Published in final edited form as:

Proc SPIE Int Soc Opt Eng. 2008 February 22; 6866(0): nihpa155701. doi:10.1117/12.782801.

Targeting the human serotonin transporter (hSERT) with quantum dots

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Abstract

In this paper we report our work on the development of a human serotonin transporter (hSERT) antagonist that can be conjugated to quantum dots. This approach has been used to target and visualize the human serotonin transporter protein (hSERT). We demonstrate that labeling is blocked by the addition of high affinity hSERT antagonists such as paroxetine. This approach may be useful for the development of fluorescent assays to study the location and temporal dynamics of biogenic amine transporters and also holds promise for the development of plate-based high throughput assays used to identify novel transporter antagonists.

Keywords

Quantum dots; serotonin; hSERT; antagonist; paroxetine

1. Introduction

A wide variety of quantum dots have been reported in the literature.¹⁻⁵ Of these the most widely studied are quantum dots that are composed of cadmium selenide core encapsulated by a shell of cadmium doped zinc sulfide. The core has a diameter that is between 2 and 10 nm and the surrounding zinc sulfide shell is several layers thick. As the band gap of the shell is wider than the core it enables the quantum confinement of an electron-hole pair (exciton) in the core after excitation with the appropriate wavelength. This electron-hole pair ultimately recombines, resulting in a fluorescent emission in the visible region of the spectrum.⁶ The energy of the emitted photon is determined by the size of the size of the quantum dot. Smaller quantum dots emit blue light and larger ones emit red light. Quantum dots have several inherent advantages when compared to conventional fluorescent dyes

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edited by Marek Osinski, Thomas M. Jovin, Kenji Yamamoto

these include; increased photo stability, increased brightness, quantum yields are in excess of 80-90%^{7,8}, and a narrow width of emission spectrum (less than 30nm full width at half maximum in commercial products).⁹⁻¹² Also their multivalent surfaces enable the attachment of more than one type of ligand to a single quantum dot.

The first applications of biological imaging with quantum dots were reported in 1998^{13,14} and since these early experiments numerous applications have been reported in the literature. Amongst these imaging applications live cell imaging¹⁵ and whole animal imaging¹⁶ have received a great deal of interest. Near infrared quantum dots have also found applications in the clinic as tools for imaging sentinel lymph nodes during surgery.¹⁷ Many of the imaging applications reported in the literature have utilized quantum dots that are conjugated to large macromolecules such as viruses¹⁸, proteins¹⁹⁻²⁶, peptides²⁷⁻²⁹, DNA³⁰⁻³⁹, RNA⁴⁰ peptide nucleic acid (PNA)⁴¹, cytokines⁴², and in particularly antibodies.⁴³⁻⁴⁶ The antibody approach has yielded many interesting results, however this approach is limited by the supply of available antibodies with high affinity for the wide variety of targets that interest the research community. Antibodies are expensive and in some instances difficult to produce; in addition antibodies have several functional groups that may react with functionalities on the surfaces of the quantum dots following conjugation. This can result in a mixture of conjugates⁴⁷ some of which will have affinity for the desired target and some of which will have low or no affinity.

Our approach differs from the antibody approach in that we are developing small molecule conjugates of quantum dots. This approach has greater potential than the antibody approach since the number of ligands that may be synthesized is infinite. These ligands may be synthesized using known chemical transformations and are less expensive than antibodies. They may be designed to higher affinity and selectivity than antibodies and these ligands may contain a single reactive functionality that can be conjugated to the surface of a quantum dot, ensuring the ligand retains activity once bound and is in the correct orientation for biological activity. Also due to their smaller sizes it is possible to increase the number of ligands conjugated to a single quantum dot which may be important for strong interactions between the receptors of interest and the quantum dot. Using this approach we wish to develop ligand nanocrystal conjugates that may be used to image biologically relevant targets from the central nervous system. In particular we are interested in the biogenic amine transporter proteins that include the serotonin transporter (SERT), the dopamine transporter (DAT) and the norepinephrine transporter (NET). In earlier work we outlined the synthesis of a pegylated derivative of serotonin that could be conjugated to quantum dots via an acid base interaction with a thiol and the surface of the zinc sulfide shell. Using this conjugate we demonstrated that HEK cells expressing the human serotonin transporter (hSERT) may be labeled with serotonin conjugated quantum dots (Figure 1.).⁴⁸

