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**Development of photopharmacophores for light mediated control of
metalloenzymes in *in vitro* and *in vivo* systems**

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**Development of photopharmacophores for light mediated control of
metalloenzymes in *in vitro* and *in vivo* systems**

by

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Dedication

To my Mummy and Papa, Mohit, Bhawna, Ushank and Nidhi

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Abstract

Development of photopharmacophores for light mediated control of metalloenzymes in *in vitro* and *in vivo* systems

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Biological systems consist of complex and highly dynamic networks of molecular processes carried out by the enzymes. About one third of the enzymes need a metal cofactor for their activity, and hence, are called as metalloenzymes. One such metalloenzyme is carbonic anhydrase (CA), a Zn^{2+} containing protein, that catalyzes the reversible hydration of carbon dioxide, thereby playing an important role in acid base homeostasis. There are multiple isoforms of CA and their activity, localization, and expression level vary in different cell types and also differ between normal and disease states. CA is known to be overexpressed in hypoxia, which leads to the greater pH imbalance providing a positive pathway for the cancerous tumors to metastasize. Therefore, it is imperative to get precise on-demand control over these isoforms without perturbing the whole system in order to understand their role in biological systems, especially in the therapeutic context. Light serves as a wonderful external trigger due to these advantages: non-invasive; easy to control the wavelength, duration, and intensity of light; and exhibits high spatiotemporal precision. My doctoral research focuses on

developing photopharmacophores for light mediated control of CA in *in vitro* and *in vivo* systems.

We designed carbonic anhydrase azobenzene photoswitch (**CAP-1**) that can isomerize between two isomeric forms, *trans* and *cis*. This probe contains a metal binding group which interacts with the Zn^{2+} in the active site only in *trans* form, hence, inhibiting the protein activity.

We further tuned the design with photoswitch **CAP-5**, which allowed the visible light mediated bidirectional control over CA in an isolated protein, intact live cells, and *in vivo* with zebrafish during embryo development. Additionally, we explored the prospect of photoregulation of a specific membrane-associated isoform of CA (CAIX) by modifying **CAP-F5** to confine the probe to extracellular matrix.

Correspondingly, we used the photoremovable protecting group (PPG) to achieve spatiotemporal control over the mitochondrial isoform of carbonic anhydrase (CAV). The probe **PCE-M** consists of three parts: inhibitor, PPG and a mitochondrial-directing group. The photocleaving process is triggered with 410 nm light to get free inhibitor that can inhibit CAV without interference from other isoforms of CA.

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Chapter 1: Photobiochemistry- light mediated control over enzyme activity

1.1 LIGHT AND MEDICINE: PHOTOPHARMACOLOGY

Photopharmacology is an emerging field in medicinal chemistry where drugs (pharmacophores) are activated or deactivated by light. The pharmacophores were originally developed by Paul Ehrlich during the late 1800s, but the official term was coined by Schueler in 1960s. Along with all the desired features of a traditional drug molecule, traditional pharmacophores often produce undesirable and sometimes intolerable side effects due to poor selectivity for the target tissue/site as the molecule is always present in the “active” form. Photopharmacology provides a means of ameliorating off-target toxicity by providing control of *when* and *where* drugs are active. Light serves as an external stimulus/modulator. The ease of regulating the duration, intensity, and wavelength makes light a great modulator to achieve control over a drug (and hence, the biomolecular target) with high spatiotemporal resolution. This concept of photocontrol over biomolecules can be explored further to understand a protein of interest in both healthy and diseased contexts at a cellular level. This chapter discusses the relevant background and previous work conducted for the development of a chemical toolbox for photomodulation of enzyme activity.

1.1.1 Photocontrol over protein activity: Inspiration from nature

Light can play important roles in controlling various biochemical processes. Owing to its high spatiotemporal resolution and non-invasive nature, light-based activation can be utilized as a smart trigger to mediate the biological activity of proteins and other biomolecules. Light of a specific wavelength can be used to gain control over

protein function (Figure 1.1) either by (1) directly changing its activity state, (2) modifying its effective cellular concentration via controlling its production rate, (3) inducing protein degradation, or (4) inducing its relocation.

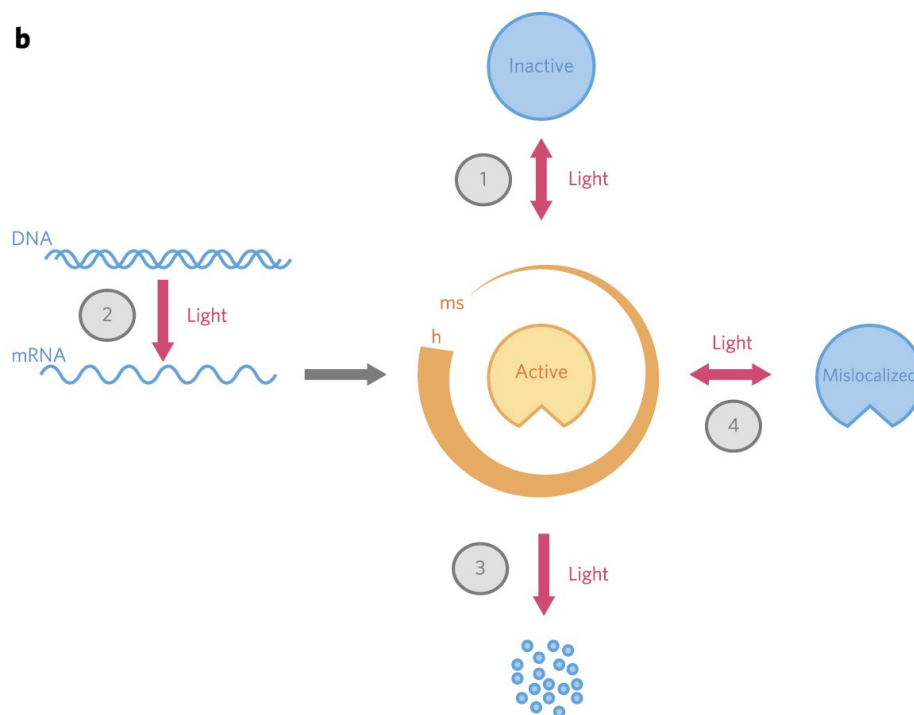


Figure 1.1 Different ways to use light to control proteins in living organisms, as adapted from Vriza et al.

Light has been a key tool for understanding protein function since the beginning of evolution. All organisms from bacteria to mammals have acquired an autonomic system in order to detect light and respond to it. Key examples of naturally occurring photo-responsive proteins include bacteriorhodopsin that moves protons across the membrane out of the cell in Archaea, PSII for oxygenic photosynthesis in plants and algae, and opsins in the photoreceptor cells of retina for vision in humans. The inspiration to develop chemical tools to gain control over protein function can easily derive from

photoreceptors found in the nature including rhodopsins (Figure 1.2 A, B, C), flavoproteins (Figure 1.2 D, E), and phytochromes (Figure 1.2 F).

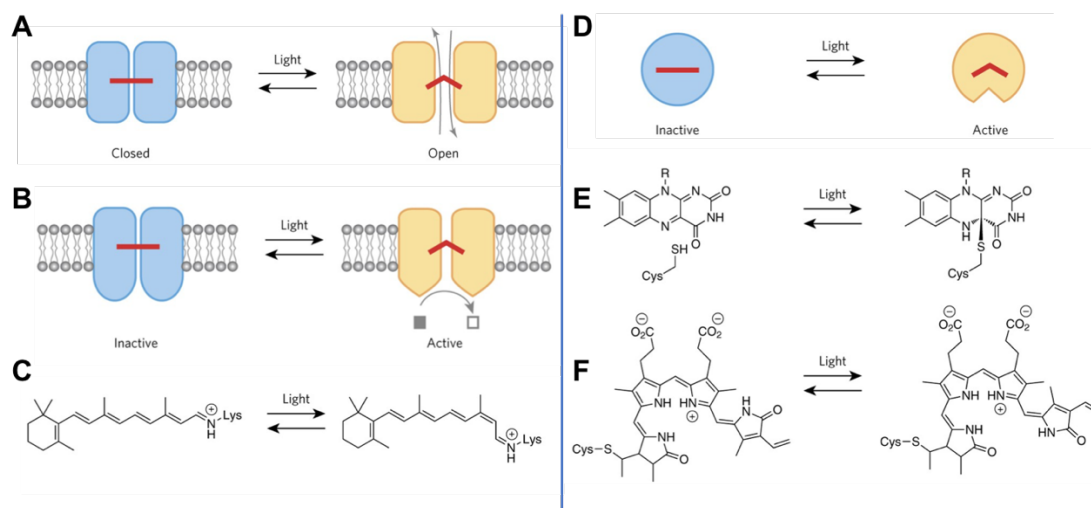


Figure 1.2 Nature-inspired examples to gain control over the activity of a protein: Rhodospin Type I (A) and Rhodopsin Type II (B) and corresponding retinal chromophore (C); Flavoprotein (D) and the associated chromophore (E); bilin chromophore for phytochromes (F).¹

1.1.2 Strategies to control proteins with light

In photo-regulated proteins, photoirradiation of the associated chromophore induces an overall conformational change in the protein, which can further be rerouted to manipulate the protein's activity. This has inspired scientists to develop a tool box of exogenous chemical chromophores that can be used to target any protein of interest. These photoactive groups function mainly through these two approaches, irreversible² (Figure 1.3 A) and reversible³ (Figure 1.3 B).

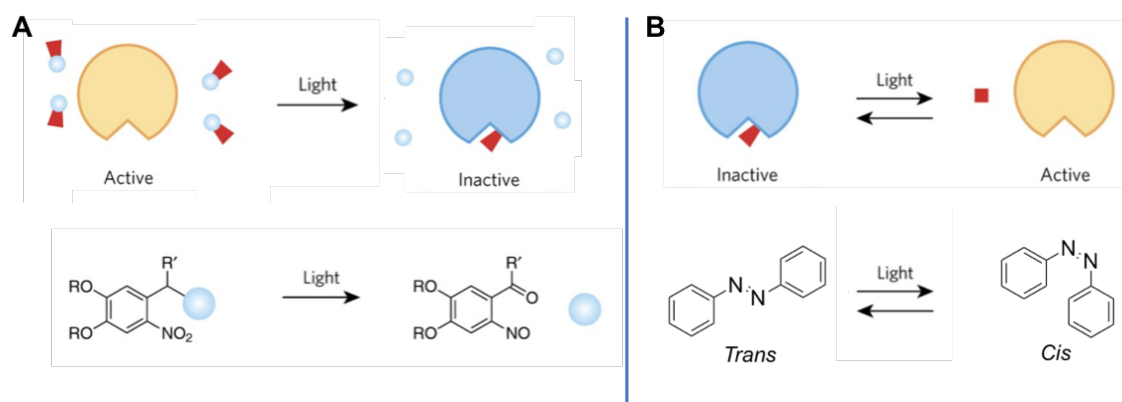


Figure 1.3 Irreversible (A) and reversible (B) approaches to modulate the activity of the protein using light.

The irreversible approach uses the concept of photocaging, where the molecule of interest (inhibitor/ligand, activator or substrate) is modified with a photoprotecting group (PPG) which blocks the interaction of the molecule with the biological target. Upon photolysis, the active molecule is released that can now interact with the protein and affect the properties of the target, either directly or indirectly, with high spatiotemporal resolution. This approach has found applications in neurobiology,⁴ biomedicine,⁵ protein folding/unfolding,⁶ regulation of protein activity in vitro and in vivo,^{7, 8} etc.

The reversible approach, on the other hand, is based on the structural or chemical differences between two conformations of a molecule upon light irradiation in a reversible manner. This reversibility is valuable when we need to study the cellular processes under both active and inactive enzyme within a small timeframe. From a pharmacological perspective, the reversible modulation could also minimize the downstream effects of altered protein activity that could occur with the irreversible approach. Altogether, the reversibility and temporal control can help us to understand the effect of an active compound when turned on/off at different time periods with respect to the response at the time of introduction.

In this dissertation, due to the additional advantages of reversible modality in chemical biology, we focus mainly on exploring such reversible tools to photomodulate the activity of metalloenzymes.

1.1.3 Chemical tools for the reversible control of protein activity

There are various types of photochromic groups that, upon photoirradiation, can undergo reversible chemical rearrangement causing structural as well as electronic changes in the molecule. They can be subcategorized by the photoreaction path they follow: *trans-cis* isomerization, photocleavage of bonds, electrocyclization and cycloreversion reactions, and intermolecular cycloaddition and cycloreversion reactions. The most common route to gain photocontrol over protein activity is by using *trans-cis* isomerization based photoswitches (Figure 1.4).

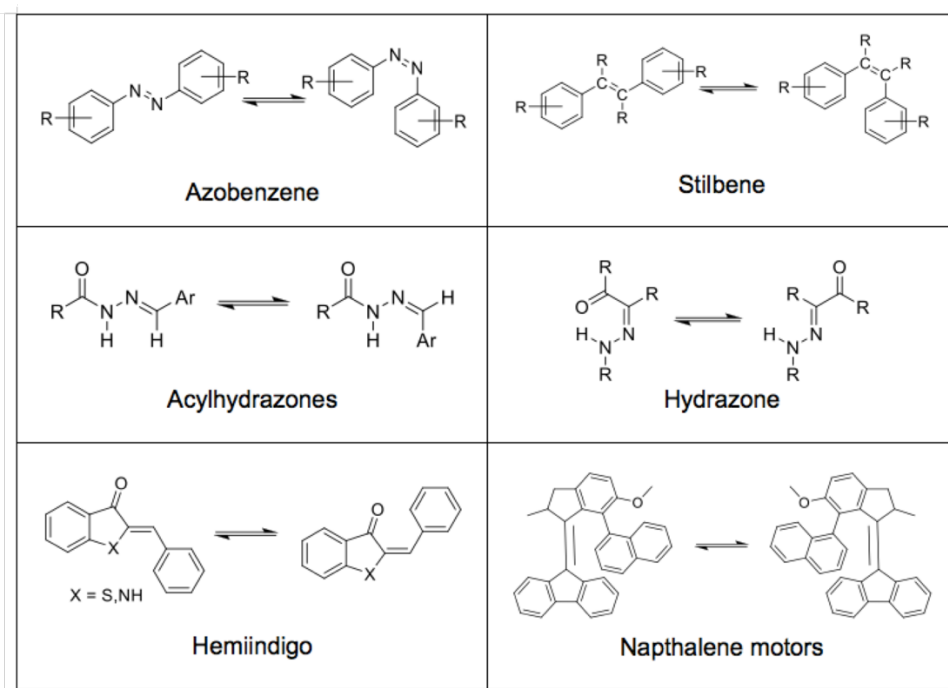


Figure 1.4 Examples of *trans-cis* isomerization based photoswitches

Traditionally, they can also be classified as being T-Type (thermally reversible) and P-type (thermally irreversible), depending on the thermal stability of the photogenerated product. In T-type molecules, the product generated is thermally unstable, which results in the reversion of product to the initial form; this occurs with azobenzene and spiropyrans, for example. On the other hand, the photogenerated species in P-type molecule is also thermally stable, and hence, does not produce the original configuration thermally, for example, fulgides and diarylethenes.

There has been an extensive amount of work done to explore these photochromic molecules in the context of biomedicine: photoactivated control of imaging, on-demand cell attachment, photoactivated control of drug delivery, and photopharmacological chemotherapy.⁹ More interestingly, they have also been applied to photoregulate the activity of proteins, where the ligand can have higher or lower binding affinity as a result of the transition between different configurations (structural or chemical) of the molecule. Although stilbenes, hemithioindigos and diarylethenes have been used as switches, azobenzenes shows the most promise in achieving a larger range of activation wavelengths and greater photoactivated state stabilities.¹⁰ For purposes of this dissertation, focus is on the azobenzene photoswitch, as will be described below.

1.2 AZOBENZENE: AN OVERVIEW

1.2.1 Background

Azobenzenes are one of the most characterized photoswitches. They are a derivative of diazene ($\text{HN}=\text{NH}$), where both hydrogens are replaced by the phenyl rings. They can exist in the *trans* or *cis* conformations, depending on the relative positions of the phenyl groups with respect to the diazo bond (Figure 1.5). The *trans*→*cis* photoisomerization can be induced by the irradiation with UV-visible light. The *cis* form

obtained is not thermally stable, and hence, thermal *cis*→*trans* relaxation process occurs in the dark. This process of *cis*→*trans* isomerization can also be achieved by visible light irradiation. Azobenzene exhibits remarkable photostability and can be photoisomerized multiple times with negligible photodegradation.

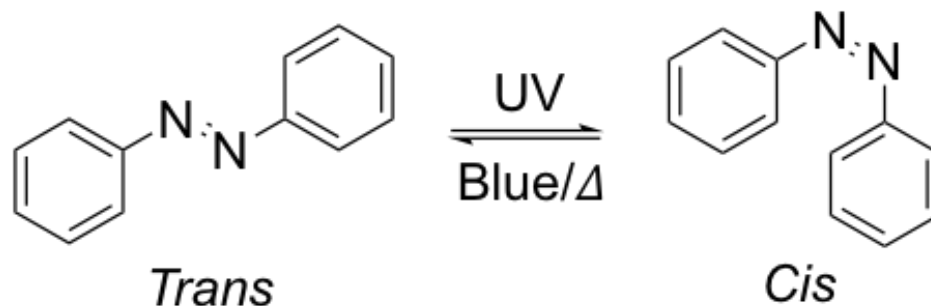


Figure 1.5 The structure of *trans* and *cis* azobenzene

1.2.2 Photochemistry of isomerization process

There are four possible isomerization pathways for azobenzene: rotation, inversion, concerted inversion, inversion-assisted rotation (Figure 1.6).¹¹ The key differences in these mechanisms are:

1. Rotation: This pathway involves the rupture of the N=N π -bond to allow the free rotation about the N-N bond.
2. Inversion: This mechanism results in a fixed C-N=N-C dihedral angle of 0°, while one of the N=N-C angle increases to 180°.
3. Concerted inversion: In this pathway, both N=N-C bond angles increase to 180° generating a linear transition state.
4. Inversion-assisted rotation: This pathway results in large changes in the C-N=N-C dihedral angle and small changes in the N=N-C angles at the same time.

Each pathway has a transition state, and the relaxation from those states can yield to either the *trans* or *cis* conformation. Hence, at any given photostationary state (PSS), there are both *trans* and *cis* isomers present in the solution. The sum of quantum yields of the isomerization process ($\phi_{t \rightarrow c} + \phi_{c \rightarrow t}$) is not equal to 1, suggesting multiple pathways for isomerization for any azobenzene system. Hence a single mechanism cannot explicitly explain all aspects of the photoisomerization process, even for the parent azobenzene.

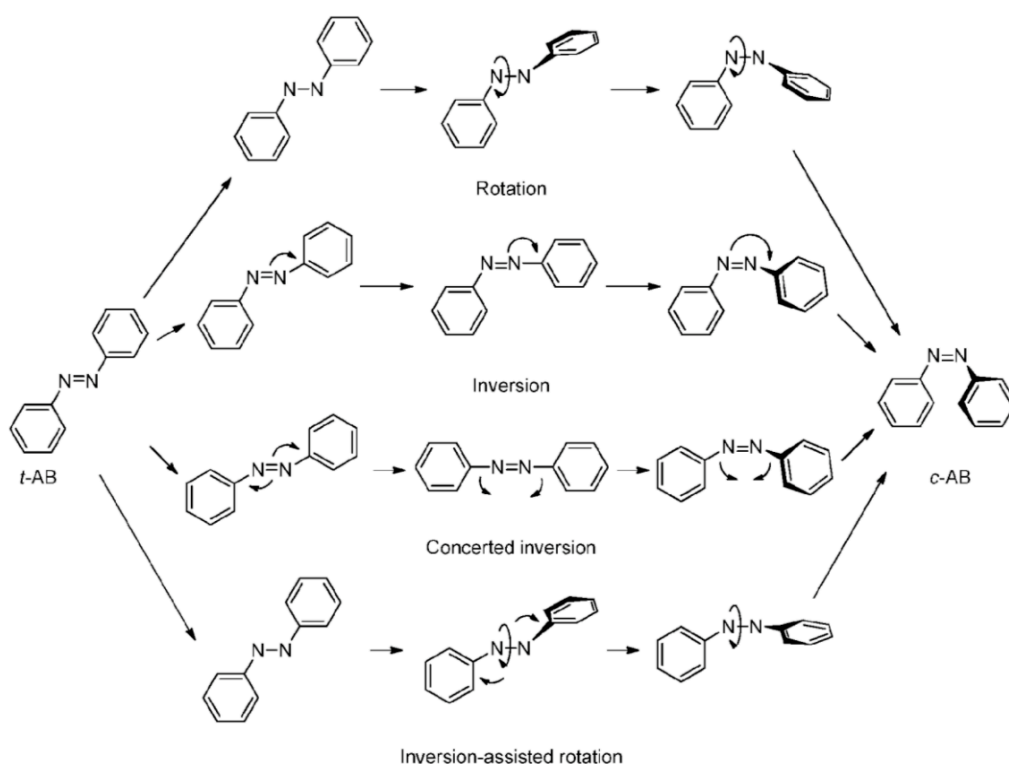


Figure 1.6 Possible mechanisms for the photoisomerization of azobenzene, as adapted from Bandara et al.¹¹

An understanding of the basic photochemistry of the isomerization process is very important to understand azobenzene photophysical properties such as the required isomerization wavelengths and the stability of the isomers generated. The ground state of azobenzene is a singlet state (S_0), and S_1 and S_2 are the first and second singlet excited

states (Figure 1.7 B). The S_1 and S_2 states generated by the *trans* isomer differ in terms of the energy and conformation from the analogous excited states generated by the *cis* isomer. The photoisomerization from *trans*→*cis* and *cis*→*trans* can happen following S_0 → S_2 and S_0 → S_1 excitation.

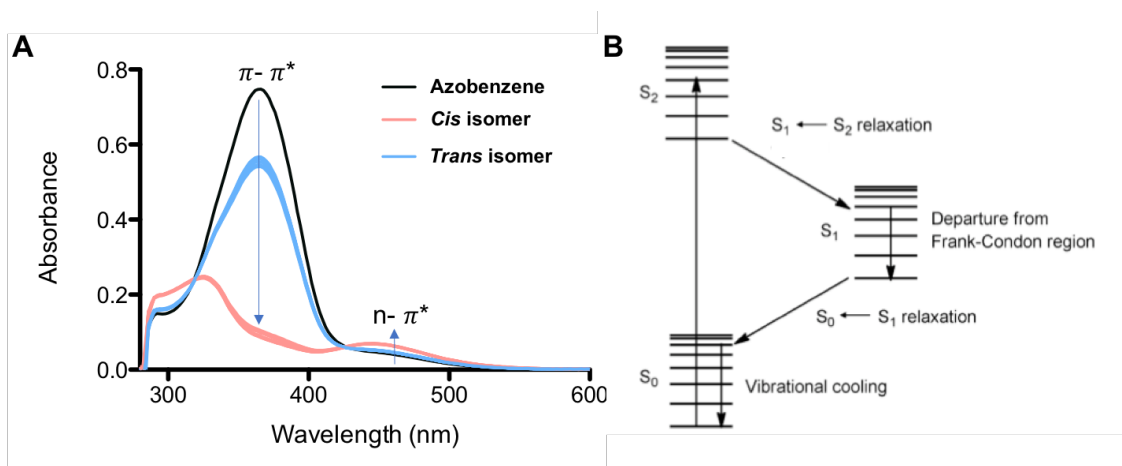


Figure 1.7 The spectral differences between *trans* and *cis* isomer of azobenzene (A). The energy level corresponding to the observed bands in the absorbance spectrum of azobenzene (B).

The photoisomerization process can be studied by UV-visible spectroscopy. The *trans* isomer has a $\pi-\pi^*$ transition band (S_2) in the UV region and an $n-\pi^*$ transition band (S_1) in the visible region (Figure 1.7 A). Upon photoisomerization to the *cis* isomer, the absorbance profile changes dramatically, with a decrease in the $\pi-\pi^*$ band and an increase in the $n-\pi^*$ band.¹¹ The isomerization mechanism and the quantum yield of photoisomerization of azobenzene depends on various factors such as the isomeric form, excitation mode, irradiation wavelength, solvent system, temperature, and finally, the substituents on the phenyl rings. Hence, designing the azobenzene system for a specific task requires the manipulation of the photoisomerization properties such as wavelength, efficiency, and the rate of thermal relaxation.

1.2.3 Different Classes of Azobenzene

Substitution on the azobenzene ring has a drastic effect on the absorption, emission and photophysical properties of the system.¹¹ Depending on the relative energies of π - π^* and n- π^* transitions, azobenzenes are broadly classified into three different classes (Figure 1.8):

1. Azobenzenes (ABn):

For these azobenzenes, the absorption profile looks very similar to the parent all H-substituted azobenzene, i.e. the π - π^* transition band is in the UV region and is well separated from the n- π^* band in the visible region. Hence, ABns have very similar photoisomerization properties to the parent molecule. Examples include AB derivatives with alkyl, amide, nitrile groups.

2. Aminoazobenzenes (aAB):

Azobenzenes with one or more amino or hydroxyl groups at *ortho*- or *para*-position are classified as aABs. These electron-donating substituents increase the electron density of the system, and shift the π - π^* transition band to a higher wavelength. This shift results in the partial overlap between the π - π^* and n- π^* transition band, which makes it difficult to predict properties like quantum yield of photoisomerization. However, this class of azobenzenes has a higher rate of cis→trans thermal relaxation due to the decreased thermal isomerization barrier as compared to ABns.

3. Pseudostilbenes (pSB):

In pSBs, the overlap between the π - π^* and n- π^* bands is so high that they are almost degenerate in energy and occur in the visible region. Examples include protonated azobenzene (pAB) and push-pull azobenzenes (ppAB). In the case of pAB, even though protonation produces no significant change in the quantum

yield, it increases the rate of thermal relaxation of *cis*→*trans* to a great extent.¹² In ppAB, the 4- and 4'- positions of the phenyl rings are occupied by a strong electron donor and a strong electron acceptor, resulting in the overall shift of electron density from one end to the other. This results in a poor quantum yield for the *trans*→*cis* photoisomerization due to the extremely fast competing process of *cis*→*trans* thermal isomerization, usually at a scale of milliseconds to seconds.

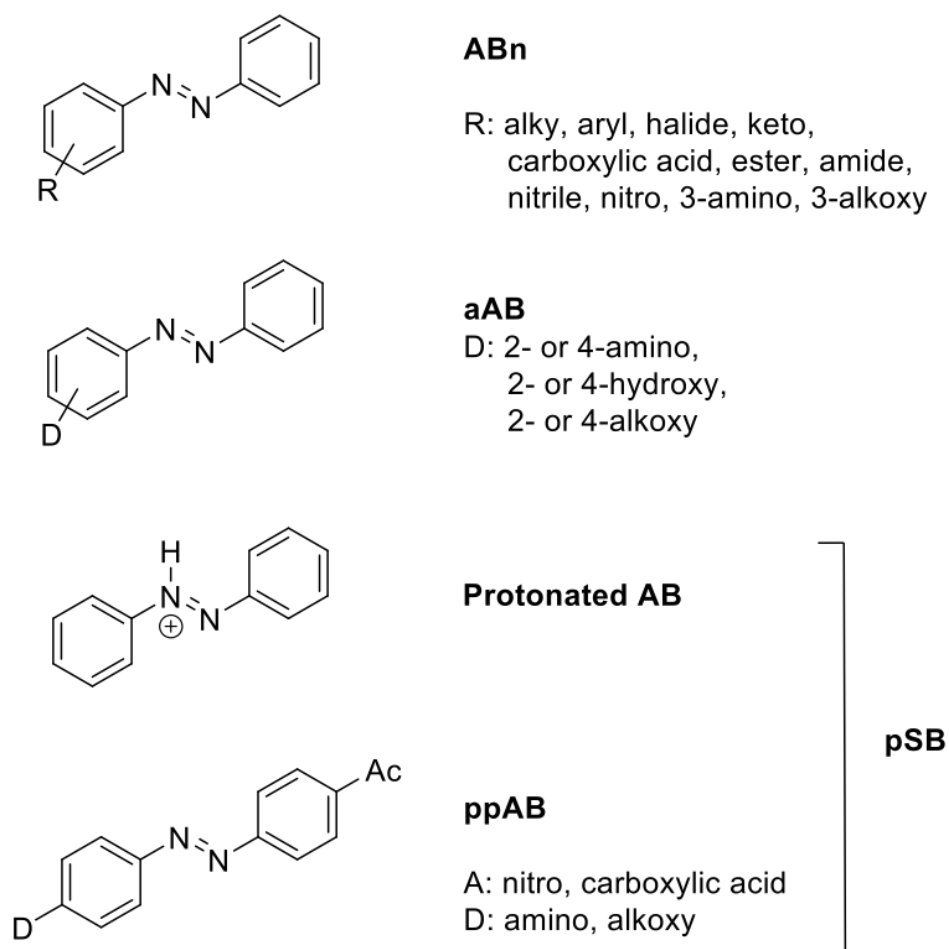


Figure 1.8 Different classes of azobenzene

1.2.4 Application of azobenzenes as photoswitches in biomedicine

Azobenzene photochromic moieties have been applied in a number of biological contexts¹³, including regulation of protein expression¹⁴ and enzyme folding,¹⁵⁻¹⁷ optical control over beta cell functions,^{18, 19} DNA conformation and transcription,²⁰⁻²⁴ mRNA translation,²⁵ chemotherapeutics,^{26, 27} and in neurobiology.²⁸⁻³² The application of azobenzene in the field of photoregulation of enzyme activity has also been studied in much detail.^{5,21, 33-38} There are mainly three ways to achieve it:

1. Direct modification of proteins/peptides with azobenzene

The peptide/protein modified via reaction with residues like lysine can reversibly change its conformation and/or activity with a change in excitation wavelength.³⁹⁻⁴¹ For example, using carboxyl derivatives for the acylation reaction with a lysine residue on the surface of papain.⁴²

2. Designing photoregulated enzyme substrates or inhibitors

In this approach, the azobenzene analogues of substrates or inhibitors/ligands that change their properties upon illumination are used. For example, the reversible modification of cysteine and serine by using aldehyde and arylboronic acid residue for cysteine proteases⁴³ and serine proteases⁴⁴, respectively.

3. Using photoswitchable affinity labeling

In this method, the azobenzene derivatives are designed with an electrophilic group on one end of the phenyl ring, and ligand on the other ring. The ligand interacts with the binding site of the protein, which fixes the photoswitch and allows the electrophile to interact covalently with amino acid residues near the binding site (Figure 1.9). Upon irradiation, the molecule converts from a configuration with high affinity for the enzyme into a low affinity configuration. One such example is the generation of a light-activated potassium channel.⁴⁵

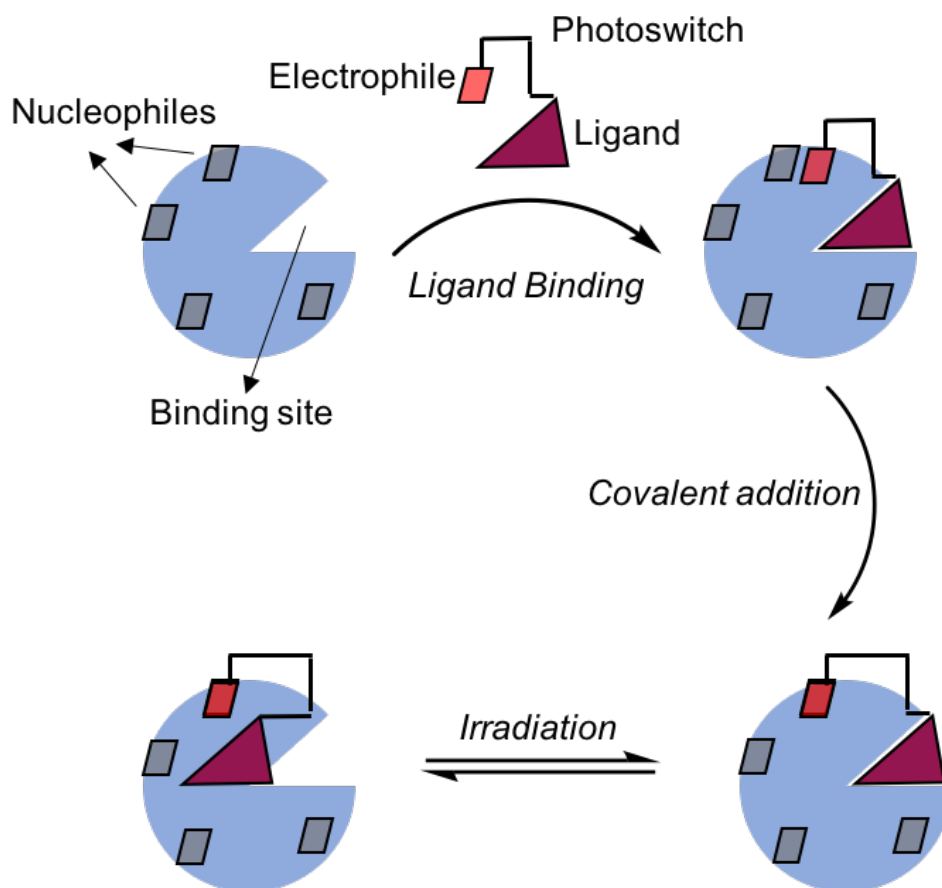


Figure 1.9 Representation of photoswitchable affinity labeling (PAL)

The last approach has gained a particular interest because of its advantage that it enables making native proteins in cells photoresponsive. This is very useful, especially for cellular systems where introduction of mutant protein genes is difficult. Furthermore, the labeling directed by ligand binding to protein reduces the non-specific interactions that are often observed using the first two methods, which makes it a bit easier to predict the behavior of the enzyme afterwards. Recently, this approach was used to photocontrol carbonic anhydrase (CA)-the protein of interest in this dissertation, where the azobenzene derivative containing an oxirane ring was used to inhibit the protein in the *trans* form only.³³ The majority of these methods require chemical modification on the

surface of the protein, but there have been a couple of examples where the activity modulation was achieved by non-covalent, yet specific, interactions of the photochromic moiety with the protein. In 2015, Dr. Feringa reported the azobenzene inhibitor that is structurally related to the clinically approved HDAC inhibitors, Panobinostat and Belinostat, for a range of HDACs, opening up avenues of photopharmacology in the area of chemotherapy.⁴⁶ In this dissertation, we will explore such possibility to controlling carbonic anhydrase activity without the need of any tethering or labeling group.

1.3 CARBONIC ANHYDRASE: AN OVERVIEW

1.3.1 Metalloenzyme

Metalloenzyme are the enzymes that contains a metal ion cofactor that is required to carry out the enzymatic function through various means.^{47, 48} They can serve as substrate binding site and help in the storage and transportation of small molecules, such as hemoglobin and hemerythrin that carries oxygen. The metal ion can serve as a reducing or oxidizing agent and helps in carrying out reduction and oxidation reactions such as cytochromes. They can facilitate the transport of electrons for other protein catalysis, such as rubredoxin and plastocyanin. These proteins also can be used to store and transfer metal ions such as ferritin for Fe(III) storage and transferrin for iron transportation. They can participate in signal transductions such as troponin in skeletal and cardiac muscles regulated by intracellular calcium concentrations. They can also serve as catalytic reaction centers to facilitate certain physiological reactions and metabolic processes, such as zinc containing carbonic anhydrase for carbon dioxide hydration and copper containing superoxide dismutase.

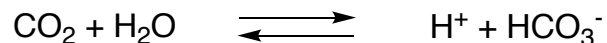
The metal ions in metalloenzymes are usually coordinated by N, O or S atoms on the side chain of amino acid residues in the protein. Some of the coordinating ligand are

imidazole substituent in His residues, thiolate in Cys, and carboxylate in Asp. The peptide backbone can also serve as donor groups by coordinating through deprotonated amides and the amide carbonyl oxygen centers. In addition to natural amino acid as ligands, many organic cofactors could also function as the ligands. One such example is tetradentate N₄ macrocyclic ligand in Fe-containing heme proteins.

As an inorganic chemist, we are highly interested in understanding the versatile nature of such metals in metalloenzymes and their correlation with the specific role of the enzymatic activity. For this dissertation purpose, we are targeting carbonic anhydrase, which is a zinc containing metalloenzyme.

1.3.2 CA and function

The carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous zinc-dependent metalloenzymes present in prokaryotes and eukaryotes that catalyze a simple physiological reaction, the reversible conversion of carbon dioxide to bicarbonate and protons.⁴⁹



Although this reaction may occur without a catalyst, it is too slow under physiological conditions to meet the metabolic needs of an organism. Thus, CAs have evolved in a convergent/divergent evolution phenomenon with six CA enzymatic families, the α -, β -, γ -, δ -, ζ -and η - CAs. The families are classified on the basis of where they are found in nature (prokaryotes vs eukaryotes), the coordination sphere of the Zn²⁺ ion and the nature of active site (open vs closed). CAs are involved in the maintenance of acid-base equilibrium and CO₂ homeostasis. CAs are also involved in many important physiological and pathological processes, including transport of metabolic wastes from cellular respiration, maintenance of acid–base equilibrium, CO₂ homeostasis,

biosynthesis reactions such as glucogenesis and lipogenesis, bone resorption and calcification. Interference with the activity (by inhibition or activation) of CAs leads to a pharmacological response, and hence, they serve as potential drug targets in vertebrates, fungi and bacteria.

1.3.3 Structure and mechanism of action

The active site of most CAs is a complex space that includes three functional features:

1. The primary coordination sphere with a catalytic Zn^{2+} ion bound to three imidazole groups deriving from histidine residues and one water molecule (or hydroxide ion), in a distorted tetrahedral geometry (Figure 1.10 A, B).
2. The hydrophobic face that helps the coordination of ligands and substrate to the zinc center (Figure 1.10 C).
3. The hydrophilic face that helps regenerate catalytic activity by facilitating the proton shuttle (Figure 1.10 C).

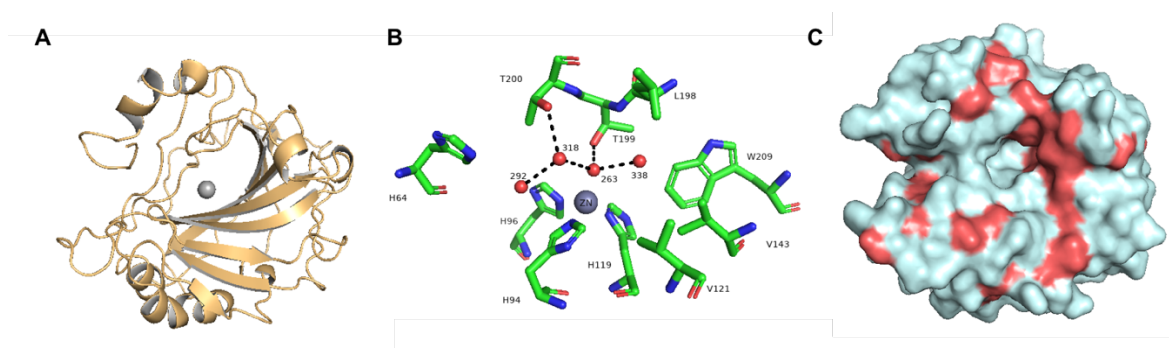


Figure 1.10 The structure of carbonic anhydrase PDB 1CA2 (A). The active site residues and water in the hydrogen bonded network (B). The hydrophobic and hydrophilic surface of CA showed by red and cyan color, respectively (C).

This conical binding cavity is roughly 15 Å deep and around 15 Å in diameter at the opening. There is a network of hydrogen bonds among the amino acids side chains and water molecules within the active site, which contributes to the catalytic activity of the enzyme (Figure 1.10 B). One of the most important features of this network is the His64 residue, which acts as a “proton shuttle” that aids in the transfer of protons in the final step of the CO₂ hydration mechanism. The structure of the binding pocket is highly conserved among different CA isozymes, which suggests that each residue serves a specialized and optimized function in the catalytic process.

CA catalyzes the reversible hydration of CO₂ to bicarbonate in a two-step ping-pong mechanism (Figure 1.11). The first step involves nucleophilic attack of a CO₂ molecule by the Zn²⁺-bound OH⁻ ion to form a Zn²⁺-bound bicarbonate (HCO₃⁻) intermediate, which is followed by the displacement of the bicarbonate by a H₂O molecule. Computational studies suggest that CO₂ binds weakly ($K_d \approx 100$ mM) in a hydrophobic region 3-4 Å away from the Zn²⁺ complex. The second step involves transfer of a proton from the Zn-bound water molecule to regenerate the Zn²⁺-OH⁻ form of CA. It is generally considered that the proton is shuttled to buffers in solution by a series of intramolecular and intermolecular proton-transfer cascades. The whole catalytic process is extremely efficient ($k_{cat}^{CO_2} \approx 10^6$ s⁻¹) with the second order rate constants approaching the limit of CO₂ diffusion ($k_{cat}/K_m^{CO_2} \approx 10^8$ M⁻¹s⁻¹), which makes CA one of the most active enzymes.

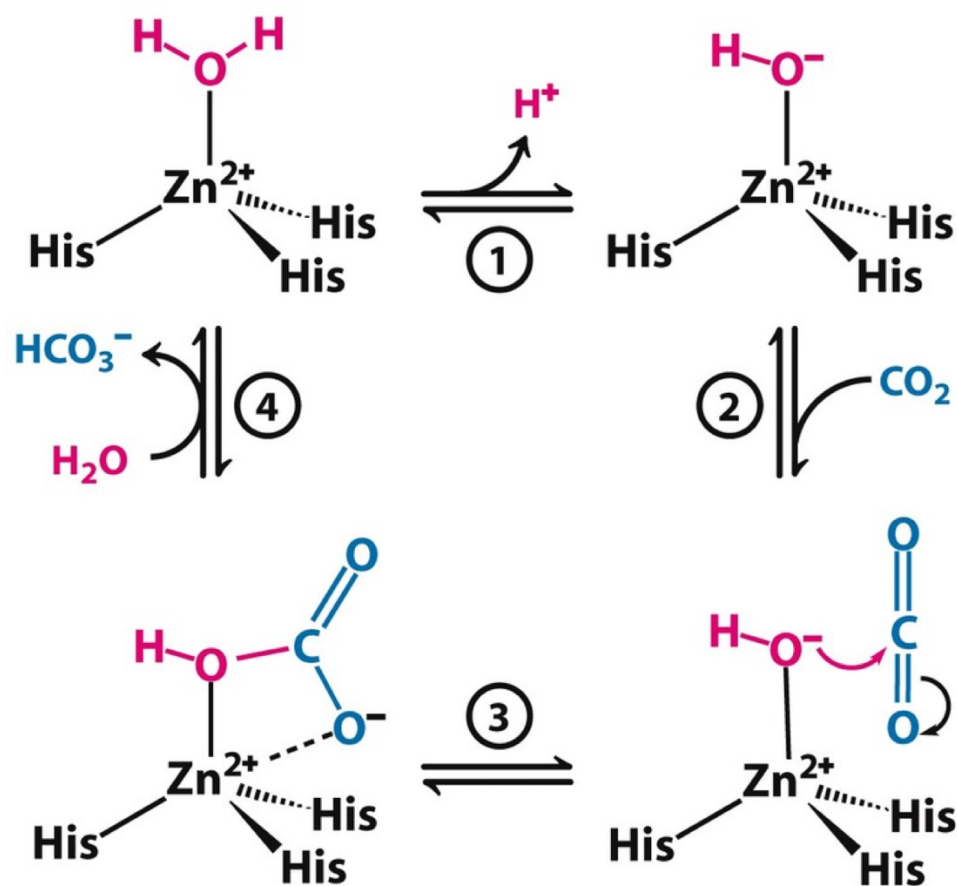


Figure 1.11 Mechanism of action of CA

1.3.4 Human CA isoforms: Physiological and Pathological roles

CAs are expressed in specific tissues and cellular organelles that drive the contributions of catalyzed CO_2 reactions in many physiological processes. In humans, there are 15 encoded CA isoforms (CAI, II, III, IV, V (A and B), VI, VII, VIII, IX, X, XI, XII, XIII, XIV and XV), out of which only 12 coordinate a zinc in the active site making them catalytically active. These active isoforms exhibit specific cellular localization and catalytic efficiency for CO_2 hydration.⁵⁰

1.3.4.1 Cytosolic CA isoforms: Expression, Distribution and Function

The human isoforms in this category are: CAI, II, III, VII and XIII. CAII is a high-activity isozyme with a maximum turnover rate for CO₂ hydration of 10⁶ s⁻¹. This isoform is most widely distributed in virtually every tissue and organ. CAII is expressed various cell types including osteoclasts in bone, epithelium of the choroid plexus and ciliary body and lens of the eye.^{51, 52} CAII has diverse physiological role in these cells, but it majorly contributes towards the acid base homeostasis.⁵³⁻⁵⁵

1.3.4.2 Mitochondrial CA isoforms: Expression, Distribution and Function

The mitochondrial CA isoform is CA V (A and B) which plays an important role in the cellular metabolic processes such as glycogenesis, lipogenesis and ureagenesis.⁵⁶⁻⁵⁹ CAV provides the bicarbonate as substrate for the essential enzymes in these biosynthetic pathways such as pyruvate carboxylase that replenishes intermediate in the synthesis of fatty acids. Amino acids, neurotransmitters and porphyrins.

1.3.4.3 Membrane associated CA isoforms: Expression, Distribution and Function

The membrane associated CA isoforms are: CAIV, IX, XII, XIV and XV. CAIV is anchored to the membrane by a glycosylphosphatidylinositol anchor and is expressed on the membrane of various type of cells such as the apical surfaces of epithelial cells of some segments of the nephron. Due to their expression on the kidney cell membrane, their physiological role is to facilitate bicarbonate reabsorption. CAIX is an exceptional isoform of the CA; in addition to its classical role in pH regulation, it has also been shown to participate in cell adhesion, proliferation and tumorigenic processes.^{60, 61}

In this dissertation, we will discuss all these three categories individually and design the photopharmacophore to target them specifically for photomodulation of these classes of CA isoforms.

1.3.4.4 Role of CA in cellular physiology

CA plays a crucial role in cellular respiration as it catalyzes the reaction of CO₂ and H₂O to produce bicarbonate, which is otherwise not fast enough to meet the respiration requirements of living organisms at 37 °C. These reactions are important for a variety of biological processes such as regulation of respiration and gas exchange, regulation of acid-base equilibrium, production of saliva and pancreatic juices, signaling and memory, and several biosynthetic reaction pathways in metabolism. CA is also important in vision, bone development and function, calcification, and cellular adaptation to external stress. Bicarbonate, a primary substrate of CA, serves as a counter ion in Na⁺ transport and as a CO₂ carrier. It also serves as a substrate for a variety of enzyme-catalyzed carboxylation reactions. CA is present in the eye lens, vitreous body, cornea, and retina, where it contributes to vision. Within the ciliary body, CA-catalyzed formation of bicarbonate is the primary mechanism for the influx of sodium ion into the eye, which is accompanied by the transport of water, a required process to maintain the aqueous humor.⁶²

1.3.4.5 Role of CA in pathology

CAs are important therapeutic targets, as they are overexpressed in diseases, including cancer, glaucoma, obesity, and epilepsy.⁶³ They are also responsible for the acidification of the extracellular environment around hypoxic tumors. Interference with enzyme activity and pH regulation in tumor cells using CA inhibitors has been identified as a therapeutic strategy.⁶⁴

1.3.5 CA inhibition: Therapeutic Target

Inhibition of CA decreases the production of bicarbonate, which subsequently lowers the intraocular pressure⁶⁵, and is effective in the treatment of glaucoma. In

addition to that, CA inhibition can help in treating both macular degeneration and macular edema –disorders that affect the central retina.⁶⁶ The strategy of CA inhibition is used in controlling various brain functions such as temporary suppression of memory⁶⁷ and prevention of episodic hypokalemic periodic paralysis.⁶⁸ CA inhibitors are also used as anticonvulsant and antiepileptic agents.⁶⁹ They are very well studied in renal pharmacology as diuretic agents, where they aid the increase in excretion of Na^+ and HCO_3^- by preventing the reabsorption of bicarbonate.⁷⁰ They can also act as potent diuretics by affecting intracellular pH, and thereby, decreasing the transportation of Na^+ ions across the luminal membrane.⁷⁰ Hence, they are used to treat hypertension and congestive heart failure.^{71, 72} CA inhibitors are important in treating ulcerogenesis where they suppress the secretion of gastric acid, and normalizing severe metabolic alkalosis.⁷³ CA inhibition has been shown to reduce the bone loss in postmenopausal osteoporosis.⁷⁴ CA inhibitors are also used to treat acute mountain sickness^{75, 76} and to improve the arterial oxygenation of patients suffering from chronic obstructive pulmonary disease (COPD).^{77, 78}

It has been recently observed that inhibition of CA inhibits the growth of several types of tumors in cell culture and *in vivo* by decreasing the acidification around these cells, a cause for the increased invasive behavior of cancer cells.⁷⁹⁻⁸² Several inhibitors are currently being tested clinically for this effect.^{83, 84}

1.3.6 Development of CA Inhibitors

There is a myriad of CA inhibitors developed over the past couple of decades, and they are mainly characterized in these three groups:

1. Sulfonamide ($\text{R-SO}_2\text{NH}_2$)
2. Other sulfonic acid derivatives ($\text{R-SO}_2\text{-X}$; $\text{X} \neq \text{NH}_2$)

3. Small monoanions (X^- ; N_3^- , SCN^-)

The sulfonamides and sulfonic acid derivatives usually have binding constants (K_d) in the pM to μ M range, whereas the inorganic monoanions have K_d in the μ M to mM range. There are various existing inhibitors with different chemical structure and binding affinity, there are still a lot of factors to consider in terms of physiology and *in vivo* pharmacology when translating a ligand design to developing a *drug*.⁸⁵

1.3.6.1 Arylsulfonamides: Great ligands for CA

Aryl sulfonamides serves as the superior class of inhibitors to CA owing to the favorable primary and secondary interactions in the active site. Upon binding, the ionized $-SO_2NH^-$ nitrogen displaces the Zn bound water ($Zn^{2+}-OH/Zn^{II}-OH_2^+$) and interacts directly with the Zn^{2+} cofactor. The sulfonamide $-NH$ interacts with O_γ of Thr199 through hydrogen bonding. This arrangement mimics the structure of CA bound to HCO_3^- (or putative CO_2 binding), where the bicarbonate oxygen coordinates to the Zn^{2+} cofactor, and the bicarbonate OH donates a hydrogen bond to the O_γ of Thr199 (Figure 1.12). This resemblance in the transition states makes sulfonamides a great binding ligand to CA.⁸⁶ The binding affinity is further enhanced by the favorable interactions between the aryl ring and the hydrophobic pocket of CA, which makes arylsulfonamides exquisitely specific for CA.⁸⁷

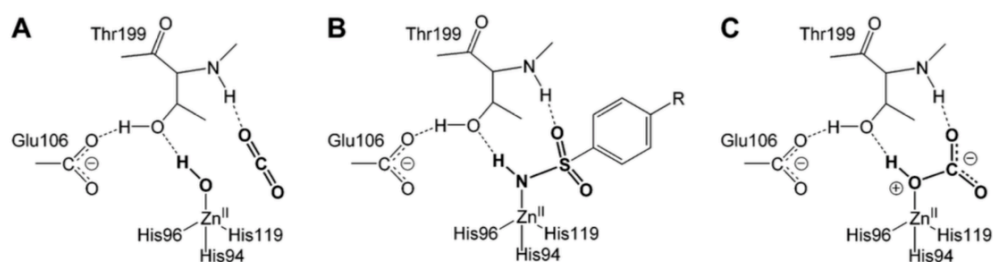


Figure 1.12 Comparison of putative CO₂ interaction (A), an arylsulfonamide (B), and bicarbonate (C) bound to the active site of CA, adapted from *Krishnamurthy et al.*⁸⁶

One example of such CA inhibitor is acetazolamide, sold under the trade name Diamox (Figure 1.13). This drug is used to alleviate headache, nausea, and dizziness and helps in reducing the symptoms of altitude sickness. Moreover, this drug has shown promise in decreasing the relative invasion rates of various renal carcinoma cells. As the inhibitory properties of this molecule is very well studied, this molecule will be used as a control molecule to compare the binding affinities of our designed probes in this dissertation.

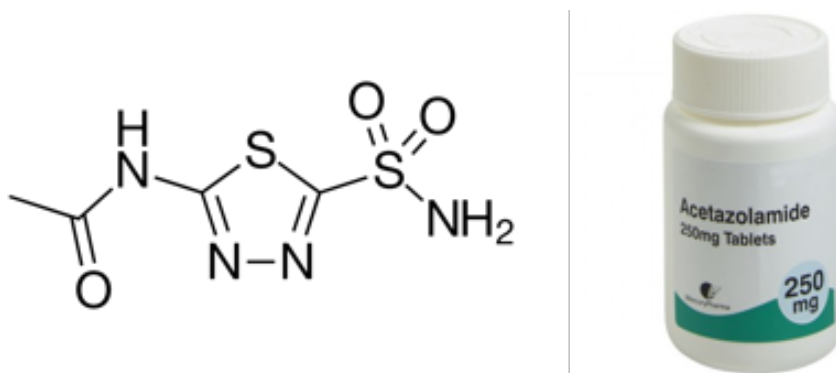


Figure 1.13 Structure of acetazolamide

1.3.7 CA: Protein of choice for basic research

In addition to the important physiological and pathological roles, CA serves as a great model system for various studies in a research laboratory for the following reasons:

1. It is an inexpensive and readily available protein which is stable under standard laboratory conditions. It is also easy to prepare site-specific mutants from facile overexpression in *E.coli*.
2. The structure of CA and its isoforms has been studied in detail using high resolution single crystal X-ray diffraction.
3. The mechanism of action is well-characterized by various biophysical and biochemical assays which makes it easy to establish probe structure-function relationship. Furthermore, CA does not undergo gross conformational changes upon sulfonamides binding which allows for a relatively straightforward interpretation of thermodynamic and kinetic data.
4. There is enough literature precedence to design an inhibitor (or activator) with new functionalities.

We mainly focus on hCAII (Human CAII) and bCAII (bovine CAII) because there is a wealth of information available for them, which makes them particularly well-suited for biophysical assays. Moreover, bCAII is the least expensive pure commercial variant of CA. While bCAII is not as well characterized, there is a high degree of sequence homology (Figure 1.14 B) between bCAII and hCAII, indicating a very similar global and active site architecture (Figure 1.4 A). They have very similar physical properties as well as ligand binding properties, which makes it easy to tune the molecular design and establish structure-function relationships. In this dissertation, we report studies with hCAII and bCA, unless specified otherwise.

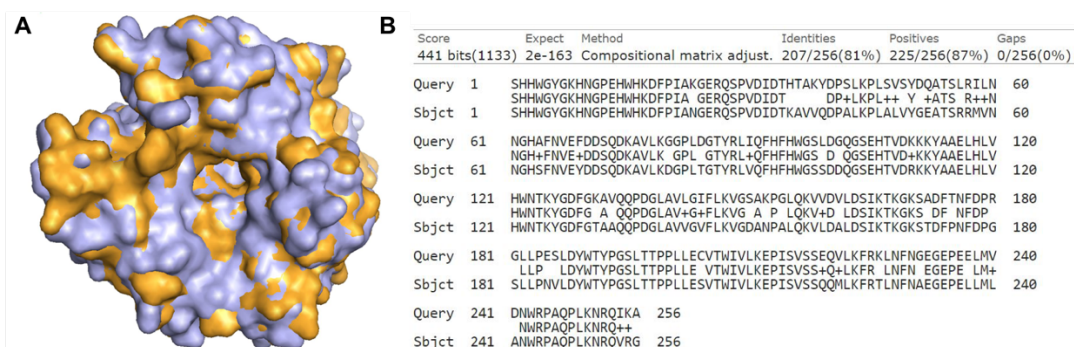


Figure 1.14 Structural (A) and sequence (B) alignment overlap of hCAII (PDB 1CA2) and bCAII (PDB 1V9E chain A). hCAII is shown as purple and labelled as query sequence. bCAII is shown as orange and labelled as subject sequence

These proteins are also very to use a proof-of-concept prtoeins as there are myriad of choices of biophysical and biochemical assays that can be used to determine the thermodynamic parameters (fluorescence and luminescence spectroscopy; Absorption spectrophotometry; Affinity Capillary Electrophoresis; Calorimetry; Magnetic Resonance Spectroscopy; Circular Dichroism Spectroscopy; Mass Spectrometry), kinetic parameters (Fluorescence and Absorbance Spectroscopy; Affinity Capillary Electrophoresis; Surface Plasmon Resonance Spectroscopy) of protein-ligand binding as well as the effect of ligand binding on the enzyme catalytic activity (detection and release of protons; measurement of consumption of CO₂; Observation of esterase activity).⁸⁶

1.4 FOCUS OF THE DISSERTATION

There have been a few other studies of photoregulation of carbonic anhydrase activity with azobenzene derivatives. The Trauner group reported the use of Photoswitchable Affinity Labeling (PAL) method where the azobenzene derivative containing the oxirane ring was used to covalently label CA and inhibit it in the *trans* form.³³ They also reported an *in silico* screening process to identify suitable cysteine sites on CA for azobenzene ligand tethering.⁸⁸ In 2017, a water soluble adhesive photoswitch

(Glue₅-Azo-SA) was reported, where the azobenzene was modified with a “glue” functionality (Glue_n) that utilizes its multiple guanidinium ion pendants to adhere to the CA surface.⁷⁴ This was the first example where a non-covalent adherence method was used as opposed to covalent tethering. Still, prior to our work, there had been no reports of photocontrol of CA without the need for tethering strategies. Further, the reported applications of previously reported probes were limited to isolated proteins and cell lysates. For any practical application, it is really important to understand and control the behavior of probes in live cells, and if possible, in a complex *in vivo* system.

