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Effects of Multifunctional Antioxidants on Mitochondrial Dysfunction and Amyloid-β Metal Dyshomeostasis

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Abstract.

Background: Redox-active metal dyshomeostasis and oxidative stress are associated with mitochondrial dysfunction and amyloid-β (Aβ) neurotoxicity that are linked to both the development of age-related macular degeneration (AMD) and Alzheimer's disease (AD). As potential therapeutic agents, orally active multifunctional antioxidants (MFAOs) possessing two independent functional groups capable of binding redox-active metals and scavenging free radicals have been synthesized.

Objective: To determine whether MFAOs affect mitochondrial function and reduce the presence of Aβ plaque formation.

Methods: The MFAOs were evaluated in cultured SH-SY5Y cells and ARPE-19 cells. MFAO effects on mitochondrial function were investigated using rhodamine 123 staining after 2 hour exposure to $MnCl_2$. MFAO effects on Aβ:Zn complex formation were evaluated with Zinquin staining and the ability of the Aβ:Zn complex to be degraded by matrix metalloproteinase-2 (MMP-2). The ability of MFAOs to reduce Aβ plaque in the brain was determined by orally feeding MFAO for one year to *B6*;*129-Psen1tm1Mpm Tg(AβPPSwe,tauP301L) 1Lfa/Mmjax* transgenic mice. Aβ levels were determined by ELISA.

Results: MFAOs neither adversely affected mitochondrial signaling nor labile cytoplasmic zinc levels. MFAOs protected cells against Mn^{2+}-induced mitochondrial dysfunction. MFAOs also removed zinc from the Aβ:Zn complex so that Aβ plaque could be degraded by MMP-2. Zinquin staining indicated that the removed zinc was present in the cytoplasm as labile zinc. Orally administered MFAOs reduced the brain levels of both $Aβ_{40}$ and $Aβ_{42}$ isoforms of Aβ.

Conclusion: These studies demonstrate that these MFAOs have metal attenuating properties with therapeutic potential in the treatment of both AMD and AD.

Keywords: Age-related macular degeneration, Alzheimer's disease, amyloid-β, clioquinol, JAX transgenic Alzheimer mouse, mitochondrial dysfunction, multifunctional antioxidants

INTRODUCTION

In our aging population, the development of Alzheimer's disease (AD) is becoming an increasing problem, with 115 million expected to develop AD by 2050 [1]. Currently, no treatment is available to prevent, significantly slow, or reverse this disease. To find a cure, research has recently focused on the amyloid hypothesis [2, 3] with target treatments designed to reduce the formation and/or presence of this protein. However, inhibitors of amyloid-β (Aβ) production, Aβ immunotherapy that reduces the levels of Aβ, and

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enhancers of Aβ clearance have all failed to show significant clinical efficacy [4]. This has led some investigators to question the appropriateness of Aβ-targeted therapy and seek other research directions [5].

An alternate avenue is targeting the role of oxidative stress and metal dyshomeostasis that is altered by the presence of Aβ. Aβ forms an insoluble complex with redox-active metals such as iron, copper, and especially zinc, which abnormally accumulate in the brain of AD patients [6, 7]. This Aβ:metal complex facilitates not only Aβ aggregation and increases protease-resistance, especially against matrix metalloprotease-2 (MMP2) [8], but also reactive oxygen species (ROS) generation [9, 10]. Aβ:metal complex formation also results in the lowered bioavailability of essential metals such as zinc. A number of metal chelators and antioxidants have been investigated for targeting metal dyshomeostasis and the increase of ROS in AD. Most of these drugs have failed to show *in vivo* efficacy; however, orally administered clioquinol (PBT1) has demonstrated the ability to not only decrease Aβ levels by nearly 50% without severe toxic effects but also to improve cognition in animal studies [11, 12] and pilot clinical studies [13, 14]. Because clioquinol does not reduce overall systemic metal concentrations [12, 15] but decreases abnormal metal accumulation in select cells or tissues by redistributing the metals so that they again become bioavailable [16], clioquinol is believed to possess metal attenuating rather than metal chelating activity. The clinical development of clioquinol, however, has been abandoned because of difficulties in removing a toxic intermediate formed during large scale synthesis required for clinical studies [6, 13]. As a result, this compound has been replaced by PBT2, a direct analog of clioquinol. PBT2 demonstrates good bioavailability and moderate affinity for redox-active metals [11, 12]. In a Phase IIa 12 week clinical trial, a 250 mg daily dose of PBT2 reduced Aβ levels in the cerebrospinal fluid while improving cognitive performance and being well tolerated [17, 18]. However, additional large scale clinical studies are required to validate the clinical results on cognition and memory in AD patients treated with PBT2 [19].

To target oxidative stress and metal dyshomeostasis, drugs possessing multiple functional groups with multiple mechanisms of action should have greater therapeutic potentials than drugs possessing only a single mechanism of action [20]. We have recently synthesized a new series of multifunctional antioxidants (MFAOs) possessing functional groups that are capable of independently binding redox-active metals and scavenging free radicals. They are orally active and readily cross both the blood-retinal barrier and blood-brain barrier [21]. This new series HK-2 and HK-4 were derived from an initial series of orally active MFAOs JHX-4 and JHX-8 [22] that readily crossed the blood-aqueous barrier and blood-retinal barrier to delay ROS generated diabetic and γ-irradiation induced cataracts [23] as well as light induced retinal degeneration in rats, which is an animal model for dry age-related macular degeneration (AMD) [24]. These MFAOs (HK-2, HK-4, JHX-4, and JHX-8) protect human retinal pigmented epithelial (RPE) cells and human neuroblastoma (NB) cells, which are therapeutic targets for age-related neurodegenerative diseases, in a dose-dependent manner against ROS from hydroxyl radicals and superoxide. These compounds also selectively bind redox active metal ions. Stoichiometry studies using Job plots show that these MFAOs bind Cu^{+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, and Mn^{2+} with a compound:metal ratio of 1:2; however, they do not bind Ca^{2+} or Mg^{2+}.

