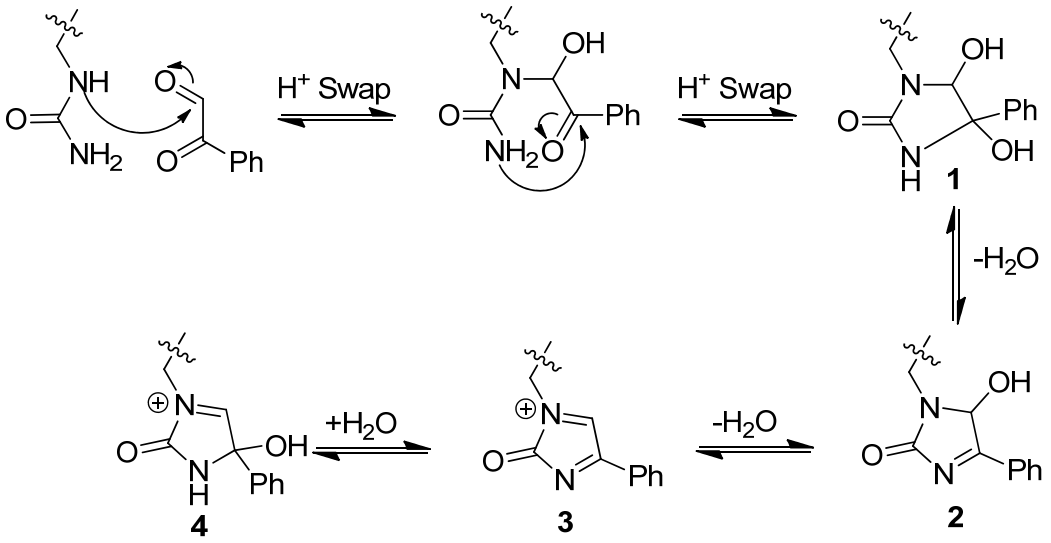
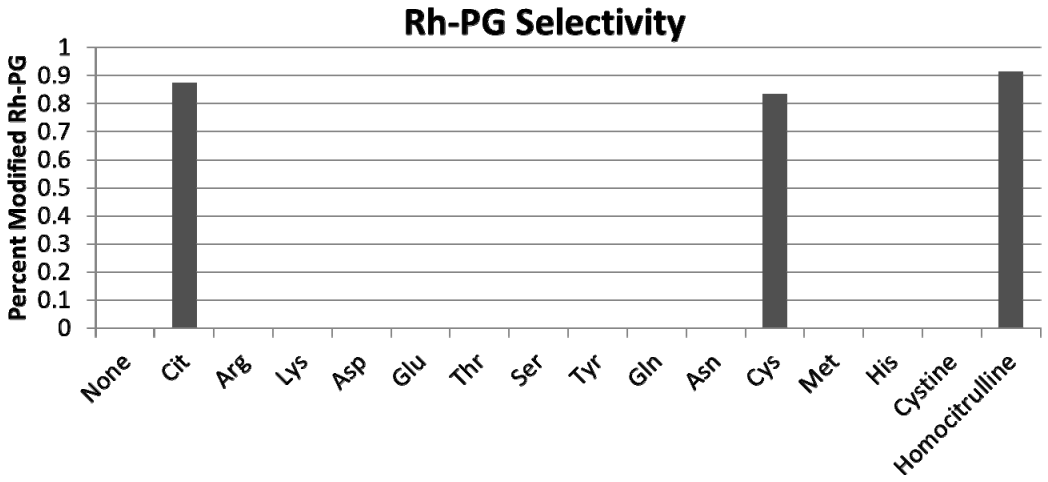


Figure S4. Proposed reaction mechanism between citrulline and phenylglyoxal. Note that four possible structures, all in equilibrium, are possible. Mass spectral analysis and previous reports[1] indicate that structure **2** is the most likely structure.



