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Synthesis of A Photocaged Tamoxifen for Light-dependent Activation of Cre-ER Recombinase-driven Gene Modification

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Abstract

We report the design of a water-soluble, quaternized tamoxifen photoprobe and demonstrate its application for 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 to 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 hr 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 approach⁶ 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

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spatiotemporal 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}

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 C₄₃H₅₃N₄O₈ 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 HCl salt (see SI for details). This design is notable in two aspects. First, it allows us to photocage tamoxifen directly without 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** (10 mg/mL) was greatly improved compared to practically insoluble tamoxifen (≈0.0002 mg/mL water²¹). 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 control of the constitutively-expressed human Ubiquitin C (UBC) promoter²² 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} × mT/mG (UT) MEFs for reporter induction upon tamoxifen exposure (Fig. 2, S5). One day after 8 μM TAM treatment, we examined MEFs by 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 to detect TAM-dependent recombination due to its extremely low levels of spontaneous recombination.

Validation of TAM-ONB4 release

We next tested whether the photocaged tamoxifen molecule (TAM-ONB4, **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 at 365 nm which is strongly absorbed by the ONB **1** ($\lambda_{\text{max}} = 340 \text{ nm}$, $\epsilon = 2750 \text{ M}^{-1}\text{cm}^{-1}$).¹ 24 Hr after adding TAM-ONB **4**, MEFs treated with unexposed **4** had 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 hr 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 activated.

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 hr, then treated UT MEFs with the uncaged products at 8 μ M. 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 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 μ M and 64 μ M 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 μ M 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 hr exposure to TAM was sufficient for near maximum recombination frequency (Fig. S6). However, the presence of serum dramatically reduced TAM-mediated recombination, requiring treatments to be in serum-free media (Fig. S6). UV had little effect on MEF viability or background recombination of UT MEFs (Fig. S7), and we could expose **4** to UV directly on MEFs with no loss in recombination efficiency or cell viability (Fig. S8). Uncaging also worked whether the UV source was above cells, or when cells (in plastic dishes) were placed directly onto the UV source (Fig. S9). We then compared recombination frequency in UT MEFs treated for 1 hr with **4** either prior to (pre) or after (post) UV exposure (Fig. 4). In both cases, UT MEFs were washed after 1 hr treatments to remove unbound **4**. Compared to 24 hr treatment (24 hr post) of uncaged **4**, we observed a slight reduction in recombination efficiency when UT MEFs were treated for 1 hr (1 hr post) of uncaged **4**. For UT MEFs treated with caged **4** for 1 hr prior to UV uncaging (1 hr pre), there was clear evidence of recombination and GFP activation, though substantially less than 1 hr treatment with uncaged **4**, and efficiency depended critically on the absence of serum during treatment (Fig. S10). Nevertheless, our data clearly indicate that the caged, inactive form of **4** can bind to cells and mediate recombination once released by exposure to UV light.

In summary, we developed an efficient method for synthesis of a water soluble photocaged tamoxifen by using a *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 tissue 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 are 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 on a specific tissue.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Notes and references

1. Feil R. *Handb Exp Pharmacol*. 2007; 3
2. Metzger D, Clifford J, Chiba H, Chambon P. *Proc Natl Acad Sci U S A*. 1995; 92:6991–6995. [PubMed: 7624356]
3. Feil R, Brocard J, Mascres B, LeMeur M, Metzger D, Chambon P. *Proc Natl Acad Sci U S A*. 1996; 93:10887–10890. [PubMed: 8855277]
4. Feil R, Wagner J, Metzger D, Chambon P. *Biochem Biophys Res Commun*. 1997; 237:752–757. [PubMed: 9299439]
5. Nakamura E, Nguyen MT, Mackem S. *Dev Dyn*. 2006; 235:2603–2612. [PubMed: 16894608]
6. Mayer G, Heckel A. *Angew. Chem. Intl. Ed*. 2006; 45:4900–4921.
7. Wieboldt R, Gee KR, Niu L, Ramesh D, Carpenter BK, Hess GP. *Proc Natl Acad Sci U S A*. 1994; 91:8752–8756. [PubMed: 8090718]
8. Walker JW, McCray JA, Hess GP. *Biochem*. 1986; 25:1799–1805. [PubMed: 3707910]
9. Sonia PGS, Pollitt K. *Angew. Chem. Intl. Ed. Engl*. 1998; 37:2104–2107.
10. Karginov AV, Zou Y, Shirvanyants D, Kota P, Dokholyan NV, Young DD, Hahn KM, Deiters A. *J. Am. Chem. Soc*. 2010; 133:420–423. [PubMed: 21162531]
11. Lemke EA, Summerer D, Geierstanger BH, Brittain SM, Schultz PG. *Nat. Chem. Biol*. 2007; 3:769–772. [PubMed: 17965709]
12. Pinheiro AV, Baptista P, Lima JC. *Nucl. Acids Res*. 2008; 36:e90. [PubMed: 18586819]
13. Mal NK, Fujiwara M, Tanaka Y. *Nature*. 2003; 421:350–353. [PubMed: 12540896]

