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A Roadmap to Success in Photopharmacology

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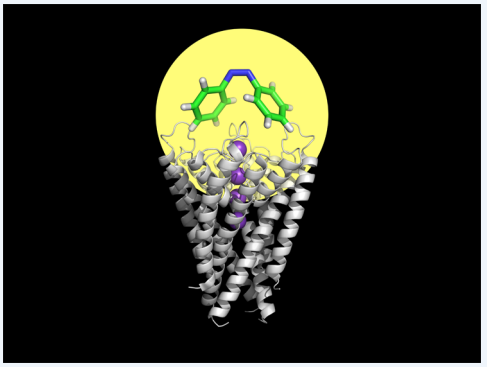
CONSPECTUS: Light is a fascinating phenomenon that ties together physics, chemistry, and biology. It is unmatched in its ability to confer information with temporal and spatial precision and has been used to map objects on the scale of tens of nanometers (10^{-8} m) to light years (10^{16} m). This information, gathered through super-resolution microscopes or space-based telescopes, is ultimately funneled through the human visual system, which is a miracle in itself. It allows us to see the Andromeda galaxy at night, an object that is 2.5 million light years away and very dim, and ski the next day in bright sunlight at an intensity that is 12 orders of magnitude higher.



Human vision is only one of many photoreceptive systems that have evolved on earth and are found in all kingdoms of life. These systems rely on molecular photoswitches, such as retinal or tetrapyrrols, which undergo transient bond isomerizations or bond formations upon irradiation. The set of chromophores that have been employed in Nature for this purpose is surprisingly small. Nevertheless, they control a wide variety of biological functions, which have recently been significantly increased through the rapid development of *optogenetics*. Optogenetics originated as an effort to control neural function with genetically encoded photoreceptors that use abundant chromophores, in particular retinal. It now covers a variety of cellular functions other than excitability and has revolutionized the control of biological pathways in neuroscience and beyond.

Chemistry has provided a large repertoire of *synthetic* photoswitches with highly tunable properties. Like their natural counterparts, these chromophores can be attached to proteins to effectively put them under optical control. This approach has enabled a new type of synthetic photobiology that has gone under various names to distinguish it from optogenetics. We now call it *photopharmacology*.

Here we trace our involvement in this field, starting with the first light-sensitive potassium channel (SPARK) and concluding with our most recent work on photoswitchable fatty acids. Instead of simply providing a historical account of our efforts, we discuss the design criteria that guided our choice of molecules and receptors. As such, we hope to provide a roadmap to success in photopharmacology and make a case as to why synthetic photoswitches, properly designed and made available through well-planned and efficient syntheses, should have a bright future in biology and medicine.

INTRODUCTION

The ability to respond to light, the ultimate provider of energy and information throughout the environment, is one of the oldest and most basic features of life on earth. Nature achieves this through a small set of chromophores, such as retinal, flavins, and tetrapyrrols that are attached, usually covalently, to a protein envelope. These chromophores are produced through ancient biochemical pathways and have proven so successful that they appear to have remained unaltered over the course of evolution. Their absorbance covers almost the entire solar spectrum from the UV-A range to the near-infrared. Functionally, these chromophores carry out a wide variety of tasks, ranging from the establishment of ionic gradients to the triggering of heterotrimeric G-protein dissociation. Some of them directly activate enzymes or ion channels, such as guanylyl cyclases or channelrhodopsins. In recent years, additional functions have been added through clever protein engineering, giving rise to a field now called Optogenetics.[1]

In contrast, synthetic photoswitches are less than a hundred years old. Attempts to control biological activity with these molecules are even younger and have only recently become a vibrant field. After experimenting with several names, we have termed it *photopharmacology*.[2,3] Photopharmacology is an attempt to control biological function with *synthetic* photoswitches that act on native biopolymers or membranes. The photoswitches used can either be covalently attached to their target like in the majority of the natural photoreceptors or be tightly bound through noncovalent interactions. We call the former PTLs (as in photoswitchable tethered ligands) and the latter PCLs (as in photochromic ligands). Taken together, we simply call them photoswitchable ligands or photopharmaceuticals, both of which have been applied to a wide variety of biological targets. Today, these include ion channels, G-protein

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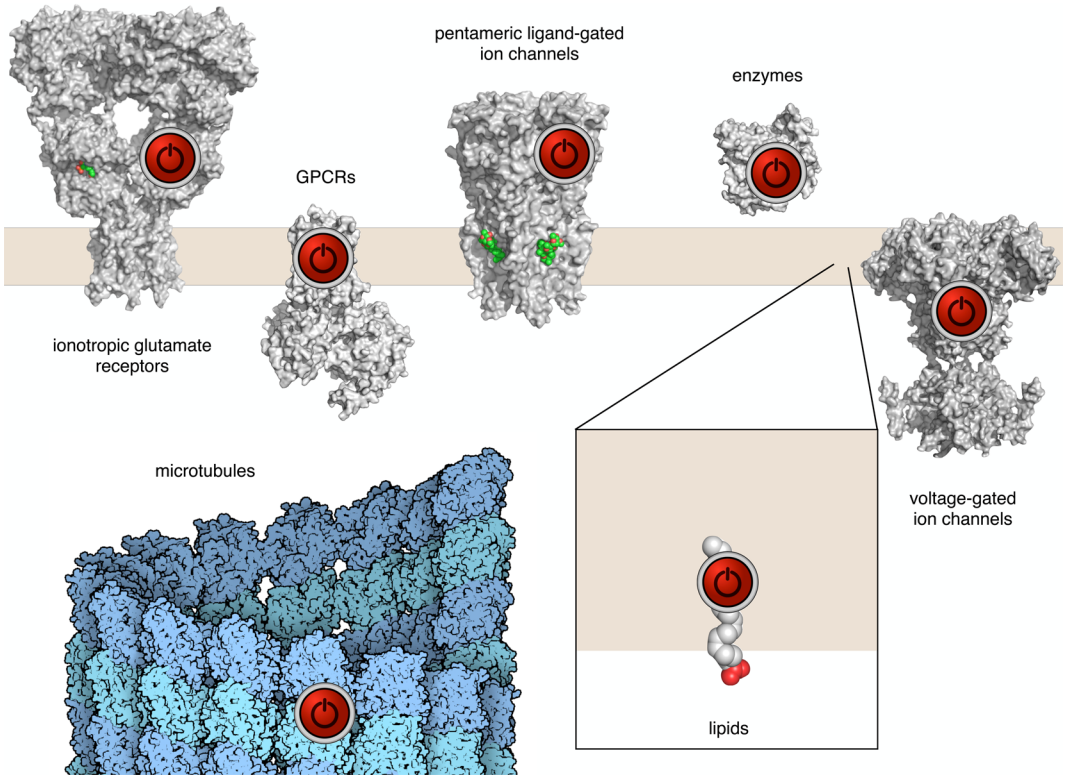

Figure 1. Targets of photopharmacology. Ion channels, GPCRs, transporters, enzymes, components of the cytoskeleton, and lipids have been placed under optical control with photoswitchable ligands.

coupled receptors (GPCRs), transporters, enzymes, elements of the cytoskeleton, and lipids (Figure 1).[2−4]

As if drug development were not difficult enough, photopharmacology adds a few extra dimensions to the design process. First, a suitable photoswitch needs to be chosen. The switching process cannot be associated with phototoxicity and must take place reliably over many cycles. After initial photoisomerization by irradiation at a certain wavelength, the reverse isomerization must also be considered. Depending on the biological question at hand, it might be more suitable to use a thermally unstable photoswitch that spontaneously turns itself OFF after the irradiation is halted. Alternatively, a bistable photoswitch, which requires light of one wavelength to turn ON and another one to turn OFF, could be more suitable toward the desired application. The ideal photoswitch toggles completely between two distinct states. This can be achieved if one isomer absorbs with a high molar absorptivity (ε) at a wavelength where the other is completely transparent and *vice versa*. As such, the absorbing isomer can be completely and quickly depleted, provided the quantum yields are high. While this ideal scenario is rare, photostationary states (PSSs) in the range of 95% in favor of each isomer are not unheard of, and quantum yields in the rage of 0.2−0.5 are common. It should be noted, however, that even with a poorly defined PSS, for example, in the 40/60 range, pronounced biological effects can still be triggered upon switching due to the inherently nonlinear nature of biological systems. This is more obvious in neural systems but also applies to single cells, for example, with respect to their dynamic cytoskeleton. Another case in point are G-protein coupled receptors, which typically have baseline activity that can be influenced by inverse agonists (such as covalently bound 11-*cis*-retinal or β blockers), agonists (such as neuromodulators or hormones), and antagonists (usually drugs). The misconception that synthetic photoswitches are useless in biology because of their poor photostationary states has largely been overcome.