Figures S5. Selectivity of the Rh-PG probe. Rh-PG treated with excess amino acid under acidic conditions for 30 min at 37 °C followed by analysis by LC-MS to determine the percent of Rh-PG modified by the amino acid.

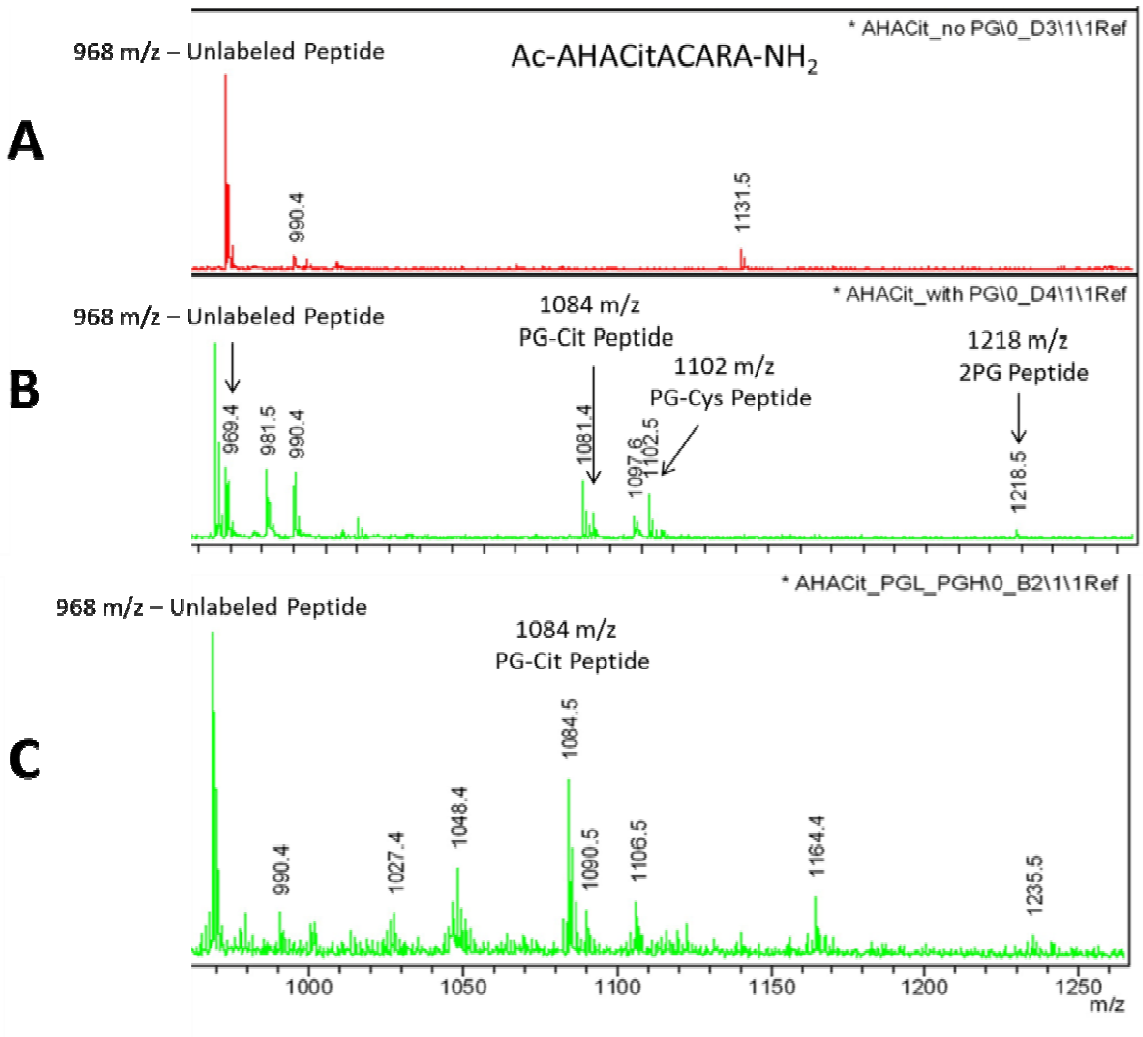


Figure S6. MALDI spectra of either unlabeled (**A**), PG labeled and analyzed under acid conditions (**B**), or PG labeled under acid conditions and analyzed under neutral conditions (**C**) peptide containing both citrulline and cysteine. Note that when analyzed under acidic conditions, the cysteine residue remains modified by PG, while neutral condition analysis results in only PG modified citrulline. This is apparent from the loss of signal at an m/z of 1102.

