



Cite this: *Metallomics*, 2015, 7, 743

Received 3rd January 2015,
Accepted 1st April 2015

DOI: 10.1039/c5mt00003c

www.rsc.org/metallomics

Disruption of zinc and copper interactions with A β (1–40) by a non-toxic, isoniazid-derived, hydrazone: a novel biometal homeostasis restoring agent in Alzheimer's disease therapy?†

R. A. Hauser-Davis,^a L. V. de Freitas,^a D. S. Cukierman,^a W. S. Cruz,^a M. C. Miotto,^b J. Landeira-Fernandez,^c A. A. Valiente-Gabioud,^b C. O. Fernández^b and N. A. Rey^{*a}

Disruptions of biometal–A β (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 the frequency expected to double every 20 years until 2040.¹ In 2014, reports indicated 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 β -amyloid peptide (A β) are characteristic of the senile plaques typically present in AD patients.^{3,4} Elevated amounts of physiological metal ions such as Zn²⁺ and Cu²⁺ are present in these plaques, indicating that A β 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 A β for binding with redox-active metal ions and zinc, preventing A β 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, Fig. 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.

The present study describes the *in vitro* effects of INHHQ on biometal interactions with A β 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 ¹⁵N isotopically enriched A β (1–40) samples were prepared using an alkaline dissolution protocol¹⁰ (ESI†). 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 ¹H-NMR experiments were acquired on 50 μ M unlabeled A β (1–40) dissolved in TRIS buffer 20 mM, pH 7.4, at 5 °C. Heteronuclear ¹H–¹⁵N HSQC NMR experiments were performed with pulsed-field gradient enhanced pulse sequences on 50 μ M ¹⁵N isotopically enriched peptide samples in TRIS buffer 20 mM, pH 7.4, at 5 °C (Fig. S1, ESI†). Amide cross-peaks affected during Zn²⁺ or Cu²⁺ 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 (*I*₀). For the mapping experiments, the *I*/*I*₀ ratios of well-resolved cross-peaks were plotted as a function of the peptide sequence to obtain the intensity profiles. Spectra

^a Laboratory of Organic Synthesis and Coordination Chemistry Applied to Biological Systems (LABSO-BIO), Department of Chemistry, Pontifical Catholic University of Rio de Janeiro (PUC-Rio), Rua Marquês de São Vicente, 225, Gávea, 22453-900, Rio de Janeiro, RJ, Brazil. E-mail: nicoarey@puc-rio.br

^b Max Planck Laboratory for Structural Biology, Chemistry and Molecular Biophysics of Rosario (MPLBioR), Universidad Nacional de Rosario, 27 de Febrero 210 bis, S2002LRK Rosario, Argentina

^c Nucleus of Clinical and Experimental Neuropsychology, Department of Psychology, PUC-Rio, Rua Marquês de São Vicente, 225, Gávea, 22453-900, Rio de Janeiro, RJ, Brazil

† Electronic supplementary information (ESI) available: Preparation of A β samples for NMR experiments; assignment of backbone amide A β (1–40) peptide resonances; 1D ¹H-NMR spectra of INHHQ and A β (1–40); effect of EDTA on A β –Zn²⁺ and A β –Cu²⁺ interactions; scheme indicating the INHHQ atoms most susceptible to oxidation by different isoforms of the CYP superfamily of enzymes, as calculated using the SMARTCyp V. 2.4.2 software package and the UV-Vis spectra proving the stability of INHHQ in a 10% DMSO/saline solution vehicle. See DOI: 10.1039/c5mt00003c

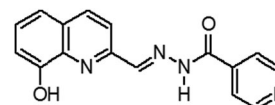


Fig. 1 Chemical structure of INHHQ.

acquisition, processing and visualization were performed 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 A β samples were centered on residues Asp1, His6, His13, and His14, clearly indicating their involvement as metal coordinating moieties (Fig. 2A and C for zinc, and Fig. 2D and F, for copper). The affected A β (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 Fig. 2B and C for zinc, and Fig. 2E and F, for copper, increasing amounts of INHHQ efficiently compete for Zn^{2+} and Cu^{2+} ion binding, completely removing the metal-induced perturbations in A β 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 (Fig. S2, ESI[†]), indicating that it does not interact directly with A β . This clearly demonstrates that INHHQ is able to disrupt zinc and copper interactions with the A β -peptide by a mechanism that most likely involves metal ion sequestering.