Once the best photoswitch has been chosen, it needs to be incorporated into the molecule of interest in such a way that the efficacy of the new ligand changes significantly upon photoswitching. Ideally, the ligand is active as an agonist, antagonist, blocker, etc. in one configuration and completely inactive in the other. In a version of photopharmacology that uses covalently attached photoswitches, protein engineering is required as another skill, and the confines of bioconjugation techniques must be considered. Additionally, one should ensure that the molecule remains soluble and stable in the environment of its target. If the target is intracellular, membrane permeability is a concern unless the molecule can be introduced through active transport across the lipid bilayer, by microinjection, or via a patch pipet. Economic considerations must also be taken into account, as one has to ensure that the photopharmaceuticals can be made within a reasonable time frame and at a justifiable expense. Synthetically, a molecule should be designed so that its molecular structure can be easily diversified, preferably at a late synthetic stage.

Obviously, the biology at hand and the biological assays have to be compatible with irradiation. This is not a trivial matter and needs to be carefully considered before a photopharmacological program is started. In our work on neurobiology, for instance, we often find it more convenient to interface electrophysiology with photoswitching than fluorescence imaging assays. This does not mean, however, that the latter cannot be used under the right circumstances, that is, when switching wavelengths and fluorophore excitation/emission wavelengths are orthogonal, or upon observation of a slower, sometimes delayed, biological response. Even live-cell imaging can be readily combined with photoswitching. Going *in vivo*, light penetration through tissue

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must be taken into account, especially in larger organs and animals. Given the success of photodynamic therapy, where singlet oxygen, a rather crude molecule, is produced, and the explosive development of optogenetics, this has become less of a concern than one might expect.

In this Account, we will only briefly touch upon the biological aspects of photopharmacology and focus mostly on the molecular level. We will provide a recipe for the design and a roadmap for the syntheses of small molecules that function as photoswitchable ligands. Using selected examples, mostly from our own laboratory, we will show that photopharmacology has a high likelihood of success provided certain criteria are met, criteria that, in our opinion, are not too stringent. Of course, this is a highly personalized Account, and many will have a different take on the field, especially with respect to the photoswitches used. Still, we hope that colleagues from all walks of chemistry, pharmacology, physiology, and medicine will find this account useful and feel inspired to add a little ON/OFF switch to their favorite molecular tool.

FIND THE RIGHT PHOTOSWITCH

As stated in the Introduction, Nature relies on a relatively small repertoire of photoswitches to drive biological activity with light. Chemistry, by contrast, has come up with a large number of synthetic photoswitches covering a vast number of structural types. In our own research, we strongly favor the azobenzene molecule, which is one of the oldest, if not the oldest, synthetic photoswitch.[5] We find it very flexible, both in a literal and figurative sense.

Figure 2 summarizes some of the basic features of azobenzenes and demonstrates their malleability. When the parent photo-

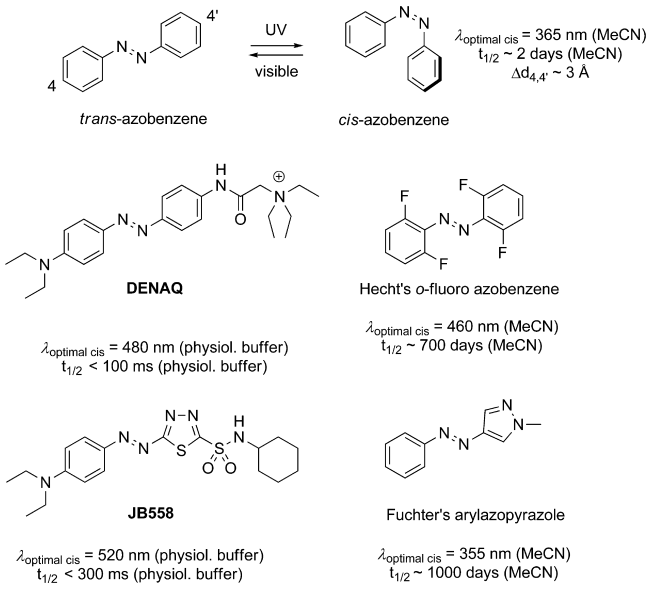

Figure 2. Some functional features of azobenzenes. The photostationary states (PSS) and thermal bistability ($t_{1/2}$) of azobenzenes are highly tunable. $\lambda_{optimal\text{-}cis}$ denotes the wavelength at which the *cis*-content in the PSS is maximized.

switch is irradiated with 365 nm light, a PSS containing 95% *cis*-azobenzene can be achieved (Figure 2). The *cis*-isomer is relatively stable and isomerizes back to the *trans*-form thermally at room temperature with a half-life of about 2 days. Irradiation of azobenzenes with visible light (e.g., 450 nm) quickly achieves a PSS that is largely *trans*. The difference in energy between the two isomers is high (ca. 22 kcal/mol); therefore it can be assumed that most azobenzene derivatives will reside overwhelmingly in the *trans*-form once thermal equilibrium is reached. How fast this can be achieved is very much a function of the electronic and steric nature of the azobenzene, and this property can be finely tuned through synthetic modifications. Other types of azobenzenes, such as Hecht's *o*-fluoroazobenzes[6] or Fuchter's arylazopyrazoles,[7] are even more bistable. The solvent can also have a large influence. For biological applications, the solvent of choice is of course an aqueous, physiological solution (such as Ringer solution or phosphate buffered saline) at pH 7.4, which can speed up thermal relaxation through hydrogen bonding. Red-shifted, water-soluble azobenzenes, such as **DENAQ** or **JB558**, attain their maximum *cis*-PSS with visible light (480 and 520 nm, respectively) and fall back to their thermodynamically more stable *trans*-state within tens or hundreds of milliseconds. Whether bistability or rapid thermal back-relaxation is preferred and whether a "regular" or "red-shifted" azobenzene is better suited depends on the biological question at hand. Of course, the kinetics of both the photoswitch isomerization and the biological response greatly depend on whether the photoswitch is covalently attached as a PTL or freely diffusible as a PCL.[8]

Another important factor in photopharmacology is the efficiency of the photochemical isomerization. This property depends both on the molar absorptivity of the photoswitch and the quantum yield of the isomerization. Once in the excited state, azobenzenes undergo very fast photoisomerizations, on the time scale of a picosecond. This prevents the formation of triplet states and directly translates into light fastness (as a dye), fatigue resistance (as a switch), and most importantly biocompatibility, as the formation of singlet oxygen is avoided. As a highly reactive molecule, singlet oxygen would not only destroy the photoswitch but also stress or kill the nearby cells.

Azobenzenes are relatively small, which makes them easy to incorporate into a drug-like molecule of low molecular weight. The azobenzene moiety itself has a molecular weight of 182 Da and extending a benzene ring to an azobenzene adds 104 Da to a molecule. Azoarenes with five-membered aromatic heterocycles can be even smaller, if not lighter. The isomerization of azobenzenes accompanies well-defined changes in geometry, which allows the two isomeric forms to show different efficacies toward their target. Upon isomerization from the *trans*-form to the *cis*-form, the distance between the 4 and 4′ positions shortens by about 3 Å. This remarkably large change (the *trans*-azobenzene molecule itself is 9 Å long) can be amplified with appropriate substitution. As the azobenzene isomerizes, its dipole moment increases, causing its hydration sphere to undergo changes as well, which will have an influence on the K_d of the ligand.

Azobenzenes can be synthesized through a wide variety of methods ranging from classical azo-coupling to Mills reactions or the cross-couplings of hydrazine derivatives followed by oxidation.[9] This repertoire has significantly increased in recent years, and it is not uncommon nowadays to find a new azobenzene synthesis in a prominent journal. Even difficult substitution patterns can be realized with these new methods. The chemistry of heterocyclic azoarenes is in its infancy in comparison to the diphenyl diazenes, and many new types of photoswitches with improved solubility and interesting pharmacology are bound to be discovered.

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Despite these advantages, certain hesitations exist to develop azobenzenes as drugs. Being colored and "photoreactive", they are often considered unsuitable for fluorescence-based high-throughput screening assays.[10] As a consequence, a large number of drugs that could rival prontosil or sulfasalazine in importance remain undiscovered. Of course, the metabolic stability and potential long-term toxicity of azobenzenes has to be taken into account. This is not a major point of concern for photopharmaceuticals that function as research tools but needs to be addressed for clinical candidates. As with any drug, both properties depend on the exact makeup of the molecule. Stating that compounds are undruggable because they contain azobenzenes is akin to saying that every drug that contains a benzene ring should be dismissed because benzene itself is a known carcinogen. In our experience, electron-rich azobenzenes are quite stable toward bacterial azoreductases, which produce the anilines held accountable for most of the toxic effects associated with azobenzenes in the gut.[11] Although their numbers are small, there are some approved azobenzene drugs and several food colorants, such as tartrazine (Figure 3). While it

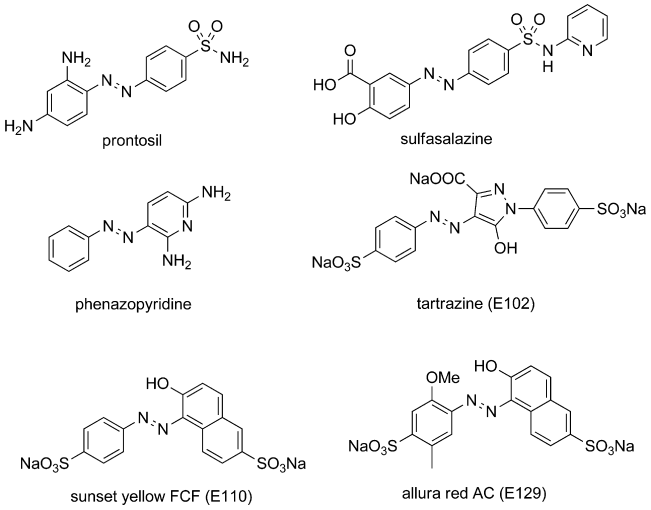

Figure 3. Azobenzenes that have been used as drugs (prontosil, sulfasalazine, and phenazopyridine) and are approved food colorants (tartrazine, sunset yellow FCF, and allura red AC).

may be a questionable practice to use synthetic dyes for human consumption on a massive scale, these examples underscore the fact that azobenzenes are not inherently toxic to the extent that they should be dismissed as pharmaceuticals, especially when given parenterally.

