Gene Function

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A Caged Doxycycline Analogue for Photoactivated Gene Expression**

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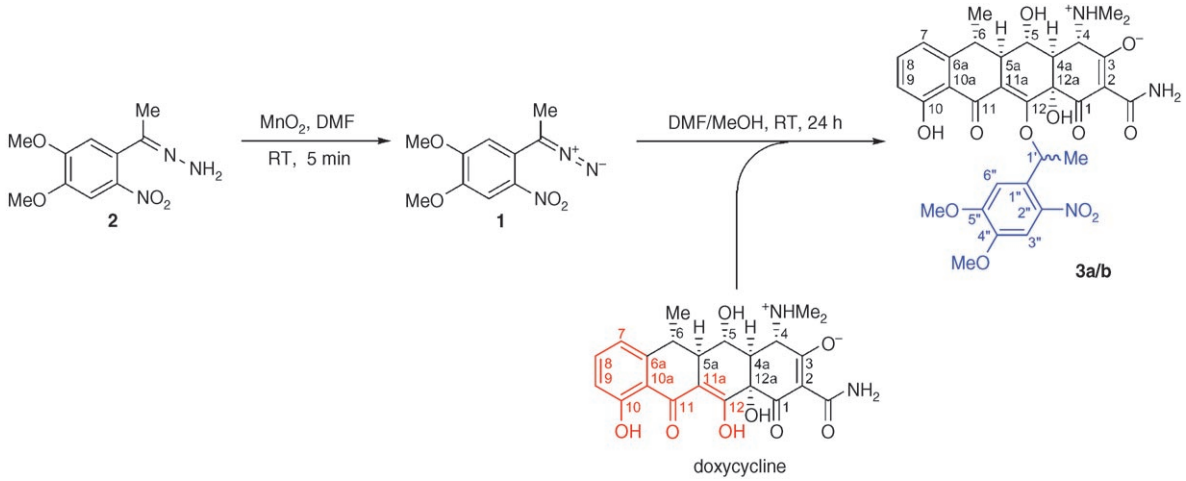
Conditional gene-expression paradigms are crucial tools for the study of genes and gene functions. However, none of the currently available paradigms permits transgene expression with high spatial and temporal resolution; rather, they usually rely on specific expression patterns enabled by endogenous promoters. To improve the resolution of transgene expression, we synthesized a photosensitive ("caged") doxycycline analogue for precise light-controlled activation of genes based on the "Tet-on" (Tet = tetracycline) system.[1] Because of the ease and precision with which light can be manipulated, this approach should make it possible to target subsets of cells for transgene expression which can range from single cells and tissue patches to whole organs.[2]

To implement a photoactivated gene-expression system that is generally applicable in any organism at any stage, we based our approach on a conditional gene-expression paradigm that uses a small, membrane-permeant molecule for induction. The most prominent inducible system by far is the Tet system, which is commonly used for the induction of transgenes in cell culture, tissues, and whole organisms.[1] The most potent analogue for the Tet system is doxycycline, which can bind to a modified and mutated version of the Tet repressor fused to a transcriptional activation domain (rtTA) and induce transgenes under the control of the Tet promoter.[3] Because doxycycline (Scheme 1) has several functional groups that may be derivatized, it was necessary to specifically target a group that is essential for transcriptional activity and block this activity by "caging" with a photosensitive protecting group. It was previously shown that the phenolic β-diketone system (highlighted in red, Scheme 1) is important for the formation of the doxycycline–magnesium complex, which binds to the rtTA protein with high affinity.[4] We therefore attempted to generate a 1-(4,5-dimethoxy-2-nitrophenyl)ethyl (DMNPE) ether of doxycycline (DMNPE-caged doxycycline) from the commercially available doxycycline hyclate (hydrochloride hemiethanolate hemihydrate) with the DMNPE moiety attached at the phenolic β-diketone system.



Scheme 1. Synthesis and structure of caged doxycyclines **3a** and **3b**. The phenolic β-diketone system (ketone enol form) is highlighted in red in doxycycline, and the caging group in blue in **3a**/**3b**. DMF = *N*,*N*-dimethylformamide.

Doxycycline hydrochloride was neutralized with KOH and then treated with an approximate fivefold excess of freshly prepared 1-(4,5-dimethoxy-2-nitrophenyl)diazoethane (**1**) obtained from 4,5-dimethoxy-2-nitroacetophenone hydrazone (**2**) by oxidation with MnO_2. The alkylation

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of doxycycline with **1** resulted in two stable diastereomers of DMNPE-caged doxycycline (**3a** and **3b**) as main products and a few unstable caged side products. Because the unstable by-products complicated the separation of the stable isomers, they were selectively hydrolyzed by brief treatment of the reaction mixture with diluted trifluoroacetic acid (TFA) at 40 °C prior to preparative HPLC separation. The stable diastereomers **3a** and **3b** were then isolated by reversed-phase C-18 HPLC, whereby the yield of each isomer was about 8 %.

