Experimental focal cerebral ischemia
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Effect of SUL121, a novel chromanol-based compound in a rat model of focal cerebral ischemia-reperfusion

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ABSTRACT

**Background:** SUL121, a novel 6-chromanol-substituted compound has been shown to exert protective effects in various *in vitro* and *in vivo* models of oxidative stress and inflammation. We therefore hypothesized a similar effect to mitigate focal cerebral ischemia-reperfusion in a rodent model.

**Methods:** Transient middle cerebral artery occlusion (tMCAO, 2/22h) using an intravascular filament approach was induced in adult Sprague-Dawley rats. Intravenous bolus (200 μg/kg) followed by a slow infusion initiated 30 min before MCAO (25 μg/kg/min) of either saline or SUL121 was administered until reperfusion under low isoflurane anesthesia. All animals were euthanized 24 h post MCAO. Ischemic damage was quantified using silver nitrate staining. Oxidative stress was assessed by the malondialdehyde assay. Immunoblotting was carried out to evaluate brain damage markers and H₂S synthesizing enzymes (CBS, 3-MST). Contractile and relaxant effects were studied in aorta using isometric vessel set up.

**Results:** SUL121 mitigated cerebral ischemic damage and hemispheric swelling in comparison to the controls (*p*<0.01). However, the variable infarction that developed in both saline and SUL121 treated rats did not produce significant differences at the level of ROS, ER stress, early immediate gene induction and anti-apoptotic pathways, and effects of SUL121 on those could thus not be assessed. With respect to H₂S-synthesizing enzymes, infarction lowered the levels of 3-MST, while the expression level of CBS was upregulated by SUL121 in the ischemic brain. Finally, SUL121 treatment alleviated the impairment of ACh-evoked, NO-mediated aortic relaxation observed in saline treated animals.

**Conclusion:** The variable induction of ischemia in both study groups precluded the firm assessment of SUL121 in our model. Nevertheless, lower infract volume, upregulation of CBS and improvement of ACh-mediated relaxation, possibly by maintenance of the H₂S signaling cascade and its interaction with NO, could have potential therapeutic implications and thus needs further exploration.
INTRODUCTION

Ischemic stroke is one of the most frequent causes of adult disability worldwide. The currently available thrombolytic therapy using recombinant tissue plasminogen activator (rtPA) has been the mainstay in treating this condition (1), albeit with a narrow time window of up to 4.5 h from symptom onset (2). Several novel cerebroprotective agents have been tested and proved successful in various pre-clinical models of ischemic stroke in the quest to alleviate cerebral infarction and thereby to improve neurological outcome (3). However, translation to clinical care has met little success due to the complex pathophysiological mechanisms of infarct development and repair, warranting the exploration of novel therapeutic approaches.

Hibernating animals endure ischemia/reperfusion-like phases. These phases are primarily cycles of suppressed metabolism along with a reduced core body temperature and cerebral blood flow to near-ischemic levels (4), interspersed with short duration of complete reversal of metabolism and body temperature. Nevertheless, animals endure hibernation without signs of organ damage. Hydrogen sulfide (H\textsubscript{2}S) signaling and its substrates have emerged as key protective compounds in hibernation, possibly by serving as an electron donor to replenish ATP in the presence of reduced oxygen uptake (5,6). Cystathionine β-synthase (CBS), 3-mercaptoppyruvate sulfurtransferase (3-MST) and cystathionine γ-lyase (CSE) are the main enzymes involved in the production of H\textsubscript{2}S in mammals. Notably, the CBS/H\textsubscript{2}S signaling cascade has been shown to protect cells from cooling and rewarming injury (7). Further, CBS is upregulated in lung during the hypometabolic phase of hibernation (7) and has been implicated in reversible lung tissue remodeling during hibernation (5).

