Published in final edited form as:

Toxicol Appl Pharmacol. 2012 September 15; 263(3): 303–314. doi:10.1016/j.taap.2012.07.002.

Inhibition of Aminoacylase 3 Protects Rat Brain Cortex Neuronal Cells from the Toxicity of 4-Hydroxy-2-nonenal Mercapturate and 4-Hydroxy-2-nonenal

Kirill Tsurulnikov^a, Natalia Abuladze^a, Anatol Bragin^{b,c}, Kym Faull^{c,d,e}, Duilio Cascio^f, Robert Damoiseaux^g, Matthew J. Schibler^g, and Alexander Pushkin^{a,*}

^aDepartment of Medicine, University of California at Los Angeles, CA 90095

^bDepartment of Neurology, University of California at Los Angeles, CA 90095

^cBrain Research Institute, University of California at Los Angeles, CA 90095

^dDepartment of Psychiatry and Biobehavior Science, University of California at Los Angeles, CA 90095

^ePasarow Mass Spectrometry Laboratory, University of California at Los Angeles, CA 90095

^fInstitute of Genomics and Proteomics, University of California at Los Angeles, CA 90095

^gCalifornia NanoSystems Institute, University of California at Los Angeles, CA 90095

Abstract

4-Hydroxy-2-nonenal (HNE) and acrolein are highly reactive neurotoxic products of lipid peroxidation that accumulate in Alzheimer's and Parkinson's disease brain and are implicated in the pathogenesis and progression of these neurological diseases. Conjugation with glutathione (GSH) initiates the HNE and acrolein detoxification pathway, which generates the mercapturates of HNE and acrolein that can be excreted. Prior work has shown that the efficiency of the GSH-dependent renal detoxification of haloalkene derived mercapturates is significantly decreased upon their deacetylation. Moreover the deacetylated products are rapidly transformed via β -lyases into toxic compounds responsible for the nephrotoxicity of the mercapturates. Because the enzymes of the GSH-conjugation pathway and β -lyases are expressed in brain, we hypothesized that a similar toxicity mechanism may be initiated in brain by the deacetylation of HNE and acrolein mercapturates. The present study was performed to identify an enzyme(s) involved in HNE and acrolein mercapturate deacetylation, characterize the brain expression of this enzyme and determine whether its inhibition decreases HNE and HNE mercapturate toxicity in neurons. We demonstrated that of two candidate enzymes known to deacetylate mercapturic acids, aminoacylases 1 (AA1) and 3 (AA3), only AA3 efficiently deacetylates both HNE and acrolein mercapturates. AA3 was further localized to neurons and blood vessels. Using a small molecule screen we further generated high-affinity AA3 inhibitors. Two of them completely protected rat

*Corresponding author: A. Pushkin, Division of Nephrology, David Geffen School of Medicine at UCLA, 10833 Le Conte Avenue, 7-155 Factor Building, Los Angeles, CA 90095-1689, USA.

Conflict of interests statement

A.P., K.T. and N.A. are inventors on a patent application that relates to the subject matter of this paper. Other authors do not have conflict of interest to declare.

brain cortex neurons expressing AA3 from the toxicity of HNE mercapturate. The results suggest that AA3 mediated deacetylation of HNE mercapturate may be involved in the neurotoxicity of HNE.

Keywords

Aminoacylase 3; 4-Hydroxy-2-nonenal; acrolein; mercapturates; deacetylation; Alzheimer's disease; Parkinson's disease

Introduction

Alzheimer's disease (AD)¹ is a neurodegenerative disorder with the prevalence drastically increasing with age (Davignus et al., 2010). While the exact mechanism of AD pathogenesis remains unknown, numerous studies indicate that the products of lipid peroxidation, 4-hydroxy-2-nonenal (HNE) and acrolein, are involved (Abdul et al., 2008; Baideiras et al., 2010; Butterfield et al., 2010; Dumont, Bell, 2011; Juhasz et al., 2011; Lovell et al., 1997; Montine et al., 1997, 1998; Picklo et al., 2002; Pratico, 2010; Sayre et al., 1997; Sidell et al., 2003; Siegel et al., 2007; Sultana et al., 2009). These highly reactive aldehydes react with and modify different macromolecules including proteins, DNA, RNA and lipids, whereby inducing toxic effects eventually involved in AD pathology (Coppede, Migliore, 2009; Lovell, Markesbery, 2007, 2008; Markesbery et al., 2005; Nunomura et al., 2007; Pratico, 2010; Sultana et al., 2009). The level of free and protein-bound HNE is significantly higher in the brain and cerebrospinal fluid of AD patients in comparison with age matched controls (Lovell et al., 1997; Montine et al., 1997, 1998; Sayre et al., 1997; Williams et al., 2006). Acrolein levels are also increased in AD brain, and protein-bound acrolein is mostly associated with neurons (Lovell et al., 2001; Williams et al., 2006). Furthermore acrolein adducts have been detected in neurofibrillary tangles and dystrophic neuritis surrounding senile plaques in AD (Calingasan et al., 1999). HNE promotes amyloid β ($A\beta$) aggregation and may play a crucial role in the formation of neurofibrillary tangles (Chen et al., 2007; Hardy, Allsop, 1991; Siegel et al., 2007), the process implicated in AD (Hardy, Allsop, 1991; Sultana et al., 2009).

HNE protein adducts are also significantly increased in nigral neurons of patients with Parkinson's disease (PD) (Yoritaka et al., 1996) and stimulated *in vitro* aggregation of alpha-synuclein (Qin et al., 2007). Alpha-synuclein was modified with acrolein in the dopamine neurons of the substantia nigra from PD patients (Shamoto-Nagai et al., 2007).

In addition to neuronal damage, HNE and acrolein may damage blood vessels and thereby induce vascular dysfunction that includes both the reduced cerebrovascular flow and cerebral $A\beta$ angiopathy (de la Torre, 2004; Marchesi, 2011; Murray et al., 2011). Vascular

¹Abbreviations: AA1, aminoacylase 1; AA3, aminoacylase 3; AD, Alzheimer's disease; $A\beta$, amyloid β ; Ebselen, 2-phenyl-1,2-benzisoselenazol-3-one; ECL, enhanced chemiluminescence; EPMS, 2-(4-ethoxyphenyl)-2-methylsuccinic acid; NA-Cys, N-acetyl-L-cysteine; NA-DCVC, N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine; NA-Tyr, N-acetyl-L-tyrosine; GSH, reduced glutathione; HNE, 4-hydroxy-2-nonenal; IC_{50} , half maximal inhibitory concentration; k_{cat} , catalytic constant; K_m , Michaelis constant; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

dysfunction disrupts vascular architecture and is linked to AD pathology (de la Torre, 2004; Marchesi, 2011), PD pathology and other central nervous system disorders (Grammas et al., 2011). Vascular dysfunction may decrease A β clearance; it precedes AD pathology, and may be related to atherosclerotic lesions and blood flow restriction of arterial vessels of the brain (Murray et al., 2011). Synergistic effects between A β deposition and vascular dysfunction on neuronal degeneration have been proposed (Carlsson, 2010).

Experimental data and general considerations indicate that an effective detoxification mechanism of HNE and acrolein is necessary to protect the brain and other organs from their toxicity. Montine and colleagues demonstrated the presence of the glutathione (GSH) dependent NHE detoxification pathway in mammalian cerebrum, although its was estimated to play a less significant role than in liver, the major site of HNE detoxification (Montine et al., 1997, 1998; Piclo et al., 2002; Sayre et al., 1997; Sidell et al., 2003). Importantly, the rate of NHE detoxification via this pathway significantly increased in frontal cortex of AD patients in comparison with controls (Sidell et al., 2003). This increase correlated with the elevated level of HNE in AD patients (Sayre et al., 1997).

