Synthesis and application of conditionally activatable photolabile linkers for light controlled targeted delivery of bioactive molecules

The aim of the proposed research was to design and synthesize so far unprecedented, multi-functional systems capable of delivering biologically active molecules to a specific location, where they can be activated or released using external light stimulus. This concept is based on bioorthogonally functionalized photoremovable protecting groups (PPGs, or photolabile linkers or photocages) that become photoresponsive solely upon a specific chemical reaction. This novel concept, termed "conditional photoactivation" requires the chemical modification of photocages with a bioorthogonal functionality that quenches photoactivity until it is transformed in a bioorthogonal ligation reaction. The results of this project were expected to give tools for chemical biologists for light-assisted manipulation of biological systems with unprecedented precision as well as propose a novel approach in light-controlled drug delivery. During the course of this research, in addition to the proof-of-concept demonstration and extended applications of bioorthogonally-assisted photoactivation, we have introduced and published a set of green- to red light activatable xanthenium PPGs. We have also developed photoactivatable chemotherapeutic agents based on coumarin and xanthenium PPGs with excellent light-dependent toxicities in cell cultures. These results will be discussed below in detail.

I. Proof-of-concept of conditionally activatable photocages

Before the beginning of the project, we have already established and published the concept of conditional uncaging in *J. Am. Chem. Soc.* **2020**, *142*, 15164. (therefore, it does not appear in the associated publications), which already received around 50 citations. We have synthesized a bioorthogonally functionalized coumarin photoremovable protecting group that becomes photoresponsive solely upon a specific chemical reaction, and also presented the applicability of a fluorogenic construct in living cells using mitochondrial pretargeting (Figure 1)

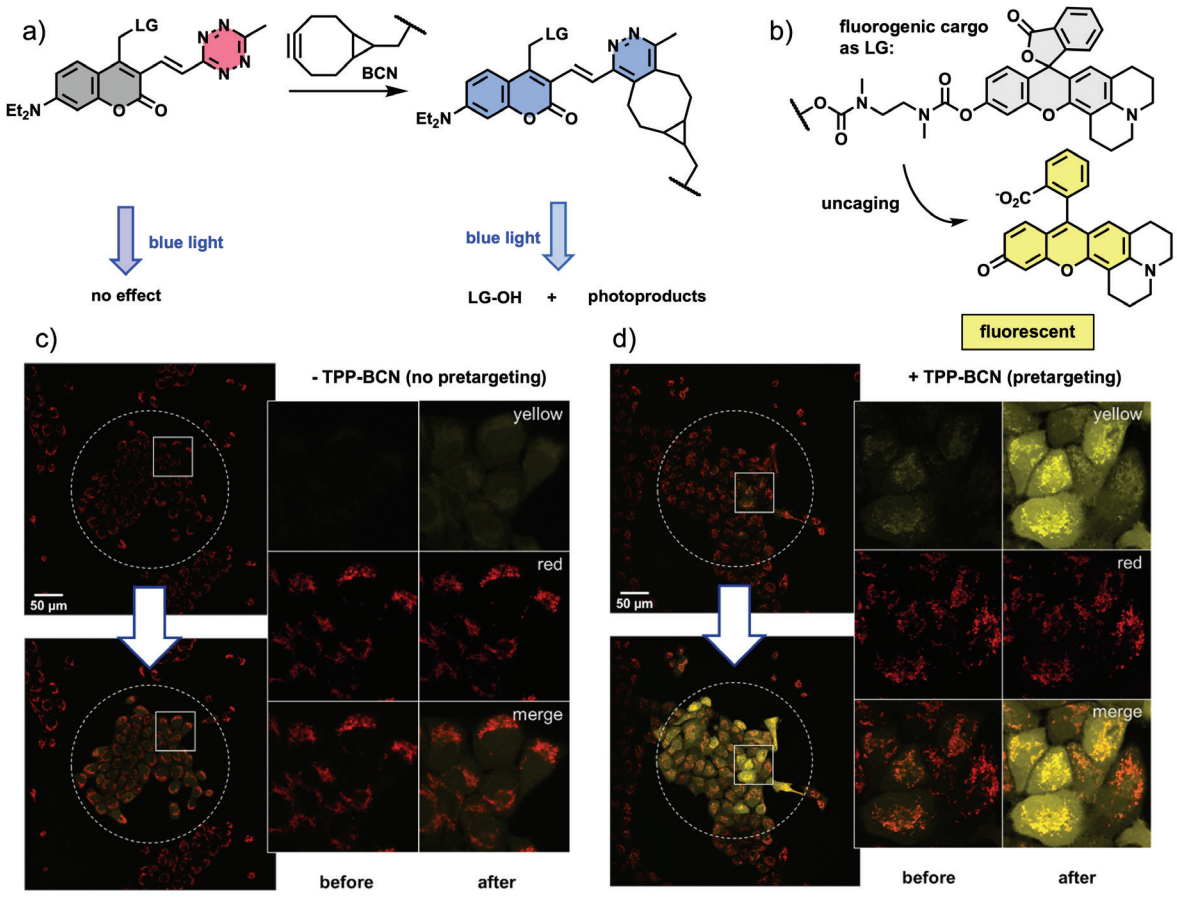

Figure 1 *a) Scheme of bioorthogonally-assisted conditional photocaging (LG = leaving group) b) structure of the fluorogenic payload attached to the PPG and the released fluorogenic probe after uncaging c) tile-scan confocal microscopy images of live A-431 cells treated with the PPG-fluorogenic probe conjugate in the absence of triphenylphosphonium-BCN (TPP-BCN) with subsequent irradiation of the dotted circle with blue light d) same images in the presence of TPP-BCN*

