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Specific triazine herbicides induce amyloid β 42 production

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Running title: Triazine herbicides induce amyloid-β42 production

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CONFLICT OF INTEREST STATEMENT

LM and HG are co-founders of ManRos Therapeutics. HZ reports no conflicts of interest.

ABSTRACT

Background. Proteolytic cleavage of the amyloid precursor protein (APP) by secretases leads to extracellular release of amyloid β (Aβ) peptides. Increased production of Aβ42 over Aβ40 and aggregation into oligomers and plaques constitute an Alzheimer's disease (AD) hallmark.
Objectives. Identifying products of the 'human chemical exposome' (HCE) able to induce Aβ42 production is key to understand the initiating causes of AD and to generate non-genetic animal models of AD.
Methods. A cell model was used to screen chemical libraries for Aβ42 inducers. Active molecules were extensively characterized.
Results. Six herbicides triazines induced a 2-10 fold increase in the production of extracellular Aβ42 in various cell lines, primary neuronal cells and neurons differentiated from human induced pluripotent stem cells (iPSCs). Induced Aβ42 production by triazines requires active secretases. Immunoprecipitation/mass spectrometry analyses showed enhanced production of Aβ peptides cleaved at positions 42 and 43, and reduced production of peptides cleaved at positions 38 and lower. Neurons derived from iPSCs obtained from a familial AD (FAD) patient (APP K724N) produced more Aβ42 vs. Aβ40 than neurons derived from healthy controls iPSCs (APP WT). Triazines further enhanced Aβ42 production in both control and AD neurons. Triazines also shifted the cleavage pattern of alcadeins, another family of γ-secretase substrates, suggesting a direct effect of triazines on γ-secretase.
Conclusions. Some widely used triazines enhance the production of toxic, aggregation-prone Aβ42/Aβ43 amyloids, suggesting the possible existence of environmental 'Alzheimerogens' which may contribute to the initiation and propagation of the amyloidogenic process in AD.

INTRODUCTION

Proteolytic processing of amyloid precursor protein (APP) by β- and γ-secretases leads to the production of various Aβ peptides, including the 42 amino acid form which plays a crucial role in Alzheimer's disease (AD) (Huang and Mucke 2012; Selkoe et al. 2012; Vinters 2015). The action of β-secretase, or beta-site amyloid precursor protein cleaving enzyme 1 (BACE1), first leads to a soluble extracellular fragment (sAPPβ) and a membrane bound fragment (βCTF, β-carboxyl-terminal fragment). γ-Secretase then acts on βCTF, leading to the generation of Aβ peptides of various lengths and release of the APP intracellular domain (AICD). Aβ peptides tend to aggregate as extracellular oligomers and ultimately as plaques, one of the clinical hallmarks of AD.

Aβ40 is the most abundantly produced Aβ peptide. Considerable data indicates that generation of the aggregation-prone Aβ42 strongly correlates with the onset and development of AD. In early onset AD (EOAD) (<1% of all cases), mutations in APP, or the γ-secretase subunits PSEN1 & PSEN2 (review in Bateman et al. 2011), all lead to enhanced Aβ42 production and/or increased Aβ42/Aβ40 ratio, a critical factor in AD pathology initiation (Kuperstein et al. 2010). Increased Aβ42/Aβ40 ratio is also found in brain tissue in late onset AD (LOAD) (>99% of AD cases). Aβ42 is more toxic than Aβ40, a consequence of its higher stability and strong tendency to oligomerize and to aggregate in plaques (McGowan et al. 2005; Findeis 2007; Gouras et al. 2014). Aβ43 is also enriched in AD patients' brains and has been reported as a toxic, aggregation-prone amyloid, inducing strong AD phenotypes in mice (Welander et al. 2009; Saito et al. 2011; Sandebring et al. 2013; Conicella et al. 2014).

