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Photoisomerization in Cyclic Dipeptide Derivatives: Photoswitchable Supramolecular Hydrogels and the Discovery of Hemipiperazine Photoswitches

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ABSTRACT

Cyclic dipeptides (CDPs) are ubiquitous biological motifs and pharmacophores. Through their defined structure and rich hydrogen bonding network they are prone to forming a large variety of supramolecular systems in aqueous environment. The merger of CDPs with molecular photoswitches, described here, enables photocontrol over bioactivity, as well as the self-assembly and macroscopic properties. Such systems can find broad range of applications, from smart photoresponsive materials to photopharmacology and precise control of complex biological systems. In particular, we demonstrate here two concepts—CDP-based photoswitchable hydrogels, and our journey towards discovery of a novel CDP-derived photoswitchable motif—hemipiperazine—which complements established photoswitches in terms of biocompatibility and visible-light triggering.

1 | Introduction

Cyclic dipeptides (CDP) based on the 2,5-diketopiperazine skeleton ("glycine anhydride") (Figure 1a,b) commonly occur in Nature [2]—as secondary metabolites, components of fermentation broths [3], yeast cultures, fragments of larger natural products, and bioactive compounds [4]. CDPs are also common pharmacophores [5] with broad range of physiological activities [6]—like the blockbuster tadalafil, retosiban [7], or a promising anticancer drug candidate plinabulin [8] derived from another CDP phenylahistin [9]. Some CDPs can penetrate living cells [10]. In particular, their structural rigidity and extensive hydrogen bonding network steaming from the ring amide groups efficiently trigger self-assembly processes [11]. Many simple CDPs build nanofibers in water and form hydrogels [1], or show cooperative catalytic activity [12]. Numerous CDP-based hydrogels [13, 14] supramolecular assemblies [15, 16], and other functional materials [17]—some also with therapeutic applications perspective [18]—have been recently demonstrated.

All these properties and applications are already extremely interesting in their static version. Yet, the ability to reversibly modulate the properties of CDPs would provide exciting opportunities to build smart materials or regulate bioactivity. Thus, we decided to merge CDPs with molecular photoswitches [19–21]—compounds that reversibly convert the energy of light into molecular changes (shape, polarity) [22], which in turn can further influence the original function of the adjacent CDP. Molecular photoswitches operate using various mechanisms. Among them, the most common are *E*-*Z*-isomerizations of double C=C, C=N, or N=N bonds—as in stilbenes [23], imines [24], azobenzenes [25], or indigoids [26]. Other common mechanism is reversible ring opening—like in diarylethenes [27], spiropyrans [28], or dihydropyrenes [29]. These established photoswitchable motifs have been recently complemented with a range of novel photochromic scaffolds [30].

In this concept article, we discuss two embodiments of CDP photocontrol—light-responsive low-MW supramolecular hydrogels,

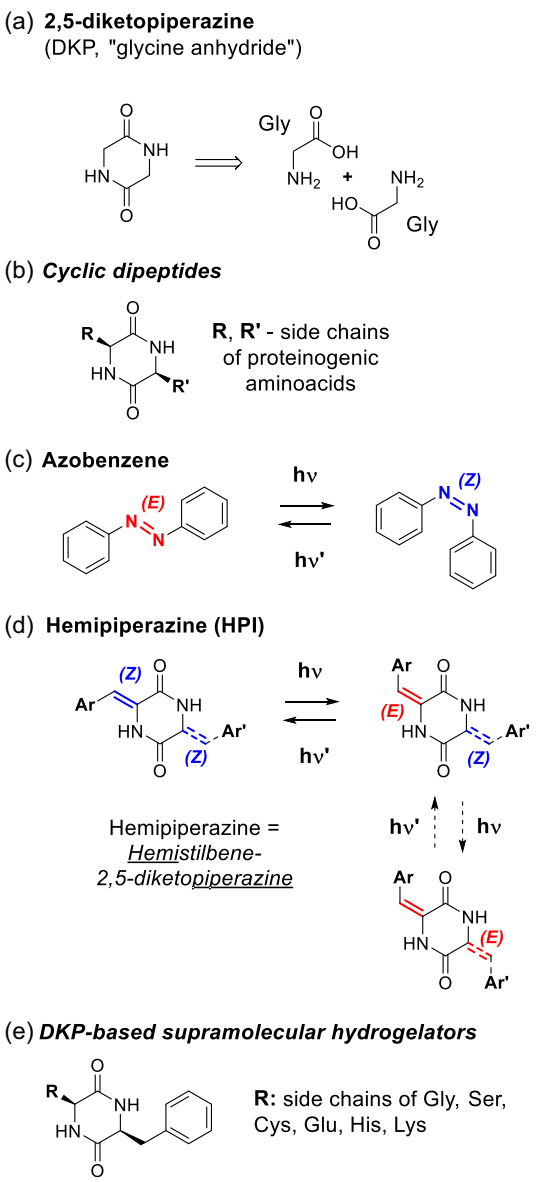

FIGURE 1 | Common molecular motifs discussed in the concept. (a) 2,5-diketopiperazine (DKP) is a product of glycine dehydratation and a prototype of (b) cyclic dipeptides (CDP)—common secondary metabolites and condensation products of proteinogenic aminoacids; (c) azobenzene is by far the most commonly applied molecular photoswich; (d) recently discovered hemipiperazine (HPI) photoswitch derived from CDPs can complement azobenenes in particular applications, especially in biological systems; and (e) amphiphilic CDPs are excellent supramolecular hydrogelators (ref. [1]).

and photomodulation of bioactivity. As the latter led our group to describe a new class of CDP-derived molecular photoswitches—hemipiperazines (HPIs), we also use this opportunity to describe the scope of its applications.

2 | Photoisomerization of Azobenzenes and Hemipiperazines

Photoswitches are defined as a group of two or more related isomers with distinct absorption spectra, which can be interconverted (at least in one direction) by irradiation with light. Photochromism—color change upon irradiation—is an external manifestation of this conversion in solution or in the bulk of material [22]. While the switching can be realized using variable photoisomerization reactions (electrocyclization, cycloaddition, dissociation, group/electron transfer) [31], the systems discussed in this concept are based on *E/Z*-photoisomerization of double bonds. The diverse applications of photoswitches—optical filters, imaging, holography, solar energy conversion, molecular electronics, drug delivery, and photopharmacology—attracted much attention in the recent decades.

The *E*- and *Z*-azobenzenes (Figure 1c) have similar and partially overlapping absorption spectra. They are classified as T-type switches (where the metastable isomer generated upon initial irradiation can spontaneously reisomerize to the initial form via thermal relaxation) [32]. This process for a typical azobenzene takes several hours [33], however structural modifications can provide switches with the thermal half-life in the span of years [34], or picoseconds [35].

The unmodified *E*-azobenzene is about 12 kcal/mol more stable than the *Z*-isomer, with the energy bareer of about 23 kcal/mol. The $E \rightarrow Z$ isomerization occurs upon exposure to UV light (most efficiently with 320–350 nm), and the $Z \rightarrow E$ photoisomerization can be quickly achieved with blue light (400–460 nm) [25]. Upon photoisomerization, the end-to-end distance (between carbon atoms located in the *para* positions in respect to the azo bond) varies between 9 Å (*E*-isomer) and 5.5 Å (*Z*-isomer). In the *Z*-isomer, one of the aryl rings twists out of plane by 50°, to accommodate the steric congestion. For that reason, the twisted *Z*-isomer is more polar—it shows $\mu = 3.0$ D, while the planar *E*-isomer has $\mu = 0$ D. The UV–vis spectrum of *E*-azobenzene shows an intense $\pi \rightarrow \pi^*$ transition band at 320 nm and a weak $n \rightarrow \pi^*$ transition band at 440 nm. The *Z*-isomer has a stronger $n \rightarrow \pi^*$ transition band around 440 nm and two absorption bands at around 280 nm and 240 nm [33].

HPIs [36], (Figure 1d) are arylidene-substituted CDPs, which share the basic *E/Z*-photoisomerization mechanism with azobenzenes. Yet, in case of HPIs the isomerization occurs on the C=C double bond, flanked on one side by an aromatic system, and on the other—by the 2,5-diketopiperazine, which is a cyclic diamide. There are increasing evidences, that the simple *E/Z*-switching in many cases is complemented with tautomerism of the conjugated amide system, which makes it more similar to indigoids—a switch that is known to be mesomerically active [37–39].

Opposite to azobenzenes, the thermodynamically stable isomer is usually the *Z*-HPI, which is also the one typically obtained from synthesis—using base-catalyzed condensation of an aryl aldehyde to bis-*N*, *N*-acetyl-2,5-diketopiperazine (acetylated glycine anhydride). The acetyl, as an auxiliary leaving group, increases the speed and yields of the condensation (which also occurs, albeit slower and with lower yields, with unsubstituted 2,5-diketopiperazine). The following point of diversity is the use of heteroarylidene substituents instead of the carbocyclic arylidene groups.

