Thesis approved by

Date: 08-14-2013

Catherine A. Opere, Ph.D

Manzoor Khan, Ph.D

Somnath Singh, Ph.D

Dean, Graduate School
Effect of Hydrogen Sulfide donors on Sympathetic Neurotransmission and Intraocular pressure of the Eye

by

ANKITA ABHAY SALVI

A THESIS
Submitted to the faculty of the Graduate School of the Creighton University in partial fulfillment of the requirements for the Degree of Master of Science in the Department of Pharmacy Sciences

Omaha, NE (August 2013)
ABSTRACT

Hydrogen sulfide (H$_2$S) has been identified to be one of the gaseous transmitters in the body. There are studies enumerating its physiological significance in regulation of different processes in various organ systems. Identification of enzymes involved in the endogenous synthesis of the gas in the eye led to the investigation of its role in the eye. It was found that H$_2$S is not only required at physiological concentrations, but its deficiency can lead to some ocular conditions.

Glaucoma is an ocular disorder that involves progressive damage to the optic nerve. The condition is associated with an increase in the intraocular pressure (IOP). Its levels are maintained with a balance in the inflow and outflow of aqueous humor, a transparent fluid responsible for maintaining IOP. Existing therapies include medications either reducing aqueous humor production or strategies improving its drainage in order to restore the normal IOP. However these therapies are associated with many side effects and there is a need to improve the therapy for glaucoma.

The present study investigated the role of H$_2$S donors in decreasing sympathetic neurotransmission in the anterior uvea in the eye and reducing IOP. There is evidence that decrease in levels of norepinephrine (NE), the major sympathetic neurotransmitter causes relaxation of pre-contracted iris muscles that increases space between the trabecular meshwork. This improves drainage of aqueous humor which can help alleviate IOP.
In this study, the effect of two H\textsubscript{2}S donors, GYY 4137 and ACS67 and L-cysteine, the substrate for endogenous H\textsubscript{2}S production on electrically evoked [\textsuperscript{3}H]NE release in isolated bovine iris-ciliary bodies (ICB) \textit{in vitro} was determined. GYY 4137 (1-30 \textmu M), ACS67 (10 nM-10 \textmu M) as well as L-cysteine (10 nM-10 \textmu M) attenuated field-stimulated [\textsuperscript{3}H]NE release in bovine ICB in a concentration-dependent manner. The inhibition was in the order: ACS67 > L-cysteine > GYY 4137. GYY 4137 elicited a maximum inhibition of 20.8% (n=3; p<0.001) at 30 \textmu M while ACS67 achieved a maximum inhibition of 37.26% (n=5; p<0.001) at 10 \textmu M. For L-cysteine, the maximum inhibition achieved was 26.13% (n=5; p<0.5) at a dose of 10\textmu M. It was also found that at high doses, endogenously produced prostaglandins (PGs) contributed to the effect of the drugs. In order to eliminate this effect, endogenous PG synthesis was blocked using flurbiprofen (FBF) which is a PG synthesis inhibitor.

To determine the mechanism of action of the drugs, antagonists were used. These included aminooxyacetic acid (AOA), a cystathionine-β-synthase (CBS) inhibitor and glibenclamide, a K\textsubscript{ATP} channel blocker. Although AOA (3mM) and glibenclamide (300 \textmu M) had no effect (p>0.05) on [\textsuperscript{3}H]NE release, they both reversed the inhibitory action of GYY 4137 (10-30 \textmu M), ACS67 (0.1-1 \textmu M) and L-cysteine (10 \textmu M) on the neurotransmitter release. This led to the conclusion that the inhibitory action on neurotransmission is partially dependent on \textit{in situ} release of H\textsubscript{2}S and possibly on the activation of K\textsubscript{ATP}-channels.

Finally \textit{in vivo} experiments were performed to study the effect of the drugs on IOP in male normotensive rabbits. GYY 4137 (0.1-2\%) and ACS67 (0.005-001\%) reduced IOP in both treated and untreated eyes. GYY 4137 (2\%) exhibited a maximum inhibition of 27.76\%
(n=5; p< 0.001) 6h after treatment, ACS67 (0.005%) achieved a maximum effect of 23.27%
(n=5; p<0.001) 3h after treatment.

Thus the final conclusion drawn from the studies was that H\textsubscript{2}S donors attenuate sympathetic neurotransmission in the anterior uvea. This effect could be primarily related to the release of H\textsubscript{2}S from the drugs which possibly act via activation of K\textsubscript{ATP} channels. This alteration in neurotransmission can be co-related to the ability of these H\textsubscript{2}S donors in alleviating IOP, however this hypothesis merits further investigation.

One approach to confirm this hypothesis could be investigating the effect of AOA and glibenclamide on the activity of drugs in vivo. Studying the effect of the H\textsubscript{2}S donors on IOP of glaucomatous rabbits could further confirm the efficacy of these drugs as a potential therapy for glaucoma.
PREFACE

Abstracts:


Salvi, Ankita; Bankhele, Pratik ; Jamil, Jamal ; Njie-Mbye, Ya Fatou ; Kulkarni, Madhura S. ; Ohia, Sunny E. ; Opere, Catherine A. “Regulation of Mammalian Sympathetic Neurotransmitter Release and Intraocular Pressure by Hydrogen Sulfide Donors” ARVO (The Association for Research in Vision and Ophthalmology), Seattle, held in 5-9 May 2013.

DEDICATION

To my loved ones
I would like to thank my advisor, Dr. Catherine Opere for her immeasurable support, guidance and inspiration during the tenure of my research work. I am extremely grateful to her for giving me an opportunity to be a part of her group.

I would like to thank my committee members Drs. Catherine Opere, Manzoor Khan and Somnath Singh for their guidance and support through my masters training.

I am sincerely grateful to Dr. Manzoor Khan, program director and Dr. Alekha Dash, Chair of Department of Pharmaceutical Sciences for giving me an opportunity to be a part of this master’s program.

I would also like to extend my sincere appreciation to Dr. Aimee Limpach and Dr. Harsh Chauhan for their invaluable guidance through journal club presentations.

My sincere thanks to my lab mates Pratik Bankhele and Jamal Jamil, this task would have been impossible without their constant support.

I would like to extend my gratitude to the faculty and staff of the Department of Pharmaceutical Sciences for their help.

To merely thank my friends and family in the United States and back home in India would not be enough. I am extremely grateful for all their support and encouragement. Finally, I am grateful to God for making it all possible.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>iii</td>
</tr>
<tr>
<td>Preface</td>
<td>vi</td>
</tr>
<tr>
<td>Dedication</td>
<td>vii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>viii</td>
</tr>
<tr>
<td>Table of contents</td>
<td>ix</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xiii</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>xv</td>
</tr>
</tbody>
</table>

## I. INTRODUCTION

### A. GENERAL CONSIDERATIONS

1. **Hydrogen sulfide**
   1.1 H$_2$S: a gasotransmitter .................................................. 2
   1.2 Enzymatic synthesis of H$_2$S .................................................. 2
   1.3 Release ..................................................................................... 4
   1.4 Metabolism and elimination ....................................................... 5
   1.5 Mechanism of action of H$_2$S ..................................................... 6
   1.6 Physiological functions of H$_2$S .............................................. 9
   1.7 Potential role of H$_2$S in the eye .......................................... 13

2. **Glaucoma**
   2.1 Clinical manifestations of glaucoma......................................... 14
   2.2 Aqueous humor dynamics ........................................................... 15
B. SPECIFIC CONSIDERATIONS

1. Hypothesis and Specific Aims

2. Literature review of Specific Aims

   2.1 Aim # 1

   2.2 Aim # 2

   2.3 Aim # 3

II. MATERIALS AND METHODS

A. General Methods

1. In vitro studies
   1.1 Preparation of Kreb’s buffer
   1.2 Preparation of Bovine Iris-Ciliary bodies
   1.3 Studies on [^3H]NE Release

2. In vivo studies

3. Data Analysis

4. Sources of drugs and chemicals

B. Specific Methods

1. Effect of H₂S donors on sympathetic neurotransmission, in vitro
2. Effect of antagonists- AOA and glibenclamide on the action of H₂S donors.... 31

3. Effect of H₂S donors on IOP, in vivo ................................................................. 32

III. RESULTS

Specific Aim # 1: To investigate the effect of H₂S donors on sympathetic neurotransmission, in vitro

1.1 Effect of agonists on [³H]NE release ........................................................................ 34

1.2 Role of endogenous PG synthesis .......................................................................... 35

1.3 Effect of H₂S donors on sympathetic neurotransmission, in vitro ....................... 37

Specific Aim # 2: To determine the mechanism of action of H₂S donors.............. 41

Specific Aim # 3: To study the effect of H₂S donors on IOP, in vivo .......... 45

IV. DISCUSSION

1. Effect of H₂S donors on sympathetic neurotransmission, in vitro .................... 53

2. Determination of mechanism of action.............................................................. 57

3. Effect of H₂S donors on IOP, in vivo ................................................................. 59

V. SIGNIFICANCE OF STUDY ............................................................................. 63

VI. CONCLUSION .................................................................................................. 64

VII. FUTURE DIRECTIONS .................................................................................. 65

VIII. REFERENCES ................................................................................................. 66
LIST OF TABLES

1. Composition of Kreb’s buffer solution................................................................. 25

2. Sources of $\text{H}_2\text{S}$ ................................................................................................. 40

3. Peak activity, duration of hours and maximum % IOP reduction of different concentrations of GYY 4137 and ACS67 ................................................................. 50
LIST OF FIGURES

1. Enzymatic synthesis of H\textsubscript{2}S .......................................................... 3

2. Release of H\textsubscript{2}S ........................................................................ 5

3. Role of H\textsubscript{2}S as an anti-oxidant ...................................................... 8

4. Different signaling pathways in cardiovascular system modulated by H\textsubscript{2}S ........... 9

5. Normal eye versus glaucomatous eye .................................................................. 15

6. Blockade of peripheral vision in glaucoma ...................................................... 15

7. Aqueous humor dynamics.............................................................................. 16

8. Aqueous humor outflow.............................................................................. 17

9. Effect of control on [\textsuperscript{3}H]NE release ........................................ 35

10. Effect of GYY 4137 (30 µM) on [\textsuperscript{3}H]NE release .................................. 35

11. Effect of ACS67 with or without FBF on electrically evoked [\textsuperscript{3}H]NE release in isolated bovine ICB, \textit{in vitro} ................................................................. 36

