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Multipath Natural Product Supplement Suppresses Dementia Symptoms in Amyloid-β and Tau Transgenic *Drosophila*

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Abstract

Background: Numerous reports on Alzheimer's Disease (AD) indicate that AD has many biochemical pathways. However, most AD treatments have targeted a single pathway such as beta amyloid or phosphorylated tau and have not been very successful in stopping AD progression. To address the multipath nature of AD, we have used multiple natural products that target critical aging and independent AD pathways to test whether a multipath approach might be more effective than the single pathway approaches typically used.

Methods and findings: We have constructed *Drosophila melanogaster* models of AD with inducible transgenic human Amyloid-β and tau mutations. Induction of either AD mutant gene in the transgenic flies after emergence from pupal stage leads to the early onset of slowed mobility that was tracked by assaying crawl times as a function of age. The transgenic Amyloid-β and tau *Drosophila* assays were utilized to test various natural products that target separate biological pathways involved in brain aging and dementia. Screening a library of natural products, we identified a group of 7 natural substances (MX100A) that synergistically prevented nearly all early mobility difficulties and reverses the life-shortening effects of Amyloid-β and tau. The favorable results on AD symptoms in *Drosophila* with MX100A were observed in animals treated from youth and from later adult ages. MX100A had no observed negative side effects.

Conclusion: Treatment of transgenic AD *Drosophila* with the multipath MX100A dietary supplement prevents nearly all early mobility difficulties and reverses the life-shortening effects of Amyloid-β and tau. In patients with AD, progressively more severe mobility difficulties and heightened risks of mortality are common symptoms of the later stages of AD. While successful animal results have not typically translated into effective treatments for AD, the multipath MX100A treatment results are promising enough to warrant further testing.

Keywords: Dementia; *Drosophila*; Tau Transgenic *Drosophila*

Abbreviations: AD: Alzheimer's Disease; Amyloid-β: Beta-amyloid; ptau: Phosphorylated tau; MAPT: Microtubule-Associated Protein Tau

Introduction

Alzheimer's Disease (AD) is a complex neurodegenerative disease that causes most of all cases of human dementia. Most of the drugs that are currently being tested to treat Alzheimer's Disease in FDA clinical trials target beta amyloid (Amyloid-β) or phosphorylated tau (p_{tau}), but not both simultaneously.

The currently available drugs also can cause serious adverse events, such as amyloid related imaging abnormalities (ARIA) [1,2] which sometimes lead to life threatening cerebral hemorrhages. As these single-path therapeutics have disappointed in clinical trials, interest in multi-target approaches to AD has grown [3,4].

While ptau and Amyloid-β have been the dominant focus of research on AD pathophysiology, other mechanisms are clearly involved. The risk of AD increases exponentially with age [5,6]. Thus, age-related processes like mitochondrial dysfunction, neural vascular aging, chronic inflammation, and astrocyte aging are other potential factors in the complex changes in brain function that occur over the decades preceding actual AD diagnosis [7-14]. This apparent complex etiology of AD suggests that many biochemical pathways should be altered simultaneously for a more successful intervention into AD.

In devising our AD intervention strategy, our overall aim was to test the hypothesis that AD progression can be significantly slowed by acting on multiple longevity genes while boosting mitochondrial function and stress resistance.

To carry out this strategy, we first conducted genetic and machine learning work on age-related databases for *Drosophila melanogaster* and humans. Using genetic databases from these two very divergent species, we were

able to identify many common neural-specific genetic and biochemical pathways involved in longevity. We also added gene targets known to be important to human AD, such as Amyloid-β and tau.

To identify potential botanical drugs, we initially screened natural products that act on identified longevity and AD-specific pathways. We next screened both individual and combinations of natural products in both Amyloid-β and ptau transgenic *Drosophila* models of AD. We then identified the combinations that provided the best protection against the development of neural dysfunction in the flies, as assayed by slowed mobility in our crawl tests and early death.

Our screening led to a seven-component botanical drug, MX100A, that was effective in preventing neural motor dysfunction in our transgenic *Drosophila* AD models.

Materials and Methods

MX100A treatment

The 7 natural products in MX100A are *Astragalus* membranaceus extract, Berberine HCl, *Pterocarpus marsupium* extract, L-Theanine, Genistein, Lithium Orotate (1.5 mg Lithium), and Selenium Glycinate (70 mcg Selenium). MX100A is protected by US Patent 9744204 [15]. The formulation is available for testing from Genescient, Inc. Please contact Genescient at science@genesceint.com.

Transgenic *Drosophila* models

We first screened individual botanical components and optimized combinations for their potential in slowing AD motor dysfunction in transgenic flies expressing mutant human AD-inducing beta-amyloid or tau genes that were obtained from human patients with early onset Alzheimer's disease. The transgenic *Drosophila* constructs were created by crossing a nervous system specific gene-switch driver line of flies (Elav-GS) [16] with upstream activation sequence (UAS) promoter fly lines for both Amyloid-β and tau (UAS-Amyloid-β 42 and UAS-MAPT, respectively).

