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Design and Synthesis of a New Fluorescent Probe for Cytochrome P450 3A4 (CYP 3A4)

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Abstract—Inhibition of CYP 3A4 catalytic activity is a principal mechanism for in vivo drug–drug interactions, sometimes leading to severe toxic effects. Rapid in vitro testing for CYP 3A4 high affinity/high inhibition potential has become part of the standard investigations for new drug candidates. Unfortunately, the complexity of the kinetics associated with CYP 3A4 catalyzed reactions (multiple substrates binding, non Michaelis–Menten kinetics) make these tests either inaccurate or tedious. We have designed and synthesized a new fluorescent probe, a testosterone substituted at the 6β- position with a fluorescent deazaflavine moiety which is able to inhibit to the same extent the hydroxylation of compounds known to bind to different sites in the CYP 3A4 active site. Furthermore, the binding of this compound and its displacement from the active site can be followed by fluorescence measurements, which allows a rapid evaluation of the CYP 3A4 affinity of any new drug candidate.

Cytochrome P450 3A4 (CYP 3A4), a heme–thiolate protein, is one of the most important P450s in human liver.[1] The ability of CYP 3A4 to metabolize >50% of administered therapeutic agents accounts for the large number of documented drug–drug interactions associated with CYP 3A4 inhibition.[2] It is therefore of great importance to know as early as possible the affinity of new drug candidates to CYP 3A4, in order to determine their potential as inhibitors, and to evaluate their influence on the metabolism of co-administered drugs.

Some particular features of CYP 3A4 make it difficult to achieve this goal. First, X-ray structures of this membrane-bound enzyme are not available yet. Second, several distinct binding sites have been described for testosterone **1**, midazolam **2** and nifedipine **3**, the prototype substrates of CYP 3A4 (Fig. 1).[3] Further, it has been shown that more than one substrate molecule can be accommodated by the active site.[4] Partial inhibition and even activation have been observed when pair of drugs were co-incubated.[5] Our aim is to develop compounds blocking the different binding sites of CYP 3A4 such that the affinity of drug candidates could be evaluated by displacement of a single probe, without performing tedious enzyme inhibition studies. Favourably these compounds should display an IC_{50} between 1 and 10 μM, which is considered to be a cut-off value to eliminate drugs binding too strongly to CYP 3A4. Further, probes should be compatible with high-throughput technology such that a photochemical interaction of the molecule with the active site's heme–thiolate complex can be rapidly measured.

The design of compound **4** (Scheme 1) was guided by the following requirements

(i) the putative binding sites of testosterone to the protein, carbonyl at C-3 and OH at C-17 are maintained
(ii) the 6β position that is usually hydroxylated is substituted by a linker
(iii) the linker is long enough to provide flexibility for adjustment in the active site
(iv) the linker connects the testosteryl moiety to a deazaflavin, a well known fluorophore,[6] whose fluorescence should be quenched when interacting with the iron porphyrin (Fig. 2).

The synthesis of **4** is depicted in Scheme 1. The 6β-substituted diester **6**[7] yielded after decarboxylation and hydrolysis the acid **8**,[8,9] ready for coupling with the deazaflavinyl alcohol **9**. The latter was prepared from commercially available 6-chloro uracil **10** following established procedures.[10] Coupling of **9** with **8** gave **4** as a 94/6 mixture of the 6β/6α isomers.[11]

