ChemComm

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COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *Chem. Commun.*, 2013, **49**, 4971

Received 26th March 2013,
Accepted 10th April 2013

DOI: 10.1039/c3cc42179a

www.rsc.org/chemcomm

Synthesis of a photocaged tamoxifen for light-dependent activation of Cre-ER recombinase-driven gene modification†

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We report the design of a water-soluble, quaternized tamoxifen photoprobe and demonstrate its application in light-controlled induction of green fluorescent protein expression *via* a Cre-ER recombinase system.

In vivo lineage tracing has been revolutionized by the Cre–loxP system, whereby tissue-specific promoter activation of the Cre-recombinase leads to permanent activation of a reporter gene in a cell and all its downstream progeny.[1] Fusion of the Cre enzyme with the estrogen receptor (ER), which prevents nuclear localization of the Cre-ER protein unless bound by an estrogen analog (*e.g.* tamoxifen), allows an additional level of temporal control of reporter activation.[2–4] Tamoxifen (TAM) and its active metabolite form 4-hydroxytamoxifen (4-OHT) have brief half-lives upon injection into mice, thus restricting reporter induction to a 24–48 h window when tamoxifen is active.[5] Despite the utility of this system, tamoxifen diffuses rapidly *in vivo*, preventing precise spatial activation of a specific location or region within an organism (*e.g.* femur *vs.* tibia; left *vs.* right).

In this study, we investigated a photochemical approach[6] for the controlled expression of mutant genes using a mammalian Cre-ER recombinase in combination with light-triggered release of tamoxifen, an estrogen receptor antagonist. The mechanism for the drug release is based on the design of photocaged tamoxifen in which the tamoxifen molecule is temporarily inactivated through its covalent attachment to a photocleavable *ortho*-nitrobenzyl (ONB) group (Fig. 1). Upon exposure to UV irradiation, the ONB group of the caged molecule (TAM-ONB) is irreversibly cleaved, leading to release of free tamoxifen. This photocaging strategy has been previously employed in other systems for the precise spatio-temporal control of small molecule mediated biological processes such as ion channel gating,[7,8] protein–protein interactions,[9,10] protein phosphorylation,[11] transcription,[12] and payload release in targeted drug delivery.[13–16] In addition, tamoxifen-related molecular photoprobes have previously been utilized for studying gene expression with Cre-ER fusion proteins.[2,17,18]

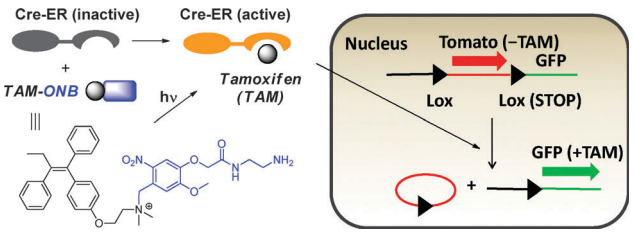
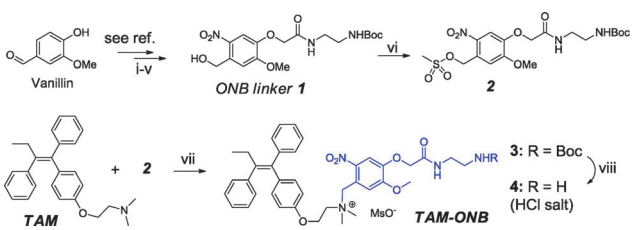


Fig. 1 Schematic for the release of tamoxifen from its photocaged form by UV light, and control of Cre-ER recombinase-mediated GFP expression in mouse embryonic fibroblast (MEF) cells.