More precisely, the well-established coumarin PPG was modified with a vinylene-tetrazine unit in position 3. By showing experimental evidence and theoretical calculations, we have **demonstrated that the presence of the tetrazine motif efficiently quenches the excited state of the coumarin necessary for photolysis resulting in disabled photoresponsivity** (both in terms of photocaging and fluorescence). Transformation of the tetrazine moiety in a bioorthogonal click reaction with bicyclononyne (BCN) fully restored its sensitivity for light, therefore the **photo-uncaging reaction only occurred in the presence of the corresponding bioorthogonalized pretargeting element**. We have presented this concept using 1) small molecule-based intracellular targeting, namely TPP-BCN which targets mitochondria (*already published*); 2) using genetically encoded HaloTag fusion proteins in various intracellular targets (e.g., vimentin, lamin) with Halo-BCN 3) using genetic code expansion techniques, namely BCN-lysine encoded in intracellular target proteins (*unpublished results*). Nevertheless, our attempts towards cell-specific extracellular targeting remained elusive – these will be discussed in the next two sections.

Furthermore, our original choromophore concept – vinylenetetrazine in position 3 of the coumarin core – was also exploited in the design of **bioorthogonal ligation-activated fluorogenic dyads** reported in *Angew. Chem. Int. Ed.* **2022**, *61*, e202111855. Although these were not PPGs, rather fluorogenic dyes, it was essentially the same chromophore that provided the fluorogenicity in this 'relayed fluorogenicity' concept. This energy transfer-based relay mechanism resulted in improved

cyanine fluorogenicities together with increased photostabilities and large apparent Stokes-shifts for enhanced fluorescence imaging in live cells.

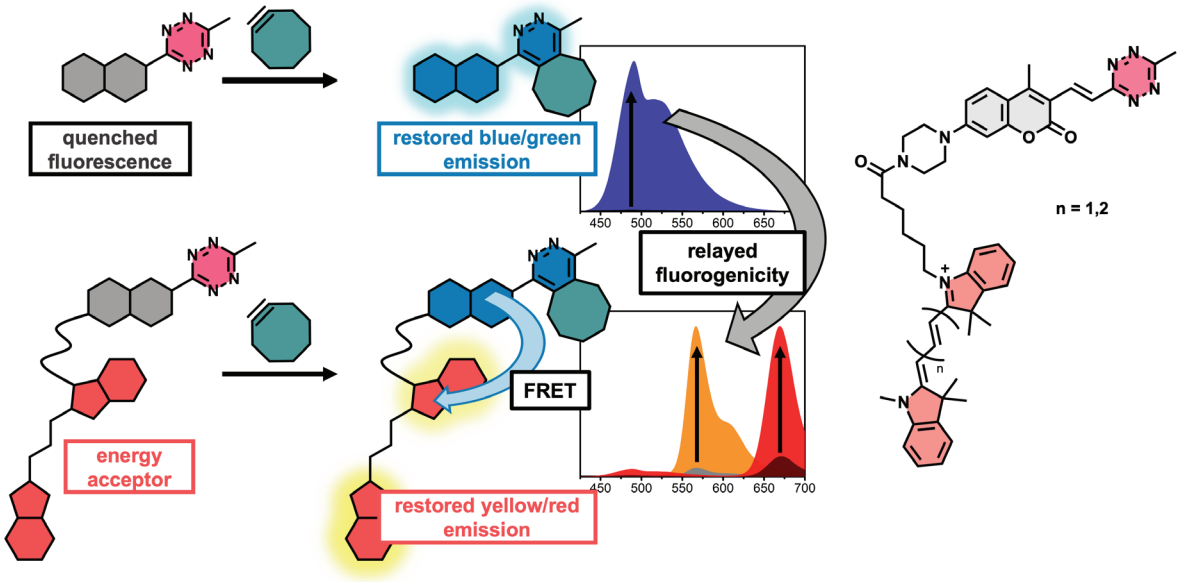

Figure 2 *Scheme of bioorthogonally activated relayed fluorogenicity and the structure of some fluorogenic FRET dyads*

We have also compiled a review in *Angew. Chem. Int. Ed. 2023, 62, e202303198*. that discusses recent advances in bioorthogonally-assisted phototherapeutic approaches as well as it also includes some thoughts about the field's potential future.