We recently reported that some tri-substituted purines, the Aftins (**A**myloid β **F**orty-**T**wo **In**ducers), trigger a robust, secretases-dependent increase in extracellular Aβ42 production in cultured cells (Bettayeb et al. 2012; Hochard et al. 2013). Under these conditions Aβ38 levels dropped while Aβ40 remained relatively stable. These results suggest that (i) such molecules might constitute new pharmacological tools to investigate the mechanisms underlying increased Aβ42/Aβ40 ratio observed in AD, (ii) these molecules might contribute to generate a chemically induced animal model of AD (Meunier et al. 2015) and (iii) some simple, low molecular weight (LMW) products in our environment might shift the Aβ42/Aβ40 ratio similarly to what is seen in AD patients and might thus contribute to the development, acceleration or even initiation of LOAD.

We therefore screened for potential Aβ42 inducing molecules in libraries of human chemical exposome (HCE) products (Rappaport 2011; Wild 2005, 2012; Juarez et al. 2014; Vrijheid et al. 2014; Wishart et al. 2015). We here report that a subset of the widely used triazine herbicides is able to shift Aβ production towards longer, aggregation-prone amyloid peptides (Aβ42/Aβ43) at the expense of shorter variants (Aβ37, Aβ38, Aβ40). In addition, production of the shorter Aβ1-16 and Aβ1-17 peptides that are generated by sequential β- and γ-secretase cleavages (Portelius et al. 2011; Pérez-Grijalba et al. 2015) was also enhanced. This effect is observed in various cell lines, primary neuron cultures and neurons differentiated from iPSCs obtained from healthy or AD patients. Triazines shift the cleavage pattern of alcadeins, another family of γ-secretase substrates (Araki et al. 2007; Hata et al. 2009; Kamogawa et al. 2012; Piao et al. 2013; Omori et al. 2014), in a way similar to the APP

cleavage shift, suggesting a direct effect on γ-secretase rather than on its substrates. Altogether these data support our hypothesis that the HCE contains products able to modulate γ-secretase activity towards the production of high MW, aggregation prone, AD-associated amyloids. Such products could be qualified as potential “Alzheimerogens”. Their identification and regulation might constitute a key step in AD prevention.

METHODS

Triazines and other reagents, cell lines and primary neuron cultures, cell viability, transient transfections with APP truncation mutants, human iPSCs-derived neuronal cultures, amyloids sample preparation and ELISA capture assays: see Supplementary Material.

Mass spectrometric quantification of amyloids by selected reaction monitoring (SRM)

Solid phase extraction, liquid chromatography and SRM analysis of Aβ species was performed as described previously (Leinenbach et al. 2014; Pannee et al. 2013) with the following modifications. Standard curves for Aβ38 and 42 were prepared at 0.15, 0.5, 1, 2, 3 and 4 ng/mL while Aβ40 was prepared at 15, 50, 100, 200, 300 and 400 ng/mL using unlabeled peptides (rPeptide) in DMEM/F12 supplemented with 0.5% FBS. Uniformly labeled ^{15}N-Aβ38, 40 and 42 peptides (rPeptide) were added to a final concentration of 1.6 ng/mL in calibrators and unknown samples as internal standards. Standard curves were constructed using the unlabeled to ^{15}N-Aβ peak area ratios and fitted using linear regression. All standard curves were linear and had an R^2 value greater than 0.998. Concentrations of unknowns were extrapolated from the standard curves using the peak area ratio of endogenous to ^{15}N-Aβ.

Amyloids profile analysis by immunoprecipitation / mass spectrometry (IP-MS)

Immunoaffinity capture of Aβ species was combined with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS for analyzing a variety of Aβ peptides in a single analysis as described (Portelius et al. 2007). In brief, the anti-Aβ antibodies 6E10 and 4G8 were separately coupled to magnetic beads. After washing of the beads, the 4G8 and 6E10 coated beads were used in combination for immunoprecipitation. After elution of the immune-purified Aβ peptides, analyte detection was performed on an UltraFlextreme MALDI TOF/TOF instrument (Bruker Daltonics). For each peak the areas were normalized against the sum for all the Aβ peaks in the spectrum followed by averaging of results for separately determined duplicate samples (Brinkmalm et al. 2012; Portelius et al. 2013).

