**Aptamer Selection against the Dopamine Transporter (DAT) to Alleviate Symptoms of Parkinson’s Disease**

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Pool N58, RNA, DAT

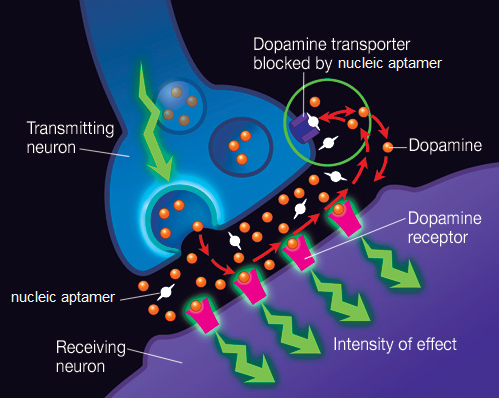
**Abstract**

The dopamine transporter (DAT) protein is responsible for controlling the level of dopamine in the body by taking it out of the synapse and putting it back into presynaptic neurons, where the neurotransmitter is then stored and released by vesicles. Returning dopamine to the transmitting neuron terminates the neurotransmitter’s signal (Huang and Zahn 2007). All dopamine-mediated behaviors and motor activities are dependent on DAT and its ability to successfully bind with adequate amounts of the neurotransmitter. Any irregularities in dopamine or DAT concentration could be the result of dopamine related disorders, including Parkinson’s disease, attention deficit hyperactivity disorder (ADHD), Tourette’s syndrome, schizophrenia, and depression. The latter four disorders elevate the amount of DAT, while Parkinson’s usually results in DAT and dopamine depletion over time (Miller and Madras 2002). The direct cause of Parkinson’s is unknown, but is thought be the result of mutations on the DAT and other various genes (Ritz et al. 2009).

To alleviate the more severe symptoms of Parkinson’s, researchers have developed DAT inhibitors. Even though a DAT inhibitor would further decline the amount of dopamine that could be recycled and used, these inhibitors would prevent detrimental neurotoxins from entering transmitting neurons through DAT. Scientists have used rhesus monkeys with the disease to study the effects of inhibitors. Monkey’s treated with inhibitors had reduced bradykinesia, rigidity, and tremor symptoms (Madras et al. 2006). All inhibitors used had a high affinity for the DAT protein, but research with more specific aptamers is needed to draw any concrete conclusions about the potential of DAT inhibitors and what other affects they might cause.

Specific Aim 1: Selection of RNA aptamers against DAT.

Using a high affinity and specific binding nucleic acid aptamer would be an ideal approach to studying DAT inhibitors and their beneficial effects on dopamine related disorders such as Parkinson’s. In its case, inhibiting the protein might improve severe symptoms by preventing neurotoxins from travelling through DAT and slowing, possibly even stopping, the degradation of this transmembrane protein. Using specific amounts of a therapeutic aptamer could also regulate other disorders in which there are high levels of DAT in the body. The possibility at finding a high affinity DAT inhibitor could have a huge and positive impact on the lives of those suffering with dopamine related disorders, specifically Parkinson’s disease.

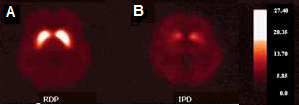
**Figure 1.** Using a specific nucleic aptamer to inhibit the dopamine transporter would initially create a buildup of dopamine in the synaptic cleft and increase receptor activity. Over time the amount of dopamine released would decrease, detrimental neurotoxins would be unable to enter presynaptic cells through DAT, and the symptoms of dopamine related disorders may be alleviated. Picture adapted from the “Dopamine Receptors” website.

This transport protein is one of the “available” targets and can be found in the PAI 2.14 -80˚C freezer, but there is only 600 pmol of this target available. One of the only websites that sold DAT was Abnova. 2 ug of recombinant DAT with a GST tag was $249.00. The catalog number is H00006531-P01 and the company can be reached at 909-839-7620.

