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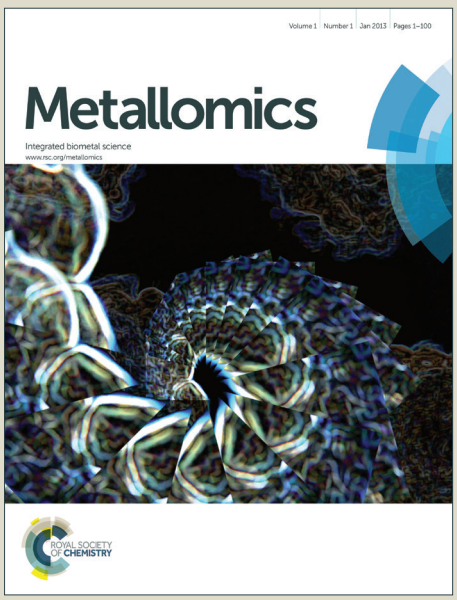

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Disruption Of Zinc And Copper Interactions With Aâ(1-40) By A Non-Toxic, Isoniazid-Derived, Hydrazone: A Novel Biometal Homeostasis Restoring Agent In Alzheimer's Disease Therapy?

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Disruptions of biometal-Aâ(1-40) interactions by an isoniazid-derived hydrazone, INHHQ, were demonstrated via *in vitro* NMR titrations. The compound has adequate theoretical BBB absorption properties, assessed by *in silico* studies. *In vivo* acute toxicity assays indicate that INHHQ is innocuous up to 300 mg kg^{-1}, showing potential as an anti-Alzheimer's drug.

Alzheimer's disease (AD) is currently the most frequent form of dementia. This disorder was estimated to affect as much as 24 million people in 2012, with frequency expected to double every 20 years until 2040[1]. In 2014, reports indicate that 5.2 million Americans of all ages present AD. Of these, 5 million are over the age of 65, accounting for 11% of all Americans in this age group[2]. Fibrillary deposits of the â-amyloid peptide (Aâ) are characteristic of the senile plaques typically present in AD patients[3, 4]. Elevated amounts of physiological metal ions such as Zn^{2+} and Cu^{2+} are present in these plaques, indicating that Aâ interactions with these biometals are key in the AD pathology. Iron has also been implicated as a contributor to the oxidative processes that occur in nerve cells[3, 5, 6]. Metal-protein attenuating compounds (MPACs) are an emerging class of therapeutic agents for the treatment of neurodegenerative disorders[7]. They compete with Aâ for binding with redox-active metal ions and zinc, preventing Aâ oligomerization[5, 8], as well as restoring metal homeostasis and decreasing oxidative stress. This strategy has been proposed as a way of slowing or even reversing AD progression. Recently, our research group reported a novel potential MPAC based on the anti-tuberculosis drug isoniazid, namely, 8-hydroxyquinoline-2-carboxaldehyde isonicotinoyl hydrazone (INHHQ, Figure 1)[9]. This compound has now been the target of a Brazilian (BR 10 2013 033006 0) and an international (PCT/BR2014/000186) patent application.

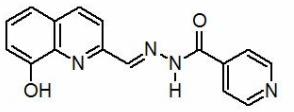

Figure 1. Chemical structure of INHHQ.

The present study describes the *in vitro* effects of INHHQ on biometal interactions with Aâ and reports pharmacological and pharmacokinetic characteristics assessed through *in silico* analyses. Evaluations of acute toxicity effects on a small group of healthy Wistar rats were also conducted.

For the NMR titration experiments, non-labeled and ^{15}N-isotopically enriched Aâ(1-40) samples were prepared using an alkaline dissolution protocol[10] (Supplementary Information). NMR spectra were acquired on a Bruker Avance II 600 MHz spectrometer using a triple-resonance probe equipped with z-axis self-shielded gradient coils. 1D ^{1}H-NMR experiments were acquired on 50 µM unlabeled Aâ(1-40) dissolved in TRIS buffer 20 mM, pH 7.4, at 5 °C. Heteronuclear $^{1}H–^{15}N$ HSQC NMR experiments were performed with pulsed-field gradient enhanced pulse sequences on 50 µM ^{15}N isotopically enriched peptide samples in TRIS buffer 20 mM, pH 7.4, at 5 °C (Figure S1, Supplementary Information). Amide cross-peaks affected during Zn^{2+} or Cu^{2+} titrations were identified by comparing their intensities (I) with those of the same cross-peaks in the data set of samples lacking the divalent metal ions (Io). For the mapping experiments, the I/Io ratios of well-resolved cross-peaks were plotted as a function of the peptide sequence to obtain the intensity profiles. Spectra acquisition, processing and visualization were performed by using the software packages TOPSPIN 2.0 (Bruker) and Sparky.

