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**The hydrogen sulfide-releasing compounds "ATB-346 and Diallyltrisulfide"
attenuate Streptozotocin induced cognitive impairment, neuro-inflammation and
oxidative stress in rats: involvement of Asymmetric dimethylarginine**

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Abstract:

Hydrogen sulfide (H₂S) has attracted interest as a gaseous mediator involved in diverse processes in the nervous system, particularly with respect to learning and memory. However, its therapeutic potential in Alzheimer disease (AD) is not fully explored. Therefore, the effect of H₂S-releasing compounds against AD like behavioural and biochemical abnormalities was investigated. Memory deficit was induced by intracerebroventricular injection of Streptozotocin (STZ, 3mg/kg). Animals were randomly assigned into 5 groups (12 rats each): normal control, STZ and 3 drug treated groups receiving Naproxen, H₂S-releasing naproxen (ATB-346), and Diallyltrisulfide in 20, 32, 40 mg/kg/day, respectively. Memory function was assessed by passive avoidance and T-maze tasks. After 21 days, hippocampal IL-6, malondialdehyde, reduced glutathione (GSH), Asymmetric dimethylarginine (ADMA), and acetylcholinesterase activity were determined. ATB-346 and Diallyltrisulfide ameliorated behavioural performance and reduced malondialdehyde, ADMA and acetylcholinesterase activity while increased GSH. This study demonstrates the beneficial effects of H₂S release in STZ-induced memory impairment by modulation of neuro-inflammation, oxidative stress and cholinergic function. It also delineates the implication of ADMA to the cognitive impairment induced by STZ. These findings draw the attention to H₂S-releasing compounds as new candidates for treating neurodegenerative disorders which have prominent oxidative and inflammatory components as AD.

Keywords:

Hydrogen sulfide, Alzheimer disease, neuro-inflammation, oxidative stress, ATB-346, Diallyltrisulfide

1. Introduction

Hydrogen sulphide (H_2S), the rotten egg smelling gas, has gained much interest in the past decade as the third endogenous gaseous mediator. In the human body, H_2S is generated mainly by four enzymes, cystathionine β -synthase (CBS), cystathionine γ -lyase (CSE), 3-mercaptopyruvate sulfurtransferase (3MST) and cysteine aminotransferase (CAT). Endogenously generated H_2S has been found to have multiple functions in the nervous system (Kimura 2013). In the CNS, H_2S is generated mainly by CBS in astrocytes and 3MST in neurons, and it appears to participate in cognition, memory and neuroprotection. Meanwhile, abnormal generation and metabolism of H_2S are involved in most of neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease, and vascular dementia (Hu et al. 2011). A large body of evidence supports an *in-vitro* anti-inflammatory, anti-apoptotic and antioxidant activity of H_2S in neuronal cells (Kimura and Kimura 2004; Lee et al. 2009, 2010; Nakao et al. 2009; Tang et al. 2008, 2010; Whiteman et al. 2004). Moreover, similar effects of H_2S have been reported in animal models of several inflammatory disorders (Dief et al. 2015; Distrutti et al. 2006; Fiorucci et al. 2007, Wallace et al. 2007, 2010; Zanardo et al. 2006). Therefore, it is hypothesized that H_2S -releasing drugs may have a therapeutic potential in neurodegenerative disorders of aging which have prominent oxidative and inflammatory components such as AD, an assumption that yet needs to be confirmed in animal models of this increasingly prevailing neurodegenerative disease.

Since there is significant evidence for a central role of inflammation in the development of AD, a protective effect of anti-inflammatory drugs has long been

appreciated (Moore et al. 2010; Shi et al. 2012). Many epidemiological studies indicate that chronic use of non-steroidal anti-inflammatory drugs (NSAIDs) reduces the risk of developing AD in healthy aging populations (Breitner et al. 2011; Leoutsakos et al. 2012; Szekely et al. 2004). Accordingly, it can be assumed that an anti-inflammatory drug which increases H₂S level would be more promising.

Thus, the aim of this work is to investigate the possible protective role of two H₂S-releasing compounds: an H₂S-releasing hybrid NSAID [the H₂S-releasing Naproxen (ATB-346)], as compared to Naproxen [an NSAID], and a garlic-derived polysulfide compound [Diallyltrisulfide (DATS)], against AD-like cognitive dysfunction. It also aims at delineating their potential to prevent or modify the neurochemical alterations associated with this form of dementia. Contrary to the early-onset familial AD which is caused by mutations in genes related to amyloid pathology and occurs only in less than 5% of cases of AD, the late-onset sporadic AD (sAD) is the type prevailing in the majority of cases. In sAD, more general and common factors like aging, peripheral insulin resistance and environmental toxins have been implicated as possible risk factors (Lee et al. 2006). Large body of evidence points to abnormalities in brain glucose/energy metabolism and insulin signaling as pivotal initiators in early sAD pathology. In this respect, intracerebroventricular (ICV) administration of the diabetogenic drug, Streptozotocin (STZ) in rats is proposed as a suitable experimental model of sAD that show many similarities to sAD. ICV STZ induces impaired cerebral glucose and energy metabolism, oxidative stress and cholinergic dysfunction. Further, impaired insulin signaling leads to insulin resistant state that consequently promotes amyloid β (A β) accumulation and tau hyperphosphorylation with subsequent neuroinflammation. This is associated with cell damage and loss that result in a progressive deterioration of

learning and memory. (Correia et al. 2011; Salkovic-Petrisic et al. 2007). The STZ-induced cognitive impairment model was thus chosen in this study because it displays numerous behavioural, biochemical and structural features that resemble those found in human sAD.