As there are two possible arylidene attachment sites, and the condensation proceeds stepwise, it is possible to obtain mono-arylidene-HPIs, homo-bis-arylidene-HPIs, or hetero-bis-arylidene-HPIs, which possess two, three, or four photoisomers, respectively.

The mono-arylidene HPIs (shortly mono-HPIs) with carbocyclic arylidenes show low-to-moderate photoconversions, except for

arylidenes substituted with strongly electrodonating groups (NMe_2, NPh_2).

The absorption maxima of *Z*-isomers are situated in the UV part of the spectrum, with the slightly bathochromically shifted peaks of the *E*-isomers, which enables forward switching with 365 nm (UV), and backward—with 410 nm (violet). The heteroarylidene mono-HPIs show higher photoconversions and their spectra are bathochromically shifted toward visible frequencies. While the photoisomerization parameters for bis-arylidene-HPIs (short "bis-HPIs") is more complex, and depends on several parameters, they typically show good photoconversions, and many can be reversibly photoisomerized without using UV light, solely with visible light frequencies.

A common feature for all arts of HPIs investigated until now is their very high thermal stability, even though they are still T-type switches, and their *E*-isomers undergo thermal relaxation to the respective *Z*-forms, the typical lifetime at ambient conditions is counted in months (while standard azobenzenes vary between minutes and days). Therefore, for most experiments, especially biological, HPIs are treated as a bistable system, tuned solely with light.

3 | Light-Triggered Supramolecular Hydrogels

3.1 | Photoliquefaction with UV Light

Amphiphilic CDPs undergo efficient self-assembly in water, yielding supramolecular hydrogels [13]. In 2013, Kleinsmann and Nachtsheim [1] demonstrated that CDPs containing phenylalanine and a polar aminoacid residue (Figure 1e) are biocompatible hydrogelators with lowest molecular weight, which efficiently form hydrogels in broad pH and concentration ranges and are promising materials for drug delivery.

The gelation properties have been credited to the hydrophobic interactions between neighboring phenyl rings, which form the core of supramolecular fibers, complemented with the extensive hydrogen bonding network between the 2,5-diketopiperazine rings.The polar side chains pointing outwards supposedly enhance the water structuring process on the polar surface of the fibers.

Other literature sources [2] indicate that in 2,5-diketopiperazine with an aromatic side chain the phenyl ring overlaps with the heterocyclic ring due to stabilizing CH–π interactions [40, 41], which presence is revealed by NMR [42] and single-crystal XRD structures [40], and supported by computational results [43]. However, in the discussed cases the tendency of amphiphilic CDPs to form linear supramolecular polymers due to the regular spatial orientation of H-bond donors and acceptors on the heterocyclic ring might be decisive for the formation of supramolecular fibers observed by electron microscopy [40].

It is known, that azobezene photoswitches significantly change their geometry and polarity upon photoisomerization [44]. We have hypothesized that replacing the statically hydrophobic and flat phenyl ring in the structure of dipeptide hydrogelators with an azobenzene will provide a system with geometry and polarity photomodulation at the molecular level, which might be reflected by photomodulation of its macroscopic properties.

For that, we prepared a previously reported [45] photoswitchable aminoacid PAP ((*S*)-4-phenylazo-phenylalanine)—an azobenzene analog of (*S*)-phenylalanine. It was incorporated into a series of CDPs with polar proteinogenic aminoacids (Ser, Glu, His, Cys, Lys). Most of them either show good water solubility, or precipitated from aqueous solutions. Yet, the CDP **1**—cyclo(PAP-Lys)—was an efficient supramolecular hydrogelator (Figure 2 *left*). It formed hydrogels in the range of 15–30 g/L (1.5–3.0 wt%) in aqueous media (3.0 wt% was the solubility limit). The resulting supramolecular hydrogels were rapidly (<1 min) self-healing, and their mechanical strength could be tuned by addition of sodium chloride. The hydrogelator also strongly bound to dsDNA, which was reflected in rheology measurements.

Most importantly, hydrogels composed from **1** underwent reversible liquefaction upon exposure to UV (365 nm) light (<30 min

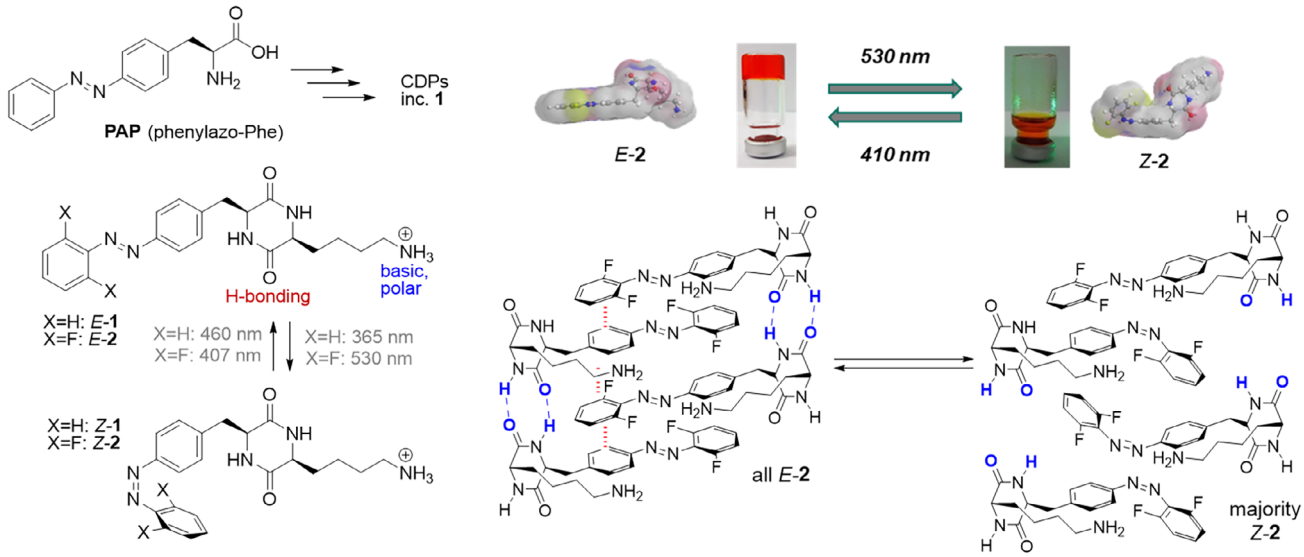

FIGURE 2 | The photoswitchable aminoacid **PAP** (*S*-phenylalanine analog) can be incorporated into CDPs. The CDP **1** (PAP + *S*-lysine) is an efficient hydrogelator triggered with UV light [46]. Its bis-*ortho*-fluorinated analog **2** is bidirectionally switched with visible light frequencies [47] (*left*); the hydrogels formed by **1** and **2** can be reversibly liquefied upon irradiation (*top right*); the reason is reversible destabilization of supramolecular fibers upon light-induced transition from the hydrophobic *E*-azobenzene with efficient mutual π–π stacking to its more polar *Z*-isomer devoid of efficient hydrophobic interactions (*bottom right*). Adapted from ref. [47] with permission. Copyright 2018 John Wiley and Sons.

for a 1 mL-vol sample). The resulting nonviscous fluid could be gelated back upon exposure to 460 nm light (30 min) followed by a curation period of 4–6 h in darkness). Alternatively, the gelation occurred spontaneously after 10–14 days of storing the nonviscous fluid in darkness, due to thermal azobenzene back-isomerization. This behavior was explained by the assumption, that the nanoscopic fibers of the nonirradiated hydrogel are primarily stabilized by the $\pi-\pi$ stacking of the flat, nonpolar *E*-azobenzene residues, supported by the hydrogen bonding network of the 2,5-diketopiperazine rings. Upon irradiation, the majority of azobenzene isomerizes to the bent and more polar *Z*-form, which destabilizes the hydrophobic core of the fibers to the degree where they fall apart and do not sustain anymore the 3D-structure of the gel. Thus, the resulting composition becomes a nonviscous fluid at the given concentration range. The $Z \rightarrow E$ back-isomerization (light-induced or thermal) restores >95% of the amphiphilic *E*-isomer, which undergoes prompt self-assembly. This restores the inner fibrous structure upon the curation phase. When cross-linking of the fibers spans over the whole volume of the material, it restores the macroscopic appearance as hydrogel and the respective mechanical parameters.

With such a biocompatible photoresponsive material in hands, we have demonstrated its applicability for light-controlled drug release by encapsulating an anticancer agent doxorubicin, as well as long dsDNA chains, and releasing the cargo in a controllable manner using UV light. While the selectivity of dsDNA release was high (possibly due to additional electrostatic stabilization), we have observed however that doxorubicin shows significant leaking from our material in the darkness—the feature that has been optimized in the course of further experiments [46].