12. Effect of L-cysteine with or without FBF on electrically evoked [\textsuperscript{3}H]NE release in isolated bovine ICB, \textit{in vitro} ................................................................. 36

13. Inhibitory effect of fast donor, ACS67 and latanoprost on electrically evoked release of [\textsuperscript{3}H]NE release in isolated bovine ICB, \textit{in vitro} ................................................................. 38
20. Effect of GYY 4137 (0.1-2%) on IOP in normotensive conscious albino rabbits, in vivo ...................................................................................... 46
21. Summary of effect of GYY 4137 (0.1-2%) on IOP in normotensive conscious albino rabbits, in vivo ...................................................................................... 47
22. Effect of ACS67 (0.005-0.01%) on IOP in normotensive conscious albino rabbits, in vivo ...................................................................................... 48
23. Summary of effect of ACS67 (0.005-0.01%) on IOP in normotensive conscious albino rabbits, in vivo ...................................................................................... 49
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$S</td>
<td>Hydrogen sulfide</td>
</tr>
<tr>
<td>IOP</td>
<td>Intraocular pressure</td>
</tr>
<tr>
<td>ICB</td>
<td>Iris-Ciliary body</td>
</tr>
<tr>
<td>FBF</td>
<td>Flurbiprofen</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>AOA</td>
<td>Aminooxyacetic acid</td>
</tr>
<tr>
<td>CBS</td>
<td>Cystathionine-β-synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>CO</td>
<td>Carbon monoxide</td>
</tr>
<tr>
<td>CSE</td>
<td>Cystathionine-γ-lyase</td>
</tr>
<tr>
<td>3MST</td>
<td>3-Mercaptopuruvate sulfurtransferase</td>
</tr>
<tr>
<td>CAT</td>
<td>Cysteine aminotransferase</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic Adenosine monophosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>LTP</td>
<td>long term potentiation</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic Guanosine monophosphate</td>
</tr>
<tr>
<td>PKG</td>
<td>Protein kinase G</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>NaHS</td>
<td>Sodium hydrogen sulfide</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>RGC</td>
<td>retinal ganglion cells</td>
</tr>
<tr>
<td>Na$_2$S</td>
<td>Sodium disulfide</td>
</tr>
<tr>
<td>PGs</td>
<td>prostaglandins</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
</tbody>
</table>
INTRODUCTION
A. GENERAL CONSIDERATIONS

1. Hydrogen sulfide (H$_2$S)

1.1 H$_2$S a gasotransmitter

H$_2$S, a colorless, flammable and water-soluble gas (Wagner et al., 2009), has been known to be a toxic pollutant for centuries (Tan et al., 2010). Though its hazardous effects on the environment cannot be denied, its importance as a gasotransmitter in the cardiovascular system, inflammatory system and the nervous system has been documented (Wagner et al., 2009). Gasotransmitters are gaseous constituents that are produced endogenously in the body. They perform physiological functions in various organ systems by directly acting on cellular targets (Pae et al., 2009). The first gas to be discovered amongst gasotransmitters was nitric oxide (NO) while carbon monoxide (CO) and H$_2$S were later discovered (Pae et al., 2009). Being a gasotransmitter, H$_2$S is enzymatically synthesized in the body and after acting on its targets, it is metabolized and then excreted from the body.

1.2 Enzymatic synthesis of H$_2$S

H$_2$S is synthesized endogenously in various tissues from the sulfur-containing amino acid L-cysteine. The synthesis of this gasotransmitter occurs in the presence of either of the two enzymes: cystathionine-β-synthase (CBS) or cystathionine-γ-lyase (CSE), with pyridoxal 5’-phosphate (vitamin B$_6$) working as a co-factor. CBS and CSE are expressed in the liver, kidney, brain as well as the other tissues like ileum, uterus and placenta (Kimura, 2011). Apart from its
production from L-cysteine, H$_2$S is also produced from the condensation reaction between homocysteine and cysteine in the presence of CBS and CSE (Chen X, Jhee KH, 2004; Chiku et al., 2009). Approximately 70% of H$_2$S is produced from cysteine and the remaining 30% from homocysteine (Kimura, 2011). 3-Mercaptopyruvate sulfurtransferase (3MST) is another H$_2$S producing enzyme belonging to the class of sulfurtransferases; which along with cysteine aminotransferase (CAT) produces the gas from cysteine in the presence of α-ketoglutarate (Cooper, 1983; Frendo J, 1997; Kuo SM, Lea TC, 1983). 3MST is expressed in liver, kidney, heart, lung, thoracic aorta and the brain (Nagahara et al., 1998; Shibuya et al., 2009). H$_2$S is present in micromolar concentrations in the extracellular space and has been detected in mammalian tissues were in the range of 50-160 μM. It is permeable through cell membranes and thus reaches its target without the need of specific transporters (Wagner et al., 2009).

**Fig. 1:** Enzymatic synthesis of H$_2$S (Predmore et al., 2012)
1.3 Release

After its production, H$_2$S is either immediately released or stored until a physiological response is received. It is stored in the tissues in either of the two forms: acid-labile sulfur or bound sulfane-sulfur (Toohey, 1989; Ogasawara et al., 1993). Acid labile sulfur comprises of sulfur atoms in iron-sulfur complexes. It is involved in redox reactions occurring in the mitochondria and release H$_2$S under acidic conditions with pH below 5.4 (Ishigami et al., 2009). Since this is not the physiological pH, these complexes do not release H$_2$S under physiological conditions.

Another form is the bound sulfane-sulfur that involves covalently bound sulfur to other units like outer sulfur atoms of persulfides and inner chain atoms of polysulfides. H$_2$S is release from this form under reducing conditions (Ogasawara Y, Isoda S, 1994). H$_2$S produced via 3MST and CAT enzymes is stored in this form. Thus intracellular levels of bound sulfane-sulfur depend on H$_2$S producing activity of these enzymes. Additionally, exogenously applied free H$_2$S gets incorporated into bound sulfane-sulfur units (Ishigami et al., 2009).

H$_2$S is released at a pH around or higher than 8.4 from neurons and astrocytes (Ishigami et al., 2009). Upon excitation of neurons, there is an entry of sodium ions into the cells whereas potassium ions exit the cell. This causes increase in the extracellular potassium ion concentration which leads to depolarization of the membrane of surrounding astrocytes and activation of their Na$^+$/HCO$_3^-$ co-
transporters. Thus HCO₃⁻ enter the cells causing alkalization of the cells and hence release of H₂S (Brookes N, 1994). A stimulus of approximately 10 mM K⁺ has been found to shift the pH to 8.4 and cause release of H₂S in 10% of cultured astrocytes (Ishigami et al., 2009).

![Diagram of H₂S metabolism and elimination]

**Fig. 2: Release of H₂S.** Bound sulfane sulfur is a store of H₂S (modified from Kimura, 2011).

### 1.4 Metabolism and elimination

H₂S undergoes metabolism via one of the three pathways: mitochondrial oxidation to thiosulfate which is further converted to sulfite and sulfate; cytosolic methylation to dimethylsulfide; and binding to hemoglobin to form sulfhemoglobin (Lowicka E, 2007). One of the major routes of elimination of H₂S is its exhalation. There is a possibility of using exhaled H₂S as a diagnostic
measurement. H$_2$S is also excreted in the urine as free sulfate or thiosulfate. In feces, it is excreted in the form of sulfide (Wang, 2012).

1.5 Mechanism of action of H$_2$S

So far, several pathways and targets that have been postulated to account for the mechanism of action of H$_2$S in different tissues of the body:

a) Activation of K$_{ATP}$ channels: H$_2$S acts on the extracellular loop of the SUR1 subunit of K$_{ATP}$ channels. Activation of these channels help H$_2$S regulate the processes of inflammation, vasorelaxation, nociception, pain and cell death. Opening of these channels also play a role in exerting protective effects against ischemia damage, apoptosis etc. H$_2$S mediated neuroprotection and suppression of glutamate toxicity have also been correlated to activation of K$_{ATP}$ channels (Wang, 2012).

b) cAMP and PKA pathway: H$_2$S leads to activation of cAMP/PKA pathway since it inhibits the enzyme phosphodiesterase (PDE). These enzymes cause decomposition of cyclic adenosine monophosphate (cAMP) to adenosine monophosphate (AMP). Thus their inhibition increases levels of cAMP which then activates Protein kinase A (PKA) which then further phosphorylates different signaling or acceptor proteins. In the CNS, this activation leads to phosphorylation of N-methyl-D-aspartate (NMDA) receptors that lead to potentiation of early and late phases of long term
potentiation (LTP). On the other hand, in muscle cells, activation of the cAMP/PKA pathway inactivates myosin light-chain kinase. Thus muscle contraction cannot be triggered leading to relaxation (Wang, 2012).

c) **cGMP and PKG pathway**: Due to its ability to inhibit PDE, H$_2$S is thought to elevates the levels of cyclic guanosine monophosphate (cGMP), thus activating cGMP/PKG pathway. This pathway appears to account for the cardioprotective effects of H$_2$S in cultured rat aortic smooth muscle cells (Wang, 2012).

d) **MAPK Family**: The mitogen activated protein kinase (MAPK) superfamily, which regulates various processes such as cell proliferation, apoptosis, differentiation, inflammation and cycle progression, is activated by H$_2$S. In human aortic smooth muscle cells, activation of this pathway induces apoptosis via activating ERK, one of the members of the MAPK superfamily (Yang et al., 2010)(Yang G, Sun X, 2004). Additionally, H$_2$S induced cell survival of human polymorphonuclear cells by inhibiting phosphorylation of p38, another member of the MAPK family, affirming the involvement of MAPK-superfamily in H$_2$S–mediated effects (Rinaldi et al., 2006).

e) **Cell cycling checking points**: H$_2$S affects cell proliferation or death by altering the fate of the cell going through cell cycle (Wang, 2012). For
instance 1mM of sodium hydrogen sulfide (NaHS) causes proliferation of nontransformed intestinal IEC-18 cell by facilitating cell cycle entry (Deplancke B, 2003).

f) **Antioxidant and Reducing capacity**: \( \text{H}_2\text{S} \) functions as an antioxidant since it has an SH group that causes reduction of disulfide bonds and radical scavenging of reactive oxygen species and nitrogen species (Ganster et al., 2009). \( \text{H}_2\text{S} \) also increases cellular glutathione (GSH) levels. This indirectly contributes to its antioxidant property since GSH is a major intracellular antioxidant (Wang, 2012).