HEK293 cell culture and Alcadein fragments analysis

The full length human Alcadeinα1 (Alcα) open reading frame (Araki et al. 2003) was subcloned into the HindIII and XbaI sites of pcDNA3.1 (Hygro+) vector (Invitrogen), transfected into HEK293 cells with Lipofectamine 2000, and cells stably expressing Alcα were cloned. The cells cultured in dish coated with poly-L-lysine were treated with Aftin-5 or triazines (100 μM) for 24 h. The secreted p3-Alcα were recovered from the cultured medium by immunoprecipitation with anti-p3-Alcα UT175 antibody, an antibody raised to a antigen peptide composed of Cys plus the human Alcα1 839-851 sequence, using Protein G-Sepharose beads. The beads were sequentially washed and samples were eluted with trifluoroacetic acid/acetonitrile/water (1:20:20) saturated with sinapinic acid, and subject to MALDI-TOF/MS analysis using an Ultraflex II TOF/TOF (Bruker Daltonics). Molecular masses were calibrated using the peptide calibration standard (Bruker Daltonics) (Hata et al.

174 2009).

RESULTS

Screening the HCE reveals triazines as Aβ42 inducers

A library of 3500+ LMW products representative of the HCE was assembled. All compounds were tested for their ability to trigger extracellular Aβ42 production by N2a-APP695 cells at 1, 10 and 100 µM (not shown). In parallel, cell viability assays were run to assess cell survival at these concentrations. The vast majority of products were unable to induce Aβ42 production. Among the few active products we identified several triazines. Triazines are widely used as herbicides, anti-fouling agents or flame retardants (reviews in Lebaron et al. 2012). We next tested a library of 37 triazines representing the most produced triazines worldwide (**1-37**, Supplementary Table S1), along with Aftin-5 (**38**) as a positive control, on both N2a-APP695 and CHO-7PA2-APP751 cells for their ability to trigger Aβ42 production initially at 1, 10 and 100 µM (Supplementary Table S2). Six triazines were found to induce more than a 3-fold change in Aβ42 (Figure 1A, 1B): Ametryn, Prometryn, Dipropetryn, Terbutryn, Cybutryne, Dimethametryn. As observed with Aftins (Bettayeb et al. 2012; Hochard et al. 2013), Aβ42 production was strongly inhibited by inhibitors of β- (inhibitor IV) and γ-secretases (BMS 299897, DAPT) and by a γ-secretase modulator ('Torrey Pines' compound) (Figure 1C). Similarly, Aβ38 production was strongly reduced, while Aβ40 levels were only modestly affected (less than 2 fold increase) (not shown). Most of the triazines are metabolized in the environment. We thus tested some of the Cybutryne/Terbutryn metabolites (**39-44**) (Supplementary Figure S1) for their ability to trigger Aβ42 production in N2a-APP695 and CHO-7PA2 cells. None of the tested metabolites was active as an inducer of Aβ42 production (not shown). We nest tested a library of 236 triazines that had been synthesized for affinity chromatography, for their ability to induce Aβ42 production (Ahn et al. 2007; Lee et al. 2009). Twenty-one of these (**45-65**) showed significant enhancement of Aβ42 production (Supplementary Table S3), showing that Aβ42 induction is an intrinsic property of some triazines. Affinity chromatography attempts with immobilized triazines did not allow us to purify specific targets, because of unselective hydrophobic interactions (not shown).

Results were confirmed with HEK293-APPsw (not shown) and neurons derived from human iPSCs (see below). We also analyzed the effects of triazines on primary neuronal cultures prepared from E18 OFA rat embryo brains. Neurons were exposed to 100 µM of each triazine for 18 h, and the supernatants were collected for Aβ determination by ELISA assays. Results show that triazines also induce an increase in Aβ42 production by primary neurons. The Aβ-42/Aβ40 ratios were strongly increased (Figure 1D).

