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Synthesis and characterization of a pegylated derivative of 3-(1,2,3,6-tetrahydro-pyridin-4yl)-1*H*-indole (IDT199): A high affinity SERT ligand for conjugation to quantum dots

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Abstract—Quantum dots consisting of a cadmium selenide core encapsulated in a shell of cadmium doped zinc sulfide have the potential to revolutionize fluorescent imaging of live cell cultures. In order to utilize these fluorescent probes it is necessary to functionalize them with biologically active ligands. In this paper we report the design and synthesis of a ligand that has a high affinity for the serotonin transporter (SERT) that may be conjugated to quantum dots.

Quantum dots composed of a cadmium selenide core encapsulated by a shell of cadmium doped zinc sulfide are novel fluorescent markers that are increasingly finding a wide range of applications.[1–6] Their fluorescent characteristics are superior to conventional fluorescent dyes. These characteristics include increased brightness, narrow emission spectra, increased photostability, and large extinction coefficients (of the order of 1×10^6).[7,8] Commercially available quantum dots have quantum yields in excess of 80–90%[9–11] and have size tunable emission. Small dots emit at the blue end of the spectrum whilst large dots emit at the red end. The absorbance of quantum dots is a continuum above the first absorption feature, enabling a range of different colored dots to be excited with a single excitation source.

Since their introduction as biological imaging agents in the 1998[12,13] quantum dots have been used to image a wide variety of targets. Biological specificity has been achieved by attaching biologically active molecules to their surfaces. Antibodies, DNA, proteins, and peptides have all been employed to introduce specificity.[14–19] The multivalent surfaces of quantum dots enable the attachment of multiple copies of a single ligand, thus enabling enhanced sensitivity and lower detection limits for an analyte.

Despite the advances made in macromolecule conjugated quantum dot imaging relatively few accounts of small molecule conjugated quantum dots have been reported in the literature.[20–22] Our group is interested in developing drug and neurotransmitter derivatives that may be conjugated to quantum dots. We hope to use these conjugates to image cell cultures and tissue cultures in vitro. In particular we are interested in agonists of $5HT_{2A}$ and antagonists of the serotonin and dopamine transporters (SERT and DAT).[23–28] In a prior publication we demonstrated that a pegylated version of serotonin (**1**) may be conjugated directly to the surfaces of quantum dots via an acid–base interaction. This probe had an IC_{50} of 115 μM, against HeLa cells transiently transfected with human serotonin transporters (hSERT).[21] The low affinity of this ligand and poor colloidal stability of the ligand–nanocrystal conjugates lead us to search for a more potent ligand that gave conjugates with better colloidal stability. We have identified 3-(1,2,3,6-tetrahydro-pyridin-4-yl)-1*H*-indole (**2**) as a potential compound that may be conjugated to quantum dots and retain biological activity in the nano molar range.[29] In earlier work we demonstrated that biological activity was retained when biotin was attached to **2** via a short alkyl spacer.[26]

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We have demonstrated that biotinylated ligands bound to streptavidin coated quantum dots retained biological activity[26] as measured by the inhibition of the uptake of tritiated serotonin in SERT expressing HeLa cells. However these conjugates gave non-specific binding of the quantum dots to cellular membranes. In order to understand this binding we have recently studied nanocrystals coated in an amphiphilic polymer (AMP) and their non-specific binding characteristics to cell surfaces. The AMP dots used in these studies were obtained from the Quantum Dot Corporation and are now available from Invitrogen Corporation. The AMP dots consisted of dots coated in a modified polyacrylamide polymer terminated in carboxylic acid functional groups. Their overall diameter was of the order of 15–20 nm and they had a maximum fluorescent emission of 605 nm. Pegylation of the nanocrystal surface significantly reduced non-specific binding when the PEG chain had a length of 12 repeat units or longer.[30] From these results we hypothesized that a SERT antagonist bound to a long PEG chain may retain biological activity and reduce non-specific binding to cellular membranes upon conjugation to AMP quantum dots. In this paper we present the synthesis of such a ligand and demonstrate that this ligand may be conjugated to the surfaces of AMP quantum dots. This ligand was found to have a high affinity for SERT and the activity was retained after conjugation (Fig. 1).