![Fig. 3](image)

**Fig. 3**: Role of \( \text{H}_2\text{S} \) as an anti-oxidant

g) **Protein S-sulfhydration**: This is one of the most recently discovered mechanisms. \( \text{H}_2\text{S} \) causes covalent modification of cysteine residues in proteins through S-sulfhydration. This involves conversion of cysteine –SH groups to hydrosulfide i.e., -SSH groups (Mustafa et al., 2009). S-
sulphydration contributes to increased activity of the modified proteins (Wang, 2012).

![Diagram of signaling pathways in cardiovascular system modulated by H₂S. AC, adenylyl cyclase; sGC, soluble guanylyl cyclase; PDEs, phosphodiesterases. The inhibitory effect is denoted with (-), and the stimulatory effect is denoted with (+).](image)

**Fig. 4:** Different signaling pathways in cardiovascular system modulated by H₂S. AC, adenylyl cyclase; sGC, soluble guanylyl cyclase; PDEs, phosphodiesterases. The inhibitory effect is denoted with (-), and the stimulatory effect is denoted with (+)

1.6 Physiological functions of H₂S

H₂S has been found to be effective in various organ systems of the body. Free H₂S reaches different targets and performs functions.

a.) Neuromodulation: H₂S functions as a neuromodulator at low concentrations (< 130μM) and has been shown to facilitate induction of LTP in rat hippocampus via activation of NMDA receptors. LTP is significant for learning and memory. Thus H₂S is an important neuromodulator in the CNS (Zhou Cheng-fang, 2011). It also suppresses synaptic potentials and modifies K⁺ channels (Abe K, 1996).
b) Regulation of GABA (B) receptors: γ-amino butyric acid (GABA) is the major inhibitory neurotransmitter in the brain. GABA (B) receptors are located on the pre-synaptic ends of neurons and are involved in regulation of release of GABA and glutamate. H₂S increases expression of the pre-synaptic GABA (B) receptor subunits 1 and 2. This is beneficial in seizures accompanied by loss of these pre-synaptic receptors (Zhou Cheng-fang, 2011).

c) Vasorelaxation: H₂S shows anti-nociceptive and blood pressure lowering effects by activating K<sub>ATP</sub> channels that causes relaxation of smooth muscles (Tang G, Wu L, 2010). This effect of relaxation is seen in thoracic aorta, portal vein and ileum. Oxygen modulates sensitivity of the vessels to relaxation produced by H₂S (Kimura, 2011).

d) Regulation of intracellular calcium and pH homeostasis in neurons and glial cells: H₂S regulates calcium homeostasis. It increases the influx of intracellular Ca²⁺ in microglial cells and astrocytes, thus increasing levels of intracellular Ca²⁺. H₂S also controls intracellular pH (pH<sub>i</sub>) homeostasis as seen in rat primary cultured glial cells. This balance of pH<sub>i</sub> is significant in regulation of ion channel conductance, synaptic transmission and gap junctions (Zhou Cheng-fang, 2011).
e) **Cytoprotection:**

i. **Nervous system:** H$_2$S prevents neurodegeneration, neuron apoptosis and gliosis in mice (Kida et al., 2011). It prevents neurodegeneration by upregulation of genes encoding anti-inflammatory and anti-oxidant properties (Predmore et al., 2012).

ii. **Cardiovascular system:** H$_2$S protects cardiac muscle from ischemia/reperfusion injury on heart as observed in murine models. This effect is seen since H$_2$S increases GSH levels thus activating reperfusion injury salvage kinase (RISK) pathway (Predmore et al., 2012).

iii. **Digestive system:** H$_2$S protects the gastric epithelium from oxidative stress. Endogenous H$_2$S is involved in regulation of acid-induced bicarbonate ion secretion and mucosal protection of duodenum. It is also involved in increasing epithelial secretion and mucosal blood flow, thus further protecting the epithelium (Predmore et al., 2012).

iv. **Anti-inflammatory and cytoprotective effects:** H$_2$S confers protection against ischemia-reperfusion injury in heart, brain, retina, liver, H$_2$O$_2$-induced damage in rat gastric epithelial cells. These effects have been attributed to the ability of H$_2$S to activate K$_{ATP}$ channels and its inhibitory action on the activation of NF-Kb and p38 MAPK pathway (Predmore et al., 2012).

v. **Anti-apoptotic effects:** Apoptotic cell death contributes to neurotoxin-induced neurotoxicity. H$_2$S has been shown to have anti-
apoptotic properties in PC12 and SH-SY5Y cells. This action is mediated via preservation of mitochondrial function, reducing loss of mitochondrial membrane potential (MMP), attenuating activation of reactive oxygen species (ROS) and decreasing release of cytochrome C (Zhou Cheng-fang, 2011).

vi. **Antioxidant and free radical scavenger:** the anti-oxidant action of H\textsubscript{2}S is shown via three mechanisms:

- **Free radical scavenger:** H\textsubscript{2}S dissociates to hydrosulfide ions which are powerful one-electron chemical reductants, capable of quenching free radicals by hydrogen atom transfer and 1 electron transfer. Thus H\textsubscript{2}S donors act as free radical scavengers and have also shown cytoprotective effects in rats (Predmore et al., 2012).

- **Increase in GSH levels:** GSH is the major potent anti-oxidant in the body. H\textsubscript{2}S increases synthesis of GSH by increasing cellular concentration of cysteine, the primary substrate for GSH synthesis; increasing activity of γ-glutamylcysteine synthase (γ-GCS), the limiting enzyme in GSH synthesis; and by increasing glutamate uptake, since glutamate inhibits cysteine transportation and hence GSH synthesis. These effects of H\textsubscript{2}S have been studied in primary cultured rat cortical astrocytes and neurons (Zhou Cheng-fang, 2011).
Increase in levels of endogenous anti-oxidants: H₂S activates nuclear-factor-E2-related factor-2 (Nrf2) dependent signaling pathway that regulates gene expression of the number of enzymes that detoxify pro-oxidative stressors. Thus H₂S increase levels of endogenous anti-oxidants (Zhou Cheng-fang, 2011).

1.7 Potential role of H₂S in the eye

The enzymes involved in biosynthesis of H₂S, namely CBS and CSE are present and enzymatically active in mammalian retina. Moreover, deficiency of CBS has been associated with many eye disorders like retinal degeneration, retinal detachment, optic atrophy and glaucoma (Nije-Mbye et al., 2010). These observations suggest that though lethal concentrations of H₂S at mucus membrane can produce deleterious effects like keratoconjunctivitis, H₂S could be beneficial for normal vision at physiological concentrations.

H₂S inhibits sympathetic neurotransmission in porcine and bovine iris ciliary bodies (ICB) (Ohia et al., 2012). In the posterior segment of the eye, attenuates excitatory neurotransmitter release (Opere et al., 2009) and serves as a neuroprotective function by preventing light-induced retinal deterioration in retina of mice (Mikami et al., 2011). Thus H₂S and its donors present a novel and potential approach for management of ocular conditions like glaucoma.
2. Glaucoma

Glaucoma refers to a group of eye conditions that lead to progressive damage to the optic nerve. There are two main types of glaucoma: open angle glaucoma or wide angle glaucoma; and angle-closure glaucoma or narrow angle glaucoma. Primary open angle glaucoma (POAG) is the most common type of glaucoma affecting 1-2% of the world population every year (“Glaucoma,” 2011).

2.1 Clinical Manifestations of Glaucoma

The symptoms of glaucoma vary according to the type of glaucoma. Most people suffering from POAG have no symptoms. Loss of vision starts with a peripheral block of vision which when advanced leads to complete blindness. Patients suffering from angle-closure glaucoma feel a sudden severe pain in their eyes along with a cloudy vision. This is also accompanied with nausea and vomiting and redness in the eye (“Health Guide-http://health.nytimes.com/health/guides/disease/glaucoma/overview.html#Symptoms, ” 2013)

In most cases, damage to the optic nerve occurs due to increased pressure in the eye, also known as intraocular pressure (IOP) (“Glaucoma,” 2011). In the eye, the IOP is maintained by a watery fluid called aqueous humor. This transparent fluid that supplies nutrients to the eye is present in the anterior and posterior chambers of the eye. It is continuously replaced every few hours.
Aqueous humor present in the anterior and posterior chamber of the eye undergoes constant turnover. These dynamics play a major role in the maintenance of normal IOP. Aqueous humor is secreted into the posterior chamber of the eye by the ciliary processes of the ciliary body, a process known as aqueous humor inflow. Aqueous humor then flows from the posterior chamber into the anterior chamber via the pupil and exchanges its contents with cornea and lens. Finally it is recycled by exiting the eye and returning to systemic circulation in a process defined as aqueous humor outflow.
The conventional pathway of aqueous humor outflow, which is responsible for about 85-90% of aqueous humor outflow, is also known as the trabecular pathway. In this pathway, aqueous humor flows through the trabecular meshwork into the Schlemm’s canal, and drains directly into the intrasceral venous plexus (Fig. 8). Non-conventional pathways consists of several pathways, of which the main pathway is known as the uveoscleral pathway. In the uveoscleral pathway, the aqueous humor exits the anterior chamber by diffusion through intercellular spaces among ciliary muscle fibers.