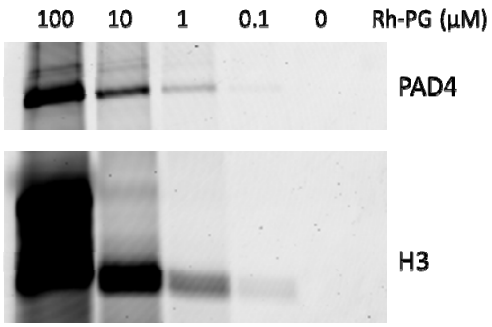


Figure S7. The Rh-PG probe labels autodeiminated PAD4 and citrullinated histone H3 in a concentration dependent manner. Histone H3 and PAD4 were deiminated by PAD4, followed by treatment with varying concentrations of Rh-PG under acidic conditions for 30 min at 37 °C. Samples were separated by SDS-PAGE and analyzed on a fluorescent scanner.

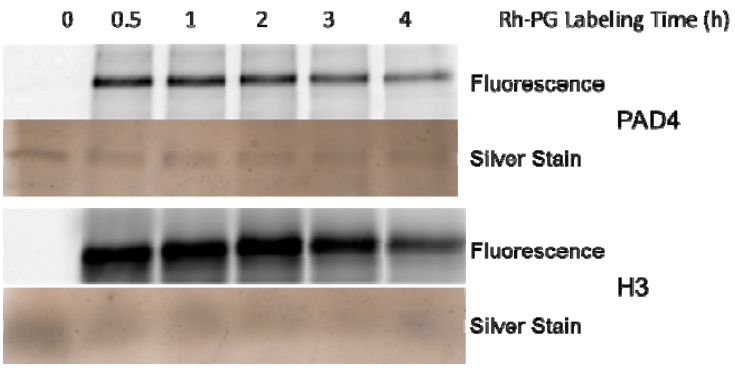


Figure S8. Rh-PG labeling of autodeiminated PAD4 and deiminated histone H3. Samples were treated with Rh-PG under acidic conditions at 37 °C for varying times, separated by SDS-PAGE, and imaged. Rh-PG labeling is essentially complete after 0.5 h and extended labeling times lead to decreased protein loading on the gel.

