☐ Research method

1. Zebrafish larvae

- For the control and experimental groups, AB strain zebrafish fry managed by Zefit were selected and used.

2. Group setting for amyloid-β (Aβ) -injected Alzheimer's disease (AD) model (Table 1)

- To induce AD model, 1 mg/ml Beta-Amyloid (1-42), made into oligomer form by incubation at 37 degrees for 7 days, HiLyteTM Fluor 488-labeled (Anaspect), was injected into the cerebral ventricle of zebrafish fry at 2 dpf. 2nl injection
- Donepezil, used as a positive control, was exposed to zebrafish fry at a concentration of 10μM for 7 days from 0dpf to 7dpf, and the drug was changed at 24-hour intervals.
- Drug candidates were exposed to zebrafish fry at the final experimental concentration for 7 days from 0 dpf to 7 dpf, and the drugs were replaced at 24-hour intervals.

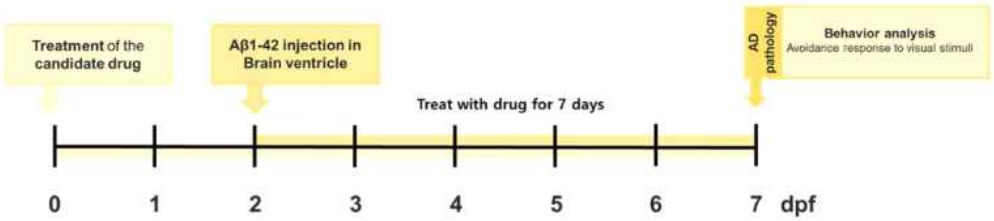

Figure 1. Diagram shows the shedule of amyloid-β (Aβ) -injected Alzheimer's disease (AD) model

Table 1. Group Setting for amyloid-β (Aβ) -injected Alzheimer's disease (AD) model

	Group name	Drug concentration	Animal	measurement stage	Sample number for behavior analysis
1	Vehicle + Vehicle		AB	7dpf	N=20

				larvae		
	Aβ + Vehicle			AB larvae	7dpf	N=20
	Aβ + Donepezil (P.C)			AB larvae	7dpf	N=20
2	Aβ	Drug candidate	30mg/kg(L)	AB larvae	7dpf	N=20
			150mg/kg(L)	AB larvae	7dpf	N=20

*** Behavior analysis (AB(n=100))**

: Avoidance responase to visual stimuli (% of larvaes in non-stimuli area)

- 1 (Positive control group) : Control and Aβ, Donepezil groups (AB(n=60))
- 2 (Experimental group) : Aβ and Drug candidate groups (AB(n=40))

3. Group setting for MPTP-induced Parkinson (PD) model (Table 2)

- To induce PD model, zebrafish fry were exposed to MPTP at a concentration of 200μM for 4 days from 2dpf to 6dpf, and the drug was changed at 24-hour intervals.
- Rasagiline, used as a positive control, was exposed to zebrafish fry at a concentration of 2μM by co-treatment with 200μM MPTP for 4 days from 2dpf to 6dpf, and the drug was changed at 24-hour intervals.
- Drug candidates were exposed to zebrafish fry through co-treatment with 200μM MPTP for 4 days from 2dpf to 6dpf at the final experimental concentration, and the drugs were replaced at 24-hour intervals.

