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### **► To cite this version:**

Anna de Falco, Grasielle Kincheski, Elena Atrián-Blasco, Christelle Hureau, Sergio Ferreira, et al.. The aroylhydrazone INHHQ prevents memory impairment induced by Alzheimer's-linked amyloid- $\beta$  oligomers in mice. *Behavioural Pharmacology*, 2020, 31 (8), pp.738-747. <10.1097/FBP.0000000000000578>. <hal-03412868>

**HAL Id: hal-03412868**

**<https://hal.science/hal-03412868v1>**

Submitted on 15 Nov 2021

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**The aroylhydrazone INHHQ prevents memory impairment induced by  
Alzheimer's-linked A $\beta$  oligomers in mice**

INHHQ prevents A $\beta$ -induced memory impairment

(short title to be used as “running head”)

Anna DE FALCO<sup>a</sup>, Grasielle C. KINCHESKI<sup>b,c</sup>, Elena ATRIÁN-BLASCO<sup>d</sup>,

Christelle HUREAU<sup>d</sup>, Sergio T. FERREIRA<sup>b,c\*</sup>, Nicolás A. REY<sup>a\*</sup>

<sup>a</sup>Department of Chemistry, Pontifical Catholic University of Rio de Janeiro, Brazil;

<sup>b</sup>Institute of Medical Biochemistry Leopoldo de Meis, Federal University of Rio de Janeiro, Brazil;

<sup>c</sup>Institute of Biophysics Carlos Chagas Filho, Federal University of Rio de Janeiro, Brazil;

<sup>d</sup>LCC-CNRS, Université de Toulouse, CNRS, Toulouse, France.

\*Joint corresponding authors:

Nicolás A. Rey, Department of Chemistry, Pontifical Catholic University of Rio de Janeiro, Rio de Janeiro, RJ 22453-900, Brazil; nicoarey@puc-rio.br; or Sergio T. Ferreira, Institute of Biophysics Carlos Chagas Filho and Institute of Medical Biochemistry Leopoldo de Meis, Federal University of Rio de Janeiro, Rio de Janeiro, RJ 21944-590, Brazil; ferreira@bioqmed.ufrj.br.

**Author contributions**

*In vivo* experiments were designed by ADF, GCK, STF and NAR, conducted and analyzed by ADF and GCK. ROS generation experiments were designed by EAB and CH, conducted and analyzed by EAB. The manuscript was initially written by ADF, GCK, EAB and CH; and revised by STF and NAR.

**Notes**

The Pontifical Catholic University of Rio de Janeiro holds granted patents in the United States of America (US 10,189,811 B2 and US 10,316,019 B2) related to the compound INHHQ, under the unified title “Method for Preparing the Anhydrous Crystalline Form of Isoniazid-Derived Hydrazone, Thus Produced Crystalline Polymorph of the Anhydrous Form, Use Thereof for the Treatment of Alzheimer's Disease, Parkinsonism and Other Neurodegenerative Disorders, and Pharmaceutical Composition”, in which NAR is the main inventor.

Part of the results described in this manuscript was extracted from Anna De Falco's PhD thesis work, and the author has clearly authorized the use of the data for the purpose of publication.

## ABSTRACT

Converging evidence indicates that neurotoxicity and memory impairment in Alzheimer's disease (AD) are induced by brain accumulation of soluble amyloid- $\beta$  oligomers (A $\beta$ Os). Physiological metals are poorly distributed and concentrated in the senile plaques typical of AD, where they may be coordinated to the amyloid- $\beta$  peptide (A $\beta$ ). Indeed, zinc and copper increase A $\beta$  oligomerization and toxicity. Metal-Protein Attenuating Compounds (MPACs) represent a class of agents proposed for AD treatment, as they reduce abnormal interactions of metal ions with A $\beta$ , inhibit A $\beta$  oligomerization and prevent deleterious redox reactions in the brain. The present work investigates the protective action of an isoniazid-derived aroylhydrazone, INHHQ, on A $\beta$ O-induced memory impairment. Systemic administration of a single dose of INHHQ (1 mg kg<sup>-1</sup>) prevented both short- and long-term memory impairment caused by A $\beta$ Os in mice. *In vitro* studies showed that INHHQ prevents Cu(A $\beta$ )-catalyzed production of reactive oxygen species. Although the mechanism of protection by INHHQ is not yet fully understood at a molecular level, **the results reported herein certainly** point to the value of aroylhydrazones as promising neuroprotective agents in AD and related disorders.

## Keywords

Alzheimer's disease, A $\beta$  oligomers, memory impairment, hydrazones, MPACs, ROS.

## Abbreviations

A $\beta$ , amyloid- $\beta$  peptide; A $\beta$ Os, A $\beta$ <sub>1-42</sub> oligomers; HMW-A $\beta$ Os, high molecular weight A $\beta$ Os; INHHQ, 8-hydroxyquinoline-2-carboxaldehyde isonicotinoyl hydrazone; i.p., intraperitoneal(ly); LMW-A $\beta$ Os, low molecular weight A $\beta$ Os; LTM, long-term memory; MPAC, metal-protein attenuating compound; NOR, novel object recognition; OF, open field; ROS, reactive oxygen species; STM, short-term memory.

## INTRODUCTION

Alzheimer's disease (AD) is the most prevalent form of dementia in the elderly. Recent reports indicate that 35 million people are currently affected by AD, with a predicted increase to 50 million cases by 2030 (Alzheimer's Association, 2018). Presence of extracellular senile plaques formed by fibrillar aggregates of the amyloid- $\beta$  peptide ( $A\beta$ ) is a major pathological feature of AD (Mayeux and Stern, 2012). In addition to insoluble fibrils,  $A\beta$  forms soluble oligomers ranging from dimers to 24-mers and even larger species. Robust evidence accumulated in the past two decades supports the hypothesis that  $A\beta$  oligomers ( $A\beta$ Os) are largely responsible for synaptic toxicity and memory impairment in AD (Ferreira and Klein, 2011, Mucke and Selkoe, 2012, Ferreira et al., 2015, Selkoe and Hardy, 2016). Previous studies have shown that endogenous metal ions mediate  $A\beta$  oligomerization and potentiate its toxicity (Bush et al., 1994, Lovell et al., 1998, Miura et al., 2000). It has been proposed that deregulation of metal homeostasis in AD promotes  $A\beta$  aggregation and leads to the accumulation of redox-active biometals within or in the vicinity of neurons, producing reactive oxygen species (ROS) and causing oxidative damage and neurodegeneration (Bush, 2013).