Draft

2. Materials and Methods:

2.1. Experimental Animals:

This study was conducted on 60 adult male Wistar rats, 250-280 g. Animals were maintained under standard laboratory conditions and 12:12 light/dark cycles with free access to food and water ad-libitum. After 1 week acclimatization period, animals were subjected to operative procedure for induction of cognitive impairment by ICV administration of STZ. All the experiments were carried out between 09:00 AM and 15:00 PM and were conducted in accordance with the Canadian Council on Animal Care (CCAC 1984, 1994) guidelines, after approval of the Ethics Committee of the Faculty of Medicine, Alexandria University, Egypt.

2.2. Drugs and chemicals:

Streptozotocin (STZ), Diallyltrisulfide (DATS), protease inhibitor cocktail were purchased from Sigma-Aldrich, USA. ATB-346 was purchased from Antibe Therapeutics Inc., Canada. Naproxen was obtained from the Nile Co for pharmaceutical and chemical industries, Egypt. All other chemicals were analytical grade commercial products.

2.3. Experimental design:

Cognitive impairment was induced in all rats by bilateral ICV administration of STZ (3 mg/kg, b.wt) in artificial cerebrospinal fluid CSF [(aCSF): 147 mM NaCl; 2.9 mM KCl; 1.6 mM MgCl₂; 1.7 mM CaCl₂ and 2.2 mM dextrose], on day 1 of the study (Sharma and Gupta 2001; Ishrat et al. 2006). STZ solution was freshly prepared so as a total volume of 10 µl that contained the required dose was injected/site at a rate of 2 µl/min. Control rats were injected with an equal volume of aCSF on the same day instead. Drugs or vehicle (gum acacia) were administered by oral gavage, starting on the next day to STZ administration (day 2) and continued daily till the end of the

study (day 21). Drugs were prepared in 2% gum acacia so as the final solution/suspension contained the required drug dose/kg b. wt/5 ml gum and was administered to each animal as 1ml/kg.

Accordingly, rats were assigned into the following 5 groups of 12 rats each:

- Normal control group, receiving aCSF + oral Gum acacia (1ml/kg) as a vehicle to orally administered drugs.
- Vehicle treated -STZ group, receiving STZ + oral gum acacia (1ml/kg).
- Naproxen (NPX)-treated STZ group; receiving STZ + Naproxen: 20 mg/kg/day (Kumar et al. 2006)
- ATB-346-treated STZ group, receiving STZ + the H₂S-releasing naproxen derivative in equimolar dose to that of naproxen: 32 mg/kg/day (Cicala et al. 2000)
- DATS-treated STZ group, receiving STZ + DATS: 40 mg/kg/day (Pari and Murugavel 2007)

Cognitive functions were evaluated by passive avoidance (PA) task on day 17 and 18, and T-maze task on day 19 after STZ injection. On day 21, brain tissues were collected for biochemical analysis. The experimental schedule of drug administration and behavioural tests are shown in Figure 1.

2.4. Surgical procedure:

Rats were deeply anesthetized by intramuscular (IM) injections of xylazine (15 mg/kg) and ketamine (100 mg/kg) and placed in a stereotaxic apparatus (Kopf Instruments). A midline sagittal incision was made in the scalp. Burr holes were drilled in the skull on both sides over the lateral ventricles using the following coordinates: 0.8 mm posterior to bregma; 1.5 mm lateral to sagittal suture; 3.6 mm beneath the surface of brain. ICV injection of either STZ solution or aCSF (in normal

control) was done using a Hamilton® microsyringe. Following surgery, rats were housed individually, and were injected with gentamycin (5 mg/kg, i.p.) for prophylaxis against infection, while ketoprofen (Ketofan®), 5 mg/kg IM was given as single IM injection for postoperative pain relief. During the early postoperative period, animals were kept warm using a hot water bottle, and were checked regularly until recovery from anaesthesia. Following recovery, they were fed sweetened milk by oral gavage once a day till they started spontaneous feeding. (Sharma and Gupta 2001).

2.5. Behavioral Studies:

Spatial memory performance and aversive conditioning-based memory were tested by Spontaneous alternation behavior (SAB) in T-maze and Passive avoidance (PA) tasks, respectively. To avoid rat exhaustion and confounding memory behaviour, different rats in each experimental group (6 rats/group) were subjected to either T-maze or PA tasks.

2.5.1. Passive avoidance (PA) task (Kameyama et al. 1986)

Memory retention deficit was evaluated by a step through passive avoidance apparatus on day 17 and 18 after STZ injection. The apparatus consisted of a two-compartment dark/illuminated (22×21×22 cm each), with a guillotine door separating the two chambers. The dark compartment had a stainless steel shock grid floor. During the acquisition trial, rats should learn that a specific place should be avoided; since it is associated with an aversive event. Each rat was placed in the illuminated chamber for 30 s as habituation period. The guillotine door was then opened and the rat was allowed to enter the dark chamber freely. The same was repeated after 15 min. and the initial latency (IL) of the animals to enter the dark chamber was recorded. Rats with an initial latency time of more than 60 s were excluded from further

experiments. Immediately after the rat had entered the dark chamber, the guillotine door was closed and an electric foot shock (0.5 mA) was delivered to the floor grid for 3 s. Five seconds later, the rat was removed from the dark chamber and returned to its home cage. Between each training trial, both chambers were cleaned with 70% alcohol to remove any perplexing olfactory cues. After 24 h, the retention latency (RL) time was measured in the same way as in the acquisition trial, but the foot shock was not delivered and the latency time was recorded up to a maximum of 300 s. Short latencies indicate poorer retention.