With very few exceptions,[12] azoarenes have not been identified as genuine natural products despite their structural simplicity and the occurrence of many secondary metabolites that are anilines or contain N−N bonds. The evolutionary pressure to develop more photoswitches must have been small given the enormous success of the existing chromophores.

TAKE ADVANTAGE OF THE EXPLOSIVE DEVELOPMENT OF STRUCTURAL BIOLOGY

Function in neuroscience largely relies on transmembrane proteins, such as ion channels (voltage sensors and ionotropic receptors), metabotropic receptors (i.e., GPCRs), transporters, and pumps. The last two decades have seen an explosive development in the structural elucidation of these fascinating nanomachines. Starting with MacKinnon's seminal structure of the potassium KcsA,[13] the list of transmembrane proteins that have been elucidated with high, often atomic, resolution, has grown exponentially and now includes ionotropic glutamate receptors (e.g., AMPA or NMDA receptors), pentameric ligand-gated ion channels (such as $GABA_A$- or 5-HT_3 receptors), transient receptor potential channels (e.g., TRPV1), calcium channels (e.g., ryanodine receptors), and numerous GPCRs, as well as transporters and pumps (such as SERCA, Na^+/K^+-ATPase, NCX).[14] All of these biomolecules have been subject to activation or inhibition by small molecules and, as such, are excellent targets for photopharmacology.

Our initial involvement in this field was indeed motivated by the first X-ray structure of an ion channel. Quaternary ammonium ions are known to block potassium channels by binding both at the extracellular side of the pore and internally to the so-called "inner cavity". To estimate the distance of a specific cysteine from the pore, Miller and Blaustein developed molecular "tape measures", which consisted of a reactive maleimide, a spacer of increasing length, and a quaternary ammonium ion (Figure 4a,b).[15] Once anchored to the cysteine of an engineered *Shaker* potassium channel via maleimide chemistry, these blockers were ineffective when the tether was too short and became effective only after a certain tether length was reached.

To convert the *Shaker* potassium channel into a photoreceptor, we designed the photoswitchable tethered blocker **MAQ** (maleimide−azobenzene−quaternary ammonium). We reasoned that after attachment to the extracellular surface, the ammonium ion would be unable to reach and block the pore in the *cis*-form of the azobenzene, whereas in the extended *trans*-form it would be able to reach its binding site (Figure 4c,d). As such, **MAQ** would provide an additional light gate to a voltage sensitive ion channel, giving rise to SPARK, the synthetic photosomerizable azobenzene-regulated K^+-channel. Although **MAQ** could have probably been designed with the Miller−Blaustein data alone, the KcsA structure (pdb 1BL8) proved critical for the rapid development of this PTL, since both the cysteine and the TEA binding site could be mapped onto this model. The first molecule that we designed and synthesized, namely, **MAQ**, was a success. SPARK enabled the optical control of neuronal firing in dissociated neurons that heterologously expressed the engineered *Shaker* potassium channel. Incidentally, our report was not only our first contribution to photopharmacology, but also the second paper in the history of a field now coined "optogenetics".[16]

From a design point of view, the development of **MAQ** was relatively straightforward since the TEA binding site sits in a relatively shallow "outer vestibule" and the tethered molecules could be assumed as largely solvent-exposed. The situation became considerably more challenging when we turned our attention toward the ligand binding domains (LBDs) of ionotropic and metabotropic glutamate receptors. These domains close like a clamshell upon ligand binding. The degree of this closure is proportional to the degree of receptor activation.

From inspection of the structure of 4-(*R*)-methyl-glutamate bound to the clamshell-like ligand-binding domain of the kainate receptor GluK2 (pdb 1SD3), it was clear that a glutamate molecule substituted in such a manner could still function as an agonist or at least as a partial agonist. A similar structure of domoic acid bound to GluK2 (pdb 1YAE) showed that a side chain could be accommodated and would protrude through an "exit tunnel" between the lips of the clamshell. Based on these data, we designed and synthesized the diffusible photochromic ligand **4-GluAzo** (Figure 5).[17] As intended, the molecule

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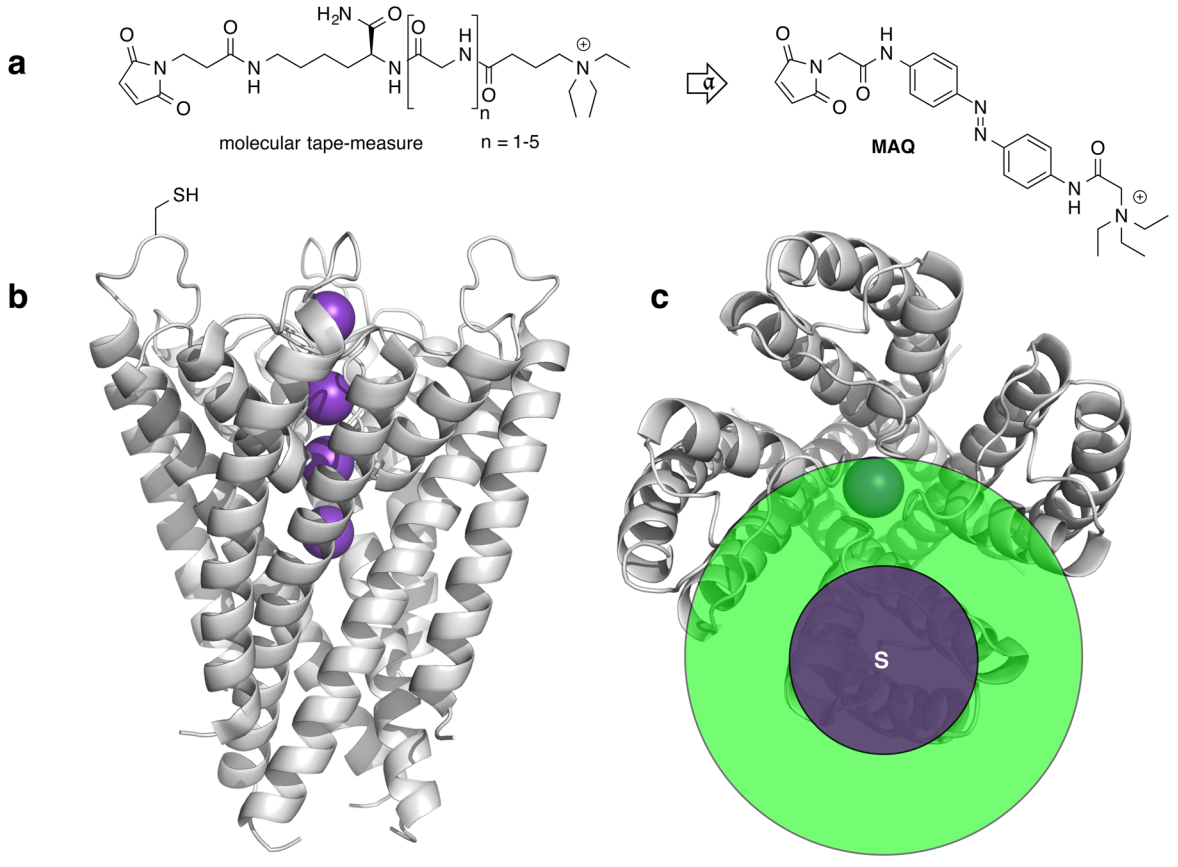

Figure 4. Optical control of a potassium channel with a photoswitchable molecule. (a) Structure of the molecular "tape measures" and of **MAQ**, the photoswitchable version. (b) Approximate position of the engineered cysteine anchor. (c) Schematic representation of the SPARK channel surface that can be reached by the ammonium ion in the *cis*-form of the azobenzene (violet) and the *trans*-form (green). S = cysteine point of attachment.