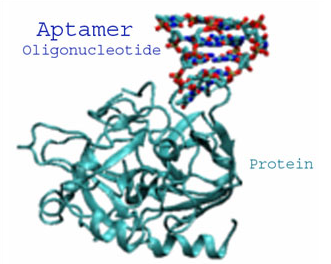
**Introduction and Background**

The dopamine transporter (DAT) is a transmembrane protein that can be found in the all vertebrate brains, but is mainly concentrated in an area of the midbrain called the substantia nigra (“Dopamine Transporter”). It is responsible for the regulation of dopamine by taking it out of the synaptic cleft and putting it back into presynaptic neurons, where the neurotransmitter is then stored and released by vesicles. Dopamine moves across the neuron membrane by coupling the movement of sodium ions going from an area of high to low ion concentration. DAT requires the co-transport of two sodium ions with the transmitter. A single chloride ion is also required to prevent the buildup of positive charge within the nerve cell. After sodium ions bind to the outside of the transporter, dopamine can bind. DAT then undergoes a conformational change that allows both sodium and dopamine to unbind on the inside of the presynaptic neuron (“Dopamine Transporter”). Removing dopamine from the synaptic cleft, where it can bind with dopamine receptors, terminates the transmitter’s signal (Huang and Zahn 2007). Dopamine affects areas of the brain that are responsible for controlling movement, cognition, and emotional responses (Erickson).

Irregularities in DAT concentration and function, which vary slightly in normal subjects, may contribute to the susceptibility of dopamine-related disorders. These disorders include schizophrenia, depression, and attention-deficit hyperactivity disorder (ADHD). In patients with Parkinson’s disease, DAT and dopamine neurons degenerate over time (Miller and Madras 2002) (**Figure 2**). As they die off, it becomes difficult to successfully produce and store dopamine in the brain. This results in the gradual loss of basic motor skills. DAT has proved itself a signifcant target for antiparkinson medications. DAT inhibitors temporarily increase extracellular dopamine levels for receptor activation in the body, thus reducing the severity of Parkinson’s symptoms. DAT blockers are also thought to function as neuroprotective agents by preventing neurotoxins from entering and building up in dopamine neurons via DAT (Madras et al. 2006).

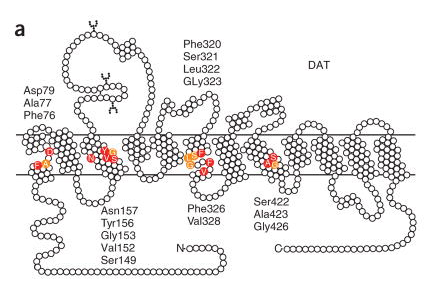
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**Figure 2.** The above figure compares the concentration of dopamine transporters in regular human subjects (A) and those with advanced Parkinson’s disease (B). Adapted from Brashear et al.

Aptamers are single stranded DNA or RNA oligonucleotides that are fifteen to sixty base pairs long. These ligands bind with high affinity to specific targets, including nucleic acids, proteins, small organisms, and organic compounds (“Aptamers from Gene Link”). The binding success of aptamers can be contributed to their three dimensional folding patterns. The specific conformations these sequences take on are stable and recognize their target (**Figure 3**). The binding affinities of aptamers can be compared to, or even exceed, regular antibodies. Aptamers can have many advantages over antibodies. It does not cost much to develop them and they do not vary from batch to batch. Under most conditions, they are more stable than antibodies, last longer, and hardly ever provoke immune responses. These advantages make aptamers a possible or even a better alternative to antibodies in molecular diagnostics and therapeutics (Holahan et al. 2011).

**Figure 3.** The figure above illustrates the binding affinity of an aptamer, colored blue and red, to specific protein (not DAT). This image was adapted from “Aptamers from Gene Link.”

Numerous DAT inhibiting molecules have been developed and the possibility of using aptamers against the transporter has been discussed, but one has yet to be synthesized. A high affinity aptamer would be ideal for Parkinson’s research. Scientists have studied the effects of non aptamer inhibitors on rhesus monkeys with Parkinson’s disease. Monkeys treated with high affinity inhibitors had reduced bradykinesia, rigidity, and tremor symptoms (Madras et al. 2006). Although a DAT inhibiting aptamer would decrease the amount of recycled and reused dopamine in the brain, it would also prevent detrimental neurotoxins from entering transmitting neurons, thus hindering the degradation of dopamine neurons and DAT (Madras et al. 2006). If Parkinson’s is detected early enough, therapeutic aptamers could preserve nearly all dopamine transporters and dopamine neurons. In combination L-DOPA, a drug that can be converted to dopamine in the brain, those inflicted with Parkinson’s can live less afflicted lives (Erickson). L-DOPA alone has been known to cause motor problems because of its short half-life. If aptamers are able to occupy all dopamine transporters, L-DOPA could be more effective (Miller and Madras 2002). DAT specific ligands could also regulate other disorders in which there are high levels of DAT in the body. Finding a high affinity aptamer could have an enormous and positive impact on the lives of those suffering from any dopamine-transport related disorders.