3.2 | Photoliquefaction with Visible Light

The promising system demonstrated above had several limitations that could potentially hamper its in vivo use as a drug delivery system. The most serious one was its activation with UV light, incompatible with biological systems due to its low cell and tissue penetration, high scattering, and—in some cases—also cytotoxicity. Visible-light-triggered systems show much higher biocompatibility [48]. Thus, we decided to replace the original photoswitchable residue with halogenated azobenzene analogs, previously reported to be reversibly switchable within the visible light range [49]. We have initially focused on *ortho*-fluorosubstituted azobenzenes triggered with green light ($E \rightarrow Z$ isomerization) [34], which have long thermal lifetime of the *Z*-isomer and showed good biostability in our hands.

We have synthesized the di-*ortho*-fluoroazobenzene analog of (*S*)-phenylalanine and incorporated it into a CDP with (*S*)-lysine. The resulting photoswitchable CDP **2** showed to be an efficient supramolecular hydrogelator in aqueous solutions (e.g., PBS buffer—phosphate-buffered saline, pH 7.4) at the concentration range of 40–70 g/L (4.0–7.0 wt%). The resulting biocompatible and self-regenerating hydrogels reversibly dissipated to nonviscous fluids upon irradiation with green light (523 nm) within 30–180 min (depending on the gelator's concentration) (Figure 2 *right*). The hydrogel has been loaded with various bioactive compounds (anticancer, antimicrobial, and anti-inflammatory drugs, as well as cytochrome C as a representative of proteins), and the cargo has been successfully released with green light upon hydrogel liquefaction. As a demonstration, we have loaded a hydrogel with an antibiotic ciprofloxacin. The gel incubated in darkness did not influence the rate of bacterial growth in a surrounding culture, yet upon green light irradiation the antibiotic was released in its active form and suppressed the bacterial growth [47]. Still, the problem of cargo leaking in the darkness was significant for some guests. We have observed, that basic cargo has higher rate of passive diffusion than the acidic ones, which can be explained by additional electrostatic interaction of the latter ones with the basic side chain of lysine fragment of our gelator. These observations have been corroborated by quantitative measurements of intramolecular cargo-gelator interactions using NMR methods [50].

The photoswitchable hydrogel system has been further optimized [51] by incorporation of a highly fluorinated switch—tetra-*ortho*-fluoroazobenzene into the (*S*)-lysine-containing CDP. The resulting gelator **3** showed higher photoconversions in solution, as well as lower critical gelator concentration (CGC) (>15 g/L), as compared with the previous gelator **2**. The supramolecular hydrogel formed from **3** in PBS buffer liquefied upon exposure to green light (523 nm) within 30 min., and could be solidified again with violet light irradiation (410 nm) of 60 min. followed by an overnight curation phase in darkness.

To reduce the undesired bioactive cargo leaking in darkness, we decided to tune the cargo structure. Based on the previous observations (Coulombic guest stabilization inside the hydrogel fibrous network), we assumed that increasing the number of supramolecular guest–gelator interactions will reduce its leaking propensity. To prove that, we have screened on bioactive compounds with structural similarity to the used hydrogelator. Finally, we came up with plinabulin **4**—a low-nM inhibitor of microtubule dynamics and a promising anticancer drug candidate, currently in the third phase of FDA clinical trials against NSCLC (nonsmall cell lung cancer) as well as several other clinical indications. We have synthesized **4** using a modular literature strategy, where the central 2,5-diketopiperazine ring is first bis-acetylated and then subsequently substituted (mechanism involves acetyl leaving groups [52]) with two respective aryl aldehydes (benzaldehyde and an imidazole-derived aldehyde) under basic conditions [8].

Indeed, hydrogels made of the gelator **3** and loaded with **4** could be efficiently dissipated using green light (523 nm) with concomitant release of the cargo in the soluble form. Yet, incubation of the gels with PBS buffer in darkness over prolonged periods of time (>24 h) did not show any measurable amounts of the cargo leaking out of the material. Even more interestingly, the hydrogels could be loaded with **4** significantly above (>40-fold) its solubility limit in aqueous media. Altogether, it strongly suggests that the cargo **4** is incorporated via hydrogen bonding and $\pi-\pi$ stacking into the fibrous structure of the hydrogel as a dopant (which explains negligible leaking in darkness), and only released to the solution upon light-induced dissipation of the hydrogel (Figure 3) [51].

3.3 | Azobenzene-Decorated CDPs Triggered with Red Light

The red and near-IR light (630–900 nm) can deeply penetrate soft human tissues, as it is not absorbed by hemoglobin. Therefore,

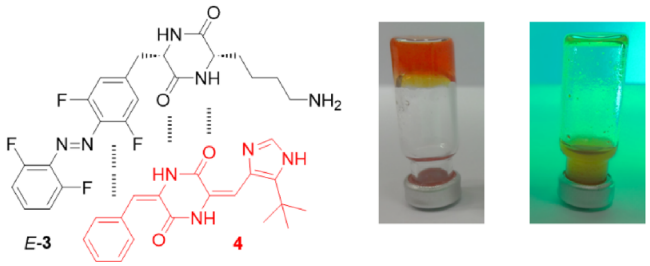

FIGURE 3 | Low-nM anticancer agent plinabulin **4** is an optimized cargo for supramolecular hydrogels based on photoswitchable CDPs, such as **3**. Due to efficient interactions (H-bonding, hydrophobic interactions), **4** can be efficiently released from the gel using light, but does not show leaking (passive diffusion out of the material) in darkness [51]. Adapted from ref. [51].

preparation of red light-triggered materials would enable general in vivo applications, such as photopharmacology. Our effort related to obtaining hydrogels capable of releasing bioactive cargo upon red light illumination is described below.

One synthetic limitation for preparing the hydrogelator **3** is the need for synthesis of a chiral fluorinated phenylalanine analog via stereoselective Negishi coupling (in contrary to the gelators **1** and **2**, where it is sufficient to functionalize naturally configured (*S*)-phenylalanine). For that reason, we wanted to attempt synthesis of a hydrogelator **5** similar to **3**, but devoid of the synthetically challenging stereocenter. The key synthetic step was base-catalyzed coupling of a cyclo(Lys-Gly) with a benzaldehyde derivative bearing an *ortho*-fluoroazobezene motif. We prepared two such aldehydes—with *para*- and *meta*-configured azobenzene residue. To our surprise, the *para*-configured azobenzene aldehyde **6** showed significant bathochromic absorption shift, which enabled efficient photoisomerization with red light (660 nm LED diode, with a cutoff filter > 630 nm). Decoration of the tetra-*ortho*-fluoroazobenzene with two *para*-located aldehyde groups made that effect even more pronounced (**6a**), while the *meta*-configured aldehyde remained inert to red light [53]. Importantly, beforehand no fluorinated azobenzenes have been reported to isomerize with light frequencies above 600 nm.

The sensitivity on red light has been maintained for the conjugated systems also upon coupling to the cyclo(Lys-Gly) CDP system. So, it can be generally stated that the extension of the π-electron system (with C═O or C═C bonds) generally confers the fluorinated azobenzene systems with the ability to photoisomerize upon exposure to >630 nm light. Sadly, the photoswitchable CDP **7** (although photoisomerized to 61% *Z*-isomer with 660 nm light) was a poor hydrogelator, and did form crystalline phases instead of the proper hydrogel in aqueous media. For that reason, the **7** could not be used to prepare fully biocompatible drug-releasing hydrogels (Figure 4 *top*). Nevertheless, we have incorporated the bis-acid analog of **6a** connected via amide bonds to two macrocyclic glucose-containing receptors of amino groups. The resulting molecular tweezers with photomodulated distance between the receptors could selectively detect methyl esters of basic aminoacids (Lys, Arg, Orn) in the *Z*-configuration, and longer biogenic amines (spermidine) in the *E*-form. While the bathochromic shift for the bis-amide was not so strong as for the bis-aldehyde, the system was still triggered with red light. It switched bidirectionally within the visible light range with high efficiency (almost quantitatively): >90% *Z*-isomer with red light (623 nm) and >95% *E*-isomer with violet light (410 nm) [55].

