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Mrinal Mandal, Pierluigi Scerbo, Ian Coghill, Jean-françois Riou, Christian Bochet, et al.. Caged Dexamethasone to Photo-control the Development of Embryos through Activation of the Glucocorticoid Receptor. *Chemistry - A European Journal*, 2024, 30 (23), pp.e202400579. 10.1002/chem.202400579 . hal-04765294

HAL Id: hal-04765294

<https://hal.science/hal-04765294v1>

Submitted on 5 Nov 2024

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Caged Dexamethasone to Photo-control the Development of Embryos through Activation of the Glucocorticoid Receptor

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Efficient tools for controlling molecular functions with exquisite spatiotemporal resolution are much in demand to investigate biological processes in living systems. Here we report an easily synthesized caged dexamethasone for photo-activating cytoplasmic proteins fused to the glucocorticoid receptor. In the dark, it is stable *in vitro* as well as *in vivo* in both zebrafish (*Danio rerio*) and *Xenopus sp.*, two significant models of

vertebrates. In contrast, it liberates dexamethasone upon UV illumination, which has been harnessed to interfere with developmental steps in embryos of these animals. Interestingly, this new system is biologically orthogonal to the one for photo-activating proteins fused to the estrogen ERT receptor, which brings great prospect for activating two distinct proteins down to the single cell level.

Introduction

There has recently been an active development of approaches and tools for interrogating biological processes involving complex

interaction networks in living systems.^[1] In particular, optogenetics where light is utilized for driving biomolecular activity has gained great importance for its high spatiotemporal resolution^[2] and opportunities for spectrally orthogonal photoactivations.^[3] Hence, photolabile protecting (caging) groups^[4] or genetically encoded photoactivable triggers have been harnessed to photoactivate the function of native or engineered proteins.^[5] Alternatively, the functions of proteins fused with a non-photoactive site can be light-driven by photo-activating a small permeant caged lipophilic inducer such as estradiol^[6] ecdysone,^[7] 4-hydroxy tamoxifen^[8] cyclofen-OH^[9] or doxycycline.^[10]

Our consortium has extensively used caged cyclofen with one,^[9a] two,^[9a] or three.^[11] photon excitation for photo-controlling gene expression. Recently, we have been interested in developing another caged inducer that would enable biologically orthogonal photo-control of the expression of two different genes. We specifically targeted caged dexamethasone (cDEX) for the photo-activation of cytoplasmic proteins fused to the glucocorticoid receptor (GR).^[12] Upon illumination, it would liberate dexamethasone (DEX) whose binding with GR would release the fused protein from its complex with heat shock proteins (Scheme 1).^[13] Importantly, whereas cyclofen-OH selectively activates proteins fused to the ERT moiety which has been evolved to display absence of activation by endogenous estrogens, DEX is active towards endogenous GRs. Yet, since GR are expressed late in development, cDEX can be used to probe the early stages of embryogenesis^[14] when endogenous GR are absent.

A caged DEX has been already reported and it has been used for spatially and temporally controlling gene expression and plant development in transgenic *Arabidopsis thaliana*.^[15] However, this caged DEX contains a carbonate group, which could be prone to hydrolysis in other living organisms. For this reason, we decided to develop an alternative caged dexamethasone that would be stable in vertebrates and could be used in studies of development and cancer research in two well-