Mass spectrometry quantification and profile analysis of induced amyloids

The amyloid peptides Aβ-38, Aβ40 and Aβ42 were quantified in the supernatants of N2a-APP695 (Figure 2A) and CHO-7PA2-APP751 (Figure 2B) using SRM (Leinenbach et al. 2014; Pannee et al. 2013). Like Aftins (Bettayeb et al. 2012; Hochard et al. 2013), the triazines induced a reduction in Aβ38 levels, a slight increase or modest decrease in Aβ40 levels and a strong increase in Aβ42 levels (Figure 2, bottom). The Aβ42/Aβ40 ratios were strongly increased (Figure 2, top).

We next analyzed, by IP-MS, the range of Aβ produced by both cell lines exposed to each of the six triazines and Aftin-5. Cell supernatants were collected, amyloid peptides were immunoprecipitated and analyzed using MALDI TOF/TOF (Brinkmalm et al. 2012; Portelius et al. 2013). Examples of spectra for N2a-APP695 and CHO-7PA2 cells exposed to Terbutryn, Aftin-5 and DMSO are provided in Figure 3A and 4A, respectively. Results show that exposure to triazines increased the production of Aβ 1-17, 11-42, 5-42 and 1-42, while the production of Aβ 1-19, 1-27, 1-33, 1-38, 1-39 was reduced (Figure 3B, 4B). Other amyloid peptides (including Aβ 1-40) showed only modest changes. Aβ 1-43, a highly neurotoxic amyloid (Welander et al. 2009; Saito et al. 2011; Sandebring et al. 2013; Conicella et al. 2014) was undetectable in supernatants of control cells but strongly induced in Aftin-5 and triazine-treated cells.

Neurons differentiated from human iPSCs from AD patients and healthy controls.

We next tested the effects of aftin-5 and the active triazines on neurons differentiated from human iPSCs derived from healthy individuals (APP WT, wild-type) or from AD patients (APP K724N mutation) (Mertens et al. 2013; Koch et al. 2012) (Figure 5). Neurons were first differentiated for either 4 or 10 weeks from iPSCs derived from healthy patient, before 24 h exposure to 100 µM Aftin-5 or Terbutryn (Figure 5A). Treatment resulted in a 2-3 fold increase in the levels of Aβ42 levels compared to neurons exposed to DMSO. Aβ40 levels remained essentially unchanged. We next tested the effects of all six triazines on neurons differentiated from iPSCs (from healthy volunteer or AD patient with APP K724N) (Koch et al. 2009, 2012) (Figure 5B). APP K724N neurons produced more Aβ42 versus Aβ40 compared to APP WT neurons. Addition of Aftin-5 or any of the six active triazines resulted in a further increase in Aβ42 production, in both APP WT and APP K724N neurons.

APP sequence requirements for Aβ42 induction by triazines

To investigate the molecular mechanisms and possible epsilon cleavage sites requirement for the induced Aβ42 production, we generated six APP truncations and expressed them in N2a cells (Figure 6A). All cell lines were the exposed first to 100 µM Aftin-5 and Aβ42 production was measured (Figure 6B). Full-length (FL) and the first three truncations displayed enhanced Aβ42 production (Figure 6B). In contrast, the three last truncations did not allow enhanced Aβ42 production when cells were exposed to Aftin-5. Cells expressing FL APP and truncations 1, 3, 4 were next exposed to 100 µM of each triazine (Figure 6C). Aβ42 production assays show that although T3 allows stimulation of Aβ42 production, T4 does not. These results reveal a strong APP structural requirement for enhanced Aβ42 production induced by Aftin-5 and triazines, which seems to correspond to the ε cleavage site of APP by γ-secretase. At least 10 residues downstream of the Aβ42 cleavage site are required for the full effect of Aftin-5 and triazines.

