Published in final edited form as:
Bioorg Med Chem Lett. 2008 November 15; 18(22): 5864–5866. doi:10.1016/j.bmcl.2008.06.015.

A highly sensitive fluorogenic probe for cytochrome P450 activity in live cells

Melissa M. Yatzeck[a], **Luke D. Lavis**[a], **Tzu-Yuan Chao**[b], **Sunil S. Chandran**[b], and **Ronald T. Raines**[a,b,*]

[a]Department of Chemistry, University of Wisconsin–Madison, Madison, Wisconsin 53706, USA

[b]Department of Biochemistry, University of Wisconsin–Madison, Madison, Wisconsin 53706, USA

Abstract

A derivative of rhodamine 110 has been designed and assessed as a probe for cytochrome P450 activity. This probe is the first to utilize a "trimethyl lock" that is triggered by cleavage of an ether bond. *In vitro*, fluorescence was manifested by the CYP1A1 isozyme with $k_{cat}/K_M = 8.8 \times 10^3$ $M^{-1}s^{-1}$ and $K_M = 0.09$ μM. *In cellulo*, the probe revealed the induction of cytochrome P450 activity by the carcinogen 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, and its repression by the chemoprotectant resveratrol.

Keywords

Carcinogen; CYP1A1 Isozyme; Cytochrome P450; Dioxin; Fluorogenic Substrate; Prodrug; Resveratrol; Rhodamine 110; Trimethyl Lock

The cytochrome P450 (P450) family of enzymes is responsible for the oxidative metabolism of a wide variety of compounds, including chemotherapeutic agents and environmental toxins.[1,2] The catalytic activity of P450 enzymes controls the rate of xenobiotic metabolism, and can produce undesirable byproducts.[3] Originally, this activity had been assessed by using HPLC or other methods to separate and quantify metabolites. In the 1970's, 7-ethoxycoumarin and 7-ethoxyresorufin were introduced as the first fluorogenic substrates for assays of P450 activity.[4] Although these and other fluorogenic substrates have been used to assay P450 activity *in vitro* and enable assays *in cellulo*,[5,6] they suffer from background fluorescence.[7] For example, alkoxycoumarins exhibit moderate fluorescence and are used frequently as fluorophores in peptidase substrates based on Forster resonance energy transfer (FRET).[8] In addition, both 7-ethoxyresorufin and resorufin fluoresce brightly.[6] This problem arises because *O*-alkylation of the hydroxyl group of fluorophores such as coumarin and resorufin does little to deter the oxygen electrons from participating in the resonance that gives rise to fluorescence.

Here, we report on a superior small-molecule probe for assessing P450 activity. Our probe employs the trimethyl lock.[9–11] The trimethyl lock is an *o*-hydroxycinnamic acid derivative in which severe crowding of three methyl groups induces rapid lactonization to form a hydrocoumarin.[12] In this strategy, the phenolic oxygen of the *o*-hydroxycinnamic acid is modified to create a functional group that is a substrate for a designated enzyme, and the carboxyl group is condensed with the amino group of a dye. Unmasking of the phenolic oxygen leads to rapid lactonization with concomitant release of the dye. An important attribute of this

*Corresponding author. Tel.:+1-608-262-8588; fax:+1-608-262-3453; e-mail: rtraines@.wisc.edu.

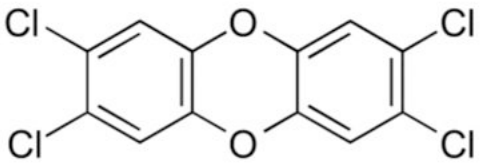
strategy is that the fluorescence/absorbance of the dye is masked completely by amidic resonance and the resulting lactonization within the rhodamine moiety.[9,10]

The human genome contains 27 genes encoding P450 isozymes, along with many pseudogenes.[13] Of these isozymes, cytochrome P450 1A1 (CYP1A1) is known to be especially important in the metabolism of xenobiotics.[14] Unlike most P450 isoforms, which are found primarily in the liver, CYP1A1 is present mainly in the lungs, where it plays an important role in the metabolic activation of chemical carcinogens.[1,15] The lung is a primary site of exposure for inhaled toxins along with carcinogens that can ultimately yield lung carcinomas.[16] CYP1A1 is strongly induced by cigarette smoking.[15] Many compounds, including some found in cigarette smoke, are not hazardous until metabolized by CYP1A1.[16,17] Accordingly, CYP1A1 levels could be correlated with human disease.