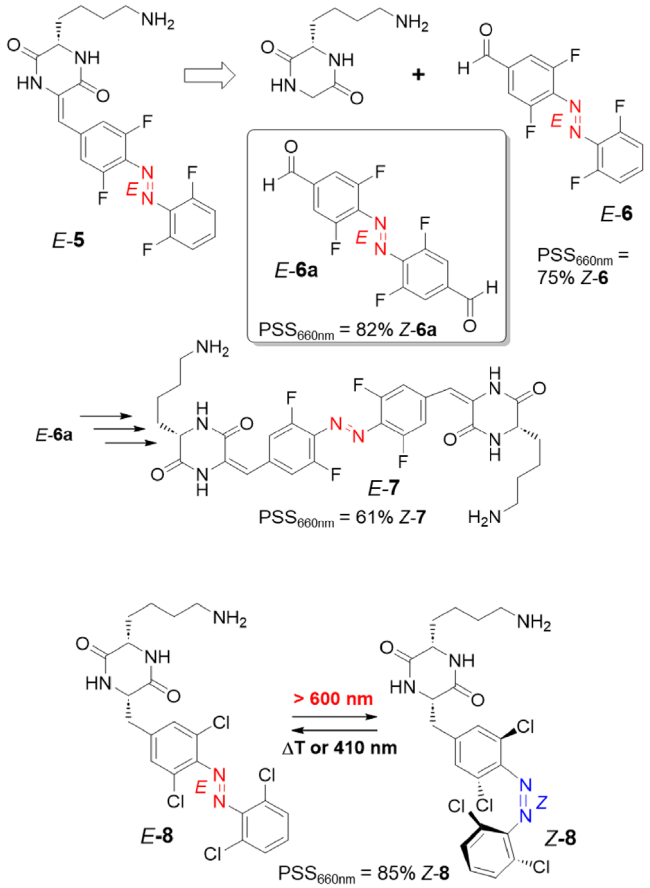

FIGURE 4 | Azobenzene-based CDPs triggered with red light. The attempts to synthesize nonchiral gelator **5** led to a discovery that *ortho*-fluoroazobenzene systems with extended conjugation (**6,7**) can be efficiently photoisomerized with red light and thus potentially applicable inside soft human tissues (penetrable with >630 nm light) (*top*) [53]; as no efficient hydrogel with that motif has been synthesized, our attention was turned to chlorinated azobenzene, which yielded hydrogels gels based on **8** that shrink upon exposure to 660 nm light (*bottom*) [54].

In order to overcome the obstacle for preparing red light-triggered hydrogels, we turned to another previously validated azobenzene system sensitive on red light—the tetra-*ortho*-chloroazobenzene, first reported by Woolley and coworkers [49]. The CDP **8** prepared from the respective photoswitchable aminoacid and (*S*)-lysine occurred to be a superhydrogelator (defined as gelators with CGC < 1 wt%). It formed mechanically stable hydrogels in PBS above 1 g/L (0.1 wt%) concentrations, which could encapsulate cargo (in that case, fluorescein). Upon exposure to red light (660 nm), the gel did not fully liquefy, but instead shrank with concomitant expulsion of the solution of the cargo molecule. Therefore, we dubbed the system a "supramolecular syringe" (Figure 4 *bottom*) [54].

3.4 | Composite Hydrogels Stabilized with Coulombic Interactions

Another interesting observation from the early encapsulation attempts was an additional hydrogel stabilization (in terms of mechanical properties and melting temperature) caused by the

addition of double-stranded DNA as cargo [46]. We rationalized it with ionic interactions between the oppositely charged lysine side chain of the gelator (positively charged) and the polyanionic DNA backbone (negatively charged). Thus, we hypothesized that other polyanionic nonphotoswitchable polymers, like sodium alginate, can significantly increase mechanical stability or reduce the input of the photoswitchable component needed to achieve the critical gelation concentration (CGC). For that, we prepared a modification of the hydrogelator **1** (with nonhalogenated azobenzene triggered with UV light), where two lysine side chains were exposed—as part of the CDP motif—on one molecule, linked with a central azobenzene fragment. The resulting compound **9** was designed as a photoswitchable cross-linker between oppositely charged alginate side chains [56].

Indeed, the compound **9** alone formed hydrogels in PBS buffer pH 7.4 at the concentrations above 10 g/L (1 wt%), while in presence of alginate (in the 1:1-1:2 mass ratio), the CGC of **9** dropped down to 4–6 g/L (0.4–0.6 wt%), respectively (Figure 5 *top*). The resulting composite hydrogels can be liquefied upon exposure to UV light (365 nm), and solidified again after irradiation with blue light (460 nm). Interestingly, as this process is fully reversible, photoequilibration slightly increases the overall stability (measured by Tm) of the material, probably upon slower and more efficient cross-linking in comparison to short boiling of the components together. The minimal concentrations of gel components using this preparation method yielding a mechanically stable material (with $T_m = 80°C$) was 0.3 wt% of **9** and 0.3 wt% of alginate. Thus, we developed an efficient hydrogelation protocol, where stock solutions of alginate and the hydrogelator **9** (previously irradiated at 365 nm) are mixed at room temperature at the concentrations below their individual CGCs, and then solidified with blue light followed by a few hours of curation in darkness. The advantage here is, that heat-sensitive cargo (labile drugs, or even living cells) could be used as cargo encapsulated inside the hydrogel, as the preparation process is accomplished at room temperature, solely with light.

Finally, due to the dynamic and supramolecular nature of stabilization in our hydrogel, it is possible to perform an exchange reaction with aqueous solutions of calcium salts. It is known, that soluble alginates form irreversibly a strong complex with calcium ions, stabilized by the so-called "Egg-box" structures [58]. Our composite hydrogel, treated with 10% aqueous solution of $CaCl_2$ underwent full exchange upon several hours of incubation—the photoswitchable component has been expelled to the aqueous solution, while the alginate remained a hydrogel (now not photoswitchable anymore) in form of its calcium salt. The exchange process was c.a. fourfold faster, if the reaction mixture was exposed concomitantly to UV light (which likely reduced the original cross-linking grade of the components and enhanced Ca^{2+} ions penetration capacity) [56].

Recently, we have expanded that concept by using the original photoswitchable hydrogelator design—the CDP **9a** made from arginine and a fluorinated azobenzene analog of phenylalanine (both in the (*S*)-configuration) formed as well a composite hydrogel with alginate (typical composition: 0.6 wt% of **9a** and 1.2 wt% of alginate). Now, due to the modified photoswitch, the composition can be liquefied with green light (523 nm) and gelated again with violet light (407 nm). There, we hypothesized that

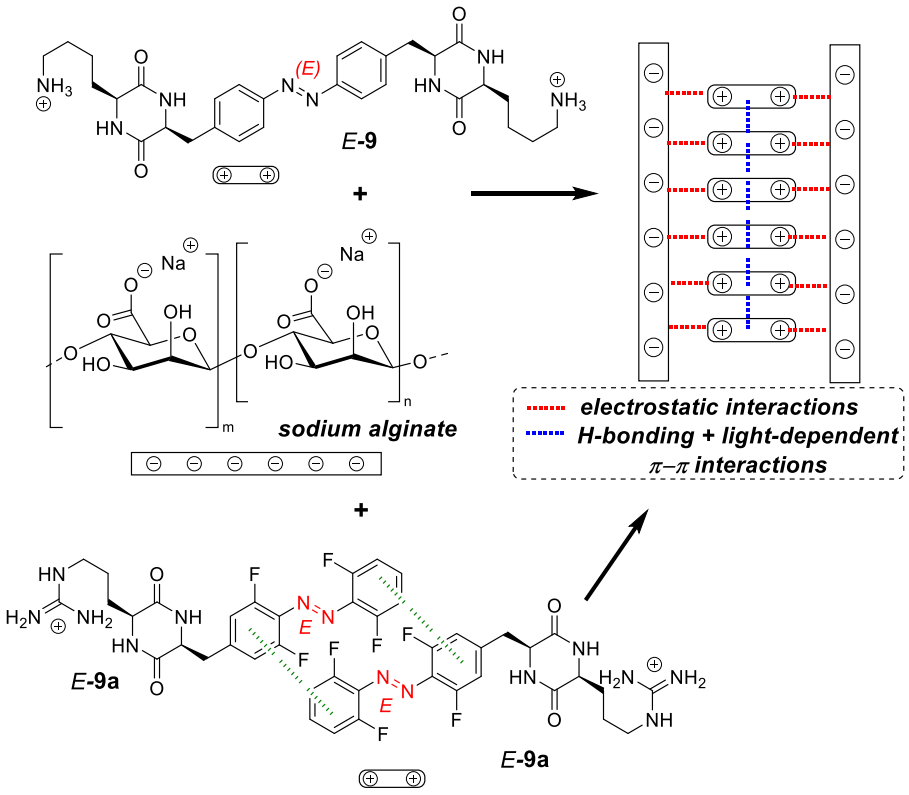

FIGURE 5 | Composite photoswitchable hydrogels stabilized with Coulombic interactions. The positively charged photoswitchable CDPs **9** and **9a** interact with negatively charged alginate polymers. The resulting stabilization and fiber formation efficiency strongly depends on the isomeric state of the CDPs. In result, reversible photoliquefaction triggered with UV [56] (for **9**) or green light [57] (for **9a**) has been observed. Adapted from ref [56].

the alginate cross-linking is achieved due to dimer or oligomer formation between the **9a** molecules, which is much more efficient in the *E*-configuration than as the *Z*-isomer (see Figure 5 *bottom*). We have demonstrated, that this composition can be used for encapsulation and light-driven release of an anticancer drug doxorubicin with green light [57].

