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Design and synthesis of a fluorescent muscarinic antagonist

Lyn H. Jones,[a,*] Amy Randall,[a] Carolyn Napier,[b] Mike Trevethick,[b] Sasha Sreckovic[b] and Jessica Watson[b]

[a]*Discovery Chemistry, Sandwich Laboratories, Pfizer Research and Development, Ramsgate Rd, Kent CT2 8DR, UK*
[b]*Discovery Biology, Sandwich Laboratories, Pfizer Research and Development, Ramsgate Rd, Kent CT2 8DR, UK*

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Abstract—The design and concise synthesis of a fluorescent tolterodine-BODIPY (boron dipyrromethene) conjugate is described which possesses potent antimuscarinic activity. This derivative illustrates proof-of-concept for the preparation of other useful fluorophoric antimuscarinic agents which have potential utility in receptor occupancy studies and high throughput screens.

Biological molecules tagged with fluorescent groups have been used for a myriad of purposes from cell imaging to automated DNA sequencing. Fluorophoric ligands have also been extensively used in drug discovery to create fluorometric assays for high throughput screening (HTS) which offer significant advantages over the radiometric variants. Radioligand-based assays suffer primarily due to radioactivity safety risks, resulting in increased safety training, radioactive waste disposal, and environmental monitoring of waste. As a result, fluorometric assays offer considerable cost savings as well as reduced environmental safety risks. The high sensitivity of fluorescence techniques also enables relatively straightforward miniaturization and as a result they are well suited to ultraHTS (10^5 assays per day or more).

One of the main issues with the design of these ligands is knowing where to place the fluorogenic tag in the probe molecule since these tags can be rather large functions which need to satisfy the structure-activity relationships (SARs) of the original scaffold molecule.[1] Obviously, this problem need not be associated with radiometric ligands, although the synthetic tractability of these derivatives can prove troublesome due to the short half-life of the incorporated radioactive isotope or the limited synthetic chemistries which are available to introduce the radioactivity at a late stage.

In this study, we report the first design and synthesis of a potent fluorescent muscarinic M3 antagonist with potential uses in HTS and receptor occupancy studies.[2] Antimuscarinics continue to generate significant interest in the drug discovery community, particularly highlighted by the recent successes of tolterodine for the treatment of urinary incontinence and tiotropium bromide, an inhaled, long-acting muscarinic antagonist for the treatment of chronic obstructive pulmonary disease (Fig. 1). Therefore, innovations in this field, particularly where methods can improve existing biological assays to enable HTS, are still highly desirable. Interestingly, a recent report by Canning et al. has detailed the fortuitous discovery of muscarinic antagonist activity associated with the fluorescent styryl dyes FM1-43 and FM2-10 (Fig. 1, Table 1).[3] These derivatives are key tools in monitoring endocytosis and exocytosis as their divalent cationic nature allows them to partition into the outer leaflet of the cell membrane. Unfortunately, their utility is limited due to non-specific background labeling of tissues and sections. However, these dyes help illustrate the potential uses of fluorogenic probes for visualizing muscarinic receptor occupancy in real time in tissues and cell preparations.

Our approach was to create a muscarinic-selective probe by linking a fluorophoric BODIPY (boron dipyrromethene) tag to an M3 antimuscarinic agent utilizing the most promiscuous region of the antagonist scaffold for conjugation. The BODIPY fluorophore was chosen due to the high extinction coefficients and quantum efficiencies associated with BODIPY derivatives. Addition-

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* Corresponding author. Tel.: +44 1304 644256; fax: +44 1304 651821; e-mail: lyn.jones@pfizer.com

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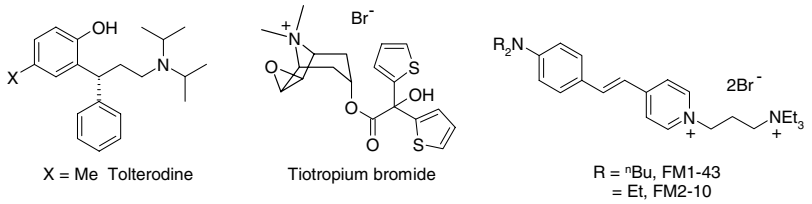


Figure 1. The structures of some antimuscarinics.

