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Inhibiting the Interaction Between Grp94 and Myocilin to Treat Primary Open-Angle Glaucoma

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Inhibiting the Interaction Between Grp94 and Myocilin to Treat Primary Open-Angle Glaucoma

by

Andrew Rigel Stothert

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy with a concentration in Neuroscience Department of Neuroscience College of Medicine University of South Florida

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DEDICATION

This work is dedicated to my wife, Alana Stothert. Her constant love and support was the driving force behind completing this work. I love you.
ACKNOWLEDGMENTS

Without the hard work and collaboration between members of our lab, and others labs, none of this work would be possible. I want to especially thank Sarah Fontaine, Jonathan Sabbagh, and Siddharth Kamath for their guidance, willingness to teach me techniques, and availability for any questions I had. Also, I want to thank Amirthaa Suntharalingam for her work on this project and aid in driving the story forward.
# TABLE OF CONTENTS

List of Tables........................................................................................................................................................................... iv

List of Figures.............................................................................................................................................................................. v

Abstract...................................................................................................................................................................................... vii

Chapter One: Determining the client-chaperone relationship between Myocilin and Grp94 during Primary Open-Angle Glaucoma ................................................................. 1

1.1 Abstract ................................................................................................................................................................................. 1
1.2 Introduction.......................................................................................................................................................................... 2
1.3 Grp94 interacts with mutant myocilin, preserving it in the ER................................................................. 4
1.4 Grp94 inhibition of interaction with mutant myocilin leads to intracellular clearance via an autophagic mechanism ........................................................................................................ 7
1.5 Conclusions.......................................................................................................................................................................... 9

Chapter Two: Exploiting the interaction between Grp94 and aggregated myocilin to treat glaucoma ......................................................................................................................... 17

2.1 Abstract ................................................................................................................................................................................. 17
2.2 Introduction.......................................................................................................................................................................... 18
2.3 Materials and Methods ......................................................................................................................................................... 20
2.3.1 Cell Culture ........................................................................................................................................................................ 20
2.3.2 cDNA and siRNA Transfection .................................................................................................................................. 20
2.3.3 Antibodies ........................................................................................................................................................................... 21
2.3.4 Pharmacological Treatment ......................................................................................................................................... 21
2.3.5 Cell Harvest ................................................................................................................................................................. 22
2.3.6 Western Blotting and Co-Immunoprecipitation ........................................................................................................ 22
2.3.7 Triton Solubility ........................................................................................................................................................... 23
2.3.8 Tetrazolium Assay (MTS) ........................................................................................................................................ 23
2.3.9 Lentiviral Production and Transduction ........................................................................................................................... 24
2.3.10 Immunofluorescence and Imaging ............................................................................................................................... 24
2.3.11 Synthesis ....................................................................................................................................................................... 25
2.3.12 Computational Modeling Based on Co-Crystal Structure ............................................................................................ 26
2.3.13 Fluorescence Polarization Assays ............................................................................................................................... 26
2.3.14 Quantification and Statistical Analysis ........................................................................................................................... 26
2.4 Results.................................................................................................................................................................................. 27
2.4.1 Intraocular mutant myocilin levels and toxicity are reduced following Grp94 knockdown using siRNA ......................................................... 27
2.4.2 Improvement of Grp94 inhibitor efficacy through structure based design ................................................................. 28
2.4.3 Grp94 inhibition reduces mutant myocilin levels and toxicity in primary human TM cells .................................................. 29
2.4.4 Post-translational modifications render wild type myocilin a client of Grp94 via misfolding .................................................. 30
2.5 Discussion ........................................................................................................... 31

Chapter Three: Topical ocular administration of the novel Grp94 inhibitor, 4Br-BnIm, treats glaucomatous pathology in the Tg-MYOCY437H mouse model .......................................................... 40

3.1 Abstract ........................................................................................................... 40
3.2 Introduction .................................................................................................... 41
3.3 Materials and Methods .............................................................................. 42
  3.3.1 Inhibitor synthesis ............................................................................. 42
  3.3.2 Study approval .................................................................................. 43
  3.3.3 Animal husbandry ........................................................................... 43
  3.3.4 Cell Culture ..................................................................................... 43
  3.3.5 Antibodies (cell culture) ................................................................. 44
  3.3.6 Pharmacological treatment (cell) ..................................................... 44
  3.3.7 Cell Harvest .................................................................................... 44
  3.3.8 Western blotting (cell) .................................................................... 45
  3.3.9 Inhibitor pharmacokinetics .............................................................. 45
  3.3.10 Inhibitor application and study duration ......................................... 46
  3.3.11 Intraocular pressure (IOP) measurements .................................... 46
  3.3.12 Electrophysiology .......................................................................... 47
  3.3.13 Eye enucleation ............................................................................ 48
  3.3.14 Whole retinal mount ...................................................................... 48
  3.3.15 Histological processing of mouse eye tissue ................................ 49
  3.3.16 Tissue deparaffinization/rehydration ............................................. 49
  3.3.17 Antigen retrieval .......................................................................... 49
  3.3.18 Tissue staining .............................................................................. 50
  3.3.19 Tissue imaging, quantification, and analysis ................................. 50
  3.3.20 Quantification and statistical analysis ........................................... 51
3.4 Results ........................................................................................................... 51
  3.4.1 The selective Grp94 antagonist 4Br-BnIm triages mutant myocilin and possess trans-scleral permeability ......................... 51
  3.4.2 Intraocular pressure elevation in Tg-MYOCY437H mice is rescued by topical ocular treatment with 4Br-BnIm .................. 53
  3.4.3 Myocilin accumulation in the trabecular meshwork of Tg-MYOCY437H mice is reduced following topical ocular treatment with 4Br-BnIm .......................................................... 54
  3.4.4 Scotopic and photopic vision is preserved in Tg-MYOCY437H mice following topical ocular treatment with 4Br-BnIm .......... 54
3.4.5 Retinal ganglion cell viability is preserved in Tg-MYOC\textsuperscript{Y437H} mice following topical ocular treatment with 4Br-BnIm .......... 56

3.5 Discussion ................................................................................................................. 56

Chapter Four: Targeting the ER-autophagy system in the trabecular meshwork to treat Glaucoma ................................................................. 65

4.1 Abstract ..................................................................................................................... 65
4.2 Introduction ............................................................................................................... 66
4.3 Normal intracellular myocilin processing ................................................................ 67
4.4 Myocilin misfolding propagates glaucomatous pathology ........................................ 71
4.5 Autophagic degradation of misfolded proteins promotes cellular homeostasis .......... 72
4.6 TM autophagic dysregulation promotes ocular disorders ......................................... 75
4.7 Grp94 prevents misfolded myocilin degradation via autophagy .............................. 76

Chapter Five: Final Considerations ................................................................................. 84

References ...................................................................................................................... 87

Appendix A: Copyright Permissions .............................................................................. 96
   A1: Journal of Biological Chemistry .......................................................................... 96
   A2: Human Molecular Genetics ............................................................................... 97
   A3: Experimental Eye Research .............................................................................. 99

Appendix B: Approved IACUC Protocols ....................................................................... 105

Appendix C: Tg-MYOC\textsuperscript{Y437H} Mouse Breeding Schematic............................... 107
LIST OF TABLES

Table 4.1: List of known mutations in the MYOC gene that lead to glaucoma........... 80
LIST OF FIGURES

Figure 1.1: Validation of inducible cell model ............................................................. 11
Figure 1.2: siRNA-mediated knockdown of Grp94 regulates levels of I477N, but not wild type, myocilin ............................................................. 12
Figure 1.3: Grp94 preserves mutant myocilin ............................................................ 13
Figure 1.4: Association of Grp94 with mutant but not wild type myocilin ................. 14
Figure 1.5: Grp94 triages mutant myocilin through ERAD, enabling autophagy degradation following Grp94 knockdown ...................................... 15
Figure 1.6: Inhibition of the HSP90 chaperone complex reduces levels of the disease-causing I477N myocilin .................................................... 16
Figure 2.1: Consequences of Grp94 knockdown or overexpression on levels of intracellular and aggregated mutant myocilin ............................ 34
Figure 2.2: Structure, modeling and activity of Grp94 inhibitors ................................ 35
Figure 2.3: Effects of specific Grp94 inhibitors on levels of intracellular mutant myocilin .................................................................................................. 36
Figure 2.4: Grp94 inhibitors reduce mutant myocilin levels and toxicity in HTM cells ..................................................................................................... 37
Figure 2.5: WT myocilin becomes a client of Grp94 when myocilin is misfolded ...... 38
Figure 2.6: Grp94 accelerates myocilin aggregation, a process that can be prevented by Grp94 inhibition ................................................................. 39
Figure 3.1: 4Br-BnIm reduces intracellular mutant myocilin and readily penetrates the eye when topically administered ................................. 59
Figure 3.2: Topical ocular 4Br-BnIm reduces intraocular pressure in Tg-MYOC^{Y437H} mice .......................................................... 60
Figure 3.3: Topical ocular 4Br-BnIm reduces intracellular myocilin accumulation in TM of Tg-MYOC^{Y437H} mice .............................................. 61
Figure 3.4:  Topical ocular 4Br-BnIm restores scotopic and photopic vision in Tg-MYOC$^{Y437H}$ mice ................................................................. 62

Figure 3.5:  Topical ocular 4Br-BnIm preserves RGC viability in Tg-MYOC$^{Y437H}$ mice .............................................................................................. 63

Figure 3.6:  4Br-BnIm inhibits Grp94 association with mutant myocilin, reducing pathologies associated with POAG. ............................... 64

Figure 4.1:  Normal intracellular processing of myocilin ......................................................... 81

Figure 4.2:  Grp94 association with mutant myocilin promotes intracellular accumulation ................................................................................................. 82

Figure 4.3:  Inhibition of Grp94 interaction with mutant myocilin promotes autophagic degradation ............................................................................... 83

Figure C1:  Tg-MYOC$^{Y437H}$ Mouse Breeding Schematic .................................................................................. 128
ABSTRACT

Glaucoma is a neurodegenerative protein misfolding disorder classified by increases in IOP, damage to retinal ganglion cells (RGCs), optic nerve (ON) head damage, and progressive irreversible blindness. Primary open-angle glaucoma (POAG) is the most common form of glaucoma, constituting over 90% of clinical cases. POAG is observed in patients where normal outflow channels, mainly the trabecular meshwork (TM), are exposed at the angle formed by the iris and cornea. However, due to TM cellular dysfunction, aqueous outflow resistance is increased preventing normal circulation of aqueous humor. Recent studies have shown that in 2-4% of POAG cases, increased intracellular levels of a secreted glycoprotein, called myocilin, are present in the TM.

Myocilin is a 504aa glycoprotein, with an unknown precise function. Recent studies have postulated the importance of myocilin in oligodendrocyte differentiation, axonal myelination, and early apoptosis of retinal cells in development, but exact function is still widely debated. However, it is important to note that pathology associated with myocilin is only seen during POAG. Also, only cells of the TM exhibit toxicity when overexpressing mutant myocilin. In the normal eye, myocilin is secreted from the ER of TM cells, however, mutations in the MYOC gene lead to an aggregation-prone form of the protein, which is inefficiently processed and degraded from the ER, leading to build-up and associated toxicity. There are over 70 known MYOC mutations
associated with glaucoma, with over 90% occurring in the C-terminal OLF domain.