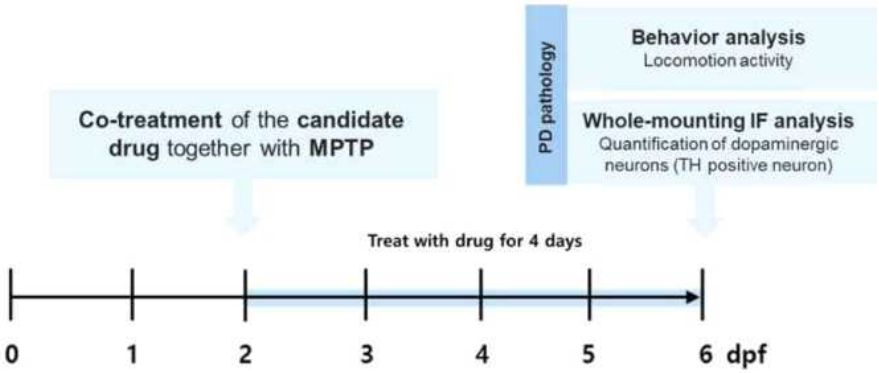

Figure 2. Diagram shows the shedule of MPTP-induced Parkinson (PD) model

Table 2. Group Setting for MPTP-induced PD model

	Group name		Drug concentration	Animal	measurement stage	Sample number for behavior analysis	Whole-mounting IF
1	Vehicle + Vehicle			AB larvae	6dpf	n=20	n=10
	MPTP + Vehicle			AB larvae	6dpf	n=20	n=10
	MPTP + Rasagiline (P.C)			AB larvae	6dpf	n=20	n=10
2	MPTP	Drug candidate	30mg/kg(L)	AB larvae	6dpf	n=20	n=10
			300mg/kg(L)	AB larvae	6dpf	n=20	n=10

*** Behavior analysis (AB(n=100))**

: Locomotion activity (total moved distance(mm), Average velocity(mm/s))

- 1 (Positive control group) : Control, MPTP and Rasagiline groups (AB(n=60))
- 2 (Experimental group) : MPTP and Drug candidate groups (AB(n=40))

*** Whole-mounting IF analysis (AB(n=50))**

: Quantification of dopaminergic neurons by anti-tyrosind hydroxylase whole-mounting IF

- 1 (Positive control group) : Control, MPTP and Rasagiline groups (AB(n=30))
- 2 (Experimental group) : MPTP and Drug candidate groups (AB(n=20))

3. Group setting for A549 (Human lung cancer cell lines) derived xenograft (CDX) model (Table 3)

- To produce A549 (Human lung cancer cell lines) zebrafish xenograft model, CM-Dil labeled A549 cells were injected into the perivitelline space (PVS) of zebrafish larvae at 2 dpf.
- Paclitaxel (PTX), used as a positive control, was exposed to zebrafish fry at a concentration of 25nM for 3 days from 3dpf to 6dpf, and the drug was replaced at 24-hour intervals.
- Drug candidates were exposed to zebrafish fry at the final experimental concentration for 3 days from 3dpf to 6dpf, and the drugs were changed at 24-hour intervals.

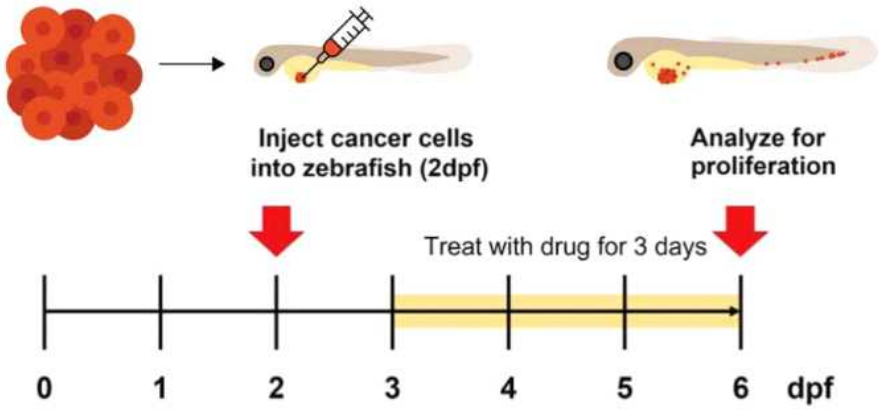

Figure 3. Diagram shows the shedule of A549 (Human lung cancer cell lines) derived xenograft (CDX) model

Table 3. Group Setting for A549 (Human lung cancer cell lines) derived xenograft (CDX) model