In pursuit of addressing these limitations, this dissertation focuses on our efforts to photomodulate carbonic anhydrase and its specific isoforms using simple azobenzene molecules that are modified with sulfonamide group (for interaction with the Zn ion in the active site) and other small groups (to achieve desired photophysical properties). In parallel, new photoswitches that can be operated with visible light and capable to show differential binding in trans and cis isomer are designed and discussed. We also explored using an irreversible approach to gain photocontrol of CA activity by using a coumarin based photoprotecting group to target the mitochondrial isoform of carbonic anhydrase. This work shows promise as a basic research tool to study CA in ‘healthy’ and ‘diseased’ cells as well as opens up the future avenues for therapeutic applications, particularly for glaucoma due to its location in the eye where light activation is very feasible.⁸⁹

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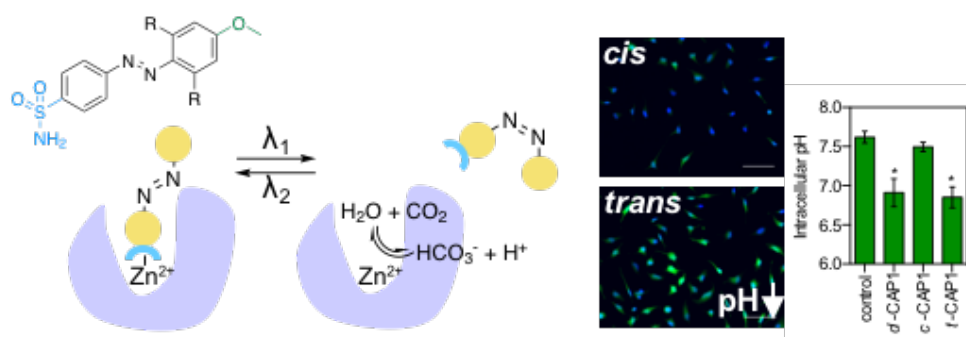
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Chapter 2: In situ photoregulation of carbonic anhydrase activity using azobenzene sulfonamides¹

2.1 ABSTRACT

We report two small molecule azobenzene sulfonamide probes, CAP1 and CAP2, capable of photomodulating the activity of carbonic anhydrase (CA) on demand. In the *trans* form CAP azobenzene probes adopt a linear shape, making them suitable to occupy the CA active site and interact with Zn^{2+} , thereby inhibiting enzyme activity. Following irradiation with either 365 nm or 410 nm light, the CAP probes isomerize to their *cis* form. Due to the change in steric profile, the probe exits the active site, and the activity of the enzyme is restored. The *cis* isomer can revert back to the *trans* isomer through thermal relaxation or *via* photoirradiation with 460 nm light and thereby inhibit protein activity again. This process can be repeated multiple times without any photodegradation and thus can be used to inhibit or activate the protein reversibly. Importantly, we demonstrate our ability to apply CAP azobenzene probes to regulate CA activity both in isolated protein solution and in live cells, where the two isomers of CAP1 differentially regulate the intracellular cytosolic pH.



¹ This chapter is based on the published work:

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Author Contribution: KA conceived and initiated the project, synthesized the molecules, acquired and analyzed the characterization data, and prepared and revised the manuscript.

2.2 INTRODUCTION

Carbonic anhydrases are ubiquitous zinc-containing metalloenzymes present in prokaryotes and eukaryotes that catalyze a simple physiological reaction: the reversible conversion of carbon dioxide to bicarbonate and protons.¹⁻³ The active site of most CAs contains a zinc ion bound to three histidine residues and one water molecule.¹⁻⁴ CAs are involved in many important physiological and pathological processes including transport of metabolic wastes from cellular respiration, maintenance of acid-base equilibrium, CO₂ homeostasis; biosynthesis reactions such as glucogenesis and lipogenesis; bone resorption; calcification; and tumorigenicity.^{5, 6} CAs are important therapeutic targets, as they are overexpressed in diseases including cancer, glaucoma, obesity and epilepsy.⁷ These disorders are treated by sulfonamide based drugs, which directly interact with the Zn²⁺ active site. In this interaction, the deprotonated sulfonamide coordinates to the Zn²⁺ cofactor. Hydrogen bonding with a nearby threonine residue and hydrophobic interactions between the aryl ring and the hydrophobic pocket of the enzyme further contribute to this interaction.^{2, 3} One example of a CA inhibitor is acetazolamide, sold under the trade name Diamox.⁸ This drug is used to decrease headache, nausea, dizziness, and helps in reducing the symptoms for altitude sickness. Moreover, this drug has shown promise in decreasing the relative invasion rates of various renal carcinoma cells.⁹

A limitation of many enzyme inhibitors, including acetazolamide, is the inability to control the location and timing of their inhibitory activity. Thus, strategies for targeting the activity of these molecules are of great interest both for therapeutic and basic science applications. Photoactivation is one strategy, in which application of specific wavelength(s) of light induces structural and functional changes in a molecule. This process can be irreversible, in which photocleavable protecting groups are incorporated to block the activity of an inhibitor.¹⁰ Alternately, a reversible process, such as

photoisomerization, can be employed such that the inhibitor can be activated and deactivated on demand.¹¹ To this end, azobenzenes are promising structures that display reversible isomerization between two different stereoisomers, *trans* and *cis*, upon light irradiation.^{12, 13} Azobenzene photoswitches have been applied in a number of biological contexts including regulation of enzyme folding¹⁴⁻¹⁶ and activity,¹⁷⁻²¹ optical control over beta cell functions,^{22, 23} DNA conformation and transcription,^{21, 24, 25} mRNA translation,²⁶ chemotherapeutics,^{27, 28} and in neurobiology.²⁹⁻³¹

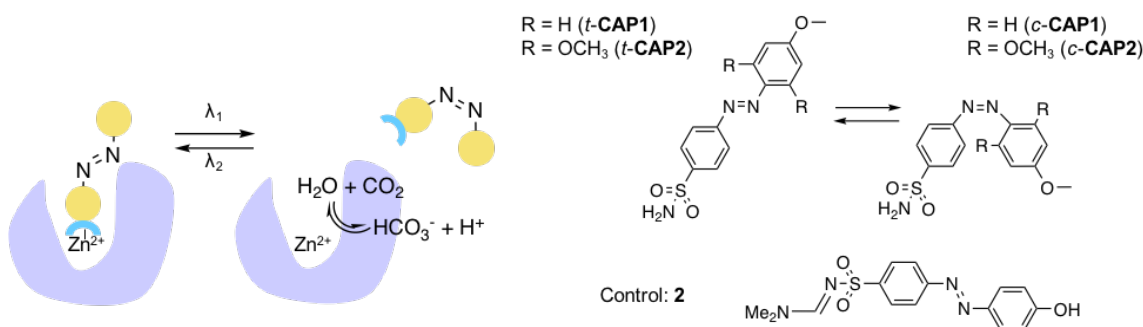


Figure 2.1 Structures of **CAP1**, **CAP2** and control compound **2** and their interactions with CA.

Given the affinity of aryl sulfonamides for the CA active site and the differing steric profiles of the *cis* and *trans* forms, we designed simple azobenzene sulfonamides, Carbonic anhydrase Azobenzene Photoswitch 1 and 2 (**CAP1** and **CAP2**), that we hypothesized would bind more tightly to CA in the *trans* form (Figure 2.1). In these molecules, the Zn^{2+} binding moiety, the sulfonamide ($-\text{SO}_2\text{NH}_2$), is found at the position *para* to the azo group. When the molecules are in their thermally stable *trans* form, they can enter the CA active site, bind to the Zn^{2+} ion and inhibit the enzyme's activity.³² After irradiation with the appropriate wavelength of light, they will isomerize to the *cis* form. As a result of the steric bulk around the sulfonamide group in the *cis* form, **CAP1** and **CAP2** will exit the CA active site, and normal CA function will return. Isomerization

back from *cis* to *trans* can then be achieved either thermally or *via* photoirradiation with a lower energy wavelength of light. In this way, different wavelengths of light can be used to change the relative concentrations of each isomer both *in vitro* and in cellular assays. Previous work has elegantly demonstrated that azobenzene sulfonamides covalently tethered to the CA protein^{17, 33} and azobenzene-sulfonamide adhesive materials³⁴ can inhibit CA activity more strongly in the *trans* form than the *cis* form in *in vitro* protein assays, however these probes were not employed in live cell experiments. Herein, we describe two small molecule azobenzene containing sulfonamides that exhibit strong CA inhibitory activity in the *trans* form without the need for molecular tethering and demonstrate their ability to differentially regulate intracellular pH in live cancer cells.

2.3 EXPERIMENTAL SECTION

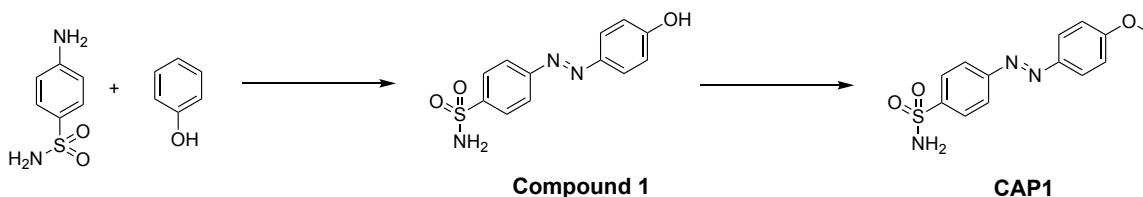
2.3.1 General Procedures

All solvents and chemicals were purchased from Sigma-Aldrich and Fisher Sci. and used as received. Bovine carbonic anhydrase was purchased from Sigma Aldrich. Human CAII was kindly provided by Dr. Joseph Emerson at Mississippi State University. For spectroscopic and protein studies, 0.05 M HEPES buffer with 0.1 M KNO₃ (pH 7.2) was used unless stated otherwise. All the studies were done under dark condition to avoid exposure of light.

The ¹H and ¹³C NMR spectroscopic measurements were collected in deuterated solvents from Cambridge Isotope Laboratories (Cambridge, MA), using an AGILENT MR 400 NMR spectrometer at 400 MHz and 500 MHz respectively. The chemical shift is calibrated with respect to the solvent peak, and is reported in ppm. Absorbance spectroscopic studies were performed using an Agilent Cary 60 UV-Vis spectrophotometer. Fluorescence spectroscopic measurements were made using an

Agilent Cary Eclipse fluorescence spectrofluorimeter. The samples were irradiated with an Asahi Xe lamp connected with bandpass filters of 365 nm (FWHM 12 nm), 460 nm (FWHM 11 nm) from Asahi Spectra, and 410 nm (band width 10 nm) from Thorlabs. Walk-up LC-MS and high-resolution Electrospray Ionization (ESI) mass spectral analyses were performed by the Mass Spectrometry Facility of the Department of Chemistry at UT Austin. The absorbance values in 96 well plates were recorded on a Tecan Infinite M200 microplate reader.

2.3.2 Synthetic Procedures

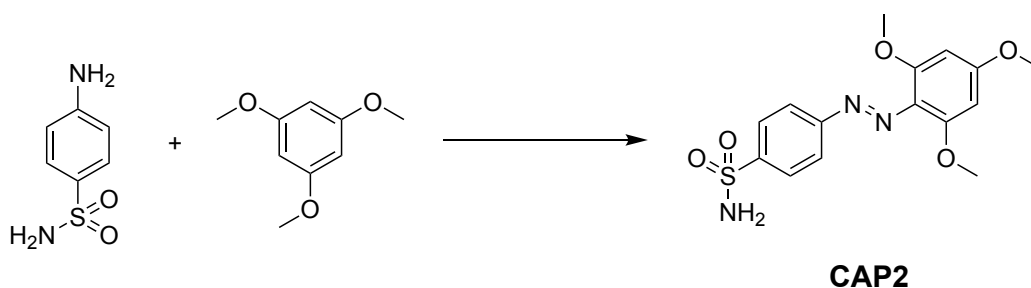


Scheme 2.1 Synthesis of **CAP1**

*Synthesis of **Compound 1***^{32,35}: 4-amino-benzenesulfonamide (200 mg, 1.16 mmol) was dissolved in 3 mL 40% HCl/H₂O in a 100 mL round bottom flask at 0°C and was stirred for five minutes. Phenol (96.2 mg, 1.39 mmol) was dissolved in a separate 250 mL round bottom flask in 3 mL of 10 M NaOH at 0°C. A solution of 2.3 M aqueous NaNO₂ (96.2 mg, 1.39 mmol) was added dropwise to the sulfonamide round bottom flask and was stirred for five minutes, forming a yellow colored solution. The sulfonamide solution was added dropwise to the phenol solution at 0°C and was allowed to stir vigorously at 0°C for 15 minutes. It was then warmed to room temperature and the pH was adjusted to slightly acidic conditions to precipitate out the product. Vacuum filtration was performed to obtain an orange powder, which was then rinsed with water. Product formation was confirmed through ¹H NMR and LCMS with a yield of 99%. ¹H NMR

(400 MHz, d_4 -MeOD) δ 8.05-8.02 (d, 2 H), δ 7.96-7.94 (d, 2 H), δ 7.88-7.86 (d, 2 H), δ 6.94-6.92 (d, 2 H). Residual water remains in spectrum. LR ESI-MS (ESI⁻, MeOH): calculated for $[C_{12}H_{10}N_3O_3S]^-$ 276.05, found 276.

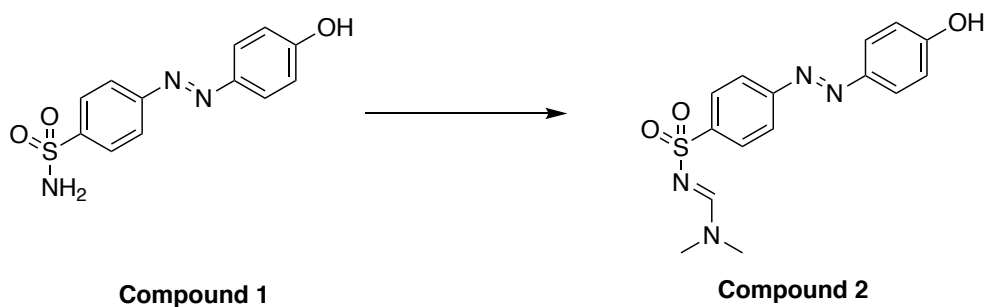
Synthesis of CAP1: Compound **1** (20.0 mg, 0.0722 mmol) and K_2CO_3 (12.0 mg, 0.0866 mmol) were added to 1 mL acetonitrile in a 10 mL round bottom flask. CH_3I (9.87 μ L, 0.156 mmol) was added to the flask. The reaction was refluxed for five hours. After evaporating the solvent under reduced pressure, the reaction was acidified to form the precipitate. Vacuum filtration was performed to obtain a red solid. The crude product was absorbed onto celite and run on a C-18 reverse phase column in MeOH/ H_2O mixture. The pure product was eluted at 70% MeOH/ H_2O with 20% yield, and confirmed through ^1H NMR and LCMS. ^1H NMR (400 MHz, d_6 -DMSO) δ 8.06-8.01 (d, 2 H), δ 8.00-7.98 (m, 4 H), δ 7.16-7.13 (d, 2 H), δ 3.93 (s, 3 H). Residual water remains in spectrum. ^{13}C NMR (400 MHz, d_4 -MeOD) δ 163.20, δ 154.54, δ 152.14, δ 146.80, δ 144.62, δ 127.08, δ 124.91, δ 123.82, δ 119.66, δ 124.17, δ 113.62, δ 54.89. HR ESI-MS (ESI⁺, MeOH): calculated for $[C_{14}H_{21}N_3O_4S]^+$ 292.0750, found 292.0753.



Scheme 2.2 Synthesis of **CAP2**

Synthesis of CAP2^{32,35}: 4-amino-benzenesulfonamide (50.0 mg, 0.290 mmol) was dissolved in 2 mL 40% HCl/ H_2O in a 25 mL round bottom flask at 0°C and was stirred for five minutes. 1, 3, 5-trimethoxybenzene (48.8 mg, 0.290 mmol) was dissolved in a

separate 50 mL round bottom flask in 2 mL of 10 M NaOH at 0°C. A solution of 2.3 M aqueous NaNO₂ (24.0 mg, 0.348 mmol) was added dropwise to the sulfonamide round bottom flask and was stirred for five minutes, forming a yellow colored solution. The sulfonamide solution was added dropwise to the phenol solution at 0°C and was allowed to stir vigorously at 0°C for 15 minutes. It was then warmed to room temperature and stirred for 24 hours. The pH was adjusted to 7 to precipitate out the product. Vacuum filtration was performed to obtain an orange powder, which was then rinsed with water. Product formation was confirmed through ¹H NMR in and LCMS. The product was absorbed onto celite and run on a C-18 reverse phase column in ACN/H₂O mixture. The product eluted at 30% ACN/H₂O with a final yield of 60%. ¹H NMR (400 MHz, d₆-DMSO) δ 8.04-8.02 (d, 2 H), δ 7.88-7.86 (d, 2 H), δ 6.40 (s, 2 H), δ 3.93 (s, 3 H), δ 3.88 (s, 6 H). Residual water remains in spectrum. ¹³C NMR (400 MHz, d₄-MeOD) δ 164.24, δ 156.00, δ 149.65, 143.89, δ 126.78, δ 126.11, δ 121.89, δ 118.72, δ 91.14, δ 90.31, δ 55.46, δ 54.78, δ 29.33. HR ESI-MS (ESI⁺, MeOH): calculated for [C₁₈H₂₇N₃O₆S]⁺ 352.0962, found 352.0963.



Scheme 2.3 Synthesis of control molecule, Compound 2

*Synthesis of **Compound 2**:* Compound 1 (65.0 mg, 0.223 mmol) was stirred with neat DMF-DMA (0.5 mL) at room temperature for 20 minutes. At the completion of reaction, as monitored by TLC chromatography, water was added to the mixture and was

acidified with 6 M HCl to form the precipitate of product at a yield 70%. The purity of the compound was checked through NMR spectroscopy. ^1H NMR (400 MHz, $\text{d}_4\text{-MeOD}$) δ 8.22 (s, 1 H), δ 7.99-7.92 (q, 4 H), δ 7.88-7.86 (d, 2 H), δ 6.94-6.92 (d, 2 H), δ 3.20 (s, 3 H), δ 3.03 (s, 3 H). Residual water remains in spectrum. LR ESI-MS (ESI⁺, MeOH): calculated for $[\text{C}_{15}\text{H}_{15}\text{N}_4\text{O}_3\text{S}]^+$ 331.1, found 331.

2.3.3 Characterization

2.3.3.1 CAP Isomerization

The photoisomerization kinetics of CAP1 and CAP2 (10mM stock in DMSO) were investigated by electronic absorption spectroscopy.¹² The absorbance spectrum was taken in methanol and aqueous buffer under dark conditions. To get the *cis* isomer absorbance spectrum, the sample was irradiated with 365 nm (CAP1) or 410 nm (CAP2), and the spectrum was collected. To get the *trans* isomer absorbance profile, the sample was further irradiated with 460 nm before collecting the absorbance spectrum.

To study the real-time isomerization kinetics of the molecules, the solution was irradiated with the desired wavelength perpendicular to the direction of spectrophotometer light source. The absorbance value was recorded at λ_{max} every 2 seconds with Cary enzyme kinetics software. The absorbance versus time profile obtained was fitted to one phase decay equation to get half-life and rate constant value:

$$y = (y_0 - y_{\text{max}})e^{-kx} + y_{\text{max}}$$

where y_0 is initial absorbance at λ_{max} ; y_{max} is absorbance at saturation (or maximum conversion); and k is the rate constant.

To get the multiple isomerization of the probes, they were irradiated to convert and *cis* and *trans* alternatively, and absorbance spectrum was collected after every irradiation. Afterwards, the absorbance at λ_{max} was plotted with the number of cycle.

The percent of *trans* and *cis* content at the PSS was obtained by irradiating the sample with required wavelength in methanol-*d*₄ and collecting the ¹H NMR data with 30 sec relaxation time to ensure the relaxation of all the spins. The *p*-OCH₃ hydrogens were integrated and compared to obtain the PSS isomeric percent.

The thermal stability of *cis* isomer at 37 °C was determined by using UV-Vis spectroscopy in aqueous buffer. The molecule was converted to *cis* by irradiating the sample with 365 nm for CAP1 and 410nm for CAP2, and the spectra was taken every 1 hour at 37 °C for 24 hours. The absorbance at λ_{max} was plotted with respect to time to get the thermal relaxation profile.

2.3.3.2 Tryptophan Quenching

Binding analysis of CAP1 and CAP2 was performed using bovine carbonic anhydrase at room temperature in black 96 well plates. A stock solution of 10 mg/mL (~350 μM) enzyme was prepared in aqueous buffer. A stock solution of 2 mM probe was prepared in DMSO under dark conditions. During titration, the enzyme concentration was kept constant (1 μM), and the *trans* or *cis* isomer (irradiated prior to the addition) concentration was increased from 0-20 μM with 1% final DMSO content. The well plate was incubated at room temperature for 15 minutes to allow the formation of E-I complex. Each well was then read for the tryptophan fluorescence (λ_{ex}=280 nm; λ_{em}= 317 nm). The quenching profile for *trans* isomer titration was fitted to quadratic equation (as [E] > K_d):

$$y = y_o + (y_{max} - y_o) \frac{\sqrt{(E + x + K_d)^2 - 4xE}}{2E}$$

The quenching profile for *cis* isomer titration was fitted to hyperbolic equation (as [E] < K_d):

$$y = \frac{y_{max}x}{(K_d + x)}$$

where y_o is initial fluorescence, y_{max} is fluorescence at plateau, K_d is dissociation constant, E is enzyme concentration.

2.3.3.3 DNSA Competition Assay

DNSA (Dansylamide) competition studies were done with bovine carbonic anhydrase (bCA).³⁶ All studies were done at room temperature under dark conditions. A stock solution of 2 mg/mL (~70 μ M) enzyme was prepared in aqueous buffer. A stock solution of 1 mM DNSA and 100 μ M of probe was prepared in DMSO. To a solution of 0.25 μ M of enzyme and 2.5 μ M of DNSA, 0.25 μ M of probe was added under dark conditions. The excitation wavelength was set at 280 nm (slit width 10), and the emission was collected over a wavelength range of 300-600 nm. The solution was irradiated with 365 nm (CAP1) or 410 nm (CAP2) for the *in situ* conversion of probe to *cis* isomer, and the emission spectrum was collected. The same solution was further irradiated with 460 nm to get the *trans* isomer and the emission was recorded.

2.3.3.4 NPA Assay using UV/vis spectroscopy

The effects of CAP1 and CAP2 binding on bCA and hCAII activity, *p*-nitrophenyl acetate (NPA) was used as the substrate.³⁷ As a result of enzymatic activity, NPA is hydrolyzed to give *p*-nitrophenolate ion, which has a λ_{max} at 400nm. Hence, the enzymatic activity in the absence and presence of probe can be determined spectroscopically by monitoring the increase in absorbance with time. In this study, a stock solution of 10 mg/mL (~350 μ M) protein in buffer, 10 mM probe in DMSO and 125 mM NPA in dry acetonitrile were prepared. During analysis, the enzyme, probe and NPA concentrations were kept constant at 2 μ M, 2 μ M and 250 μ M respectively. The absorbance was recorded at 400 nm every 60 sec for up to 10 minutes and was plotted

with respect to time. To study the effect of *cis* and *trans* isomer on the enzymatic activity, the solution of probe in buffer was irradiated to achieve the PSS prior to the addition of protein and NPA. All the studies were performed in the absence of light at room temperature.

2.3.3.5 *IC*₅₀ determination

The *IC*₅₀ values for *trans* and *cis* isomer (generated prior to the assay) was determined by using a 96 well plate and plate reader.³⁸ The enzyme concentration was kept constant at 500 nM, and the inhibitor concentration was varied from 0-8 μ M (for *trans*) or 0-16 μ M (for *cis*). The solution was incubated for 15 minutes at room temperature to allow the formation of E-I complex. NPA (final concentration of 10 mM) was added in all the wells at the same time and the hydrolysis reaction was monitored by recording the absorbance values at 405 nm with time. For all reactions, a corresponding negative control containing everything but the protein was set up. The absorbance readings from these reactions were considered as the background and subtracted from the corresponding readings of the main reactions. The corrected absorbance values were plotted with respect to time for each set of reaction, and the slope was calculated. The relative activity of enzyme was determined by dividing the slope of each probe concentration by the positive control ([CAP] = 0). The relationship between the relative activity of enzyme and the probe concentrations was fitted to the equation:

$$Y = \frac{1}{1 + (\frac{x}{IC_{50}})^H}$$

where *Y* is the relative activity of enzyme; *x* is the probe concentration, *H* is the Hill coefficient.

2.3.3.6 NPA reversibility

The instrument was set to record absorbance values at 400 nm every 20 seconds. To a solution of 2 μ M of bCA and 2 μ M of probe in aqueous buffer, 250 μ M of NPA was added under dark conditions, and the kinetics were started. After two minutes, the solution was irradiated with 365 nm (CAP1) or 410 nm (CAP2) for the *in situ* isomerization of probe to *cis* isomer and the readings were recorded for two more minutes. Then the solution was irradiated with 460 nm to generate the *trans* isomer within the protein solution, and the absorbance values were recorded for two minutes. This alternate conversion of probe to *cis* and *trans* isomer was repeated 12 times. The data was plotted as absorbance with respect to time.

2.3.3.7 CO₂ Hydration Assay

The carbon dioxide hydration activity of bCA was analyzed by monitoring the pH variation due to catalyzed conversion of CO₂ to HCO₃⁻. The substrate used was saturated carbon dioxide solution in water, which was freshly prepared every time by bubbling CO₂ to 100 mL of DI H₂O at 0 °C for an hour. It was closed and stored at 0 °C throughout the experiment. Bromothymol blue was used as an indicator of pH variation. The absorbance at λ_{max} (617 nm) is directly proportional to the pH (6.3-7.6), and was monitored spectroscopically throughout the hydration reaction. The buffer used for this assay was 20 mM Tris HCl at pH 8.3. A stock solution of 0.01mg/mL bCA in buffer, and 25 μ M probe in DMSO was prepared. To a solution of 1:1 bCA:probe in 3 mL of buffer containing pH indicator, 1 mL of CO₂-saturated water was added, and the absorbance values at 617 nm was recorded every 2 seconds. For positive control, the reaction mixture contained everything except the probe (bCA only). For negative control, the reaction mixture contained everything except protein (no bCA). The probes were photoirradiated

to generate *trans* or *cis* isomer at PSS prior to the addition in buffer. The assay was performed at 0-4 °C under dark conditions. The absorbance versus time was converted to pH versus time by using calibration plot of absorbance at various pH.

2.3.4 Cell Culture

Cell culture was performed in Dulbecco's modified Eagle's Medium (DMEM), supplemented with 4000 mg/L glucose and sodium pyruvate, 10% heat inactivated fetal bovine serum (FBS), and 1% antibiotics (200 U/cm³ penicillin and 200 µg/cm³ streptomycin) at 37 °C in atmosphere containing 5% CO₂. The cells were grown in T-75 flasks and were passaged once the confluence of cell reached 80%. For intracellular pH measurements, the cells were grown in 96 well plate at a seeding density of 6000 cells/well and were analyzed when the cells are confluent at 60-70%. Imaging was performed with Gibco live cell imaging media (LCIS) on EVOS[®] FL Auto inverted fluorescence microscope at room temperature. The images were processed and the fluorescence was quantified using FIJI software.

2.3.4.1 MTS Assay

MTS Cells proliferation colorimetric assay kit (BioVision) was used to analyze the cytotoxicity of CAP1 and CAP2 in *trans* and *cis* form in HeLa cells. The cells were plated in 96 well plate at a density of 4000 cells/well in phenol red free DMEM media, and 24 hours later, different concentrations of CAP1 and CAP2 isomers (photoirradiated prior to the addition) were added to cells to have a final volume of 150 µL media in each well. For each set of concentrations, a control well of cells was set, which had the equivalent volume of DMSO content. After 24 hours of incubation, 30 µL of MTS was added to each well, and the plate was incubated for 2 hours at 37 °C. The absorbance of

wells was read at 490nm on a plate reader and was normalized to the absorbance in control (no compound) wells. Samples were analyzed in triplicates, and the data was plotted in Prism.

2.3.4.2 Cell Experiments

The intracellular pH (pH_i) of live cells was measured by using the pHrodo™ Red AM Intracellular pH Indicator according to the manufacturer's protocol (Molecular Probes, Eugene, OR). This fluorogenic dye quantifies the cytosolic pH in the range of 4-9 with a pK_a of 6.5. For nuclear staining, Hoechst 33342 stain was used. The cells were grown in 96 well plates and were washed with Live Cell Imaging Solution (LCIS). A solution of 1 μL pHrodo, 1 μL Hoechst, 10 μL powerload (supplied with pH kit) was prepared in 1 mL LCIS, and 60 μL of it was added to each well. The plate was incubated at 37 °C for 30 minutes under dark conditions. After the incubation of cells with dye, they were washed with Live Cell Imaging Solution, and the cells were imaged directly under an EVOS® FL Auto inverted fluorescence microscope at excitation/emission of 560/580 nm (RFP light cube) for pHrodo dye and 360/447 nm (DAPI light cube) for nucleus stain. Total of 3 images per well (in triplicates) were taken at 20X.

2.3.4.3 Calibration

To quantify the intracellular pH of cells, the provided intracellular pH calibration kit was used (Molecular Probes, Eugene, OR). A cell loading solution was prepared by adding 1 μL of valinomycin/nigericin (10mM each in stock solution) in 1 mL of cellular pH calibration buffer (pH 4.5, 5.5, 6.5 and 7.5). After performing the desired experiment with pHrodo, the cells were washed 2X with LCIS, and 100 μL of prepared cell loading solution was added. The intracellular pH was allowed to equilibrate with the extracellular

buffer for 10 minutes at 37 °C prior to fluorescence reading. The process was repeated with each buffer solution, and a fluorescence vs. pH calibration curve was prepared. As the conditions (e.g. concentration of dye, lamp intensity, microscope settings) can vary, calibrations were performed for each individual experiment for both HeLa and HCT-116 cell lines.

2.3.4.4 For 30 minutes studies

The cells were plated in 96 well plate at a density of 7000 cells/well in 100 µL of growth media and were incubated at 37 °C for 24 hours. A solution of *d*-CAP1 was prepared in LCIS and was divided in to two parts. One part was added to the cells, and the other part was irradiated with 365 nm to generate *c*-CAP1 *in situ*. It was further divided in to two parts, where one part was added to the cells. The other part was irradiated with 460 nm to generate *t*-CAP2 and was added to the cells. In all the cells, pH dye and nuclear stain was added and incubated for 30 minutes at 37 °C. They were washed with LCIS 3X and analyzed for fluorescence.

2.3.4.5 For 24 hours studies

The cells were plated in 96 well plate at a density of 5000 cells/well in 100 µL of growth media. 24 hours later, the cells were washed with PBS buffer and 100 µL of 60 µM *d*-CAP1, was added, and incubated at 37 °C. After 24 hours, the cells were washed with LCIS, and pH dye and nuclear stain was added to the cells and were incubated for 30 minutes. The cells were washed and analyzed for fluorescence.

2.3.4.6 Image Processing

Cell images were processed and the fluorescence was quantified using FIJI (ImageJ). 8-bit images were thresholded to define the regions of interest (ROIs) for

individual cells and a binary image was created. The mean fluorescence (the integrated density/area for each cell) were quantified for all ROIs. Dead cells were eliminated manually following visual inspection, and the average of mean fluorescence values was calculated. Each image had ~50-80 cells and three images were taken per well. Each experimental condition had three wells to give a total of ~300-500 cells per condition. The average of mean-fluorescence for each well was converted to absolute pH_i values on the basis of their calibration curve and were reported as the $\text{mean} \pm \text{SD}$ of three wells ($N=3$). One-way ANOVA, followed by Bonferroni's *post hoc* test for multiple comparisons (Figure 5), was used to assess statistical differences (p versus control). Unpaired t test was performed for comparison between two sets (Figure S14-16) to assess the statistical difference.

2.4 RESULTS AND DISCUSSION

2.4.1 Synthesis and the photoisomerization properties of the azobenzene probes

Azobenzene photoswitches CAP1 and CAP2 were synthesized from sulfanilimide and the appropriate phenol analog using NaNO_2 and HCl .^{32,35} A subsequent methylation step was required to produce CAP1. We note that CAP1 has been synthesized previously, but its photoisomerization has not been studied and it has not been used for studies with CA.^{39,40} Control molecule **2**, which contains a protected sulfonamide group and a *para*-OH group, was also synthesized. This molecule should not be able to coordinate to the Zn^{2+} site in the protein and further, due to the presence of the $-\text{OH}$ moiety, will contain no detectable *cis* isomer in aqueous solution (Figure S1).⁴¹

2.4.1.1 Spectroscopic properties of CAP1, CAP2 and Control 2

The photoisomerization properties of **CAP1** and **CAP2** were monitored in methanol and aqueous buffer (Figure 2.2 A, C) using UV-Vis absorbance spectroscopy.

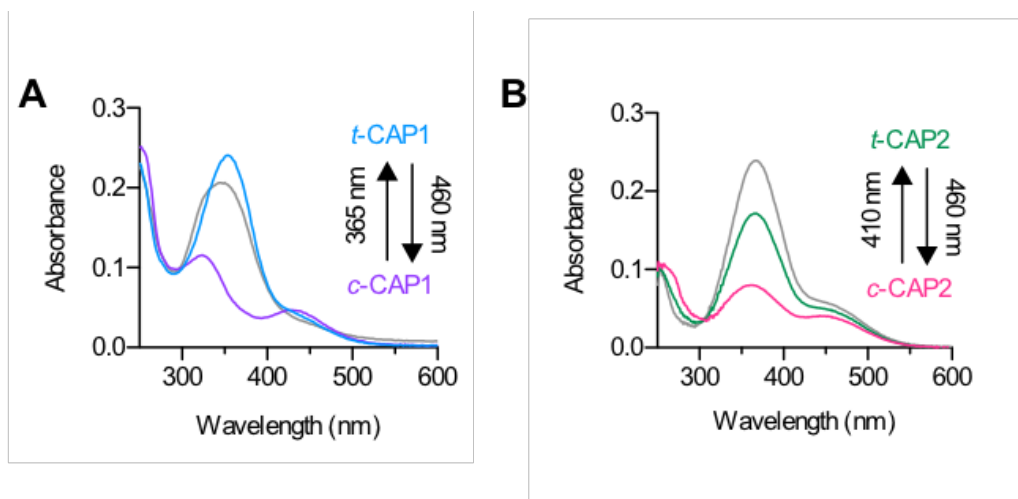


Figure 2.2 UV-vis spectrum of **CAP1** (A) and **CAP2** (B) before (grey) and after the photoirradiation of 365 nm (*c*-CAP1; 410 nm for *c*-CAP2) and 460 nm (*t*-CAP1 and *t*-CAP2). All studies performed in 50 mM HEPES, 0.1 M KNO₃ pH 7.2.

For **CAP1**, the spectrum showed a λ_{max} of 356 nm for the π - π^* transition (S_2), and 438 nm for the n - π^* transition (S_1). When irradiated with 365 nm light, the absorbance spectrum drastically shifted towards a profile consistent with the *cis* isomer (*c*-CAP1), with a decrease in the π - π^* band and an increase in the n - π^* band.¹² When *c*-CAP1 was irradiated with 460 nm light, the opposite spectral changes were observed which is consistent with restoration of the *trans* isomer (*t*-CAP1). Similarly, when **CAP2** was irradiated with 410 nm, the π - π^* band at 362 nm decreased, which was consistent with formation of the *cis* isomer (*c*-CAP2). When **CAP2** was irradiated with 460 nm, the opposite change in the π - π^* band was observed, indicating formation of the *trans* isomer (*t*-CAP2). All wavelengths are summarized in Table 2.1.

Probe	Methanol		Aqueous buffer	
	π - π^*	n- π^*	π - π^*	n- π^*
<i>t</i> -CAP1	354	442	354	408
<i>c</i> -CAP1	320	437	326	423
<i>t</i> -CAP2	360	465	365	438
<i>c</i> -CAP2	358	445	360	451

Table 2.1 λ_{max} (in nm) of π - π^* and n- π^* transitions for CAP1 and CAP2 in methanol and aqueous buffer (0.05 M HEPES buffer with 0.1 M KNO₃ at pH 7.2) as measured through UV/vis.

2.4.1.2 Photoisomerization kinetics and reversibility

We subsequently studied the real-time isomerization kinetics for both molecules in aqueous buffer (Figure 2.3 B, D).

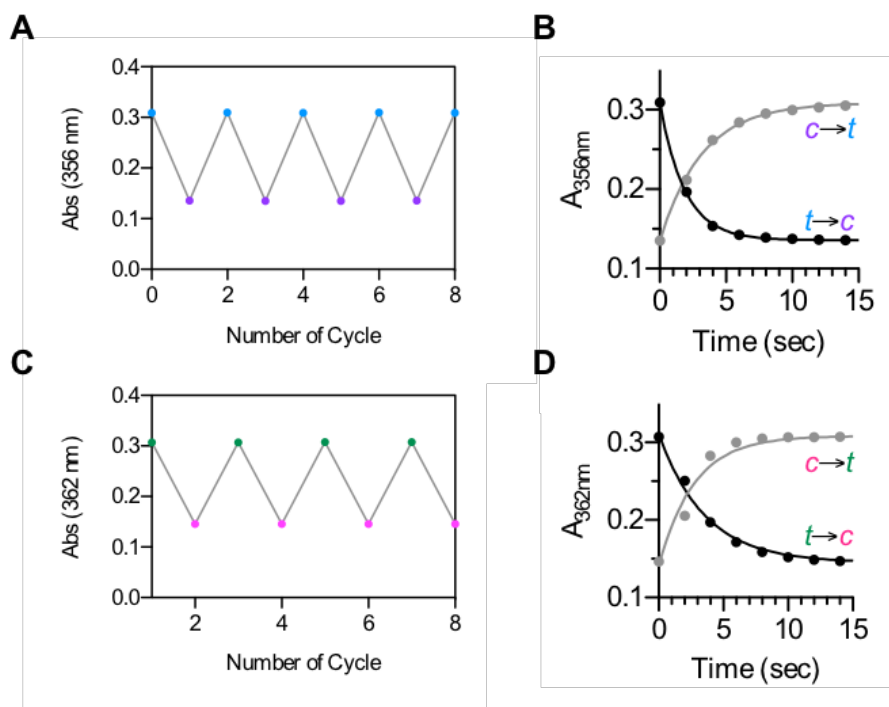


Figure 2.3 Multiple photo-isomerization of **CAP1** (A) and **CAP2** (C) as monitored by UV-vis. Kinetics of photoisomerization of **CAP1** (B) and **CAP2** (D). All studies are performed in 50 mM HEPES, 0.1 M KNO₃ pH 7.2.

Rapid isomerization was observed for both CAP1 and CAP2 in both directions. The profiles obtained were fitted to single phase decay, and the photoisomerization rate constants and half-lives for CAP1 and CAP2 are summarized in Table 2.2.

Importantly, both **CAP1** and **CAP2** reached their PSS within 15 seconds. The rapid photoisomerization is favorable to get dynamic and real-time control over their biological targets. Further, this isomerization process could be repeated across multiple cycles for both **CAP1** and **CAP2** (Figure 2.3 A, C).

Probe	Process	Rate constant (s ⁻¹)	Half-life (s)
CAP1	<i>c</i> to <i>t</i>	0.3128	2.216
	<i>t</i> to <i>c</i>	0.5370	1.291
CAP2	<i>c</i> to <i>t</i>	0.3691	1.878
	<i>t</i> to <i>c</i>	0.2815	2.462

Table 2.2 Rate constant and half-life for real-time photoisomerization of **CAP1** and **CAP2** in aqueous buffer (0.05 M HEPES with 0.1 M KNO₃ at pH 7.2).

2.4.1.3 NMR analysis of Photostationary state composition

The photo stationary state (PSS, state achieved after irradiation with a specific wavelength) isomeric percent was determined using NMR spectroscopy (Figure 2.4). While multiple ¹H NMR signals change upon photoswitching, the signals for the *p*-OMe for both molecules provided a clearly resolved signal for quantification.

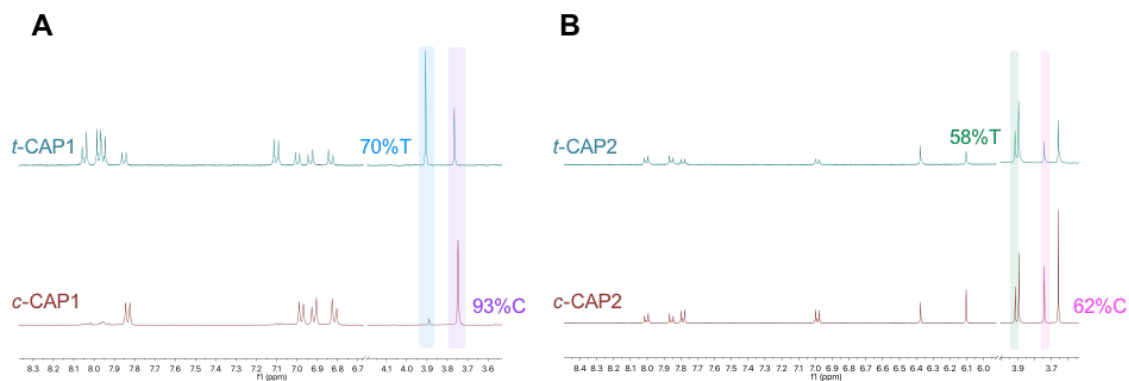


Figure 2.4 Isomeric percent at the photostationary state (PSS) for **CAP1** (A) and **CAP2** (B) after the photo-irradiation as determined by ¹H NMR spectroscopy in CD₃OD. The highlighted regions show the change in *p*-OCH₃ peaks.

The PSS generated by irradiation of **CAP1** in CD₃OD with 365 nm (*c*-**CAP1**) contained 93% *cis* isomer, and at 460 nm (*t*-**CAP1**) contained 70% *trans* isomer.

Similarly, the PSS generated by the irradiation of **CAP2** with 410 nm (*c*-**CAP2**) contained 62% *cis* isomer, and with 460 nm (*t*-**CAP2**) contained 58% *trans* isomer. We note that throughout the text, *t*-**CAP1/2** and *c*-**CAP1/2** represent the PSS described here. The higher degree of isomerization at the PSS of **CAP1** as compared to **CAP2** makes it a better candidate for biological studies.

2.4.2 Biophysical characterization of probe isomer-protein interaction

2.4.2.1 Difference in binding affinities of *trans* and *cis* isomers with CA

Azobenzenes work as quenchers for fluorescent molecules, so we exploited this property to study the binding interactions of CAP1 (Figure 2.5 A) and CAP2 (Figure 2.5 B) with bCA by monitoring the change in tryptophan (Trp) fluorescence from the protein.^{42,43} We observed that binding of *trans* and *cis* isomer of CAP1 and CAP2 results in the quenching of Trp fluorescence.

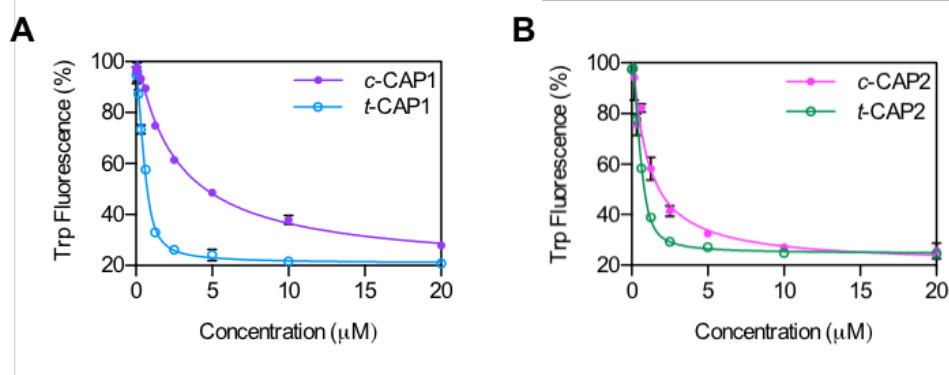


Figure 2.5 Tryptophan quenching of bCA after the incubation with different concentrations of *t*-**CAP1** and *c*-**CAP1** (A), and *t*-**CAP2** and *c*-**CAP2** (B). ($\lambda_{\text{ex}}=280$ nm, $\lambda_{\text{em}}=317$ nm). Data represents mean $\pm$ S.D.

The quenching profile was fitted to obtain apparent K_d values. The K_d values for *t*-**CAP1** and *t*-**CAP2** are 120 ± 50 nM and 102 ± 82 nM, respectively, while for *c*-**CAP1**

and **c-CAP2**, the values are $2.6 \pm 0.4 \mu\text{M}$ and $1.0 \pm 0.2 \mu\text{M}$ respectively. This yields differences in K_d for **CAP1** and **CAP2** [$K_d(\text{cis})/K_d(\text{trans})$] of 22- and 10-fold respectively. The lower K_d values for the *trans* isomers compared to the *cis* isomers for both molecules demonstrate the higher binding affinity of the *trans* isomers compared to the *cis* isomers.

2.4.2.2 Effect of *in situ* irradiation on probe-protein interaction

To further support binding of *t*-CAP1 and *t*-CAP2 to the CA active site, we performed a competition assay with dansylamide (DNSA) with bCA. DNSA is known to be a good fluorescent probe for the sulfonamide-binding site for carbonic anhydrase with μM binding affinity.³⁶ The binding is characterized by an enhancement in DNSA fluorescence emission due to a FRET process from photoexcited Trp residues ($\lambda_{\text{ex}} = 280 \text{ nm}$) near the active site.⁴⁴ When the CA•DNSA conjugate is titrated with an inhibitor of stronger affinity, DNSA comes out of the active site, resulting in a decrease in emission at 470 nm.³⁶

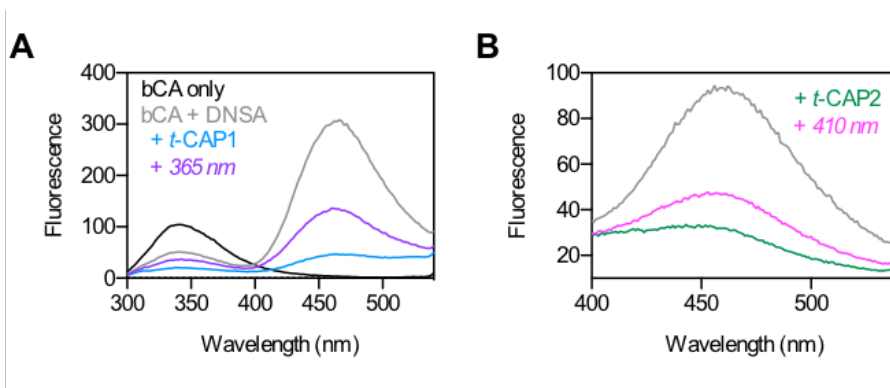


Figure 2.6 DNSA competition assay in the presence of ***t*-CAP1** (A) and ***t*-CAP2** (B), and after it is irradiated (365 nm for **CAP1** and 410 nm for **CAP2**) for *in situ* isomerization ($\lambda_{\text{ex}} = 280 \text{ nm}$). Studies performed in 50 mM HEPES, 0.1 M KNO_3 , pH 7.2.

We found that addition of ***t*-CAP1** (Figure 2.6 A) and ***t*-CAP2** (Figure 2.6 B) results in the quenching of emission at 470 nm, indicating the replacement of DNSA in the active site by the *trans* isomers. When the solution was irradiated with 365 nm (**CAP1**) or 410 nm (**CAP2**) to induce isomerization from *trans* to *cis*, the emission at 470 nm increased, showing that isomerization can occur in the presence of CA and that the *cis* isomers have lower binding affinities for the protein.

2.4.3 Application of CAP1 and CAP2 in the photomodulation of isolated CA

2.4.3.1 Inhibitory properties of *cis*/*trans* isomers of CAP1 and CAP2 on CA esterase activity

The most widely recognized biological role of CA is cellular pH regulation, but its intrinsic esterase activity is also utilized to measure its catalytic activity and to study the effect of inhibition spectroscopically. The inhibitory activity of **CAP1** and **CAP2** with bCA and hCAII was determined using a *p*-nitrophenyl acetate (NPA) hydrolysis assay (Figure 2.7 and 2.8). NPA was used as a substrate for CA, which is converted to *p*-nitrophenolate ion upon hydrolysis and gives an intense absorbance at 400 nm at pH 7.2.³⁷

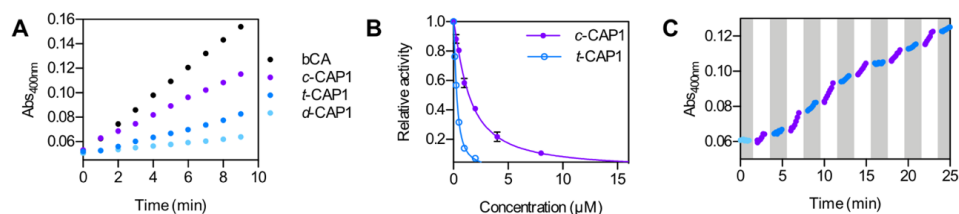


Figure 2.7 Change in absorbance at 400 nm due to NPA hydrolysis by bCA in the absence and presence of **CAP1** in different isomeric forms (A). IC₅₀ determination. Data represents mean $\pm$ S.D (B). Change in enzyme activity upon alternate *in situ* conversion of **CAP1** in *trans* (460 nm) and *cis* form (365 nm) (C). Studies performed in 50 mM HEPES, 0.1 M KNO₃ pH 7.2 at room temperature.

Figure 2.7 A shows the increase in absorbance at 400 nm over time when bCA was incubated with 1 equivalent of the different isomeric forms of **CAP1**. We performed this experiment with *c*-**CAP1**, *t*-**CAP1**, and **CAP1** that is formed after thermal isomerization in the dark (>98% *trans* isomer, *d*-**CAP1**).

In the presence of *d*-**CAP1**, the rate of NPA hydrolysis decreased, indicating inhibition of protein. When bCA was incubated with *c*-**CAP1** (after irradiation with 365 nm), the protein activity was dramatically higher than the *dark* state. Likewise, when the protein was incubated with *t*-**CAP1** (after irradiation with 460 nm), the protein activity was lowered, exhibiting the inhibition of the protein in *trans* state achieved by photoirradiation. This activity was not completely inhibited because of the presence of some *cis* isomer (~30%) at the PSS. Similar effects were observed in the case of **CAP2** for both bCA and hCAII (Figure 2.8 A). This experiment shows the difference in the inhibition properties of *trans* and *cis* isomers for both **CAP1** and **CAP2**.

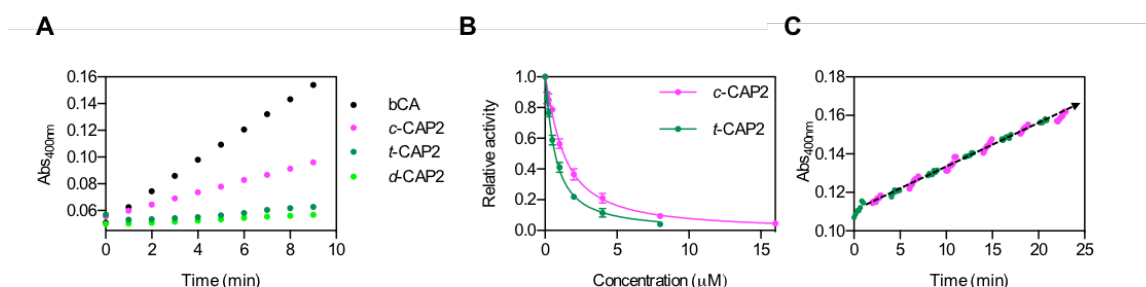


Figure 2.8 Change in the esterase activity of bCA upon incubation with **CAP2** prior to photoirradiation (*d*-**CAP2**), and after irradiation with 410 nm (*c*-**CAP2**) and 460 nm (*t*-**CAP2**) (A). IC₅₀ values in the presence of *t*-**CAP2** and *c*-**CAP2** (B). Data represents mean values $\pm$ S.D. The change in esterase activity of bCA on irradiating the solution with alternate 410 nm and 460 nm multiple times (C).

This property was further analyzed quantitatively by determining the IC₅₀ values. As shown in Figure 2.7 B and 2.8 B, the IC₅₀ for *t*-**CAP1** is 293 nM and for *t*-**CAP2** with

bCA is 688 nM, which is comparable to the IC₅₀ of acetazolamide (209 nM) determined under the same experimental conditions. The IC₅₀ for **c-CAP1** and **c-CAP2** are 1.46 μM and 1.28 μM respectively. All values are summarized in Table 2.3.

Probe	IC ₅₀ (nM) with bCA	IC ₅₀ (nM) with hCAII
Az	209	141
<i>c</i> -CAP1	1417	1465
<i>t</i> -CAP1	293	321
<i>c</i> -CAP2	1283	1435
<i>t</i> -CAP2	688	743

Table 2.3 IC₅₀ values for each isomer for both **CAP1** and **CAP2** isomers and compared to acetazolamide. Enzyme was kept constant at 500 nM in 50 mM HEPES, 0.1 M KNO₃ pH 7.2 at RT

The higher inhibitory concentrations required for the *cis* isomers as compared to the *trans* isomers shows the decreased inhibitory effect of the *cis* isomers. We note that no inhibitory activity was observed with control compound **2**, illustrating the importance of the sulfonamide-Zn²⁺ interaction in the inhibition.

2.4.3.2 *In situ* photomodulation of CA esterase activity

We then explored the possibility of reversible modulation of enzyme activity by photoswitching the probes *in situ*. The protein was incubated with **d-CAP-1** (Figure 2.7 C) or **d-CAP2** (Figure 2.8 C) under dark conditions. NPA was added to initiate the hydrolysis reaction, and the solution was irradiated with alternate 365 nm and 460 nm light (for **CAP1**) or 410 nm and 460 nm (for **CAP2**) for *in situ* isomerization. As demonstrated, the esterase activity was low prior to irradiation. After the first irradiation with 365 nm, the rate increased, which was consistent with *in situ* isomerization to **c**-

CAP1 and relief of protein inhibition. Irradiation with 460 nm light resulted in decreased NPA hydrolysis rate, due to inhibition of the protein. This process was repeated multiple times, demonstrating the reversible photomodulation of enzyme activity upon treatment with these probes.

2.4.3.3 Inhibitory properties of *cis/trans* isomers of CAP1 and CAP2 on CA hydration activity

As the biological role of CA is to catalyze the reversible hydration of CO₂ to produce protons and bicarbonate ions, the inhibitory effect of isomers of **CAP1** and **CAP2** on the CO₂ hydration activity of CA was analyzed spectroscopically in the presence of bromothymol blue as a pH indicator.^{45, 46} An aliquot of DI water saturated with CO₂ was added to buffered solutions containing bromothymol blue and the change in absorbance over time was monitored under different conditions (Figure 2.9 A). The absorbance was converted to pH by using the calibration curve.

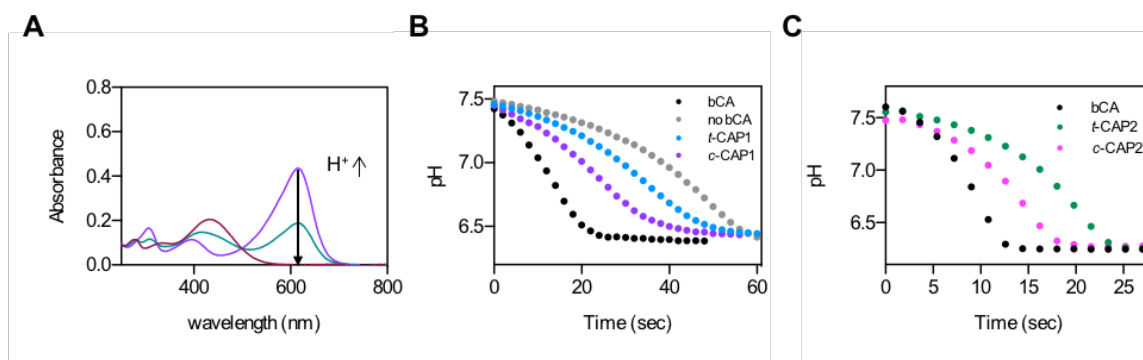


Figure 2.9 Absorbance spectrum of bromothymol blue, and changes at 617 nm as [H⁺] increases (A). Change in pH (in the presence of bromothymol blue as pH indicator) as a result of CO₂ hydration activity of bCA in the absence and presence of *t*-CAP1 and *c*-CAP (B). CO₂ hydration assay of bCA in the absence and presence of *trans* and *cis* CAP2 (C). Data represents mean values ± S.D.; in 200 mM Tris HCl pH 8.3 at 0-4 °C as determined through UV-Vis.