Metal-Protein Attenuating Compounds (MPACs) could represent an interesting therapeutic approach to inhibit metal-induced  $A\beta$  oligomerization and to decrease metal-mediated toxicity (Scott and Orvig, 2009, Sampson et al., 2012, Savelieff et al., 2018). The current MPACs repertoire is mainly based on the well-known 8-hydroxyquinoline (8-HQ) scaffold, being Clioquinol (CQ) the first example proposed (Mancino et al., 2009). Unfortunately, different studies report a correlation between treatment with CQ and the occurrence of subacute myelo-optic neuropathy, which encouraged the search for less toxic analogues (Sampson et al., 2012). PBT2, a CQ derivative, demonstrated promising therapeutic potential for management of AD, decreasing both soluble and insoluble  $A\beta$  levels, and improving cognitive performance in a transgenic mouse model of the disease (Adlard et al., 2008). However, despite positive phase II trial results (Lannfelt et al., 2008), a recent 12-month double-blinded study reported a lack of significant difference between placebo and 250 mg day<sup>-1</sup> PBT2 treatment in biochemical and cognitive analyses (Villemagne et al., 2017).

Several other 8-HQ derivatives have been studied in recent years, showing chelating capacity towards biometals and inhibition of Cu<sup>2+</sup>/Zn<sup>2+</sup>-induced  $A\beta$  aggregation. Most of these agents presented low cytotoxicity and protective effects against oxidative stress (Gomes et al., 2014, Ryan et al., 2015, Yang et al., 2018). Multitargeted and hybrid compounds have also attracted attention regarding their potential suitability for the treatment of neurological disorders (Unzeta et al., 2016, Savelieff et al., 2018). Many of them, yet, still rely on the 8-HQ pharmacophore (Kupersmidt et al., 2012, Salkovic-Petrisic et al., 2015, Wang et al., 2015, Fu et al., 2016, Prati et al., 2016, Su et al., 2016).

Aroylhydrazones present both a recognized metal chelating ability and a broad spectrum of pharmacological applications (Savini et al., 2004, Silva et al., 2005, Vicini et al., 2006, Kajal et al., 2014). This prompted us to synthesize and characterize a hybrid compound combining the 8-HQ scaffold with an aroylhydrazone moiety, 8-hydroxyquinoline-2-carboxaldehyde isonicotinoyl hydrazone (INHHQ; Figure 1), as an MPAC for the treatment of AD (de Freitas et al., 2013). INHHQ blocks the interactions of  $Zn^{2+}$  and  $Cu^{2+}$  with A $\beta$  *in vitro* (Hauser-Davis et al., 2015). The compound crosses the blood-brain barrier, as suggested initially by *in silico* studies and proved subsequently by detection of INHHQ in the brains of intraperitoneally (i.p.) injected Wistar rats (Cukierman et al., 2017). Moreover, *in vivo* acute toxicity assays indicated that INHHQ was nontoxic up to 200 mg kg<sup>-1</sup> (Hauser-Davis et al., 2015, Cukierman et al., 2017). Herein, the ability of INHHQ to prevent memory impairment in an AD mouse model was investigated using the Novel Object Recognition (NOR) task, which represents, to the best of our knowledge, the first study of this type performed for an aroylhydrazone. Additionally, INHHQ's activity on Cu(A $\beta$ )-mediated ROS production was evaluated as one of the possible mechanisms underlying its protective action.

## MATERIALS AND METHODS

### Animals

Three-month-old male Swiss mice, weighing  $35 \pm 4$  g, were used. Animals were housed in groups of five per cage with *ad libitum* access to food and water under a 12 h light/dark cycle with controlled temperature and humidity. **Independent groups of animals were used for each behavioral test, unless otherwise specified.** All procedures were approved by the Institutional Animal Care and Use Committee of the Federal University of Rio de Janeiro (protocol 001/2019).

### INHHQ

INHHQ was synthesized as reported by De Freitas *et al.* (2013).

### Preparation of A $\beta$ Os

A $\beta$  oligomers consisting of a mixture of High Molecular Weight (HMW) and Low Molecular Weight (LMW) species were prepared weekly from synthetic A $\beta$ <sub>1-42</sub> (American Peptide; Sunnyvale, CA) as previously described (Lambert et al., 1998). All preparations were routinely characterized by size-exclusion chromatography as already reported (Jürgensen et al., 2011, Sebollela et al., 2012, Figueiredo et al., 2013), and, occasionally, by western immunoblots (De Felice et al., 2007, De Felice et al., 2008, Jürgensen et al., 2011, Sebollela et al., 2012). A $\beta$ Os were kept at the temperature of 4 °C and used within 48 h of preparation.

## Treatment with INHHQ

Mice were treated with INHHQ (or vehicle) *via* i.p. injection of 10 mL kg<sup>-1</sup> saline solution containing 10% DMSO (Sigma-Aldrich; Germany) and the desired doses of INHHQ.

## Intracerebroventricular injections

Intracerebroventricular (i.c.v.) infusion of A $\beta$ Os was performed as described (Figueiredo et al., 2013, Ledo et al., 2013, Lourenco et al., 2013, Ledo et al., 2016). Animals were anesthetized with 2.5% isoflurane (Cristalia; Brazil) using a vaporizer system and were gently restrained only during the injection (total time ~ 7 min). For administration of a final volume of 3  $\mu$ L vehicle or A $\beta$ Os (10 pmol A $\beta$ , expressed as monomers), a 2.5 mm length needle was inserted unilaterally 1 mm to the right of the midline point equidistant from both eyes and 1 mm posterior to a line drawn through the anterior base of the eyes, as previously reported (Laursen and Belknap, 1986, Figueiredo et al., 2013). Mice showing any signs of misplaced injections or brain hemorrhage were excluded from further analysis.

