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Characterization of Inhibitory Potency of Compounds YYYand ZZZ Against Dopamine Transport in a Mutant Cell Line

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Author(s):	
Sponsor:	CLIENT XXX Pharmaceuticals
Performing Laboratory:	Under the supervision of NeuroDetective International, Inc. 1422 Hopeland Rd. Wyncote, PA 19095 U.S.A.
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REDACTED FINAL REPORT

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Methods and Results are described using the Work Plan included in the Services Agreement between CLIENT XXX Pharmaceuticals and NeuroDetective International, DATED. The Work Plan methods are described in sequence, and numbered. The results of each Work Plan method are then described in italics.

1) Using a human DAT cDNA construct (pcDNA host vector) capable of inducing cocaine-sensitive DA uptake in HEK-293T cells, we performed oligonucleotide-mediated site-directed mutagenesis to convert DNA sequence encoding DAT

Y335 to DAT A335. The presence of the mutation was verified by automated DNA sequencing. Both WT and mutant plasmids were then prepared to provide sufficient DNA for all transfections.

The hDAT Y335A mutant cDNA was successfully constructed in the mammalian expression vector pcDNA3 and then sequenced, to verify the intended mutation and ensure that no other mutations occurred. The construct is available to the sponsor upon request.

2) HEK-293T cells were transfected with DAT WT or DAT Y335A cDNA constructs and [^{3}H]DA transport activity assessed *in vitro* by scintillation counting of transported DA. Nonspecific [^{3}H]DA uptake was defined using 10 μM mazindol, which was then subtracted from total accumulated counts to give the level of specific [3H]DA uptake. Nontransfected cells were also assessed for mazindol-sensitive [3H]DA uptake as a control. All assays were repeated in quadruplicate with means and S.E.M. calculated and then data graphed with Prism software.

Kinetic studies were performed with increasing concentrations of DA using a mix of labeled and unlabeled DA to determine the linear range and maximal sensitivity for assays using both WT and the Y335A mutant (Fig 1). As expected, the activity of the mutant was significantly lower (~20 fold) than that of the WT hDAT (note double Y axis labels, plotted here as % of WT maximal transport activity). Therefore, a different mix of labeled and unlabeled DA was used to afford adequate sensitivity when assaying both WT and Y335A in parallel, so that the same concentration of DA (0.10 μM) could be used for all subsequent assays.

3) Four compounds were then examined for their inhibitory potency in antagonizing [3H] DA uptake using (initially at least) 8 concentrations of each drug, ranging from 10 nM to 500 μM as pertinent to the assessed potency of the specific agent, along with a no-drug control. DAT WT and DAT Y335A were tested in parallel.

The compounds so tested were a) cocaine, b) methylphenidate, c) ZZZ, and d) YYY. The resulting Inhibition data were fit by nonlinear regression methods to determine the Ki values for inhibitors. All assays were conducted in quadruplicate, and all results show the means and SEMs.of the four assays.

These studies were conducted with the results obtained in Fig 2 (cocaine), Fig 3 (methylphenidate), Fig 4 (ZZZ) and Fig 5 (YYY). The data show that all four compounds exhibit a rightward shift in the DA uptake inhibition curves, indicating reduced potency at Y335A versus WT hDAT. Log_{10}(M) IC50 values are as follows:

		IC50 WT hDAT	IC50 mutant hDAT Y335A
1)	***Cocaine (10 min)***	*hDAT: -6.24+/-0.05,*	*hDAT Y335A: -4.61+/-0.10*
2)	***Methylphenidate (10 min)***	*hDAT:-7.00+/-0.05,*	*hDAT Y335A, -5.40+/-0.13*
3)	***Compound ZZZ (10 min)***	*hDAT:-6.35+/-0.04,*	*hDAT Y335A, -4.87+/-0.06*
4)	***Compound YYY (10 min)***	*hDAT:-6.21+/-0.04,*	*hDAT Y335A, -4.38+/-0.08*
5)	***Compound YYY (1 hr)***	*hDAT:-7.23+/-0.03,*	*hDAT Y335A, -5.38+/-0.04*