4 | Hemipiperazines—A Novel Class of Peptide-Derived Molecular Photoswitches

4.1 | Plinabulin—A Potent Photopharmacology Agent

We have described above a hydrogel based on the photoswitchable CDP **3** and loaded with bioactive cargo plinabulin **4** as a good example of guest–gelator matching that eliminates the unwanted cargo leaking process in the absence of light [51]. In order to assess the likelihood of practical in vivo applications of our system, we have carefully screened the biocompatibility, cytotoxicity, and stability of all the components toward the broad spectrum of light (365–660 nm). There, we have made an interesting observation—**4** is isolated upon synthesis as the most thermodynamically stable *Z*, *Z*-isomer. This form, however, undergoes photoisomerization to the *E*, *Z*-isomer, when exposed on violet (410 nm) or UV light (365 nm). The photoconversion is far from quantitative (56%–62%, respectively), but the photoisomer is thermally metastable (below 2% back-conversion at 37°C in aqueous solution within 32 h), thus it can be efficiently purified using HPLC and stored in a fridge for weeks. The *E*, *Z*-**4** can be quickly photoconverted back to the *Z*, *Z*-form (87% at the photoequilibrium) with cyan light (490 nm). Even more importantly, the in vitro toxicity of both photoisomers (MTT assays on human HT-29 cells) differs by two rows of magnitude—IC_{50}(*Z*, *Z*-**4**) = 0.47 nM versus IC_{50}(*E*, *Z*-**4**) = 92 nM (Figure 6 *top*). Overall, these features make the metastable *E*, *Z*-isomer an attractive cytotoxic prodrug, which can be activated in the desired time and location with cyan light (including in vivo systems that can be penetrated with that light frequency), and eventually deactivated with violet or UV light, if such a process is desirable for any reason. It is a generally agreed, that modulation of bioactivity (in this case with light) that exceeds 10-fold potency increase is therapeutically useful, if the compound in question has otherwise good properties for drug formulation (ADMET, etc.), and if the stimulus can be applied inside the patient's body at the desired location [59, 60]. Thus, our observation combined with the fact that **4** (purified as the *Z*, *Z*-isomer) is already tested as an anticancer therapeutic in the third phase of FDA clinical trials, makes plinabulin an attractive photopharmacology agent candidate [36].

Apart from the discovery of a biocompatible photoswitchable inhibitor of microtubule dynamics, superior in many instances to previously reported agents based on azobenzene or similar photoswitches [61], our observation has more general implications. It is the first reported example of a reversible *E*-*Z* photoisomerization of an arylidene group installed on a CDP motif (2,5-diketopiperazine). Thus, it constitutes a prototype of a novel class of molecular photoswitches, which we named "hemipiperazines" ("hemistilbene" + "diketopiperazine"; abbreviated as "HPI")—in analogy to the known classes of photoswitchable compounds, like hemiindigos [62] or hemithioindigos [63], where an arylidene group undergoes a reversible photoisomerization on indole-derived systems [26].

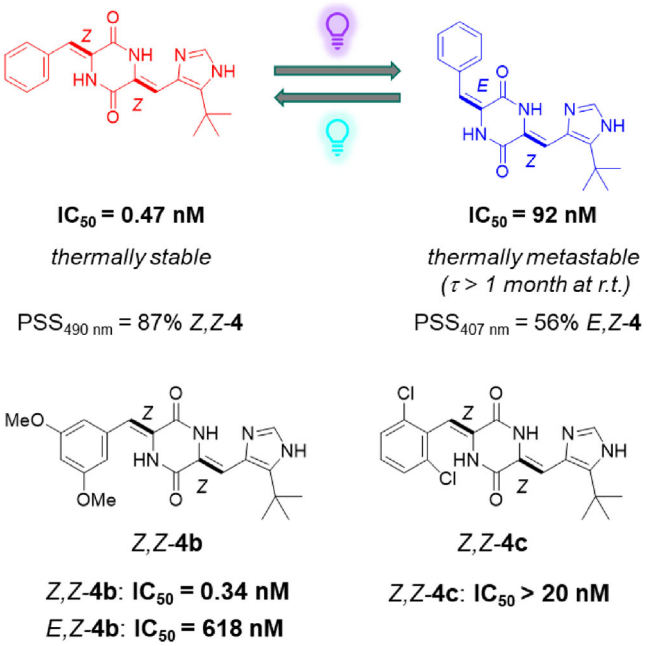

FIGURE 6 | Photoisomerization of plinabulin **4** and its derivatives. The stable active isomer (*Z*, *Z*-**4**) can be converted to the less active metastable isomer *E*, *Z*-**4** with violet or UV light. That compound can be purified with HPLC, and it remains relatively stable at or below the room temperature. It can be converted back to the active *Z*, *Z*-**4** form with blue or cyan light, and thus conceptually treated as a "prodrug" [36] (*top*); dimethoxyplinabulin **4b** shows largest activity photomodulation (>1000-fold) upon photoswitching, and the dichloroplinabulin **4c** shows sufficient thermal stability (as the *E*, *Z*-isomer) to record crystal structures of both purified photoisomers.

In order to explore the bioactivity photomodulation scope, we have synthesized a collection of plinabulin derivatives with variable substituents on the arylidene group. The best results so far have been achieved for a methoxy-derivative **4b**, where the activity difference spans upon three orders of magnitude. Interestingly, thermal stability of the *E*, *Z*-isomer of a bis-chloroderivative **4c** was so high, that the crystal structures could be obtained for both isomeric forms (Figure 7 *bottom*). Another interesting fact is, that under no irradiation circumstances we could generate the *E*, *E*-isomer of **4**, nor any of its derivatives. The heteroarylidene substituent remained inert to light. The simplest explanation would be a strong hydrogen bonding that stabilizes that substituent in the *Z*-configuration. However, the same heteroarylidene group installed on the 2,5-diketopiperazine ring in the absence of the other benzylidene group (**10**) undergoes fair photoconversion to the *E*-isomer (Figure 8 *top*). One possible explanation can be the occurrence of tautomeric structures or charged resonance structures of **4**. These would electronically couple both double bonds directly in a fully conjugated way and thus eliminate productive switching. Similar electronic coupling effects are known for other conjugated systems with dual photoswitches based on azobenzenes [65] or indigoids [37–39].

4.2 | In Vivo Photopharmacology with Plinabulin

Following our encouraging results on the in vitro cytotoxicity photomodulation of **4**, we have applied it in vivo—to reversibly

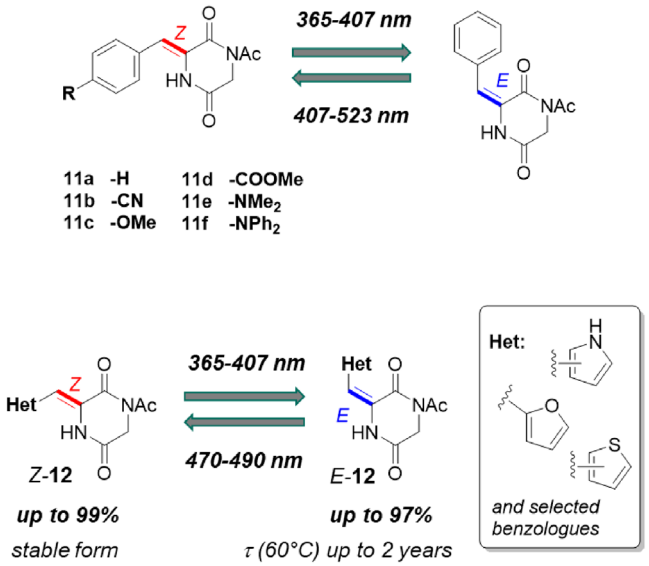

FIGURE 7 | Photoisomerization of simple HPIs—carbocyclic [36] and heterocyclic [64]. The photoconversions and isomerization wavelengths strongly depend on the substitution pattern. Yet, all HPIs show high thermal stability of the *E*-isomers and are compatible with aqueous media.