## Elevated plus-maze

The apparatus used consists of four wooden arms with black flooring arranged in a cross shape. Two arms are enclosed by black walls (30 x 5 x 15 cm) opposite to two open arms (30 x 5 cm), and the apparatus is set up 50 cm above the room floor. The test lasted 5 min and was performed in a room with fluorescent lighting, with intensity set at 44 lux. **Independent groups of animals were tested 1 or 4 h after administration of different doses of INHHQ.** The animals were placed on the central platform of the raised cross maze with the head facing one of the closed arms and the behavioral parameters recorded were: frequency of entry into the open arms and closed arms of the labyrinth, and length of permanence of the animals in the open arms and in the closed arms of the labyrinth. These data were used to calculate the percentage of open arms entries (OAE) and the percentage of time spent in open arms (OAT) using the following equations (Eq.1 and Eq.2):

$$\%OAE = \frac{\text{open entries}}{\text{Total entries}} \times 100, \quad (\text{Eq.1})$$

$$\%OAT = \frac{\text{open time}}{\text{Total time}} \times 100. \quad (\text{Eq.2})$$

General exploratory behavior was represented by the frequency of enclosed arm entries (EAE). After each test session, the maze was cleaned with a 10% solution of alcohol in water (Lister, 1990, Rodgers and Dalvi, 1997).

## Open field

The animals were placed at the center of an OF, where their spontaneous activity was recorded during 5 min. Tests were performed in independent groups of animals 1 or 4 h after administration of three different doses of INHHQ (namely, 1, 10 or 25 mg kg<sup>-1</sup>) or vehicle. The apparatus consists of a wooden arena with black floor (30 x 30 cm) and 40 cm high walls. The luminous intensity at the center of the OF was 50 lux. The total distance travelled and total time spent on a virtual center square (10 x 10 cm) were automatically quantified using Any-maze® video-tracking system (Stoelting Inc.; Kiel, WI). As before, the arena was cleaned with 10% ethanol between trials.

## Novel Object Recognition test

Mice were first habituated for 5 min in an open field arena as the one described in the previous section. The novel object recognition (NOR) test was performed by first allowing mice to freely explore the OF containing two identical objects for 5 min (training phase). The exploration times for each object were measured by an observer and expressed as the total exploration time, and percentage of exploration time for novel and familiar object were calculated.

Test sessions were performed 1, 4 or 24 h after administration of three different doses of INHHQ (1, 10 or 25 mg kg<sup>-1</sup>) or vehicle. For the sessions completed 1 and 24 h after INHHQ administration, the same set of animals was used. An independent group was employed in the NOR test performed 4 h after administration of INHHQ. Mice tested in the test sessions, animals were individually reintroduced in the same OF, and one of the objects presented during the training phase was replaced by a novel object. The exploration times for each object were measured by an observer and expressed both as the discrimination index and as percentages of the total exploration time. The discrimination index, or D index, is defined as the difference in exploration time between the novel and the familiar objects divided by the total time spent exploring the two objects in the discrimination phase (Eq. 3):

$$D \text{ index} = \frac{(\text{Novel object exploration time} - \text{Familiar object exploration time})}{\text{Total exploration time}} \quad (\text{Eq.3})$$

The arena was cleaned with 20% ethanol between trials in order to eliminate olfactory cues. All OF sessions were performed during the light phase of the cycle (7 am – 5 pm). Tests were performed, in a first stage, using four groups (N= 8-16 mice per group) of mice that received i.p. injections of saline containing 10% (V/V) DMSO with four different INHHQ doses, namely 0, 1, 10, and 25 mg kg<sup>-1</sup>. NOR was evaluated at the timepoints 1, 4 or 24 h after INHHQ injection. A similar experiment was performed using four additional groups (N= 9-10 mice per group) of mice that received an i.p. injection of saline containing 10% (V/V) DMSO with or without 1 mg kg<sup>-1</sup> INHHQ. After 1 hour, these mice received i.c.v.

infusions of A $\beta$ Os (10 pmol) or vehicle. NOR was assessed 24 or 48 h after A $\beta$ Os infusion, first allowing the animals to freely explore an OF containing two identical objects for 5 min, followed by the retention tests: 1 h (for evaluation of short-term memory, STM) or 24 h (for evaluation of long-term memory, LTM) after training, as described above. Seven days after the first test, a second training was performed, and the NOR task was repeated with different objects from those used in the first two phases of the experiment. In this case, the same mice were tested at the three different times.

#### Ascorbate consumption

Consumption of ascorbate, which mirrors the production of hydrogen peroxide (Alies et al., 2013), was determined by measuring absorbance at 265 nm ( $\epsilon = 14,500 \text{ M}^{-1} \text{ cm}^{-1}$ ) with a baseline correction at 800 nm as a function of time. A $\beta_{1-16}$  (aa sequence: DAEFRHDSGYEVHHQK,  $M = 1955.1 \text{ g mol}^{-1}$ , purchased from GeneCust – Dudelange, Luxembourg) was used as it constitutes a good model peptide for the redox activity of full-length A $\beta$  (Lu et al., 2010, Hureau, 2012, Conte-Daban et al., 2018). Conditions in the cuvette were as follows: [Cu(II)] = 9  $\mu\text{M}$ , [Zn(II)] = 0 or 10  $\mu\text{M}$ , [A $\beta_{1-16}$ ] = 0 or 10  $\mu\text{M}$ , [INHHQ] = 0, 10 or 50  $\mu\text{M}$ , [ascorbate] = 100  $\mu\text{M}$ , HEPES = 50 mM, pH 7.4. The solutions were stirred at 800 rpm and kept at 25 °C. The rate of ascorbate oxidation was calculated by considering the slope of the variation in ascorbate concentration during the first 60 s after INHHQ addition. Reference values in presence of Cu(A $\beta$ ) or Cu,Zn(A $\beta$ ) were measured at an equivalent ascorbate concentration ( $\sim 65 \mu\text{M}$ ), corresponding to  $A = 1.0$ .

