Opinion

Reversible, Spatial and Temporal Control over Protein Activity Using Light

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In biomedical sciences, the function of a protein of interest is investigated by altering its net activity and assessing the consequences for the cell or organism. To change the activity of a protein, a wide variety of chemical and genetic tools have been developed. The drawback of most of these tools is that they do not allow for reversible, spatial and temporal control. Here, we describe selected developments in photopharmacology that aim at establishing such control over protein activity through bioactive molecules with photo-controlled potency. We also discuss why such control is desired and what challenges still need to be overcome for photopharmacology to reach its maturity as a chemical biology research tool.

The Limitations of the Traditional Tools to Study Protein Function

Cells, tissues, and organisms are highly complex systems in which several thousands of proteins interact and play a role in a wide variety of processes such as metabolism, signaling, homeostasis, and cell division. To understand the function of a protein of interest in both health and disease, researchers alter its net activity and subsequently observe the resulting changes in the biological system [1–3]. To change the protein activity, a wide variety of chemical and genetic tools have been developed.

Bioactive molecules are widely used as chemical tools to modify the activity of native proteins. The main advantage is that their solutions can conveniently be added to a cell culture or injected into a model organism. For many proteins, bioactive molecules have been developed that can activate or inhibit the activity via either competitive or allosteric mechanisms. Currently, the Binding database (\url{www.bindingdb.org}) reports over 600,000 small molecules targeting over 7,000 protein targets. However, drawbacks of using bioactive molecules include the lack of reversibility and limited spatial control: the solutions are added systemically, and there is no easy way to remove the bioactive molecule in a controlled manner, once it has been added.

Genetic tools for protein activity modulation, besides controlling the activity of native proteins, can also change the concentration of the protein of interest at either the transcription level or the translation level by (single or double) knockout, knockdown, and the use of siRNA [4]. However, it is known for many proteins that knockouts in mice are lethal [5], which only demonstrates that these proteins are crucial, without elucidating their role. Decreasing the activity can also be achieved by making specific mutations in the active site, by a knockin, which results in a catalytically inactive protein that still maintains its binding properties [6]. Increasing the concentration of proteins can be achieved through overexpression, resulting in higher net activity of the protein of interest. Genetic tools, while widely applied, are elaborate in use. Yet, the rapidly growing field of clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) might allow easier modification [7]. More advanced genetic techniques are inducible expression systems in which addition of a chemical inducer such as...

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doxycycline changes the activity of a promotor and thereby the expression [8], which can be returned to its original level by washing out of the chemical inducer. In conclusion, genetic tools are mainly irreversible, that is, the concentration of knocked-out protein cannot be conveniently restored to the natural level at a given time. Furthermore, the spatial resolution of protein expression modification is limited, meaning that, for example, a protein is knocked out systemically and not in an organ- or tissue-specific manner.

**Why Is Reversible, Spatial and Temporal Control over Protein Activity Important?**

Currently, the toolbox to alter protein activity relies mainly on irreversible techniques, as discussed previously. Yet, reversibility can be of importance in elucidating the function of a protein of interest. From an experimental point of view, reversibility serves as a strong control, since the same system (cell, tissue, organism) can be studied over a short period of time with and without the altered protein activity. Also, reversibility of modulation minimizes the irreversible downstream effects, which are observed for every alteration of a biological system and depend on the duration of alteration. A well-established example is drug addiction in which long dosage of an active compound results in a different response than the initial response [9-10]. Another typical example is how tumor cells can acquire drug resistance by activating alternative pathways to bypass the inhibited pathway [11,12]. Compensation effects and their influence on the observed biological outcome could be better understood when the duration of the inhibition or activation is precisely controlled. Altogether, reversibility and temporal control over the modulation will contribute to a better understanding of protein function in a biological system with minimized compensation effect.