Some of the more common, pathological mutations are: I477N, Y437H, P370L, W286R, N480K. All of these mutations have been observed in patients with glaucoma, and all lead to build-up and aggregation of the mutant protein within the ER of TM cells.

Recently, work out of our lab has discovered an interaction between mutant forms of myocilin and the chaperone Grp94. Grp94 is the resident Hsp90 isoform of the ER. Grp94 is an important chaperone in ER quality control, aiding in the output of properly folded secretory and membrane-bound proteins. Besides protein folding, other roles of Grp94 in the ER include: calcium buffering, roles in ER quality control (including targeting misfolded proteins for ERAD), peptide binding, and roles in ER stress. Generally, terminally misfolded proteins in the ER are degraded through ERAD; the Grp94 mediated shuttling of misfolded proteins to the ER trans-membrane machinery for ubiquitination and subsequent translocation to the cytosol for proteasomal degradation. However, in the case of POAG, ERAD is inefficient in mutant myocilin degradation, causing protein accumulation within the ER.

In this study, we demonstrate that specific Grp94 inhibition of interaction with mutant or misfolded myocilin leads to myocilin degradation and subsequent lowering of protein accumulation in the TM, thus reducing downfield pathology associated with POAG. Grp94 preserves mutant myocilin in the ER of TM cells leading to protein accumulation and aggregation precipitating TM cellular dysfunction. We showed in various in vitro cell assays that Grp94 inhibition leads to a reduction in intracellular protein levels, while alleviating TM cellular toxicity. Furthermore, in the Tg-MYOC<sup>Y437H</sup> mouse model of POAG, we showed that topical ocular administration of a specific
Grp94 inhibitor alleviated glaucomatous pathologies, including elevated IOP, myocilin accumulation in the TM, reduced scotopic/photopic visual responses, and RGC health and viability. Finally, we have proven the importance of ER-stress pathway malfunction in the development of POAG pathology, while also discovering the involvement of the autophagy mechanism for myocilin degradation following Grp94 inhibition.

Overall, this work proves that Grp94 is an important regulator of myocilin pathology during POAG. While there are no current therapeutics on the market that directly target the underlying POAG disease mechanism, specific Grp94 inhibition shows great promise and should be considered for human clinical trials. If successful, specific targeted Grp94 inhibition could be the first curative therapeutic options for patients suffering from myocilin-associated POAG.
Chapter One

Determining the client-chaperone relationship between Myocilin and Grp94 during Primary Open-Angle Glaucoma

1.1 Abstract

Endoplasmic Reticulum-Associated Degradation (ERAD) is the traditional method for clearing and degrading misfolded proteins in the ER. This process involves the ubiquitination and translocation of misfolded proteins through the ER lumen to the cytosol for proteasomal degradation. A network of ER-associated chaperone proteins aid in this translocation, and are responsible for recognition of terminally misfolded proteins. In this work, we investigated whether the glaucoma-associated protein, myocilin, was processed through ERAD. Myocilin is a secretory protein that is normally transported through the ER-Golgi network, however, inherited genetic mutations in the MYOC gene lead to a protein product that readily misfolds and accumulates in the ER of trabecular meshwork (TM) cells. This protein accumulation precipitates cellular dysfunction and toxicity, ultimately leading to ER stress induced TM cell death. Using siRNA knockdown, we discovered that glucose-regulated protein 94 (Grp94), the ER-associated homolog of heat shock protein 90 (Hsp90), directly recognizes and associates with mutant forms of myocilin. This recognition prevents normal ERAD, and leads to intra-ER mutant myocilin protein accumulation. Additionally, through genetic

\(^1\) Portions of this work were previously published (Suntharalingam A et al, 2012) and are used with the permission of the publisher
manipulation, or therapeutic intervention, inhibition of Grp94 association with mutant myocilin led to intracellular clearance by autophagy, a robust degradation pathway for aggregation-prone proteins. This work suggests that therapeutic interventions, preventing Grp94 association with mutant myocilin, could be a viable option for patients suffering from cases of myocilin-associated glaucoma.

1.2 Introduction

Primary open-angle glaucoma (POAG) is a hereditary form of glaucoma closely linked to accumulation of the secretory protein, myocilin. Over 10% of juvenile-onset open angle glaucoma (JOAG) and 5% of adult-onset open angle glaucoma are associated with myocilin accumulation [1, 2]. Interestingly, although myocilin is a ubiquitous protein, the only disease-associated pathology caused by the protein appears to be related to trabecular meshwork (TM) cell dysfunction during POAG [3]. The trabecular meshwork is a specialized tissue network in the anterior chamber of the eye responsible for regulating the outflow of aqueous humor. TM cell dysfunction leads to aqueous humor outflow dysregulation causing elevated intraocular pressure (IOP). IOP elevation is a major pathological hallmark of POAG, leading to retinal cell dysfunction and irreversible blindness [4].

Recent studies have shown that mutations in the MYOC gene, encoding the myocilin protein, lead to a protein product that accumulates in the endoplasmic reticulum (ER) of TM cells [5-9]. Mutant myocilin accumulation in the ER of TM cells leads to ER-associated cellular stress causing TM tissue network dysfunction and the known glaucoma phenotypes [10]. Interestingly, individuals with missense MYOC
mutations preventing the translation of myocilin [11], as well as myocilin knock-out mice [12] do not develop glaucomatous pathologies, classifying POAG pathology as a toxic gain-of-function. To date, over 70 MYOC mutations have been documented to be associated with POAG pathology, with a majority occurring in the C-terminal olfactomedin domain [13]. Mutant myocilin products have also been shown to readily aggregate, through presence of detergent insoluble protein species, forming aggregate species containing amyloid fibrils [14, 15].

Previous myocilin-associated glaucoma research has centered on the development of therapeutics to alleviate protein aggregate toxicity by promoting myocilin secretion [7, 16-18]. Unfortunately, it is still not understood why, unlike other ER-associated mutant proteins, myocilin is inefficiently cleared through ER-associated degradation (ERAD). In the normal cell environment, terminally misfolded intra-ER proteins are chaperoned to the ER lumen where they are ubiquitinated and translocated to the cell cytosol for proteasomal degradation [19]. However, this process seems to be inadequate for degrading mutant myocilin protein. In the ER, chaperone proteins are responsible for the recognition of misfolded proteins and determining terminally misfolded proteins fate. Main intra-ER chaperones include glucose-regulated protein 94 (Grp94), the ER-associated heat shock protein 90 (Hsp90), and glucose-regulated protein 78 (Grp78), an ER-associated member of the heat shock protein 70 (Hsp70) family, also referred to as BiP. While exact intra-ER chaperone-client relationships are still incomplete, studies have shown that both Grp94 and Grp78 associate with mutant myocilin, although Grp94 seems to be more selective in its client determination [16, 20]. Interestingly, the significance of these chaperone associations with mutant myocilin still
remain unknown. Therefore, a more concise understanding of mutant myocilin mechanics during ER accumulation could lead to viable treatment options to reduce mutant myocilin accumulation by utilizing the ER quality-control machinery.

In this work, we set to determine the molecular relationship between myocilin and the intra-ER chaperone network, and show the importance of Grp94 in mutant myocilin accumulation and degradation. Mutations in the MYOC gene facilitate the interaction between mutant myocilin and Grp94, leading to inefficient protein clearance via ERAD, precipitating TM cellular dysfunction and glaucomatous pathology. By inhibiting the interaction between Grp94 and mutant myocilin, whether through genetic manipulation or pharmacological inhibition, mutant myocilin is effectively cleared and degraded through an autophagic mechanism. Inhibiting Grp94 association with myocilin could represent a viable therapeutic target for myocilin-associated glaucoma treatment.

1.3 Grp94 interacts with mutant myocilin, preserving it in the ER.

It is well documented that biologically, properly folded myocilin is a secreted protein [8, 21]. Alternatively, studies have shown that mutations in the myocilin gene lead to intracellular accumulation [22-24]. Therefore, in order to properly study myocilin mechanics, physiologically relevant cell models must be utilized. For this, our group developed and tested stably-transfected tetracycline-inducible cells, expressing WT and I477N mutant myocilin. If these cells were to represent normal bodily function, one would expect WT myocilin to be heavily secreted, while mutant myocilin to accumulate intracellularly. To validate these cell models, we performed western blot analysis of intracellular protein levels, and dot-blot analysis of secreted protein. We observed that
in cells stably overexpressing WT myocilin, all of the protein was secreted, with no myocilin observed intracellularly. Alternatively, in cells overexpressing mutant I477N myocilin, all of the protein was observed intracellularly, with none being secreted (Fig 1.1A). Additionally, we performed immunofluorescent co-localization studies on our cells stably overexpressing mutant I477N myocilin, to determine if the intracellular myocilin was accumulating within the ER. To do this, we stained for myocilin and calnexin, an ER marker, and observed co-localization. We found that intracellular mutant myocilin co-localized with calnexin, showing that mutant myocilin was accumulating within the ER (Fig 1.1B). Together, this data proved that our stably over-expressing myocilin cell models emulated physiological function, where WT myocilin was heavily secreted, while mutant myocilin accumulates in the ER.

Next, using our cell model, we wanted to determine the effect, if any, the client-chaperone relationship had on intracellular or secreted levels of myocilin. To do this, we performed a siRNA screen on known ER-associated chaperones (Fig 1.2E). We observed that in cells overexpressing WT myocilin, chaperone knockdown had no effect on intracellular levels (Fig 1.2C) or secreted levels (Fig 1.2D) of myocilin. However, in cells overexpressing the I477N mutant form of myocilin, Grp94 knockdown led to a significant reduction in intracellular levels of myocilin (Fig 1.2A), while have no effect on secreted levels (Fig 1.2B). This led us to the conclusion that Grp94 plays a significant role in mutant myocilin accumulation. To further confirm this observation, we performed immunofluorescent staining on cells overexpressing I477N mutant myocilin and transfected with either control or Grp94 siRNA. Myocilin staining showed a 63% reduction in intracellular mutant myocilin levels following Grp94 knockdown (Fig 1.2F).
This result, in concert with the previous siRNA screen results, further validates Grp94 as a major component in intracellular mutant myocilin accumulation, providing a specialized target for further research.

Next, we wanted to further determine the client-chaperone relationship Grp94 and myocilin share. For this, we first performed a cycloheximide chase experiment on cells overexpressing I477N mutant myocilin, in the presence or absence of Grp94, to determine myocilin half-life under the described conditions. We observed, in the presence of Grp94, mutant myocilin had a half-life of approximately 7.6 hours, whereas in the absence of Grp94, mutant myocilin had a half-life of 2.9 hours (Fig 1.3A). This showed the presence of Grp94 preserved mutant myocilin, leading to increased protein deposition. Furthermore, we looked at insoluble protein accumulation by performing western blots on cells overexpressing I477N mutant myocilin with Grp94 either overexpressed with cDNA or knocked down with siRNA. We observed when Grp94 was knocked down, there was a reduction of both soluble and insoluble forms of myocilin, whereas cells overexpressing Grp94 had elevated insoluble and soluble intracellular protein (Fig 1.3B&C). This further validated our conclusion that Grp94 is preserving mutant myocilin intracellularly, while also showing that Grp94 is promoting insoluble protein aggregation.