The synthesis of the our pegylated derivative of 3-(1,2,3,6-tetrahydro-pyridin-4yl)-1*H*-indole (IDT 199) is shown in Schemes 1 and 2. Initially a phthalimide protected alkyl spacer was synthesized by reacting 11-amino undecanoic acid with *N*-carbethoxy phthalimide

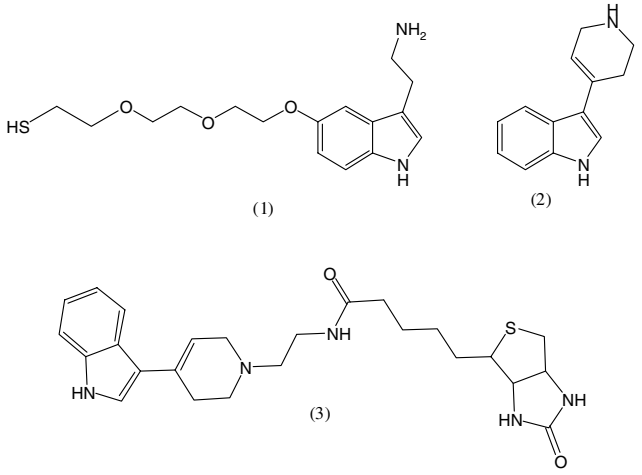


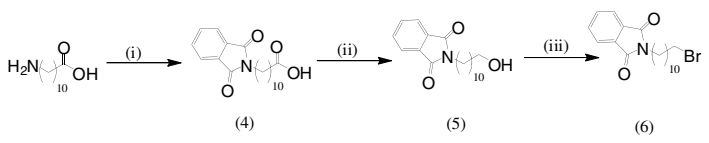
Figure 1. Compounds with affinity for the serotonin transporter (SERT).

to give 11-(1,3-dioxo-1,3-dihydro-isoindol-2-yl)-undecanoic acid (**4**) as described by Wada et al.[31] This was reduced by converting the acid into a mixed anhydride and reducing the mixed anhydride with sodium borohydride in one pot, to give an 85.9% yield of 11-(1,3-dioxo-1,3-dihydro-isoindol-2-yl)-undecanol (**5**). Compound **5** was converted to 11-(1,3-dioxo-1,3-dihydro-isoindol-2-yl)-undecyl bromide (**6**)[32] by reacting **5** with *N*-bromosuccinimide and triphenylphosphine, resulting in 11-(1,3-dioxo-1,3- dihydro-isoindol-2-yl)-undecanyl bromide (**6**)[33] in a 63% yield.

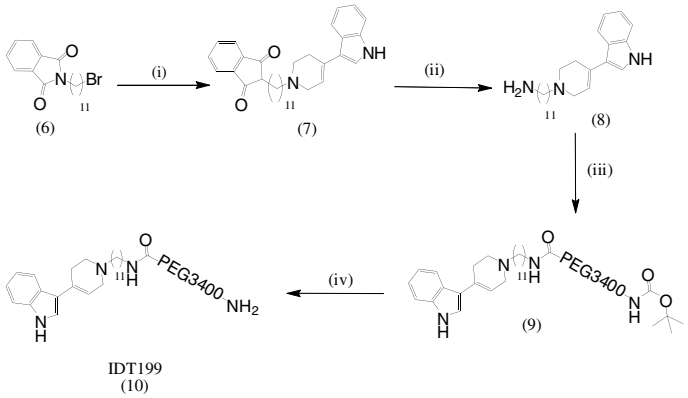
As outlined in Scheme 2 the alkyl spacer was coupled up to 3-(1,2,3,6-tetrahydro-pyridin-4yl)-1*H*-indole giving a 37.3% yield of 2-(11-(4-(1H-indol-3-yl)-5,6-dihyropyridin-1(2H)-yl)undecyl)isoindoline-1,3-dione (**7**).[34] The phalimide protecting group was removed using hydrazine monohydrate to yield 34.6% of 11-(4-(1H-indol-3-yl)-5,6-dihydropyridin-1(2H)-yl)undecan-1-amine (**8**)[35] and this was coupled to Boc-NH-PEG-NHS-3400[36] giving the pegylated derivative **9**.[37] The pegylated derivative was deprotected using trifluoro acetic acid resulting in the desired ligand IDT199 (**10**).[38]