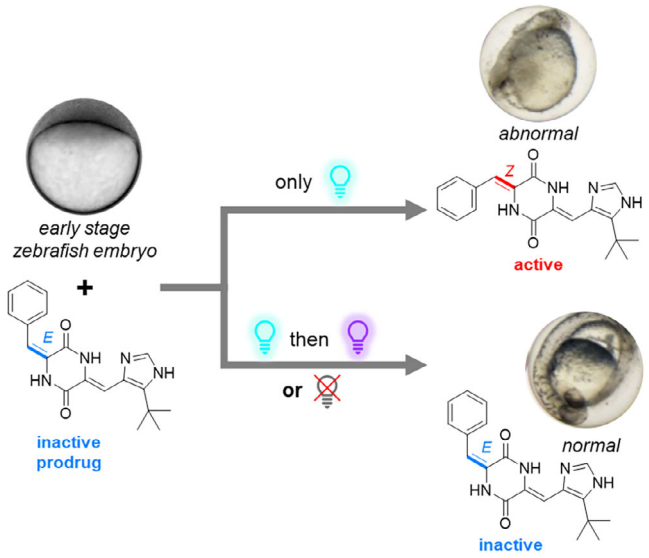

FIGURE 8 | In vivo photocontrol of microtubule dynamics in zebrafish embryos using reversible activation of **4** (plinabulin) with visible light. The embryos were treated with inactive *E*, *Z*-**4**, and irradiated with cyan light. The active *Z*, *Z*-**4** caused death or (at sublethal concentrations) deformations of the embryos. However, if quickly inactivated with UV light, the changes were reversible and yielded correctly developing embryos (as in the case of nonirradiated or untreated embryos) [66]. Adapted from the ref [66].

control the fate of zebrafish embryos. The early developmental stage of the embryos—epiboly—is orchestrated by microtubule networks which control and concert the movements of particular cells. As plinabulin **4** inhibits microtubule dynamics—on the cellular as well as organismal level—we have used this effect by applying the less-active *E*, *Z*-**4** ("prodrug") at sublethal concentrations, and its subsequent activation with cyan light. The resulting embryo-wide inhibition of microtubule dynamics led after some lag period to irreversible apoptotic effects and the abnormal development or death of the embryo. Yet, upon deactivation of **4** with UV light, the effect was reversible and correctly developed embryos have been obtained afterward (Figure 8) [66].

This demonstration shows that reversible photomodulation of activity in **4** can be applied in complex biological setups, and has a potential for therapeutic applications as well as in broad range of research on developmental biology – while tubulin is a ubiquitous target that regulates multiple cellular and developmental processes.

4.3 | Photophysical Properties of Hemipiperazines

To better understand the photoisomerization and photochromism of the newly discovered HPI photoswitch, we have synthesized a collection of simple carbocyclic arylidene-substituted 2,5-diketopiperazines without the imidazole-derived heteroarylidene (**11a–f**) (Figure 8 *top*). They were devoid of the original biological activity, as the pharmacophore is partially removed. The basic HPI motif (also denoted as mono-HPI) provides fairly poor photoconversions to the *E*-isomer (10%–34%), and only with UV light (365 nm). And the back-conversion, realized with violet light (407 nm) is close to quantitative (94%–98% *Z*-form). These compounds do not react on light above 410 nm. Thus, in its most rudimentary form, the HPI is a rather poor photoswitch. Yet, if the arylidene group is decorated with some electron-rich substituents (**11e-11f**, $-NMe_2$, $-NPh_2$), the $Z \rightarrow E$ photoconversions with UV light (365 nm) rise up to 71%, and gradually can be converted back to the *Z*-isomer with visible light frequencies, where cyan or green light are the most efficient wavelengths (up to 97% back-conversion to the *Z*-form) [36].

Inspired by literature reports on similar photoswitchable systems, where introduction of heteroaromatic substituents instead of the carbocyclic analogs improves photoconversions, stability, or elicits strong bathochromic absorption shift [67–71], we have investigated photoisomerization properties of a collection of heteroarylidene HPIs. We have initially investigated five-membered-ring heteroarenes pyrrole, furane, thiophene, and some of their benzologues (**12**) (Figure 8 *bottom*) [64]. Their photoconversions with UV light (365 nm) were consistently better as the carbocyclic HPIs (often above 80%), and the back-switching to the *Z*-isomer with violet light resulted in certain cases in >90% photoconversion. Some were also sensitive to blue and cyan light (460–490 nm). These hetero-HPIs additionally showed efficient photoisomerization in water-containing solutions, which conferred to their applicability in biological conditions [64].

Next, we begun exploring the potential of six-membered-ring heteroarenes, starting from 2-pyridine derivatives [72]. The synthesis of mono-HPI derivative **12** with Het = 2-Py was unsuccessful, as this derivative easily reacted further to the bi-substituted **13**, which was isolated as the pure (>99%) thermodynamically stable *Z*, *Z*-isomer. Its photoswitching in neutral solvents, such as DMF, was rather inefficient—it could be irradiated with a broad range of light wavelengths (365–490 nm), generating 29-37% of the *E*, *Z*-isomer, which was however difficult to convert back to the *Z*, *Z*-**13** with light, No traces of the *E*, *E*-**13** was found. The inefficient photoswitching was attributed to strong H-bonding between the cyclic amide hydrogens and opposite lone electron pairs from

pyridine nitrogen atoms. Upon dissolution in hexafluoroisopropanol (HFIP)—a solvent known for suppressing intramolecular hydrogen bonding—photoisomerization of *Z*, *Z*-**13** with 365 nm UV light was much more efficient—>70% was converted to the mixture of *E*, *Z*-**13** (58%) and *E*, *E*-**13** (13%).

And with green light, we could quantitatively recover the *Z*, *Z*-isomer. Even more evident change occurred upon dissolving the *Z*, *Z*-**13** in trifluoroacetic acid (TFA) and the following protonation of the pyridine rings: UV light (365 nm) converted 86% of the *Z*, *Z*-form to almost equal ratio of *E*, *Z*-**13** (46%) and *E*, *E*-**13** (39%). In the last case, protonation also inverted the stability order—the *E*, *E*-isomer occurred to be the thermodynamically stable form, most likely because avoids steric clashes of the additional protons on the aromatic nitrogen atoms with the amide-NH protons (Figure 9a). Due to extremely long thermal lifetime, all three photoisomers described above can be isolated by HPLC and separately characterized (Figure 9b). Their lifetime at room temperature is in the range of a few months (it was quantified at 60°C).

Furthermore, the bis-HPI **13** occurred to be a good ligand for complex formation with metal ions (Figure 9c)—it forms strongly fluorescent complexes with Zn and Cd ions in organic solvents with mid-nM affinities (Figure 9d). The complex with Zn^{2+} tends to aggregate over longer storage time (>2 h). It is also photochromic, irradiation with blue light (455 nm) results in fading of the solution (in contrary to previously described organic HPIs, this substance shows negative photochromism), which returns to its original color within 15 min at ambient conditions. Most likely, the *Z*, *Z*-**13** is a better ligand for zinc ions than the photoisomer(s).

Interestingly, the complex with Cd^{2+} does not aggregate over prolonged periods of time. It also undergoes photoisomerization upon exposure to blue (455 nm) light, which however is not thermally reversible, it requires irradiation with UV light (365 nm).

Overall, the compound **13** is a multistimuli-responsive switch, that reacts on light, acids, solvents, and metal ions. It also forms the first reported photoswitchable complexes of HPI.

4.4 | Photomodulation of Fluorescence in Plinabulin Derivatives

Due to difficulties in obtaining the *E*-isomer on the heteroarylidene substituent of plinabulin **4** (see Figure 6), and our subsequent observation that the heterocyclic mono-HPI **10** still undergoes efficient bidirectional photoisomerization, we decided to artificially lock the carbocyclic substituent of plinabulin in the *Z*-configuration by adding an extra bond between the benzylidene group and the central ring. The resulting "locked" plinabulin **14** showed increased fluorescence level (by 10-fold) with concomitant hypsochromic shift relative to the emission of **4**. The new compound efficiently photoisomerized on the last accessible double bond (to the heteroarylidene group), and the resulting *E*-**14** showed significantly lower fluorescence level at the same excitation frequency, thus constituting an interesting photoswitchable fluorophore system, which reversibly switched its fluorescence level upon at least 10 full cycles (Figure 10 *bottom*) [36].

Afterward we have synthesized [73] a collection of the "locked" HPIs bearing the indole ring (**15a–h,** Figure 11a) and the pyrrole ring (**16a,b,** Figure 11b) and determined their photophysical properties.

