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Gene Therapy

Towards a Light-mediated Gene Therapy for the Eye using Caged Ethinylestradiol and the Inducible Cre/lox System

Zoe Kiy, Juliane Chaud, Liang Xu, Eric Brandhorst, Tschackad Kamali, Carolyn Vargas, Sandro Keller, Huixiao Hong, Alexandre Specht,* and Sidney Cambridge*

Abstract: Increasingly, retinal pathologies are being treated with virus-mediated gene therapies. To be able to target viral transgene expression specifically to the pathological regions of the retina with light, we established an in vivo photoactivated gene expression paradigm for retinal tissue. Based on the inducible Cre/lox system, we discovered that ethinylestradiol is a suitable alternative to Tamoxifen as ethinylestradiol is more amenable to modification with photosensitive protecting compounds, i.e., “caging.” Identification of ethinylestradiol as a ligand for the mutated human estradiol receptor was supported by in silico binding studies showing the reduced binding of caged ethinylestradiol. Caged ethinylestradiol was injected into the eyes of double transgenic GFAP-CreERT2 mice with a Cre-dependent tdTomato reporter transgene followed by irradiation with light of 450 nm. Photoactivation significantly increased retinal tdTomato expression compared to controls. We thus demonstrated a first step towards the development of a targeted, light-mediated gene therapy for the eyes.

Systems that allow a non-invasive control of the release of bioactive or therapeutic molecules in living organisms are highly sought after.^[1] They are of interest in basic sciences to dissect dynamic biological systems, but could also be utilized

for personalized medicine. Consequently, emphasis has been placed on technologies that would allow the control of specific aspects of biological systems with highly resolved, spatiotemporal perturbations.^[2] Methods enabling light to precisely manipulate cellular processes - loosely gathered under the term of “optogenetics” - have been developed and successfully applied in living organisms.^[3] In particular, controlling transgene expression with light can be a very efficient way to regulate cellular activities during physiological or pathological conditions. Research questions across different biological fields, such as developmental biology and neuroscience, would strongly benefit from such a method, as would other biomedical applications. The photochemical regulation of gene function is therefore a rapidly advancing research field.^[4] Besides fully genetically encoded photoactivated gene expression methods,^[5] synthetic compounds as photo-responsive transgene inducers are particularly attractive for biomedical applications because these compounds can be washed in and out of the biological system, including patients. Unlike the permanently light-responsive, genetically encoded methods, washing out a photo-sensitive inducer turns off the transgene method at will, rendering the biological system insensitive to unspecific activation by incumbent light.

For biomedical applications, particularly for the photochemical regulation^[7–9] of transgenes, a straightforward approach is to reversibly inhibit small molecule inducers with photosensitive protecting compounds (“caging”) that

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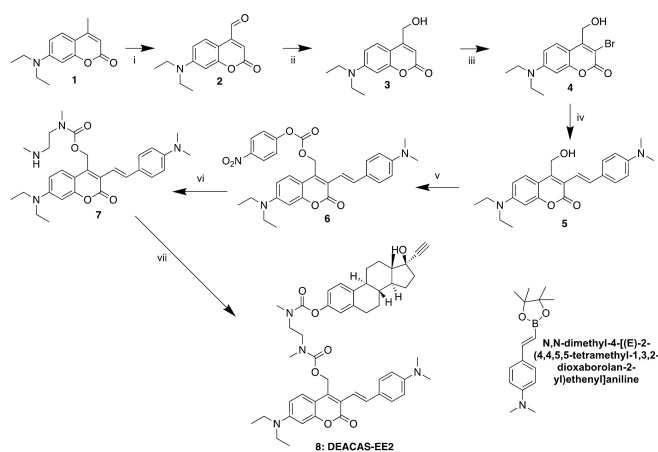
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are routinely used in combination with inducible transgene expression systems. The most prominent systems are the inducible Cre/lox system and the Tet-On/Off system using caged doxycycline analogs^[6d,10] or caged estradiol,^[11] tamoxifen-OH,^[12] and cyclofen-OH, respectively.^[13] However, to be useful for in vivo applications, light-induced release of small-molecule inducers must be highly efficient and triggered by wavelengths that can innocuously penetrate deeply into tissues.^[1,14] Light penetration depth in tissues is wavelength-dependent^[15] and most of the caged small molecule inducers depend on photolytic reactions which respond to light excitation from UV to the near visible range. For applications in eyes however, light-mediated gene therapy should be based on visible light excitation, ideally with wavelengths >450 nm.^[16] Exciting recent progress, particularly using the coumarinyl scaffolds,^[17] has extended the uncaging concept in wavelengths well into the visible range with excellent uncaging efficiencies in the blue light region.^[17]