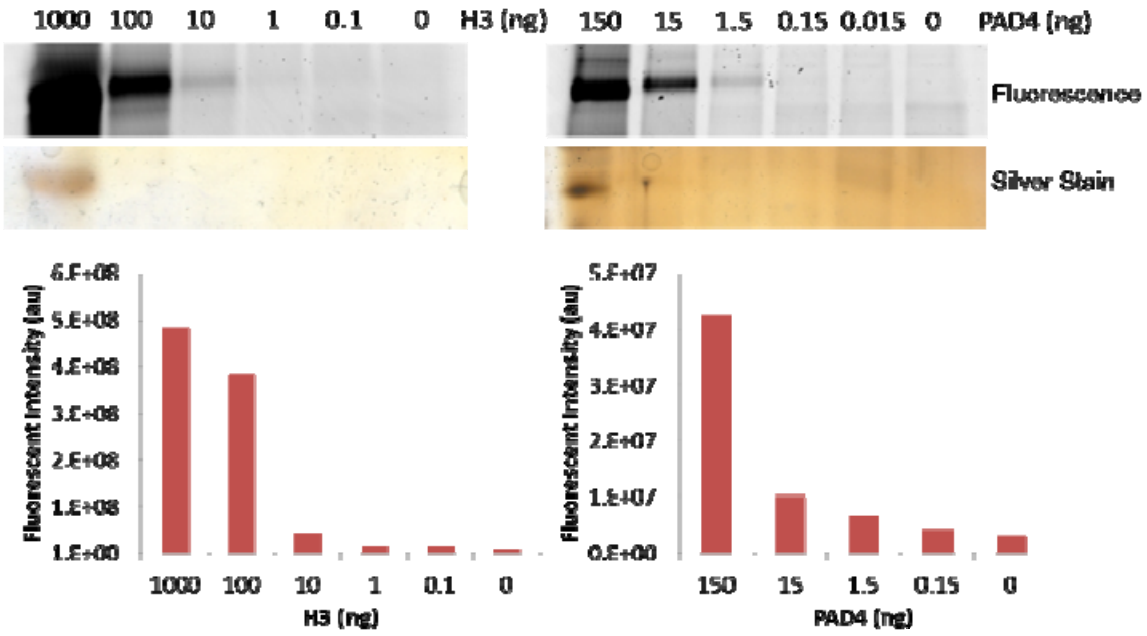


Figure S9. Rh-PG labeling limit of detection. Varying amounts of autodeiminated PAD4 and deiminated H3 were labeled with Rh-PG under acidic conditions for 30 min at 37 °C, separated by SDS-PAGE, and imaged. The Rh-PG probe is capable of detecting ~10 ng of citrullinated histone H3 and ~1 ng of autodeiminated PAD4.

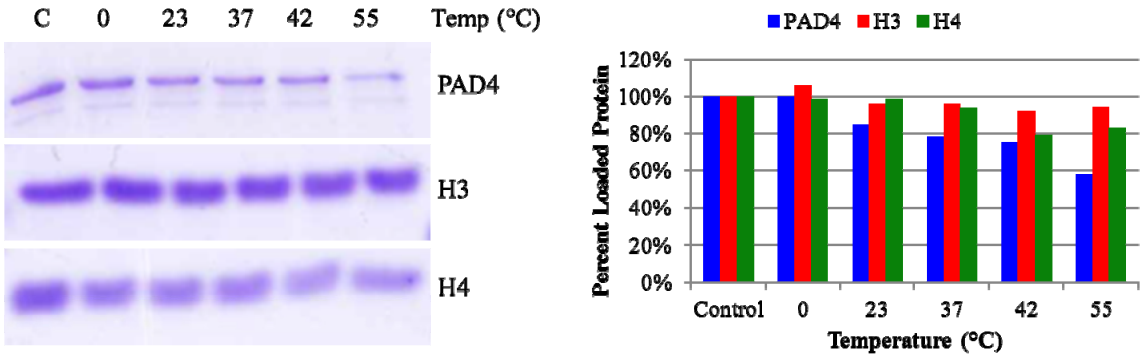


Figure S10. TCA precipitations of various proteins at different temperatures to show that increased temperature during TCA precipitations decreases protein loading. Recombinant PAD4, H3, and H4 were treated with 20% TCA at varying temperatures for 30 min. Samples were washed and analyzed by SDS-PAGE and coomassie staining.