Finally, we wanted to determine Grp94 association with myocilin. For this, we performed western blots on myocilin co-IP cell lysates overexpressing WT or I477N mutant myocilin and probing for Grp94. We observed that Grp94 only associated with cells overexpressing mutant I477N myocilin, while cells overexpressing WT myocilin showed no Grp94 association whatsoever (Fig 1.4). These data proved that Grp94 only
associates with the mutant form of myocilin, while not associating with or effecting WT myocilin. Taken together, these data show a strong association between the ER-associate chaperone Grp94 and mutant forms of myocilin. The presence of Grp94 leads to mutant myocilin sequestration within the ER, leading to protein accumulation and aggregation. However, following Grp94 knockdown, mutant myocilin is able to be degraded, albeit through an unknown mechanism.

1.4 Grp94 inhibition of interaction with mutant myocilin leads to intracellular clearance via an autophagic mechanism.

After proving that Grp94 affects intracellular levels of mutant myocilin, and knockdown of Grp94 leads to mutant myocilin clearance, we wanted to determine the specific mechanism of mutant myocilin clearance following Grp94 knockdown. Traditionally, terminally misfolded proteins in the ER are degraded through an ubiquitin-dependent proteasomal degradation pathway, known as ER-associated degradation (ERAD) [25, 26]. Therefore, we hypothesized that mutant myocilin would be degraded by ERAD following Grp94 knockdown. To test this hypothesis, we first looked at protein ubiquitination in myocilin co-IP lysates of cells overexpressing WT, P370L, or I477N mutant myocilin. We observed ubiquitin association with mutant forms of myocilin only (Fig 1.5A). This further validated our hypothesis that accumulated mutant myocilin in the ER is being targeted for ERAD. To definitively evaluate ERAD involvement we looked at the effects of proteasomal inhibition on mutant myocilin clearance. We treated cells overexpressing I477N mutant myocilin with epoxomicin, an inhibitor of proteasomal degradation, and transfected the cells with either control or Grp94 siRNA. If ERAD was
responsible for mutant myocilin degradation, we would expect epoxomicin treatment to inhibit intracellular protein clearance. However, we observed significant reductions in intracellular mutant myocilin protein levels following Grp94 knockdown and proteasomal inhibition (Fig 1.5B). This led us to believe that mutant myocilin is in fact being degraded through an alternative autophagic mechanism. To test the involvement of autophagy in mutant myocilin degradation following Grp94 knockdown, we performed western blot analysis on lysates from cells overexpressing I477N mutant myocilin, transfected with Grp94 siRNA in addition to either Beclin1 or Lamp2 siRNA. Beclin1 and Lamp2 are main components of the autophagic mechanism [27, 28], therefore, if autophagy was responsible for mutant myocilin degradation following Grp94 knockdown, we would expect the knockdown of these autophagic components to inhibit myocilin clearance. Interestingly, knockdown of both Beclin1 (Fig 1.5C) and Lamp2 (Fig 1.5D) directly inhibited mutant myocilin clearance following Grp94 knockdown. This led up to the conclusion that following Grp94 knockdown, mutant myocilin is degraded through the robust autophagic mechanism.

Finally, after showing the effects Grp94 gene knockdown had on mutant myocilin intracellular accumulation, we wanted to determine if pharmacological inhibition of Grp94 interaction with mutant myocilin would produce similar results. To do this, we performed two dose response curves using known pan-HSP90 inhibitors, 17-AAG and Celastrol, on cells overexpressing I477N mutant myocilin. We observed that these inhibitors produced a dose dependent reduction in intracellular levels of mutant myocilin (Fig 1.6A&B). Additionally, treatment with a steady concentration of 17-AAG provided a time-dependent reduction of intracellular mutant myocilin (Fig 1.6C). Interestingly,
varying concentrations of 17-AAG had no effect on WT myocilin secretion (Fig 1.6D). Finally, in collaboration with Dr. Brian Blagg at Kansas University, we received a Grp94 specific inhibitor, and tested its ability to reduce intracellular mutant myocilin in a concentration dependent manner. We observed, similar to the pan-HSP90 inhibitors, that the specific Grp94 inhibitor reduced intracellular mutant myocilin dose dependently (Fig 1.6E). Taken together, these data revealed that specific pharmacological inhibition of Grp94 interaction with mutant myocilin was sufficient to produce intracellular degradation, providing a scaffold for further research and drug development.

1.5 Conclusions

Grp94 recognition and association with mutant myocilin produces intracellular protein accumulation. Targeting Grp94 interaction with mutant myocilin has proven to be viable method of reducing accumulated intracellular pathological myocilin. However, as we are the first to discover this interaction, many unknowns remain about the complex Grp94-mutant myocilin interaction as it relates to cell biology and POAG pathology. Additionally, as Grp94 is a ubiquitous molecular chaperone, adverse effects of global Grp94 inhibition remain unclear. Our initial specific Grp94 inhibitor proved potent at reducing intracellular mutant myocilin in a dose dependent manner, however, off-target effects remain unknown. Determining the chaperone-client relationship between Grp94 and other client proteins will help to perpetuate the production of more specific pharmacological inhibitors, while also determining whether tissue-specific delivery systems would be necessary. While no current research focuses on the Grp94-myocilin interaction as it relates to POAG, Grp94 has been previously studied in terms of various
cancer phenotypes. With a relatively short client list, Grp94 has promise as a valid therapeutic target for the treatment of myocilin-associated POAG. More studies on this chaperone-client relationship, as well as the development of more potent/less toxic inhibitors, transitioning to physiologically relevant POAG cell models, effects on multiple mutant myocilin subtypes, and success in \textit{in vivo} animal models will reveal the validity of Grp94 as a target for myocilin-associated POAG treatment.
Figure 1.1 Validation of inducible cell model. (A) Dot blot and Western blot analysis of tetracycline-inducible HEK cells (iHEK) stably overexpressing either WT or I477N mutant myocilin. (B) Co-localization immunofluorescent images of iHEK cells overexpressing I477N mutant myocilin.
Figure 1.2 siRNA-mediated knockdown of Grp94 regulates levels of I477N, but not wild type, myocilin. (A) Western blot analysis of siRNA mediated knockdown of known ER-associated molecular chaperones in iHEK cells overexpressing I477N mutant myocilin. (B) Dot blot analysis of siRNA mediated knockdown of known ER-associated molecular chaperones in iHEK cells overexpressing I477N mutant myocilin. (C) Western blot analysis of siRNA mediated knockdown of known ER-associated molecular chaperones in iHEK cells overexpressing WT myocilin. (D) Dot blot analysis of siRNA mediated knockdown of known ER-associated molecular chaperones in iHEK cells overexpressing WT myocilin. (E) Western blot analysis to prove proper gene knockdown of previously mentioned chaperone siRNAs. (F) Immunofluorescent images of iHEK cells expressing I477N mutant myocilin transfected with either control or Grp94 siRNA.
Figure 1.3 Grp94 preserves mutant myocilin. (A) Western blot analysis of cycloheximide chase experiment to test half-life of I477N mutant myocilin protein following transfection with either control or Grp94 siRNA. (B) Western blot analysis of soluble and insoluble myocilin species following transfection of control or Grp94 siRNA. (C) Western blot analysis of soluble and insoluble myocilin species following transfection of empty vector or Grp94 cDNA.
Figure 1.4 Association of Grp94 with mutant but not wild type myocilin. Western blot and dot blot analysis of myocilin co-IP cell lysates and media observing Grp94 association with WT or I477N mutant myocilin.
Figure 1.5 Grp94 triages mutant myocilin through ERAD, enabling autophagic degradation following Grp94 knockdown. (A) Myocilin co-IP of cell lysates looking at ubiquitin association to various forms of myocilin. (B) Western blot analysis of iHEK I477N cell lysates treated with increasing concentration of epoxomicin to inhibit proteasomal degradation. Cells are transfected with either control or Grp94 siRNA and probed for ubiquitin. (C) Western blot analysis of iHEK I477N cells co-transfected with Grp94 siRNA and Beclin siRNA to inhibit autophagic degradation. (D) Western blot analysis of iHEK I477N cells co-transfected with Grp94 siRNA and Lamp2 siRNA to inhibit autophagic degradation.
Figure 1.6 Inhibition of the HSP90 chaperone complex reduces levels of the disease-causing I477N myocilin. (A) Dose dependent reduction of I477N mutant myocilin following treatment with the pan-Hsp90 inhibitor, 17AAG. (B) Dose dependent reduction of I477N mutant myocilin following treatment with the pan-Hsp90 inhibitor, Celastrol. (C) Time dependent reduction of I477N mutant myocilin following single concentration treatment (7.5uM) with 17AAG. (D) Dot blot analysis showing no change in WT myocilin secretion following increasing dosages of 17AAG. (E) Dose dependent reduction of I477N mutant myocilin following treatment with a novel specific Grp94 inhibitor.
Chapter Two¹:

Exploiting the interaction between Grp94 and aggregated myocilin to treat glaucoma

2.1 Abstract:
Gain-of-function mutations in the olfactomedin domain of the *MYOC* gene facilitate the toxic accumulation of amyloid-containing myocilin aggregates, hastening the onset of the prevalent ocular disorder primary open-angle glaucoma. Aggregation of wild-type myocilin has been reported in other glaucoma subtypes, suggesting broader relevance of misfolded myocilin across the disease spectrum, but the absence of myocilin does not cause disease. Thus, strategies aimed at eliminating myocilin could be therapeutically relevant for glaucoma. Here, a novel and selective Grp94 inhibitor reduced the levels of several mutant myocilin proteins as well as wild type myocilin when forced to misfold in cells. This inhibitor rescued mutant myocilin toxicity in primary human trabecular meshwork cells. Mechanistically, *in vitro* kinetics studies demonstrate that Grp94 recognizes on-pathway aggregates of the myocilin olfactomedin domain (myoc-OLF), accelerates rates of aggregation, and co-precipitates with myoc-OLF. These results indicate that aberrant myocilin quaternary structure drives Grp94 recognition, rather than

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peptide motifs exposed by unfolded protein. Inhibition of Grp94 ameliorates the effects of Grp94-accelerated myoc-OLF aggregation, and Grp94 remains in solution. In cells, when wild type myocilin is driven to misfold and aggregate, it becomes a client of Grp94 and sensitive to Grp94 inhibition. Taken together, the interaction of Grp94 with myocilin aggregates can be manipulated by cellular environment and genetics; this process can be exploited with Grp94 inhibitors to promote the clearance of toxic forms of myocilin.