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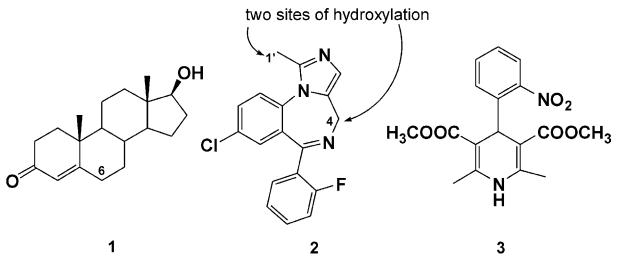
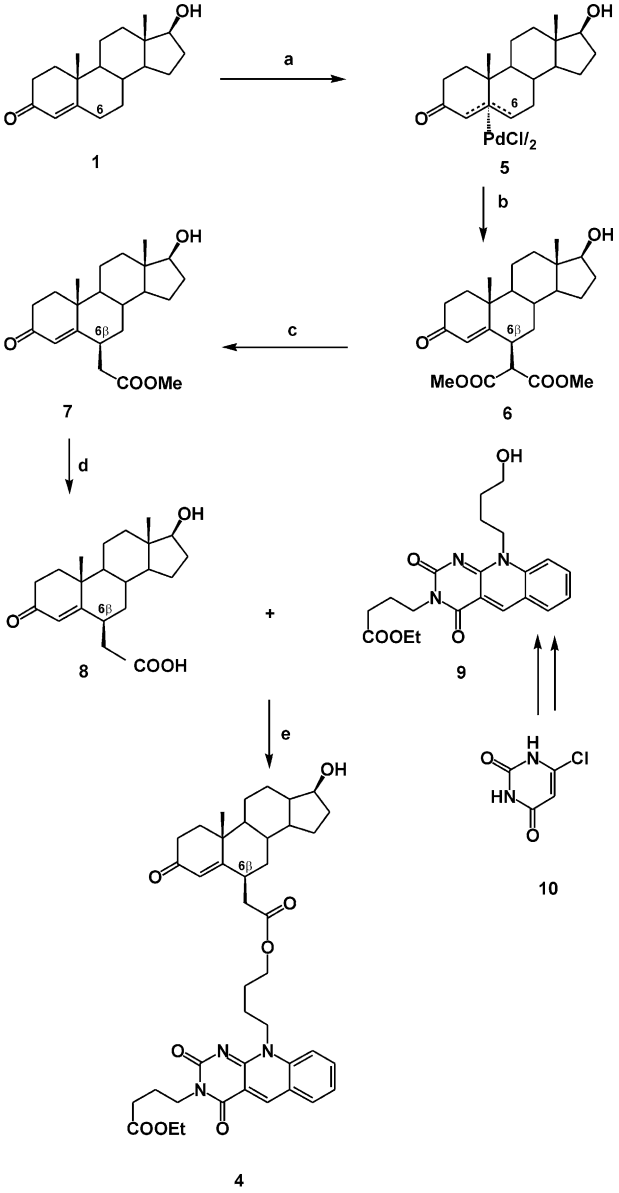


Figure 1. CYP 3A4 model substrates.



Scheme 1. Synthesis of compound **4**: (a) $PdCl_2$, NaCl, THF, reflux, 2 d, 72%; (b) NaH, DMSO, $CH_2(COOCH_3)_2$, 35 °C, 8 h, 93%; (c) LiI, DMF, reflux, 2.5 h, 57%; (d) LiOH, MeOH–H_2O 3:1, rt, 1 h, 79%; (e) DMAP, EDC, CH_2Cl_2, 0 °C, 2 h, rt, 13 h, 79%.

A typical type I spectrum was observed upon mixing **4** with solubilized CYP 3A4 (Calbiochem, Biosciences Inc, La Jolla, CA, USA) indicating the binding of **4** in the CYP 3A4 active site (Fig. 3A). A K_s of about 2 μM could be calculated from the titration curves. Compound **4** can be displaced by ketoconazole, a potent

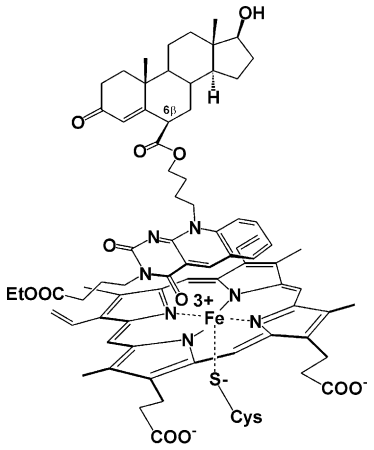


Figure 2. Proposed interaction of compound **4** with the heme-thiolate cofactor of CYP 3A4.

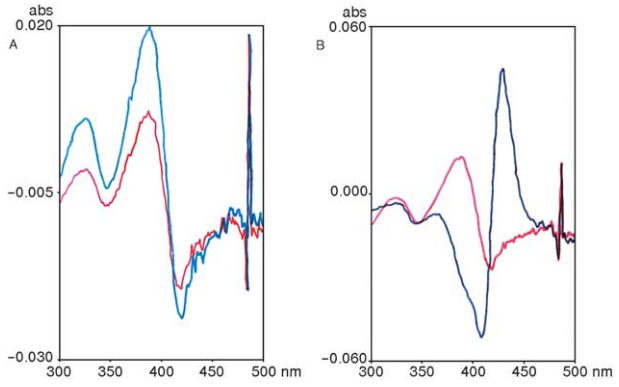


Figure 3. UV–vis difference spectra: (A) addition of compound **4** to CYP 3A4 (1.6 μM). Only two concentrations are shown (red: 1 μM; blue: 2 μM). (B) displacement of **4** bound to CYP 3A4 by ketoconazole.