The results of these first labeling proved difficult to use in multiple applications due, we believe, to the relatively low affinity of SERT for its substrate serotonin, the intramembrane localization of the binding pocket for serotonin within the transporter, and difficulties with nonspecific binding to cell surface molecules. Thus, these studies encouraged us to develop SERT ligands that have higher affinities for the SERT.^{49,50} Such ligands are outlined in

figure 2. Their affinities for SERT were measured by their ability to inhibit the uptake of tritiated serotonin in hSERT transfected HEK cells.

Once ligands were validated as high affinity antagonists for hSERT mediated serotonin transport, they were conjugated to the surfaces of quantum dots. The conjugates were purified by size exclusion chromatography and tested for biological activity. These conjugates were shown to retain biological activity using transport and current inhibition assays. Moreover, when the conjugates were pelleted, the supernatants were shown to be inactive, indicating that ligands were bound to the surfaces of the quantum dots. Unlike our early serotonin ligand, which was conjugated directly to the surfaces of quantum dots via a thiol, these second generation ligands were conjugated to the surfaces of quantum dots that were either coated with an amphiphilic polymer (AMP) via a peptide bond or to a streptavidin on the surface of the quantum dot via a streptavidin-biotin interaction. These interactions are stronger than an acid-base interaction between a thiol and the surface of the dots resulting in conjugates with enhanced stability and solubility in a wide range of biologically relevant buffers.

Non specific adsorption to biological membranes is frequently encountered when imaging with quantum dots. We have observed that nonspecific binding to cellular membranes may be reduced by pegilating the surfaces of AMP quantum dots and ligands that incorporate a polyethylene glycol chain that is longer than 12 repeat units may also reduce non specific binding to cellular membranes.⁵¹ In addition, we have shown that the choice of the host cell system also determines the degree of nonspecific adsorption. We have demonstrated that GABA_C receptors expressed in *Xenopus laevis* oocytes may be imaged with quantum dots that were conjugated to a pegilated derivative of the GABA_C agonist muscimol.⁵² These studies demonstrated that oocytes give lower nonspecific binding and we thus hypothesized that an oocyte platform may be ideal to develop ligands with high affinity for the hSERT. In this paper we describe the synthesis and biological activity of an hSERT antagonist IDT318 that may be used to image hSERT expressing oocytes with streptavidin coated quantum dots (SA QDs).

2. Methodology

Streptavidin-conjugated CdSe ZnS core/shell nanocrystals (SA QDs) with maximum fluorescence emission at 655 used in this study were purchased from Invitrogen Corporation (Carlsbad, CA) and supplied as 1 μ M solution in borate buffer (pH 8.5). Biotin-polyethylene glycol-*N*-hydroxysuccinimide ester (Biotin-PEG5000-NHS; purity > 80%) was obtained from Laysan Bio Inc. (Arab, AL). The polyethylene glycol chain in this product had an average molecular weight of 5000 (Determined by MALDI-MS) and was poly dispersed. Methylamine (33%, dissolved in methanol), hydrazine mono hydrate, ethylchloroformate, sodium borohydride, triphenylphosphine, N-Bromosuccinimide (NBS) and N-Carbethoxy-phthalimide were obtained from the Aldrich Chemical Corporation (Milwaukee, WI). 5-Methoxyindole, Indole and 4-piperidone hydrochloride monohydrate was obtained from Alfa Aesar (Lancashire, UK). Potassium hydroxide and magnesium sulfate were obtained from VWR International (West Chester, PA). Methylene chloride, acetonitrile, ethanol, and

methanol were obtained from Fischer Scientific (Fair Lawn, NJ) and used without purification.