Alteration of protein activity by genetic and chemical tools is mainly systemic. However, a protein of interest might have a specific function in an organ or tissue. The systemic alteration of the activity of a protein of interest provides observations that can be difficult to trace back to a specific local function. For example, for histone deacetylase 2 (HDAC2) it was shown that the expression in the dorsolateral prefrontal cortex in schizophrenia patients is decreased [13]. Since HDAC2 is expressed in many tissues [14], systemic inhibition of HDAC2 in an animal model does not help to elucidate the specific role of HDAC2 in this brain region. However, this limitation could be overcome by locally inhibiting HDAC2 activity, mimicking the patient situation more closely and contributing to a better understanding of the role of HDAC2 in specific brain regions and their connection to other areas. Such site-specific alterations of the activity will also have large implications in, for example, proving the site of action of drugs, studying cell signaling, and understanding adverse effects of therapeutics.

**Light Is an Emerging External Stimulus to Control Protein Activity**

To achieve reversible, spatial and temporal control over protein activity, a modulator is needed whose activity can be controlled with an external stimulus, such as photons. Light is already widely used in biological studies, for example, in optical and fluorescence microscopy, which is enabled by the orthogonality of photons toward living systems and processes within them [15]. Even UV light is, to a large extent, tolerated in cell cultures, as demonstrated by the imaging of the blue fluorescent protein [16] and DNA-labeling dye 4’,6-diamidino-2-phenylindole (DAPI) [17]. Yet, it is recommended to do control experiments in which the biological system is subjected to irradiation only, to check for any undesired effects. The key benefit of using light is that it is easily possible to regulate when, where, for how long, and with which intensity and wavelength it is used.

Currently, there are several tools available to use light to gain control over the activity of proteins. A well-established example is optogenetics, where responsive elements from photoactive
proteins are genetically engineered into other proteins, by which, for example, a receptor can be activated with light instead of a chemical ligand [18]. The field acknowledges the demand of spatial and temporal control over the activity of biological pathways [19]. However, expressing engineered proteins is challenging.

A chemical approach to acquire photocontrol is photocaging. A photocage is a photosensitive chemical group that uses the energy of a photon to break a chemical bond [20]. A photocage is placed at a functional group of a bioactive molecule [21] or amino acid of a protein [22] by which it loses its activity; upon irradiation the photocage is removed, resulting in the release of a biologically active molecule [23]. The approach of using photocaged bioactive compounds was successfully demonstrated in vivo in a mouse model [24]. A drawback is that the photochemical process of uncaging is irreversible.

A fully pharmacological, remote, and reversible control of protein activity with light is enabled through the use of molecular photoswitches, that is, small photosensitive molecules that upon irradiation change their structure [25,26] (for a detailed explanation, see Box 1), hence the name photoswitch. A widely used photoswitch is azobenzene in which the diazo bond (N=N) is connected to two phenyl rings that can be on its opposite sides (trans-azobenzene) or on the same side (cis-azobenzene). The trans isomer is thermodynamically stable and be can switched into the cis isomer by irradiation with UV light (Box 1). This process can be reversed spontaneously using heat or the molecule can be switched back using visible light irradiation. The process of switching from trans to cis and back can usually be repeated for many cycles [25,27].

The emerging field of photopharmacology utilizes the differences in shape and chemical properties between molecular isomers of a bioactive molecule that differ in activity (Figure 1, Key Figure) and that can be interconverted with light irradiation and/or spontaneous thermal relaxation [28]. Photoswitches such as azobenzene are introduced into the structure of the bioactive molecule [29]. Through this, remote control over its activity, and therefore the activity of the protein of interest, can be achieved. Photopharmacology mainly aims at developing therapeutics that are only active at the target and not in healthy tissue, to eliminate activity of drugs in healthy tissue and its consequences [30]. However, besides this potential clinical application, bioactive molecules with photocontrolled activity can serve as a powerful tool in biomedical research. These remotely controlled bioactive molecules can simply be pipetted to a cell culture or injected into a model organism; afterwards, by precise irradiation, control over protein activity is acquired. In the following, we look at examples from the protein classes of enzymes, structural proteins, and receptors for which photopharmacological control has been established either in vitro or in vivo.