Figures 2.9 B (for **CAP1**) and Figure 2.9 C (for **CAP2**) show the kinetics of change in the pH of catalyzed (bCA only) and non-catalyzed reactions (no bCA). As expected, the rate of change in pH was much higher when the enzyme was present. We incubated **t-CAP1** with the protein before adding CO₂. As shown, the rate of hydration reaction decreased significantly indicating enzyme inhibition. When the protein was incubated with **c-CAP1**, the rate of CO₂ hydrolysis increased, which shows the lesser inhibitory effect of the *cis* isomer. This experiment verifies the NPA assay and supports the claim that these photoswitches can be used to control the biological function of CA as well.

2.4.4 Application of CAP probes in the photomodulation of CA in live cells

Having demonstrated the inhibitory activity of **CAP1** and **CAP2** in enzyme solutions, we next sought to apply these probes in a cellular context. More specifically, we tested the ability of **CAP1** to modulate the activity of cytosolic CAII in cancer cells. As **CAP1** achieved higher conversions between the *trans* and *cis* isomers after photoirradiation, we used it to demonstrate the application of our system in live cells. Cytosolic CAII is overexpressed in a number of cancer cell lines and is linked with poor prognosis and metastasis.⁵ Furthermore, this isoform serves as a driving force in cytosolic pH regulation.⁷ It has been reported that the resting intracellular pH of cells is affected by CA inhibitors, and this change varies depending on the cell type.⁴⁷⁻⁵¹

In our experiment, we tested the effect of the different forms of **CAP1** on the intracellular pH of HeLa cancer cells. A solution of 60 μ M **d-CAP1** (non-cytotoxic, as determined by MTS assay) was prepared and was irradiated with 365 nm and 460 nm light to generate **c-CAP1** and **t-CAP1** *in situ*. The cells were incubated with these solutions and pHrodo Red AM (pH indicator that displays increased fluorescence at

lower pH) for 30 minutes at 37 °C. Under these conditions, the *c*-CAP1 shows only 0.12% thermal relaxation to *trans* isomer. Cells were analyzed by fluorescence microscopy and the fluorescence was quantified and converted to pH using calibration samples.

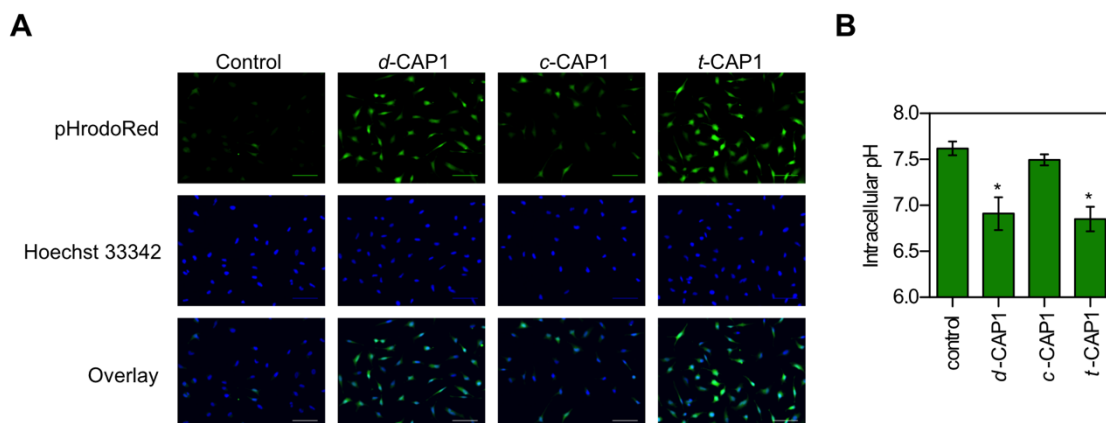


Figure 2.10 Change in the fluorescence of pHrodo red (green) as a result of different intracellular pH of HeLa cells in the absence and presence of different isomers of CAP1 for 30 minutes (A). Blue fluorescence corresponds to Hoechst stain. Scale bars = 100 μ m. Absolute pH_i values were calculated on the basis on calibration curve and were reported as the mean of individual cells from three wells (N=3, each replicate contains data from ~100 cells) (B). Data represent mean values $\pm$ S.D. Asterisks denote statistically significant differences (* $p = 0.05$, one-way ANOVA).

As shown in Figure 2.10 A, cells treated with *d*-CAP1 and *t*-CAP1 displayed much higher fluorescence compared to non-treated cells (control) and *c*-CAP1 treated cells. The corresponding pH_i values for these cells were 6.91 (*d*-CAP1), 6.85 (*t*-CAP1), 7.62 (control), and 7.50 (*c*-CAP1), as shown in Figure 2.10 B. We note that a pH_i of 6.89 was measured for cells treated with the same concentration of acetazolamide. We further tested the effects of longer inhibitor treatments on pH_i and found that treatment with *d*-CAP1 for 24 h similarly reduced pH_i (Figure 2.11 A, B, C). This effect from *d*-CAP1

was also observed in HCT-116 colon cancer cells, known to overexpress CAII (Figure 2.11 D, E, F).⁵² However, we were not able to demonstrate the long-term effects of the *cis* isomer as the half-life of *c*-CAP1 is 14 h; this probe thermally relaxes back to significant amount of *trans* isomer in ~24 hours at 37 °C.

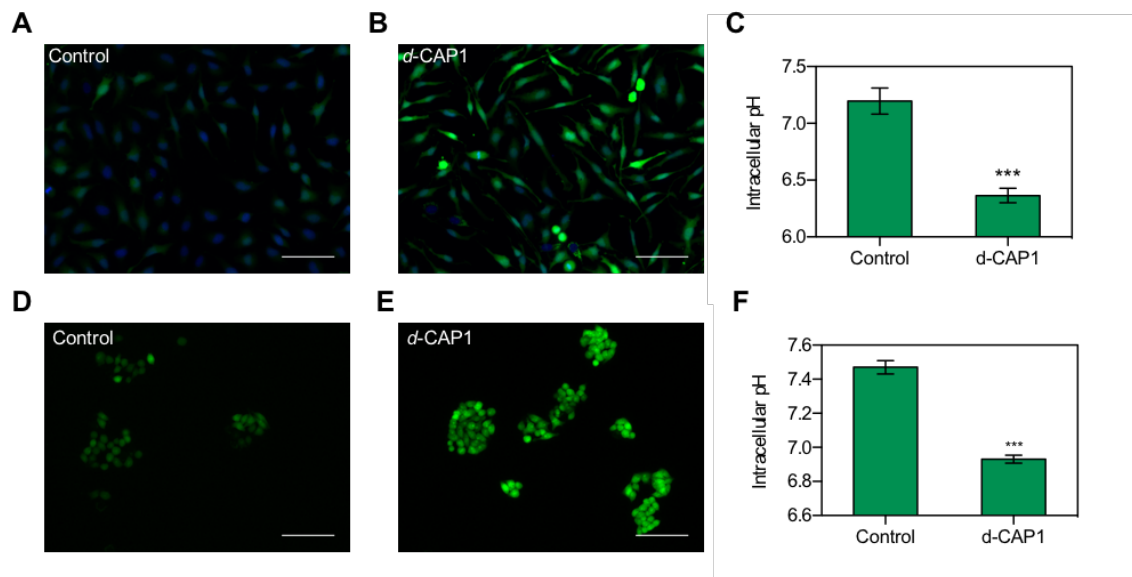


Figure 2.11 The fluorescence and intracellular pH of HeLa (A, B, C) and HCT116 (D, E, F) cells in the absence (control) and presence of *d*-CAP1 for 24 hours. Scale bars = 100 μ m. Data represents mean values $\pm$ S.D (N=3, each replicate contains data from ~100 cells). Asterisks denote statistically significant differences (***, $p = 0.0001$ unpaired t test).

2.5 CONCLUSIONS

In conclusion, two small molecule azobenzene sulfonamides, **CAP1** and **CAP2**, were developed, and the interactions of the *cis* and *trans* isomers with CA protein were studied in several contexts. *t*-CAP1 and *t*-CAP2 have increased inhibitory activity relative to their *cis* counterparts, and this activity could be modulated by photoirradiation with specific wavelengths of light. Importantly, we were able to establish the differential ability of the *cis* and *trans* isomers of **CAP1** to regulate cytosolic pH in live cells,

opening up future avenues for *in situ* pH regulation and CA inhibition. Current efforts along these lines include the development of scaffolds with longer-lived *cis* isomers and red-shifted irradiation wavelengths, both of which will expand our ability to apply these systems in live cells and organisms.

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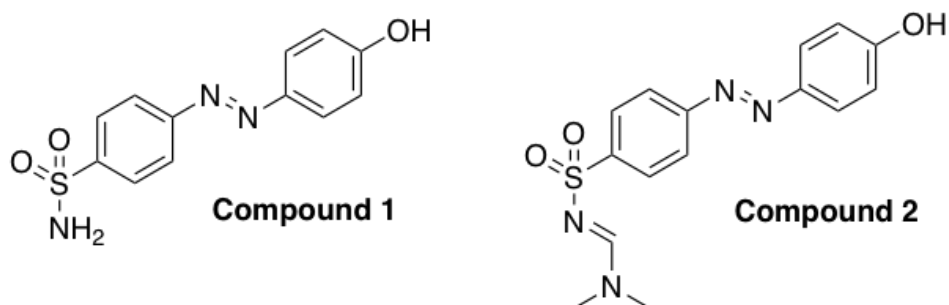
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2.7 SUPPLEMENTAL FIGURES AND DATA

2.7.1 Studies with control molecules Compound 1 and Compound 2



Scheme 2.4 Synthesis of Compound 1 and Compound 2

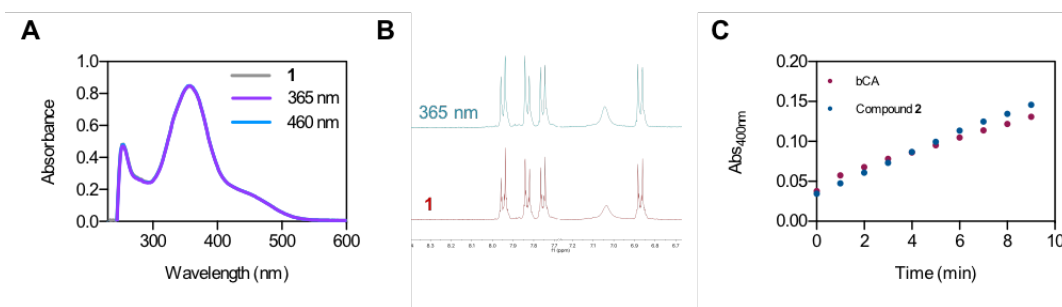


Figure 2.12 Effect of photoirradiation on Compound **1** in aqueous buffer UV-Vis (A) and CD₃OD through ¹H NMR spectroscopy (B). NPA assay of Compound **2** with bCA (C) in 50 mM HEPES, 0.1 M KNO₃ pH 7.2.

2.7.2 Studies with control molecules acetazolamide

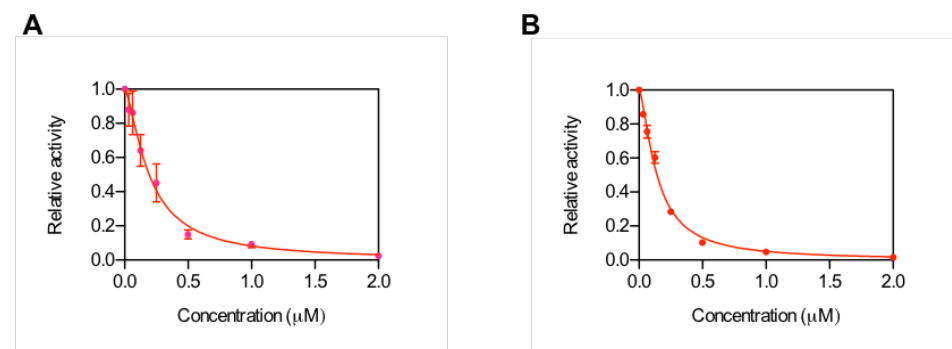


Figure 2.13 IC₅₀ determination for Az with bCA (A) and hCAII (B), as determined by NPA assay. Data represents mean values ± S.D.

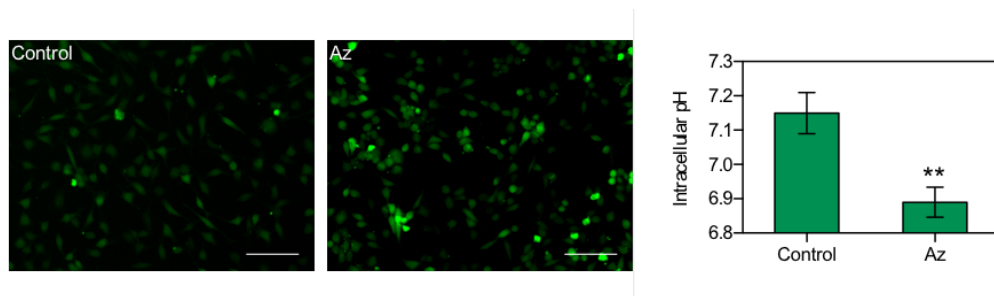


Figure 2.14 Change in HeLa cells fluorescence and intracellular pH after the incubation of cells with 60 μM of Az for 24 hours. Scale bars = 100 μm . Data represents mean values $\pm$ S.D (N=3, each replicate contains data from $\sim$ 300 cells). Asterisks denote statistically significant differences (**, $p = 0.0037$ unpaired t test).

2.7.3 Additional characterization of probes CAP1 and CAP2

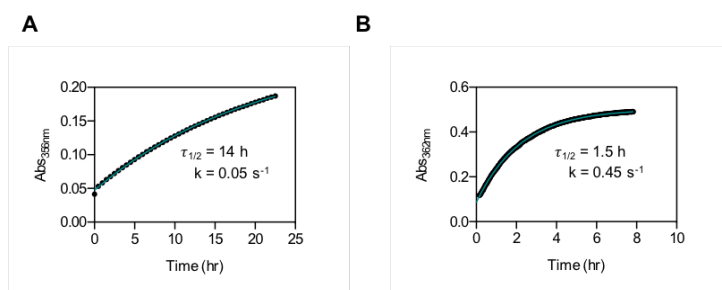


Figure 2.15 Thermal stability of **c-CAP1** (A) and **c-CAP2** (B) at 37 $^{\circ}\text{C}$ in 0.05 M HEPES buffer with 0.1 M KNO_3 at pH 7.2 as monitored by UV-vis. The half life and rate constant of thermal relaxation process is shown through one phase decay.

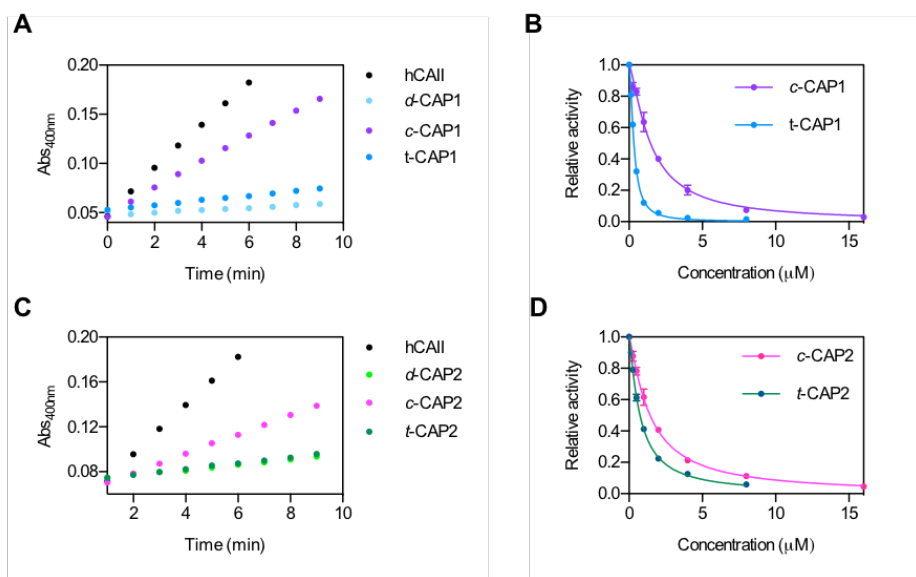


Figure 2.16 Change in the esterase activity of hCAII upon incubation with different isomers **CAP1** (A) and **CAP2** (C), and IC₅₀ values of *trans* and *cis* isomers of **CAP1** (B) and **CAP2** (D). Data represents mean values $\pm$ S.D. Studies are performed in 50 mM HEPES, 0.1M KNO₃ pH 7.2 under dark.

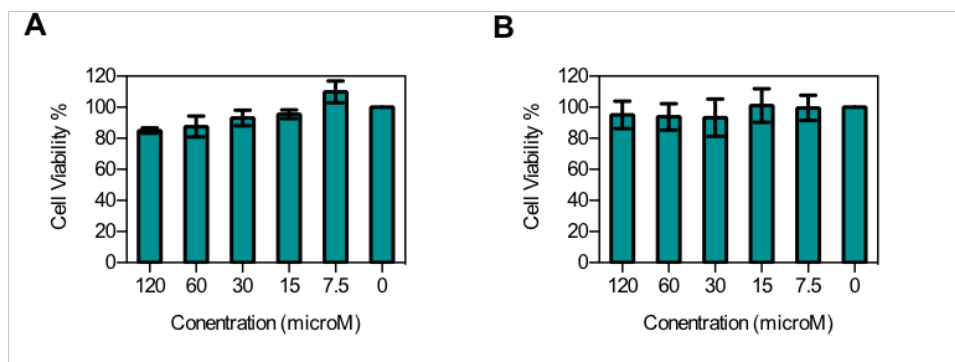


Figure 2.17 Cell viability of HeLa cells upon treatment with different concentrations of **CAP1** (A) and **CAP2** (B), as determined through MTS assay. Data represents mean values $\pm$ S.D.

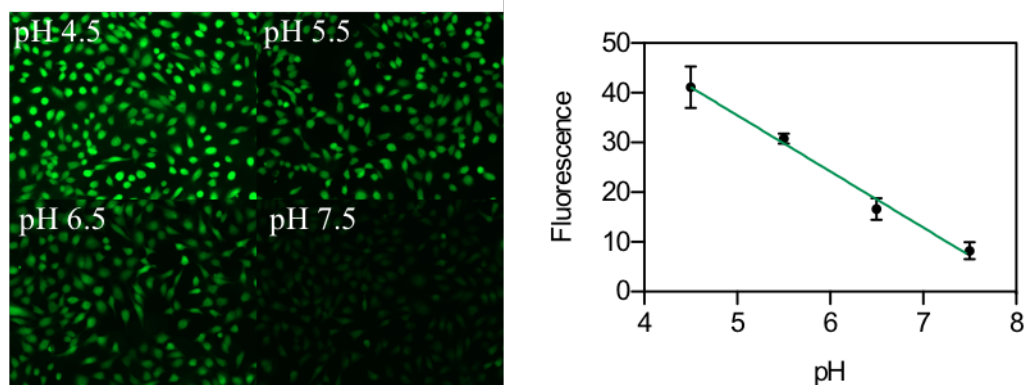
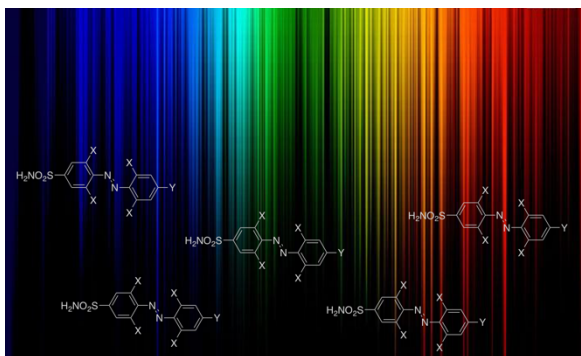


Figure 2.18 An example of calibration curve obtained by using pHrodo intracellular AM dye and calibration buffers in the presence of valinomycin and nigericin. Data represents mean values $\pm$ S.D. The calibration was performed for each individual experiment.

Chapter 3: Further explorations of azobenzene modifications for photomodulation of carbonic anhydrase²

3.1 ABSTRACT

There has been significant interest in using the azobenzene compounds to control specific biological entities in *in vivo* models. A key feature of such compounds is the wavelength of light required for photoisomerization. In addition to this, a long lifetime for the less stable *cis* isomer is desired to gain pronounced reversible photocontrol over targeted species. We developed **CAP-1** and **CAP-2** to control the activity of carbonic anhydrase (CA) using 365 nm and 460 nm, and demonstrated the application of these probes to control cellular intracellular pH (Chapter 2). However, due to limitations including isomerization wavelength, isomerization efficiency, and stability of the *cis* isomer, we were unable to apply them in *in vivo* systems. We found that small modifications on the phenyl rings of the azobenzene lead to large changes in the photophysical properties of the molecule. This chapter discusses our attempts to modify the structure of **CAP-1** through a combination of *ortho* and *para* substitutions on the azobenzene. These modifications and the resulting π - π^* or n - π^* band separation associated with the observed photoisomerization properties will be examined.



² This chapter is based on unpublished work

3.2 INTRODUCTION

Azobenzenes are one of the widely characterized photoswitches, as they can be readily synthesized, and the photophysical properties can be altered by simple substitution on the phenyl rings.¹⁻² Such characteristic features of azobenzene have resulted in their profound applications in the field of materials and chemical biology.³⁻⁴ However, azobenzene also display some limitations that may restrict the application in *in vivo* systems and ultimately may not find much application in the field of drug development and pharmacology. Some of those drawbacks are:

1. The absorption profile of *trans* and *cis* isomers of azobenzene shows significant overlap, due to which the photoisomerization process is not 100% efficient. The irradiation usually generates a photostationary state with ~20-30% of the unwanted isomer. In some cases, this drawback is further amplified that the unwanted isomer dominates, resulting in the undesired effects.
2. Next, the UV light is required to trigger the *trans* to *cis* photoisomerization of unmodified azobenzene which is strongly absorbed and scattered by skin and is not compatible to the normal tissues and living cells. Hence, such systems are not desirable for *in vivo* application.
3. Finally, the *cis* isomer usually shows limited thermal stability, and relaxes back to 100% *trans* isomer within short time periods. This property is not desirable, especially for our goal to gain control over biological functions, because it requires the continuous irradiation of tissues with high dosage of light to overcome the effect of thermal relaxation and keep the molecule in *cis* isomeric state.

The degree of these limitations does depend on the solvent conditions, but they adversely affect the application of such photopharmacophores in more biological settings.

In recent years, new photochromic molecules have been designed by performing classical aromatic chemistry on the phenyl rings of azobenzene.⁵⁻⁶ These molecular systems can be actuated with various photo-switching wavelengths and thermal conversion rates that can be easily tailored to suit the desired application.⁶ There has been considerable amount of research done to improve the design of azobenzene to overcome the limitations discussed above, with the visible light photoisomerization, in particular.⁷⁻¹¹ There are two main strategies:

3.2.1 Strategy 1: Red-shift of π - π^* absorption band

One strategy involves shifting of the π - π^* absorption band from ~350 nm to more red-shifted wavelengths to overcome the requirement for UV light for *trans* to *cis* photoisomerization. This can be achieved by increasing the electron density of the system. It can also be achieved by creating a push-pull system with electron donating groups on one ring and electron withdrawing groups on the other. However, this red-shifted π - π^* band usually overlaps with the n - π^* band, and hence, results in low photoisomerization efficiency. It also results in an extremely short lived *cis* isomer, where photoisomerization is not observed due to ultrafast relaxation of *cis* to *trans*, especially in protic polar solvents. The fast relaxation can be useful for certain cases like controlling ion channels involved in vision where rapid turn-off is critical for real-time responses.¹² Recently, BF_2 -adducts of azobenzene have been shown to demonstrate exceptional photoswitching properties by red-shifting the π - π^* to the visible region.¹³ By various alterations, it is possible to achieve the isomerization using far-red or even near-IR wavelengths.¹⁴ However, these adducts are susceptible to hydrolysis in water, forming hydrazones and limiting their applicability in biological settings.

3.2.2 Strategy 2: Separation of n- π^* absorption band of *trans* and *cis* isomer

The next strategy involves the separation of the n- π^* band in the visible region spectra of the *trans* and *cis* isomers that allows use of two distinct visible wavelengths to form a bi-stable photoswitch system. This strategy has particularly gained a lot of interest in the past couple of years, and researchers have adopted the following approaches to achieve the separation:

3.2.2.1 C-2 bridged azobenzenes

The *cis* and *trans* n- π^* transitions of C-2 bridged azobenzenes are separated by ~100 nm making photoswitching almost 100% efficient in either direction (Figure 3.1 A).¹⁵ Such probes can also be isomerized using wavelengths in the red region of the visible spectrum. The striking feature of these designs is the *cis* isomer being the thermally stable form due to the presence of the C-2 bridge.¹⁶ This becomes an important functionality when we require the more stable form to be the biologically inactive isomer. Hence, these modifications are perfect to isomerize the probe to biologically active *trans* isomer and subsequently let it relax thermally, a process that typically occurs within minutes.¹⁷ However, these designs do not meet our requirement to get a bistable system where thermal relaxation is negligible. Moreover, due to the C-2 bridge, the *trans* conformation does not yield an appropriate conformation to bind in an enzyme active site.

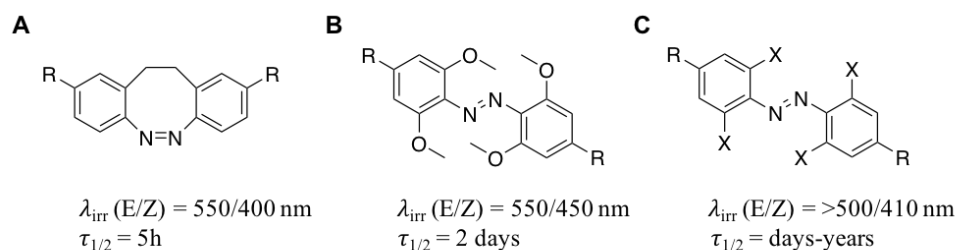


Figure 3.1 Azobenzene derivatives to get shift in n- π^* transition between *trans* and *cis* isomer

3.2.2.2 Tetra-ortho-alkoxy azobenzene

The introduction of electron donating groups such as amino or alkoxy groups at the *ortho*-positions to the azo moiety results in long wavelength photoswitching. However, one should be careful as systems with high electron density become more susceptible to photobleaching. In addition to this, the protonation of *ortho*-amino groups at physiological pH leads to shorter lifetimes of the *cis* isomer. Since methoxy groups are less electron donating than amino groups and are not protonated at biological pH, tetra-*ortho*-methoxy are preferred over *ortho*-amino substituted azobenzenes (Figure 3.1 B).⁹ In the absorbance spectra of the *trans* isomer of the tetra-*ortho*-methoxy azobenzenes, the n- π^* band is red-shifted due to twisting (non-planarity) of the *trans* isomer facilitated by the interaction of methoxy with the azo nitrogen lone pairs. In the *cis* isomer, however, the methoxy groups move away from the azo nitrogen lone pairs, so that the n- π^* transition is less affected as compared to the non-substituted azobenzene. As a result, the n- π^* bands of the *trans* and *cis* isomers are separated enough to achieve isomerization using blue and green light.⁹ Furthermore, the tetra-*ortho*-methoxy derivatives possess slower thermal relaxation of the *cis* isomer as compared to the corresponding modification at the *para*-position, opening up the avenues for pronounced applicability of these systems to control biological targets. However, the electron donating effect makes it

vulnerable to reduction in glutathione-containing buffers that reproduce the cytosolic reduction potential. Replacing the methoxy groups with methylthioethers overcomes the susceptibility to reduction while retaining the red-shifted properties, however, the lifetime of the *cis* isomer is reduced to a great extent.¹⁸

3.2.2.3 Tetra-halogenated azobenzene

Recently, *ortho*-halogenated variants of azobenzene were found to exhibit a separation between the $n\text{-}\pi^*$ band in the *trans* and *cis* isomeric forms, albeit to a lesser extent than tetra-*ortho*-methoxy derivatives, allowing photoisomerization by using two distinct visible wavelengths (Figure 3.1 C).¹⁹⁻²⁰ The presence of an electron withdrawing group at *para*-position increases the separation further, while an electron donating group will show the opposite effect. These derivatives also have more thermally stable *cis* isomers as compared to the parent azobenzene. Moreover, they are immune to reduction by glutathione, making them an exciting tool for photomodulation of biological targets.

In Chapter 2, **CAP-1** and **CAP-2** were designed to gain photocontrol over the activity of carbonic anhydrase.²¹ There were three main limitations of these probes: (1) use of UV-light for photoisomerization; (2) The low photoisomerization efficiency resulting in ~40-50% of the “*unwanted*” isomer at photostationary state; (3) the lower lifetime of *cis* isomer. Due to these reasons, we were not able to gain highly differential photocontrol over protein activity. Moreover, we were also unable to demonstrate the application of these probes for long terms treatment in cells as well as in *in vivo* systems. This chapter encompasses the journey we took to explore different modifications to **CAP-1** to achieve better properties in terms of isomerization wavelength, photoisomerization efficiency, and *cis* isomer lifetime. We will discuss the rationale

behind such modifications, and the acquired results in terms of photoisomerization properties and binding with proteins, and the possible reasons for their failure.

3.3 EXPERIMENTAL SECTION

3.3.1 General Procedures

All solvents and chemicals were purchased from Sigma-Aldrich and Fisher Sci. and used as received. Bovine carbonic anhydrase was purchased from Sigma Aldrich. For spectroscopic and protein studies, 0.05 M HEPES buffer with 0.1 M KNO₃ (pH 7.2) was used unless stated otherwise. All the studies were done under dark condition to avoid exposure of light.

The ¹H and ¹³C NMR spectroscopic measurements were collected in deuterated solvents from Cambridge Isotope Laboratories (Cambridge, MA), using an AGILENT MR 400 NMR spectrometer at 400 MHz and 500 MHz respectively. The chemical shift is calibrated with respect to the solvent peak, and is reported in ppm. Absorbance spectroscopic studies were performed using an Agilent Cary 60 UV-Vis spectrophotometer. Fluorescence spectroscopic measurements were made using an Agilent Cary Eclipse fluorescence spectrofluorimeter. The samples were irradiated with an Asahi Xe lamp connected with bandpass filters of 365 nm (FWHM 12 nm), 460 nm (FWHM 11 nm) from Asahi Spectra, and 410 nm, 520 nm, 550 nm (band width 10 nm) from Thorlabs.

3.3.2 Characterization

3.3.3.1 Photoisomerization properties

The photoisomerization kinetics of probes were investigated by electronic absorption spectroscopy. The absorbance spectrum was taken in methanol and aqueous

buffer under dark conditions. To get the *cis* or *trans* isomer absorbance spectrum, the sample was irradiated with the required wavelength and the spectrum was collected. To get the *trans* isomer absorbance profile, the sample was further irradiated with 460 nm before collecting the absorbance spectrum.

The percent of *trans* and *cis* content at the PSS was obtained by irradiating the sample with required wavelength in methanol-*d*₄ and collecting the ¹H NMR data with 30 sec relaxation time to ensure the relaxation of all the spins. The *p*-OCH₃ hydrogens were integrated and compared to obtain the PSS isomeric percent.

3.3.3.2 Tryptophan Quenching

Binding analysis of probe was performed using bovine carbonic anhydrase at room temperature in black 96 well plates. A stock solution of 10 mg/mL (~350 μM) enzyme was prepared in aqueous buffer. A stock solution of 2 mM probe was prepared in DMSO under dark conditions. During titration, the enzyme concentration was kept constant (1 μM), and the *trans* or *cis* isomer (irradiated prior to the addition) concentration was increased from 0-20 μM with 1% final DMSO content. The well plate was incubated at room temperature for 15 minutes to allow the formation of E-I complex. Each well was then read for the tryptophan fluorescence (λ_{ex}=280 nm; λ_{em}= 317 nm). The quenching profile for *trans* isomer titration was fitted to quadratic equation (as [E] > K_d):

$$y = y_o + (y_{max} - y_o) \frac{\sqrt{(E + x + K_d)^2 - 4xE}}{2E}$$

The quenching profile for *cis* isomer titration was fitted to hyperbolic equation (as [E] < K_d):

$$y = \frac{y_{max}x}{(K_d + x)}$$

where *y*_o is initial fluorescence, *y*_{max} is fluorescence at plateau, *K*_d is dissociation constant, *E* is enzyme concentration.

3.3.3.3 DNSA Competition Assay

DNSA (dansylamide) competition studies were done with bovine carbonic anhydrase (bCA).²² All studies were done at room temperature under dark conditions. A stock solution of 2 mg/mL (~70 μ M) enzyme was prepared in aqueous buffer. A stock solution of 1 mM DNSA and 100 μ M of probe was prepared in DMSO. To a solution of 0.25 μ M of enzyme and 2.5 μ M of DNSA, 0.25 μ M of probe was added under dark conditions. The excitation wavelength was set at 280 nm (slit width 10), and the emission was collected over a wavelength range of 300-600 nm. The solution was irradiated with required wavelength for the *in situ* conversion of probe to *cis* isomer, and the emission spectrum was collected. The same solution was further irradiated with 460 nm to get the *trans* isomer and the emission was recorded.

3.3.3.4 NPA Assay using UV/vis spectroscopy

The effects of isomer binding of probes on bCA activity, NPA assay was used, where *p*-nitrophenyl acetate (NPA) was used as the substrate.²³ As a result of enzymatic activity, NPA is hydrolyzed to give *p*-nitrophenolate ion, which has a λ_{max} at 400 nm. Hence, the enzymatic activity in the absence and presence of probe can be determined spectroscopically by monitoring the increase in absorbance with time. In this study, a stock solution of 10 mg/mL (~350 μ M) protein in buffer, 10 mM probe in DMSO and 125 mM NPA in dry acetonitrile were prepared. During analysis, the enzyme, probe and NPA concentrations were kept constant at 2 μ M, 2 μ M and 250 μ M respectively. The absorbance was recorded at 400 nm every 60 sec for up to 10 minutes and was plotted with respect to time. To study the effect of *cis* and *trans* isomer on the enzymatic activity, the solution of probe in buffer was irradiated to achieve the PSS prior to the addition of

protein and NPA. All the studies were performed in the absence of light at room temperature.

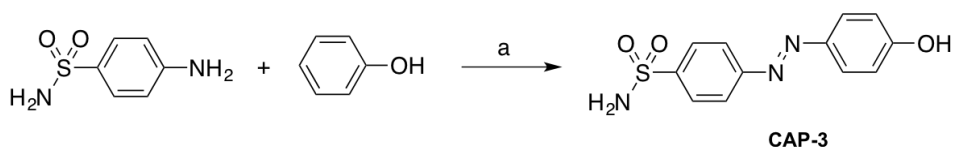
3.4 RESULTS AND DISCUSSION

With the goal of achieving the efficient photoisomerization using visible light with the long lifetime *cis* isomer, we modified the chemical structure of azobenzene sulfonamide using various design rationales.

3.4.1 Increasing the electron density to shift π - π^* band

The electron density on the azobenzene ring was increased by modifying the *para*-position with hydroxyl and amine based groups.

3.4.1.1 Hydroxyl derivatives



Scheme 3.1 Synthesis of **CAP-3**

The molecule was synthesized by coupling between an aniline analog and phenol using standard azo coupling reaction (Scheme 3.1). Aniline derivative was added in 40% hydrochloric solution in the presence of aqueous NaNO_2 , and then phenol solution in NaOH was added to the reaction mixture to get the probe **CAP-3** in 85% yield.

The photoisomerization properties of **CAP-3** were studied in different solvents through UV-vis spectroscopy. In toluene, the absorbance spectrum shows a π - π^* transition at 360 nm and a n - π^* transition at 460 nm (Figure 3.2 A). Upon irradiation with 365 nm for 5 seconds, the absorbance band in the UV region decreased and the band in visible region increased slightly. These changes correspond to the *trans*→*cis*

photoisomerization. The reversible *cis*→*trans* photoisomerization can be achieved by using 460 nm for 5 sec. The photoisomerization process was studied in different solvent systems, and was successful in aprotic polar and non-polar solvents like DCM, CHCl₃ and acetonitrile. However, no photoisomerization was observed in MeOH or HEPES buffer at pH 7.2 (Figure 3.2 B).

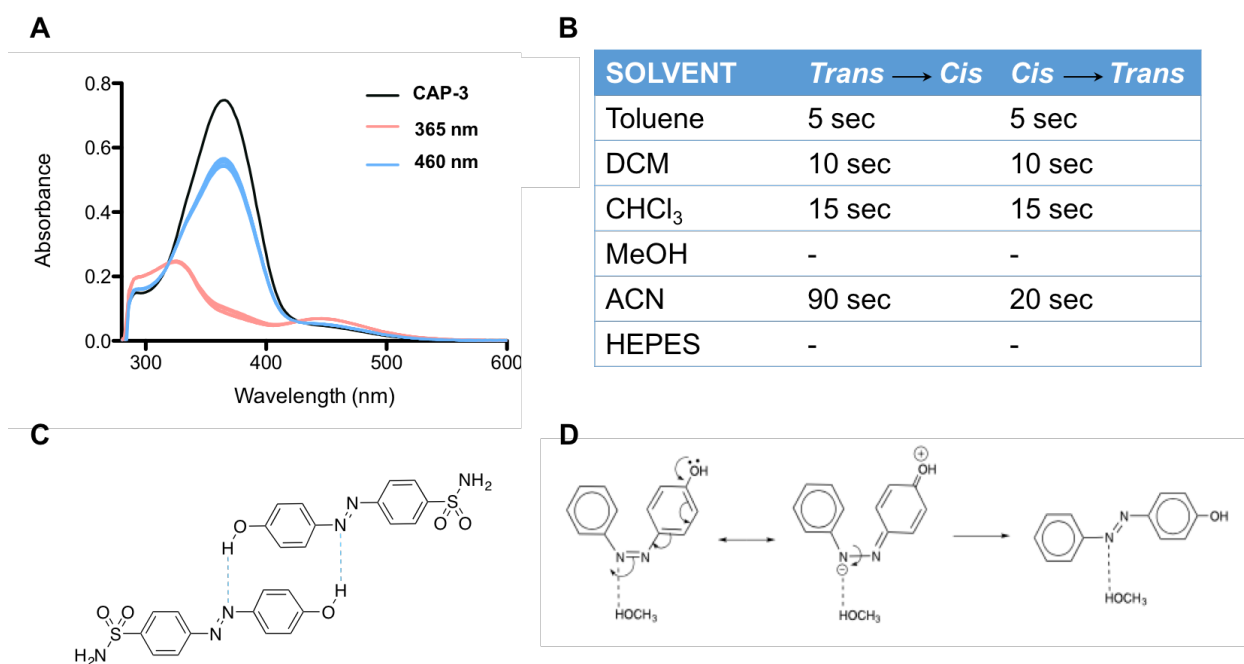
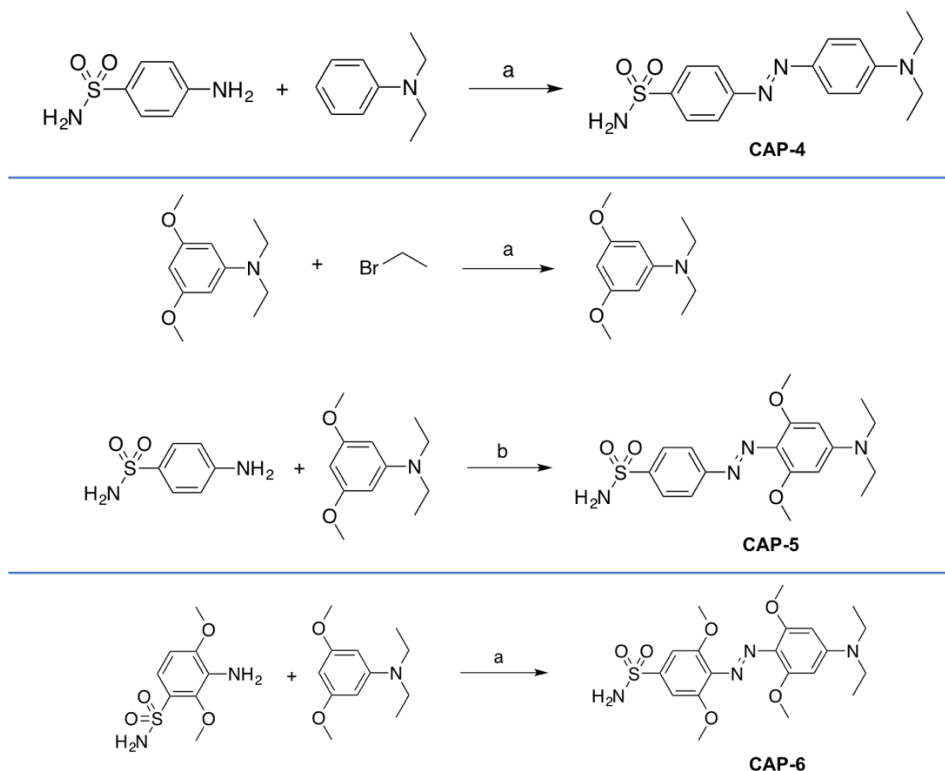


Figure 3.2 Photoisomerization of CAP-3 (A) with time required in different solvents (B). The formation of dimer (C) and tautomer (D) of CAP-3 in polar protic solvent.

Upon detailed literature search, we realized that hydroxyazobenzenes can have intermolecular hydrogen bonds (Figure 3.2 C) and tautomer formation (Figure 3.2 D) that may result in the fast thermal relaxation of the *cis* isomer to the *trans* isomer in protic polar solvents such as methanol and water.²⁴ This problem can be solved by alkylating the free hydroxyl groups, which was adapted from **CAP-1** and **CAP-2** as discussed in Chapter 1.

3.4.1.2 Amine derivatives

Alkylation of the *para*-hydroxy group helped us achieve photoisomerization in aqueous buffer using 365 nm light. As this wavelength falls in the UV range of the spectrum, we decided to try using alkylated amines instead of alkoxy at the *para*-position. As amines are better electron donating groups compared to hydroxyl due to the lower electronegativity of nitrogen vs. oxygen, we anticipated a larger red-shift in the absorbance spectrum for these amino derivatives. In order to do so, we designed three different variations (Scheme 3.2): with no (**CAP-4**), two (**CAP-5**) and four methoxy (**CAP-6**) group at the *ortho*-positions in addition to a diethyl-amino group at one *para*-position and a sulfonamide at the other.



Scheme 3.2 Synthesis of **CAP-4**, **CAP-5** and **CAP-6**

The absorbance profile of **CAP-4** was obtained and compared with that of **CAP-3**. As expected, we observed a major red-shift of ~ 100 nm in the absorbance wavelength in **CAP-4** (Figure 3.3 A). The photoisomerization of **CAP-4** was first studied in CHCl_3 where we were able to form the *cis* isomer following 365 nm irradiation, and isomerize back to the *trans* isomer using 460 nm (Figure 3.3 B). However, there was no photoisomerization observed in HEPES buffer at pH 7.2. A similar trend was also observed for **CAP-5** and **CAP-6**.

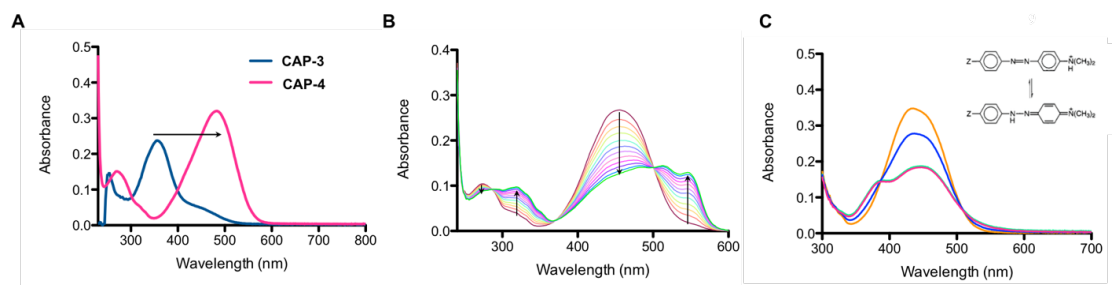


Figure 3.3 The absorbance spectra comparison of **CAP-3** and **CAP-4** (A). The isomerization of **CAP-4** in CHCl_3 (B). The isomerization of **CAP-4** in basic aqueous solution (C).

This was due to the protonation of *para*-diethyl-amino group at this pH, which results in ultrafast thermal relaxation of the *cis* to the *trans* isomer powered by a rotation-assisted mechanism. Upon adding base to the solution, we were able to observe *cis* species formation following 365 nm irradiation (Figure 3.3 C), however, these conditions do not represent biological environments, and hence, does not meet our requirements to gain control over biological enzymes in cells.

3.4.2 Tetramethoxy derivatives

As the approach of red-shifting the $\pi-\pi^*$ band came with its own challenges regarding the thermal stability of the *cis* isomer in polar protic solvents, we used next

attempted the approach of separating the $n\text{-}\pi^*$ bands to meet the photophysical requirements for our desired probe. We further modified the design of probe through the introduction of a methoxy group at the *ortho*-positions to the azo group while keeping the methoxy group at *para*-position intact to obtain **CAP-8** and **CAP-9** (Figure 3.4). These probes differ in the positioning of the sulfonamide group. Due to the separation of the $n\text{-}\pi^*$ transition band between the *trans* and *cis* isomers, they are known to photoswitch using visible light, and the *cis* isomer also has an extended thermal stability over the parent azobenzene, which made **CAP-8** our ideal molecule⁹.

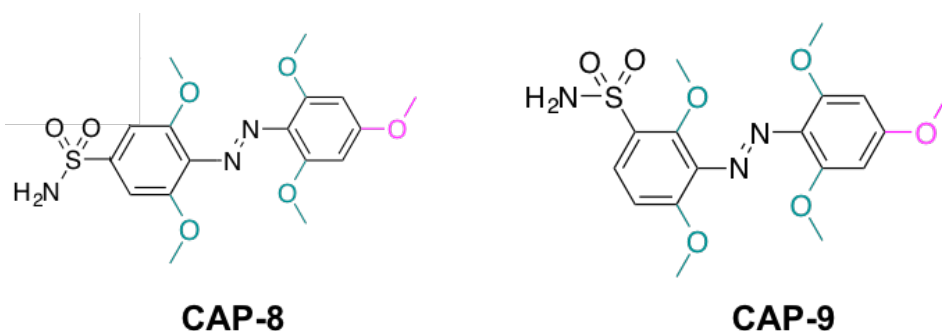
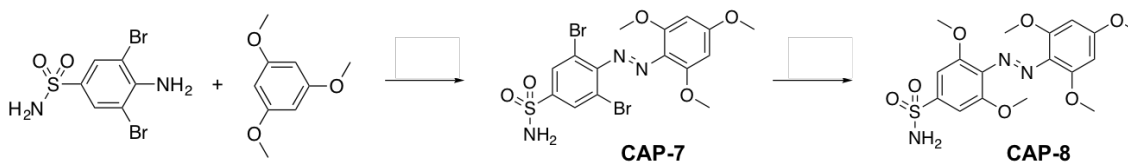


Figure 3.4 Design of tetra-*ortho*-methoxy derivatives **CAP-8** and **CAP-9**

3.4.2.1 Compound with *para*-sulfonamide **CAP-8**

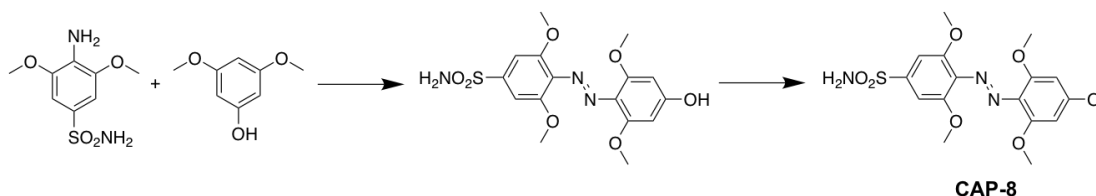
The synthetic plan for probe **CAP-8** is shown in Scheme 3.3. First, **CAP-7** was synthesized by coupling 2,6-dibromo-4-sulfonamide-aniline with 1,3,5-trimethoxybenzene in the presence of 40% HCl and NaNO₂ at 0°C followed by NaOH treatment at room temperature. The product was purified to form pure CAP-7 in 68% yield. The *ortho*-bromo groups were converted to methoxy by treatment with 5.4 M NaOCH₃ in methanol, however, the reaction was unsuccessful. Various attempts were made to get the product by using different reaction conditions such as temperature and reaction time but the reactions worked only slightly when CuI was used as a catalyst. In

that condition, however, the reaction product was highly impure with mono- and di-substitution and other unidentified species which led us to follow a longer route for **CAP-8** synthesis.



Scheme 3.3 Synthesis of **CAP-8** via **CAP-7**

In the next approach, we planned on synthesizing the probe using the same strategy as for **CAP-1** through **CAP-6**, i.e. by direct azo coupling between the final amine and phenolic derivatives (Scheme 3.4). For that, we needed to synthesize 2,6-dimethoxy-4-sulfonamide-aniline, which turned out be more challenging than anticipated.



Scheme 3.4 Proposed synthesis of **CAP-8** via azo coupling route

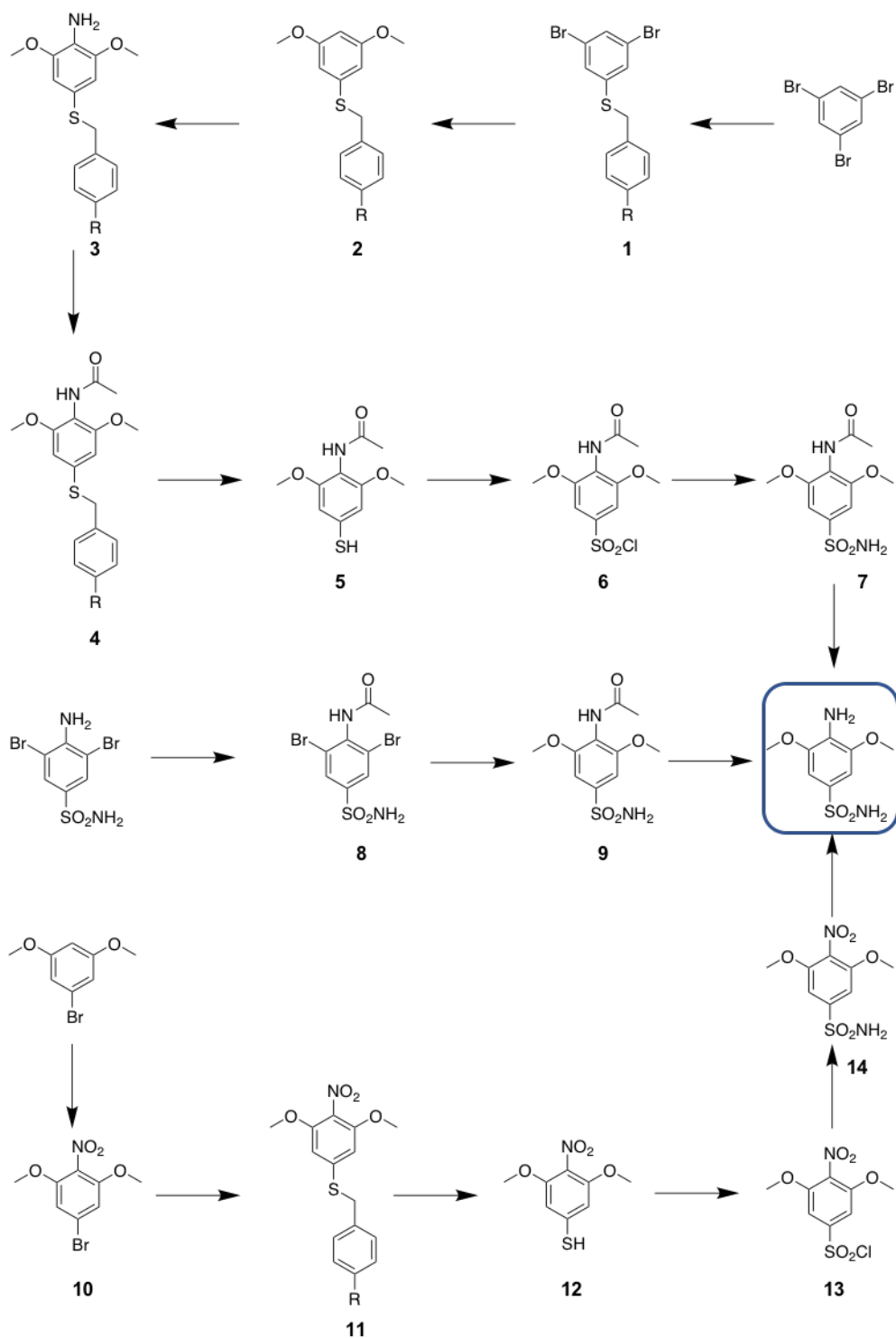
The proposed synthetic routes for precursor 2,6-dimethoxy-4-sulfonamide-aniline is shown in Scheme 3.5. In the first approach, we started with excess of 1,3,5-tribromobenzene and reacted with benzyl mercaptan (and *para*-methoxy benzyl mercaptan in parallel) in the presence of CuI and potassium tert-butoxide in DMSO at 80 °C for 18 h to form compound **1**. This was further treated with 5.4 M CH₃ONa in methanol and CuI in DMF at 110 °C for 4 h to form compound **2**. This was further treated with *n*-BuLi in the presence of B(O^{*i*}Pr)₃, HOSA, NaOH at room temperature for

16 h to get compound **3**. The formed amine group was protected with acetic anhydride in the presence of TFA at 0 °C, however, we got a mixture of multiple products, including the deprotected thiol at *para*-position and the corresponding oxidized disulfide product. We attempted to purify the thiol substituent derivative but it was unstable, which led us to change the reaction route.

In the next route, we started with 2,6-dibromo-4-sulfonamide-aniline and protected the amine group by using acetic anhydride in the presence of ZnCl₂ at room temperature for 2 days to get compound **8**. We also observed the addition of an acyl group to the sulfonamide, but decided to move forward as this would not affect the subsequent synthetic step. Compound **8** was further reacted with CH₃ONa in methanol in the presence of CuI in DMF at 110 °C to convert the di-bromo to the di-methoxy derivative. However, this reaction resulted in a mixture of multiple products: mono- and di-substituted products with protected and deprotected amine derivatives, as well as the acyl sulfonamide. The reaction mixture was highly impure and it was difficult to isolate the product needed for the azo coupling reaction.

In our last attempt to make the precursor, we started with 3,5-dimethoxy bromobenzene and reacted it with HNO₃ in the presence of acetic anhydride at 0 °C for 1 h and then at room temperature for 3 h to obtain compound **10**, which has a nitro group at *para*-position to the bromo group. We also obtained the derivative with a nitro group at the *ortho*-position to the bromo group as a side product, which was removed by column chromatography. Compound **10** was reacted with benzyl mercaptan (and *para*-methoxy benzyl mercaptan in parallel scheme) in the presence of CuI and potassium tert-butoxide in DMSO at 80 °C for 18 h to substitute the bromo group with benzyl thiol derivative. This was further reacted to form free thiol **12**, that could be converted to a sulfonamide later, but all attempts to synthesize this precursor were unsuccessful. Due to these many

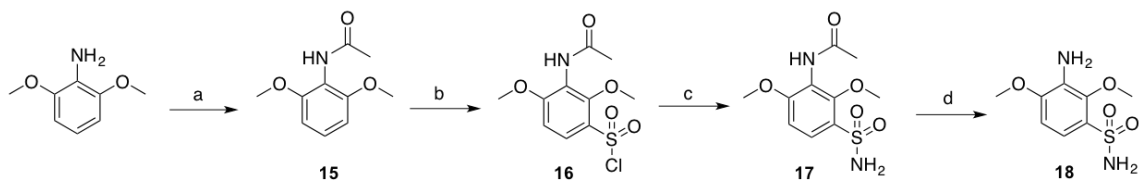
failed steps, we moved forward to future design modifications, which were relatively easier to synthesize.