Since MFAOs can bind redox active metals ions, we have investigated whether these compounds can interfere with mitochondrial function and Aβ:Zn complex degradation. Here, we demonstrate that these MFAOs protect mitochondrial function monitored by rhodamine staining and remove zinc from the Aβ:Zn complex formation monitored by zinquin staining. In addition we demonstrate that orally active MFAO have the ability to reduce Aβ levels in a transgenic mouse model of AD.

MATERIAL AND METHODS

All reagents used were of reagent grade. Multifunctional antioxidants JHX-4, HK-2, HK-4, and their corresponding nonfunctional parent compounds JHX-1, HK-9, and HK-11 were synthesized as previously described [21, 22, 25]. Final compound purities were assessed as 99+% by reverse phase HPLC using a 250 mm × 4.6 mm C18 Luna column (5μ 100 Å) eluted isocratically by a mobile phase of 75% aqueous methanol at a flow rate of 0.9 mL/min and detection at 220, 254 and 280 nm. Clioquinol (5-chloro-8-hydroxy-7-iodoquinoline) was purchased from Tokyo Chemical Industry CO., Ltd. (Tokyo, Japan) and recrystallized from ether-hexanes. Zinquin and human recombinant matrix metalloproteinase-2 (MMP-2) were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Rhodamine 123 was obtained from EMD Bioscience, Inc. (La Jolla, CA, USA). Hoechst

33342 staining was purchased from Life Technologies Corporation (Grand Island, NY, USA). $A\beta_{1\text{-}42}$ was obtained from American Peptide Company, Inc. (Vista, CA, USA). Recombinant human recombinant metalloprotease-2 (MMP2) was obtained from Sigma Aldrich. Antibodies used were: β-Amyloid (D54D2) XP® Rabbit mAb (Cell Signaling Technology, Danvers, MA, USA) - Diluted 1:1000; Phototope®-HRP Western Blot Detection System containing Anti-rabbit IgG and HRP-linked Antibody (Cell Signaling Technology) - Diluted 1:2000. Chemiluminescent reagents and pre-stained and biotinylated protein ladders were obtained from Cell Signaling Technology. 4–15% Tris-HCl Ready Gel Precast gels and Trans-Blot® PVDF membrane were obtained from Bio-Rad Laboratories (Hercules, CA, USA). X-ray film was purchased from Bio Express (Kaysville, UT, USA). The BCA™ Protein Assay Kit was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). The $A\beta_{40}$ and $A\beta_{42}$ ELISA kits were purchased from Life Technologies (Carlsbad, CA, USA).

Cell culture studies

SH-SY5Y human neuroblastoma cells (American Type Culture Collection (ATCC), Manassas, VA, USA, NB cells) and ARPE-19 human retinal pigmented epithelial cells (ATCC) (RPE cells) were cultured in cell media as follows: NB cells were cultured in mixed media of 50% of Eagles minimum essential medium with Earle's balanced salt solution (Life Technologies Corporation) and 50% of Ham's F12 (Life Technologies Corporation) (EMEM-F12) containing 10% fetal bovine serum (FBS) and 1% penicillin streptomycin solution at 37°C under a 5% CO_2 atmosphere; RPE cells were incubated in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies Corporation) containing 4% FBS and 1% penicillin streptomycin solution at 37°C under a 5% CO_2 atmosphere. The cells were passaged when 80–90% confluent by treatment with trypsin-EDTA and then plated at a density of 1×10^4 cells onto 96-well plates and 8-well microscope plates for both cell lines, and at a density of 1×10^6 cells onto 150 mm dishes for RPE cells or 60 mm dishes for NB cells.

Mitochondrial viability/toxicity

NB and RPE cells were plated at a density of 1×10^4 cells onto 8-well microscope plates (BD Falcon, 800 μL slides). Once the cells were approximately 60% confluent in each well, the media was removed and the cells were washed with PBS. The cells were then pre-incubated with 1 mM of either MFAOs JHX-4, HK-2, and HK-4, or their corresponding non-functional parent compounds JHX-1, HK-9, and HK-11 dissolved in Hank's Balanced Salt Solution (HBSS) medium (Mediatech, Inc., Manassas, VA) (200 μL) containing 0.4% DMSO without FBS for 1 h at 37°C. Then, $MnCl_2$ was added to the appropriate cell groups to give a final concentration of 1 mM and the groups were cultured for an additional 2 h. After a 2-h exposure to manganese, the media was removed and the cells were washed with PBS. The cells were then stained with 100 μL of the fluorescent Rhodamine 123 dye [26] (20 μM) and counterstained with the nuclear Hoechst 33342 staining (8 μM) per well at 37°C for 30 min [27]. After removal of the media and washing with the HBSS medium 3 times for 5 min, the wells were washed with sterilized HPLC grade water [26]. Finally, the plate shelf was removed, 10 μL of HBSS medium containing FBS (10% for NB cells, 4% for RPE cells) was added to each well and the wells were covered with a rectangular cover glass. The gap between the cover glass and slide glass was sealed with nail polish and the cells were then immediately examined with a Zeiss 510 Meta Confocal Laser Scanning Microscope (LSM) (Carl Zeiss Microscopy, LLC, Thornwood, NY). The combination of a 488 nm Argon laser and 505 long pass emission filters were used to visualize Rhodamine 123 fluorescence [28] while Hoechst 33342 was excited with a 405 nm diode laser and the emission was collected with a BP420-480-nm filter. The studies were repeated 2–4 times.