Table 1. Antimuscarinic sub-type selectivity of FM1-43, FM2-10, **3**, and tolterodine

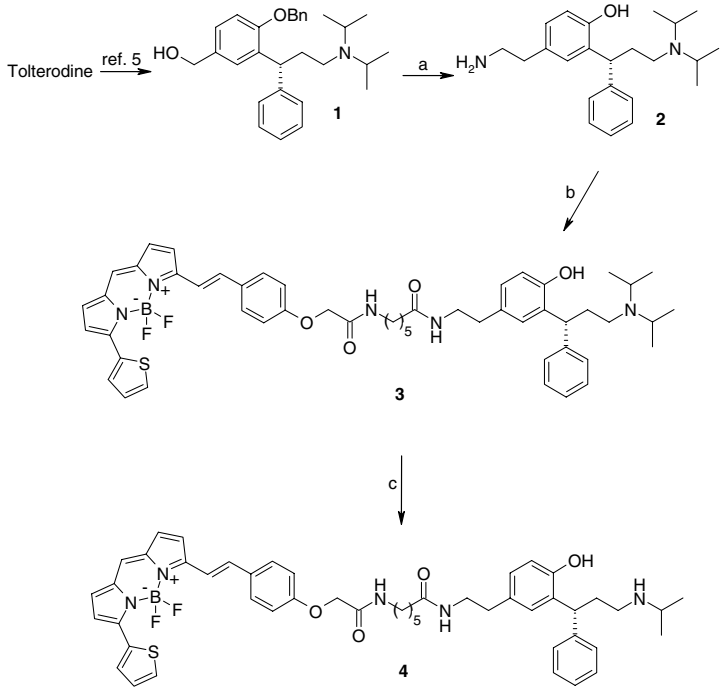
Compound	M1 K_i (nM)	M2 K_i (nM)	M3 K_i (nM)	M4 K_i (nM)	M5 K_i (nM)
FM1-43[a]	44	55	30	103	83
FM2-10[a]	34	38	24	121	228
3[b]	4.6	9.0	10.3	6.3	17.5
	(3.8–6.0)	(8.0–10.3)	(9.4–11.8)	(5.5–7.0)	(15.4–20.1)
Tolterodine[b]	1.4	2.7	3.6	3.1	2.2
	(1.0–2.0)	(2.2–3.4)	(2.9–4.1)	(2.9–3.7)	(2.1–2.3)

[a] Values are taken from Ref. 3.
[b] Values are means of four experiments.

ally, a number of BODIPY succinimidyl esters are commercially available[4] thus aiding the synthetic tractability of conjugation to our chosen scaffold. Moreover, a range of absorption/emission wavelengths are available and we decided the BODIPY D-10000 derivative was appropriate for our needs (abs 625/em 640).

Tolterodine appeared to be a well-suited antimuscarinic template for our design. It is a high affinity muscarinic antagonist and a number of analogues have been prepared within Pfizer that have generated useful SARs (data not shown). These relationships illustrate that there may be an opportunity to conjugate the fluorophoric BODIPY dye through a linker attached to position X (Fig. 1) in the tolterodine template.

Amine **2** was an ideal precursor for fluorescence tagging since it could be prepared readily from the known tolterodine benzylic alcohol **1**[5] (Scheme 1). Mesylation of **1** followed by treatment with sodium cyanide provided



Scheme 1. Reagents and conditions: (a) i—CH_3SO_2Cl, NEt_3, DCM, rt, ii—NaCN, DMF, 50 °C, iii—H_2, 60 psi, Raney nickel, c.NH_3 aq., EtOH, 40 °C, 65% over 3 steps; (b) BODIPY D-10000 succinimidyl ester (from Molecular Probes), NEt_3, DMF, 88%; (c) DMSO, rt.

the intermediate nitrile derivative which was reduced and deprotected to provide the desired amine **2**.[6] Subsequent amidation with the commercially available BODIPY succinimidyl ester derivative and purification by preparative TLC yielded pure **3**[7] which was further profiled for antimuscarinic activity.