However, the ability to produce H\textsubscript{2}S is not limited to the lung. In the central nervous system (CNS), H\textsubscript{2}S is produced predominantly by CBS and 3-MST. In conditions of hypoxia/ischemia with disrupted cellular energy homeostasis and metabolism as evident in stroke, H\textsubscript{2}S could play a key role in protecting tissue from undergoing irreversible damage. The role of H\textsubscript{2}S signaling in pre-clinical models of CNS disorders including brain ischemia and trauma has however yielded somewhat controversial results. In several models of focal cerebral ischemia, exogenous administration of H\textsubscript{2}S-donors was found to mitigate ischemic damage by inhibition of apoptosis, oxidative stress, inflammation and maintaining blood-brain barrier integrity (8-12). Previously, Qu and co-workers (2006) (13) reported increased cortical levels of H\textsubscript{2}S in a permanent model of MCA stroke under ketamine-xylazine anesthesia. However, infarct size aggravated further upon treatment with sodium hydrogen sulfide (a H\textsubscript{2}S donor). Recently, it was shown that increased brain levels of H\textsubscript{2}S appear not to be associated with an upregulation of CBS expression (14).
Recently, we developed chromanol-based compounds, including one indicated as SUL121 (Fig. 1A, Sulfateq BV, Groningen, the Netherlands), which alleviate cell damage in various conditions of oxidative stress. SUL121 has been found to prevent kidney damage in hypothermic rat and to reduce bronchoconstriction and inflammation in lipopolysaccharide (LPS)-induced lung injury in guinea pig. Moreover, we found SUL121 to improve cell survival in hypothermia-rewarming induced microglial cell injury (Shanbhag et al., unpublished). Maintenance of the function and expression of CBS and 3-MST, resulting in preserved \( \text{H}_2\text{S} \) production, has been coined as one of the potential mechanisms underlying the protective effects of SUL121, as found in hypothermic injury in various cell-lines and in kidneys of hypothermic rat (Dugbartey et al., unpublished).

**Figure 1**: (A) Chemical structure of SUL121. (B) Schematic outline of experimental design. Treatment was initiated with an I.V. bolus of saline or 200 μg/kg SUL121 via a tail vein catheter followed by a slow infusion of either normal saline or SUL121 (started 30 min before MCAO, 25 μg/kg/min, at the rate of 1 ml/hr). Animals were subjected to 2 h of brain ischemia followed by reperfusion. The whole procedure was carried out under isoflurane anesthesia. Upon reperfusion, the infusions with either saline or SUL121 were discontinued. Euthanization was carried out 24 h post MCAO by in situ perfusion with normal saline and relevant organs were isolated. (C) Frozen brain slices were obtained using a cryotome, with an interslice distance of 1 mm (20 μm thick) extending fronto-occipitally and quantified using silver nitrate staining. Tissue in between the slices at each section level were subsequently divided into ischemic and contralateral hemispheres and collected in separate vials for molecular studies. I.V., intravenous; MCAO, middle cerebral artery occlusion; REP, reperfusion.

Based on its action in these models, we hypothesized that SUL121 limits ischemic damage in a rat model of ischemic stroke. In addition to neuronal cell death, stroke *per se* can trigger a systemic inflammatory response and influence systemic vascular reactivity. The associated cytokine release and generation of reactive oxygen species (ROS) may affect endothelial responsiveness both in brain as well as in systemic vasculature and thus
influence ischemic damage. Most of the therapeutic agents tested in pre-clinical models of stroke (focal ischemic damage) have focused primarily on mitigating cerebral ischemic damage without assessing their systemic vascular and/or organ specific effects, if any. We therefore set out to study changes in cerebral expression of H\textsubscript{2}S synthesizing enzymes and investigate putative protective effects of SUL121 in a rat model of transient focal cerebral ischemia. Moreover, we studied whether stroke and the treatment influence the systemic vascular function by examining vascular reactivity in aortic rings.

MATERIALS & METHODS

Animals

Adult male Sprague-Dawley rats (280-350g) obtained from Harlan (Horst, The Netherlands) were used. The animals were kept under standardized housing conditions for at least one week before the start of the experiment, with free access to standard chow and water. All experimental procedures were performed in accordance with the Dutch animal laws and institutional guidelines (DEC 6862A).