Triazines and Aftin-5 shift the cleavage pattern of the γ-secretase substrates alcadeins/ calsyntenins

Like APP, alcadeins/calsyntenins are sequentially cleaved by secretases, first by α-secretase, leading to an N-terminal and a C-terminal fragment, the latter being then cleaved by γ-secretase to an intracellular domain and the p3-Alcs peptide, in a way similar to APP (Hata

et al. 2009; Piao et al. 20013) (Figure 7A). To investigate the effects of triazines on alcadeins cleavage, we used HEK293 cells stably expressing full length alcadein α. Alcadein α is first cleaved on the N-terminal side (two possible sites) followed by cleavage by γ-secretase leading to p3-Alcα35 and p3-Alcα 2N+35, the later representing the major peptide in cultured cells (Figure 7A). Cleavage at nearby sites (Figure 7A, blue arrows) leads to other peptides which are less abundant. HEK293-alcadein α cells were grown till 60% confluence and treated with 100 µM Aftin-5 or triazines for 24 h. The secreted p3-Alcα peptides were recovered and analyzed by MALDI TOF/MS (Figure 7B). Quantification of the different p3-Alc peptides showed that, compared to the p3-Alc peptide profile in vehicle treated cells, the concentration of the main alcadein peptide (p3-Alcα 2N+35) and the p3-37 peptide remained stable. In contrast both p3-34 and p3-36 concentrations dropped by about 50 % and the p3-38 peptide concentration increased massively (up to 28.1 fold for dimethametryn; 16.8 fold for Aftin-5) (Figure 7C). These results show that, like for APP, triazines and Aftin induce a shift in the cleavage pattern of alcadeins, another family of γ-secretase substrates, suggesting that these products are more likely to interact with γ-secretase rather than its substrates.

DISCUSSION

Induction of Aβ42 production, shift in Aβ42/Aβ40 ratio

Various drugs (fenofibrate, celecoxib, indomethacin, isoprenoids) (Kukar et al. 2005), DAPT under certain conditions (Svedružić et al. 2013; Barnwell et al. 2013), steroids (Jung et al. 2013), ceramide analogs (Takasugi et al. 2015), SIN-1 (a peroxynitrite donor) (Guix et al. 2012) have been shown to increase the Aβ42/Aβ40 ratio, mostly by increasing Aβ42 production, though never to the high level seen with Aftins (Bettayeb et al. 2012; Hochard et al. 2013). We anticipated that other chemical families able to trigger Aβ42 production would be identified. We here show that some, but not all, widely used (though mostly banned nowadays) herbicide triazines induce the massive production of AD-associated Aβ42 in a variety of cell types. Consequently the Aβ42/Aβ40 ratio is increased, as observed in both EOAD (genetic origin) and LOAD (environmental, epigenetic origin). Detailed analysis of the produced amyloids reveals a pattern clearly associated with AD onset, such as increased Aβ1-16/17 (Portelius et al. 2011; Pérez-Grijalba et al. 2015), Aβ1/5/11-42, Aβ1-43 (Welander et al. 2009; Saito et al. 2011; Sandebring et al. 2013; Conicella et al. 2014), and decreased Aβ1-33/37/38. The underlying molecular mechanisms remain unclear. However several remarks can be made:

(1) there is a clear structure/activity relationship within triazines, as also observed with Aftins: not all products of the chemical class are active. This suggests specific molecular interactions rather than unspecific effects such as detergent, hydrophobic, membrane or protein structure disrupting actions.

(2) the mechanism of action is more likely to involve an effect on γ-secretase and/or its micro-environment rather than an interaction with its substrates, as shown by the fact that Aftins and triazines also induce a shift in the cleavage pattern of alcadeins, another γ-secretase substrate. The APP truncation experiments clearly suggest a very specific molecular requirement rather than a global, non-selective effect.

(3) despite extensive proteomics studies (not shown) we were unable to detect major/significant modifications of protein expression that might be linked to the APP cleavage shift induced by triazines, suggesting that RNA or protein synthesis alterations are unlikely involved in the induction of Aβ42 production. We were also unable to identify a specific target of triazines through affinity chromatography/proteomics approaches, suggesting that either the lipid raft comprising the γ-secretase or rather hydrophobic domains of γ-secretase might constitute the real targets of triazines (and Aftins).

"Alzheimerogens" in the HCE?

The virtual organic chemistry space accessible using currently known synthetic methods is estimated to be between 10^{20} and 10^{24} molecules (Ertl 2003). The Chemical Abstracts Service (CAS) registry, the World's largest chemical database, contains more than 101 million organic and non-organic substances. About 15,000 novel substances are registered every day, representing on average one new substance every 2.5 min. since 50 years (www.cas.org). Most of these compounds will never reach market and global exposure.