**** DAT exists as a dimer and is 620 amino acids long (Hastrup et al. 2001) (**Figure 4)**. The average molecular weight of the human dopamine transporter is 68407.6406 and it has an estimated pI of 6.92, suggesting DAT has a negative charge at neutral pH (-.05). As the pH becomes more acidic, DAT takes on a more positive charge (Putnam 2006). Numerous labs are studying the dopamine transporter and its affect on other diseases, including the U.S. Department of Energy's Brookhaven National Laboratory (which discovered the correlation between low DAT levels and ADHD severity) and the Vorhee’s Lab (which studies meth and its ability to bind to dopamine transporters in maturing brains). Researchers have used numerous buffers with pHs between 6.5 and 7.5 to stabilize DAT. Major phosphorylation sites can be found within the N terminus of DAT, which contains several serines (Granas et al. 2008). 600 pmol of DAT are available in the -80˚C freezer in PAI 2.14. Currently the size and possible tags on this recombinant protein is not known. 2 ug of recombinant DAT protein with a GST tag can be bought at the Abnova site for $249.00. The catalog number is H00006531-P01 and the company can be reached at 909-839-7620.

**Figure 4.** The figure above is a two-dimensional schematic of the human dopamine transporter. Adapted from Beuming et al.

**Experimental Design, Methods, Materials**

RNA Aptamer Selection

RNA bead based selection protocol will be used on the target. The first step of this process is immobilizing the target onto magnetic beads. The beads will be prewashed to get rid of any impurities, the target immobilized, a RNA pool binding reaction created and heat denatured, and unbound proteins washed away from the beads. Once the pool binding reaction has cooled, it is added to the beads and incubated. The beads, containing the target and bound RNA, are then collected and the supernatant, full of unbound RNA, is removed and put into a tube labeled W0. The beads are then washed and incubated again. The supernatant from the wash is removed, containing weakly bound RNA and put into a tube labeled W1. This process is continued until three washes are completed. The remaining bound species are collected and placed into a tube labeled E1. The RNA in tubes W0, W3 (the last wash) and E1are then ethanol precipitated. Reverse transcription will be performed on the precipitated tubes to transform the bound and unbound RNA into ssDNA. This product is then amplified using large scale PCR after the number of cycles to correctly amplify the recovered pool is determined by cycle course PCR. ssDNA will then be transcribed back into RNA and visualized by running a PAGE gel. If the gel was successful, RNA from the gel will be eluted, precipitated, and quantized using the nanodrop program. This RNA will be used for the next round of selection. During the third round, a negative selection will be performed as well as target selection. RNA from the previous round will be selected without DAT to confirm that no contamination has occurred during the previous rounds. The lone RNA will be washed and unbound sequences removed, will be incubated, go through reverse transcription, amplified, and then transcribed again. Negative selection will continue until the desired concentration of amplified RNA (the results of running a PAGE gel) has been reached.

The average molecular weight of the human dopamine transporter is 68407.6406 and it has an estimated pI of 6.92, suggesting DAT has a negative charge at neutral pH (-.05). As the pH becomes more acidic, DAT takes on a more positive charge. DAT was not stable in any specific buffer, but NaCl will be used to combat DAT’s negative charge. DAT is stable at pHs between 6.5 and 7.5 and can be stored in the -80˚C freezer for long periods of time. A negative selection will be performed against GST-binding RNA on the recombinant DAT protein ordered from Abnova, which is available in a 50 mM Tris-HCI, 10 mM reduced Glutathione, pH=8.0 in the elution buffer. If there is a tag on the available DAT protein that has been stored in the -80˚C freezer in PAI 2.14, then negative selection will be used to remove the RNA on that tag. If there is no tag, one will have to be synthesized.

Binding Assay

After five rounds of selection have been completed, a binding assay will be performed to determine how successful the RNA ligands were at binding with DAT.

**Budget**

DAT, unknown tags and source………………………………………………………………………$0.00

600 pmols available, only 5 aliquots containing 40 pmols of DAT seen

Available in PAI 2.14 in the -80˚C freezer

If 200 pmols are used per round, then three full rounds will be conducted for a cost of $0.

DAT, GST•Tag, Human, Recombinant (optional)…………………………………….………….$249.00

2 ug

Available at Abnova Corporations at <http://www.abnova.com>

Catalog number: H00006531-P01

Given the molecular weight of DAT to be 68407.6406 grams/mol, 13.685 ug of DAT will be used per 200 pmol round. This is way more than the given from Abnova, making this project very costly. The final cost, if calculated right, of each round will be $1708. If only 100 pmols or less could be used per round, the available protein could be used for twice as more rounds.

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