IC50 values are converted to Ki values below:

		Ki WT hDAT (nM)	Ki mutant hDAT Y335A (nM)
6)	***Cocaine (10 min)***	*hDAT: 564 nM*	*hDAT Y335A: 24,056 nM*
7)	***Methylphenidate (10 min)***	*hDAT: 98 nM*	*hDAT Y335A, 3,901 nM*
8)	***Compound ZZZ (10 min)***	*hDAT: 438 nM*	*hDAT Y335A, 13,220 nM*
9)	***Compound YYY (10 min)***	*hDAT; 604 nM*	*hDAT Y335A, 40,853 nM*
10)	***Compound YYY (1 hr)***	*hDAT; 57.7 nM*	*hDAT Y335A, 4,085 nM*

4 Discussion and Conclusions

These findings indicate that the inwardly facing conformation bias of the Y335A mutant impacts both CLIENT XXX compounds in a manner similar to cocaine and methylphenidate. Due to unexpected problems with the inhibitory profile of the initial lot of compound ZZ, a new batch was obtained and tested identically to the first set of assays, with the result that this second lot behaved comparably in terms of dose-response inhibition characteristics.

While awaiting shipment of this new lot, we explored whether a more lengthy pre-incubation time (1 hr) with the compound, versus our standard pre-incubation protocol (10 min), might overcome problems encountered with this compound. Our results indicate that the problem was with the initial lot and not the pre-incubation conditions. However, we did find a 10-fold shift to higher potency of compound YYY against Y335A when a 1 hour incubation was used (Fig 5). Because the same shift occurred with WT hDAT, these data indicate that, at least for this compound, a longer pre- incubation condition will be useful in estimating the true potency of the drug against either WT or Y335A. Radioligand binding inhibition studies, where the initial potency of the CLIENT XXX compounds were initially evaluated, use equilibrium incubations that are typically longer than the initial rate studies of transport assays. In the future, it is recommended that the pre-incubation time of both controls and CLIENT XXX compounds in transport inhibition assays be extended to 1 hr.