Induction of transient ischemic stroke

Right-side transient middle cerebral artery occlusion (tMCAO) was produced as previously described (15) with some modifications. Under isoflurane anesthesia (1.5-2 %), MCAO was induced by inserting a 4-0 monofilament coated with silicon at its tip into the proximal external carotid artery (ECA), navigated into the internal carotid artery and pushed forward until the origin of MCA. The completeness of occlusion was achieved under the guidance of Doppler flow probe (Perimed Instruments, Jarfalla, Sweden) placed over a shallow drilled burr hole positioned 5 mm lateral and 2 mm posterior to the bregma. Rats were included in the study if MCAO reduced laser Doppler flow (LDF) by > 50% from baseline. Upon achieving successful occlusion, the filament was secured tightly in the ECA stump, the animal turned to a prone position and allowed to undergo 2 h of occlusion maintained under low isoflurane anaesthesia (0.8–1%) until removal of the filament to establish reperfusion. This low maintenance of anesthesia was undertaken to instil relevant therapeutic agent(s) as a slow intravenous (I.V.) infusion as stated below. Peri-operative monitoring of heart rate, peripheral oxygen saturation (Nonin Med Inc., Plymouth, MN, USA) and body temperature was carried out.

Experimental groups & drug administration

Animals undergoing transient MCA occlusion (2h) followed by reperfusion (22h) were randomly distributed between 2 groups: control (CON, n=5) and SUL121 (SUL, n=6). The
animals received either normal saline or SUL121 via a tail vein catheter. SUL121 was dissolved in normal saline and administered as a bolus (200μg/kg) followed by a slow intravenous infusion (25μg/kg/min, 1ml/hr) initiated 30 min before onset of occlusion and continued until reperfusion as shown in the scheme (Fig. 1B).

Termination of experiments

Twenty-four hours post occlusion, the animals were deeply anesthetized using isoflurane and sacrificed by severing the inferior caval vein and in situ perfusion via the abdominal aorta with ice-cold normal saline until the venous outflow was clear. Blood was withdrawn from the aorta before initiating the in situ perfusion. Brain was isolated and snap-frozen in liquid nitrogen and stored at -80°C until further analysis. In addition, aorta was collected and stored separately in Krebs solution as described below.

Ischemic damage assessment

For quantitative determination of ischemic damage coronal cryosections were obtained at regular intervals. The sections were transferred onto glass slides, air-dried, and fixed with paraformaldehyde. The presence of ischemic damage was determined in each section using silver nitrate staining as described previously (16). The slides were scanned and the extent of damage quantified using ImageJ (free-ware available under [http://rsbweb.nih.gov/ij/docs/guide/index.html](http://rsbweb.nih.gov/ij/docs/guide/index.html)). Finally the volume of hemispheric ischemic damage was calculated along the fronto-occipital axis and corrected for brain swelling (Fig. 1C).

Reactive oxygen species measurement

During cryosectioning of brain, the tissues in between the sections taken for silver nitrate staining were dissected and the ischemic and contralateral hemispheres collected in respective vials for molecular analyses. Samples from individual hemispheres were homogenised with phosphate buffered saline (PBS) containing butylated hydroxytoluene (100x BHT, Cell Biolabs, Inc. Netherlands) on ice and centrifuged (10,000 g, 5 min, 4 °C). From each sample 100 μl of the supernatant was taken and mixed with the same volume of SDS-Lysis solution. The mixture was incubated at room temperature for 5 min before addition of 250 μl of thiobarbituric acid (TBA) reagent. This mixture was incubated at 95 °C for 60 min, quickly cooled down to room temperature and centrifuged (1000 g, 15 min). The supernatants were then transferred to a 96-well black fluorescence microplate for measuring the optical density (BioTek Synergy 4 plate reader; excitation and emission wavelength, 540 and 590 nm). Appropriate malondialdehyde (MDA) standard assays were...
prepared in the same manner as described above for the tissue samples and used to generate a standard curve. Levels of reactive oxygen species were expressed as micromole of MDA per milligram of tissue protein concentration. Tissue protein concentration was determined using a BioRad assay kit, as per the manufacturer’s guidelines (Bio-Rad, Germany).

