

# Repurposing Elemental Sulfur for Alzheimer Dementia (AD) Therapeutics: Role of Hydrogen Sulfide and B-Galactosidase

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## Abstract

With the global increase in the aging population, the disease burden of Alzheimer Dementia (AD) has become a major public health challenge. Current AD drugs fail to modify the course of AD. In the mini-therapeutic perspective on AD, we highlight two promising targets: Homocysteine/Hydrogen sulfide signaling and lysosomal beta-galactosidase pathway in AD. We outline the rationale underlying our repurposing paradigm in translating Sulmedol (patented elemental sulfur) indicated for lactose intolerance, to promising drug lead for AD treatment. Our approach of designing Proof-of-concept biomarker-based placebo-controlled Phase II clinical trial of Sulmedol in early AD can accelerate AD therapeutics development.

**Keywords:** Sulfur; Lysosomes; Beta-galactosidase; Hydrogen sulphide; Alzheimer

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## Dual Hcy/H<sub>2</sub>S and Lys-GALAC Signalling in AD

Recent accumulating evidence supports the role of elevated level of homocysteine level (Hcy) as a risk factor common to both cardiovascular diseases (CVD) and AD [1,2]. Confer increase in coronary heart disease (CHD), cardiovascular disease (CVD) and all-cause mortality [3]. Homocysteine is metabolized by two pathways: re-methylation and trans-sulfuration, dependent upon availability of folate and vitamin B groups: Vitamins B-6 and vitamin B-12 (cyanocobalamin) [4-10]. Transmethylation reactions depend upon S-adenosylmethionine (SAM) as a methyl donor. Studies to examine the likely beneficial effects of folates and vitamins B supplementation to reduce hyper-homocysteinemia and alter the cardiovascular risks have met with mixed results [4,5,9,10].

There is growing body of evidence suggesting that hyper-homocysteinemia: hHcy, confers risk factor in cognitive decline progressing to AD [7,8]. It is likely that hHcy stands at the crossroads of vascular signaling regulating CVD and CHD risk factors and brain pathway regulating AD risk factor. While the Hcy level exceeded 14 mol/L, AD risk doubled. The threshold value of Hcy level for AD risk is set at 10.0 μmol/L. Inconsistent results are found in studies on folate and vitamin B group in rescuing

cognitive deficits in hHcy, suggesting complex neural networks in cognitive aging and in AD [8-10].

The discovery and characterization of three gaseous neurotransmitter: nitric oxide (NO), hydrogen sulfide (H<sub>2</sub>S) and carbon monoxide (CO) has stirred some measure of excitement in translational neurosciences [11-14]. The neuroprotective and neuromodulatory actions of H<sub>2</sub>S have only recently been recognized in diverse brain disorders ranging from AD,

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Parkinson Disease to traumatic brain injury. Studies have shown that Hcy/H<sub>2</sub>S coupling regulates oxidative stress, inflammatory and apoptosis pathways exerting feedback control over Hcy metabolism, consistent with the emerging role of H<sub>2</sub>S in regulating cerebral circulation [11-14]. H<sub>2</sub>S modulates NMDA(N-methyl-glutamate)glutamate receptors, antagonizes homocysteine-induced neurotoxicity, and regulates intracellular cAMP levels and intracellular Ca(2+). In postmortem AD brains, H<sub>2</sub>S levels were found to be markedly reduced compared with non-AD matched control [13]. Plasma level of hydrogen sulfide was significantly lower than in control and inversely correlated with AD severity [14]. A recent study found homocysteine levels correlate with the severity of neurobehavioral symptoms in AD [15]. Homocysteine neurotoxicity targets signal pathways directly related to AD: synaptic plasticity, tau phosphorylation and beta-amyloid accumulation. Taken together, brain atrophy in AD is related to dysregulation in Hcy/H<sub>2</sub>S cascade of intracellular signal transduction **Figures 1 and 2** [11,12].

