Supporting Information for:

A Gliclazide Complex based on Palladium towards Alzheimer's Disease: Promising Protective Activity against Aβ-induced Toxicity in *C. Elegans*

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1. Synthesis of Compound

All chemicals were of reagent grade and used as commercially obtained without any further purification.

First, 0.03 mmol (0.01 gr) of gliclazide ligand was dissolved in 1 mL of EtOH while slightly heated. In a separate vial, 0.03 mmol (0.008 gr) of $Pd(NO_3)_2 \cdot H_2O$ was also dissolved in 1 mL of EtOH. Rapidly, metal solution was added over ligand solution and the resulting solution was kept at 5ºC. Immediately, a powder precipitates and after a week, yellow crystals of compound [$Pd(glz)_2$] were obtained. Yield based on Pd: 17%. Anal. calcd. for $C_{30}H_{40}N_6O_6S_2Pd$: C, 47.96; H, 5.37; N, 11.19; S, 8.54%. Found: C, 47.67; H, 5.86; N, 11.11; S, 8.80%.

2. X-ray Crystallographic data

Crystal for [$Pd(glz)_2$] was mounted on a glass fibre and used for data collection on a Bruker D8 Venture with Photon100 detector equipped with monochromated MoKα radiation (λ=0.71073 Å). The data reduction was performed with the APEX3[1] software and corrected for absorption using SADABS.[2] Crystal structure was solved with SHELXT[3] and refined with SHELXL.[4] The OLEX2 software was used as a graphical interface.[5] Anisotropic atomic displacement parameters were introduced for all non-hydrogen atoms. Hydrogen atoms were placed at geometrically calculated positions and refined with the appropriate riding model, with Uiso(H) = 1.2 Ueq(C, N) (1.5 for methyl groups). Even though crystal data was collected up to 0.77 Angstroms resolution, crystals still diffracted quite weakly at high angle due to their rather low quality and data were cut off according to intensity statistics. During the refinement of [$Pd(glz)_2$], a number of RIGU restraints had to be used to obtain reasonable anisotropic displacement parameters for some non-hydrogen atoms. Crystal data and refinement details are listed in Table S1. Selected bond lengths and angles are given in Table S2. Crystallographic data (excluding structure factors) for the structure reported in this paper has been deposited with the Cambridge Crystallographic Data Centre as supplementary publication nos. CCDC 2102397 for compound. Copies of the data can be obtained free of charge on application to the Director, CCDC, 12 Union Road, Cambridge, CB2 1EZ, U.K. (Fax: +44-1223-335033; e-mail: deposit@ccdc.cam.ac.uk).

Table S1. Crystallographic data and structure refinement details for compound $[Pd(glz)_2]$

CCDC	2102397
Formula	$C_{30}H_{40}N_6O_6S_2Pd$
M	751.20
Crystal System	Monoclinic
Space group	$P2_1/c$
T [K]	100
a [Å]	16.161(2)
b [Å]	11.7499(16)
^{o}c [Å]	17.900(3)
α [deg]	90
β [deg]	109.313(6)
γ [deg]	90
V [Å^3]	3207.8(8)
Z	4
Density [gcm^{-3}]	1.555
μ [mm^{-1}]	0.761
Observed reflections	16749
R_{int}	0.1795
R1 [b] / wR2 [c] [I>2σ(I)]	0.0598 / 0.1414
R1 [b] / wR2 [c] (all data)	0.1012 / 0.1821
GoF	1.199
Largest diff. pk and hole [eÅ^{-3}]	0.600 and -0.536

[a] $S = [\Sigma w(F_0^2 - F_c^2)^2 / (N_{obs} - N_{para}m)]^{1/2}$ [b] $R_1 = \Sigma||F_0|-|F_c|| / \Sigma|F_0|$ [c] $wR_2 = [\Sigma w(F_0^2 - F_c^2)^2 / \Sigma wF_0^2]^{1/2}$
$w = 1/[\sigma^2(F_0^2) + (aP)^2 + bP]$ where $P = (max(F_0^2,0) + 2F_c^2)/3$