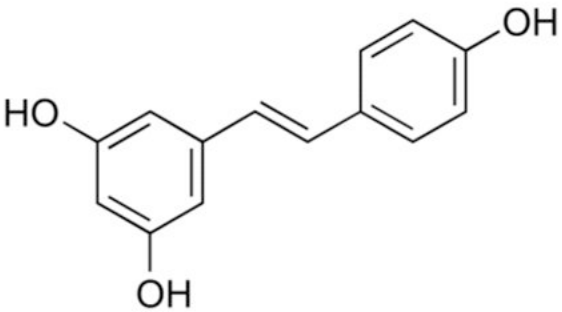
We suspected that the trimethyl lock could provide the basis for a useful probe for CYP1A1 activity. As a dye, we chose a morpholino urea derivative of rhodamine 110 (Rh_{110}) that is bright ($\varepsilon \times \Phi = 2.38 \times 10^4\ M^{-1}cm^{-1}$) but has no measurable fluorescence after *N*-acylation.[10] We installed an ethyl group on the phenolic oxygen of the trimethyl lock because ethyl ethers are especially effective substrates for CYP1A1.[15] The synthetic route to fluorogenic probe **1** is shown in Figure 1. Briefly, known intermediate **2**18 was alkylated with diethyl sulfate to give ethyl ether **3**. Removal of the silyl group followed by Jones oxidation afforded carboxylic acid **5**. Condensation with urea–rhodamine **6** gave fluorogenic probe **1** in 5% overall yield.

Fluorogenic probe **1** was first assayed as a substrate for human CYP1A1 **in vitro**. Fluorogenesis was rapid, with $k_{cat}/K_M = 8.8 \times 10^3\ M^{-1}s^{-1}$ and $K_M = 0.09\ \mu M$ (Figure 2A). These values are comparable to the highest values obtained with other fluorogenic substrates.[1,2] These data are the first to demonstrate that the trimethyl lock can be activated by the cleavage of an ether bond.

Next, fluorogenic probe **1** was assayed as a substrate for CYP1A1 in live human cells. These experiments employed human lung adenocarcinoma cell line A549, which is especially well suited for studying the expression of the pulmonary CYP system.[16,19] A low but observable level of CYP1A1 was apparent after a 1-h incubation with fluorogenic probe **1** (Figure 2B).



TCDD



resveratrol

Then, fluorogenic probe **1** was evaluated as a means to detect an increase in CYP1A1 levels. To do so, A549 cells were incubated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 10 nM), which is the notorious contaminant in the herbicide Agent Orange and the most potent known inducer of CYP1A1.[20] The effect of TCDD on fluorogenesis within A549 cells was dramatic (Figure 2C).

Finally, fluorogenic probe **1** was used to reveal a more complex modulation of P450 activity. Levels of P450 are highly variable in individuals, and there are many known P450 polymorphisms.[21] Inhibitors of P450 activity have potential as chemotherapeutic agents.[22] For example, resveratrol (3,5,4′-trihydroxystilbene), which is a natural phytoalexin present in grapes and other foods, has been proposed to have a chemoprotective effect against lung cancer by virtue of its ability to decrease CYP1A1 activity.[23] To test this hypothesis with fluorogenic probe **1**, live A549 cells were treated with both TCDD and resveratrol, along the probe. After a 1-h incubation, cells exhibited a dramatic decrease in fluorescence compared with cells treated with TCDD (Figure 2D). The levels appeared to be even lower than those in untreated cells. These and other data[23] provide direct and conclusive evidence that resveratrol decreases CYP1A1 activity *in cellulo*.

In conclusion, fluorogenic probe **1** is the first to utilize a “trimethyl lock” that is triggered by cleavage of an ether bond. This probe has numerous desirable attributes. Its chemical and photophysical properties allow for real-time imaging of P450 levels *in cellulo*. The modularity of this probe enables its extension to enzymes throughout the P450 family, and its success indicates that the trimethyl lock strategy can be applied to P450-activated prodrugs. Finally, appending the urea group with a trichloromethyl ketone or other weak electrophile would allow the probe to react with an intracellular thiol and enable its retention within a cell, providing additional utility.[24]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This paper is dedicated to Professor Benjamin F. Cravatt on the occasion of his winning the 2008 Tetrahedron Young Investigator Award. This work was supported by grant CA073808 (NIH). NMRFAM was supported by grant P41 RR02301 (NIH). M.M.Y. was a Pfizer Undergraduate Summer Fellow in Chemistry and a Hilldale Undergraduate/ Faculty Research Fellow. L.D.L was supported by Biotechnology Training Grant 08349 (NIH) and an ACS Division of Organic Chemistry Graduate Fellowship sponsored by the Genentech Foundation. T.-Y.C. was supported by the Dr. James Chieh-Hsia Mao Wisconsin Distinguished Graduate Fellowship.