II. Development of cellular model systems for the evaluation of bioorthogonally-assisted delivery systems

Our ultimate goal is to create **chemical tools that provide unprecedented precision for light-assisted manipulation of biological systems.** These chemical tools, however, need to be evaluated in relevant biological systems. Therefore, biological platform development for the assessment of bioorthogonally targeted PPGs was also included in the project. These platforms include the following (in addition to the intracellular targeting modalities discussed above) 1) a bioorthogonalized extracellular receptor 2) a targeting system based on receptor-ligand interactions with bioorthogonalized ligands and 3) antibody-based pretargeting using bioorthogonalized antibodies (Figure 3). As these are still unpublished results, they will be discussed here in more detail.

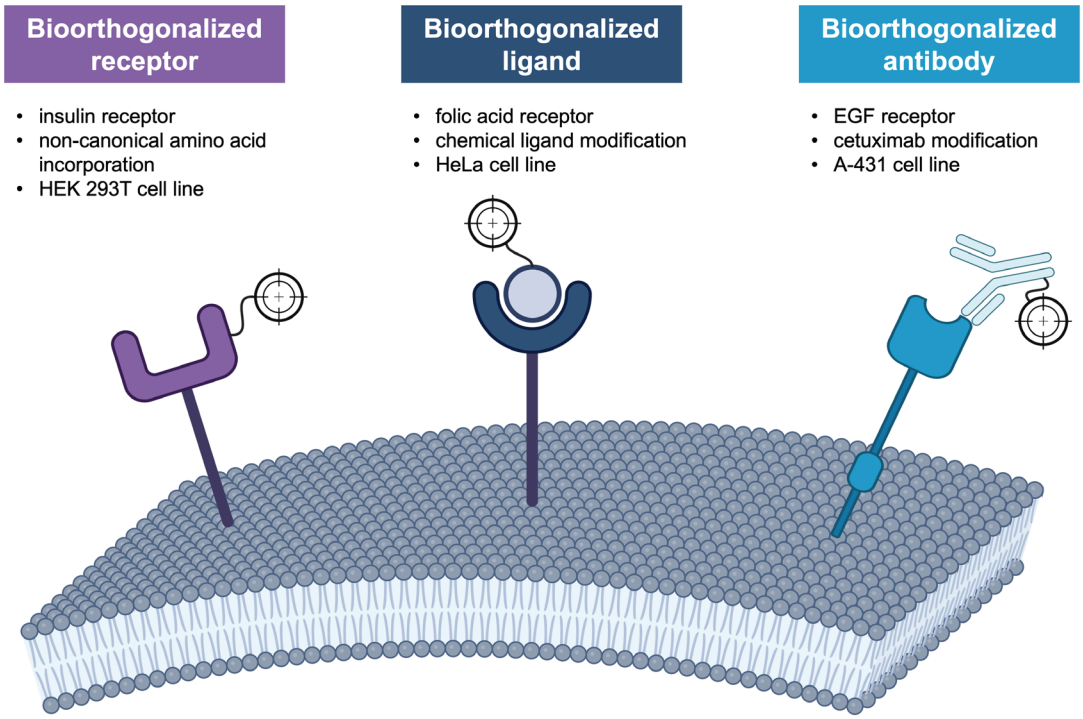

Figure 3 *Schematic representation of the different biological platforms developed for the evaluation of extracellularly pretargeted bioorthogonal delivery systems. The crosshair represents the bioorthogonal function.*

The overall workflow in each case included the development of these model systems based on an established, extracellularly applicable, fluorescent bioorthogonal probe which allowed the confocal imaging of these engineered systems after optimization. The preliminary results with the extracellularly applicable PPGs will be discussed in the next chapter. Importantly, the value of these systems is obviously beyond this project as these **models are also applicable to evaluate any type of chemical biology toolkit that utilizes bioorthogonal chemistry**.

Pretargeting of extracellular receptors using genetic code expansion

Targeting of photocaged ligands towards extracellular receptors is an appealing technique for studying their function and downstream signaling without off-target effects. Nevertheless, the only examples found in the literature for receptor-targeted photopharmacology rely on Halo/SNAP-Tag fusion protein-targeted photoswitches, likely due to the a lack of targetable PPGs. Our first model system was based on a bioorthogonalized insulin receptor (IR^{TAG}) fused to fluorescent proteins (miRFP, mOrange) which was developed using genetic code expansion (GCE). Using transfected live HEK-293T cells, this model enabled us to effectively co-localize the pretargeting bioorthogonal BCN functions with the tetrazine probes (or later, PPGs). In addition, as the nascent proteins are also present inside the cells, it was the optimal model for testing the membrane permeability of the probes and PPGs (for the confocal images, see the next section).

Receptor-ligand interaction-based pretargeting

Using bioorthogonalized ligands specific towards overexpressed receptors on a particular cell type is a popular approach in, for example, targeted therapies. Indeed, folic acid (FA) receptor is overexpressed on many cancer cell types therefore FA is often used as a targeting moiety of

chemotherapeutics and imaging probes. We selected the HeLa cell line due to its known FA receptor overexpression and used a recently reported (*Angew. Chem. Int. Ed.* **2020**, *59*, 6015.), negatively charged FA derivative for bioorthogonalization. The carboxylate functions were responsible for the reduction of aspecific internalization while the BCN group was appended to a lysine side-chain. After careful optimization of the labeling conditions (e.g., culture media, concentrations, labeling time, etc.), we performed successful selective FA receptor labeling experiments using either preassembled (preclick) or pretargeted constructs with tetrazine-appended cyanine dyes (Cy5-Tet) and PPGs (Figure 4).