	Group name		Drug concentration	Animal	measurement stage	Sample number for behavior analysis
1	A549 Xenograft + Vehicle			AB larvae	7dpf	N=10
	A549 Xenograft + Paclitaxel (P.C)			AB larvae	7dpf	N=10
2	A549 xenograft	Drug candidate	30mg/kg(L)	AB larvae	7dpf	N=10
			300mg/kg(L)	AB larvae	7dpf	N=10

* Analyze for proliferation (AB(n=40))

: Tumor growth/regression

- 1 (Positive control group) : Zebrafish xenograft model (A549) and Paclitaxel group (AB(n=20))
- 2 (Experimental group) : Zebrafish xenograft model (A549) and Drug candidate groups (AB(n=20))

☐ Research process

1. **Avoidance response analysis for amyloid-β (Aβ) -injected Alzheimer's disease (AD) model (Figure 4)**
   - Beta-Amyloid (1-42), HiLyteTM Fluor 488-labeled (Anaspect), was prepared as a 2 mg/ml solution using dimethyl sulfoxide (DMSO), diluted in PBS to a concentration of 1 mg/ml, and then incubated

in zebrafish at 2 dpf. Inject 2nl into the cerebral ventricle of fish fry using a micro-injector.

- Donepezil used as a positive control was prepared as a 10mM stock solution using DMSO, and the final concentration immediately before use in the experiment was adjusted to E3 medium (5mM NaCl, 0.17mM KCl, 0.33mM CaCl2 and 0.33mM MgSO4 and the pH was adjusted to 7.0 Used after diluting in -7.2)
- The drug candidate was used by diluting the freeze-dried product provided by Care Innovation Co., Ltd. in E3 medium to the final concentration immediately before use.
- After drug treatment, 6dpf zebrafish fry were transferred to a 6-well plate (5 larvae per well, N=20 in quadruplicate) to measure avoidance response, and then photographed for 15 minutes (5 minutes of adaptation followed by 10 minutes of red ball stimulation). Confirmed avoidance response behavior due to visual stimulation
- Movements were filmed at a frame rate of 30/s using a high quality camera (16mm Telephoto Lens for Raspberry Pi High Quality Camera), and for stable movement filming, a separate soundproof room was installed at 27.5±1°C, the optimal temperature for zebrafish breeding. Conducting experiments in chambers
- Using software developed in-house by Zefit Co., Ltd., we tracked the movement of the fry distinguishable from the background by setting a threshold, and avoided the red ball (stimuli area) moving left and right in the upper half to avoid the lower half (non-stimuli area). The average value was measured by counting the percentage of moving objects (% of larvaes in non-stimuli area) at 12-second intervals for 6 minutes.