References and notes

1. Ortiz de Montellano, PR., editor. Cytochrome P450: Structure, Mechanism, and Biochemistry. New York: Kluwer Academic/Plenum Publishers; 2005.
2. (a) Gungerich FP. Chem. Res. Toxicol 2008;21:70. [PubMed: 18052394] (b) Johnson WW. Drug Metab. Rev 2008;40:101. [PubMed: 18259986]
3. Nebert DW, Dalton TP. Nat. Rev. Cancer 2006;6:947. [PubMed: 17128211]
4. (a) Ullrich V, Weber P. Hoppe-Seyler’s X. Physiol. Chem 1972;353:1171. (b) Burke MD, Mayer RT. Drug Metab. Dispos 1974;2:583. [PubMed: 4155680] (c) Lavis LD, Raines RT. ACS Chem. Biol 2008;3:142. [PubMed: 18355003]
5. (a) Burke MD, Murray GI, Lees GM. Biochem. J 1983;212:15. [PubMed: 6870848] (b) White IN. Anal. Biochem 1988;172:304. [PubMed: 3189781] (c) Mayer RT, Netter KJ, Heubel F, Hanhemann B, Buchheister A, Mayer GK, Burke MD. Biochem. Pharmacol 1990;40:1645. [PubMed: 2222518] (d) Buters JT, Schiller CD, Chou RC. Biochem. Pharmacol 1993;46:1577. [PubMed: 8240414] (e) Mayer RT, Dolence EK, Mayer GE. Drug Metab. Dispos 2007;35:103. [PubMed: 17035601]
6. Ghosal A, Hapangama N, Yuan Y, Lu X, Horne D, Patrick JE, Zbaida S. Biopharm. Drug Dispos 2003;24:375. [PubMed: 14689466]
7. Wright AT, Cravatt BF. Chem. Biol 2007;14:1043. [PubMed: 17884636]
8. Lesner A, Wysocka M, Guzow K, Wiczk W, Legowska A, Rolka K. Anal. Biochem 2008;15:306. [PubMed: 18261971]

9. (a) Chandran SS, Dickson KA, Raines RT. J. Am. Chem. Soc 2005;127:1652. [PubMed: 15700995] (b) Huang S-T, Lin Y-L. Org. Lett 2006;8:265. [PubMed: 16408891] (c) Lavis LD, Chao TY, Raines RT. ChemBioChem 2006;7:1151. [PubMed: 16755624] (d) Levine MN, Lavis LD, Raines RT. Molecules 2008;13:204. [PubMed: 18305412]
10. Lavis LD, Chao T-Y, Raines RT. ACS Chem. Biol 2006;1:252. [PubMed: 17163679]
11. (a) Johnson RJ, Chao TY, Lavis LD, Raines RT. Biochemistry 2007;46:10308. [PubMed: 17705507] (b) Mangold SL, Carpenter RT, Kiessling LL. Org. Lett 2008;10:xxxx.
12. (a) Milstein S, Cohen LA. J. Am. Chem. Soc 1972;94:9158. [PubMed: 4642365] (b) Borchardt RT, Cohen LA. J. Am. Chem. Soc 1972;94:9166. [PubMed: 4642366]
13. Nelson DR, Zeldin DC, Hoffman SMG, Maltais LJ, Wain HM, Nebert DW. Pharmacogenetics 2004;14:1. [PubMed: 15128046]
14. Rooseboom M, Commandeur JNM, Vermeulen NPE. Pharmacol. Rev 2004;56:53. [PubMed: 15001663]
15. Liu J, Ericksen SS, Besspiata D, Fisher CW, Szklarz GD. Drug Metab. Dispos 2003;31:412. [PubMed: 12642467]
16. Hukkanen J, Lassila A, Paivarinta K, Valanne S, Sarpo S, Hakkola J, Pelkonen O, Raunio H. Am. J. Respir. Cell. Mol. Biol 2000;22:360. [PubMed: 10696073]
17. (a) Ueng TH, Hu SH, Chen RM, Wang HW, Kuo ML. J. Toxicol. Environ. Health A 2000;60:101. [PubMed: 10872632] (b) Lemm F, Wilhelm M, Roos PH. Int. J. Hyg. Environ. Health 2004;207:325. [PubMed: 15471096]
18. Nicolaou MG, Yuan C-S, Borchardt RT. J. Org. Chem 1996;61:8636.
19. Urani C, Doldi M, Crippa S, Camatini M. Chemosphere 1998;37:2785. [PubMed: 9839400]
20. Whitlock JP Jr. Annu. Rev. Pharmacol. Toxicol 1999;39:103. [PubMed: 10331078]
21. (a) Murray M, Petrovic N. Curr. Opin. Mol. Ther 2006;8:480. [PubMed: 17243482] (b) Ingelman-Sundberg M, Sim SC, Gomez A, Rodriguez-Antona C. Pharmacol. Ther 2007;116:496. [PubMed: 18001838]
22. Schuster I, Bernhardt R. Drug Metab. Rev 2007;39:481. [PubMed: 17786634]
23. (a) Ciolino HP, Daschner PJ, Yeh GC. Cancer Res 1998;58:5707. [PubMed: 9865727] (b) Mollerup S, Ovrebo S, Haugen A. Int. J. Cancer 2001;92:18. [PubMed: 11279601] (c) Chen Z-H, Hurh Y-J, Na H-K, Kim J-H, Chun Y-J, Kim D-H, Kang K-S, Cho M-H, Surh Y-J. Carcinogenesis 2004;25:2005. [PubMed: 15142886]
24. Haugland, RP.; Spence, MTZ.; Johnson, ID.; Basey, A. The Handbook: A Guide to Fluorescent Probes and Labeling Technologies. Vol. 10th ed.. Eugene, OR: Molecular Probes; 2005.