There is growing body of evidence highlighting the emerging role of autophagy in AD [16-20]. Autophagy, the “self-eating phenomenon”, represents a highly regulated intracellular signaling closely involved in the recycling and clearance of major cellular organelles and misfolded proteins by the lysosomes. In AD, faulty induction of the so-called auto-phagosomes within the neurons and around the synapses results in impaired clearance of cellular debris through the retrograde trafficking. The mode of aberrant delivery and recycling functions of lysosomes leads to the accumulation of potentially toxic Aβ amyloid and tau aggregates. Taken together, dysfunction in the maturation of auto-phagosome-lysosome and the decline in the efficiency of retrograde trafficking are the core determinants of AD pathology. It is noteworthy that changes in lysosomal enzymes and autophagy-lysosomal (auto-Lys) proteins run in parallel with the severity of AD symptoms [17,18].

In both AD and Down's syndrome, leukocytes showed a significant increase of beta-galactosidase (GALAC) activity compared with age-matched controls [20]. β-Hexosaminidase and β-galactosidase were associated with post-synaptic vesicles and increased at early and advanced stage of AD [18]. Levels of exosome-derived auto-Lysosomes sharply defined the prodromal phase of AD [16,17]. Type II diabetes mellitus (T2DM) is known to be a risk factor for AD. A recent study found evidence for upregulation of lysosomal enzymes: plasma β-Galactosidase (GALAC) and β-Hexosaminidase levels were higher in patients with AD than control [19].

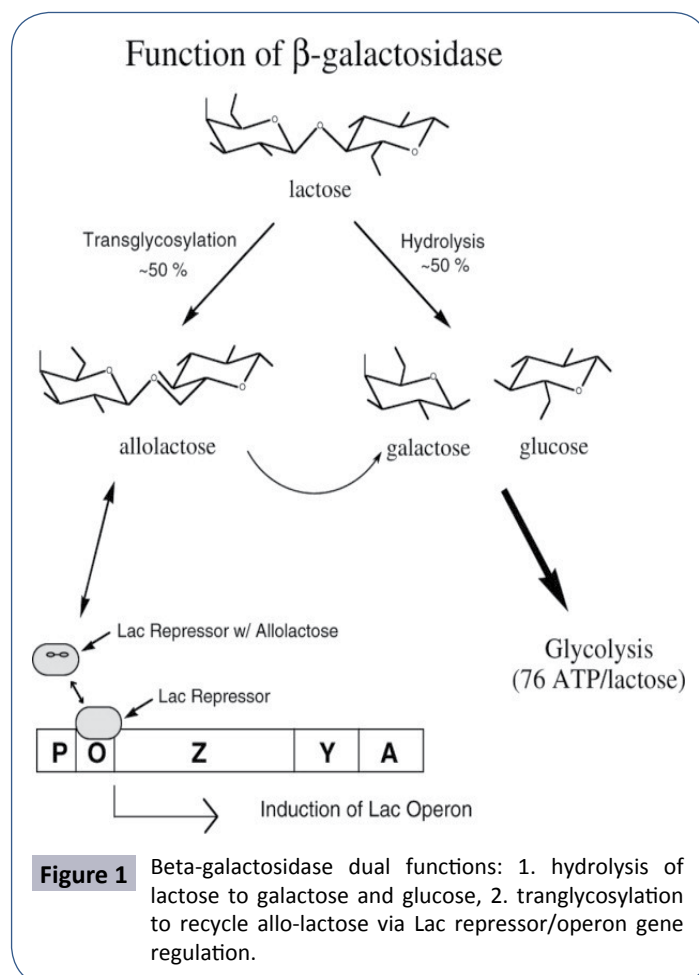
## Repurposing Elemental Sulfur as AD Drug Lead