Labile intracellular zinc level

The ability of MFAOs to release zinc from Aβ:Zn complex was examined as follows: One milligram of synthetic $A\beta_{1\text{-}42}$ was dissolved in 1 mL of 1,1,1,3,3,3-hexafluoro-2-propanol and the solution was dried by overnight vacuum centrifugation and stored at −20°C as previously described [29, 30]. For the preparation of the Aβ:Zn complex the dried $A\beta_{1\text{-}42}$ was dissolved in 20 mM NaOH and PBS to give a 400 μM solution. The 200 μM Aβ:Zn complex was prepared by mixing 10 μL of 400 μM $ZnSO_4$ solution with 10 μL of the 400 μM $A\beta_{1\text{-}42}$ solution. After incubating the mixture at 37°C for 48 h, the Aβ:Zn mixture was centrifuged at 14000× *g* for 3 min to pellet the aggregated Aβ:Zn complex. The pellet was then resuspended in 20 μL of water or 20 μL of 200 μM of compounds. The complex was diluted with HBSS medium to give a final 10 μM concentration for each component. This complex was

then used in the following studies: NB and RPE cells were seeded into 8-well microscope plates at a density of 1×10^4 cells. Once 60% confluent, the cells were pre-incubated with 200 μL of HBSS medium without FBS containing 10 μM of the MFAOs JHX-4, HK-2 or HK-4, their nonfunctional parent compounds JHX-1, HK-9 or HK-11, or clioquinol pre-dissolved in DMSO (final conc. of DMSO, 0.2%) for 1 h at 37°C. After the 1 h exposure to each drug, the media was exchanged with 200 μL of similar HBSS media containing either 10 μM of Aβ:Zn:drug (1:1:1) or, for the control groups, similar HBSS media containing Aβ:Zn, Zn:drug, Aβ:drug, or Aβ. The cells were then further cultured for 1 h at 37°C under a 5% CO_2 atmosphere. After removal of the media and washing the cells with PBS, the cells were stained with 100 μL of the fluorescent zinquin dye (10 μM) in HBSS medium per well as previously described [8, 31]. After 30 min culture at 37°C, the zinquin media was removed and replaced with PBS containing 4% paraformaldehyde. The zinquin fluorescence [8, 31] was visualized in the fixed cells with a Zeiss 510 Meta Confocal LSM using the combination of a 405 nm diode laser and BP420–480-nm emission filter. Image analysis (fluorescent intensity) was achieved using the commercial Carl Zeiss ZEN lite software (2011 Ver.1.0.0.0.). The studies were repeated 3–4 times.

MMP2 proteolysis assays of Aβ:Zn complexes in western blot analysis

The ability of MFAOs to modify the degradation of the Aβ:Zn complex was examined using recombinant human MMP-2. Ten μM of Aβ, Aβ:Zn, or Aβ:Zn:drug (HK-2, HK-4, HK-9, HK-11, or clioquinol) were dissolved in 2.2 μL of HPLC grade water containing 0.4% DMSO and mixed with 1.1 μL of MMP-2 (25 ng) dissolved in 6.7 μL of TCNB buffer composed of 150 mM NaCl, 50 mM Tris, 10 mM $CaCl_2$, 0.05% Brij-35™, pH 7.5 [29] which was pre-warmed at 37°C. Each reaction mixture was incubated at 37°C for 1 h and the reactions were terminated as previously described [29]. Two μL of each reaction mixture was then added to 35.5 μL of Milli-Q water and 12.5 μL of 4x denaturating buffer (100 mM Tris, 10% glycerol, 4% SDS, 4% β-mercaptoethanol and 0.01% bromophenol blue). The samples were then denatured at 98°C in an Eppendorf Mastercycler (Eppendorf N.A., Hauppauge, NY, USA). After 5 min, the samples were cooled to 4°C and loaded into 10-well (50 μL) 4–15% Tris-HCl Ready Gel Precast gels in with freshly prepared SDS-PAGE running buffer (14.4 grams of glycine, 3.03 grams of Tris, and 1 gram of SDS diluted to 1 liter) in a Mini-PROTEAN® Tetra cell vertical mini gel electrophoresis system (Bio-Rad, Hercules, CA, USA). After electrophoresis in a cold room at 90V, each gel was placed into freshly prepared transfer buffer (3.025 grams of Tris, 14.42 grams of glycine, 200 mL of methanol, and 800 mL of distilled water) for 30 min and then the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane using the Mini Trans-blot® system set at 90V for 90 min in a cold room. Following transfer, the membranes were blocked at room temperature with 5% nonfat dry milk in tris-buffered saline containing 0.1% Tween-20 (TBST). After 1 h each membrane was washed 3x for 5 min with TBST and then incubated overnight at 4°C with x1000 diluted primary Aβ XP Rabbit antibody. The membranes were again washed 3x with TBST and then incubated for 1 h at room temperature with a mixture of an anti-biotin, horseradish peroxidase (HRP)-linked secondary antibody to detect the protein ladder and the appropriate HRP-conjugated secondary antibody. The Aβ bands were visualized by incubating the membrane with luminol reagent and then exposing the membrane to X-ray film. The film images were digitized and the Aβ levels were quantified by calculating total pixel volume (pixel density X area) using NIH ImageJ software program (http://rsbweb.nih.gov/ij/). The studies were repeated 2–4 times.

