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High affinity inhibitors of the dopamine transporter (DAT): Novel biotinylated ligands for conjugation to quantum dots

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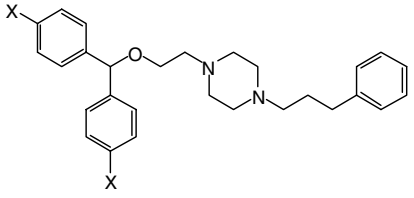
Abstract—Compounds capable of inhibiting the dopamine transporter protein (DAT) that can be conjugated to cadmium selenide/zinc sulfide/core shell nanocrystals may be used to image the location and distribution of the DAT in neuronal cell membranes. This letter describes the synthesis of biotinylated analogs of the DAT antagonists GBR 12909 and GBR 12935 that can be attached to streptavidin coated cadmium selenide/zinc sulfide/core shell nanocrystals.

Quantum dots are a new type of fluorescent markers that are increasingly finding novel applications in biology. Many groups have reported imaging applications based upon quantum dots conjugated to peptides,[1–6] nucleic acids,[7–13] proteins,[14,15] and antibodies.[16–21] Our research focus involves conjugating ligands that bind to neuronal proteins to quantum dots.[22–26] We are particularly interested in drugs that have high affinity for neuronal transporter proteins including the serotonin transporter protein (SERT) and the dopamine transporter protein (DAT). Both SERT and DAT exhibit regulated trafficking that controls transport capacity. Imaging transporter movements in neuronal membranes is a challenging but important objective.

Quantum dots have the potential to significantly improve the sensitivity and duration of in vitro biological imaging experiments that are currently performed with fluorescent dyes. Their increased photostability, brightness, and narrower emission spectra should enable the development of imaging studies that can be performed for longer durations and with lower concentrations of analyte.

Abnormalities in the dopaminergic system have been observed in the etiology of a wide range of diseases including Alzheimer's and Parkinson's diseases. The dopaminergic system is also thought to have a role in the reinforcing effects of cocaine addiction. We hope to be able to use DAT antagonist quantum dot conjugates to provide more information about the biological mode of action of dopamine reuptake inhibitors. In addition by using static and dynamic biological imaging techniques it is hoped that these conjugates may reveal information about the location and distribution of the DAT within neuronal cells.

In our initial studies we synthesized derivatives of the high affinity DAT inhibitors GBR 12909 and GBR 12935.



GBR 12909 X = F
GBR 12935 X = H

Ligands **1** and **2** were selected for their ease of synthesis, potency, and specificity for the DAT over SERT. They

Keywords: Dopamine transporter; Quantum dot; Cadmium selenide; Reuptake inhibitor.
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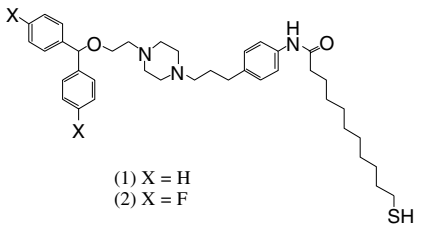
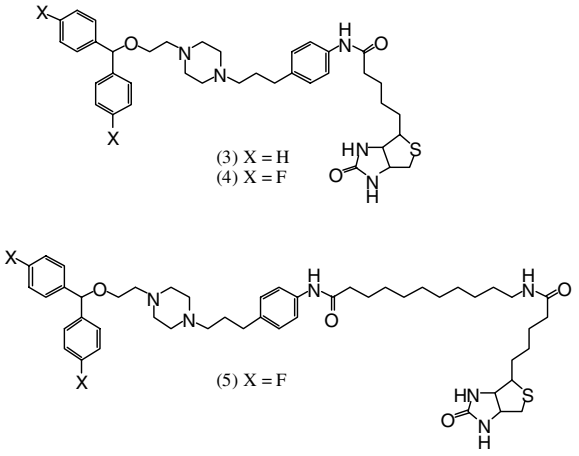
were bound to the surface of the quantum dot via an acid–base interaction between a thiol and the zinc on the surface of the quantum dot.[25]

