Applications of *ortho*-Nitrobenzyl photocage in cancer-biology

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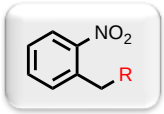
Abstract: The ability to achieve spatial and temporal control over biological processes and drug activation has led to many applications of "photocages". By definition, photocages are chemical structures that are light-sensitive and is used to "cage" or restrict the activity of any molecule. Upon illumination of light at appropriate wavelength, the protecting group of the molecule is cleaved releasing the active entity. Many recent reports in research areas including molecular biology, neurosciences and drug delivery have demonstrated the applications of photocages. In this write-up, we provide a brief overview on commonly used photocages, *ortho*-Nitrobenzyl (*o*-NB) derivatives that are relevant to cancer-biology, and emphasize upon the usefulness and advantages of using these photosensitive molecules.
Keywords: Photocages, ortho-nitrobenzyl, opto-genetics, cancer-biology, biomolecules

Photocages or photocleavable-protecting groups are being used extensively in biomedical research to temporarily quench activity of either biomolecules [1] or drugs[2,3]. Some of the most commonly used photocages are ortho-nitrobenzyl (*o*-NB) derivatives that have many applications in opto-genetics [4] and drug-release[5,6] research areas. A general approach to use a photocage is to couple it with a functional group, commonly amine (-NH_2) or hydroxyl (-OH) that may be central to the activity of a given molecule; thereby restricting its activity. But, under light-irradiation and at an appropriate wavelength (commonly 360 nm), photocages can be cleaved, uncloaking the active functional group. Multiple cellular events that occur in mammalian cells have been studied using *o-NB* derivatives[1]. Additionally, there are several reports that demonstrated optical control of both cas9 and guide RNAs (gRNAs) in CRISPR-cas9 gene-editing technique using *o-NB*[7]. Although, most events were described in normal immortalized cells, a similar strategy is helpful to understand analogous or novel events in cancer cells.

One of the key challenges to achieve spatio-temporal control in cells has been the ability to incorporate photocages into proteins. In one of the initial approaches, photocaged entities were microinjected directly into cells to study relevant cellular events [8,9]. Later, Schultz and coworkers demonstrated, via genetic code expansion, the introduction of photosensitive unnatural amino acids into protein sequences using orthogonal aminoacyl-tRNA synthetase (aaRS)/$tRNA_{CUA}$ pairs. This allowed for conditional regulation of protein functions



R = Functional groups such as -NH_2; -OH, -SH

Figure:1 Chemical structure of *ortho*-Nitrobenzyl photocage

including those associated with migration, apoptosis and other events in cells and animal models[10,11]. This approach was also helpful in understanding cancer related pathways[12]. An attempt to elucidate all relevant examples describing applications of caged molecules in understanding cellular processes is beyond the scope and theme of this article, however in an effort to highlight the relevance of this approach to cancer, we briefly describe a few representative examples here. To understand the techniques and applications of this approach in biological systems and animal models, please refer to the works of Alexander Deiters at Pittsburgh, David Lawrence at UNC and Ellis-

Davies at Mt. Sinai[13–15]. However, a caveat associated with this technique is that the applications are mostly demonstrated *in vitro,* while *in vivo* applications remain limited.

Genetic Code Expansion

Although life has chosen the 20 proteogenic amino acids to perform all of its necessary biochemistry, scientists have developed mechanisms to force *in vitro* and *in vivo* systems