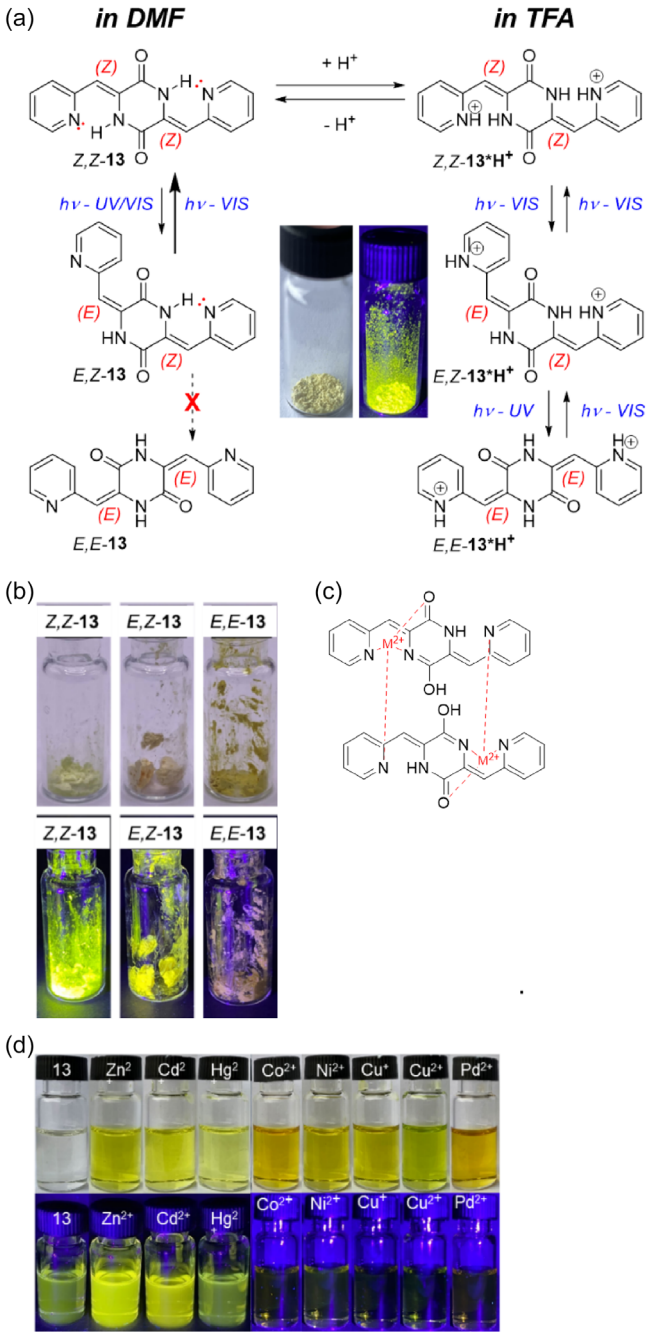

FIGURE 9 | Heterocyclic HPIs. (a) The symmetric bis-HPI **13** bearing two 2-pyridil substituents is a rather inefficient switch (up to 37% photoconversion) due to stabilizing effect of hydrogen bonds in the thermally stable *Z*, *Z*-isomer. Yet, upon protonation its photoconversion is much more efficient, and all three isomers can be isolated by HPLC; (b) due to high thermal stability, the isolated protonated isomers can be neutralized and stored under ambient conditions; they also differ in their fluorescence levels; (c) the neutral **13** is a good ligand for transition metal ions, resulting in complexes with 1:1 ligand:metal ratio; and (d) while most transition metals quench the faint fluorescence of *Z*, *Z*-**13**, Zn and (to somewhat less extense) Cd ions strongly enhance its fluorescence. Thus, **13** can be used as a mid-nM optical sensor for these ions [72]. Adapted from the ref. [72].

The largest emission photomodulation has been observed for the imidazole-containing **15c** and **16a**, most likely because of the hydrogen bonding disturbance upon photoisomerization.

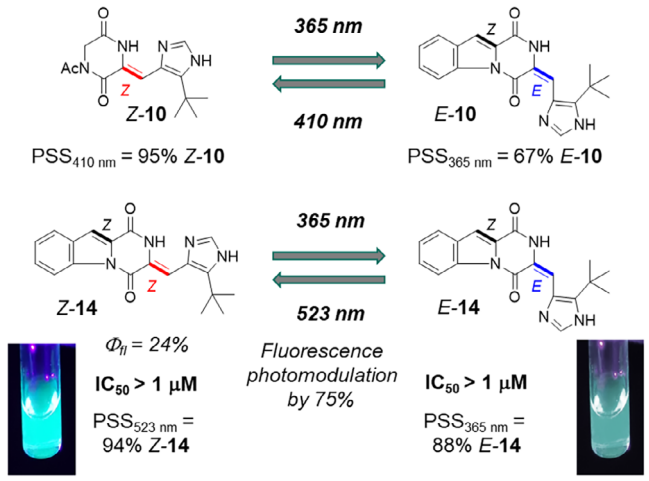

FIGURE 10 | The heteroarylidene substituent of plinabulin **4** cannot be isomerized with any tested wavelength. Yet, the same isolated heteroarylidene HPI system **10** undergoes efficient photoisomerization. Moreover, plinabulin with locked benzylidene configuration—the "locked" plinabulin **14** is a photoswitchable fluorophore (>75% reversible photomodulation of the fluorescence level between respective photostationary states at 365 and 523 nm) [36].

Next, we have tested compounds switchable bidirectionally with visible light ($Z \rightarrow E$ with 410 nm, $E \rightarrow Z$ with > 460 nm) as potential photoswitchable fluorophores inside living cells. Out of our collection, we selected compounds **15d** and **15h**, which had sufficient cell penetrability and showed reversible changes of fluorescence intensity upon irradiation.

In particular, upon irradiation of **15d** inside cells with violet light (405 nm laser) a strong decrease of signal intensity (larger than in solution) was observed. We have also seen rapid thermal relaxation to the more fluorescent state (*Z*-isomer) in darkness (Figure 12). While this contradicts the properties of **15d** in solution (irradiation with green light was needed for the $E \rightarrow Z$ switching, otherwise the *E*-isomer was thermally metastable), we detected that glutathione (present in cells in milimolar concentrations) catalyzes the back-isomerization with similar rates, also in solutions outside cells. Therefore, we have demonstrated an in cellulo photoswitchable fluorophore that can reversibly operate with a single light wavelength. As compounds **15d** and **15h** show no cytotoxicity below 2 μM concentrations, they could be considered, e.g., as fluorescent labels for superresolution microscopy.

5 | Summary and Perspectives of Applications

5.1 | Photoswitchable Supramolecular Hydrogels

Several generations of photoswitchable supramolecular hydrogels based on azobenzene-decorated CDPs have illustrated the flexibility of molecular design and the scope of modifications. Below, we have assembled a table that collects most important properties of the aforementioned hydrogel systems (Table 1).

In general, the supramolecular structure provides our materials with faster response to external stimuli (full dissipation often achieved in less than 1 h for 0.5–1 mL gel volume), yet on the expense of mechanical stability, which remains inferior to hydrogels based on covalent polymers.

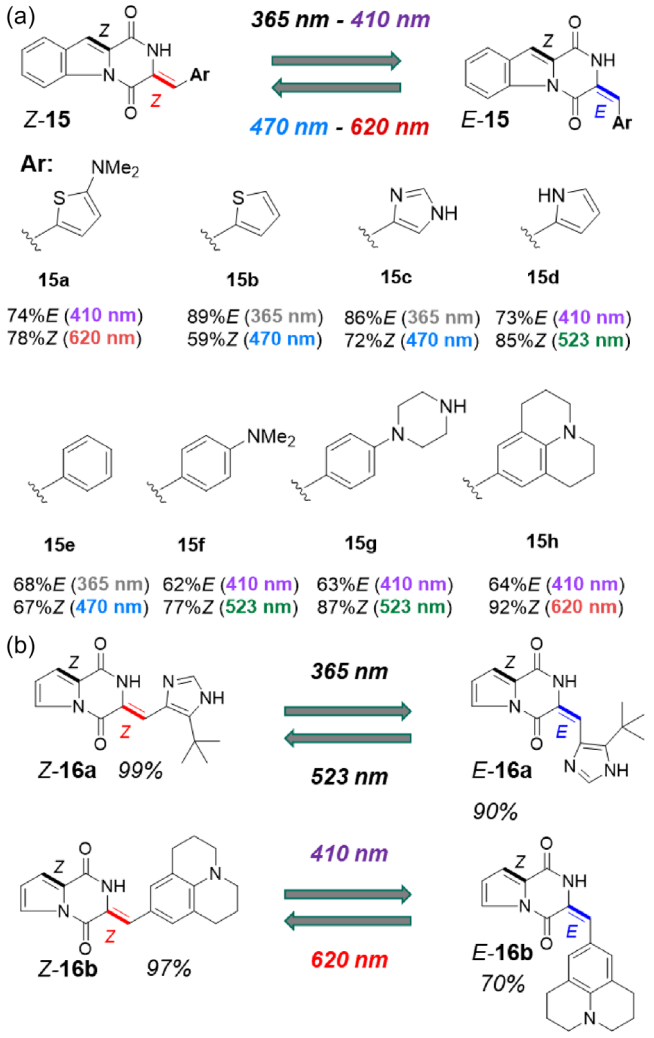

FIGURE 11 | HPIs with extended aromatic system. (a) Indole-derived HPIs and (b) pyrrole-derived HPIs, both with thermal lifetime of the *E*-isomers in the order of years, can be switched in the backward direction ($E \rightarrow Z$, towards the thermodynamically stable isomer) with blue, green, or even red light (470–620 nm) [73].

We can indicate two types of potential applications—photoresponsive media for 3D cell cultures, and light-controlled drug delivery.

Most of the gels demonstrated above, particularly the ones triggered with visible light, can be applied as media in 3D cell cultures, as penetration of skin and tissues with light is not necessary in that case. The advantage of using our materials versus commercially available gel matrices such as M ccv atrGel is the possibility of easy cell isolation after growing—the material can be simply irradiated and the dissipated monomeric gelator washed away, while the commercial media often require enzymatic or chemical digestion, which is harmful for the cells themselves.