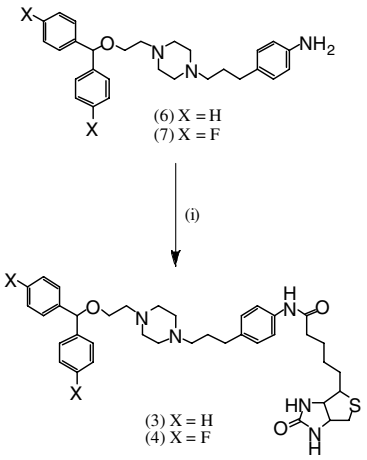


We observed that when the ligands **1** and **2** were bound to the surface of quantum dots the fluorescence of the dots diminished. This reduction in quantum yield could be due to the thiols acting as traps. In addition to reduced quantum yields these conjugates had a limited shelf life. The thiols were not irreversibly bound to the surfaces of quantum dots and they dissociated from the surface over time. This dissociation caused the dots to aggregate and precipitate from solution.

As biotin has a high affinity for avidin ($K_a \sim 10^{15}$ mol^{-1}) and streptavidin, we decided to synthesize a new generation of DAT ligands that incorporated a biotin moiety for use with streptavidin coated quantum dots. Our initial ligands had biotin bound directly to the GBR derivatives **3** and **4**. Steric hindrance between the cell surface and the dot's surface may reduce the affinity of these conjugates. Therefore we also designed a GBR derivative that incorporated an alkyl linker between the GBR derivative and the biotin **5** to reduce steric effects.



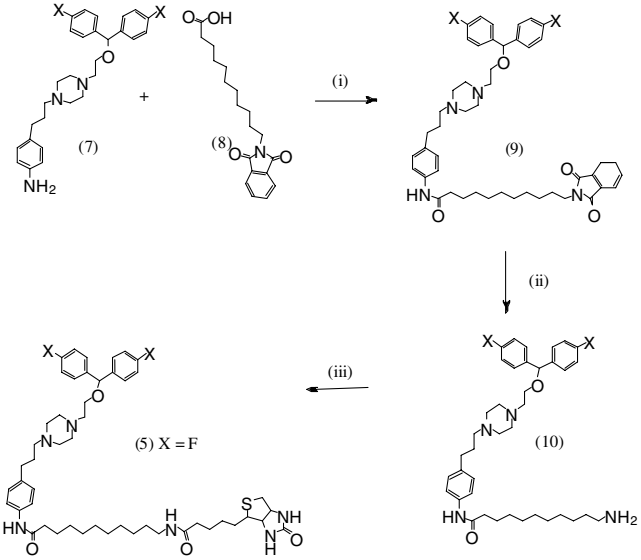
The synthesis of compounds **3** and **4** is outlined in Scheme 1. Either 1-[2-[bisphenylmethoxy]ethyl]-4-(3-(4-aminophenyl)propyl)piperazine **6** or 1-[2-[4,4′-flourobenzhydryloxy]ethyl]-4-(3-(4-aminophenyl)propyl)piperazine **7** was biotinylated in dry DMF using CDI. This resulted in a 29.7% yield of **3**[27] and a 16.4% yield of **4**.[28] The synthesis of the amino analogs of GBR 12909 and GBR 12935 has previously been described in an earlier publication.[25]



Scheme 1. Reagents: (i) Biotin, CDI.

The synthetic route used to prepare compound **5** is outlined in Scheme 2. 1-[2-[4,4′-Flourobenzhydryloxy] ethyl]-4-(3-(4-aminophenyl)propyl)piperazine **7** was coupled to the phthalimide protected 11-aminoundecanoic acid **8** using two different methods. In the first method the acid was converted to the acid chloride with thionyl chloride and reacted it with **7** in dry dichloromethane resulting in a 69% yield of **9**. The second method coupled **8**–**7** using CDI in dry DMF resulting in a 33% yield of **9**.[29] The phthalimide protecting group was removed using hydrazine in ethanol giving **10**[30] in a 72% yield. Biotin was coupled to the terminal amino functionality using DCC in dry DMF resulting in a 12.4% yield of **5**.[31]

Compounds **3**, **4**, and **5** were conjugated to strepatvidin coated quantum dots as follows; 0.1 ml of a 8.5 μM solution of streptavidin coated quantum dots that had a maximum fluorescence emission of 605 nm was added



Scheme 2. Reagents: (i) Method A $SOCl_2$, CH_2Cl_2; Method B CDI; (ii) a—hydrazine, ethanol; b—CH_2Cl_2; (iii) Biotin, NHS, DCC, DMF.

to 0.9 ml of borate buffer at pH 8.4. Compounds **3**, **4**, and **5** were dissolved in DMSO to give an 850 μM solution of each compound. 0.1 ml of this solution was added to the solution of quantum dots in borate buffer and the mixture was stirred at 25 °C for 18 h. Excess ligand was removed from this solution by dialyzing the borate solution for 18 h. The concentration of the resultant solution was determined by UV/visible spectrophotometry by measuring the absorbance at 600 nm and using an extinction coefficient of 650,000.