Table S2. Bond Distances and Angles for compound $[Pd(glz)_2]$

Bond Distances	Bond Angles
Pd1 N1A 2.062(15)	N1B Pd1 N1A 168.8(6)
Pd1 N1B 2.057(15)	N3A Pd1 N1A 79.3(7)
Pd1 N3A 2.044(16)	N3A Pd1 N1B 102.3(7)
Pd1 N3B 2.022(16)	N3B Pd1 N1A 98.9(7)
	N3B Pd1 N1B 79.1(8)
	N3B Pd1 N3A 177.2(6)

Table S3. Hydrogen Bonds for compound $[Pd(glz)_2]$

Atom1	Atom2	Lenght
O1A	O3A	2.936
O1B	O2B	3.026
O1B	N2A	2.889
N2B	O1A	2.790

3. Cell conditions

C2C12 cells were purchased by American Type Culture Collection ATCC collection. Cells were maintained at fibroblast cells were grown at 37 ◦C, 5% (v/v) CO2 and 99% (v/v) relative humidity in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Waltham, Massachusetts) supplemented with Fetal Bovine Serum (FBS) 10% (Gibco, Waltham, Massachusetts) and L-Glutamine 2mM (Gibco, Waltham, Massachusetts). For the differentiation process, 2000 cells/ cm^2 were seeded in 96 well plates (VWR Int., Radnor, Pennsylvania); when cells were totally confluent, medium was refreshed by DMEM supplemented with horse serum 2% (Gibco, Waltham, Massachusetts) during 5 days.

4. Glucose uptake

The day before the assay, medium was replaced by DMEM glucose-free. Subsequently, cells were washed two times using Krebs-Ringer-HEPES (KRH) glucose-free at 37ºC and incubated for 30 minutes with 60 µM of the compound and the glz (insulin 100 nM was used as positive control and cells treated with Dimethylsulfoxide (DMSO) 1% (v/v) was the negative control). Then, cells were washed with KRH glucose-free at 37ºC two times and incubated with 2-NBDG 300 µM (Cayman Chemical, Ann Arbor, Michigan) for 15 minutes at 37ºC and washed with KRH supplemented 20 mM glucose. Finally, fluorescence was determined using a microplate reader (Biotek, VT, USA), excitation 465 nm-emission 540 nm.

5. Antidiabetic studies

The exposition of C2C12 muscle cells to this novel palladium compound did not increase the uptake of glucose, since no statistical ($p<0.05$) differences were found between the cells incubated with glz and [$Pd(glz)_2$] (60 µM, 30 min), and untreated cells (negative control, Figure S2). In addition, these results showed lower values of glucose uptake than cells treated with insulin (positive control). These negative results should be related with the fact that the activity antidiabetic of glz is related with the use of high doses of the drug. For example, Zhang and co-workers, that showed the potential prevention of vascular obstructive disease and the antidiabetic effect employing 2000 µM of glz. The dose that we evaluated was obtained from other experiments developed using the present

compound that exhibited an antitumoral effect. It should be noted that the treatment with [Pd(glz)$_2$] did not affect the glucose uptake, suggesting the biocompatibility this compound.

6. Inhibition of AChE and BuChE

We followed the original Ellman's assay with minor modifications to measure the cholinesterases kinetics.[6] Comercially-available (Sigma Aldrich) enzymes were used (acetylcholinesterase type V-S from *electrophorus electricus* and butyrylcholinesterase from equine serum).

Stock solutions of [Pd(glz)$_2$] were prepared in DMSO, being the solvent content in the final assay medium 1.25% (V/V). Kinetics were measured using a Hitachi U-2900 spectrophotometer with PS cuvettes (final volume 1.2 mL) containing phosphate buffer (0.1 M, pH 8.0), Ellman's reagent in buffer solution (5,5'-dithiobis(2-nitrobenzoic acid, DTNB, 0.88 mM), acetylthiocholine (ATChI) or butyrylthiocholine iodide (BuTChI) as substrates, inhibitor, and a properly diluted enzyme solution (aq.). The latter was prepared considering that the reaction rate should be in the range 0.12-0.15 Abs/min when [S] = 4 x K_M (4 x Michalis-Menten constant). Formation of the chromophore was monitored during 125 s at 405 nm and 25 ºC.