Aβ levels in B6;129-Psen1tm1Mpm Tg(AβPPSwe,tauP301L) mice

Two-month-old transgenic AD model mice, obtained from the Mutant Mouse Regional Resource Center (MMRRC) at the Jackson Laboratory, were divided into two groups. The untreated control group received standard rodent chow while the second group received similar rodent chow containing 0.05% of the MFAO HK-2 (Bio-Serve, Flemington, NJ). After 12 months of feeding, the mice were perfused under terminal anesthesia and the brains were removed. One hemisphere was immediately frozen while the other half was formalin fixed. The frozen brain tissues were subsequently thawed and homogenized in 2% SDS-containing solubilization buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 1% Triton X-100, protease inhibitor cocktail), followed by centrifugation at 100,000× *g* at 16°C for 1 h. The supernatant was used as the SDS-soluble fraction. The remaining pellet was briefly sonicated on ice in 5 M guanidine thiocyanate and used as a SDS-insoluble fraction. Protein concentrations in the supernatants

were determined using the Micro BCA Protein Assay and the amounts of $A\beta_{40}$ and $A\beta_{42}$ were measured using the $A\beta_{40}$ and $A\beta_{42}$ ELISA kit according to manufacturer's instructions (Life Technologies, Carlsbad, CA, USA).

Statistical analysis

Statistical comparisons between groups were performed with ANOVA test (Origin 8.1, OriginLab Corporation, Northampton, MA, USA). Differences with $p < 0.05$ were considered significant.

RESULTS

Mitochondrial viability/toxicity

Mitochondrial function is very important for maintaining the physiological homeostasis of cells. Since the present multifunctional antioxidants can bind iron, these compounds can potentially alter mitochondrial function. To investigate this possibility, cultured NB and RPE cells were exposed to 1 mM MFAOs and the mitochondrial function of these exposed cells was monitored using red rhodamine 123 staining. In normal NB and RPE cells (Fig. 1A, I), mitochondrial function can be observed by the presence of red rhodamine 123 staining which contrasts to the presence of DNA in the nuclei that stains blue with Hoechst 33342 stain. When 1 mM $MnCl_2$ was added to these cells, the mitochondrial rhodamine 123 staining disappeared resulting in the presence of blue stain in the nuclei (Fig. 1B, J). This loss of red stain is indicative of mitochondrial dysfunction. No change in rhodamine 123 staining was observed when MFAOs JHX-4 HK-2 and HK-4 and their non-functional parent analogs JHX-1, HK-9, HK-11 were added to both cell lines (Fig. 1C–H, K–P). Since MFAOs bind select transition