**Western blotting**

Frozen brain tissue samples were homogenized in 450 μl RIPA buffer, containing protease inhibitor cocktail, sodium orthovanadate, sodium fluoride and β-mercaptoethanol (all from Sigma Aldrich, The Netherlands) prepared according to the manufacturer’s instructions (Roche, The Netherlands). After 30 min of incubation on ice, all homogenized samples were centrifuged at 14,000 g for 20 min at 4 °C. Supernatants were collected and protein concentrations determined as mentioned above. Samples were boiled for 5 min before loading onto the pre-casted SDS-polyacrylamide gel. The gel electrophoresis was carried out at 100 V for 70 min using 30 μg of each protein sample. Proteins were subsequently blotted onto nitrocellulose membranes using a transfer buffer solution containing 0.25 mM Tris (pH 8.5), 192 mM glycine and 10 % v/v methanol at 4 °C for 60 min at 0.3 mA. Thereafter, the membranes were blocked in 5% w/v skimmed milk dissolved in TBST buffer (containing 50mM Tris-HCl, pH 6.8, 150mM NaCl, 0.05 % v/v Tween-20). Subsequently, the membranes were incubated overnight at 4 °C with one the following antibodies diluted in 5% BSA (w/v) + TBST solution: anti-phospho-eIF2α (ab5369, Abcam, UK), anti-phospho c-Jun (#9261, Cell Signaling Technology), anti-Iba1 (016-20001, Wako), anti-CBS (sc-271886, Santa Cruz), anti-3MST (HPA 001240, Sigma) and anti-Mn SOD (ADI-SOD-111D, Enzo). After overnight incubation, the membranes were washed 3 times in TBST buffer and incubated with HRP-linked polyclonal secondary antibodies (Dako, Denmark) in 5% BSA-TBST solution for 1 h. Blots were developed using the Western Lighting Ultra (Perkin Elmer Inc., USA) substrates according to the manufacturer’s guidelines. Protein bands were visualized using the Gene Genome system (Westburg BV, The Netherlands) and intensities quantified using Gene Tools software. β-actin was used as house keeping protein to normalize protein expression.

**Vascular reactivity**

Vascular responses were measured in rat thoracic aortic rings (n=3 in each of the study groups) using an isometric vessel setup. Aorta was cleaned from the surrounding fat tissue and dissected into 1.5-2 mm long rings and mounted into baths containing Krebs solution, containing (in mM): 120.4 NaCl, 5.9 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 25.0 NaHCO₃, 1.2 NaH₂PO₄, 11.5 glucose (Merck, Darmstadt, Germany) at 37 °C, and bubbled continuously.
with 95% O₂/5 % CO₂ at pH 7.4. All vascular reactivity studies were carried out in aortic rings with intact endothelium. Before initiating the measurements, aorta rings were allowed to equilibrate for an hour, followed by two consecutive exposures to potassium chloride (KCl, 60 mM prepared in physiological salt solution maintaining its osmolarity with NaCl). Vascular contraction was assessed by obtaining cumulative dose response curves to phenylephrine (PE, \(10^{-9}\) to \(10^{-3}\) mol/L) and expressed as percentage of the second KCl Krebs-induced contraction. Vascular relaxation and contribution of different vasodilators was assessed in rings pre-contracted with PE (\(10^{-6}\) mol/L) by obtaining cumulative dose response curves to acetylcholine (ACh, \(10^{-9}\) to \(10^{-5}\) mol/L), in absence and presence of L-N\(^\circ\)-Nitroarginine methyl ester (LNAME, a NO-synthase blocker, \(10^{-5}\)) and/or indomethacin (INDO, a cyclooxygenase inhibitor, \(10^{-5}\)). Following the final dose of ACh, maximal endothelial-independent relaxation was measured in the aorta by application of NO-donor sodium nitroprusside (SNP, \(10^{-5}\)). Vascular relaxation was expressed as percentage of SNP relaxation.