In designing our photocaged tamoxifen molecule, we performed the *N*-quaternization reaction of tamoxifen at its tertiary amine nitrogen with an *o*-nitrobenzyl (ONB)-based alkylating molecule[15,19,20] **2** (Scheme 1). This reaction afforded TAM-ONB **3**: ESI HRMS (*m*/*z*): calcd for $C_{43}H_{53}N_4O_8$ 753.3858 $[M]^+$, found 753.3856; purity ≥99% (anal. HPLC); UV-vis absorption peaks at 350, 270 nm.[1] After deprotection of its *N*-Boc, TAM-ONB **4** was obtained as a HCl salt (see ESI† for details). This design is notable in two aspects. First, it allows us to photocage tamoxifen directly without



Scheme 1 Synthesis of photocaged tamoxifen. *Reagents and conditions*: (i) ethyl bromoacetate, K_2CO_3, DMF, RT; (ii) NaOH, THF, MeOH, H_2O, RT; (iii) conc. HNO_3, AcOH, 0 °C to RT; (iv) *N*-Boc-1,2-diaminoethane, DCC, DMAP, DMF, 0 °C to RT; (v) $NaBH_4$, THF, MeOH, 0 °C to RT; (vi) methanesulfonyl chloride (MsCl), Et_3N, CH_2Cl_2, 0 °C, 88%; (vii) tamoxifen, acetone, 37 °C, 3 days, 27%; (viii) 6 M HCl, MeOH, rt, 6 h, 96%.

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† Electronic supplementary information (ESI) available: Experimental details for synthesis and characterization of **3** and **4**; details for photocleavage experiments and cell studies (PDF). See DOI: 10.1039/c3cc42179a

making any structural modifications or derivatizations to the drug molecule. It therefore differs from earlier methods reported for preparing tamoxifen photoprobes which are made using tamoxifen derivatives or analogs.[17,18] Second, the aqueous solubility of TAM-ONB **4** ($\geq$10 mg mL^{-1}) was greatly improved compared to practically insoluble tamoxifen ($\approx$0.0002 mg mL^{-1} water[21]). We believe that its quaternary salt form improves the solubility as does the hydrophilic amine-terminated side chain tethered to the ONB group.

Generation of a tamoxifen-inducible reporter MEF line: to validate TAM-ONB **4**, we generated tamoxifen-inducible reporter mouse embryonic fibroblasts (MEFs) by crossing a transgenic mouse line that expresses Cre-ER^{T2} under the control of the constitutively-expressed human ubiquitin C (UBC) promoter[22] to a transgenic reporter line with a reporter cassette (mTmG) inserted into the constitutive Rosa26 locus.[23] This cassette initially expresses the red fluorescent protein TdTomato (Tomato), but will permanently recombine and switch expression to green fluorescent protein (GFP) when Cre-recombinase is active. We first tested UBC-Cre-ER^{T2} $\times$ mT/mG (UT) MEFs for reporter induction upon tamoxifen exposure (Fig. 2, Fig. S5, ESI†). One day after 8 μM TAM treatment, we examined MEFs using flow cytometry for GFP and Tomato expression. After treatment, 35% of UT MEFs had recombined and activated GFP expression, although our timepoint was too early to observe Tomato fluorescence diminish after its excision. Thus UT MEFs are a highly sensitive tool for detecting TAM-dependent recombination due to its extremely low levels of spontaneous recombination.

*Validation of TAM-ONB **4** release*: we next tested whether the photocaged tamoxifen molecule (TAM-ONB, **4**) is released by a photochemical mechanism, causing Cre activation and GFP induction in UT MEFs (Fig. 2b). Here, the MEF cells were treated with **4** (8 μM) that was either unexposed, or exposed for 20 min to UV-A light with a mean wavelength of 365 nm which is strongly absorbed by the ONB **1** (λ_{max} = 340 nm, ε_{340} = 2750 M^{-1} cm^{-1}).[1] 24 h after adding TAM-ONB **4**, MEFs treated with unexposed **4** showed less than 1% GFP induction. In contrast, 20 min UV exposure was sufficient to activate recombination and induce GFP expression in 21% of UT MEFs. While this is somewhat less than the 35% of GFP$^+$ UT MEFs we observed with 24 h of TAM treatment, our data clearly indicate that TAM can be rendered inactive by photocaging with ONB, and this caging can be released by UV exposure and recombination activation.