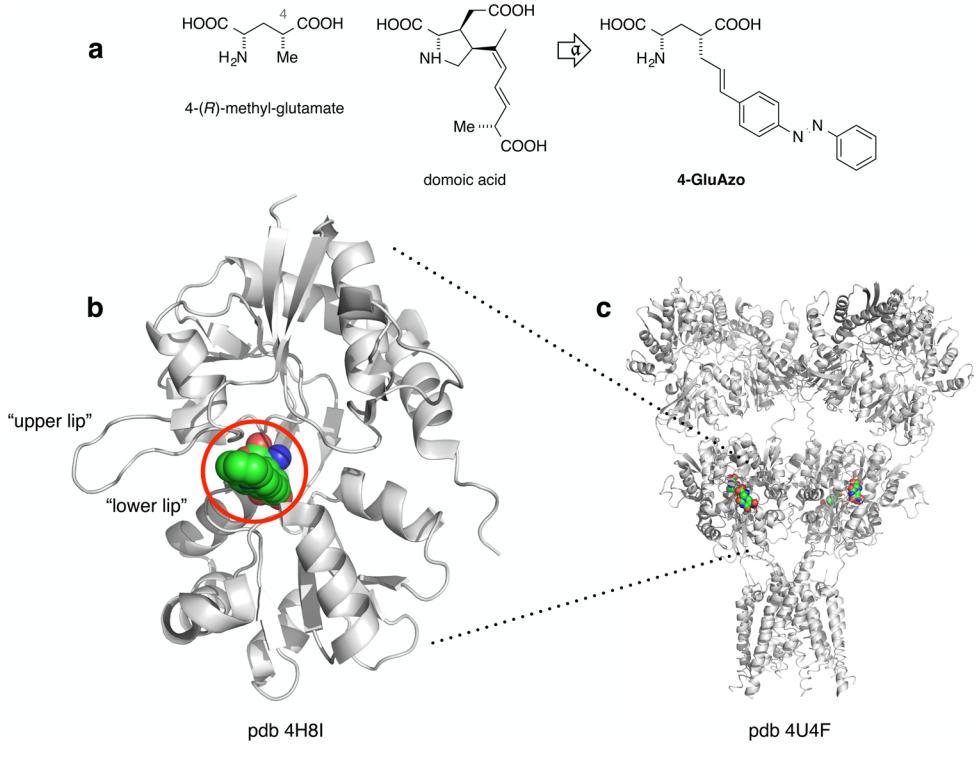

Figure 5. Optical control of an ionotropic glutamate receptor. (a) Structures of partial agonists for the kainate receptor GluK2. (b) X-ray structure of **4-GluAzo** bound to the GluK2 ligand-binding domain (LBD) . The azobenzene moiety projects toward the viewer. The "exit tunnel" is outlined in red. (c) Relative size and position of the LBD in a glutamate receptor tetramer.

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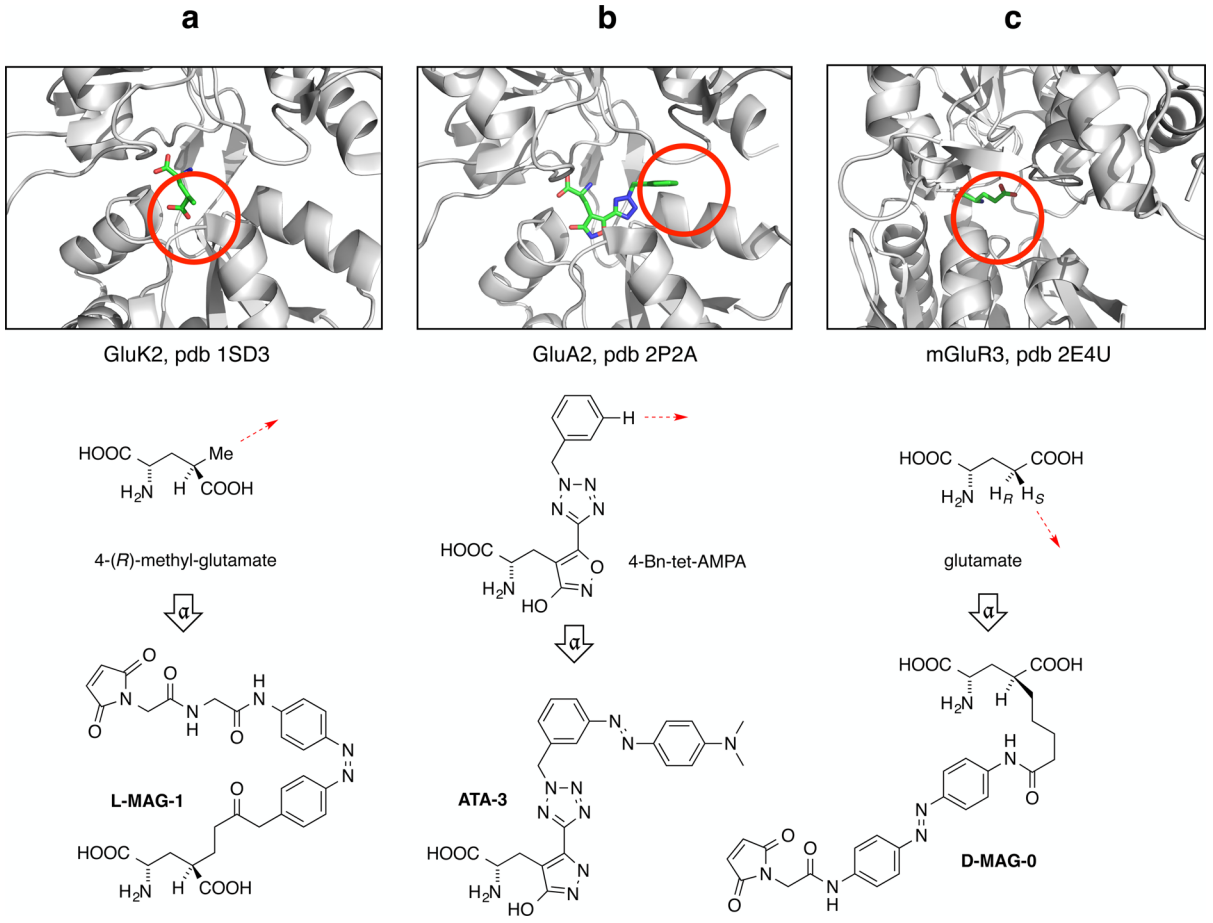

Figure 6. Photopharmaceuticals for kainate receptors, AMPA receptors, and metabotropic glutamate receptors. (a) X-ray structure of 4-(*R*)-methyl glutamate bound to a kainate receptors and the structure of **L-MAG-1**. (b) X-ray structure of 4-Bn-tet-AMPA bound to GluA2 and the formula of **ATA-3**, a photoswitchable version thereof. (c) X-ray structure of glutamate bound to a class III metabotropic glutamate receptor and the formula of **D-MAG-0**, a photoswitchable version thereof. The red arrow indicates the trajectory of the "exit tunnel" that could be occupied by a tether or an azobenzene moiety. The red circle indicates the mouth of this tunnel.

functioned as an agonist for kainate receptors with the photoswitch in its *trans*-form, that is, in the dark or at 500 nm but became inactive in its *cis*-form when irradiated with deep violet light (380 nm). Our subsequent X-ray structure of **4-GluAzo** bound to GluK2 (pdb 4H8I) confirmed that the azobenzene moiety indeed occupies an exit tunnel in its *trans*-form and could not be accommodated as the *cis*-isomer without prying open the clamshell (Figure 5b).[18] Looking at the relative size of **4-GluAzo** and a glutamate receptor (Figure 5c), it is remarkable how a small photoswitchable molecule can control the whole nanomachine.

In addition to **4-GluAzo**, we designed and synthesized the maleimide **L-MAG-1** as a PTL.[19] **L-MAG-1** bears a slim, flexible linker that would occupy the "exit tunnel" between the "lips" of the clamshells (Figure 6a). In contrast to **4-GluAzo**, the azobenzene itself would be largely solvent exposed. After tethering to an engineered cysteine via its maleimide, the photoswitch would hold the glutamate at arms length in its *trans*-form but allow it to occupy the binding site after photoisomerization to its *cis*-form. Our design turned out to be valid, giving rise to the light-gated ionotropic glutamate receptor, LiGluR.[19] In subsequent studies, we developed tethered glutamates that functioned as photoswitchable antagonists.[20]

The application of the PTL concept to AMPA receptors, the workhorses of synaptic transmission, was less straightforward than originally anticipated. It required the identification of a different exit tunnel, since the lips of the AMPA clamshell are more tightly closed compared with the kainate receptors (Figure 6b). Only after inspecting a variety of ligands bound to the most important AMPA receptor, GluA2, were we able to identify a pathway that penetrates the lips of the closed and activated clamshell more like a cigarette (i.e., sideways) than like a straw (i.e., straight). Our PTL **ATA-3** was designed based on the X-ray structure of 4-Bn-tet-AMPA, which is an improved and more selective version of AMPA and glutamate.[21] Upon inspection of its binding mode to the LBD of GluA2, it appeared that the benzyl group could be extended to an azobenzene, provided the requisite phenyldiazene unit was attached to the existing benzyl ring in a *meta* fashion. This idea turned out to be valid because the red-shifted azobenzene **ATA-3** lost its activity when irradiated with 460 nm light, pushing it to the *cis*-form, but regained activity within fractions of a second when the light was turned off.[21]

