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Supporting Information

A new probe for detecting zinc-bound carbonic anhydrase in cell lysates and cells

Radhika Mehta,[a] Munaum H. Qureshi,[a] Meredith K. Purchal,[a] Sylvester M. Greer,[a] Shanzhong Gong,[b] Chinh Ngo,[a] Emily L. Que*[a]

[a] Department of Chemistry, University of Texas at Austin, 105 E 24th St Stop A5300, Austin, TX 78712

[b] Department of Molecular Biosciences, University of Texas at Austin, 2506 Speedway, Austin, TX 78712

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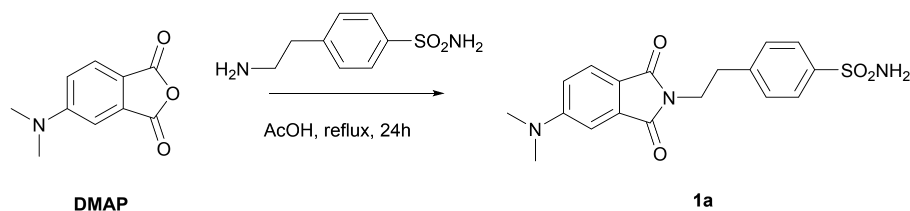
Experimental Section:

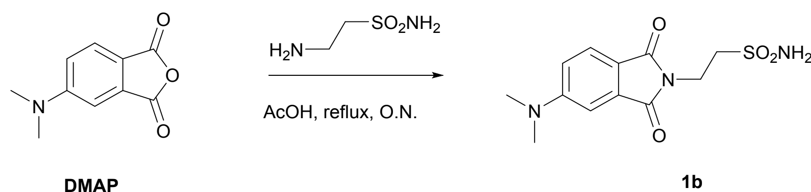
General Procedures:

All synthesis reagents were purchased from TCI America, ACROS or Sigma Aldrich. Bovine carbonic anhydrase, myoglobin, lysozyme and catalase were purchased from Sigma Aldrich. Human CAII was kindly provided by Dr. Joseph Emerson at Mississipi State University. Human CAIX was used from the R&D CA IX ELISA kit. New Delhi metallo-beta-lactamase 1 was kindly provided by Dr. Walter Fast at UT Austin. Bovine red blood cells (10%) in PBS was purchased from Innovative Research and the lysate prepared as needed. For spectroscopic studies, 0.05 M HEPES buffer with 0.1 M KNO_3 (pH 7.2) and 0.1 M ammonium acetate were prepared. ^{1}H and ^{13}C NMR spectra were acquired on 400 MHz Agilent MR spectrometers. NMR samples were prepared in CD_3CN, $CDCl_3$ or DMSO-d^6 and chemical shifts are reported in ppm. Spectroscopic studies were performed using Agilent Cary 60 UV-Vis spectrophotometer. Fluorescence spectroscopic measurements were made using an Agilent Cary Eclipse fluorescence spectrofluorimeter. Confocal imaging was performed on Zeiss 710 Laser Scanning Confocal Microscope using a 40X lens. Live-cell imaging was performed using ibidi μ-slide 8-well glass bottom dishes.

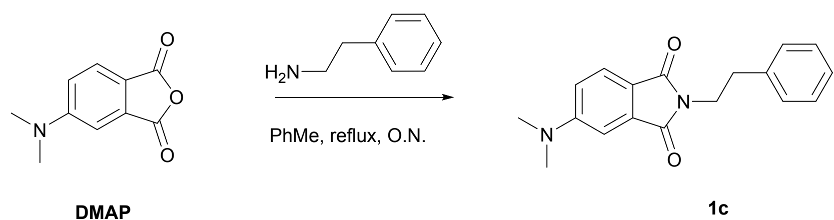
Synthetic Procedures:



*Synthesis of **1a***: To a solution of **DMAP** (80 mg, 0.4 mmol) in acetic acid (5 mL), was added 4-(2-aminoethyl)benzenesulfonamide (80 mg, 0.4 mmol). The reaction was refluxed for 24 hours and the formation of the product was monitored *via* TLC in 2% methanol-DCM mixture. The solvent was then evaporated on the rotary evaporator and the product was purified by silica gel chromatography using DCM followed by 1% methanol-DCM mixture. Yellow solid (30% yield). HRMS (m/z, positive): 374 ($M+H^+$), 396 ($M+Na^+$). ^{13}C NMR (DMSO- d^6, δ): 40.5, 41.2, 45.4, 106.7, 110, 126.1, 129.6, 142.7, 143, 167.



*Synthesis of **1b***: To a solution of **DMAP** (50 mg, 0.26 mmol) in acetic acid (5 mL) was added 2-aminoethanesulfonamide hydrochloride (45 mg, 0.28 mmol). The reaction was refluxed overnight and the solvent was evaporated on the rotary evaporator. The product was purified *via* silica gel chromatography using DCM and 1% methanol-DCM mixture. Yellow solid (33% yield). Mass (m/z, positive): 298. ^{13}C NMR (, DMSO-d^6, δ): 32.8, 42.3, 61.4, 106.6, 115.9, 117.4, 121.8, 128.1, 132.3, 154.5, 164.7.



*Synthesis of **1c***: To a solution of **DMAP** (70 mg, 0.36 mmol) in toluene (4 mL), was added phenethylamine (60 mg, 0.4 mmol). The reaction was refluxed overnight and the formation of the product was monitored *via* TLC using 1:1 DCM-hexanes mixture. The solvent was then evaporated on the rotary evaporator and the product was purified using silica gel chromatography using DCM followed by 1:1 DCM-hexanes mixture. Yellow solid (60% yield). Mass (m/z, positive): 295. ^{13}C NMR (DMSO- d^6, δ): 34.8, 39.0, 40.4, 105.7, 114.5, 117.7, 124.7, 126.5, 128.5, 128.9, 134.7, 138.4, 154.3, 168.6, 169.