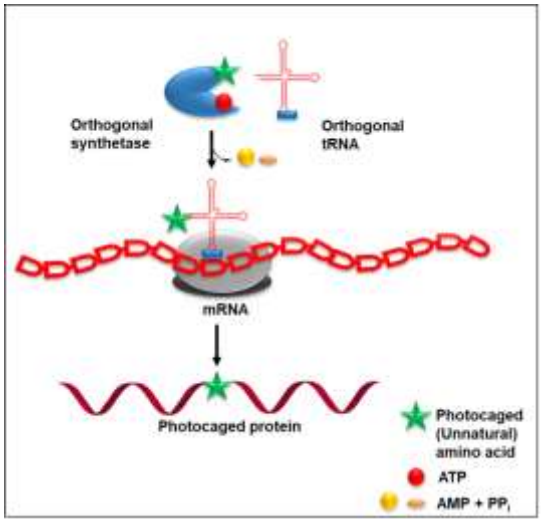


Figure: 2 Mechanism of incorporating a photocaged amino acid into a protein sequence using genetic code expansion technique

to utilize amino acids outside of these 20, dubbed non- canonical amino acids (ncAAs)[16]. The ability to create proteins with amino acids with unusual functionality allows researchers to create novels systems to probe protein functionality. Creation of ncAA-containing proteins *in vivo* is dependent on supplementation of an orthogonal aminoacyl tRNA synthetase/tRNA pair capable of reading through a stop codon[17]. An especially powerful application of this system is to introduce photocaged amino acids into proteins *in vivo*[10]. This allows researchers a powerful spatio-temporal platform to control conditional regulation of proteins. For example, Schultz and coworkers have impressively demonstrated that introduction of photosensitive ncAAs can be used to elucidate complex cellular mechanisms involved in migration and apoptosis.[18,19] In addition several groups applied this technique to understand the importance of a certain protein, in the context of cancer. Below, we highlight few representative examples.

Understanding Kinome

Protein phosphorylation is a well studied post-translational modification that occurs in both healthy and diseased cells alike[20]. It is a reversible reaction facilitated by a kinase wherein a side chain of an amino acid with a hydroxyl group (Ser, Thr and Tyr) is tagged with phosphate group. This simple change can initiate a cascade of signaling events in cancer cells leading to differentiation, survival, apoptosis etc[21]. Hence, kinases have been the focus of many small molecule drug discovery campaigns that have lead to robust treatments[22]. Currently, 37 inhibitors have received approval from US-FDA to target different kinases such as tyrosine kinases. With these successes, the momentum to understand kinases to find better inhibitors has been well supported.

In addition to the classical techniques such as site-directed mutagenesis and proteomics, multiple chemical biology tools, including photosensitive tools are also being used to understand activity of these proteins[1]. A literature survey yielded multiple examples of application of photocages in studies related to kinases and other enzymes, wherein a general strategy seems to be photocaging residues responsible for its activity in the active motif. A model example could be found in an approach by Chin and co-workers that described a common strategy to photocontrol activity of kinases by incorporating photocaged lysine (a universally conserved lysine in all kinases) in ATP binding pocket of MEK1 using PCKRS/tRNA (photocaged Lysyl-tRNA synthetase)[23]. Using this approach, the kinetics of the signaling cascade of Raf/MEK/ERK was studied in HEK293T cells. In a different application, Allbritton and co-workers developed a cell-permeable, photoactivatable reporter that could be used as a sensor of Akt activity (Protein kinase B), an oncogenic kinase relevant in multiple cancer types[24]. The reporter

molecule, a peptido-mimetic Akt specific substrate, was synthesized with an inclusion of DMNB (2-4,5-dimethoxy 2-nitrobenzyl) that caged a threonine residue, simultaneously enhancing its permeability. Besides increasing the permeability, DMNB protected the peptide from Akt phosphorylation. Using a fully automated system for serial single-cell capillary electrophoresis, the authors were able to quantify Akt activity in pancreatic cancer cells (PANC-1), but only after light irradiation. According to the authors, this approach of monitoring a kinase activity superseded the ones that were previously reported.