Statistical analysis

Data are expressed as mean ± SD. Hemispheric damage and edema between control and treated groups were compared with two-tailed t-test. Two-way ANOVA analysis was used in comparisons for infarcted/contralateral hemispheres versus saline/SUL121 treatment. Vascular contractility and relaxation responses were compared using repeated measures ANOVA.

RESULTS

Mortality

One out of the 6 CON animals died due to subarachnoid hemorrhage at the base of the brain during the filament insertion as evident from the course of the laser Doppler flowmetry signal and confirmed on post-mortem examination. All of the other animals survived the 2 h occlusion followed by 22 h reperfusion period. One out of 7 animals in the SUL121 group showed a drop of LDF smaller than 50 % from the baseline upon MCAO. Both animals were excluded from the study. Consequently, animal groups constituted of n=5 in CON and n=6 in the SUL121 group.

Perioperative characteristics

Body temperature, oxygen saturation and heart rate remained within physiological limits during the perioperative period in both groups (Table 1). Occlusion of the MCA origin
resulted in a significant and comparable reduction of laser Doppler flow (LDF) in both groups (Fig. 2).

**Table 1: Peri-operative monitoring of body temperature, oxygen saturation and heart rate**

<table>
<thead>
<tr>
<th></th>
<th>Body temperature (°C)</th>
<th>SaO2 (%)</th>
<th>Heart rate (bpm)</th>
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<tbody>
<tr>
<td></td>
<td>CON</td>
<td>SU121</td>
<td>CON</td>
</tr>
<tr>
<td>Pre-MCAO</td>
<td>37.1 ± 0.4</td>
<td>37.2 ± 0.4</td>
<td>97.4 ± 0.9</td>
</tr>
<tr>
<td>MCAO</td>
<td>37.8 ± 0.4</td>
<td>37.4 ± 0.5</td>
<td>95.8 ± 1.9</td>
</tr>
<tr>
<td>1h post MCAO</td>
<td>38.2 ± 0.5</td>
<td>37.8 ± 0.4</td>
<td>95.6 ± 3.0</td>
</tr>
<tr>
<td>Pre-REP</td>
<td>37.8 ± 0.8</td>
<td>37.5 ± 0.4</td>
<td>96.8 ± 1.9</td>
</tr>
<tr>
<td>Post-REP</td>
<td>37.8 ± 0.6</td>
<td>37.4 ± 0.3</td>
<td>94.8 ± 1.8</td>
</tr>
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**SUL121 mitigates cerebral ischemic/reperfusion-induced damage**

In our study, tMCAO led to small infarct sizes with a considerable variation in both groups. Two animals in the CON (2/5) and 4 animals in SU121 (4/6) groups did not show a detectable ischemic damage as assessed by silver nitrate staining. This difference proved to be statistically significant. In addition, there was also a significantly reduced degree of brain swelling in the animals treated with SU121 (Fig. 3A, B, p<0.01, 2-tailed t-test).

**Figure 2**: Blood flow measurements by laser Doppler flowmetry (LDF) prior to, during and after the MCAO. Various time points depicted (1-5) are as follows: 1= baseline, 2= upon MCAO, 3= 1 h post MCAO, 4= before reperfusion, 5= upon reperfusion. A drop ≥ 50 % of the baseline was considered as a successful occlusion. MCAO, middle cerebral artery occlusion; CON, control; SUL, SUL121-treated group. Values are calculated as percentage drop from the initial baseline and represented as mean ± SD.

**Figure 3**: Hemisperic ischemic damage (A) and brain swelling (B) in control (CON) and SU121-treated groups. *p<0.01 vs. CON group. Values are represented as mean ± SD.
**Effect of SUL121 on cerebral oxidative stress, immediate early gene induction & inflammation**

Due to a potential interference of the use of isoflurane leading to a variable ischemic damage in our study groups, the analyses of infarcted versus non-infarcted animals would have led to a too low number of animals in the respective groups failing to identify possible treatment effects on various molecular markers of brain damage. We therefore employed two-way ANOVA to reveal treatment effects by comparing both treatment groups and infarcted versus contralateral hemispheres.