Steroid hormones and their receptors have extensively been used for transgene expression methods,^[18] in particular the inducible Cre/lox^[19] system which is based on a protein fusion between Cre recombinase and a mutated form of the estrogen receptor (ER), designated Cre-ERT2.^[20] The mutations ensure that endogenous estradiol does not bind to the mutated human ER (hER) and unspecifically induce transgene expression via Cre recombinase. Instead, the estradiol analog Tamoxifen-OH can efficiently be used for induction. Together with standard, non-inducible Cre recombinase expression (i.e., without ER), this method is now the leading approach for in vivo transgene activation in mice as witnessed by almost one thousand different Cre-related transgenic mouse lines (JAX mice resource). Photoactivatable, i.e., caged Tamoxifen-OH, analogs have been developed,^[12] but the photochemical reactivity of unmodified Tamoxifen-OH is still an important limitation for accurate and clean control of ERT2 with light.^[21] We therefore decided to test several non-endogenous estrones in order to identify alternative small molecules that could potentially activate Cre-ERT2. 17 α -ethinylestradiol (EE2), oral contraception, exhibited the best chemical and biological profile. We then modified this compound with a recently developed photo-removable protecting group (PPG)^[17d] in order to synthesize a blue light sensitive photoactivatable inducer. Importantly, this caged EE2 could be used for efficient induction of retinal transgene expression in vivo after photoactivation of mouse eyes with 450 nm (blue) light. For personalized medicine in ophthalmology, this constitutes a key first step in the development of light-mediated gene therapies for the retina.

To synthesize a caged EE2 analog that could be photoactivated by blue light, we employed the following strategy. Starting from commercially available 7-(diethylamino)-4-methyl-2H-chromen-2-one (**1**, Scheme 1), 7-(diethylamino)-2-oxo-2H-chromene-4-carbaldehyde (**2**, scheme 1) was synthesized with 78 % yield in two steps using an enamine intermediate.^[17] Noteworthy, this latter strategy was chosen instead of the direct oxidation of **1** by selenium oxide^[17g] since it allowed a larger scale synthesis of **2**. The aldehyde **2**



Scheme 1. Synthesis of DEACAS-EE2: i) a) DMF-DMA, DMF, reflux, 16 h, quant. b) NaIO₄, THF/H₂O, 0 °C to RT, 1 h, 78 %, over two steps, ii) NaBH₄, THF, 0 °C to RT, 3 h, 70 %, iii) NBS, ammonium acetate, anhydrous acetonitrile, RT, 40 min, 81 %, iv) N,N-dimethyl-4-[(E)-2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)ethenyl]aniline, K₂CO₃, Pd(PPh₃)₄, H₂O/DME, in the microwave at 105 °C, 45 min, 79 %, v) *p*-nitrophenyl carbonate, DIPEA, DMF, RT, 16 h, 51 %, vi) N,N'-dimethylethylenediamine, DIPEA, DMAP, THF, 0 °C to RT, 3 h, no purification due to the instability of the compound, vii) a) Triphosgene, DIPEA, CH₂Cl₂, 5 °C, 1 h, b) EE2, DMAP, DIPEA, DMF, RT, 30 h, 61 % over two steps.

was reduced to the corresponding alcohol (**3**, scheme 1) and converted to the brominated derivate (**4**, scheme 1) using N-bromosuccinimide. Then, a Suzuki–Miyaura cross-coupling reaction was carried out using freshly prepared (*E*)-N,N-Dimethyl-4-(2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)vinyl)aniline^[22] leading to the DEACAS PPG (**5**, scheme 1) in 82 % yield. Of note, we were not able to directly couple this PPG through a conventional ether linkage to EE2 using a methanesulfonate analog of **5**. Therefore, we decided to use an extension of the linkage through a self-immolative carbamate linker previously reported by the groups of Krummel and Choi.^[12e] The DEACAS-EE2 caged compound was synthesized with 31 % yield in four steps using alcohol **5**, commercially available EE2 and N,N'-dimethylethylenediamine. Therefore, **5** was first converted to the corresponding *p*-nitro-phenyl-carbonate (**6**, scheme 1). The *p*-nitrophenol group was then substituted by N,N'-dimethylethylenediamine leading to the formation of **7**. This latter unstable compound was converted in situ to the corresponding chloroformate intermediate leading to the formation of the DEACAS-EE2 caged compound after treatment with 1.5 equivalents of EE2 in the presence of 4-dimethylaminopyridine (DMAP).