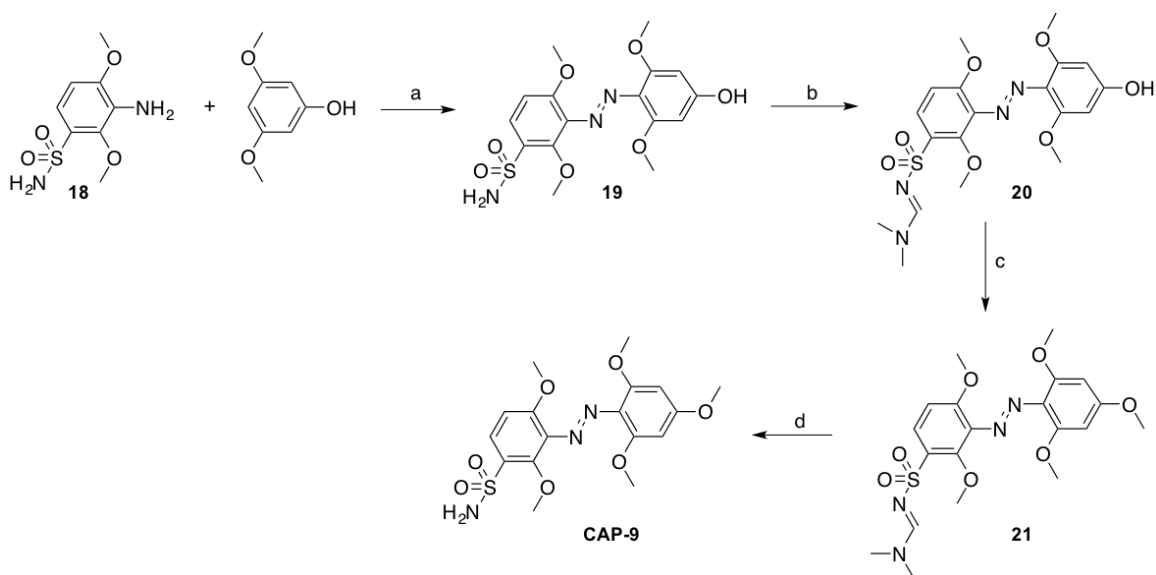
Scheme 3.5 Different synthesis routes for **CAP-8**

3.4.2.2 Compound with meta-sulfonamide CAP-9

As **CAP-8** possessed some synthetic challenges, we modified the design to **CAP-9** where the sulfonamide group is at the *meta* position rather than the *para* position to the azo group. In order to synthesize **CAP-9**, the precursor **18** carrying the sulfonamide group was prepared as shown in Scheme 3.6. The aromatic amine group on 2,6-dimethoxyaniline was protected using neat acetic anhydride at room temperature to furnish compound **15** in 90% yield. The amine protected compound **15** was then treated with chlorosulfonic acid at 0 °C for 3 h to get the sulfonyl chloride derivative **16** in 80-85% yield, which was then converted to sulfonamide derivative **17** using NH₄OH in methanol at room temperature. The amine protecting group was removed using acidic conditions in methanol at 60 °C to yield the precursor **18** with 83% yield.



Scheme 3.6 Synthesis for the precursor of **CAP-9**



Scheme 3.7 Synthesis of **CAP-9**

The precursor **18** was coupled with 3,5 dimethoxy phenol in the presence of 40% HCl and NaNO₂ followed by NaOH treatment to get the compound **19** in 60% yield (Scheme 3.7). The sulfonamide group was then protected using DMF-DMA (N, N-Dimethylformamide dimethyl acetal) at room temperature to obtain compound **20** in 89% yield. The *para*-hydroxy group of compound **20** was methylated using CH₃I in acetonitrile in the presence of K₂CO₃ as a base under refluxing conditions to get compound **21** in 75% yield. The sulfonamide group was then deprotected using acidic conditions in methanol at 60 °C to get pure **CAP-9** in 70% yield.

The photoisomerization properties of **CAP-9** were studied in HEPES buffer using UV-vis absorbance spectroscopy. The absorbance spectrum of **CAP-9** contains a π - π^* band at 342 nm and a n- π^* band at 444 nm (Figure 3.5 A). Upon 550 nm (yellow light) irradiation, *trans*→*cis* photoisomerization was observed, where the π - π^* decreased in the intensity and n- π^* band shifted to 436 nm. Time-dependent density functional theory calculations suggested that the red-shift of the n- π^* band of the *cis* isomer arose due to a

destabilization of the HOMO centered on the azo nitrogen lone pairs because of the close proximity of the methoxy oxygen lone pairs.²⁵

The *cis*→*trans* photoisomerization of **CAP-9** can be achieved using 460 nm (blue) light. The shift in the n-π* band between the *trans* and *cis* isomer enabled us to use wavelengths in the visible region for both photoisomerization processes. **CAP-9** showed excellent stability and multiple photoisomerization with no photodegradation in aqueous condition at pH 7.2, which is ideal for protein and cellular studies (Figure 3.5 B).

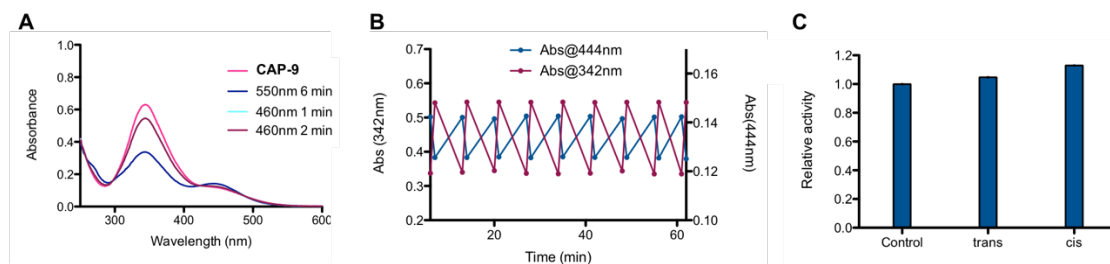


Figure 3.5 The absorbance profile of **CAP-9** before and after photoirradiation with 550 nm and 460 nm in aqueous buffer (A). The reversibility of isomerization process of **CAP-9** in buffer, as determined by UV-vis absorbance spectroscopy (B). The esterase activity of bCA in the absence and presence of *trans* and *cis* isomer of **CAP-9** as determined by NPA assay (C).

Next, we studied the binding of *trans* and *cis* isomers of **CAP-9** to carbonic anhydrase by analyzing the esterase activity of carbonic anhydrase using the NPA assay. As shown in Figure 3.5 C, the activity of the protein was not affected by either the *trans* or the *cis* isomer, suggesting no interaction of the probe with the active site of the protein. This was a bit surprising, but not completely unexpected. The presence of -SO₂NH₂ at the *meta* position instead of the *para* position may not allow for binding to the Zn^{II} ion at the depth of the active site.

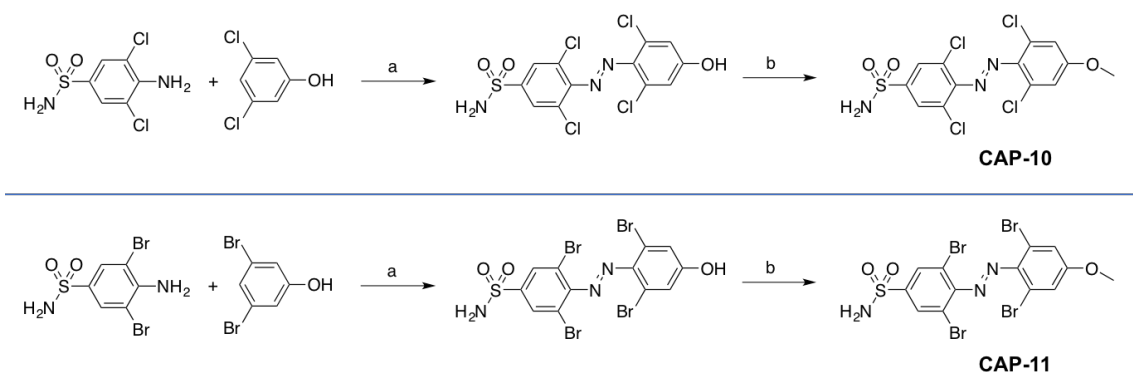
These efforts to design and synthesize different variants of tetra-*ortho*-methoxy azobenzene along with many synthetic challenges were cumbersome. However, in

retrospect, this molecule might have lacked the desired stability under biological conditions. Along with the visible wavelength for photocontrol, the chemical and metabolic stability of the photoswitch in terms of photobleaching, reduction by intracellular glutathione (GSH), or enzymatic metabolism is very important. It has been reported that such molecules are more sensitive to the reduction under biological concentration of GSH, and usually undergo slow reduction and photo-bleaching, limiting the application to only extracellular or short-term intracellular uses only.^{9-10, 25}

3.4.3 Tetrachlorinated and Tetrabrominated derivatives

In pursuit of a molecule that could meet at least some of the required photophysical properties and has a more straightforward synthesis, we explored tetra-*ortho*-chloro (**CAP-10**) and tetra-*ortho*-bromo substituents (**CAP-11**). One other significant advantage of these derivatives is that they are known to be resistant to reduction by GSH in both isomeric forms, and are stable towards photobleaching. This is due to the lower effective pK_a of the azo group in tetra-*ortho*-chloro azobenzene leading to less protonation at physiological pH, which ultimately results in the probe being less susceptible to thiol attack as compared to tetra-*ortho*-methoxy azobenzene.²⁵

CAP-10 was synthesized by azo coupling between 2,6-dichloro-4-sulfonamide aniline with 3,5-dichloro phenol using NaNO_2 and 40% HCl in CH_3OH to get the coupled product in 60% yield (Scheme 3.8). This was further reacted with CH_3I in acetone in the presence of K_2CO_3 as the base at 56°C to get final **CAP-10** in 40% yield. The probe **CAP-11** was synthesized using a similar route of azo coupling between 2,6-dibromo-4-sulfonamide aniline with 3,5-dibromo phenol using NaNO_2 and 40% HCl in CH_3OH to get the coupled product in 20-40% yield. This was further reacted with CH_3I in acetone in the presence of K_2CO_3 at 56°C to get final **CAP-11** in 50% yield.



Scheme 3.8 Synthesis of **CAP-10** and **CAP-11**

The photoisomerization properties of **CAP-10** and **CAP-11** were studied in methanol (Figure 3.6 A, D) and aqueous buffer (Figure 3.6 B, E). Both the probes can easily be isomerized to the *cis* isomer using 550 nm light (green) and back to *trans* isomer by using 460 nm light (blue). This was promising as we were able to achieve both directiona of photosomerization using visible light.

The ability of differential interaction between both isomers of these probes with carbonic anhydrase was studied using a NPA assay. The activity of bCA towards NPA hydrolysis was monitored in the absence (control) and presence of the *trans* and *cis* isomers as generated by 460 nm and 510 nm photoirradiation respectively. As shown in the figure 3.6 C and Figure 3.6 F for **CAP-10** and **CAP-11**, the activity of protein is inhibited in the presence of probes pre- and post-photoirradiation with 460 nm, which shows the binding of the *trans* isomers of both the probes to the active site of the protein. However, no activity was regained following conversion to the *cis* isomer using 520 nm. This was quite unexpected and shocking especially when we had evidence of the formation of *cis* isomer as determined by ^1H NMR spectroscopy (Figure 3.7). There could be two possible theories for such behavior: 1) the probe interacts with the Zn ion through sulfonamide in the *cis* conformation; 2) there are other non-covalent interactions

of the azobenzene core with essential amino acids in the active site that affect the catalytic cycle or restrict access of substrate or affect catalysis.

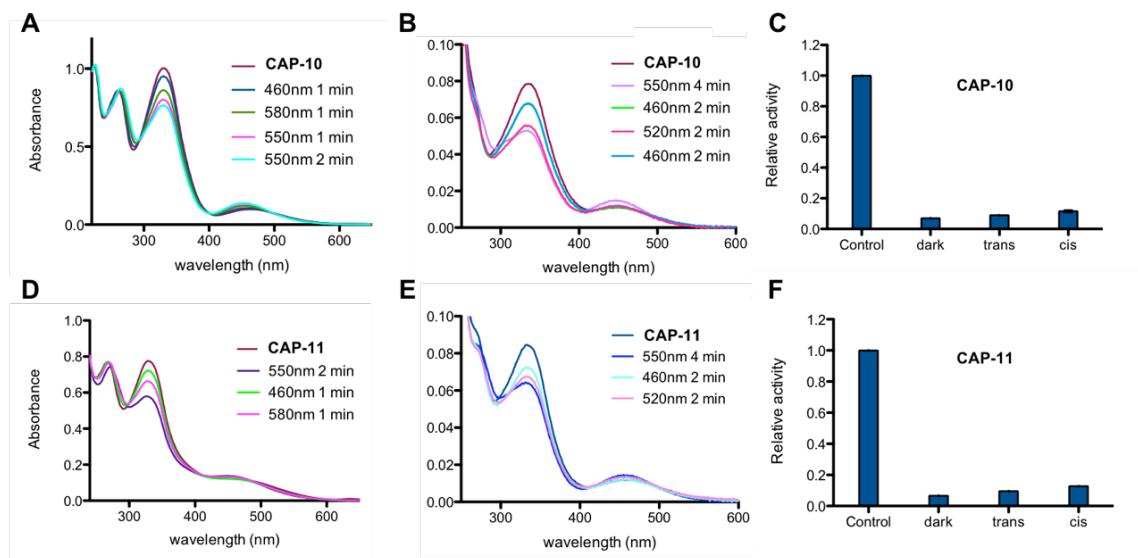


Figure 3.6 The isomerization of **CAP-10** in methanol (A) and aqueous buffer (B), and the effect of *trans* and *cis* isomer of **CAP-10** on the esterase activity of bCA as analyzed by NPA assay (C). The isomerization of **CAP-11** in methanol (D) and aqueous buffer (E), and the effect of *trans* and *cis* isomer of **CAP-11** on the esterase activity of bCA as analyzed by NPA assay (F).

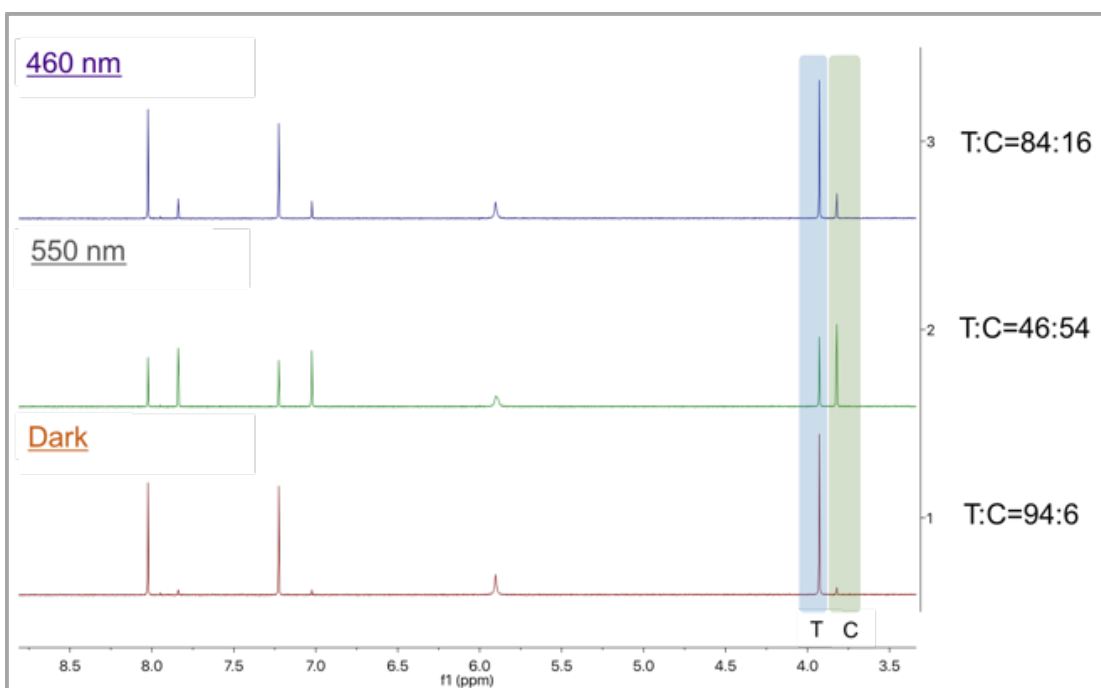


Figure 3.7 The isomeric content of **CAP-11** at PSS generated by 460 nm and 550 nm photoirradiation, as determined by ^1H NMR spectroscopy.

We tested different hypotheses to understand this behavior such as: in-depth structural analysis for the possible interaction of the probe with residues at the entrance of protein active site that might hinder the access of substrate leading to protein inhibition; establishing the difference in protein stability due to different interactions with the *trans* and *cis* isomers as measured by differential scanning calorimetry (DSC); DNSA competition assay to study the different binding affinities of our probes; monitoring the intrinsic tryptophan quenching by different interactions of the *trans* and *cis* isomers with the protein in the active site. However, none of these efforts provided an explanation for the peculiar CA activity data.

In a final attempt to understand the behavior of these molecules, we designed a control molecule for **CAP-11** where the sulfonamide group was replaced by a hydrogen atom to get **CAP-11-control**. The synthesis was similar to **CAP-11** where 2,6-

dibromianiline was coupled with 3,5-dibromophenol in the presence of 40% HCl in CH₃OH and NaNO₂ to get the product in 40% yield. The phenolic group was methylated by treatment with CH₃I in K₂CO₃ and acetone under refluxing condition to obtain **CAP-11-control** in 50% yield (Figure 3.8 A).

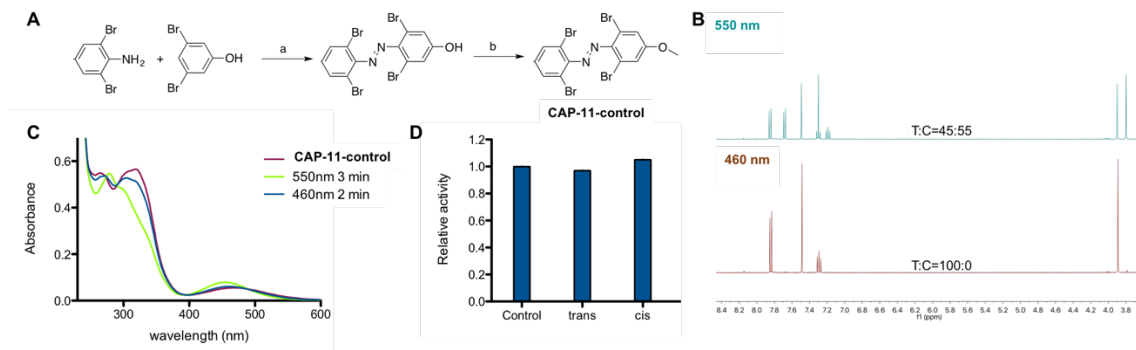


Figure 3.8 Synthesis of **CAP-11-control** (A). The percent of *cis* and *trans* species formed at PSS generated by 460 nm and 550 nm photoirradiation, as determined by ¹H NMR spectroscopy in CH₃OD (B). The absorbance profile of the probe before and after irradiation with 460 nm and 550 nm, as determined by UV-vis spectroscopy in aqueous buffer (C). The esterase activity of bCA in the presence of *trans* and *cis* isomer of the probe, as determined by NPA assay (D).

The photosomerization of **CAP-11-control** was studied through UV-vis absorbance spectroscopy. As shown in Figure 3.8 C, the probe can be isomerized to *cis* using 550 nm light and back to *trans* using 460 nm light with adequate photoisomerization efficiency as shown in Figure 3.8 B. The binding of the probe to the protein was studied by an NPA activity assay. As shown in Figure 3.8 D, the probe does not inhibit the protein activity in either the *trans* or *cis* conformations, eliminating the theory where non-covalent interaction of the azobenzene core with the protein were the reason for activity inhibition of **CAP-11**. The difference in behavior of **CAP-11** and its control molecule is due to interaction of –SO₂NH₂ with Zn²⁺ in the active site. In our

general scheme, the *cis* conformation is predicted to not interact with Zn^{2+} in the active site due to the large change in dihedral angle of azo group and phenyl rings when molecule isomerize from *trans* to *cis* isomer. However, if this angle change is low, we might not get differential interaction of the probe with different isomers. As $-\text{Cl}$ and $-\text{Br}$ are bulky groups, we believe that even though the probe is undergoing photoisomerization to produce the *cis* isomer, the change in conformation is not enough to avoid the interaction of $-\text{SO}_2\text{NH}_2$ with Zn^{2+} ion, thereby inhibiting the protein in *cis* conformation of tetra-*ortho*-chloro and tetra-*ortho*-bromo derivatives of azobenzene sulfonamide.

3.4.4 Fluorinated Derivatives

As the approach of tetra-halogenation helped in obtaining photoisomerization process by using visible light, we decided to switch to a halogen of smaller size: fluorine!²⁶⁻²⁸ Due to the similar size of fluorine to hydrogen, we hoped to gain differential control over the protein activity by manipulating the number of fluorine atoms on the benzene groups. Further, the *para*-methoxy group was replaced by electron withdrawing groups, as that is known to work in the favor of getting the desired photophysical properties of azobenzene, such as high lifetime of the *cis* isomer, which is discussed in detail in Chapter 4.

3.5 CONCLUSION

We attempted to synthesize a number of azobenzene sulfonamides **CAP-1** to **CAP-11**, and were successful for a subset of our synthetic targets. The successfully achieved chemical modifications either resulted in a red-shift of the π - π^* band (**CAP-3** to **CAP-6**) or a large separation of the n - π^* band between *trans* and *cis* isomers (**CAP-8** to

CAP-11) that allowed us to achieve the photoisomerization using two distinct visible wavelengths. These molecular systems were actuated with various photo-switching wavelengths and thermal conversion rates tailored to suit the desired photophysical properties to photomodulate the activity of carbonic anhydrase.

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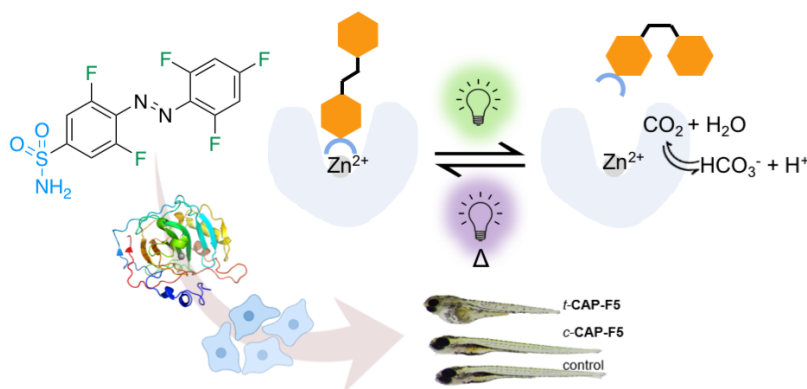
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Chapter 4: Visible light mediated bidirectional control over carbonic anhydrase activity in cells and in vivo using azobenzene sulfonamides³

4.1 ABSTRACT

Two azobenzene sulfonamide molecules with thermally stable *cis* configurations resulting from fluorination of positions *ortho* to the azo group are reported that can differentially regulate the activity of carbonic anhydrase in the *trans* and *cis* configurations. These fluorinated probes each use two distinct visible wavelengths (520 nm and 410 nm or 460 nm) for isomerization with high photoconversion efficiency. Correspondingly, the *cis* isomer of these systems is highly stable and persistent (as evidenced by structural studies in solid and solution state), permitting regulation of metalloenzyme activity without continuous irradiation. Herein, we use these probes to demonstrate the visible light mediated bidirectional control over the activity of zinc-dependent carbonic anhydrase in solution as an isolated protein, in intact live cells and *in vivo* in zebrafish during embryo development.



³ This chapter is based on published work:

Aggarwal, K.; Kuka, T.P.; Banik, M.; Medellin, B.; Ngo, C.; Xie, D.; Fernandes, Y.; Dangerfield, T.L.; Ye, E.; Bouley, B.; Johnson, K.; Zhang, Y.J.; Eberhart, J.; Que E.L. Banik, M.; Medellin, B.; Que, E. L. *J. Am. Chem. Soc.*, **2020**, *142*, 34, 14522-14531.

Author Contribution: KA conceived and initiated the project, synthesized the molecules, acquired and analyzed the characterization data, and prepared and revised the manuscript.

4.2 INTRODUCTION

Photopharmacology, in which the activity of pharmacophores can be controlled via application of specific wavelengths of light, has emerged as an attractive method for controlling the behavior of biological species on demand. Light serves as an ideal external control element for *in situ* biochemical manipulation because it offers a high level of spatiotemporal resolution, it is minimally invasive, and its wavelength and intensity can be readily tuned. One important strategy used in the development of photoactivable probes is alteration of the properties of a molecule (chemical structure, shape, polarity) upon light irradiation resulting in different affinity towards the desired target. Recent years have seen great advances in this field where the control over biomacromolecular function has been attained either by using a photoprotecting group¹ (irreversible approach) or small photochromic moieties² (reversible approach). The latter approach uses a photoswitchable bond that allows access to different molecular structure induced by irradiation of light. Although stilbenes, hemithioindigos and diarylethenes have been used as switches, azobenzenes shows the most promise in achieving a larger range of activation wavelengths and greater photoactivated state stabilities.³ They have been readily used to manipulate a wide variety of biological processes⁴ including regulation of protein expression,⁵ enzyme folding⁶ and activity^{7, 8}, and DNA conformation and transcription,^{9, 10} and have been explored in the fields of chemotherapeutics^{11, 12} and neurobiology^{13, 14}.

Carbonic anhydrase (CA) is a promising target for photoregulation due to its physiological and pathological roles.¹⁵ The active site of most CAs contains a zinc ion bound to three histidine residues and one water molecule. This protein catalyzes a simple physiological reaction, the reversible conversion of carbon dioxide and water to bicarbonate and protons. CAs are involved in the maintenance of acid-base equilibrium

and CO₂ homeostasis. However, they can be highly overexpressed in diseases, including cancer, epilepsy, and glaucoma, and hence, are important therapeutic targets.¹⁶ Effective photoswitchable inhibitors of CA have promise as research tools as they can be activated in a spatially and temporally specific manner. Therapeutic applications are also possible, particularly for glaucoma due to its location in the eye where light activation is very feasible.¹⁷

Aryl sulfonamides show exquisite selectivity for CA inhibition, combining metal coordination, hydrogen bonding, and hydrophobic interactions to achieve this specificity.¹⁸ Others have reported azobenzene-based photoswitches for CA, however studies have been limited to isolated protein or cell lysates.¹⁹⁻²¹ Recently, we reported small molecule azobenzene sulfonamide **CAP1** that demonstrated differential regulation of intracellular pH in the *trans* and *cis* isomeric forms in live cancer cells.²² However, this probe had three major limitations: use of UV light for photo-isomerization, poor photoconversion efficiency, and importantly, poor thermal stability of the *cis* isomer, which limited long-term applications in live cells and organisms.

To address these limitations, we identified *ortho*-fluorination as a strategy to improve the performance of azobenzene-based probes. Fluorination *ortho* to the azo moiety of the azobenzene results in separation of the n- π^* absorption band in the *trans* and *cis* isomers.²³ This splitting of absorption bands can further be increased by the incorporation of an electron withdrawing group at the position *para* to the azo unit. This large separation of n- π^* bands enables use of distinct visible wavelengths to achieve higher isomer conversion in the photostationary state when compared to other azobenzene derivatives. Remarkably, the *cis* isomers of *ortho*-fluorinated azobenzenes are exceptionally thermally stable with half-lives as long as several days.²⁴ Furthermore, unlike other visible light sensitive and stable *cis*-azobenzene derivatives, *ortho*-

fluorinated systems are immune to the cytosolic reducing environment making them more biocompatible.²⁵ *Ortho*-fluorinated azobenzenes have been extensively used for materials applications^{13, 26-29} and have gained interest recently in biological applications³⁰⁻³⁴, demonstrating the robustness of this system in the context of biomolecules and cells. Herein, we report the first demonstration of the *ortho*-fluorination strategy for controlling the activity of a metalloenzyme. Importantly, we demonstrate the efficacy of this approach for dynamic modulation of the activity of Zn-dependent carbonic anhydrase *in vitro* with isolated protein, in live cells, and for the first time, *in vivo* in live zebrafish.

4.3 EXPERIMENTAL SECTION

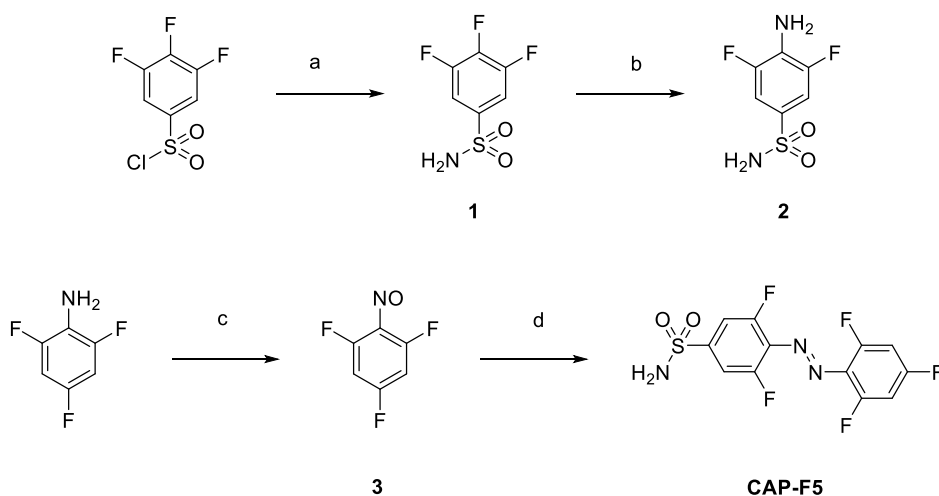
4.3.1 General Procedures

All solvents and starting materials were purchased from Sigma-Aldrich and Fischer Sci. and used as is. For protein studies, bovine carbonic anhydrase was purchased from Sigma-Aldrich. For spectroscopic and protein studies, 0.05 M HEPES buffer with 0.1 M KNO₃ (pH 7.2) was used as aqueous buffer, unless stated otherwise. All characterization studies were performed under dark conditions.

The NMR spectroscopic measurements were collected in deuterated solvents from Cambridge Isotope Laboratories (Cambridge, MA), using an Agilent MR 400 NMR spectrometer at 400 MHz for ¹H and ¹⁹F, Burkert AVIII HD liquid nitrogen Cryoprobe at 500 MHz for ¹³C, and AutoXDB PFG at 600 MHz for 2D NMRs. The chemical shift is calibrated with respect to solvent peak, and is reported in ppm. UV-Vis absorbance spectroscopic studies were performed using an Agilent Cary 60 UV-Vis spectrophotometer. The absorbance values in 96 well plates were recorded on a Tecan Infinite M200 microplate reader. Fluorescence spectroscopic measurements were collected using an Agilent Cary Eclipse fluorescence spectrofluorimeter. The samples

were irradiated with an Asahi Xenon lamp fitted with collimator and bandpass filters of 410 nm (band width 10 nm; 5.20 mW/cm²), 460 nm (FWHM 11 nm; 14.12 mW/cm²), and 520 nm (band width 10 nm; 9.18 mW/cm²) from Asahi spectra and Thorlabs. Walk-up LC-MS and high-resolution Electrospray Ionization (ESI) mass spectral analyses were performed by the Mass Spectrometry Facility of the Department of Chemistry at UT Austin. X-Ray crystallography was performed on an Agilent Technologies SuperNova Dual Source diffractometer, using a μ -focus Cu K α radiation source ($\lambda = 1.5418\text{\AA}$) with collimating mirror monochromators. An EVOS[®] FL Auto inverted fluorescence microscope was used to image the cells. The flow cytometry analysis was done on BD Accuri Cytometer (BD Biosciences, San Jose, CA) using 488 nm laser to provide excitation, while collecting emitted photons through a 575/25 nm bandpass filter. The confocal z-stacks of otolith of zebrafish were collected on a Zeiss LSM 710 using Zen software. Analytical and semi-preparative RP-HPLC were performed on a Thermo Scientific Dionex Ultimate 3000 instrument equipped with a PDA detector and Synchronis C18 column, 5 μ m, 4.6 x 250 mm.

4.3.2 Synthetic Procedures



Scheme 4.1 Synthesis of **CAP-F5**

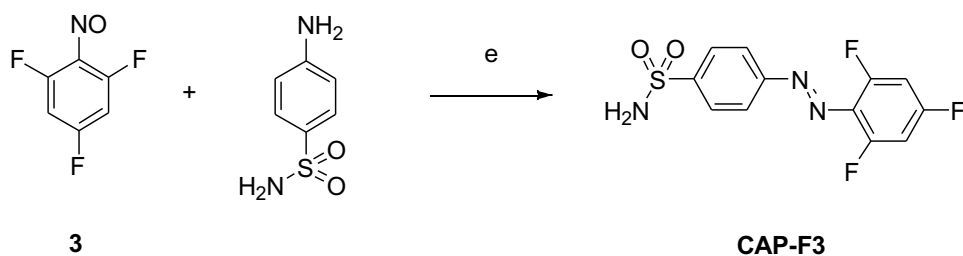
Synthesis of Compound 1: To 25% aqueous ammonium hydroxide (25 mL solution, 178 mmol) in 100 mL round bottom flask, trifluorobenzene sulfonyl chloride (1.5 g, 6.5 mmol) was added and stirred for 2 hours at room temperature. The product was extracted with ethyl acetate three times, and the combined organic layer was dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure to obtain the product as a white powder (1.26 g, 92% yield). ^1H NMR (400 MHz, d-CDCl_3) δ 7.59-7.62 (m, 1 H), δ 4.97 (s, 1 H). ^{19}F NMR (400 MHz, d-CDCl_3) δ 129.38-129.45 (m, 2 F), δ 151.72 (s, 1 F). LR ESI-MS (ESI, MeOH): calculated for $[\text{C}_6\text{H}_3\text{F}_3\text{NO}_2\text{S}]^-$ 209.99, found 210.1.

Synthesis of Compound 2: **Compound 1** (700 mg, 3.315 mmol) was dissolved in a Parr high pressure reactor with 15 mL of 7 M NH_3/MeOH . The reactor was sealed and heated at 120 °C for 2 days. After cooling the system to room temperature, the reaction mixture was concentrated under reduced pressure. The crude mixture was loaded on celite and purified through reverse phase column chromatography to elute the product at 4% acetonitrile/water to obtain the product as a white powder (414 mg, 60% yield). ^1H NMR (400 MHz, $\text{d}_6\text{-DMSO}$) δ 7.27-7.30 (m, 2 H), δ 7.21 (s, 1 H), δ 6.01 (s, 1 H). ^{19}F NMR (400 MHz, $\text{d}_6\text{-DMSO}$) δ 130.13-130.23 (m, 2 F). LR ESI-MS (ESI, MeOH): calculated for $[\text{C}_6\text{H}_5\text{F}_2\text{N}_2\text{O}_2\text{S}]^-$ 207.01, found 207.1.

Synthesis of Compound 3: 2,4,6-trifluoroaniline (500 mg, 3.39 mmol) was dissolved in a ratio 1:2 DCM: H_2O (10 mL DCM: 20 mL H_2O), and Oxone (2.09 g, 6.79 mmol) was added at room temperature. After vigorous stirring for 3 hours, the organic phase was washed with 1 N HCl and brine and dried with anhydrous sodium sulfate. The solvent was removed in vacuo and the unreacted reactant was removed from the crude

mixture through normal phase column chromatography with 100% CHCl₃, and the product mixture was then directly used for the next reaction.

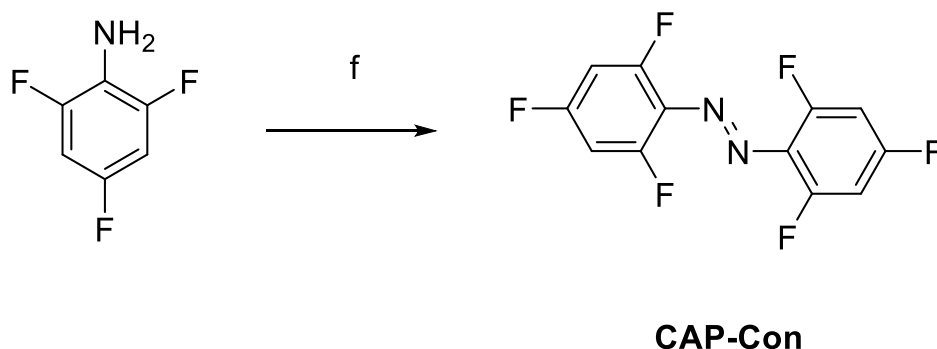
Synthesis of Compound CAP-F5: **Compound 2** (100 mg, 0.48 mmol) was taken in acetic acid:TFA:toluene (6:1:6) mixture, and **Compound 3** (116.1 mg, 0.721 mmol) was added to it, and stirred at 45 °C for a week. The compound was extracted in ethyl acetate, washed with saturated NaHCO₃, water, and brine, dried over anhydrous sodium sulfate, and concentrated under vacuum. The crude product was loaded on a normal phase silica column, and the product eluted at 2.5% MeOH/CHCl₃. The solvent was evaporated and the compound was dried under vacuum to obtain a red solid (42.2 mg, 25% yield). ¹H NMR (400 MHz, d₆-DMSO) δ 7.66-7.68(d, 2 H), δ 7.11-7.16 (t, 2 H). Residual water remains in spectrum. ¹⁹F NMR (600 MHz, CD₃OD) δ 120.50-120.52 (d, 2 F), δ 117.45-117.48 (t, 2 F), δ 102.56-102.63 (m, 1 F). HR ESI-MS (ESI+, MeOH): calculated for [C₁₂H₇F₅N₃O₂S]⁺ 352.0174, found 352.0177.



Scheme 4.2 Synthesis of **CAP-F3**

Synthesis of CAP-F3: 4-aminobenzenesulfonamide (200 mg, 1.16 mmol) was dissolved in 10 mL acetic acid, and **Compound 3** (545.2 mg, 3.38 mmol) was added to it. The reaction was stirred at 60 °C for 24 hours. The compound was extracted in ethyl acetate, washed with saturated NaHCO₃, water, and brine, dried over anhydrous sodium sulfate, and concentrated under vacuum. The crude product was loaded on normal phase silica column, and the product eluted at 5% MeOH/CHCl₃. The solvent was evaporated

and the compound was dried under vacuum to obtain the red product (146.3 mg, 40% yield). ^1H NMR (400 MHz, d_6 -DMSO) δ 7.98-8.04 (dd, 4 H), δ 7.56 (s, 1 H), δ 7.26-7.29 (t, 1 H). Residual water remains in spectrum. ^{19}F NMR (600 MHz, CD_3OD) δ 117.98-118.03 (t, 2 F), δ 104.47-104.56 (m, 1 F). HR ESI-MS (ESI+, MeOH): calculated for $[\text{C}_{12}\text{H}_7\text{F}_5\text{N}_3\text{O}_2\text{S}]^+$ 316.0362, found 316.0369.



Scheme 4.3 Synthesis of **CAP-Con**

Synthesis of CAP-Con: 2, 4, 6-trifluoroaniline (500 mg, 3.39 mmol) was dissolved in 34 mL DCM. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (1.69 g, 6.11 mmol) was grounded in a mortar and added to the reaction. Then KMnO_4 (1.69 g, 10.8 mmol) was added to the reaction, which refluxed overnight at 45 °C. The compound was extracted in ethyl acetate, washed with saturated NaHCO_3 , water, and brine, dried over anhydrous sodium sulfate, and concentrated under vacuum. The crude product was loaded on normal phase silica column, and the product eluted at 10% EtOAc/hexanes. After solvent evaporation under vacuum, an orange/red solid was obtained in quantitative yield. ^1H NMR (400 MHz, d_4 -MeOD) δ 7.06-7.12 (m, 4 H). ^{19}F NMR (400 MHz, d_4 -MeOD) δ 118.56-118.76 (m, 4 F), δ 104.65-104.74 (m, 2 F). HR ESI-MS (ESI+, MeOH): calculated for $[\text{C}_{12}\text{H}_5\text{F}_6\text{N}_2]^+$ 291.0351, found 291.0354.

4.3.3 Characterization

4.3.3.1 CAP photoisomerization

The photoisomerization of **CAP-F3** and **CAP-F5** (10 mM stock in DMSO) was investigated by electronic absorption spectroscopy. The absorbance spectrum was collected in methanol and aqueous buffer (50 mM HEPES, 0.1M KNO₃, pH 7.2) under dark conditions. To get the *cis* isomer profile, the sample was irradiated with 520 nm for 5 minutes, and spectrum was collected. To isomerize the *cis* isomer back to *trans* isomer, the sample was irradiated with 460 nm (**CAP-F3**) or 410 nm (**CAP-F5**) for two minutes before collecting the absorbance spectrum.

The real-time isomerization kinetics of the probes was studied by irradiating the solution with the desired wavelength perpendicular to the direction of spectrophotometer light beam. The absorbance value was recorded at $\lambda_{\max}$ every 2 seconds with Cary enzyme kinetics software. The absorbance versus time profile obtained was fitted to one phase decay equation to get the rate constant

$$y = (y_0 - y_{\max})e^{-kx} + y_{\max}$$

where y_0 is the initial absorbance at $\lambda_{\max}$; $y_{\max}$ is the absorbance at saturation (or maximum conversion); and k is the rate constant.

The lifetime of the *cis*-isomer at 37°C was determined by using UV-Vis spectroscopy in aqueous buffer. The sample was prepared by diluting 10 mM DMSO stock solution of CAP-F3 and CAP-F5 in aqueous buffer. The probe was photoisomerized to *cis* isomer by irradiating the sample with 520 nm, and the absorbance spectrum was collected every 1 hour at 37 °C for 48 hours under dark. The absorbance value at $\lambda_{\max}$ was plotted with respect to time to get the % thermal relaxation.

The *trans* and *cis* ratio at PSS was obtained by irradiating the sample in CD₃OD with 410/460 nm and 520 nm before collecting ¹⁹F NMR and ¹H spectra with 30 seconds relaxation delay to ensure the relaxation of all spins for quantitative measurement.

The pure *cis* and *trans* isomers of **CAP-F3** and **CAP-F5** were separated using HPLC method.

4.3.3.2 Structural Characterization

The probe was dissolved in minimum amount of solvent (MeCN/H₂O for *trans* and methanol for *cis*) and stored in dark at room temperature until crystals were obtained.

Empirical formula	C ₁₂ H ₆ F ₅ N ₃ O ₂ S
Formula weight	351.26
Temperature/K	100
Crystal system	monoclinic
Space group	P2 ₁ /n
a/Å	6.9237(7)
b/Å	26.744(3)
c/Å	7.0056(7)
α/°	90
β/°	95.630(5)
γ/°	90
Volume/Å ³	1290.9(2)
Z	4
Tmin/Tmax	0.741/1.000
ρ _{calc} /g/cm ³	1.807
μ/mm ⁻¹	0.327
F(000)	704.0
Crystal size/mm ³	0.187 × 0.181 × 0.087
Radiation	MoKα (λ = 0.71073)
2θ range for data collection/°	6.038 to 50.14
Index ranges	-8 ≤ h ≤ 8, -31 ≤ k ≤ 31, -8 ≤ l ≤ 8

Reflections collected	8777
Independent reflections	2280 [$R_{\text{int}} = 0.0622$, $R_{\text{sigma}} = 0.0692$]
Data/restraints/parameters	2280/0/214
Goodness-of-fit on F^2	1.033
Final R indexes [$I \geq 2\sigma(I)$]	$R_1 = 0.0419$, $wR_2 = 0.0760$
Final R indexes [all data]	$R_1 = 0.0754$, $wR_2 = 0.0858$
Largest diff. peak/hole / $e \text{ \AA}^{-3}$	0.34/-0.44

Table 4.1 The crystal data and structural refinement for *t*-CAP-F5.

Empirical formula	$\text{C}_{12}\text{H}_6\text{F}_5\text{N}_3\text{O}_2\text{S}$
Formula weight	351.26
Temperature/K	100.0(5)
Crystal system	triclinic
Space group	P-1
$a/\text{\AA}$	6.0686(3)
$b/\text{\AA}$	10.2512(5)
$c/\text{\AA}$	21.8804(13)
$\alpha/^\circ$	85.750(4)
$\beta/^\circ$	86.889(5)
$\gamma/^\circ$	86.402(4)
Volume/ $\text{\AA}^3$	1353.09(12)
Z	4
Tmin/Tmax	0.71186/1.00000
$\rho_{\text{calc}}/\text{g cm}^{-3}$	1.724
μ/mm^{-1}	2.859
F(000)	704.0
Crystal size/ mm^3	$0.119 \times 0.066 \times 0.057$
Radiation	$\text{CuK}\alpha$ ($\lambda = 1.54184$)
2Θ range for data collection/ $^\circ$	8.114 to 150.15
Index ranges	$-7 \leq h \leq 7$, $-12 \leq k \leq 12$, $-25 \leq l \leq 27$
Reflections collected	15032
Independent reflections	5394 [$R_{\text{int}} = 0.0679$, $R_{\text{sigma}} = 0.0611$]

Data/restraints/parameters	5394/2/427
Goodness-of-fit on F ²	1.038
Final R indexes [I>2σ (I)]	R ₁ = 0.0906, wR ₂ = 0.2537
Final R indexes [all data]	R ₁ = 0.1166, wR ₂ = 0.2866
Largest diff. peak/hole / e Å ⁻³	0.79/-0.46

Table 4.2 The crystal data and structural refinement for *c*-**CAP-F5**.

4.3.3.3 DNSA Competition Assay

Binding analysis of *trans* and *cis* isomer of **CAP-F5** was performed using DNSA competition assay with bovine carbonic anhydrase (bCA) at room temperature in black flat bottom 96 well plates. A stock solution of 10 mg/mL (~350 μM) enzyme was prepared in 50 mM HEPES, 100 mM KNO₃, pH 7.2. The stock solutions of 10mM **CAP-F5** under dark and 1mM DNSA were prepared in DMSO. The dissociation constant of DNSA was obtained by titrating enzyme (100nM) with DNSA varying from 0-20 μM with 0.4% final DMSO content. The well plate was incubated at room temperature for 5 minutes to allow the formation of the E-DNSA complex. Each well was then read for FRET emission of DNSA (λ_{em}= 458 nm) from Trp excitation (λ_{ex} = 280 nm). The profile obtained was fitted to the equation:

$$F_{Total} = \frac{F_{obs} - F_{ini}}{F_{end} - F_{ini}} = \frac{1}{1 + K_d/[DNSA]}$$

where, F_{total} is the total fluorescence, F_{obs} is the observed fluorescence, F_{ini} is the fluorescence in the absence of DNSA, F_{end} is the fluorescence at saturation and K_d is DNSA dissociation constant.

The dissociation constant of isomers with enzyme was obtained by competition with DNSA binding. A solution of 100 nM enzyme and 10 μM DNSA was prepared and incubated at RT for 5 minutes. The stock solution of **CAP-F5** was irradiated with 520 nm to get *cis* isomer and 410 nm to get *trans* isomer. The E-DNSA complex was titrated with

CAP-F5 varying from 0-8 μM (*c*-**CAP-F5**) and 0-0.5 μM (*t*-**CAP-F5**). After 5 minutes of incubation at RT, the plate was read for fluorescence emission at 458 nm upon excitation at 280 nm. The quenching profile obtained due to the replacement of DNSA by the probe in active site was fitted to the following equation to get the K_d of the probe:

$$F_{Total} = \frac{F_{obs} - F_{end}}{F_{ini} - F_{end}} = \frac{1}{1 + (K_{DNSA}/[DNSA])(1 + [probe]/K_{probe})}$$

where, F_{total} is the total fluorescence, F_{obs} is the observed fluorescence, F_{ini} is the fluorescence in the absence of probe, F_{end} is the fluorescence at saturation, K_{DNSA} is DNSA dissociation constant, as analyzed earlier and K_{probe} is dissociation constant of probe.

To study the reversibility of CA inhibition by isomerizing the probe *in-situ* in the enzyme solution, 1 eq of *c*-**CAP-F5** (irradiated with 520 nm prior to addition) was added in a solution of 100 nM enzyme and 10 μM DNSA, and the fluorescence was read at 458 nm. The solution was irradiated with alternate cycles of 410 nm and 520 nm to get *trans* and *cis* isomer in the solution, and the fluorescence was read in between each cycle.

4.3.3.4 Probe Docking

The PDB 5BYI of hCAII complexed with an azobenzene ligand was used to perform all docking simulations. The model was then obtained with the ligand placed into the active site with L configuration as initial reference. The binding mode is then optimized with Maestro (Schrödinger, LLC)³⁵ which has a minimization routine based on OPLS_2005 Forcefield^{36, 37}. Utilizing the Maestro from Schrodinger, the initial position of the ligand was obtained by the superimposition of ligands into the position of L as a reference and then conducting energy minimization in Maestro. Steric clashes were analyzed using the Pymol bump script^{38, 39}. Retrieved *c*-**CAP-F5** was modeled manually

while keeping the same orientation as the L in the crystal structure through rigid movement. All figures were prepared with Pymol.

4.3.3.5 Stopped Flow Assay

The inhibitory effect of *trans* and *cis* isomer of **CAP-F5** on the CO₂ hydration activity of CA was measured and compared by the changing pH-indicator method in Kintek SF-300X at 25 °C. The aqueous buffer of HEPES at 50 mM concentration was maintained at an ionic strength of 0.1M KNO₃ and pH 7.2 was used for the assay.

A stock solution of 350 µM bCA was prepared and diluted to 200 nM in aqueous buffer. A stock solution of 50 mM phenol red was prepared in water and diluted in enzyme solution to a final concentration of 0.2 mM. The stock solution of 100 µM **CAP-F5** was prepared in DMSO, and irradiated by 410 nm to get *t*-**CAP-F5** and 520 nm to get *c*-**CAP-F5**. The probe was diluted in the assay buffer to get at least 10 different concentration in the range of 0-1600 nM, and was incubated with enzyme solution at 25 °C for 30 minutes to get the E•I complex. The substrate solution for bicarbonate dehydration activity was prepared by dissolving KHCO₃ in water to get 30 mM solution. The substrate solution for carbon dioxide hydration activity was prepared by bubbling CO₂ through water to get saturated solution at 25 °C (for Carbon dioxide hydration activity). The E•I complex and substrate were mixed in a 1:1 ration in the instrument, with light at 557 nm and the absorbance was recorded for 120 seconds (30 seconds for uninhibited reactions). The absorbance of phenol red was scaled by addition or subtraction so all traces starts at either 1 (CO₂ hydration) or 0 (HCO₃⁻ dehydration). The initial rate of the enzyme was determined by the slope of the linear part of absorbance versus time, and plotting with varying concentration of the probe (in replicates of five). Although in literature it has been shown that sulfonamides bind to CA in non-competitive

manner, the data here is insufficient to determine well-constrained parameters (K_{ii} and K_{is}) simultaneously for this model:

$$v = \frac{k_{cat}[S]}{K_m \left(1 + [I]/K_{ii}\right) + [S] \left(1 + [I]/K_{is}\right)}$$

As these parameters (K_{cat} and K_m) will be constant for both *t-CAP-F5* and *c-CAP-F5* incubation, this equation can be used to extract apparent K_i (a function of K_{ii} and K_{is}) by fitting the data in the following simplified equation:

$$V = \frac{V_0}{\left(1 + \frac{[I]}{K_i'}\right)}$$

where V is the reaction velocity at a particular inhibitor concentration, V_0 is the reaction velocity in the absence of inhibitor, $[I]$ is inhibitor concentration in the flow cell, K_i' is the apparent inhibitory constant.

Five experiments were done for each inhibitor concentration, and the values are reported as the mean of such results.

4.3.3.6 Data Fitting in Kintek Explorer

To fit the bicarbonate data in Kintek Explorer, the stopped flow data following the reaction without added protein was first used to come up with estimates for the $S = P$ step in the model. Next, the reaction with added protein (no inhibitor) was used to get a fit for the enzyme catalyzed steps in the model ($E+S = ES = EP = E+P$). Because a high, saturating substrate concentration was used, the rate constants chosen for this part of the model are individually not defined by the data, however they fit the experimental data without inhibitor and when locked in the fitting, allowed well defined determination of the inhibition parameters for the two forms of the inhibitor. The second order rate constants for substrate and product binding ($E+S = ES$, and $E+P = EP$) were locked at $100 \mu\text{M}^{-1} \text{s}^{-1}$ and the off rates were locked at an arbitrary value of $\sim 250,000 \text{s}^{-1}$. Rate

constants for the chemistry step ($ES = EP$) in both directions were then adjusted to fit the enzyme catalyzed reaction data and subsequently locked. Next, inhibition by the *cis* and *trans* isomer of the probe was modeled with the $E+I = EI$ (for *c*-CAP-F5) and $E+I_{trans} = EI_{trans}$ (for *t*-CAP-F5) steps. The second order inhibitor binding steps are not defined by the data and were therefore locked at $100 \text{ uM}^{-1} \text{ s}^{-1}$. Finally, all data were fit together to the model, only allowing the inhibitor dissociation rates to float in the fitting. The results of the global fit show that the trans inhibitor binds $\sim 100\text{x}$ tighter than the cis inhibitor.

4.3.4 Cell Culture

The cell culture media and bio supplies were purchased from Sigma Aldrich, unless specified otherwise. HeLa cell culture was performed in sterile bio cabinet using Dulbecco's modified Eagle's Medium (DMEM), supplemented with 4000 mg/L glucose and sodium pyruvate, 10% heat inactivated fetal bovine serum (FBS), and 1% antibiotics (200 U/cm³ penicillin and 200 $\mu\text{g}/\text{cm}^3$ streptomycin). The cells were grown in T-75 Corning Flasks and incubated at 37 °C in atmosphere containing 5% CO₂. They were passaged once the confluency of the cells reached 80% using trypsin.

4.3.4.1 Multiple isomerization in cell media

The fatigue resistance of CAP-F5 was determined by preparing a solution of CAP-F5 in cell culture media (DMEM without phenol red) and irradiating with alternate 520 nm and 410 nm wavelength for 5 min and 2 min respectively. The absorbance spectrum was collected in between the irradiation for 20 cycles of photoisomerization.

4.3.4.2 MTS Assay

The cytotoxic effects of *t*-CAP-F5 and *c*-CAP-F5 on HeLa cells were analyzed using MTS Cells proliferation colorimetric assay kit (BioVision). The cells were plated in

96 well plate at a density of 4000 cells/well in phenol red free DMEM media with same composition of glucose, sodium pyruvate, glutamine, FBS and antibiotics. 24 hours later, different concentrations of *trans* and *cis* isomer of **CAP-F5** (generated by photoirradiation with 410 nm and 520 nm prior to the addition) were added to have a final volume of 150 μ L media in each well. A control set of wells was set, which had an equivalent volume of DMSO content. After 24 hours of incubation, 30 μ L of MTS was added to each well, and the plate was incubated for 2 hours at 37 °C. The absorbance of wells was read at 490 nm on a plate reader and was normalized to the absorbance in control (DMSO only) wells. Samples were analyzed in triplicates, and the dose response curve was plotted in Prism and fitted to the following equation:

$$Y = \frac{100}{1 + 10^{(\text{LogEC}_{50} - X)H}}$$

where X is log of concentration of the probe, Y is normalized cell viability (0-100%), EC_{50} is concentration of probe at half-maximal response, Log EC_{50} is same log units as X, H is hill slope (unit less)

4.3.5 Intracellular pH measurements

4.3.5.1 Live Cell Imaging:

The intracellular pH (pH_i) of live cells was measured by using the pHrodoTM Red AM Intracellular pH Indicator according to the manufacturer's protocol (Molecular Probes, Eugene, OR). This fluorogenic dye quantifies the cytosolic pH in the range of 4-9 with a pK_a of 6.5. The cells were plated in 96 well plate at a density of 5000 cells/well in 100 μ L of growth media. The next day, cells were washed with PBS buffer and 100 μ L of 25 μ M **CAP-F5** prepared in DMEM was added, and incubated at 37 °C for 24 hours.

After 24 hours, a solution of 1 μ L pHrodo and 10 μ L powerload (supplied with pH kit) was prepared in 1 mL Live Cell Imaging Solution (LCIS). The cells were washed

with LCIS and 60 μ L of dye solution was added to each well. The plate was incubated at 37 °C for 30 minutes under dark conditions. After the incubation of cells with dye, they were washed with LCIS, and the cells were imaged directly under an EVOS® FL Auto inverted fluorescence microscope at excitation/emission of 560/580 nm (RFP light cube) for pHrodo dye. Total of 3 images per well (in triplicates) were taken at 20X.

Calibration: To quantify the intracellular pH of cells, the provided intracellular pH calibration kit was used (Molecular Probes, Eugene, OR). A cell loading solution was prepared by adding 1 μ L of valinomycin/nigericin (10mM each in stock solution) in 1 mL of cellular pH calibration buffer (pH 4.5, 5.5, 6.5 and 7.5). After performing the desired experiment with pHrodo, the cells were washed 2X with LCIS, and 100 μ L of prepared cell loading solution was added. The intracellular pH was allowed to equilibrate with the extracellular buffer for 10 minutes at 37 °C prior to fluorescence reading. The process was repeated with each buffer solution, and a fluorescence vs. pH calibration curve was prepared. As the conditions (e.g. concentration of dye, lamp intensity, microscope settings) can vary, calibrations were performed for each individual experiment.