The structures of **3a** and **3b** were clarified by comprehensive NMR spectroscopic analysis (see Supporting Information). Heteronuclear multiple-bond correlation (HMBC) spectra of **3a** and **3b** showed cross-correlation between H1′ and C12 in both cases, and also between the three protons of the methyl group at C1′ and C12 in the case of **3a**. Consequently, the DMNPE-caging group must have been conjugated with C12, which is part of the phenolic β-diketone system. This analysis strongly suggests that **3a** and **3b** are two diastereomers which only differ in their configuration of the asymmetric C1′ center of the DMNPE moiety. In addition, the etherification of the C12 hydroxy group of doxycycline is substantiated by a strong shift in the signals of C12 in the ^{13}C NMR spectra of **3a** and **3b** by $\Delta\delta = 15$ and 18 ppm, respectively, to higher fields relative to the chemical shift of C12 in doxycycline (see Supporting Information for the ^{1}H and ^{13}C NMR data of doxycycline).

Both diastereomers are characterized by a notable long-wavelength absorption maximum at around 345 nm (see Table 1 and Supporting Information), which is caused by the overlap of the long-wavelength absorption bands of the phenolic β-diketone system in doxycycline and the DMNPE chromophore. Irradiation of **3a** and **3b** between 300 and 400 nm in aqueous buffer solution led to the photorelease of doxycycline, as confirmed by HPLC analysis (data not shown), and the 4,5-dimethoxy-2-nitrosoacetophenone by-product of the caging group, which did not show any harmful effect in the biological experiments. Furthermore, **3a** and **3b** displayed acceptable photochemical quantum yields (Table 1).

Table 1: Properties of **3a** and **3b**.

Caged doxycycline	Absorbance maximum[a] λ_{max} [nm]	Extinction coefficient[a] ε_{max} [$M^{-1}cm^{-1}$]	Photolysis quantum yield ϕ[b]	Solubility[a] c_{sat} [μM]
3a	≈347	10500	0.013	380
3b	345.5	11400	0.075	770

[a] In HEPES buffer solution, pH 7.2. [b] In HEPES buffer solution (pH 7.2)/acetonitrile (80:20, v/v).

Compounds **3a** and **3b** were relatively soluble in aqueous 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer solution at pH 7.2 (Table 1). This solubility is essential for applications of caged doxycycline in sensitive tissues, such as the nervous system, which do not tolerate alcohols or other organic solvents as vehicles.[5] Thus, concentrated stock solutions can be readily prepared and maintained in water. In addition, **3a** and **3b** were tested for their hydrolytic stability in aqueous buffers. Incubation for 2 days at 37 °C did not lead to release of doxycycline, as determined by HPLC analysis (data not shown). Pilot experiments to assess the suitability of **3a** and **3b** for photoactivated gene expression showed that **3a** had only little background activity, whereas **3b** produced significant transgene expression even in the absence of irradiation. We currently do not understand the reason for this difference especially as both molecules are stable isomers. However, given that both isomers displayed surprisingly different physicochemical properties, differences in their biological properties were therefore not unexpected. Consequently, **3a** was used for all further experiments.

Caged doxycycline should ideally be membrane permeant so that photoactivated gene expression is able to control transgene expression in single cells. Although in many applications doxycycline may be photoreleased extracellularly to subsequently diffuse into the cytoplasm for transgene induction, this scenario will probably not permit single-cell resolution. The membrane permeability of **3a** was therefore assessed by isothermal titration calorimetry (ITC).[6,7] A comparison of ITC uptake and release protocols demonstrated that **3a** can quickly cross the membranes of large unilamellar vesicles (LUVs) composed of the zwitterionic phospholipid 1-palmitoyl-3-oleoyl-*sn*-glycero-2-phosphocholine (POPC).

Figure 1 a shows a good simultaneous fit (red lines) to the heats of the reaction Q obtained in uptake (diamonds) and release (circles) experiments. This fit was based on the assumptions of ideal mixing and full lipid accessibility of the caged compound, that is, complete transbilayer equilibration within the time needed for a single injection. In contrast, based on the assumption that **3a** cannot traverse membranes, the fit in Figure 1 b failed to describe the experimental data, as there were significant deviations at low injection numbers n (see Supporting Information for the ITC experimental data). Repetition of the experiment again with **3a** and once with a mixture of **3a**/**3b** produced almost identical results (data not shown). The validity of using ITC to test for membrane permeability was demonstrated in another set of experiments