As previously reported,[11] the spectral changes observed upon addition of Zn^{2+} or Cu^{2+} to the Aâ samples were centered on residues Asp1, His6, His13, and His14, clearly indicating their involvement as metal coordinating moieties (Figures 2A,C and 2D,F for zinc and copper, respectively). The affected Aâ(1-40) resonances were severely broadened by the addition of substoichiometric concentrations of the divalent metal ions (0.2-0.5 equivalents), whereas further addition caused the Glu3, Phe4, Arg5, Asp7, Glu11, Val12, His13, Gln15 and Lys16 signals to be broadened beyond detection, indicative of a system undergoing intermediate exchange on the NMR chemical shift timescale. Interestingly, as shown in Figure 2B,C for zinc and 2E,F for copper, increasing amounts of INHHQ efficiently compete for Zn^{2+} and Cu^{2+} ion binding, completely removing the metal-induced perturbations in Aâ backbone amides upon addition of 5 equivalents of the compound. Moreover, the ^1H spectrum of the peptide is not modified in the presence of INHHQ (Figure S2, Supplementary Information), indicating that it does not interact directly with Aâ. This clearly demonstrates that INHHQ is able to disrupt zinc and copper interactions with the Aâ-peptide by a mechanism that most likely involves metal ion sequestering.

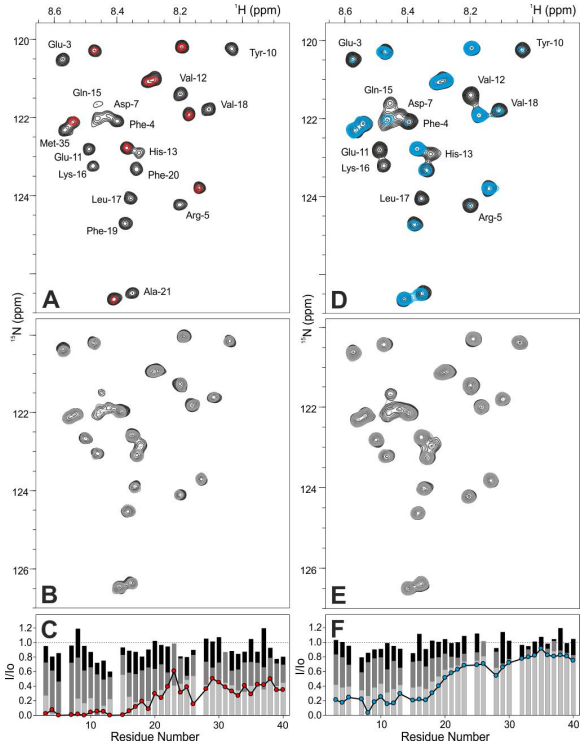

Figure 2. Effect of INHHQ on Aâ-Zn^{2+} (left) and Aâ-Cu^{2+} (right) complexes. (A) and (D): Overlaid contour plots of the ^1H–^{15}N HSQC spectra of Aâ (50 μM) in the absence (black) and presence of 1 eq. of Zn^{2+} (red) and Cu^{2+} (blue). The most affected residues are labelled. (B) and (E): Overlaid contour plots of the ^1H–^{15}N HSQC spectra of Aâ (50 μM) (black) and that recorded in the presence of 1 eq. of Zn^{2+} and Cu^{2+}, followed by the addition of 5 eq. of INHHQ (grey). (C) and (F): I/Io intensity profiles for the Aâ resonances in the presence of 1 equivalent of Zn^{2+} (red circles) and Cu^{2+} (blue circles) ions before and after the addition of 1 (grey bars), 3 (dark grey bars) and 5 (black bars) equivalents of INHHQ.