Understanding aberrant metabolism

An essential feature of many cancers is their aberrant metabolic profile[25]. Multiple enzymes that partake in cell metabolism have altered metabolic activities [26]. Isocitrate dehydrogenase 2 (IDH2)[27] encoded by the *IDH2* gene is a mitochondrial enzyme that plays an intermediary role in metabolism by facilitating the conversion of isocitrate (ICT) to 2-oxoglutarate (2-KG) in the presence of NADP(+). Monoallelic mutations in *IDH2* (predominantly Arg172) have diminished abilities to oxidize, but gain neomorphic enzymatic activity in converting 2-KG to 2-Hydroxy glutarate (2-HG), a putative oncometabolite and an important biomarker in different cancer types including glioma, AML, etc. 2-HG also modulates the activity of TET (Ten-Eleven translocation) enzyme family that facilitate conversion of 5mc (5-methylcytosine) to 5hmC (5-hydroxymethylcytosine), which has been proposed to promote the demethylation of DNA. Currently, research is underway to identify better inhibitors of IDH2[28].

Chin and coworkers used genetic code expansion approach to generate photocaged lysine and to study IDH2 activity [29]. In this study, Arg172 of IDH2 was replaced with photocaged Lysine (PCK), which, unlike inducible-systems like doxycycline, circumvents the entire transcriptional and translational processes and activates protein right after light irradiation. In their work, upon light-mediated uncaging of PCK there was a rapid increase in 2-HG levels comparable to those found in patient samples. Simultaneously, the levels of 5-hmc dropped due to excessive 2-HG levels. An important finding from this work was that the low levels of 5-hmc was consistent even after 72 h of light irradiation, indicating that the depletion is an early consequence of mutant IDH2 activity.

Altering Transcriptional regulation

Light has been recognized as an ideal external trigger to alter gene expression[30]. Specifically, the *o*-NB derivatives are being repeatedly used to cage nucleo-bases, transcription regulating proteins, and even proteins that partake in epigenetic modifications, thereby fostering a photo-controlled gene-expression system[13]. The installation of photocages are already being demonstrated as substitutes to small-molecule dependent inducible systems, which carry its own disadvantages. Several groups have efficiently described the application of photocages in altering gene expression in animal models, which indicates that it is perhaps possible to use this approach in treating human diseases[1]. For example, Chen and coworkers illustrated an example describing photocontrolled gene silencing wherein *ntl* (no tail), a zebrafish homolog of the mouse T (Brachyury) gene, was targeted using photocaged morpholino unit[31]. The derivative of *o*-NB served as a photo-immolative linker that was used to couple *ntl* RNA blocking morpholino unit (*ntl* MO) and a complementary oligonucleotide that inhibits the activity of *ntl* MO. The *Ntl* transcription factor is essential for the development of tail and notochord. Hence, when a caged moiety was injected into wild-type zebrafish embryos at one-cell stage and irradiated at the sphere stage, the development of the embryo was impaired assimilating embryos with mutant *ntl* gene. The dark-adapted embryo unsurprisingly appeared similar to the wild-type *ntl* gene expression. In an illustrative example of photocontrolled gene overexpression in animals, Deiters and coworkers microinjected a EGFP (Enhanced Green Fluorescent Protein) plasmid that had

caged thymidine nucleobases in TATA box of promoter region in zebrafish embryos at one-cell stage[32]. In dark adapted stage, there was absolutely no expression of EGFP despite the presence of genetic content, but under the irradiation of light the expression of protein is restored. These, and other referenced examples indicate that the transcriptional and translational machinery could be manipulated with the help of photocage units.

Conclusion: One of the reasons for the success of photosensitive molecules in biomedical research has been its ability to "alter" or "pause" the activity of a gene or a protein, in a cancer-associated pathway, which further augmented the understanding of disease related phenomenon. In addition, photosensitive molecules have also been used as drug-mimetics or in drug-delivery system designs, to target oncogenic activities. In this write-up, we have described generic approaches that employs photocages especially *o*-NB, to understand certain pathways in cancer biology. Instead of site-directed mutagenesis, researchers can resort to this approach to conditionally "switch-off/on" the protein's activity under light-irradiation.

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