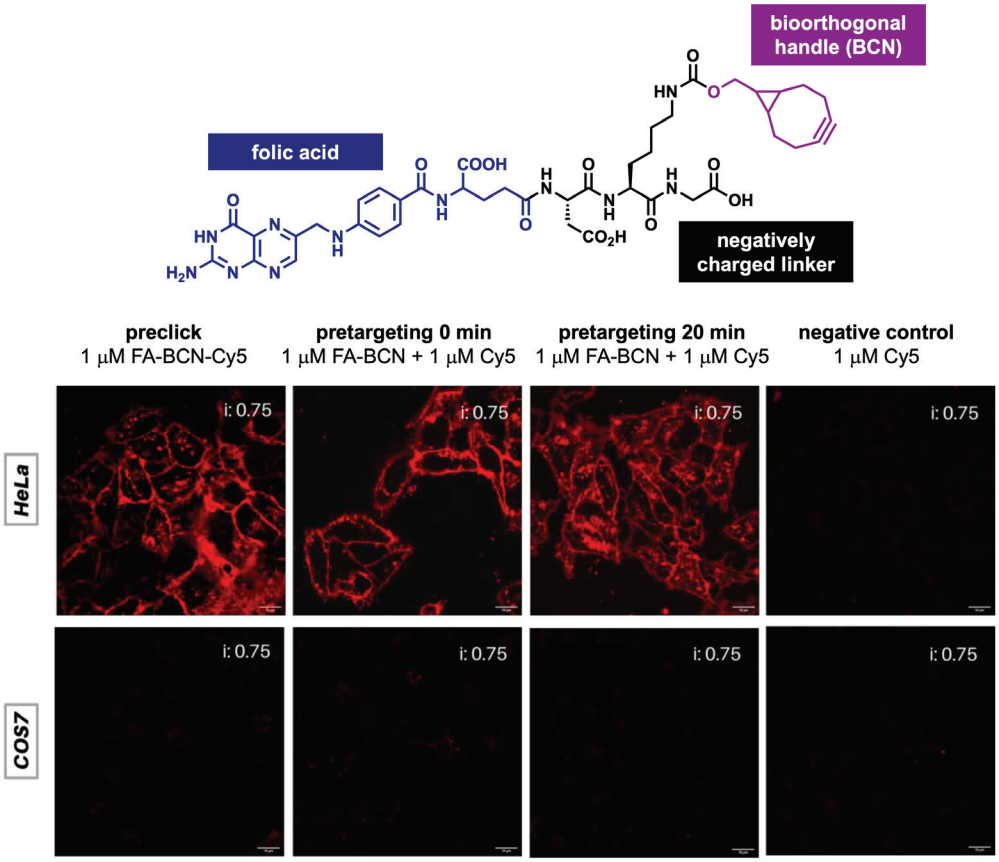

Figure 4 *Structure of the BCN-appended folic acid derivative and confocal images upon different treatment protocols with the ligand and tetrazine-Cy5. HeLa cells overexpress FA receptors while COS7 cells are the negative control*

Pretargeting of overexpressed receptors using bioorthogonalized antibodies

From a therapeutic perspective, this approach is probably the most relevant as pretargeting-dependent conditional uncaging with bioorthogonalized antibodies might **enable dual control of drug delivery**. This approach would offer several advantages over antibody-drug conjugates (ADCs), such as the controlled release of the payload by light, longer circulation times of the nontoxic (BCNylated) antibodies without side-effects, enhancement of the bystander effect and importantly, the unnecessity of biomolecule internalization – obviously with the limitation of light accessibility. Antibody-based pretargeting was developed by using BCN-appended, commercially available Cetuximab antibodies. These target the epidermal growth factor receptor (EGFR), often overexpressed in cancer cells, however these receptors are also present on healthy cells, therefore represent a challenging target for ADCs due to their extremely high toxicities. Using light-activatable ADCs with the pretargeting approach would add a further layer of control and could reduce off-site

effects as EGFR-overexpressing tumors are often accessible to light (e.g., colorectal cancer, head and neck tumors (https://go.drugbank.com/drugs/DB00002)). After careful optimization of the conjugation using BCN-NHS and the cellular labeling protocol, we were able to pretarget EGFR overexpressing A-431 cancer cells using BCN-Cetuximab and label them with tetrazine-cyanine dyes. Furthermore, EGFR labeling using a tailored nanobody construct (molecular weight approx. 10% of the antibody) was also realized.

In summary, although these results are still unpublished they represent a biological platform of profound importance not just as part of this project but as a **relevant bio-evaluation toolkit for bioorthogonal targeting approaches in chemical biology and targeted therapies**. We have therefore presented examples to 1) target organelles using BCN-appended small molecules 2) target intra- and extracellular proteins using GCE/fusion proteins 3) target receptors using their BCN-appended ligands 4) target overexpressed receptors using antibodies and nanobodies.