Table 1. Inhibition of CYP 3A4 catalyzed reactions by compound **4**

CYP 3A4 substrate	**4**/IC_{50} (μM)
Testosterone **1**	2.5±0.6
Midazolam **2** (MDZ)	1±0.5 (4-OH)
	1.1±0.5 (1′-OH)
Nifedipine **3**	4.1±0.2

inhibitor of CYP 3A4 as shown in Figure 3B.[12] Consequently, with formation a type II spectrum is produced as expected when imidazole derivatives like ketoconazole bind to CYP 3A4.[13]

Compound **4** was tested as inhibitor of three characteristic CYP 3A4 reactions: testosterone **1** 6β-hydroxylation, midazolam **2** 1′ and 4-hydroxylations and nifedipine **3** oxidation using CYP 3A4 coexpressed with P450 reductase and b_5 (BD-Gentest, Woburn, MA, USA) and well established incubation and analysis methods.[3,14] Each assay corresponds to a different substrate binding mode in the active site of the enzyme.[4] The results, summarized in Table 1, show that the IC_{50} obtained with the three substrates are all of the same order of magnitude and in the desired range (1–5 μM).[15] These values indicate that compound **4** prevents access of **1**, **2** and **3** to their putative binding sites. When midazolam **2** was used as substrate, both 1′- and 4-hydroxylations were inhibited with the

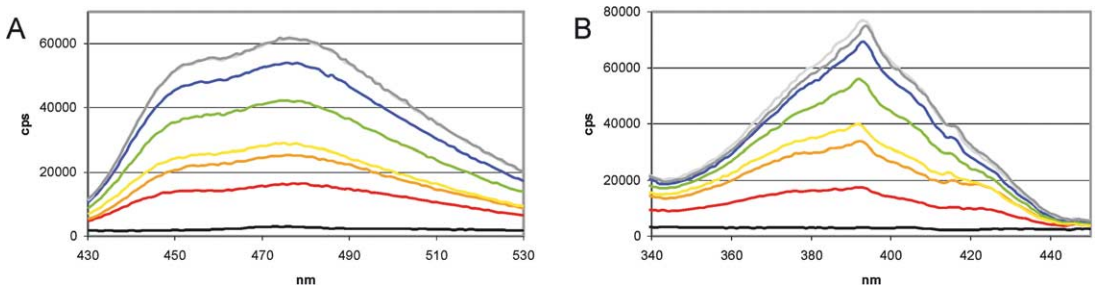


Figure 4. Fluorescence spectra of compound **4**: A: emission spectrum ($\lambda = 400$ nm) and B: excitation spectrum ($\lambda = 480$ nm). Black: CYP 3A4; red: enzyme + **4** (0.8 μM); orange to light-grey: enzyme + **4** (0.8 μM) + increasing concentrations of ketoconazole 1, 3, 7, 11, 15, 19 μM (final concentration).

same IC_{50}. This result is especially remarkable since it is reported that testosterone **1** inhibits the midazolam 1′-hydroxylation but has no effect on the 4-hydroxylation.[3] Therefore a displacement of compound **4** by a new drug candidate **X** at a ca. equimolar concentration would be an indication of a high CYP 3A4 affinity for **X**. This property could eventually translate in vivo to drug–drug interactions in the case where **X** is co-administered with other drugs metabolized by CYP 3A4.

This concept was tested with ketoconazole. As expected, a fluorescence quenching was observed upon mixing **4** (0.8 μM) and CYP 3A4 (Calbiochem) at a 3.2 μM concentration (Fig. 4). The fluorescence could be restored by addition of ketoconazole, in good agreement with the high affinity of this compound for CYP 3A4 as reported in the literature.[12]

In summary, the method presented here allows for rapid evaluation of the affinity of any compound to CYP 3A4.[16] The method has several advantages over the conventional screening methods which rely on turnover: (i) no time consuming inhibition tests are required, (ii) assays can be conducted in the absence of reductase and NADPH, (iii) no HPLC analysis of the metabolites formed is needed. This concept can be used to rapidly screen a series of new drug candidates and identify compounds whose high affinity to CYP 3A4 may lead to dangerous drug-drug interactions when administered together with other CYP 3A4 substrates.