The conjugation with GSH catalyzed by GSH transferase (GST) initiates HNE detoxification (Fig. 1). The following steps catalyzed by γ -glutamyltransferase, dipeptidase and N-acetyl transferase (Fig. 1), generate the N-acetylcysteine conjugate of HNE (HNE mercapturate), which after release into the circulation is available for subsequent renal excretion. Although the release and renal excretion of HNE mercapturate has to be studied in detail, *in vivo* rodent experiments using i.p. and i.v. routes of HNE administration have identified HNE mercapturate in the urine (Alary et al., 2003). However, prior to release and excretion, HNE and other mercapturates are available for deacetylation (Fig. 1), a process that bioactivates them for transformation by ubiquitous β -lyases into other highly reactive toxic/mutagenic compounds (Anders et al., 1988; Cooper, 1994, 2004; Dekant et al., 1994; Koob, Dekant, 1991; Lash et al., 2006; Newman et al., 2007; Pushkin et al., 2004; Tsirulnikov et al., 2009; Uttamsingh, Anders, 1999; Uttamsingh et al., 1998). For example, deacetylation of N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine (NA-DCVC), a mercapturate formed in the GSH detoxification pathway of an environmental contaminant trichloroethylene, bioactivated it for the toxic transformation by β -lyase into other highly reactive toxic compounds, which caused acute renal failure (Newman et al., 2007; Tsirulnikov et al., 2009).

Two deacetylases have been shown to deacetylate mercapturic acids, aminoacylase 1 (AA1; EC 3.5.1.14) and a recently discovered aminoacylase 3 (AA3) with a significantly different substrate specificity (Anders, Dekant, 1994; Newman et al., 2007; Pushkin et al., 2004; Tsirulnikov et al., 2009; Uttamsingh, Anders, 1999; Uttamsingh et al., 1998). In addition to mercapturates, AA1 deacetylates N-acetyl aliphatic amino acids and AA3 deacetylates N-acetyl aromatic amino acids (Anders, Dekant, 1994). Although the data is less complete for AA3, these proteins appear to have different but partially overlapping cellular distributions. AA1 is ubiquitously expressed in different mammalian organs including brain (Anders, Dekant, 1994); in rat brain it is immunolocalized to cortical and hippocampal CA1, CA2 and CA3 pyramidal neurons (Uttamsingh et al., 2000). AA3 expression in mouse brain has been verified by Northern blotting (Pushkin et al., 2004), although the precise neuroanatomical

distribution has yet to be defined. Both proteins are expressed in primary neuroblastoma tumors (Long et al., 2010).

The atomic structures of AA1 (Lindner et al., 2003) and AA3 (Hsieh et al., 2010) have been studied by X-ray crystallography. An important structure/functional property of AA1 and AA3 is that they both are metalloenzymes inactivated by chelating agents (Anders, Dekant, 1994; Kördel, Schneider, 1977; Lindner et al., 2003, 2008; Perrier et al., 2005; Tsirulnikov et al., 2009; Wu, Tsou, 1993). Human AA1 expressed in *E. coli* contained 2 zinc atoms in the active site (Lindner et al., 2003) although Co^{2+} and Mn^{2+} substituted for zinc in AA1 without a substantial loss of activity. Mouse and human AA3 expressed in *E. coli* contained only 1 zinc atom per active site (Hsieh et al., 2004; Tsirulnikov et al., 2009), which substitution with Co^{2+} resulted in several-fold activation. The metal-dependence of AA1 and AA3 suggests that chelating agents should be effective inhibitors of these enzymes.

AA1 deficiency is characterized by accumulation of N-acetylated aliphatic amino acids and is variably accompanied by febrile seizures, delay of psychomotor development, moderate mental retardation and other pathologies (Sass et al., 2006; Van Coster et al., 2005). No human disease has been associated with an AA3 deficit, but it is unclear if this is due to the rarity of the condition, *in utero* lethality of a deficiency or the operation of effective compensatory mechanisms.

In the present study we determined whether AA1 or/and AA3 deacetylates HNE and acrolein mercapturates. Given that AA3 not AA1 was shown to deacetylate both mercapturates, we immunolocalized AA3 in the mouse brain. Further high-affinity inhibitors of AA3 were generated that completely abolished the neurotoxicity of HNE mercapturate.

Materials and Methods

Generation of AA3 Specific Antibody

This antibody (CH-4) was generated in chicken co-immunized with mouse AA3 peptides NH_2 -EPAGTETFSVESISK (Pep 1) and NH_2 -RYWLQNPGE LQRPS (Pep 2) coupled to keyhole limpet hemocyanin. Pep 1 is specific for mouse AA3 whereas Pep 2 is identical in mouse, rat and human AA3. Therefore the antibody was expected to react with AA3 from all three species. The immunoglobulin Y fraction from eggs was purified using a commercial kit (Gallus Immunoresearch, Fergus, Ontario, Canada). The antibody was effective during immunohistochemistry of both unfixed and paraffin embedded (fixed with 10% neutral buffered formalin) mouse kidney cryosections where it specifically labeled proximal tubules (Fig. 2A, B). This pattern is identical to the pattern previously reported in unfixed mouse kidney cryosections using a mouse AA3 C-terminus specific antibody (MR-C1) raised in rabbits and affinity purified (Pushkin et al., 2004). On immunoblots of mouse kidney extracts they both identified a ~35 kDa protein (Fig. 2C). The CH-4 antibody was used in this study because the MR-C1 antibody was not effective on cryosections from animals perfused with neutral buffered formalin.

Immunolocalization of AA3 in Mouse Brain

Brain coronal frozen sections in the hippocampus, thalamus and hypothalamus area of CD1 mice were purchased from Zymen (San Diego, CA). The animals had been perfused with 10% neutral buffered formalin before sacrifice. Brains were fixed for 12 h in neutral buffered formalin, cryoprotected in 30% sucrose in PBS, snapped frozen on OCT freezing medium, sectioned (7 μ m), and mounted on positively charged slides. They were then incubated at room temperature in PBS for 10 min with one buffer change, and then for 15 min with 5M sodium formate, pH 5.9, for antigen retrieval. Free formalin was quenched by 20 min incubation with 0.1 M Tris-HCl, pH 7.5, containing 0.3 M glycine. After 2 washes with PBS, the sections were incubated for 1 h at 37°C with CH-4 antibody diluted 1:50 in PBS. CH-4 antibody preincubated with both immunizing peptides (1:2 molar ratio) and diluted 1:50 in PBS was used as control. After five washes in PBS, a secondary CY3-conjugated donkey anti-chicken antibody (Jackson ImmunoResearch, West Grove, PA) at 1:500 dilution was applied at room temperature for 20 min. After five washes with PBS the slides were mounted in Crystal/Mount (Biomedex, Foster City, CA). To co-immunolocalize mouse brain neurons, a mouse monoclonal antibody anti-NeuN (clone 60, Millipore) was used at 1:100 dilution together with CH-4 antibody. Then a secondary Alexa Fluor 488 conjugated goat anti-mouse antibody (Molecular Probes, Eugene, OR) was used at 1:500 dilution for neuronal detection and a secondary CY3-conjugated donkey anti-chicken antibody was used for AA3 detection (see above). Additional controls were done on each of the secondary antibodies.

Confocal images were taken sequentially on a Leica TCS-SP2 AOBS inverted Confocal Microscope (Mannheim, Germany) equipped with an 405 nm blue diode laser, argon laser (488 nm: blue excitation: Lasos), and three helium neon lasers (543 nm: green, 594 nm: near red, and 633 nm: far red excitations).

Synthesis of HNE, HNE Mercapturate and Acrolein Mercapturate

HNE was prepared by acid hydrolysis of HNE dimethyl acetal (Sigma, Milwaukee, WI) according to the manufacturer's protocol. HNE concentrations were determined by measuring HNE solution absorbance at 224 nm using the molar extinction coefficient value of 13,750 M⁻¹cm⁻¹ (Humphries et al., 1998). HNE and acrolein mercapturates were synthesized by incubating HNE or acrolein with 2-fold excess of N-acetyl-L-cysteine (NA-Cys) in 100 mM sodium-phosphate buffer, pH 7.5. The mercapturates were purified by HPLC and their identity was confirmed by mass-spectrometry.

Expression and Purification of Mouse and Human AA3

Mouse and human AA3 were expressed in *E. coli* and purified as described (Tsirulnikov et al., 2009; Hsieh et al., 2010). Both purified enzymes were homogeneous by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with MR-C1 and CH-4 antibodies (data not shown). Purified enzymes were frozen in liquid nitrogen and stored at -80°C. Before use, the enzymes were thawed and centrifuged at 200,000g for 1 h at 4°C in to precipitate aggregated protein.

Porcine Kidney AE1

Purified AA1 from porcine kidney was purchased from Sigma (St. Louis, MO).