Image Processing: Cell images were processed and the fluorescence was quantified using FIJI (ImageJ). 8-bit images were thresholded to define the regions of interest (ROIs) for individual cells and a binary image was created. The mean fluorescence (the integrated density/area for each cell) were quantified for all ROIs. Dead cells were eliminated manually following visual inspection, and the average of mean fluorescence values was calculated. Each image had ~50-80 cells and three images were taken per well. Each experimental condition had three wells to give a total of ~300- 500 cells per condition. The average of mean-fluorescence for each well was converted to absolute pH_i values on the basis of their calibration curve and were reported as the

mean $\pm$ SD of three wells (N=3). One-way ANOVA, followed by Bonferroni's post hoc test for multiple comparisons was used to assess statistical differences (p versus control).

4.3.5.2 FACS measurements

HeLa cells were grown in T25 flasks until 80% confluency, washed with PBS, trypsinized, and centrifuged to obtain a cell pellet, which was further washed with LCIS (2X). A solution of probe and dye was prepared by adding 1 μ L pHrodo and 10 μ L powerload (pH kit) in 2 mL of 25 μ M of *t*-**CAP-F5** or *c*-**CAP-F5** (DMSO for control) prepared in LCIS. The cells were suspended in this solution (same cell density in between samples) and incubated at 37 °C for 30 min with occasional homogenizing. The cells were centrifuged and were washed with LCIS (2X) to remove the excess probe and dye.

pH measurements: The cell pellet incubated with dye and probe was suspended in LCIS and injected into the flow cytometer instrument. The cells were separated from the debris and sorted into single cells. Each acquisition measured 20,000 cells after the gating and pHrodo fluorescence histogram was collected by using 488 nm laser for excitation and 575/25 nm band pass filter for emission.

To quantify the intracellular pH, a cell loading solution was prepared by adding 1 μ L of valinomycin/nigericin (10mM each in stock solution) in 1 mL of cellular pH calibration buffer (pH 4.5, 5.5, 6.5 and 7.5). The cell pellet was suspended in this solution and incubated for 15 minutes to equilibrate with the buffer solution. The cells were injected in the same way, and median fluorescence was plotted with respect to pH to obtain the calibration curve. This curve was generated for each individual experiment and used to calculate the absolute pH_i of unknown sample.

Response to perturbations in CO₂: The cells were treated with **CAP-F5** and pHrodo in the similar way as described earlier and the pellet obtained was stored over ice.

A solution of saturated CO₂ was prepared by bubbling CO₂ gas in MilliQ water at 4°C over a period of 2 hours. The cells were suspended in 300 µL ice-cold LCIS and an injection of 10,000 cells were made in FC every 15 seconds. After three such injections, 20 µL of ice-cold CO₂ was added, and the readings were continued. The calibration curve was plotted in the same way to determine absolute pH_i of each injections, which was further plotted with respect to time.

In situ isomerization of probe in cells: The sample was prepared in a similar way by incubating the cells with *c*-**CAP-F5**, and dividing them into two equal parts after washing out the excess probe. One part was kept as it is, while the second part was irradiated with 410 nm to *insitu* photoisomerize the probe to *trans* configuration in the cells. The samples were analyzed for responses to external perturbations to CO₂ in the same way as described earlier.

4.3.6 In Vivo Studies

4.3.6.1 Drug treatment and end point morphological analysis

Embryonic zebrafish (*Danio rerio*, AB strain) were maintained in accordance with IACUC-approved protocols at UT Austin and were staged as described in Kimmel et al. At 6hpf, the varied concentrations of *trans* and *cis* isomer of **CAP-F5**, as formed by the irradiation with 410 and 520 nm, were added in the medium with 1% final DMSO concentration. Embryos treated with 1% DMSO were used as negative control. The embryos were incubated at 28.5 °C in the dark. At 48hpf, the fish were transferred to fresh media and raised to 5dpf. The viability of the fish was analyzed and recorded. For morphology, the images were captured with an Olympus stereomicroscope with a DP22 2.8 MP color camera, DP2-SAL stand-alone kit (Olympus).

4.3.6.2 Swimming behavior

Zebrafish swimming behavior was assayed at 5 days post fertilization using a 6 well plate. Each well had a 3-centimeter diameter. Individual zebrafish were placed into a well containing 5 milliliters of embryo medium. The behavior of each fish was recorded for 20 minutes. Given that the fish were being placed into a novel environment, i.e., the well, the first 10 minutes of the trial were provided as a habituation period. Swimming behavior over the final 10 minutes of the trial were used to compare locomotor activity across treatments. The total distance moved was measured to compare locomotor activity.

4.3.6.3 Otolith development

To assay morphological defects at 5dpf, zebrafish were fixed and processed using a procedure that we routinely use for bone and cartilage analyses.⁴⁰ In short, embryos were incubated in Alcian Blue stain overnight, then rehydrated and washed in 25% glycerol. They were then incubated with Alizarin Red for 30 min, then washed and stored in 50% glycerol. They were imaged on a Zeiss Axio Imager-AI microscope with AxiovisionLE software.

4.3.6.4 Insitu photoactivation

The embryos were raised and treated with *c*-**CAP-F5** as before. The fish embryos were irradiated by 410 nm (0.50 mW/cm^2) for 3 min for *in situ* conversion of the *cis* isomer to *trans* isomer at 12 hpf, 24 hpf and 30 hpf stages of embryogenesis. The irradiation itself did not affect the development of the fish, as analyzed by irradiating the embryos with DMSO only. For comparison purposes, *t*-**CAP-F5** (generated by irradiation of probe *ex situ*) was added to the embryos at the similar time points of drug activation. After transferring the hatched fish to fresh media at 48 hpf, the embryos were raised to 5dpf, and analyzed for morphology and otolith development as described above.

4.4 RESULTS AND DISCUSSION

4.4.1 Synthesis and the photoisomerization properties of the azobenzene probes

We synthesized and characterized two fluorinated azobenzene sulfonamide probes containing either three (**CAP-F3**) or five (**CAP-F5**) fluorines. We anticipated that these probes would achieve red-shifted wavelengths of isomerization relative to non-fluorinated derivatives with improved switching fidelities and increased thermal lifetime of the *cis* isomer (Figure 4.1). Full experimental details are provided in the Experimental Section.

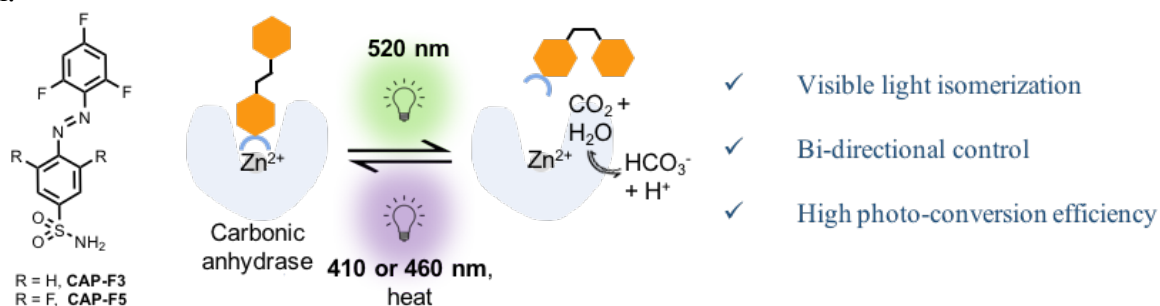


Figure 4.1 Structures of **CAP-F3** and **CAP-F5**, and photoswitching in the presence of carbonic anhydrase

4.4.1.1 Spectroscopic properties of **CAP-F3** and **CAP-F5**

The photoisomerization properties of **CAP-F3** and **CAP-F5** were monitored in aqueous buffer (Figure 4.2 A, C) and methanol (Figure 4.2 B, D) using UV-vis absorption spectroscopy.

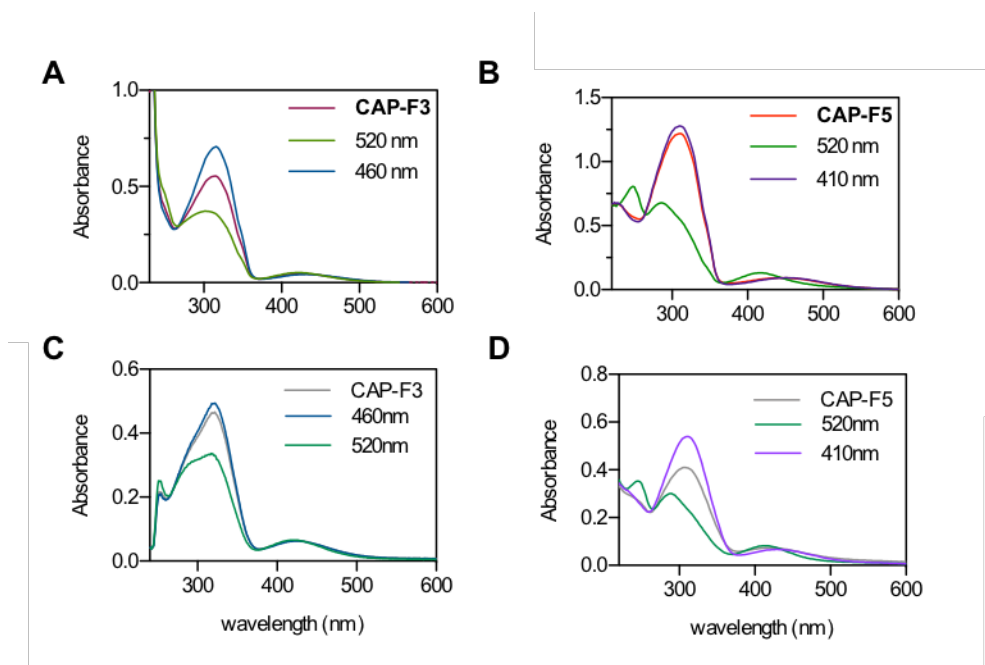


Figure 4.2 UV-vis spectra of **CAP-F3** and **CAP-F5** in aqueous buffer (A, C) and methanol (B, D) before and after photoirradiation (*cis* 520 nm for 5 minutes; *trans* 460/410 nm for 2 min. The power densities for 410, 460 and 520 nm were 5.20 mW/cm², 14.12 mW/cm² and 9.18 mW/cm², respectively.

All relevant wavelengths are summarized in Table 4.1. The spectra display absorption bands corresponding to a π - π^* transition (S_2) with a λ_{max} of 312 nm for **CAP-F3** and 306 nm for **CAP-F5**. Furthermore, moderate absorbance bands at 436 nm for **CAP-F3** and 450 nm for **CAP-F5** were observed, which correspond to n - π^* transitions (S_1). Irradiation of **CAP-F3** and **CAP-F5** with 520 nm light shifted their absorption profiles to ones consistent with *cis*-isomer formation, including a significant decrease in the intensity of the π - π^* band. While the π - π^* bands underwent a slight hypsochromic shift in both cases, there was a large hypsochromic shift in the n - π^* transition by 16 nm for **CAP-F3** and 37 nm for **CAP-F5** upon photoisomerization (Table 4.1).

Probe	CALCULATED					EXPERIMENTAL				
	$\pi \rightarrow \pi^*$		$n \rightarrow \pi^*$		$\Delta\lambda_{\pi \rightarrow \pi^*}$ /nm	$\pi \rightarrow \pi^*$		$n \rightarrow \pi^*$		$\Delta\lambda_{\pi \rightarrow \pi^*}$ /nm
	Trans- λ /nm	Cis- λ /nm	Trans- λ /nm	Cis- λ /nm		Trans- λ /nm	Cis- λ /nm	Trans- λ /nm	Cis- λ /nm	
CAP-F1	354	309	491	478	13	-	-	-	-	-
CAP-F3	348	312	512	468	44	312	306	436	420	16
CAP-F5	338	319	518	458	60	306	284	450	413	37

Table 4.3 The experimental and calculated values of absorbance bands and hypsochromic shift in $n-\pi^*$ transition for **CAP-F3** and **CAP-F5**

The photoisomerization kinetics were studied by monitoring the real-time absorbance changes at $\lambda_{\max}$ upon photoirradiation. The profiles obtained were fitted to single phase decay, and the photoisomerization rate constants for **CAP-F3** and **CAP-F5** were noted (Figure 4.3). As observed, rapid isomerization was observed for both the probes which is favorable to get dynamic and real-time control over their biological targets.

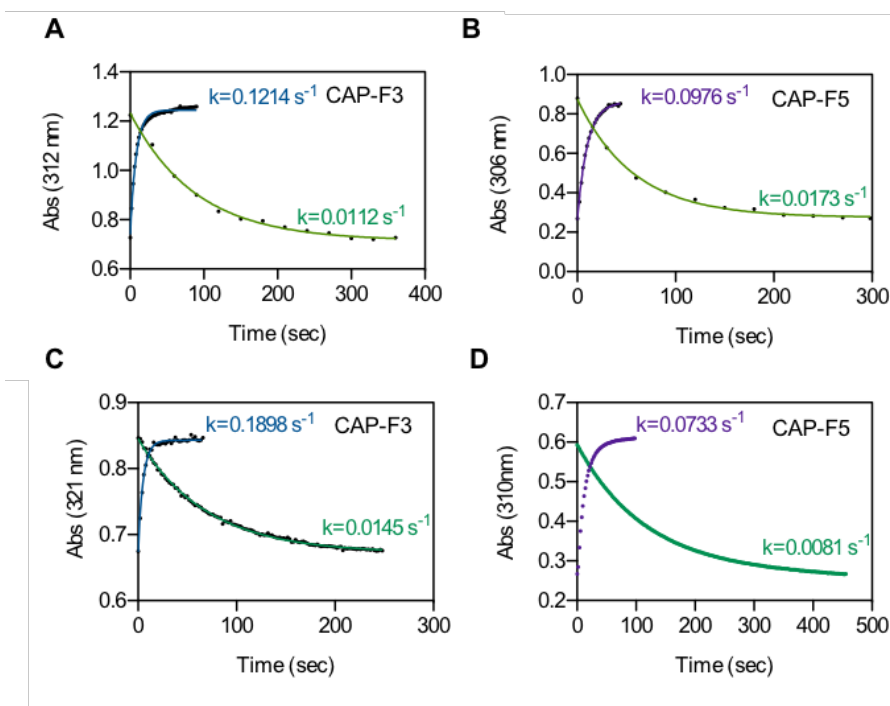


Figure 4.3 Isomerization kinetics of **CAP-F3** and **CAP-F5** in methanol (A and B) and aqueous buffer (C and D) as monitored by UV-vis spectroscopy.

The large separation of the $n-\pi^*$ transition bands between *trans* and *cis* isomers should result in high photoisomerization conversions.²³ Indeed, irradiation of solutions with 520 nm light yielded high conversions to *c*-**CAP-F3** (71%) and *c*-**CAP-F5** (87%) (¹⁹F NMR, CD₃OD, Figure 4.4). Isomerization from *cis* to *trans* can subsequently be achieved by irradiating with 460 nm and 410 nm light for **CAP-F3** (58 %) and **CAP-F5** (82 %) respectively.

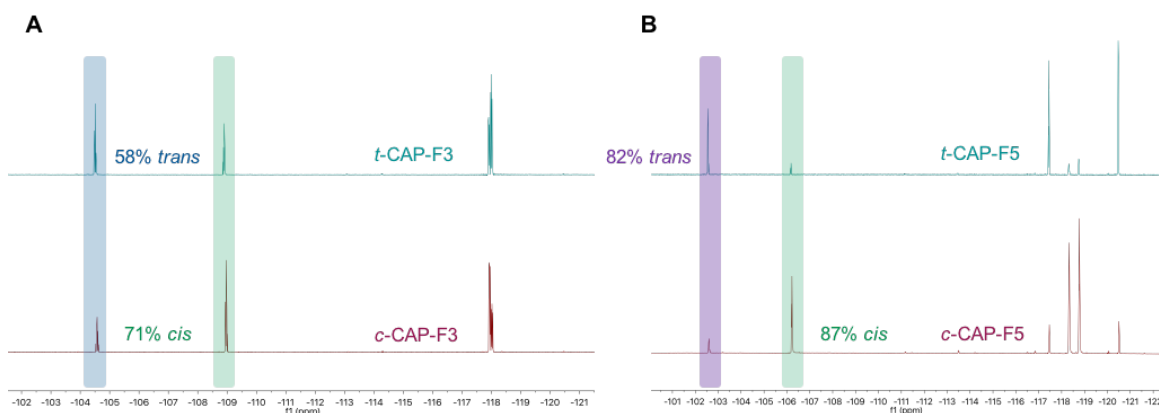


Figure 4.4 The % of isomer at PSS for **CAP-F3** (A) and **CAP-F5** (B), as analyzed by ^{19}F NMR spectroscopy with 30 sec relaxation time in CD_3OD solvent. The highlighted region corresponds to F atom at *p*-position of the probes.

The high conversion of **CAP-F5** in aqueous buffer is better than our previously reported probes (**CAP1** and **CAP2**),²² and is on the same order as other *cis-trans* isomerizing photoswitches studied in buffer solution.²

4.4.1.2 Understanding the exceptional thermal stability of *cis* isomer

The *cis* isomers of **CAP-F3** and **CAP-F5** both have increased thermal stability compared to non-fluorinated analogues,²² with exceptional thermal stability exhibited for *cis*-**CAP-F5**. The thermal conversion from the *cis* to *trans* isomer of **CAP-F3** and **CAP-F5** at 37 °C in aqueous buffer was studied under dark conditions (Figure 4.5 A). We observed a low ~15% conversion of *c*-**CAP-F5** to *t*-**CAP-F5** over 48 hours, and a ~40% conversion of *c*-**CAP-F3** to *t*-**CAP-F3**. This difference is unsurprising giving the $n\text{-}\pi^*$ separation trend. The stability driven by *ortho*-fluorination is better than what has been observed for *ortho*-methyl substituted bistable azobenzenes.⁴¹ Previous reports have indicated that intramolecular interactions may also contribute to the stability of the *cis* isomer of *ortho*-fluorinated derivatives of azobenzene.^{42, 43}

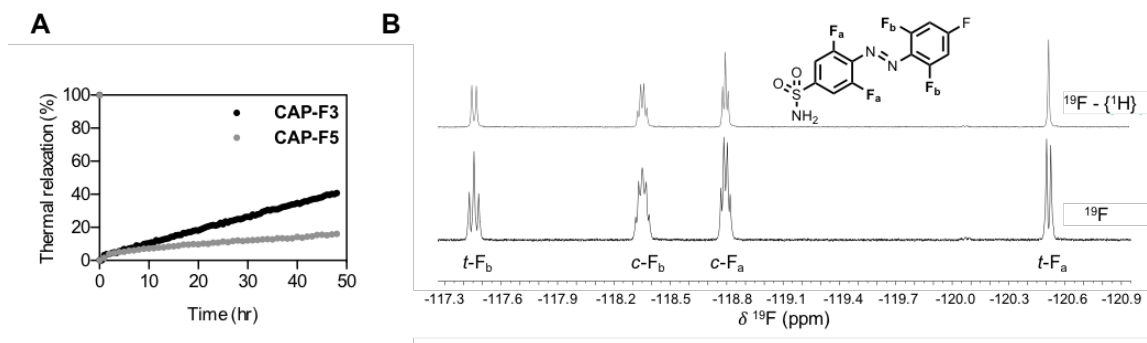


Figure 4.5 Thermal relaxation of *c*-CAP-F3 and *c*-CAP-F5 in aqueous buffer at 37 °C (A). ^{19}F and $^{19}\text{F}-\{^1\text{H}\}$ spectra for CAP-F5 collected in CD_3OD (B).

We examined potential interactions using NMR spectroscopy in a polar protic solvent. In the ^{19}F NMR spectrum of CAP-F5, we observed that the peak splitting pattern for the *cis* isomer was more complex than the *trans* isomer (Figure 4.5 B), and could not be accounted for solely by through-bond couplings. The decoupled $^{19}\text{F}-\{^1\text{H}\}$ spectrum was collected and revealed coupling between F_a and F_b only in the *cis* isomer.⁴³ As shown in Figure 4.5 B, the peaks corresponding to F_a and F_b in the *trans* isomer appeared as singlet and doublet (due to the *para*-F), respectively, and hence, there was no evidence of through-space coupling between the *ortho*-fluorine moieties in this isomeric form. The peaks corresponding to F_a and F_b in the *cis* isomer appear as a triplet and quartet, respectively, as a result of through-space coupling between the *ortho*-fluorines ($^{\text{TS}}J_{\text{FF}} = 5.64$ Hz for F_a , 5.67 Hz for F_b).⁴⁴

Coupling between F_a and F_b in the *cis* isomer was further supported by correlation peaks observed in its ^{19}F - ^{19}F COSY spectrum. In figure 4.6 A, there is a significant correlation between F_a and F_b in the *cis* isomer, and no such correlation between these fluorines in the *trans* isomer. To eliminate the effects of limited concentration of *trans* isomer on the signal intensity, same experiment was repeated where the sample was

irradiated with 410 nm to get ~85% of *trans* isomer in the solution (Figure 4.6 B). The same correlation pattern witnessed here as well suggests the presence of favorable interaction between *ortho*-fluorines in *cis* isomer only.

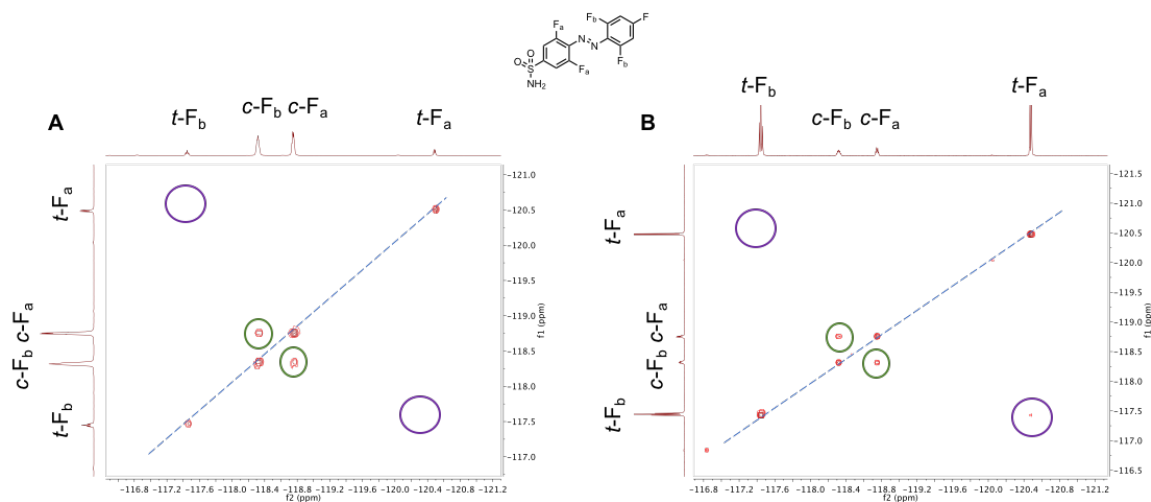


Figure 4.6 The ^{19}F - ^{19}F COSY spectrum of **CAP-F5** collected at PSS generated by 520 nm (A) and 410 nm (B) irradiation in CD_3OD .

To examine the presence of any such interaction between the *ortho*-atoms of **CAP-F3**, ^1H - ^1H gCOSY NMR was collected (Figure 4.7). The circled signal in blue and green represents the 2-bond coupling ($^1\text{H}_\text{a}$ -C- $^1\text{H}_\text{b}$) for *trans* and *cis* isomer, respectively. The large separation of H_a and H_b in *cis* isomer is due to the interaction with *ortho*-fluorine on the other ring, which is absent in the *trans* configuration.

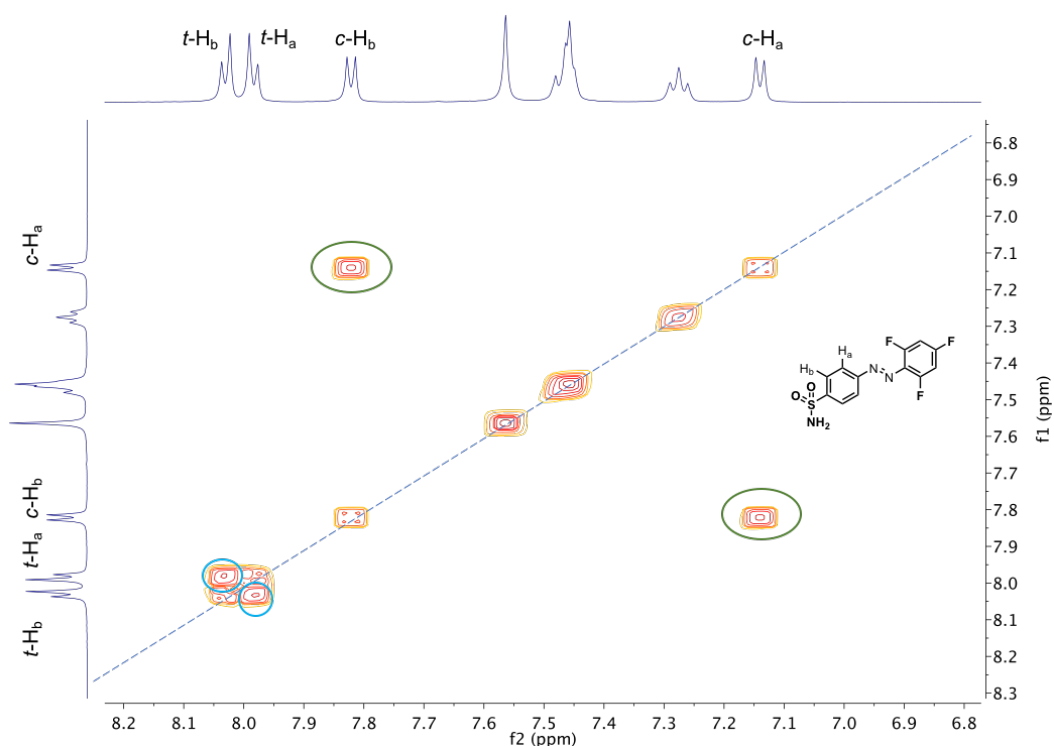


Figure 4.7 ^1H - ^1H gCOSY NMR of **CAP-F3** in CD_3OD .

Finally, we investigated how the rotation of the ring bearing F_b is affected by the absence (**CAP-F3**) or presence (**CAP-F5**) of F_a on the other phenyl ring using transverse relaxation time (T_2) measurements.⁴⁵ The shorter T_2 values of F_b in the *cis* isomer (1.19 sec for *c*-**CAP-F3**; 0.73 sec for *c*-**CAP-F5**) indicate that ring rotation is more constrained in *c*-**CAP-F5**; this constriction likely stabilizes *c*-**CAP-F5** in solution greater than *c*-**CAP-F3**. All these experiments confirm the presence of stabilizing interaction between the *ortho*-atoms in the *cis* isomer of **CAP-F3** and **CAP-F5** which results in the high lifetime of *cis* isomer as compared to non-fluorinated azobenzene.

The persistence of *c*-**CAP-F5** was further demonstrated in the solid state via single-crystal X-ray diffraction, a rare example of a *cis*-azobenzene structure.⁴² In the crystal structure, the distances between the *ortho*-fluorine atoms and *ipso* carbons are

2.78 Å and 2.76 Å (Figure 4.8 A). Both distances are shorter than the C-F van der Waals distance (3.17 Å), demonstrating the presence of C•••F interactions in the *cis* isomer. Such favorable interactions were not observed in the solid-state structure of *t*-CAP-F5 (Figure 4.8 B).

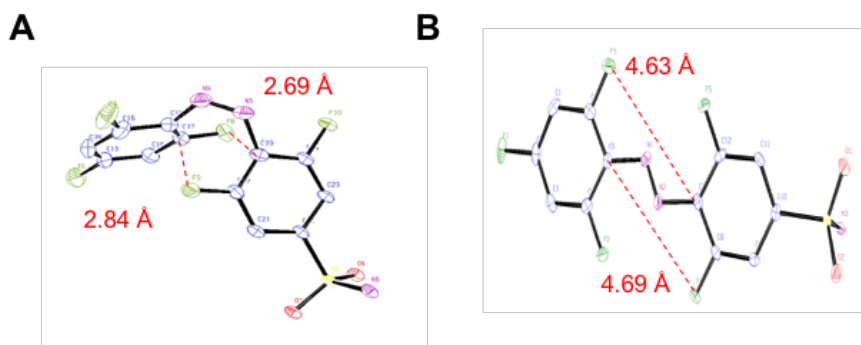


Figure 4.8 Single crystal structure of *c*-CAP-F5 (A) and *t*-CAP-F5 (B). Thermal ellipsoid plots at 50% probability are shown. Solvent molecules and hydrogen atoms are omitted for clarity.

4.4.1.3 Understanding the photocharacteristics through computational analysis

To further understand the spectroscopic properties of fluorinated azobenzene probes, **CAP-F3** and **CAP-F5**, we performed TD-DFT calculations to compute the energies of the HOMO (n) and LUMO (π^*) orbitals for both the *trans* and *cis* isomers of **CAP-F3** and **CAP-F5** at the B3LYP/6-31G* level. For a better comparison, we also performed the calculations for a **CAP-F1** analogue, where the *ortho*-fluorine atoms are all substituted by hydrogen atoms. and also included calculations for a **CAP-F1** analogue, where the *ortho*-fluorine atoms are all substituted by hydrogen atoms (Figure 4.9).

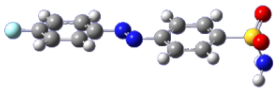
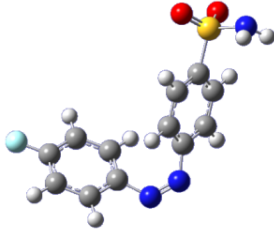
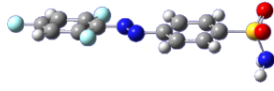
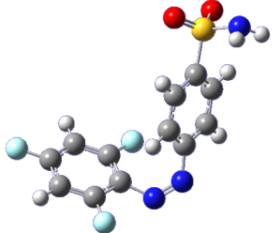
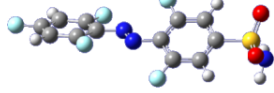
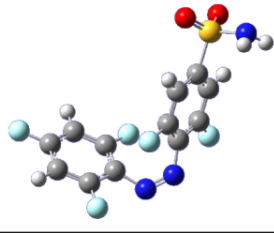
Probe	<i>Trans</i> isomer	<i>Cis</i> isomer
CAP-F1		
CAP-F3		
CAP-F5		

Figure 4.9 The optimized structure of **CAP-F1**, **CAP-F3** and **CAP-F5** as predicted by DFT calculations.

TD-DFT calculations reveal that the S_1 excited states of **CAP-F3** and **CAP-F5** isomers are mainly composed of HOMO $\rightarrow$ LUMO transitions, which are conventionally recognized as $n\text{-}\pi^*$ transitions (Figure 4.10). The π^* orbitals are delocalized across the whole molecule, with reduced delocalization on the cis-isomers due to the large twist of the aromatic rings with respect to each other. Therefore, the π^* orbitals of *c*-**CAP-F3** and *c*-**CAP-F5** are higher in energy than those of *t*-**CAP-F3** and *t*-**CAP-F5**.

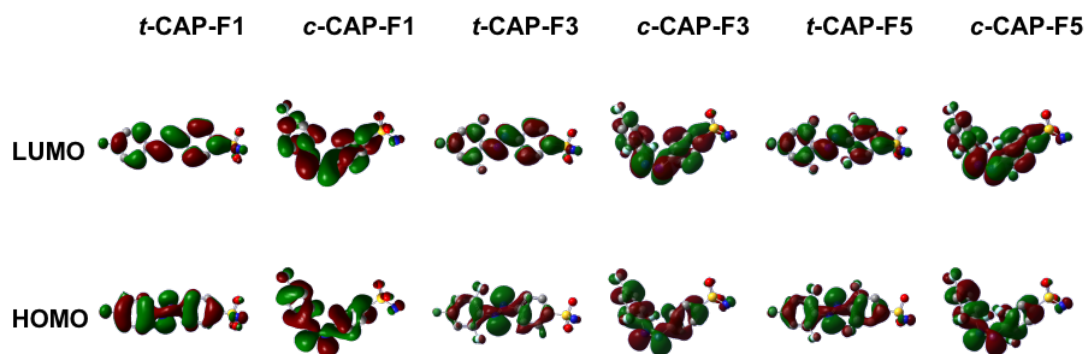


Figure 4.10 The electron density plot of LUMO and HOMO orbitals for *trans* and *cis* isomers of **CAP-F1**, **CAP-F3** and **CAP-F5**, as analyzed through DFT calculations.

The n orbitals could be viewed as a linear combination of lone pair orbitals of the adjacent nitrogen atoms. These two orbitals interact through-space in the *cis*-structures, which presents a much stronger repulsion than in the *trans*-structures, where the interaction is through-bond. Thus, the interaction would increase the n-orbital's energy more in the *cis* isomers than in the *trans*-isomers. On the other hand, the σ -electron-withdrawing effect of the fluorine atoms on the aromatic rings effectively attenuate the n-electron density, thereby lowering its energy level. The overall effect is well seen in **CAP-F3** and **CAP-F5**, where *c*-**CAP-F3** shows an n-orbital 0.18 eV *higher* in energy than *t*-**CAP-F3**, while *c*-**CAP-F5** bearing more fluorine atoms shows a *lower* n orbital than its *trans*-isomer by 0.11 eV (Figure 4.11).

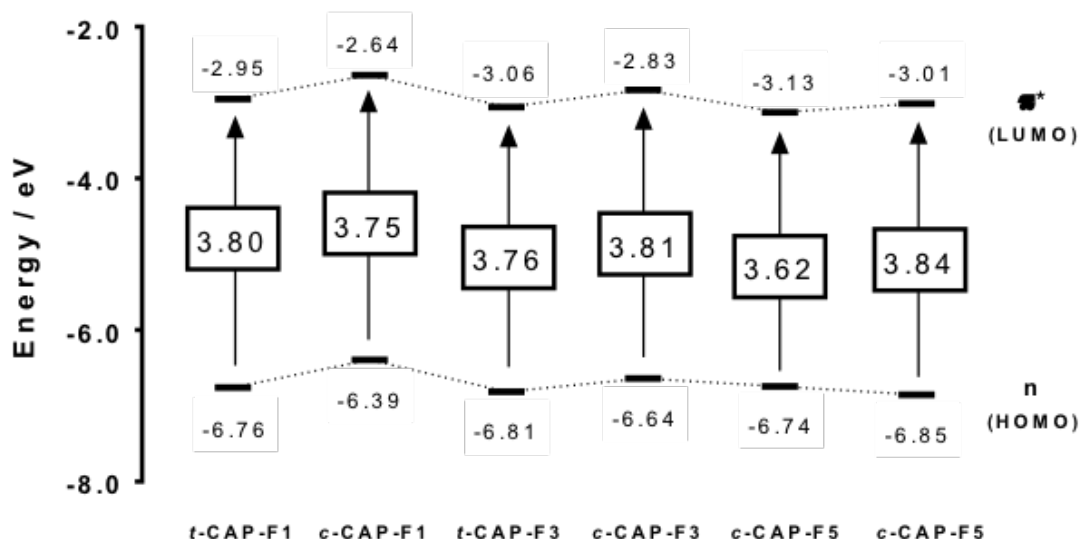


Figure 4.11 The energy level of HOMO and LUMO of *trans* and *cis* isomer of **CAP-F1**, **CAP-F3** and **CAP-F5** as calculated through DFT calculations. The trend represents the stabilization of orbitals, especially for *cis* isomer as the number of fluorine increases on the probe.

This distinct difference led to a prediction of larger separation of $n\text{-}\pi^*$ transition bands between *trans*- and *cis*- isomers ($\Delta\lambda_{n\text{-}\pi^*}$) for **CAP-F5** than for **CAP-F3**. Indeed, this prediction agreed with experimental results that showed a 16 nm spectral separation for **CAP-F3** and a 37 nm spectral separation for **CAP-F5**. A higher photoisomerization of **CAP-F5** than **CAP-F3** at their photostationary states (^{19}F NMR), further demonstrates that an introduction of *ortho*-fluorine atoms helps separate the S_1 states between *cis*- and *trans*-isomers and is important in our molecular design.

In order to understand the effect of fluorine in obtaining the thermally stable *cis* isomer, the obtained solid-state structure of *c*-**CAP-F5** through single-crystal X-ray diffraction was compared with the optimized structure. We note that the observed single-crystal structure resembles well the optimized theoretical structure of *c*-**CAP-F5**. In the optimized structure, the spatial distances between the *ortho*-fluorine atom and *ipso*

carbons are 2.89 Å and 2.81 Å, respectively. Both of them are shorter than the C-F van der Waals distance (3.17 Å), which matches with the 2.69 and 2.84 Å measured in the crystal structure. These favorable C•••F interactions provide further stabilization to the *cis*-structure and results in a smaller Gibbs free energy difference ($\Delta G_{cis-trans}$) between the *trans*- and *cis*- configurations, as predicted by DFT calculations by around 14.2 kcal/mol for **CAP-F1**, 11.5 kcal/mol for **CAP-F3** and 8.7 kcal/mol for **CAP-F5**, respectively (Table 4.2).⁴² This trend is consistent with the longer half-life of *c*-**CAP-F5** compared to *c*-**CAP-F3** (Table 4.2). Through a first approximation, the stabilization energy because of the favorable interaction between *ipso*-carbon and *ortho*-fluorine was calculated by subtracting $\Delta G_{cis-trans}$ of *c*-**CAP-F3** and *c*-**CAP-F5** from $\Delta G_{cis-trans}$ of *c*-**CAP-F1**, respectively, which reveals a 2.7 kcal/mol stabilization for *c*-**CAP-F3** and 5.5 kcal/mol stabilization for *c*-**CAP-F5**.

Probe	$\Delta G_{cis-trans}$ / kcal mol ⁻¹	% thermal relaxation (37 °C, 48h)
CAP-F1	14.2	~ 100% ^a
CAP-F3	11.5	40%
CAP-F5	8.7	15%

Table 4.4 Calculated $\Delta G_{cis-trans}$ for probes containing 1 (**CAP-F1**), 3 (**CAP-F3**) and 5 (**CAP-F5**) fluorine atoms, and their relationship with experimental thermal relaxation (*cis* to *trans* conversion) at 37 °C over a period of 48 h in aqueous buffer (50 mM HEPES with 0.1 M KNO₃ at pH 7.2) under dark conditions.^a denotes the value determined for structurally similar molecules.

The crystal structure of *t*-**CAP-F5** was obtained and was compared with theoretical structure. The optimized *t*-**CAP-F5** isomer displays a large dihedral angle

(38°) between the two aromatic rings, which is quite different from its single-crystal structure (4.7°). We considered that the near-planar structure of *t*-**CAP-F5** in the crystal is due to its packing artifacts, and that the calculated *trans*- structure with the C-N=N-C moiety has C₂-symmetry, representing the lowest energy configuration. This non-coplanarity partly results from the repulsion between the *ortho*-fluorine electrons and nitrogen lone pairs and is consistent with earlier publications.

4.4.2 Biophysical characterization of probe isomer-protein interaction

4.4.2.1 Comparison of binding affinities of CAP-F isomers with CA

Due to the increased thermal stability and higher photoisomerization efficiency of *c*-**CAP-F5** over *c*-**CAP-F3**, we focused on **CAP-F5** for more in-depth studies. The binding interactions of *t*-**CAP-F5** and *c*-**CAP-F5** were studied with bovine carbonic anhydrase (bCA) using a dansylamide (DNSA) competition assay. DNSA, a sulfonamide based CA-inhibitor, exhibits an enhanced fluorescence at 458 nm upon binding to the active site of CA (apparent K_d = 1.6 μM, under our experimental conditions), and a weakened fluorescence when an external ligand displaces it from the active site. A solution of bCA•DNSA was titrated with *trans* and *cis* isomers of **CAP-F5** and the apparent K_d values were calculated. As shown in Figure 4.12 A, the binding profiles of the *trans* and *cis* isomers are drastically different, yielding K_d' values of 9 ± 1.3 nM for *t*-**CAP-F5** and 106 ± 16 nM for *c*-**CAP-F5**, demonstrating an order higher binding affinity of the *trans* isomer.

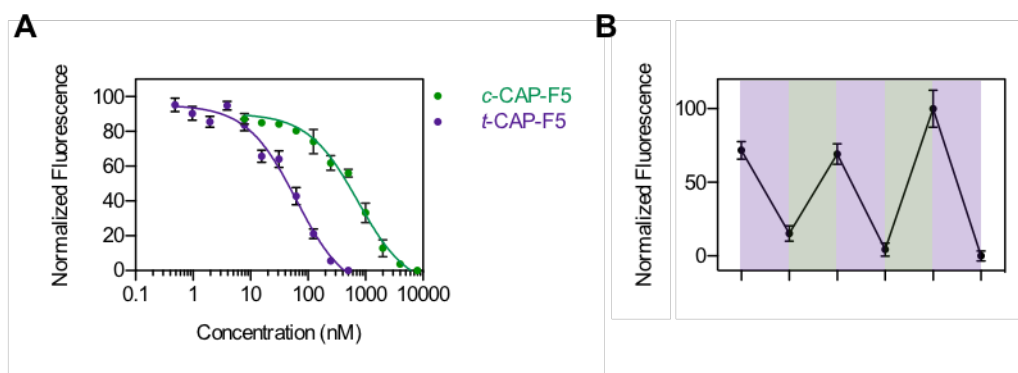


Figure 4.12 Change in fluorescence emission ($\lambda_{\text{ex}} = 280 \text{ nm}$; $\lambda_{\text{em}} = 458 \text{ nm}$) of bCA-DNSA mixture in the presence of different concentrations of *t*-CAP-F5 and *c*-CAP-F5 to determine apparent K_d values (A). The binding of CAP-F5 is reversible in nature as shown by irradiating the sample of CA, DNSA and probe with alternate 520 nm and 410 nm for *in situ* isomerization and monitoring DNSA fluorescence (B). Data represents mean $\pm$ standard deviation. Studies conducted in 50 mM HEPES, 0.1 M KNO₃, pH 7.2, 298K, in the dark.

A control molecule lacking the Zn²⁺-binding *p*-sulfonamide group (CAP-con, Scheme S3) induces almost no change in the fluorescence emission. We further employed DNSA to investigate the isomerization of CAP-F5 in the active site of CA, using CAP-F5, bCA, and an excess of DNSA. Solutions of bCA, DNSA and CAP-F5 were irradiated with 520 nm (*trans* $\rightarrow$ *cis*) and 410 nm (*cis* $\rightarrow$ *trans*) alternately with fluorescence measured after each irradiation (Figure 4.12 B). DNSA fluorescence intensity decreased after irradiation with 410 nm and increased after irradiation with 520 nm through several cycles. These experiments demonstrate the reversible and robust CAP-F5 photoisomerization even in the presence of CA.

4.4.2.2 Understanding the probe-protein interaction by molecular docking

To better understand the difference in binding preference between the *cis* and *trans* isomers of CAP-F5 towards CA, we analyzed the X-ray structure of hCAII with a

related azobenzene sulfonamide ligand, (E)-4-(4-aminophenyldiazenyl)benzenesulfonamide (**L**), bound to the active site (Protein Data Bank: 5BYI).⁴⁶ In this crystal structure, **L** forms extensive hydrophilic and hydrophobic interactions with hCAII at the Zn active site (Figure 4.13 A). The primary interaction between the protein and **L** is between the deprotonated nitrogen of the -SO₂NH₂ and the Zn²⁺ center, forming a tetrahedral coordination along with the three active site histidines (H94, 96 and 119). The sulfonamide group of **L** also hydrogen bonds to the backbone and side chain of Thr198 while the adjoining benzene ring sits in a hydrophobic pocket formed by residues W208, L197 and L142. The other phenyl ring hydrophobically stacks with P201 and forms a 6 Å T-shape π - π stacking with F130 (Figure 4.13 A).

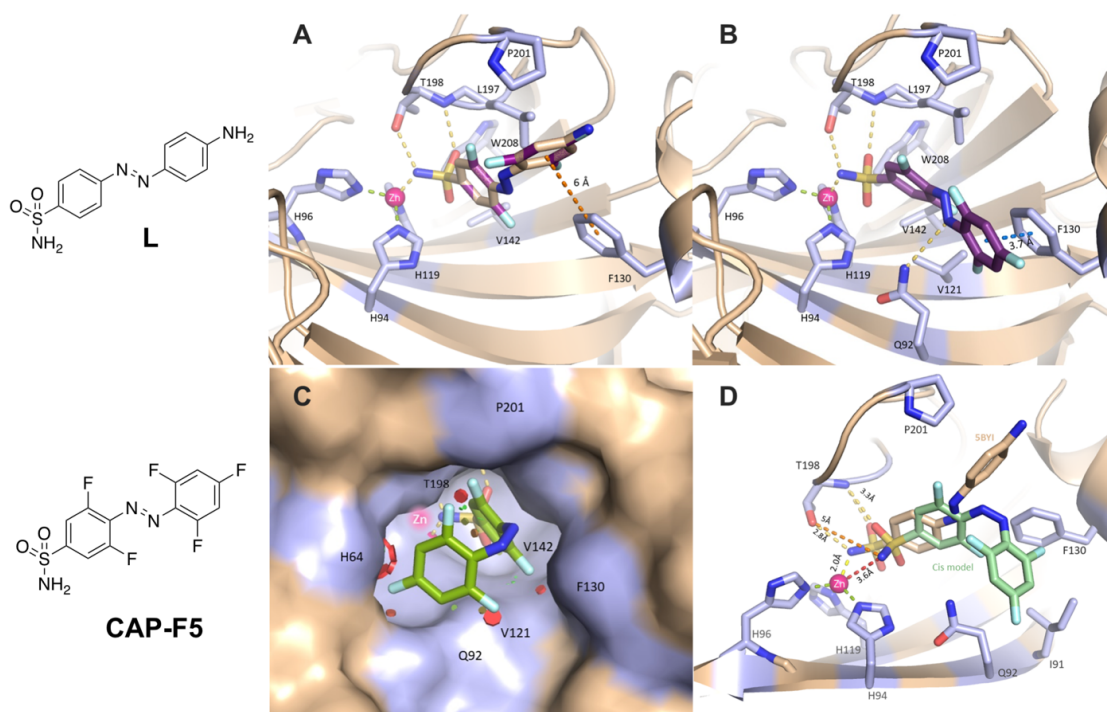


Figure 4.13 Docking of **CAP-F5** in the active site of hCAII structure obtained with azobenzene ligand **L** bound to the active site (PDB 5BYI). The secondary Protein (PDB 5BYI) is shown with side chains of active site residues in sticks. The favorable hydrogen bonds and π - π^* interactions are shown in yellow and orange dotted lines, respectively. (A) Docking of *t*-**CAP-F5** into the hCAII active site after superimposing it onto the ligand **L**. (B) Alternative binding mode for *t*-**CAP-F5** due to the presence of fluorine atoms. (C) Docking of *c*-**CAP-F5** when interactions between $-\text{SO}_2\text{NH}_2$ and Zn^{2+} are kept intact, steric clashes are shown as red disks. (D). Potential orientation of *c*-**CAP-F5** when steric clashes are removed, showing the probe retreats from the active site.

The molecular structure of **CAP-F5** highly resembles that of **L** with the only alteration at the benzyl ring extending away from the Zn^{2+} coordination site. We conducted molecular modeling and docking using Maestro (Schrodinger Suite).³⁵ Due to the high structural similarity of *t*-**CAP-F5** to **L**, the same coordination to Zn^{2+} is expected to be conserved. The model is then energy minimized in Maestro.^{47, 48} Two configurations of *t*-**CAP-F5** are identified with favorable energy, both maintaining

similar binding mode to **L** without any steric clashes (Figures 4.13 A, B). The first configuration is identical to the crystal structure of **L** binding with all favorable interaction maintained (Figure 4.13 A). The addition of fluorine atoms in **CAP-F5**, however, decreases the electron-richness of the benzene rings compared to **L**. Thus, an additional binding mode is also possible for *t*-**CAP-F5** when the azo bond flips by 180° (Figure 4.13 B). In this alternative configuration with similar favorable binding energy, the fluoro-benzene ring rotates to a configuration that forms a parallel π - π stacking of 3.7 Å with F130 while maintaining almost all other non-covalent interactions. This orientation can be further stabilized by a potential hydrogen bond between the azo group and Q92 with no steric clashes with residues forming the active site pocket (Figure 4.13 B). Thus, *t*-**CAP-F5** forms extensive favorable contacts with the enzyme in two possible poses.

When docking *c*-**CAP-F5**, the favorable interaction between the Zn^{2+} and the sulfonamide nitrogen of the ligand cannot be retained (Figure 4.13 C). In order to maintain the same coordination to Zn^{2+} , the distal phenyl ring would be in close contact with the active site pocket due to the *cis*-configuration of the azo group, causing clashes with Q92, V121, V142 and H64 (Figure 4.13 C). To avoid steric clashes, *c*-**CAP-F5** has to adapt to a configuration retreating from the deep active site pocket containing the Zn^{2+} . As a result, the distance between the sulfonamide nitrogen and the Zn^{2+} increase from 2.0 Å (as found in **L** and more likely in *t*-**CAP-F5**) to 3.6 Å, which is no longer optimal for Zn^{2+} coordination (Figure 4.13 D). Furthermore, the hydrogen bond between T198 and the sulfonamide group's nitrogen in the azobenzene ligand is unlikely to form in this configuration (Figure 4.13 D). Thus, *c*-**CAP-F5** cannot show any favorable interaction with Zn^{2+} in the active site, which makes it more weakly binding than *t*-**CAP-F5**.

4.4.3 Spectroscopic and kinetic analysis of inhibitory properties of CAP-F5 with CA

CA catalyzes the hydration of CO₂ and dehydration of HCO₃⁻ reversibly. The inhibitory effect of the *trans* and *cis* isomers of **CAP-F5** on CO₂ hydration activity of bCA was measured by tracking changes in solution pH using phenol red as a pH indicator, and a solution of saturated CO₂ as a substrate (Figure 4.14 A).⁴⁹ The observed rates for CO₂ conversion by bCA in the absence and presence of known CA inhibitor acetazolamide were 0.113 s⁻¹ and 0.027 s⁻¹ respectively. Correspondingly, the initial velocities of the catalytic reaction in the presence of *trans* and *cis* isomers of **CAP-F5** were found to be 0.025 s⁻¹ and 0.082 s⁻¹ respectively, demonstrating the superior ability of the *trans* isomer to inhibit CA enzymatic activity. Using this same stopped flow method, the apparent K_i values for *t*-**CAP-F5** and *c*-**CAP-F5** were calculated to be 36 ± 2 nM and 164 ± 8 nM respectively (Figure 4.14 B), indicating ~5 times greater potency of *t*-**CAP-F5**.

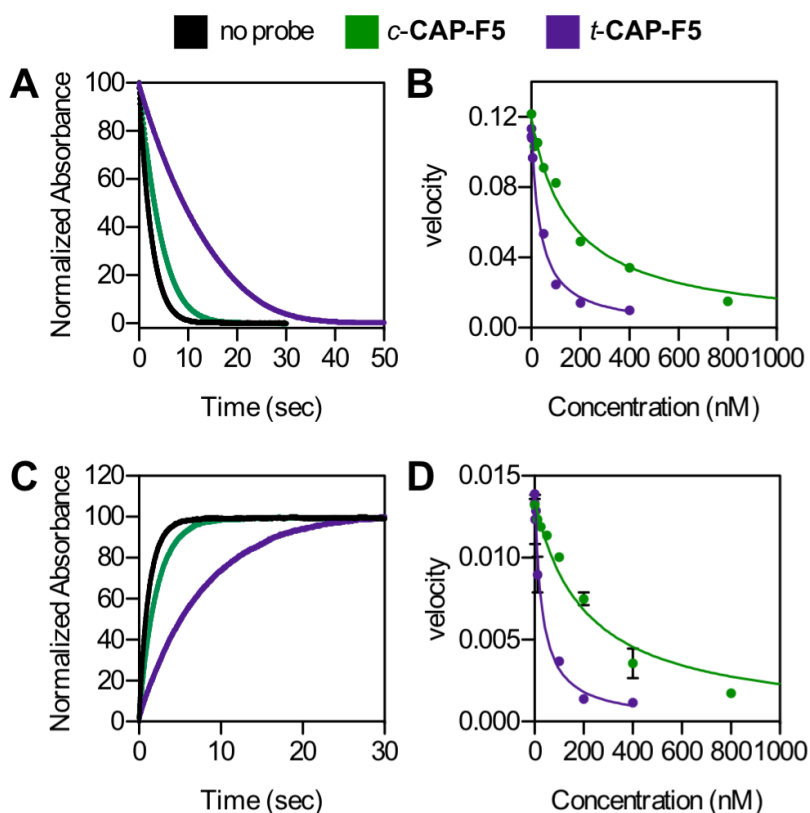


Figure 4.14 Change in absorbance of phenol red ($\lambda=557\text{nm}$) due to CO₂ hydration activity (A) and HCO₃⁻ dehydration activity (C) of CA in the absence and presence of 1eq of *trans* and *cis* **CAP-F5**; Change in CO₂ hydration (B) and HCO₃⁻ dehydration (D) catalytic velocity as a function of *trans* and *cis* isomer concentration. Studies are conducted in 50 mM HEPES, 0.1M KNO₃ pH 7.2 at 25 °C using stop flow apparatus. Data represents mean $\pm$ the standard deviation.

As CA can also catalyze bicarbonate dehydration, we also investigated **CAP-F5** in the context of this reaction, where aqueous KHCO₃ was used as the substrate. The initial velocity of the CA-catalyzed reaction was $0.36 \times 10^{-2} \text{ s}^{-1}$ in the presence of *t*-**CAP-F5** and $1.0 \times 10^{-2} \text{ s}^{-1}$ in the presence of *c*-**CAP-F5**, similar to the reaction rates in the presence ($0.10 \times 10^{-2} \text{ s}^{-1}$) and absence of acetazolamide ($1.3 \times 10^{-2} \text{ s}^{-1}$) (Figure 4.14 C).

The apparent K_i values of *t*-**CAP-F5** and *c*-**CAP-F5** were calculated to be 30 ± 3 nM and 205 ± 14 nM respectively (Figure 4.14 D). These studies show that **CAP-F5** isomers can be used to modulate both the CO₂ hydration and bicarbonate dehydration activity of CA.

4.4.4 Application of CAP-F5 in the photomodulation of CA in living cells

Having demonstrated the efficacy of **CAP-F5** to differentially interact with isolated CA protein in the *trans* and *cis* isomeric forms, we next applied our photoswitch to control the activity of CA in cell culture. We note that **CAP-F5** isomerization is reversible over several cycles in cell culture media. CAII is the cytosolic isoform of CA responsible for maintaining intracellular pH, and is known to be overexpressed in certain cancer cells.¹⁶ It has been reported that the resting intracellular pH of cells is affected by CA inhibitors, and this change highly depends on the cell type and environment.⁵⁰⁻⁵²

4.4.4.1 Controlling intracellular steady state pH

Previously, we showed differential effects of *trans* and *cis* isomers of **CAP1** on intracellular pH, however thermal instability of *cis* isomer prevented applications over longer time periods.²² With our improved probe design with highly stable *cis* isomer, we compared the ability of *trans* and *cis* isomers of **CAP-F5** to modulate intracellular pH in HeLa cells at both short (30 min) and long (24h) incubation times. We performed a cellular cytotoxicity assay to determine probe concentrations at which minimal toxicity would be observed for the *cis* isomer (Figure 4.15 A). We observed that cells incubated with the *cis* isomer maintained a similar pH to control cells after both short and long incubation times, whereas cells incubated with the *trans* isomer displayed decreased intracellular pH in both scenarios (Figure 4.15 B and C). Furthermore, we noticed that the intracellular pH response due to *cis* (or *trans*) isomer of **CAP-F5** generated at PSS is

similar to the cells treated with 100% *cis* (or *trans*) isomer purified by HPLC (Figure 4.15 C). This result shows promises in the biological application of **CAP-F5** at PSS to get the best response possible.

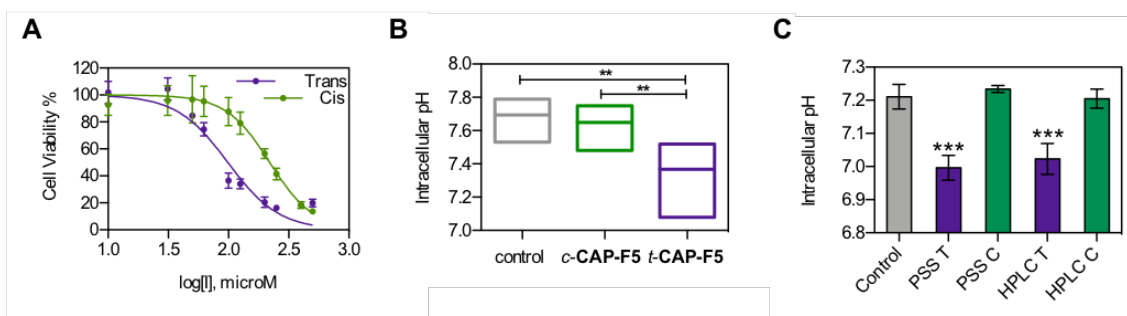


Figure 4.15 Cell viability (A) of HeLa cells upon treatment with different concentrations of *trans* and *cis* isomer of **CAP-F5** for 24 hours, as determined through MTS Assay. The intracellular pH of HeLa cells as a result of incubation of *t*-**CAP-F5** and *c*-**CAP-F5** for 30 minutes (B) and 24 h (C), as analyzed by flow cytometry and cellular imaging. The results are shown as an average of six independent experiments. Box plot indicates the range and average values for each group. Asterisks denote statistically significant differences ($p < 0.05$; one-way analysis of variance). Data represents mean $\pm$ standard deviation.