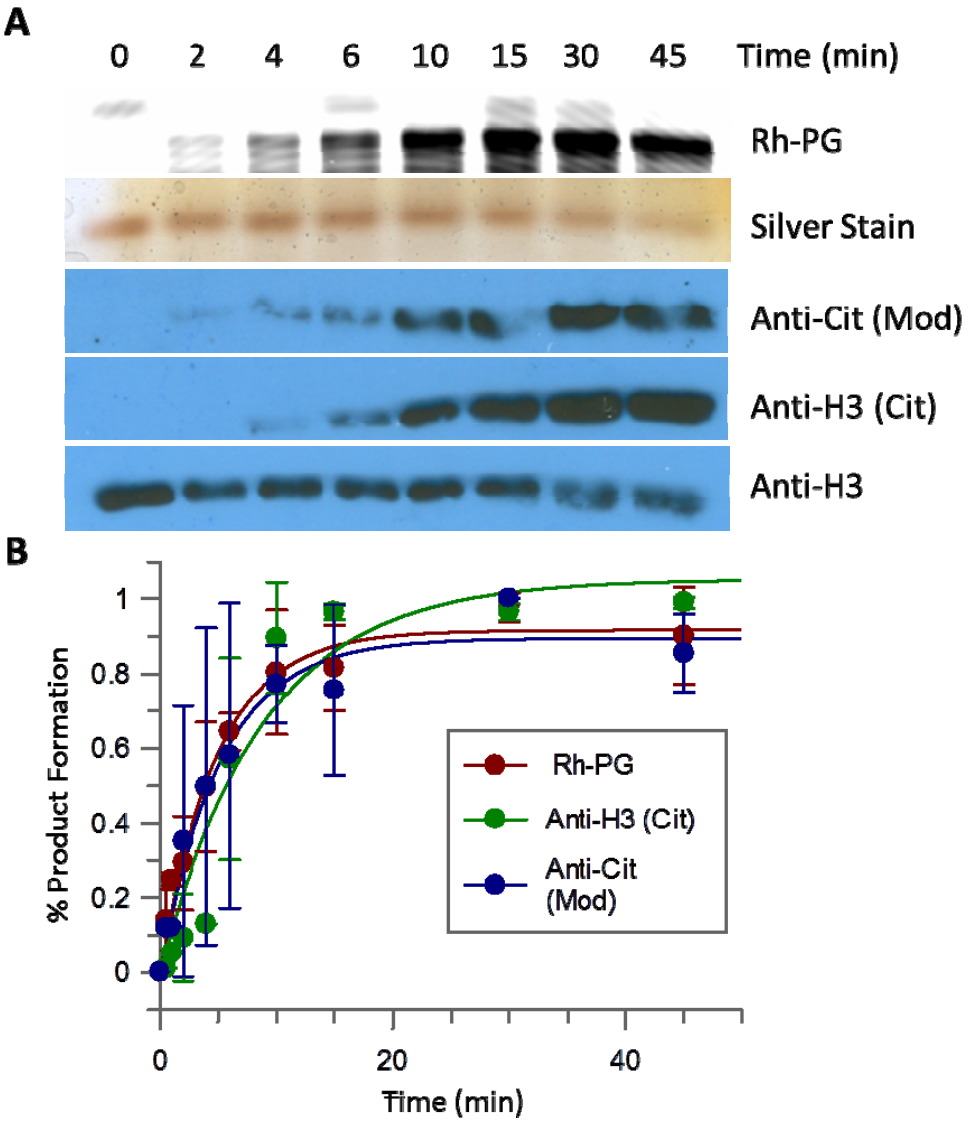


Figure S11. (**A**) Time course of histone H3 citrullination by PAD4 analyzed by the Rh-PG method (top), ACM kit (middle), and anti-H3 (cit) antibody (lower middle). Silver staining (upper middle) confirms equal protein loading for the Rh-PG method of detection and probing by an anti-H3 antibody (bottom) confirms equal protein loading for the antibody based analysis. (**B**) Analysis of all three methods for analysis of H3 citrullination, showing that they compare favorably.

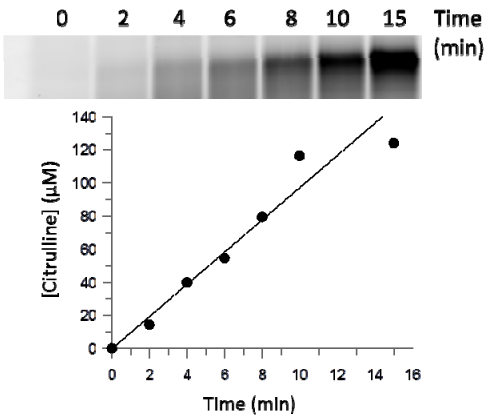


Figure S12. Time course of H3 citrullination by PAD4 to assure that subsequent K_m assays were conducted in the linear range.