However the US EPA Toxic Substances Control Act lists over 84,000 chemicals that are manufactured or imported at levels >10 tons per year, not including pesticides, cosmetics, food stuffs and food additives which are covered by other legislations (www.epa.gov). It is estimated that man is exposed to over 85,000 products. The REACH initiative assembles all products which are produced/imported at >100 tons/year (>1 ton/year by May 2018). All these products, along with all natural substances to which we are exposed from conception to death constitute the HCE (Wild 2005, 2012; Egeghy et al. 2012; Goldsmith et al. 2014).

The impact of environment on health has been known since antiquity. Carcinogens have only been discovered in the last few decades. More recently the existence of endocrine disruptors and obesogens has been recognized. It is therefore no surprise that a small number of products may enter the human body, cross the blood brain barrier (BBB), alter specific molecular pathways in some of the human brain 10^{11} neurons and 10^{12} glia cells and thereby induce or contribute to specific CNS diseases. Identification of environmental factors involved in neurodegeneration and neurodegenerative diseases is in its infancy (reviews in Grandjean and Landrigan 2006, 2014; Cannon and Greenamyre 2011). The nervous system may be exposed to neurotoxic agents acutely (hours, days) or chronically (weeks, years, decades) before disease symptoms appear. Epidemiology studies are particularly difficult for neurodegenerative diseases since causes and effects are often separated by decades. These studies have therefore provided only few examples of environmental agents linked to the onset of neurodegenerative diseases. Pesticides, organic solvents, metals and some natural toxins (cyanobacteria) constitute the most frequently proposed neurotoxic agents. Two recently published books (Grandjean 2013; Demeneix 2014) review the impact of early age and even *in utero* exposure to environmental chemical entities on brain development and cognitive abilities.

AD is one of the most prevalent and worrying CNS disease[1]. EOAD is clearly a genetic disease due to specific APP or PSEN1/2 mutations leading to overproduction of Aβ42 over Aβ40. However EOAD represents <1% of all AD cases. The origin of LOAD (sporadic AD) (>99% of all AD cases) remains a mystery unsolved by epidemiological studies or by genome-wide association studies, which only revealed a few, low impact genetic risk factors (Lambert et al. 2013). The most prominent risk alleles, *APOE* ε4 and clusterin/ApoI link AD to lipid metabolism. , and aging together with several environmental factors also impose an increased risk.. Exposure to numerous industrial and agricultural chemicals correlate with neurotoxicity (Grandjean an Landrigan 2006, 2014; Julvez et al. 2009; Cannon and Greenamyre 2011; Zeliger 2103). Elevated serum pesticides levels, in particular DDE, the major DDT metabolite, are associated with increased risk for AD (Richardson et al. 2014). DDT increases Aβ levels (Li et al. 2015). There are epidemiological links between exposure to pesticides and AD (Hayden et al. 2010).

[1] According to the AD International Association the number of AD patients is expected to almost double in 20 years in the world, from 35.6 million in 2011 to 65.7 million in 2035. In Europe, the prevalence of AD is ~6.4% over 65 years and ~20% over 80 years (EURODEM estimates). Women are three times more affected than men. Life expectancy of patients at diagnosis is estimated at 3-8 years. AD is one of the most costly diseases for developed economies. The global total estimated cost for dementia (of which AD is the most common form) is 604 billion $ in 2010 (70% in Western Europe and North America) (cost of illness EU27: 160 billion € (1.3% of GDP) in 2008 of which 55% as informal care; 2.2 million life years lost due to disability; considerable weight for patients caregivers; annual cost of 22 K€ per patient (2005), including 26% in medical expenses). (AD facts & figures 2015).

Continuous sub-cutaneous injection of Aftin-5 in mice triggers robust dose-dependent increase in brain Aβ42 levels (unpublished data). Similar results were obtained with Aftin-4 (Meunier et al. 2014) and celecoxib or FT-1 (Kukar et al., 2005). Although orally administered triazines readily cross the BBB, their short half-life in mice prevented any accumulation, and consequently any effects on Aβ42 production *in vivo* (not shown).