#### Statistics

Mice that did not explore the objects used in the NOR test in either the training or test phases, animals showing any signs of sickness or stress, or, as already pointed out previously, mice showing misplaced i.c.v. injection or brain hemorrhage were excluded from further analysis. Additional details of mice exclusion are provided as Supplementary Digital Content.

All values are expressed as mean  $\pm$  SEM. Normal distribution of data was checked using the D'Agostino & Pearson *omnibus* normality test. All data were evaluated using Grubbs' outlier test. Data were analyzed by one- or two-way ANOVA followed by the Bonferroni *post hoc* test. Data from the NOR task were additionally analyzed using a one-sample *t*-test comparing the percentage of exploration of the novel object against the chance value of 50%, as previously described (Figueiredo et al., 2013, Ledo et al., 2013, Lourenco et al., 2013, Ledo et al., 2016, Lourenco et al., 2019). The significance level in all analyses was  $p < 0.05$ . All tests were performed using the GraphPad Software (version 5.00 for Windows).



## RESULTS

Statistical analyses showed that INHHQ doses up to 25 mg kg<sup>-1</sup> did not cause fear or anxiety as assessed by the elevated plus-maze test and by the open field test either 1 or 4 h after administration of the compound (Figure 2 a-h). There was a trend of decreased locomotor activity and increased percentage of time spent at the center of the arena 4 h after treatment with 25 mg kg<sup>-1</sup> INHHQ (Figure 2 g and h, respectively). However, the decrease in locomotor activity was statistically significant (Bonferroni's multiple comparison test with  $P=0.0101$ ) only when the 25 mg kg<sup>-1</sup> INHHQ group was compared to the other dose groups, 1 and 10 mg kg<sup>-1</sup> ( $t=2.914$ , and  $t=3.088$ , respectively). The difference was not statistically significant when the comparison was performed with the group treated only with vehicle ( $t=1.535$ ). The complete statistical analysis is provided as Supplementary Digital Content (Table SDC1).

Results from the NOR test (Figure 2 i-q) showed that INHHQ, at doses of 10 and 25 mg kg<sup>-1</sup>, but not at 1 mg kg<sup>-1</sup>, caused acute memory impairment detected when the test was done 1 hour after i.p. administration (Figure 2 i) [no INHHQ:  $t=3.582$ ; 1 mg kg<sup>-1</sup> INHHQ:  $t=3.229$ ; 10 mg kg<sup>-1</sup> INHHQ:  $t=0.1085$ ; 25 mg kg<sup>-1</sup> INHHQ:  $t=0.4865$ ]. Nonetheless, performance in the NOR test was normal 4 or 24 h after administration of up to 25 mg kg<sup>-1</sup> INHHQ (Figure 2 j and k), indicating that memory impairment induced by the highest drug doses was transient, as confirmed through the D index analysis (Figure 2 m and n). Although there was a trend of decrease in D index 1 h after treatment with 10 and 25 mg kg<sup>-1</sup> INHHQ doses (Figure 2 l) [ $F(3,33)=3.213$ ,  $P=0.0354$ ], this difference was not statistically significant when the comparison was made with the group treated only with vehicle [ $t=0.2737$  and  $t=0.2448$ , respectively]. A statistically significant difference was detected in total exploration time 4 h after INHHQ injection (Figure 2 p) (Bonferroni's multiple comparison test with  $P=0.0073$ ) only when the 25 mg kg<sup>-1</sup> INHHQ group was compared to the other dose-receiving groups, 1 and 10 mg kg<sup>-1</sup> ( $t=3.100$  and  $t=2.937$ , respectively). On the other hand, total exploration time showed no statistical differences amongst tested doses and the control group at 1, 4 or 24 h after INHHQ injection (Figure 2 o-q). The complete statistical analysis can be found in supplementary Tables SDC1 and SDC2.

Figure 3 illustrates the outcomes of the NOR test performed 24 h, 48 h or 7 days after i.c.v. infusion of A $\beta$ Os. Systemic treatment with a single dose of 1 mg kg<sup>-1</sup> INHHQ prevented both STM and LTM impairment induced by A $\beta$ Os, and memory protection was maintained for up to one week. Although in LTM analysis the differences between exploration times for novel and familiar objects did not yield statistically significant differences in the discrimination index (Figure 3 b) [ $F(1,35)$  interaction = 11.74,  $F(1,35)$  INHHQ factor = 3.161 and  $F(1,35)$  A $\beta$ Os factor = 3.659], in all NOR tests mice treated with INHHQ showed a clear preference for the novel object, expressed by percentages of exploration higher than the chance value of 50% (Figure 3 d-f) [24 h test:  $t(9)=6.301$ ,  $P=0.0001$ ; 48 h test:  $t(8)=2.882$ ,  $P=0.0181$ ; 7 days test:  $t(9)=8.428$ ,  $P<0.0001$ ]. Full statistical analysis results of these data are available

as Tables SDC2 and SDC3. A difference in exploration time was detected during the training phase in the NOR tests when 25 mg kg<sup>-1</sup> INHHQ were administrated 4 h before the assay (see Supplementary Digital Content, Tables SDC4 and SDC5, as well as Figure SDC1), indicating that this higher dose may affect animal behavior.

Additionally, we have verified that INHHQ possesses the *in vitro* ability to decrease ascorbate consumption induced by Cu(A $\beta$ ): in the presence of 1 equivalent of this compound, the rate of ascorbate consumption was reduced by ~ 30% (Figure 4 a and b). A further increase in INHHQ concentration up to 5 equivalents led to an even larger (~ 50%) reduction. The presence of Zn<sup>2+</sup> ions slightly increased Cu(A $\beta$ )-induced ROS production (Figure 4 a and c), but did not alter the effectiveness of INHHQ.