SDS-PAGE and Immunoblotting

15% polyacrylamide gels (Bio-Rad, Hercules, CA) were used for SDS-PAGE. Protein bands were visualized with Coomassie blue R (Sigma) or electrotransferred onto a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Piscataway, NJ) for immunoblotting. Affinity purified rabbit anti-mouse and anti-human AA3 antibodies MR-C1 and HR-C1 were diluted 1:1,000 in PBS. A secondary horseradish peroxidase-conjugated mouse anti-rabbit antibody (Jackson Immunoresearch) was used at 1:20,000 dilution. Protein bands were visualized using an ECL kit and Hyperfilm ECL (GE Healthcare).

AA3 Activity Assay

AA3 activity was determined by measuring the amount of a deacetylated product in a fluorescence assay (Pushkin et al., 2004) with fluorescamine (Sigma; 390 nm excitation, 475 nm emission).

The K_m and K_{cat} values were calculated by fitting data to the Michaelis-Menten equation using GraphPad Prism software (GraphPad Software, San Diego, CA) or EZ-Fit software (Perella Scientific, Amherst, NH).

Generation of AA3 Inhibitors

For preliminary detection of AA3 inhibitors, we used high-throughput small molecule screening. AA3 activity was assayed with N-acetyl-L-tyrosine (NA-Tyr) in 384-well plates. In each well 20 μ l of 0.6 μ M mouse AA3 in 20 mM sodium phosphate, pH 7.5, was incubated for 20 min at room temperature with 0.5 μ l of 1 mM tested compound dissolved in DMSO. Then 20 μ l of 2 mM N-acetyl L-tyrosine in 20 mM sodium phosphate, pH 7.5, was added and incubated for 30 min followed by 20 μ l of 3 mM fluorescamine in acetonitrile. Fluorescence of the solution was measured at 475 nm (excitation wavelength 390 nm). Degree of inhibition (%) was calculated using the equation:

$$\% \text{ inhibition} = 100 - (F_e - F_p) / (F_n - F_p) \times 100,$$

F_e – experimental fluorescence value,

F_b – background fluorescence after complete inhibition of the enzyme with 1 mM benzylsuccinic acid (Sigma, positive control),

F_n – fluorescence of the reaction assay without added compound (negative control).

IC_{50} was the inhibitor concentration, which decreased AA3 activity by 50%.

Primary Rat Cortex Neurons

Primary rat cortex neurons from Invitrogen (Carlsbad, CA) were used to study the effect of AA3 inhibitors on HNE and HNE mercapturate neurotoxicity. Neuronal cells in NeurobasalTM/B27 medium (Invitrogen) were plated in poly-D-lysine coated 96-well plates

at a density of 5×10^4 cells per well and grown at 37°C in the presence of 5% CO_2 in air, as recommended by the manufacturer.

Mouse AA3 has a high sequence homology with rat and human AA3, and the identical amino acid residues form their active sites (Newman et al., 2007; Hsieh et al., 2010). Therefore the compounds generated in this study for mouse AA3 are expected to also inhibit rat AA3 and human AA3 at similar rates. Indeed $20 \mu\text{M}$ 2-phenyl-1,2-benzisoselenazol-3-one (ebselen) and 2-(4-ethoxyphenyl)-2-methylsuccinic acid (EPMS) completely inhibited both the rat and human AA3 (data not shown).

Cytotoxicity Measurements

The effect of AA3 inhibitors, ebselen and EPMS, on the HNE and HNE mercapturate induced toxicity was studied in rat brain cortex neurons (Invitrogen) cultivated for 2 weeks at 37°C in the presence of 5% CO_2 in 96-well plates. Cells were incubated for 20 h with 20, 50, 100 and $200 \mu\text{M}$ HNE, or 20 and $50 \mu\text{M}$ HNE mercapturate in the presence of a $20 \mu\text{M}$ AA3 inhibitor (EPMS or ebselen) added to the growth media simultaneously with HNE and HNE mercapturate. Cell viability was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay (Mosmann, 1983). Cells were incubated during 4 h at 37°C with $150 \mu\text{l}$ of fresh media containing $0.5 \text{ mg} \cdot \text{ml}^{-1}$ MTT. Then $50 \mu\text{l}$ of 20% SDS in 25 mM HCl was added to the wells and incubated 4 h. The optical density of solution at 570 nm was measured on a VMax Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA).

Data Analysis

All values are means $\pm$ S.E. of measurements of at least three separate experiments unless specifically indicated. Statistical significance of differences was determined by the unpaired Student *t* test. A probability (*p*) level of < 0.05 was considered as significant.

Results

The Deacetylation of HNE and Acrolein Mercapturates Is Mediated by AA3

Purified mouse AA3 deacetylated HNE and acrolein mercapturates (Table 1). Because Co^{2+} has been shown to drastically stimulate AA3 (Tsirulnikov et al., 2009), the kinetics of deacetylation of acrolein and HNE mercapturates by mouse AA3 was measured both with and without Co^{2+} . The k_{cat} values for HNE mercapturate (0.27 and 1.67 s^{-1} without and with Co^{2+} respectively) and acrolein mercapturate (0.40 and 3.0 s^{-1} without and with Co^{2+} respectively), and the K_{m} values for HNE mercapturate (without and with Co^{2+} respectively) and acrolein mercapturate (without and with Co^{2+} respectively) were similar to the k_{cat} and K_{m} values for NA-Tyr and NA-DCVC (Newman et al., 2007; Tsirulnikov et al., 2009). The efficiency of deacetylation ($k_{\text{cat}}/K_{\text{m}}$) of HNE mercapturate ($1,350$ and $3,150 \text{ s}^{-1} \cdot \text{M}^{-1}$ without and with Co^{2+} respectively) and acrolein mercapturate (49 and $4,270 \text{ s}^{-1} \cdot \text{M}^{-1}$ without and with Co^{2+} respectively) was significantly greater than the efficiency of deacetylation of a typical substrate NA-Tyr (100 and $1,930 \text{ s}^{-1} \cdot \text{M}^{-1}$ without and with Co^{2+} respectively) and comparable with the efficiency of deacetylation of a mercapturate NA-DCVC (620 and $7,600 \text{ s}^{-1} \cdot \text{M}^{-1}$ without and with Co^{2+} respectively). The cobalt induced

AA3 activation was mostly mediated by a drastic increase of k_{cat} , whereas the effect on K_{m} was significantly less (Table 1). Cobalt may play an important role in AA3 mediated deacetylation of NHE and acrolein mercapturates *in vivo* (Tsirulnikov et al., 2009).

AA1 Does not Deacetylate HNE and Acrolein Mercapturates

The ability of AA1 to deacetylate HNE and acrolein mercapturates was assayed using purified AA1 from porcine kidney (Sigma). Since Co^{2+} does not activate AA1 (Wu, Tsou, 1993), AA1 activity was assayed without this metal ion. The enzyme was homogeneous under SDS-PAGE and had the K_{cat} value with a typical AA1 substrate N-acetyl-L-methionine of $\sim 20 \text{ s}^{-1}$, which is similar to the reported values for porcine and human AA1 (Lindner et al., 2008). HNE and acrolein mercapturate deacetylating activity of AA1 was low, 0.004 and 0.01 s^{-1} respectively, which represented 1.5 and 2.5% respectively of the deacetylating activity of AA3 with these substrates (Table 1). We were unable to precisely determine the K_{m} values of AA1 for HNE and acrolein mercapturates because of the very weak deacetylating activity of AA1 with both mercapturates.

Our data suggest that the deacetylation of HNE and acrolein mercapturates *in vivo* is likely mediated by AA3.

Immunolocalization of AA3 in Mouse Brain

AA3 was mainly immunolocalized to neurons (Fig. 3). The inter-neuronal localization of AA3 was confirmed using the Millipore antibody NEUronal Nuclei, Clone A60 (Millipore, Billerica, MA) that specifically recognizes the DNA-binding neuron-specific protein NeuN. Merged images (Fig. 3D) demonstrated that AA3 was colocalized with the neuronal staining in larger neurons. AA1 was also immunolocalized to rat brain cortex and hippocampus neurons (Uttamsingh et al., 2000). The immunolocalization of AA3 in mouse brain neurons supports the hypothesis of the potential involvement of AA3 in AD and PD pathogenesis.

Our data demonstrated (Fig. 3) that AA3 is also expressed in blood vessels; AA1 was not shown to localize in blood vessels in brain (Uttamsingh et al., 2000). The presence of AA3 in brain blood vessels correlates with the hypothesized involvement of the oxidative stress-induced damage of small blood vessels in early steps of AD (de la Torre, 2004; Marchesi, 2011; Murray et al., 2011) and PD (Grammas et al., 2003).