2.1 Synthetic outline

The structure of IDT 318 may be divided into 3 components, these are (a) the 5-methoxy tetrahydroindole moiety that has the biological activity (IDT312); (b) the undecyl spacer and; (c) the biotin-PEG5000 chain. The PEG biotin chain was obtained from commercial suppliers and the other 2 components were synthesized in our laboratories. The synthetic route used to synthesize IDT318 is outlined in schemes 1, 2 and 3. Initially the parent drug (IDT312) was synthesized as outlined in scheme 1 and coupled to 11-(1,3-Dioxo-1,3-dihydro-isoindol-2-yl)-undecyl bromide (IDT160) to yield IDT314 as shown in scheme 2. The phthalimide protecting group was removed using hydrazine monohydrate to give IDT316 and this was coupled to Biotin-polyethylene glycol-*N*-hydroxysuccinimide ester to give IDT318 as shown in scheme 3.

2.1.1 IDT312 synthesis—Initially the parent molecule IDT312 was synthesized by reacting 5-methoxyindole (5g, 34 mmols) with 4-piperidone hydrochloride monohydrate (14g, 91 mmols) by refluxing in methanolic potassium hydroxide solution (2M, 200ml) for 18 hours. The resulting solution was cooled to ambient temperature and poured into deionised water (1000ml). 5-Methoxy-3-(1,2,3,6-tetrahydropyridin-4-yl)-1*H*-indole (IDT312) crystallized upon standing at ambient temperature for 2 days this gave 3g of IDT312 in a 50% yield as a brown solid that was removed by filtration air dried and used without further purification.

2.1.2 IDT314 synthesis—The phthalimide protected alkyl spacer was synthesized by reacting 11-amino undecanoic acid with *N*-carboxethoxy phthalimide to give 11-(1,3-Dioxo-1,3-dihydro-isoindol-2-yl)-undecanoic acid (IDT129) as described by Wada et al.⁵³ This was reduced by converting the acid into a mixed anhydride and reducing the mixed anhydride with sodium borohydride in one pot, yielding a 85.9% yield of 11-(1,3-Dioxo-1,3-dihydro-isoindol-2-yl)-undecanol (IDT159). IDT159 was converted to 11-(1,3-Dioxo-1,3-dihydro-isoindol-2-yl)-undecyl bromide (IDT160) by reacting IDT159 with *N*-bromosuccinamide and triphenyl phosphine, resulting in 11-(1,3-Dioxo-1,3-dihydro-isoindol-2-yl)-undecanyl bromide (IDT160) in a 63% yield. Finally 11-(1,3-Dioxo-1,3-dihydro-isoindol-2-yl)-undecanyl bromide (IDT160) was coupled to 5-Methoxy-3-(1,2,3,6-tetrahydropyridin-4-yl)-1*H*-indole (IDT312) by heating these reagents in acetonitrile (100ml) at reflux for 2 hours in the presence of triethylamine (10ml). The solution was cooled to ambient temperature and evaporated under reduced pressure to yield a yellow solid that was recrystallised from acetonitrile to give 2-(11-(4-(5-methoxy-1*H*-indol-3yl)-5,6-dihydropyridin-(2*H*)-yl)undecyl)isoindoline-1,3-dione (IDT314) as a pale yellow solid in a 36% yield.