14. Agasti SS, Chompoosor A, You C-C, Ghosh P, Kim CK, Rotello VM. *J. Am. Chem. Soc.* 2009; 131:5728–5729. [PubMed: 19351115]
15. Choi SK, Thomas T, Li M, Kotlyar A, Desai A, Baker JR Jr. *Chem. Commun. (Cambridge, U.K.)*. 2010; 46:2632–2634.
16. Shamay Y, Adar L, Ashkenasy G, David A. *Biomaterials*. 2011; 32:1377–1386. [PubMed: 21074848]
17. Link KH, Shi Y, Koh JT. *J. Am. Chem. Soc.* 2005; 127:13088–13089. [PubMed: 16173704]
18. Shi Y, Koh JT. *ChemBioChem*. 2004; 5:788–796. [PubMed: 15174161]
19. Choi SK, Thomas TP, Li M-H, Desai A, Kotlyar A, Baker JR. *Photochem. Photobiol. Sci.* 2012; 11:653–660. [PubMed: 22234658]
20. Choi SK, Verma M, Silpe J, Moody RE, Tang K, Hanson JJ, Baker JR Jr. *Bioorg. Med. Chem.* 2012; 20:1281–1290. [PubMed: 22225916]
21. Meylan WM, Howard PH, Boethling RS. *Environ. Toxicol. Chem.* 1996; 15:100–106.
22. Ruzankina Y, Pinzon-Guzman C, Asare A, Ong T, Pontano L, Cotsarelis G, Zediak VP, Velez M, Bhandoola A, Brown EJ. *Cell stem cell*. 2007; 1:113–126. [PubMed: 18371340]
23. Muzumdar MD, Tasic B, Miyamichi K, Li L, Luo L. *Genesis*. 2007; 45:593–605. [PubMed: 17868096]
24. Schroeder A, Goldberg MS, Kastrup C, Wang Y, Jiang S, Joseph BJ, Levins CG, Kannan ST, Langer R, Anderson DG. *Nano Letters*. 2012; 12:2685–2689. [PubMed: 22432731]
25. Deiters A, Garner RA, Lusic H, Govan JM, Dush M, Nascone-Yoder NM, Yoder JA. *J. Am. Chem. Soc.* 2010; 132:15644–15650. [PubMed: 20961123]
26. Beverage JN, Sissung TM, Sion AM, Danesi R, Figg WD. *J Pharm Sci.* 2007; 96:2224–2231. [PubMed: 17518364]

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.

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). (a) 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–b) Flow cytometry analysis of MEF cells exposed to UV light as a function of time after treated with either free TAM or TAM-ONB 4. Plots for induction of GFP-positive cells following UV exposure after treated with TAM-ONB 4 at a single concentration 14 μ M (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 hr (left) or 1 hr (middle) after 5 min UV uncaging (“post”), or pre-treated with caged TAM-ONB 4 for 1 hr (“pre”, right) prior to 5 min UV exposure. After 1 hr treatments (either pre- or post-UV exposure), UT MEFs were washed to remove unbound TAM-ONB 4.

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) 6M HCl, MeOH, rt, 6 h, 96%.