The inhibition of α-synuclein aggregation using marine-derived bacterial metabolites as a novel neuroprotective approach for Parkinson's Disease

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Introduction

Objectives

Parkinson's Disease (PD) is characterized by inclusions of aggregated α-synuclein (α-syn), a protein found abundantly in neurons. Soluble high-molecular-weight oligomers, formed through the multimerization of α-syn monomers, are thought to compromise neuronal viability. Targeting these toxic α-syn species for therapeutic intervention is crucial, given the absence of a definitive cure and the limited symptomatic relief current treatments provide. The marine environment is rich in biodiversity and represents a largely untapped resource for bioactive compounds with novel chemical structures and functional groups, which could have potent biological activity and therapeutic potential.

Methods

This study involved screening marine bacterial extracts for compounds that could act as potent inhibitors of α-syn aggregation. A structured five-step screening pipeline was employed, including inhibition of seed elongation, MTT assay, measurement of α-syn aggregation, enzymatic evaluation of cellular degradation and structural interference analysis.

Results

A specific fluorescence assay based on thioflavin T was established to select bacterial homogenates that hinder the elongation of pre-formed α-syn fibrils. The most effective extracts were tested on SH-SY5Y cells, a human-derived model that, upon induced α-syn expression, leads to oligomer buildup and cell death. Levels of aggregated α-syn were then measured using an ELISA assay that specifically detects aggregated a-syn. Secondary metabolites isolated from potent extracts were further tested to pinpoint compounds with anti-aggregation properties in cells. Through this approach, the metabolite BIO904-09, a 2,5-diketopiperazine, was identified as a promising α-syn aggregation inhibitor, acting to enhance proteasomal activity in cells burdened by cytoplasmic α-syn aggregates. SEM experiments indicated that this compound can also affect the structural properties of these toxic assemblies.

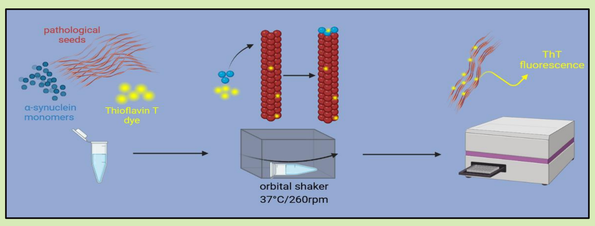
Conclusions

We successfully established a straightforward pipeline to identify small-molecule inhibitors of α-syn aggregation in SH-SY5Y cells. Our strategy indicated a marine-derived diketopiperazine as a potent α-syn aggregation inhibitor that could drive future pharmaceutical interventions in PD.

Materials and Methods

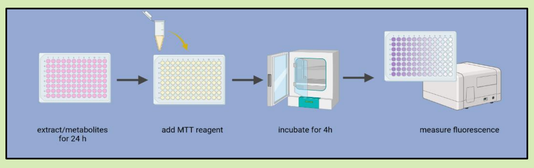
A 5-step pipeline for assessing the anti-aggregation capacity of small organic compounds against α-syn

1. Seed Elongation Assay We incubated 50 nM of preformed fibrils (PFFs), derived from recombinant α-syn, with 0.75 μM monomeric α-syn, in the presence of 10 μM Thioflavin T (ThT), which emits fluorescence upon binding to β-sheets. The assay monitors the increase in ThT fluorescence due to fibril elongation during the addition of monomeric α-syn to PFFs.



2. MTT assay

Cell viability was assessed through the MTT assay on α-syn expressing cells using various concentrations of the tested compounds.



3. Administration of compounds in SH-SY5Y cells and measurement of aggregated α-synuclein

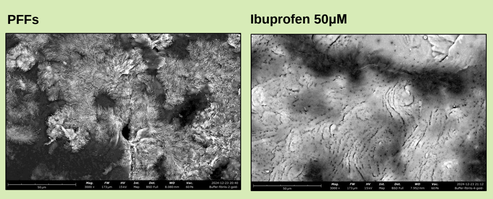
SH-SY5Y cells were treated with the most effective extracts and metabolites as suggested by the Seed Elongation Assay. The impact of marine extracts or their respective metabolites on the levels of oligomeric α-syn was evaluated by using a conformation-specific ELISA assay developed in house and western blotting.

4. Enzymatic assessment of cellular degradation systems

SH-SY5Y cells were treated with the most effective metabolite. The proteasome and lysosome activities were assessed by measuring the chymotrypsin-like and cathepsin B-like enzymatic activities using fluorogenic peptide substates.

5. Structural interference assessment

PFFs were incubated with a hit compound (or vehicle as control) and the assessment of conformational changes was performed by Scanning Electron Microscopy (SEM). Ibuprophen was used as a positive control for fibril degradation.