2.1.3 Synthesis of IDT318—The synthetic route used to synthesize IDT318 is outlined in scheme 3 briefly 2-(11-(4-(5-methoxy-1*H*-indol-3yl)-5,6-dihydropyridin-(2*H*)-yl)undecyl)isoindoline-1,3-dione (IDT314) was dissolved in ethanol and 10 equivalents of hydrazine mono hydrate were added this was stirred for 2 hours evaporated and added to

methylene chloride the methylene chloride solution was stirred for 18 hours filtered and evaporated to yield crude 11-(4-(5-methoxy-1*H*-indol-3-yl)-5,6-dihydropyridin-1(2*H*)-yl)undecan-1-amine (IDT316) which was purified by recrystallisation from acetonitrile to give the pure product in a 100% yield as a yellow solid. This was dissolved in methylene chloride and an equimolar amount of Biotin-polyethylene glycol-*N*-hydroxysuccinimide ester was added. The solution was stirred for 18 hours then evaporated to yield crude IDT318 which was purified by washing with diethyl ether (3 × 100ml) and used without further purification.

2.2 hSERT expression in *Xenopus laevis* oocytes

Xenopus oocytes and hSERT cRNA were prepared and isolated as previously described.⁵⁴⁻⁵⁶ Briefly, stage V-VI oocytes were harvested from *Xenopus laevis* (Nasco, Medesto, CA). After harvest, the follicle cell layer was removed by incubation with 2 mg/ml collagenase in Ringer's buffer (in mM, 96 NaCl, 2 KCl, 5 MgCl₂, 5 HEPES, pH 7.4) for an hour. cRNA injections were performed on the day of harvest. hSERT cRNA was transcribed from *NotI* (New England BioLabs, Beverly, MA)-digested cDNA in pOTV vector (a gift of Dr. Mark Sonders, Columbia University) using Ambion mMessage Machine T7 kit (Ambion, Austin, TX). The cRNA concentrations were confirmed by UV spectroscopy and gel electrophoresis. Each oocyte was injected with 3 ng cRNA and incubated at 18 °C for 3-6 days in Ringer's buffer supplemented with 550 µM/ml sodium pyruvate, 100 µg/ml streptomycin, 50 µg/ml tetracycline, and 5 % dialyzed horse serum. Healthy oocytes for subsequent electrophysiological and fluorescence assays were selected by visual inspection.

2.2.1 Two-electrode voltage-clamp—Whole-cell currents were measured with two-electrode voltage clamp techniques using a GeneClamp 500 (Molecular Devices, Palo Alto, CA). Microelectrodes were pulled using a programmable puller (Model P-87, Sutter Instrument, Novato, CA) and filled with 3 M KCl (0.5-5 MΩ resistance). A 16-bit A/D converter (Digidata 1322A, Molecular Devices) interfaced to a PC computer running Clampex 9 software (Molecular Devices) was used to control membrane voltage and to acquire data. To induce hSERT-associated current, serotonin was dissolved (typically 10 µM) in a buffer solution (in mM, 120 NaCl, 5.4 potassium gluconate, 1.2 calcium gluconate, 7.2 HEPES, pH 7.4) and applied to oocytes using a gravity-flow perfusion system (4-5 ml/min flow rate). Serotonin-induced current is defined by subtraction of current in the presence of serotonin from current in the absence of serotonin. For recordings, data were low-pass filtered at 10 Hz and digitized at 20 Hz. All analyses were performed using Origin 7 (OriginLab, Northampton, MA).

2.3 Microscopy

Confocal images were obtained on a Zeiss LSM 510META confocal imaging system (Carl Zeiss Microimaging, Inc., Thornwood, NY). Images were collected using a Zeiss Plan-Apochromat 5×/0.16 numerical aperture (NA) objective lens and excited by Argon laser at 458 nm. All images were 512×512 pixels in size and had an 8-bit pixel depth. Fluorescence signal was collected on photomultiplier-tube (PMT) detector after passing through a 650 nm cutoff filter to ensure the transmission of only the QD signal. Wide-field fluorescent images were acquired using a Zeiss Axiovert 200 M inverted fluorescence microscope equipped

with a Photometrics Cool-SnapHQ electrically cooled CCD camera, a Zeiss Plan-Neofluar 20×/0.4 numerical aperture (NA) objective lens and QD655 filter set (XF 1002 filter, Omega Optical, Brattleboro, VT). Exposure time was set at 200 ms for all fluorescent imaging. Image acquisition and analysis was processed using Metamorph® 7 imaging software (Molecular Devices Corp.; Downingtown, PA).