Characterization:

Spectroscopic studies: Spectroscopic studies were performed using 5 mM stock solutions of the probes in DMSO. Solvent dependent studies were performed for probe concentrations ranging from 1 to 50 μM with 0.1-2% DMSO (v/v). Quantum yields (Φ) were measured using quinine sulfate in 0.1 M H_2SO_4 (Φ = 0.54) as standard. The absorbance and fluorescence was recorded for each probe (**1a-c**) in different solvents with 375 nm as the absorbance and excitation wavelength.The integrated fluorescence was plotted against the absorbance and the slope of the graph (m) was used to calculate the quantum yields for the probes in different solvents using the formula:

$$\Phi x = \Phi std \left(\frac{mx}{mstd}\right)\left(\frac{{\eta_x}^2}{{\eta_{std}}^2}\right)$$

Where *x* and *std* denote sample and standard (quinine) respectively. Φ denotes the quantum yield, *m* denotes the slope of the graph of integrated fluorescence *vs* absorbance and η denotes the refractive index of the solvent.

Density Functional Theroretical calculations: DFT and Time–dependent DFT were performed using using the Gaussian program 09 (B3LYP functional using 6-31G-(d, p) orbital base) courtesy B.J Holliday. Further details provided in Figure S2.

Binding studies with carbonic anhydrase: Binding studies were performed using bovine carbonic anhydrase (bCA). A 10 mg/mL (~350 μM) stock solution of the enzyme was prepared in HEPES buffer pH 7.2. The enzyme concentration was kept at 5 μM with varying equivalents of probe as needed. All studies were conducted at room temperature.

Preparation of apo-bCA: Apo-enzyme was prepared by treating bovine CA with 0.05 M dipicolinic acid, 0.2 M KH_2PO_4 pH 6.9 in a dialysis casette for 4 hours at 0 ℃.[1]The buffer was then changed and the dialysis was continued overnight. Thereafter, the buffer was discarded and the apo-CA was incubated in 10 mM HEPES, pH 7.5. Formation of apo enzyme was confirmed using the NPA assay.

Preparation of Co-bCA, Ni-bCA and Cd-bCA: Different metalated forms of CA were prepared as described previously.[2]

4-Nitrophenolacetate (NPA) assay: The enzymatic activity of CA was monitored using 4-nitrophenyl acetate (NPA) as the substrate.[3] On hydrolysis, 4-nitrophenolate ion is produced that can be followed spectroscopically at 400 nm. In this assay, the activity of CA is measured as a function of time by following the increase in absorbance at 400 nm in the absence and presence of the synthesized probes. The enzyme, probe and 4-nitrophenolacetate concentration was kept constant at 2 μM, 2 μM and 125 μM respectively. The absorbance at 400 nm was recorded as a function of time- at 1.5 minutes intervals upto 5 readings. The increase in absorbance A_{400} was plotted against time and the slope calculated. The probe activity was then normalized with respect to CA activity (100%).

Mass spectrometry study: 10 μM of bovine carbonic anhydrase was incubated with equimolar concentration of **1a** in 100 mM ammonium acetate buffer with <5% DMSO for ligand solubility. Approximately 5 μL of solution was loaded into an in-house silver-coated pulled tip static emitter

and sprayed with an applied voltage of 1.1 kV. Analysis was performed on a ThermoFisher Scientific Instruments Elite Orbitrap mass spectrometer (Bremen, Germany.) Mass spectra of the intact protein were acquired using the Orbitrap mass analyzer at a resolving power of 120,000. The raw spectra were decharged and deisotoped using the EXTRACT algorithm embedded in the Thermo Excalibur software.

Kinetic studies using stop-flow instrument: Kinetic experiments were performed using a stop-flow instrument with the λ_{ex} set at 420 nm. The probe concentration was kept constant at 1 μM while the bCA concentration was varied from 0.1-1 μM. The fluorescence upon CA-probe binding was collected over 20 seconds for each concentration with 2000 data points and atleast 3-5 overlaying spectra. The kinetic fitting was done using Kintek explorer[4, 5] Pro V5 with a one-step fitting model [E]+[S]=[ES] to give the k_{on}, k_{off} and K_d.

Selectivity studies: Selectivity was tested by monitoring the fluorescence turn-on with respect to **1a** (5 μM) in Hepes buffer for λ_{ex}= 420 nm and λ_{em}= 430-800 nm at equimolar ratios going upto twice the concentration of the probe. The fluorescence turn-on was monitored with bovine CA, and two isoforms of human CA- hCAII and hCAIX. Selectivity was also tested with free zinc ($ZnSO_4$), lysozyme, bovine serm albumin (BSA) and other metalloproteins including myoglobin (MY), carboxypeptidase A (CPA) and New Delhi metallo-beta-lactamase 1 (NDM1).

Red blood cell lysates: 10% bovine erythrocytes in PBS were obtained from Innovative Research. The lysates were prepared according to an adapted protocol[6] to obtain red blood cell lysates free of hemoglobin. First, 4 mL of the bovine blood solution in PBS was concentrated down using 10 kDa centrifugal filters to 0.25 mL at 4 ℃. Then, 1 mL of milliQ water was added to

the bovine blood cells and shaken intermittently for 10 minutes while keeping it on ice. To the above solution, 0.6 mL of chloroform and 0.4 mL of ethanol was added and it was again shaken intermittently for 5 minutes. The tube was then centrifuged at 1000g for 5 minutes and the top aqueous layer containing the lysates was collected. 0.8-1 mL of water was added to the pellet, the solution shaken again for 5 minutes followed by centrifugation at 1000g and collection of the aqueous layer. This step was repeated 5 times. Finally, the aqueous layers were lyophilized and the dry lysates containing proteins were reconstituted in 50 mM HEPES at approximately 20 mg/mL.