## DISCUSSION

In 2013, we implemented i.c.v. infusion of A $\beta$ <sub>1-42</sub> oligomers in Swiss mice as a model to directly investigate A $\beta$ Os' deleterious impact *in vivo* (Figueiredo *et al.*, 2013). This model has been shown to recapitulate key neuropathological features and memory deficits of Alzheimer's disease. In the present work, three different doses of the potentially neuroprotective hydrazone INHHQ, namely 1, 10 and 25 mg kg<sup>-1</sup>, which are well below the nontoxic dosage of 200 mg kg<sup>-1</sup> previously reported in rats (Hauser-Davis *et al.*, 2015, Cukierman *et al.*, 2017), were administered i.p. to some of the Swiss mice groups studied.

INHHQ doses of 10 or 25 mg kg<sup>-1</sup> induced transient memory impairment. This effect may be due to temporary brain dyshomeostasis of metals that play relevant roles in synaptic functions in response to the mild chelation therapy with INHHQ. We note, however, that memory performance returned to normal 4 hours after administration of the compound, suggesting self-equilibration of brain metal homeostasis. We further note that no lethality or sign of toxicity was observed at any INHHQ dose tested.

Based on the results obtained, we chose the safe working dose of 1 mg kg<sup>-1</sup> INHHQ to assess the protective effect of this compound against memory impairment induced by A $\beta$ Os. Thus, INHHQ was administered i.p. 1 h before (i.e., focusing on a preventative approach) the i.c.v. infusion of A $\beta$ Os. As previously reported (Figueiredo *et al.*, 2013, Ledo *et al.*, 2013, Lourenco *et al.*, 2013, Brito-Moreira *et al.*, 2017, Lourenco *et al.*, 2019), A $\beta$ O-infused mice presented memory impairment detectable as early as 24 h post-infusion of A $\beta$ Os and this deleterious effect persists for at least 7 days.

Given its good acute toxicity profile (Hauser-Davis *et al.*, 2015, Cukierman *et al.*, 2017), our results suggest that INHHQ holds potential to combat memory impairment in AD, warranting further *in vivo* investigations of its efficacy in additional AD models, *e.g.*, investigating its beneficial actions after onset of memory deficits. It is worth noting that Adlard *et al.* reported that two other MPACs, namely PBT2 and CQ, improved learning and memory in APP<sup>swe</sup>PS1 $\Delta$ E9 (APP/PS1) mice, a transgenic model

272 of familial AD (Adlard et al., 2008). Tests with PBT2 or CQ were performed with 30 or 10 mg kg<sup>-1</sup> per  
273 day doses administered during 11 or 35 days, respectively (Adlard et al., 2008). In more recent studies,  
274 Kupershmidt *et al.* (2012) reported that the multifunctional chelator M30 attenuated anxiety and memory  
275 deficits in APP/PS1 mice. M30 (1 or 5 mg kg<sup>-1</sup>) was administered by gavage four times a week beginning  
276 at 3 months of age and continued for 9 months (Kupershmidt et al., 2012). We found that treatment with  
277 a single dose of 1 mg kg<sup>-1</sup> INHHQ prevents both short- and long-term memory impairments in an  
278 experimental model of sporadic AD.

279 The efficacy of INHHQ in preventing memory impairment can further be compared to that of  
280 memantine, a well-known NMDA receptor blocker and the only drug introduced in clinical practice for  
281 AD treatment during the past two decades. We previously showed that administration of memantine (10  
282 mg kg<sup>-1</sup>) by gavage for 7 days prevents memory impairment induced by HMW-A $\beta$ O<sub>s</sub>, but not by LMW-  
283 A $\beta$ O<sub>s</sub> (Figueiredo et al., 2013). Our current findings demonstrate that a single i.p. administration of a 10-  
284 fold lower dose of INHHQ prevents memory impairment induced by A $\beta$ O preparations containing both  
285 HMW and LMW A $\beta$  oligomers. Therefore, the results presented herein suggest that INHHQ is much more  
286 effective than memantine in preventing memory impairment induced by AD-related A $\beta$  oligomers.

287 In order to investigate a possible, albeit probably not the only one, mechanism of protection by  
288 INHHQ, we evaluated the compound's activity on Cu(A $\beta$ )-mediated ROS production. Oxidative stress  
289 constitutes a hallmark of several amyloidogenic diseases, including AD, but its link to disease progression  
290 is only partly understood (Atrián-Blasco et al., 2018b, Cheignon et al., 2018). A $\beta$ O<sub>s</sub> have been shown to  
291 induce marked neuronal oxidative stress (De Felice et al., 2007, Decker et al., 2010, Saraiva et al., 2010).  
292 The production of reactive oxygen species (ROS) by Cu(A $\beta$ ) complexes, together with brain metal ion  
293 imbalance, may be one of the mechanisms underlying oxidative stress and neuroinflammation in AD.  
294 Thus, targeting Cu(A $\beta$ )-mediated ROS production appears as an important goal in metal-based therapy of  
295 AD. Investigation of the effect of INHHQ on Cu(A $\beta$ <sub>1-16</sub>)-induced ROS production was performed using  
296 a well-validated ascorbate consumption assay (Jiang et al., 2010, Cheignon et al., 2016, Atrián-Blasco et  
297 al., 2018a). The high affinity metal binding site of A $\beta$ <sub>1-42</sub> is entirely comprised between amino acid  
298 residues 1 and 16. Therefore, A $\beta$ <sub>1-16</sub> is frequently used as a model for the metal coordination properties  
299 and ROS generation ability of the full-length peptide (Conte-Daban et al., 2018). Ascorbate consumption  
300 mirrors the production of ROS: a faster consumption rate corresponds to an increased ROS generation  
301 (Alies et al., 2013). While the rate of ascorbate consumption is lower when copper is coordinated to A $\beta$   
302 in comparison to the free metal, contribution to ROS production by the Cu(A $\beta$ ) complex is not negligible  
303 (Atrián-Blasco et al., 2018a).

The results obtained agree with previous data which determined that up to 5 equivalents of INHHQ are needed to effectively compete with A $\beta$ <sub>1-40</sub> for binding of Cu<sup>2+</sup> (Hauser-Davis et al., 2015). Moreover, zinc is not an obstacle to the inhibition of ROS production by INHHQ, in contrast to previously reported metal chelators (Conte-Daban et al., 2016, Conte-Daban et al., 2018). This constitutes an important feature of INHHQ, considering the ubiquitous presence of zinc in the synaptic cleft (Atrián-Blasco et al., 2017).