2.2 Introduction:

Primary open-angle glaucoma (POAG) is a degenerative eye disease characterized by retinal ganglion cell loss and optic nerve head damage. This is often preceded by increased intraocular pressure (IOP) due to impaired aqueous humor outflow through the anterior anatomical segment called the trabecular meshwork (TM), leading to irreversible vision loss [16, 29-31]. Mutations in the MYOC gene, which encodes the myocilin protein, cause ~5% of hereditary POAG in populations throughout the world, including approximately 100,000 people in the US [32, 33]. As a result, the pathogenic mechanisms of mutant myocilin and its corresponding native biological function(s) have been subjects of intensive investigation over the past decade [22, 34-36]. Myocilin, composed of an N-terminal coiled-coil and 31 kDa C-terminal olfactomedin domain (myoc-OLF), is generally believed to be a secreted protein [37]. The majority of the non-synonymous MYOC gene lesions known to cause POAG are found within myoc-OLF [2, 38], leading to intracellular sequestration and aggregation [5, 8, 9, 14]. These observations suggest a functional link between this domain and pathogenicity. The vast majority of work in the field indicates that mutations in myocilin do in fact cause a toxic gain-of-function for the protein [11, 39].
Our team has recently provided a molecular basis for this toxicity: mutations in the OLF domain of myocilin promote the toxic aggregation of the myocilin protein into amyloid aggregates [38, 40]. This non-native structure is known to be nearly impossible to disaggregate, likely explaining why, unlike other proteins, mutant myocilin cannot be efficiently cleared by endoplasmic reticulum-associated degradation (ERAD) and instead accumulates in the ER, leading to activation of ER stress pathways and TM cell death. Indeed, we recently showed that knocking down or inhibiting the ER chaperone Grp94 facilitates the degradation of mutant myocilin in cells via autophagy. Grp94 preserves mutant myocilin in the ER by attempting to force it through the ERAD pathway that involves the valosin containing protein (VCP/p97) and the proteasome.

Here, we have identified a potent and selective Grp94 inhibitor that lowers the levels of several mutant myocilin species. Grp94 inhibition reduces the toxicity of mutant myocilin in human primary TM cells. The mechanism for Grp94 association with myocilin is through its recognition of myocilin aggregates, not the folded mutant protein as originally hypothesized. Based on this observation, we show that even wild type (WT) myocilin, if misfolded and aggregated, can become a Grp94 client, and therefore can be successfully degraded by inhibiting Grp94. Since ocular hypertension in glaucoma is thought to be a result of the overexpression of WT myocilin [41, 42], Grp94 inhibition could be more broadly applicable to glaucoma treatment than just for individuals with inherited POAG.
2.3 Materials and Methods:

2.3.1 Cell Culture

HEK 293T cells were grown and maintained in Dulbecco’s modified Eagle’s Medium supplemented with 10% FBS (Invitrogen), penicillin (100 units/mL), streptomycin (100 µg/mL) and 1% GlutaMAX (Invitrogen) at 37 °C under 5% CO₂.

Stably-transfected iHEK cells expressing myocilin were grown and maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS (Invitrogen), penicillin (100 units/mL), streptomycin (100 µg/mL) and 1% GlutaMAX (Invitrogen) at 37 °C under 5% CO₂. For cell selection, the cells were supplemented with hygromycin B (200 µg/mL) (InvivoGen) and G418 (100 µg/mL) (Gibco). To induce myocilin expression in these cells, they were treated with 5 µg/uL tetracycline 24 h prior to transfection, or 48 h prior to treatment.

Human trabecular meshwork cells (ScienCell) were plated on poly-L-Lysine coated plates at a ratio of 1:10 poly-l-lysine to ddH₂O. Cells were kept and maintained in Fibroblast Medium (Sciencell) supplemented with 2% fetal bovine serum (ScienCell cat. 0010), 1% fibroblast growth supplement (ScienCell cat. 2352), and 1% penicillin/streptomycin solution (ScienCell cat. 0503). HTM cells were maintained at 37 °C under 5% CO₂.

2.3.2 cDNA and siRNA Transfection

All myocilin cDNA constructs were a generous gift from Dr. Vincent Raymond (Laval University Hospital (CHUL) Research Hospital). Grp94 (SI02663738) and control (1027281) siRNA was purchased from Qiagen (Valencia, CA) [43].
Plasmid transfections were carried out in serum-free opti-mem (Invitrogen) medium. cDNA was mixed with Lipofectamine 2000 (Invitrogen) transfection reagent at a ratio of 1 µg cDNA: 2.5 µL Lipofectamine 2000. cDNA was left on the cells for 48 h before harvest. siRNA transfections were carried out in serum-free opti-mem (Invitrogen) medium. siRNA was mixed with siLentFect (BioRad) lipid reagent for RNAi transfection. Next, 40 nM of siRNA was added to cells, so that the siRNA to siLentFect ratio was 1 µL siRNA to 2 µL siLentFect. siRNA was kept on the cells for 24 h prior to cDNA transfection or drug treatment.

2.3.3 Antibodies

Myocilin monoclonal antibody was purchased from R&D Systems (Minneapolis, MN (MAB3446)). Grp94 (2104S), Grp78 (3177S), Hsp70 (4873S), Hsp27 (2402S), and DYKDDDK (Flag) (8146S) monoclonal antibodies were purchased from Cell Signaling Technologies. Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) antibody (H86504M) was purchased from Meridian Life Science (Saco, ME). Actin antibody was purchased from Sigma (A5316). Secondary antibodies were all HRP-linked and purchased from Southern Biotechnologies (Birmingham, AL). All antibodies were added to blots in a 1:1000 dilution in 7% milk.

2.3.4 Pharmacological Treatment

Grp94 inhibitors were solubilized in DMSO (Sigma), and added to cells at varying concentrations. All drug treatments were carried out for 24 h prior to cell harvest, and DMSO never exceeded 1% of total volume of cell medium.
2.3.5 Cell Harvest

For all cell harvesting, culture media was aspirated and cells were washed 2x in ice cold phosphate-buffered solution (pH 7.4) (PBS). Mammalian Protein Extraction Reagent (M-PER) buffer (Pierce) containing protease inhibitor mixture (Calbiochem), 100 mM phenylmethylsulfonyl fluoride (PMSF), and phosphatase inhibitor II and III mixtures (Sigma) were added to the cells at a 1:100 dilution and cells were scraped. Cells in lysis buffer were collected and incubated on ice for 10 min to allow lysis to occur. Cell lysates were spun down at 16,000 x g to remove nuclear supernatant, and a bicinchoninic acid assay (BCA) reaction was carried out to determine protein concentration.

2.3.6 Western Blotting and Co-Immunoprecipitation

Western blotting and Co-Immunoprecipitation were performed as previously described [22]. Cell lysates were prepared with 2x Laemmli sample buffer (Bio-Rad) and denatured by boiling for 5 min at 100 °C. Prepared lysates were then loaded onto a 10-well 10% Tris-glycine gel (Invitrogen) or 18-well, 10% criterion gel (Bio-Rad). After running, gels were transferred onto PVDF membranes (Millipore) and blocked for 1 h at room temperature with 7% milk.

Co-Immunoprecipitation cell harvest was achieved in M-PER buffer supplemented with protease inhibitor mixture, PMSF, and phosphatase inhibitor II and III mixtures. A BCA reaction was performed to determine protein concentration, and the lysates were incubated with myocilin antibody by rocking for 4 h to overnight at 4 °C. Next, 50 μL of Protein G Dynabeads® (Novex by Life Technologies) were added to the samples and
incubated by rocking overnight at 4 °C. Samples were washed with ice-cold PBS and loaded onto gels for Western blot analysis.

2.3.7 Triton Solubility

HEK 293T cells were transfected with either Grp94 siRNA or Control siRNA 24 h prior to mutant myocilin transfection. After a 48-h mutant myocilin transfection, cells were washed twice with ice-cold PBS and lysed in buffer containing 100 mM Tris-HCl (pH 7.4), 3 mM EGTA, 5 mM MgCl₂, 0.5% Triton X-100, protease inhibitor cocktail Calbiochem), and 1 mM PMSF and incubated for 2 minutes on ice. Cells were spun down in a tabletop centrifuge at 16,000 x g for 10 min. Supernatant was removed and kept as the Triton soluble fraction. The pellet was washed with ice-cold PBS, and then re-suspended in 2x Laemmli sample buffer with 9 M urea. The pellet was sonicated and denatured. Soluble fractions were prepared with 2x Laemmli sample buffer, denatured, and run on Western blot for analysis.

2.3.8 Tetrazolium Assay (MTS)

Assay is performed using CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega) kit. Cells are plated in a 96-well plate, and transfection/drug treatment is carried out as previously reported. Manufacturer’s protocol was followed to complete assay. Assay is read at 490 nm on a standard plate reader. The tetrazolium in the MTS solution will react with live cells to form formazan, which is detectable at 490 nm. The more formazan detected, the more viable the cells.
2.3.9 Lentiviral Production and Transduction

Lentiviral vectors were created by sub-cloning c-terminal RFP-tagged cDNA from a mammalian expression vector to the pLEX lentiviral vector. Second generation lentiviral production required both a packaging plasmid (pPax2) and an envelope plasmid (VSV-G). Lentiviral vector, packaging plasmid, and envelope plasmid were transfected into HEK 293T cells using Lipofectamine 2000 transfection reagent (Invitrogen) in serum-free media. Cells with transfection reagent were placed in a 37 °C incubator with 5% CO₂ for 4 h. After 4 h, viral supernatant was removed and fresh serum-free media was added to the cells. After 48-72 h the media containing viral particles was collected. The virus-containing media was then centrifuged to remove cell debris, and filtered with a 0.45 µM syringe filter. Virus-containing media was aliquotted and frozen at -80 °C for long-term storage.

For lentiviral transduction, virus-containing media was added to cells in a 1:1 dilution with complete media. Polybrene (Santa Cruz) was added to the lentiviral mixture at 8 µg/mL. HTM cell transduction was achieved after 7 days.

2.3.10 Immunofluorescence and Imaging

HTM Cells were plated on poly-l-lysine coated coverslips, and transduced with RFP-tagged lentivirus, treated as described above. Cells were then fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 in PBS, and glycine-fixed in 0.1% glycine in PBS. DAPI was used for a nuclear stain at 1:20,000 dilutions in PBS and incubated for 5-10 min. Coverslips were then glued to glass tissue slides using ProLong® Gold Antifade Reagent (Invitrogen). Images of cells were taken using the Olympus
FV1000 MPE Multiphoton Laser Scanning Microscope. Image analysis of intensity was carried out using ImageJ64 software (NIH).