We conducted further control experiments using a strong metal chelator, EDTA, instead of INHHQ (Figure S3, Supplementary Information). Compared to the rather moderate binding features of the Aâ peptide to both Zn^{2+} and Cu^{2+} ions (K_d ~ 2 μM), the addition of only 1 equivalent of EDTA completely abolishe metal-Aâ interactions. Overall, these results demonstrate that the affinities of INHHQ or Aâ for the studied metal ions are comparable, consistent with INHHQ performing as a potential metal-protein attenuating compound.

Computational pharmacological analyses include, as a key process, the investigation of a compound's absorption by the organism. This was done by calculating certain parameters by a 1D-QSAR methodology and then applying the Lipinski rule of five[12]. pKa and log D calculations were performed using the Database for Pharmacokinetic Properties, USP. Log P, druglikeness, drug-score, theoretical solubility and toxicity calculations were conducted using the Osiris® Property Explorer software. The Wave Function Spartan 10 v. 1.1.0 software was used for the calculation of the surface electrostatic potential, and structural and QSAR analyses. The SMARTCyp v. 2.4.2 program was employed for cytochrome P450-mediated metabolism predictions. Table 1 compares INHHQ with four of the most representative MPACs to date: clioquinol (PBT1), PBT2, DFO, and deferiprone.

Table 1. Comparison of *in silico* parameters for INHHQ and other representative MPACs.

Parameters	INHHQ	Clioquinol	PBT2	DFO	Deferiprone
MW	292.0	305.0	243.9	560.7	111.1
HBD	2	1	1	5	2
HBA	6	2	3	14	3
PSA (Å^2)	66.077	24.910	48.919	183.34	46.107
Calcd. Log P	2.34	3.54	2.90	1.66	0.14
Calcd. Log S	3.36	3.78	3.04	2.23	1.05
Rotatable bonds	4	0	2	>10	0
Drug Score	68%	19%	81%	12%	97%

MW – molecular weight; HBD – H-bond donors; HBA – H-bond acceptors; PSA – polar surface area.

For INHHQ, all parameters are in accordance with ideal values. The theoretical pK_as indicate that, at physiological pH, INHHQ is in its neutral form, facilitating passive transport through biological membranes. Studies regarding blood-barrier drug transportation report that drugs targeted to the central nervous system should present a PSA of either less than 90 Å^2 [13] or lower than 60–70 Å^2 [14]. These results indicate that INHHQ shows structural properties that may allow it to reach the brain. Finally, based on all these parameters, the Drug Score of each compound, an estimate of the probability the substance has to become an actual drug, can be calculated. INHHQ displays a Drug Score of almost 70%.

Up to 90% of drug metabolism occurs through oxidation by the cytochrome P450 (CYP) superfamily of enzymes. The C16 carbon atom of INHHQ was calculated as the most reactive site concerning oxidation by the main CYP isoforms (Figure S4, Supplementary Information). On this basis, potential oxidation break-down products were proposed. Theoretical comparisons with over 3,000 commercial drugs and 15,000 chemicals indicate that all the predicted oxidation metabolism products are completely non-toxic, as is the INHHQ molecule itself.