Generation of AA3 Inhibitors

High affinity AA3 inhibitors are required to explore the hypothesis that AA3 inhibition may decrease HNE and HNE mercapturate neurotoxicity. For inhibitor selection commercially available small molecule libraries were analyzed in Molecular Screening Shared Resource facility (California Nanosystems Institute at UCLA). Amongst 90,000 screened chemicals, approximately 100 compounds were identified that provided close to complete inhibition of mouse AA3 in the low micromolar range. Potentially toxic compounds (e.g. containing heavy metals or highly reactive groups) were excluded from consideration. Several of the most and least effective AA3 inhibitors are shown in Table 2.

Clioquinol ($IC_{50} \sim 10 \mu M$), a metal chelator, has been shown to markedly decrease soluble interstitial brain A β and improve cognitive performance in transgenic AD mice (Adlard et al., 2008; Grossi et al., 2009) and AD patients (Ritchie et al., 2003). Clioquinol were also neuroprotective in the MPTP PD mouse model (Kaur et al., 2003). The reported effects in mice and human patients may be at least in part mediated by the inhibition of AA3.

The metal chelating properties of clioquinol indicated that this compound has multiple cellular targets and its use as an AA3 inhibitor may induce adverse effects; therefore for the HNE and HNE mercapturate neuronal cell toxicity studies other AA3 inhibitors were selected, namely ebselen and EPMS, without metal chelating properties and with more favorable affinities to AA3 ($IC_{50} \sim 1 nM$). EPMS has never been used in any study, whereas ebselen is being investigated as a possible treatment for reperfusion injury and stroke (Parnham et al., 2000; Yamagata et al., 2010; Yamaguchi et al., 1998). Ebselen is a seleno-compound that easy reacts with reactive oxygen species and other cellular components, for example cysteine residues in proteins. Mouse AA3 has a cysteine residue (Cys175) in proximity to the active site (Hsieh et al., 2010), which modification by ebselen may decrease catalytic activity via restriction of substrate access to the active site. This possibility was excluded from consideration because purified human AA3 that has a glycine residue instead of cysteine (Pushkin et al., 2004; Tsirulnikov et al., 2009) was inhibited by ebselen to the same extent as mouse AA3 (data not shown).

Inhibition Constants of Selected AD Drugs

We also determined the K_i values for several AD drugs, and also for compounds used in human, animal and cellular AD studies (Arakawa et al., 2007; Dumont, Beal, 2011; Huang et al., 2010; Wilkinson et al., 2012; Yang et al., 2008, 2010), namely tacrine, memantine, donepezil, ibuprofen and N-acetyl-L-cysteine (NA-Cys) (Table 3). Some of the selections were based on the reported substrate specificity (Newman et al., 2007; Tsirulnikov et al., 2009) and the active site structure of AA3 (Hsieh et al., 2010), and were expected to inhibit (ibuprofen) or not to inhibit (tacrine, memantine, donepezil) AA3. Indeed tacrine, memantine, and donepezil did not inhibit AA3 (Table 3), and NA-Cys was a very weak inhibitor ($K_i = 3 mM$). Therefore the reported protective effect of NA-Cys in animal and cell model studies (Dumont, Bell, 2011) appears not to be mediated by AA3 inhibition but rather the stimulation of GSH biosynthesis.

Ibuprofen inhibited mouse AA3 with a moderate affinity ($K_i = 40 \mu M$) that is ~ 5 – 10 times higher than HNE and acrolein mercapturates (Table 1). Assuming this inhibition operates *in vivo*, it may be involved in the reported clinical effects of ibuprofen on AD (Dumont, Beal, 2011) and also PD because use of ibuprofen has been recently reported to decrease risk of PD (Gao et al., 2011).

Molecular Docking of Inhibitors to AA3

We further performed molecular docking of selected inhibitors to mouse AA3 in order to confirm that these compounds mediate AA3 inhibition via binding to the active site. Both a high affinity inhibitor EPMS, and a lower affinity inhibitor ibuprofen occupied the positions in the active site very close to the substrates, NA-Tyr and NA-DCVC (Fig. 4), in the AA3–

NA-Tyr and AA3-NA-DCVC crystallographic structures (Hsieh et al., 2010). Ibuprofen (Fig. 4A) forms a coordination bond with a metal ion whereas EPMS (Fig. 4B) forms hydrogen bonds with Asn70 and Arg71 in the active site. Van der Waals interactions hypothesized to participate in substrate binding (Hsieh et al., 2010) are probably also involved in the inhibitor binding although the importance of specific residues should be further evaluated by mutational studies and screening of modified inhibitors.

AA3 Inhibitors EPMS and Ebselen Decrease HNE Mercapturate and HNE Toxicity in Rat Brain Cortex Neurons Expressing AA3

HNE and HNE mercapturate were toxic to cultured rat cortex cerebral neurons (Fig. 5A). Low concentrations (20 and 50 μ M) of HNE decreased neuronal viability by ~30%, whereas 20 μ M ebselen or EPMS added to the media simultaneously with HNE demonstrated some protection that was not significant. Higher HNE concentrations, 100 and 200 μ M, killed respectively ~70 and 100% of cells. Ebselen and at a lesser extent EPMS partially protected neurons at 100 μ M HNE dose, whereas at the highest HNE dose (200 μ M) only EPMS slightly (~12%) but significantly protected neurons.

Both 20 and 50 μ M HNE mercapturate decreased neuronal viability at a higher extent than corresponding concentrations of HNE (Fig. 5A). Importantly, ebselen and EPMS completely protected neurons from HNE mercapturate toxicity at both mercapturate concentrations.

Our data suggested that the toxic effects of both compounds may be induced at least in part by the deacetylation of HNE mercapturate mediated by AA3. To confirm this hypothesis we determined whether rat cortex cerebral neurons used in our experiments express AA3. To this goal immunoblotting with the chicken anti-mouse AA3 antibody CH-4 was used. This antibody has been successfully used in immunoblotting and immunohistochemistry studies of AA3 in human brain and neuroblastoma (Long et al., 2010). Given a higher homology of mouse AA3 with rat than human AA3 (Newman et al., 2007), it is expected that the CH-4 antibody will also recognize rat AA3. Two predominant AA3 bands of ~75 kDa were detected on immunoblots of rat cortex neurons resolved by SDS-PAGE (Fig. 5B). These bands were twice the apparent molecular weight of kidney AA3 (Newman et al., 2007; Pushkin et al., 2004) and twice the molecular mass of AA3 calculated on base of the amino acid composition (~35 kDa). A weak band of ~35 kDa corresponding to AA3 monomer was also present. Comparison of the kinetic characteristics of HNE mercapturate deacetylation by kidney and brain extracts demonstrated their identity (data not shown) suggesting that the larger AA3 monomer size in brain does not affect its kinetic characteristics. It should be also noted that two predominant ~75 kDa AA3 bands were also reported in neuroblastoma cells (Long et al., 2010); these cells also expressed at a significantly fewer level a ~35 kDa band corresponding to AA3 monomer.

The neurotoxicity of HNE has been already reported whereas the neurotoxicity of HNE mercapturate is a novel finding. Our results demonstrated that AA3 inhibitors protect neurons from HNE mercapturate toxicity, and suggested that AA3 inhibition may be beneficial in the protection from AD and PD.

Discussion

Our study for the first time demonstrated that AA3 deacetylates mercapturic acids formed in the GSH conjugation metabolism of HNE and acrolein, and that AA1 has a low affinity for these mercapturates. Since we further demonstrated that AA3 is localized to brain neurons and blood vessels, the AA3 mediated deacetylation of HNE and acrolein mercapturates in neurons and blood vessels might initiate a chain of reactions leading to transformation of macromolecules and organelles in neurons and blood vessels that might further participate in AD and PD pathology. Accumulation of free and protein/DNA/RNA bound acrolein and HNE and their metabolites in the brain has been hypothesized to participate in AD and PD pathology (Butterfield et al., 2010; Chen et al., 2007; Dumont, Beal, 2011; Lovell et al., 1997, 2001; Magni et al., 2002; Montine et al., 1997, 1998; Nunomura et al., 2007; Sayre et al., 1997; Siegel et al., 2007; Shamoto-Nagai et al., 2007; Williams et al., 2006; Yoritaka et al., 1996). Highly reactive compounds, whose formation may be initiated by the deacetylation of HNE mercapturate and acrolein mercapturate, may damage different targets, e.g. cross-link A β , tau, and other proteins, bind and modify DNA, membranes, whereby damaging cellular organelles (e.g. mitochondria), cells, and tissues.