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References and Notes

1. Guengerich, F. P. Cytochrome P450: Structure, Mechanism, and Biochemistry. In *Biochemistry*, Ortiz de Montellano, P. R., Ed.; Plenum Press: New York, 1995; p 473–525.
2. (a) Lilja, J.; Kivisto, K.; Neuvonen, P. *Clin. Pharmacol. Ther*. **2000**, *68*, 384. (b) Lin, J. H.; Lu, A. Y. H. *Pharmacol. Rev*. **1997**, *49*, 403. (c) Guengerich, F. P. *Ann. Rev. Pharmacol. Tox*. **1999**, 1.
3. Wang, R. W.; Newton, D. J.; Liu, N.; Atkins, W. M.; lu, A. Y. H. *Drug Metab. Dispos*. **2000**, *28*, 360.
4. (a) Hosea, N. A.; Miller, G. P.; Guengerich, F. P. *Biochemistry* **2000**, *39*, 5929. (b) Dabrowski, M. J.; Schrag, M. L.; Wienkers, L. C.; Atkins, W. M. *J. Am. Chem. Soc*. **2002**, *124*, 11866.
5. Kenworthy, K. E.; Clarke, S. E.; Andrews, J.; Houston, J. B. *Drug Metab. Dispos*. **2001**, *29*, 1644.
6. Sun, M.; Song, P.-S. *Biochemistry* **1973**, *12*, 4663.
7. Collins, D. J.; Jackson, W. R.; Timms, R. N. *Aust. J. Chem*. **1977**, *30*, 2167.
8. The β-configuration can be distinguished from its α-epimer by ^{1}H NMR, most significant is the shift difference of H at C-4: 5.80 ppm (β) and 5.51 ppm (α): Collins, D. J.; Jackson, W. R.; Timms, R. N. *Tetrahedron Lett*. **1976**, *6*, 495.
9. Jones, C. D.; Mason, N. R. *Steroids* **1975**, *23*, 323.
10. Carell, T.; Schmid, H.; Reinhard, M. *J. Org. Chem*. **1998**, *63*, 8741.
11. **4**: UV–vis ($CHCl_3$): λ_{max} (relative intensity) = 266 nm (100), 322 nm (21), 402 nm (26), 422 (21). Fluorescence: excitation $\lambda = 410$ nm, emission $\lambda_{max} = 447$, 470 nm. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 8.86$ (s, 1H), 7.86–7.95 (m, 2H), 7.63–7.65 (m, 1H), 7.47 (t, 1H), 5.73 (s, 1H), 4.76 (s, 2H), 4.06–4.17 (m, 6H), 3.62 (t, 1H), 2.96 (q, 1H), 2.56–2.60 (m, 1H), 2.44–2.47 (m, 1H) 2.35–2.40 (m, 4H), 1.96–2.10 (m, 4H), 1.86–1.90 (m, 6H), 1.60–1.72 (m, 5H), 1.38–1.44 (m, 2H), 1.22–1.30 (m, 7H), 1.02–1.10 (m, 1H), 0.80–0.97 (m, 3H), 0.76 (s, 3H). Anal. calcd for $C_{42}H_{53}N_3O_8$ (727.91): C 69.30, H 7.34, N 5.77, found: C 68.93, H 7.57, N 5.32. The amount of the 6α-epimer of **4** (6%) can be estimated from integration of the resonance of H at C-4 at 5.59 ppm, d, $J = 1.5$ Hz, compared to the corresponding resonance of the 6β isomer (5.73 ppm, s). It is important to note that the IC_{50} values of 6α-**4** are very similar to 6β-**4** such that inhibition pursued with 94% 6β-**4**/6% 6α-**4** gives IC_{50} values that are within experimental error indistuingishable from 100% 6β-**4**.
12. Wandel, C.; Kim, R. B.; Guengerich, F. P.; Wood, A. J. J. *Drug Metab. Dispos*. **2000**, *28*, 895.
13. Jefcoate, C. R. *Methods Enzymol*. **1978**, *52*, 258.
14. Stresser, D. M.; Blanchard, A. P.; Turner, S. D.; Erve, J. C. L.; Dandeneau, A. A.; Miller, V. P.; Crespi, C. L. *Drug Metab. Dispos*. **2000**, *28*, 1440.
15. If the testosteryl moiety is omitted IC_{50} values are larger by two orders of magnitude, for example **9** has an $IC_{50} = 240$ μM for testosterone **1** as a substrate.
16. Stoessel, C.; Chougnet, A.; Woggon, W.-D.; In preparation.