Native SDS Page: The procedure for native SDS page was adapted from previously reported method by Nowakowski et. al[7]. Protein-dye samples were prepared and incubated with sample buffer (100 mM Tris HCl, 150 mM Tris Base, 0.01875% Coomassie G-250 and 20% glycerol) for 15 minutes at 4 ℃ before loading onto precast 7.5% Mini-Protean TGX (Biorad). The running buffer was made with 50 mM MOPS, 50 mM Tris Base and 0.011% SDS, pH 7.3, to avoid any protein unfolding or demetalation. Samples run at 0.0375% as reported by Nowakowski resulted in partial loss of protein due to unfolding. The running buffer was chilled to 4°C prior to the run. Samples were loaded into the gel and electrophoresis was conducted at 180 V at 4°C for 30 mins until the dye front reached the end of the gel. Fluorescence was recorded under UV light (short wave, 254 nm) to excite the CA-bound fluorophore. This is owing to excitation of trytophan residues that undergo FRET transfer to the fluorophore bound in the CA active site.[3] The gels were then stained with coomassie overnight to visualize all the proteins.

Cell work: Cell culture was performed in Dulbecco's modified Eagle's Medium (DMEM), containing 4000 mg/L glucose and sodium pyruvate, supplemented with 10% heat inactivated

fetal bovine serum (FBS), and 1% antibiotics (200 U/cm^3 penicillin and 200 μg/cm^3 streptomycin) at 37 °C with 5% CO_2. HeLa cells were grown in T-75 and T-150 flasks. HEK 293 cells were grown in EMEM media, supplemented with 10% FBS and 1% antibiotics. For imaging, the cells were seeded onto ibidi 8-well μ-slides (0.3 mL capacity) and grown to 70% confluency prior to imaging. Imaging was performed with Gibco live cell imaging media on the Zeiss 710 Laser Scanning Microscope at 37 °C.

Cell Imaging experiments : For studies with **1a**, λ_{ex}= 405 nm and λ_{em}= 505-565 nm was used and the cells were imaged with 1 μM probe after 20 minutes of incubation. For Fluozin3-AM, λ_{ex}= 488 nm and λ_{em}= 500-565 nm was used, and the cells were incubated for 20 minutes at 2.5 μM, washed and imaged after another 15 minutes. Post-treatment with the dyes, cells were treated with 10 μM AZA, 1 μM ZnPT and 10 μM TPEN in separate wells and imaged after 25, 5 and 15 minutes of incubation respectively. Image processing was conducted using Fiji (ImageJ) where the **1a** treated cells were represented with the Fire-processing and the Fluozin3-AM cells were represented in green.

Table S1: Measured spectroscopic properties of solvatochromic fluorophores **1a-c** and TD-DFT predicted wavelengths for **1a** (in brackets†). Fluorescence quantum yield for **1c** in HEPES buffer could not be recorded.

		Toluene	**$CHCl_3$**	**DMSO**	**MeOH**	**HEPES buffer**
1a	λ_{abs} (nm)	388 (388)†	398	400 (401)†	397	420 (402)†
	ε (M^{-1} cm^{-1})	11800	9100	4800	5000	4800
	λ_{em} (nm)	470	485	512	550	575
	Φ	0.9	0.81	0.13	0.014	0.004
1b	λ_{abs} (nm)	391	398	398	397	417
	ε (M^{-1} cm^{-1})	5400	4600	5000	4800	5100
	λ_{em} (nm)	477	496	516	560	580
	Φ	0.97	0.88	0.14	0.018	0.003
1c	λ_{abs} (nm)	386	395	399	397	420
	ε (M^{-1} cm^{-1})	8000	8000	7200	6500	7200
	λ_{em} (nm)	465	485	512	548	590
	Φ	0.9	1	0.23	.03	-