After synthesis, IDT199 was characterized using MALDI TOF mass spectroscopy.[39] The mass spectrum confirmed that IDT199 had a narrow polydispersity based on the Gaussian shape of the oligomer peak envelope. Two series of peaks were present; each series of oligomer peaks was separated by 44 *m*/*z* which is the mass of poly(ethylene glycol) repeat unit. The series of peaks with the highest intensities (*m*/*z* = 3668, 3712, and 3756) representing PEG oligomers terminated the SERT specific ligand on one end and an ethylamine end group on the other end. The second series of oligomer peaks represented the addition of an extra CO to the oligomer chain; the difference between the two series of oligomer peaks is *m*/*z* = 28. No other series of peaks were present in the spectrum suggesting that IDT199 consisted of a mixture of polyethylene glycol chains derivatized with the desired ligand. The MALDI TOF mass spectrum indicated that the PEG had been derivatized and was not contaminated with underivatized starting material. From this we reasoned that the yield in the reaction between **8** and Boc-PEG3400-NHS was close to 100%. The purity of this product was estimated to be approximately 80%, as the Boc-PEG3400-NHS was obtained from the supplier with a purity greater than 85%.

The ligand IDT199 (**10**) was attached to the surface of AMP dots[40] with a maximum fluorescent emission of 605 nm using an EDC coupling. One thousand five hundred equivalents of EDC and NHS was added to dots dissolved in borate buffer at pH 8.5. This was followed by



Scheme 1. Reagents: (i) *N*-carbethoxy phthalimide, 56%; (ii) a—ethyl chloroformate; b—sodium borohydride, methanol, 85.9%; (iii) triphenylphosphine, *N*-bromosuccinimide, 63%.



Scheme 2. Reagents: (i) 3-(1,2,3,6-tetrahydro-pyridin-4yl)-1*H*-indole, cesium carbonate, 37.5%; (ii) hydrazine monohydrate, 34.6%; (iii) Boc-NH-PEG-NHS-3400, 100% (estimated using MALDI TOF-MS); (iv) TFA, 100% (estimated using MALDI TOF-MS).

addition of 2000 equiv of IDT 199 and the mixture was stirred at ambient temperature for 1 h. The ligand conjugated dots were purified by column chromatography on a Sephadex column (G50) eluted with borate buffer at pH 8.5. The concentration of the conjugates was determined spectrophotometrically based upon an extinction coefficient of 650,000 M^{-1} cm^{-1}. To verify effective ligand conjugation the dots were loaded onto a 1% agarose gel in TAE buffer, and the electrophoretic mobility was compared with unconjugated AMP dots (Fig. 2).

IC_{50} values against the serotonin transporter protein were obtained for the unbound ligand and ligand covalently attached to dots by using a competition assay with tritiated serotonin that was previously reported by Rosenthal et al.[22] Table 1 shows the IC_{50} values for **2**, **3**, **8**, and IDT199; additionally the IC_{50} of IDT199 conjugated to AMP dots was measured relative to the concentration of dots and is also shown in Table 1. (Streptavidin coated quantum dots were conjugated to compound **3**, via an avidin–streptavidin interaction.)

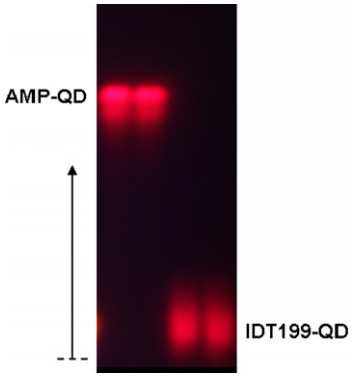


Figure 2. A comparison of IDT199 conjugated AMP dots (50 nM) and AMP (50 nM) dots on a 1% agarose gel.

Table 1. IC_{50} values for free ligands and ligand conjugated AMP quantum dots against the serotonin transporter SERT

Compound	IC_{50} (nM) of free ligand	IC_{50} (nM) of ligand conjugated dots
2	80[a]	na
3	2	30
8	25	nc
10 (IDT199)	30	10

na, not applicable.
nc, not conjugated.
[a] Literature value.

As IDT199 had a high affinity for SERT it was necessary to determine that no free ligand was in solution after conjugation to quantum dots. Free ligand would bind to SERT in the displacement assay and affect the magnitude of IC_{50} value. To determine if there was free ligand in solution after conjugation of IDT199 to AMP dots, the dots were pelleted using a centrifuge at 5000 rpm and the supernatant was tested for biological activity. Since the supernatant showed no activity against SERT, we were able to determine that no free ligand was present.