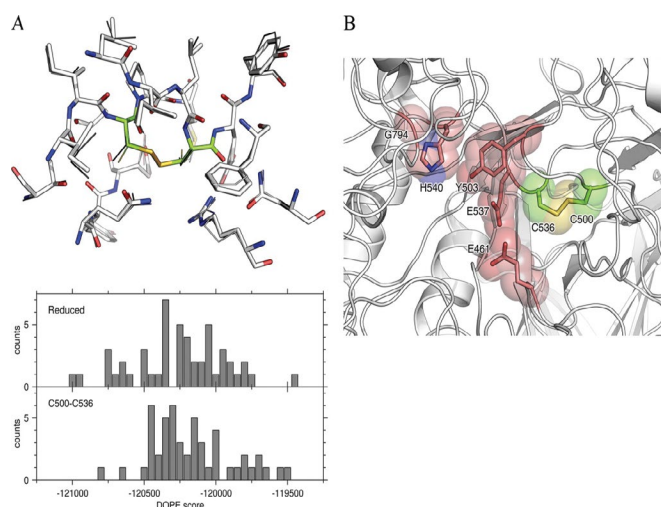
We adopt the repurposing paradigm towards developing novel AD therapeutics. Our research group is interested in the patented product: Sublimed Sulfur [SS] (patent and biotechnology inventor: A Khan) formulated as oral compound and approved by Health Canada Ottawa Canada for treatment of lactose intolerance: Lac-INT. We focus primarily on the dual signaling pathways in AD: I. Homocysteine (Hcy)/Hydrogen sulfide(H<sub>2</sub>S) signaling; II.

Beta-galactosidase (GALAC)-mediated autophagy signaling. For the first time, in a controlled study Khan showed] showing that Sulmedol was efficacious in reducing the symptoms of Lac-INT in a cohort of Lac-INT [21,22]. We translated the findings from preclinical studies of elemental sulfur in modulating the activities of galactose in both the gut and in the brain [23-25] to the clinical arena.

Sulfur activates beta-galactosidase responsible for hydrolyzing lactose to glucose and galactose via the disulfide bond of gut GALAC for reducing the gastro-intestinal symptoms of lac -INT. The importance of disulfide linkage as the site of sulfur's action of beta-galactosidase can hardly be emphasized. It appears that the bidirectional loop connecting the brain and the gut hinges heavily on the disulfide bond of GALAC found in the lymphocytes, the brain and in gastrointestinal tract **Figure 3**.

For the past few years, our research team has succeeded in navigating simple inorganic non-metal: sulfur through the maze of pharmaceutical development to advance to the CNS drug candidate for neurodegenerative disorders. The multiple steps consist of a) testing for physico-chemical stability and purity; b) organizing clinical trial in well-characterized cohort of patients diagnosed as lactose intolerance and submitting the results to regulatory boards for human use; c) repurposing the formulated sulfur product for CNS disorders. It is noteworthy that a very recent study found hydrogen sulfide-containing sulfurous water