III. Towards extracellular targeting and monitoring of photo-uncaging

Simultaneously with the biological platform development, we have soon realized that the original construct reported in the 2020 JACS paper was not suitable for extracellular pretargeting due to its low solubility and high membrane permeability. Furthermore, our approach toward novel proof-of-concept studies always includes **seeing the entire process first**, often using fluorescence microscopy and labeling of particular structures. This was feasible for intracellular targets, however, using extracellular labeling it presented an enormous challenge. The first difficulty was the further functionalization of the PPG with a side-chain that renders the entire construct membrane impermeable. Significant synthetic efforts were made towards adding another, orthogonal clickable function in addition to the tetrazine and payload that resulted in several molecular designs (Figure 5) and reproducible synthetic strategies. The strategies employ the incorporation of a propargyl group in the coumarin frame in addition to the tetrazine that can be modified easily with a water-soluble side-chain using its azide derivative (e.g., polyethylene-glycol-N_3). Furthermore, payload selection here was significantly problematic as photo-uncaging of fluorogenic probes (like in the "inside-cell" experiments) on the outer cell surface was not applicable due to rapid diffusion to the culture medium. We came up with the idea of using organelle-labeling probes as cargoes to be liberated from the coumarin PPG after conjugation to the extracellular targets. These included a silicon-rhodamine far-red dye (MitoSiR, capable of labeling mitochondria), a cyanine dye (also for mitochondria) and a Hoechst-type nuclear stain. These compounds were synthesized with the only aim to visualize 1) bioorthogonal labeling of the extracellular targets followed by confocal microscopy (and possible colocalization with fluorescent proteins fused to such targets) 2) uncaging followed by the 'relocation' of the probes from the outer cell membrane to their corresponding targets (e.g., the mitochondria). An example of these experiments is shown in Figure 5.

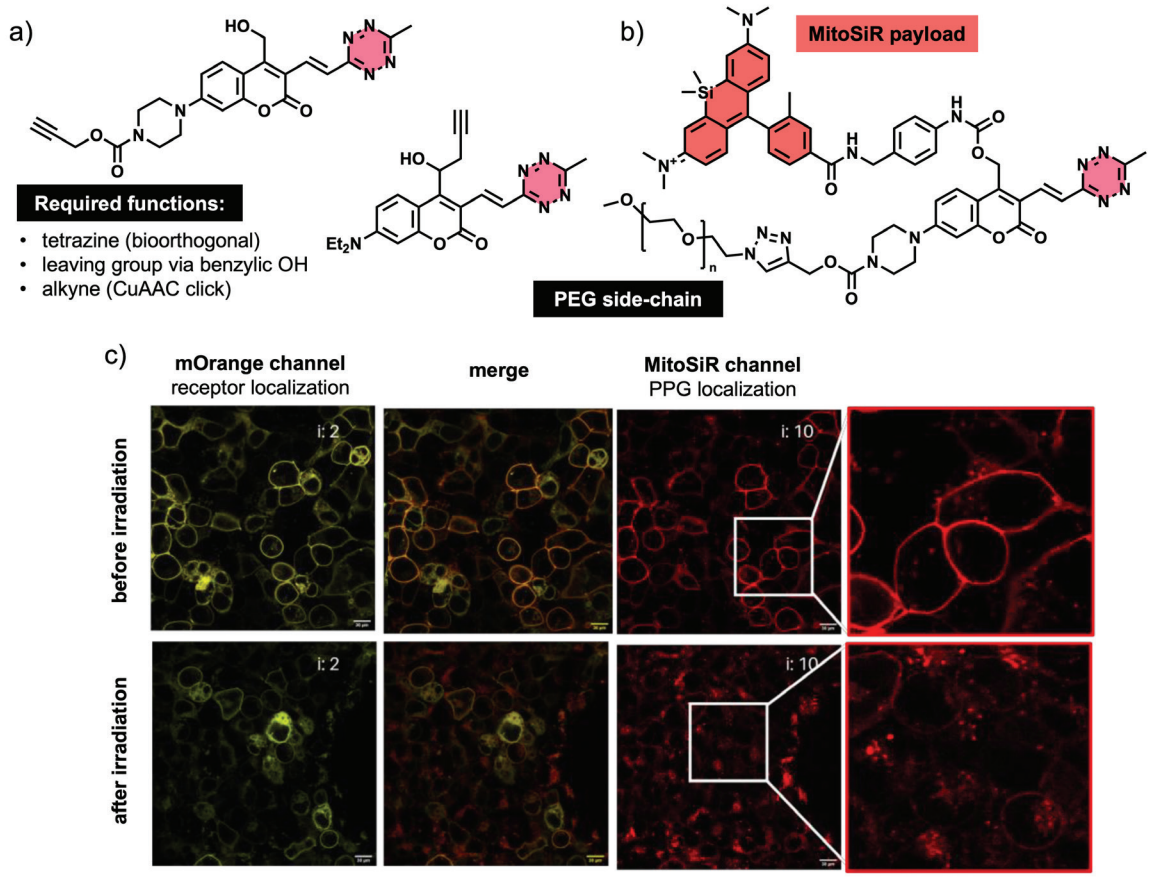

Figure 5 *a) Structure of the key functionalized PPG precursors b) Structure of the PEGylated tetrazine-coumarin PPG linked to the MitoSiR payload c) confocal images HEK cells transfected with IRT^{AG}-mOrange and treated with the MitoSiR conjugate before and after blue light irradiation. Note the high level of membrane signal overlap only before the irradiation as the cargo is liberated from the membrane after blue light*