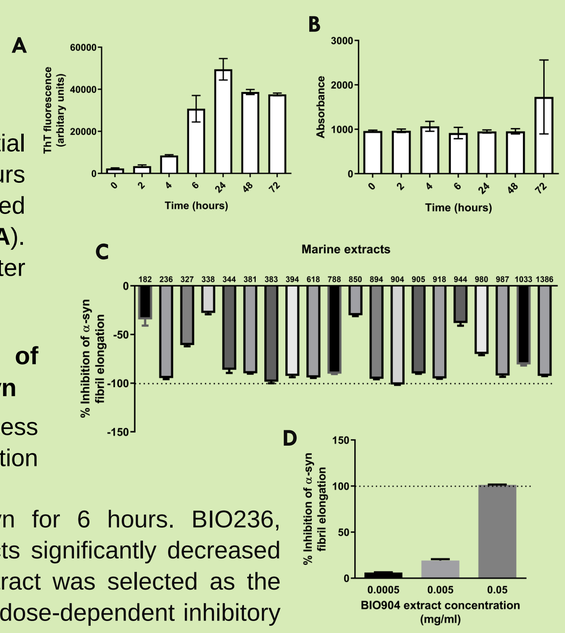
Results

1. Kinetics of PFFs elongation

The elongation process exhibited an exponential increase and reached its peak at 24 hours incubation. The rate of elongation remained stable from 24 to 72 hours of incubation. (**A**). Background fluorescence was stable even after 48 hours of incubation (**B**).

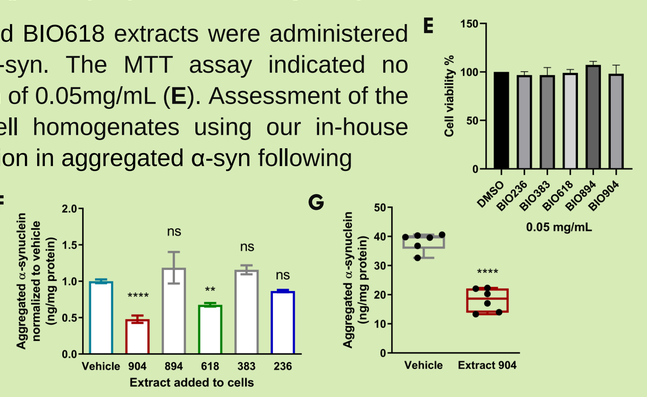
2. *In vitro* investigation of the impact of marine extracts on the aggregation of α-syn

Marine-derived extracts were screened to assess their potential inhibitory effects on fibril formation by using the seed elongation assay following incubation with PFFs and monomeric α-syn for 6 hours. BIO236, BIO383, BIO894, BIO904 and BIO918 extracts significantly decreased α-syn aggregation by ≥95% (**C**). BIO904 extract was selected as the most potent aggregation inhibitor exhibiting a dose-dependent inhibitory effect in PFF elongation (**D**).



3. Marine extracts exhibit anti-aggregation properties for α-syn expressed in SH-SY5Y cells

BIO236, BIO383, BIO894, BIO904, and BIO618 extracts were administered to SH-SY5Y cells overexpressing α-syn. The MTT assay indicated no significant cell death at a concentration of 0.05mg/mL (**E**). Assessment of the levels of α-syn oligomers/fibrils in cell homogenates using our in-house ELISA (**F**) revealed a significant reduction in aggregated α-syn following administration of BIO904 and BIO618 extracts (**G**). Extract 904 showed the most potent inhibition in α-syn aggregation and was selected for further investigation.

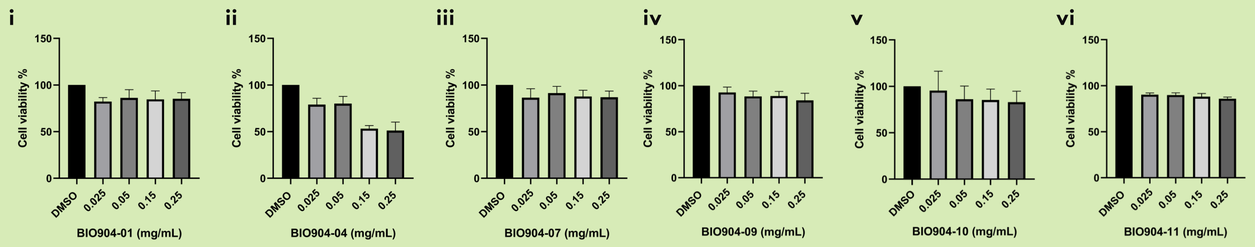


Conclusions

- We have optimized a simple pipeline for the discovery of small inhibitors for α-syn aggregation in human SH-SY5Y cells.
- Application of such approach allowed us to identify one naturally occurring α-syn aggregation inhibitor, BIO904-09 that acts to boost proteasomal activity under conditions of cytoplasmic accumulation of α-syn aggregates, but also affects the conformation of α-syn fibrils as demonstrated by SEM.