The flow of aqueous humor through the trabecular meshwork is largely affected by the ciliary muscle tone, amongst others. When the ciliary muscle contracts, its ligamentous insertions widen the intercellular spaces in the trabecular meshwork, subsequently increasing trabecular meshwork permeability while simultaneously decreasing uveoscleral outflow. The opposite scenario occurs when the ciliary muscle relaxes. The ligament insertions loosen, causing a narrowing of the trabecular pores, and a reduction in aqueous humor outflow. At the same time, flow through the uveoscleral pathway increases. Thus, aqueous fluid outflow is distributed between the trabecular and uveoscleral pathways depending on the tone of the ciliary muscle (Llobet et al., 2003).
### 2.3 Etiology of Glaucoma

Several factors modulate trabecular meshwork permeability and have been hypothesized to contribute to glaucoma. For example, specific receptors for neurotransmitters and neuropeptides such as epinephrine, acetylcholine have been identified on the trabecular meshwork cells. These factors are active at low concentrations and might control the activity of trabecular meshwork cells in a paracrine manner. The cells also contain ion channels namely, L-type Ca\(^{2+}\) channels, inwardly rectifying K\(^+\) (K\(_{ir}\) 2.1 channels and swelling activated chloride channels. These channels are modulated by intracellular mediators like cAMP, NO and are involved in volume-regulatory responses and cell contraction, and thus could modulate the flow of aqueous humor. Additionally, alterations in the juxtacanalicular regions or decrease in trabecular meshwork cells and could contribute to increased resistance to the aqueous humor outflow from the meshwork. In general, inadequate drainage of the aqueous humor leads to its build up in the eye, consequently increasing IOP and giving rise to glaucomatous conditions (Llobet et al., 2003).

![Aqueous humor outflow](image)

**Fig. 8:** Aqueous humor outflow (Llobet et al., 2003)
et al., 2003). For instance, in POAG, the irido-corneal angle is open and inadequate drainage of aqueous humor outflow through the trabecular meshwork is primarily responsible for the build up and increase in IOP. Whereas in angle-closure glaucoma, irido-corneal angle is closed due to bulging of the iris that leads to build up of aqueous humor increasing IOP. In addition to factors affecting aqueous humor dynamics, diet, genetic factors, prolonged use of certain medications also contribute to glaucoma. Ocular hypertension is the major risk factor for glaucoma.

2.4 Current glaucoma management

Vision loss associated with glaucoma is irreversible. However, various strategies and medications employed in glaucoma therapy target the only modifiable risk factor: elevated IOP. Reduction in IOP can be achieved either by reducing aqueous humor inflow or by increasing its drainage or outflow to within acceptable IOP range. According to American Academy of Ophthalmology (AAO), the target for IOP in patients is 25% of the pretreatment IOP (“http://one.aao.org,” 2013) Commonly used medications include prostaglandins, beta-blockers, alpha-adrenergic agonists, carbonic anhydrase inhibitors and miotic or cholinergic agents. However, use of these drugs is associated with untoward effects on entering systemic circulation.

In addition to medical intervention, other measures used in glaucoma management involve surgical options such as laser surgery or filtering surgery or use of drainage implants (“Glaucoma-treatment and drugs,” 2012). An additional therapeutic strategy could involve targeting the retinal ganglion cells (RGCs) for neuroprotection to promote
cell survival (Teague et al., 2012). This is necessary because current therapies decrease IOP without promoting survival of RGCs. In patients suffering from normotensive glaucoma, RGC degeneration occurs in spite of IOP falling within the normal range (15-21 mm/Hg). This cohort of patients could benefit from a therapy that both lowers IOP and provides neuroprotection. Thus there is need for additional therapeutic options in glaucoma management.

3. H$_2$S and glaucoma

H$_2$S is produced endogenously and has been reported to show biological functions in ocular mammalian tissues (Predmore et al., 2012). There is evidence that sodium NaHS decreases sympathetic neurotransmission in the anterior uvea of the eye. It has also shown to decrease IOP in male albino rabbits (Ohia et al., 2012). Interestingly, sympathetic neurotransmission have been reported to affect trabecular meshwork drainage pathway (Llobet et al., 2003). Indeed, decrease in nor-epinephrine levels lead to relaxation of pre-contracted iris-muscle strips and trabecular meshwork cells that support drainage of aqueous humor, thus decreasing IOP. Therefore, use of H$_2$S in glaucoma therapy can serve as an effective as well as safer approach to reducing IOP levels. In addition to its IOP-lowering action, H$_2$S has been reported to confer neuroprotection against light-induced retinal degeneration in salamander retina (Mikami et al., 2011). Thus H$_2$S donors are a promising, new class of drugs that can provide a dual-targeted approach to glaucoma therapy.
B. SPECIFIC CONSIDERATIONS

Hypothesis

H$_2$S donors regulate mammalian sympathetic neurotransmitter release and decrease IOP in the anterior segment of the eye.

Specific Aims

1. To investigate the effect of H$_2$S donors on sympathetic neurotransmission, in vitro

   a. To investigate the role of endogenous prostaglandin (PG) synthesis

   Initially, I will examine the role of endogenous PGs on the activity of H$_2$S donors.

   b. To investigate the effect of H$_2$S donors on sympathetic neurotransmission, in vitro

   In order to investigate the effect of H$_2$S donors on sympathetic neurotransmitter release, I will compare the effect of various H$_2$S donors on electrically-evoked [$^3$H]NE release.

2. To determine the mechanism of action of H$_2$S donors.

   a. I will examine the role of enzymes involved in synthesis of endogenous H$_2$S

   b. I will determine the role of K$_{ATP}$ channels.
3. To study the effect of H₂S donors on IOP, in vivo

I will determine the effect of the H₂S donors on IOP in normotensive New Zealand albino rabbits.

LITERATURE REVIEW OF SPECIFIC AIMS

AIM 1: To investigate the effect of H₂S donors on sympathetic neurotransmission, in vitro

There is preliminary data available on the effect of H₂S donors, NaHS and sodium sulfide (Na₂S) on amino acid neurotransmission. In these experiments, [³H]D-aspartate was used as a biomarker for glutamate. Studies performed on isolated superfused porcine and bovine retinae have demonstrated that NaHS and Na₂S cause inhibition of K⁺-evoked [³H] D-aspartate release (Opere et al., 2009). Confirmation of the effect of the H₂S donors on amino acid neurotransmission led to studying the effect of NaHS on sympathetic neurotransmission in porcine ICB. It was observed that NaHS produced a dose-dependent inhibition of electrically evoked [³H] NE release (Kulkarni et al., 2009). Decrease in NE release caused relaxation of pre-contracted iris strips and trabecular meshwork cells, that improves aqueous humor outflow (Llobet et al., 2003). However there are many other drugs that donate H₂S and thus could be beneficial in altering sympathetic neurotransmission. In the present study I will compare the effect of three categories of H₂S donors on electrically-evoked [³H] NE release. There is evidence that PGs are involved in H₂S -mediated effects. Hu et al (2008) showed that cyclooxygenase-2 (COX-2) delayed H₂S-induced cardioprotection preconditioning in isolated rat
cardiomyocytes (Li-Fang et al., 2008). In order to delineate the role of PGs on the activity of the H₂S donors, I will also investigate the role of PG biosynthesis on the H₂S donor mediated inhibition in neurotransmitter release.

**AIM 2: To determine the mechanism of action of H₂S donors**

H₂S is synthesized endogenously from L-cysteine in biological tissues. Aminooxyacetic acid (AOA) is an inhibitor that targets CBS, one of the enzymes involved in the biosynthesis is H₂S (Asimakopoulou et al., 2013). There is evidence that AOA reverses NaHS-mediated inhibition of field-stimulated [³H]NE release (Kulkarni et al., 2009). Thus this antagonist could be used to determine the involvement of endogenous biosynthesis of H₂S in the activity of H₂S donors. In the present study I will test the effect of AOA on the activity of the donors.

Various biological functions of H₂S like inflammation, vasorelaxation, nociception and pain are shown to be mediated via activation of Kₐₕₚ channels. For instance, H₂S facilitated neuroprotection and suppression of glutamate toxicity that was co-related to activation of Kₐₕₚ channels (Wang, 2012). Hence there is a possibility that Kₐₕₚ channels might be involved in the regulation of sympathetic neurotransmission by H₂S donors. Glibenclamide is known to be to be a competitive Kₐₕₚ channel blocker (Pompermayer et al., 2005). Furthermore, glibenclamide has been shown to reverse the vasodilatory effects of H₂S on vascular smooth muscles of mice (Liang et al., 2011). In the present study I will investigate the effect of glibenclamide on the regulation of neurotransmitter release by the H₂S donors.
AIM 3: To study the effect of H$_2$S donors on IOP, *in vivo*

Glaucoma is an ocular condition involving progressive damage to optic nerve. Since IOP is the major modifiable risk factor for glaucoma management, it still remains the primary target for development of new therapies. Preliminary studies on H$_2$S donors like NaHS have shown reduction of IOP in normotensive rabbits (Ohia et al., 2012). However, the study demonstrated the effect of just one H$_2$S donor on IOP. In the present study I will compare the effect of two different categories of H$_2$S donors on IOP in normotensive male New Zealand albino rabbits.
MATERIALS AND METHODS
A. General Methods

1. In vitro studies

i. Preparation of buffers

The bovine tissues were incubated in and superfused with Kreb’s buffer solution. Kreb’s buffer solution used was an isotonic buffer solution containing dextrose, ascorbic acid (10mg/ml) and flurbiprofen (FBF) (3µM). The pH of the solution was maintained at 7.45

Composition of Kreb’s buffer solution

Buffer solution was prepared as follows:

Table 1: Composition of Kreb’s buffer solution I. Solution A; II. Solution B; III. Solution C

<table>
<thead>
<tr>
<th>SALT grams/l (10x)</th>
<th>GRAM/%</th>
<th>mM</th>
<th>GRAMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>6.9</td>
<td>118</td>
<td>69</td>
</tr>
<tr>
<td>KCl</td>
<td>0.39</td>
<td>4.8</td>
<td>3.9</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.16</td>
<td>1.2</td>
<td>1.6</td>
</tr>
<tr>
<td>MgSO$_4$, 7H$_2$O</td>
<td>0.3</td>
<td>2.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Stock solution A was prepared by weighing out 10x grams of the above salts and dissolving them in 1 litre of distilled water.
II. SOLUTION B

<table>
<thead>
<tr>
<th>SALT</th>
<th>Grams/l (10x)</th>
<th>Gram/%</th>
<th>mM</th>
<th>Grams</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaHCO₃</td>
<td>2.1</td>
<td>25</td>
<td>21</td>
<td></td>
</tr>
</tbody>
</table>

Stock solution B was prepared by weighing out 10x grams of the above salts and dissolving them in 1 litre of distilled water.