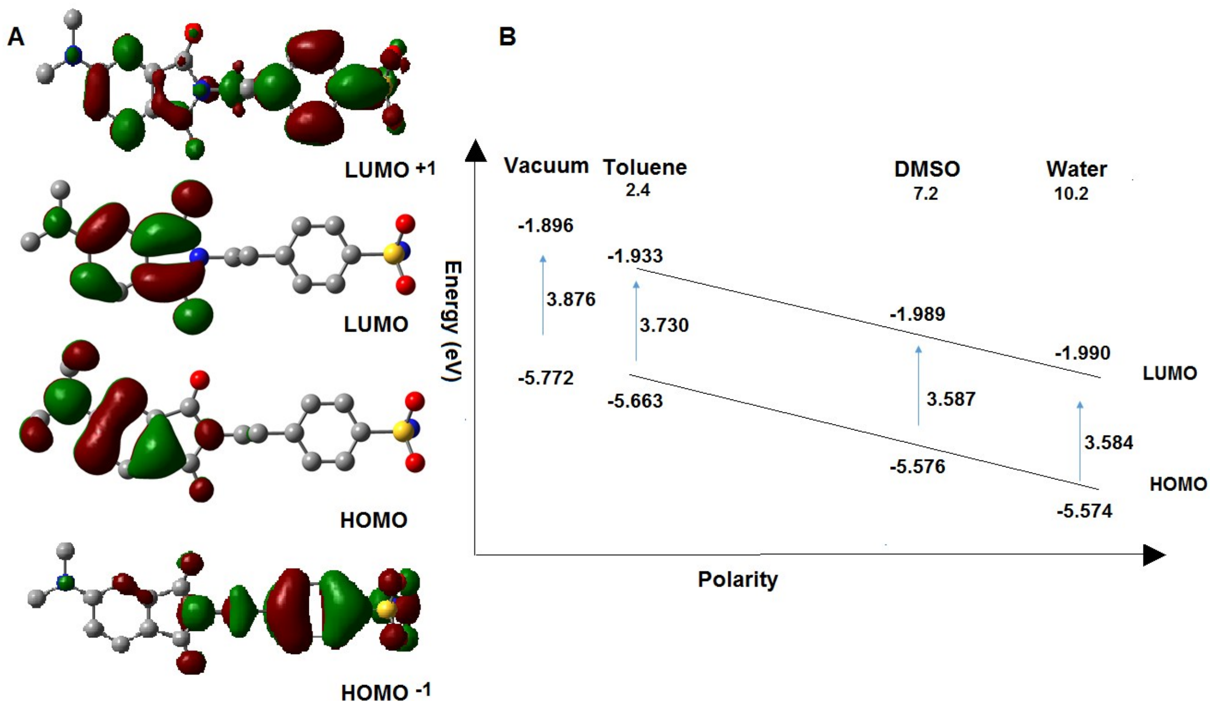


Figure S1: (A) Optimized structure of **1a** showing its HOMO -1, HOMO, LUMO and LUMO +1 density states. (B) Energies (in eV) for the HOMO to LUMO transition in various solvents as a function of the polarity.

Explanation of DFT calculations:

The ground-state structures for **1a** were optimized by density functional theory (DFT) calculations and the vertical electronic transitions were predicted by time-dependent DFT (TD-DFT) methods, shown in Figure S1. Three solvents, including toluene, water and DMSO were considered, together with the vacuum as a control. The HOMOs and the LUMOs of the molecule were all localized on in-plane π orbitals of the fluorophore moiety. The S_0-S_1 transition stems from HOMO to LUMO, which thus corresponds to an charge transfer from the electron donating amino-group to the electron withdrawing-imide ring in the molecule as shown in Figure S1A. A decrease in the energy gap between the HOMO and LUMO was predicted on increasing polarity, consistent with a red shift in absorbance as observed (Table S1).

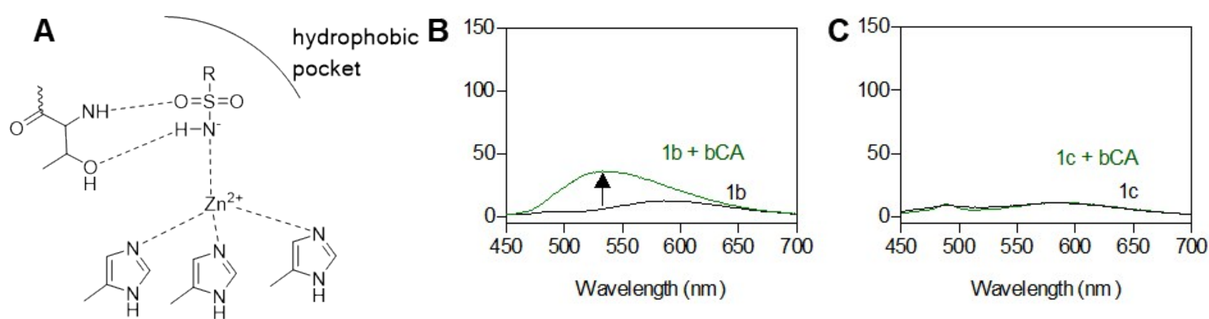


Figure S2: (a) Proposed interactions between sulfonamide and Zn^{2+} in the CA active site where the sulfonamide group is stabilized *via* hydrogen bonds to nearby residues. Adapted from *Carbonic Anhydrase: Its Inhibitors and Activators, CRC Press, 2004, edited by Claudiu T Supuran, Andrea Scozzafava and Janet Conway.* (b,c) Fluorescence spectra of probes **1b** and **1c** (5 μM each) upon incubation with equimolar bCA in 50 mM HEPES, 0.1 M KNO_3 buffer (pH 7.2) at room temperature at λ_{ex}= 420 nm. For **1b**, a 3-fold increase in fluorescence was observed while **1c** exhibited no fluorescence turn-on.