Percentage of inhibition were obtained for [S] = K_M (121 μM and 112 μM for AChE and BuChE, respectively).

IC_{50} values were obtained from the plot of %I vs. log[I], adjusting the curve to a second order equation, with 6 different inhibitor concentrations, ranging from 1.0-100 μM, being run in duplicate.

For the calculation of the kinetic parameters of the free enzyme, and in the presence of [Pd(glz)$_2$], five different substrate concentrations, ranging from ¼ K_M to 4 x K_M were used. Cornish-Bowden plots[7] provided the mode of inhibition of [Pd(glz)$_2$]: 1/V *vs.* [I] (Dixon plot) and [S]/V *vs*. [I].

Kinetic parameters (K_M, V_{max}, $K_{M\ app}$, $V_{max\ app}$) were obtained using the least square fit (nonlinear regression analysis, GraphPad Prism 8.01); inhibition constants were calculated using the following equations for four different inhibitor concentrations (0, 12.5, 25.0 and 50.0 μM):

$$K_{M\ app} = K_M \frac{1 + \frac{[I]}{K_{ia}}}{1 + \frac{[I]}{K_{ib}}}$$

$$V_{max\ app} = \frac{V_{max}}{1 + \frac{[I]}{K_{ib}}}$$

Data are expressed as the mean ± SD.

7. C. elegans strains and maintenance conditions.

C. elegans strains used in this work were N2 Bristol (wild type), CL4176 (dvls27 [marker gene mio-3/Aβ minigene + rol-6 (su1006)] X), and CL802 (smg-1(cc546) I; rol-6(su1006) II). All *C. elegans* strains were obtained from the Caenorhabditis Genetics Center (Minneapolis, USA). N2 was maintained at 20 °C, meanwhile CL4176 and CL802 at 16 °C. Worms were grown on nematode growth medium (NGM) agar plates with *Escherichia coli* OP50 as food source. For experiments, age-synchronized animals were obtained by isolating embryos from gravid hermaphrodites using bleaching solution.

8. Short-term lethal toxicity assay.

Wild-type N2 synchronized L4 larvae were exposed to raising concentrations of the compound and unconjugated glyclazide dissolved in NGM (0, 0.1, 1, 10 and 100 μg/mL) without food for 24 h at 20 C. Nematodes were scored as dead when there was no response to repeated touches with platinum wire. Experiment was performed in triplicate with three independent assays for each concentration were made.

9. Pharyngeal pumping assay.

Synchronized embryos were lifelong incubated at 20 °C in NGM plates supplemented with or without different concentrations of the compounds for 96 h. At young adult state, worms were moved to different NGM plates to count the number of contractions per minute of the terminal bulb of the pharynx using a Motic microscope (Motic Inc. LTD. Hong Kong, China). Experiment was performed in triplicate and ten worms were studied per group (n = 10).

10. Body length measurement.

Young N2 animals were cultured from embryos as described for the pharyngeal pumping assay. In this case, animals were washed 3 times with M9 buffer to remove *E. coli*. Next, time of flight (TOF) was measured with COPAS BioSorter® flow cytometer (Union Biométrica, Belgium, Europe) as a representative measure of body length. A minimum of 150 worms were studied per experiment and the experiment was performed in triplicate.

11. β-amyloid (Aβ)-induced paralysis assay.

Aβ-muscle toxicity was evaluated in CL4176 strain and CL802 was used as negative control. Worms were lifelong cultured on NGM containing or not different concentrations of the compounds. In this case, synchronized embryos were incubated for 48 h at 16°C to L3 larvae. Then, to initiate Aβ-induced paralysis, worms were temperature-up-shifted from 16 to 25 °C. After 20 h, paralysis was scored at 2 h intervals. Nematodes were scored as paralyzed when there was no response to repeated touches with platinum wire. Results are expressed as percentage (%) of non-paralyzed worm. The experiment was performed in triplicate with, at least, 20 worms per group.