4.4.4.2 Controlling cellular CA machinery to CO₂ stress

We next explored the effect of the isomers of **CAP-F5** on the response of the cytoplasmic CA machinery to exogenous perturbations of CO₂. When the level of extracellular CO₂ increases, it passively diffuses into the cytoplasm through the cell membrane where it is converted to carbonic acid by cytoplasmic CAs, resulting in decrease in intracellular pH (Figure 4.16 A).⁵³ Inhibition of cytoplasmic CA reduces the rate of intracellular acidification following increases in extracellular CO₂ concentration.^{50, 51, 53, 54} We used this rate of change of intracellular pH to demonstrate the real-time inhibition of cytosolic CA by *t*-**CAP-F5**.

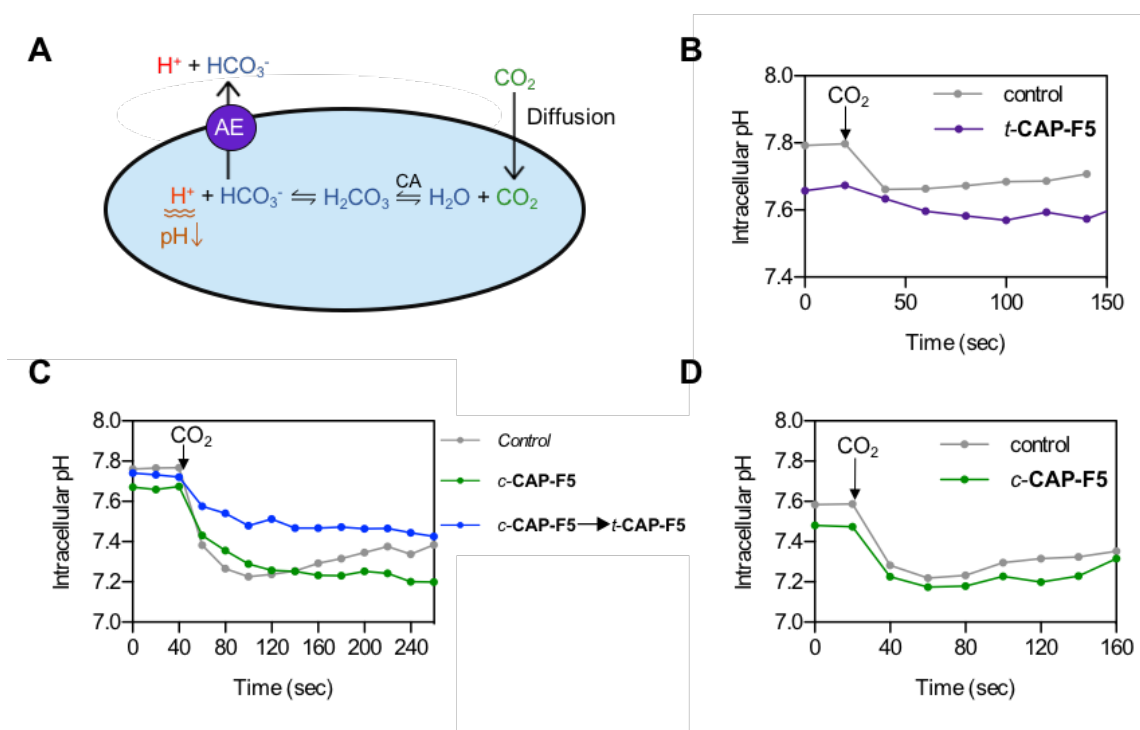


Figure 4.16 The change in intracellular pH with respect to time as a result of addition of CO₂ in the presence of *t*-CAP-F5 (B) and *c*-CAP-F5 (D) due to the mechanism shown in (A). The change in intracellular pH of cells incubated with *c*-CAP-F5 before and after irradiating with 410 nm to isomerize *cis* isomer to *trans* isomer *in situ* (C).

In our experiment, HeLa cells were loaded with pHrodo and incubated with 25 μ M *t*-CAP-F5, *c*-CAP-F5, or a DMSO control for 30 minutes. A bolus of saturated aqueous CO₂ was added to the cell suspension and intracellular pH changes were monitored using flow cytometry. As shown in Figure 4.16 D, *c*-CAP-F5 treated cells had similar kinetic changes in intracellular pH over time as compared to DMSO-treated control cells, signifying that cytosolic CA remains active in both groups. However, *t*-CAP-F5 treated cells displayed a slower rate of change in pH upon CO₂ addition, consistent with inhibition of the cytosolic CA machinery of CO₂ hydration (Figure 4.16 B).

4.4.4.3 *In situ* photocontrol of cellular CA using CAP-F5

Intracellular *c*-CAP-F5 can further be photostimulated *in situ* to activate CA inhibition. Cells were treated with pHrodo dye, *c*-CAP-F5 or DMSO in the similar way as described earlier. The *c*-CAP-F5 treated cells were divided into two groups, where only one group was irradiated with 410 nm light for 3 minutes (no phototoxicity observed) to isomerize the *cis* form to *trans* form *in situ* right before the addition of CO₂, and were compared with non-irradiated cells. As shown in Figure 4.16 C, the kinetics of intracellular pH change in *cis*-treated cells was faster than cells irradiated with 410 nm (converting *cis* to *trans*) prior to the addition of CO₂. This experiment demonstrates that probe isomerization can be achieved within the complex cellular environment, and indeed could be used to inhibit the CA activity upon light irradiation.

4.4.5 Application of CAP-F5 in the photomodulation of CA in *in vivo* systems

Next, we applied CAP-F5 in an *in vivo* system. Embryonic zebrafish (*Danio rerio*) are an excellent animal model for studying drug induced toxicity due to their high fecundity, external fertilization, rapid development, and the ease of maintaining large numbers in a small space.⁵⁵ Substances of interest can be administered in the media and then are rapidly up taken into the embryo. Importantly, the embryos are also optically transparent, which allows for study of photo responsive materials. CA is a vital component in acid-base regulation as well as ionic and osmotic balance in vertebrates.⁵⁶ Just like mammals, fish possess an abundance of CA isoforms that vary in molecular sequence, tissue distribution and subcellular localization.⁵⁷ They also have different kinetic properties and active site structure and thus different susceptibility to inhibitors. They are necessary during the early stages of development in zebrafish for effective CO₂ excretion.⁵⁸ Inhibition of CA by inhibitors like acetazolamide has a great effect on

zebrafish embryogenesis; embryos treated with CA inhibitor can display small otoliths, an irregular jaw, enlarged heart and yolk sac, and impaired locomotion.^{59, 60}

4.4.5.1 Effect of cis and trans CAP-F5 on developmental and behavioral properties of fish

We used phenotypic analysis to differentiate the behavior of *cis* and *trans* **CAP-F5** in zebrafish. Wild-type (AB strain) embryos were collected and transferred into standard embryo media⁶¹ and sorted by developmental stage.⁶² At six hours post fertilization (6hpf), zebrafish were exposed to media containing 1% DMSO and varying concentrations of **CAP-F5** in both *trans* and *cis* forms. Embryos were incubated in a light-proof container at 28.5 °C until 48hpf. They were then transferred to fresh media and raised to 5 days post fertilization (5dpf), when they were assessed for viability (Figure 4.17 A). As expected, *t*-**CAP-F5** induces higher toxicity than *c*-**CAP-F5** (Table 4.5).

Concentration (μM)	<i>t</i>-CAP-F5	<i>c</i>-CAP-F5
30	100% Lethal	100% Lethal
15	100% Lethal	Partial survival; Morphological defects
7.5	100% Lethal	100% survival; no obvious defects
2.5	Partial survival; Morphological defects	100% survival; no obvious defects

1.25	100% survival; no obvious defects	100% survival; no obvious defects
0.62	100% survival; no obvious defects	100% survival; no obvious defects
0.31	100% survival; no obvious defects	100% survival; no obvious defects
0	100% survival; no obvious defects	100% survival; no obvious defects

Table 4.5 The toxic effects on the viability of zebrafish at 5dpf due to the incubation of different concentrations of *t*-**CAP-F5** and *c*-**CAP-F5**. The highlighted concentration was used for all the further studies.

Further, the distinct effect of the isomers on embryogenesis was evaluated. For morphological endpoints, the zebrafish were treated with 2.5 μ M of *trans* and *cis* isomers of **CAP-F5**, and were raised to 5dpf as described above. As shown in Figure 4.17 B, zebrafish treated with *t*-**CAP-F5** showed multiple morphological abnormalities, including failure to form a swim bladder, pectoral fin defects, and cardiac edema, consistent with previous reported effects of CA inhibitors.^{59, 60} 100% of embryos (n=30) treated with the same concentration of *c*-**CAP-F5** developed normally, signifying the normal function of CA in *cis*-treated fish.

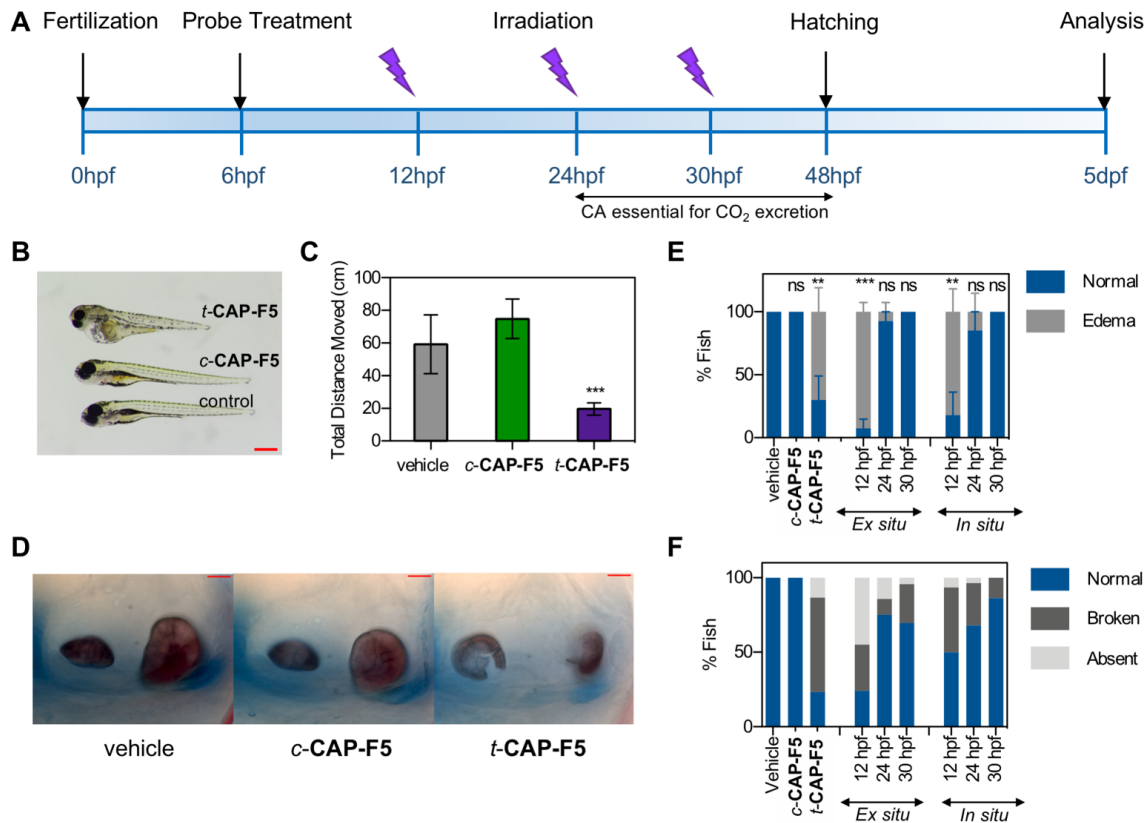


Figure 4.17 The timeline representing probe treatment and fish analysis (A). The morphological appearance (B), swimming behavior (C) and otolith development (D) in fish in the absence (vehicle) and presence of *t*-CAP-F5 and *c*-CAP-F5. The data represents mean values $\pm$ the standard deviation. Asterisks denote statistically significant differences ($p < 0.0001$; one-way analysis of variance). Scale bars represents 500 μ m and 50 μ m for B and D respectively. The morphological (E) and otolith (F) development as a result of in situ activation of probe from *cis* to *trans* isomer by irradiating the fish with 410 nm at different time point during embryo development. These developments are compared with *ex situ* generated *trans* isomer treatment at the respective time points. The representative images for normal, broken and absent otoliths can be found in supplementary. The results are represented as mean of 30 fish in three independent experiments. Data represents mean values $\pm$ SEM. Asterisks denote statistically significant differences (** $p < 0.005$, *** $p < 0.0001$; one-way analysis of variance).

Furthermore, the zebrafish were fixed and processed for otolith analysis. The *t*-CAP-F5 treated zebrafish had hollow and underdeveloped otoliths as opposed to *c*-CAP-

F5 treated fish, where 100% (n=30) had normally developed otoliths (Figure 4.17 D). This is likely caused by the inhibition of CA isoforms present in inner ear hair cells by *t*-**CAP-F5** only, disrupting the supply of HCO_3^- to the endolymph for otolith calcification.⁵⁶ Potentially as a result of poor otolith development or other developmental defects, fish treated with *t*-**CAP-F5** showed poor locomotion, while *c*-**CAP-F5** treated fish exhibited normal locomotive behavior (Figure 4.17 C). Our results signify that the isomers of **CAP-F5** are capable of differential effects in this complex multicellular system that are associated with CA inhibition.

4.4.5.2 In situ activation of CAP-F5 inhibitory properties for CA in fish

After establishing that different isomers of **CAP-F5** can have different effects on the developmental and behavioral properties of fish, we investigated if the probe could be activated *in situ* within the embryo for CA inhibition during development. Embryos were treated with *c*-**CAP-F5** as described above, but the probe was activated to the *trans* isomer *in situ* (within the fish) through photoirradiation with 410 nm (0.50 mW/cm²; 3min) at different time points during embryogenesis (12 hpf, 24 hpf, 30hpf), delineated in Figure 4.17 A. These irradiation conditions do not affect the development of embryos at any given irradiation time point. As CA isoforms play an important role in meeting the requirements for CO₂ excretion between 24hpf and 48hpf in developing fish, these time points were chosen to assess the effect of inhibition before and during that window.⁵⁸ The results were compared to fish that were treated with *ex situ* generated *trans* isomer (positive control) and vehicle (negative control) at all the respective time of *cis* activation. The degree of edema observed in the photoactivated fish are similar to the *t*-**CAP-F5** treated fish at all points of photoirradiation, signifying the capability of activating the probe within the embryos (Figure 4.17 E). Moreover, as we progress

further along the post-fertilization timeline for probe activation, the inhibitor efficacy appears to be decreasing. These results are consistent with the known crucial role of CA role in the initial phases of fish development.⁵⁸ Otolith development at various stages of probe activation shows a similar trend (Figure 4.17 F), further corroborating the relation of phenotypic changes with *in situ* CA inhibition in this live organism model system. These experiments represent a key demonstration of using *cis* stabilized *ortho*-fluorinated azobenzenes in an *in vivo* system and achieving *in situ* photoactivation over extended time periods (up to 48 h).

4.5 CONCLUSIONS

In conclusion, we have demonstrated the *in vitro*, cellular, and *in vivo* application of an azobenzene photopharmacophore that targets the active site of the metalloenzyme carbonic anhydrase. *Ortho*-fluorination serves to red-shift photoisomerization wavelengths, improve photoconversion efficiency, and importantly, stabilize the *cis* isomer. These properties enable longitudinal studies in cells and, for the first time, *in vivo*, allowing *in situ* temporal control of CA activity and demonstrating the potential use of this strategy for spatially and temporally regulated metalloenzyme activity. These strategies should not be limited to carbonic anhydrase and current efforts are focused on targeting metalloenzyme classes with relevance to cancer and other diseases.

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4.7 SUPPLEMENTAL DATA AND FIGURES

4.7.1 Studies with control molecule Acetazolamide (Az)

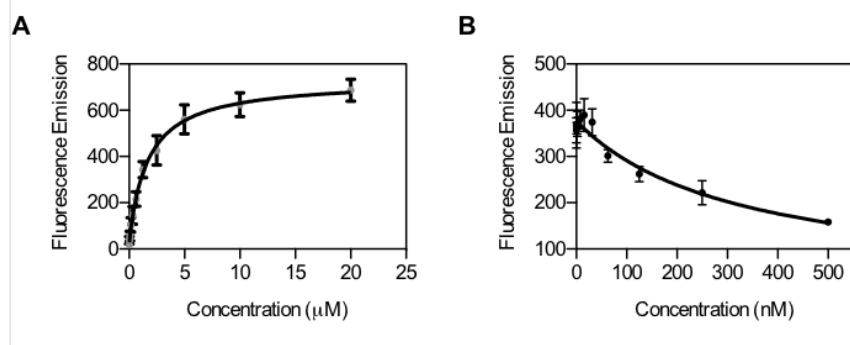


Figure 4.18 Determination of K_d for DNSA binding to bCA (A). Determination of K_d for acetazolamide binding to bCA in the presence of DNSA (B) $\lambda_{\text{ex}} = 280 \text{ nm}$, $\lambda_{\text{em}} = 470 \text{ nm}$. Studies are performed in 50 mM HEPES containing 0.1M KNO_3 pH 7.2 at RT. Data represents mean $\pm$ standard deviation.

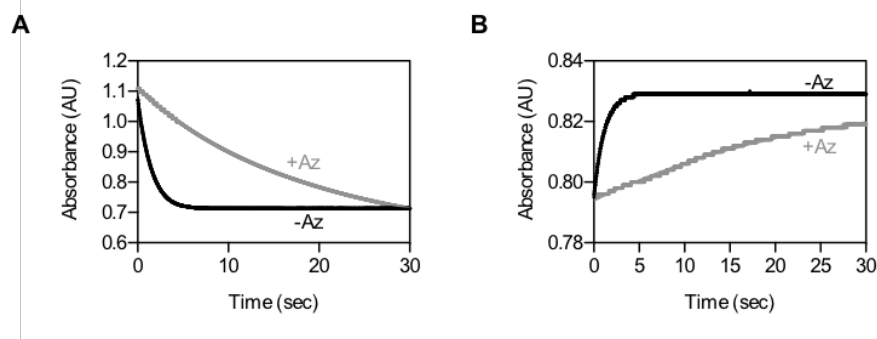


Figure 4.19 The change in absorbance of phenol red ($\lambda=557\text{nm}$) during CO_2 hydration (A) and HCO_3^- dehydration (B) activity of CA in the absence (-Az) and presence of 1 eq Acetazolamide (+Az). Studies are conducted in 50 mM HEPES, 0.1M KNO_3 pH 7.2 at 25 $^\circ\text{C}$.

4.7.2 Studies with control molecule CAP-con

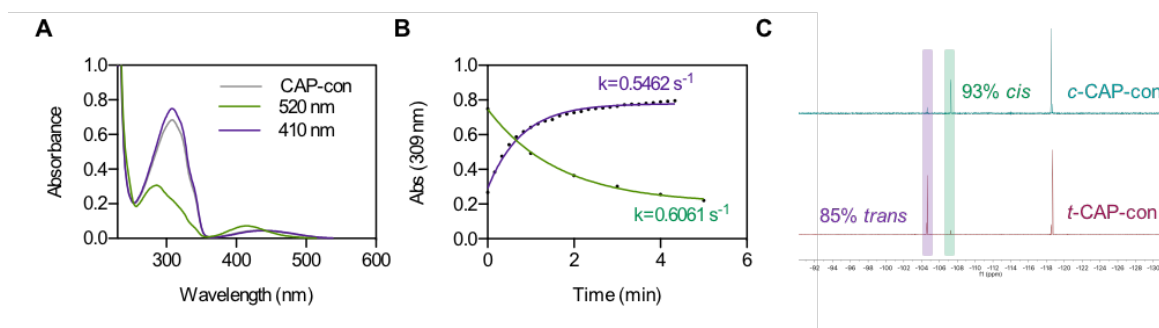


Figure 4.20 Absorbance spectra (A) and photoisomerization kinetics (B) of **CAP-con** in methanol solvent as monitored by UV-vis spectroscopy. The % of isomer at PSS (C) as analyzed by ¹⁹F NMR spectroscopy with 30 sec relaxation time in CD₃OD solvent. The highlighted region corresponds to F atom at *p*-position.

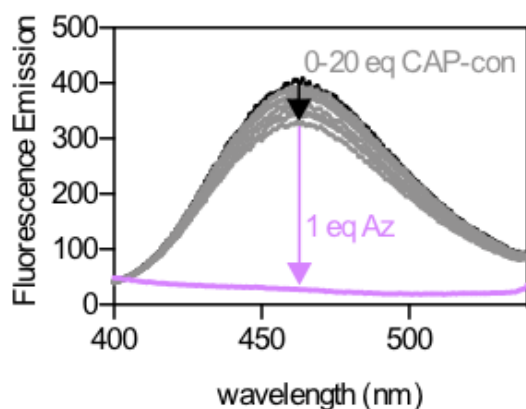


Figure 4.21 The fluorescence emission ($\lambda_{\text{ex}} = 280 \text{ nm}$) of bCA.DNSA adduct after the addition of 1-20 eq of **CAP-con** (grey) and then of 1 eq of acetazolamide (Az, purple), representing the negligible binding of **CAP-con** to the Zn²⁺ ion in the active site of CA. Studies are performed in 50 mM HEPES containing 0.1M KNO₃ pH 7.2 at RT.

4.7.3 Additional data for CAP-F5

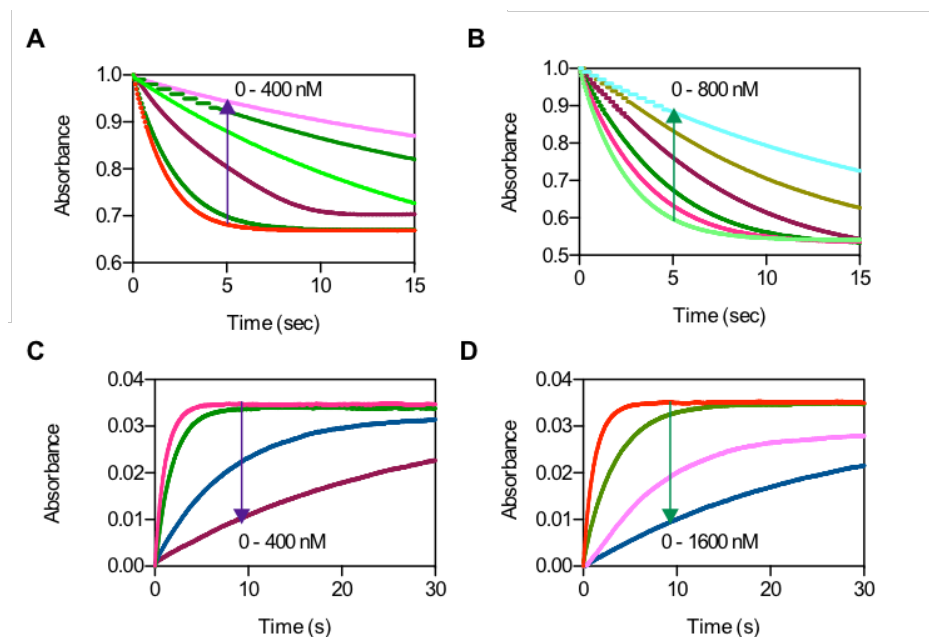


Figure 4.22 The relative absorbance profile of phenol red ($\lambda=557\text{nm}$) during CO_2 hydration by bCA in presence of varying concentration of *t*-CAP-F5 (A) and *c*-CAP-F5 (B). The profile obtained for HCO_3^- dehydration process in the presence of *t*-CAP-F5 and *c*-CAP-F5 is shown in C and D, respectively. Studies are conducted in 50 mM HEPES, 0.1M KNO_3 pH 7.2 at 25 °C.

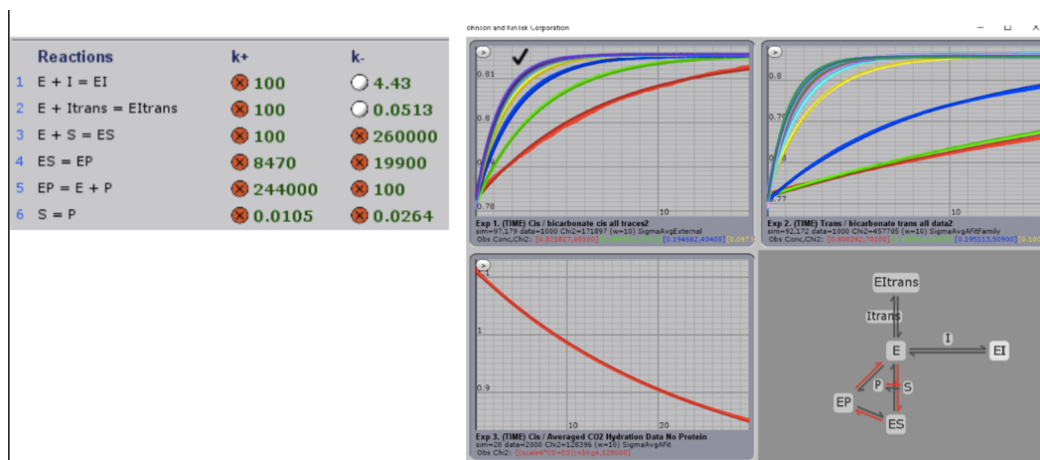


Figure 4.23 The fitting of experimental data of HCO_3^- dehydration of bCA in the presence of CAP-F5 in kintek explorer.

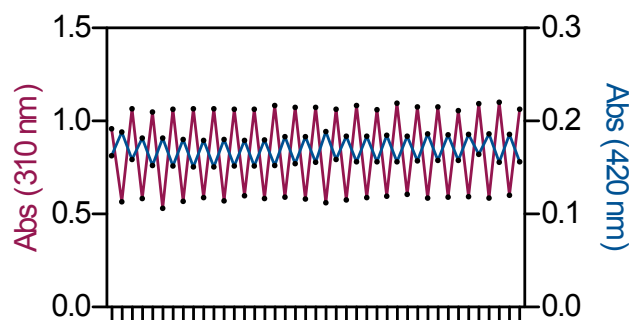


Figure 4.24 The fatigue resistance of CAP-F5 measure by changes in absorbance over 20 cycles of photoisomerization. The sample was irradiated with alternate 520 nm (5 min; 9.18 mW/cm²) and 410 nm (2 min; 5.20 mW/cm²). Studies are performed in phenol red free DMEM cell culture media.

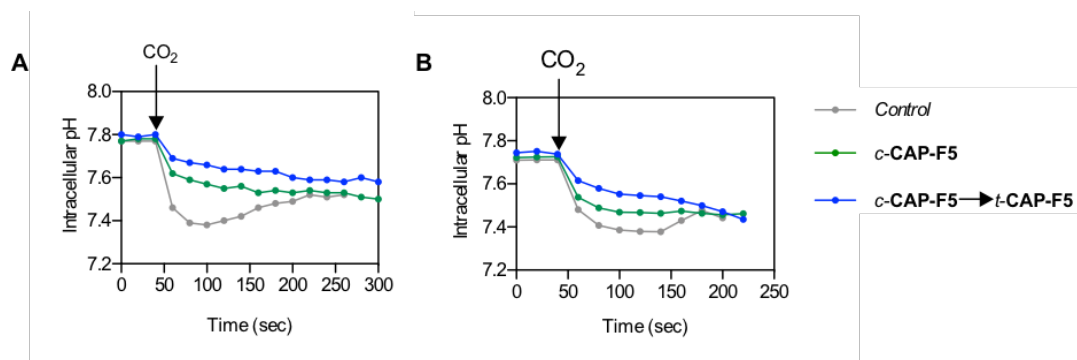


Figure 4.25 The change in intracellular pH of cells incubated with *c*-CAP-F5 before and after irradiating with 410 nm to isomerize *cis* isomer to *trans* isomer. These studies performed in Live Cell Imaging Solution at 4°C. These are replicate experiments from Figure 4.16 C.

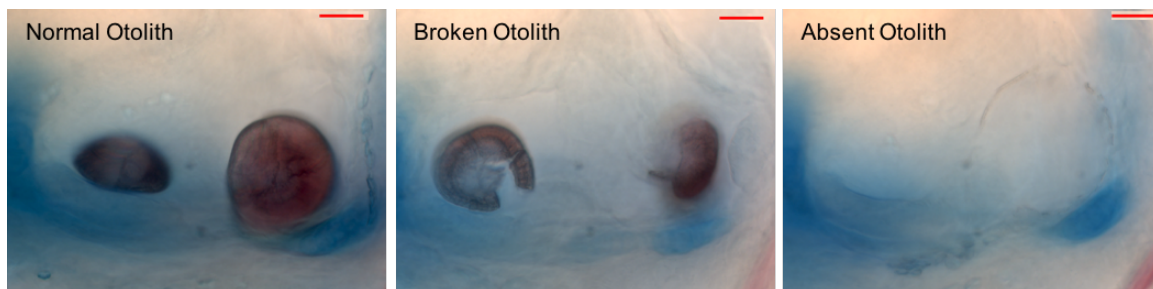


Figure 4.26 The representative images for normal otolith, broken otolith and absent otolith as a result of probe treatment during zebrafish development from Figure 4.17 F. Scale bar: 50 μm

4.7.4 Parameters set for in vitro studies

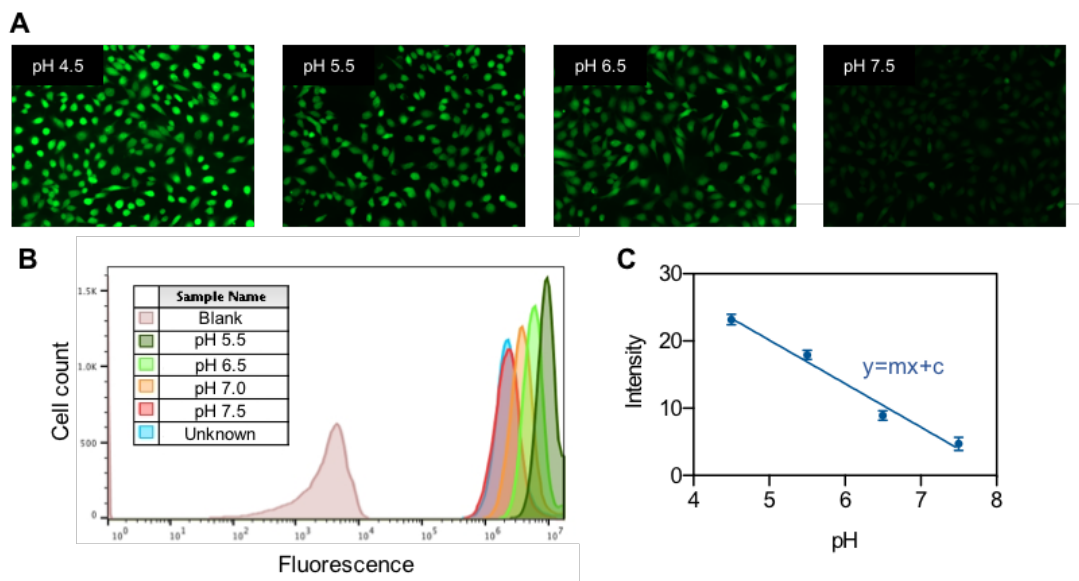


Figure 4.27 An example of calibration curve (C) obtained by using pHrodo intracellular AM dye and calibration buffers in the presence of valinomycin and nigericin. Data represents mean $\pm$ standard deviation. The calibration was performed for each individual live cell imaging (A) and flow cytometry (B) experiments.

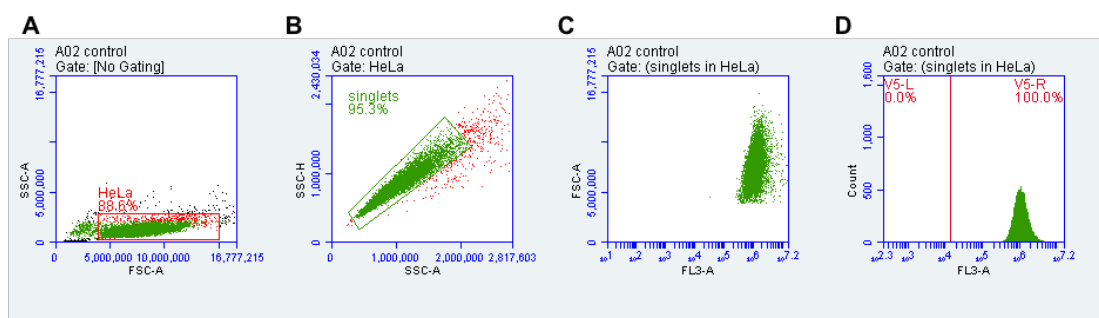
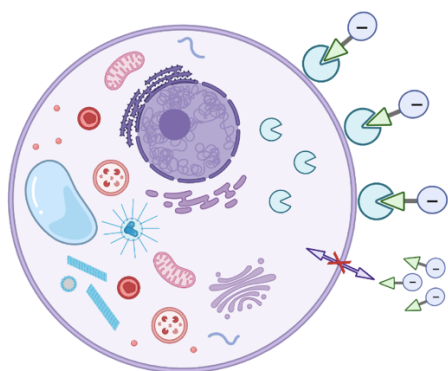


Figure 4.28 The gating channels used to separate cells from debris (A), singlets from cluster of cells (B) when passing the cells through Flow Cytometry. These gates were fixed for all FC experiments. The fluorescence of pHrodo red AM ester dye in the cells is determined with respect to forward scatter area density plot (C) and population of cells distribution (D).

Chapter 5: Design and synthesis of photoswitchable azobenzene probes for the reversible control of Carbonic Anhydrase IX activity using visible light⁴

5.1 ABSTRACT

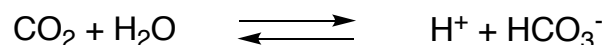
Carbonic anhydrase catalyzes the reversible conversion of carbon dioxide and bicarbonate, and thus, plays a very important role in cellular pH homeostasis. There are 15 unique mammalian known isoforms of this protein, with some isoforms overexpressed in cancer cells, like CA IX and CA XII, resulting in a pH imbalance. This forms a positive feedback pathway for malignant tumors to metastasize. While various sulfonamide-based inhibitors have been developed for inhibiting the activity of the over expressed protein, there are two major issues: 1) they are not selective to a particular isoform; 2) they permanently alter protein function. Because of this protein's importance to the functioning of both normal and cancerous cells, we need a chemical tool to modulate the activity of specific isoforms of this protein through external control. In this chapter, we describe a strategy to use light to switch between the biologically active and inactive conformations of an inhibitor designed to specifically access cell membrane-bound CAIX that is associated with hypoxic tumors.



⁴ This chapter is based on the following work:
Aggarwal, K.; Ye, E.; Kolli, D.; Banik, M. *In preparation*

5.2 INTRODUCTION

The reversible regulation of enzymes has profound importance in biology and may be used to tune the activity of over-expressed proteins in cancer cells.¹ One of the proteins of interest is carbonic anhydrase, a zinc-containing metalloenzyme. The role of this protein is to catalyze the reversible conversion of bicarbonate and carbon dioxide:



Carbon dioxide is the final and major product of oxidative phosphorylation and is immediately converted to bicarbonate by carbonic anhydrase, which helps in maintaining the $\text{CO}_2\text{-HCO}_3^-$ pH buffer system in all living organisms.² Due to the high energy needs of living systems and the slow un-catalyzed process, many different isoforms of carbonic anhydrase have evolved to meet physiological pH balance needs.³ There are currently 15 known homologous mammalian carbonic anhydrase isoforms localized to different cellular organelles.⁴ Of these, carbonic anhydrase IX (CA IX), a membrane bound protein, is of major interest due to its high level of overexpression in many tumors.⁵ CA IX is encoded by the *CA9* gene in human and facilitates acid secretion in the gastrointestinal tract.⁶⁻⁷ The CA IX enzyme, along with the CA II enzyme, binds to Anion Exchanger 2 (AE2) which increases bicarbonate transport and maximizes the rate of acid secretion by gastric parietal cells.⁸

CA IX is one of the most active isoforms of CA and has a dimeric structure, as compared to the other isoforms, which are all monomeric (Figure 5.1 A). Both units of the dimer are stabilized by two hydrogen bonds between the side chain guanidinium of Arg-137 and the carbonyl oxygen of Ala-127, along with many Van der Waals interactions (Figure 5.2 B). There is extra stabilization in one of the unit which is offered by the disulfide bridge formed by two cysteine residues.⁹

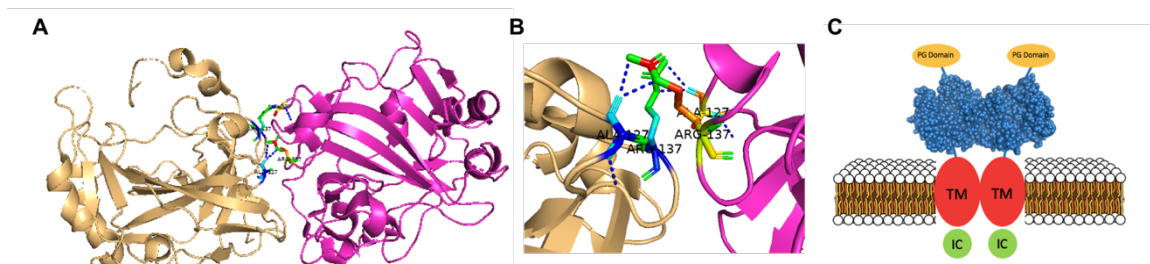


Figure 5.1 CA IX dimer (A) and representation of two hydrogen bonds between Arg-137 and Ala-127 carbonyl oxygen that stabilizes the CAIX dimer (B). Four domains of CA IX dimer: proteoglycan domain (PG), catalytic domain (blue), transmembrane segment (TM) and the intracellular tail (IC) (C).

One face of each dimer contains proteoglycan (PG) domains, and the opposite faces contain the C-termini which facilitate enzyme attachment to the cell membrane (Figure 5.1 C).¹⁰ The active site of this protein is exposed to the extracellular environment, enabling this isoform to be more easily targeted by cell membrane impermeable probes.

In general, carbonic anhydrase plays a hypocritical role in contributing to the processes of life; CA is a hallmark of all cancer cells, as it is highly overexpressed under hypoxic conditions.¹¹ CA IX specifically is overexpressed in cancer cells, including clear cell renal cell carcinoma (RCC) as well as carcinomas of the cervix, breast and lung where it promotes tumor growth by enhancing tumor acidosis (Figure 5.2).¹²⁻¹⁴ An indication of cancerous cells is a high rate of anaerobic respiration, even when there is enough oxygen to carry out aerobic respiration and produce more ATP. During anaerobic respiration, cells break down glucose into pyruvate and then into lactic acid. Due to this shift in metabolism, cancer cells pump acidic catabolites out to maintain normal alkaline intracellular pH (pH_i) levels for their survival.¹⁵ As a result of the lactic acid's high extrusion, the extracellular pH (pH_e) decreases. Moreover, CA IX over-expression further acidifies the extracellular environment.¹⁶ This causes upregulation of angiogenic factors

and proteases, damaged immune functions, and faster metastasis, forming a positive feedback pathway for the invasion of cancer.¹⁷ In combination, these factors suggest the important role of CA IX in cancer proliferation and make it an appropriate marker for cancer prognosis.^{12, 18-19}

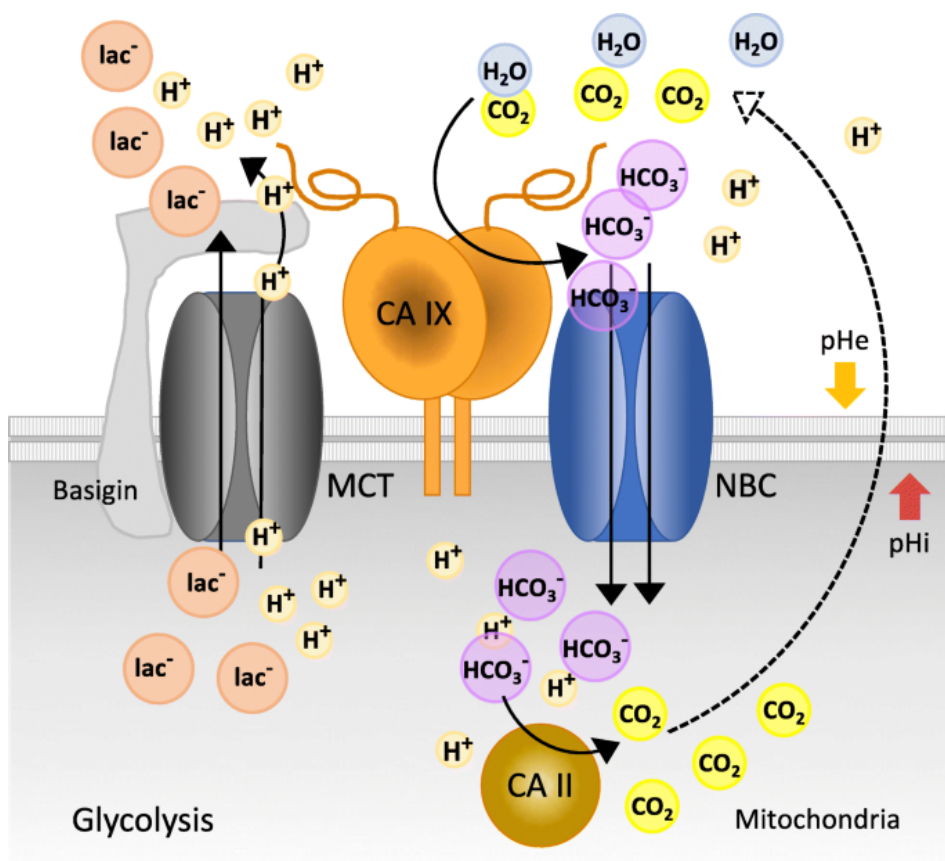


Figure 5.2 Graphic representation of role of CAIX in creating an acidic extracellular pH (pH_e)¹³

Over the past couple of decades, several sulfonamide-based inhibitors have been developed, where they interact with the zinc ion in the active site of the protein, and hence inhibit the catalytic activity.²⁰⁻²¹ A select few of them, for example, acetazolamide, methazolamide, ethoxazolamide, dorzolamide, are used as drugs to treat certain

conditions, including altitude sickness.²²⁻²⁶ However, due to the abundance of hCAII and the lack of isoform specificity of the inhibitors, most of the effects of these inhibitors will be due to hCAII inhibition. Thus, for example, when trying to target other CA isoforms clinically, most of the observed side effects of such clinically used sulfonamides are probably due to the undesirable inhibition of hCAII in certain tissues and organs.²⁷ Hence, it is highly desirable to develop isoform specific inhibitors. The high degree of sequence and structural similarity among CA isoforms, however, hinders this strategy.²⁸ Recently, there have been some developments made where the inhibitor is made bulkier in order to preferentially interact with the wider active site of CA IX than CA II.²⁹⁻³¹ A limitation of these developed inhibitors is the inability to control the location and timing of their inhibitory activity. Thus, strategies for targeting the activity of these molecules are of great interest both for therapeutic and basic science applications.

Photoactivation is one strategy, in which application of specific wavelength(s) of light induces structural and functional changes in a molecule. This process can be irreversible, in which photocleavable protecting groups are incorporated to block the activity of an inhibitor.³²⁻³³ Alternately, a reversible process, such as photoisomerization, can be employed such that the inhibitor can be activated and deactivated on demand.³⁴⁻³⁵ To this end, azobenzenes are promising structures that display reversible isomerization between two different stereoisomers, *trans* and *cis*, upon light irradiation.³⁶⁻³⁹

In this dissertation, we have discussed the development of Carbonic Anhydrase Azobenzene Probes (CAPs) to gain control over carbonic anhydrase. In our scheme, the azobenzene photoswitch is linked to a sulfonamide (-SO₂NH₂) group at the *para*-position, which serves as the metal binding group to the Zn²⁺ ion in carbonic anhydrase. In its thermally stable *trans*-form, the ligand sits in the protein's active site, thus binding to the Zn²⁺ ion, and inhibiting the protein's activity, as shown in Figure 5.3. When the inhibitor

is irradiated with light of an optimal wavelength, it isomerizes to its *cis*-form. Due to steric bulk around the sulfonamide group, the *cis*-azobenzene comes out of the protein's active site, activating the protein's normal functions. The *cis*-isomer can be isomerized back to *trans* through light of another wavelength, or through the thermal relaxation process. This will inhibit the protein again, making the process a reversible system.⁴⁰ We reported **CAP-F5** which shows all the optimal properties required to gain photocontrol over protein activity: visible light isomerization, high photoisomerization efficiency, increased thermal stability of both *trans* and *cis* isomer.⁴¹

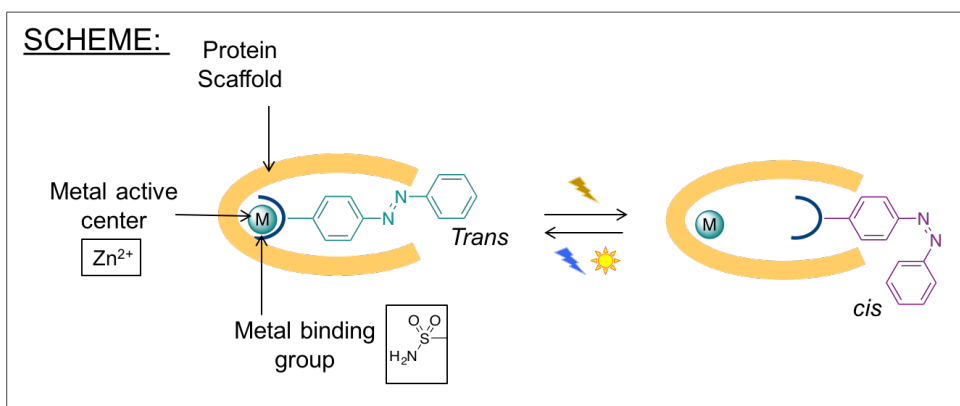


Figure 5.3 Schematic representation of using azobenzene as a photoswitch to gain control over CA using light as the trigger.

In this chapter, we will use these fluorinated systems and modify them with charged functional groups to limit the access of the probe to the extracellular environment only, and hence achieving exclusive photomodulation over the membrane bound isoforms of CA, such as CA IX. We will discuss the design rationale and the synthetic challenges faced to develop the final target molecule.

5.3 EXPERIMENTAL

5.3.1 General Procedures

All solvents and chemicals were purchased from Sigma-Aldrich, VWR, and Fisher Sci. and used as received. ^1H and ^{19}F spectra are collected in deuterated solvents from Cambridge Isotope Laboratories (Cambridge, MA), using an AGILENT MR 400 NMR spectrometer at 400 MHz. The chemical shift is calibrated with respect to the solvent peak, and is reported in ppm. Walk-up LC-MS mass spectral analyses were performed by the Mass Spectrometry Facility of the Department of Chemistry at UT Austin. Absorbance spectroscopic studies were performed using an Agilent Cary 60 UV-Vis spectrophotometer. The samples were irradiated with an Asahi Xe lamp connected with bandpass filters of 365 nm (FWHM 12 nm), 460 nm (FWHM 11 nm) from Asahi Spectra, and 410 nm (band width 10 nm) and 520 nm (band width 10 nm) from Thorlabs. For spectroscopic studies, 0.05 M HEPES buffer with 0.1 M KNO_3 (pH 7.2) was used unless stated otherwise. All the studies were done under dark condition to avoid exposure of light.

5.3.2 Synthetic Procedures

Synthesis of Compound 1: To a 20-mL solution of concentrated aqueous ammonium hydroxide at 0°C in a 100 mL round bottom flask, pentafluorosulfonyl chloride (0.56 mL, 3.75 mmol) was added dropwise. The reaction was brought to room temperature and stirred for 1 hour. The product was extracted with ethyl acetate ten times, with the combined organic layers dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure to obtain the product in a 96% yield as a white powder.

Synthesis of Compound 2: Compound **1** (1.00 g, 4.40 mmol) was dissolved in 40 mL acetone in a 100-mL round bottom flask. NaN₃ (263 mg, 4.05 mmol) were added and the solution was refluxed overnight. The product was extracted with water and ether three times, with the combined organic layers dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure to obtain the product in a 95% yield as a white powder.

Synthesis of Compound 3: Compound **2** (1.18 g, 4.4 mmol) was dissolved in 17.5 mL THF and 6.56 mL saturated ammonium chloride in a 100-mL round bottom flask. Zinc powder was added (1.71 g, 26.3 mmol) to the solution, which was refluxed for one hour. The product was extracted with ethyl acetate and water three times, with the combined organic layers dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure. The product was then absorbed onto celite and was eluted at 60% acetonitrile/water on a reverse phase C18 column to get a pure white-yellow powder in a 46% yield.

Synthesis of Compound F8-OH: Compound **3** (516.5 mg, 2.12 mmol) was dissolved in 25 mL 50% HCl/MeOH in a 100 mL round bottom flask at 0°C and was stirred for five minutes. Tetrafluorophenol (351.4 mg, 2.12 mmol) was dissolved in a separate 250 mL round bottom flask in 25 mL 10 M NaOH at 0°C. A solution of 2.3 M aqueous NaNO₂ (438.2 mg, 6.35 mmol) was added dropwise to the sulfonamide round bottom flask and was stirred for five minutes, forming a yellow colored solution. The sulfonamide solution was added dropwise to the phenol solution at 0°C, and was allowed to stir vigorously at room temperature overnight. The solvent was evaporated, and the solution was acidified with 2 N HCl and then extracted with ethyl acetate three times. The combined organic layers were dried over anhydrous sodium sulfate, and the solvent was evaporated under reduced pressure. The crude product was then absorbed onto celite

and run on a reverse phase C18 column in a water acetonitrile mixture. The product was obtained at 60% CH₃CN/H₂O an 43% yield as a red powder.

Synthesis of Compound F8-OEtOH: Compound **F8-OH** (128.8 mg, 0.305 mmol) was dissolved in 6.5 mL acetonitrile in a 50 mL round bottom flask. K₂CO₃ (126.8 mg, 0.918 mmol) and 2-bromoethanol (65.1 μ L, 0.918 mmol) were added. The reaction was refluxed for 24 hours. The product was precipitated with 2 N HCl, extracted with ethyl acetate. The unreacted reactant was removed by washing the ethyl acetate layer with 1M K₂CO₃ solution. The ethyl acetate layer was dried over anhydrous sodium sulfate and was concentrated under reduced pressure. The product was absorbed on celite and run on a reverse phase C18 column in a water methanol mixture, with the product eluting at 80% MeOH/H₂O as a red powder in a 90% yield.

Synthesis of Compound F8-Boc: First the linker was prepared by taking DIPEA (1.60 mL, 4.57 mmol) in 20 mL THF and 10 mL DCM in a 50 mL round bottom flask. 3-bromopropylamine hydrobromide (1 g, 4.57 mmol) and di-tert-butyl dicarbonate (1.99 g, 9.14 mmol) were added to the reaction mixture. The reaction was stirred for 16 hours at room temperature and then extracted and washed with 5% citric acid, DCM, and brine. The product was obtained in an 85% yield as a clear yellow liquid. Product formation was confirmed with NMR. Compound **F8-OH** (5 mg, 0.0117 mmol) was dissolved in 0.5 mL acetonitrile. K₂CO₃ (4.93 mg, 0.010 mmol), KI (catalytic amount) and Compound 4 (1 mg, 0.010 mmol) were added to the reaction mixture. The reaction refluxed overnight, resulting in a darker red opaque solution. The acetonitrile was evaporated off under reduced pressure, and the crude product was extracted twice with ethyl acetate and water. The aqueous layer was then basified with 1 M K₂CO₃ and extracted with ethyl acetate to obtain unreacted reactant back. The solvent was evaporated under reduced pressure. A

manual column was performed with ethyl acetate and methanol, with the product eluting at 5% MeOH/EtOAc.

Synthesis of Compound F8-OEtBr: Compound **F8-OH** (10 mg, 0.024 mmol) was dissolved in mL dry acetonitrile. K_2CO_3 (5.91, 0.043 mmol), 18-crown-6 (0.25 mg, 0.000946 mmol), and 1,2-dibromoethane (0.005 mL, 0.112 mmol) were added to the flask. The solution was refluxed for 3.5 days. The solution was extracted with 2 N HCl, ethyl acetate, and water, with the combined organic layers dried over anhydrous sodium sulfate. The product was evaporated under reduced pressure.

Synthesis of Compound 4: 3-bromo-2,5-difluoroaniline (2 g, 9.62 mmol) was added to acetic anhydride (3 mL) and let stir for 1 hour at room temperature. A white solid formed and an ethyl acetate/water extraction was performed 7 times. The organic layers were dried over sodium sulfate and evaporated under reduced pressure. No additional purification was required. The yield was 99%, with the product as a white solid.

Synthesis of Compound 5: Compound **4** (200 mg, 0.743 mmol) was dissolved in degassed 3 mL EtOH in a purged Schlenk tube with nitrogen flowing. NEt_3 (156.6 μ L, 1.114 mmol) and $P(OEt)_3$ (106.8 μ L, 0.892 mmol) were then added to the solution. After 5 minutes of stirring at room temperature, $Pd(OAc)_2$ (20.01 mg, 0.089 mmol) and PPh_3 (31.2 mg, 0.119 mmol) were added as one mixture and the reaction was set to reflux overnight. The palladium catalyst was filtered off, the ethanol was evaporated, the product was extracted into ethyl acetate three times, and then the organic layer was dried over sodium sulfate. The product was absorbed onto silica and a normal phase column was run in 0% MeOH/DCM to 3% MeOH/DCM, resulting in a product yield of 29% as a white solid. It is important to note that upon initial evaporation of the solvent containing

the product, the product seemed to be a very light oil. After being on the high vac for more than 24 hours, a white mold-like solid formed.

Synthesis of Compound 6: Compound **5** (200 mg, 0.65 mmol) was dissolved in 25 mL EtOH. Concentrated H₂SO₄ (1.25 mL) was added dropwise and the reaction was set to reflux for 18 hours. The ethanol was evaporated under reduced pressure, and the reaction was worked up with ethyl acetate and water, with the organic layers dried over sodium sulfate and evaporated under reduced pressure. No further purification was needed, and the pure product formation was confirmed through NMR and LCMS with a 99% yield.

Synthesis of Compound 7: Compound **6** (190 mg, 1 eq) and oxone (857.9 mg, 4 eq) were dissolved in 4.1 mL DCM (1 eq) and 8.2 mL H₂O (2 eq). The reaction was stirred at room temperature over 2 days and slowly turned from colorless to green. The solution was extracted with DCM and water and then dried over sodium sulfate. Upon evaporation under reduced pressure, a brown-orange solid formed. Purification with column chromatography (silica gel, 3% methanol/chloroform) gave compound **7** as a white solid in a 40 to 90% yield.

Synthesis of Compound 8: To a 100-mL solution of concentrated aqueous ammonium hydroxide at 0°C in a 100 mL round bottom flask, trifluorosulfonyl chloride (5 g, 21.7 mmol) was added dropwise. The reaction was brought to room temperature and stirred for 1 hour. The product was extracted with ethyl acetate five times, with the combined organic layers dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure to obtain the product in a 96% yield as a white powder.

Synthesis of Compound 9: Compound **8** (1.5 g) was dissolved in a Parr high pressure reactor with 15 mL of 7 M NH₃/MeOH. The reactor was sealed and heated at

120°C for 2 days. After cooling the system to room temperature, the reaction mixture was concentrated under reduced pressure. The crude mixture was loaded on celite and purified through reverse phase column chromatography to elute the product at 4% acetonitrile/water to obtain the white compound in 60% yield as a white powder.

Synthesis of Compound 10: Compound **9** (9.9 mg, 0.048 mmol) was taken in acetic acid:TFA:toluene (6:1:6) mixture, and Compound **7** (20 mg, 0.072 mmol) was added to it, and stirred at room temperature for one day and then at 45°C for one day. The compound was extracted in ethyl acetate and washed with water, dried over anhydrous sodium sulfate, and concentrated under vacuum. No reactants or product were identified after work-up.

Synthesis of Compound 11: To a 20-mL solution of concentrated aqueous ammonium hydroxide at 0°C in a 100 mL round bottom flask, pentafluorosulfonyl chloride (0.56 mL, 3.75 mmol) was added dropwise. The reaction was brought to room temperature and stirred for 1 hour. The product was extracted with ethyl acetate ten times, with the combined organic layers dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure to obtain the product in a 96% yield as a white powder.