In another variation of the tethered glutamate theme, metabotropic glutamate receptors (mGluRs) could be converted into photoreceptors as well.[22] These family C GPCRs bear a clamshell-like or "Venus fly trap"-like extracellular LBD, which is linked to the canonical seven transmembrane helix domain via a cysteine-rich domain. Again, X-ray structures of the LBD bound to glutamate and its analogs, in particular the X-ray structure of the extracellular domain of mGluR3 (pdb 2E4U), a group II

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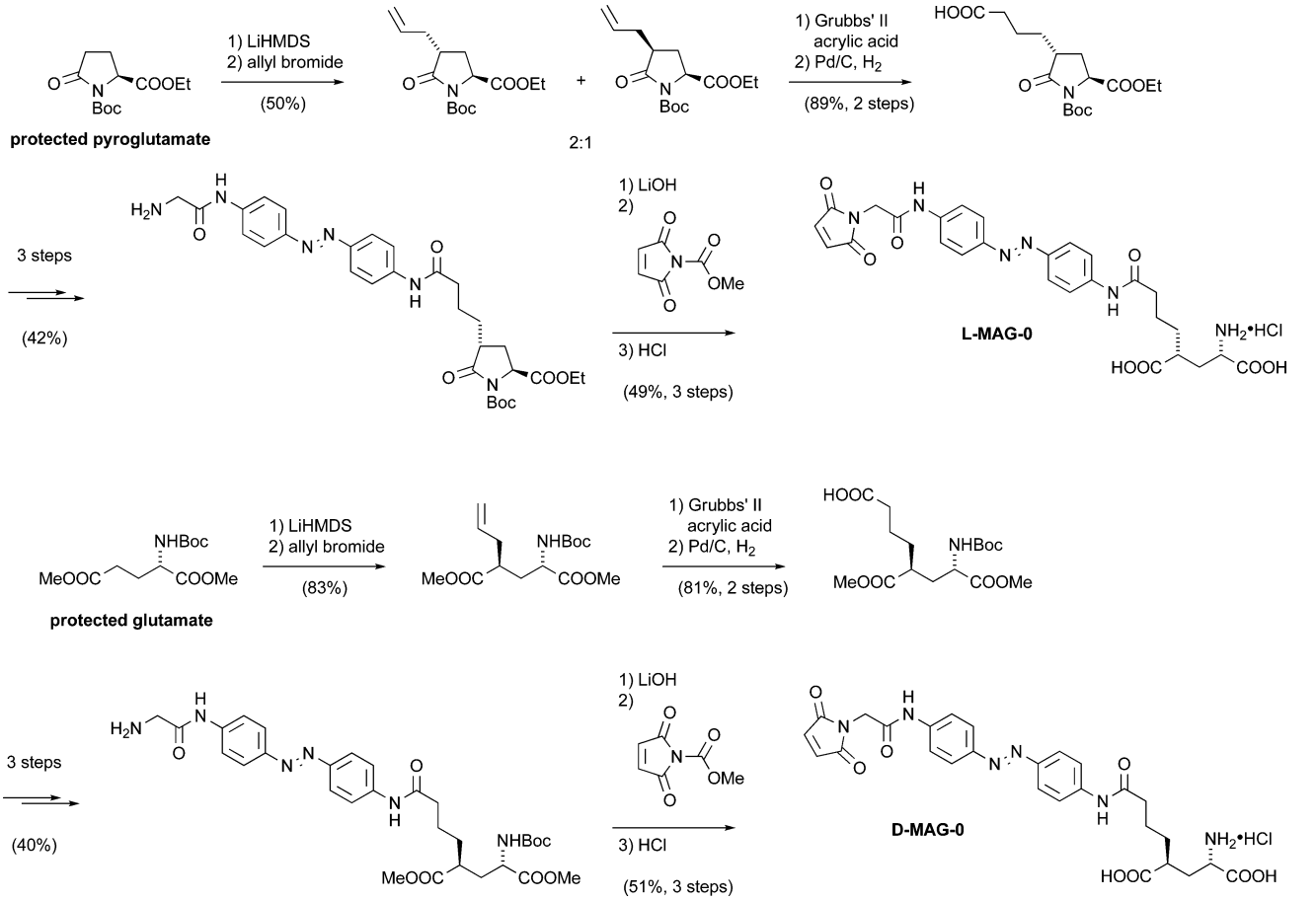
Scheme 1. Comparison of the Synthesis of L-MAG-0 with D-MAG-0[a]



[a](a) Synthesis of **L-MAG-0** starting from protected pyroglutamate via unselective and low-yielding allylation. (b) Stereoselective Fráter–Seebach allylation greatly improves accessibility to **D-MAG-0**.

receptor, were crucial for our design (Figure 6c). From the analysis of this structure, we could conclude that the stereochemistry at the glutamate ligand would have to be inverted from 4-(*R*) to 4-(*S*) when going from iGluRs to mGluRs. This reflects the fact that glutamate adopts a different conformation when bound to a metabotropic receptor than when ligated to an ionotropic receptor. The corresponding tethered ligand, **D-MAG-0**, was synthesized much more efficiently than its isomer **L-MAG-0** and was shown to be an excellent PTL for class II and III mGluRs. The resulting light-gated metabotropic glutamate receptor, LimGluR, could be expressed in dissociated neurons and nervous tissues, where it is used to control neuronal excitability and cAMP levels with light.

Our synthetic route to **D-MAG-0** owes its effectiveness to the implementation of a highly stereoselective Fráter–Seebach alkylation (Scheme 1b). The analogous alkylation of pyroglutamate derivatives proceeded with poor yield and diastereoselectivity (Scheme 1a).[17,19] To date, more than 10 g of each molecule has been synthesized for continuing biological studies. Variants of **L-MAG-0** and **D-MAG-0** that have red-shifted action spectra have also been developed and synthesized in relatively large quantities.[23]

It should be noted that molecular dynamics simulations have facilitated the design of LiGluR[20] and LimGluR[22] and have helped us understand the changes in efficacy upon photoswitching of **ATA-3**.[24] They were less crucial for the choice of ligand and identification of the "exit tunnels" but were highly useful for picking the cysteine residue for attachment of the tethered ligands. Calculations of the conformational space that could be reached by the photoswitches in their *cis*- and *trans*-configurations were also instrumental for the development of LiNaChR, the light-gated nicotinic acetylcholine receptor.[25] This system used **MAACh** and **MAHoCh** as photoswitchable tethered agonist and antagonist, respectively (Figure 7). Both could be tethered to the same engineered cysteine residue despite their functional differences (E61C). Structurally, the PTLs were derived from the agonist acetylcholine and the antagonist phenyl homocholine, respectively.

Many other receptors and targets of interest have now been characterized through X-ray crystallography, and cryo-electron microscopy promises to deliver additional high-resolution structures of very large complexes. In most cases, a low molecular weight ligand is bound to these structures and very often a binding cleft or an "exit tunnel" can be discerned. This could accommodate an azobenzene switch in one conformation but not in the other, or would allow a slim tether to protrude to the surface of the protein. A particularly attractive class of transmembrane proteins in this regard is the family A GPCRs, which include adrenergic receptors, muscarinic acetylcholine receptors, and dopamine, histamine, or serotonin receptors. It is likely that photopharmaceuticals will emerge for these GPCRs as they already have for A2 adenosine receptors[26] and μ-opioid receptors.[27]

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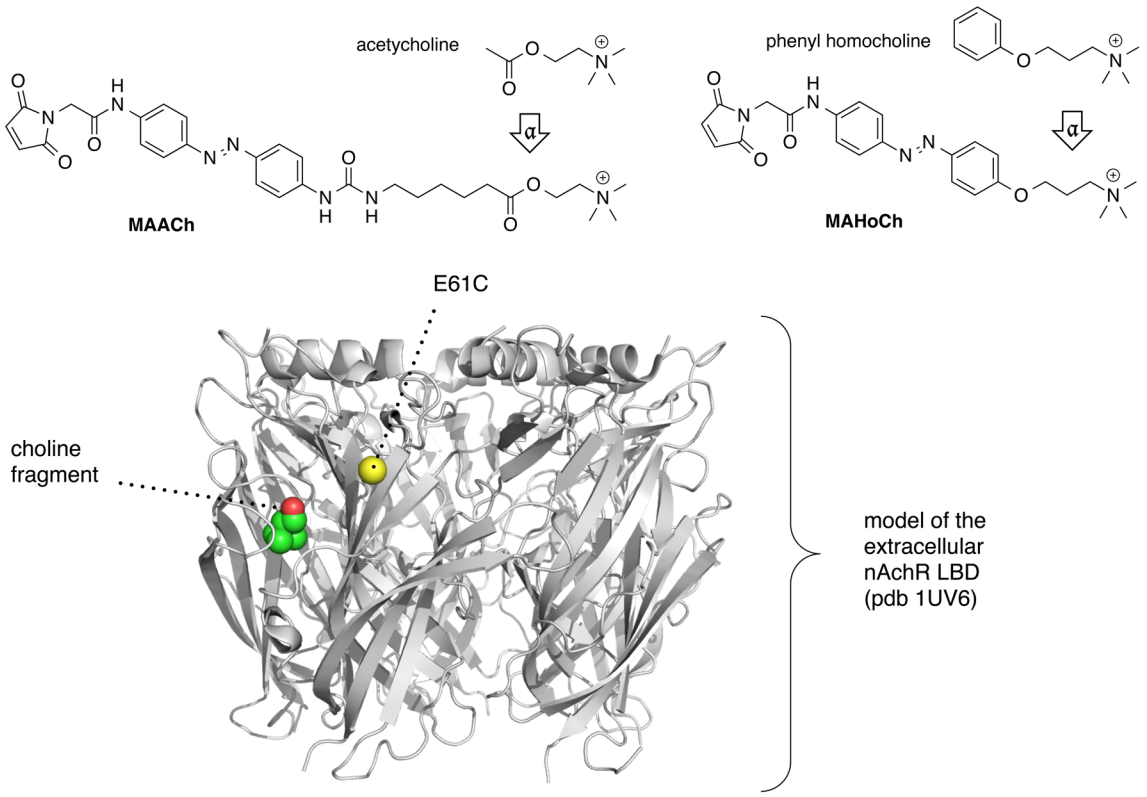