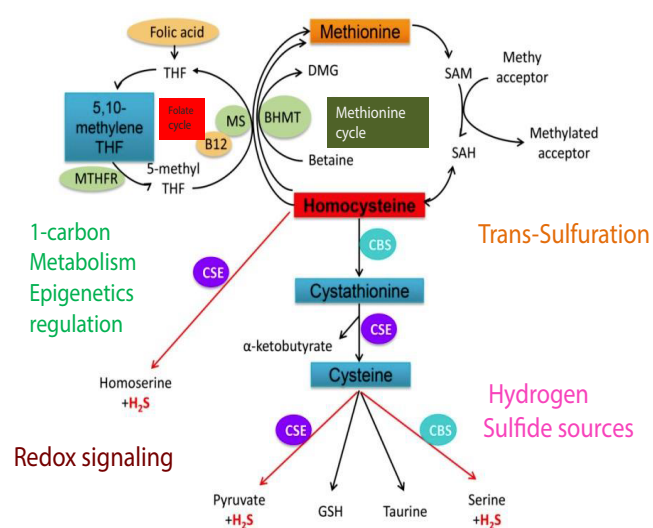


**Figure 2**  $\beta$ -Galactosidase modeling under oxidizing conditions. From Joaquin Seras-Franzoso et al. Appl. Environ. Microbiol. 2012;78:2376-2385.  $\beta$ -Galactosidase modeling under oxidizing conditions. (A) (Top) Illustration of the conformational changes involved in the formation of a disulfide bond between cysteines C500 and C536. Oxygen, nitrogen, and sulfur atoms are colored red, blue, and yellow, respectively. Thick sticks represent the best model (according to its DOPE potential) with the disulfide bond formed; thin sticks represent the best model of the reduced form. The template structure (PDB entry 1JYV) is drawn with black lines. (Bottom) Histograms of the DOPE scores of the 50 models obtained for reduced  $\beta$ -galactosidase and of the 50 models obtained with the disulfide bond between C500 and C536. (B) Illustration of the close proximity between the C500-C536 disulfide bond and the active-site residues (E461, Y503, E537, H540, G794). The  $\beta$ -galactosidase backbone is drawn as a white cartoon representation. The side chains of the two cysteine residues involved in the disulfide bond (green) and of the active-site residues (light red) are shown as solid sticks surrounded by semitransparent spheres. Oxygen, nitrogen, and sulfur atoms are colored red, blue, and yellow, respectively.

(SW) containing hydrogen sulfide to be neuroprotective in the *in vitro* clinical model of AD [23]. An *in vitro* therapeutic trial of SW was carried out in peripheral blood mononuclear cells from AD patients compared with matched controls using the comet assay (to measure oxidative DNA damage) and the MTT assay (as an indicator of cell viability). Hydrogen peroxide and homocysteine were used to induce oxidative DNA damage. SW protected them against enhanced oxidative stress. H<sub>2</sub>S confers to SW a more potent antioxidant capacity than other antioxidants.

From the perspective of GALAC-mediated autophagy, we have to demonstrate whether in AD, the changes in lysosomal enzyme, GALAC are reversed with SS treatment and correlate with the clinical responses in AD. Modulation of autophagy has entered to the main stage of AD drug development [16,25]. The efficacy of Sulmedol is most likely related to its action in restoring redox balance and buffering the oxidative stress load, as well as in

shifting the host defense towards anti-inflammatory phenotype. The pharmacological actions of Sulmedol is reminiscent of the findings of the old compound, methylthioninium chloride (methylene blue: MB) inhibits Tau aggregation through inducing autophagy [26,27]. At nanomolar concentrations, MB can reduce Tau levels in both organotypic brain slices from the mouse model of frontal-temporal dementia (FTD). MB altered levels of cathepsin D and BECN1 the mammalian ortholog of the yeast autophagy-related gene 6, consistent with MB's action as the potent inducer of autophagy. Besides, MB has also been shown to inhibit mitochondrial caspase through oxidation (sulfenation) of the active site: 163 cysteine of Caspase 6. MB shares with elemental sulfur in targeting GALAC. The molecular mechanism of action of MB is reminiscent of the specific role of conformational changes related to the disulfide formation between cysteine residues: C500 and C536, in close proximity to the active site of GALAC. GALAC is a model enzyme to illustrate the known evolutionary progression from the microbial world: *E. coli* to the mammalian system including humans. GALAC regulating brain-gut nexus across life cycle represents novel landmark in creative drug development. These mechanistic considerations of the



**Figure 3** Homocysteine/H<sub>2</sub>S link to One-Carbon Metabolism. Metabolic map showing how homocysteine is related to methionine primarily through the epigenetics-driven activated Methyl donor reaction indexed by SAM/SAH, with minor contribution from the branched reaction of DMG regulated by BHMT. Folate cycle is coupled with the homocysteine through the METFR under epigenetics control via DNA methylation. Transulfuration pathway regulated by two key enzymes: CBS and CSE, provides the sources of the gaseous neurotransmitter: Hydrogen Sulfide: H<sub>2</sub>S from which potent sulphydryl containing antioxidants with anti-inflammatory actions are derived. **Abbreviations:** CBS: Cystathionine  $\beta$ -Synthase; CSE: Cystathionine Y-lyase; METFR: Methyl-tetrahydrofolate Reductase; SAM: S-Adenoyl-Methionine; BHMT: Betaine-Homocysteine S-Methyltransferase; THF: Tetrahydrofolate; SAH: S-Adenoyl-Homocysteine; DMG: Dimethylglycine.

overlapping molecular sites may suggest that elemental sulfur is similar to MB in inhibiting hyper-phosphorylated Tau and thereby stabilizing microtubules as well as activating autophagy.