AD motor dysfunction symptoms were then inducible in the resulting flies with the administration of RU486 (Mifepristone) [16]. The detailed animal testing of the 7 component MX100A used the same specially engineered Amyloid-β and tau *Drosophila* animal models.

In each treatment group, 100 adult transgenic flies were placed in male-female pairs in 50 vials immediately after emerging from their pupae. Each vial contained 1.5 g of banana-agar food media, and the drug treatment solutions were painted on top of the food and allowed to dry. Four days after the flies were placed in vials, gene expression was induced in the transgenic flies using 0.16 mg of RU486 in 50 microliters of 100% ethanol per vial to over-express either mutant human beta-amyloid (Amyloid-β) or tau. Two days after mutant Amyloid-β or tau induction, 150 mL of a deionized water solution containing 0.0164 mg of MX100A was added to each vial of the early MX100A treatment groups.

In delayed treatment groups, MX100A was given 8 days after Amyloid-β or tau induction. The flies were moved into fresh vials every day. As a measurement of the progression of neural dysfunction, the flies were knocked to the bottom of their vials and then timed on how fast they could crawl back up to the top of the vial. These crawl tests are metrics of mobility, which in both humans and animals declines with age and with the progression of AD in humans. The crawl tests were performed three times per week until the flies died or reached a crawl time greater than one minute.

Fly media

All studies employed transgenic lines of *D. melanogaster* that were maintained in vials containing a banana medium at about 25°C.

The banana medium (BM) is a low protein diet containing a boiled mixture of (i) 100 g agar in 6.6 L distilled water, (ii) 900 g peeled bananas, 165 mL Eden barley malt syrup, 110 mL light and 110 mL dark Karo corn syrup blended into 400 mL distilled water, and (iii) 240 g Fleischmann's instant dry yeast and 160 mL 95% ethanol blended into 460 mL water. After cooling to 48°C, 15.6 g methyl-4-hydroxybenzoic acid (10% w/v in 95% ethanol) was added as an antifungal agent. Life spans on this medium are nearly as long as on dietary restriction diets [17].

Fly longevity assays – Vials

Flies were housed in small vials with 1 male and 1 female flies/vial and enough vials to have 50 flies of each sex. The vials were changed every 2-3 days or 3 times per week, with flies combined into new vials to preserve the 2 flies per vial as flies died. Sexual selection (1 of each sex or 2 of one sex if not enough of each sex) was preserved until all flies were dead.

Statistical analysis

All statistical analyses were carried out using Microsoft Excel.

Results

MX100A is composed of 7 components

Our AD transgenic fly screening of herbal extracts and purified natural products led us to a botanical mix of 7 components that target multiple AD and longevity pathways, which included inflammation, acetylcholinesterase, AMPK, ptau, Amyloid-β, mitochondria and metabolic dysfunction, genomic instability, adrenergic receptors, NMDA receptors, GABA receptors, autophagy (mTOR), and epigenetic factors. The list of 7 herbal extracts and natural products in MX100A is

given in **Table 1** along with the known actives and targets [18-57].

Table 1 Composition, known actives, and targets of MX100A.

Components	Known Active(s)	Targets	References
Astragalus membranaceus (extract)	Astrogalosides I-VII Flavenoids, HDTICs	Telomerase, Mitochondria, ptau, mTOR, TNF-α, ERK, AMPK	[18-24]
Berberine HCL	Berberine (98%)	Acetylcholinesterase, AMPK, α-adrenergic receptors, β-Amyloid	[25-30]
Vaccinium uliginosum or *Pterocarpus marsupium* (extracts)	Resveratrol Analogs	PPARα, PGE2, AMPK, phosphodiesterase, Mitochondria	[31-35]
L-Theanine	L-Theanine (98%)	NMDA receptors, EAATs, GABA receptors, eNOS, mitochondria	[36-42]
Genistein	Genistein (98%)	ERα, AMPK, p450c21	[43-45]
Lithium Orotate	Lithium	NCS-1/Frequenin, Amyloid-β, NMDA, GSK3B, ptau	[46-54]
Selenium Glycinate	Selenium	PRPF8, ERCC1, Selenoproteins	[55-57]

MX100A prevents nearly all the early mobility and mortality effects from beta-amyloid or tau expression in our AD *Drosophila* model