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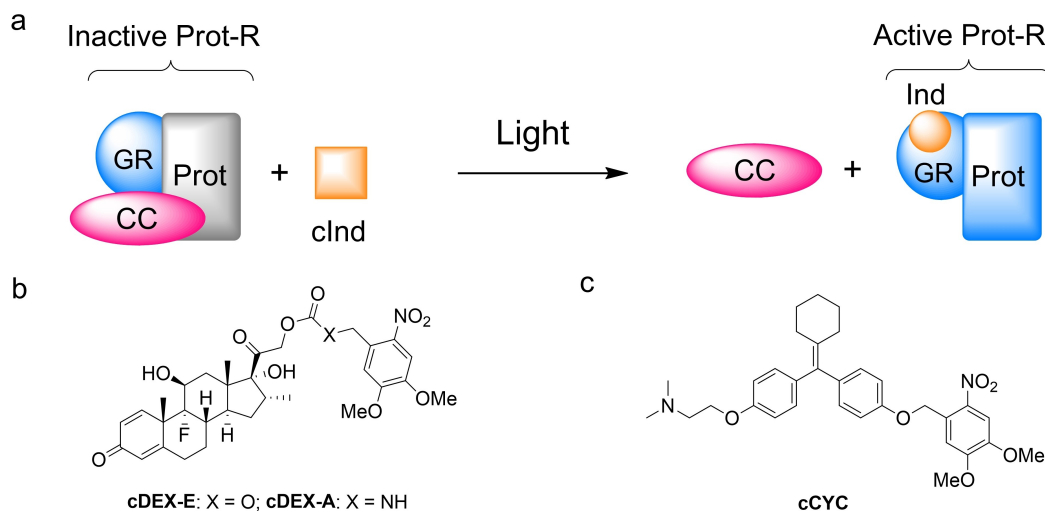
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Supporting information for this article is available on the WWW under
<https://doi.org/10.1002/chem.202400579>

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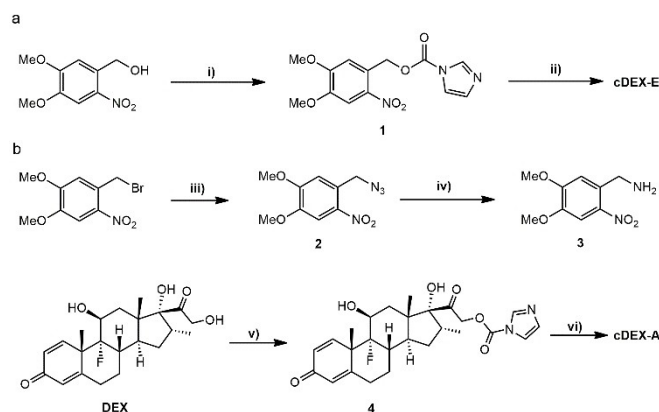
Scheme 1. The strategy used to photo-activate a protein. **a:** A protein of interest (**Prot**) fused to a receptor (**R**) is inactivated by its assembly with a chaperone complex (**CC**). Upon photoactivation of a caged precursor (**cInd**), the inducer (**Ind**) is released and binds to the receptor. Its concomitant conformational change causes assembly disruption and activates the **R**-fused protein; **b:** implementation of this strategy with caged dexamethasone (**cDEX-E**, **cDEX-A**) to photoactivate a GR-fused protein; **c:** implementation of this strategy with caged cyclofen (**cCYC**)^[9a] to photoactivate an ERT-fused protein.

known vertebrate models: zebrafish (*Danio rerio*)^[16] and *Xenopus*.^[17] We thus report on the synthesis and characterization of a caged DEX, which has been effective to photo-control the development of their embryos. We further evidence that this new system for photo-controlling gene expression is endowed with biological orthogonality with the established caged cyclofen approach for protein photoactivation, which paves the way for spatiotemporal control of activation of two proteins down to the single cell level.

Results and Discussion

To design our caged dexamethasone, we adopted the following criteria: (i) be chemically stable under physiological conditions; (ii) passively enter the cells; (iii) be biologically inactive and nontoxic; (iv) be uncaged at light levels that do not damage the embryos. Hence, we first chose the robust nitroveratryl caging group. Then, in view of the preceding realization of a caged dexamethasone,^[15] we linked the latter caging group to the dexamethasone backbone with the carbonate functional group and first targeted a caged carbonate-containing dexamethasone **cDEX-E** (Scheme 1). Anticipating a lower risk of hydrolytically- or enzymatically-driven leakage *in vivo* in the absence of any illumination, we then adopted the carbamate functional group and targeted a caged carbamate-containing dexamethasone **cDEX-A** (Scheme 1) anticipated to photorelease **DEX** from previous investigations.^[18]