Although the mechanisms of pathogenesis in AD are still incompletely known, a large body of evidence implicates A $\beta$  in cellular toxicity. The hypothesis that A $\beta$  neurotoxicity is explained by the neuronal impact of soluble A $\beta$  oligomers has gained considerable support in the past two decades (Ferreira and Klein, 2011, Cline et al., 2018). Converging evidence also supports a link between biometals, A $\beta$  oligomerization and toxicity. In this scenario, MPACs could be a promising avenue in the search for new anti-AD drug candidates. While other concurrent modes of action, such as those involving suppression of metal-mediated aggregation, as well as the inherent antioxidant potential of phenol-containing compounds cannot be ruled out, our results suggest that the mechanism of protection by INHHQ may involve a reduction in Cu(A $\beta$ )-induced ROS production, even in the presence of competing Zn<sup>2+</sup> ions.

## CONCLUSIONS

The “state-of-the-art” in Metal-Protein Attenuating Compounds research includes a variety of substances, with the most representative ones belonging to the 8-HQ derivatives type. Although the hybrid compound INHHQ contains the 8-HQ group as well, we have shown that the aroylhydrazone moiety is the actual metal-binding unit in it (Cukierman et al., 2018). Therefore, results presented here on the prevention of memory impairment induced by A $\beta$  oligomers in mice suggest that aroylhydrazones warrant attention as a potential new class of MPACs. In fact, INHHQ displays more potent activity than memantine in the animal model of sporadic AD we have studied. In pursuing the molecular basis behind this interesting activity, we demonstrated that INHHQ is capable of inhibiting the production of reactive oxygen species catalyzed by Cu(A $\beta$ ). The investigation of other potential INHHQ mechanisms of action is underway.

## Acknowledgements

This work was supported by National Institute for Translational Neuroscience/Brazil (to STF) and by the Brazilian agencies Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, to NAR and STF). ADF was recipient of a fellowship from CNPq. GCK was recipient of a post-doctoral fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). CH and EAB thank the European Research Council (ERC) projet StG-638712 (aLzINK) for financial support.

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Figure 1. Chemical structure of the aroylhydrazone INHHQ.

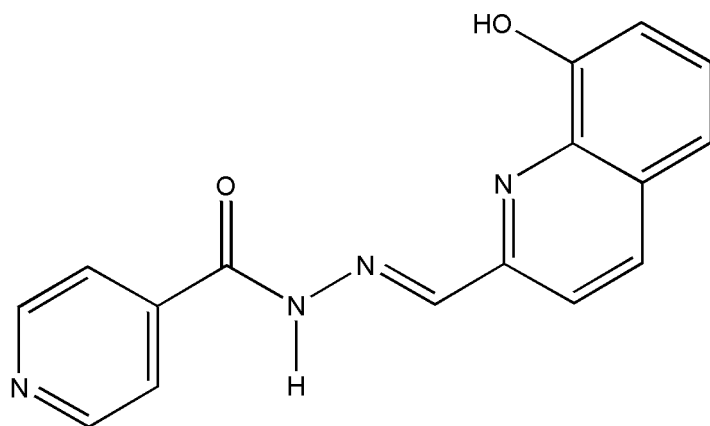
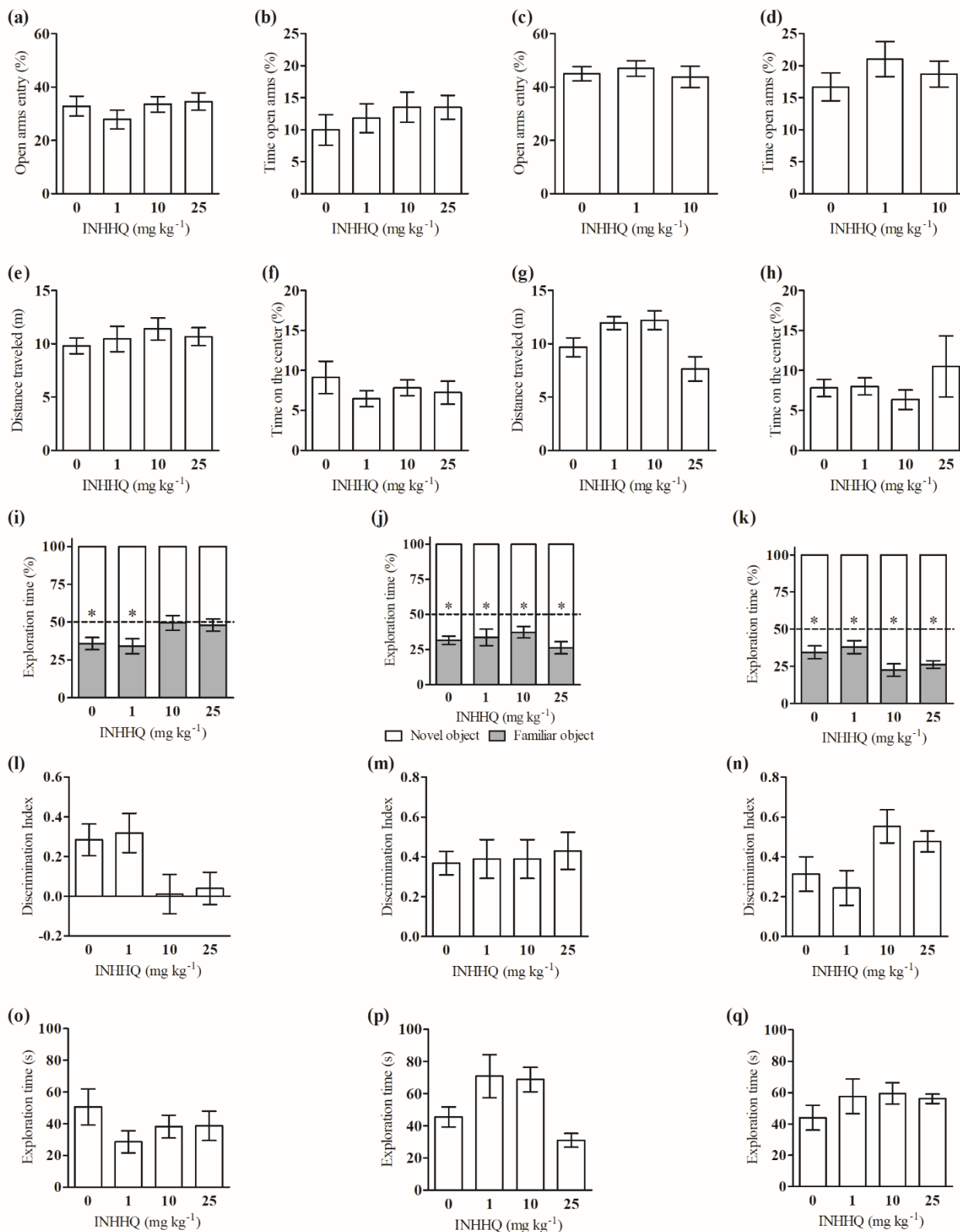