2.5.2. *T-maze task* (Spowart-Manning and van der Staay 2004)

SAB is a quick and simple measure of retention that reflects the operation of spatial working or short-term memory without the need for extensive training or the use of conventional re-enforcers. In this test, the animal must remember which arm it had entered on a previous occasion to enable it to alternate its choice on a following trial (Hughes 2004). The T-maze consists of a central (start) arm (50 cm long) and 2 goal arms (each of 40 cm long), mounted onto a central square. The maze arms are 10 cm wide and the walls are 20 cm height. Manual guillotine doors are used to close specific arms in forced choice trials. On day 19, training consisted of one session, which started with one forced-choice trial, followed by 14 free-choice trials. In the first trial, the 'forced-choice trial', either the left or right goal arm was blocked by the door. The animal was released from the start arm and was allowed to explore the maze, entering the open goal arm, and return to the start position where it was confined for 5 s by lowering the guillotine door. During the following 14 'free-choice' trials and after opening the door, the animal was free to choose between the left and right goal arm. As soon as it entered one goal arm, the other goal arm was closed and once it returned to the start arm, the next free-choice trial started after 5-s restraint in

the start arm. A session was terminated and the animal was removed from the maze as soon as 14 free-choice trials were performed or 15 min. elapsed. The series of arm entries was recorded visually. The percentage of alternations was calculated as (actual alternations/total possible alternations) $\times$ 100. Animals that did not finish the test within 15 min. were discarded as considered poorly explorative. The T-maze arms were cleaned with 70% alcohol between tests to remove odors and residues.

2.6. Biochemical analysis

2.6.1. Collection of brain tissue samples:

At the end of the experimental period (on day 21), animals were euthanized and the brains were rapidly removed and rinsed with ice-cold phosphate buffered saline (PBS) solution, pH 7.4 to remove any blood clots. The hippocampi were then dissected. Briefly, the brain was cut along the longitudinal fissure of the cerebrum using a surgical knife. The posterior part of the brain (midbrain, hindbrain, and cerebellum), as well as the olfactory pulp were removed. The cerebral hemisphere was then placed with the medial side up, and the diencephalon (thalamus and hypothalamus) was carefully separated to expose the medial side of the hippocampus. The tip of a spatula was placed under the ventral part of hippocampus rolling it up to separate it from the cortex (Hagihara et al. 2009; Sitges et al. 2014). The hippocampi are then homogenized at 4 °C in PBS (pH=7.4) containing protease inhibitor cocktail to prevent auto-oxidation of the dissected brain parts. The homogenates were then centrifuged at 3,000 rpm for 15 min., and aliquots of supernatant were stored at -80 °C until further biochemical testing. Total protein content was assayed using the Folin-Lowry's method (Lowry et al. 1951), based on the amount of aromatic amino acids in protein which, after pre-treatment with copper ions in an alkaline solution,

produce a blue purple colour complex, with maximum absorption at 750 nm wavelength, with Folin - Ciocalteu's phenol reagent. Bovine Serum Albumin (BSA) was used as a standard protein.

2.6.2. Brain homogenate assays:

2.6.2.1. Interleukin-6 (IL-6) assay:

IL-6 levels were determined using rat enzyme linked immunosorbant assay (ELISA) kit (WKEA MED SUPPLIES CORP, USA; Rat Interleukin-6) according to the manufacturer's protocol. Colour change was measured spectrophotometrically at a wavelength of 450 nm. The concentrations of IL-6 were calculated based on standards and expressed in ng/mg of total protein.

2.6.2.2. Brain oxidative stress state:

The brain tissue reduced glutathione (GSH) (mg/g tissue), was measured as an endogenous antioxidant, while malondialdehyde (MDA) (nmol/g tissue) was assessed as an index for lipid peroxidation during oxidative damage. For both assays, colorimetric technique using commercial kits (Biodiagnostic, Egypt), was applied according to the manufacturer's instructions.

2.6.3. Brain Asymmetric (NG,NG) dimethylarginine (ADMA) assay:

Being intimately related to oxidative stress, the implication of the endogenous Nitric oxide synthase (NOS) inhibitor, ADMA into AD and possible interaction with H₂S was explored. Tissue ADMA was determined using a commercial ELISA kit (WKEA MED SUPPLIES CORP, USA; Rat ADMA) following the manufacturer's instructions on a spectrophotometric reader at a wavelength of 450 nm. The concentrations of ADMA were calculated based on standards and expressed in $\mu\text{mol/g}$ protein.

2.6.4. Assay of acetyl cholinesterase (AChE) activity (Ellman et al. 1961):

The assay for acetyl cholinesterase (AChE) activity is based on Ellman's method using an alternative substrate acetylthiocholine and 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB). The reaction results in production of 5-thio-2-nitrobenzoate that has yellow colour due to the shift of electrons to the sulfur atom. Briefly, the reaction mixture consisted of 125 μ l of 3 mM Dithionitrobenzoic acid (DTNB), 25 μ l of 15 mM acetylthiocholine iodide (ATCh) and 50 μ l of 50 mM Tris-HCl, pH 8.0 in a microplate. After incubation for 10 min, 25 μ l of tissue sample was added and the reaction was then scanned at 405 nm for 10 min. at 2 min. intervals for the absorbance per minute using a microplate reader. AChE activity was calculated using *the following formula* and finally expressed as μ mol of ACh/min/mg protein.

$$R = \frac{\$ OD \times volume\ of\ assay}{E \times mg\ of\ protein} \times 1000$$

Where,

R = Rate of enzyme activity in moles of substrate hydrolysed/ min/mg protein.