The downstream 'end-point' can slow or reduce the deleterious consequences of neuronal loss in AD. The future challenge consists of conducting biomarker-based clinical trials: Phase II and Phase III to test the clinical efficacy and long term safety of Sulmedol in the AD cohort.

## Roadmap of Catalysing Sulmedol as AD Therapeutics

To the best of our knowledge, none of the AD pipeline drugs has explored the dual approach in targeting the gut-brain GALAC axis related to GALAC autophagy and Hcy/H<sub>2</sub>S signaling in AD. Our research group has completed a controlled Sulmedol in a group of cognitively normal cohort subjects stratified with respect to fasting Hcy levels. In study I [24], we examined the 30-day treatment effects of SS at daily dosage of 200 mg to be administered at in altering the plasma Hcy levels in a small cohort of cognitive normal subjects. We stratified the cohort of cognitively normal control subjects to be administered SS into three groups with pre-determined range of basal fHcy levels as follows: Group 1 (n=16): range of fHcy 10.0-22.1  $\mu$ mol/L; Group 2 (n=15) fHcy range: 7.1-9.9  $\mu$ mol/L; Group 3 (n=15); range of fHcy 2.3-7.0  $\mu$ mol/L. In Study I, Group I subjects, fHcy decreased significantly by 36.1%, 24 hours. After treatment and by 29.3%, 30 days later: in Group 2 fHcy did not change significantly. Group 3 exhibited paradoxical non-significant increase in fHcy, we have also examined whether in AD patients, Sulmedol exerted beneficial effects on AD. In Study II, analysis of the clinical vignette analysis of two AD patients revealed that 3-month SS treatment ameliorated cluster of AD symptoms: nocturnal wandering, paranoid delusions, disorientation and confusion [24]. Sulmedol exhibits a high degree of safety and tolerability. No serious adverse events are observed in Lac-INT and in Hcy studies.

We propose to examine the efficacy and safety of Sulmedol (SS) in biomarker-based RCT trial in AD. We hypothesize that SS exerts dual modes of action: modulating GALAC dysregulation involved in autophagy pathway and resetting Hcy/H<sub>2</sub>S signaling in AD. We propose that elemental sulfur to function *in vivo* as a potential hydrogen sulfide releasing action to exert concerted anti-oxidant, anti-inflammatory and anti-apoptotic actions in synaptic remodeling in aging and in AD. We anticipate that SS, through the GALAC-mediated autophagy, will enhance the clearance of both beta-amyloid and Tau hyper-phosphorylation. We consider our paradigm to be unique in developing anti-TAU compounds through the GALAC/autophagy avenue. Sulmedol, in hitting the Hcy/H<sub>2</sub>S signaling involved in regulating the neuro-vascular coupling and cardio-vascular-brain nexus, represents new approach in probing inflammation cascade model in AD therapeutics [28]. Peripheral inflammation mediators can reflect microglial neuroinflammation via immune interface. The results of our proposed study will clarify the role of homocysteine metabolite, homocysteine-thiolactone as the triggering factor in neurodegeneration through the mechanism of "protein

N-homocysteinylation", resulting in dynamics of cellular toxicity and death and neurodegeneration [29].

Thiolactone functions as a highly reactive cyclic thioester in biological systems. During the process of protein N-homocysteinylation, a host of deleterious cellular events: protein denaturation, key enzyme inactivation and amyloid formation and tau hyper-phosphorylation, contributes towards cellular death. It is likely that protein N-homocysteinylation perturbs the homeostasis of Hcy/H<sub>2</sub>S signaling in AD.

From the therapeutic perspective, the molecular footprint of "protein N-homocysteinylation", may provide the opportunity for sulfur to antagonize the activities of protein N-homocysteinylation prior to the final step in the neurodegeneration.