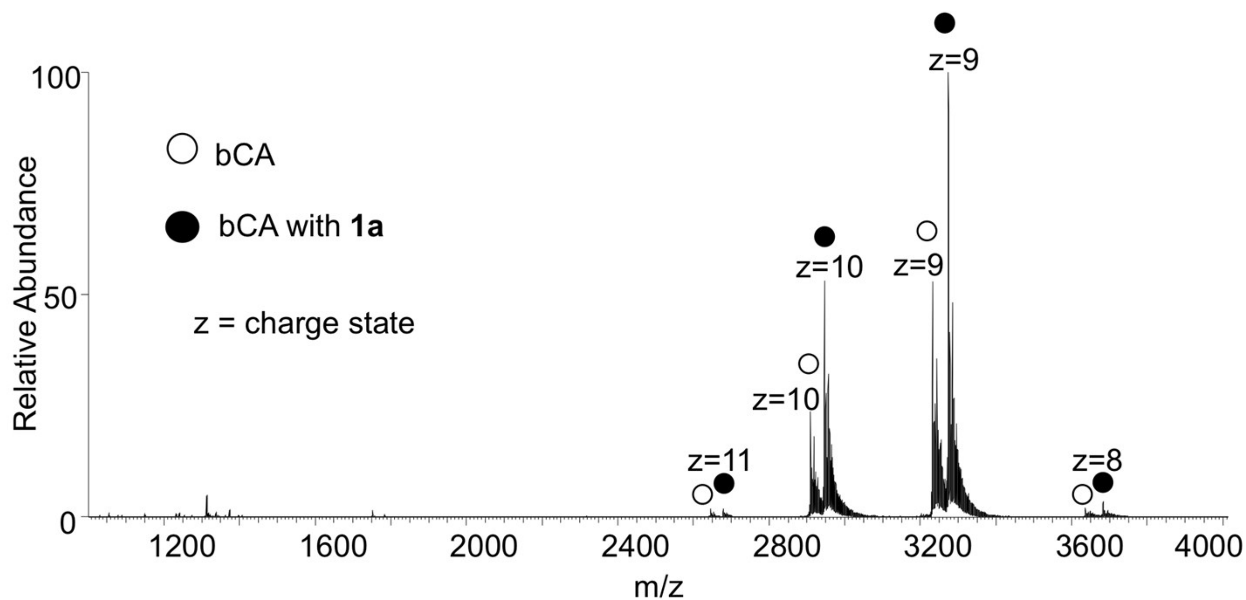


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Figure S3: High resolution mass spectrum for the solution containing bovine carbonic anhydrase and ligand **1a** showing: (a) the ESI mass spectrum (b) the deconvoluted mass spectrum with an inset that displays an expanded region of the deconvoluted mass spectrum from 28,800 Da to 30,300 Da showing the mass shift corresponding to the attached ligand.

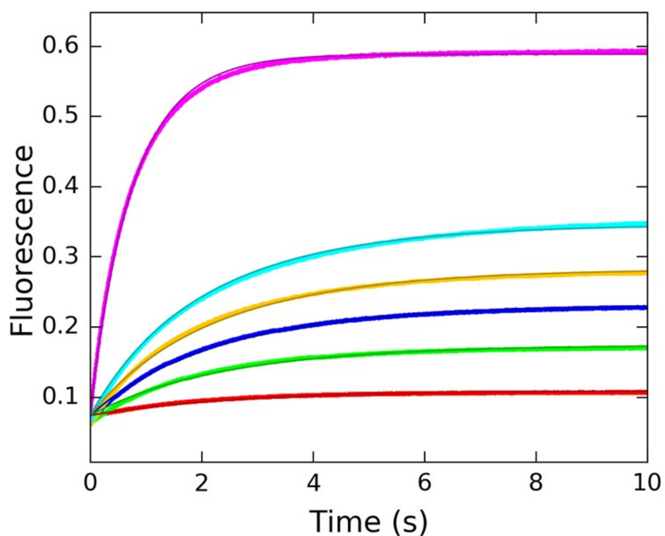


Figure S4: Kintek plot for probe **1a** (1 μM) with increasing amounts of CA: 0.1 (red), 0.3 (green), 0.5 (blue), 0.7 (yellow), 1(cyan) and 5 μM (pink) as monitored *via* stopped-flow measurements. The fluorescence was measured for λ_{ex}= 420 nm and the data was fit using Kintek explorer as described in the experimental section above. Both data points and fit are shown.

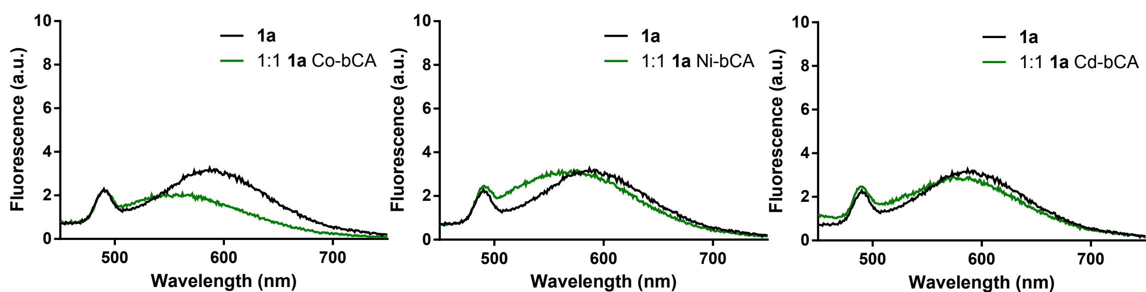


Figure S5: Fluorescence spectra of probes **1a** (5 μM) upon incubation with equimolar amounts of Co-bCA, Ni-bCA and Cd-bCA in 50 mM HEPES, 0.1 M KNO_3 buffer (pH 7.2) at room temperature (λ_{ex}= 420 nm). With Co^{2+} and Ni^{2+}, we observed no fluorescence increase –this is expected since the unpaired electrons on the metal is expected quench the fluorescence signal. With Cd^{2+}, we observed no change in fluorescence.

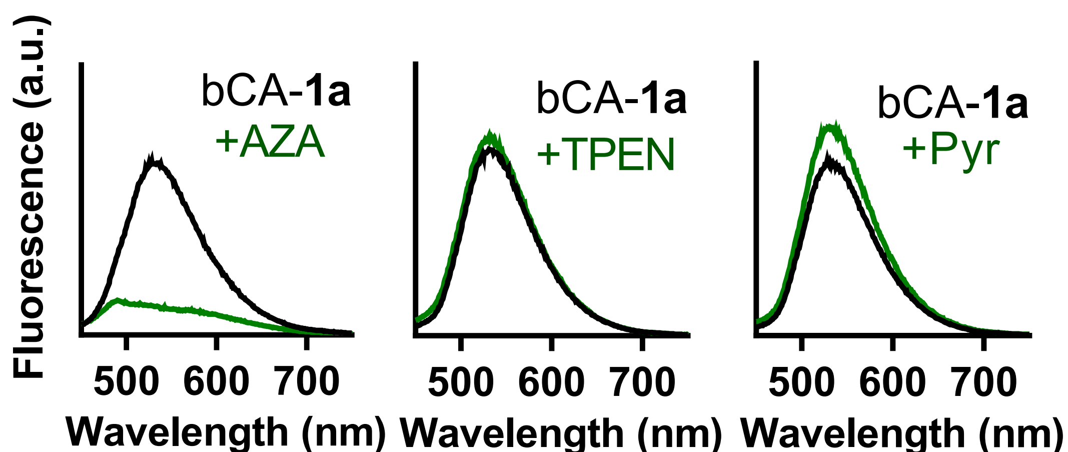


Figure S6: Competition studies of **1a**-CA binding was done at 2 μM bCA and 2 μM **1a**, with (a) AZA (10 μM), (b) TPEN (10 μM) and (c) pyrithione (10 μM) in 50 mM HEPES, 0.1 M KNO_3 buffer (pH 7.2) at room temperature. λ_{ex} = 420 nm.