In conclusion, the addition of a longer alkyl chain to **2** does not reduce the potency of **2** as was demonstrated by the nanomolar affinity of **8**. Upon attachment to a long PEG chain, no significant reduction in biological activity was observed. Conjugation of IDT199 to the surface of AMP quantum dots resulted in retained biological activity. These conjugates demonstrated reduced non-specific binding to HEK cells. As non-specific binding is also cell type specific conjugates such as these will ultimately be useful in fluorescent imaging using the appropriate biological platforms that give lower non-specific binding with pegylated quantum dots.[41] IDT199 may have also other applications that require immobilized SERT antagonists.

Acknowledgments

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32. 11-(1,3-Dioxo-1,3-dihydro-isoindol-2-yl)-undecanoic acid (4.5 g, 17 mmol) was dissolved in tetrahydrofuran (80 ml). The solution was cooled to −10 °C and triethylamine (4.1 ml) was added, followed by ethyl chloroformate (2.37 ml), and the mixture was stirred at −10 °C for 20 min. The resulting mixed anhydride was reduced by adding sodium borohydride (2.27 g) all at once. Then methanol (20 ml) was added dropwise over 20 min. After this the solution was allowed to warm to room temperature and hydrochloric acid (1 M, 20 ml) was added. The solution was concentrated under reduced pressure and extracted with ethyl acetate (3× 50 ml). The ethyl acetate solution was dried over magnesium sulfate, filtered, and evaporated to give crude product. This was purified on a silica column eluted with ethyl acetate yielding 3.6 g (85.9%) of 11-(1,3-dioxo-1,3-dihydro-isoindol-2-yl)-undecanol as a colorless oil. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.83 (m, 2 H), 7.71 (m, 2H), 3.65 (m, 4H), 2.13 (s, 1H), 1.62 (t, J = 6 Hz, 2H), 1.52 (t, J = 6 Hz, 2H), 1.28 (m, 14H); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 168.40, 133.75, 132.06, 123.05, 62.88, 37.96, 32.68, 29.42, 29.32, 29.27, 29.04, 28.81, 28.47, 26.73, 25.62.
33. 11-(1,3-Dioxo-1,3-dihydro-isoindol-2-yl)-undecanol (2.54 g, 10 mmol) was dissolved in dichloromethane (100 ml). Triphenylphosphine (2.9 g, 11 mmol) and *N*-bromosuccinimide (1.9 g, 11 mmol) were added to this solution. The mixture was stirred at ambient temperature for 18 h and evaporated under reduced pressure. The crude 11-(1,3-dioxo-1,3-dihydro-isoindol-2-yl)-undecanyl bromide was purified using silica gel chromatography eluted with an ethyl acetate/hexanes 1:1 giving 1.95 g (63%) of pure 11-(1,3-dioxo-1,3-dihydro-isoindol-2-yl)-undecanyl bromide as a colorless solid mp 57–57.5 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.72 (m, 2H), 7.63 (m, 2H), 3.57 (t, J = 7 Hz, 2H), 3.27 (t, J = 6 Hz, 2H), 1.74 (m, 2H), 1.58 (t, J = 7 Hz, 2H), 1.22 (m, 14H); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 168.20, 133.65, 132.01, 122.94, 37.86, 33.85, 32.67, 29.23, 29.20, 28.97, 28.56, 28,50, 28.41, 27.99, 26.66.
34. 11-(1,3-Dioxo-1,3-dihydro-isoindol-2-yl)-undecanyl bromide (1.42 g,7.2 mmol) and 3-(1,2,3,6-tetrahydro-pyridin-4yl)-1*H*-indole (2.74 g, 7.2 mmol) were mixed in a 250 ml round-bottomed flask. Acetonitrile (100 ml) was added followed by cesium carbonate (3 g, 9 mmol). The mixture was heated at gentle reflux with stirring for 2 h and then cooled to ambient temperature. Solvent was removed under reduced pressure and the resulting crude product was leached into boiling ethyl acetate. The ethyl acetate was filtered to remove cesium bromide and the solvent was evaporated under reduced pressure. The 2-(11-(4-(1*H*-indol-3-yl)-5,6-dihyropyridin-1(2*H*)-yl)undecyl)isoindoline-1,3-dione was purified by recrystallization from acetonitrile giving 1.3 g (37.5%) of the product as a pale yellow solid mp 89–90 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.5 (s, 1H), 7.86 (m, 3H), 7.72 (m, 2H), 7.36 (d, J = 6 Hz, 1H), 7.16 (m, 3H), 6.20 (s, 1H), 3.63 (t, J = 6 Hz, 2H), 3.24 (s, 2H), 2.75 (s, 2H), 2.60 (s, 2H), 2.50 (t, J = 6 Hz, 2H), 1.66 (m, 4H), 1.32 (m, 14H); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 168.49, 136.78, 133.82, 132.13, 129.73, 125.22, 123.13, 122.04, 121.32, 120.67, 119.90, 118.98, 117.89, 111.29, 58.70, 53.14, 50.44, 38.06, 29.56, 29.52, 29.48, 29.44, 28.93, 28.58, 27.67, 27.08, 26.84.
35. 2-(11-(4-(1*H*-Indol-3-yl)-5,6-dihydropyridin-1(2*H*)-yl)-undecyl)-isoindoline-1,3-dione (1.25 g, 2.6 mmol) was dissolved in ethanol (100 ml) and hydrazine monohydrate (5 ml) was added. The mixture was stirred at ambient temperature for 18 h and then evaporated under reduced pressure. The resulting colorless solid was dissolved in dichloromethane (100 ml) and washed with water (2× 50 ml). The organic solution was dried over magnesium sulfate, filtered, and evaporated to yield crude product. This was purified by recrystallization from a mixture of ethyl acetate and hexanes to give 0.41 g (34.6%) of the product as a colorless solid, mp 108–109 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.79