**Photo-control over Enzymatic Activity**

Enzymes are the workhorses of the cell and harbor many regulatory functions and processes that are often dysregulated in disease. To demonstrate photopharmacological control over enzyme activity, the specific case of HDAC2 is discussed here. This enzyme is a member of the histone deacetylase family, which is involved in epigenetic regulation of gene expression [31]. In several cancers, increased expression of HDAC2 is observed, resulting in decreased expression of genes with antitumor activity [14]. Therefore, inhibition of HDAC2 has been shown to be effective in killing tumor cells [32], like, for example, the FDA-approved HDAC2 inhibitor vorinostat for the treatment of metastatic melanoma [33].

Traditional genetic and chemical toolboxes have been used to study the specific role of HDAC2. Unfortunately, HDAC2 knockout mice die of cardiac malfunction the first day after birth [32],
Box 1. Understanding Light-Controlled Drugs: Molecular Structure and Photochemistry

Azobenzene (A) is the most-often-used molecular photoswitch in photopharmacology and serves here as an example to introduce the behavior of molecular photoswitches. Azobenzene has two isomers: the thermally stable trans isomer (blue) and the thermally unstable cis isomer (orange). These two forms differ in structure, polarity, solubility, and many other features.

Importantly, their UV-visible spectra are also different (B), which leads to the possibility of selectively addressing each of the forms with light. The trans form shows a strong absorption band at low wavelengths (denoted as \( \lambda_1 \), typically, UV light of 320–370 nm), where the absorption of the cis form is lower. At higher wavelengths (denoted as \( \lambda_2 \), typically, visible light of 420–480 nm) the cis form absorbs more strongly than the trans form. Using \( \lambda_1 \), it is usually possible to selectively switch the trans form to the cis form. With \( \lambda_2 \), the cis form can selectively be switched back to trans.

The first of these processes is discussed in more detail in (C). When light of \( \lambda_1 \) is applied, the trans form absorbs the photon and enters the excited state, from which it can relax to the ground state of the cis form. The kinetics of this process depends on (i) the probability of absorbing the photon, represented by the extinction coefficient \( \varepsilon_{\lambda_1} \); and (ii) the probability that, once in the excited state, it will fall to ground state with isomerization, represented by the trans-to-cis isomerization quantum yield \( \phi_{\text{trans-cis}} \). While the concentration of the cis form increases, it also absorbs light, with extinction coefficient \( \varepsilon_{\lambda_2} \), and with the quantum yield of \( \phi_{\text{cis-trans}} \), it can isomerize back to trans. In time, a dynamic equilibrium is established between the two processes. Assuming negligible thermal cis–trans reisomerization on the timescale of the experiment, the position of this equilibrium is described by the photo-stationary state (PSS), which is simply the percentage of compounds that are in the cis state at equilibrium under irradiation.

Once the light is switched off (D), the molecular photoswitch returns to its original state, which is usually 99% of the stable trans form. This recovery is a first-order process, and the time needed to isomerize half of the cis compounds back to trans is described as half-life \( t_{1/2} \). This value depends both on the structure of the photoswitch and on its environment (solvent, temperature, etc.) and can range from microseconds to years.

(A) Photoswitchable drug (B) UV-visible spectra

\[ \text{Trans} \quad \text{Thermally stable} \quad \text{cis} \quad \text{Thermally unstable} \]

(C) Photoisomerization

\[ \text{Conc.} \quad \text{PSS} = \frac{[\text{Cis}]}{[\text{Trans}] + [\text{Cis}]} \times \frac{\varepsilon_{\lambda_1} \phi_{\text{trans-cis}}}{\varepsilon_{\lambda_2} \phi_{\text{cis-trans}}} \]

(D) Thermal relaxation

\[ \text{Conc.} \quad [\text{Cis}] = \frac{[\text{Cis}]}{2} \]

Demonstrating the importance of the protein, but not its specific function. To decrease the HDAC2 activity pharmacologically, a wide variety of inhibitors have been developed with selectivity for HDAC2 over other HDACs from the same protein family [33]. Recently, a photocaged variant of vorinostat was developed by which spatial and temporal control over HDAC2 activity can be achieved [34], however irreversibly.
To achieve the desired reversible, spatial and temporal control over HDAC2 activity, our lab developed HDAC2 inhibitors with photo-controlled activity [35], as shown in Figure 2. For compound 1, the cis isomer is 39 times more active than the trans isomer. The difference in cytotoxic activity between trans and cis was also observed in HeLa cells, even showing a larger difference in cell viability than for the individual HDAC2 inhibitor. Also, reversibility and temporal control over the activity of HDAC2 were demonstrated, overcoming the limitations of the current chemical and genetic toolbox.