2.3.11 Synthesis

Synthesis performed by Dr. Brian Blagg’s group at Kansas University. 4-Br-BnIm 4-bromo-benzylamine (32 μL, 0.26 mmol) was added dropwise to a stirred solution of readily available aldehyde [44] (125 mg, 0.26 mmol) in MeOH (2 mL) followed by addition of ammonium bicarbonate (20 mg, 0.26 mmol) and dropwise addition of 40% w/v glyoxal (29 μL, 0.26 mmol). The reaction was stirred at 25°C for 12 h. Upon consumption of the starting aldehyde, as seen by thin-layer chromatography, tetrabutylammonium fluoride (0.52 mL, 1.0 M solution in THF) was added and the reaction was allowed to stir for an additional 30 minutes. The reaction was then quenched with saturated ammonium chloride (5 mL) and then extracted with EtOAc (10 mL x 3). The organic layers were combined, dried over Na₂SO₄ and concentrated under reduced pressure. The resulting residue was subjected to flash chromatography (95:5 CH₂Cl₂/MeOH) to provide 4-Br-BnIm as an off white solid (45 mg, 38% yield). \(^1\)H NMR (400 MHz, CDCl₃/MeOD) 7.45-7.41 (m, 2 H), 7.00 (d 1.39, 1 H), 6.88 (d 8.37, 2 H), 6.82 (d 1.38, 1 H), 6.45 (s, 1 H), 5.00 (s, 2 H), 3.86 (s, 3 H), 3.50-3.44 (m, 2 H), 2.88-2.82 (m, 2 H). \(^1\)C NMR (500 MHz, CDCl₃/MeOD) 171.9, 163.5, 159.3, 148.8, 142.6, 136.1, 133.5 (2 C), 129.6 (2 C), 127.6, 123.5, 121.4, 116.0, 107.3, 104.07, 54.0, 50.4, 32.1, 27.2 HRMS (ESI) calculated for C₂₀H₁₉BrClN₂O₄ (M + H\(^+\)) 465.0217, found 465.0237
2.3.12 Computational Modeling Based on Co-Crystal Structure

Molecular docking studies, performed by Dr. Blagg’s group were done using Surflex-Dock module in Sybyl v8.0 from Tripos International (St. Louis, Mo). Co-crystal structures of radamide bound to Grp94 and yeast Hsp82 were used for docking the designed molecules [45]. Visual interpretation and rendering of the images were carried out using the PyMOL Molecular Graphics System v1.5.0.4. from Schrödinger, LLC.

2.3.13 Fluorescence Polarization Assays

Fluorescent polarization assays were performed by Dr. Blagg’s group. Increasing concentrations of compound were incubated in 96-well plate (black well, black bottom) format with recombinant cGrp94 (Enzo Life Sciences, 10 nM) and FITC-labeled geldanamycin (Enzo Life Sciences, 6 nM) in assay buffer (20 mM HEPES pH 7.3, 50 mM KCl, 5 mM MgCl₂, 2 mM DTT, and 20 mM Na₂MoO₄, 0.01% NP-40, and 0.1 mg/mL BGG) at a final volume of 100 μL per well. Plates were incubated for 24 h at 4 °C. Fluorescence polarization values were measured using excitation and emission filters of 485- and 528-nm, respectively. Polarization values were then normalized to DMSO (100% tracer bound) and apparent Kₐ values determined using GraphPad Prism software.

2.3.14 Quantification and Statistical Analysis

Quantification of Western blots was carried out using ImageJ64 software. Graphs are plotted based on Intensity Density/Area values. Statistical analysis was performed as indicated in the figure legends.
2.4 Results:

2.4.1 Intracellular mutant myocilin levels and toxicity are reduced following Grp94 knockdown using siRNA.

We previously showed that Grp94 could regulate the levels of I477N-mutant myocilin, but we sought to determine if modulating Grp94 levels could be generalized to other disease-associated variants. HEK cells were transfected with myocilin harboring I477N, Y437H, P370L, W286R or N480K substitutions and the effects of Grp94 over-expression (Fig. 2.1A) or siRNA knockdown (Fig. 2.1B) on their intracellular levels were evaluated by Western blot. Over-expression of Grp94 preserved mutant myocilin in cells. Conversely, Grp94 knockdown dramatically reduced the levels of each mutant myocilin species. Optimal Grp94 knockdown of >60% was achieved with one of 3 siRNAs tested.

We then fractionated lysates from cells transfected with each myocilin mutant and either control or Grp94 siRNA to determine the impact of Grp94 suppression on mutant myocilin solubility, as previously described [14, 43]. Briefly, cells were lysed in a buffer containing 0.5% Triton, centrifuged at 16,000 x g for 10 minutes and supernatant was recovered for the “soluble” fraction. The pellet from the spin was re-suspended in loading buffer and 9 M urea to produce the “insoluble” fraction. Grp94 knockdown reduced both soluble and insoluble myocilin species, suggesting, as expected, that Grp94 suppression was facilitating myocilin clearance, not forcing it into an insoluble state (Fig. 2.1C&D). Quantification was achieved from 4 independent experiments, including those shown in figures 2.1B and C. These findings further imply that Grp94 modulation is broadly applicable to POAG cases resulting from different myocilin mutations.
2.4.2 Improvement of Grp94 inhibitor efficacy through structure-based design.

We previously determined that the Grp94 inhibitor, BnIm 01, could reduce mutant myocilin levels [43]. Based on molecular modeling studies of the pan-Hsp90 inhibitor radamide bound to Grp94, Dr. Blagg’s group proposed the imidazole ring as a bioisosteric replacement for the cis-amide moiety (Figs. 2.2A&B). Consistent with computational predictions, the first compound synthesized, BnIm 01, exhibited ~100:1 selectivity for Grp94 versus cytoplasmic Hsp90α/β, as determined by inhibition of Grp94-mediated Toll-like receptor protein presentation at the cell surface versus Western blot analyses of Hsp90-dependent proteins found in the cytoplasm [44]. On the basis of our computational model, Dr. Blagg’s group speculated that creating a molecule with increased hydrophobicity at the C4-position of the N-benzyl group would improve drug interaction with Grp94 and further prevent its interaction with Hsp90 proteins, thereby enhancing selectivity [45].

To test this hypothesis, Dr. Blagg’s group synthesized 4-Br-BnIm (Fig. 2.2A); modeling predicts this molecule fits more tightly into the hydrophobic pocket of Grp94 than the parent BnIm 01 (Fig. 2.2C). While in vitro fluorescence polarization binding assays indicate its potency against Grp94 is only slightly increased (1.5 µM with the BnIm 01 compound compared to 1 µM with the 4-Br-BnIm compound) (Fig. 2.2D), 4-Br-BnIm had an approximately 10-fold better effect on I477N mutant myocilin in HEK cells stably over-expressing tetracycline-regulatable FLAG-tagged myocilin (iHEK) compared to BnIm 01 (Fig. 2.3A&B). This improved potency in cells was likely due to better selectivity for Grp94 over other Hsp90 proteins in cells [44]. We observed no changes in Grp78, suggesting that ER stress was not activated by either inhibitor [46], nor did we observe
an increase in heat shock proteins 27 or 70, indicating that our compound was selectively targeting Grp94 over the cytosolic Hsp90 isoforms [47]. We also did not see any change in Grp94 expression levels, suggesting that the inhibitors are reversibly binding to Grp94. In addition, similar to BnIm 01, 4-Br-BnIm did not cause degradation of other Hsp90-dependent clients Akt and Ras (Fig. 2.3C) [44]. We then evaluated whether 4-Br-BnIm showed improved potency over BnIm 01 against other mutant myocilin species. Indeed, 4-Br-BnIm had greater efficacy against total myocilin levels of each mutant species tested compared to BnIm 01 (Fig. 2.3D).

2.4.3 Grp94 inhibition reduces mutant myocilin levels and toxicity in primary human TM cells.

We next sought to evaluate the efficacy of the selective and potent 4-Br-BnIm in primary human TM cells because mutant myocilin is not known to be toxic to cells other than TM cells. Lentiviral vectors were generated to express RFP-tagged WT and Y437H mutant myocilin, along with RFP only as a control. TM cells were transduced with each viral vector for 7 days and subsequently treated with 30 µM BnIm 01, 4-Br-BnIm, or an equivalent volume of DMSO (vehicle) for 24 hours. Cells were counterstained with DAPI and imaged using confocal microscopy (Fig. 2.4A). Quantification of RFP density revealed that BnIm 01 and 4-Br-BnIm significantly reduced Y437H mutant myocilin while WT myocilin and the RFP-only vector were largely unaffected by Grp94 inhibition (Fig. 2.4B). As was the case with the HEK cell system, 4-Br-BnIm resulted in better clearance of Y437H-mutant myocilin than did BnIm 01. We then evaluated whether Grp94 inhibition could be cytoprotective in TM cells ectopically expressing mutant myocilin using an MTS
assay. Y437H, but not WT myocilin, significantly reduced TM cell viability (Fig. 2.4C). Impressively, both Bnlm 01 and 4-Br-Bnlm rescued this toxicity (Fig. 2.4C).

2.4.4 Post-translational modifications render wildtype myocilin a client of Grp94 via misfolding.

In vitro studies have described several ways in which WT myocilin can aggregate in the absence of a mutation, raising the possibility that misfolding of WT myocilin could be occurring in sporadic cases of POAG, for example by induction of misfolding in relation to post-translational modifications. Impaired glycosylation can prevent protein secretion, but it is also known to cause protein misfolding. For myocilin, we predict this misfolding could lead to production of early aggregates recognized by Grp94. Therefore, we tested the effects of tunicamycin, a reagent known to inhibit N-linked glycosylation [48], on WT myocilin and its subsequent ability to associate with Grp94 in cells. Indeed, transiently over-expressed WT myocilin, which is not fully secreted [43], did become insoluble in cells treated with tunicamycin (Fig. 2.5 A), consistent with our cell-free data. Then, using the iHEK cell model of WT myocilin over-expression, in which all WT myocilin is secreted, we found that Grp94 associated with WT myocilin only in cells treated with tunicamycin, further corroborating our in vitro observations (Fig. 2.5B). Conversely, WT myocilin did not interact with Grp94 in these cells when treated with brefeldin A (BFA), a chemical that blocks ER/Golgi transport and secretion without affecting myocilin folding, further suggesting that the effects of tunicamycin on the myocilin/Grp94 interaction are due to myocilin misfolding, and not impaired secretion (Fig. 2.5C). Then, using the same iHEK cell model of WT myocilin over-expression, we found that tunicamycin blocked myocilin
secretion, but this myocilin was sensitized to Grp94 inhibition (Fig. 2.5D&E), further suggesting that misfolded myocilin becomes a Grp94 client.

2.5 Discussion:

Here, we have demonstrated the effectiveness of a second-generation Grp94-selective inhibitor in clearing a number of POAG-relevant mutant myocilins in several cell lines, including, importantly, primary human TM cells. Mechanistically, in vitro studies indicate that Grp94 recognizes on-pathway aggregation intermediates, suggesting that aberrant quaternary myocilin structure drives Grp94 client recognition rather than exposed peptide motifs of individual misfolded or mutant proteins. When forced to misfold, WT myocilin becomes an intracellular client of Grp94 that is then sensitized to Grp94 inhibition. Taken together, our work demonstrates that reducing mutant or aggregated WT myocilin via inhibiting its interaction with Grp94 represents a viable therapeutic strategy for myocilin-associated forms of glaucoma (Fig. 2.6).