Figure 7. Optical control of a nicotinic acetylcholine receptor (LiNAChR). **MAACh** and **MAHoCh**, tethered to the same mutant cysteine (E61C), function as a photoswitchable agonist and antagonist of nAChRs, respectively.

LOOK OUT FOR "AZOSTERES" IN STRUCTURAL DATABASES AND IN THE LITERATURE

An obvious pathway to success in photopharmacology is to search for azobenzenes bound to a target in the protein database (PDB). It can be safely assumed that the biological activity of these compounds, if there is any, changes upon photoisomerization between the two distinct configurations. Indeed, a limited but not insignificant number of such azobenzenes can be found. This number can be vastly increased if certain isosteres of azobenzenes, which we call "azosteres", are taken into account. Figure 8 shows a set of structural motifs that we consider azosteres and are primed for "azologization".[28] They include styrenes, *N*-phenyl benzamides, other types of (hetero)aryl−(hetero)aryl amides, benzyl anilines, benzyl phenyl (thio)ethers, 1,2-diaryl ethanes, and related structures. The prevalence of these motifs within drugs and drug candidates may stem from the fact that they are relatively easy to synthesize with standard synthetic methodology in medicinal chemistry.

As of early 2015, there are about 30 styrenes, 200 *N*-phenyl benzamides, 50 benzyl anilines, and 80 benzyl phenyl ethers registered as ligands in the PDB and bound to a wide range of biological targets. This collection alone should provide research opportunities in photopharmacology for years to come. One of the advantages of the azologization approach is that it can be pursued with a high level of confidence even in the absence of X-ray structures. A quick survey of databases listing established drugs and drug candidates, such as the IUPHAR database[29] or DrugBase[30] shows that there are hundreds of additional azosteres, even within top-selling drugs. If one allows for diaryl amides, diaryl ethers, and other regions of flatness that could be replaced by (heterocyclic) azobenzenes, this number is even higher.

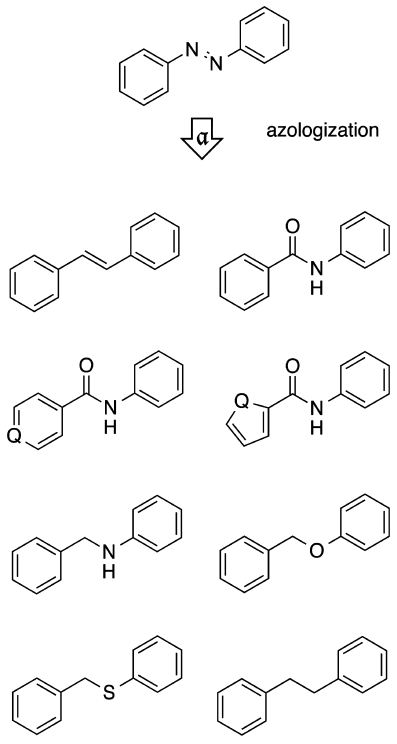

Figure 8. The "Azologization" approach. The azobenzene can mimic structural motifs ("azosteres") found in drugs or drug candidates such as stilbenes, (heterocyclic) *N*-aryl benzamides, benzyl phenyl (thio)ethers, benzyl anilines, and 1,2-diaryl ethanes.

Several published photopharmaceuticals have been designed using the azologization approach (Figure 9). Our group

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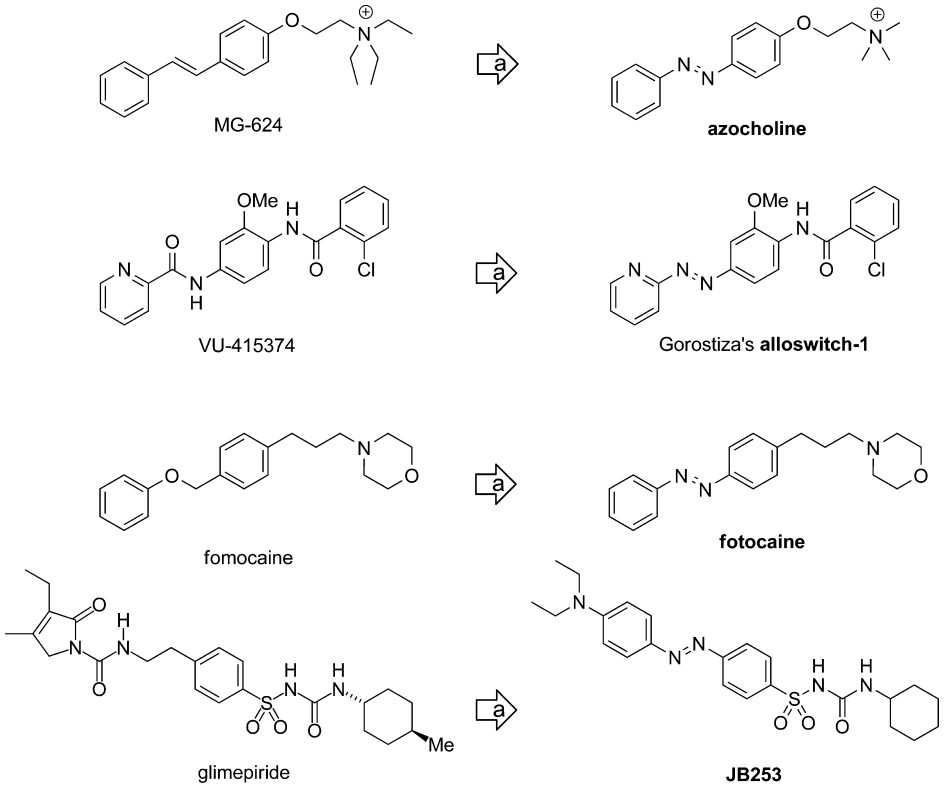

Figure 9. "Azologization" approach exemplified.

introduced **azocholine**, a photoswitchable agonist with selectivity for α7 nAChRs that was based on the antagonist MG-624.[31] Gorostiza developed **alloswitch-1**, a photoswitchable positive allosteric modulator of metabotropic glutamate receptors, which is an azostere of VU-415374.[26] The photoswitchable analgesic **fotocaine** was designed as an azostere of the benzyl phenyl ether fomocaine.[28] More recently, we have converted a well-established sulfonylurea antidiabetic drug, glimepiride, into **JB253**.[32] Glimepiride features a benzene ring connected to a "heterocycle", if one counts a hydrogen bond, through a two-carbon linker, which reminded us of an azobenzene. The corresponding photoswitchable sulfonylurea, **JB253**, could be used to control the membrane potential in pancreatic β cells (via K_{ATP}) and consequently insulin release with light. A red-shifted version of **JB253**, namely **JB558**, was also developed (cf. Figure 1).[11]

READ STRUCTURE–ACTIVITY–RELATIONSHIP TABLES

Even in the absence of a clear azologization motif, photoswitchable ligands and drugs can be rationally developed with relative ease provided structure–activity tables are available. Often, one can conclude from these data that certain substituents can be varied without completely abrogating the ligand's biological activity. This structural variation is usually associated with a change in efficacy, which bodes well for a switchable substituent in the same position. Figure 10 shows a variety of compounds where this "azo-extension" strategy has been successfully implemented. Feringa noted that the morpholino substituent in ciprofloxacin and related fluoroquinolones is highly variable. Replacement of this amine with an aryldiazenyl residue gave **quinolone-2**, which enabled the optical control of antibacterial activity.[33] Based on literature reports, we reasoned that the anesthetic propofol, a structurally simple $GABA_A$ receptor potentiator, could be substituted in the 4-position with relatively large substituents. Extension of propofol to the azobenzene **AP-2** provided a photoswitch for the optical control of inhibitory GABA currents.[34] Amiloride and its more lipophilic congener phenamil are clinically used blockers of epithelial sodium channels (ENaC). Our photoswitchable version, **PA-1**, could be used to optically control the $\delta\beta\gamma$-isoform of these important channels.[35] Tacrine is a centrally acting inhibitor of acetylcholinesterase that has been used for the treatment of Alzheimer's disease. Based on available SAR data and X-ray structures (e.g., pdb 1DX4), we designed **AzoTHA**, thus extending our program on the optical control of neural activity to enzymes involved in synaptic transmission.[36] Capsazepine antagonizes activation of the TRPV1 ion channel with heat or small molecules, such as the natural product capsaicin. Realizing that its chlorine residue could be replaced with a variety of different substituents, we developed **AC-4**, a photoswitchable antagonist of ion channel activity.[37]