Synthesis of Compound 12: Compound **11** (1.00 g, 4.40 mmol) was dissolved in 40 mL acetone in a 100-mL round bottom flask. NaN₃ (263 mg, 4.05 mmol) were added and the solution was refluxed overnight. The product was extracted with water and ether three times, with the combined organic layers dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure to obtain the product in a 95% yield as a white powder.

Synthesis of Compound 13: Compound **12** (1.18 g, 4.4 mmol) was dissolved in 17.5 mL THF and 6.56 mL saturated ammonium chloride in a 100-mL round bottom

flask. Zinc powder was added (1.71 g, 26.3 mmol) to the solution, which was refluxed for one hour. The product was extracted with ethyl acetate and water three times, with the combined organic layers dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure. The product was then absorbed onto celite and was eluted at 60% acetonitrile/water on a reverse phase C18 column to get a pure white-yellow powder in a 46% yield.

Synthesis of Compound 14: Compound **13** was taken in a round bottom with cyclohexylamine and DMSO was added. While stirring, NEt_3 was added and heated at 100 °C for two days. The product was extracted in ethyl acetate and concentrated under vacuum. The product was obtained in 94% yield.

Synthesis of Compound 15: 2,5-difluoro-4-bromo-aniline was taken in a seal tube and dry DMF was added. CuCN was slowly added to the mixture and it was heated at 160 °C for 14 h. The reaction mixture was then quenched by adding aqueous NH_4OH and the product was extracted in ethyl acetate. The organic layer was further washed with basic water, and was then concentrated under vacuum. The product was purified by silica gel column. To obtain the product in 46% yield.

Synthesis of Compound 16: Compound **15** was taken in 1M NaOH and was refluxed for 14 h. The reaction mixture pH was changed to acidic by using 2M HCl, and the pure product was collected via precipitate filtration in 96% yield.

Synthesis of Compound 17: Compound **16** was dissolved in a round-bottom flask with ethanol. Sulfuric acid was added, and the reaction was refluxed at 78°C overnight. After cooling the system to room temperature, the reaction mixture was concentrated under reduced pressure. The crude mixture was purified via normal-phase column chromatography in MeOH/DCM to obtain the white compound in 87% yield as a white powder.

Synthesis of Compound 18: Compound **17** was dissolved in a round-bottom flask with 1:1 ratio of DCM and water. Oxone was added to the solution mixture, and the reaction was stirred vigorously at room temperature overnight. After cooling the system to room temperature, the reaction mixture was concentrated under reduced pressure. The crude mixture was purified via normal-phase column chromatography in MeOH/DCM to obtain the white compound in 45% yield as a white powder.

Synthesis of Compound 20: Compound **9** (80 mg, 0.385 mmol) and compound **18** (124 mg, 0.578 mmol) were dissolved 6.2 mL toluene in a round-bottom flask and stirred at 45 °C. 6.2 mL of acetic acid and 1 mL TFA were added to the solution mixture and the solution was refluxed for 72 hr. The color of the solution changed from green to red over the course of the reaction, and the product was purified via normal phase column chromatography with MeOH/DCM.

Synthesis of Compound F4-COOH: Compound **20** was dissolved 2 mL ethanol and 50 μ L NaOH in a round-bottom flask. The solution mixture was left at room temperature overnight, and the product was purified via normal phase column chromatography with MeOH/DCM. Product formation was confirmed through LCMS.

Synthesis of Compound 21: Compound **F4-COOH** was dissolved in DMF, and SOCl_2 was added and stirred at 45 °C overnight, and the product was purified via normal phase column chromatography with MeOH/DCM.

Synthesis of Compound F4-Am-SO₃: Compound **21** (25 mg, 0.154 mmol) was dissolved in a mixture of 1 mL DMF and 0.15 mL water and stirred. Aminomethylsulfonic acid (12 mg, 0.154 mmol) was added to the solution, and the solution mixture was stirred at 45 °C overnight. Upon completion of the reaction, the solution was acidified using HCl to pH $\sim$ 2, and the reaction was filtered using a vacuum

filtration system. The product was then purified via normal phase column chromatography with MeOH/DCM.

5.3.3 Photoisomerization Characterization

The photoisomerization of **F4-COOH** was investigated by electronic absorption spectroscopy. The absorbance spectrum was collected in methanol and aqueous buffer (50 mM HEPES, 0.1 M KNO₃, pH 7.2) under dark conditions. To get the *cis* isomer profile, the sample was irradiated with 520 nm for 5 minutes, and spectrum was collected. To isomerize the *cis*-isomer back to *trans*-isomer, the sample was irradiated with 430 nm for two minutes before collecting the absorbance spectrum.

The lifetime of the *cis*-isomer at 37°C was determined by using UV-Vis spectroscopy in aqueous buffer. The sample was prepared by diluting 10 mM DMSO stock solution of **F4-COOH** in aqueous buffer. The probe was photoisomerized to *cis* isomer by irradiating the sample with 520 nm, and the absorbance spectrum was collected every 1 hour at 37 °C for 24 hours under dark. The absorbance value at λ_{max} was plotted with respect to time to get the thermal relaxation profile.

5.3.4 Cell Culture

Cell culture was performed in Dulbecco's modified Eagle's Medium (DMEM), supplemented with 4000 mg/L glucose and sodium pyruvate, 10% heat inactivated fetal bovine serum (FBS), and 1% antibiotics (200 U/cm³ penicillin and 200 µg/cm³ streptomycin) at 37 °C in atmosphere containing 5% CO₂ and 2% O₂ (for Hypoxia). The cells were grown in T-75 flasks and were passaged once the confluence of cell reached 80% under normoxic conditions. For extracellular pH measurements, the cells were

grown in 6 well plate at a seeding density of 10^5 cells/well and allowed to grow in hypoxic conditions until they reach at confluency level of 60-70% at the time of analysis.

At the duration of 48 hours and 72 hours, the cell media was collected, and the pH was measured using pH probe. As the HCO_3^- based cell media was used, it was essential to measure the pH in the hypoxic chamber itself to avoid fluctuations in pH due to change in CO_2 level.

5.4 RESULTS AND DISCUSSION

To gain control over CA IX, we developed four different classes of azobenzene based probes over a period of two years (Figure 5.4). These probes differ in terms on number of fluorine atoms on the azobenzene for better photophysical properties and different functionalization at the *para*-position to make the probe resistant to cell permeation. We will briefly discuss each class individually and state the challenges faced that led us to develop the design and improve it further.

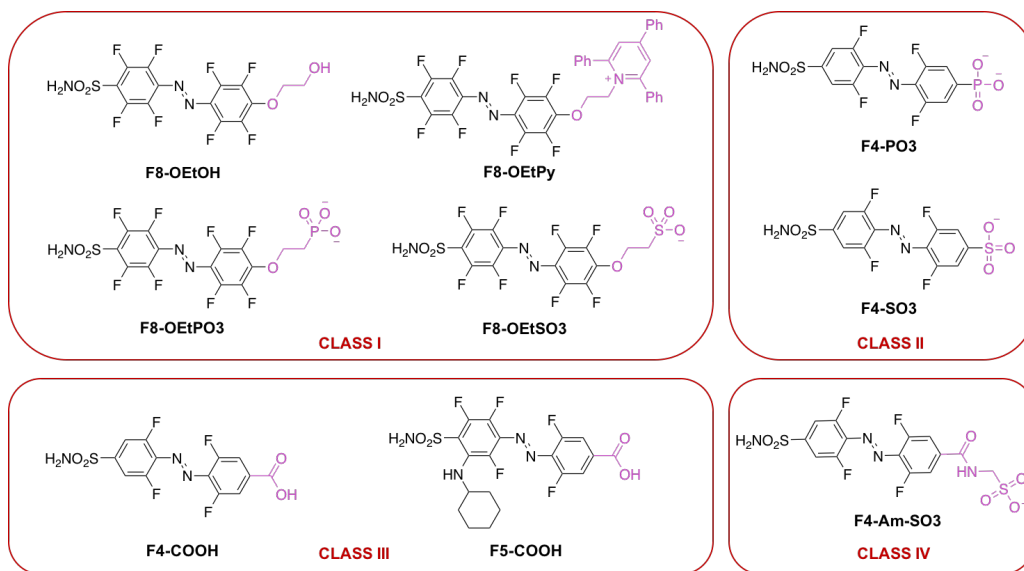


Figure 5.4 The four classes of azobenzene probes proposed to gain reversible photocontrol over CAIX.

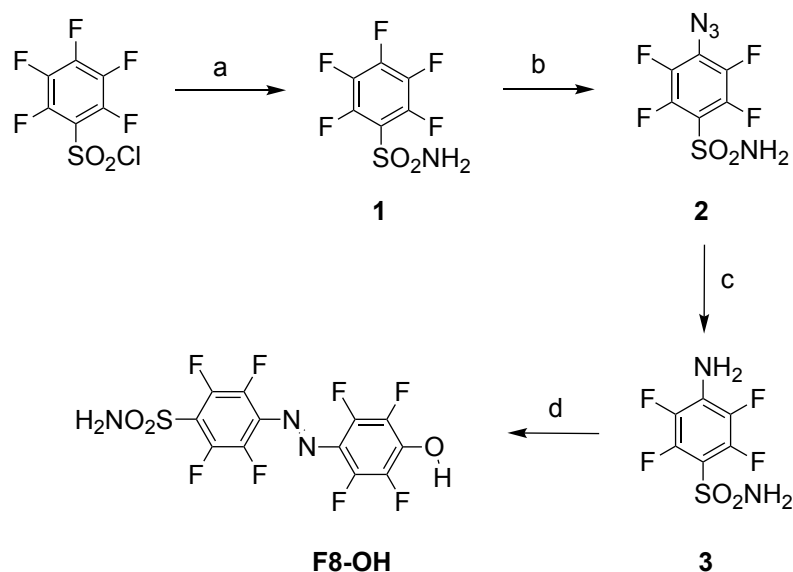
5.4.1 Class I azobenzene probes

5.4.1.1 Design rationale

The proposed probes in Class I (**F8-OEtOH**, **F8-Py**, **F8-SO3** and **F8-PO3**) in Figure are designed on the basis of previous results with **CAP-F5** and are further functionalized with fluorine atoms at the *ortho* and *meta* positions, with respect to the azo group. It has been reported that having eight fluorines on the azobenzene rings results in a greater percentage of *trans* and *cis* isomers at photostationary states (PSS) due to the larger separation of the $n-\pi^*$ absorbance band, as compared to four fluorine systems.⁷ The position of the sulfonamide group was kept the same, as it is in the optimal orientation to bind the active site's zinc ion.

On the other *para*-position of the second aromatic ring, four final molecules were made. Each of these had two or three carbon alkyl chains linking the azobenzene to one of four different ionizable groups—alcohols, pyridinium, sulfonic acids, and phosphonic acids. These groups were chosen either for their inherent charged nature or low pK_a values to ensure their membrane impermeability at physiological pH. However, **F8-OEtOH** will remain protonated due to its high pK_a , and was designed as a control molecule to study the effect of just the eight-fluorine substitution.

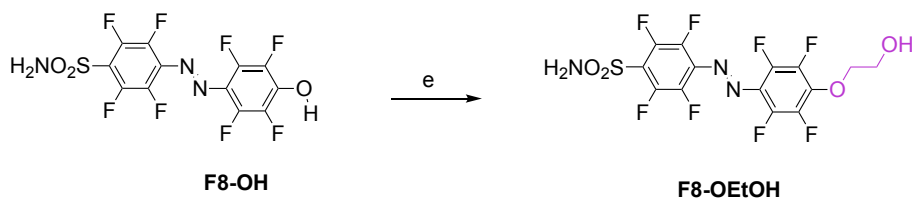
5.4.1.2 Synthesis and challenges



Scheme 5.1 Synthesis of compound **F8-OH**

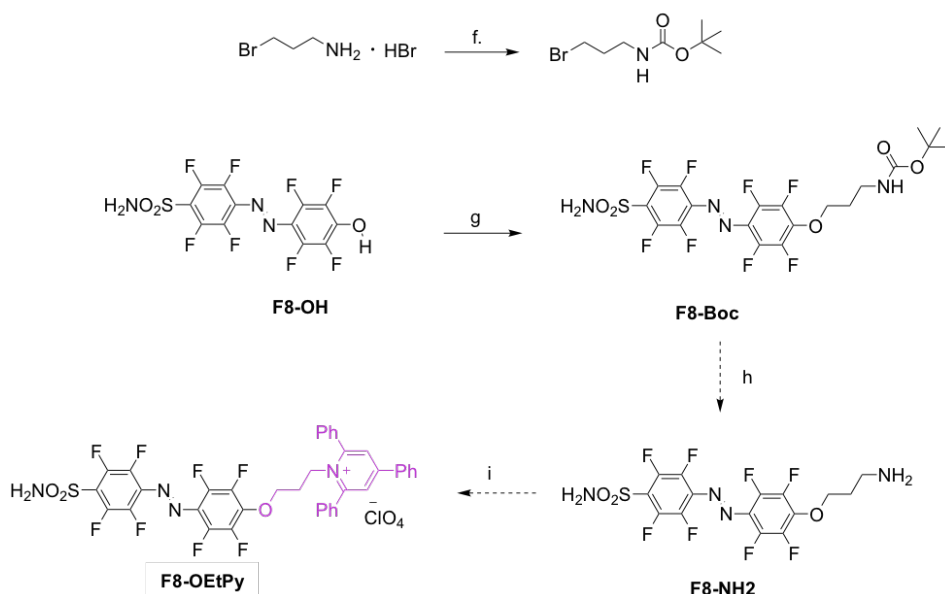
In order to make the final target molecules, **Compound F8-OH** was first prepared (Scheme 5.1). The starting sulfonyl chloride derivative was converted to the sulfonamide using aqueous NH_4OH at room temperature. This reaction was very simple, and yielded the product in good yield and high purity. However, using neat NH_4OH is very important as the reaction forms a sulfonic acid substituted substrate as the hydrolyzed product, whose amount varies depending on the chosen solvent. Under these conditions, it can be separated by an aqueous wash. The azide was then formed using NaN_3 as the reagent. The azide reduction was performed with saturated NH_4Cl in the presence of Zn dust as the catalyst, forming the aniline substituted product. Initially, several attempts to do this 2-step process in just one step were performed using 7 M NH_3/MeOH in a pressure reactor at high temperature, which was successful in the past for **CAP-F3** and **CAP-F5** (Chapter 4). However, these conditions resulted in polymerization in this case, as demonstrated by the observation of molecular weight values double that of the intended

product (via LC/MS). The azobenzene (**F8-OH**) was then formed through a classic azo coupling with tetrafluorophenol in the presence of NaNO₂.



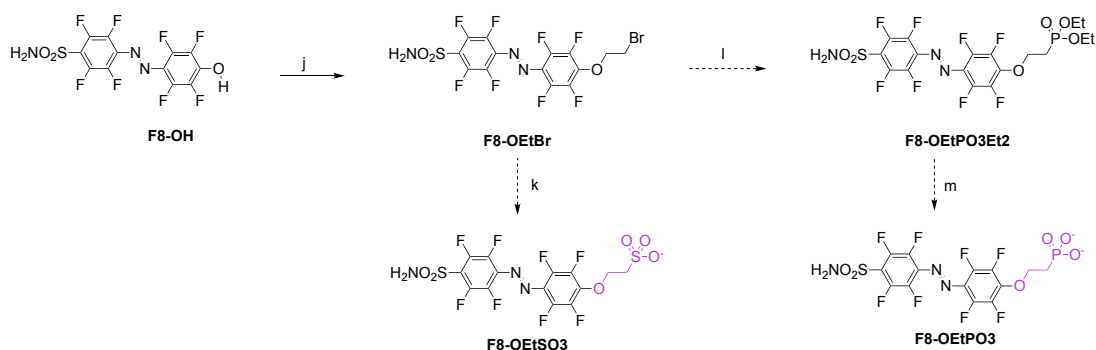
Scheme 5.2 Synthesis of **F8-OEtOH**

Compound F8-OEtOH was synthesized by reacting **Compound F8-OH** with 2-bromoethanol under basic conditions (Scheme 5.2). Initially, 2-chloroethanol was used; however, chlorine was a weaker leaving group, resulting in a poorer yield and higher percentage of unreacted **F8-OH**. The unreacted **F8-OH** was separated from the product using a basic wash with 1M K₂CO₃ and other impurities were separated through reverse phase and normal phase chromatographies. However, even after multiple columns, impurities remained present according to NMR, and it appeared that the product may degrade over time.



Scheme 5.3 Synthesis of **F8-OEtPy**

We attempted to make our second Class I molecule, **F8-OEtPy** per the scheme shown in Figure 5.3. First, the -NH_2 group on 3-propylamine was protected with a boc group using Boc_2O under anhydrous conditions. Then it was reacted with **Compound F8-OH** in the presence of K_2CO_3 as the base, and KI as the catalyst. Progress toward the final pyridinium molecule was halted due to uncertainty about the success of the reaction. As we have seen previously, substitution can occur at both the -OH and $\text{-SO}_2\text{NH}_2$ positions in the presence of base. In order to confirm the substitution site, we performed preliminary UV/Vis spectroscopy experiments in methanol with **F8-Boc**. The probe failed to isomerize under multiple wavelengths (365 nm, 410 nm, 460 nm, and 520 nm), which suggested the presence of a free -OH group. As we discussed in Chapters 2 and 3, probes containing -OH groups undergo rapid *cis* $\rightarrow$ *trans* thermal relaxation in protic polar solvent conditions. Due to these challenges, we focused on synthesizing the last two molecules in Class I, which were negatively charged to ensure cellular membrane impermeability of the probes.



Scheme 5.4 Synthesis of **F8-OEtSO3** and **F8-OEtPO3**

Starting from **Compound F8-OH**, the phenol functional group was coupled with 1,2-dibromoethane (Scheme 5.4). However, the reaction did not work under different basic conditions. The reason could be the poor nucleophilic nature of the phenolic group, or the *vic*-substrate, which makes it less stable. These challenges coupled with the fact that azobenzene system with fluorine at all the positions resulted in the very unexpected reactivity and decreased stability of products led us to design the Class II probes.

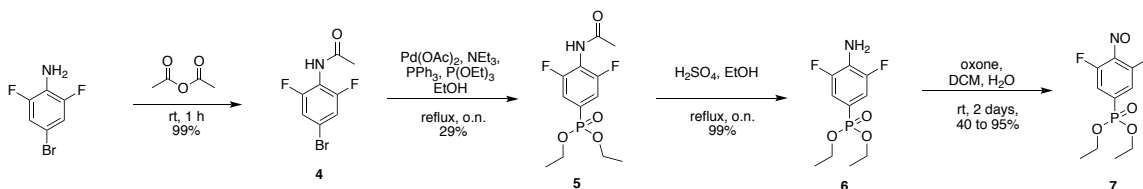
5.4.2 Class II azobenzene probes

5.4.2.1 Design Rationale

We decided to proceed with the azobenzene system containing only four fluorines, and to modify the *para*-position directly with charged species rather than via an alkoxy group. Ar-SO₃H groups have low pK_a values around -2.8, while Ar-PO₃H₂ groups are known to have pK_a values of approximately 1.1-2.3.⁸ These acidic groups will ensure that these species will be negatively charged under all biological pH conditions and will thus be unlikely to diffuse into the cell.

5.4.2.2 Synthetic Challenges

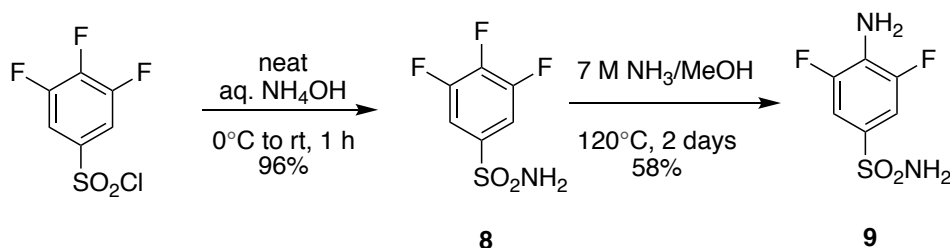
To synthesize **F4-PO3**, the precursors **7** and **9** required for azo coupling were synthesized individually. For the precursor **7** containing the phosphonic acid group, 3-bromo-2,5-difluoroaniline was used as the starting material and the boc protection was performed to get disubstitution on the aromatic amine, however, the subsequent reactions were unsuccessful, likely due to too much steric bulk on the reactant and the catalyst $\text{Pd}(\text{PPh}_3)_4$. Hence, we hypothesized that using a smaller protecting group that was singly substituted on the aniline would make the subsequent reactions more facile. Thus, the aromatic amine was protected using acyl group to get compound **4** (Scheme 5.5).



Scheme 5.5 Synthesis of precursor **7** for **F4-PO3**

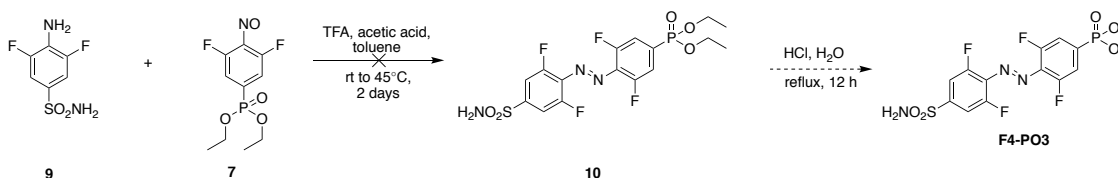
The next reaction of substitution of bromo group with phosphonic ester was optimized in terms of the choice of phosphonic acid reagent, catalyst, base and temperature. Finally, we were able to obtain compound **5** by reacting compound **4** with $\text{P}(\text{OEt})_3$, $\text{Pd}(\text{OAc})_2$ in the presence of NEt_3 as base in degassed ethanol and refluxing overnight. However, this reaction was not consistent due to the presence of three major impurities: unreacted reactant, one ester, and both esters hydrolyzed on the phosphonic group. Even though we need to hydrolyze the ester in the end for our final product, the nitroso formation reaction with non-esters were unsuccessful. Hence, the percent yield of these unwanted impurities was reduced significantly by using rigorous reaction conditions (water free) and multiple parallel reactions. The aromatic amine of compound **5** was then deprotected under acidic conditions, and the nitroso species **7** was formed by

oxone treatment in a biphasic DCM/H₂O with vigorous stirring at room temperature, which was used immediately for the final coupling reaction.



Scheme 5.6 Synthesis of precursor **9** for **F4-PO3**

Another precursor Compound **9** carrying the sulfonamide moiety was prepared using a similar synthetic protocol for **CAP-F5** (Chapter 4). The sulfonyl chloride derivative was converted to sulfonamide **8** in the presence of NH₄OH which was finally converted to Compound **9** by reacting **8** with 7M NH₃/MeOH at 120°C for two days (Scheme 5.6).



Scheme 5.7 Synthesis of **F4-PO3**

Finally, precursors **9** and **7** were coupled together in the presence of TFA/acetic acid and toluene at room temperature for different durations of time (Scheme 5.7). Due to observation of no reaction progress, the temperature was slowly increased to 45°C, which resulted in the degradation of nitroso species with no product formation. As nitroso derivatives are generally less stable and need to be used immediately following formation, the high temperature likely expedited degradation, especially under the given acidic conditions.

The synthesis of molecule **F4-SO₃** was then attempted and met with similar unsuccessful results. The substitution of sulfonic acid onto the benzene ring proved difficult, and the azo-coupling reaction was also likely unsuccessful due to the instability of nitroso derivative. These synthetic challenges led us to modify the design once more to make it more synthetically facile.

5.4.3 Class III azobenzene probes

5.4.3.1 Design Rationale

Instead of using highly acidic species, we chose a –COOH group as our choice for the ease of synthesis of making **F4-COOH**. We also designed **F5-COOH** as it is known that the CA-IX active site is wider and more accessible as compared to any other CA isoform (Figure 5.5).

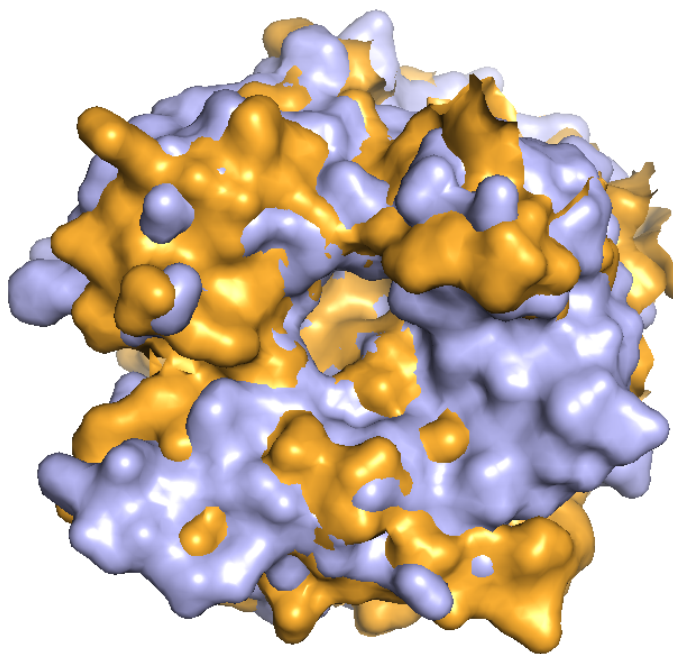


Figure 5.5 The structure overlay of hCAII (1ca2 in light purple) and hCAIX (chain 4 of 3iai in light orange)

There are certain examples in literature where they have multiple fluorine groups and a bulky group next to sulfonamide on the ring (Table 5.1).²⁹ Due to the large size of the molecule and wide open active site of CAIX, we can get selective preferential binding for CAIX as shown by K_d values shown in Figure.

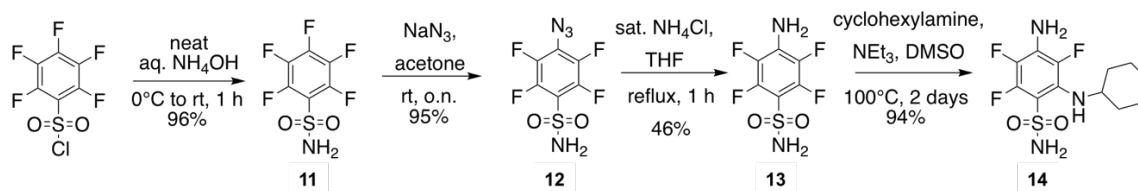
CA isoform	$K_d (K), \text{nM}$			
	VR16-09	VR16-10	VD11-4-2*	VD12-09*
CA I				
CA I	$\geq 200\ 000$	5000	710	50 000
CA II	$\geq 200\ 000$	1000	60	1 300
CA III	$\geq 200\ 000$	$\geq 200\ 000$	40 000	$\geq 200\ 000$
CA IV	$\geq 200\ 000$	1820	25	1 700
CA VA	$\geq 200\ 000$	5000	2 500	3 300
CA VB	45000	100	5.6	210
CA VI	$\geq 200\ 000$	26300	95	4 300
CA VII	37 000	100	9.8	330
CA IX	0.16 (<1)	0.20 (<1)	0.05	1.1
CA XII	710 (100)	370	3.3	330
CA XIII	20	40	3.6	140
CA XIV	170	170	1.6	170

Table 5.1 Inhibitors example for selective inhibition of CAIX.²⁹

Incorporating such functionality is highly important for our design as the aromatic carboxylic acid has a pK_a of around 4.7, which means that in an acidic extracellular environment ($pH \sim 6.4-6.6$), there will be some ($\sim 1\%$) non-charged $-\text{COOH}$ species that would have the potential to enter the cell. Our hypothesis is that even if they enter the cells, they won't interact and inhibit the cytosolic CAs, and hence, allowing us to photomodulate the membrane associated isoforms of CA only by using **F5-COOH**. We also designed **F4-COOH** to use as the control molecule to compare the intracellular pH (pH_i) changes with **F5-COOH**.

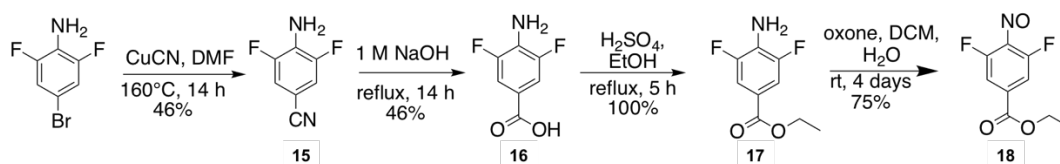
5.4.3.2 Synthesis

The probe **F5-COOH** was synthesized by preparing the precursors required for azo coupling. Precursor **14** was synthesized by using pentafluorobenzenesulfonyl chloride as the starting material and reacting with NH_4OH (Scheme 5.8). The fluorine at the *para*-position was substituted by an azide using NaN_3 to yield aromatic azide **12**, which was converted to aromatic amine **13** by treatment with NH_4Cl . Final precursor **14** was synthesized by treatment of **13** with cyclohexylamine in the presence of NEt_3 as the base.



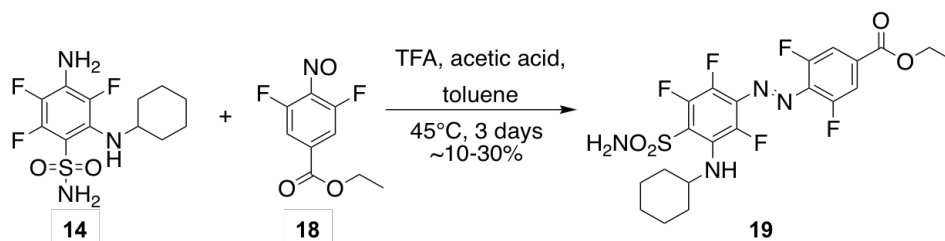
Scheme 5.8 Synthesis of precursor **14** for **F5-COOH**

The other precursor **18** required for azobenzene coupling was synthesized from 4-bromo-3,5-difluoroaniline as the starting material (Scheme 5.9). The bromo group was converted to a cyano group by treatment with CuCN in DMF to yield compound **15**. The cyano group was converted to carboxylate by treatment of compound **15** with 1M NaOH to get compound **16**, which was further converted to an ester derivative **17** by treatment with ethanol under acidic conditions. The final nitroso derivate **18** was prepared by reacting **17** with oxone, and was used immediately for the final azo coupling.



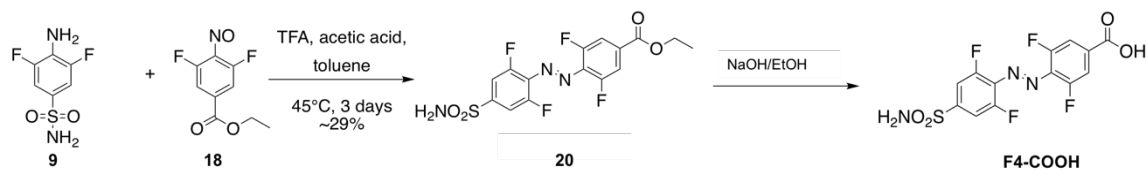
Scheme 5.9 Synthesis of precursor **18** for **F5-COOH**

The final azo-coupling reaction between compound **14** and **18** was performed in the presence of TFA and acetic acid in toluene (Scheme 5.10). However, the reaction yield was low, with many unidentified impurities. Further, the product mixture was unstable over time.



Scheme 5.10 Synthesis of **F5-COOH** ethyl ester **19**

Due to the challenges faced, we focused on the synthesis of **F4COOH**. The probe was synthesized by azo coupling between compound **9** and **18** in the presence of TFA/acetic acid in toluene to get compound **20** (Scheme 5.11). The ester was then hydrolyzed in the presence of NaOH to obtain final product **F4-COOH** successfully.



Scheme 5.11 Synthesis of **F4-COOH**

5.4.3.3 Preliminary characterization

After obtaining pure **F4-COOH**, we conducted studies to understand its photoisomerization properties using UV-vis spectroscopy in methanol (Figure 5.6 A) and aqueous buffer (Figure 5.6 B). Upon irradiation with 520 nm, the π - π^* band decreased in intensity and n - π^* band shifted to a lower wavelength, consistent with the *trans* $\rightarrow$ *cis*

isomerization. It is important to note that **F4-COOH** shows an $n-\pi^*$ shift of 41 nm, as compared to 37 nm in **CAP-F5** (Chapter 4). The larger shift results in a more efficient photoisomerization process, which is highly desirable to get photocontrol over protein activity. The reversible *cis*→*trans* isomerization can be achieved by using 430 nm in both the solvents. Finally, the thermal stability of the *cis* isomer of **F4-COOH** was studied in aqueous buffer at 37°C over a period of 24 hours. As shown in figure 5.6 C, the *cis* isomer is thermally stable and does not relax back to *trans* isomer, just like **CAP-F5**.

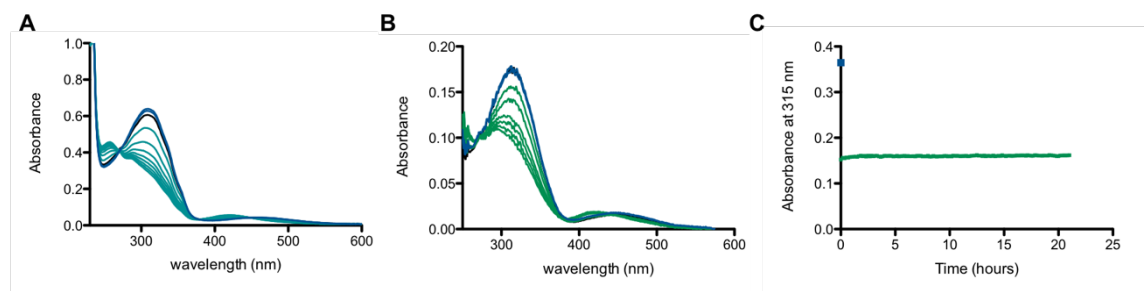
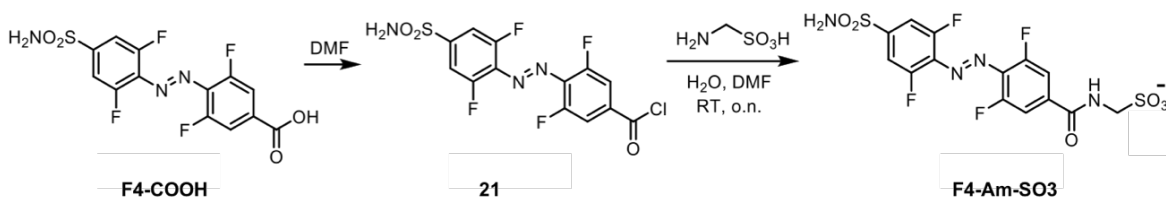


Figure 5.6 Photoisomerization of **F4-COOH** using 520 nm and 430 nm in methanol (A) and aqueous buffer (B) studied through UV-vis spectroscopy. (C) The thermal stability of *cis* isomer of **F4-COOH** in aqueous buffer at 37°C. The buffer is 50 mM HEPES, 0.1 M KNO₃ at pH 7.2

5.4.4 Class IV azobenzene probes

After the successful synthesis and characterization of **F4-COOH**, we decided to link the charged species (-SO₃H) that could not be obtained using our Class II strategy. In order to do so, we modified the design to **F4-Am-SO₃**, and employed amide coupling strategies.



Scheme 5.12 Synthesis of **F4-Am-SO3**

The carboxyl group of **F4-COOH** was converted to acyl chloride **21**, which was reacted further with aminomethane sulfonic acid to obtain **F4-Am-SO3** (Scheme 5.12). The reaction yield is very low (5-10%) and inconsistent for this coupling. We need to optimize this reaction, and possibly consider other derivatives of sulfonic acid with different linkers or different acidic groups.

5.5 FUTURE WORK

After optimizing the final reaction, we will characterize the photoisomerization properties and thermal stability of the probe using UV/vis, NMR spectroscopy, and HPLC. We will further study the binding interactions of the *trans* and *cis* isomers with the isolated protein (bCA and hCAIX) and the translation of such interactions to get differential esterase and hydration/dehydration activity of the protein. Finally, we will perform cell experiments to show the applicability of these probes to get differential regulation of CA-IX in a complex cellular environment. We will monitor the extracellular pH (pH_e) of the cells under hypoxic conditions in the absence and presence of *trans* and *cis* isomers, and compare pH_e with cells grown under normoxia. Preliminary studies were conducted to assess this method by using **CAP-F5**. As shown in Figure 5.7, we get increased pH_e when cells are treated with *trans* as compared to *cis* isomer, which may suggest the reduction of extracellular acidosis due to CA-IX inhibition following treatment with the *trans* isomer.

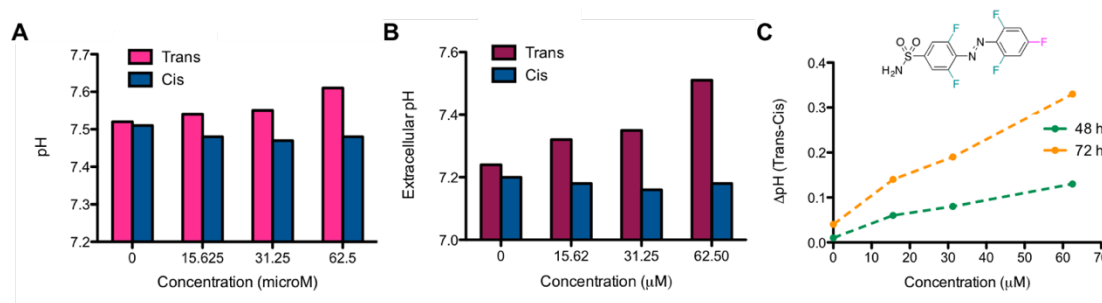


Figure 5.7 Extracellular pH (pH_e) of HeLa cells grown in hypoxia (2% O₂ and 5% CO₂) and changes due to treatment with different concentrations of *trans* and *cis* isomer of **CAP-F5** for 48 h (A) and 72 h (B). The difference in pH_e due to the *trans* and *cis* isomer at each concentration is shown in (C).

As **CAP-F5** has the capability to diffuse in to the cells and inhibit cytosolic CAs along with membrane bound CAs, it would be interesting to study these changes by using **F4-Am-SO3** which will only inhibit CA IX. More complex model systems, such as spheroids, may also be used in conjugation with CA IX fluorescence-based probes to show the differential binding of our probe, as well as the possibility of *in situ* photoisomerization.

5.6 CONCLUSION

In this chapter, we discussed the use of azobenzene photoswitches to gain control over CA IX specifically, an isoform of CA which is highly overexpressed in hypoxic tumor cells. We discussed the design of different classes of probes and attempts and challenges to synthesize them. In the end, we were able to obtain **F4-COOH**, which is easy to synthesize and shows all the desired properties in terms of photoisomerization, however may not be cell impermeable. One can argue that it has the potential to be a better photoswitch than **CAP-F5** in terms of photophysical properties owing to its larger shift in $n-\pi^*$ band. To make it negatively charged at physiological pH, we modified the design further to **F4-Am-SO3**. As the groups directly linked to the azobenzene are

identical in these molecules, we predict minimal differences in photoisomerization properties between these two probes. We are currently in the process of making progress towards our goals, but we believe that this approach of using photoswitch to gain reversible control over a specific isoform of a protein will be a great contribution in the field of optobiochemistry.

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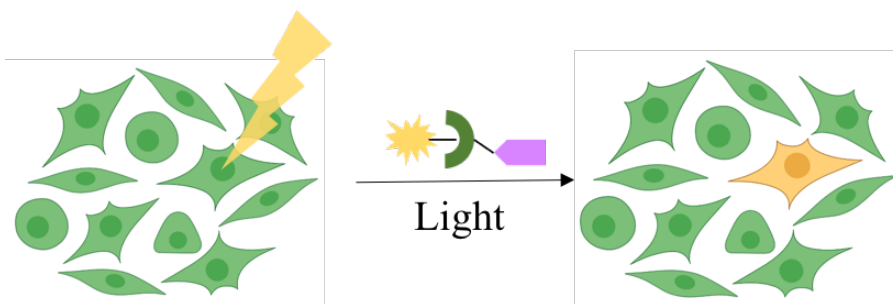
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Chapter 6: Photoactivatable probes to target the mitochondrial isoform of carbonic anhydrase, CAV⁵

6.1 ABSTRACT

Biological systems consist of complex and highly dynamic networks of molecular processes, and getting precise control over one process without perturbing the system is important for understanding biological problems, especially in the therapeutic context. We have established that using light as an external trigger to achieve control is key due to these advantages: it is non-invasive; is facile to control the wavelength, duration, and intensity of light; and exhibits high spatiotemporal precision. As discussed in chapter 1, there are two general approaches for photoregulation: reversible and irreversible. The first approach relies on using a molecular photoswitch, such as azobenzene, to alter the properties of the probe in a reversible manner. The second approach uses a photoremovable protecting group (PPG). In this chapter, we will discuss the application of the second approach to gain control over a specific isoform of carbonic anhydrase (CAV) localized in the mitochondria of the cell. The probe consists of three parts: CA inhibitor, PPG and a mitochondrial-directing group. We will discuss in detail the concept behind the probe design and the studies conducted thus far.

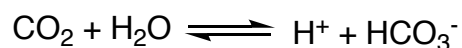


⁵ This chapter is based on following work:
Aggarwal, K.; Ye, E.; Kolli, D. *In preparation*

6.2 INTRODUCTION

6.2.1 Mitochondrial Isoforms of CA- CAVA and CAVB

Carbonic anhydrase (CA) is a zinc metalloenzyme that catalyzes the reversible hydration of carbon dioxide and bicarbonate.



There are 15 different isoforms of CA that differ in tissue-specific expression, subcellular localization, and biological significance.¹ Certain isoforms such as CAII and CAIX have been extensively studied for their structural and inhibitory properties, inhibition as well as have been identified as therapeutic targets.²⁻³ However, there are some isoforms that are significantly less studied, and one such example is CAV – the only mitochondrial isoform known to date. This isoform is primarily localized in hepatocytes and adipocytes in the body.⁴ CAVA and CAVB are two types of CAV encoded by the *CA5A* and *CA5B* gene. CAVA is localized in the mitochondria and expressed primarily in the liver. CAVB is also localized in mitochondria but has a wider tissue distributions than CAVA. CAVA and CAVB shows high sequence similarity as well as specific catalytic activity and sensitivity to sulfonamide based inhibitors.

6.2.1.1 Physiological roles of CAV

Pharmacological studies have implicated CAV in several metabolic pathways, including gluconeogenesis, ureagenesis, and lipogenesis.⁵ Key mitochondrial enzymes in the aforementioned pathways require HCO_3^- , which, in contrast to CO_2 , does not freely cross the mitochondrial membrane. CAV then serves the essential role of converting CO_2 to HCO_3^- , providing the precursor molecule for important biosynthetic pathways.⁶ CAV provides bicarbonate for pyruvate carboxylase in liver and kidney, and has been linked to the regulation of insulin secretion in beta cells.⁷

6.2.1.2 Pathological roles of CAV

Dysregulation of CAV has been linked to a number of illnesses including obesity, diabetes, hyperammonemia, growth deficiencies, and oxidative stress-related illnesses of the brain.⁸ Carbonic Anhydrase VA (CAVA) is directly associated with glucose homeostasis and is considered as a promising target to combat obesity.⁹ CAVA deficiency has been linked to the differential diagnosis of hyperammonemia in the neonate and young child.¹⁰ Like other CA isoforms, overexpression of CAV is associated with a number of cancers, with the highest expression observed in acute myeloid leukemia, prostate, and hepatocellular carcinomas. While it is well-recognized that CAV regulation is of therapeutic significance, there have been limited studies on the specific role of CAV in the progression of cancer and other illnesses.⁹ This is largely due to the lack of methods to selectively target CAV *in situ*.

6.2.1.3 Methods to study CA V

Current methods to study CAV generally fall under three categories. The first approach is phenotype analysis of knockout (KO) mouse models produced by targeted mutagenesis. This method, while providing good evidence for the detrimental effects of complete CA V deficiency in whole organisms, fails to answer when, how, and why CA V dysregulates, such as whether CA V dysregulation is the cause or result of mitochondrial dysfunction—a key hallmark of cancer.

In attempt to answer these questions more specifically, the second general approach is the inhibition of CA V in intact, isolated mitochondria.¹¹ This method gives us kinetic and biochemical data on CAV, but since the mitochondria are extracted from the cell, how CAV interacts within the wider cellular environment remains unknown.

The third approach is the non-specific inhibition of CAV in cells. This approach is rarely used, due to the low relative concentration of CAV when compared to other CA isoforms localized in the cytoplasm. Therefore, when a non-specific CA inhibitor enters the cell, the majority of the inhibitor binds to cytoplasmic CA before even entering the mitochondria, causing it to be difficult if not impossible to distinguish the effect of CAV inhibition.

6.2.1.4 *Our approach*

In order to target CAV specifically, we need to develop a chemical tool that can be directed to mitochondria without binding with other CA isoforms along the way. We propose to block the metal binding group ($-\text{SO}_2\text{NH}_2$) of a CA inhibitor with a photoreactive protecting group (PPG). Using light as an external trigger for the release of active ligand, we hope to not only gain spatiotemporal control over the protein activity, but also target CAV specifically without the need for designing a new class of isoform-specific inhibitors.

6.2.2 The Photocaging concept

The general way of achieving photocontrol over biomolecules via PPG is by rendering the biologically active molecule (ligand or inhibitor) temporarily inactive through chemical modification with a photo-removable group, also known as the “caging group”. The active functionality is revealed upon photoirradiation which triggers the photocleaving reaction in a highly precise spatial and temporal manner (Figure 6.1).

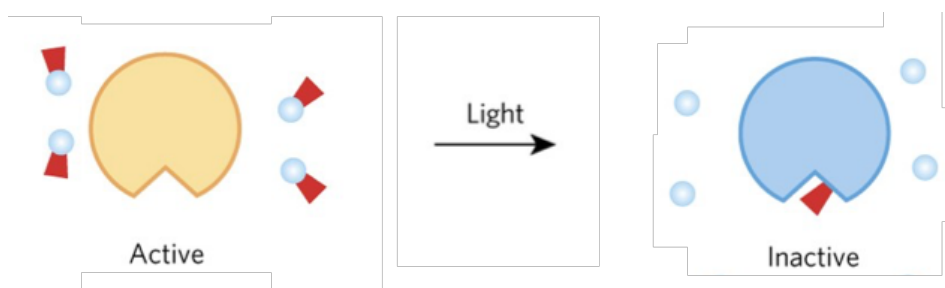


Figure 6.1 Working principle behind photocaging approach. The PPG is represented by the light blue circle and the ligand is represented by red trapezoid.

6.2.2.1 Development of PPGs

Several types of PPGs have been developed over the years that have found applications in the field of organic synthesis¹²⁻¹³ and material science.¹⁴ The first introduction of photocleavable groups in the field of chemical biology was the photorelease of cyclic adenosine monophosphate (cAMP)¹⁵ and ATP¹⁶. Since then, there have been a plethora of examples to demonstrate the application of PPGs in biomedicine, biochemistry and neurobiology (Table 6.1).¹⁷⁻²¹ In particular, the introduction of a PPG that can be photocleaved by longer wavelengths of light, such as *ortho*-nitrobenzyl derivatives, has made it possible to control biological processes with more biocompatible light wavelengths with deep tissue penetrating properties.²²

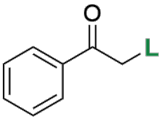
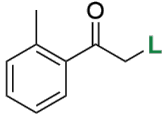
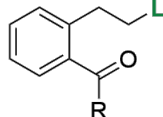
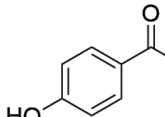
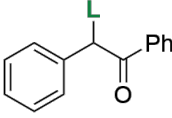
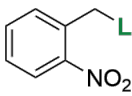
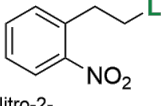
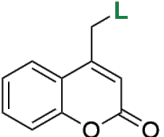
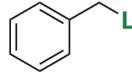
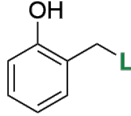
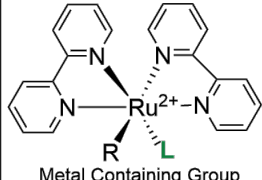
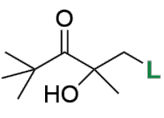
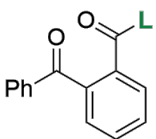
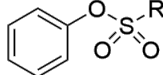
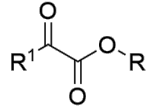
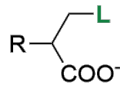
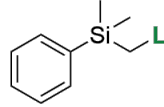
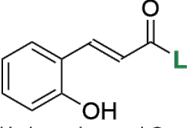
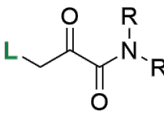
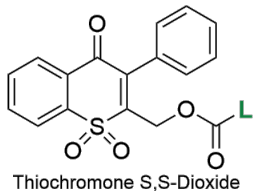
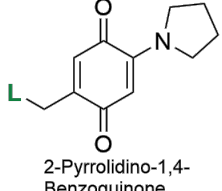
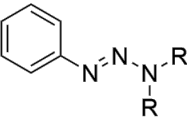
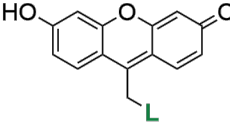
 Phenacyl Group	 o-Alkylphenacyl Group	 o-Alkylphenacyl Group	 p-Hydroxyphenacyl Group
 Benzoin Group	 o-Nitrobenzyl Group	 o-Nitro-2-phenethyloxycarbonyl Group	 o-Nitroanilides
 Coumarin-4-ylmethyl Group	 Arylmethyl Group	 o-Hydroxyarylmethyl Group	 Metal Containing Group
 Pivaloyl Group	 Carboxylic Acid Ester Group	 Arylsulfonyl Group	 Ketone Group
 Carbanion Mediated Group	 Silicon-based Group	 2-Hydroxycinnamyl Group	 α-Ketoamide Group
 Thiochromone S,S-Dioxide	 2-Pyrrolidino-1,4-Benzoquinone	 Triazine Group	 Xanthene Group

Table 6.1 Different types of photocleavable protecting groups (PPG).

6.2.2.2 PPGs for biological applications

While designing a probe with a PPG for biological applications, there are certain benchmarks to consider:

1. In order to avoid interference from absorbance of endogenous biological chromophores, the PPG should have strong absorbance above 300 nm.
2. The photocleavage reaction should be clean with high quantum efficiency.
3. The PPG should not give any detectable response or intrinsic activity before and after the photocleaving reaction.
4. The PPG should be soluble in aqueous media and stable under biological conditions.
5. The photocleavage products (ligand or any other byproduct) should not exhibit absorbance at the irradiation wavelength.
6. The photocleaving reaction should be rapid, with the need of short duration of pulses for excitation.

Even though not a single PPG can satisfy all the requirements, we selected a coumarin-based photocleavable group for our work.

6.2.3 Coumarin PPGs

Coumarin derivatives have been widely applied in chemical biology research due to their ease of synthesis and high biocompatibility.²³ They also provide additional advantages over other PPGs: large molar absorption coefficients at longer wavelengths, fast release rates and improved stability. Moreover, the additional functionality, owing to the fluorescence properties of coumarin, is that this PPG serves as a tag for convenient monitoring of the molecule as well as the reaction progress.

6.2.3.1 Development and derivatives

Coumarin offers multiple sites for structural modifications which can lead to large changes in the chemical properties (such as pK_a , solubility) and spectral properties (such as fluorescence and absorbance). The substitution at the C6 or C8 positions of the coumaryl group results in a red-shift of the absorption maximum and improves the water solubility. Introduction of an amino group at C7 further improves the spectroscopic and photochemical properties by inducing a large shift in absorbance wavelength (Figure 6.2). This has also been shown to give the highest quantum yields (0.21-0.28) among the other coumarin analogues. Any such small change to the structure lead to a wide range of deprotection wavelengths (300-500 nm) when used as a photocage.

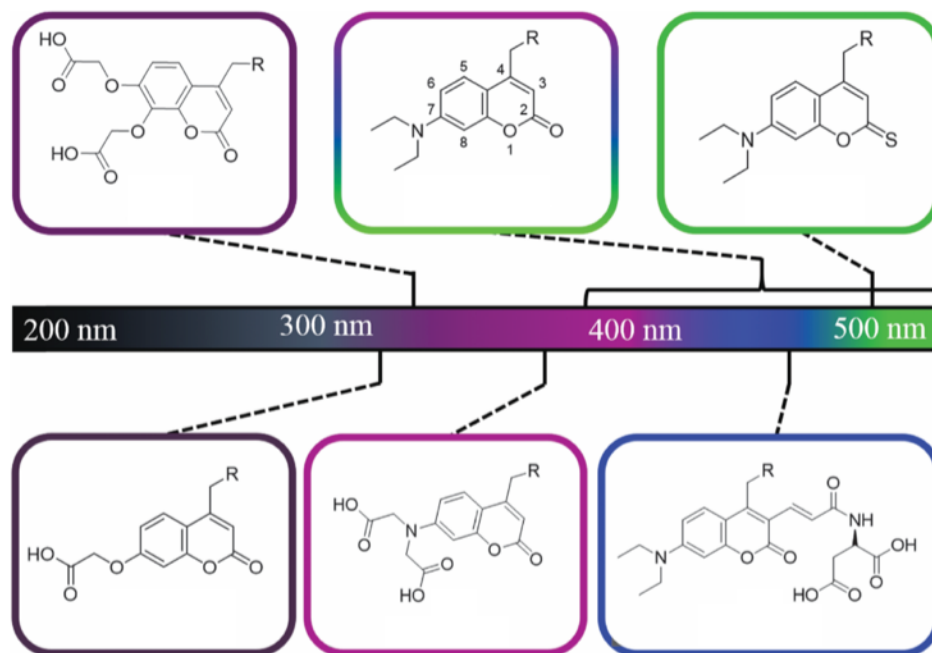
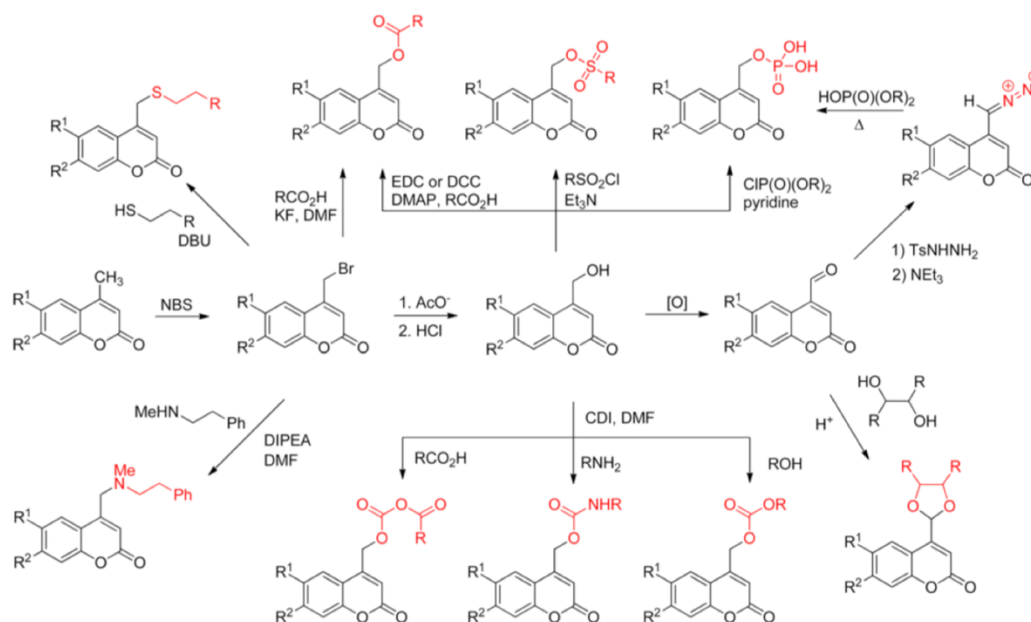


Figure 6.2 Effect of coumarin modifications on the absorption maximum wavelength (λ_{max}), adapted from Hansen *et al.*²⁴

6.2.3.2 Types of Ligands photoprotecting by coumarin-derivatives

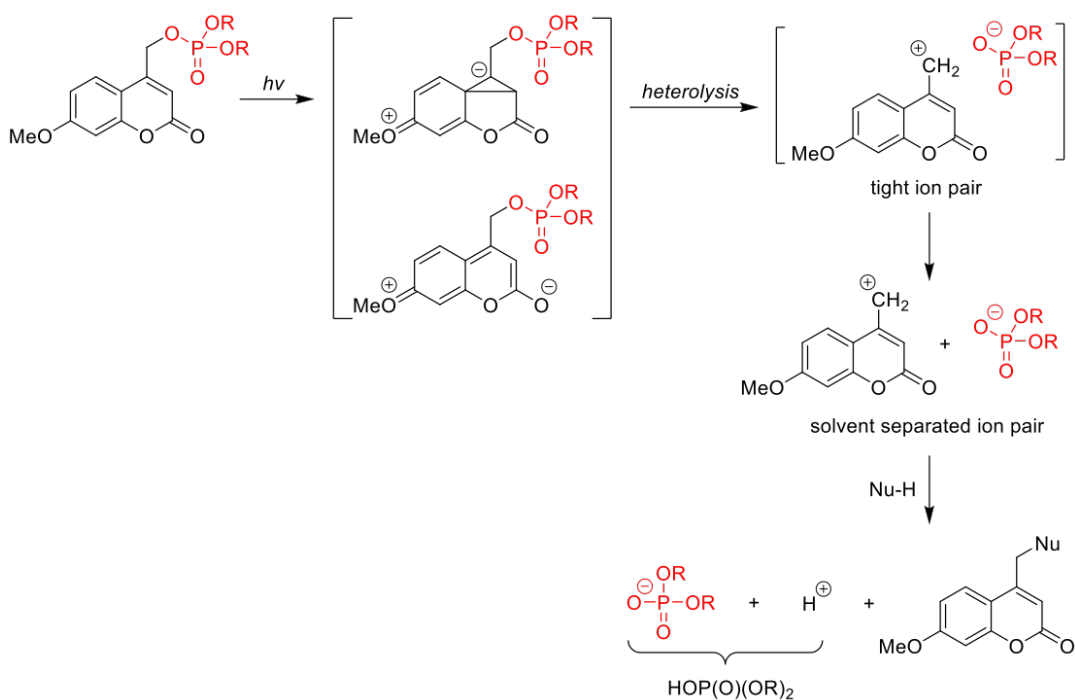
Various nucleophiles and ligands, including esters, phosphates, carboxylates, sulfonates, carbonate and carbamates can be coumarin-caged by using simple precursors (Scheme 6.1).²³



Scheme 6.1 Different types of ligands photocaged by coumarin, and their general synthesis pathways

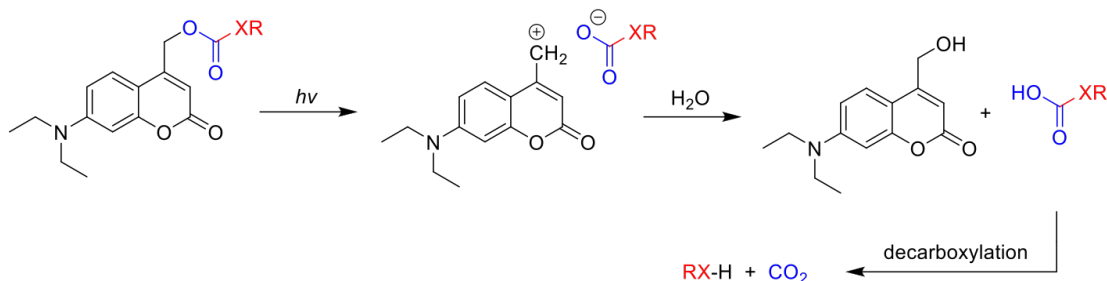
6.2.3.3 Mechanism of photorelease

There is a general mechanism for the photorelease of good leaving groups such as phosphates, sulfates and carboxylic acids, where light irradiation leads to heterolytic C-X bond cleavage, resulting in the formation of a tight ion pair as an intermediate. This ion pair then escapes the solvent cage and reacts with available nucleophiles to yield free ligand and a different coumarin analog (Scheme 6.2).²⁵



Scheme 6.2 The general mechanism of photorelease of coumarin-caged compounds

However, poor leaving groups such as alcohols and thiols ($X = O, S$) are caged through a carbonate linkage with the (6-bromo-7-hydroxy/ alkoxy coumarin-4-yl)methyl moiety for efficient decaging. After photoirradiation, the carbonic or thiocarbonic acid released is unstable and undergoes decarboxylation to give the free alcohol or thiol (Scheme 6.3).²⁶⁻²⁷ This process is susceptible to both acid and base catalysis, and is usually quite slow ($k_{-CO_2} = 10^{-3} \text{ s}^{-1}$). In the case of amine release ($X = NH$), the process could be much slower, and strongly depends on the pH and the nature of amine being released.²⁸⁻²⁹



Scheme 6.3 The photocleaving reaction mechanism for alcohols, amines, and thiols by using coumarin by as the photoprotecting group

6.2.4 Our work

In this chapter, we describe the development of a photocaged probe to target CAV in the mitochondria of live cells. In this design, the metal binding group (sulfonamide) is protected by a photoremovable protecting group on one end and linked to a mitochondrial directing group on the other end. This strategy relies on the mitochondrial directing group to lead the molecule into the mitochondria while preventing binding to extracellular and cytoplasmic CAs such as CA II and CA IX. Once localized in the mitochondria, photoirradiation will cleave off the photoremovable protecting group, exposing the sulfonamide group to the CA V active site and leading to CA V inhibition (Figure 6.3). By providing spatial and temporal control over the activation of the inhibitor, this design enables the selective inhibition of specific CA isoforms in their natural cellular environment and opens doors for a range of studies and applications that were previously infeasible.