We first produced the activated intermediate **1** from 4,5-dimethoxy-2-nitrobenzyl alcohol and carbonyldiimidazole (CDI).^[19] Its subsequent reaction with dexamethasone produced **cDEX-E** with 62% yield (Scheme 2a). To produce **cDEX-A**, we first designed a new pathway for synthesizing 4,5-dimethoxy-2-nitrobenzyl amine^[18,20] **3** in two steps involving the reaction of 4,5-dimethoxy-2-nitrobenzyl bromide with sodium azide fol-



Scheme 2. Synthesis of the caged-dexamethasone derivatives, **cDEX-E** (a) and **cDEX-A** (b). i) CDI, DCM, RT, 2 h; ii) **DEX**, DMAP, THF, reflux, 48 h; iii) NaN_3 , acetone/water (1/1; v/v), RT, 18 h; iv) PPh_3 , water, THF, RT, 18 h; v) CDI, THF, reflux, 48 h; vi) 4,5-dimethoxy-2-nitrobenzylamine, THF, reflux, 48 h.

lowed by reduction of the azide **2** with triphenylphosphine. In parallel, dexamethasone was reacted with carbonyldiimidazole (CDI) to form the activated intermediate **4**, which was subsequently coupled to the 4,5-dimethoxy-2-nitrobenzyl amine **3** to obtain **cDEX-A** with 46% yield (Scheme 2b).

cDEX-E, and **cDEX-A** are fairly soluble in dimethyl sulfoxide. Their 10 mM stock solutions have been diluted in aqueous buffers for all experimental studies. At physiological pH, the free **DEX** exhibits a strong absorption band in the 210–300 nm wavelength range with absorption maximum at 244 nm whereas the caging group generates a new absorption band in the 300–400 nm range for **cDEX-E** and **cDEX-A** (see Figure S1 and Table S1). Then we characterized uncaging of **cDEX-E** and **cDEX-A** *in vitro* by analyzing the time evolution of the composition of solutions of **cDEX-E** and **cDEX-A** under illumination at 365 nm by using HPLC coupled to mass spectrometry (Figure 1).

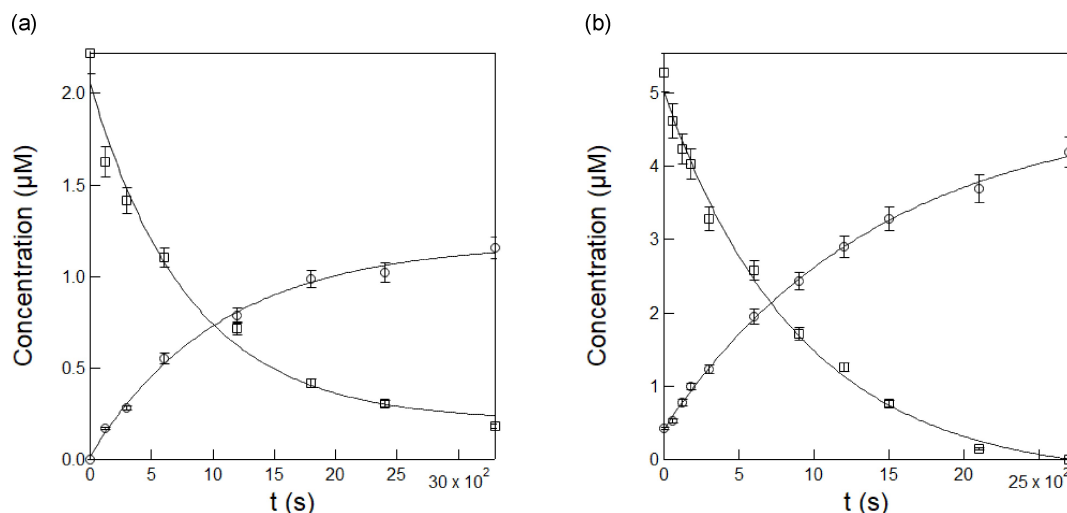


Figure 1. Kinetics and yield of **cDEX-E** and **cDEX-A** uncaging at 365 nm as evidenced by HPLC. Time evolution of the composition of 3 μM **cDEX-E** (a) and 7 μM **cDEX-A** (b) solutions upon 365 nm illumination at 1.6×10^{-4} (a) and 1.1×10^{-4} (b) $\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Markers: concentrations of the caged compounds **cDEX-E** or **cDEX-A** (squares) and photoreleased **DEX** (circles) as extracted from the peak areas in the HPLC chromatograms; Solid lines: Monoexponential fit with Eq. (S1) respectively yielding 800 and 904 s for the characteristic times of **cDEX-E** and **cDEX-A**, and 1061 s and 1434 s for **DEX**. Solvent: 10 mM PBS pH = 7.4. $T = 293$ K.