The ability of compounds **3**, **4**, and **5** to inhibit the uptake of dopamine was measured using a competitive transport assay. Briefly, 12 concentrations of each compound (10^{-5} and 10^{-12} M) were competed against tritiated dopamine (50 nM). Nonspecific uptake was defined with 1 mM GBR and subtracted from total radiolabeled uptake. Subtracted values were plotted versus logarithmic values of each compound and fitted with a one-site competition curve to determine IC_{50} values using Graphpad prism.

Table 1 shows the potencies of the ligands **1**–**5** against dopamine transport as well as the potency of conjugated nanocrystals. When ligands **3**–**5** were conjugated to quantum dots, no significant reduction in fluorescence was observed; however when ligands **1**–**2** were conjugated to quantum dots, a reduction in the fluorescent intensity of the dot solution was observed. No attempts were made to quantify this reduction. The proximity of the quantum dot to compounds **3** and **4** appears to reduce the affinity of these conjugated nanocrystals for the DAT. When a spacer is introduced between the biotin and the drug as in compound **5**, no significant reduction in potency was observed. The measured IC_{50}s of the unconjugated ligands were between 5 and 36 nM, whilst the IC_{50}s of the conjugates differed by a factor of a hundred-fold, indicating that the ligand is bound tightly to the surface of the quantum dot and not dissociating in solution. These conjugates were stable and could be kept at 4 °C for a period of several weeks with no precipitation of aggregates.

In conclusion we have synthesized three new DAT ligands that have high affinity for hDAT. These ligands may be conjugated to the surfaces of streptavidin coated quantum dots with little or no reduction in affinity. In addition, unlike earlier conjugates, no significant reduction in quantum yield was observed when these ligands were conjugated to quantum dots. The unbound ligands have high affinity for the DAT suggesting that there is significant open space leading to the binding site. This methodology may also be applied to ligands that have specificity for other transporters such as SERT.

Table 1. Inhibition of dopamine reuptake by compounds **1**–**5** and their nanocrystal conjugates

Compound	IC_{50} of unbound ligand (nM)	IC_{50} of ligand conjugates[a] (nM).
1	10	32
2	18	140
3	11	637
4	36	300
5	5	1

[a] IC_{50} was measured relative to quantum dot concentration.