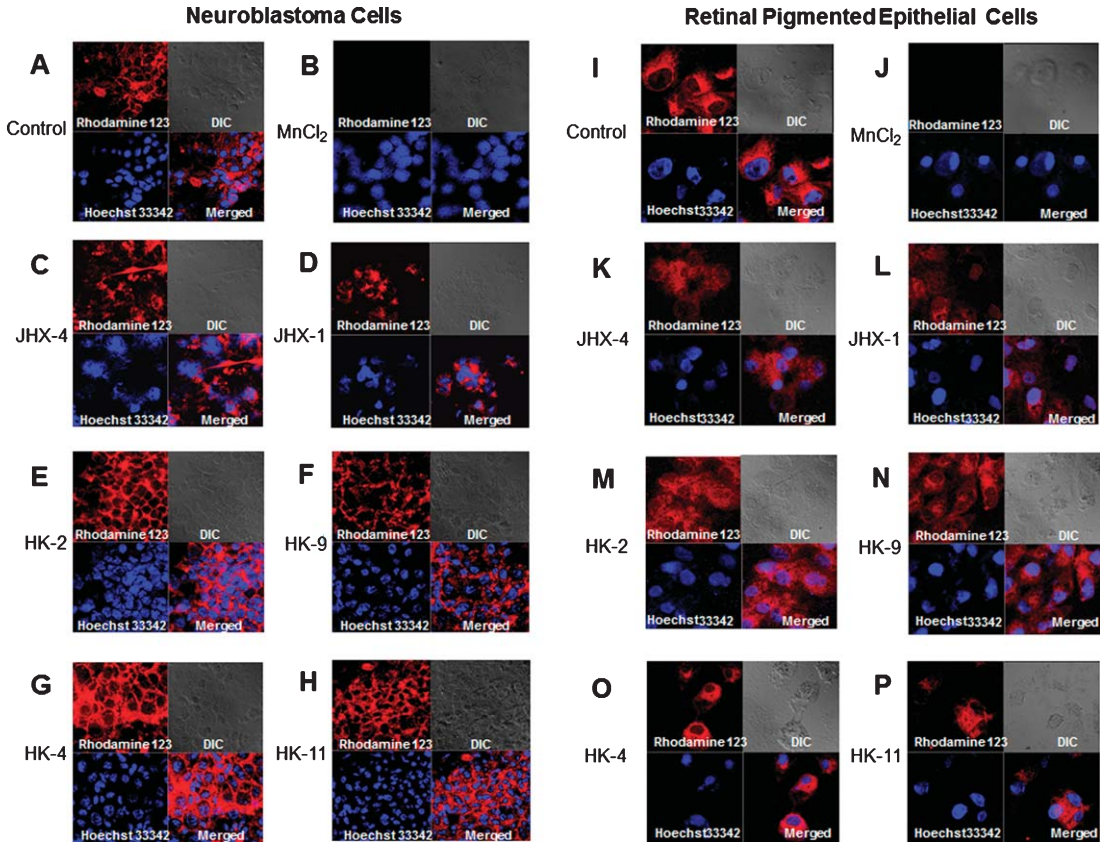

Fig. 1. Mitochondrial function, monitored with rhodamine 123 staining in NB and RPE cells exposed for 2 h to either 1 mM $MnCl_2$, MFAOs, or their nonfunctional parent analogs. Normal mitochondrial function results in the appearance of red fluorescence in the cytoplasm of NB cells (A) or RPE cells (I), while their nuclei appear blue due to Hoechst 33342 staining. Addition of 1 mM $MnCl_2$ results in the loss of cellular rhodamine 123 staining indicative of mitochondrial dysfunction (B, J). No loss of mitochondrial staining in the NB or RPE cells was observed when the cells were exposed to the MFAOs JHX-4 (C, K), HK-2 (E, M), or HK-4 (G, O) or their nonfunctional parent analogs JHX-1 (D, L), HK-9 (F, N), or HK-11 (H, P).

metals, their effects on $MnCl_2$ induced mitochondrial dysfunction were also examined. While the presence of $MnCl_2$ rapidly results in loss of mitochondrial activity (Fig. 1B, J), mitochondrial dysfunction induced by 1 mM $MnCl_2$ was prevented by the presence of 1 mM of MFAOs JHX-4, HK-2, and HK-4 (Fig. 2A, C, E, G, I, K). In contrast, the non-functional parent analogs JHX-1, HK-9, and HK-11 did not prevent the decreased red mitochondrial rhodamine 123 staining induced by $MnCl_2$ (Fig. 2B, D, F, H, J, L). This indicates that the MFAOs prevent mitochondrial dysfunction by chelating Mn^{+2}.

Intracellular labile zinc levels

The metal dyshomeostasis hypothesis in the development of AD states that the bioavailability of zinc is a very important parameter in neuronal cells. When NB or RPE cells are cultured in media containing 10 μM zinc sulfate, free Zn^{2+} ions present in the cell cytoplasm can be detected by zinquin staining which develops a blue fluorescence with labile Zn^{2+} ions. Since MFAOs can also bind Zn^{2+}, the ability of MFAOs JHX-4, HK-2, or HK-4, their non-functional parent analogs HK-9 or HK-11 or clioquinol to reduce zinquin staining in NB and RPE was investigated. As illustrated in Fig. 3, zinquin staining resulting from culturing the cells in the presence of 10 μM zinc sulfate was not reduced by the presence of 10 μM of either MFAOs, their nonfunctional parent analogs, or clioquinol.

Aβ also tightly binds Zn^{2+} to form a neurotoxic Aβ:Zn complex that becomes less susceptible to degradation by metalloproteases such as MMP2 [8]. As Zn^{2+} becomes co-localized with Aβ, the bioavailability of cellular Zn^{2+} not only decreases, but the neurotoxicity of the Aβ:Zn complex increases [7, 16, 29, 32–34]. In contrast to 10 μM of zinc sulfate, culturing NB and RPE cells with 10 μM of Aβ:Zn complex results in a significant reduction of zinquin staining fluorescence (Fig. 4A, B). This indicates that Zn^{2+} is so tightly bound to Aβ that its availability for zinquin binding as labile Zn^{2+} is reduced. This loss of zinquin staining was not observed when MFAOs JHX-4, HK-2, HK-4, or clioquinol were cultured with the Aβ:Zn complex. This indicates that the MFAOs and clioquinol similarly extract Zn^{2+} from the Aβ:Zn complex and then redistribute it to the cytoplasm in both NB and RPE cells where the labile Zn^{2+} becomes available for zinquin binding. No increase in zinquin fluorescence was observed when Aβ:Zn complex was added to cells exposed to the non-functional parent analogs