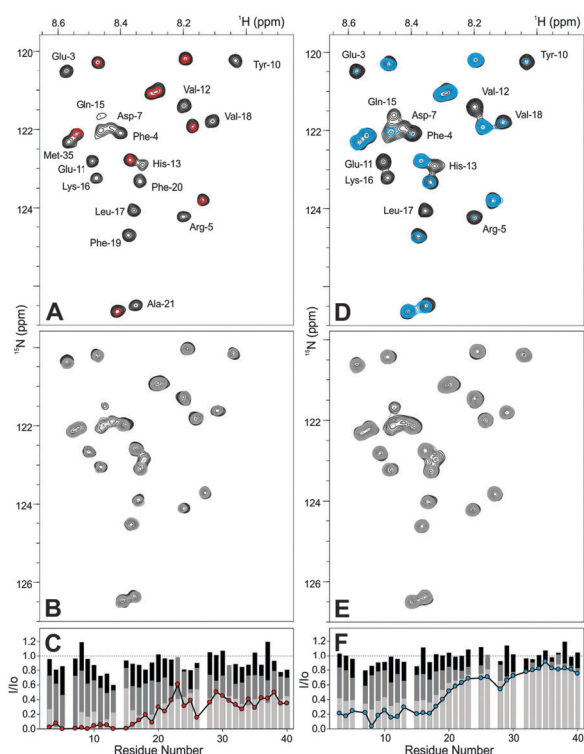


Fig. 2 Effect of INHHQ on A β - Zn^{2+} (left) and A β - Cu^{2+} (right) complexes. (A) and (D) Overlaid contour plots of the 1H - ^{15}N HSQC spectra of A β (50 μ M) in the absence (black) and presence of 1 eq. of Zn^{2+} (red) and Cu^{2+} (blue). The most affected residues are labelled. (B) and (E) Overlaid contour plots of the 1H - ^{15}N HSQC spectra of A β (50 μ M) (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/I_0 intensity profiles for the A β resonances in the presence of 1 equivalent of Zn^{2+} (red circles) and Cu^{2+} (blue circles) 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 (Fig. S3, ESI[†]). Compared to the rather moderate binding features of the A β peptide to both Zn^{2+} and Cu^{2+} ions ($K_d \sim 2 \mu$ M), the addition of only 1 equivalent of EDTA completely abolishes metal-A β interactions. Overall, these results demonstrate that the affinities of INHHQ or A β 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 pK_a and logD calculations were performed using the Database for Pharmacokinetic Properties, USP. LogP, 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.

For INHHQ, all parameters are in accordance with ideal values. The theoretical pK_a s 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 exhibit a PSA of either less than 90 \AA^2 ¹³ or lower than $60\text{--}70 \text{ \AA}^2$.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 (Fig. S4, ESI[†]). On this basis, potential oxidation break-down products were proposed. Theoretical comparisons with over 3000 commercial drugs and 15000 chemicals indicate that all the predicted oxidation metabolism products are completely non-toxic, as is the INHHQ molecule itself.

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

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 (\AA^2)	66.077	24.910	48.919	183.34	46.107
Calcd logP	2.34	3.54	2.90	-1.66	0.14
Calcd logS	-3.36	-3.78	-3.04	-2.23	-1.05
Rotatable bonds	4	0	2	>10	0
Drug score (%)	68	19	81	12	97

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

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*. The 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 proved to be stable in this medium, with only 4% of the compound being hydrolyzed after 12 h, as verified by UV-Vis spectroscopy (Fig. S5, ESI[†]). 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 an inert atmosphere (nitrogen), followed by centrifugation at 13 500 rpm. The total reduced glutathione content was determined using GSH as an 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 Vivaspın 3 kDa MWCO concentrators for glutathione removal.¹⁸ Concentrations were determined using GSH as an external standard at 412 nm by UV-Vis spectrophotometry¹⁶ and the MT content was estimated by assuming the relationship of 1 mol MT equaling 20 mol GSH. For the biometal determinations, tissue samples (approximately 0.1 g) were acid-digested with sub-distilled HNO₃ 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 adequate 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, ¹⁰³Rh was used as an 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 an 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 noninjected and INHHQ-injected animals. The vehicle-injected subjects, on the other hand, showed a statistically significant increase in MT levels (Fig. 3A). Conklin and co-workers reported similar results of 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. For brain GSH concentrations, no significant differences between INHHQ-injected animals and both the vehicle- and non-injected rats were observed (Fig. 3B).