Next, the photoinduced liberation of EE2 from DEACAS-EE2 was monitored by UV/Visible spectroscopy and HPLC. Photolysis was carried out by irradiation of samples (11 or 20 μ M) at 430 nm in 0.1 M PBS buffer pH 7.3 or a 1:1 (v/v) mixture of MeOH/ 0.1 M PBS buffer pH 7.3, respectively (see Supporting Information). During the photolysis of DEACAS-EE2, a new broad absorbance at 385 nm appeared (i.e., indicating the formation of the expected uncaging side-product^[17d]) whilst the initial absorbance at

446 nm gradually diminished. Additionally, the appearance of an isosbestic point at 405 nm was observed, indicating homogeneous reactions. The release of EE2 was also measured by HPLC (Supplementary Figure S2) together with the formation of the DEACAS alcohol **5** and its methyl ether analogs (see Supporting Information). The hydrolytic stability was explored by HPLC in a 0.1 M PBS buffer pH 7.3 at room temperature (in the dark) indicating no significant hydrolysis after 5 days (see Supporting Information). The quantum yield for EE2 release was assessed to be 0.12 by competition with the *p*-methoxybenzoic acid (*p*-MBA) caged DEACAS analog^[17d] as a reference molecule (see Supporting Information and Figure S3), producing an uncaging efficiency of 4900 M⁻¹ cm⁻¹ for the DEACAS-EE2 compound.

In combination with the inducible Cre/lox system, EE2 has so far not been described as a suitable substitute for Tamoxifen. To confirm that EE2 exhibited a high affinity for the mutated ER in comparison to the reduced affinity of DEACAS-EE2, we used in silico modeling of molecular docking to the receptor.^[23] The predicted binding affinity values from the molecular docking for estradiol, 4-hydroxytamoxifen, EE2, and DEACAS-EE2 are shown in Table 1. The positive binding energies for the DEACAS-EE2 confirmed that the caged compound could not bind to the ligand-binding domain (LBD) of hER containing three mutations (G440V, M543A, and L544A).^[20] Conversely, EE2 displayed the same low binding energy (−9.5 kcal/mol) as 4-hydroxytamoxifen indicating that both compounds strongly bind to the mutated hER. Notably, the mutations in hER were originally introduced to reduce binding of endogenous estradiol.^[20] This modeling analysis corroborates the reduced binding affinity of estradiol.

Figure 1 shows the binding poses of EE2 and caged EE2 in the LBD of the mutated hER. EE2 occupies the binding pocket through interacting with residues Met343, Leu346, Leu349, Ala350, Glu353, Leu384, Leu387, Met388, Leu391, Arg394, Phe404, Gly420, Met421, Ile424, His524, and Leu525 of the mutant hER. However, the EE2 motif of the DEACAS-EE2 could not occupy the same binding pocket.

We first confirmed in dissociated neurons in vitro that 4-hydroxytamoxifen and EE2 indeed exhibit comparable efficiencies in activating transgene expression via the inducible Cre/lox system (Figure S4). Next, we demonstrated that caging of EE2 did not interfere with its ability to passively enter cells. Taking advantage of the innate fluorescence of the coumarin-based photoprotection group, we detected bright green cellular fluorescence with DEACAS-EE2 compared to control cells (Figure S5A). The cellular uptake of a steroid such as EE2 is facilitated by an increase in the steroid's hydrophobicity, which promotes both binding to the lipid bilayer and membrane translocation (flip-flop).^[24] We therefore measured and compared

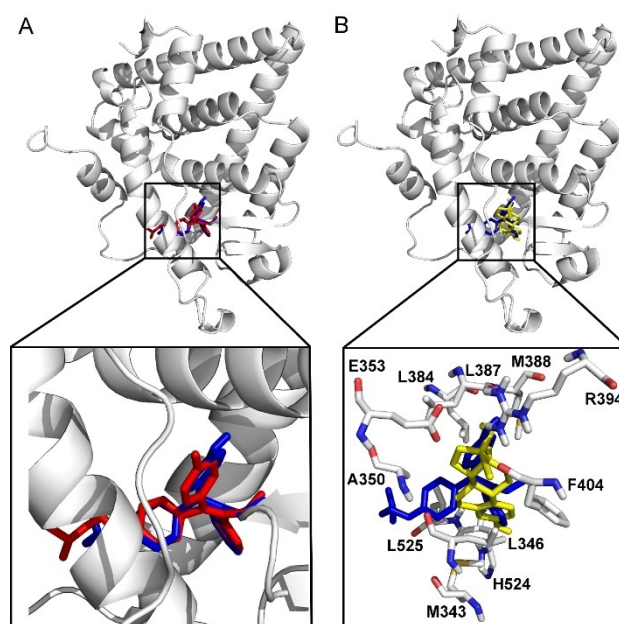


Figure 1. (A) Superimposition of the binding pose of 4-hydroxytamoxifen in the crystal structure of the LBD of hER (red) with the docked 4-hydroxytamoxifen (blue). (B) Binding of 4-hydroxytamoxifen (blue) and EE2 (yellow) in the binding pocket. Residues interacting with 4-hydroxytamoxifen and EE2 are shown and labeled.