This assay first enabled us to evidence **DEX** photorelease with 60 and 91% chemical yields. These values are consistent with previously reported ones for nitroveratryl-caged-fluorophores.^[21] Moreover, we satisfactorily fitted the temporal dependence of the concentrations in **DEX** and caged **DEX** with a monoexponential function (see Eq. S1 in the Supporting Information). From the retrieved relaxation time, we extracted 6.7 and 8.6 $\text{m}^2 \cdot \text{mol}^{-1}$, and 6.7×10^{-3} and 8.2×10^{-3} for the uncaging cross-sections and quantum yields of uncaging with one-photon excitation at 365 nm of **cDEX-E** and **cDEX-A** respectively,^[23] in line with reported values for the nitroveratryl caging group.^[22] The latter derivation was further confirmed from analyzing the time evolution of the **cDEX-A** absorption spectrum upon UV illumination at 365 nm (Figure S2a): After exponential fitting of the time dependence of the absorbance at 350 nm (Figure S2b), we extracted 10.7 $\text{m}^2 \cdot \text{mol}^{-1}$ and 10.2×10^{-3} for the uncaging cross-section and quantum yield of uncaging with one-photon excitation at 365 nm respectively, in fair agreement with the HPLC derivation.

Having characterized the relevance of **cDEX-E** and **cDEX-A** for photoreleasing **DEX** upon illumination, we turned to experiments on biological samples. We here aimed at demonstrating that a caged dexamethasone could be used for photoactivating Ventx-GR. This protein is produced from fusing a fragment of GR gene coding for the ligand binding domain of the glucocorticoid receptor (aminoacids 512–777) with Ventx which belongs to the Ventx/nanog gene family and is involved in the development of vertebrates.^[24] In a first step, we quantitated the impact of **DEX** on Ventx-GR activation from analyzing the development of zebrafish embryos. When a capped mRNA coding for the Ventx-GR gene was injected in zebrafish zygotes just after fertilization (at one-cell stage), the embryos were observed to grow normally over 24 h (end of somitogenesis) without any anomalies, thereby suggesting Ventx-GR to be inactive so as to generate no alteration in the development of the zebrafishes. In contrast, when similar Ventx-

GR expressing siblings were incubated with 10 μM **DEX** for 60 min (from 4 h post fertilization - 4hpf - sphere stage until epiboly stage), washed and further grown for 24 h, they exhibited expected anomalies in the body structures (e.g. smaller or lacking head, shorter tail, or delayed development), which were classified into three different classes based on the severity of their penetrance (Figure S3 and Tables S2 and S3). These phenotypes are noteworthy specific since **DEX** treatment in non-injected (control) embryos does not induce developmental defects.

Then we investigated the stability of **cDEX-E** and **cDEX-A**. Their solutions were first shown to be stable in 10 mM pH = 7.4 PBS buffer over 24 h at room temperature in the dark (*data not shown*). Then, we examined their stability *in vivo*. Ventx-GR-injected zebrafish embryos at one-cell stage and growth until 4 hpf were incubated with solutions of **cDEX-E** and **cDEX-A** at both 7 and 15 μM concentrations for 1 h. They were subsequently washed three times and grown for 24 h in the dark. Embryos incubated at both investigated concentrations of **cDEX-E** exhibited a phenotype evidencing **DEX**-mediated Ventx-GR activation, which suggested that this caged dexamethasone liberates **DEX** in zebrafish embryos thereby activating Ventx-GR even without UV illumination (see Figure S5 and Table S2). Thus, we discarded **cDEX-E** for further investigations. In contrast, similar embryos incubated with both 7 and 15 μM **cDEX-A** solutions did not exhibit any **DEX**-mediated Ventx-GR phenotype, which demonstrated the stability of **cDEX-A** in zebrafish embryos (see Figures 2a, 2c, and S6, and Table S3). We similarly evaluated the stability of **cDEX-A** in Ventx-GR-injected *Xenopus* embryos incubated with **DEX** or **cDEX-A** at blastula stage from analyzing the emergence of any **DEX**-induced Ventx-GR activation on their development at tailbud stage. Whereas **DEX**-incubated embryos showed strong anomalies in the formation of the body axes and head development, **cDEX-A**-incubated ones exhibited a wild-type like phenotype without any anomalies (Figures S4, S7, S8 and Table S4), thereby demonstrating the absence of any leakage and **cDEX-A** stability in