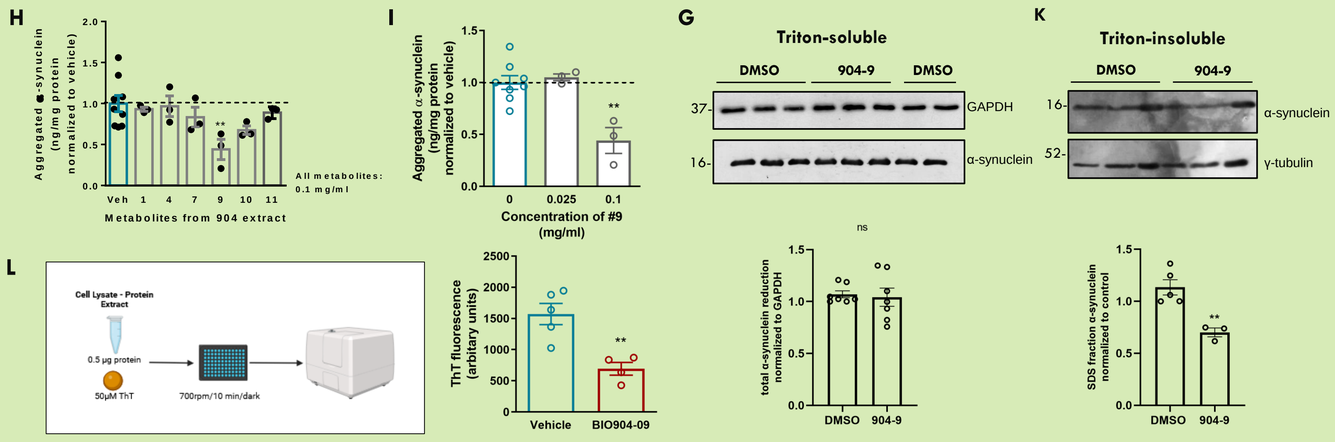
4. Evaluation of the optimal concentration of BIO904 and its metabolites by using the MTT assay

Metabolites #1-10 were extracted and purified from BIO904 extract. SH-SY5Y cells were treated with 0.025-0.25 mg/mL of each metabolite for 24 hours and cell viability was assessed by the MTT assay (**i-vi**). We selected 0.1 mg/ml as the highest concentration that did not compromise cell viability.



5. The metabolite BIO904-09 potently decreases the levels of aggregated α-syn in SH-SY5Y cells

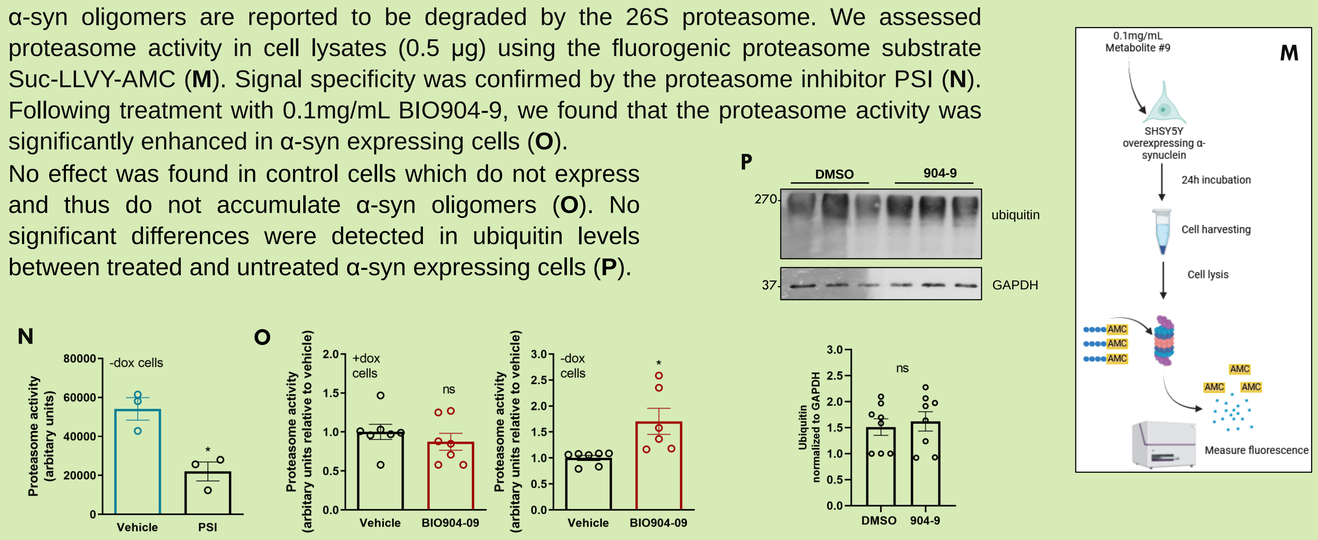
Purified metabolites (0.1 mg/ml) were added in cells for 24 hours. Assessment of the levels of α-syn aggregates revealed that BIO904-09 acted as a potent aggregation inhibitor (**H**). The inhibitory action of #9 was dose-dependent (**I**) further suggesting a specific action on aggregated α-syn. Western blotting analysis showed a significant reduction only on Triton-insoluble/SDS-soluble α-synuclein levels in cell homogenates (**G, K**) which also displayed reduced β-sheet content as demonstrated by ThT fluorescence measurements (**L**).