For these images, we used the pegylated PPG with the MitoSiR cargo and our bioorthogonalized IR^{TAG}-mOrange fusion systems to provide colocalization images of the fluorescent protein and the silicon rhodamine payload. As can be seen in the images, the red fluorescence of our PPG-MitoSiR conjugate overlaps with the mOrange reporter protein only on the cell membarne, demonstrating excellent targeting abilities. However, after blue light irradiation, it is not entirely clear that whether the released MitoSiR is relocated in the mitochondria. Similar or worse results were obtained with different cargos and targeting modalities. Therefore, it can be concluded **that visualizing the entire process in extracellular (e.g., cell-type specific) targeting schemes remains elusive**. The main reason is possibly the lack of appropriate signal originating from the uncaged probes due to their ultra-low concentration. As bioorthogonal chemistry is stoichiometric, the maximum theoretical number of the released payload is in fact limited by the number of targeted receptors, which is very low, around 10^5-10^6 per cell (this, obviously, also applies for classical ADCs). Nevertheless, as the synthetic strategy is already established now we will focus our efforts on **the assembly of these PPGs with highly potent cytotoxic compounds**, such as monomethyl auristatin E. Even with the low number of uncaged payload molecules these are highly effective and indirect responses (such as pretargeting and ligh-dependent toxicities) can be observed in the future.

IV. Development of xanthenium-based photocages

Although not present in the original work plan, one of the most important published results from this project is the **introduction of xanthenium-based PPGs**. Among the emerging phototherapeutic approaches, photoactivated chemotherapy (PACT) has received increasing attention. Despite its advantages and the availability of the directly transferable advanced technology of light delivery from photodynamic therapy, clinical translation of PACT is still hindered by the lack of PPGs suitable for in vivo applications. To be suitable for clinical use, photocage platforms featuring strong one-photon absorption above 600 nm with efficient uncaging cross sections are urgently needed. Often, visible light activatable PPGs are based on classical chromophores used in fluorescent dyes such as coumarins, BODIPYs, porphyrins, or cyanines. (*Chem. Rev.*, **2020**, *120*, 13135.) This palette, however, lacks one of the most popular scaffolds used in fluorescent imaging schemes: xanthenium dyes, such as rhodamines – an absence that is rather surprising. Our concept was that the **conversion of xanthene-derived fluorescent dyes to photocages would have a substantial impact on the phototherapeutic landscape**. With this in mind, we started to investigate how this class of chromophores can be turned into PPGs. While this project presented significant challenges and is certainly an interesting journey, it will only discussed here briefly since it was published entirely in *J. Am. Chem. Soc.* **2023**, *145*, 4026. We have also submitted a PCT patent application (on 08 Aug 2023) about these PPGs.

In short, we have demonstrated that it is possible to **convert certain rhodol-, xanthenium- and carboxanthenium chromophores to photocages by the 9-subsitution with a 2-hydroxyethyl group offering a set off PPGs activatable from green to red (> 650 nm) light**. The PPGs were conjugated to various model cargos in order to demonstrate the versatility of the caging of various functional groups, such as carboxylic acid (caged as ester), amine (as carbamate) and phenol (as carbonate). **Efficient uncaging cross sections without the need of oxygen, excellent water solubility and dark stability was obtained in the conjugates**. As these compounds possess "near ideal" properties as PPGs, they are certainly good candidates for in vivo demonstration of PACT (for the preliminary cellular experiments, see the next section) and novel applications in chemical biology. Our current and future efforts will include the improvement, functionalization and further demonstration of their applicability in light-assisted manipulation techniques.

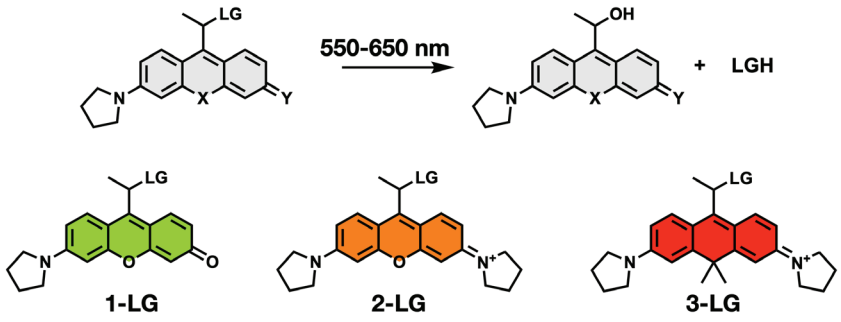

Figure 6 *Structure of the new xanthenium PPGs*

V. Photoactivated chemotherapy across the visible spectrum

Using the set of novel PPGs developed in this project and before (for our redshifted coumarin-based PPGs see: *Org. Lett.* **2019**, *21*, 9410.), we compiled and tested a set of photoactivatable chemotherapeutic agents using several well-known cytotoxic drugs (e.g., doxorubicin, combretastatin A-4, chlorambucil and SN38). As only a part of these were published in the 2023 *J. Am. Chem. Soc.* paper, some further details will be elaborated here. First an irradiation setup was strongly needed for in vitro and in cellulo experiments. Therefore, a series of LED panels with

different emission wavelength were developed that are compatible with the current plates for the biology experiments. These tools became indispensable in our laboratory (and for others in the Institute as well) and are excellent tools for testing visible light absorbing photoresponsive materials. The best results were obtained with the xanthenium PPGs (*published*) and red-shifted coumarin photocages (*unpublished*) for photoactivated chemotherapy using SN38, a topoisomerase inhibitor (for their structure and in cellulo data, see Figure 7).