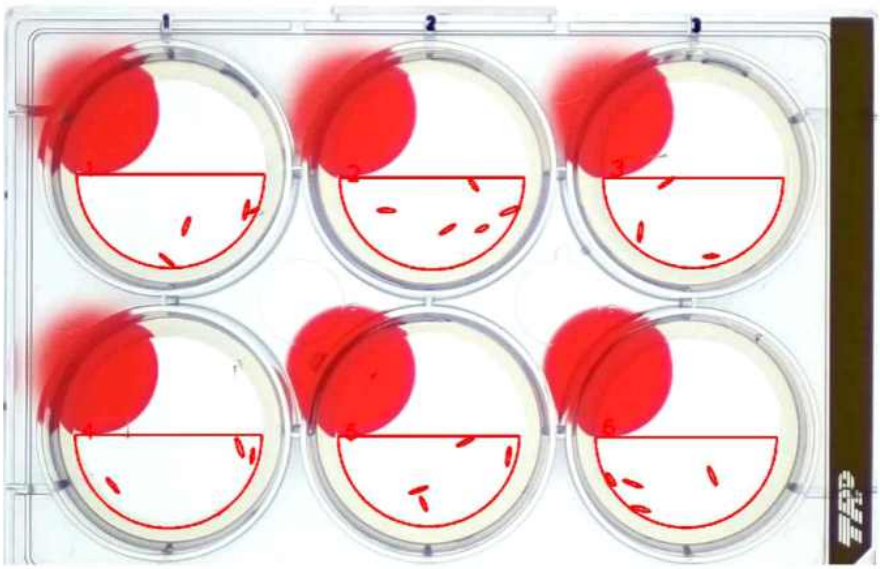

Figure 4. analysis of avoidance behavior in the five-fish red bounding ball

2. Locomotor activity analysis for MPTP-induced PD model (Figure 5)

- MPTP was prepared as a 100mM stock solution using dimethyl sulfoxide (DMSO), and was diluted in E3 medium to the final concentration immediately before use in the experiment.
- Rasagiline used as a positive control was prepared as a 10mM stock solution using DMSO, and was diluted in E3 medium to the final concentration immediately before use in the experiment.
- The drug candidate was used by diluting the freeze-dried product provided by Care Innovation Co., Ltd. in E3 medium to the final concentration immediately before use.
- After drug treatment, 6dpf zebrafish fry were transferred to 96 well plates to measure locomotor activity, and then photographed for 10 minutes to analyze total moved distance (mm).
- Movements were filmed at a frame rate of 30/s using a high quality camera (16mm Telephoto Lens for Raspberry Pi High Quality Camera), and an infrared (IR) light source that zebrafish cannot see was used to track movements in the dark. LV-BL-R 150/120 IR24) was used as a backlight to detect movement with an IR filter (850nm narrow bandpass filter)
- The distance from the 96 well plate to the camera is 35cm. Using a Fresnell lens (IYF-240-1), image distortion at the edge of

the plate is eliminated and all wells are photographed in a vertical direction.

- To capture stable movements, the experiment was conducted in a separate soundproof chamber at 27.5±1°C, which is the appropriate temperature for zebrafish breeding.
- Using software developed in-house by Jefit Co., Ltd., the movement of fry that was distinguished from the background was tracked using a threshold setting method.
- During 10 minutes of video analysis, if the speed of the larvae moving for 1 frame is less than 2mm/s, it is judged that the larvae did not move a distance but instead turned, trembled and floated in the water, etc., and moved at a speed of 2mm/s or more. Only cases were included in Total moved distance (mm)

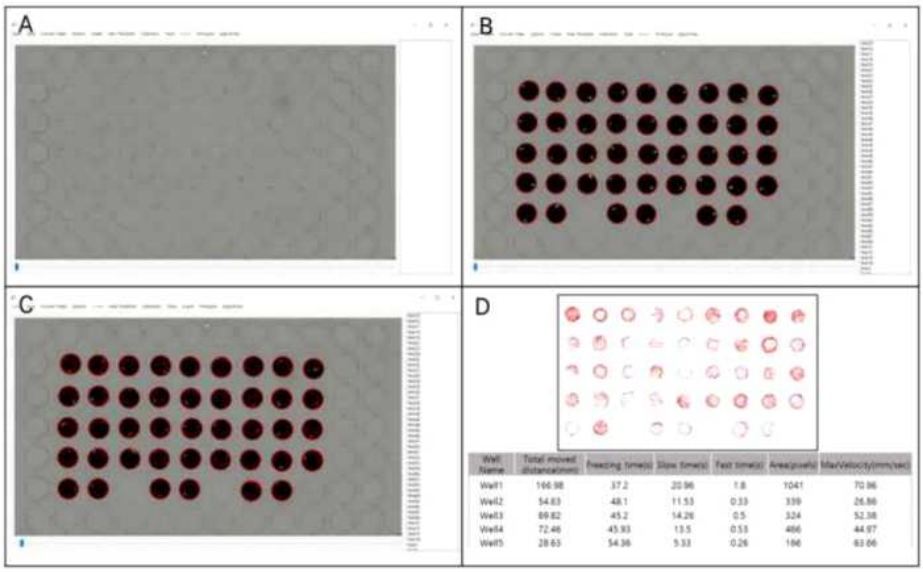

Figure 5. Automated analysis for locomotion activity of zebrafish larvae in multiwell plate