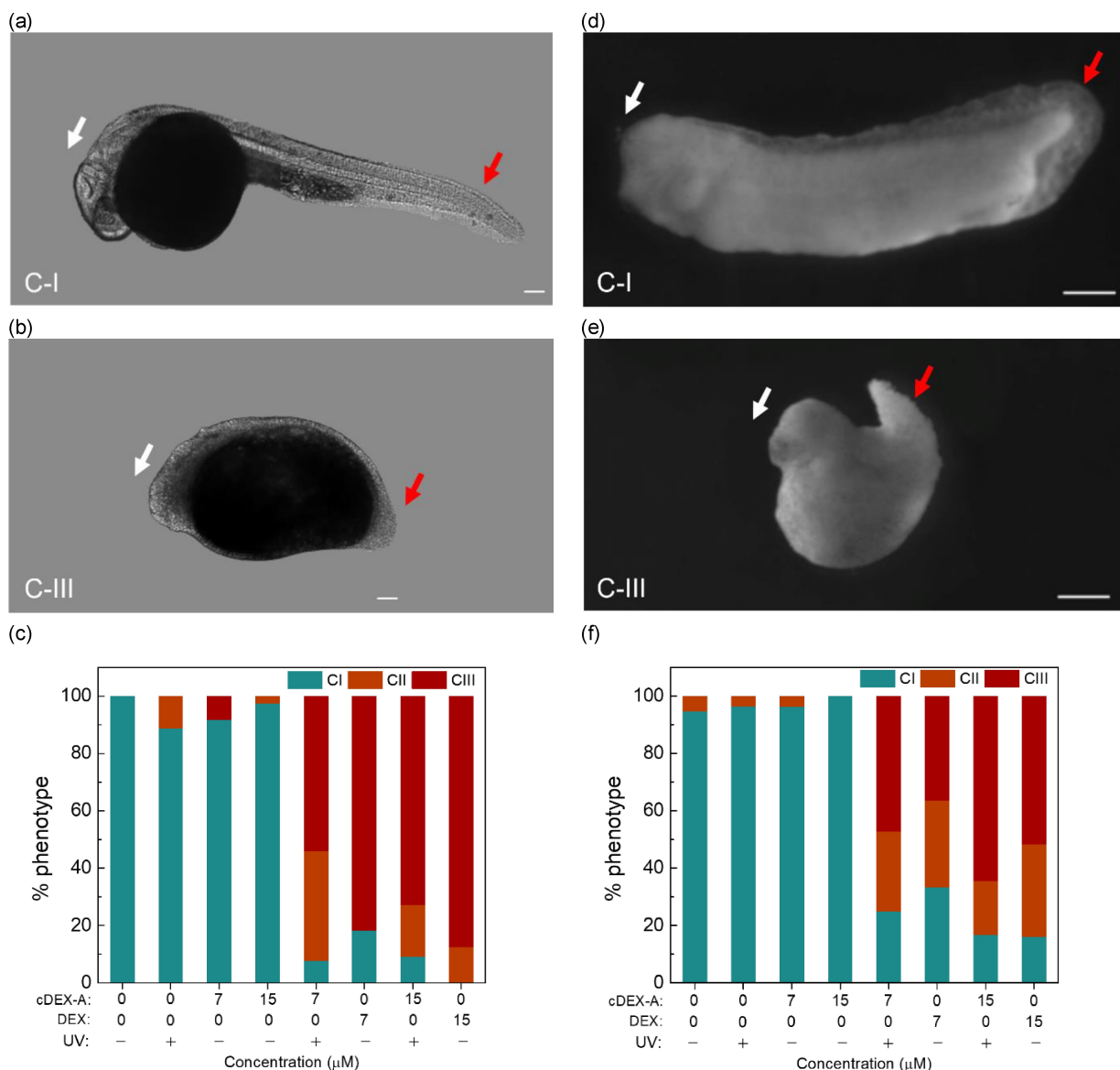


Figure 2. *cDEX-A* photoactivation in Ventx-GR-injected zebrafish and *Xenopus* embryos. Whereas the zebrafish (a) and *Xenopus* (d) embryos injected with Ventx-GR mRNA and incubated in the dark with 15 μM *cDEX-A* at 4 hpf (a,b; inset; scale bar: 100 μm) and blastula stage (d,e; inset; scale bar: 500 μm) exhibit normal phenotypes at 24 hpf and tailbud stage respectively, upon illumination at 365 nm for 600 s these zebrafish (b) and *Xenopus* (e) embryos reveal strong phenotypes at 24 hpf and tailbud stage (e); c,f. Extent of Ventx-GR-induced phenotype determined at 24 hpf (c) and tailbud stage (f) in zebrafish (c) and *Xenopus* (f) embryos incubated (+) or not (–) at 4 hpf (c) and blastula stage (f) with 7 or 15 μM DEX or *cDEX-A* and illuminated (+) or not (–) at 4 hpf (c) and blastula stage (f) at 365 nm with $5.7 \times 10^{-4} \text{ E.m}^{-2}.\text{s}^{-1}$ for 600 s (see Tables S3 and S4 for zebrafish and *Xenopus* embryos respectively). Bars: Extent of phenotypes C-I (dark cyan), C-II (orange), and C-III (dark red) (see Figures S3 and S4 and Tables S3 and S4). $T = 293 \text{ K}$.

Xenopus embryos (Figure 2d and f) and the specificity of the Ventx-GR-mediated phenotype.