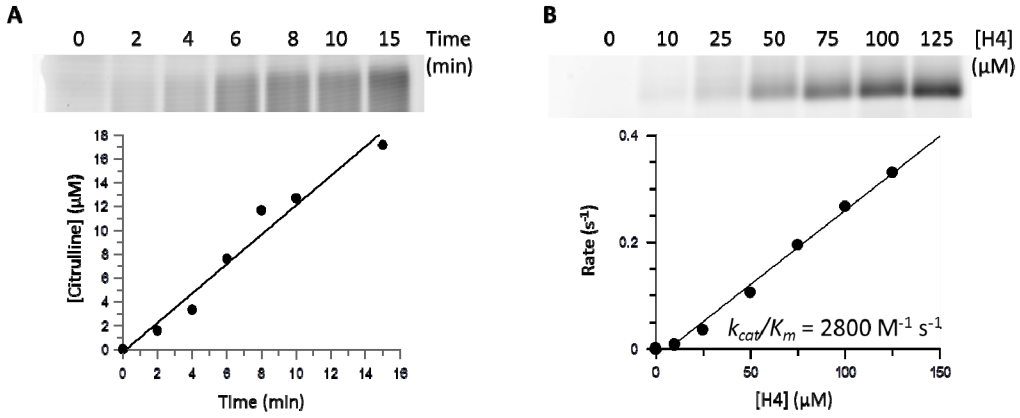


Figure S13. (**A**) Time course of H4 citrullination by PAD4 to assure that subsequent K_m assays were conducted in the linear range. (**B**) k_{cat}/K_m analysis of histone H4 citrullination by PAD4 using the Rh-PG method produces a value of 2800 M^{-1} s^{-1}.