(A) The software for automated analysis of larval locomotion activity, (B) identification of larvae from the background with a threshold for dark objects (C) Tracking of larval locomotion. The red line indicates a movement of larvae during 10frame (D) The results file of locomotion activity, which including the trajectory image, total moved distance (mm), Freezing time(s)(<2mm/s), Slow time(s)(2~20mm/s), Fast time(s)(>20mm/s), and Max velocity(mm/s)

4. **Quantification of dopaminergic neurons by anti-tyrosine hydroxylase whole-mounting IF (Figure 6)**

- After drug treatment, 6dpf zebrafish fry were anesthetized on ice for 10 minutes and fixed in 4% paraformaldehyde (PFA) for whole-mounting IF.
- For fixed samples, the hair shell was removed to allow penetration of antibodies, and blocked with 10% goat serum + 2% BSA in PBT buffer.
- Blocked samples were incubated with mouse monoclonal anti-tyrosine hydroxylase (TH) antibody (Millipore, Billerica, MD, USA), and after PBT washing, they were incubated with Alexa Fluor 488 goat anti-mouse antiboddy.
- Stained zebrafish were mounted with 3.5% methylcellulose and imaged with a fluorescence microscope (Nikon, Edipse TI-E).
- The area (um2) of the TH+ neurons cell cluster in the brain region corresponding to the substantia nigra of zebrafish fry was measured using Image J.
- The results were expressed as a percentage of the area of TH+ neurons cells compared to the Control group, and statistical analysis was performed using T-test based on 10 animals in each group.

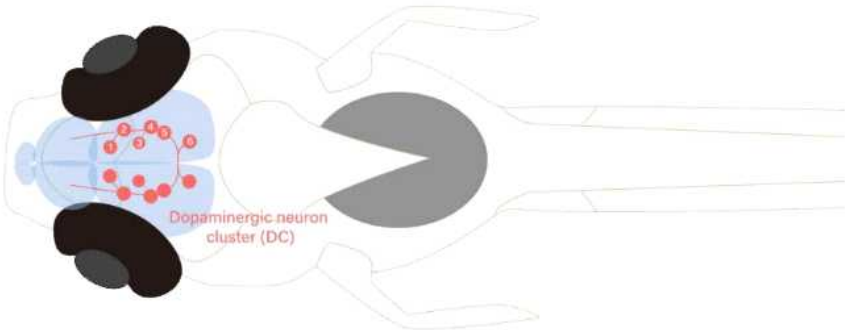

Figure 6. Schematic overview of dopaminergic neuron cell cluster in zebrafish larvae.

4. **Proliferation analysis for A549 (Human lung cancer cell lines) derived xenograft (CDX) model (Figure 7)**

- Human lung cancer cell lines, A549, were cultured in DMEM medium containing 10% FBS + 1% Penicillin streptomycin.

- Cultured A549 cancer cells were labeled with 0.5 μL/mL CM-Dil (Invitrogen, USA) before injection.
- Cell suspension prepared at a density of 2X107/ml was diluted in 30% matrigel matrix Injected into the perivitelline space (PVS) of 2dpf zebrafish larvae using a microinjector.
- The injected zebrafish xenograft was cultured at 34 °C
- 24 hours after cell injection, zebrafish that underwent imaging were transferred to 6-well plates and exposed to medium containing the drug.
- Paclitaxel used as a positive control was prepared as a 100 μM stock solution using DMSO, and was diluted in E3 medium to the final concentration immediately before use in the experiment.
- The drug candidate was used by diluting the freeze-dried product provided by Care Innovation Co., Ltd. in E3 medium to the final concentration immediately before use.
- 1, 4 days postinjection (dpi) zebrafish larvae were mounted on 1.2% low-melting agarose gel for imaging, imaged with inverted fluorescent microscopy (Nikon, Eclipse Ti-E), and tumor size was measured using Image J software. was quantified
- Tumor cell proliferation was calculated as the ratio of tumor size at 4dpi and 1dpi.