Derivative **3** is the first tolterodine-BODIPY conjugate prepared in our laboratory. Ligand binding affinity was assessed through conventional radiometric [^{3}H] *N*-methyl scopolamine competition experiments[3] and its potent activity against the muscarinic receptors (similar to that of tolterodine itself—Table 1) vindicates our original strategy. Interestingly, we noticed that **3** was unstable when stored in solution. In light, degradation ($t_{1/2}$ 1 week) resulted in the formation of a complex mixture which presumably results from the photosensitizing effects of the BODIPY fluorophore.[8] However, even in the dark, slow degradation occurred to give the de-isopropyl derivative **4** as the only observed degradant ($t_{1/2}$10 weeks).[9]

This approach, culminating in the preparation of tolterodine-BODIPY derivative **3**, serves as an excellent proof-of-concept for the design of fluorescent muscarinic antagonists. Fine tuning of the conjugates can be realized through changes in the linker and/or fluorophoric tag, which could provide a series of useful derivatives suited to individual needs, including HTS and receptor occupancy studies. Further profiling of **3** and generation of further fluorescent conjugates will be reported in due course as this is ongoing work within our group.

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References and notes

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2. Fluorescent M1-selective muscarinic antagonists have been prepared, for example Tahtaoui, C.; Parrot, I.; Klotz, P.; Guillier, F.; Galzi, J.-L.; Hibert, M.; Ilien, B. *J. Med. Chem.* **2004**, *47*, 4300.
3. Mazzone, S. B.; Mori, N.; Burman, M.; Palovich, M.; Belmonte, K. E.; Canning, B. J. *J. Physiol.* **2006**, *575*, 23.
4. The Handbook—A Guide to Fluorescent Probes and Labeling Technologies, Molecular Probes web edition, chapter 1.4.
5. Kumar, Y.; Prasad, M.; Nayyar, K.; Sharma, N. WO2005012227.
6. Data for **2**: ^{1}H NMR (CD_3OD, 400 MHz) δ 7.32–7.20 (m, 4H), 7.12 (m, 1H), 6.95 (m, 1H), 6.83 (m, 1H), 6.66 (d, 1H), 4.32 (t, 1H), 3.00 (m, 2H), 2.75 (m, 2H), 2.59 (m, 2H), 2.41 (m, 2H), 2.13 (m, 2H), 0.93 (d, 12H); LCMS ESI *m/z* 355 $(M+H)^+$, >95%.
7. Data for **3**: ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.14 (bs, 1H), 8.04 (m, 2H), 7.83 (m, 2H), 7.78 (bs, 1H), 7.74 (d, 1H), 7.61 (m, 1H), 7.41 (d, 1H), 7.29 (m, 1H), 7.28 (d, 2H), 7.26 (m, 2H), 7.22 (m, 2H), 7.11 (m, 1H), 7.09 (m, 1H), 7.07 (m, 1H), 7.01 (s, 1H), 6.92 (d, 2H), 6.79 (m, 1H), 6.65 (m, 1H), 4.53 (s, 2H), 4.27 (m, 1H), 3.15 (m, 2H), 3.13 (m, 2H), 2.91 (m, 2H), 2.55 (m, 2H), 2.26 (m, 2H), 2.02 (m, 2H), 2.01 (m, 2H), 1.46 (m, 2H), 1.42 (m, 2H), 1.20 (m, 2H), 0.84 (d, 12H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 171.9, 167.1, 155.7, 153.0, 152.8, 145.3, 139.9, 132.4, 130.9, 130.3, 130.1, 129.6, 128.9, 127.7, 127.7, 127.7, 126.5, 125.9, 125.3, 118.1, 115.8, 115.3, 115.3, 114.8, 114.8, 66.8, 42.7, 40.2, 38.0, 35.7, 35.6, 34.4, 28.7, 25.7, 24.8, 22.2, 20.5, 4.05; ^{19}F NMR (DMSO-d_6, 376 MHz) δ −138.4 (q, J 33 Hz, 2F); ESI *m/z* 900 $(M+H)^+$; HRMS *m/z* $C_{52}H_{61}F_2N_5O_4SB$ $(M+H)^+$ found 899.4550 (error 2.2 ppm).
8. Yogo, T.; Urano, Y.; Ishitsuka, Y.; Maniwa, F.; Nagano, T. *J. Am. Chem. Soc.* **2005**, *127*, 12162.
9. One milligram of **3** was dissolved in 1 mL of DMSO and kept in the dark at room temperature. The appearance of **4** was determined by the increase of a peak in the LCMS corresponding to de-isopropylation [ESI *m/z* 858, $(M+H)^+$] and a corresponding reduction in the isopropyl integration in the proton NMR [^{1}H NMR (DMSO-d_6, 400 MHz) 0.84 ppm].