We next compared the length of UV exposure to recombination efficiency (Fig. 3a). We exposed either regular tamoxifen (TAM) or TAM-ONB **4** to UV light for 1 min up to 1 h, then treated UT MEFs with the uncaged products at 8 μM. One min UV exposure was sufficient to activate low level reporter expression, and 5 min was sufficient for near peak recombination efficiency. There was a mild increase in recombination efficiency between 5 min and 20 min, though beyond 20 min, there was no improvement. TAM induction remained around 35% regardless of the length of UV exposure. Thus, 5 min UV exposure is sufficient for robust uncaging of **4** and reporter activation.

Time-dependent control of GFP expression: we next tested different concentrations of TAM-ONB **4** to optimize the dose and UV exposure time for TAM release (Fig. 3b). We found that the concentration of **4** had a significant impact on recombination frequency, with 32 μM and 64 μM treatments approaching the recombination efficiency of unmodified tamoxifen (35%). With each concentration, there was only a modest increase in recombination efficiency by increasing the UV exposure from 5 min to 20 min. We conclude the ideal concentration to be 16–32 μM with 5–20 min UV exposure.

*Pre-treatment with TAM-ONB **4** prior to UV uncaging*: In order to allow precise labeling *in vivo*, TAM-ONB **4** will have to be loaded efficiently into cells in its caged, inactive state prior to UV release. We next tested the loading capacity of **4** in UT-MEFs prior to UV uncaging. Preliminary experiments revealed that 1 h exposure to TAM was sufficient for near maximum recombination frequency (Fig. S6, ESI†). However, the presence of serum

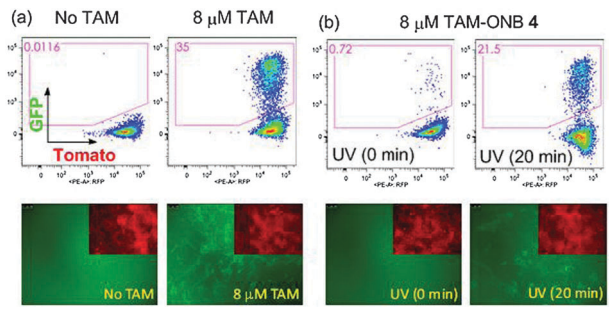

Fig. 2 Tamoxifen (TAM)-controlled GFP expression in mouse embryonic fibroblast (MEF) cells that constitutively express UBC-promoter Cre-ER mTmG fusion protein (UT MEFs). Flow cytometry analysis of MEF cells treated with free TAM (–, +) (a), or with TAM-ONB **4** pre-exposed to 0 or 20 min of UV irradiation at 365 nm (b). Fluorescent microscopic images of the same cells treated as above are shown below respectively: green fluorescence protein (GFP), and Tomato fluorescence protein (Tomato, inset).

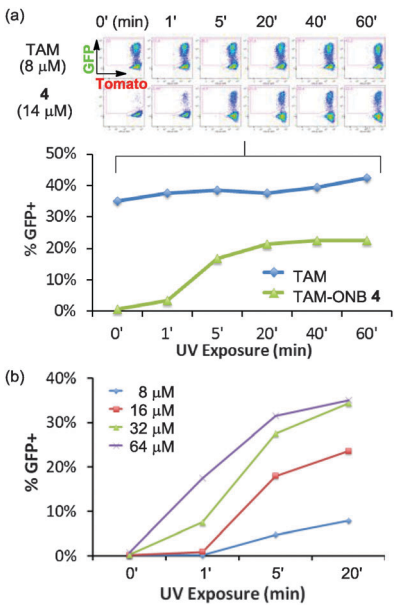

Fig. 3 Time-dependent, UV light-controlled activation of TAM-ONB **4** and induction of GFP-positive MEF cells. (a and b) Flow cytometry analysis of MEF cells exposed to UV light as a function of time after treatment with either free TAM or TAM-ONB **4**. Plots for induction of GFP-positive cells following UV exposure after treatment with TAM-ONB **4** at a single concentration of 14 μM (a, bottom), or at four different concentrations (b). Induction of GFP-positive cells quantified in each of the plots is based on flow cytometry data.