![Brain damage markers in control and SUL groups.](image)

**Figure 4:** Brain damage markers in control and SUL groups. (A) Oxidative stress (MDA levels), (B) ER stress (phospho-eIF2α), (C) immediate early gene activation (phospho-c-Jun), (D) microglial activation (Iba-1), and (E) anti-apoptotic phenotype (Mn-SOD) did not show any significance difference between the study groups. MDA, malondialdehyde; PC, positive control (LPS-stimulated microglial cells); ER, endoplasmic reticulum; Mn-SOD, manganese superoxide dismutase; Iba-1, ionized calcium-binding adaptor molecule 1. *p<0.05 ischemic vs. contralateral hemisphere. ISCH, ischemic hemisphere; CONTRA, contralateral hemisphere.

Reactive oxygen species (ROS) could not be quantified, as the level of MDA was below its detection level in both treatment modalities and in both hemispheres (Fig. 4A). In line with undetectable ROS levels, the ischemic animals did not show induction of ER stress as assessed by the level of phospho-eIF2α (Fig. 4B). The expression of the immediate early gene, phospho-c-Jun was observed to be significantly increased upon SUL121 treatment in ischemic as compared to the contralateral hemisphere (Fig. 4C). Nonetheless, the microglial activation marker (Iba-1) was unaffected, as was the expression level of Mn-SOD, a key component of mitochondrial matrix (Fig. 4D, E). Taken together, extensive analysis of brain damage markers failed to disclose marked differences between infarcted and uninfarcted brain or differences between SUL and CON groups.
Regulation of H$_2$S during stroke and the effect of SUL121

In addition to damage markers, 2 key enzymes involved in H$_2$S production in the brain were quantified using western blotting, i.e. CBS and 3-MST. SUL121 treatment significantly upregulated CBS expression in the ischemic brain compared to CON group (Fig. 5A, p<0.05, two-way ANOVA). The intra and inter-hemispheric differences were not evident in both the groups which can be attributed to a variable infliction of ischemic damage. On the other hand, infarction lowered the expression of 3-MST when compared to contralateral hemispheres without an effect of treatment (Fig. 5B, p<0.05, two-way ANOVA).

**Figure 5:** CBS and 3-MST expression level in brain following transient MCAO. SUL121 treatment resulted in a significant upregulation of CBS in comparison to vehicle treated groups (A). Contrary, infarction downregulated 3-MST level in comparison to contralateral hemispheres in both the groups (B). **p<0.05 for CBS in SUL121 (SUL) vs. vehicle-treated (CON) groups (two-way ANOVA with Bonferoni’s test). #p<0.05 vs CON-ischemic hemisphere; ##p<0.05 vs SUL-ischemic hemisphere (repeated measures ANOVA). Values are represented as mean ± SD. ISCH, ischemic hemisphere; CONTRA, contralateral hemisphere.

SUL121 improves aortic relaxation function

Vascular contraction and relaxation was assessed in aortic rings by obtaining cumulative dose response curves to phenylephrine (PE) and acetylcholine, respectively. Treatment with SUL121 did not affect contraction to PE (Fig. 6A), but significantly improved ACh-induced dilatation in rat aorta precontracted with PE (Fig. 6B).
Figure 6: Effect of SUL121 treatment on contractile and relaxant response in aorta of tMCAO rats. (A) SUL121 treatment did not affect PE-mediated contractile responses in comparison to vehicle treated (CON) group, while it improved the ACh-mediated aortic relaxation (B). Preincubation of aortic rings with L-NAME, in absence (C) and presence of INDO (D) normalize relaxation of SUL121 treated rat to that of vehicle infused rats. *p<0.05 vs 1.0 x 10^-5 M, **p<0.05 vs 3.0 x 10^-5 M CON+VEH groups. Values are represented as mean ± SD. Statistical significance was determined by a two-way repeated measures ANOVA with Bonferroni’s test. PE, phenylephrine; ACh, acetylcholine; L-NAME, L-N-Nitroarginine methyl ester; INDO, indomethacin. n=3 in each of the study groups.