$\$$ OD = change in absorbance /min.

E = Extinction coefficient 13,600/M/cm, which evaluates how much light will be absorbed by 1 cm of a 1 M solution of the yellow chemical product.

3. Data analysis

For biochemical and behavioural results, data were analysed by one-way analysis of variance (ANOVA) followed by Least significance Difference (LSD) criterion for multiple comparison. Data were analysed using Statistical Toolbox for MATLAB (Matrices Laboratory software version R2008b) and were expressed as means $\pm$ S.E.M. Correlations between two quantitative variables were assessed using Pearson coefficients. Statistical significance was set at $p < 0.05$.

4. Results

4.1. Memory improving effect of the studied H₂S-releasing compounds:

4.1.1. ATB-346 and DATS prevented STZ-induced impaired performance in PA

task: $F(4, 25)=396.720, p<0.001$

In the acquisition trial, only 2 rats (one from the vehicle-treated STZ group and the other from the DATS-treated group) had mean initial latency time (IL) of more than 60 s. These animals were excluded from the whole study and were replaced by another 2 rats which meet the criterion, one in each group. For the animals included in the study, the mean IL recorded on day 17 did not significantly differ among the 5 groups ($F(4, 25)=0.221, p=0.924$). However, on day 18, when aversive conditioning-based long term memory was evaluated, there was a significant shortening in the mean retention latency (RL) of rats in the vehicle-treated STZ group (up to 10% of that of control rats ($p=0.0000$), indicating memory deficit. On the other hand, the mean RL of three drug-treated STZ groups were significantly longer versus STZ group signifying memory enhancing effect ($p=0.000$ for each of the 3 groups). Notably, ATB-346 and DATS treatment almost prevented memory decline with significant difference to NPX group ($p=0.000$ for either groups). (Figure 2a)

4.1.2. ATB-346 and DATS ameliorated STZ-induced reduction in SAB in T-maze

task: $F(4, 25)=44.900, p<0.001$

To investigate whether co-treatment with H₂S-releasing compound can lead to functional improvement in short term memory, we examined SAB in T-maze. Data shown in Figure 2b demonstrate a significant decrease in SAB in all STZ injected rats versus normal control ($p<0.05$). NPX treatment did not significantly alter the reduced SAB ($p=0.501$), while the percentage of alteration was significantly higher with both ATB-346 and DATS treatment versus the vehicle treated-STZ rats ($p=0.0000$ for either

groups). However, ATB-346 treated rats showed significant improved performance in comparison to DATS treated group ($p=0.003$).

4.2. ATB-346 and DATS attenuated STZ-induced neuro-inflammation: $F(4, 55)=68.804, p<0.001$

ICV injection of STZ was associated with significant increase in hippocampal IL-6 in comparison to normal control rats ($p=0.0000$). Data from the three drug treated groups showed significant reduction of IL-6 versus the vehicle-treated STZ rats ($p<0.001$). However, the mean value obtained from the NPX-treated rats was significantly inferior to both ATB-346 and DATS groups ($p=0.0000$ versus each of them). Importantly, IL-6 in the ATB-346 treated rats was reduced down to level that was in-significantly different from that of normal control ($p=0.105$). (Figure 3a)

4.3. ATB-346 and DATS attenuated STZ-induced oxidative stress: $F(4, 55)=144.219, p<0.001$ and $F(4, 55)=124.238, p<0.001$ for MDA and GSH, respectively

The STZ-induced brain oxidative stress was confirmed by the observed marked increase in MDA with corresponding reduction of GSH in vehicle-treated STZ rats versus normal control ($p=0.0000$ for both MDA and GSH). Both H_2S -releasing compounds were able to partially inhibit the STZ-induced oxidative stress as shown by the significant decrease of MDA ($p=0.0000$ for either groups) and increase of GSH ($p=0.0000$ and $p=0.0008$ for ATB-346 and DATS, respectively). It is to be noted that NPX lacked any significant effect on GSH ($p=0.076$) and although it significantly reduced MDA relative to the vehicle-treated STZ rats ($p=0.000$), its effect was significantly less than that of H_2S -releasing NPX ($p=0.0003$). (Figure 3b and 3 c)

4.4. ATB-346 and DATS decreased brain ADMA: $F(4, 55)=11.754, p<0.001$

As shown in Figure 3d, ADMA concentration was significantly elevated in vehicle treated STZ rats versus normal control ($p=0.0000$). Both ATB-346 and DATS treated rats almost restored normal ADMA level ($p=0.89$ and $p=0.07$ for ATB-346 and DATS, respectively, versus normal control), while no statistical difference was observed between the NPX and the vehicle treated STZ groups ($p=0.067$).