**Can Bioactive Molecules with Photo-controlled Activity Be Developed for Every Protein?**

Currently, there are hundreds of thousands of small molecule compounds that can modulate the activity of several thousands of target proteins. In contrast, only several dozens of bioactive molecules with photo-controlled activity have been developed [30]. However, the number is rapidly growing, and the list of protein targets is expanding. Photo-control over the activity of members of protein families such as enzymes [36,37], receptors [38–42], transporters [43], and structural proteins [44–47] has been achieved, demonstrating the generality of this approach. The design is usually based on known protein modulators that do not harbor photo-control. As shown by two examples in Figure 3, chemical structures similar to azobenzene are replaced by an azobenzene photoswitch in a photopharmacological
Figure 2. Photo-control over the Activity of Histone Deacetylase 2 (HDAC2). (A) Based on known HDAC2 inhibitor vorinostat, compound 1 was designed. Upon irradiation, compound 1 switches from trans to cis form, becoming 39-fold more active as an HDAC2 inhibitor. (B) Dose-response curve for compound 1 in trans (blue) and cis (orange) form on cell viability of HeLa cells. Reproduced, with permission, from [35].

Figure 3. Examples of Bioactive Molecules with Photo-controlled Activity. (A) Light control of a structural protein: formation of microtubule. Based on tubulin polymerization inhibitor combretastatin A4, compounds 2a and 2b were designed. Upon irradiation with UV light, compound 2b becomes 550 times more active, which can be reversed using visible light irradiation [46]. (B) Compound 2a induced the breakdown of tubulin (green) and fragmentation of the nucleus upon irradiation with 390 nm to the active cis isomer and 20-h incubation, while irradiation without inhibitor and the trans isomer of compound 2a do not change the physiology of the cell. Adapted from [45]. (C) Light control of receptor activity; metabotropic glutamate receptor 5 (mGlur5). Based on negative allosteric modulator VU0414374, compound 3 was designed. Upon irradiation with UV light, compound 3 becomes 5.1 times less active, which can be reversed using visible light [49]. (D) Persistent inflammatory pain was induced in a mouse model, and after 10 days the number of paw lifts was recorded (naive) and normalized to healthy mice (vehicle) with and without irradiation in the amygdala. Injection of compound 3 resulted in the same behavior in the mouse as in naive mice; upon irradiation to the cis isomer, this effect could be abolished, to the same level as in the vehicle mice. Adapted from [40].
approach called azologization [48]. This approach has been extended to other chemical structures with less similarity to the structure of the photoswitch, guided by structure–activity relationship studies and computational support [40,42,49]. So far, the development of bioactive molecules with photo-controlled activity is limited by the availability of known modulators and the existence in those modulators of structural features that can be replaced by a photoswitch without a major loss in potency.

The replacement of a fragment of a molecule by a photoswitch has been convincingly demonstrated by taking advantage of the structural similarity of natural compound combretastatin A4 and cis-azobenzene [44,47] (Figure 3A). Combretastatin A4 is an inhibitor of microtubule formation. Microtubules belong to the family of structural proteins and are an important compartment of the cytoskeleton, playing a role in mechanical processes such as the intracellular transport of vesicles and separation of chromosomes in mitosis [50]. Azologization of combretastatin A4 resulted in an inhibitor with photo-controlled activity (Figure 3A), where irradiation of the inactive trans isomer to the cis isomer increases the potency in HeLa cells in vitro by an impressive factor of 550 for compound 2b [47].