Figure 1: DA transport kinetics for WT hDAT and hDAT Y335A

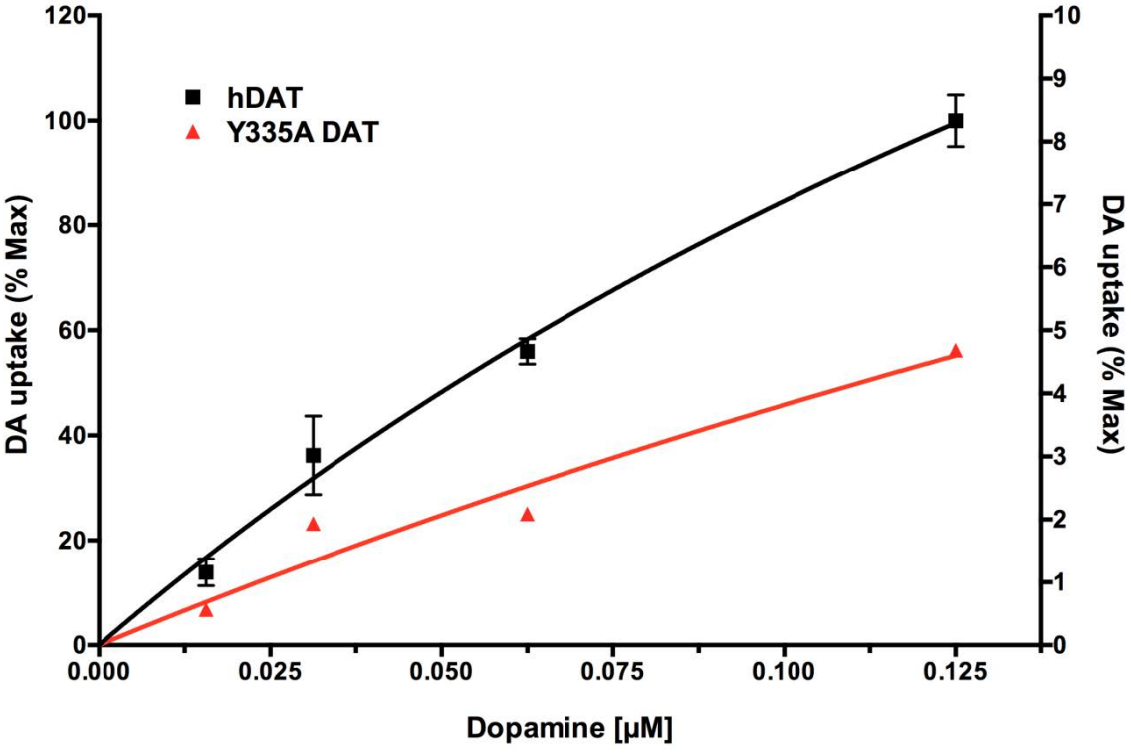

Figure 2: DA transport inhibition by Cocaine

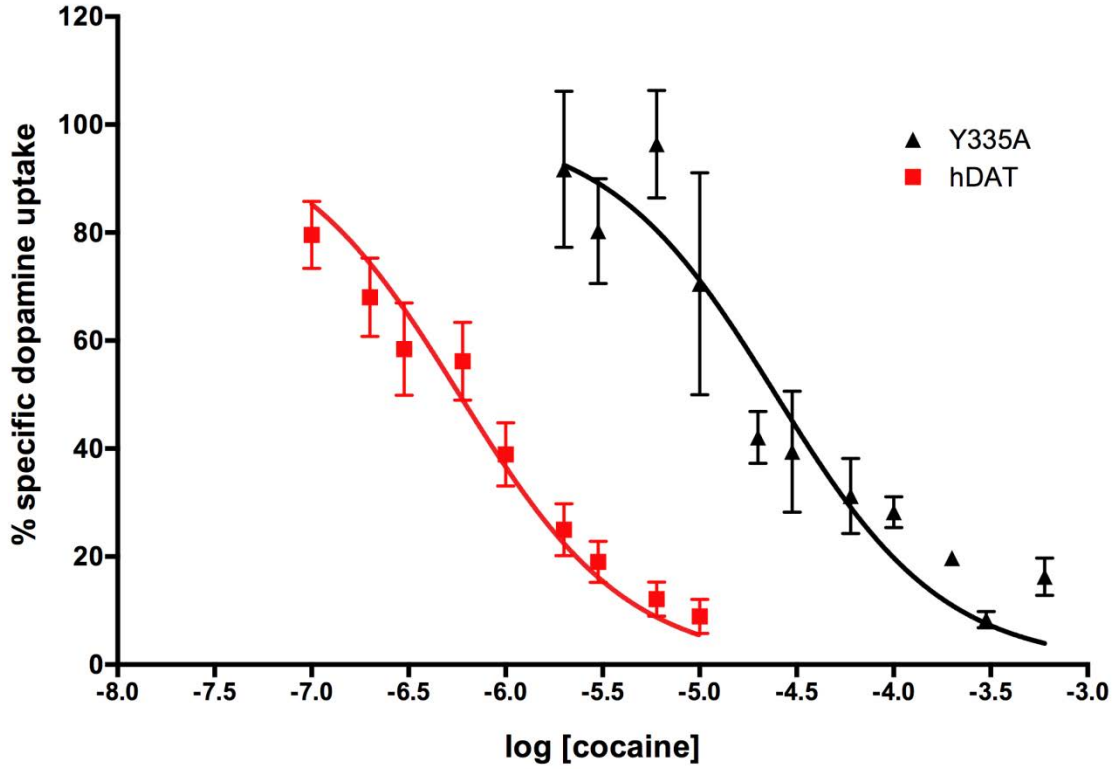