In agreement with our hypothesis, AA3 inhibition completely protected cultured neuronal cells from HNE mercapturate toxicity. A number of high affinity inhibitors of AA3 was identified in this study. Two of these inhibitors with the IC_{50} value of ~1 nM, ebselen and EPMS, demonstrated complete protection from HNE mercapturate neurotoxicity and had partially protective effect from HNE toxicity at a high (100 μ M) dose. The insignificant neuroprotective effects of ebselen and EPMS at low doses of HNE (20 and 50 μ M) suggest that most of the damage at these HNE concentrations was induced by free HNE and HNE metabolites other than HNE mercapturate. It is interesting that EPMS partially (~12 %) protected neurons from the highest dose of HNE (200 μ M) that without this inhibitor completely killed all neurons. Although the neuroprotective effect of AA3 inhibitors might be attributed to their interaction with other cellular targets, their high affinity to AA3 and results of the docking experiments supported the hypothesis that AA3 inhibition protects neurons from HNE mercapturate toxicity.

Rat cortex neurons were shown in this study to contain AA3 forms of ~75 kDa, which are significantly different in size from the renal AA3 (Pushkin et al., 2004). Because there were no differences in kinetics of brain and renal AA3, the physiological significance of the size difference between these AA3 forms is currently unclear. Our results suggest that brain AA3 is a product of dimerization of ~35 kDa monomer via a covalent non-S-S type bond(s).

The expression of AA3 in endothelial cells demonstrated that this enzyme may deacetylate HNE and acrolein mercapturates crossing the blood–brain barrier thus initiating a chain of toxic reactions immediately upon entering the brain. It is important to note that β -lyases that transform deacetylated mercapturates into highly reactive toxic compounds (Fig. 1) are known to be expressed in many organs and tissues including the brain and blood (Cooper, 1994, 2004). It has been proposed (Marchesi, 2011) that AD dementia begins as a disease of small blood vessels damaged by oxidative-induced inflammation, and endothelial dysfunction was also proposed to be involved in PD and other neurodegenerative diseases

(Grammas et al., 2011). Our study provides a potential mechanism for this hypothetical blood vessel damage.

It is surprising that some of the AA3 inhibitors reported in this study have been already used in AD and PD research by other groups. Their results using cell and animal models are in a good correlation with the hypothetical role of AA3 in AD and PD pathogenesis. Promising results obtained with one of the AA3 inhibitor (ibuprofen) in lowering PD incidence in human studies also support the proposed role of AA3 in PD pathology (Gao et al., 2011). Controversial results of ibuprofen in AD human studies (Dumont, Beal, 2011) may reflect an important difference between human and animal studies; namely, human studies include AD patients and patients with mild cognitive impairment that is considered as a pre-AD condition, which already had significant pathological changes in their brains that could not be reversed by AA3 inhibition. We hypothesize that this approach could be rather used for the prophylactics of AD disease not for AD symptoms treatment, and/or could be used in AD patients in combination with other AD drugs.

A metal chelator clioquinol that demonstrated decrease of cognitive impairment in AD patients and animal models, decreased A β burden in animal models (Adlard et al., 2008; Grossi et al., 2009; Ritche, 2003), and rescued the pathogenic phenotype in the MPTP Parkinson's disease mouse model (Kaur et al., 2003), was shown in this study to significantly inhibit AA3 ($IC_{50} = 10 \mu M$). It has been hypothesized that metal ions (Fe^{2+} , Zn^{2+} , Cu^{2+}) are involved in AD and PD pathology via different mechanisms; e.g. their complexation may be beneficial in reducing A β -induced free radical generation and metal-catalyzed A β aggregation (Doraiswamy, Finefrock, 2004; Dumont, Beal, 2011). We have previously demonstrated (Tsirulnikov et al., 2009) that chelating agents inactivate AA3 via depletion of the active site metal ion; therefore the protective effect of chelating agents on AD pathogenesis may also be explained by their inhibition of AA3. Given that metal chelators are expected to inhibit a significant number of metalloenzymes, the benefits of their use in AD and PD prevention/treatment may be outweighed with side effects.

We have demonstrated that Co^{2+} drastically activates mouse AA3 *in vitro* (Tsirulnikov et al., 2009). The mechanism of the mouse AA3 activation by cobalt is not completely understood given that the enzyme may bind up to 4 cobalt ions per monomer (Tsirulnikov et al., 2009) although only 1 metal ion is coordinated to the active site (Hsieh et al., 2010). Although the activating effect of cobalt may be mediated by the Co^{2+} ion coordinated to the active site, the involvement of other cobalt ions in AA3 activation via different mechanisms cannot be excluded. Given a reported drastic increase of cobalt level in rat brain during aging (Maynard et al., 2002), a possible similar increase of cobalt in aging human brain may activate AA3 and therefore play a role in AD and PD pathogenesis. Comparison of cobalt levels in young and old brains, and in normal and AD, and PD brains is necessary to explore this hypothesis.

Ibuprofen ameliorated AD and PD pathology (Dumont, Beal, 2011; Imbimbo et al., 2010; Gao et al., 2011; Kotilinek et al., 2008; Wilkinson et al., 2010), and is an effective AA3 inhibitor ($K_i=40 \mu M$). Previously the protective effect of ibuprofen has been attributed to decrease of inflammation mediated by inhibition of cyclooxygenase 1 (Cox-1) and 2

(Cox-2) involved in formation of prostaglandins, and decrease of A β load (Dumont, Bell, 2011). An important question stemming from the results of these studies concerns the *in vivo* concentration of ibuprofen in animal and human brain, and to what extent these concentrations inhibit Cox-1, Cox-2, and AA3? The steady-state level of ibuprofen in rat brain and plasma was estimated to be ~3.5 and ~180 μ M (Mannila et al., 2005), and the level in human plasma reached ~100 μ M after single dose of 600 mg (Jabor et al., 2002). Assuming that humans have a similar ratio of serum to brain ibuprofen, its level in human brain may be roughly estimated at ~1–3 μ M. Although different results for the affinity of Cox-1 and Cox-2 to ibuprofen have been published, the K_i value for both Cox-1 and Cox-2 may be estimated as ~1 μ M (Ouellet et al., 2004). Therefore, ibuprofen doses used in clinical trials (Imbimbo et al., 2010) are expected to inhibit Cox enzymes at >50%. The minimal cognitive improvement rates in AD patients treated with ibuprofen (Dumont, Beal, 2011) are therefore not likely to be caused by inhibition of Cox enzymes. AA3 inhibition in these patients at the doses achieved is predicted to be ~ 10–20% and may be playing a role.

Although inhibition of individual enzymes of the GSH-dependent pathway of HNE (and acrolein) merapturate formation, and inhibition of β -lyase could be additional therapeutic approaches in prevention of AD and PD, AA3 inhibition has certain hypothetical advantages since AA3, unlike the aforementioned enzyme systems has a very restricted expression pattern (Pushkin et al., 2004) and therefore its inhibition could be associated with less adverse side effects. As reported in this study, AA3 inhibitors may potentially be used as a basis for generation of AD and PD drugs.

In summary, we report here a new potential mechanism for neuronal damage in AD that involves AA3. This previously unrecognized mechanism may also function in PD. Future studies to determine the clinical value of AA3 inhibition in preventing the pathologic abnormalities in AD and PD are warranted.

Acknowledgments

The authors thank Marie-Francoise Chesselet, Greg Cole and Lisa Kurtz for critical discussions during this work, and Michael Jung and Kenneth Bradley for the advice with small molecule screen. A.P. was supported in part by the AHA Western State Affiliate grant 10GRNT4140074.