the interactions of EE2 vs. caged EE2 with phospholipid bilayers using high-sensitivity isothermal titration calorimetry (ITC).^[25–26] Our ITC experiments confirm that the increased hydrophobic nature of caged EE2 compared to EE2 results in strong interactions with phospholipid membranes, suggesting straightforward cellular uptake (Figure S5B). We also verified that DEACAS-EE2 administration, irradiation, or both did not affect neuronal morphology (Figure S6).

Next, we validated the potential of DEACAS-EE2 for photoactivated gene expression in ex vivo hippocampal organotypic cultures (OTCs)^[27] obtained from double transgenic mice. In combination, the two transgenes GFAP-CreERT2^[28] and Cre-dependent tdTomato (line Ai14)^[29] produce robust red-fluorescent tdTomato expression in astrocytic glia cells upon activation of CreERT2. Incubation of transgenic hippocampal OTCs with DEACAS-EE2 for 24 hours in the dark followed by brief irradiation (15 sec) with 470 nm substantially induced tdTomato expression (Figure 2A, left). Control, unirradiated cultures did not exhibit any transgene expression under these conditions indicating that DEACAS-EE2 in combination with the inducible Cre/lox system can provide a robust on/off system for transgene induction (Figure 2A, right). We observed a sharp threshold between 10 μM and 30 μM, as photoactivation only produced substantial transgene expression at the higher 30 μM concentration (Figure 2B). While molecular modeling suggested a reduced affinity of DEACAS-EE2 for the mutant hER, we observed at high DEACAS-EE2 concentrations (>100 μM) residual tdTomato expression,

Table 1: Predicted binding affinity from molecular docking studies

Ligand	4-OH Tamoxifen	EE2	Estradiol	DEACAS-EE2
Binding Energy (kcal/mol)	−9.5	−9.5	−9.2	8.8

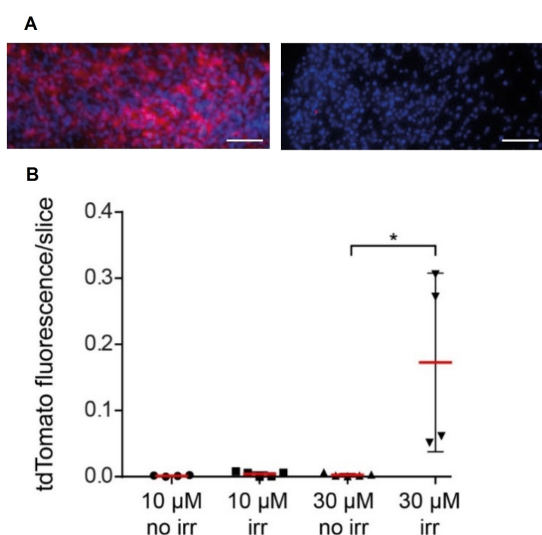


Figure 2. Photoactivation of DEACAS-EE2 in P8 OTCs from GFAP-CreERT2: Ai14 mice **A:** Example images of OTCs incubated with 30 μ M DEACAS-EE2 (tdTomato staining in red, DAPI in blue, scale bar: 75 μ m). Left: widespread tdTomato expression 7 days after irradiation. Right: no transgene expression was induced without irradiation. **B:** OTCs were incubated with DEACAS-EE2 at the indicated concentrations for 24 h, irradiated ("irr"), and induced tdTomato fluorescence quantified and compared to control slices that were not irradiated. Each data point corresponds to the overall fluorescence from one slice (Mann-Whitney test; mean in red with SD error bars, $P < 0.05$).

similar to the residual transgene expression seen with high concentrations of caged doxycycline^[10a] (Figure S7).

For photoactivated transgene induction in retinas in vivo, we employed the same double transgenic GFAP-CreERT2: Ai14 mice. In control experiments, retinal transgene expression was robust upon direct, intravitreal injection of EE2 into the eyes (Figure 3A, left). Mice were thus injected with 1 μ l of 10 mM DEACAS-EE2, incubated for 24 hours, and eyes irradiated for 30 min using an ophthalmoscope equipped with a 450 nm laser. This protocol reproducibly induced significant retinal tdTomato expression (Figure 3A, right) compared to injected, but not irradiated eyes (Figure 3A, middle). Quantification of tdTomato fluorescence of whole retinas from successful photoactivation experiments (Material and Methods) showed that irradiation roughly doubled transgene expression which is considerable for inducible conditional transgene expression systems^[30] (Figure 3B, Figure S8).