Based on results obtained with products belonging to various chemical classes, we propose the existence, in the HCE, of products able to increase the production of the AD-associated Aβ42 and Aβ43 peptides. Such products might be classified as potential "Alzheimerogens" if long exposure, slow turn-over, low elimination and high BBB permeability allow long-term accumulation in the brain and action on brain cells. It is difficult to predict whether very long term, daily exposures of humans to the triazines described here might have resulted in sustained increase in Aβ42 production. We are now investigating other Aβ42 inducers which have a long half-life both in the environment and in the body, which accumulate in adipose tissues and which cross the BBB. We believe that such products may contribute to the onset, development and acceleration of sporadic LOAD. It is intriguing that both Aftin and triazines were able to stimulate Aβ42 production in human cells displaying a pathological APP mutation and already showing enhanced Aβ42 production. This suggests that environmental factors may synergize with genetic/epigenetic factors in enhancing Aβ42 production and triggering AD. Identification of such potential "Alzheimerogens" in the HCE and regulation of human exposure to them should open the way to innovative AD prevention strategies.

CONCLUSIONS

Like Aftins, and a few other chemicals of various structures, some widely used triazines trigger massive production of AD-associated Aβ42. These results suggest that HCE may contain other products to which humans are exposed on a long-term basis and which may contribute to the initiation, development or acceleration of AD. Identification and regulation of such potential "Alzheimerogens" should be a priority for the implementation of effective strategies to prevent the very common sporadic AD. In addition, some of these products might be turned into pharmacological tools to develop a chemically-induced animal model of AD, with fundamental and applied potential similar to the MPTP -induced Parkinsonism model (Fox and Brotchie 2010).

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FIGURE LEGENDS

Figure 1. Some triazines trigger β- and γ-secretase dependent production of extracellular Aβ42. A. Effect of 37 triazines on extracellular Aβ42 production by N2a-APP695 and CHO-7PA2-APPsw cells. Cells were treated with 100 µM of each compound for 18 h and cell supernatants were collected for Aβ42 levels measurement by ELISA. Aftin-5 was used as a positive control and the corresponding volume of vehicle (DMSO) as a negative control. Levels are expressed as fold change, ± SE, of Aβ42 levels over those of control, vehicle-treated cells. Average of two experiments performed in triplicate (representative of four independent experiments). Horizontal dotted lines indicate levels for 1 and 3 fold changes in Aβ42 concentration. **B.** Structure of the six active triazines and of Aftin-5. **C.** Extracellular Aβ42 production induced by triazines is inhibited by β-secretase inhibitor IV, γ-secretase inhibitors DAPT & BMS 299897 and γ-secretase modulator 'Torrey Pines' compound. N2a-APP695 cells were exposed to 10 µM of each inhibitor. 1.5 h later cells were exposed to 100 µM of each active triazine or 50 µM Aftin-5. Extracellular Aβ42 levels were measured after 18 h. Representative of two independent experiments performed in triplicates. **D.** Triazines trigger Aβ42 production in primary rat neuron cultures. Cells were exposed to DMSO, 100 µM of each triazine or Aftin-5 for 18 h. Cell supernatants were collected and the levels of Aβ38, Aβ40 and Aβ42 (bottom panel) were determined by ELISA assays (average of triplicate values). The Aβ-42/Aβ40 ratios were calculated (top panel). The horizontal dotted line refers to the basal ratio in control cells.

Figure 2. Mass spectrometry quantification of Aβ38, Aβ40 and Aβ42. Levels of the three amyloid peptides were determined by mass spectrometry in supernatants of N2a-APP695 (**A**) and CHO-7PA2-APPsw (**B**) cells following 18 h treatment with DMSO, 100 µM of each triazine or Aftin-5. Amyloid levels are expressed as percentage of levels in vehicle-treated cells (bottom panels; average ± SE of triplicate values; absolute values in control cell supernatants are indicated under the bottom panels) and Aβ42/Aβ40 ratios (top panels; horizontal dotted lines refer to the basal ratios in control cells).