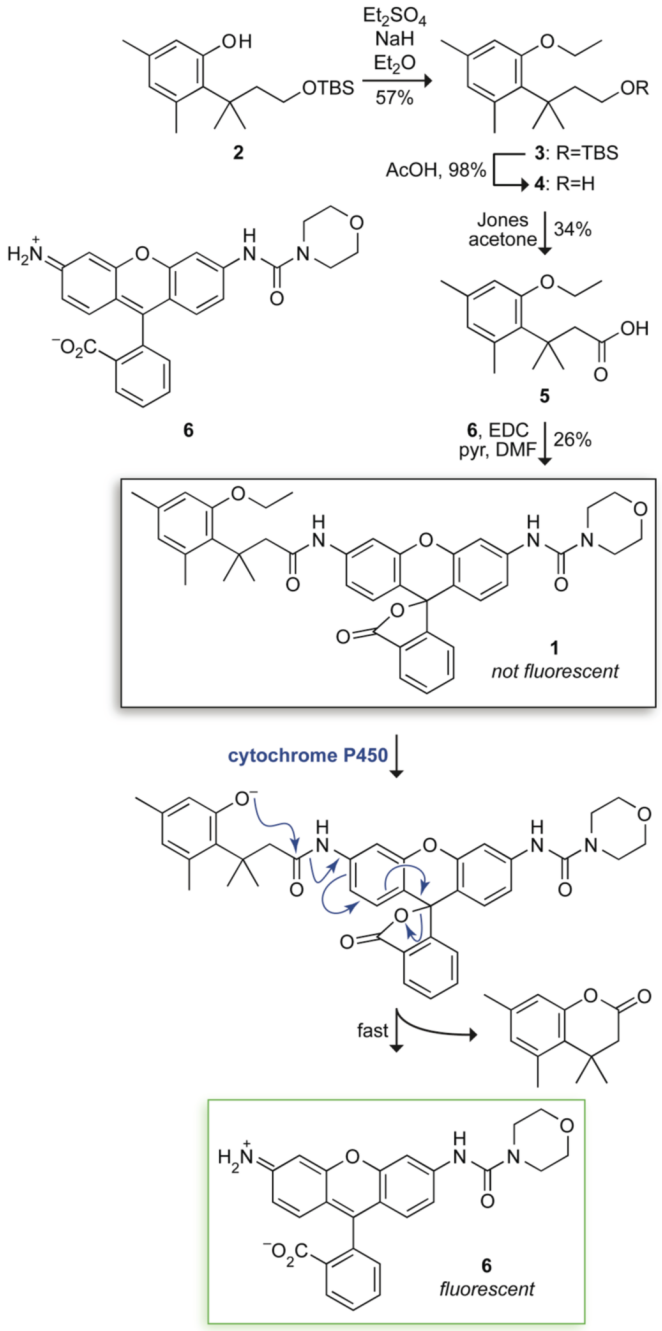


Figure 1.
Scheme for the synthesis and use of fluorogenic probe **1**.