4.5. ATB-346 and DATS prevented STZ-induced increase in AChE activity: $F(4, 55)=32.631, p<0.001$

ICV injection of STZ was associated with disruption of cholinergic function as evidenced from the significant increase of AChE activity in comparison to normal control rats ($p=0.0000$). Both ATB-346 and DATS prevented such increase as their mean values were insignificantly different from that of normal rats ($p=0.98$ and $p=0.28$ for ATB-346 and DATS, respectively). Though NPX treatment was also associated with significant decrease in enzyme activity versus vehicle-treated STZ rats ($p=0.005$), this effect was significantly inferior to that of ATB-346 ($p=0.02$). Figure 4

4.6. Correlation of biochemical parameters to cognitive impairment:

Analysis of data presented in Table 1 revealed that IL-6, oxidative stress and ADMA negatively correlated with improvement of behavioural performance ($p<0.001$). On the other hand, positive correlation was observed between ADMA with both IL-6 and MDA. ($p<0.001$)

5. Discussion:

As an almost ubiquitous bioactive molecule, H_2S was found to exert important regulatory effects in several biological functions in the CNS. Remarkably, a high expression of CBS in the rat hippocampus was observed, and the production of H_2S in brain homogenates was also proved long time ago (Abe and Kimura 1996). Moreover, disturbed neuronal H_2S synthesis is claimed to be implicated in AD, and systemic

plasma H₂S levels were reported to be negatively correlated with its severity (Cheng-fang and Xiao-qing 2011; Liu et al. 2008). Furthermore, a dramatic decrease of CBS activity and consequent drastic fall in H₂S levels (about 50%) have been detected in the brains of patients affected by AD (Kimura 2013). Therefore, identifying the neuro-protective potential of H₂S-releasing compounds in animal models of neurodegenerative diseases is not only of basic interest, but also may have important clinical implications.

Herein, we investigated the effects of administration of two different H₂S-releasing compounds, ATB-346 and DATS on a representative model of sAD. ATB-346 belongs to a long list of H₂S-releasing NSAIDs obtained through the conjugation of the “parent” NSAIDs with an H₂S-releasing moiety. These hybrid NSAIDs show anti-inflammatory effects, at least, comparable to those exhibited by the parent drug, and a greatly improved profile of gastric safety. In ATB-346, Naproxen is conjugated with an H₂S-releasing carbamoylic moiety. On the other hand, convincing evidence has been provided that organic polysulfide derivatives of garlic act as H₂S-releasing compounds and, indeed, the biopharmacological properties of H₂S well explain most of the beneficial effects promoted by garlic. DATS is one of garlic derived organosulfur stable compounds that act as true H₂S-donors, and release H₂S with a relatively slow mechanism which requires the cooperation of endogenous thiols (such as reduced GSH) (Martelli et al. 2012).

In accordance with previous studies (Correiaa et al. 2011; Ishrat et al. 2006; Özkay et al. 2012; Pachauri et al. 2013), ICV administration of STZ induced evident deficit in learning and memory that may be attributed to the observed severe neuro-inflammation and oxidative stress. This was also associated with disrupted cholinergic function as reflected by increased AChE activity. These abnormalities have been

previously reported to be secondary to induction of “insulin resistant brain state” responsible for the increased A β burden and tau hyperphosphorylation (Plaschke et al. 2010).

Administration of either the H₂S-releasing NPX or DATS improved performance in behavioural tests involving short and long term memory as depicted from the results of SAB and PA task, respectively. It is noteworthy that NPX treatment, though produced some improvement of long term memory, had significantly inferior effect to that of H₂S-releasing NPX. This difference thus emphasizes the role of H₂S moiety rather than the parent compound, NPX.

While numerous studies demonstrated a neuroprotective effect of H₂S *in-vitro* (Lee et al. 2010; Tang et al. 2008, 2010), it is only recently that the effect of exogenous H₂S on memory impairments is being addressed *in-vivo*. In line with our findings, Giuliani et al. (2013), He et al. (2014) and Xuan et al. (2012) reported that NaSH (the prototype hydrogen sulfide donor) significantly attenuated impaired learning and memory (spatial memory in MWM task) in different models of AD. Encouraging data were also retrieved in other forms of memory deficit as that associated with stroke or cerebral hypoxia (Wen et al. 2014; Wang et al. 2013).

As a matter of fact, AD is a multifaceted disorder with contribution of several distinct neuropathological alterations in the development of impaired cognition. Given the similarity of the STZ model to the pathogenic changes in AD (Salkovic-Petrisic et al. 2007), several mechanisms can possibly explain the ameliorative effect of H₂S on memory performance.

First, a cardinal feature of the neuropathology of most AD brains is an active neuro-inflammatory process which is detectable in the earliest stages of the disease. Moreover, there is biochemical evidence for elevated levels of cytokines such as IL-1,

IL-6 and tumour necrosis factor- α (TNF- α) in human AD brain (Glass et al. 2010; Heneka et al. 2010). Accordingly, an anti-inflammatory activity of H₂S could be proposed as an underlying mechanism. In our study, both H₂S-releasing compounds were shown to lower the elevated IL-6 in rat brain homogenate. Importantly, reduction in IL-6 was recently reported to be correlated with cognitive/behavioural improvement in mice model of AD (Lin et al. 2014), a finding that was also confirmed in our study. A large body of evidence supports an anti-inflammatory effect of endogenous H₂S (Lee et al. 2009) or H₂S donors in different *in-vitro* models of neuro-inflammation (Lee et al. 2010, 2013). Yet, to our knowledge, *in-vivo* studies are particularly scarce. Only Fan et al. (2013) reported, in accordance to our results, that NaHS attenuated inflammatory response in Amyloid- β induced AD, but the impact of this anti-inflammatory effect on cognitive impairment was not examined. Suppression of neuro-inflammation was also shown to protect against neurodegeneration and cerebrovascular dysfunction in another recent *in-vivo* study (Kamat et al. 2013). Nevertheless, clinical evidence now available does not clarify whether or not, the anti-inflammatory drugs per-se can be an efficacious option in AD. For example, the results of a recent clinical trial provided some unexpected contrasts in effects of NPX treatment at different times. It was concluded that NPX may attenuate cognitive decline in slow decliners while accelerating decline in fast decliners (Leoutsakos et al. 2012). Furthermore, final results based on follow up of AD anti-inflammatory prevention trials showed no benefit of 1-3 years treatment of NPX in older adults with a family history of AD. (ADAPT-FS Research Group 2015). However, our data demonstrated a more anti-inflammatory effect of H₂S-releasing NPX and to a lesser extent DATS versus NPX, and therefore more promising clinical benefits from these H₂S-releasing compounds may be expected.