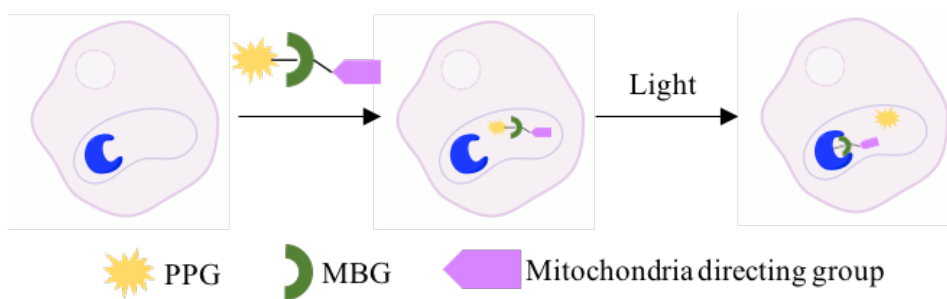


Figure 6.3 Representation of the working mechanism of our probe in live cells.

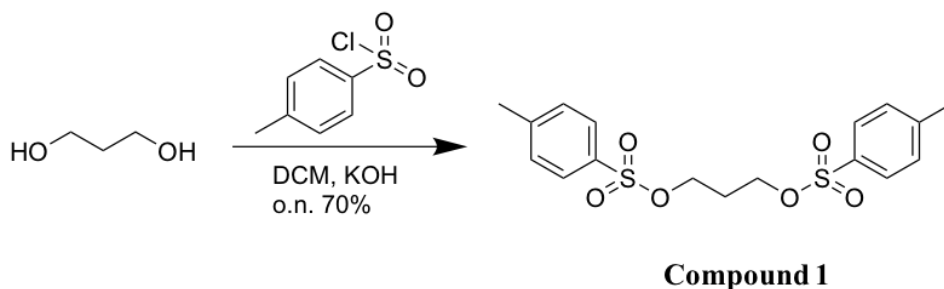
6.3 EXPERIMENTAL

6.3.1 General procedures

All solvents and chemicals were purchased from Sigma-Aldrich and Fisher Sci. and used as received. Bovine carbonic anhydrase was purchased from Sigma Aldrich. For spectroscopic and protein studies, 0.05 M HEPES buffer with 0.1 M KNO₃ (pH 7.2) was used unless stated otherwise. All the studies were done under dark condition to avoid exposure of light.

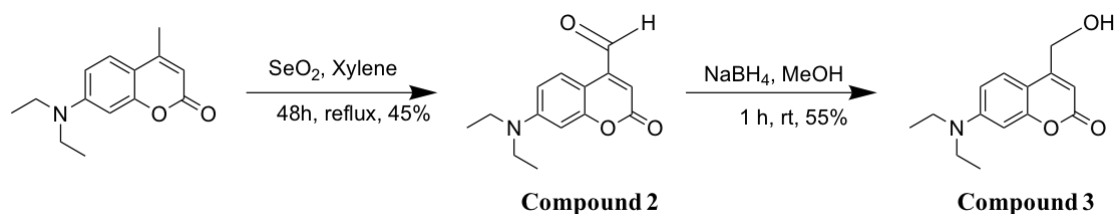
The ¹H spectroscopic measurements were collected in deuterated solvents from Cambridge Isotope Laboratories (Cambridge, MA), using an AGILENT MR 400 NMR spectrometer at 400 MHz. The chemical shift is calibrated with respect to the solvent peak, and is reported in ppm. Absorbance spectroscopic studies were performed using an Agilent Cary 60 UV-Vis spectrophotometer. Fluorescence spectroscopic measurements were made using an Agilent Cary Eclipse fluorescence spectrofluorimeter. The samples were irradiated with an Asahi Xe lamp connected with bandpass filters of 365 nm (FWHM 12 nm), 460 nm (FWHM 11 nm) from Asahi Spectra, and 410 nm (band width 10 nm) from Thorlabs. Walk-up LC-MS and high-resolution Electrospray Ionization (ESI) mass spectral analyses were performed by the Mass Spectrometry Facility of the Department of Chemistry at UT Austin.

6.3.2 Synthetic procedures



Scheme 6.4 Synthesis of linker for **PCE-M**

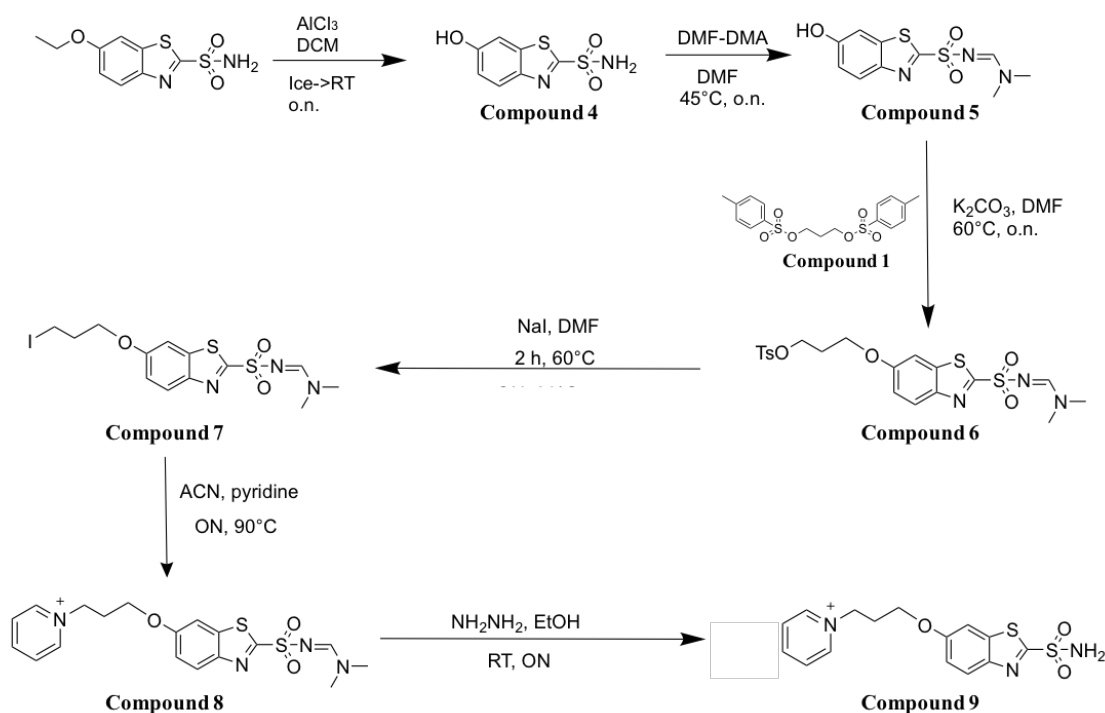
Synthesis of Compound 1: Propanediol (2 g, 26.3 mmol) was added to pyridine (5.26 mL). Tosyl chloride (12.02 g, 63.2 mmol) was then added to the solution mixture in an ice bath, and the solution was slowly brought to room temperature and stirred overnight. After the reaction was complete, the solution was brought to 0 °C using an ice bath, then concentrated under reduced pressure. The crude product was then purified on silica through normal phase column chromatography using Acetone/DCM.



Scheme 6.5 Synthesis of coumarin precursor (PPG) for **PCE-M** and **PCE**

Synthesis of Compound 2: 4-dimethylamino-4-methylcoumarin (0.928 g, 4 mmol) and SeO₂ (0.664 g, 6 mmol) were added to 24 mL dioxane in a round-bottom flask. The solution mixture was stirred and refluxed at 110 °C for 48 hr. The crude product was then purified on silica through normal phase column chromatography using Acetone/DCM. The product was obtained as an orange/red solid as 45% yield.

Synthesis of Compound 3: Compound 2 (450 mg, 1.8 mmol) and NaBH₄ (102 mg, 1.5 mmol) was added to a solution mixture of 6 mL THF and 6 mL EtOH. The solution mixture was stirred in the dark by wrapping aluminum foil around the round-bottom flask at room temperature overnight. The crude product was then purified on silica through a MeOH/DCM normal phase column chromatography.



Scheme 6.6 Synthesis of ethoxazolamide precursor (MBG) for **PCE-M**

Synthesis of Compound 4: 6-ethoxy-1,3-benzothiazole-2-sulfonamide (500 mg, 1.94 mmol) was added to 10 mL DCM in a round-bottom suspended in an ice bath. AlCl₃ (904.2 mg, 6.79 mmol) was added to the solution mixture and the round-bottom was removed from the ice bath. The reaction was stirred at room temperature overnight. Upon completion of the reaction, the reaction mixture was cooled in an ice bath and quenched with 5 mL 3M HCl. The solid precipitate was filtered out as the product and washed with

water with 60% yield. The product was then purified on silica through a EtoAC/Hex normal phase column. Product formation was confirmed through NMR and LCMS.

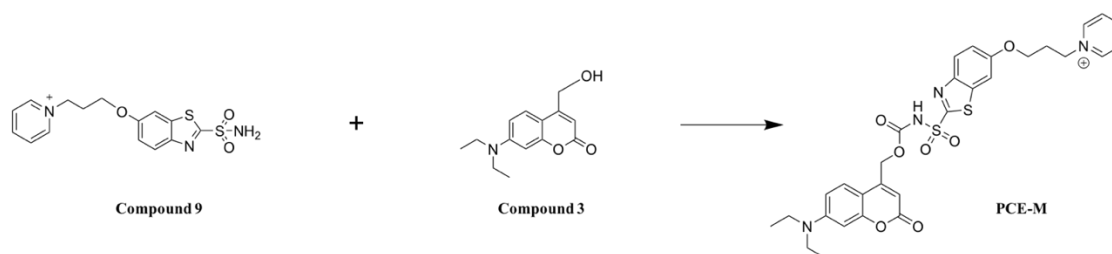
Synthesis of Compound 5: Compound **4** (300 mg, 1.3 mmol) was added to a solution with DMF-DMA (1.125 mL, 8.45 mmol) and 0.45 mL DMF in a sealed tube. The solution mixture was stirred at 45 °C overnight. The crude reaction mixture was purified on a MeOH/DCM silica normal phase column. 470 mg product was collected as 88% yield.

Synthesis of Compound 6: Compound **5** (470 mg, 1.65 mmol) and K₂CO₃ (314.5 mg, 2.475 mmol) were added to 8.28 mL DMF in a round-bottom flask. The solution was stirred at room temperature for 20 minutes, then Compound **1** (634 mg, 1.65 mmol) was added to the reaction mixture, and the flask was heated to 60 °C and left overnight. The crude reaction mixture was purified on a MeOH/DCM silica normal phase column. Product was collected as a white powder in 88% yield.

Synthesis of Compound 7: Compound **6** (950 mg, 1.91 mmol) and NaI (1.22 g, 9.55 mmol) was added to 60 mL DMF in a round-bottom flask. The reaction mixture was stirred at 60 °C for 2 h. The organic layer was then separated via an EtoAC/Hexane extraction and concentrated using reduced pressure, and used directly for the next step.

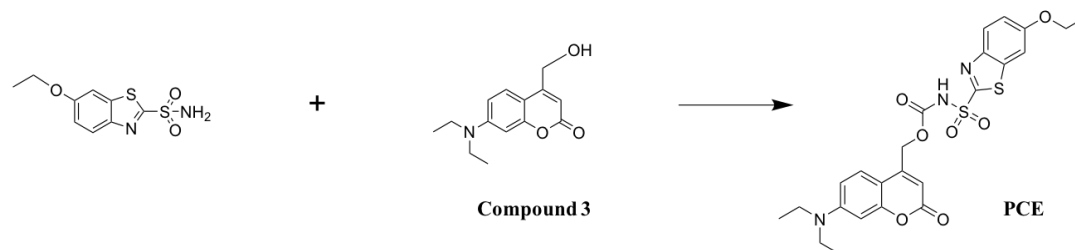
Synthesis of Compound 8: The concentrated product from the last step was suspended with 20 mL DMF and pyridine (2 mmol) was added to the solution. The crude reaction mixture was purified on a acetone/DCM silica normal phase column. The product was collected as an orange/red solid in 88% yield.

Synthesis of Compound 9: Compound **8** (30 mg, 0.051 mmol) and 10 µL hydrazine were added to 1 mL ethanol and stirred at room temperature overnight. The crude reaction mixture was via reverse-phase column chromatography. The product was collected with quantitative yield, and product formation was confirmed through LCMS.



Scheme 6.7 Synthesis of **PCE-M**

Synthesis of Compound PCE-M: Compound **3** was added in dry DCM along with DMAP and 4-nitrophenyl chloroformate in the dark. The solution mixture was stirred for 2 days until the reactant was reacted completely, as monitored by TLC. The reaction mixture was concentrated under vacuum and suspended in acetonitrile. Compound **9** and DMAP was added to the mixture and refluxed overnight. The solvent was then evaporated and product was separated through reverse phase column chromatography to obtain the product in quantitative yield. Currently we are working on anion exchange methods and purifying it further to use a more biocompatible anion.



Scheme 6.8 Synthesis of probe **PCE**

Synthesis of Compound PCE: Compound **3** was added in dry DCM along with DMAP and 4-nitrophenyl chloroformate in the dark. The solution mixture was stirred for 2 days until the reactant was reacted completely, as monitored by TLC. The reaction mixture was concentrated under vacuum and suspended in acetonitrile. Ethoxazolamide and DMAP was added to the mixture and refluxed overnight. The solvent was then

evaporated and product was separated through reverse phase column chromatography to obtain the product in quantitative yield.

6.3.3 Photospectral properties

The photocleaving process of molecules (10mM stock in DMSO) were investigated by electronic absorption spectroscopy. The absorbance and fluorescence spectrum was taken in methanol and aqueous buffer at different pH under dark conditions. To initiate the photoreaction, the samples was irradiated with 410 nm, and both the spectrum was collected. This process was repeated until there was no change in the spectrum. The kinetics of photocleavage was studied by plotting absorbance versus time to obtain the profile which was further fitter to one phase decay equation to get half-life and rate constant value:

$$y = (y_0 - y_{max})e^{-kx} + y_{max}$$

where y_0 is initial absorbance at λ_{max} ; y_{max} is absorbance at saturation (or maximum conversion); and k is the rate constant.

6.3.4 Activity with bCA – NPA Assay using UV/vis spectroscopy

The effects of probe binding with on bCA before and after photocleaving reaction was studied by monitoring the esterase activity of bCA in the presence of *p*-nitrophenyl acetate (NPA) as the substrate. As a result of enzymatic activity, NPA is hydrolyzed to give *p*-nitrophenolate ion, which has a λ_{max} at 400nm. Hence, the enzymatic activity in the absence and presence of probe can be determined spectroscopically by monitoring the increase in absorbance with time. In this study, a stock solution of 2 mg/mL (~70 μ M) protein in buffer, 10 mM probe in DMSO and 125 mM NPA in dry acetonitrile were prepared. During analysis, the enzyme, probe and NPA concentrations were kept constant

at 2 μ M, 2 μ M and 250 μ M respectively. The absorbance was recorded at 400 nm every 60 sec for up to 10 minutes and was plotted with respect to time. To study the effect of photoirradiation on the enzymatic activity, the solution of probe in buffer was irradiated with 410 nm for 20 minutes before the addition of protein and NPA. All the studies were performed in the absence of light at room temperature.

6.3.5 Homology modeling for human CAV

Using the SwissModel program, a homology model for hCAV was made to provide a more accurate protein working model for structural comparison and molecular docking. The User Template method was used through the SwissModel program which uses a target sequence for hCAV and the coordinates of a template crystal structure to make the homology model. Structural information is taken from the template crystal structure and used to create the homology model in regions where the sequences of the template and the target are identical. Areas of sequence misalignments are solved using structural databases, statistical potentials, and energy minimization through the ProMod3 comparative modeling engine in the SwissModel software. Homology models were made using various templates. Model 1 was formed using template 1CA2 (carbonic anhydrase II), model 2 was formed using template 6FE2 (carbonic anhydrase IX), and model 3 was formed using template 1URT (murine carbonic anhydrase V). Model 3 was used for subsequent docking due to a higher similarity between murine CAV and human CAV. Model 3 (template PDB: 1URT) has a GMQE score of 0.72, a QMEAN Z-score of -0.75, with a sequence identity of 78.90%. The sequence identity is well above that recommended for a homology model (50%), and the GMQE score and QMEAN Z-score indicate average scores for quality of the structure. Rotamer and angle quality is low in

this model but mostly apparent in regions other than the active site. The relatively well conserved active site provided confidence that this model can be used for docking.

All-Atom Contacts	Clashscore, all atoms:	0.53		99 th percentile* (N=1784, all resolutions)
	Clashscore is the number of serious steric overlaps (> 0.4 Å) per 1000 atoms.			
Protein Geometry	Poor rotamers	12	5.91%	Goal: <0.3%
	Favored rotamers	185	91.13%	Goal: >98%
	Ramachandran outliers	1	0.43%	Goal: <0.05%
	Ramachandran favored	219	93.59%	Goal: >98%
	MolProbity score^	1.69		90 th percentile* (N=27675, 0Å - 99Å)
	Cβ deviations >0.25Å	2	0.90%	Goal: 0
	Bad bonds:	0 / 1962	0.00%	Goal: 0%
	Bad angles:	16 / 2678	0.60%	Goal: <0.1%
Peptide Omegas	Cis Prolines:	2 / 15	13.33%	Expected: ≤1 per chain, or ≤5%
Additional validations	Chiral volume outliers	0/284		
	Waters with clashes	0/0	0.00%	See UnDowser table for details

Table 6.2 Quality assessment of Model 3 homology structure of hCAV (template PDB: 1URT) from Duke University Molprobity all-atom structure assessment software.

SwissModel Assessment Score information:

GMQE: global model quality estimate, how accurate the structure is (scale of 1)

QMEAN Z-score: how similar model is to experimental structures (needs to score above -4)

6.4 RESULTS AND DISCUSSION

6.4.1 CA V homology

As human CAV does not have a resolved crystal structure, *in silico* methods were used to predict the protein structure, and hence, to improve the chemical structure for our probe for optimal binding. Using the SwissModel program, a homology model for hCAV was created as the protein working model for structural comparison and molecular docking. The User Template method was used where model 1 was formed using template 1CA2 (carbonic anhydrase II), model 2 was formed using template 6FE2 (carbonic

anhydrase IX), and model 3 was formed using template 1URT (murine carbonic anhydrase V). Model 3 was used for subsequent docking due to a higher similarity between murine CAV and human CAV. The global model quality estimation was found to be 0.72, and the QMean Z-score was found to -0.75. These are relatively average scores—in comparison, a QMean Z-score of -4 or below would be considered low homology., and the local quality plot only has one residue with a bad score near the active site due to bond angle differences from rotamers. Overall, the sequence identity has a 78.9% exact residue match, which suggests good structural homology between mCAV and hCAV (Figure 6.4).

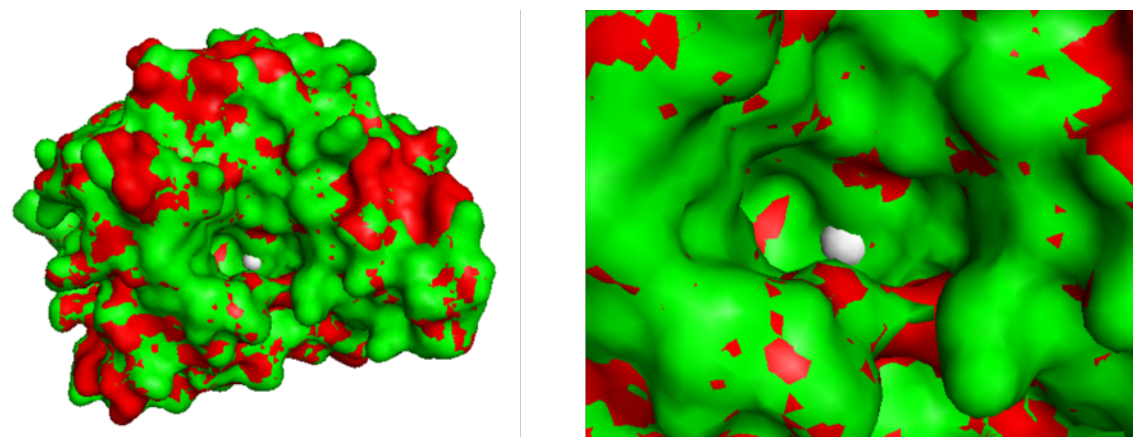


Figure 6.4 Homology Modeling for hCAV using a murine CAV template (PDB: 1URT). 1URT template shown in red as surface, homology model for hCAV shown in green as surface, catalytic zinc atom shown in active site as white sphere. The second photo shows a close up of the hCAV active site

The active site residues are highly conserved between hCAV and murine CAV whereas the surface residues are less so, which led to more divergence in the proteins' surface features away from the active site. The active site is comprised of many loops which leads to a more flexible active site and thus higher variance, however, this cannot be modelled accurately in a static crystal structure. The structure developed was

determined to be useful enough for preliminary docking studies to determine any optimization needed in probe structure and design.

6.4.2 Development of the molecule

6.4.2.1 Scheme and mechanism

The target molecule design for CAV is comprised of four parts: a fluorescent photo-removable protecting group (PPG), a CA active-site inhibitor (MBG), a mitochondrial directing-group (TAG), and a linker connecting the metal binding group and the tag (LINKER) as shown in Figure 6.5.

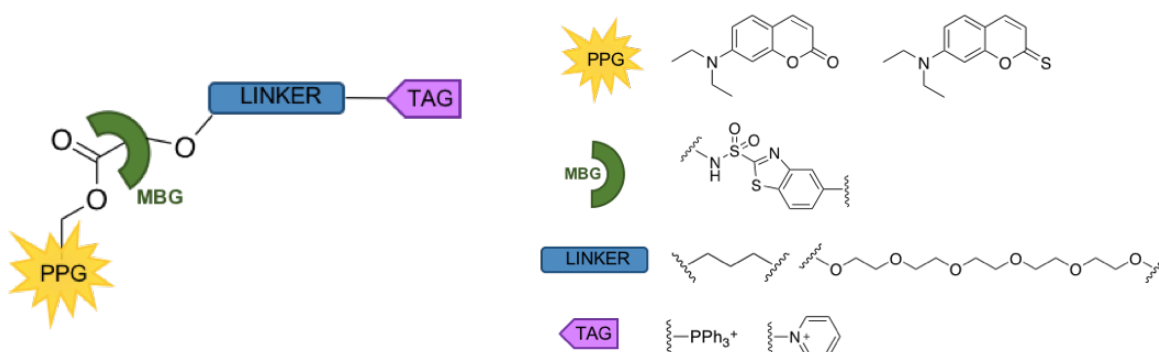
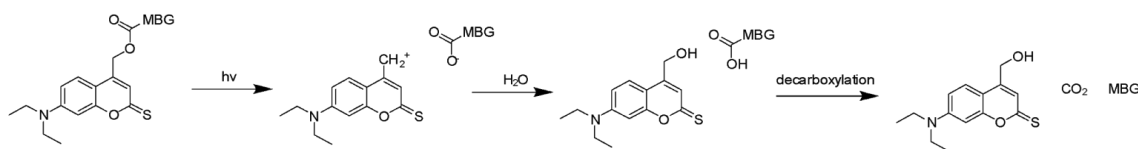


Figure 6.5 The proposed components design for photocaged probe

The MBG is taken from the structure of a known general CA inhibitor, ethoxzolamide, with high CA inhibition potency specifically reported. Ethoxzolamide is an aromatic compound containing sulfonamide as the metal binding group. This group interacts with the Zn^{2+} ion in the active site of CA and inhibits the enzyme's activity. A coumarin derivative was chosen as the photoremovable protecting group (PPG), and it serves a two-fold purpose. First, coumarin is a well-characterized fluorophore, and will allow us to visualize the localization of the probe within the cell using live cell fluorescence microscopy, which will help us to both qualitatively and quantitatively

assess the effectiveness of targeting. Second, it is a reported photoremovable protecting group with high quantum yield and red shifted activation wavelength to make it biocompatible. We used two different variations of coumarin with modification at C2. The mitochondrial directing group (TAG) is a positively charged hydrophobic group that will drive the probe across the mitochondrial membrane. We used two different type of groups: a triphenylphosphonium ion group, very well-known as a mitochondrial directing group, and a pyridinium ion, which is less bulky and may be a better fit for the enzyme pocket. Finally, we connected the MBG with TAG using a linker of different lengths and hydrophobic properties.

As there is no report of a sulfonamide group protected with coumarin as a photocage, we hypothesized that the photocleaving reaction will proceed in the similar fashion as for amines. Upon photoirradiation with a specific wavelength of light, the coumarin will be cleaved following the mechanism outlined in Scheme 6.9, resulting in the formation of an ion pair. In the presence of water, the formed carboxylate derivative of sulfonamide will undergo decarboxylation to yield the free active metal binding group, which will be available to interact with the zinc ion in the active site. As we have the tag attached to the inhibitor, we believe the photocleaved product will stay localized in the mitochondria and will not pass through the membrane to interact with other CA isoforms present in the cell. One concern of note in this design, however, is the production of one equivalent of carbon dioxide as a side product of the photoreaction since CO_2 is a known substrate of CA. Appropriate controls will be required in biological studies to mitigate this concern.



Scheme 6.9 Proposed Mechanism for the photocleavage reaction

6.4.2.2 Design optimization: molecular docking

Using the hCAV structure generated from the homology model, the UCSF Chimera docking program was used to model protein-probe interactions between CAV and the proposed probe. Upon docking the probe with R-PPh₃⁺ as the tag, no interactions between the probe molecule and the protein active site were found. We tried shorter and longer linker lengths, but we observed no binding of the probe likely due to the bulkiness of the triphenylphosphonium group that clashes with the amino acids at the opening of the CA active site. Furthermore, the molecule was synthesized in the meantime and had poor solubility in aqueous solution.

To improve the design in terms of bulkiness and hydrophobicity, we replaced the tag with a pyridinium group. Furthermore, the polyethylene glycol (PEG) linker was changed to an alkyl linker to avoid the unwanted micelle-like structures. Using Chimera, the optimal linker length was determined to be three carbons long, which both allows sufficient length for the probe to show good binding with the Zn²⁺ in the active site, but also not too long such that the whole probe becomes too hydrophobic to be soluble in aqueous and other biologically compatible solvents. A distance of 1.798 Å was found between probe and the zinc in the active site for the conformation with the lowest RMSD and highest score (Figure 6.6). Typically, distances lower than 2 Å suggest strong binding, distances between 2 and 4 Å suggest medium binding, and larger than 4 Å suggest weak or no binding. These results suggest that theoretically, the target probe

design with pyridinium as the mitochondrial directing group would have very strong binding with the CAV active site.

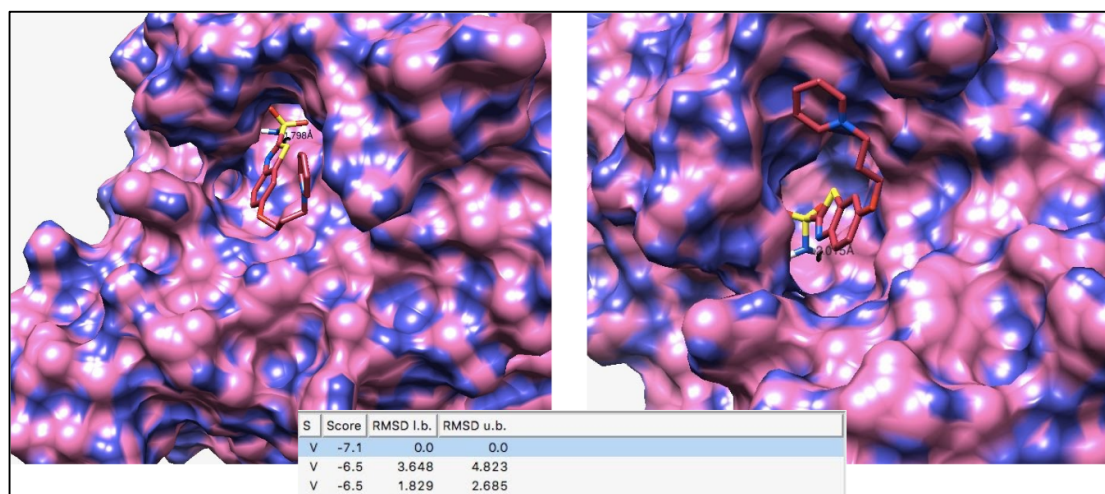


Figure 6.6 Top two docking results for CAV probe with pyridinium ion mitochondrial directing group. Distances shown between nitrogen of probe and catalytic zinc atom in active site.

Finally, the coumarin moiety with an oxygen atom instead of a sulfur atom (thiocoumarin) at C2 was chosen for the final probe design. Even though thiocoumarin has been shown to have better photophysical properties, the molecule is not very stable and undergoes slow conversion to coumarin.

6.4.3 Design and synthesis

Based on the rationale detailed above, the probe design was finalized to **PCE-M** (Photo Caged Ethoxazolamide with Mito tag). A control molecule without the mito tag, **PCE**, was also designed (Figure 6.7).

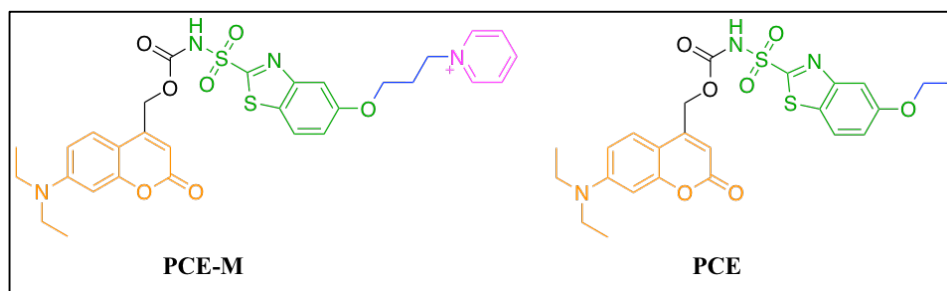


Figure 6.7 Chemical design probes **PCE-M** and **PCE**

The probes were synthesized by preparing each precursor corresponding to PPG, MBG, Linker and Tag individually and then coupling them together in the end to yield **PCE-M** and **PCE**.

The linker was synthesized by tosylating propanediol in pyridine. The PPG was synthesized by oxidizing the methyl group on coumarinmethyl derivative to an aldehyde using SeO_2 and then reducing it to an alcohol derivative using NaBH_4 as the reducing agent. The MBG was prepared by converting the ethoxy group to an alcohol to ethoxazolamide in the presence of AlCl_3 . The sulfonamide group was then protected in the presence of DMF-DMA and linker was attached to the probe. The tosylated group was substituted by an iodide group in the presence of NaI , which was further reacted with pyridine to tag the probe with pyridinium ion. The final precursor was prepared by deprotecting the sulfonamide in the presence of hydrazine. The formed species was coupled with coumarin in two steps in the presence of 4-nitrophenylchloroformate and DMAP to yield the final product in quantitative yield. The detailed synthetic scheme and procedure is shown in the experimental section.

6.4.4 Photocleaving properties

6.4.4.1 UV-vis and Fluorescence Spectroscopy

The photophysical properties of **PCE** was studied using absorbance and fluorescence spectroscopies in aqueous buffer.

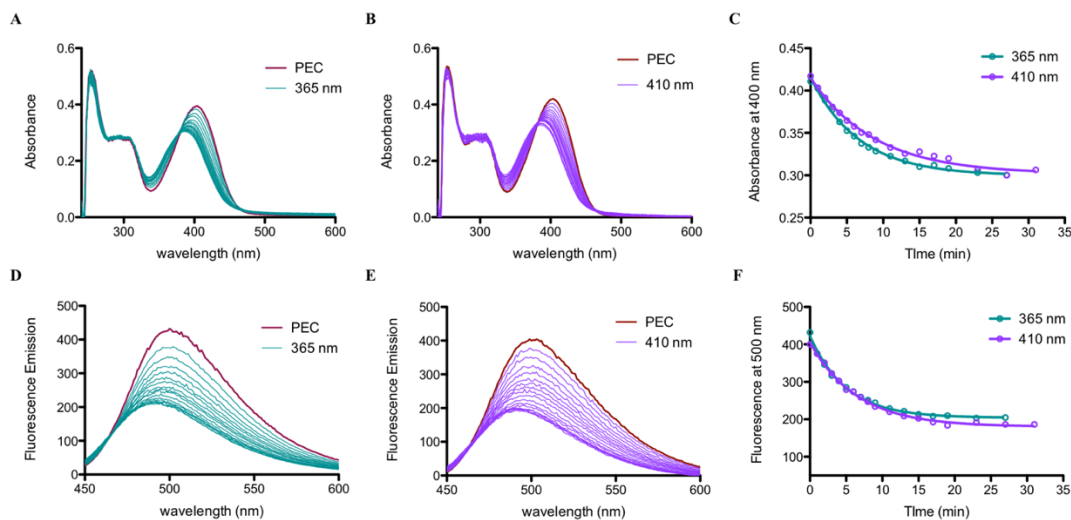


Figure 6.8 The absorbance profile of **PCE** before and after the irradiation with 365 nm (A) and 410 nm (B), and kinetics of photocleaving reaction (C) due to photoirradiation. The fluorescence emission profile ($\lambda_{\text{ex}}=410$ nm) of **PCE** before and after the irradiation with 365 nm (D) and 410 nm (E), and kinetics of photocleaving reaction (F) due to photoirradiation. The studies are conducted in 50 mM HEPES with 0.1 M KNO_3 pH 7.2 at room temperature under dark.

As shown in Figure 6.8, the absorbance spectra of **PCE** shows an intense absorption band at 400 nm, which reduces after the irradiation with 365 nm (Figure 6.8 A) and 410 nm (Figure 6.8 B). The kinetics of the photocleavage reaction are shown in Figure 6.8 C where the absorbance at λ_{max} was plotted with respect to time. Similar pattern was observed in the fluorescence emission where the peak at $\lambda_{\text{em}} = 500$ nm ($\lambda_{\text{ex}} = 410$ nm) was reduced following photoirradiation with 365 nm (Figure 6.8 D) and 410 nm light (Figure 6.8 E), with kinetics in fluorescence changes shown in Figure 6.8 F. As the

photodeprotection kinetics were similar in terms of rates and saturation level, we decided to use 410 nm for further studies.

6.4.4.2 Mass Spectrometry

As the profile obtained by UV-vis and fluorescence spectroscopies suggested that photodeprotection was occurring, we used LCMS to analyze the product formed after photoirradiation. A sample was prepared in a mixture of water (required for photocleaving mechanism) and acetonitrile (required for solubility), and was run through neutral H₂O/ACN in LCMS under dark conditions. The sample was then irradiated with 410 nm for 30 min and was run through same method in LCMS again under dark conditions.

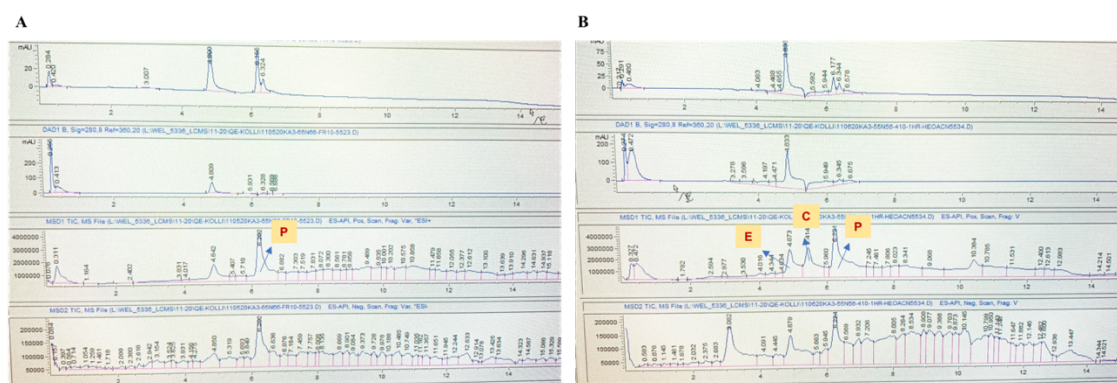


Figure 6.9 LCMS spectra collected for **PCE** before and after 410 nm irradiation. The P, C and E stands for mass corresponding to **PCE**, Coumarin and Ethoxazolamide product after photocleaving reaction.

As shown in Figure 6.9, the positive scan had a peak at 6.3 min retention time, which corresponds to the mass of **PCE** (**P**). After photoirradiation, we see peaks at 4.6 and 5.4 minutes, which correspond to the masses of the ethoxazolamide (**E**) and coumarin (**C**) products, respectively, that we proposed to form after photocleaving reaction. This result suggests the applicability of pursuing coumarin as PPG for

sulfonamide based ligands, which is the first example so far. This also suggests we can get a clean reaction to obtain ethoxazolamide as the product, which makes the system biocompatible for protein and cellular studies.

6.4.5 Studies with bCA

The most widely recognized biological role of CA is cellular pH regulation, but its intrinsic esterase activity is also utilized to measure its catalytic activity and to study the effect of inhibition spectroscopically. The inhibitory activity of **PCE** before and after photoirradiation with 410 nm light with bCA was determined using a *p*-nitrophenyl acetate (NPA) hydrolysis assay. NPA was used as a substrate for CA, which is converted to *p*-nitrophenolate ion upon hydrolysis and gives an intense absorbance at 400 nm at pH 7.2.³⁰ Figure 6.10 shows the increase in absorbance at 400 nm over time when bCA was incubated with 1 equivalent of **PCE** before and after irradiation with 410 nm to yield deprotected **PCE** (*d*-**PCE**). We performed this experiment with 1 equivalent of ethoxazolamide as well for comparison purposes.

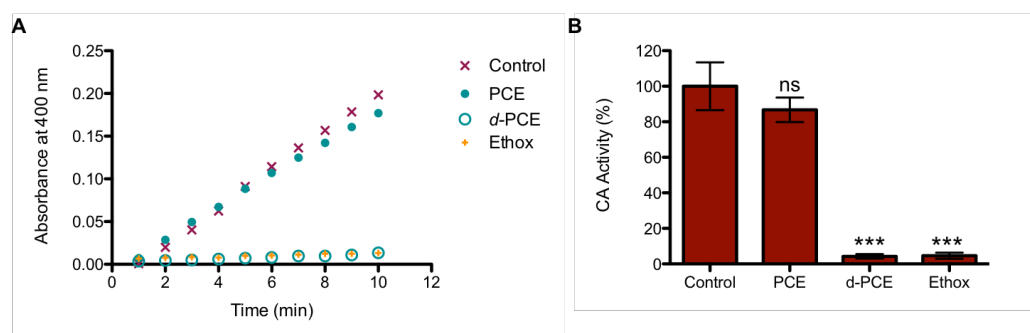


Figure 6.10 Change in absorbance (A) at 400 nm and CA activity (B) due to NPA hydrolysis by bCA in the absence and presence of **PCE**, deprotected **PCE** (*d*-**PCE**) and ethoxazolamide (Ethox). (B) IC₅₀ determination. Data represents mean $\pm$ S.D. Statistics analysis are done by one way analysis of variance (***) represents $P < 0.05$). Studies performed in 50 mM HEPES, 0.1 M KNO₃ pH 7.2 at room temperature.

As shown in Figure 6.10, the incubation of **PCE** did not decrease the protein activity significantly (non-significant as compared to control), showing the non-inhibitory properties of **PCE** due to the photocage present on the sulfonamide moiety. After photoirradiation with 410 nm light, *d*-**PCE** inhibited the protein activity drastically, similar to the treatment with ethoxazolamide. This result demonstrates that we were able to photocleave the coumarin group upon photoirradiation, and were able to successfully release the free inhibitor to interact with the protein active site.

6.5 FUTURE DIRECTIONS

After showing these successful results with the molecule **PCE**, we need to characterize our main probe **PCE-M** which carries the mitochondrial directing group. Currently, we are working on figuring out the appropriate counter anion for the pyridinium ion, which can lead to significant changes in the stability of molecule in biological conditions. We plan to characterize the probes through other binding and activity based assays, including DNSA competition and CO₂ hydration assays, with different variants of carbonic anhydrase (bCA, hCAII, CAV). Cellular studies will also be performed to determine the biocompatibility, reactivity, and effectiveness of the molecule in cellular contexts. For these cellular studies, we will perform three key experiments: an MTS assay, a citrulline assay, and a urea/glucose assay. First, a MTS cell viability assay will be performed to assess the concentration at which the target molecule is toxic to cells, which will help us to determine the probe concentration for further assays. To study CAV activity, we will monitor the production of citrulline as the first two enzymatic steps of the ureagenesis pathway take place in the hepatic mitochondria, and results in the production and exportation of citrulline into the cytosol.³¹ We will study the changes in the rates of citrulline production due to the probe treatment on

isolated mitochondria, extracted from bovine cells using a mitochondria isolation kit. Finally, we will perform cellular functional assay for the selective CA V inhibition using intact bovine hepatocytes. Utilizing CA V's role in the ureagenesis and gluconeogenesis pathways, we will measure 1) urea production, determined calorimetrically using the diacetyl monoxime method, and 2) glucose production, determined calorimetrically by the toluidine technique. Control studies using **PCE** and ethoxzolamide will be considered as well.

6.6 CONCLUSION

In conclusion, we have proposed a rationale design to achieve spatiotemporal control over the activity of CAV using coumarin as a photocleavable protecting group (PPG) and executed key initial experiments. A homology model was created to model human CAV using template murine CAV for docking studies of the proposed variants of the probe, which were used to further improve the design to finalize **PCE** and **PCE-M** probes. We were able to successfully synthesize these molecules and studied them for their photophysical and photocleavage properties. The photocleavage reaction was monitored by absorbance and fluorescence spectroscopies and mass spectrometry. Finally, we used a NPA-activity based assay to demonstrate the application of these probes to inhibit of CA using 410 nm light to trigger the photocleavage reaction. To the best of our knowledge, this will be the first example of a sulfonamide photocaged with coumarin derivatives as well as the first application of photopharmacology to gain on-demand regulation over a specific isoform of CA in isolated as well as in intact live cells.

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Chapter 7: Conclusion and Future Perspectives

Light serves as an ideal external control element for *in situ* biochemical manipulation because it offers a high level of spatiotemporal resolution, it is minimally invasive, and its wavelength and intensity can be readily tuned. Our research work focused on the development and application of light activated pharmacophores in the regulation of carbonic anhydrase, a zinc containing metalloenzyme. One important strategy used in the development of such photopharmacophores is the alteration of molecular properties (chemical structure, shape, polarity) upon light irradiation resulting in different affinity towards the desired target. This can be attained either by using a small photochromic moiety (reversible approach) or photoprotecting group (irreversible approach). The first section of this chapter focuses on efforts to gain reversible control over cytosolic and membrane associated isoforms of CA using the reversible approach by using azobenzene as photoswitches. The second section of this chapter discusses the application of the irreversible approach to gain specific control over mitochondrial isoform of CA. The last section discusses the challenges faced in the field of photopharmacology and the outlook based on the insights gained during this doctoral research for future project development.

7.1 REVERSIBLE CONTROL OVER METALLOENZYME ACTIVITY

We developed carbonic anhydrase azobenzene photoswitches, **CAP1** and **CAP2**, and demonstrated the interactions of the *cis* and *trans* isomers with CA protein in several contexts. Both ***t*-CAP1** and ***t*-CAP2** have increased inhibitory activity relative to their *cis* counterparts, and this activity can be modulated by photoirradiation with specific wavelengths of light. Importantly, we were able to establish the differential ability of the

cis and *trans* isomers of **CAP1** to regulate cytosolic pH in live cells, opening up future avenues for *in situ* pH regulation and CA inhibition.

We further tuned the scaffold of **CAP1** to attain longer-lived *cis* isomers and red-shifted irradiation wavelengths to expand our ability to apply these systems in live cells and organisms. Through the extensive investigation of azobenzene derivatives, we identified *ortho*-fluorination as a strategy to improve the performance of azobenzene-based probes owing to the separation of the $n\text{-}\pi^*$ absorption band in the *trans* and *cis* isomers. This large separation of $n\text{-}\pi^*$ bands enabled us to use distinct visible wavelengths (520 nm and 410 nm) to achieve higher isomer conversion in the photostationary state when compared to other azobenzene derivatives. Moreover, the *cis* isomers of *ortho*-fluorinated azobenzenes are exceptionally thermally stable, enabling us to obtain the single crystal structure.

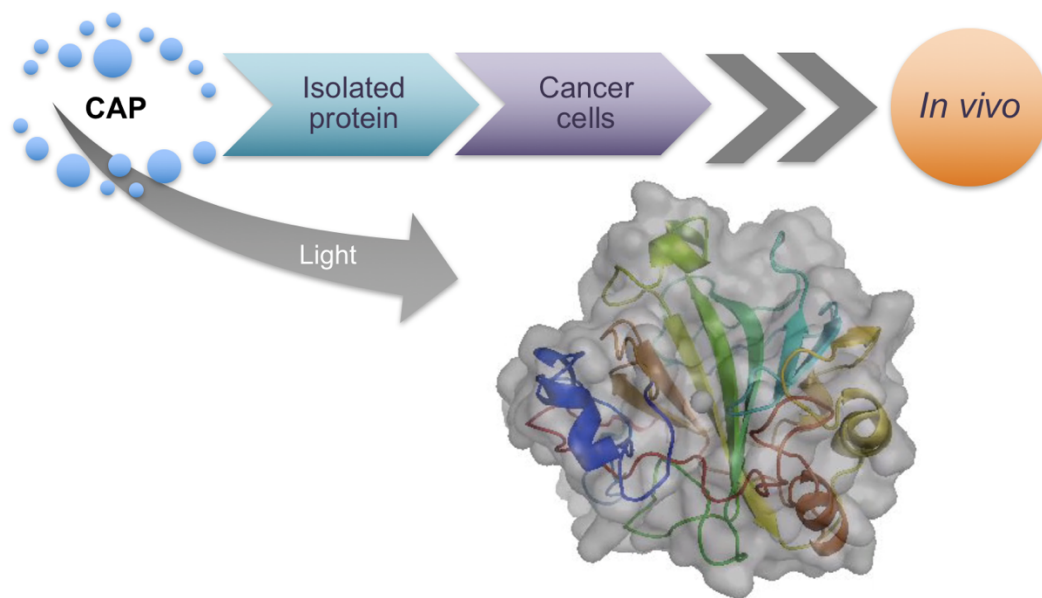


Figure 7.1 The graphic representation of **CAP** to get control over CA in isolated protein, in cancer cells and in vivo systems by using light as an external trigger.

We presented the first demonstration of the *ortho*-fluorination strategy for controlling the activity of a metalloenzyme by using **CAP-F3** and **CAP-F5**. Importantly, we were able to establish the efficacy of this approach for dynamic modulation of the activity of Zn-dependent carbonic anhydrase *in vitro* with isolated protein, in live cells, and for the first time, *in vivo* in live zebrafish (Figure 7.1). Specifically, we were able to photoactivate the **CAP-F5** *in situ* to track cytosolic CA machinery for CO₂ hydration in HeLa cells as well as modulate the role of CA in phenotypic development of zebrafish in the initial phases of embryogenesis.

After the success of **CAP-F5** in photomodulation of cytosolic CA, we explored the prospects of these photoswitches in regulating the activity of membrane bound isoform CAIX, which is known to be overexpressed in cancer cells leading to the metastasis of tumors. We modified the design of **CAP-F5** with a negatively charged sulfonate group to get **F4-Am-SO₃**, rendering the probe impermeable to the cellular membrane and achieving the selective control over CAIX. Current efforts are ongoing to find the application of these probes in cellular context.

7.2 IRREVERSIBLE CONTROL OVER METALLOENZYME ACTIVITY

There are multiple isoforms of carbonic anhydrase and their localization, expression level, and physiological role vary in different cell types and also differ between normal and disease states. Therefore, it is imperative to get precise inhibition of a specific isoform without perturbing other isoforms in order to understand their function in biological systems, especially in the therapeutic context. However, it is difficult to development isoform specific inhibitors due to the high similarity in the sequence and structure of active site of different isoforms.

To that end, we used photocaging to develop a chemical tool that can be directed to mitochondria and target the mitochondrial isoform CAV, without binding other CA isoforms in the cytosol or cellular membrane. We developed the probes **PCE** and **PCE-M** with the metal binding group protected by a photoremovable protecting group (PPG) and linked to a mitochondrial directing group. The photocleaving reaction can be triggered once the probe is localized in the organelle by using 410 nm light, relieving the active inhibitor, thereby inhibiting only CAV. By providing spatial and temporal control over the activation of the inhibitor, this design enables the selective inhibition of specific CA isoforms in their natural cellular environment and opens the door for a range of studies and applications that were previously unfeasible.

7.3 CHALLENGES AND OUTLOOK

As discussed in this dissertation, light can be used as a versatile method to perturb and manipulate enzyme activity with high spatiotemporal resolution. Despite the progress made thus far in designing new photochromic molecules, several problems and challenges still exist such as:

The first one is understanding the meaning behind spatiotemporal resolution. As the biological system is highly complex and dynamic processes happen on short timescales, the temporal criterion of our approach needs to match the relevant biological question to fully exploit the light potential. On the other hand, when we are dealing with slow biological phenomena, one may have to consider leakage of the activated product out of the organelle or the cell. In terms of spatial control, we need to consider the likelihood of selectively photocontrolling subcellular component which are typically on nm dimensions.

The second one is lacking understanding and quantification of various forms of photopharmacophores in living systems. It is comparatively easier to control the concentration of photoactivable probes during the experiment conducted exogenously. However, it is difficult to quantify the relative concentration of the “inactive” and “active” probe and control the photoactivation rate when conducting experiments endogenously.

The third one concerns the versatility and robustness of the photopharmacophore in biology. Their properties in terms of toxicity and interference with other cellular processes are vital to assess their biocompatibility. Additionally, it is important to gauge the bioavailability in terms of cell permeation, localization of the photopharmacophore as well as the targeted protein.

Finally, one of the main hurdles to overcome is the complicated process of the development of chemical tools for light mediated control over biological molecules. Designing and implementing lengthy and complex synthetic procedures is a cumbersome process requiring much time and efforts demonstrated very well in the chapters 3, 4 and 5 of this dissertation demonstrating the need to target simple synthetic designs. The rational design of a single photoresponsive moiety, such as tetra-*ortho*-methoxy-azobenzene posed many synthetic challenges, while other synthetic targets including tetra-*ortho*-chloro and tetra-*ortho*-bromo azobenzene, did not give the desired interaction with the protein, despite of desired photophysical properties

Along with these challenges, the field of photopharmacology does raise some questions for consideration: is there a possibility to independently control several proteins within the same cell or at different locations? As visible light is not penetrable through tissues, is there any other photoresponsive moiety that utilizes the ideal therapeutic window of 600-900 nm? Finally, if not photopharmacology, are there other triggers, such

as heat, acoustic waves, magnetic fields, electric field and so on that can be used to develop different fields of pharmacology for protein control? These questions notwithstanding, the work presented herein clearly demonstrates the utility of using photopharmacophores for on demand control of metalloenzyme activity and has paved the way for future work in this area.

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Vita

The author, Kanchan Aggarwal, belongs to Ghaziabad, India. She completed her Integrated Masters in Science as an INSPIRE fellow from Indian Institute of Technology, Roorkee with the Institute Silver Medal and then moved to USA in 2015 to begin her doctoral expedition at UT Austin. As a part of Que lab, she established the photopharmacology research in the group, and served as biosafety officer of the lab. She published two first authors and four secondary manuscripts, and had two additional ongoing projects led by her at the time of her graduation.

During her time at UT, she served as a TA for the department of Chemistry, and mentored three undergraduate students for research who all went to pursue PhD from Top US universities. She also mentored girls through other organizations such as Bold Women in Chemistry (BWIC), My Science My Life (sponsored by L'Oréal) and McNair Intellectual Entrepreneurship Internship Program (St. Edward's University).

Kanchan was a founding member of Chemistry Student Safety Organization (CSSO), and has served as graduate student representative in numerous committees and societies, including Council of Graduate Chemists. She is a strong promoter of science communication among people of all ages *via* outreach activities. She won the Three Minute Thesis (3MT) competition at UT and represented the university at regional level, and was interviewed for UT graduate school promotional video.

Her passion to implement solutions to improve the lives of fellow students and the efficacy of the graduate program was recognized with many accolades, including Safety-First Leadership Fellowship and Chemistry Department Graduate Student Excellence Award. She can be reached *via* the following email address:

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