Our findings also help explain the paradox of the failure of ERAD to triage misfolded myocilin. Previously, we showed that mutant, but not wildtype, myocilin, was recognized by Grp94, and that the degradation rate of mutant myocilin was increased when Grp94 was depleted or inhibited [43]. This result was surprising because it suggested that Grp94 was interfering with mutant myocilin clearance, rather than facilitating its removal through the default ERAD pathway. Grp94 attempts to triage mutant myocilin through ERAD but is unsuccessful, leading to its accumulation and mis-sorting, which results in ER stress [16, 43]. Our current work shows that Grp94 cannot triage mutant myocilin through ERAD because it recognizes an already-aggregated state
of myocilin, and this association further accelerates aggregation into an amyloid structure known to be resistant to resolubilization and refolding. This is not the first time that an Hsp90 protein was shown to accelerate the aggregation of an amyloid-prone protein: Hsp90α was previously shown to stimulate the aggregation of tau [49]. The evolutionary reason for accelerated aggregation by a chaperone protein remains unclear, but one possibility is that amyloid fibrils are typically less toxic than amorphous protein aggregates [50, 51]. Thus, perhaps chaperones can stimulate amyloid structure to avoid the production of amorphous structures that are more prone to non-specific interactions with other proteins in the cell. However, in the case of myocilin amyloid, this innate function of chaperones triggers an ER stress response, that culminates in TM cell death. Thus, in this case, pharmacological inhibition of Grp94 reduces the interaction with aggregated myocilin, and, as we showed previously, engages autophagic mechanisms that are better equipped for handling protein aggregates while avoiding ER stress activation.

The BnIm scaffold holds considerable promise for selective Grp94 inhibition without significant cellular side effects. New analogs that further exploit the hydrophobic pocket occupied by the C4 position are currently under development. Efforts are also currently underway to better understand the complex interaction between Grp94 and myoc-OLF. Although the N-terminal ATP-binding domain of Grp94 contains the site of 4-Br-BnIm inhibition, other domains might provide the client protein recognition site; inhibition may simply lock Grp94 in a configuration that occludes binding. Such structural information could be valuable in developing new inhibitors remote from the N-terminal ATP binding site. Finally, the cellular mechanisms that lead to clearance of myocilin after Grp94 inhibition are still not clear, but once defined, these too could be
important targets for therapeutic development against myocilin and allow for a better understanding of how aggregated proteins are removed from the ER.
Figure 2.1 Consequences of Grp94 knockdown or over-expression on levels of intracellular and aggregated mutant myocilin. A, HEK cells were co-transfected with each indicated myocilin mutant and either Grp94 or Control 6TR cDNA. Western blot analyses of cell lysates were performed 48 h after cDNA transfection. B, prior to transfection with each myocilin mutant, cells were transfected with either Grp94 or Control siRNA. Western blot analysis of cell lysates were performed 72 h following siRNA transfection and 48 h after cDNA transfection. C, prior to transfection with each myocilin mutant, cells were transfected with either Grp94 or Control siRNA. Triton solubility fractions of cell lysates were analyzed by Western blot. D, Quantification of C shows significant reductions in each myocilin mutant caused by Grp94 suppression by siRNA as a percent of control siRNA following normalization to loading control. * indicates p<0.05.
Figure 2.2 Structure, modeling and activity of Grp94 inhibitors. All inhibitor synthesis and binding performed by Dr. Brian Blagg’s Lab at Kansas University. A, Structures of radamide, BnIm 01 and 4-Br-BnIm. B, BnIm 01 docked in a hydrophobic pocket within the ATP binding site of Grp94 (based on radamide-bound structure, PDB: 2GFD). C, 4-Br-BnIm docked in Grp94 shows how the hydrophobic pocket can be occupied with the hydrophobic substitution at C4 (indicated by arrow), which likely improves specificity. D, Fluorescence polarization (FP) assay of recombinant Grp94 pre-incubated with FITC-labeled geldanamycin and then increasing concentrations of each compound. Plates were incubated for 24h and FP measured at 485 ex and 528 em. FP values were normalized to DMSO (100% tracer bound).
Figure 2.3 Effects of specific Grp94 inhibitors on levels of intracellular mutant myocilin. A, Western blot analysis of HEK cells stably over-expressing tetracycline-regulatable FLAG-tagged I477N mutant myocilin lysates and treated with indicated doses of BnIm 01 or 4-Br-BnIm for 24 h. Western blots were probed for myocilin levels, as well as levels of other heat-shock proteins (Hsp27, Hsp70) and ER chaperones (Grp78, Grp94). B, Quantification of A shows the half maximal effective concentration (EC50) of BnIm 01 and 4-Br-BnIm for reducing mutant myocilin. C, Western blot analysis of HEK cells stably over-expressing tetracycline-regulatable FLAG-tagged I477N mutant myocilin lysates and treated with indicated doses of 4-Br-BnIm for 24 h. Akt and Ras levels were unaffected by treatment. D, Western blot of lysates from HEK cells transiently overexpressing five forms of mutant myocilin and treated with 30 µM BnIm 01, 4-Br-BnIm, or equivalent volume of vehicle (Veh/DMSO) for 24 h.
Figure 2.4 Grp94 inhibitors reduce mutant myocilin levels and toxicity in HTM cells. A, Immunofluorescence imaging of human trabecular meshwork (HTM) cells transduced with RFP-tagged lentivirus expressing RFP control, RFP-tagged WT myocilin, or RFP-tagged Y437H myocilin and treated with 30 µM BnIm 01, 4-Br-BnIm, or equivalent volume of DMSO vehicle. Myocilin is shown in red and the nuclear marker DAPI shown in blue (40x magnification). B, Quantification of immunofluorescence images detecting RFP density ± standard deviation (n=3). Significance was measured using the student’s t test; * indicates p<.05, ** indicates p<0.01. C, MTS assay of HTM cells overexpressing WT or Y437H myocilin following treatment with 30 µM BnIm 01, 4-Br-BnIm, or vehicle equivalent (DMSO) for 6 h. Cell viability is shown prior to treatment (Pre-Tx) and following treatment (Post-Tx) ± standard deviation (n=3). Significance was measured using the student’s t test; * indicates p<.05, ** indicates p<0.01.
Figure 2.5 WT myocilin becomes a client of Grp94 when myocilin is misfolded. A, Western blot analysis of Triton soluble/insoluble fractions from iHEK WT myocilin cell lysates treated with 6µM tunicamycin for 24 h. B, Co-IP of myocilin and subsequent Western blot analysis of iHEK WT myocilin cell lysates treated with 6µM tunicamycin; Inputs indicates Western blot of lysates without co-IP. C, Co-IP of myocilin and subsequent Western blot analysis of iHEK WT myocilin cell lysates treated with 3µM concentration of Brefeldin A; Inputs indicates Western blot of lysates without co-IP. D, Western blot analysis of iHEK WT myocilin cells treated with 6µM concentration of tunicamycin followed by a dose response of Bnlm 01, 4-Br-Bnlm, or equivalent volume of DMSO vehicle. E, Quantification of Western blot analysis on dose response from D showing myocilin levels as a percent of vehicle following normalization to loading control (Gapdh).
Figure 2.6 Grp94 accelerates myocilin aggregation, a process that can be prevented by Grp94 inhibition. Myocilin misfolding causes it to form small aggregates that bind Grp94. Grp94 facilitates amyloid formation, preventing clearance of myocilin and causing toxicity. Grp94 inhibitors prevent Grp94 from interacting with myocilin, allowing a secondary autophagic pathway to facilitate clearance.
Chapter Three:
Topical Ocular Administration of a Selective Grp94 Inhibitor Rescues Functional Toxicities in a Mouse Model of Hereditary Primary Open Angle Glaucoma

3.1 Abstract
Over 70 mutations in exon 3 of the MYOC (myocilin) gene have been linked to development of primary open-angle glaucoma (POAG). This genetic link has led many to believe myocilin plays a vital role in both disease initiation and progression. Recent work has demonstrated a direct interaction between mutant forms of myocilin and the Hsp90 homolog, Grp94. This interaction preserved mutant myocilin within the endoplasmic reticulum (ER) of trabecular meshwork (TM) cells, precipitating TM cell dysfunction and downstream glaucomatous pathology. Here, we report the treatment of myocilin-associated glaucomatous pathology in the Tg-MYOC<sup>Y437H</sup> mouse model through topical ocular administration of the specific Grp94 inhibitor, 4Br-BnIm. Following chronic treatment with 4Br-BnIm, we observed a rescue of multiple pathologies commonly associated with myocilin-induced glaucoma, including elevated intraocular pressure, myocilin accumulation in the TM, reduced scotopic and photopic visual responses, and retinal ganglion cell death. These results demonstrate the validity of targeted Grp94 inhibition as a novel disease-modifying treatment for patients with POAG.
3.2 Introduction

Primary open-angle glaucoma (POAG) is a neurodegenerative optic neuropathy, characterized by progressive irreversible vision loss [31] [52] [53] [54] [55]. Mutations in the myocilin gene (MYOC) are the most common genetic link to clinical cases of POAG, including the less common but more aggressive juvenile-onset open-angle glaucoma (JOAG) [56]. There are over 70 documented MYOC mutations that have been linked with glaucomatous pathology. Recent studies have shown that MYOC mutations lead to a protein product that readily misfolds and accumulates within the endoplasmic reticulum (ER) of trabecular meshwork (TM) cells [5, 8, 9, 14]. This accumulation causes TM cell dysfunction, leading to reduced aqueous humor outflow, elevated intraocular pressure (IOP), retinal ganglion cell (RGC) dysfunction, and loss of optic nerve (ON) axons [57].

Clinically, treatment of POAG is best initiated when diagnosed early in the disease progression. For patients with POAG, initial treatment options include pharmaceuticals, such as alpha agonists, beta blockers, and prostaglandin analogs, to reduce aqueous humor production at the ciliary body (CB) and promote alternative outflow pathways [58] [59] [60] [61]. As the disease continually progresses, more substantial surgical filtration therapies become necessary, such as sclerectomy or trabeculectomy [62] [63] [64] [65]. While these therapies have proven to be effective at reducing IOP and slowing progression of the disease, they are merely palliative in nature and do not prevent the disease endpoint of irreversible blindness. To develop a curative therapy, the underlying disease mechanisms must be targeted; however, this has proven to be difficult, as a defined disease mechanism still remains a mystery.
Our previous work has explored this disease mechanism and discovered a link between mutant or misfolded forms of myocilin and the ER-associated Hsp90 homolog glucose-regulated protein 94 (Grp94). We have shown that Grp94 recognition of mutant/misfolded myocilin preserves the protein within the ER of TM cells, leading to protein accumulation and aggregation, precipitating TM cell dysfunction [43,54]. When the Grp94-misfolded myocilin interaction is inhibited, whether through gene knockdown or pharmacological inhibition, the TM cell machinery regains the functional ability to degrade and remove the misfolded protein, likely through a robust autophagic mechanism [43, 54]. This has led us to hypothesize that Grp94 is directly responsible for mutant myocilin pathology in POAG.

The current study initially investigated the myocilin interactome following treatment with the Grp94 specific inhibitor 4Br-BnIm in cells to determine pathways associated with myocilin triage following Grp94 inhibition. The efficacy of this compound in vivo was then explored using topical ocular administration to evaluate glaucomatous pathologies in these mice such as IOP, RGC function, and myocilin accumulation. Our results indicate the validity of specific Grp94 inhibition as a potential first curative therapeutic for patients suffering from myocilin-associated POAG.

3.3 Materials and Methods

3.3.1 Inhibitor synthesis

4Br-BnIm was synthesized and provided by Dr. Brian Blagg’s group at the University of Kansas as previously described [54].
3.3.2 Study Approval

All institutional guidelines for the care and use of animals were followed. All animal handling and procedures were carried out in accordance with the University of South Florida’s Institutional Animal Care and Use Committee (IACUC) in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care International (AALAC) regulations.