To confirm the accuracy of the *in silico* toxicological analyses, and taking into account that the first step in clinical trials are safety evaluations of potential pharmacologically active compounds, an initial approach concerning pre-clinical *in vivo* analyses consisting in the investigation of acute toxicity effects on a small group of healthy male Wistar rats was performed. All experiments were approved by the ethics committee at PUC-Rio and collaborating universities (CEUA/036/2013) and conformed to the US Society of Neuroscience and Behavior Guidelines for Care and Use of Laboratory Animals[15]. The subjects were housed in polycarbonate cages measuring 18×31×38 cm, with food and water provided *ad libitum*. Room temperature was controlled (24 ± 1 °C) and the light-dark cycle was maintained at 12 h. All experiments took place during the light phase of the cycle. Two groups of male Wistar rats (approximately 200 days old, 250-350 g) were intraperitoneally injected with 200 and 300 mg kg^{-1} of INHHQ (n=8 and n=6, respectively), in a 10% DMSO/saline solution vehicle. The hydrazone was proven stable in this medium, with only 4% of the compound being hydrolyzed after 12 h, as verified by UV-Vis spectroscopy (Figure S5, Supplementary Information). The rats were monitored daily for signs of distress or toxicity such as the state of the fur and eyes, lethargy, diarrhea and tremor. Food and water intake were also observed. Subjects were sacrificed after 72 h. Two control groups for each concentration were used: one not injected at all (n=4) and one injected only with the vehicle (n=4). The aim of the 300 mg kg^{-1} injections was to verify possible subject mortality, while the 200 mg kg^{-1} injected group was used to further investigate reduced glutathione (GSH) and metallothionein (MT) levels, both oxidative stress indicators, and some physiological metals (i.e. Zn, Cu and Fe), all determined *post-mortem* in the brain. MT is also responsible for Zn and Cu homeostasis, thus being of further interest in the context of this study. GSH extraction was conducted, in triplicate, by homogenization of the brain samples in 0.1 M sodium phosphate buffer, pH 7.0, containing 0.25 M sucrose under inert atmosphere (nitrogen), followed by centrifugation at 13,500 rpm. Total reduced glutathione content was determined using GSH as external standard at 412 nm by UV-Vis spectrophotometry[16]. MT extraction, in triplicate, followed the protocol proposed by Erk and collaborators[17]. The resulting supernatants were centrifuged in Vivaspin 3 kDa MWCO concentrators for glutathione removal[18]. Concentrations were estimated using GSH as external standard at 412 nm by UV-Vis spectrophotometry[16] and MT content was estimated by assuming the relationship of 1 mol MT equaling 20 mols GSH. For the biometal determinations, tissue samples (approximately 0.1 g) were acid-digested with sub-distilled HNO_3 overnight. Digestion was completed by heating the samples on a heating block at 80 °C for approximately 4 h. The samples were then appropriately diluted and Zn, Cu and Fe concentrations were determined, in triplicate, using external calibration by appropriate dilutions of a mixed standard solution (Merck IV). Metal determinations were conducted on an ICP-MS (ELAN DRC II model, Perkin-Elmer Sciex, Norwalk, CT, USA) in standard mode, without the use of a reaction cell. The sample introduction system consisted of a Meinhard-type nebulizer with a twister cyclonic chamber. During the analysis, ^{103}Rh was used as internal standard at a concentration of 20 mg L^{-1}. The accuracy of the analytical procedure was verified with procedural blanks and by the parallel analysis of certified reference materials (DORM-2, dogfish muscle tissue, and DOLT-3, dogfish liver; National Research Council of Canada), in triplicate. A one-way ANOVA test was applied to verify possible statistical differences between metal, MT and GSH concentrations between the groups. A p value of less than 0.05 was considered as indication of statistical significance. The standard deviation of the intra-group variations of each parameter was combined with the inter-group variations of the same parameter, resulting in a combined standard deviation.

No mortality was observed during the 72 h assay period, not even in animals injected with 300 mg kg^{-1} (> 1000 µmol kg^{-1}) of INHHQ. No alterations in the monitored subject parameters were verified throughout the experiments and no macroscopical anomalies were observed in major organs (brain, liver, kidneys, heart) for the compound-injected group. Differences in MT levels were statistically non-significant between the non-injected and INHHQ-injected animals. The vehicle-injected subjects, on the other hand, showed a statistically significant increase in MT levels (Figure 3A). No significant differences between INHHQ-injected animals and both the vehicle- and non-injected rats were observed for brain GSH concentrations (Figure 3B). Conklin and co-workers reported similar results, increased MT mRNA levels following exposure of neonatal rat primary astrocyte cultures to DMSO[19]. After the INHHQ administration, however, MT concentrations dropped to almost exactly the same as the non-injected controls.

This indicates that INHHQ seems to aid in restoring normal MT levels in the brain after alterations induced by the presence of DMSO. As observed for GSH, biometal concentrations did not show statistically significant variations when comparing the non-injected, vehicle- and INHHQ-injected individuals (Figure 3B), demonstrating that INHHQ does not act as a traditional chelating agent, simply eliminating metals from the organism, but indeed performs as expected for an MPAC, redistributing and assisting in the restoration of brain biometal homeostasis.