EMBRACE THE LIPOPHILIC NATURE OF AZOBENZENES

Most of the photopharmaceuticals shown in Figure 10 are lipophilic in nature or amphiphilic when charged, which is usually the case under physiological conditions. This lipophilicity reflects the fact that the basic azobenzene moiety itself is nonpolar, especially in its *trans*-form. Although the diazene functional group has some Lewis-basic properties, it is usually not protonated at physiological pH (as a point of reference protonated methyl orange, a highly electron-rich azobenzene, has a pK_a of 3.47 at 25 °C).[38] Of course, the polarity of the azobenzene core and hence the solubility of the whole molecule

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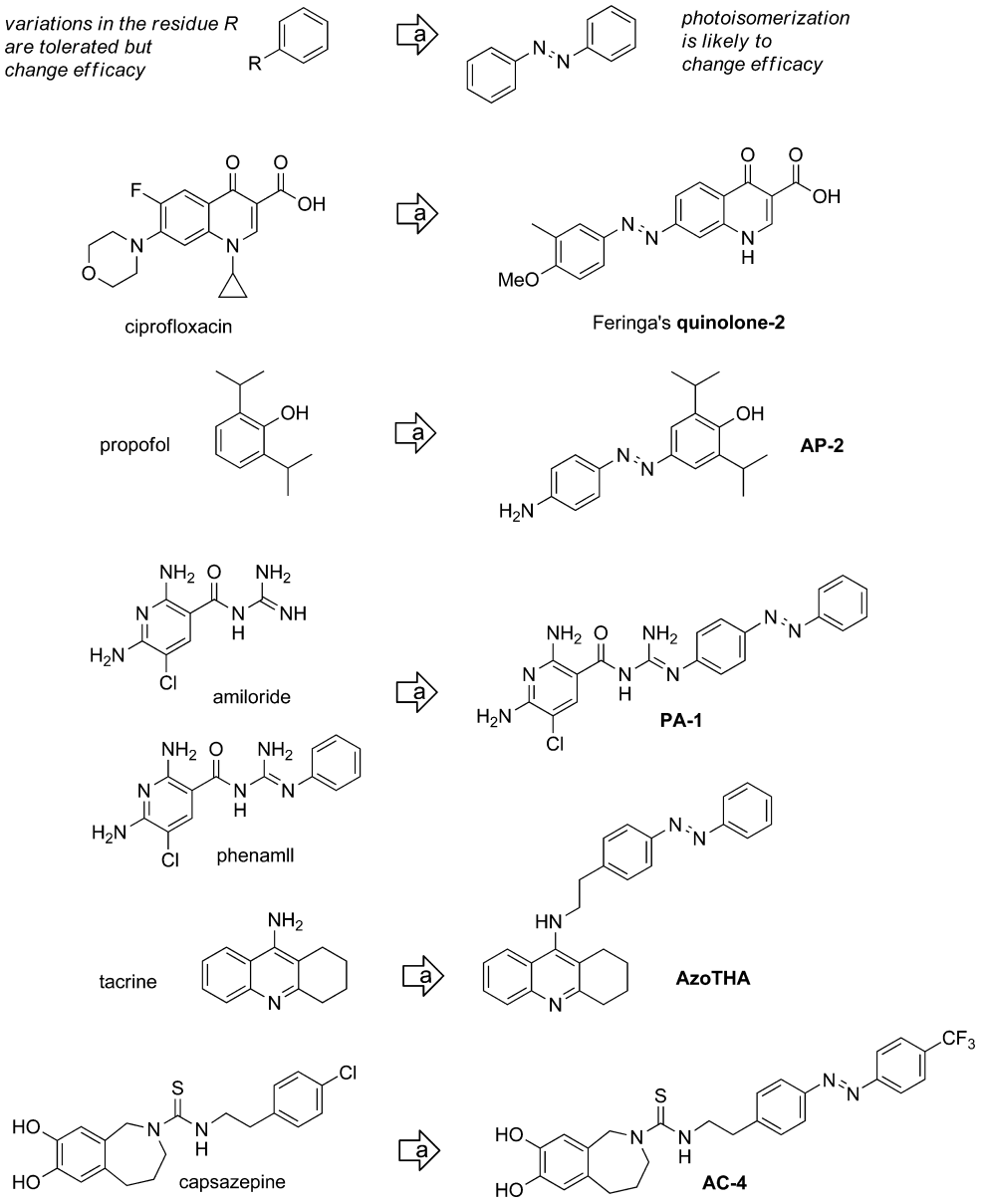

Figure 10. "Azo-extension" approach.

can be fine-tuned through the implementation of heteroaromatic variants or substitution patterns that disturb planarity.

At first sight, the lipophilic nature and flatness of *trans*-azobenzenes may appear to be a drawback. However, while the solubility in physiological buffer solution becomes a concern, the lipophilic nature of some azobenzenes can also be advantageous, especially when transmembrane proteins are involved. In these cases, ligands often partition between the membrane and a cavity within the protein. This mode of action is well established in so-called use-dependent open channel blockers of voltage gated sodium and potassium channels. They include popular local anesthetics, such as novocaine or lidocaine. Our photoswitchable versions of these molecules, **BENAQ** and **DENAQ**,[39] indeed resemble protonated lidocaine but are permanently charged and more lipophilic due to an azobenzene linked to additional nonpolar substituents (Figure 11). They were derived from **AAQ**, an acrylamide that was originally designed as a tethered blocker,[40] but were subsequently found to block voltage-gated channels as a diffusible PCL.[41] They have been used to restore visual function to blind mice and were shown to persist for days due to their lipophilicity.[42,43] By contrast, **QAQ**, a bis-quaternary cation derived from **QX-314**, the permanently charged version of lidocaine, was not cell-permeable and required the activation of additional import channels, such as P2X receptors or TRPV1 channels, to become efficacious as a photoswitchable analgesic.[44]

In a more recent direction of our program, we synthesized a photoswitchable version of capsaicin, the pungent ingredient of chili peppers.[45] Capsaicin strongly activates TRPV1 channels and is in essence a fatty acid amide of vanillamine. Its synthetic analogs arvanil and olvanil demonstrate that a variety of fatty acids can be incorporated into the molecule without a decrease in potency. To systematically explore the photopharmacology of vanilloids, we synthesized eight isomeric photoswitchable fatty acids that correspond to stearic and oleic acid in their *trans*- and *cis*-forms, respectively (Figure 11). The molecules, **FAAzo1−8**, were all linked to vanillamine and tested for their ability to activate TRPV1 channels upon irradiation. Among the different azo-vanilloids evaluated, **AzCA4** emerged as the most effective