2.3.1 Labeling protocol—Parental and hSERT transfected oocytes were first incubated with a solution of the biotinylated ligand (1 μM) in PBS for 60 minutes. After which they were treated for 5 minutes with a 2.5 nM SA QDs treatment. When a paroxetine pre-block was required the oocytes were first incubated with 1 μM paroxetine in PBS for 60 min, followed by incubation with a ligand/paroxetine mixture (1 μM /1 μM) in PBS for further 60 minutes. Then the oocytes were subsequently incubated with 2.5 nM solution of SA QDs for 5 min. Single oocytes were transferred to an 8-well Lab-Tek chamber slides (NUNC, Roskilde, Denmark) and excess ligand and dots was removed by two washes with PBS.

3. Results

3.1 IC₅₀ measurement and electrophysiology studies

The ability of IDT318 to inhibit the uptake of serotonin was measured by incubating oocytes in the presence of increasing concentrations of IDT318 and 50nM tritiated serotonin. The accumulated radioactivity was plotted against concentration of IDT318 and the IC₅₀ was found to be $3.4 \pm 1.4 \mu\text{M}$. The leak current for 10 μM IDT318 in the presence of 10 μM serotonin was measured and found to be approximately 20% of the magnitude of the serotonin-induced influx current. When oocytes were washed after incubating with IDT318, they still displayed this leak current indicating that the IDT318 ligand binds tightly to hSERT and does not readily wash away. Such behavior is typical of much higher affinity hSERT antagonists such as paroxetine. The IC₅₀ value is in good agreement with the reported literature value for the parent drug which has been reported to be 730nM.⁵⁷ The inhibition profile of IDT318 is steeper than expected for a simple competitive antagonist, suggesting the presence of positive cooperativity in ligand binding and that may account for the reduced dissociation of the ligand during washout.

3.1 Oocyte-quantum dot labeling experiments

To assess the usefulness of IDT318 as a probe for quantum dot labeling of hSERT in oocytes, we used a 2 step labeling protocol involving an initial incubation with 1 μM of IDT318 in PBS with either a hSERT expressing oocyte or a control, saline-injected oocyte. After incubating with IDT318 for 60 minutes, the oocyte was incubated with a 2.5nM solution of SA QDs in PBS for 5 minutes. Finally the oocyte was washed twice with PBS and then visualized. Representative results of this 2 step labeling protocol for the incubation with IDT318 and SA QDs are shown in Figure 5. Panel A and B show the results obtained by incubating hSERT transfected oocytes with a 1 μM solution of IDT318 followed by incubating with a 2.5nM solution of SA QDs. The image in panel B shows a thin fluorescent halo the intensity of which is greater than the background. For comparison the bright field image of the hSERT expressing oocyte is shown in panel A. To determine whether or not this binding was due to specific binding labeling or non specific binding of the SA QDs

binding to the hSERT expressing oocyte membrane, we incubated hSERT-expressing oocytes with a 2.5nM solution of SA QDs for 5 minutes. Bright field and fluorescent images obtained from this incubation are shown in panels C and D. The fluorescent intensity of the image shown in panel D is significantly lower than that shown in panel B indicating that the binding of SA QDs to hSERT transfected oocytes is not due to non specific interactions between the oocytes membrane and the SA QDs. To confirm that this binding is due to a non specific interaction between the oocyte membrane and the ligand, control, noninjected oocytes were incubated with a 1 μ M solution of IDT318 for 60 minutes followed by a 5 minute incubation with a 2.5nM solution of SA QDs. The results obtained from this incubation are shown in panels E and F. The fluorescent intensity shown in panel F is significantly lower than panel B, indicating that the labeling of hSERT injected oocytes is specific for the transporter. Panels G and H show the result of a blocking experiment in which hSERT transfected oocytes were incubated with a 1 μ M solution of paroxetine for 60 minutes followed by incubation with a ligand/paroxetine mixture (1 μ M/1 μ M) in PBS for further 60 minutes. The fluorescent halo in panel H is significantly lower than panel B indicating that paroxetine competes for the oocyte binding of conjugated IDT318. As a negative control we also synthesized IDT364 that consists of biotin attached to PEG5000 that has a methyl group at the end of the PEG (rather than the SERT ligand). No fluorescent halos were observed when hSERT expressing oocytes were treated with this ligand and SA QDs.