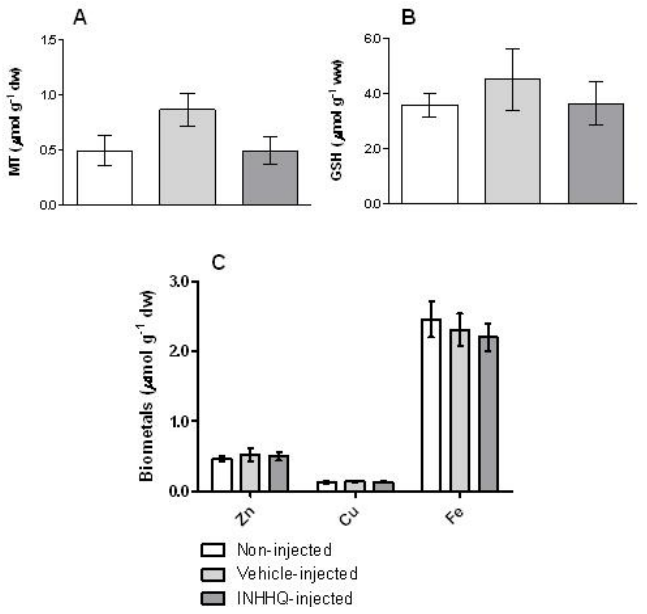

Figure 3. (A) Metallothionein (MT) and reduced glutathione (GSH). (B) Biometal (Zn, Cu and Fe) levels determined in the non-injected, vehicle-injected and INHHQ-injected rats. Results are displayed as μmol g^{-1}, wet weight (ww) for GSH and dry weight (dw) for MT and biometals. Error bars represent the combined standard deviations.

Conclusions

Even with all the effort dispended on research regarding AD, no effective treatment is currently available. Because of this, INHHQ is a promising compound, since it effectively inhibits Aâ interaction with biometals and is atoxic in concentrations up to 300 mg kg^{-1} in a mammal model organism, as predicted by the *in silico* pharmacological analyses. Furthermore, the *in vivo* studies demonstrate that INHHQ does not significantly affect the monitored biochemical parameters and biometal levels that could indicate brain redox and/or metal dyshomeostasis. Also, hydrazones are known as presenting anti-inflammatory activity, which could counteract the typical neuroinflammation present in the brains of AD patients[20]. In sum, the results obtained in this study suggest that this isoniazid-derived hydrazone is an excellent candidate for further trials. Additional comprehensive biochemical studies concerning other major organs (liver, kidneys, heart) in the model animals investigated herein are underway and will be the subject of future reports.

Finally, despite the recent study published by Beraldo and collaborators[21], proving the modulation of Cu-mediated Aâ(1-42) aggregation by an 8-hydroxyquinoline-derived hydrazone, INHHQ remains the first representative of this class to be proposed as a suitable MPAC[9]. Altogether, these studies open new investigation possibilities in the search for novel drugs to compose the chemical arsenal against Alzheimer's disease.

Notes and references

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LVF synthesized and characterized the target hydrazone; RAH-D, COF and NAR conceived and designed the experiments; RAH-D, DSC and MCM executed the experiments; WSC performed the *in silico* analyses; RAH-D, WSC, JLF, AAV-G, COF and NAR analyzed the data; RAH-D, COF and NAR wrote the paper.

Conflict of interest: The authors declare no conflict of interest.

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Electronic Supplementary Information (ESI) available: preparation of Aâ samples for NMR experiments; assignment of backbone amide Aβ(1-40) peptide resonances; 1D ^{1}H-NMR spectra of INHHQ and Aβ(1-40); effect of EDTA on Aâ-Zn^{2+} and Aâ-Cu^{2+} interactions; scheme indicating the INHHQ atoms most susceptible to oxidation by different isoforms of the CYP superfamily of enzymes, as calculated by the SMARTCyp V. 2.4.2 software package and the UV-Vis spectra proving the stability of INNHQ in a 10% DMSO/saline solution vehicle.

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