We then turned to evidencing photo-activation of *cDEX-A* in zebrafish and *Xenopus* embryos (see Tables S3 and S4 for results and numbers of embryos). Ventx-GR-injected zebrafish and *Xenopus* embryos grown until 4 hpf and blastula stage respectively were incubated with *cDEX-A* for 1 h before illumination at 365 nm for 600 s. Both zebrafish embryos at 24 hpf and *Xenopus* embryos at tailbud stage displayed strong anomalies in the formation of the body axes and head development (Figures 2b,c,e,f, and S6–S8 and Tables S3 and S4), which led us to conclude that *cDEX-A* uncaging led to photo-activation of Ventx-GR *in vivo*.

Lastly, we performed a series of experiments to demonstrate that *cDEX-A* was biologically orthogonal with caged cyclofen (*cCYC*; see Scheme 1c, which exhibits cross section and chemical yield of uncaging similar to *cDEX-A*^[9a]) for protein photoactivation. We first evidenced that photoreleased cyclofen (*CYC*) did not activate a GR-fused protein of interest from applying 365 nm light on Ventx-GR-injected zebrafish embryos conditioned with 10 μM *cDEX-A* or *cCYC*. Whereas the *cDEX-A* treated embryos observed at 24 hpf exhibited the development anomalies following Ventx-GR activation (Figure 3a,c and Table S5), the *cCYC* treated ones revealed a wild-type like phenotype, which led us to conclude that the photoreleased cyclofen does not activate Ventx-GR (Fig-