The second mechanism by which H₂S has the potential to improve cognition is its well established antioxidant activity. In fact, the key pathogenic role of oxidative stress in AD is uncontested (Yan et al. 2013), and convincing evidence demonstrated the initiation of an oxidative process even as an earlier event to A β aggregation (Lecanu et al. 2006). As oxidative stress is a prominent feature of the STZ induced impaired cognition, this model easily uncovered the H₂S antioxidant activity as shown by the decreased MDA with both H₂S-releasing compounds. Since the antioxidant effect of H₂S was first reported by Kimura and Kimura (2004), there is a rapid growth of literature delineating possible mechanisms beyond this antioxidant activity. As confirmed by current data, replenishment of the potent intracellular antioxidant, GSH is one of these important mechanisms. This was reported to be due to increasing GSH synthesis by enhancing the activity of γ -glutamyl cysteine synthase, the limiting enzyme for GSH synthesis as well as cystine/glutamate antiporter, an important substrate transporter (Kimura et al. 2010). The notion that organic polysulfide derivatives require endogenous thiols for H₂S release (Benavides et al. 2007) may explain the relatively lower GSH in the DATS-treated in comparison to the ATB-treated STZ rats, though not significant. Remarkably, H₂S does not only augment antioxidant defenses, but also scavenges reactive oxygen species (ROS) and suppresses nitrosative stress by either inactivation of NO or inhibition of its synthesis (Cheng-fang and Xiao-qing 2011, Kamat et al. 2013). Indeed, the interaction between the two gaseous transmitters H₂S and NO is complex, and a mutual relationship is generally accepted. NO was proved to bind to CBS and inhibit the H₂S synthesis and thus decrease its neuronal antioxidant activity (Tang et al. 2013). Conversely, H₂S donors were shown to inhibit the 3 NOS isoforms (Kubo et al. 2007). However, the observed effect of both ATB-346 and DATS on ADMA can unravel another aspect of

H₂S antioxidant and neuroprotective potential. ADMA is present in plasma and cells where it can inhibit both constitutional and inducible NOS that generates NO as well as the cationic amino acid transporters that supply intracellular NOS with its substrate, L-arginine from the plasma. Therefore, ADMA and its transport mechanisms are strategically important in regulation of cellular function and have considerable clinical impact in many disease states (Teerlink et al. 2009).

In the present study, ADMA was found to be elevated with STZ injection and its level was reduced by administration of both H₂S-releasing compounds. Moreover, ADMA level negatively correlated with cognitive performance while it showed positive correlation with both oxidative stress and neuro-inflammation. These findings give the impression at first inspection that the ADMA might be involved in the pathogenesis of STZ induced cognitive deficit, and its reduction by H₂S release may contribute to its beneficial effects in AD. To our knowledge, the implication of ADMA in this model of cognitive deficit was not explored so far, and its relation to H₂S is not yet fully elucidated. Since oxidative stress has been reported to activate arginine methylating enzymes and inhibit dimethylarginine dimethylaminohydrolase (DDAH, the ADMA degrading enzymes), it is thus not surprising for ADMA level to be elevated with STZ injection (Sydow and Münzel, 2003). In line with current finding, rats with STZ induced diabetes showed elevated serum and renal ADMA concentrations secondary to inhibition of DDAH (Lin et al. 2002; Tain et al. 2013). Now, a point that deserves to be raised is how ADMA can adversely affect cognition though, being a NOS inhibitor, it is expected to inhibit the STZ induced nitrosative stress. Nevertheless, it has been shown that ADMA not only inhibits nNOS but also converts this enzyme from an NO to a superoxide generator (Cardounel et al. 2005; Druhan et al. 2008). More clearly, ADMA stimulates superoxide production by

uncoupling of NOS during depletion of tetrahydrobiopterin (BH₄), an important NOS co-factor. Oxidative stress can thus oxidize BH₄ to BH₂, which further uncouples NOS. Since ROS increases intracellular ADMA levels, this is a potential positive feedback mechanism to perpetuate a vicious circle of oxidative damage (Teerlink et al. 2009). Furthermore, there is accumulated evidence suggesting a link between ADMA and neuro-inflammation. It was demonstrated that lowering of ADMA through DDAH modulation, might be beneficial in different *in-vivo* and *in-vitro* models of acute and chronic inflammation (Chen et al. 2012). In support of this concept, a positive co-relation of ADMA to IL-6 was also observed in our study.

However, diverse reports of literature have addressed ADMA in relation to AD, and its role appears to be very multiplex and controversial (Arlt et al. 2008; Asif et al. 2013; Canpolat et al. 2014; Chen et al. 2010; Gubandru et al. 2013; Huang et al. 2010; McEvoy et al. 2014; Luo et al. 2014). Therefore, this relation must be studied further before any firm conclusions can be drawn.