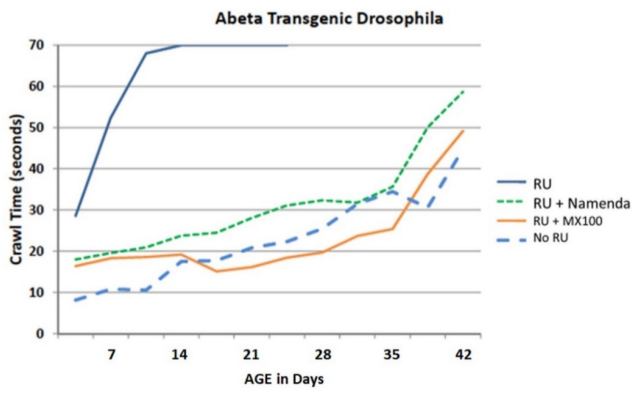

Figure 1 MX100A (MX100) suppresses AD symptoms in *Drosophila* expressing the AD-inducing human beta-Amyloid gene. The crawl tests for each treatment group measures the time in seconds (Y axis) that it takes to reach the top of the vial from the bottom using 50 vials with 2 flies (1 male and 1 female) in each vial. The control flies (dashed blue line) are not treated with the drug RU486, which triggers the human transgenic beta-Amyloid gene. The RU Control flies (solid blue line), Namenda treated (dashed green) RU flies, MX100 (orange) treated RU flies were each treated with RU486 starting at two days after hatching to induce continuous expression of the transgenic human beta-amyloid gene. MX100 flies are treated with MX100A two days after treatment with RU486, while Namenda flies are treated with Namenda two days after treatment with RU486.

The detailed testing of our botanical drug MX100A used the same genetically engineered transgenic AD *Drosophila* model that we developed for our preliminary screens testing the individual botanical components and optimized combinations of the best components for their potential in slowing the progression of motor dysfunction in the transgenic flies. The transgenic flies were induced to express either mutant human beta-amyloid (Amyloid-β) or tau and then tested for lifespan and how fast they could crawl up the side of their housing vial, which is a mobility assay.

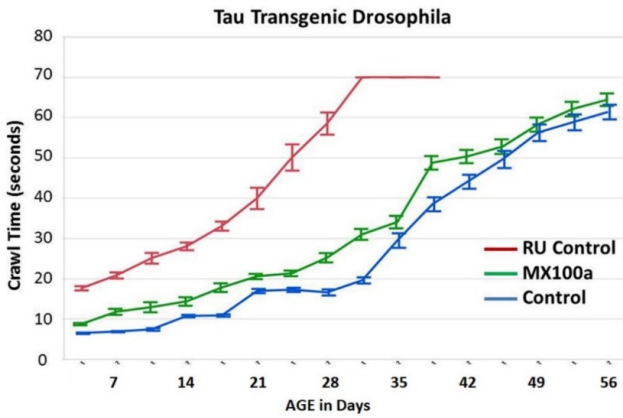

Figure 2 MX100A^{TM} (MX100a) suppresses AD symptoms in *Drosophila* expressing the AD-inducing human tau gene. The crawl tests for each treatment group measures the time in seconds (Y axis) that it takes to reach the top of the vial from the bottom using 50 vials with 2 flies (1 male and 1 female) in each vial. The control flies (blue line) are inducible transgenic tau flies that were not treated with the drug RU486, and thus do not express human tau. The RU Control flies (red line) and MX100A RU flies (green line) were each treated continuously with RU486 two days after hatching to induce expression of the human tau gene. MX100a flies were also treated with MX100A beginning two days after treatment with RU486 was started.

Figure 1 shows our typical *Drosophila* fly results with the botanical supplement MX100A (labeled MX100 in **Figure 1**) on the Amyloid-β Transgenic *Drosophila* model using 100 flies for each group housed in 50 vials with one male and one female in each vial. The **Figure 1** assay is a climbing or crawling test measuring the time in seconds (Y axis) that it takes each fly to climb to the top of the vial from the bottom. With age (X axis), the flies naturally take longer to climb or crawl to the top of the vial. The Control flies (dashed blue line) have not been induced to express the beta-Amyloid gene and represent the natural progression of longer crawling times with advancing age. The RU Control flies (solid blue line) are induced with the drug RU486 to express the beta-amyloid gene, which greatly increases the crawling time at every age and induces early death. In contrast, MX100A flies expressing the beta-amyloid gene and treated with MX100A (solid orange line) have crawling times with age that are like those of control flies and slightly better than those flies expressing the beta-amyloid gene and treated with the drug Namenda (dashed green line), which is an existing FDA-approved AD pharmaceutical. The MX100A-treated Amyloid-β -expressing *Drosophila* also reverts to the lifespan of Control flies.

Figure 2 shows similar results for MX100A treatment of transgenic *Drosophila* mutants that have an inducible human mutant tau gene. As with **Figure 1**, the Control flies (blue line) carry the mutant gene, but have not been treated with RU486 to induce expression of tau. These flies demonstrate the normal slow increase in climbing time with age.