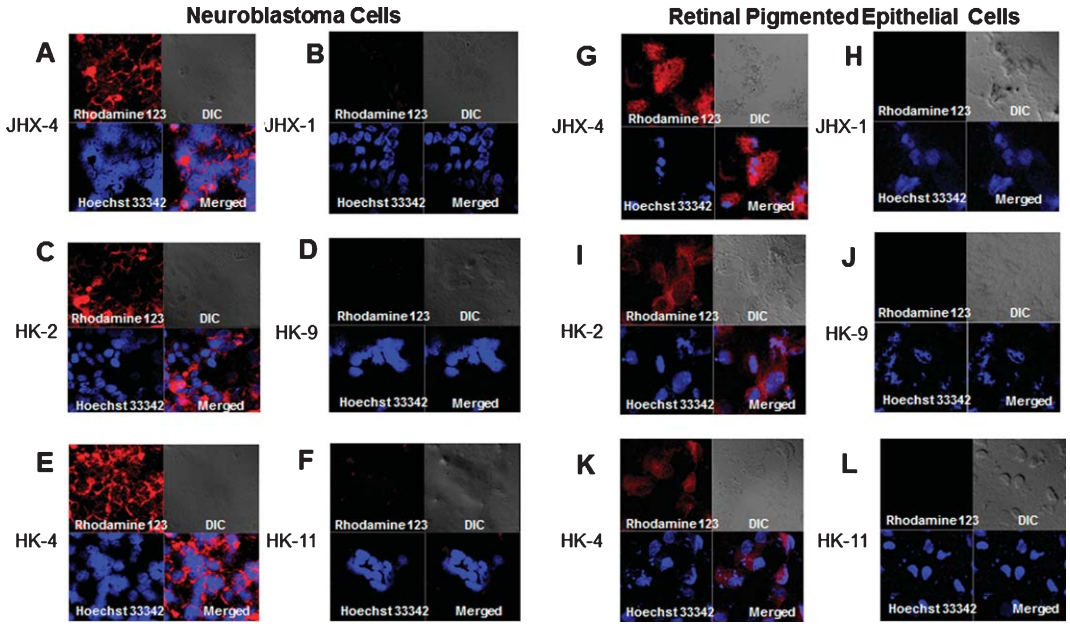

Fig. 2. Effect of MFAOs on $MnCl_2$ induced loss of mitochondrial fraction in NB and RPE cells. Red cytoplasmic rhodamine 123 fluorescence indicative of mitochondrial function remains when cells are cultured with 1 mM of $MnCl_2$ and either MFAOs JHX-4 (A, G), HK-2 (C, I), or HK-4 (E, K). When the cells are similarly cultured with 1 mM of $MnCl_2$ and either nonfunctional parent analogs JHX-1 (B, H), HK-9 (D, J), or HK-11 (F, L), only the blue nuclear staining from Hoechst 33342 is present while the absence of rhodamine 123 staining indicate that mitochondrial function is absent. These results indicate that MFAOs protect cells against mitochondrial dysfunction by binding Mn^{2+}.

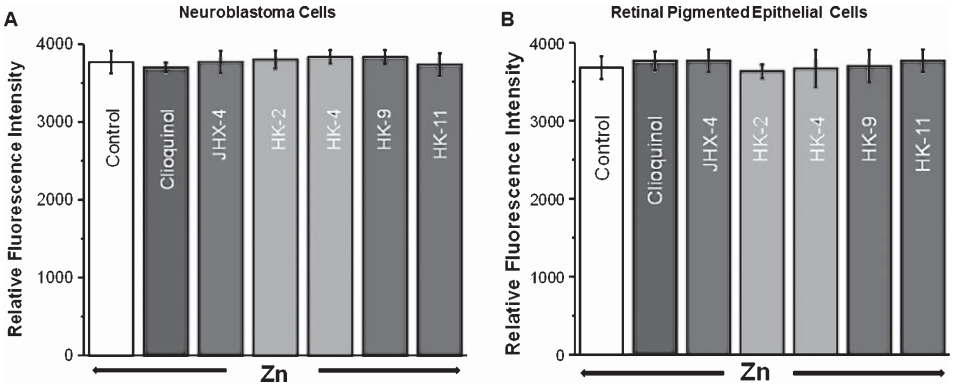

Fig. 3. Zinquin staining of NB (A) and RPE (B) cells cultured for 1 h in medium containing 10 μM zinc sulfate. The relative fluorescence of zinquin staining, which is indicative of the cytoplasmic presence of labile Zn^{2+}, was not significantly reduced by the presence of MFAOs JHX-4, HK-2, or HK-4, the nonfunctional parent analogs HK-9 and 11, or clioquinol. This indicates that these compounds do not reduce the cytoplasmic presence of labile Zn^{2+}. The results represent the mean ± SEM, $n = 5$.

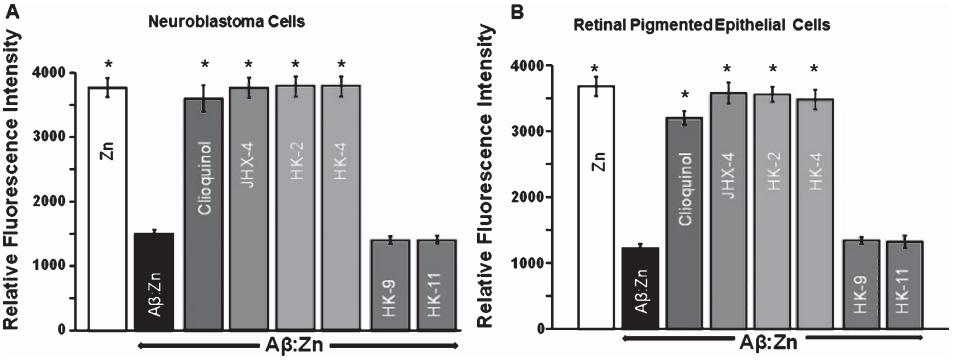

Fig. 4. Cytoplasmic zinquin staining of NB (A) and RPE (B) cells cultured for 1 h in medium containing 10 μM zinc sulfate or 10 μM of Aβ:Zn complex with or without MFAOs, nonfunctional analogs, or clioquinol. The relative fluorescence of zinquin staining was significantly lower when cells were cultured in 10 μM of Aβ:Zn compared to 10 μM zinc sulfate. Co-culture of cells with 10 μM of Aβ:Zn complex and either MFAOs JHX-4, HK-2, or HK-4 resulted in zinquin fluorescence similar to that observed with 10 μM zinc sulfate; however, zinquin fluorescence was not increased by the presence of the nonfunctional analogs HK-9 or HK-11. These results indicate that the MFAOs and clioquinol are able to extract labile zinc from the Aβ:Zn complex and redistribute it in the cytoplasm as labile Zn^{2+}. The results represent the mean ± SEM, $n = 5$. *$p < 0.01$ compared to Aβ:Zn.

HK-9 and HK-11 because they are unable to bind metals. Cytoplasmic zinquin staining was also absent in both NB and RPE cells cultured in the absence of 10 μM zinc sulfate or Aβ protein that was not complexed to zinc. This further supports the observation that the source of labile zinc observed in the presence of MFAOs or clioquinol was the Aβ:Zn complex (data not shown).