Acknowledgments

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27. Mp = 139–140 °C. ^{1}H NMR ($CDCl_3$) δ 1.4–1.50 (m, 4H), 1.51–1.56 (m, 6H), 2.37–2.81 (m, 17H), 3.01–3.1 (m, 1H), 3.48–3.53 (m, 2H), 4.21 (br s, 1H), 4.40 (br s, 1H), 5.37 (s, 1H), 6.18 (s, 1H), 6.70–7.08 (m, 4H), 7.24–7.34 (m, 8ArH), 7.54 (d, 2ArH), 8.99 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 15.10, 25.64, 27.86, 28.04, 32.79, 36.58, 40.31, 52.44, 52.81, 55.59, 57.58, 60.08, 61.49, 65.64, 66.66, 83.71, 119.89, 126.77, 127.24, 128.16, 128.46, 136.43, 137.08, 141.97, 164.31, 172.02; *m/z* ESI (M+H) 656.35990.
28. Mp = 146–147 °C. ^{1}H NMR ($CDCl_3$) δ 1.44–1.51 (m, 2H), 1.62–1.78 (m, 7H), 2.34–2.70 (m, 16H), 2.87 (dd, J = 4.5 Hz, 1H), 3.12 (q, J = 7.11 Hz, 1H), 3.57 (t, J = 5.85 Hz, 2H), 4.29 (m, 1H), 4.84 (m, 1H), 5.34 (s, 1H), 5.80 (s, 1H), 6.91 (s, 1H), 7.0 (m, 4ArH), 7.09 (d, 2ArH), 7.27 (m, 4ArH), 7.51 (d, 2ArH), 8.55 (br s, NH); ^{13}C NMR ($CDCl_3$) δ 15.23, 25.76, 27.97, 28.16, 33.00, 36.78, 40.50, 53.10, 53.43, 55.73, 57.75, 60.22, 61.65, 65.81, 66.83, 82.47, 115.09, 115.37, 119.97, 128.49, 128.59, 128.68, 136.34, 137.52, 137.74, 137.78, 160.47, 163.73, 164.29, 171.91; *m/z* ESI (M+Na) 714.32345.
29. ^{1}H NMR ($CDCl_3$) δ 1.12–1.15 (m, 10H), 1.53–1.67 (m, 6H), 2.21–2.44 (m, 12H), 2.53 (t, J = 5.58 Hz, 2H), 3.44 (t, J = 5.58 Hz, 2H), 3.53 (t, J = 7.11 Hz, 2H), 5.22 (s, 1H), 6.82–6.88 (m, 4ArH), 6.96 (d, 2ArH), 7.16–7.18 (m, 4ArH), 7.40 (d, 2ArH), 7.51–7.55 (m, 2ArH), 6.63–7.67 (m, 2ArH), 8.70 (br s, NH); ^{13}C NMR ($CDCl_3$) δ 25.22, 26.25, 28.01, 28.54, 28.73, 28.78, 28.82, 30.28, 32.48, 36.91, 37.41, 52.61, 53.06, 57.26, 57.32, 66.28, 81.83, 114.50, 114.79, 119.52, 122.51, 128.01, 128.11, 131.51, 133.34, 135.91, 137.04, 137.42, 137.46, 159.87, 163.12, 167.79, 171.44, 206.41; *m/z* ESI (M+H) 779.42864, (M+Na) 801.41052.
30. ^{1}H NMR ($CDCl_3$) δ 1.16–1.32 (m, 12H), 1.60 (t, J = 6.12 Hz, 2H), 1.68 (t, J = 7.41 Hz, 2H), 2.21–2.62 (m, 22H), 3.45 (t, J = 5.73 Hz, 2H), 6.85–6.91 (m, 4ArH), 6.97 (d, 2ArH), 7.16–7.22 (m, 4ArH), 7.35 (d, 2ArH), 8.50 (br s, NH); ^{13}C NMR ($CDCl_3$) δ 25.43, 26.52, 28.25, 28.98, 29.09, 29.20, 32.77, 32.91, 37.13, 41.61, 52.81, 53.26, 57.52, 57.57, 66.47, 82.15, 114.78, 115.06, 119.82, 128.21, 128.32, 135.96, 137.35, 137.51, 137.55, 160.15, 163.40, 171.66; *m/z* ESI (M+H) 649.425188, (M+Na) 671.407087.
31. Mp = 147–149 °C. ^{1}H NMR (CD_3OD) δ 1.23–1.29 (m, 12H), 1.32–1.37 (m, 4H), 1.52–1.53 (m, 4H), 1.70–1.65 (m, 2H), 2.09 (t, J = 7.26 Hz, 2H), 2.25 (t, J = 7.26 Hz, 2H), 2.49–2.69 (m, 18H), 2.78–2.80 (m, 1H), 3.02–3.08 (m, 4H), 3.19–3.21 (m, 1H), 3.49 (t, J = 5.34 Hz, 2H), 4.16–4.19 (m, 1H), 4.35–4.39 (m, 1H), 5.32 (s, 1H), 6.94 (m, 4ArH), 7.05 (d, 2ArH), 7.24 (m, 4ArH), 7.36 (d, 2ArH), 7.85 (br s, NH); ^{13}C NMR ($CDCl_3$) δ 9.24, 26.95, 27.96, 28.41, 29.50, 29.76, 30.29, 30.35, 30.35, 30.41, 30.50, 30.57, 33.60, 36.83, 37.95, 40.35, 41.06, 47.86, 53.14, 53.22, 57.01, 58.30, 61.59, 63.35, 67.19, 83.77, 115.96, 116.24, 121.45, 129.72, 129.83, 129.94,137.98, 138.32, 139.46, 139.50, 161.94, 165.19, 166.05, 174.61, 175.90; m/z ESI (M+Na) 897.492111.