12. Thioflavin T Aβ staining

CL4176 worms were cultured with or without the compounds from embryos as described for the paralysis assay. Briefly, worms were collected and washed 3 times with M9 buffer at 26 h after temperature raised from 16 °C to 25 °C. Next, worms were fixed with 4% paraformaldehyde/M9 buffer (pH 7.4) for 24 h at 4 °C. Permeabilization of the worms were made with 5% fresh β-mercaptoethanol, 1% Triton X-100 and 125 mM Tris (pH 7.4) at 37 °C for 24 h. Subsequently, worms were washed 2 times with M9 buffer and stained with 0.125% thioflavin T (Sigma) in 50% ethanol for 2 min. Fading was made with sequential ethanol washes (50%, 75%, 90%, 75%, and 50% v/v) for 2 min each. Finally, worms were washed with M9 and transferred to slides using a drop of M9. Thioflavin-T stained worms were observed under a Nikon epi-fluorescence microscope (Eclipse Ni, Nikon, Tokyo, Japan) and images were acquired at 10X magnification using the GFP filter with a Nikon DS-Ri2 camera (Tokyo, Japan). Fluorescence intensity analysis of the images was performed using the software NIS-Elements BR (Nikon, Tokyo, Japan).

13. Additional Figures

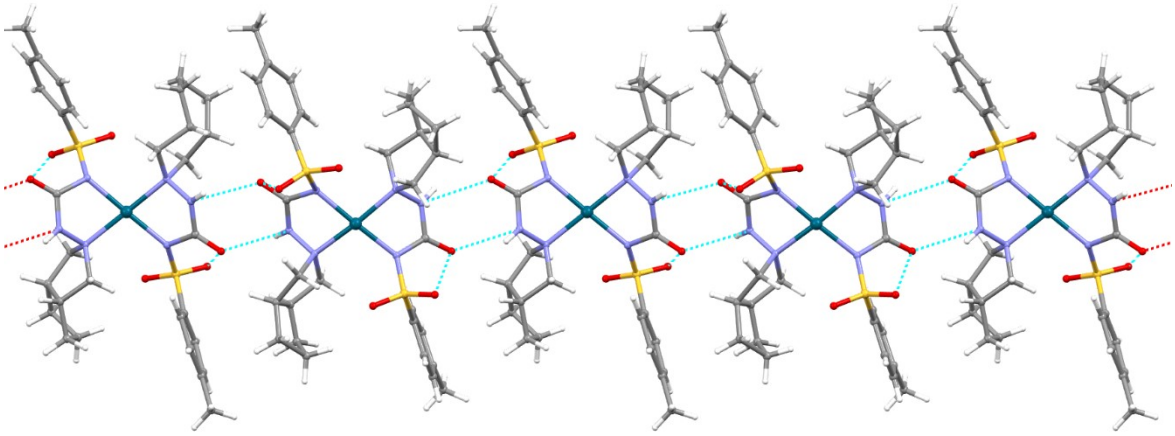

Figure S1. One-dimensional supramolecular chains formed by hydrogen bonds along the crystallographic *c* axis.