MMP2 proteolysis assays of Aβ: Zn complexes

While Aβ protein can be degraded by metalloproteases such as MMP2, this degradation is significantly reduced when Zn^{2+} is bound to Aβ to form the neurotoxic Aβ:Zn complex [8]. To confirm that MFAOs increased zinquin staining by removing Zn^{2+} from the Aβ:Zn complex, the ability of these compounds to alter the susceptibility of the Aβ:Zn complex to proteolysis by MMP2 was investigated. Proteolysis was performed by incubating 10 μM of either Aβ, Aβ:Zn, or Aβ:Zn with MFAOs HK-2 and HK-4, and the nonfunctional parent analogs and clioquinol with MMP2 (25 ng at 37°C for 1 h) and then analyzed the proteolysis products by western blots with Aβ antibody. As summarized in Fig. 5, Aβ was degraded 95% compared to 40% degradation of the Aβ:Zn complex by MMP2. However, in the presence of MFAOs or clioquinol, the Aβ:Zn complex degradation was increased to levels similar to Aβ where Zn^{2+} binding to protein is absent. Surprisingly, no degradation of the Aβ:Zn complex was observed in the presence of the non-functional parent analogs HK-9 and HK-11.

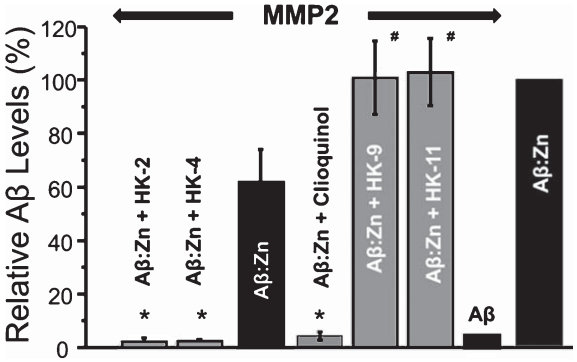

Fig. 5. MMP2 catalyzed degradation of Aβ and Aβ:Zn complex with/without the presence of MFAOs, nonfunctional analogs, or clioquinol. In the presence of MMP2 for 1 h, Aβ is rapidly reduced while Aβ:Zn complex is only partially degraded. Degradation of the Aβ:Zn complex increased to levels observed with Aβ when the Aβ:Zn complex was cultured in the presence of MFAOs HK-2, HK-4 or clioquinol. No degradation of the Aβ:Zn complex occurred in the presence of the nonfunctional analogs HK-9 or HK-11. Relative Aβ levels were obtained by western blots using Aβ antibody. The results represent the mean ± SEM, $n = 6$. $^{*}p < 0.01$, $^{\#}p < 0.05$) compared with Aβ:Zn cultured with MMP2.

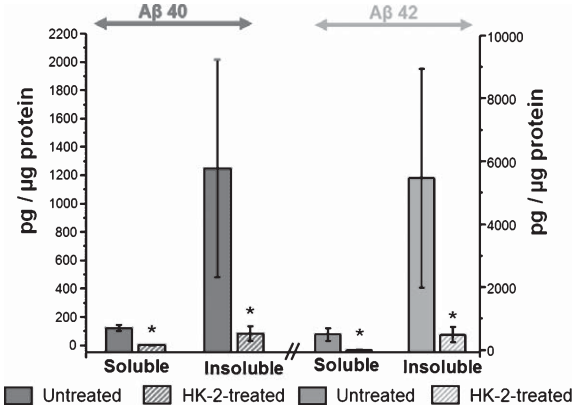

Fig. 6. $A\beta_{40}$ and $A\beta_{42}$ levels in both SDS-soluble and insoluble fractions are reduced in the brains of transgenic AD mice treated for 12 months with the MFAO HK-2. Mean ± SEM, $n = 3$–8; $^{*}p < 0.05$ (3 control and 8 HK-2 treated mice).

Aβ levels in transgenic mice

The MFAO HK-2 was orally administered into AD mice starting at 2 months through 12 months of age. The drug was administered in standard rodent chow at a concentration of 0.05% that corresponded to an average ingested dosage of 80 mg/kg/day. During this period, no adverse effects on body weight, food consumption, or mortality were observed. As summarized in Fig. 6, administration of the MFAO resulted in a significant reduction in both SDS-soluble and insoluble forms of $A\beta_{40}$ and $A\beta_{42}$ when equal variance is assumed. These results suggest that MFAO treatment reduce the production of both oligomeric and fibrilar Aβ.

DISCUSSION

The present multifunctional antioxidants JHX-4, HK-2, and HK-4 are compounds that independently reduce ROS and selectively bind the redox active metal ions that include Cu^{+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, and Mn^{2+} [21, 22]. The dual functionality of these compounds has been designed to address the roles of increased ROS and dyshomeostasis of metals observed in age related diseases. In rats, the orally active MFAO JHX-4 has been observed to delay ROS-associated diabetic, γ-irradiation, and UV radiation induced cataracts as well as light induced retinal (photoreceptor) degeneration [23, 24]. The present studies have used human NB and RPE cells to evaluate the ability of MFAOs to modulate metal bioavailability because biometals play a role in the pathogenesis of AD and AMD [16, 35, 36] and Aβ plaque develops in both the brain and the retina of patients with AD or AMD [37, 38].