References

- Abdul HM, Sultana R, St Clair DK, Markesbery WR, Butterfield DA. Oxidative damage in brain from human mutant APP/PS-1 double knock-in mice as a function of age. *Free Radic Biol Med*. 2008; 45:1420–1425. [PubMed: 18762245]
- Adlard PA, Cherny RA, Finkelstein DI, Gautier E, Robb E, Cortes M, Volitakis I, Liu X, Smith JP, Perez K, Laughton K, Li QX, Charman SA, Nicolazzo JA, Wilkins S, Deleva K, Lynch T, Kok G, Ritchie CW, Tanzi RE, Cappai R, Masters CL, Barnham KJ, Bush AI. Rapid restoration of cognition in Alzheimer's transgenic mice with 8-hydroxy quinoline analogs is associated with decreased interstitial Abeta. *Neuron*. 2008; 59:43–55. [PubMed: 18614028]
- Alary A, Guéraud F, Cravedi JP. Fate of 4-hydroxynonenal in vivo: disposition and metabolic pathways. *Mol Aspects Med*. 2003; 24:177–187. [PubMed: 12892995]
- Anders MW, Dekant W. Aminoacylases. *Adv Pharmacol*. 1994; 27:431–448. [PubMed: 8068563]
- Anders MW, Lash L, Dekant W, Elfarra AA, Dohn D. Biosynthesis and biotransformation of glutathione S-conjugates to toxic metabolites. *Crit Rev Toxicol*. 1988; 18:311–341. [PubMed: 3288445]

- Arakawa M, Ishimura A, Arai Y, Kawabe K, Suzuki S, Ishige K, Ito Y. N-acetylcysteine and ebselen but not nifedipine protected cerebellar granule neurons against 4-hydroxynonenal-induced neuronal death. *Neurosci Res.* 2007; 27:220–229.
- Baldeiras I, Santana I, Proenca MT, Garrucho MH, Pascoal R, Rodrigues A, Duro D, Oliveira CR. Oxidative damage and progression to Alzheimer's disease in patients with mild cognitive impairment. *J Alzheimers Dis.* 2010; 21:1165–1177. [PubMed: 21504121]
- Calingasan NY, Uchida K, Gibson GE. Protein-bound acrolein: a novel marker of oxidative stress in Alzheimer's disease. *J Neurochem.* 1999; 72:751–756. [PubMed: 9930749]
- Carlsson CM. Type 2 diabetes mellitus, dyslipidemia, and Alzheimer's disease. *J Alzheimers Dis.* 2010; 20:711–722. [PubMed: 20413858]
- Chen K, Kazachkov M, Yu PH. Effect of aldehydes derived from oxidative deamination and oxidative stress on beta-amyloid aggregation; pathological implications to Alzheimer's disease. *J Neural Transm.* 2007; 114:835–839. [PubMed: 17401529]
- Cooper AJL. Enzymology of cysteine S-conjugate β -lyases. *Adv Pharmacol.* 1994; 27:71–113. [PubMed: 8068566]
- Cooper AJ. The role of glutamine transaminase K (GTK) in sulfur and alpha-keto acid metabolism in the brain, and in the possible bioactivation of neurotoxicants. *Neurochem Int.* 2004; 44:557–577. [PubMed: 15016471]
- Coppede F, Migliore L. DNA damage and repair in Alzheimer's disease. *Curr Alzheimer Res.* 2009; 6:36–47. [PubMed: 19199873]
- Daviglius ML, Bell CC, Berrettini W, Bowen PE, Connolly ES, Cox NJ, Dunbar-Jacob JM, Granieri EC, Hunt G, McGarry K, Patel D, Potosky AL, Sanders-Bush E, Silberberg D, Trevisan M. NIH State-of-the-Science conference statement: preventing Alzheimer's disease and cognitive decline. *NIH Consens State Sci Statements.* 2010; 27:1–30.
- Dekant W, Vamvakas S, Anders M. Formation and fate of nephrotoxic and cytotoxic glutathione S-conjugates: cysteine conjugate β -lyase pathway. *Adv Pharmacol.* 1994; 27:114–162.
- de la Torre JC. Is Alzheimer's disease a neurodegenerative or a vascular disorder? Data, dogma, and dialectics *Lancet Neurol.* 2004; 3:184–190. [PubMed: 14980533]
- Doraiswamy PM, Finefrock AE. Metals in our minds: therapeutic implications for neurodegenerative disorders. *Lancet Neurol.* 2004; 3:431–434. [PubMed: 15207800]
- Driesen HPC, De Jong WW, Tesser GI, Bloemendal H. The mechanism of N-terminal acetylation of proteins. *Crit Rev Biochem Mol Biol.* 1985; 18:281–325.
- Dumont M, Beal MF. Neuroprotective strategies involving ROS in Alzheimer disease. *Free Radic Biol Med.* 2011; 51:1014–1026. [PubMed: 21130159]
- Gao X, Chen H, Schwartzschild MA, Ascherio A. Use of ibuprofen and risk of Parkinson disease. *Neurology.* 2011; 76:863–869. [PubMed: 21368281]
- Grammas P, Martinez J, Miller B. Cerebral microvascular endothelium and the pathogenesis of neurodegenerative diseases. *Expert Rev Mol Med* 2011 Jun. 2011; 10:13, e19.
- Grossi C, Francese S, Casini A, Rosi MC, Luccarini I, Fiorentini A, Gabbiani C, Messori L, Moneti G, Casamenti F. Clioquinol decreases amyloid-beta burden and reduces working memory impairment in a transgenic mouse model of Alzheimer's disease. *J Alzheimers Dis.* 2009; 17:423–440. [PubMed: 19363260]
- Hardy J, Allsop D. Amyloid deposition as the central event in the etiology of Alzheimer's disease. *Trends Pharmacol Sci.* 1991; 12:383–388. [PubMed: 1763432]
- Hsieh JM, Tsirulnikov K, Sawaya MR, Magilnick N, Abuladze N, Kurtz I, Abramson J, Pushkin A. Structures of aminoacylase 3 in complex with acetylated substrates. *Proc Natl Acad Sci USA.* 2010; 107:17962–17967. [PubMed: 20921362]
- Huang Q, Aluisse CD, Joshi G, Sultana R, St Clair DK, Markesbery WR, Butterfield DA. Potential in vivo amelioration by N-acetyl-L-cysteine of oxidative stress in brain in human double mutant APP/PS-1 knock-in mice: toward therapeutic modulation of mild cognitive impairment. *J Neurosci Res.* 2010; 88:2618–2629. [PubMed: 20648652]
- Humphries KM, Yoo Y, Szweda LI. Inhibition of NADH-linked mitochondrial respiration by 4-hydroxy-2-nonenal. *Biochemistry.* 1998; 37:552–557. [PubMed: 9425076]