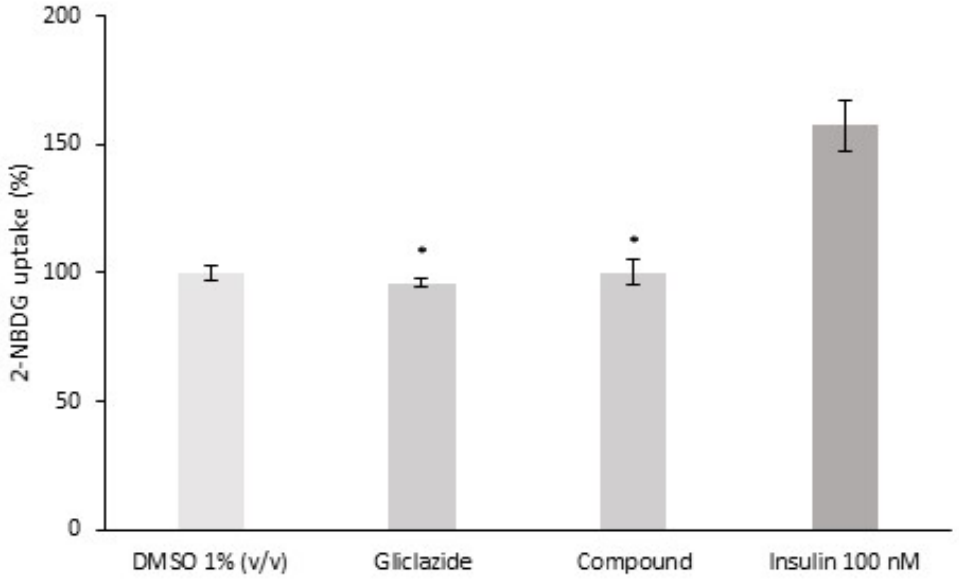

Figure S2. 2-NBDG uptake after incubation C2C12 cells with compound/gliclazide 60 µM. * vs Insulin 100 nM. $P<0.05$.