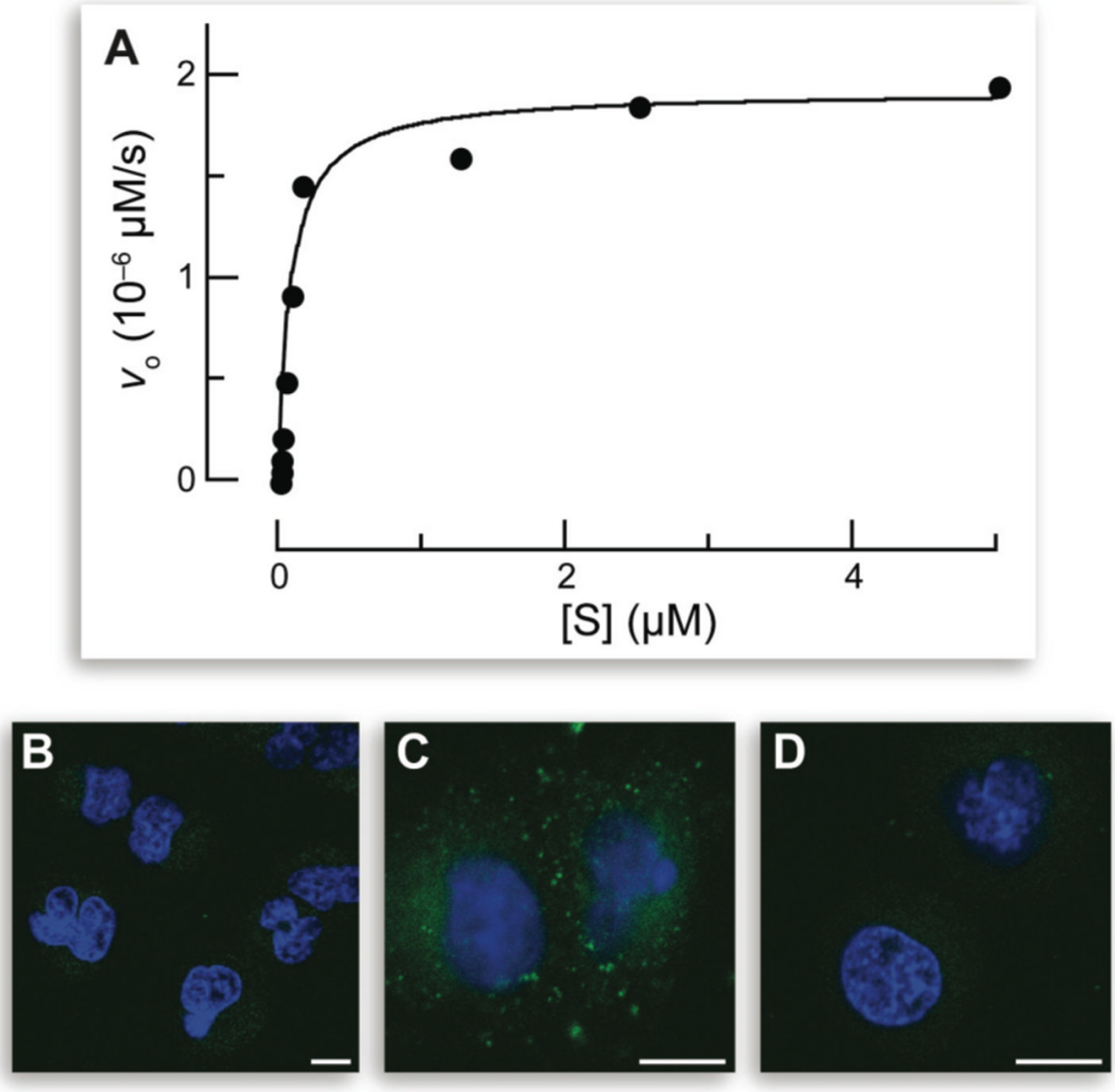


Figure 2.

Fluorogenesis from fluorogenic probe **1** *in vitro* and *in cellulo*. (A) Data for the *in vitro* cleavage of fluorogenic probe **1** by human CYP1A1 (5.0 pM) in PBS containing NADPH (8 mM) and $MgCl_2$ (8 mM). (B–D) Images of the *in cellulo* cleavage of fluorogenic probe **1**. A549 cells were incubated with fluorogenic probe **1** (10 μM) and an additive for 1 h and counterstained with Hoechst 33342. (B) No additive. (C) TCDD (10 nM). (D) TCDD (10 nM) and resveratrol (50 μM). Scale bars: 10 μm.