Mitochondrial function is very important for maintaining regular physiological homeostasis of cells because oxidative phosphorylation and ATP synthesis by mitochondrial respiration consumes approximately 90% of total oxygen content in the cell [39]. Mitochondrial function is sensitive to cellular iron levels and both excess iron levels and iron deprivation can reduce mitochondrial function [40, 41]. Mitochondrial function can be monitored at physiological pH with rhodamine 123 stain, a membrane potential-specific dye for monitoring mitochondrial activity at physiological pH [26, 42]. The present studies show that MFAOs do not adversely affect mitochondrial function (Fig. 1). Mitochondrial dysfunction can also result from increased levels of Mn^{2+} [43–46]. This was observed in both NB and RPE cells by the rapid reduction of rhodamine 123 fluorescence after 2 h culture with 1 mM $MnCl_2$ (Fig. 2). This reduction of rhodamine 123 staining fluorescence was not observed in the presence of MFAOs, indicating that JHX-4, HK-2, and HK-4 can protect mitochondrial function by binding Mn^{2+} (Fig. 2). This premise is supported by the failure of their nonfunctional analogs JHX-1, HK-9, and HK-11 to prevent the reduction of rhodamine 123 staining. Increased concentrations of Mn^{2+} can also result in neurotoxicity [47] and increased exposure to high Mn^{2+} levels is clinically associated with "manganism", a characteristic syndrome that involves psychiatric

symptoms and the development of neurodegenerative diseases [45].

The MFAOs can also bind Zn^{2+} and based on the metal dyshomeostasis hypothesis for the progression of AD, the bioavailability of zinc is a very important parameter for monitoring AD progression in neuronal cells. This is because Zn readily complexes Aβ to form a neurotoxic Aβ:Zn complex which cannot be degraded as well by proteases, especially MMP2 [8]. Using blue zinquin staining, which is a Zn-specific detector [31, 48, 49], no decrease in cytoplasmic blue fluorescence was observed upon addition of either MFAOs, their nonfunctional analogs, or clioquinol (Fig. 3). This indicates that MFAOs do not adversely deplete the cellular availability of labile Zn^{2+}. Nevertheless, these MFAOs can remove highly bound Zn^{2+} from the protease-resistant Aβ:Zn complex and restore it to the cytoplasm as labile Zn^{2+} (Fig. 4). Removal of Zn^{2+} from the Aβ:Zn complex also restores the ability of MMP2 to degrade the remaining Aβ protein (Fig. 5). In the present studies, the metal complexing activity of MFAOs and clioquinol were observed to be similar. Clioquinol does not reduce the systemic metal concentrations [12, 15] but reduces abnormal metal accumulation in select cells and tissues by redistributing the metals so that they are again bioavailable [16]. Therefore, clioquinol has been proposed to possess metal attenuating rather than metal chelating activity. The observed similar behavior of MFAOs suggests that these compounds also possess metal attenuating rather than metal chelating activity.

While MFAOs and clioquinol restored the ability of MMP2 to degrade the Aβ:Zn by removing Zn^{2+} from the complex, the presence of the nonfunctional analogs HK-9 and HK-11 which do not bind Zn^{2+} prevented even the partial degradation of the Aβ:Zn complex by MMP2. It is possible that these nonfunctional compounds bind to the Aβ:Zn complex to either sterically restrict or conformationally prevent the ability of MMP2 to digest the complex. Alternatively, these compounds may directly affect MMP2 activity. In addition to attenuating Zn^{2+} from the Aβ:Zn complex, an alternate mechanism for clioquinol in the reduction of Aβ deposition in the brains of treated animals and potentially AD patients has been proposed that is based on the observation that secreted Aβ is rapidly degraded through upregulation of MMP2 and MMP3 after the addition of clioquinol and Zn^{2+} or Cu^{2+} [50]. This alternate mechanism suggests that MMP activity is upregulated through activation of phosphoinositol 3-kinase and JNK because clioquinol has been observed to promote the phosphorylation of glycogen synthase kinase-3 which potentiates activation of JNK. Additional studies are required to elucidate the exact mechanism(s) of how clioquinol, these MFAOs, and their nonfunctional analogs restore or totally prevent the degradation of the Aβ by MMP2.

The present studies demonstrate that these novel MFAOs attenuate transition metal ions to prevent mitochondrial dysfunction and enhance Aβ degradation in both NB and RPE cells. The similar metal attenuating activity of these compounds to that of clioquinol suggests that MFAOs may have potential therapeutic activity based on their modulation of metal bioavailability.

The ability of a MFAO HK-2 to reduce Aβ protein was also examined in a mouse model of AD. In preliminary studies in C57BL6 mice (not shown), HK-2 readily enters the brain and after oral administration of 80 mg/kg/day achieves levels of 206 ± 48 ng/mg protein (SEM). Moreover, HK-2 appears to be well-tolerated in no adverse effects on body weight, food consumption, or mortality observed when these mice receive up to 1,800 mg/kg/day by gavage for 5 days. In the present study, administration of approximately 80 mg/kg/day resulted in a marked reduction of both SDS-soluble and insoluble forms of $A\beta_{40}$ and $A\beta_{42}$ levels which represent oligomeric Aβ and Aβ fibrils, respectively. The effect of HK-2 on cognitive function, however, will be evaluated in future studies.

In summary, *in vitro* studies demonstrated that these MFAOs show antioxidant and metal attenuating activity with no adverse observed effects observed on either mitochondrial signaling or labile cytoplasmic zinc levels. Similar to clioquinol, MFAOs remove zinc from the Aβ:Zn complex so that Aβ plaques can be subsequently degraded by MMP-2. Orally administered to transgenic JAX Alzheimer's mice, MFAOs can enter the mouse brain and reduce the levels of both SDS-soluble and insoluble $A\beta_{40}$ and $A\beta_{42}$ isoforms. Based on their biological activity and their ability to be well-tolerated with an apparent 225-fold safety margin, MFAOs are clinical candidates that merit further development.

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P. Kador is inventor of the patents on multifunctional antioxidants in this manuscript and is President of Therapeutic Vision, Inc.

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