3.3.3 Animal Husbandry

The transgenic-Y437H mouse model (Tg-MYOCY437H) was a generous gift from Val Sheffield at the University of Iowa. Characterization of the Tg-MYOCY437H mouse was previously described [16,18]. Mice were housed and bred at the University of South Florida Byrd Alzheimer’s Institute onto the C57BL/6 background. Mice heterozygous for the Tg-MYOCY437H mutation were bred together to produce a 1:2:1 ratio of homozygous transgenic: heterozygous transgenic: wild type littermates. Heterozygous littermates were maintained for breeding, and homozygous transgenic and wild type littermates were used for the study. Genotyping for the transgene was performed as previously described by the Sheffield Lab [16,18].

3.3.4 Cell culture

Cell culture was performed as previously described [43]. Stably transfected iHEK cells expressing I477N mutant myocilin were grown and maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS (Invitrogen), penicillin (100
units/ml), streptomycin (100 mg/ml) and 1% GlutaMAX (Invitrogen) at 37°C under 5% CO2. For cell selection, the cells were supplemented with hygromycin B (200 mg/ml) (InvivoGen) and G418 (100 mg/ml) (Gibco). To induce myocilin expression in these cells, they were treated with 5 mg/ml tetracycline 24 h prior to transfection, or 48 h prior to pharmacological treatment.

3.3.5 Antibodies (cell culture)

    Myocilin polyclonal antibody (1:1500) was a generous gift from W.D. Stamer at Duke University [150]. Actin antibody (1:1000) was purchased from Sigma (A5316). Secondary antibodies for western blots were all HRP-linked and purchased from Southern Biotechnologies (Birmingham, AL). Primary and secondary antibodies were added to western blots at a 1:1000 dilution in 7% milk.

3.3.6 Pharmacological treatment (cell)

    Grp94 inhibitors were solubilized in DMSO (Sigma Aldrich), and added to cells at the indicated concentrations. All drug treatments were carried out 24h prior to cell harvest, and DMSO concentration never exceeded 1% of total volume of cell medium.

3.3.7 Cell Harvest

    Cell harvest was performed as previously described [54]. Culture medium was aspirated and cells were washed 2× in ice cold phosphate-buffered solution (pH 7.4) (PBS). Mammalian Protein Extraction Reagent (M-PER) buffer (Pierce) containing protease inhibitor mixture (Calbiochem), 100 mM phenylmethylsulfonyl fluoride (PMSF)
and phosphatase inhibitor II and III mixtures (Sigma) were added to the cells at a 1:100 dilution and cells were scraped. Cells in lysis buffer were collected and incubated on ice for 10 min to facilitate lysis. Cell lysates were spun down at 16000xg for 10 min and a bicinchoninic acid assay (BCA) reaction was carried out to determine protein concentration for biochemical assessment of protein abundance.

3.3.8 Western blotting (cell)

Western blotting was performed as previously described [22]. After gel run was complete, gels were transferred onto PVDF membrane (Millipore) at 100 volts for 1 hour. After transfer, blots were blocked with 7% milk in TBS for 1 hour at room temperature before addition of primary antibody.

3.3.9 Inhibitor pharmacokinetics

PK studies performed by Dr. Brian Blagg’s group at Kansas University. Wild type C57BL/6 mice were treated with topically administered 4Br-BnIm (~10 µL) at 100 µM concentration. Eye drop remained on mouse eye for 1 minute before being irrigated off with sterile PBS. Mice were then immediately euthanized, eyes enucleated, and flash frozen. Isolated eye samples were diluted (0.1g/g in water) then homogenized by vortexing in the presence of glass beads. Organics were extracted using EtOAc. Following removal of EtOAc by evaporation, samples were diluted in MeCN/H₂O (1:1) for HPLC analysis. Concentrations of samples were determined after generating a standard curve for 4Br-BnIm at known concentrations. HPLC Conditions: Agilent 1100 series
quaternary pump, 1.0 mL/min, 50% MeCN/50% H$_2$O, detection at 254nm. Column: Agilent C-18 column, 4.6x150mm, 5µm. $R_t = 6.5$ min.

3.3.10 Inhibitor application and study duration

4Br-BnIm dose concentration was determined by HPLC PK analysis observing concentration of drug to cross the corneal epithelial barrier when applied topically. Approximately 10% of the compound readily crossed the eye, therefore 300 µM stocks were made up, which ensured a concentration of 30 µM would contact intraocular tissues in the anterior chamber. Drugs were solubilized in DMSO and diluted in sterile saline, so that the concentration of DMSO was 1%. Once a day, mice were restrained and 1 drop (~10 µL) of drug was applied topically via eye drop. The drop was allowed to sit on the eye for 1 minute before the mouse was returned to its cage. Mice were treated 1x/day for 14 weeks.

3.3.11 Intraocular Pressure (IOP) Measurements

Intraocular pressure (IOP) levels in the mouse eye were obtained using the Icare TonoLab rebound tonometer (Icare, Finland). Bi-weekly, mice were briefly anesthetized with Isoflourane at a 3-4% flow rate. Once induced, mice were quickly placed in a tube restraint, and 6 IOP measurements were taken per eye. The 6 measurements were then analyzed to determine standard deviation to determine consistency of the measurements. If the standard deviation was within the parameters determined by the machine, the mouse was removed from the restraints and returned to its cage. Animals were anesthetized for no more than 2 minutes during the IOP measurement process.
3.3.12 Electrophysiology

Electroretinograms (ERGs) were recorded from both eyes of each mouse post-treatment in a lightproof/soundproof booth. Animals were dark adapted for 24 hours prior to data collection. Prior to ERG recordings, mice were anesthetized using a Ketamine (100 mg/kg) and Acepromazine (1 mg/kg) mixture delivered via intraperitoneal (IP) injection. Mouse health and vitals were monitored visually throughout the experiment, with additional anesthesia given as needed. A custom ring-shaped gold electrode was placed on the corneal limbus of each eye. Platinum needle electrodes (Natus Neurology Inc., Warwick, RI) inserted in the temples and tail served as references and ground readings, respectively. Recorded signals were differentially amplified (2000x) and filtered (0.1-1000 Hz) by a multi-channel bio-amplifier (Xcell-3x4, FHC Inc., Bowdoin, ME) and digitized at 1000 Hz. Light stimuli were produced by a green LED (Vishay TLCTG5800, Newark Electronics, Palatine, IL) with an 8° emittance angle positioned 1 cm perpendicularly to each cornea. The LEDs had a peak wavelength of 520 nm and peak output of 2100 cd/m2 measured with a calibrated photometer (UDT Instruments Inc., Baltimore, MD). Full-field scotopic ERGs (n35-p50) were recorded for a series of 200 brief (10ms) flashes (1.32 log cd·s/m2) delivered to both eyes simultaneously. LED light intensity was gradually increased to determine lowest possible intensity to evoke an ERG response. After scotopic recording, mice were exposed for 30 min to ambient room light (15 cd/m2) from an LED strip that lined the booth ceiling. After light-adaption, full-field photopic ERGs (p50-n95) were recorded. LED flash sequences were separated by a 3 second interval, to allow sufficient time for
mice to fully recover from the previous LED flash, ensuring validity of ERG recordings. Light-evoked signals were quantified in terms of scotopic (n35-p50)/photopic (p50-n95) wave amplitude. Wave amplitudes were measured from the trough-to-peak (n35-p50) or peak-to-trough (p50-n95). Statistical analysis was performed using SigmaPlot 13.0 software (San Jose, CA) and graphs were generated with GraphPad Prism 5.0 software.

3.3.13 Eye enucleation

Mice were euthanatized with a .2% Somnasol (50 mg/kg) in saline solution. Eyes were gently removed from the skull preserving the morphology of the eye globe. Once removed from the skull, eyes were either fixed in Davidson's (1:3:2:3, glacial acetic acid: 95% ethanol, 10% neutral buffered formalin, distilled water) solution for histology, or micro-dissected to remove the retinas.

3.3.14 Whole retinal mount

Post-enucleation, mouse eyes were micro-dissected to remove the intact retina. Eyes were placed in an isotonic solution, and the cornea was removed using surgical micro-scissors. The iris was gently pulled away from the lens, and the lens was removed using tweezers. The remaining eye cup contained the retina and sclera. Gently, the retina was peeled away from the sclera, producing a whole intact retina. Retinas were placed in 10% neural buffered formalin for fixation for 48 hours and then moved to a dPBS+ Sodium Azide solution for storage until tissue staining.
3.3.15 Histological processing of mouse eye tissue

Enucleated mouse eyes were immediately fixed in Davidson’s Solution for 48 hours. Davidson’s solution was used to maintain good nuclear detail without formalin pigmenting. After 48 hour fixation, eyes were switched to 10% neutral buffered formalin for shipping. Fixed eyes were shipped to HistoWiz Inc. (Brooklyn, NY, USA) where tissue was processed, paraffin embedded, sectioned, and mounted on slides. Sections were cut longitudinally at a thickness of 10 microns. Slides were returned to our lab for deparaffinization and tissue staining.

3.3.16 Tissue deparaffinization/rehydration

Modified from an existing Abcam protocol (www.abcam.com/protocols/ihc-deparaffinization-protocol). Slides were placed in a glass slide rack, and deparaffinized/rehydrated using the following steps: 2x 3min wash in Xylenes, 1x 3min wash in 1:1 Xylenes:100% ethanol, 2x 3min wash in 100% ethanol, 1x 3min wash in 95% ethanol, 1x 3min wash in 75% ethanol, 1x 3 min wash in 50% ethanol, keep in ddH2O until antigen retrieval. After rehydration, at no point were the slides/tissue allowed to dry out.

3.3.17 Antigen Retrieval

After deparaffinization, slides were submersed in a Sodium Citrate Buffer (10mM Sodium Citrate, 0.05% Tween 20, ph 6.0) in a microwave safe plastic coplin jar. The coplin jar was placed in a water bath, to maintain a constant temperature, and microwaved at 2 minute increments, replacing water and sodium citrate buffer after
each heating cycle. This process was repeated three times, ensuring heat-induced epitope retrieval.

3.3.18 Tissue staining

After deparaffinization and antigen retrieval, whole eye section slides were fluorescently stained as previously described [67]. Briefly, following permeabilization, tissue was incubated with myocilin (Santa Cruz (21243), Dallas, TX, 1:50) primary antibody overnight. The following day, sections underwent PBS washing, and tissue was incubated with Alexa Fluor 594 donkey anti-goat IgG (Invitrogen (11058), Grand Island, NY; 1:500) secondary antibody for 2 hours.

For whole mount retinas, tissue sections were free-floating fluorescently stained as previously described above. Briefly, following permeabilization, tissue was incubated with γ-synuclein (Abcam (55424), Cambridge, UK, 1:1000) primary antibody overnight. The following day, sections underwent PBS washing, and tissue was incubated with Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen (11008), 1:1000) secondary antibody for 2 hours. Whole retinas were mounted and both whole eye and whole retina slides were cover slipped using ProLong Gold Antifade Reagent (Invitrogen).