Based on the results of the NPA assay, we tested the competition binding of the commercially available CA inhibitor, **AZA** with **1a**. As seen in Figure S6, there is a significant decrease in fluorescence intensity (up to 66%) for the bCA-**1a** complex suggesting that **AZA** displaced **1a** in the bCA active site. This suggests partial replacement of **1a** with **AZA** in the CA active site, with the appearance of a shoulder at 490 nm which is consistent with the emission profile of aggregated **1a** molecules.

Addition of TPEN and pyrithione displayed negligible effect on bCA-**1a** fluorescence- 5% and 15% respectively.

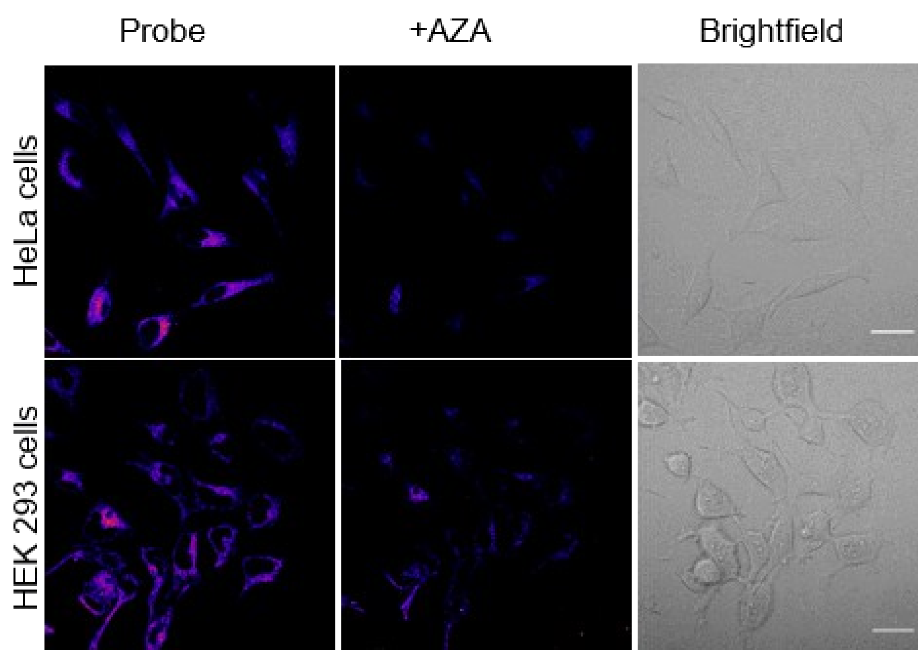


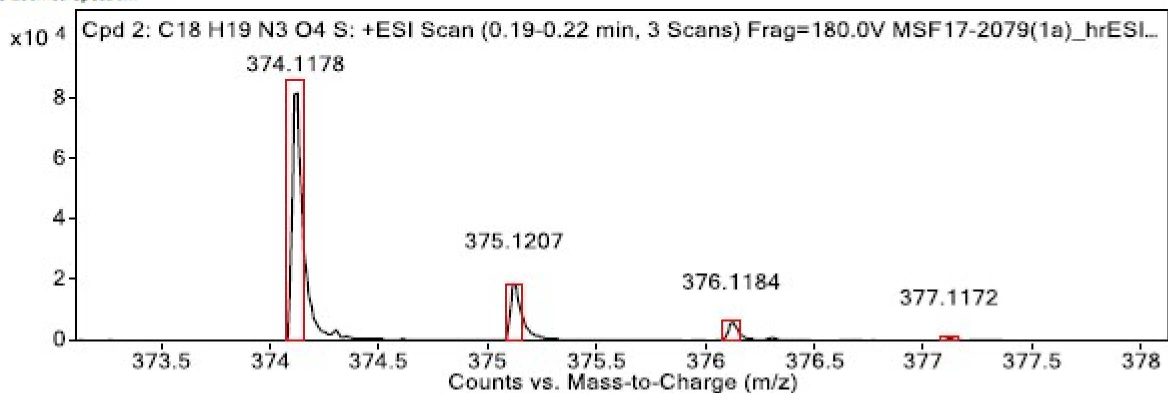
Figure S7: Confocal images taken in live-cell imaging media at 37 ℃ showing fluorescence response with **1a** (1 μM; λ_{ex}: 405 nm, λ_{em}: 505-565 nm) in HeLa and HEK 293 cells. Following dye incubation, cells were subjected to AZA treatment (10 μM) for 25 min. Image processing was conducted using Fiji (ImageJ). Scale bars: 30 μm.