Figure 3: DA transport inhibition by Methylphenidate

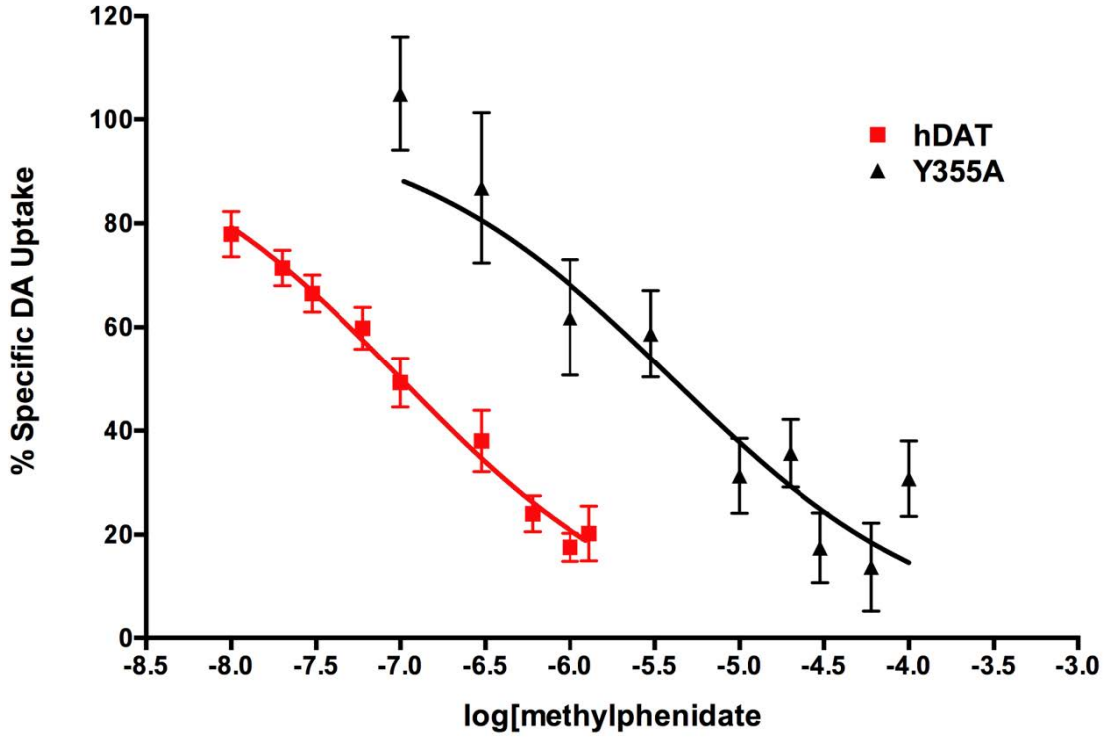

Figure 4: DA transport inhibition by CLIENT XXX compound ZZZ