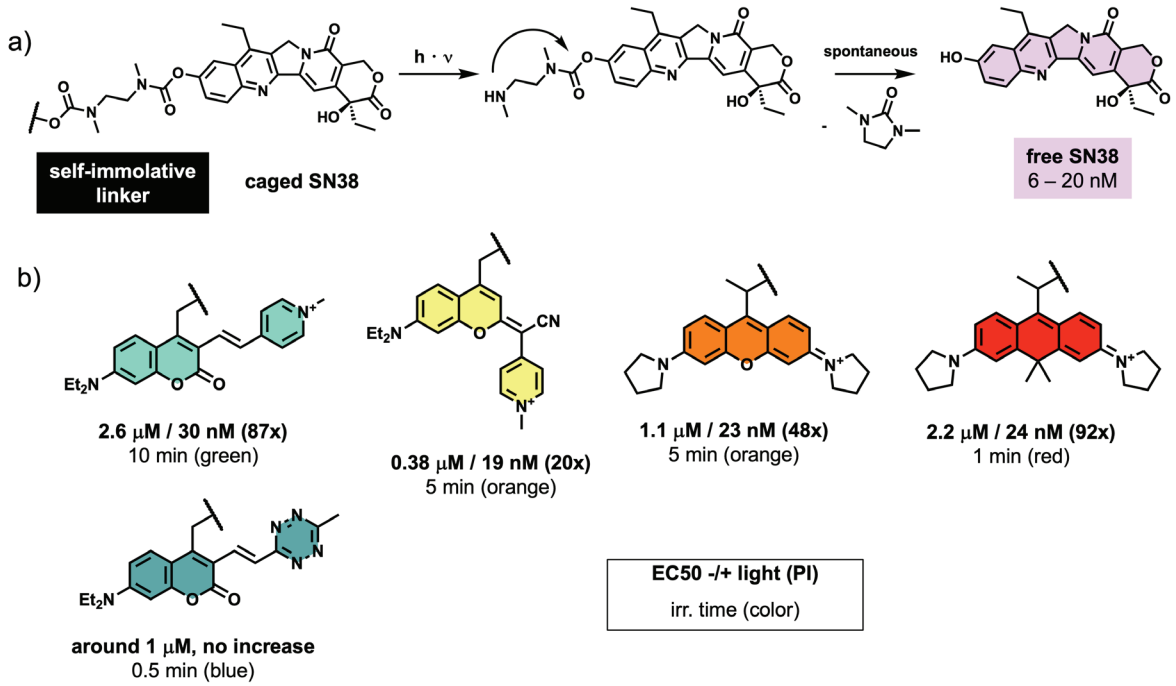

Figure 7 *a) Structure and uncaging mechanism of the blocked SN38 b) structure of some of the photocages that were conjugated to SN38 (connection via the wavy lines). The EC_{50} data for each PPG is shown without/with light irradiation using the custom-made LED panels. PI refers to phototoxicity index*

SN38 in itself is inapplicable in the clinics due to its toxicity, however, can be used in caged prodrugs and special formulations. Although the performance of almost all of these prodrug conjugates were excellent (reaching up to 100-fold increase in toxicity upon green or orange light irradiation for the coumarins, and also 100-fold increase upon red light for the best-performing carboxanthenium PPGs), we partially postponed their publication in order to elaborate the biology experiments (in vitro testing, spheroids or organoids). We have also synthesized conditionally photoactivatable, tetrazine-coumarin-based prodrugs, however, so far only intracellular targeting to mitochondria was tested without significant photo-assisted toxicity increase. Notably, we have also observed that blue light in higher doses can also be toxic for several cell lines, such as SK-OV-3 cells. Further work is currently focused on testing the SN38 derivatives in 3D cell cultures (spheroids) which experiments could also demonstrate the benefits of increased irradiation wavelength. Moreover, the current set of PACT agents activatable from green to red light is ready to be tested in vivo, such as in xenograft tumor models. This ambitious goal certainly requires further funding, collaboration and technology development (e.g., light delivery techniques), however, with these well-performing PPGs in hand, it is now only a question of resources rather than a still unresolved chemistry challenge.

VI. Summary

During the course of this research project several important key results were achieved according to the original work plan and beyond. These will be compiled in a list format together with their impact. As the first proof-of-concept demonstration was already published a month before the beginning of this project, it is not included in the list.