Apart from antioxidant and anti-inflammatory activity, this study explored also possible modulatory effect of H₂S on the cholinergic system as the third mechanism for improving cognition. Both ATB-346 and DATS mitigated the STZ-induced increase in AChE activity that is strongly implicated in memory impairment. Importantly, NPX also partially inhibited AChE, but the significant difference to ATB-346 points to the role of the H₂S-releasing moiety. The mechanism by which H₂S inhibited AChE is not clear, but substantial evidence suggests that the change in the cytokine milieu toward physiological levels helps in reshaping the differentiation of the neurons by promoting microenvironment favoring improvement of cholinergic signaling (Malmsten et al. 2014). Data concerning the interaction of H₂S with cholinergic functions in AD are particularly lacking. Only Pari et al. (2007) reported

restoration of AChE activity by DATS. Counterintuitively, no effect on AChE in a different model of neurodegeneration treated by NaSH was demonstrated by Kamat et al. (2013). Despite the encouraging results reproduced by NaSH, the prototypical H₂S donor, there is a general agreement that it is not the ideal H₂S donor for clinical use due to lack of specificity, rapid release of H₂S in massive amounts that may cause adverse or toxic effects, and also because of their relatively short effective residence time in tissues. On the other hand, compounds that generate H₂S with slow releasing rates are more suitable for therapeutic purposes. There is convincing evidence that this pharmacological feature is exhibited by some natural compounds, such as organic polysulfide derivatives of garlic or through the conjugation of parent drugs with a H₂S-releasing moiety as for example the H₂S-releasing NSAIDs (Caliendo et al. 2010; Kashfi et al. 2013). In a previous work (Dief et al. 2015), we detected increased plasma H₂S concentration at least 3 hours following ATB-346 administration, a finding that may support the slow release of H₂S from its carbamoylic moiety. On reviewing literature addressing the neuroprotective role of H₂S, most studies utilized NaSH as a donor. Only, similar to the present study, Lee et al. (2010) and Campolo et al. (2013, 2014) examined the *in-vitro* and *in-vivo* effects of ATB-346, respectively, and both yielded promising results in concordance to our data with respect to the anti-inflammatory or behavioural functions. Given the previously verified safety of this hybrid molecule (Wallace et al. 2010), these compounds should come to the forefront of clinical attention.

In conclusion, the present study provided preclinical evidence on the neuroprotective role of exogenous H₂S in STZ-induced model of AD. Both DATS and ATB-346 prevented STZ induced memory impairment that is attributed to their anti-inflammatory and antioxidant activity, as well as their favourable modification of

cholinergic functions. Interestingly, both H₂S-releasing compound negatively regulated ADMA level, revealing the implication of this endogenous molecule in STZ induced memory deficit. The superior benefits of H₂S-releasing naproxen, relative to Naproxen, confirms that these effects are due to the H₂S-releasing moiety. Given the pleiotropic effects of H₂S and the multifactorial nature of AD, H₂S-releasing compounds could thus lay the ground for a multi-targeted approach that could overcome some of the major limitations of the currently available drugs used in treatment of AD.

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Table 1: Correlation between different parameters in total sample

		Plasma ADMA ($\mu\text{mol/g}$ protein)	MDA (nmol/g tissue)	GSH (mg/g tissue)	Passive avoidance (seconds)	T-maze % of SAB
IL-6 (ng/mg protein)	<i>r</i>	0.655*	0.857*	-0.708*	-0.844*	-0.825*
	<i>p</i>	<0.001	<0.001	<0.001	<0.001	<0.001
Plasma ADMA ($\mu\text{mol/g}$ protein)	<i>r</i>		0.676*	-0.585*	-0.742*	-0.682*
	<i>p</i>		<0.001	<0.001	<0.001	<0.001
MDA (nmol/g tissue)	<i>r</i>			-0.842*	-0.790*	-0.818*
	<i>p</i>			<0.001	<0.001	<0.001
GSH (mg/g tissue)	<i>r</i>				0.665*	0.801*
	<i>p</i>				<0.001	<0.001

r, Pearson coefficient ; *,statistically significant at $p \leq 0.05$; ADMA, Asymmetric (NG,NG) dimethylarginine MDA, Malondialdehyde; GSH, reduced glutathione; SAB, spontaneous alternation behaviour

Fig.1: Experimental schedule of testing the effect of H₂S-releasing compounds on STZ-induced memory deficit in rats

Naproxen, H₂S-releasing naproxen or DATS were administered daily in doses of 20 mg/kg, 32 mg/kg and 40 mg/kg respectively, starting from the 2nd day after ICV injection of 3 mg/kg STZ. PA and T-maze tasks were used to evaluate rats' behavioural memory on selected time points.

ICV; intracerebroventricular, STZ; Streptozotocin, PA; passive avoidance, DATS; Diallyltrisulfide

Fig. 2: Effect of treatment with H₂S-releasing compounds on Streptozotocin induced memory impairment (a) Retention latency of passive avoidance task was

measures as the time to enter the dark chamber 24 h after exposure to an electric foot shock. Both ATB-346 (32mg/kg/d) and DATS (40 mg/kg/day) prevented the STZ induced shortening of RLs which were comparable to that of normal control rats. In (b) vehicle treated-STZ group showed marked impairment of SAB examined by the T-maze task. ATB-346 treated rats showed significant improvement in the percentage of alternation, an effect that was lacking with Naproxen treatment. DATS also significantly ameliorated SAB but to a lesser extent relative to ATB-346.