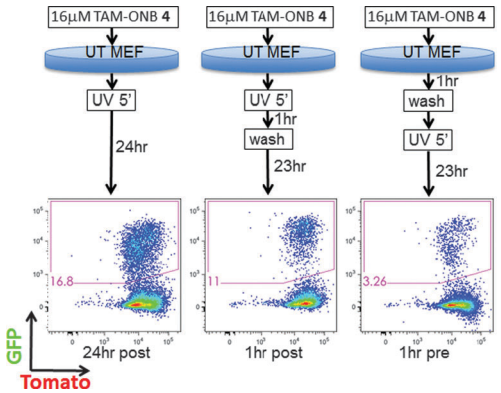

Fig. 4 Pre-treatment with TAM-ONB **4** prior to UV uncaging. UT MEFs were treated with TAM-ONB **4** for 24 h (left) or 1 h (middle) after 5 min UV uncaging (''post''), or pre-treated with caged TAM-ONB **4** for 1 h (''pre'', right) prior to 5 min UV exposure. After 1 h treatments (either pre- or post-UV exposure), UT MEFs were washed to remove unbound TAM-ONB **4**.

dramatically reduced TAM-mediated recombination, requiring treatments to be in serum-free media (Fig. S6, ESI†). UV had little effect on MEF viability or background recombination of UT MEFs (Fig. S7, ESI†), and we could expose **4** to UV directly on MEFs with no loss in recombination efficiency or cell viability (Fig. S8, ESI†). Uncaging also worked whether the UV source was above the cells, or when cells (in plastic dishes) were placed directly onto the UV source (Fig. S9, ESI†). We then compared recombination frequency in UT MEFs treated for 1 h with **4** either prior to (pre) or after (post) UV exposure (Fig. 4). In both cases, UT MEFs were washed after 1 h treatment to remove unbound **4**. Compared to 24 h treatment (24 h post) with uncaged **4**, we observed a slight reduction in recombination efficiency when UT MEFs were treated for 1 h (1 h post) with uncaged **4**. For UT MEFs treated with caged **4** for 1 h prior to UV uncaging (1 h pre), there was clear evidence of recombination and GFP activation, though substantially less than 1 h treatment with uncaged **4**, and efficiency depended critically on the absence of serum during treatment (Fig. S10, ESI†). Nevertheless, our data clearly indicate that the caged, inactive form of **4** can bind to cells and mediate recombination once released upon exposure to UV light.

In summary, we developed an efficient method for the synthesis of a water soluble photocaged tamoxifen by using an *N*-quaternization approach with an ONB photolabile moiety. Its water solubility may also facilitate applications where standard tamoxifen solvents (EtOH, DMSO, oil) may be too harsh for sensitive cells or tissues. The tamoxifen release was achieved by a single exposure to UV light in a time- and dose-dependent manner. This tamoxifen photoprobe proved to be a valuable molecular tool for controlling GFP reporter induction *via* Cre-ER activation in a transgenic cell model. This is the first report of photo controlled GFP expression using a photocaged tamoxifen. Unlike other enzyme-based reporter systems,[17,18] the expression of fluorescent proteins allows direct detection, quantification and imaging of intact biological tissues and live cells. We believe that this photochemical release strategy is of potential value for *in vitro* and *in vivo* applications that require spatiotemporal and non-invasive gene expression or deletion.[24,25]

It should be noted that tamoxifen must be converted to 4-hydroxytamoxifen (4-OHT) in order to bind Cre-ER^{T2} efficiently.[26] MEFs are a heterogeneous mixture of cells, and likely only 35% of our UT MEF line is able to mediate this conversion. Our future efforts will focus on the generation of a caged 4-OHT molecule for *in vivo* applications of 4-OHT-ONB in a transgenic mouse model such as real-time tracking of GFP-expressing Cre-ER(+) cells activated locally by irradiation of a specific tissue.

This work was supported by MNIMBS, University of Michigan Medical School, the Ellison Medical Foundation, the California Institute for Regenerative Medicine (grant RT2-02060); and the NIH (grants R01HL058220 and R01CA86065). We thank Mr Ankur Desai for his contribution in HPLC analysis and Ivan Dimov, Shah Ali, and Humberto Contreras-Trujillo for technical discussion.

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