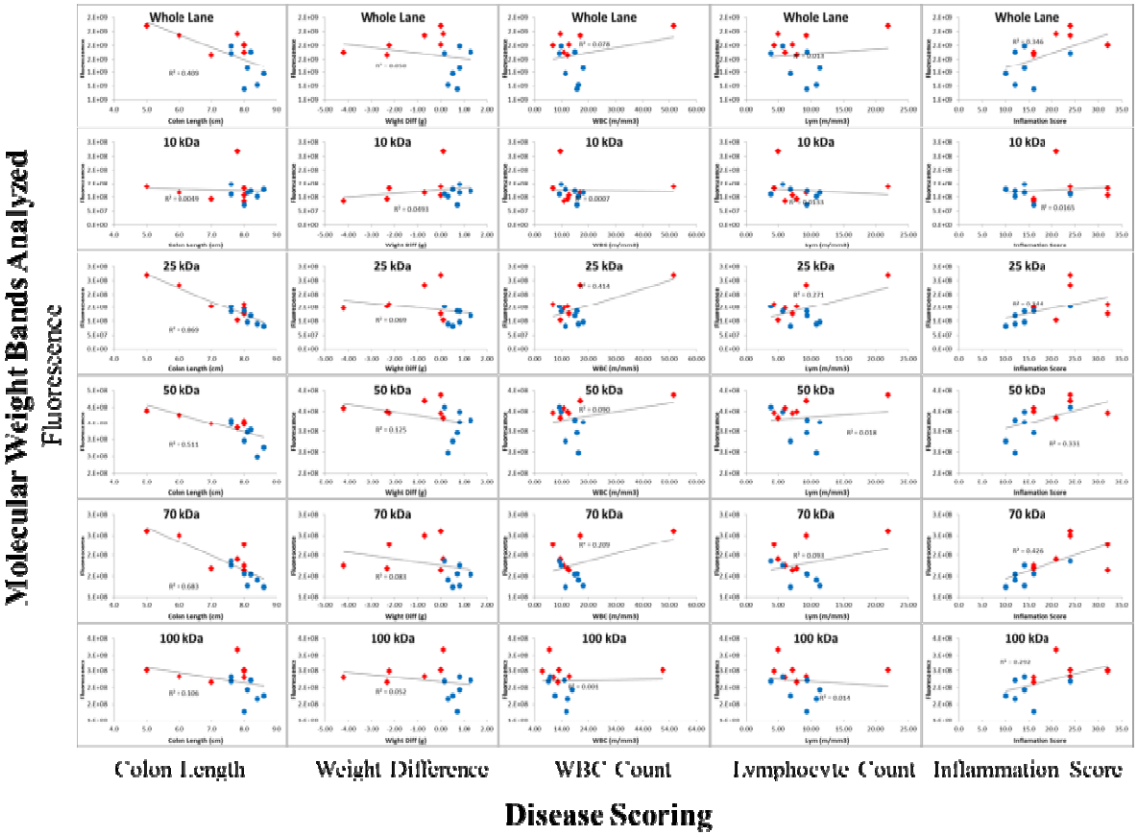


Figure S14. Correlation plots between protein fluorescence and disease scoring. Each row is a different analyzed protein band. Each column is a different disease scoring (i.e., colon length, weight difference, white blood cell count, lymphocyte count, and inflammation score).

Table S1. P-values between DSS and DSS + Cl-amidine samples.						
	Whole Lane	10 kDa	25 kDa	50 kDa	70 kDa	100 kDa
P-values	0.00613	0.436	0.046	0.0195	0.0112	0.00823

Table S2. Disease scoring of mice from the DSS induced ulcerative colitis study.

Group	##/Sex	Wight (start)	Wight (end)	Colon Length (cm)	Wight Diff. (g)	WBC (m/mm^3)	Lym (m/mm^3)	Inflam. Score	Treatment
Group 7	7.1M	20.2	20.3	7.8	0.1	9.43	4.99	21	2% DSS + PBS
WT	7.2M	21.6	19.4	8	-2.2	6.82	4.37	32	
	7.3M	20.3	20.3	5	0	51.7	21.92	24	
	7.4M	22.1	21.4	6	-0.7	17.13	9.28	24	
	7.5M	23.9	23.9	8	0	12.97	7.19	32	
	7.6M	21	18.7	7	-2.3	12.52	7.85	16	
	7.7M	24.6	20.4	8	-4.2	10.96	6.04	16	
Average		**22.0**	**20.6**	**7.1**	**-1.3**	**17.4**	**8.8**	**23.6**	
Group 8	8.1M	22.6	23.4	7.6	0.8	9.75	5.68	14	2% DSS + Cl-Amidine @ 75 mg/kg
WT	8.2M	21.9	22.05	7.6	0.15	9.08	3.87	24	
	8.3M	21.9	22.7	8.1	0.8	18.14	11.35	14	
	8.4M	22.8	24.1	8.2	1.3	15.12	9.4	12	
	8.5M	24.5	25	8.6	0.5	11.46	6.87	10	
	8.9M	21.8	22.5	8	0.7	15.91	9.35	16	
	8.10M	23.3	23.6	8.4	0.3	16.41	10.83	12	
Average		**22.7**	**23.3**	**8.1**	**0.7**	**13.7**	**8.2**	**14.6**	

References

[1] A. Holm, F. Rise, N. Sessler, L. M. Sollid, K. Undheim, B. Fleckenstein, *Analytical Biochemistry* **2006**, *352*, 68-76; A. E. V. Tutturen, A. Holm, M. Jørgensen, P. Stadtmüller, F. Rise, B. Fleckenstein, *Analytical Biochemistry* **2010**, *403*, 43-51.

[2] W. C. Chan, P. D. White, *Fmoc solid phase peptide synthesis. A practical approach.*, Oxford University Press Inc., New York, New York, **2000**.

[3] A. A. Chumanevich, C. P. Causey, B. A. Knuckley, J. E. Jones, D. Poudyal, A. P. Chumanevich, T. Davis, L. E. Matesic, P. R. Thompson, L. J. Hofseth, *American Journal of Physiology - Gastrointestinal and Liver Physiology* **2011**, *300*, G929-G938.