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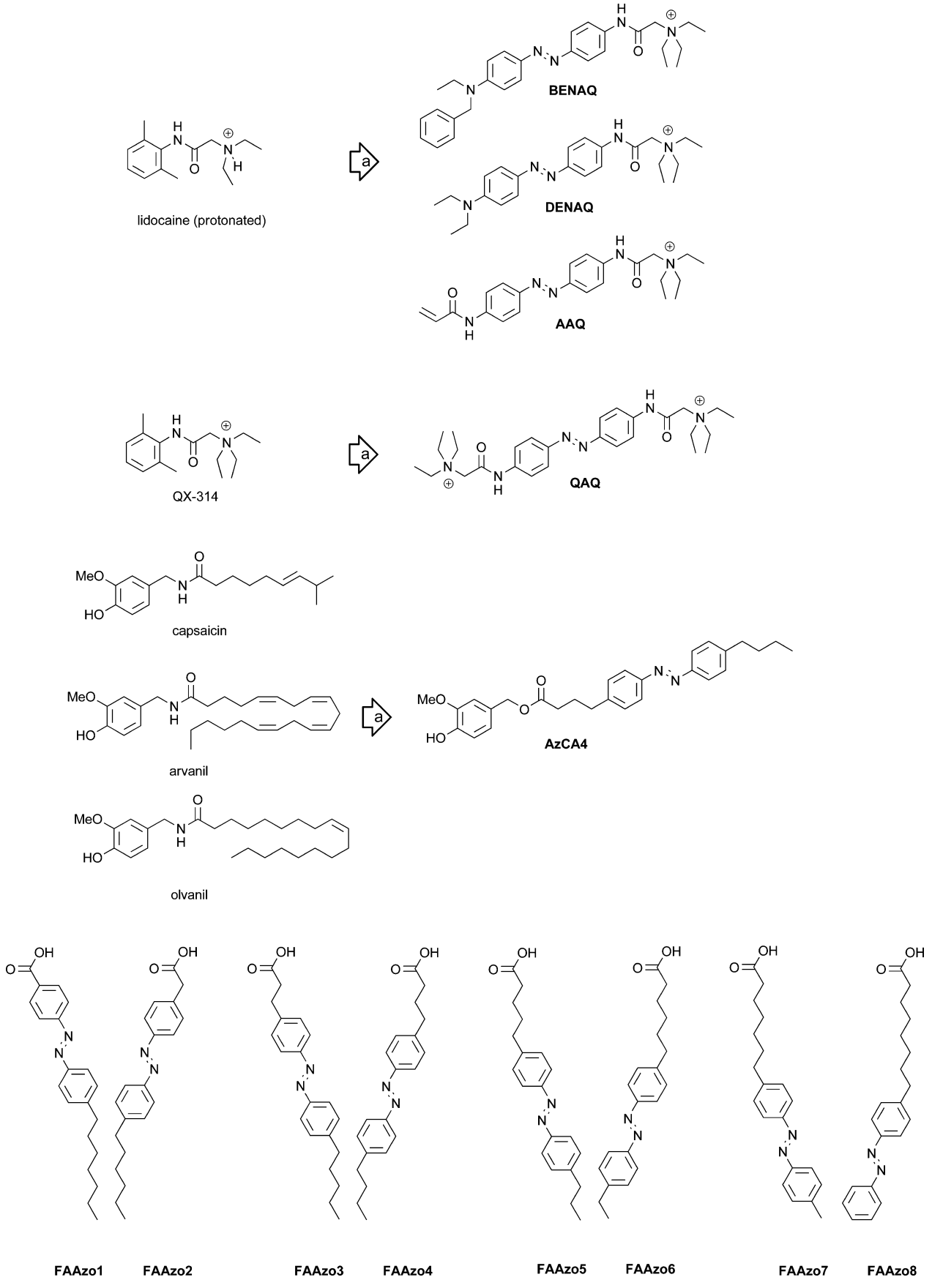

Figure 11. Photoswitchable amphiphiles to optically control biological activity.

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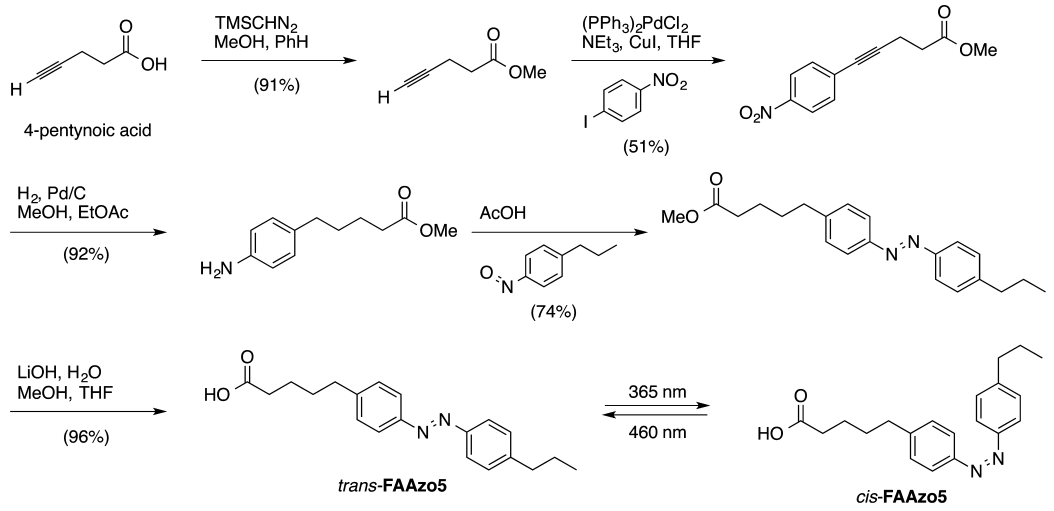
Scheme 2. Synthesis of the Photoswitchable Fatty Acid FAAzo5



TRPV1 photoswitch. Since **AzCA4** is relatively inactive in the dark and only becomes an effective agonist after isomerization to the *cis*-form, it can be used to activate TRPV1 channels with unmatched speed and precision.

Given the significance of *cis*-double bonds in lipids, we do believe that fatty acids and **FAAzos** are a natural match. The **FAAzos** have been incorporated into a variety of glycerolipids and sphingolipids, giving rise to several photoswitchable molecules that are currently undergoing biological testing. Although they are not always particularly complex molecules, they do pose synthetic challenges since they need to be procured in large quantities to support our systematic studies and further derivatization. As an example, the synthesis of **FAAzo5** is shown in Scheme 2. It involves a Sonogashira coupling and a Mills reaction to assemble the target molecule from commercially available materials in five steps.

In principle, photoswitchable lipids and amphiphiles ("photolipids") can exert bioactivity in three different ways. First, they can have a direct effect on the biophysical properties of a biological membrane, such as fluidity, curvature, raft formation, impedance, and capacitance. At present, this aspect remains largely unexplored, and it is a major focus of our current research. Second, photolipids can operate at the interface of the lipid bilayer and a membrane protein. Indeed, the lipid composition of the surrounding membrane is known to have a large influence on the dynamics of transmembrane proteins. Voltage-gated ion channels, for instance, are well documented to be sensitive to their lipid environment, and there are numerous X-ray structures, for instance, of G-protein coupled receptors that show lipids tightly bound to transmembrane helices (an example is provided by pdb 4OR2). The protein−lipid interface sometimes extends to the inner cavity of ion channels, as is the case for voltage-gated potassium and sodium channels sensitive to use-dependent open channel blockers. Finally, amphiphilic molecules can function as more conventional ligands that bind deeply within a protein or at the protein−cytosol interface. These targets include a variety of transmembrane, membrane-associated, and cytosolic proteins, such as TRP channels, protein kinase C, or nuclear hormone receptors, to name a few.

CONCLUSION

It is fair to say that photopharmacology is now a well developed field that is far from mature. Its foundations have been laid, but the extent to which it will grow remains to be determined. We hope that with this Account we have been able to demonstrate that photopharmaceuticals can be developed with a high level of confidence and that they are more likely to be successful than not, justifying a significant synthetic investment. Moving from rational design based on three-dimensional receptor structures to "ChemDraw modeling" and simple rules of thumb, we have attempted to lay out a recipe for success in photopharmacology. These ideas may be helpful for accelerating the pace of discovery in this budding field. While the usefulness of photoswitchable ligands as a research tool has evolved beyond question, their benefits in human therapy need to be demonstrated. Once this has been achieved, the combination of photoswitches and light will be embraced by the pharmaceutical industry and the medical community, as was done with photodynamic therapy, and is likely to happen with optogenetics.

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Notes

The authors declare no competing financial interest.

Biographies

Johannes Broichhagen (born on March 27th, 1984, in Würzburg, Germany) studied chemistry at the Friedrich-Alexander-University Erlangen-Nuremberg, Germany. After a one-year (2008−2009) research stay with Prof. Dr. Marcus Weck at the New York University, NY, USA, he continued his studies in Germany and received his Diploma under supervision of Prof. Dr. Ivana Ivanovic-Burmazovic in 2010 on the reaction of hydrogen sulfide with iron-containing heme-mimics. He then joined the research group of Prof. Dr. Dirk Trauner at LMU Munich to obtain his Ph.D. in 2014 for his work on "Photopharmacology of Enzymatic Activity and Transmembrane Protein Function". He will continue his endeavors in Chemical Biology in the laboratories of Prof. Dr. Kai Johnsson (EPFL, Switzerland).

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James Allen Frank (born on July 21st, 1989, in Edmonton, Canada) received his B.Sc. in Chemistry at the University of British Columbia in Vancouver, Canada (2012). Following one year training as a technician at Corden Pharma Switzerland LLC in Liestal, Switzerland, he joined the group of Prof. Dr. Dirk Trauner at the Ludwig Maximilians University in Munich, Germany, as a graduate student. His current research involves the development of photoswitchable lipids for the optical control of the cell signaling machinery. His broader research interests and future goals involve the development of tools to untangle complex neural networks and their relationships to behavior.

Dirk Trauner (born on April 17th, 1967, in Linz, Austria) conducted studies in biology and biochemistry at the University of Vienna, Austria, before he joined Prof. Dr. Johann Mulzer's group at the Free University of Berlin, Germany, to pursue natural product synthesis. Subsequently, he became a postdoctoral fellow with Prof. Dr. Samuel J. Danishefsky at the Memorial Sloan-Kettering Cancer Center in New York City, NY, USA. In 2000, Dirk joined the University of California, Berkeley, USA, where he rose through the ranks to become an Associate Professor of chemistry (with tenure). In the summer of 2008, he moved to the University of Munich, Germany, where he currently resides as a Professor of Chemistry and Chemical Biology. His research interests range from organic synthesis and natural product chemistry to chemical neurobiology, optogenetics, and photopharmacology.

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