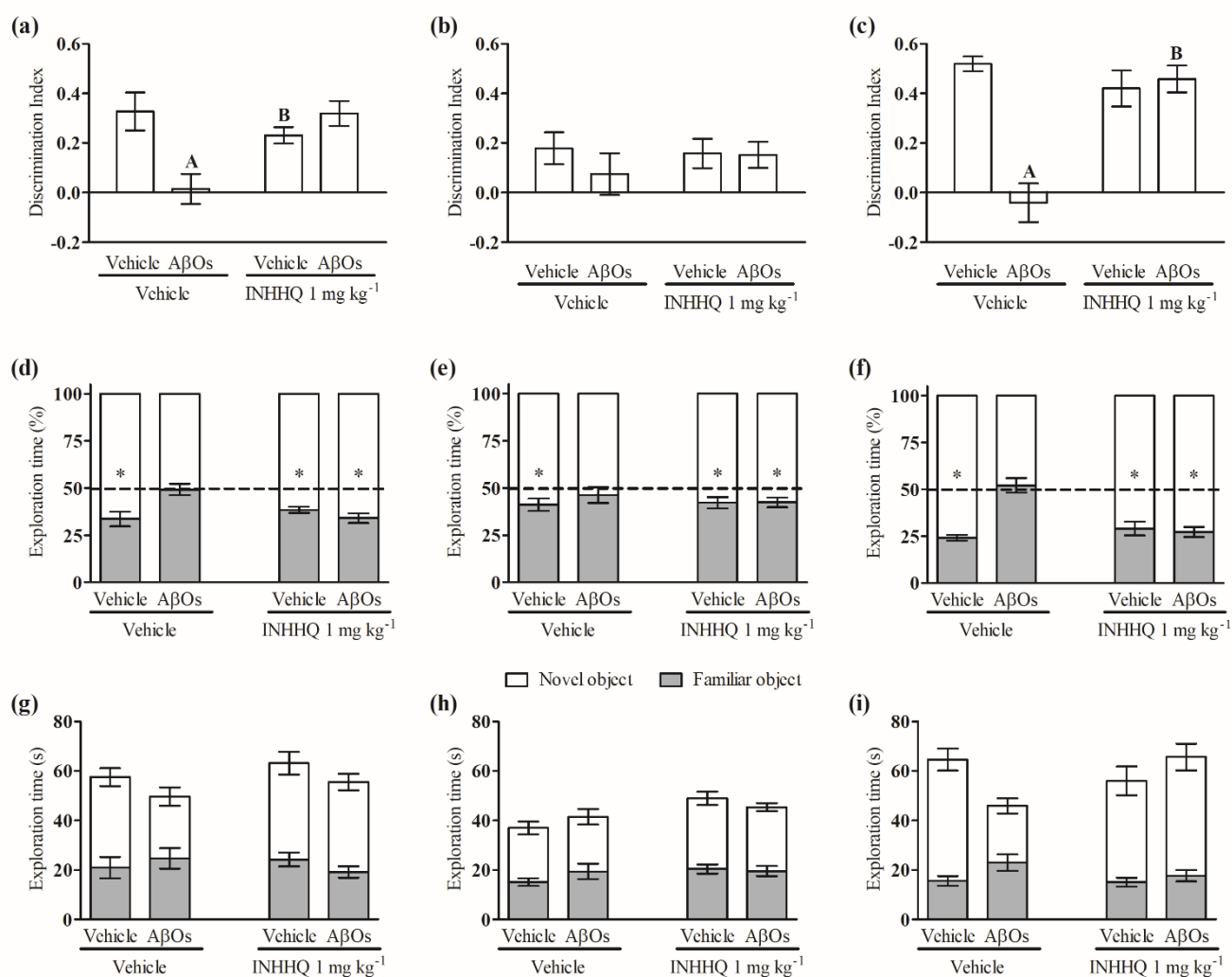
Figure 2. **INHHQ does not induce fear/anxiety behavior or affect locomotor activity or memory in mice.** Fear/anxiety behavior assessed in the elevated plus-maze (a-d). Testing was performed 1 h (a, b) or 4 h (c, d) after administration of INHHQ (i.p.) at the indicated doses. Bars represent means  $\pm$  SEM ( $N= 8-10$  mice per group). Locomotor activity (e, g) and anxiety behavior (f, h) assessed in the OF test. Testing was performed 1 h (e, f) or 4 h (g, h) after administration of INHHQ. Bars represent means  $\pm$  SEM ( $N= 8-16$  mice per group). Effect of INHHQ on novel object recognition (NOR) memory (i-q). INHHQ was administered i.p. at the indicated doses 1 h (i, l, and o), 4 h (j, m, and p) or 24 h (k, n, and q) before the NOR test. Data are reported as percentage of exploration time of familiar and novel objects (i-k), discrimination index (l-n), and total exploration time (o-q). White bars refer to novel object exploration, and gray bars refer to familiar object exploration, respectively, and correspond to means  $\pm$  SEM ( $N= 8-14$  mice per group). Dashed lines indicate chance value (50%). \* $p < 0.05$ , one-sample Student's  $t$ -test comparing to the fixed value of 50%.