To explore if and which endothelial component contributed to the improved relaxation, dose response curves to ACh were obtained in the presence of the NO synthase blocker (L-NAME, 10^-4 mol/L) and in the presence of L-NAME and the COX inhibitor, indomethacin (INDO, 10^-4 mol/L). Endothelial blockade of NO with L-NAME decreased the vasorelaxant reactivity in CON and SUL groups and abrogated the difference in relaxation between both groups (Fig. 6C). Preincubation with INDO, in presence of L-NAME, appeared to reduce vasorelaxation more in the aortas of SUL121-treated animals in comparison with vehicle-treated group (Fig. 6D). Thus, cerebral ischemia resulted in impairment of NO-mediated vasorelaxant reactivity in rat aortas, which was precluded by SUL121. Further, maintenance of vasorelaxation in SUL121-treated animals may involve relaxant prostanoids.
DISCUSSION

In the current study we explored the putative protective action of SUL121 in a rat focal cerebral ischemia/reperfusion model and also assess its impact on systemic vascular function. SUL121 belongs to a novel class of chromanol-based compounds which was proven to mitigate oxidative stress and inflammation in cellular injury models (such as hypothermia-rewarming) as well as in in vivo models of bronchial inflammation, forced hypothermia-rewarming and diabetes. In most instances, SUL121 has been shown to influence H₂S signaling, particularly maintaining the expression of the enzymes involved in its production. Here we found SUL121 to mitigate the cerebral ischemic damage and hemispheric swelling in comparison to the controls. However, infarction itself did not produce significant differences at the level of ROS, ER stress, early immediate gene induction and anti-apoptotic pathways, and effects of SUL121 on those could thus not be assessed. With respect to H₂S-synthesizing enzymes, infarction lowered the levels of 3-MST, while the expression level of CBS was upregulated in the ischemic brain globally by SUL121 treatment. Finally, SUL121 increased endothelium dependent vascular relaxation by preserving its NO component. At this end, further exploration to ascertain positive effects of SUL121 in focal brain ischemia is highly warranted.

In this study, tMCAO resulted in incomplete infarction in a substantial number of animals. As in previous studies by us and others this technique resulted in reproducible ischemic damage, the question arises why infarction was highly variable in this cohort. Importantly, the filament occlusion produced an adequate and similar MCAO occlusion in all animals, as evident from the similar drop in laser Doppler flow. Moreover, ischemic damage varied highly between animals within the control group, suggesting that the variance was not produced by SUL121 treatment. The key difference between the current and our previous studies (17,18) concerns the length of isoflurane anesthesia. Previously, isoflurane anesthesia was halted upon achieving successful MCAO and the animals gained full consciousness during the entire 2 h occlusion period. Thereafter, the animals were re-anesthetised using isoflurane to carry out the reperfusion procedure. In contrast, ethical considerations led by speculation on a high discomfort level during the occlusion period in the absence of anesthesia, imposed constraints on the current experiment to maintain isoflurane anesthesia throughout the 2 h occlusion period. Although isoflurane has been used in several pre-clinical studies with transient and/or permanent MCAO, hitherto these protocols allowed the animal to recover during the occlusion period. Indeed, several studies have shown isoflurane treatment to mitigate infarction and neuroinflammation in ischemic stroke models when administered either as a pre- or postconditioning treatment (19-23). Such an effect of isoflurane may have been prominent in our study protocol comprising a two hours exposure to isoflurane, i.e. throughout the whole occlusion.
period. Thus, the experimental protocol turned out to eventually exert a significant effect of its own on the issues of the current project.

Despite the putative interfering effect of isoflurane our data suggest that SUL121 treatment mitigated focal cerebral infarction and the accompanying swelling. Focal cerebral ischemia is known to upregulate and/or to induce various tissue reaction patterns including oxidative stress, early gene activation, apoptosis and inflammation (24). We found no significant difference in the level of oxidative stress, ER stress or activation of immediate early gene markers. This lack of difference can likely be attributed to the prolonged exposure of isoflurane and its potential neuroprotective effects as described above and therefore warrants detailed exploration by excluding its usage during the occlusion period.