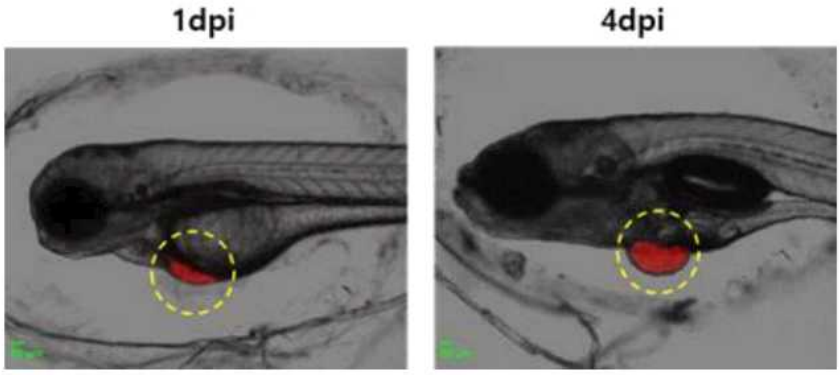

Figure 7. Representative images for tumor in zebrafish xenograft (CDX) model

□ **Result**

1. Avoidance response analysis for amyloid-β (Aβ) -injected Alzheimer's disease (AD) model (Figure 8)

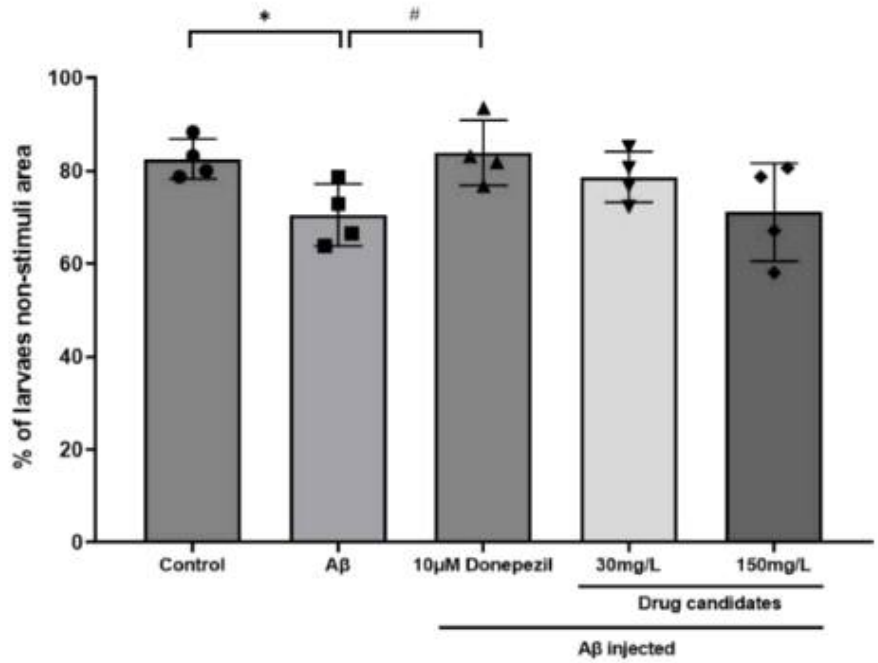

Figure 8. % of larvaes in non-stimuli area (Final concentration of DMSO: 0.1%) in amyloid-β (Aβ)-injected zebrafish AB larvae treated with donepezil and drug candidates at the indicated concentration. ; * versus Control; # versus Aβ group (T-test)

Table 4. % of larvaes in non-stimuli area (Final concentration of DMSO: 0.1%) in amyloid-β (Aβ)-injected zebrafish AB larvae treated with donepezil and drug candidates at the indicated concentration. Raw data.