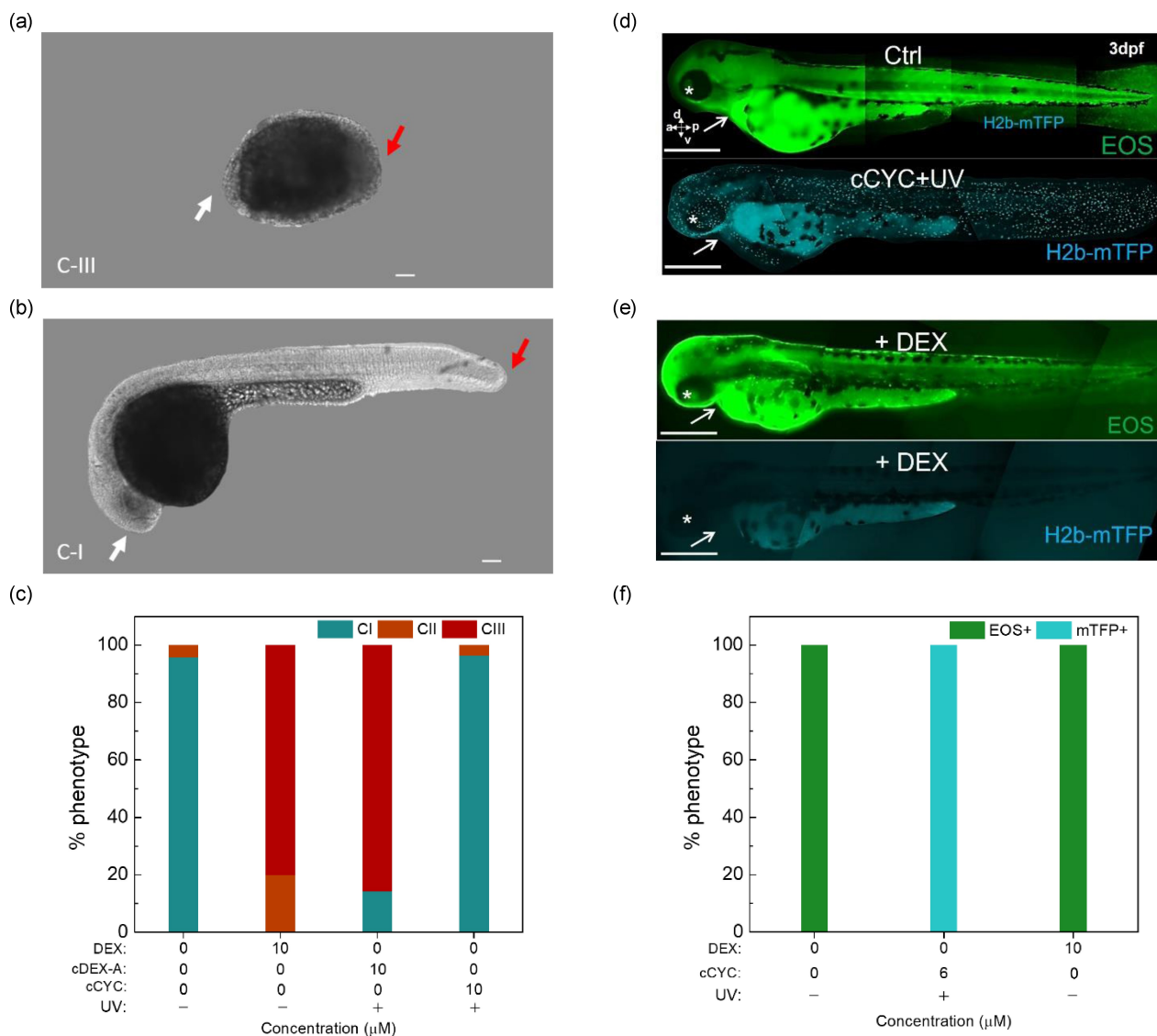


Figure 3. Towards orthogonal light-driven phenotype generation from using caged dexamethasone and caged cyclofen. **a–b:** Evidence that photoreleased cyclofen **CYC** does not activate the GR receptor activated by photoreleased dexamethasone **DEX** *in vivo*. Representative phenotypes generated at 24 hpf from illuminating Ventx-GR-injected zebrafish embryos conditioned with 10 μM solution of **cDEX-A** (**a**) and **cCYC** (**b**) at 4 hpf and illuminated with 365 nm light to fully release **DEX** promoting GR activation (inset; scale bar: 100 μm). White and red arrow represent head and tail part of the zebrafish embryos respectively; **c:** Phenotype extents from **a,b** evaluated as reported in Figure S3 and Table S5; **d–e:** Evidence that **DEX** does not activate the ERT receptor targeted by photoreleased cyclofen **CYC** *in vivo*. **d:** Observed at 3 dpf, Tg(β-actin:loxP-EOS-stop-loxP-KRASG12V-T2A-H2B-mTFP; ubi:Cre-ERT; myl7:EGFP) zebrafish transgenic growing embryos ubiquitously express the EOS fluorescent protein in the cell cytoplasm in the absence of any CRE-ERT activation (no **cCYC** and UV); it results in the observation of cytoplasmic green fluorescence (top). In contrast, when these embryos have been further conditioned at 24 hpf with 6 μM **cCYC** and illuminated with 365 nm light to fully liberate **CYC** that promotes CRE-ERT activation, they express the H2B-mTFP fluorescent protein in their nuclei (bottom). **e:** Upon 10 μM **DEX** treatment at 24 hpf, the zebrafish transgenic growing embryos do not exhibit any CRE-ERT activation as evidenced by the observation of ubiquitous green fluorescence from EOS (top) and by the absence of any nuclear blue fluorescence from TFP (bottom). In **d,e**, the asterisk (*) indicates the eyes and the white arrow the heart, whereas a (anterior), p (posterior), d (dorsal), and v (ventral) indicate the body axes of the zebrafish transgenic growing embryos (inset; scale bar: 250 μm); **f:** Phenotype extents determined from **d,e** in the zebrafish transgenic growing embryos (see Table S6). *T* = 301 K.

ure 3b,c and Table S5). We then demonstrated that **DEX** does not activate an ERT-fused protein, that is targeted by uncaged cyclofen. Here we used transgenic zebrafish embryos ubiquitously expressing the green fluorescent protein EOS in the absence of ERT activation and expressing the blue fluorescent protein mTFP in their nucleus after ERT activation (Figure 3d). When such embryos conditioned with 6 μM **cCYC** were illuminated with 365 nm light, their fluorescence turned from green to blue (Figure 3d,f and Table S6), as expected from **CYC**-mediated