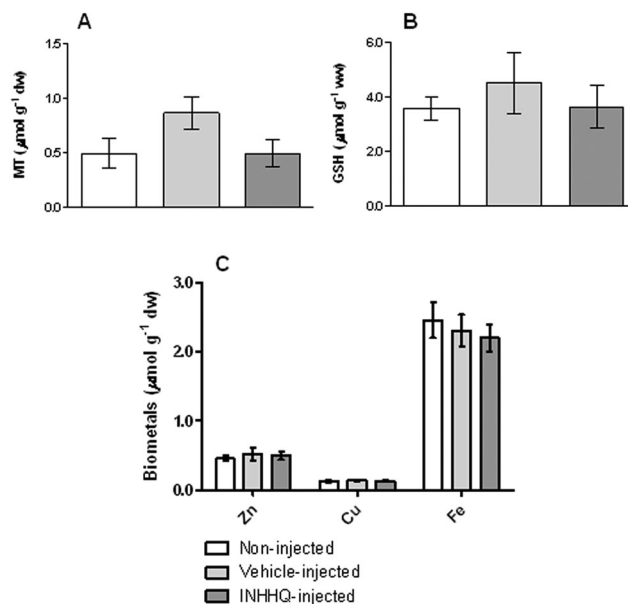


Fig. 3 (A) Metallothionein (MT), (B) reduced glutathione (GSH) and (C) biometal (Zn, Cu and Fe) levels determined in the non-injected, vehicle-injected and INHHQ-injected rats. Results are displayed as μmol g⁻¹, wet weight (ww) for GSH and dry weight (dw) for MT and biometals. Error bars represent the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

As observed for GSH, biometal concentrations did not show statistically significant variations when comparing the non-injected, vehicle- and INHHQ-injected individuals (Fig. 3C), 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.

Conclusions

Even with all the efforts dispensed on research regarding AD, no effective treatment is currently available. Because of this, INHHQ is a promising compound, since it effectively inhibits A β interaction with biometals and is atoxic at 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 to exhibit 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 A β (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.

Conflict of interest

The authors declare no conflict of interest.

Author contribution

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.

Acknowledgements

NAR wishes to thank CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil) for the research fellowship awarded. RAH-D is grateful to CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for the post-doctoral grant awarded. The authors would also like to thank Rafael C. Chávez Rocha for his invaluable help in the ICP-MS analyses, and Prof.

Tatiana Saint'Pierre for allowing the use of the LABSPECTRO facilities.