Exploration of expression of H$_2$S synthesizing enzymes showed SUL121 to significantly upregulate CBS expression in the ischemic brain globally without affecting 3-MST levels. Such an effect of SUL121 on CBS is known from our previous (7) and ongoing work. For instance, Dugbartey et al (unpublished) observed that SUL121 protected kidneys from injury induced by forced hypothermia-rewarming while maintaining the levels of H$_2$S-synthesising enzymes. Moreover, permanent MCAO (using a craniectomy approach) in ketamine-xylazine anesthetized rats has been shown to increase the cortical H$_2$S levels in the infarcted hemisphere, while exogenous administration of H$_2$S-donors aggravated the infarction with an unchanged gene expression of CBS or CSE (13). Later, studies by the same group observed a downregulation of 3-MST in the infarcted hemisphere in comparison to the contralateral side, indicating that 3-MST is probably not responsible for the increased H$_2$S production in the ischemic brain (25). Instead upregulation of CBS in the infarcted hemisphere might underlie the increased production of H$_2$S in ischemia (26). In our study, CBS expression was significantly upregulated by SUL121 treatment in the ischemic brain. On the other hand, the lowering of 3-MST in the infarcted hemispheres in both untreated and SUL121 groups matches the observations by Zhao et al (2013). In conclusion, differences in the type of anesthetic regime and the model of focal cerebral ischemia induction may have influence on the expression of CBS and 3-MST. Whether isoflurane alone could affect the baseline levels of H$_2$S synthesizing enzymes in brain is still unknown.

Finally, the current study showed that SUL121 treatment increased the ACh-mediated relaxation in rat aorta after tMCAO. Ischemic stroke per se can elicit a systemic inflammatory response (27-29) which could also alter the systemic vascular reactivity in various vascular beds. The differential vascular responses in our study upon L-NAME treatment revealed NO as the major contributor to ACh-mediated relaxation in rat aorta.
H$_2$S has been recognized recently as an important EDHF (30,31), with substantial influence on NO signaling, synergizing its effects and even increasing the NO bioavailability (32-34). As SUL121 treatment significantly affected the H$_2$S synthesizing enzyme CBS in brain, it would be apt to speculate on a potential cross-talk between NO and H$_2$S (35,36). The effect of SUL121 in alleviating the reduction in NO-mediated relaxation upon tMCAO might well be related to its maintenance of the H$_2$S synthesizing enzymes. In this regard, blockade of endogenous H$_2$S production by inhibitors such as AOAA could possibly unravel the link and thus needs further exploration by having a larger cohort to ascertain its role.

**CONCLUSION**

In this study, SUL121 mitigated the cerebral ischemic damage and hemispheric swelling in a transient MCAO model in comparison to saline treated controls. However, the degree of infarction produced varied markedly in both groups, likely representing the influence of extended isoflurane exposure in our study. As the infarction itself did not depict significant changes at the level of ROS, ER stress, early immediate gene induction and anti-apoptotic pathways, the effects of SUL121 on those could thus not be assessed. With respect to H$_2$S-synthesizing enzymes, infarction lowered the levels of 3-MST, while SUL121 increased the expression level of CBS. Finally, SUL121 preserved the ACh-mediated vasorelaxant reactivity in rat aorta, possible mediated by its action on NO via H$_2$S signaling cascade. Further studies are warranted to explore the neuroprotective effects of SUL121 in a detailed manner.

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Figure: Western blots depicting expression level of various markers employed in the study groups between ischemic and contralateral hemispheres. Infarct (+) and infarct (−) samples from ischemic and contralateral hemispheres were loaded in 2 separate gels as depicted in the upper and lower panel with the inclusion of a positive control on each of the gel (PC, using LPS stimulated BV2 microglial cells) respectively. CON, vehicle-treated; SUL, SUL121-treated.