Figure 3. Pattern of amyloid peptides produced by N2a-APP695 cells exposed to triazines. Cells were treated for 18 h with DMSO, 100 µM of each triazine or Aftin-5. Cell supernatants were collected and analyzed as described. **A.** Example spectra of supernatants amyloid profiles from N2a-APP695 cells exposed to DMSO, Aftin-5 or Terbutryn. **B.** Quantification of all amyloid peptides in N2a-APP695 cell supernatants (Log of fold change in triazine or Aftin-5 treated cells over control, DMSO-treated cells).

Figure 4. Pattern of amyloids peptides produced by CHO-7PA2-APPsw cells exposed to triazines. Cells were treated for 18 h with vehicle, 100 µM of each triazine or Aftin-5. Cell supernatants were collected and analyzed as described. **A.** Example spectra of supernatants amyloid profiles from CHO-7PA2-APPsw cells exposed to DMSO, Aftin-5 or Terbutryn. **B.** Quantification of all amyloid peptides in CHO-7PA2-APPsw cell supernatants (Log of fold change in triazine or Aftin-5 treated cells over control, DMSO-treated cells).

Figure 5. Triazines trigger enhanced production of Aβ42 versus Aβ40 in neurons differentiated from human iPSCs. A. iPSCs-derived neurons were differentiated for 4 or 10 weeks and then exposed to DMSO or 100 µM Aftin-5 or Terbutryn for 24 h. **B.** Neurons were derived from iPSCs obtained from healthy donor (APP WT) or from an AD patient (APP K724N mutation). They were exposed for 24 h to DMSO, 100 µM Aftin-5 or the six triazines. In both experiments cell supernatants were collected for extracellular Aβ levels measurement by ELISA. Levels are expressed as Aβ42/Aβ40 ratios ± SE of triplicate values.

Figure 6. Effect of APP C-terminal truncations on triazines' efficacy. A. Only the C-terminal aa sequences of APP full length (WT) and C-terminal truncations mutants (T1-T6) are shown. The γ and ε cleavage sites are indicated in orange and blue respectively. Numbers indicate the position of the residues involved in those cleavages and refer to the α cleavage site. **B**. Mutants T1 to T6 were expressed transiently in N2a cells which were exposed to DMSO or Aftin-5 (100 µM) for 24 hrs and the levels of released Aβ42 was measured by ELISA. **C**. Mutants T1, T3 and T4 expressing N2a cells were exposed for 24 hrs to DMSO (D), Aftin-5 or the six triazines (100 µM). Aβ42 level were measured and are expressed as fold-increase vs. untreated cells.

Figure 7. Triazines alter the cleavage pattern of alcadein α, leading to increased p3-Alcα38 production. A. Schematic representation of the production of p3-Alcα peptides from Alcadein α. The full length protein is cleaved primarily by α-secretase at His814 or Ala816 (purple arrows). It is then cleaved by γ-secretase at Thr851 (orange arrow) leading to the two main Alcadein α peptides p3-Alcα35 and p3-Alcα2N+35 ('2N' denotes the two additional, N-terminal amino acids). Alternative cleavage sites (blue arrows) generate additional p3-Alcα peptides of different sizes. **B**. Immunoprecipitation/mass spectrometry resolution of p3-Alcα peptides produced by HEK-Alcadein α cells exposed to various triazines, Aftin-5 or DMSO. Cells were treated for 24 h with 100 µM of each reagent and p3-Alc peptides were analyzed by MALDI-TOF/MS. Representative profiles for each product (top) and zoom on the p3-Alcα34, p3-Alcα35 and p3-Alcα38 peaks (bottom). **C**. Quantification of p3-Alcα peptides produced by cells exposed to all triazines and Aftin-5. Levels of each peptide are presented as fold change of ratios over p3-Alcα35 versus corresponding peptide ratios for DMSO-treated cells. Horizontal dotted lines indicate levels for 1 fold change in p3-Alcα/p3-Alcα35 ratio in treated vs. control cell supernatant. Note the change of scale for p3-Alcα38/p3-Alcα35 treated/control ratio.