Data are expressed as Means $\pm$ SEM, * $p < 0.05$ significant versus normal control, ** $p < 0.001$ significant versus vehicle-treated STZ group, # $p < 0.001$ significant versus Naproxen-treated rats and § $p < 0.05$ significant versus ATB-346 treated rats. STZ; Streptozotocin, ICV; Intracerebroventricular, ATB-346; H₂S-releasing Naproxen, DATS; Diallyltrisulfide, RL; Retention latency

Fig. 3: Effect of treatment with H₂S-releasing compounds on Streptozotocin induced neuro-inflammation and oxidative stress and increase in ADMA

Streptozotocin (STZ) administration was associated with significant increase in hippocampal IL-6 (**a**) and MDA (**b**) with corresponding decrease of GSH (**c**) versus the normal control. Both ATB-346 and DATS significantly reduced neuro-inflammation and oxidative stress versus vehicle treated STZ rats. In (**d**) Hippocampal ADMA was also significantly increased by ICV STZ. Both ATB-346 and DATS inhibited the increase in ADMA while Naproxen treatment was associated with non-significant alteration compared to vehicle-treated STZ rats. Data are expressed as means $\pm$ SEM. * $p < 0.05$ versus normal control, ** $p < 0.05$ versus vehicle treated STZ rats, and # $p < 0.05$ versus-Naproxen treated rats. MDA, Malondialdehyde; GSH, reduced glutathione; ADMA; Asymmetric dimethylarginine, ICV; intracerebroventricular, ATB-346, H₂S-releasing naproxen; DATS, Diallyltrisulfide.

Fig. 4: Effect of treatment with H₂S-releasing compounds on Streptozotocin induced increase in Acetylcholinesterase activity

AChE specific activity was determined in relation to protein content and expressed as μmol of ACh/min/mg protein. Data are expressed as means $\pm$ SEM, * $p < 0.001$ significant versus normal control, ** $p < 0.05$ significant versus vehicle-treated STZ group and # $p < 0.05$ significant versus Naproxen treated rats. AChE; Acetylcholinesterase, ACh; Acetylcholine, STZ; Streptozotocin, ATB-346 =H₂S-releasing Naproxen, DATS; Diallyltrisulfide

Fig.1: Experimental schedule of testing the effect of H₂S-releasing compounds on STZ-induced memory deficit in rats

Naproxen, H₂S-releasing naproxen or DATS were administered daily in doses of 20 mg/kg, 32 mg/kg and 40 mg/kg respectively, starting from the 2nd day after ICV injection of 3 mg/kg STZ. PA and T-maze tasks were used to evaluate rats' behavioural memory on selected time points.

ICV; intracerebroventricular, STZ; Streptozotocin, PA; passive avoidance, DATS; Diallyltrisulfide

170x63mm (300 x 300 DPI)

Fig. 2: Effect of treatment with H2S-releasing compounds on Streptozotocin induced memory impairment (a) Retention latency of passive avoidance task was measures as the time to enter the dark chamber 24 h after exposure to an electric foot shock. Both ATB-346 (32mg/kg/d) and DATS (40 mg/kg/day) prevented the STZ induced shortening of RIs which were comparable to that of normal control rats. In (b) vehicle treated-STZ group showed marked impairment of SAB examined by the T-maze task. ATB-346 treated rats showed significant improvement in the percentage of alternation, an effect that was lacking with Naproxen treatment. DATS also significantly ameliorated SAB but to a lesser extent relative to ATB-346. Data are expressed as Means $\pm$ SEM, * $p < 0.05$ significant versus normal control, ** $p < 0.001$ significant versus vehicle-treated STZ group, # $p < 0.001$ significant versus Naproxen-treated rats and § $p < 0.05$ significant versus ATB-346 treated rats. STZ; Streptozotocin, ICV; Intracerebroventricular, ATB-346; H2S-releasing Naproxen, DATS; Diallyltrisulfide, RL; Retention latency

126x189mm (300 x 300 DPI)

Draft

Fig. 3: Effect of treatment with H₂S-releasing compounds on Streptozotocin induced neuro-inflammation and oxidative stress and increase in ADMA

Streptozotocin (STZ) administration was associated with significant increase in hippocampal IL-6 (a) and MDA (b) with corresponding decrease of GSH (c) versus the normal control. Both ATB-346 and DATS significantly reduced neuro-inflammation and oxidative stress versus vehicle treated STZ rats. In (d) Hippocampal ADMA was also significantly increased by ICV STZ. Both ATB-346 and DATS inhibited the increase in ADMA while Naproxen treatment was associated with non-significant alteration compared to vehicle-treated STZ rats. Data are expressed as means $\pm$ SEM. * $p < 0.05$ versus normal control, ** $p < 0.05$ versus vehicle treated STZ rats, and # $p < 0.05$ versus-Naproxen treated rats. MDA, Malondialdehyde; GSH, reduced glutathione; ADMA; Asymmetric dimethylarginine, ICV; intracerebroventricular, ATB-346, H₂S-releasing naproxen; DATS, Diallyltrisulfide.

151x135mm (300 x 300 DPI)

Hippocampal Acetylcholinesterase activity

Fig. 4: Effect of treatment with H₂S-releasing compounds on Streptozotocin induced increase in Acetylcholinesterase activity
AChE specific activity was determined in relation to protein content and expressed as μmol of ACh/min/mg protein. Data are expressed as means ± SEM, * p<0.001 significant versus normal control, ** p<0.05 significant versus vehicle-treated STZ group and # p<0.05 significant versus Naproxen treated rats. AChE; Acetylcholinesterase, ACh; Acetylcholine, STZ; Streptozotocin, ATB-346 =H₂S-releasing Naproxen, DATS; Diallyltrisulfide

63x46mm (300 x 300 DPI)