III. SOLUTION C

<table>
<thead>
<tr>
<th>SALT</th>
<th>Grams/l (10x)</th>
<th>Gram/%</th>
<th>mM</th>
<th>Grams</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂</td>
<td>0.19</td>
<td>1.3</td>
<td>1.9</td>
<td></td>
</tr>
</tbody>
</table>

Stock solution C was prepared by weighing out 10x grams of the above salts and dissolving them in 1 litre of distilled water.
RECONSTITUTION OF KREB’S SOLUTION

Solutions A, B and C were prepared as above. Stock solution of ascorbic acid (Mol.wt 176.12) was prepared by dissolving 500 mg ascorbic acid in 50 ml distilled water (10mg/ml) giving a concentration of $5.67 \times 10^{-2}$ M. FBF, (Mol.wt. 244.3) stock solution was prepared by dissolving 48 mg FBF in 20 ml ethanol thus giving a concentration of $10^{-2}$ M.

Kreb’s buffer solution was reconstituted by mixing together 100 ml solution A, 100ml solution B, 100 ml solution C, 1.8ml ascorbic acid stock solution and 300 μl of FBF solution. Volume was made up to 1 litre with distilled water and pH was adjusted to 7.45 using pH meter. The buffer solution was warmed to 37°C in water bath.

ii. Preparation of Bovine Iris-ciliary bodies (ICB)

Buffer preparation was followed by dissection of the bovine ICB. Bovine eyes were obtained from Greater Omaha Packing Co., Omaha, Nebraska within 4 hours following enucleation and shipped to the laboratory in an ice bucket. Four eyeballs were used per experiment to mount on four chambers. An incision was made along the equator of each eye and aqueous humor, cornea and lens was removed. ICB were isolated and then incubated in 4ml of buffer solution containing 10μl of $[^3]$HNE (2.5μCi/ml) (New England Nuclear, Boston, MA; 40.8 Ci/mmol) for 1 hour. The tissues were provided with sufficient aeration during this time and the temperature was maintained at 37°C.
iii. Studies on \[^3\text{H}\]NE Release

After incubation the tissues were rinsed twice in 25ml buffer to remove any excess radioactive substance. These were then mounted between nylon mesh cloth (200 µM pore size) and placed in thermostatically-controlled Superfusion Chambers (Warner Instrument Corp., CT). After mounting a pre-stimulus of 300 d.c electrical pulses (5Hz, 2msec p.d., 60 sec, supramaximal voltage) was provided at the onset of superfusion. This was followed by 60 min. of superfusion at the speed of 2ml/min to establish a stable baseline of spontaneous tritium efflux. The buffer used for superfusion as well as fractional collection succeeding it also contained desipramine (1μM) which is a NE reuptake blocker. This was added to enhance the release of NE.

Tissues were superfused at 2 ml/min with oxygenated Krebs solution, with superfusates collected every 4 minutes. 3 ml aliquots of each fraction was combined with 9 ml of aqueous scintillation cocktail (Ecolume, ICN, Radiochemicals, CA) and analyzed for radioactivity by liquid scintillation spectrometry. Stimulation-evoked release and overflow of tritium was estimated by subtraction of the extrapolated basal tritium efflux from total tritium released during the 28-min period after the onset of stimulation. Basal (unstimulated) tritium efflux declined linearly between pre- and post-stimulation fractions. All tissues received two stimulations (S\(_1\) and S\(_2\)). One set of experiments involved S\(_1\) and S\(_2\) being 28 minute apart, whereas the other had the two stimulations 36 minutes apart after the onset of superfusion. Stimulation-evoked \[^3\text{H}\]NE release during S\(_1\) and S\(_2\) was determined graphically, and the ratio of two peaks (S\(_2\)/S\(_1\)) was calculated and compared with untreated control preparations. In both studies drug was added 29
minutes after the onset of superfusion. When used, antagonists were present throughout the experiment.

2. In vivo studies

New Zealand normotensive male albino rabbits (about 2 kg weight) were purchased from Charles River Laboratories and conditioned to 12-hr light-dark cycles and divided into two groups of five animals each. On the day of the experiment, baseline IOP was measured 30 min before and 0 hour after topical application of proparacaine 0.5% (local anesthetic). Measurements were taken using a pneumatonometer (model 30 classic; Reichert Ophthalmic Instruments, Depew, NY). At 0 hour, 50 µl of drug was applied topically to one eye of each animal while the contralateral eye received the same quantity of vehicle (saline). IOP was measured hourly until baseline readings resumed.

3. Data Analysis

For in vitro studies, results were expressed as the absolute \( S_2/S_1 \) ratios and/or as percentage inhibition of \([^3]H\)NE outflow relative to untreated controls. For in vivo studies, results were expressed as change in IOP (mm of Hg) and/or percentage inhibition of IOP. Except where indicated otherwise, values given are arithmetic means ± S.E.M. Significance of differences between control and agent-treated preparations was evaluated using analysis of variance (ANOVA) followed by Tukey’s post test. Differences with P values < 0.05 were accepted as statistically significant.
4. Sources of drugs and chemicals

Table 2: Sources of H$_2$S

<table>
<thead>
<tr>
<th>Substrates for endogenous synthesis of H$_2$S</th>
<th>H$_2$S-fast delivering compounds</th>
<th>H$_2$S-slow delivering compounds</th>
<th>Hybrid molecules releasing H$_2$S</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-cysteine</td>
<td>NaHS</td>
<td>GYY 4137</td>
<td>ACS67</td>
</tr>
<tr>
<td>N-acetyl-L-cysteine</td>
<td>Na$_2$S</td>
<td></td>
<td>ACS14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ACS15</td>
</tr>
</tbody>
</table>

In this study, I will examine the activity of three categories of drugs: GYY 4137, slow donor of H$_2$S (Lee et al., 2011); ACS67, fast donor of H$_2$S (Osborne et al., 2010); and L-cysteine, substrate for endogenous H$_2$S synthesis (Kimura, 2011). GYY 4137 [morpholin-4-iium-4-methoxyphenyl(morpholino)phosphinodithioate] is a water soluble compound that releases H$_2$S slowly and steadily. L-cysteine is an amino acid present in the body. ACS67 is a hybrid of latanoprost, a F$_{2a}$ agonist and a H$_2$S donating moiety, dithiolethione (ACS1). It is a fast donor of H$_2$S (Wang, 2012).

Antagonists used are AOA and glibenclamide.
GYY 4137 and ACS67 were purchased from Cayman Chemical Co., Ann Arbor, MI. L-cysteine, AOA and gibenclamide were purchased from Sigma Chemical Co., St. Louis, MO.

Stock solutions of GYY 4137, ACS67 and glibenclamide were prepared in dimethyl sulfoxide (DMSO). Stock solutions of L-cysteine and AOA were prepared in water.

B. Specific Methods

1. Effect of H₂S donors on sympathetic neurotransmission, in vitro

In the initial experiments, effect of ACS67 and L-cysteine on electrically evoked \([^{3}H]NE\) release was studied in the absence of the COX-enzyme inhibitor, FBF. Thus Kreb’s buffer solution was prepared without FBF. In the subsequent experiments, FBF (3µM) was added in the buffer solution to inhibit biosynthesis of PGs.

For experiments involving GYY 4137 and ACS67, the first stimulation (\(S_1\)) was applied at 13 min and \(S_2\) at 45 min. For L-cysteine, \(S_1\) was applied at 13 min and \(S_2\) at 53 min. Drug solutions were instilled 29 min after the start of fractional collection.

2. Effect of antagonists- AOA and glibenclamide on the action of H₂S donors

Role of enzymes was studied by using AOA as an antagonist. To study the action of \(K_{ATP}\) channels, glibenclamide was used as an antagonist. In these experiments, antagonists were added 10 minutes before starting the fractional collection and were
present throughout the experiments. Apart from that, the remaining experimental conditions remained the same.

3. Effect of H$_2$S donors on IOP, \textit{in vivo}

Effect of GYY 4137 (0.1-2%); and ACS67 (0.005-0.01%) on IOP of normotensive male albino rabbits was determined. All drug solutions were dissolved in sterile saline solution. 50 µl of drug solution was added to one eye and same quantity of normal saline (vehicle) was applied to the contralateral eye. IOP measurements were taken using a pneumatonometer (model 30 classic; Reichert Ophthalmic Instruments, Depew, NY) every hour until baseline readings resumed.
RESULTS
AIM 1

1. To investigate the effect of H_2S donors on sympathetic neurotransmission, \textit{in vitro}

1.1 Effect of agonists on [^3H]NE release

Electrical field stimulation was applied to fresh isolated bovine ICB that were incubated with tritium-labelled NE. Application of electric stimuli caused an increase in tritium efflux over basal levels. Since the stimulus was applied twice within the duration of fractional collection, two peaks of evoked [^3H]NE release were produced. Fig. 9 depicts a control experiment in which no drug was present. Area under curve (AUC) for both S_2 and S_1 were almost the same. Hence, the S_2/S_1 ratio obtained from different control experiments in the absence of chemical agents or drugs was 0.998 ± 0.004 (n=12).

As shown in Fig.10, GYY 4137 (30µM) attenuated electrically-evoked [^3H]NE release overflow without affecting basal tritium efflux. The drug was administered 29 min after onset of fractional collection, followed by second electrical stimulus (S_2). The H_2S donor, GYY 4137 (30µM) decreased basal efflux of tritium release following application of S_2. The S_2/S_1 ratio for experiments including drugs was less than 1.
1.2 To investigate the role of endogenous PG synthesis

There is evidence that PGs are involved in H₂S-mediated effects (Li-Fang et al., 2008). Therefore, I first examined the effect of ACS67 and L-cysteine in the presence and absence of flurbiprofen (FBF), a COX inhibitor on electrically evoked [³H]NE release in isolated bovine ICB. FBF (3 µM) has been shown to be sufficient to inhibit biosynthesis of endogenous PG synthesis (Graham et al., 2000). As demonstrated in Fig. 11, attenuation of electrically-induced [³H]NE release by ACS67 (0.1 – 10 µM) was higher in the presence of FBF, suggesting that endogenous PGs contribute to H₂S-mediated effect on [³H]NE release. Similarly, L-cysteine (0.1 – 10 µM) (Fig. 12) elicited a stronger inhibition in [³H]NE release in the presence of FBF than in the absence of FBF.
**Fig. 11:** Effect of ACS67 with or without FBF on electrically evoked $[^3]$H NE release in isolated bovine ICB *in vitro*. Vertical bars represent means ± SEM. Number of observations is in parenthesis. *** p< 0.001 significantly different from the control.