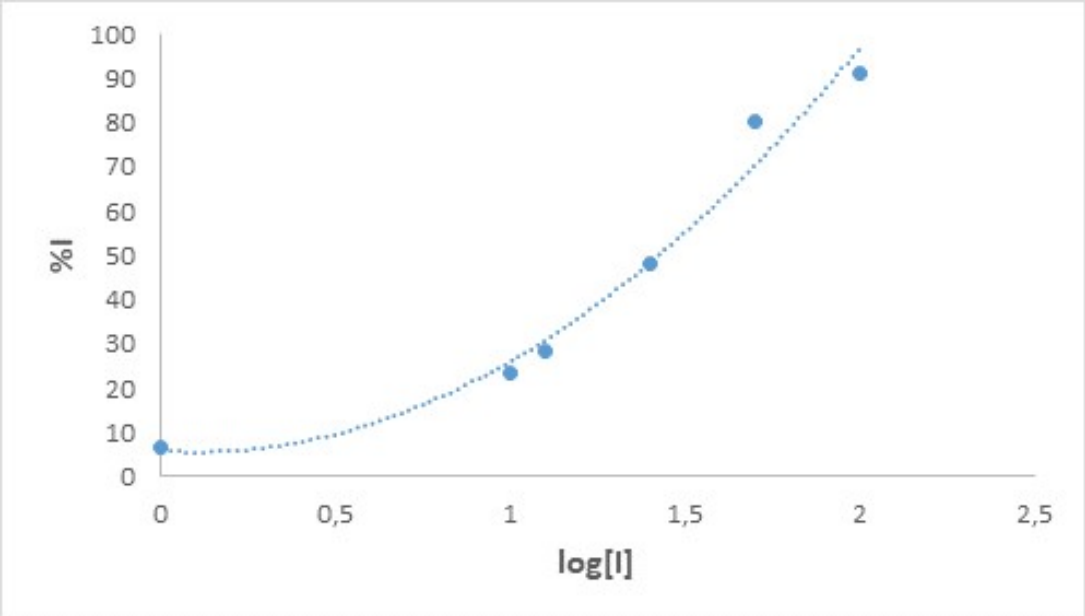

Figure S3. Plot for the IC_{50} calculation of $[Pd(glz)_2]$ against BuChE

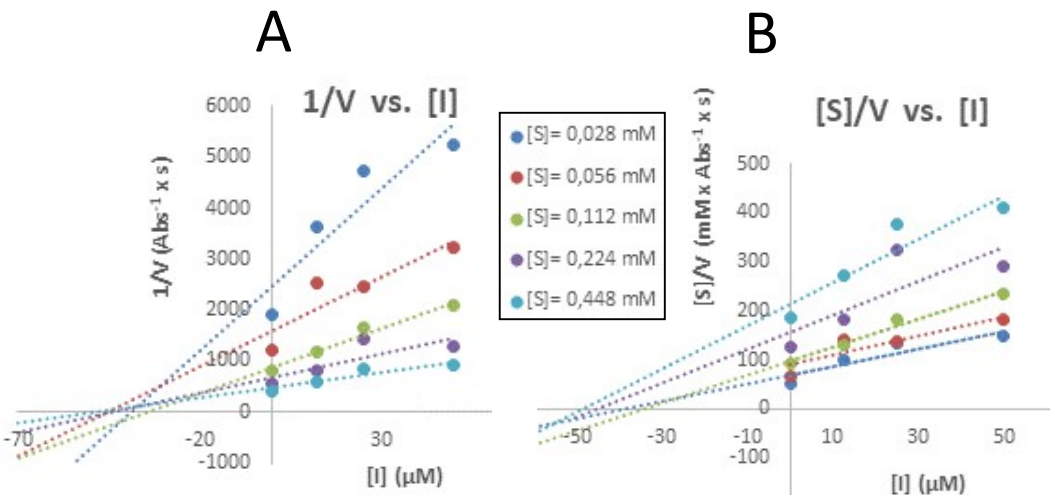

Figure S4. Cornish-Bowden plots for the calculation of mode of inhibition of [Pd(glz)2] complex, where V: reaction rate; [I]: Inhibitor concentration; [S]: Substrate concentration.