Of note, retinal transgene expression in unirradiated eyes injected with DEACAS-EE2 was significantly higher than in unirradiated control eyes injected with vehicle alone. Since we did not detect any DEACAS-EE2 hydrolysis for 48 h at room temperature and also did not observe tdTomato expression in unirradiated ex vivo OTCs, we believe that optimization of the in vivo photoactivation protocol, i.e., injection of less caged EE2 and/or better shielding of post-injection mice from ambient light, will further reduce unspecific transgene expression in unirradiated eyes. Also, this irradiation scheme did not lead to

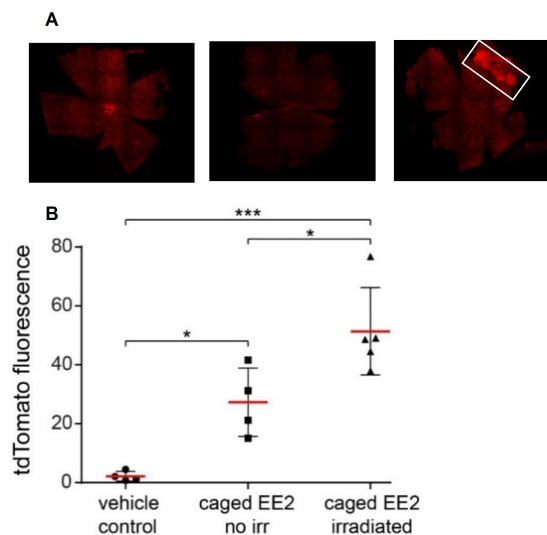


Figure 3. Photoactivation of DEACAS-EE2 in mouse eyes in vivo induced retinal tdTomato expression **(A)** Example images of retinas from eyes injected with unmodified EE2 (left), DEACAS-EE2 but not irradiated (middle), and DEACAS-EE2 and irradiated (right). Note the grid-like pattern in all images because of assembly from tiled images; the artefact within the white rectangle was not quantified (right) (scale bar: 1 mm). **(B)** Each data point represents one retina. Control injections without DEACAS-EE2 did not produce any fluorescence. Irradiation of eyes injected with DEACAS-EE2 increased tdTomato fluorescence (number of tdTomato fluorescence positive cells over threshold per mm² retinal area) compared to unirradiated eyes (one-way ANOVA, multiple comparisons, $P = 0.003$).

significant apoptosis as independently verified in control experiments (Figure S9).

We show here that in vivo photoactivated transgene expression in retinas is now possible. By introducing EE2 as a suitable substitute for Tamoxifen and modifying EE2 with a caging moiety that can be photolyzed with blue light, we tailored our transgene expression approach towards future applications in humans. EE2 as the active ingredient of oral contraceptives is of course approved for use in humans. Intravitreal injections of compounds were most efficient for retinal transgene expression although we found that topical administration through eye drops would have been possible as well. Ultimately, we envision a fully non-invasive procedure based on topical application of the viral gene therapy plus the caged compound, followed by harmless irradiation for transgene induction.

We also showed that in silico molecular docking studies of EE2 and caged EE2 can accurately predict binding differences between unmodified parent compounds and their caged analogs. Especially for parent compounds with several side groups, prior docking studies could help to guide organic synthesis efforts by quickly highlighting which side group will produce the most effective inactivation after the caging modification. Overall, our data highlight the need for productive interdisciplinary efforts towards the goal of developing "optogenetics" for future personalized medicine.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Caged Compounds • Inducible Cre/Lox System • Molecular Docking • Photoactivated Transgene Expression • Retina

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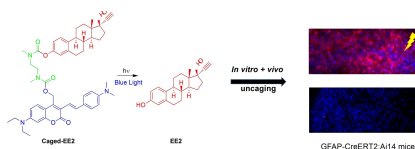
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Communications

Gene Therapy

Z. Kiy, J. Chaud, L. Xu, E. Brandhorst,
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Towards a Light-mediated Gene Therapy for
the Eye using Caged Ethinylestradiol and
the Inducible Cre/lox System



A blue light sensitive caged ethinylestradiol (EE2) was used in combination with the inducible Cre/lox system for photo-activated transgene expression in the living eye. Following intravitreal injection of caged EE2 and irradiation with light at 450 nm, tdTomato reporter gene expression was robustly induced in retinal tissue.