6. Treatment with BIO904-09 enhances proteasome activity in SH-SY5Y cells expressing α-syn

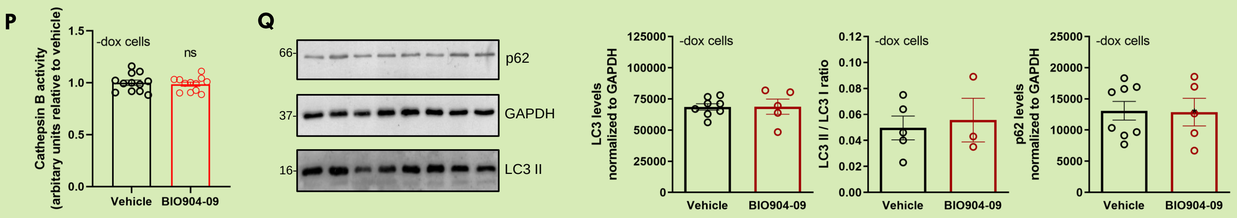
α-syn oligomers are reported to be degraded by the 26S proteasome. We assessed proteasome activity in cell lysates (0.5 μg) using the fluorogenic proteasome substrate Suc-LLVY-AMC (**M**). Signal specificity was confirmed by the proteasome inhibitor PSI (**N**). Following treatment with 0.1mg/mL BIO904-9, we found that the proteasome activity was significantly enhanced in α-syn expressing cells (**O**).

No effect was found in control cells which do not express and thus do not accumulate α-syn oligomers (**O**). No significant differences were detected in ubiquitin levels between treated and untreated α-syn expressing cells (**P**).



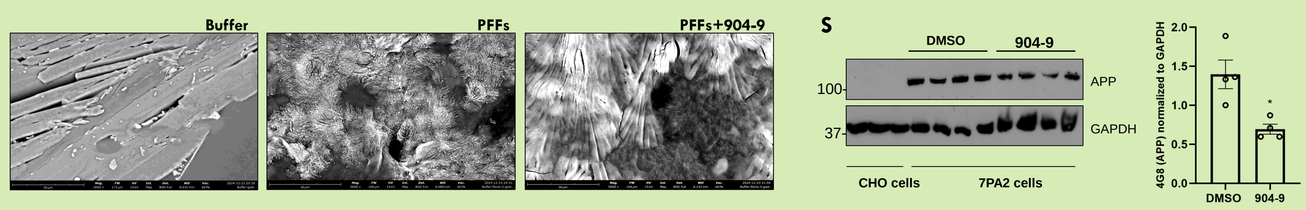
7. BIO904-09 does not enhance lysosome activity in α-syn expressing SH-SY5Y cells

We evaluated lysosomal function by measuring the activity of cathepsin B, a lysosomal enzyme that plays a critical role in autophagy. BIO904-9 caused no significant change in Cathepsin B activity in SH-SY5Y –dox cells (**P**). Similar results were obtained from the expression levels of p62 and LC3 (**Q**), two important autophagy markers, in the presence of #9, as determined by western blot analysis.



8. BIO904-09 interferes with α-syn fibril structure

SEM analysis showed that PFFs treated with #09 have a flattened linear structure compared with vehicle treated PFFs (**R**). Preliminary data indicate that compound #09 significantly reduces APP levels, the precursor of Aβ, in 7PA2 cells (**S**).



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