**Fig. 12:** Effect of L-cysteine with or without FBF on electrically evoked $[^3]$H NE release in isolated bovine ICB *in vitro*. Vertical bars represent means ± SEM. Number of observations is in parenthesis, * P<0.05 significantly different from the control.
1.3 To investigate the effect of H\textsubscript{2}S donors on sympathetic neurotransmission, \textit{in vitro}

Having delineated the role of endogenous PGs, I next examined the effect of the different H\textsubscript{2}S donors on NE release. There is evidence that the fast H\textsubscript{2}S donors, NaHS attenuate \textsuperscript{3}H]NE in porcine ICB (Kulkarni et al., 2009). Therefore I examined the effect of different categories of H\textsubscript{2}S producing compounds on NE release.

ACS67 is a hybrid of latanoprost and a H\textsubscript{2}S donor moiety. Latanoprost is a prostanoid FP-agonist that is an ocular hypotensive, first-line drug used in management of glaucoma. To determine whether the action of ACS67 on sympathetic neurotransmission is a result of its latanoprost component or H\textsubscript{2}S donor moiety, I initially compared the activity of ACS67 to latanoprost (positive control). As depicted in Fig. 13, both ACS67 (10 nM - 10µM) and latanoprost (10 nM - 10µM) elicited an inhibitory effect on electrically evoked \textsuperscript{3}H]NE release. Moreover, ACS67 exhibited a higher potency (approximately 10-fold higher) (EC\textsubscript{25}: 0.97 µM) than latanoprost (EC\textsubscript{25}: 8.5 µM), suggesting that ACS67 acts via other mechanism, in addition to the PG pathway. ACS67 achieved a maximum inhibition of 37.62\% (n=5, p<0.001) at 10 µM concentration of the drug.
I next examined the effect of the slow H₂S donor, GYY 4137 on electrically evoked [³H]NE release on isolated bovine ICB. Similar to ACS 67, GYY 4137 (1 µM – 30 µM) elicited a dose-dependent inhibition of [³H]NE release in isolated bovine ICB without affecting basal [³H]NE efflux, with a maximum inhibition of 21.21% (n=4, p<0.001) being achieved at a concentration of 30µM (Fig. 14).

**Fig. 13:** Inhibitory effect of fast H₂S donor, ACS67 and the prostanoid FP-agonist, latanoprost on electrically evoked release of [³H] NE from isolated, superfused bovine ICB *in vitro*. Vertical bars represent means ± SEM. Number of observations is in parenthesis.***P<0.001 significantly different from control.
Similarly, the substrate for endogenous production of H\textsubscript{2}S, L-cysteine (10nM-10\muM) attenuated electrically-stimulated [\textsuperscript{3}H]NE release, achieving a maximum inhibition of 26.13\% (n=5, p<0.05) at 10\muM (Fig.15).

At the equimolar concentration of 10 \muM, the rank order of activity of the three H\textsubscript{2}S producing compounds was: ACS67 > L-cysteine > GYY 4137 (Figs.16-17). However, EC\textsubscript{25} values for the H\textsubscript{2}S donors, ACS67, GYY 4137 and L-cysteine was 0.97 \muM, 35.34 \muM and 9.56 \muM respectively.

**Fig. 14:** Effect of the slow H\textsubscript{2}S donor, GYY 4137 (1-30 \muM) on electrically evoked [\textsuperscript{3}H]NE release in isolated bovine ICB in vitro. Vertical bars represent means ± SEM. Number of observations is in parenthesis. *** p<0.001 significantly different from the control.
**Fig. 15:** Effect of L-cysteine (10nM-10μM) on electrically evoked $[^3]$H NE release in isolated bovine ICB *in vitro*. Vertical bars represent means ± SEM. Number of observations is in parenthesis,* P<0.05 significantly different from the control.

**Fig. 16:** Equipotent Effect of H$_2$S donors, GYY 4137, ACS67 and L-cysteine on electrically evoked release of $[^3]$H NE from isolated, superfused bovine ICB *in vitro*. Vertical bars represent means ± SEM. Number of observations is in parenthesis. **P<0.01; ***P<0.001 significantly different from control.
To determine the mechanism of action of H₂S donors

Having established the inhibitory effect of the three H₂S donors on sympathetic neurotransmitter release, I next investigated the mechanism that may be responsible for this inhibitory action of H₂S donors. Therefore I examined the role of enzymes involved in H₂S synthesis and of K<sub>ATP</sub> channels on the activity of the drugs.

There is evidence that AOA, a CBS inhibitor, reverses the effect of NaHS on NE release (Kulkarni et al., 2009). Therefore, to study the role of enzymes, AOA was used to antagonize the effect of H₂S donors. Interestingly, AOA reversed the inhibitory action of GYY 4137 (Fig. 18, Top Panel), ACS67 (Fig 18, Middle Panel) and L-cysteine (Fig 18, Bottom Panel) on of electrically evoked [³H]NE release in isolated bovine.
Next, I determined the role of $K_{ATP}$ channels using the antagonist, glibenclamide on H$_2$S donors-mediated actions. Glibenclamide has been reported to reverse the activity of NaHS on NE release in porcine ICB (Kulkarni et al., 2009). In this study, glibenclamide reversed the inhibition of electrically evoked [$^3$H]NE release in isolated bovine ICB mediated by GYY 4137 (Fig 19, Top Panel), ACS67 (Fig 19, Middle Panel) and L-cysteine (Fig.19, Bottom Panel), suggesting the involvement of $K_{ATP}$ channels in the H$_2$S donor effect on sympathetic neurotransmission.
**Fig. 18:** Effect of AOA on inhibition of field-stimulated $[^3]$H]NE release in isolated bovine ICB *in vitro*. Vertical bars represent means ± SEM. Number of observations is in parenthesis * P<0.05, *** p < 0.001 significantly different from the control.
Fig. 19: Effect of glibenclamide on inhibition of field-stimulated $[^3$H]NE release in isolated bovine ICB *in vitro*. Vertical bars represent means ± SEM. Number of observations is in parenthesis * p< 0.05 *** p< 0.001 significantly different from the control.
AIM 3:

To study the effect of H$_2$S donors on IOP, in vivo

Having examined the effect of H$_2$S donors on anterior uvea sympathetic neurotransmitter release, in vitro, I next examined the effect of H$_2$S donors, GYY 4137 and ACS67 on IOP in male New Zealand Albino rabbits, in vivo. Elevated IOP is the major modifiable risk factor for glaucoma. Thus, drugs that reduce IOP can be potentially developed for management of the disease. In this study, drug solutions were made in sterile normal saline. 50 μl of the drug solution was instilled in one eye of normotensive male New Zealand albino rabbits whereas the contralateral eye received 50 μl normal saline (control vehicle). IOP measurements were recorded prior to treatment and at regular time intervals until baseline readings resumed.

Both H$_2$S-donors tested, GYY 4137 and ACS67 attenuated IOP in the male New Zealand albino rabbits, in vivo. A parallel but sometimes smaller decrease in IOP was observed in the vehicle-treated eye as well. GYY 4137 (0.1% - 2%) decreased IOP, with the 2% concentration exhibiting the highest effect (Fig.20, Top, Middle and Bottom Panels; Fig. 21). Consistent with its slow- H$_2$S releasing property, GYY 4137 (2%) exhibited a peak activity of 27.84% (n=5, p<0.001) six hours after treatment (Table 3) and a duration of action of up to 9 hours.
Fig. 20: Effect of GYY 4137 (0.1-2%) on IOP in normotensive conscious albino rabbits \textit{in vivo}. Vertical bars represent means ± SEM, N=5, *P<0.05, **P<0.01, ***P<0.001, significantly different from baseline IOP.
Similar to GYY 4137, ACS67 (0.005%; Fig 22, Top Panel and 0.01%; Bottom panel) reduced IOP in normotensive New Zealand Albino rabbits. ACS67 (0.005%) exhibited a maximum percent inhibition in IOP of 23.27% (n=5, p<0.001) three hours after treatment (Fig.22, 23; Table 3), affirming its fast, H₂S releasing property. As summarized in Table 3, the duration of action of ACS67 was shorter (up to 7 hours) than that exhibited by GYY 4137.

**Fig. 21:** Summary of effect of GYY 4137 on IOP in normotensive conscious albino rabbits *in vivo*: Treated eye. GYY 4137 (0.1-2%) Vertical bars represent means ± SEM of data obtained from five rabbits. *P<0.05, **P<0.01, ***P<0.001, significantly different from baseline IOP.
Fig. 22: Effect of ACS67 (0.005-0.01%) on IOP in normotensive rabbits in vivo. Vertical bars represent means ± SEM, N=5; *P<0.05, **P<0.01, ***P<0.001, significantly different from baseline IOP.
Fig. 23: Summary of effect of ACS67 on IOP in normotensive conscious albino rabbits *in vivo*: Treated eye. ACS67 (0.005-0.01%) Vertical bars represent means ± SEM of data obtained from five rabbits. *P<0.05, **P<0.01, ***P<0.001, significantly different from baseline IOP.
Table 3: Peak activity, duration of hours and maximum % IOP reduction of different concentrations of GYY 4137 and ACS67.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (%</th>
<th>Peak Activity (hrs)</th>
<th>Duration of Action (hrs)</th>
<th>Max % IOP Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>GYY4137</td>
<td>0.1</td>
<td>5</td>
<td>9</td>
<td>16.34</td>
</tr>
<tr>
<td>GYY4137</td>
<td>1</td>
<td>5</td>
<td>9</td>
<td>17.04</td>
</tr>
<tr>
<td>GYY4137</td>
<td>2</td>
<td>6</td>
<td>9</td>
<td>27.84</td>
</tr>
<tr>
<td>ACS67</td>
<td>0.005</td>
<td>3</td>
<td>7</td>
<td>23.27</td>
</tr>
<tr>
<td>ACS67</td>
<td>0.01</td>
<td>3-4</td>
<td>7</td>
<td>22.84</td>
</tr>
</tbody>
</table>
DISCUSSION
H₂S is a recently discovered gasotransmitter which is synthesized in various tissues of the body. L-Cysteine is the primary substrate for endogenous production of H₂S, using either of the three enzymes: CBS, CSE and 3-MST (Wagner et al., 2009). The concentrations of these enzymes vary in different tissues. CBS is primarily localized to the central nervous system, CSE is found mainly in the cardiovascular system, whereas 3MST is mainly found in the neurons. H₂S is present in mammalian tissues in the micromolar range. It is released from its stored form on receiving a stimulus to exert its effects directly on cellular targets. It is metabolized and released via renal excretion.