HRMS and ^{1}H NMR spectra:

Results Acquired by The University of Texas at Austin Mass Spectrometry Facility

Data File	MSF17-2079(1a)_hrESIpos1.d	**Sample Name**	2079(1a)	**Comment**	2079(1a)
Position	P1-B4	**Instrument Name**	Instrument 1	**User Name**	
Acq Method	pos.m	**Acquired Time**	12/1/2017 5:04:24 PM	**DA Method**	KS.m

MS Zoomed Spectrum



MS Spectrum Peak List

Obs. m/z	Calc. m/z	Charge	Abundance	Formula	Ion Species	Tgt Mass Error (ppm)
374.1178	374.1169	1	84285	C18H19N3O4S	(M+H)+	-2.33
375.1207	375.1198	1	19967	C18H19N3O4S	(M+H)+	-2.34
376.1184	376.1166	1	6441	C18H19N3O4S	(M+H)+	-4.87
377.1172	377.1181	1	1221	C18H19N3O4S	(M+H)+	2.24
396.0997			122812			

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Figure S8: High-Resolution mass spectrometry data for **1a**

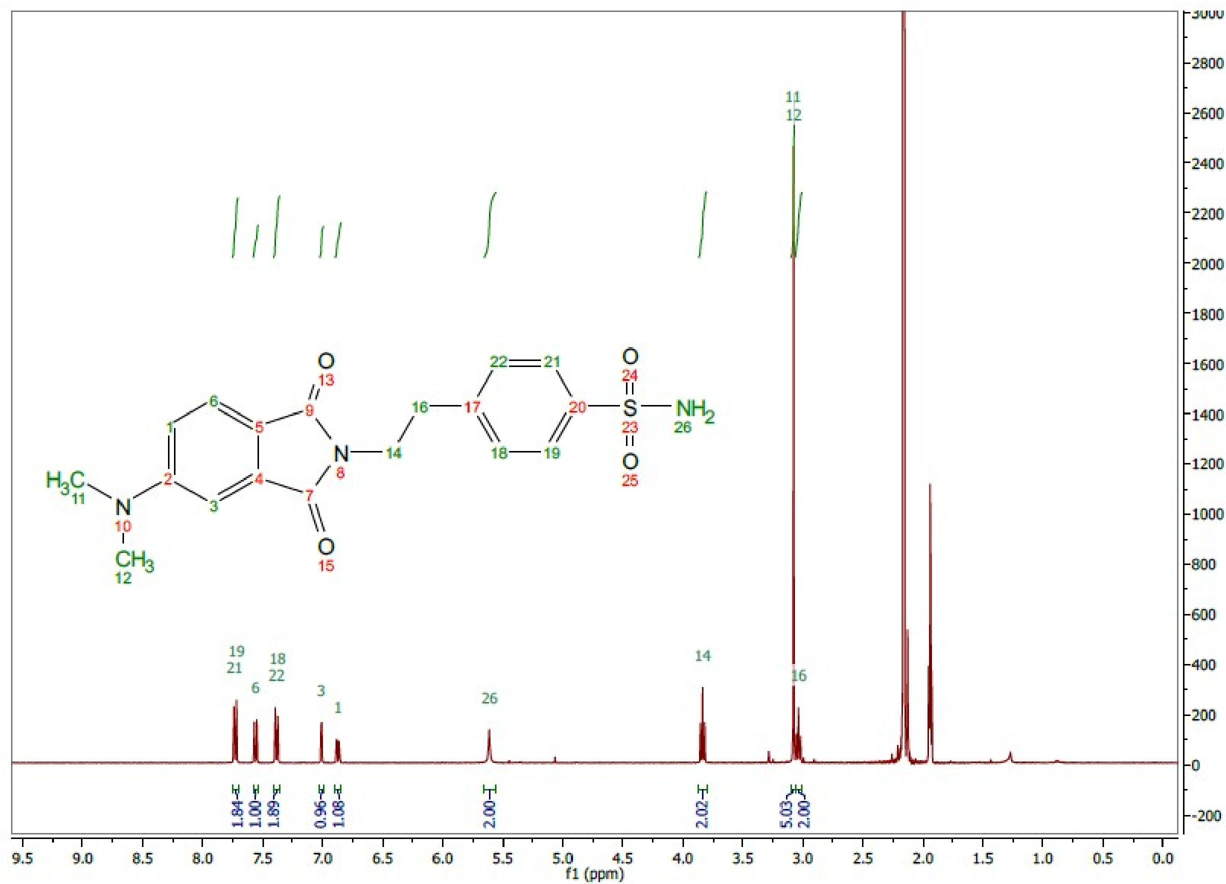

Figure S9: ^{1}H-NMR spectrum for **1a** in CD_3CN

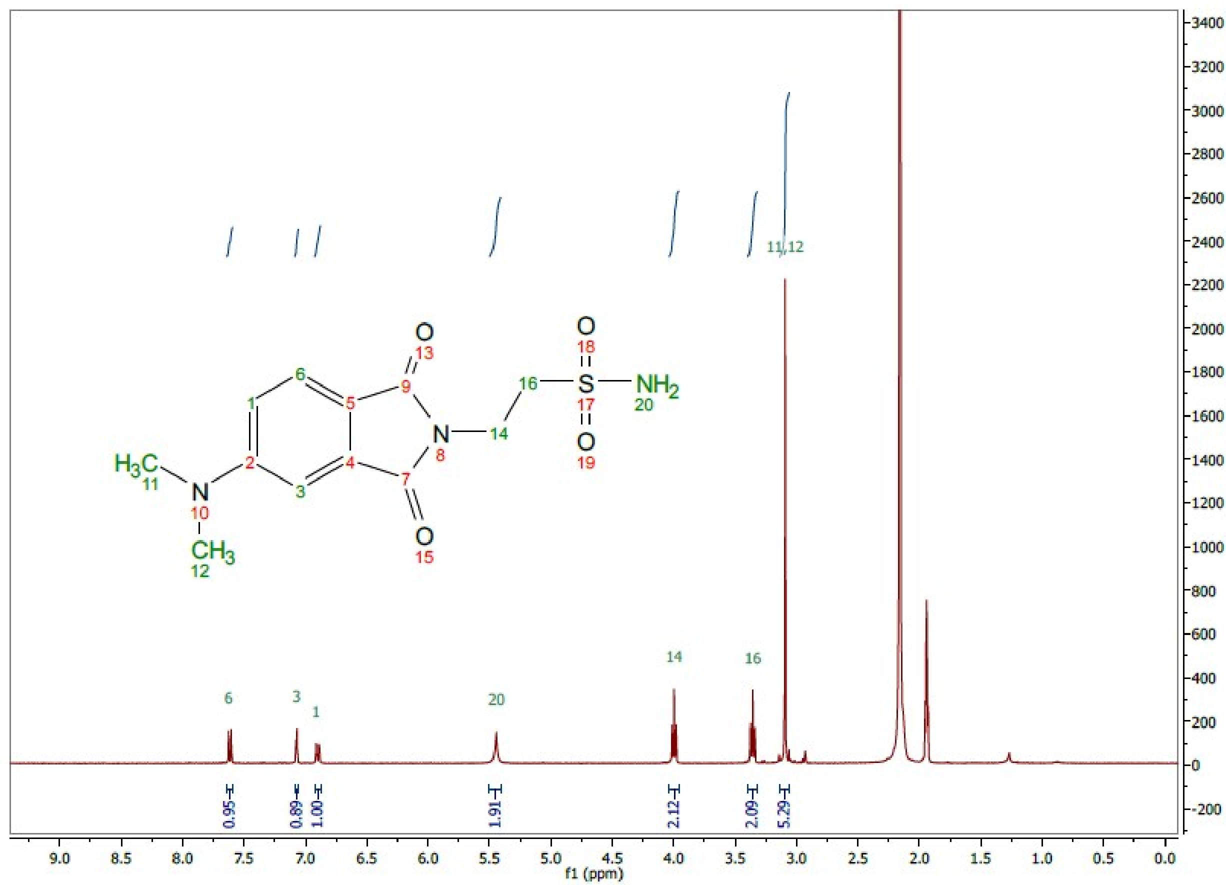

Figure S10: ^{1}H-NMR spectrum for **1b** in CD_3CN

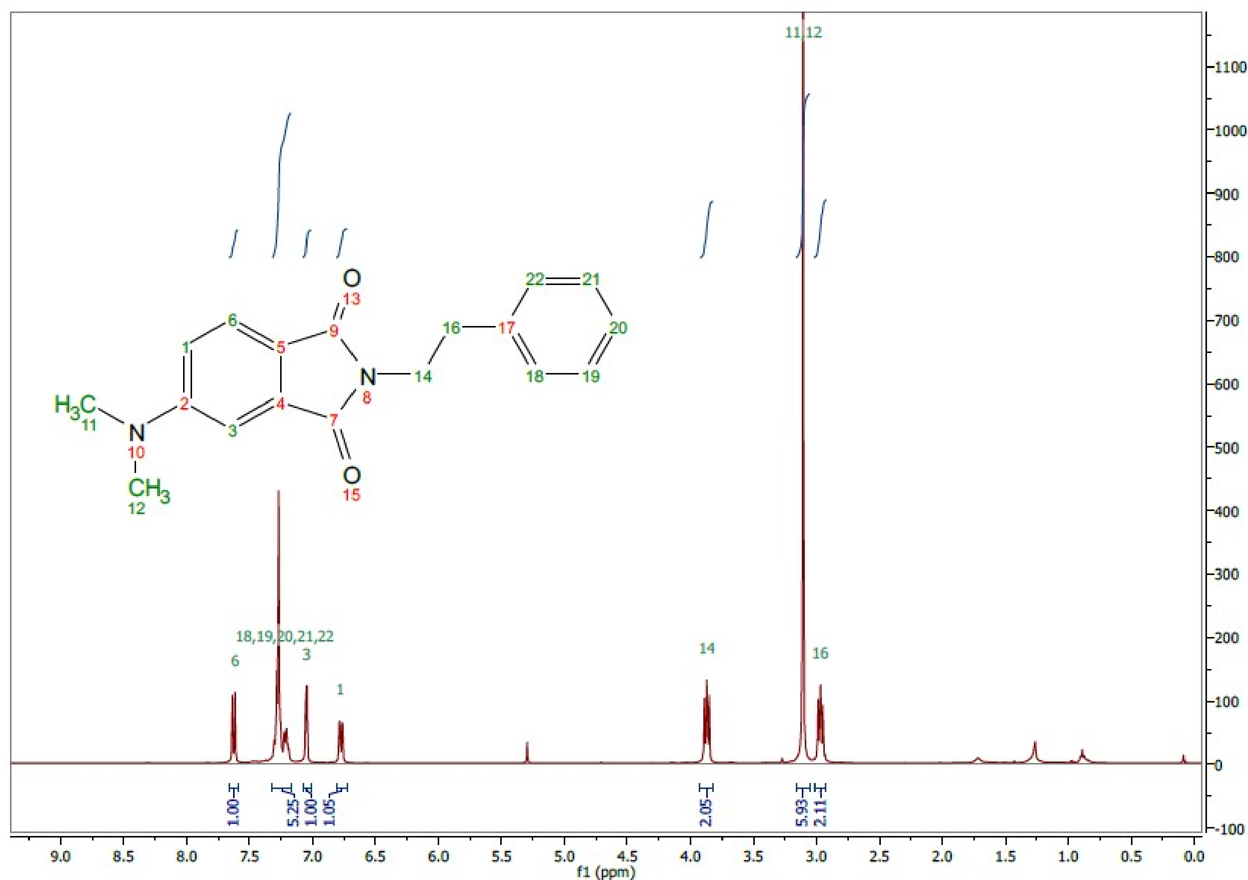

Figure S11: ^{1}H-NMR spectrum for **1c** in $CDCl_3$

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