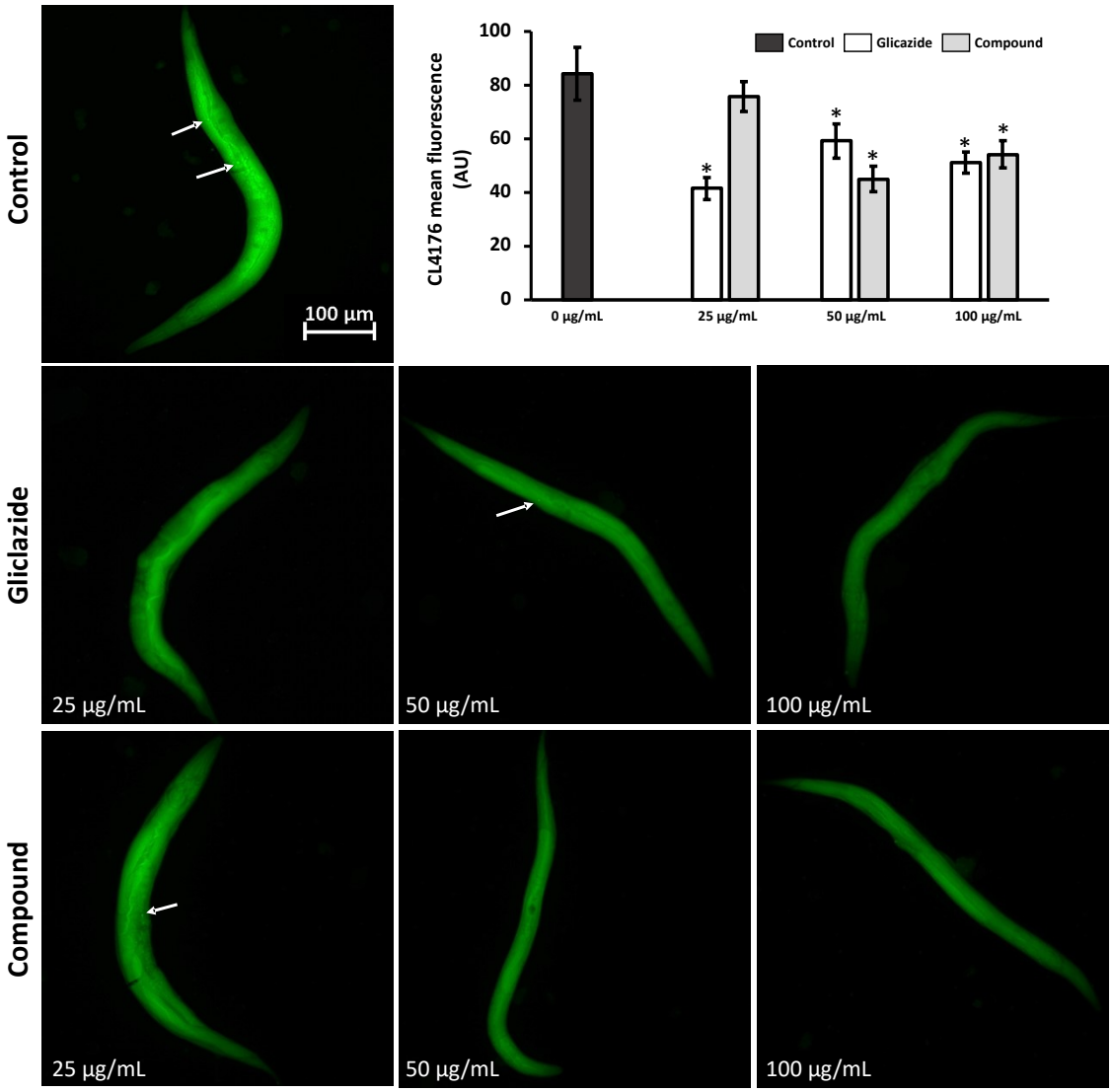

Figure S5. Effect of glz or [Pd(glz)$_2$] compound treatment on Aβ accumulation. Aβ accumulation was identified in transgenic *C. elegans* (CL4176) stained with thioflavin T. Arrows indicate representative Aβ aggregation deposits in untreated worms. Bar chart represents fluorescence intensity of representative worms from each group (n = 15). * Means statistical differences ($P<0.059$ with respect to 0 µg/mL control group.

14. ^{1}H NMR and ^{13}C NMR Studies

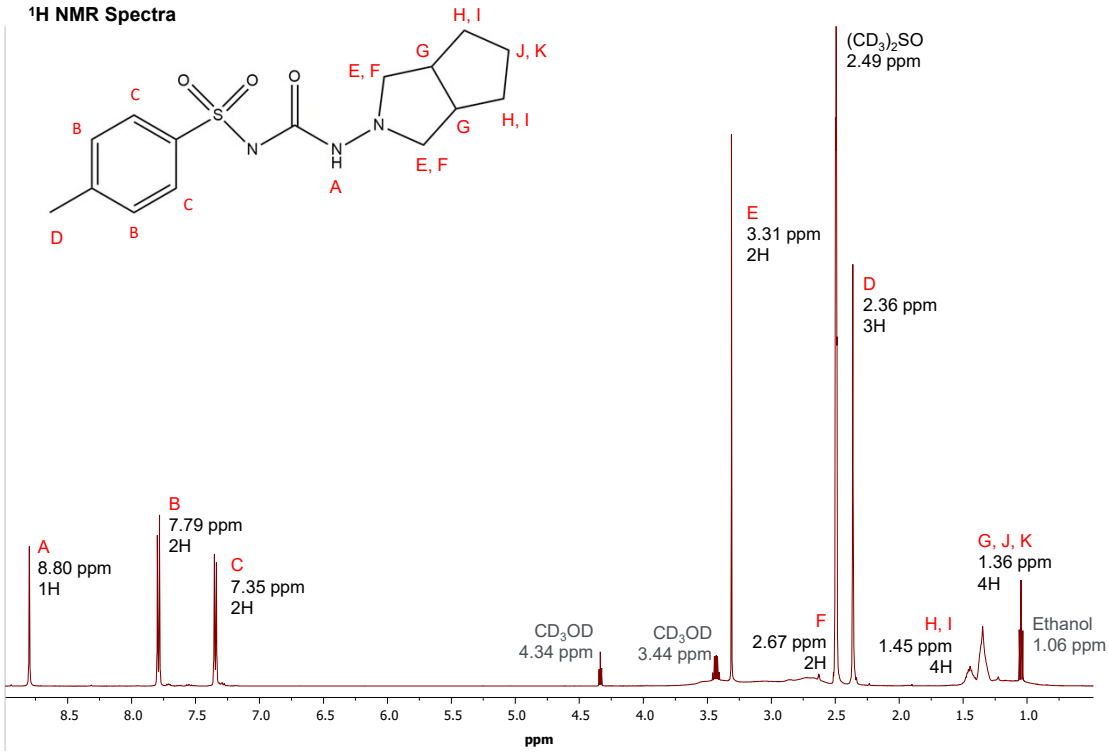

Figure S6. ^{1}H NMR Spectra of palladium compound.