Notes and references

- 1 R. Mayeux and Y. Stern, *Epidemiology of Alzheimer Disease, Cold Spring Harbor Perspect. Med.*, 2012, **2**, a006239.
- 2 Alzheimer's. Association, 2014 Alzheimer's Disease Facts and Figures. *Alzheimer's & Dementia* 2014, **10**.
- 3 S. Chen, Y. Chen, Y. Li, S. Chen, J. Tan, T. Ou, L. Gu and Z. Huang, Design, synthesis, and biological evaluation of curcumin analogues as multifunctional agents for the treatment of Alzheimer's disease, *Bioorg. Med. Chem.*, 2011, **19**, 5596–5604.
- 4 A. M. Mancino, S. S. Hindo, A. Kochi and M. H. Lim, Effects of Clioquinol on Metal-Triggered Amyloid- β Aggregation Revisited, *Inorg. Chem.*, 2000, **48**, 9596–9598.
- 5 L. E. Scott and C. Orvig, Medicinal Inorganic Chemistry Approaches to Passivation and Removal of Aberrant Metal Ions in Disease, *Chem. Rev.*, 2009, **109**, 4885–4910.
- 6 A. Budimir, Metal ions, Alzheimer's disease and chelation therapy, *Acta Pharm.*, 2011, **61**, 1–14.
- 7 K. J. Barnham and A. I. Bush, Metals in Alzheimer's and Parkinson's diseases, *Curr. Opin. Chem. Biol.*, 2008, **12**, 222–228.
- 8 A. Gaeta, F. Molina-Holgado, X. L. Kong, S. Salvage, S. Fakhri, P. T. Francis, R. J. Williams and R. C. Hiderl, Synthesis, physical-chemical characterisation and biological evaluation of novel 2-amido-3-hydroxypyridin-4(1H)-ones: Iron chelators with the potential for treating Alzheimer's disease, *Bioorg. Med. Chem.*, 2011, **19**, 1285–1297.
- 9 L. V. de Freitas, C. C. da Silva, J. Ellena, L. A. Costa and N. A. Rey, Structural and vibrational study of 8-hydroxyquinoline-2-carboxaldehyde isonicotinoyl hydrazone—a potential metal-protein attenuating compound (MPAC) for the treatment of Alzheimer's disease, *Spectrochim. Acta, Part A*, 2013, **116**, 41–48.
- 10 L. M. Hou, H. Y. Shao, Y. B. Zhang, H. Li, N. K. Menon, E. B. Neuhaus, J. M. Brewer, I. J. L. Byeon, D. G. Ray, M. P. Vitek, T. Iwashita, R. A. Makula, A. B. Przybyla and M. G. Zagorski, Solution NMR studies of the A β (1–40) and A β (1–42) peptides establish that the met35 oxidation state affects the mechanism of amyloid formation, *J. Am. Chem. Soc.*, 2004, **126**, 1992–2005.
- 11 Y. Mekmouche, Y. Coppel, K. Hochgrafe, L. Guilloueu, C. Tallmard, H. Mazarguil and P. Faller, Characterization of the Zn- π binding to the peptide amyloid-beta(1–16) linked to Alzheimer's disease, *ChemBioChem*, 2005, **6**, 1663–1671.
- 12 C. A. Lipinski, F. Lombardo, B. W. Dominy and P. J. Feeney, Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings, *Adv. Drug Delivery Rev.*, 1997, **23**, 3–25.
- 13 H. van de Waterbeemd, G. Camenisch, G. Folkers, J. R. Chretien and O. A. Raevsky, Estimation of blood-brain barrier crossing of drugs using molecular size and shape, and H-bonding descriptors, *J. Drug Targeting*, 1998, **6**, 151–165.

- 14 J. Kelder, P. D. J. Grootenhuis, D. M. Bayada, L. P. C. Delbressine and J. P. Ploemen, Polar molecular surface as a dominating determinant for oral absorption and brain penetration of drugs, *Pharm. Res.*, 1999, **16**, 1514–1519.
- 15 L. L. Alvarez and H. G. Pardo, Guide for the care and use of laboratory animals – Natl-Res-Council, *Psicothema*, 1997, **9**, 232–234.
- 16 G. L. Ellman, Tissue Sulfhydryl Groups, *Arch. Biochem. Biophys.*, 1959, **82**, 70–77.
- 17 M. Erk, D. Ivankovi, B. Raspor and J. Pavicic, Evaluation of different purification procedures for the electrochemical quantification of mussel metallothioneins, *Talanta*, 2002, **57**, 1211–1218.
- 18 B. Belda-Palazón, M. A. Nohales, J. L. Rambla, J. L. Aceña, O. Delgado, S. Fustero, M. C. Martínez, A. Granell, J. Carbonell and A. Ferrando, Biochemical quantitation of the eIF5A hypusination in *Arabidopsis thaliana* uncovers ABA-dependent regulation, *Front. Plant Sci.*, 2014, **5**, 1–11.
- 19 D. R. Conklin, K. H. Tan and M. Aschner, Dimethyl sulfide, but not acidosis-induced metallothionein mRNA expression in neonatal rat primary astrocyte cultures is inhibited by the bioflavonoid, quercetin, *Brain Res.*, 1998, **794**, 304–308.
- 20 A. Kajal, S. Bala, N. Sharma, S. Kamboj and V. Saini, Therapeutic Potential of Hydrazones as Anti-Inflammatory Agents, *Int. J. Med. Chem.*, 2014, 1–11.
- 21 L. M. Gomes, R. P. Vieira, M. R. Jones, M. C. Wang, C. Dyrager, E. M. Souza-Fagundes, J. G. Da Silva, T. Storr and H. Beraldo, 8-Hydroxyquinoline Schiff-base compounds as antioxidants and modulators of copper-mediated A β peptide aggregation, *J. Inorg. Biochem.*, 2014, **139**, 106–116.