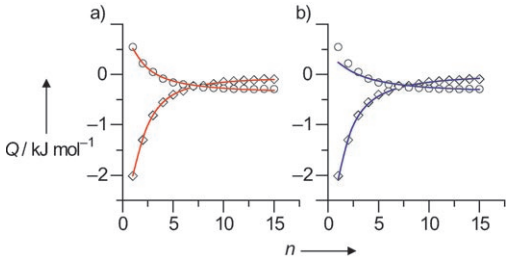

Figure 1. ITC analysis of translocation of **3a** across POPC membranes. The heats of the reaction of water–membrane partitioning Q obtained in uptake (diamonds) and release (circles) experiments are plotted versus the injection number n. a) The fit (red lines) matches the experimental data well (based on the assumption of complete membrane permeation). b) A poor fit (blue lines) is obtained particularly for the critical early injections (assuming no detectable membrane permeation on the experimental timescale).

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which showed the differential temperature-dependent membrane partitioning of sodium dodecyl sulfate (SDS; see Supporting Information).[7] Taken together, the data strongly suggest that the chemically modified doxycycline retained the ability to passively cross membranes, which in turn should aid the photoactivation of single cells.

To determine if caged doxycycline could be used for photoactivated gene expression, two different expression paradigms were tested. First, as a proof-of-principle experiment, we employed stably transfected Chinese hamster ovary (CHO) cells which express M2-rtTA[8] and contain a tetracycline-dependent enhanced green fluorescent protein (EGFP) construct. Incubation of these cells with unmodified doxycycline led to widespread green-fluorescent-protein (GFP) fluorescence (Figure 2a). Conversely, incubation with **3a** did not produce any fluorescence, thus indicating that its transcriptional activity was inhibited (Figure 2b). However, irradiation of cells in the same dish with long-wavelength UV light induced significant EGFP expression similar to EGFP levels seen with unmodified doxycycline. These data demonstrated that caged doxycycline can be used for photoactivated gene expression as a tool for localized transgene expression.

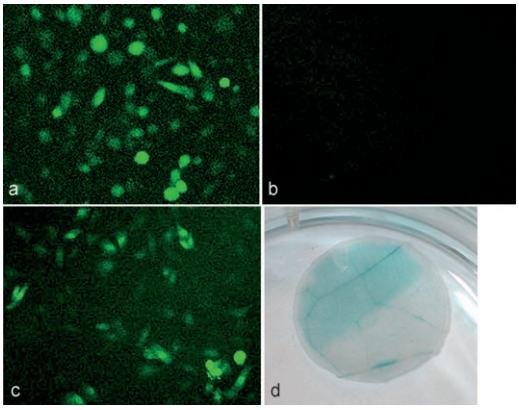

Figure 2. Spatially restricted photoactivated gene expression. a) CHO cells (M2-rtTA: tetEGFP) incubated with unmodified doxycycline. b) CHO cells incubated with **3a**, no irradiation. c) Same dish as in Figure 2b; irradiation-induced, widespread EGFP fluorescence. d) A sharp boundary between irradiated and non-irradiated areas was revealed by photoactivation of a GUS reporter gene in one half of transgenic tobacco tissue.

To test photoactivation in a three-dimensional tissue, a second paradigm was employed using transgenic tobacco leafs which harbored a quasi Tet-on system based on de-repression of a constitutive cauliflower mosaic virus (CaMV) 35S promoter which drives a β-glucuronidase (GUS) reporter gene.[9] Thus, in the absence of doxycycline, the Tet repressor prevents transcription from a modified CaMV promoter that contains three repressor binding sites. Incubation of leaf tissue with **3a** followed by irradiation produced a sharp boundary of GUS expression between the irradiated and non-irradiated areas (Figure 2d). To our knowledge, this method is the first that uses photoactivation based on an inducible gene expression paradigm to direct transgene expression with high precision in compact tissue as opposed to dispersed cells in culture.

In conclusion, we demonstrated that transgene expression can be accurately manipulated by simple irradiation with UV light. The doses of UV light needed for induction were not harmful, as no signs of cell damage were apparent (see Supporting Information). Previously, small-molecule-based photoactivated gene expression was demonstrated in cell culture with caged tamoxifen by global irradiation of the entire cell-culture medium.[10] Although this approach did not yield any spatial resolution, a different approach by Lawrence and co-workers in which caged ecdysone was applied followed by local photoactivation indeed induced transgene expression only in irradiated 293T cells.[11] The photoactivated gene-expression system described herein is based on the popular Tet system, which provides a rich infrastructure of Tet-dependent transgenes in various organisms that range from yeast to plants and mice. Thus, we have established a paradigm for the photoactivation of genes with single-cell resolution. We predict that this approach will be a tremendously powerful tool for various research areas, including single-cell lineage tracing during the development or implementation of a better cancer model by induction of oncogenes in single cells surrounded by a wild-type background.

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