Key results - *impact and implications*

1. **Extended application of intracellularly targeted, conditionally activatable PPGs** – *it is possible to deliver photoactivatable small molecules to targetable subcellular compartments or locations*
2. **Multiple cellular model systems for intra- and extracellular targeting and evaluation of bioorthogonal probes and PPGs** – *we now have several modalities we can use to investigate the performance of bioorthogonal chemical tools, either photoactivatable constructs or probes; we can also use antibody-based pretargeting to cancer cells*
3. **Synthetic access to membrane-impermeable tetrazine-PPGs** – *it is possible to synthesize bioorthogonal PPG-highly potent drug conjugates that can be used with the bioorthogonalized small-molecule ligands or antibiodies; this synthetic approach can be applied to other PPGs, such as xantheniums for their functionalization and membrane impermeabilization*
4. **Development of xanthenium PPGs** – *a novel set of "near ideal" photocages can have a transformative effect on the phototherapeutics landscape as well as provides a chemical toolbox to the scientific community with unique means for highly controlled manipulation of biological systems*
5. **Photoactivated SN38 prodrugs with high photo-assisted toxicity indices**– *it is now possible to activate the highly toxic topoisomerase inhibitor using green/orange and even red light; the best performing visible light-activatable SN38 derivatives are suitable for in vivo experiments to facilitate clinical translation of PACT*

Furthermore, the present results were the subject of 4 BSc theses, 5 MSc theses, a PhD thesis (in part, Egyed Alexandra) and 3 awards at the University/National Scientific Competition (TDK, OTDK)

VII. Dissemination of results

Publications (total IF: 47.0)
(note that the 2020 J. Am. Chem. Soc. paper is not included here)

- Kozma, E.; Bojtár, M.; Kele, P. Bioorthogonally Assisted Phototherapy: Recent Advances and Prospects. *Angew. Chem. Int. Ed.* **2023**, *62* (33), e202303198. https://doi.org/10.1002/anie.202303198. IF: 16.686 independent citations: 1
- Egyed, A.; Németh, K.; Molnár, T. Á.; Kállay, M.; Kele, P.; Bojtár, M. Turning Red without Feeling Embarrassed—Xanthenium-Based Photocages for Red-Light-Activated Phototherapeutics. *J. Am. Chem. Soc.* **2023**, *145* (7), 4026–4034. https://doi.org/10.1021/jacs.2c11499. IF: 15.0 independent citations: 3
- Albitz, E.; Kern, D.; Kormos, A.; Bojtár, M.; Török, G.; Biró, A.; Szatmári, Á.; Németh, K.; Kele, P. Bioorthogonal Ligation-Activated Fluorogenic FRET Dyads. *Angew. Chem. Int. Ed.* **2022**, *61* (6), e202111855. https://doi.org/10.1002/anie.202111855. IF: 15.336 independent citations: 3

Note that these publications were made Open Access.

Submitted publications under review with acknowledgement of NKFIH-PD-135121:

- Bálint, D.; Póti, Á. L.; Alexa, A.; Sok, P.; Albert, K.; Torda, L.; Földesi-Nagy, D.; Csókás, D.; Imre, T.; Szarka, E.; Bento, I.; Bojtár, M.; Palkó, R.; Pápai, I.; Soós, T.; Reményi, A. Reversible covalent c-Jun N-terminal kinase (JNK) inhibitors targeting a specific cysteine by precision-guided Michael-acceptor warheads. *Submitted to Nature Communications on 22nd Aug 2023*
- *Hessz, D.; Kiss, E.; Bojtár, M.; Kunfi, A; Mester, D.; Kállay, M.; Kubinyi, M. Photochemistry of a water-soluble coumarin-based photoswitch. Submitted to Dyes and Pigments on 18 Jul 2023*

Important oral and poster presentations where NKFIH-PD-135121 is acknowledged:

- Bojtár Márton, Egyed Alexandra, Németh Krisztina, Albitz Evelin, Csorba Zsóka, Molnár Tibor, Kele Péter: Új generációs fényre hasítható védőcsoportok (oral). Heterociklusos és Elemorganikus Kémiai Munkabizottsági ülés, 01 Jun 2023, Balatonszemes (2023)
- Evelin Albitz, Dóra Kern, Attila Kormos, Márton Bojtár, György Török, Adrienn Biró, Ágnes Szatmári, Krisztina Németh, Péter Kele – Bioorthogonal ligation-activated fluorogenic FRET dyads (poster). Cell Bio An ASCB / EMBO Meeting, 03-07 Dec 2022, Washington DC, USA
- Márton Bojtár, Evelin Albitz, Zsóka Csorba, Krisztina Németh, Péter Kele – Conditional Uncaging Based on Bioorthogonal Chemistry (poster). Bay Area Chemical Symposium, 10 Nov 2022, Berkeley, CA, USA
- Evelin Albitz, Dóra Kern, Attila Kormos, Márton Bojtár, György Török, Adrienn Biró, Ágnes Szatmári, Krisztina Németh, Péter Kele – Bioorthogonal ligation-activated fluorogenic FRET dyads (poster). EMBO/EMBL Workshop Chemical Biology, 05-08 Sept 2022, Heidelberg, Germany