There is ample evidence supporting a physiological role for H₂S in ocular tissues. Using molecular biology techniques, CBS and CSE enzymes have been detected in inner and outer plexiform layers of the salamander retina (Pong et al., 2007). Furthermore, H₂S is present in porcine and bovine ocular tissues (Kulkarni et al., 2009), where have been found to increase cAMP production (Nije-Mbye et al., 2010). Furthermore, deficiency of CBS due to mutation in the gene encoding the enzyme has been linked to many eye disorders such as retinal degeneration, retinal detachment, optic atrophy and glaucoma in humans, affirming the physiological significance of H₂S in the eye (“Hereditary Ocular Disease,” 2010). Despite data supporting its significant physiological role in mammalian ocular tissues, the pharmacological effect of H₂S in ocular tissues has not been completely investigated. Therefore, I sought to further evaluate the pharmacological activity of H₂S donors in bovine and rabbit mammalian ocular tissues.
Effect of H₂S donors on sympathetic neurotransmission, in vitro

The ability of H₂S donors, NaHS and Na₂S to regulate neurotransmitter release in ocular tissues has been demonstrated in mammalian retina and ICB. For instance, H₂S donors, NaHS and Na₂S caused inhibition of K⁺-evoked [³H] D-aspartate release in isolated porcine and bovine retina (Opere et al., 2009). Furthermore, NaHS inhibited electrically evoked [³H]NE release in porcine ICB (Kulkarni et al., 2009). To confirm the role of H₂S gas on sympathetic neurotransmitter release, I examined the ability of three different categories of H₂S donors on electrically evoked [³H]NE release in bovine ICB. Electrical stimulation was selected because it is faster and enables an easy and quick recovery of tissues without detrimental effects (Opere et al., 1997). It also resembles the neuronal excitation observed in the body (Vezzani et al., 2006). The three drugs under consideration were GYY 4137, a slow donor of H₂S (Lee et al., 2011); ACS67, fast donor of H₂S (Osborne et al., 2010); and L-cysteine, substrate for endogenous H₂S synthesis (Kimura, 2011). These drugs were chosen as each compound releases H₂S gas by a different mechanism and rate. GYY 4137 and ACS67 are donors that release H₂S in aqueous solutions whereas L-cysteine is a substrate that is involved in the biosynthesis of H₂S.

Initially, I sought to delineate the role of PGs in the H₂S response in these tissues. There is evidence that PGs are involved in H₂S-mediated effects. For instance, COX-2 delayed H₂S-induced cardioprotection preconditioning in isolated rat cardiomyocytes (Li-Fang et al., 2008). Thus, it is conceivable that PGs may be involved in the H₂S donors’ effects on ICB. In this study, the presence of FBF enhanced the inhibitory action
of higher concentrations of both ACS 67 (0.1-10 µM) and L-cysteine (10 µM), suggesting that excitatory, intramural prostanoids modulate the inhibitory action of these H₂S donors at higher concentrations. It was also interesting to note that, intramural PGs played a minor role on the L-cysteine response (10 µM only). It is possible to speculate that the enzymatic, intramural release of H₂S from L-cysteine minimizes its interaction with the PG pathway and therefore accounts for the minimal and delayed contribution of PGs to the inhibitory effect of L-cysteine on sympathetic neurotransmitter release. Interestingly, FBF has been reported to modulate pharmacological actions of H₂S in mammalian ocular tissues (Monjok et al., 2008, Mbye et al, 2010, Ohia et al., 2010; Kulkarni et al., 2012). For instance, pretreatment of tissues with FBF enhanced the effect elicited by GYY4137 on bovine ciliary artery (Kulkarni et al., 2012). Similarly, effect of NaHS on cAMP levels in rat retinal pigment epithelial cells was enhanced significantly (p<0.01) in the presence of FBF (Nije-Mbye et al., 2012). Taken together, these data support a modulatory function for PG pathway on the pharmacological effects of H₂S on mammalian tissues. Based upon the apparent involvement of endogenous PGs in the inhibitory effect of H₂S donors, subsequent experiments were conducted in presence of FBF.

The H₂S donors, ACS67, GYY 4137 and L-cysteine elicited an inhibitory effect on electrically stimulated [³H]NE release in isolated bovine ICB in a concentration-dependent manner without affecting tritium overflow. ACS67 is a hybrid of latanoprost and a H₂S releasing moiety, dithiolethione (ACS1) (Wang, 2012). Thus there was an uncertainty whether the action of ACS67 was due to its latanoprost component or due to
its ability to release H$_2$S. Hence I used latanoprost as a positive control to compare its effect to that of ACS67. It was interesting to note that both ACS67 and latanoprost attenuated sympathetic neurotransmitter release. However, the effect of ACS67 was more potent (EC$_{25}$: 0.97 µM) than that exhibited by latanoprost (EC$_{25}$: 8.5 µM), suggesting that the H$_2$S-contributing moiety was responsible for the additional response elicited by ACS67 on sympathetic release. Interestingly, Osborne et al (2010) reported that ACS67 but not latanoprost attenuated the H$_2$O$_2$-induced toxicity to cultured RGCs, thereby confirming the higher activity displayed by this hybrid molecule.

Similar to ACS67, GYY 4137, the slow H$_2$S donor and L-cysteine, the substrate for endogenous production of H$_2$S elicited an inhibitory action on the sympathetic neurotransmitter release, with the following rank order at the 10 µM concentration: ACS67 > L-cysteine > GYY 4137. Interestingly, the maximum inhibitory action of each drug was observed as follows: ACS67 (10 µM) - 37.62% (n=5, p<0.001); GYY 4137 (30µM) - 21.21% (n=4, p<0.001) and L-cysteine (10 µM) - 26.13% (n=5, p<0.05). Because ACS67 is a hybrid of two components, with each individual component exhibiting an ability to attenuate [$^3$H]NE release, it is conceivable that ACS67 represents a summation of activity of the two components, hence the higher potency. Indeed, prostanoid FP-agonists such as latanoprost have been reported to attenuate [$^3$H]NE release in rabbit isolated ICB (Ohia SE, 1990). Additionally, the fast-releasing H$_2$S property of ACS67 could further account for its superior potency, compared to other H$_2$S donors tested.
In addition to composition of the H$_2$S donors, it is possible that the rate of H$_2$S release from its donors further contributed to the varying potency displayed by the three drugs on neurotransmitter release. For instance, GYY 4137 is a slow H$_2$S donor that releases the gas in aqueous solutions in a slow and sustained manner without the burst release (Lee et al., 2011). Indeed, Lee et al (2011) showed that incubation of GYY 4137 (400 µM) in culture medium led to the generation of sustained release of low, (<20 µM) cumulative quantity of H$_2$S over a period of 7 days. In my experimental design, tissues were exposed to drugs (GYY 4137 and ACS67) for 16 min. only, prior to electrical stimulation, a factor that could explain the low potency achieved by GYY 4137 (EC$_{25}$: 35.34 µM). It could be postulated that a longer treatment period could have improved the efficacy of GYY 4137 in inhibiting [$^3$H]NE release. Contrary to GYY 4137, ACS67 is a fast donor that shows a rapid release of H$_2$S in aqueous solutions (Osborne et al., 2010). Thus, the short treatment time (16 min) was sufficient to elicit a more potent inhibitory response (EC$_{25}$: 0.97 µM) of the drug. The third drug, L-cysteine, does not merely release H$_2$S but is a substrate for in situ production of H$_2$S (Kimura, 2011). In anticipation of its release process, tissues were pretreated with L-cysteine for a longer duration of time (24 min). In spite of the longer duration of treatment, L-cysteine exhibited a lower potency of action (EC$_{25}$: 9.56 µM) than ACS 67. It is therefore apparent that rate of release of H$_2$S from its donor affected the potency of action displayed by the three H$_2$S donors.

Although the ability of H$_2$S donors to inhibit sympathetic neurotransmission in mammalian ICB have been previously reported (Ohia et al., 2012), this study compares the involvement of H$_2$S donors from a variety of sources and further corroborates the
regulatory role of H₂S on sympathetic neurotransmission. To the best of my knowledge, this study is the first study in literature to rank the activity of the three sources of H₂S.

**Determination of mechanism of action**

Several mechanisms, including endogenous generation of H₂S and K<sub>ATP</sub> channels have been implicated in the pharmacological actions of H₂S on biological systems. For instance, the CBS inhibitor, AOA reversed the protection provided by H₂S to hypoxia-induced radioresistance on human hepatoma cells (Zhang et al., 2011). Therefore, to determine the involvement of intramural biosynthesis of H₂S on the H₂S donor-mediated responses, tissues were pretreated with AOA prior to electrical stimulation. In the present study, AOA reversed H₂S donor-mediated inhibition of [³H]NE release, suggesting that the activity of these H₂S donors depend on *in situ* release of H₂S. Though the reversal shown by AOA to L-cysteine inhibition is self-explanatory; its reversal to the effect of GYY 4137 and ACS67 was unexpected. Because GYY 4137 and ACS67 are H₂S donors, disruption of endogenous synthesis of the same should not affect their activity. It is conceivable that this released H₂S might not be the only source of H₂S contributing to the effect on neurotransmission. A study on mouse cerebral cortical slices confirms that when H₂S donors are administered, application of electrical stimulus triggers endogenous H₂S formation (Teague et al., 2002). It is therefore possible that this endogenously produced H₂S contributes to the activity of these drugs and could account for the loss in efficacy on inhibition of endogenous synthesis. Additionally, AOA exhibits inherent activity of inhibiting CBS. This blockade could
lead to an increase in L-cysteine levels. L-Cysteine is an excitotoxin like glutamate (Tan et al., 2010). Hence there is a probability that it might increase NE levels. However, this is not plausible because, as observed under our experimental conditions, L-cysteine on its own does not increase $[^3]H\text{NE}$ release. Taken together, in situ release of H$_2$S contributes to the inhibitory action of H$_2$S donors on sympathetic neurotransmission.