- Imbimbo BP, Solfrizzi V, Panza F. Are NSAIDs useful to treat Alzheimer's disease or mild cognitive impairment? *Front Aging Neurosci.* 2010; 2:1–19. [PubMed: 20552041]
- Jabor VAP, Lanchote VL, Bonato PS. Enantioselective analysis of ibuprofen in human plasma by anionic cyclodextrin-modified electrokinetic chromatography. *Electrophoresis.* 2002; 23:3041–3047. [PubMed: 12207314]
- Juhasz G, Földi I, Penke B. System biology of Alzheimer's disease: How diverse molecular changes result in memory impairment in AD. *Neurochem Int.* 2011 Feb 17. Epub ahead of print.
- Kaur D, Yantiri F, Rajagopalan S, Kumar J, Mo JQ, Boonplueang R, Viswanath V, Jacobs R, Yang L, Beal MF, DiMonte D, Volitaskis I, Ellerby L, Cherny RA, Bush AI, Andersen JK. Genetic or pharmacological iron chelation prevents MPTP-induced neurotoxicity in vivo: a novel therapy for Parkinson's disease. *Neuron.* 2003; 37:899–909. [PubMed: 12670420]
- Koob M, Dekant W. Bioactivation of xenobiotics by formation of toxic glutathione conjugates. *Chem Biol Interact.* 1991; 77:107–136. [PubMed: 1991332]
- Kördel W, Schneider F. Renal aminoacylase, a zinc enzyme. *Z Naturforsch.* 1977; 32C:342–344.
- Kotilinek LA, Westerman MA, Wang Q, Panizzon K, Lim GP, Simonyi A, Lesne S, Falinska A, Younkin LH, Younkin SG, Rowan M, Cleary J, Wallis RA, Sun GY, Cole G, Frautschy S, Anwyl R, Ashe KH. Cyclooxygenase-2 inhibition improves amyloid- β -mediated suppression of memory and synaptic plasticity. *Brain.* 2008; 131:651–664. [PubMed: 18292081]
- Lash LH, Putt DA, Parker JC. Metabolism and tissue distribution of orally administered trichloroethylene in male and female rats: identification of glutathione- and cytochrome P450-derived metabolites in liver, kidney, blood and urine. *J Toxicol Environ Health A.* 2006; 69:1285–1309. [PubMed: 16754541]
- Lindner HA, Lunin VV, Alary A, Hecker R, Cygler M, Menard R. Essential roles of zinc ligation and enzyme dimerization for catalysis in the aminoacylase-1/M20 family. *J Biol Chem.* 2003; 278:44496–44504. [PubMed: 12933810]
- Lindner HA, Täfler-Naumann M, Röhm KH. N-acetylamino acid utilization by kidney aminoacylase-1. *Biochimie.* 2008; 90:773–780. [PubMed: 18222180]
- Long PM, Stradecki HM, Minturn JE, Wesley UV, Jaworski DM. Differential aminoacylase expression in neuroblastoma. *Int J Cancer.* 2010; 129:1322–1330.
- Lovell MA, Ehmann WD, Mattson MP, Markesberry WR. Elevated 4-hydroxynonenal in ventricular fluid in Alzheimer's disease. *Neurobiol Aging.* 1997; 18:457–461. [PubMed: 9390770]
- Lovell MA, Markesberry WR. Oxidative DNA damage in mild cognitive impairment and late-stage Alzheimer's disease. *Nucleic Acids Res.* 2007; 35:7497–7504. [PubMed: 17947327]
- Lovell MA, Markesberry WR. Oxidatively modified RNA in mild cognitive impairment. *Neurobiol Dis.* 2008; 29:169–175. [PubMed: 17920285]
- Lovell MA, Xie C, Markesberry WR. Acrolein is increased in Alzheimer's disease brain and is toxic to primary hippocampal cultures. *Neurobiol Aging.* 2001; 22:187–194. [PubMed: 11182468]
- Magni F, Galbusera C, Tremolada L, Ferrarese C, Kienle MG. Characterisation of adducts of the lipid peroxidation product 4-hydroxy-2-nonenal and amyloid beta-peptides by liquid chromatography/electrospray ionisation mass spectrometry. *Rapid Commun Mass Spectrom.* 2002; 16:1485–1493. [PubMed: 12125026]
- Mannila A, Rautio J, Lehtonen M, Savolainen J. Inefficient central nervous system delivery limits the use of ibuprofen in neurodegenerative diseases. *Eur J Pharm Sci.* 2005; 24:101–105. [PubMed: 15626583]
- Marchesi VT. Alzheimer's dementia begins as a disease of small blood vessels, damaged by oxidative-induced inflammation and dysregulated amyloid metabolism: implications for early detection and therapy. *FASEB J.* 2011; 25:5–13. [PubMed: 21205781]
- Markesberry WR, Kryscio RJ, Lovell MA, Morrow JD. Lipid peroxidation is an early event in the brain in amnesic mild cognitive impairment. *Ann Neurol.* 2005; 58:730–735. [PubMed: 16240347]
- Maynard CJ, Cappai R, Volitakis I, Cherny RA, White AR, Beyreuter K, Masters CL, Bush AI, Li QX. Overexpression of Alzheimer's disease amyloid- β opposes the age-dependent elevations of brain copper and iron. *J Biol Chem.* 2002; 277:44670–44676. [PubMed: 12215434]

- Montine KS, Kim PJ, Olson SJ, Markesbery WR, Montine TJ. 4-hydroxy-2-nonenal pyrrole adducts in human neurodegenerative disease. *J Neuropathol Exp Neurol.* 1997; 56:866–871. [PubMed: 9258256]
- Montine KS, Reich E, Neely MD, Sidell KR, Olson SJ, Markesbery WR, Montine TJ. Distribution of reducible 4-hydroxynonenal adduct immunoreactivity in Alzheimer disease is associated with APOE genotype. *J Neuropathol Exp Neurol.* 1998; 57:415–425. [PubMed: 9596412]
- Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Meth.* 1983; 65:55–63.
- Murray IV, Proza JF, Sohrabji F, Lawler JM. Vascular and metabolic dysfunction in Alzheimer's disease: a review. *Exp Biol Med.* 2011; 236:772–782.
- Newman D, Sholtz K, Dekant W, Abuladze N, Tsuprun V, Ryazantsev S, Bondar G, Sassani P, Kurtz I, Pushkin A. Specificity of aminoacylase III-mediated deacetylation of mercapturic acids. *Drug Metab Dispos.* 2007; 35:43–50. [PubMed: 17012540]
- Nunomura A, Moreira PI, Takeda A, Smith MA, Perry G. Oxidative RNA damage and neurodegeneration. *Curr Med Chem.* 2007; 14:2968–2975. [PubMed: 18220733]
- Ouellet M, Falgoutyret JP, Percival MD. Detergents profoundly affect inhibitor potencies against both cyclo-oxygenase isoforms. *Biochem J.* 2004; 377:675–684. [PubMed: 14510637]
- Parnham M, Sies H. Ebselen: prospective therapy for cerebral ischaemia. *Expert Opin Investig Drugs.* 2000; 9:607–619.
- Perrier J, Durand A, Giardina T, Puigserver A. Catabolism of intracellular N-terminal acetylated proteins: involvement of acylpeptide hydrolase and acylase. *Biochimie.* 2005; 87:673–685. [PubMed: 15927344]
- Perrson B, Flinta C, von Heijne G, Jörnvall H. Structures of N-terminally acetylated proteins. *Eur J Biochem.* 1985; 152:523–527. [PubMed: 4054119]
- Picklo MJ Sr, Montine TJ, Amarnath V, Neely MD. Carbonyl toxicology and Alzheimer's disease. *Toxicol Appl Pharmacol.* 2002; 184:187–197. [PubMed: 12460747]
- Pratico D. The neurobiology of isoprostanes and Alzheimer's disease. *Biochim Biophys Acta.* 2010; 1801:930–933. [PubMed: 20116452]
- Pushkin A, Carpenito G, Abuladze N, Newman D, Tsuprun V, Ryazantsev S, Motemoturu S, Sassani P, Solovieva N, Dukkupati R, Kurtz I. Structural characterization, tissue distribution, and functional expression of murine aminoacylase III. *Am J Physiol.* 2004; 286:C848–C856.
- Qin Z, Hu D, Han S, Reaney SH, Di Monte DA, Fink AL. Effect of 4-hydroxy-2-nonenal modification on alpha-synuclein aggregation. *J Biol Chem.* 2007; 282:5862–5870. [PubMed: 17189262]
- Ritchie CW, Bush AI, Mackinnon A, Macfarlane S, Mastwyk M, MacGregor L, Kiers L, Cherny R, Li QX, Tammer A, Carrington D, Mavros C, Volitakis I, Xilinas M, Ames D, Davis S, Beyreuther K, Tanzi RE, Masters CL. Metal-protein attenuation with iodocholehydroxyquin (clioquinol) targeting Abeta amyloid deposition and toxicity in Alzheimer disease: a pilot phase 2 clinical trial. *Arch Neurol.* 2003; 60:1685–1691. [PubMed: 14676042]
- Sass JO, Mohr V, Olbrich H, Engelke U, Horvath J, Fliegauf M, Loges NT, Schweitzer-Krantz S, Moebus R, Weiler P, Kispert A, Superti-Furga A, Wevers RA, Omran H. Mutations in ACY1, the gene encoding aminoacylase 1, cause a novel inborn error of metabolism. *Am J Hum Genet.* 2006; 78:401–409. [PubMed: 16465618]
- Sayre LM, Zelasko DA, Harris PL, Perry G, Alomon RG, Smith MA. 4-hydroxynonenal-derived advanced lipid peroxidation and products are increased in Alzheimer's disease. *J Neurochem.* 1997; 68:2092–2097. [PubMed: 9109537]
- Shamoto-Nagai M, Maruyama W, Hashizume Y, Yoshida M, Osawa T, Riederer P, Naoi M. In parkinsonian substantia nigra, alpha-synuclein is modified by acrolein, a lipid-peroxidation product, and accumulates in the dopamine neurons with inhibition of proteasome activity. *J Neural Transm.* 2007; 114:1559–1567. [PubMed: 17690948]
- Sidell KR, Montine KS, Picklo MJ Sr, Olsen SJ, Amarnath V, Montine TJ. Mercapturate metabolism of 4-hydroxy-2-nonenal in rat and human cerebrum. *J Neuropathol Exp Neurol.* 2003; 62:146–153. [PubMed: 12578224]