activation of the photoactivable CRE-ERT leading to produce nuclear mTFP. In contrast, their treatment with 10 μM **DEX** did not yield any fluorescence change (Figure 3e,f and Table S6), thus demonstrating no specific ERT activation by **DEX**. Altogether, this series of photoactivation experiments evidenced the specificity of **CYC** and **DEX** for their respective estrogen and glucocorticoid receptor leading to protein activation.

Conclusions

We introduced the caged dexamethasone **cDEX-A**, which has been shown to be relevant for the photocontrol of protein activity in two important vertebrate models: zebrafish and *Xenopus*. **cDEX-A** can be easily synthesized in large amounts and it liberates the GR inducer **DEX** upon UV illumination. **cDEX-A** does not induce any leakage of protein activity in zebrafish and *Xenopus* in the absence of illumination. In contrast, UV light has been shown to drive the photoactivation of a GR-fused protein in both model organisms. Importantly, this new system for photocontrol of GR-fused protein activity has been shown to exhibit biological orthogonality with respect to caged cyclofen (**cCYC**), which has been well-established for the photocontrol of many ERT-fused proteins. The present combination of the **cDEX-A** and **cCYC** systems already opens attractive perspectives for sequential photoactivation of two distinct proteins with precise spatial-temporal control down to single cell resolution, which should be further enhanced by implementing spectral orthogonality^[3] for uncaging **cDEX-A** and **cCYC**, which was out of the scope of this report.

Supporting Information

The authors have cited additional references within the Supporting Information.

Acknowledgements

This work has received financial support from ITMO Cancer of Aviesan within the framework of the 2021–2030 Cancer Control Strategy, on funds administered by Inserm.

Conflict of Interests

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: Optogenetics · Caging group · Photo-control of protein activity · Caged-dexamethasone · Glucocorticoid receptor · GR-activation · Gene expression · Orthogonal inducer

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Manuscript received: February 12, 2024

Accepted manuscript online: February 13, 2024

Version of record online: February 29, 2024