The challenge for satisfactory cell growth in that case is the optimization of gel stiffness, and its permeability for nutrients.

The hydrogels capable of releasing cargo with red light are additionally promising materials for therapeutic applications, in particular for light-induced drug release in vivo. However, such materials need to be formulated into injectable microgels [33] (or eventually inhalable nanogels [74]) in order to efficiently

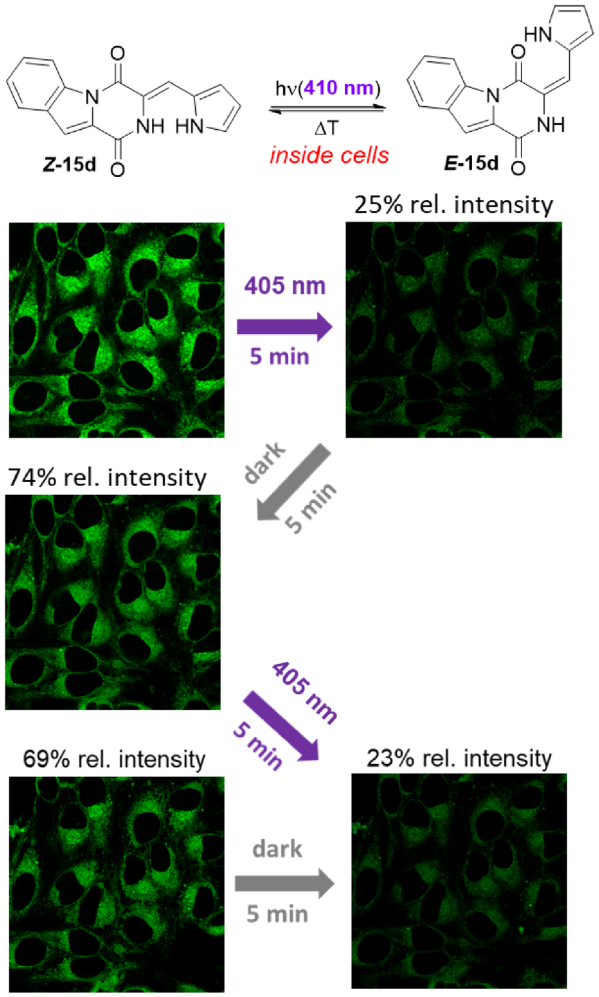

FIGURE 12 | Photoisomerization of compounds **15d** and **15h**—bidirectionally switchable with visible light frequencies—can efficiently penetrate living mammalian cells. Their emission intensity can be reversibly photomodulated. Interestingtly, the in vivo thermal back-isomerization of the *E*-form is catalyzed by glutathione and occurs in the timeframe of minutes, which requires only monochromatic irradiation (410 nm) for the "forward" ($Z \rightarrow E$) isomerization media [73]. Adapted from ref. [73].

deliver the bioactive cargo via bloodstream in proximity of the desired location (such formulations may be likely used for anticancer therapies). Luckily, there are numerous well-developed strategies for micro/nanogel formulation [75, 76], which can be likely applied to our materials.

We have to underline here, that in case of the drug delivery application, the hydrogels will be loaded with bioactive cargo outside of the organism, and the cargo will be released once upon irradiation with red light in vivo. If the (micro)gel dissipates to non-viscous fluid in the course of that process, there is no possibility to reconstitute the gel inside of an organism, even upon back-isomerization of the switch, due to fast dilution of the gelator (its concentration quickly drops below the CGC value under these conditions).

5.2 | Plinabulin and Hemipiperazines (HPI)

Plinabulin as a prototype of a new peptide-derived class of molecular photoswitches has shown promising scope of biological applications. The demonstrated in vitro tests on mammalian cells and in vivo use to photomodulate epiboly in zebrafish embryos are good starting points to assess the scope of activity photomodulation of this potent compound in other living systems and particular applications. As microtubules are omnipresent in many cell types across various living species, efficient photocontrol of their dynamics opens broad perspectives in molecular and developmental biology. It can provide control over microtubule dynamics and following processes with high spatial and temporal resolution. It means that selected parts of an organism (e.g, specific tissues or even single cell areas) can be irradiated with laser light at the desired moment of development, and that would perhaps enable dissecting of previously unseparable biological processes.

The progress in understanding the correlation between structure and photophysical properties in the HPI chromophore may enable optimization of the plinabulin's structure toward the activity switching within therapeutic window of light (630–900 nm). This would enable general photopharmacology applications, e.g., against solid tumors deep inside soft human tissues. One could also identify other HPI-containing pharmacophores and use light for modulation of their activity.

Last, but not least, understanding photoisomerization of HPIs would enable rational design of smart materials (polymers, soft materials, nanomaterials) which could complement the existing set of photoswitchable materials, e.g., based on azobenzene, in

TABLE 1 | Collected physical and photophysical properties of the hydrogels.

Gelator	1	2	3	8	9	9a
Optimal concentration, g/L	20	50	17	3	6 (+12 g/L alginate)	6 (+12 g/L alginate)
Solvent	H_2O, 50 mM aq.NaCl	PBS buffer pH 7.4	PBS buffer pH 7.4	PBS buffer pH 7.4	PBS buffer pH 7.4	PBS buffer pH 7.2
G'/G", Pa	$3*10^4/2*10^3$	$1*10^5/1*10^4$	$1*10^4/5*10^2$	$1*10^3/2*10^2$	$1.5*10^2/3*10^1$	$3*10^3/6*10^2$
Wavelength of dissipation, nm	365	523	523	660 (shrinking)	365	523
Time of dissipation (1 mL sample), min	30	180	30	240 (shrinking)	15	5
Cytotoxicity IC_{50}, μM	n.d.	>500	>100	>10	1000 (for **9**)	>100 (for **9a**)

terms of biocompatibility or ability for forming supramolecular interactions.

6 | Outlook

In this concept, the author demonstrated multidirectional perspectives of implementing photoisomerization into CDPs. In more generic aspect, exemplified in the first part by azobenzene-decorated analogs of chiral CDPs (mainly the cyclic Phe-Lys dimer), it was demonstrated that introduction of a light-dependent motif can reversibly modify macroscopic properties of soft materials—supramolecular hydrogels. Due to their biocompatibility, it opens up broad application perspectives in biological setups. The most interesting are: formulation into injectable microgels capable of transporting low-MW drugs or sensitive macromolecular cargo (oligonucleotides, therapeutic antibodies) inside an organism via bloodstream, and releasing them with high spatiotemporal precision using light beam; using the gels as responsive media for growing living cells and organoids; or exploring the applicability of such materials for biocompatible 3D-photoprinting under physiological conditions, which—upon loading with living cells—would enable, e.g., preparation of tailor-made implants for regenerative medicine.

Exploration of the aforementioned subject let our group to discover a specific molecular photoswitch—HPI—derived from the CDP structure upon replacing the stereocenter with a double bond. The respective carbocyclic and heterocyclic arylidene derivatives undergo reversible *Z-E* photoisomerization. Importantly, comparing with established *E/Z*-photoswitchable scaffolds, the HPI system shows higher biocompatibility (operational in aqueous media, resistant to reducing agents including glutathione) and bioactivity, due to the fact that CDPs—as secondary metabolites—are ubiquitous and privileged pharmacophores. Our prototypical demonstration (in vitro and later in vivo) of reversible activity photomodulation in plinabulin paves the way to exploring photopharmacology of other CDP-derived bioactive compounds. The HPI motif is explicitly present in barettin, nocazines, or albonoursin, where bioactivity photomodulation can be directly explored. Furthermore, the structure of countless other CDP-derived bioactive compounds can be slightly modified to introduce the proper HPI motif into the original system.

Due to the ubiquitous presence and various functions of microtubules in eukaryotic cells, plinabulin activity photomodulation can be further used to control developmental processes in model organisms (zebrafish, fruit flies) with increasing spatiotemporal resolution. And structural plinabulin modifications that enable cytotoxicity activation with red light would find likely expanded applications as general anticancer photopharmacology agents applicable in solid human tumors.

Other than that, self-reporting "locked plinabulin" derivatives should be further explored for in vivo applications, comprising confocal fluorescence microscopy and superresolution microscopy.

In broader perspective, the HPI photoswitch can be implemented in numerous systems, such as soft, porous, or liquid-crystalline materials, to enable light-driven control of their macroscopic properties. Its applicability in systems for solar thermal energy storage, favorable due to their absorption in the visible light range and long lifetime of the metastable isomers, should be also investigated. Finally, HPIs may become modules in various molecular machines—as light-responsive elements complementary to azobenzenes, stilbenes, or indigoids [77–83].

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Conflicts of Interest

The author declares no conflicts of interest.

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Biographies

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