It is likely that elemental sulfur may modify the course of "protein N-homocysteinylation" through sulfur-homocysteine-thiolactone exchange, and simultaneously can reset the GALAC/autophagy pathway in aging and thereby modify the course of AD as well as in cognitive aging. Biomarker-based clinical studies in AD can clarify and delineate the complexity of the mechanistic details of elemental sulfur in various stages of AD: the prodromal phase, Mild-cognitive impairment (MCI) phase and early, mid- and advanced stage of AD.

## Conclusion and Future Directions

In organizing the strategic plan for repurposing Sulmedol as earmarked for lactose intolerance to drug lead in Alzheimer dementia, we adopt the Bayesian adaptive design in terms of "Go or No-Go" decision. In clinical studies of Sulmedol in lactose intolerance and in AD, we found the drug lead has been highly favorable in the adverse events profile. For Phase II/III randomized clinical trials in AD, after we launch the Phase Ib clinical study. We define our specific objectives as follows:

- 1) To evaluate the safety and efficacy of 6-month treatment with a fixed dosage of Sulmedol (200 mg po daily) in reducing the cognitive burden and AD neuropsychiatric symptom profile;
- 2) To correlate the changes in cognitive and related AD symptoms with biomarker measures: plasma Hcy/H<sub>2</sub>S and lymphocyte GALAC and inflammatory cytokines (interleukins: IL-1, IL-4, IL-6, IL-10, IFN- $\gamma$ , and TNF- $\alpha$ );
- 3) To correlate the changes in AD core and related symptoms with volumetric MRI brain changes.

We conclude that Sulmedol in behaving as a multi-functional ligand, may emerge as the drug lead with increased likelihood of meeting the rather stringent therapeutic endpoint as "disease modifying pipeline AD drug", with well-defined spinoffs in relieving the global disease burden and improving the quality of life in the aging population. In reviewing the safety and adverse events profile of Sulmedol submitted to Health Canada, we fail to identify any allergy reaction resembling sulfa allergy.

We are fully aware that an estimated 3-6% of the general population exhibits authenticated allergic reaction to sulfonamide antibacterial agents and hence at risk for type I and related hypersensitivity reactions to sulfamethoxazole antibiotics [30-32].

Accumulating evidence strongly suggest that the arylamine functional group of the sulfonamide anti-microbials mediates the hypersensitivity. The arylamine group is oxidized by the cytochrome CYP2C9 to hydroxylamine, followed by the auto-oxidation to the highly reactive intermediate: [-nitroso-] species recognized as the absolute requirement for sulfa-induced hypersensitivity reaction [29].

Recent clinical evidence supports the concept of cross-reactivity between sulfonamide antimicrobials and non-microbial sulfonamides: loop and thiazide diuretics/ sulfonyleureas, and cyclooxygenase-2-delective nonsteroidal anti-inflammatory agents and certain anti-viral agents [31,32]. Our preliminary clinical studies of Sulmedol in lactose intolerance and AD demonstrates that Sulmedol does not induce Sulfa allergy. It is likely that sulmedol becomes the primary substrate for the transulfuration reaction producing a host of sulfhydryl-group-containing compounds exhibiting dual anti-oxidant and anti-inflammatory properties. Further studies are required to elucidate the apparent lack of hypersensitivity reaction to elemental sulfur.

In summary, there is existing body of evidence in support of the patented product: Sulmedol formulated from elemental sulfur as a unique drug lead targeting both homocysteine/hydrogen sulfide and beta-galactosidase pathways known to be related to the etiology of AD. Currently, US FDA offers fast track for any drug lead exhibiting clear-cut evidence to slow the rate of cognitive and functional decline in AD. The AD drug development

challenge consists of translating the gains of therapeutic targets: homocysteine/hydrogen sulfide and lysosome-beta-galactosidase, towards promising AD therapeutics. Our brief therapeutics perspective on AD highlights how Sulmedol belongs to the class of multi-targeted drug (MTD) candidate in hitting the quadruple therapeutic targets in AD:

1. Reducing homocysteine and resetting hHcy/H<sub>2</sub>S coupling;
2. Releasing or activating H<sub>2</sub>S;
3. Catalysing autophagy clearance;
4. Activating beta-galactosidase.

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