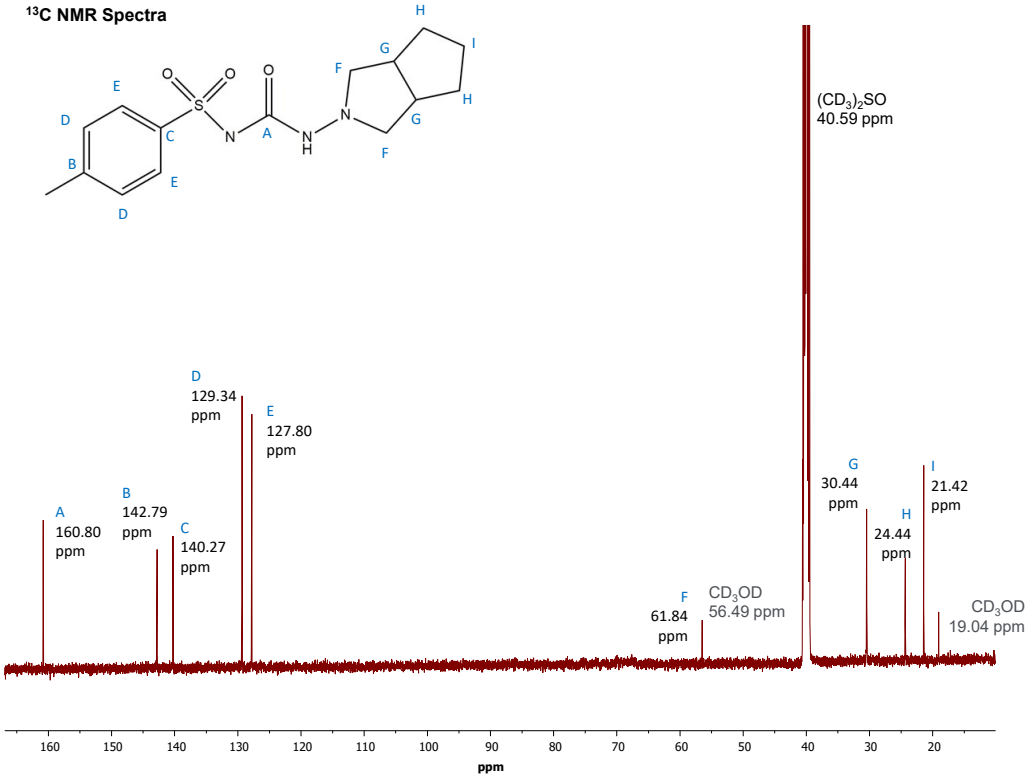

Figure S7. ^{13}C NMR Spectra of palladium compound.

15. Stability of this material

In order to confirm the potential of $[Pd(glz)_2]$ as protector against Aβ-induced toxicity in C. elegans, the stability of this material was monitored by quantifying the release of the glz constitutive organic moiety to the medium by UV-Vis spectroscopy. The results have been added to the ESI file.

Firstly, 5.3 mg of $[Pd(glz)_2]$ was suspended in 100 mL of phosphate buffered saline (PBS, pH = 7.2) to obtain an initial concentration of 70 μM. At different incubation times (0.5, 1, 3, 8.75, 24, 48, and 72 hours), 50 mL of supernatant was recovered by centrifugation (3,700 rpm, 10 min) and replaced with the same volume of PBS at 37°C (considering the poor solubility of $[Pd(glz)_2]$, always working under sink conditions). Studies were carried out in triplicate (n=3). After 72 h, less than 1% of degradation was observed for $[Pd(glz)_2]$. These results prove the large stability of the coordination compound and, therefore demonstrate that the good activity of $[Pd(glz)_2]$ against Aβ-toxicity in C. elegans is due to the formation of the coordination compound rather than the activity of free ligand.

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