Reversible spatial and temporal control over protein activity shows its full potential in an in vivo model. Recently, several in vivo studies of photopharmacological agents have been reported, mainly for neurological targets, such as restoring the visual function of the blind retina [51], and metabotropic glutamate receptors [40,52]. An impressive example of an in vivo-tested bioactive molecule with photo-controlled activity was reported by the groups of Gorostiza and Liebaresia, targeting metabotropic glutamate receptor 5 [40,49,53–55], which is a potential target for the treatment of anxiety, depression, and schizophrenia [56,57]. Inspired by negative allosteric modulator VU0414374, compound 3 was designed (Figure 3C) and tested in an in vivo system using hybrid optic and fluid cannulas that were implanted in the amygdala of persistent inflammatory pain mouse model. The mouse was injected with compound 3 in the amygdala in the active trans configuration, resulting in an analgesic effect. This pain-relieving effect could be abolished by irradiation to the inactive cis isomer [40]. By this, photo-control over pain in a rodent model was achieved, which opens opportunities in studying pain, its development, and its treatment.

The Current Limitation of Photoswitchable Bioactive Molecules as a Research Tool

A challenge in the development of bioactive molecules with photo-controlled activity is to acquire large differences in activity between the photo-isomers. As shown in Figure 1B, at a precisely chosen concentration, |Δ|_{max}, one isomer does not change the activity of the protein of interest, while the other isomer results in complete inhibition of protein activity; hence, the protein can be switched fully on and fully off. However, this optimal situation of fully switching is rarely achieved. For example, for compound 1, a 39-fold difference in activity between the trans and cis isomer is not yet sufficient to allow for switching between fully active HDAC2 and fully inhibited HDAC2 [35]. In the optimization of photopharmacological agents, every chemical modification of the bioactive molecule potentially not only changes the biological activity but also the chemical properties and important photochemical properties such as the absorption maxima, half-life of the cis isomer, quantum yield, and the photo-stationary states (PSSs). This optimization process is challenging; yet, to reach full potential as a research tool, differences in the activity between isomers should be enhanced.

Another challenge is that most of the photopharmacological agents need UV light in the region of 350–400 nm to switch [30]. Such light has a limited penetration depth of only a few millimeters in
soft tissue [58]. This is sufficient for experiments in monolayer cell culture, but not for animal models, since most inner organs cannot be reached in a noninvasive manner. However, red and near-IR light has deeper penetration depth in soft tissue, up to several centimeters [58]. Therefore, red-light-responsive photoswitches and photopharmacological agents are in development [59–61]. Recently, an elegant example was published by the Feringa group [62], where an antibiotic was developed that increases eight times in potency upon irradiation with red light.

Concluding Remarks and Future Prospects
In addition to the three examples described here, for many other proteins, bioactive molecules with photo-controlled activity have been developed in recent years. Besides their potential clinical applications in photopharmacology, these are powerful tools for biomedical research, because light is orthogonal with biological systems, no genetic modifications are required, and spatial and temporal control can be achieved in a reversible manner. The broad range of proteins that can be altered by photopharmacology and especially the reversibility of the modification can make it a superior tool compared to the existing toolbox.

More bioactive molecules with photo-controlled activity will be developed, with a focus on visible light switching and optimization of the difference in activity between isomers. In parallel, new photoswitches that can be operated with visible light or that have enlarged differences in structure between isomers are being discovered. These developments will, more and more, allow photo-controlled bioactive molecules in biomedical research to contribute to the understanding of the role of a protein of interest in health and disease.

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Appendix A Supplemental Information
Supplemental information associated with this article can be found, in the online version, at https://doi.org/10.1016/j.tibs.2018.05.004.

References

Outstanding Questions
Currently, most bioactive molecules with photo-controlled activity have a difference in activity between the photo-isomers of about 10–50-fold. So, how do we rationally design new compounds with a larger difference in activity?

Even though there is a difference in activity between both photo-isomers, usually both can bind to the target. Does the bioactive molecule with photo-controlled activity switch while bound, or does it first have to dissociate from the target protein?

How can effective and preferably noninvasive irradiation of bioactive molecules with photo-controlled activity be achieved in deep organs of model organisms?

How does diffusion of bioactive molecules compromise the spatial resolution provided by light? And in which range should the half-life of the bioactive molecules be to prevent this?

Since the photochemical properties of a compound strongly depend on its environment, how can we determine the ratio of photo-isomers in vivo?
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