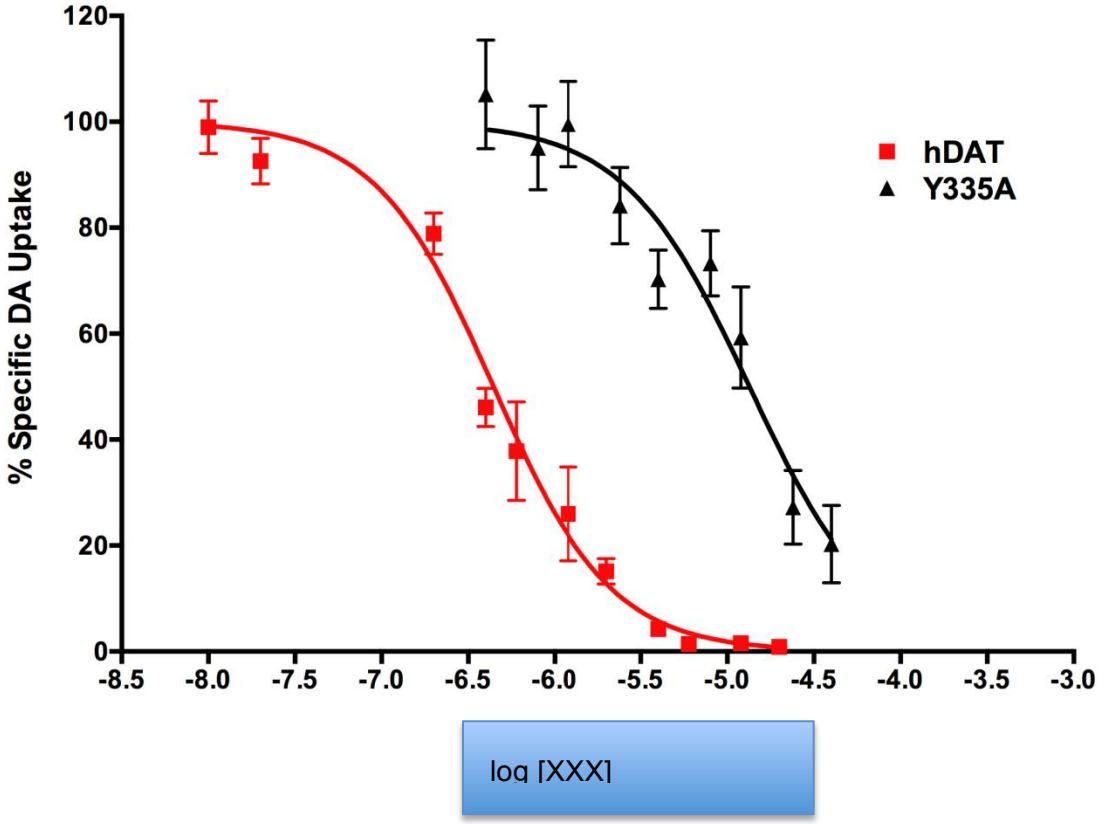

Figure 5. DA uptake inhibition by CLIENT XXX Compound YYY at 10 min vs 1 hr preincubation.

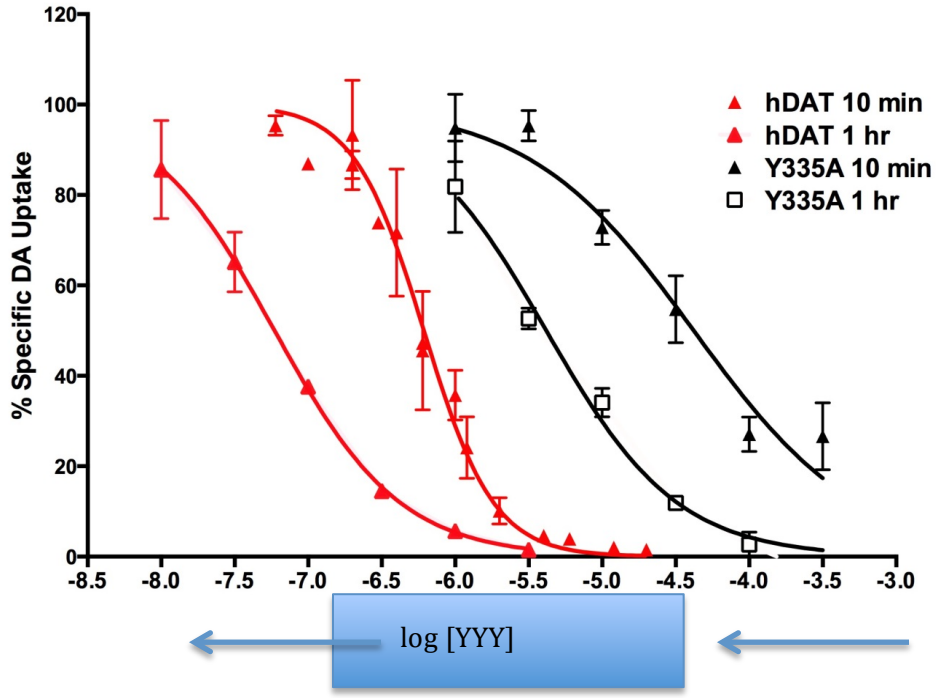

Figure 6. Composite Graph