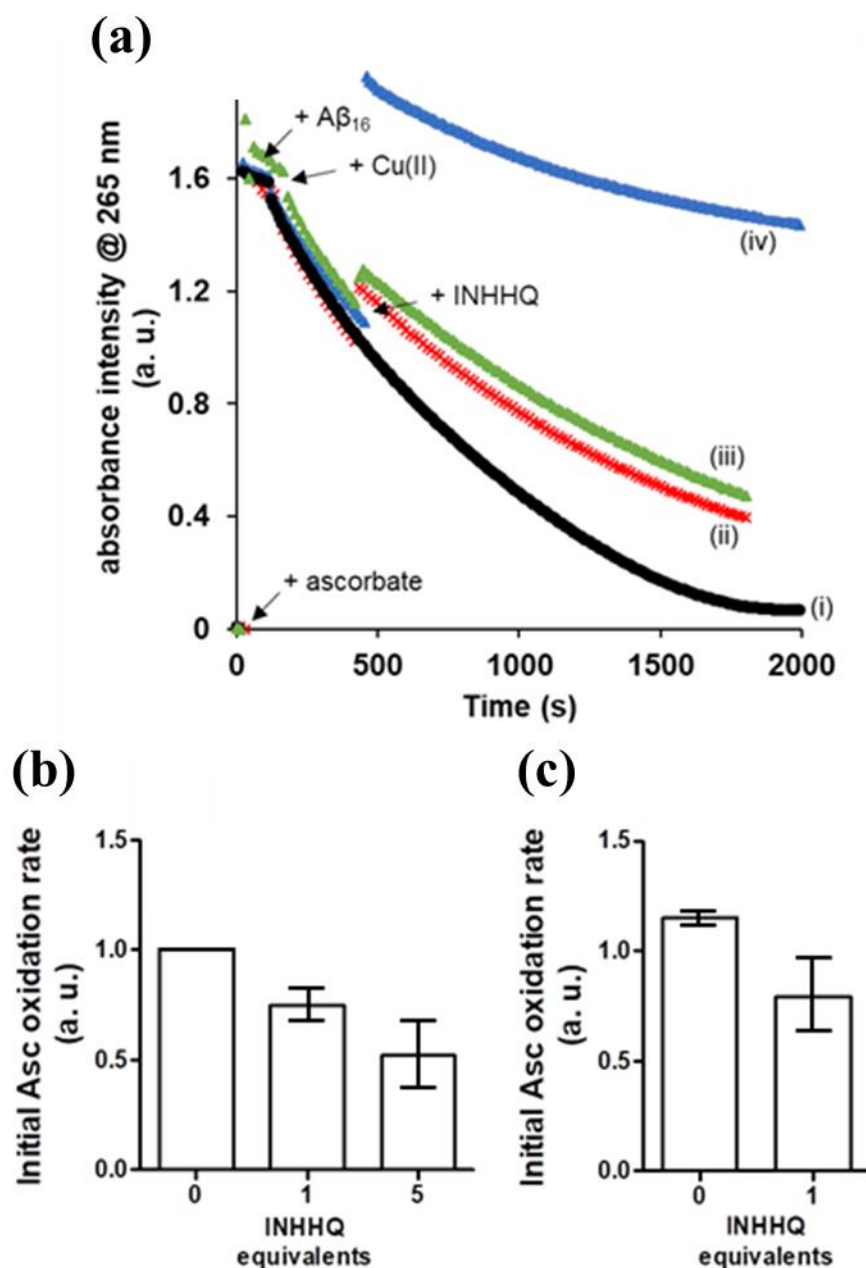
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546 Figure 3. INHHQ prevents memory deficit induced by A $\beta$ Os. A $\beta$ Os (10 pmol) were infused i.c.v. in mice 1 h after  
 547 i.p. treatment with INHHQ (1 mg kg<sup>-1</sup>). For assessment of STM, the retention test was performed 1 h after training,  
 548 whereas for assessment of LTM the retention test was performed 24 h after training. Tests for STM were performed  
 549 24 h (a, d, and g) or 7 days (c, f, and i) after infusion of A $\beta$ Os. Tests for LTM were performed 48 h after infusion  
 550 of A $\beta$ Os (b, e, and h). Data are reported as discrimination index (a-c), as percentage of exploration time of familiar  
 551 and novel objects (d-f) and as total exploration time (g-i). White bars refer to novel object exploration, and gray  
 552 bars refer to familiar object exploration, respectively, and correspond to means  $\pm$  SEM (N= 9-10 mice per group).  
 553 Dashed lines indicate the chance value (50%). **A**  $p < 0.05$ , two-way ANOVA followed by Bonferroni's *post-hoc*  
 554 *test* comparing with vehicle-vehicle group. **B**  $p < 0.05$ , two-way ANOVA followed by Bonferroni's *post-hoc*  
 555 *test* comparing with vehicle-A $\beta$ Os group. Results are from one representative experiment from a total of three  
 556 independent trials that yielded similar results. \* $p < 0.05$ , one-sample Student's *t*-test comparing to the fixed value  
 557 of 50%.



560 Figure 4. **Cu(A $\beta$ )-induced ascorbate consumption.** (a) Plots showing ascorbate consumption rate for: (i) Cu(A $\beta$ ),  
561 (ii) Cu(A $\beta$ ) + 1 equivalent INHHQ, (iii) Cu,Zn(A $\beta$ ) + 1 equivalent INHHQ, (iv) Cu(A $\beta$ ) + 5 equivalents INHHQ.  
562 The sharp increase observed after INHHQ addition is due to the intrinsic absorbance of the resulting Cu(INHHQ)  
563 complex. Rates of ascorbate consumption induced by Cu(A $\beta$ ) were measured in the presence of increasing INHHQ  
564 concentrations (b). (c) Ascorbate consumption induced by Cu,Zn:A $\beta$  with or without one equivalent of INHHQ.



565