	% of larvaes in non-stimuli area				
	Control	Aβ	10μM Donepezil	Drug Candidate	
				30mg/L	150mg/L
1	78.71	63.87	81.94	76.77	78.71
2	80.00	66.45	76.77	80.65	67.10
3	88.39	78.71	83.23	72.26	80.65
4	83.23	72.90	93.55	85.16	58.06

2. Locomotor activity analysis for MPTP-induced PD model (Figure 9)

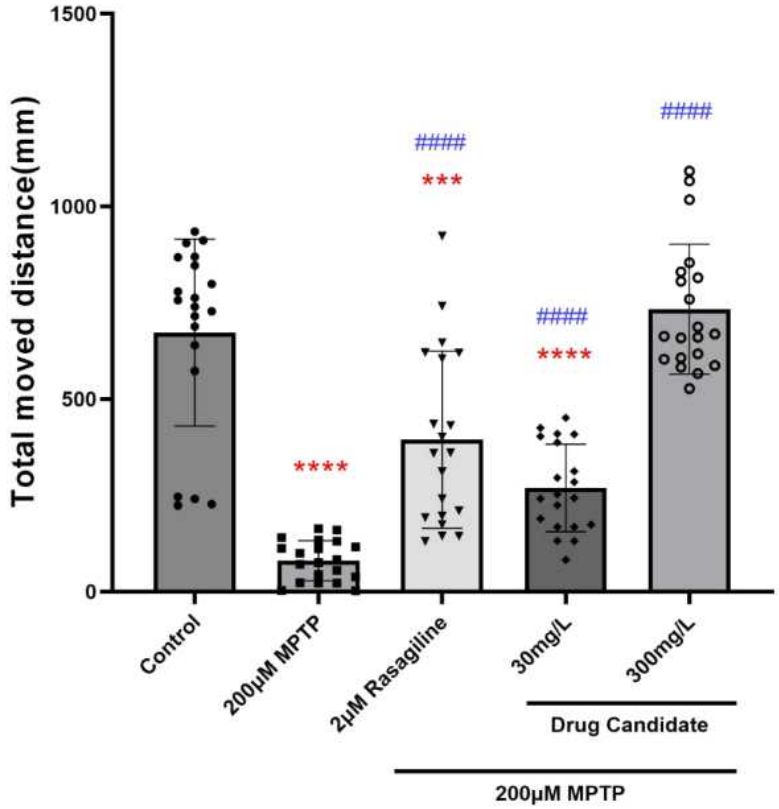

Figure 9. Total moved distance (mm) in zebrafish AB larvae treated with MPTP and Rasagiline, Drug candidate (Final concentration of DMSO: 0.02%) at the indicated concentration; * versus Control; # versus MPTP group (T-test)

Table 5. Total moved distance (mm) in zebrafish AB larvae treated with MPTP and Rasagiline, Drug candidate (Final concentration of DMSO: 0.02%) at the indicated concentration. Raw data.

	Total moved distance (mm)				
	Control	MPTP	2μM Rasagiline	Drug Candidate	
				30mg/L	150mg/L
1	798.46	44.84	431.59	253.31	805.64
2	688.51	23.01	196.93	451.16	815.33
3	715.05	99.73	311.75	173.91	1091.67
4	573.26	71.61	434.92	132.09	607.08
5	223.80	75.94	145.12	295.74	663.22
6	779.41	2.52	240.93	403.48	582.53
7	246.32	134.28	923.35	168.08	1017.74
8	227.98	3.47	401.78	168.04	687.60
9	240.97	55.93	360.77	408.87	660.65
10	911.85	38.79	359.89	189.36	527.47
1	868.14	83.31	131.31	410.22	759.40
2	728.36	116.69	605.42	312.71	603.25
3	869.02	111.81	210.24	243.49	668.83
4	904.88	163.65	192.59	387.77	830.33
5	739.76	23.16	174.28	284.83	566.39
6	934.95	160.39	144.61	82.96	617.48
7	762.47	130.79	620.07	224.72	658.89
8	639.92	140.55	646.63	425.02	587.20
9	846.43	23.08	741.70	241.50	1065.98
10	756.62	112.76	621.15	131.45	854.25