I further examined the effect of glibenclamide, a selective K$_{ATP}$ channel blocker on H$_2$S-mediated sympathetic inhibition. There is evidence that H$_2$S mediates many biological functions in the body via activation of K$_{ATP}$ channels (Wang, 2012). H$_2$S has been reported to facilitate neuroprotection and suppression of glutamate toxicity that has also been co-related to activation of K$_{ATP}$ channels (Wang, 2012). It was therefore possible that activation of K$_{ATP}$ channels might play a role in the ability of H$_2$S donors to alter sympathetic neurotransmission. In the present study, field-stimulated $[^3]H\text{NE}$ release induced by GYY 4137, ACS67 and L-cysteine was reversed by glibenclamide, suggesting that K$_{ATP}$ channels that activation of K$_{ATP}$ channels plays a significant role in the activity of GYY 4137, ACS67 and L-cysteine. Thus activation of K$_{ATP}$ channels could be partly responsible for effects mediated by H$_2$S donors. In summary, these observations support the involvement of K$_{ATP}$ channels in the inhibitory action of H$_2$S donors on neurotransmitter release.
**Effect of H$_2$S donors on IOP, *in vivo***

Glaucoma is a progressive, irreversible ocular neuropathy that is characterized by elevated IOP in most patients, RGC degeneration and corresponding visual deficits. Although ideal therapy for this condition should target IOP and promote survival of RGCs, reduction in IOP still remains the most direct indication of the effectiveness of a drug for glaucoma. Therefore, in the next series of experiments, I examined the effect of the H$_2$S donors, GYY 4137 and ACS67 on IOP in White New Zealand Albino rabbits. The rabbit model is an advantageous non-primate that has been used in physiology and pharmacology of IOP and is often used in the evaluation of new drugs and surgical procedures for glaucoma. It is therefore one of the most practical candidates that has been used for the past few years as a model for measuring IOP effects and studying the pharmacological effect of drugs on aqueous humor dynamics (Goldblum D, Garweg JG, 2000; Reitsamer HA, Posey M, 2006; Taniguchi et al., 1996a, 1996b; Vaajanen et al., 2008; Wallace et al., 1988). H$_2$S donor drugs were effective on topical administration, confirming that these drugs permeate the cornea and other ocular membranes. Thus topical administration could be a target for application of these drugs in therapy.

In the present study, both GYY 4137 and ACS67 produced a decrease in IOP in treated eyes of normotensive rabbits. A parallel but sometimes relatively less reduction in IOP was also observed in the contralateral, vehicle (normal saline)-treated eye. Since it is not possible to employ nasolacrimal occlusion in animals, the lacrimal drainage system
may partly account for the delivery of drugs into the contralateral eye. Interestingly, Chu et al (2000) provided explanation for the contralateral effect of IOP-lowering drugs. These investigators observed a contralateral effect of the drug, 7-Hydroxy-2-dipropylaminotetralin on IOP in normotensive rabbits and concluded that redistribution of the drugs in the systemic circulation or prevalence of a centrally-mediated response possibly accounted for the contralateral effects (Chu et al., 2000).

Although ACS67 exhibited the highest potency at inhibiting sympathetic neurotransmitter release, its magnitude of action on IOP [ACS67 (0.005%); 23.27%; n=5, p<0.001] was less than that exhibited by GYY 4137 [(2%); 27.84% (n=5, p<0.001]. It is pertinent to note that ACS67 is a hybrid of latanoprost and a H$_2$S-donating moiety. Therefore it would be expected to exhibit a higher magnitude of effect, compared to GYY 4137 due to the concomitant contribution of both latanoprost and the H$_2$S moiety to its IOP-lowering effect. However, there is evidence that rabbits are insensitive to the IOP-lowering effect of prostanoid FP-agonists (Orihashi et al., 2005; Woodward et al., 1989), a factor that may account for the lower IOP-lowering effect of ACS67 in the rabbit animal model.

In addition to the difference in magnitude of IOP-lowering action, the two H$_2$S donors displayed differences in duration of action and peak activity that was consistent with the rate of H$_2$S release. In general, ACS67 elicited maximal IOP-lowering effect after 3-4 hrs with duration of action up to 7 hours after administration of drug. On the contrary, GYY 4137 exerted its maximal effect after 6 hours and displayed a longer duration of
action of up to 9 hours. This observation is consistent with the physical properties of the two H₂S donors. Thus, ACS67 being a fast releasing H₂S donor, attains peak activity earlier (3-4 hours) and displays a shorter duration of action, presumably due to a burst release that involves a fast H₂S overshoot surge followed by rapid decline (Osborne et al., 2010). On the contrary, GYY 4137, which is a slow releasing H₂S donor, displayed its peak activity at 6 hours and a longer duration of action (9 hours). These observations further confirm that rate of release of H₂S determines onset of peak activity and duration of action of the parent H₂S donors, thereby affirming that H₂S is indeed responsible for the hypotensive action observed.

Although these H₂S donors decreased IOP, the magnitude of effect was not great – compared to published data (Perrino et al., 2009). For example, Perrino et al (2009) observed an IOP reduction of 30.8% magnitude for ACS67. However, these investigators used a carbomer-induced glaucoma model as opposed to normotensive animals used in this study. Be that as it may, the American Academy of Ophthalmology’s recommended target for IOP reduction in glaucoma patients is 25% of the pretreatment IOP. Thus results obtained in this study are close to the target IOP reduction and therefore this class of drug could offer a viable alternative for glaucoma management.

So far, the mechanism by which H₂S donors reduce IOP has not been fully elucidated. It is known that sympathetic neurotransmitters such as NE are present in ICB and trabecular meshwork cells, where they regulate the activity and ability of these cells to
drain aqueous humor (Llobet et al., 2003). Reduction in levels of NE in these cells causes relaxation that improves the drainage of aqueous humor (Llobet et al., 2003). Thus, by decreasing NE release in the anterior uvea, H₂S donors could induce relaxation of iris muscles (Llobet et al., 2003) and therefore increase intracellular space between the trabecular meshwork, improve aqueous humor outflow, subsequently reducing IOP. However, this hypothesis waits to be confirmed. In summary, the ability of H₂S donors to reduce NE release could have potential beneficial therapeutic implication in glaucoma reduction of IOP.
V. SIGNIFICANCE OF STUDY

Elevated IOP is the major, modifiable risk factor for glaucoma, and therefore is the main target for glaucoma therapy. This elevation in IOP is generally attributed to an imbalance in the inflow and outflow of aqueous humor (Llobet et al., 2003). However, because glaucoma is also accompanied by retinal neural degeneration, the ideal medication therapy for glaucoma should not only reduce IOP but also confer neuroprotection to retinal neurons and the optic nerve. Neuroprotection is especially beneficial to patients suffering from normotensive glaucoma wherein retinal degeneration persists in spite of IOP falling within the normal range (“Glaucoma-treatment and drugs,” 2012). Unfortunately, none of the drugs currently available in the market target both parameters and are associated with several side effects, warranting the search for better therapeutic options. H₂S presents a potential advantage over the available drugs because it is (1) produced endogenously in various tissues of the body and is thus less likely to exert severe side effects at therapeutic doses, (2) It can reduce IOP (3) H₂S has been shown to be neuroprotective in retinal neurons, thereby providing an additional therapeutic advantage to management of glaucoma (4) it is a small hydrophobic molecule that can easily diffuse through the membranes and may therefore easily reach the posterior segment of the eye to exert neuroprotection with minor structural modifications. Thus, this study provides evidence for potential therapeutic application of H₂S in ocular neuropathies such as glaucoma.
VI. CONCLUSION

In conclusion, I evaluated the pharmacological actions of three distinct H$_2$S donors, GYY 4137, a slow donor of H$_2$S; ACS67, a fast donor of H$_2$S; and L-cysteine, a substrate for endogenous production of H$_2$S on mammalian ocular tissues. All the three H$_2$S donors attenuated field-stimulated [$^3$H]NE release in isolated bovine ICB, in vitro with the following rank order of activity at an equimolar (10 µM) concentration: ACS67 > L-cysteine > GYY 4137. The activity of these drugs was partially dependent on the in situ release of H$_2$S and possibly on the activation of K$_{ATP}$ channels. Furthermore, both ACS67 and GYY 4137 decreased IOP in normotensive rabbits, in vivo. The peak and duration of action of the H$_2$S donors could be directly correlated to the rate of H$_2$S release from its donors. Thus, H$_2$S donors present a novel therapeutic approach for glaucoma management. Based upon published data, there is a possibility that H$_2$S donors reduce IOP via inhibition of sympathetic neurotransmission. However, a confirmation of this premise was beyond the scope of my study and merits further investigation.
VII. FUTURE DIRECTIONS

In order to confirm that attenuation of sympathetic neurotransmission accounts for the IOP-lowering effect of H₂S donors, *in vivo* studies should be conducted using the antagonists that inhibited NE release in *in vitro* experiments. Secondly, this project demonstrated that H₂S donors reduce IOP in normotensive vertebrate models. The next set of experiments should be conducted to demonstrate this IOP-lowering effect in a glaucomatous animal model. Thirdly, for this project to be translated into clinical use, IOP studies should be conducted in primates before these H₂S donors can be tested in human subjects. Additionally, H₂S has been reported to protect retinal neurons from toxicity. Therefore, simultaneous, experiments should be conducted to demonstrate this effect on retinal neurons, *in vitro* and *in vivo* using appropriate animal models for neuroprotection. Additional studies should be conducted to formulate H₂S donor-preparations with sustained release of the gas and for delivery into the posterior segment. Finally, toxicity and stability studies should be done to select the most potent and least toxic H₂S donor that is capable of releasing an adequate amount of H₂S for a sustained period of time with minimal side effects.
VIII. REFERENCES


Hereditary Ocular Disease. (2010). Retrieved from disorders.eyes.arizona.edu/category/alternate-names/cbs-deficiency