- Siegel SJ, Bieschke J, Powers ET, Kelly JW. The oxidative stress metabolite 4-hydroxynonenal promotes Alzheimer protofibril formation. *Biochemistry*. 2007; 46:1503–1510. [PubMed: 17279615]
- Sultana R, Perluigi M, Butterfield DA. Oxidatively modified proteins in Alzheimer's disease (AD), mild cognitive impairment and animal models of AD: role of Abeta in pathogenesis. *Acta Neuropathol*. 2009; 118:131–150. [PubMed: 19288120]
- Tsirulnikov K, Abuladze N, Newman D, Ryazantsev S, Wolak T, Magilnick N, Koag M-C, Kurtz I, Pushkin A. Mouse aminoacylase 3: a metalloenzyme activated by cobalt and nickel. *Biochim Biophys Acta*. 2009; 1794:1049–1057. [PubMed: 19362172]
- Uttamsingh V, Anders MW. Acylase-catalyzed deacetylation of haloalkene-derived mercapturates. *Chem Res Toxicol*. 1999; 12:937–942. [PubMed: 10525269]
- Uttamsingh V, Baggs RB, Krenitsky DM, Anders MW. Immunohistochemical localization of the acylases that catalyze the deacetylation of N-acetyl-L-cysteine and haloalkene-derived mercapturates. *Drug Metab Dispos*. 2000; 28:625–632. [PubMed: 10820133]
- Uttamsingh V, Keller DA, Anders MW. Acylase I-catalyzed deacetylation of N-acetyl-L-cysteines. *Chem Res Toxicol*. 1998; 11:800–809. [PubMed: 9671543]
- Van Coster RN, Gerlo EA, Giardina TG, Engelke UF, Smet JE, De Praeter CM, Meersschant VA, De Meirleir LJ, Seneca SH, Devreese B, Leroy JG, Herga S, Perrier JP, Wevers RA, Lissens W. Aminoacylase I deficiency: a novel inborn error of metabolism. *Biochem Biophys Res Commun*. 2005; 338:1322–1326. [PubMed: 16274666]
- Wilkinson BJ, Cramer PE, Varvel NH, Reed-Geaghan E, Jiang Q, Shabo A, Herrup K, Lamb BT, Landreth GE. Ibuprofen attenuates oxidative damage through NOX2 inhibition in Alzheimer's disease. *Neurobiol Aging*. 2012; 33:197, e21–e32.
- Wu HB, Tsou CL. A comparison of Zn(II) and Co(II) in the kinetics of inactivation of aminoacylase by 1,10-phenanthroline and reconstitution of the apoenzyme. *Biochem J*. 1993; 296:435–441. [PubMed: 8257435]
- Yamagata K, Tagami M, Yamori Y. Neuronal vulnerability of stroke-prone spontaneously hypertensive rats to ischemia and its prevention with antioxidants such as vitamin E. *Neuroscience*. 2010; 170:1–7. [PubMed: 20633610]
- Yamaguchi T, Sano K, Takakura K, Saito I, Shinohara Y, Asano T, Yasuhara H. Ebselen in acute ischemic stroke: a placebo-controlled, double-blind clinical trial. Ebselen Study Group Stroke. 1998; 29:12–17.
- Yang X, Dai G, Li G, Yang ES. Coenzyme Q10 reduces beta-amyloid plaque in an APP/PS1 transgenic mouse model of Alzheimer's disease. *J Mol Neurosci*. 2010; 41:110–113. [PubMed: 19834824]
- Yang X, Yang Y, Li G, Wang J, Yang ES. Coenzyme Q10 attenuates beta-amyloid pathology in the aged transgenic mice with Alzheimer presenilin 1 mutation. *J Mol Neurosci*. 2008; 34:165–171. [PubMed: 18181031]
- Yoritaka A, Hattori N, Uchida K, Tanaka M, Stadtman ER, Mizuno Y. Immunohistochemical detection of 4-hydroxynonenal protein adducts in Parkinson disease. *Proc Natl Acad Sci USA*. 1996; 93:2696–2701. [PubMed: 8610103]

Fig. 1.

The GSH conjugation pathway of HNE. The conjugation with GSH mediated by GSH transferase (GST) initiates HNE detoxification. Following steps catalyzed by γ -glutamyltransferase (γ -GT), dipeptidase (DP) and N-acetyl transferase (AT), form NA-Cys conjugate of HNE (HNE-NA-Cys or HNE mercapturate). Acylase mediates deacetylation of HNE-mercapturate, which generates a substrate for β -lyase forming a highly reactive toxic product(s).

Fig. 2.

Characterization of CH-4 chicken anti-mouse AA3 antibody. Paraffin embedded mouse kidney sections were incubated for 1 h at 37°C with CH-4 antibody diluted 1:100 in PBS (A) or with CH-4 antibody (diluted 1:100 in PBS) preincubated with immunizing peptides (B). After wash with PBS sections were incubated for 20 min at room temperature with a CY3 conjugated donkey anti-chicken antibody (Jackson ImmunoResearch). Images were collected on an Olympus BH2 fluorescent microscope. A typical cytoplasmic expression of AA3 in S2 proximal tubules (A) is shown. The identical pattern of AA3 expression in unfixed frozen mouse kidney sections has been shown using our MR-C1 rabbit anti-mouse AA3 affinity purified antibody (Pushkin et al., 2004). (C): Immunoblotting of mouse kidney proteins with CH-4 antibody (-P) and with CH-4 antibody preincubated with immunizing peptides (+P).

Fig. 3.

Immunolocalization of AA3 in mouse brain. AA3 in frozen mouse brain coronal sections in the hippocampus, thalamus and hypothalamus area was immunolocalized with CH-4 chicken anti-mouse AA3 antibody and a secondary CY3-conjugated donkey anti-chicken antibody (A). CH-4 antibody preincubated with immunizing peptides were shown as control (B). To immunolocalize neurons (C), a mouse monoclonal antibody anti-NeuN (Clone 60, Millipore) was used at 1:100 dilution. Alexa Fluor 488 conjugated goat anti-mouse antibody (Molecular Probes) was used at 1:500 dilution. Merged images are shown in (D). Confocal images were collected on a Leica TCS-SP2 AOBS inverted confocal microscope

Fig. 4.

(A) Docking of ibuprofen (olive) to the active site of mouse AA3. The position of NA-Tyr (green) in the crystallographic structure of mouse AA3 complexed with NA-Tyr (Hsieh et al., 2010) is shown for comparison. (B) Docking of EPMS (olive) to the active site of mouse AA3. The position of NA-DCVC (green) in the crystallographic structure of mouse AA3 complexed with NA-DCVC (Hsieh et al., 2010) is shown for comparison.

Fig. 5A.

Fig. 5B.

Fig. 5.

(A) The effect of ebselen (grey columns) and EPMS (black columns) on the toxicity of HNE and HNE mercapturate in primary rat brain cortex neurons (Invitrogen). Ebselen or EPMS (20 μ M each) were added to media simultaneously with HNE, HNE mercapturate or PBS (control). After 20 h incubation, cell viability was determined using the MTT cell viability assay (Mosmann, 1983). (B) Immunoblotting of rat brain cortex neurons with CH-4 antibody (-P) and with the same antibody preincubated with immunizing peptides (+P).

Table 1

Kinetic characteristics of mouse AA3.

AA3 activity was assayed with (+) and without (–) 0.1 mM Co^{2+} . Kinetic characteristics of mouse AA3 with NA-DCVC and NA-Tyr have been previously reported (Newman et al., 2007; Tsirulnikov et al., 2009).

Substrate	Co^{2+}	K_m , mM	K_{cat} , s^{-1}	K_{cat}/K_m , $\text{s}^{-1}\cdot\text{M}^{-1}$
HNE mercapturate	–	0.20	0.27	1,350
	+	0.53	1.67	3,150
Acrolein mercapturate	–	0.82	0.40	490
	+	0.71	3.03	4,270
NA-DCVC	–	0.45	0.28	620
	+	0.30	2.28	7,600
NA-Tyr	–	1.30	0.13	100
	+	0.56	1.09	1,930

Table 2

Selected AA3 inhibitors identified during small molecule screen.

Inhibitor name	Inhibitor structure	IC ₅₀ , nM
EPMS		0.63
Ebselen		1.00
5158512		3.20
T5836794		40.0

Inhibitor name	Inhibitor structure	IC ₅₀ , nM
T5660792		79.0
5649910		1,600
Clioquinol		10,100
5311633		63,000

Table 3

The K_i values of ibuprofen, NA-Cys and selected AD drugs for mouse AA3.

Inhibitor	K_i , mM
Ibuprofen	0.04
Tacrine	>10
Memantine	>10
Donepezil	>10
NA-Cys	3.0