(d, J = 6 Hz, 1H), 7.36 (m, 2H), 7.05 (m, 2H), 6.10 (s, 1H), 3.30 (s, 2H), 2.59 (t,J = 3 Hz, J = 5 Hz, 2H), 2.51 (m, 4H), 2.36 (t, J = 7 Hz, 2H), 1.47 (t,J = 6 Hz, 2H), 1.25 (s, 18H); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 136.96, 129.64, 124.68, 122.66, 121.20, 120.8, 119.20, 117.71, 115.94, 111.74, 58.78, 53.23, 50.51, 42.21, 33.81, 29.59, 29.55, 29.46, 29.04, 27.70, 27.17, 26.86.

36. Biotin-PEG3400-NHS was obtained from the Nektar Corporation, Huntsville, Alabama, and was found to have a purity of >85% by MALDI mass spectroscopy. The poly(ethylene glycol) (PEG) chains of this product had a molecular weight (determined by MALDI) of 3446 Da (approximately 78 ethylene glycol units) and a polydispersity of 1.00.
37. The pegiylated derivative of 11-(4-(1H-indol-3-yl)-5,6-dihydropyridin-1(2*H*)-yl)undecan-1-amine compound (**8**) was synthesized by adding Boc-NH-PEG-NHS-3400 (0.1 g) to a solution of 11-(4-(1H-indol-3-yl)-5,6-dihydropyridin-1(2*H*)-yl)undecan-1-amine (0.1 g, 0.27 mmol) dissolved in dichloromethane (50 ml). The mixture was stirred at ambient temperature for 18 h and then evaporated under reduced pressure. The product was washed with diethyl ether and used without further purification.
38. 0.08 g of compound (**9**) was dissolved in methylene chloride (50 ml) and trifluoroacetic acid (10 ml) was added. The mixture was stirred at ambient temperature for 4 h and the solvent was removed under reduced pressure. The product was washed with diethyl ether and dried under reduced pressure at ambient temperature for 96 h, to yield 0.06 g of IDT199 (**10**) as a tar.
39. MALDI-TOF mass spectra were recorded on an Applied Biosystems Voyager mass spectrometer equipped with a 337 nm nitrogen laser. The acceleration voltage was 25 kV, and 30–64 scans were averaged for each spectrum. For sample preparation, a saturated matrix stock solution of 2,5-dihydroxybenzoic acid (DHB) and a 0.01 M sodium iodide solution were prepared in methanol. Sample stock solutions of IDT 199 (5×10^{-3} M) in methanol in water were prepared. The stock solutions were mixed in a 2:5:2 ratio of sample to matrix to salt by volume. A 1 μL aliquot of each sample solution was placed on the sample plate. A PEG standard prepared in the same manner as the other samples was used for mass calibration of the instrument.
40. AMP coated quantum dots may be obtained commercially from the Invitrogen Corp. (Qdot 605 ITK carboxyl quantum dots 8 μM solution).
41. 'Targeting the human serotonin transporter (hSERT) with antagonist conjugated quantum dots', Tomlinson, I. D.; Chang, J.; Iwamoto, H.; Warnerment, M. R.; DeFelice, L. J.; Blakely, R. D.; Rosenthal; S. J., submitted for publication.