These results show that IDT318 is a poorly dissociating antagonist of hSERT that can be used to label hSERT expressing oocytes in a 2 step labeling protocol with SA QDs. IDT318 has specificity for hSERT since the binding may be blocked by paroxetine. When the drug is removed from the end of the PEG chain (IDT364), biological activity was lost demonstrating lack of functional antagonism by the polyethylene glycol chain.

Conclusions

Our work demonstrates to the feasibility of labeling biogenic amine transporters such as hSERT with biotinylated antagonists when hSERT is expressed in oocyte membranes and when binding is detected with SA QDs. This binding is specific for SERT and may be blocked by well-studied hSERT inhibitors. The oocyte platform is an excellent platform for ligand development and optimization as non specific binding of SA QDs is minimal. The affinity of IDT318 for hSERT is similar to the parent drug indicating that the addition of a pegilated long alkyl spacer does not significantly reduce the biological affinity of this compound for hSERT, nor does the spacer have any biological activity on its own. In addition IDT318 dissociates slowly from hSERT in a manner similar to higher affinity inhibitors such as paroxetine, suggesting the presence of a time-dependent, induced fit-mechanism or positive cooperativity, features that may aid the use of the conjugate for cell labeling. We hope to extend this technology in the future to label hSERT expressing mammalian cells and to allow for the analysis of hSERT location and mobility in neurons. In addition this approach appears amenable to high throughput assay systems that can facilitate the identification of novel transporter antagonists.

Acknowledgments

We would like to thank the National Institute of Health (NIH RO1EB0003728-02) for funding this project.

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Figure 1.
Serotonin-coated quantum dots used to label SERT-expressing cells.

Figure 2.
Ligands with affinity for SERT

Figure 3.
IDT318 a biotinylated hSERT antagonist that can be conjugated to streptavidin-coated quantum dots.

Figure 4.

Plots showing the IC_{50} value of IDT318 and the leak current generated by IDT318

Figure 5.

(A-B) Incubation of the hSERT expressing oocyte with 1 μM IDT318 prior to 2.5 nM SA QDs treatment; (C-D) Incubation of the hSERT expressing oocyte with 2.5 nM SA QDs; (E-F) Incubation of the non-expressing oocyte with 1 μM IDT318 prior to SA QDs treatment; (G-H) hSERT expressing oocyte exposed to the 1 μM paroxetine before subsequently exposed to the ligand/paroxetine mixture (1 μM /1 μM) prior to 2.5 nM SA QDs treatment.

Scheme 1.

(i) Methanolic KOH, 4-piperidone hydrochloride monohydrate, reflux 18hours

Scheme 2.

(i) 11-aminoundecanoic acid; (ii) (a) ethylchloroformate, (b) Sodium borohydride; (iii) NBS, triphenylphosphine; (iv) IDT312, triethylamine

Scheme 3.

(i) Hydrazine mono hydrate; (ii) Biotin-PEG5000-NHS

Table 1

IC₅₀ values of ligands that have been synthesized and conjugated to quantum dots (* = literature value³⁵).

Compound No	IC ₅₀ (nM)	Conjugate IC ₅₀ (nM)
1	115,000	99,000
2	80*	not applicable
3	2	30
4	1	100
5	2,000	not conjugated
6	30	10
7	25	not conjugated