The RU flies (red line) were treated with RU486 to express the transgenic human tau gene, which greatly increases the crawling time beginning at a very early age. In contrast, RU flies treated with MX100A (RU + M100a - green line) have crawling times that are similar to flies not treated with RU (Control flies - blue line). As before, the MX100A treated RU flies also revert to the lifespan of the non-induced Control flies. In the **Figure 2** graph, we also show the 95% confidence error bar for the mean climbing times for each treatment group of 100 flies.

MX100A may reverse some early damage in transgenic *Drosophila* carrying a human tau gene

Another question that we wanted to explore with the transgenic tau flies was whether early damage from tau expression in our animal model could be repaired. **Figure 3** addresses this issue by delaying the MX100A treatment of mutant tau transgenic flies for 4 days after RU486 induction of tau. The delayed start of treatment by MX100A (green line) does lead to poorer initial function of the MX100A treated flies in **Figure 3** (compare to **Figure 2**), but crawl function then reverts to the typical progression of MX100A treated flies after MX100A treatment was added at 7 days. The delayed-start MX100A treated RU flies in **Figure 3** also revert to the lifespan of the non-induced Control flies and do not appear to have any shortened lifespan due to the late start of MX100A treatment.

Discussion

We have constructed two *Drosophila* models of Alzheimer's Disease (AD) using flies with inducible transgenic mutant Amyloid-β or tau genes taken from human patients that died from early onset AD. With the use of these two inducible transgenic *Drosophila* models of AD, we screened many purified natural products and herbal extracts for their efficacy in reversing the apparent mobility dysfunction symptoms of AD in both the Amyloid-β and tau models.

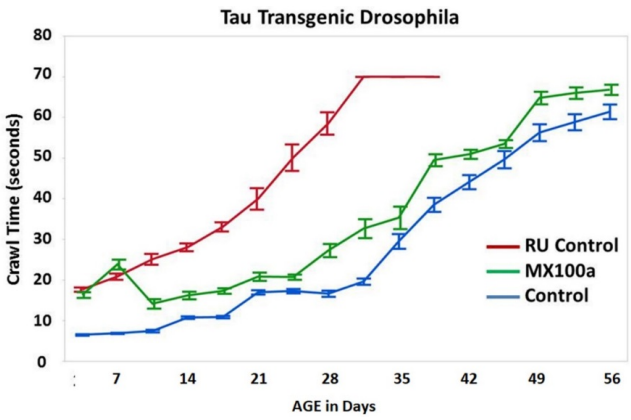

Figure 3 MX100A™ (MX100a) may repair early motor damage in *Drosophila* expressing the AD-inducing human tau gene. The crawl tests for each treatment group used 50 vials with 2 flies (1 male and 1 female) in each vial as in **Figures 1 and 2**. The Control flies (blue line) were not treated with RU486, and thus did not express mutant tau. The RU Control flies (red line) and MX100a flies (green line) were both treated with RU486 starting two days after hatching to induce expression of the mutant human tau gene. MX100a flies were treated with MX100a beginning four days after treatment with RU486, which permits sufficient time for the transgenic tau to begin damaging neural connections before treatment intervention.

Using these transgenic screens, we were able to identify a seven-component natural product supplement MX100A that reverses the AD gene-induced symptoms (i.e., mobility loss and shortened lifespan) in both the Amyloid-β (**Figure 1**) and tau (**Figure 2**) transgenic AD flies. Therefore, this AD treatment is multipath and works with high efficacy on both the Amyloid-β and tau pathways.

We also note that MX100A may act on many other longevity pathways (**Table 1**) such as inducers of: telomerase, mitochondria efficiency (e.g. AMPK and PPAR), and autophagy (e.g. mTOR). MX100A also helps reduce inflammation (e.g. TNFα) and stress (e.g. NMDA and GABA receptors in brain neurons).

Figure 3 suggests that MX100A may be able to reverse some of the early neural motor damage in tau transgenic flies, as MX100A can be added 4 days after the induction of tau and is able to reverse the early tau damage. Since Alzheimer 's disease is usually diagnosed after extensive brain damage has

already occurred, the potential ability of MX100A to reverse some of the neural damage is another hopeful sign that MX100A may be useful in the treatment of Alzheimer's Disease patients.

Conclusion

Drosophila models of AD, as described here, are far less expensive than mammal models and can yield results faster with larger N values. The use of *Drosophila* animal models in drug discovery could be a valuable tool for identifying and testing multipath natural product mixes of 7 to 20 compounds. Since Alzheimer's Disease, like most age-related diseases and disorders, is likely caused by multiple factors [7-14], the *Drosophila* models of AD could be a game changer for identifying effective multipath drugs for Alzheimer's and other neurodegenerative diseases.

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