3.3.19 Tissue imaging, quantification, and analysis

Both myocilin stained whole eye sections and γ-synuclein stained whole retinas were imaged using the Olympus FV1200 MPE Multiphoton Laser Scanning Microscope using a 40x objective (scale bar = 40 µM). All fluorescent quantification was carried out using ImageJ analysis software (National Institutes of Health). Myocilin was quantified in
the TM of whole eye slices for fluorescence intensity. Gamma-synuclein was quantified for the entire retina section for both fluorescence intensity, and positively stained cells. To determine positively stained cells, a fluorescence threshold was determined. Particle analysis was performed on areas between 0 and infinity pixels² and a circularity of 0.00-1.00, which allowed for the analysis of all positive gamma-synuclein staining. For whole eye sections, each mouse had 2 TMs analyzed. These values were then averaged to determine average myocilin levels in each mice. For whole mount retinas, analysis was taken of the retina as a whole, measuring total number of positive cells and intensity of positively stained cells.

3.3.20 Quantification and Statistical Analysis

Statistical analysis of IOP, ERG, and fluorescent images was performed using student’s t-test, comparing each individual treatment group. Values were considered significant if p<0.05. Graphs were generated using GraphPad Prism 5.0 analysis software.

3.4 Results

3.4.1 The selective Grp94 antagonist 4Br-BnIm triages mutant myocilin and possesses trans-scleral permeability

We have previously shown that the specific Grp94 inhibitor, 4Br-BnIm, was effective at clearing intracellular mutant myocilin, inhibiting in vitro myocilin protein aggregation, and alleviating TM cell dysfunction [54]. To properly translate these results to an in vivo mouse model for glaucoma, we set out to determine if 4Br-BnIm would be
effective as an ocular treatment (Fig. 3.1A). First, to confirm intracellular mutant myocilin reduction by 4Br-BnIm treatment, iHEK cells overexpressing I477N mutant myocilin were treated with increasing concentrations of 4Br-BnIm, and intracellular myocilin levels were evaluated by western blot (Fig. 3.1B). Densitometry quantification revealed 4Br-BnIm reduced intracellular mutant myocilin levels, with almost 50% reduction at a 30 µM concentration (Fig. 3.1C).

However, to definitively determine the effectiveness of this compound as a topical ocular treatment, the pharmacokinetic (PK) efficiency of crossing the corneal epithelial barrier when applied topically was tested. PK studies performed by Dr. Brian Blagg’s Lab at Kansas University. One drop (~10 µL) of 4Br-BnIm was applied to the eye and allowed to permeate for 1 minute. Mouse eyes were subsequently harvested, homogenized and analyzed through high performance liquid chromatography (HPLC) to determine the intraocular concentration of the compound. Quantification of these results revealed 13.1±2.7% of 4Br-BnIm could effectively cross the corneal epithelial barrier. These data demonstrated that 4Br-BnIm was a viable candidate for topical ocular administration, as the bioavailability of current ophthalmic medications is considered acceptable at a rate of approximately 5% [68]. Therefore, we determined a dose of 300 µM, with approximately 10% of the compound effectively crossing the corneal epithelial barrier, would ensure approximately a 30 µM concentration of 4Br-BnIm was reaching intraocular tissues in the anterior chamber of the eye when applied topically.
3.4.2 Intraocular pressure elevation in Tg-MYOC\textsuperscript{Y437H} mice is rescued by topical ocular treatment with 4Br-BnIm

In our previous \textit{in vitro} work, we showed that inhibition of Grp94 association with misfolded or mutant myocilin was sufficient to induce intracellular protein clearance [43] [54]. Therefore, we sought to determine if this could be translated to pathology reduction \textit{in vivo} in the Tg-MYOC\textsuperscript{Y437H} mouse model of glaucoma. Mice were aged to 4 months to ensure the development of pathology, and then separated into 4 distinct treatment groups: wild–type (WT) + vehicle (n=6), WT + 4Br-BnIm (n=6), transgenic (Tg) + vehicle (n=12), and Tg + 4Br-BnIm (n=14). Animals received daily topical ocular treatment as described in the methods. Bi-weekly, IOP measurements were recorded on each individual eye (Fig. 3.2A). Baseline readings for the WT mice were measured at 13.83 ± .55 and 13.5 ± .70 mm Hg for the vehicle and 4Br-BnIm groups, respectively. Baseline readings for the Tg mice were measured at 19.17 ± .61 and 19.86 ± .43 mm Hg for the vehicle and 4Br-BnIm treated groups, respectively. There was no significant difference between transgenic treatment groups prior to treatment phase (p=.3721) (Fig. 3.3B). Post-treatment IOP readings for WT mice were measured at 12.83 ± .28 and 13.17 ± .28 mm Hg for the vehicle and 4Br-BnIm treated groups, respectively. Post-treatment readings for Tg mice were measured at 17.5 ± .28 and 14.0 ± .34 mm Hg for the vehicle and 4Br-BnIm treated groups, respectively. Tg mice treated with vehicle had significantly higher IOP readings than Tg mice treated with 4Br-BnIm following the treatment period (p=.0001)(Fig. 3.2B). These findings imply that topical ocular treatment of 4Br-BnIm is sufficient to reduce elevated IOP levels in the Tg-MYOC\textsuperscript{Y437H} mouse model of glaucoma.
3.4.3 Myocilin accumulation in the trabecular meshwork of Tg-MYOC\textsuperscript{Y437H} mice is reduced following topical ocular treatment with 4Br-BnIm

As we have previously shown, 4Br-BnIm is effective at reducing intracellular levels of mutant myocilin in \textit{in vitro} primary TM cell models [54]. To determine if 4Br-BnIm had the same effect in the Tg-MYOC\textsuperscript{Y437H} mouse model, we performed immunohistological staining of myocilin in the TM (Fig. 3.3A). Quantification of immunostaining for fluorescent intensity revealed no significant difference in myocilin levels when comparing WT + vehicle (n=4) and WT + 4Br-BnIm (n=6) treatment groups (p=.9588) (Fig. 3.3B). This allowed us to group all WT mice together for comparison with Tg-MYOC\textsuperscript{Y437H} treatment groups. Tg-MYOC\textsuperscript{Y437H} + vehicle (n=14) mice had significantly more myocilin accumulation in the TM compared to WT mice (p=.0002). Importantly, Tg-MYOC\textsuperscript{Y437H} + 4Br-BnIm (n=10) mice showed no significant difference in myocilin levels in the TM compared to WT mice (p=.5933) (Fig. 3.3C). Collectively, these data indicate that topical ocular administration of 4Br-BnIm had a profound effect on TM tissue in Tg-MYOC\textsuperscript{Y437H} mice, further validating 4Br-BnIm as a viable therapeutic targeting pathology associated with POAG.

3.4.4 Scotopic and photopic vision is preserved in Tg-MYOC\textsuperscript{Y437H} mice following topical ocular treatment with 4Br-BnIm

In order to determine retinal cell health, we evaluated the scotopic visual response of Tg-MYOC\textsuperscript{Y437H} mice, using dark-adapted electroretinography (ERG). Mice were dark adapted for 24 hours prior to ERG readings. Representative tracings of dark-
adapted ERG show scotopic (n-35-p50) wave amplitudes (Fig. 3.4A). Quantification of these dark-adapted ERG tracings showed no significant difference between WT treatment groups n35-p50 wave amplitudes (p=.8255) (Fig. 3.4B). As previously reported [18], Tg mice treated with vehicle had significantly reduced n35-p50 wave amplitudes compared to WT mice (p=.0134). However, Tg mice treated with 4Br-BnIm displayed no significant difference in n35-p50 wave amplitudes compared to WT mice (p=.3652) (Fig. 3.4C), once again demonstrating the effectiveness of the compound.

To determine the photopic visual response of Tg-MYOC\textsuperscript{Y437H} mice, we performed light-adapted ERG, exposing the mice to ambient room light for 30 minutes following dark-adapted ERG recordings. Representative tracings of light-adapted ERG photopic (p50-n95) wave amplitudes are depicted in Fig. 3.4D. Quantification of these light-adapted ERG tracings showed no significant difference between WT treatment groups p50-n95 wave amplitudes (p=.6850) (Fig. 3.4E). Similar to the scotopic response, Tg mice treated with vehicle displayed significantly reduced p50-n95 wave amplitudes compared to WT mice (p=.0140). Again, Tg mice treated with 4Br-BnIm had no significant difference in p50-n95 wave amplitudes compared to WT mice (p=.5310) (Fig. 3.5F). These data suggest that topical ocular administration of 4Br-BnIm can preserve both scotopic and photopic visual responses, thus effectively preserving retinal neuronal cell activation.
3.4.5 Retinal ganglion cell viability is preserved in Tg-MYOC\textsuperscript{Y437H} mice following topical ocular treatment with 4Br-BnIm

RGC viability was evaluated by whole-mount $\gamma$-synuclein staining of mouse retinas comparing WT mice to each Tg treatment group. First, total RGC cell count was determined using ImageJ software to quantify the number of positive cells (Fig. 3.5A). Quantification showed a significant reduction (69\%) in positively stained RGC cells in Tg mice treated with vehicle as compared to WT mice ($p=.0002$). There was no significant difference between Tg mice treated with 4Br-BnIm and WT mice ($p=.7945$) (Fig. 3.5B). Next we looked at intensity of fluorescent $\gamma$-synuclein staining to determine overall viability of healthy RGCs (Fig. 3.5C). Quantification showed a significant reduction in fluorescent intensity (90\%) in Tg mice treated with vehicle as compared to WT mice ($p=.0023$). There was no significant difference in fluorescent intensity of $\gamma$-synuclein staining in Tg mice treated with 4Br-BnIm compared to WT mice ($p=.5846$) (Fig. 3.5D). These findings, taken together with the ERG data, suggest that topical ocular treatment with 4Br-BnIm preserved retinal cell health and function in the Tg-MYOC\textsuperscript{Y437H} mouse model, allowing normal signal transduction and conversion of light waves into visual signals in the visual cortex of the brain.

3.5 Discussion

Here, we have further validated specific Grp94 inhibition as a viable therapy for treating myocilin-associated POAG by restoring TM cell function and reducing glaucomatous pathology in the Tg-MYOC\textsuperscript{Y437H} mouse model. Previously, we have shown that specific Grp94 inhibition leads to a reduction in myocilin accumulation and
cellular dysfunction in primary human TM cells [54]. Additionally, we have shown that in vitro myocilin aggregation is significantly reduced following protein incubation with 4Br-BnIm [54]. The current study demonstrated that these previous in vitro cell and protein assay results translate to effective in vivo treatment of myocilin-associated POAG pathologies. These findings underscore the potential therapeutic benefit of specific Grp94 inhibition as a treatment for patients with myocilin-associated POAG and further define a specific disease mechanism of POAG pathology. The results from the mass spectrometry also elucidate the intracellular mechanisms of mutant myocilin degradation, which will provide valuable insight into Grp94-mediated myocilin triage.

TM tissue is the main component of the conventional outflow pathways of aqueous humor. Several studies have shown that accumulation of myocilin in the TM precipitates TM cellular dysfunction [16, 18, 54, 77], leading to the pathologies commonly associated with POAG, including increased IOP, RGC cell death, and eventually irreversible blindness. Therefore, if effective, by reducing mutant myocilin accumulation in the TM, normal TM cell function can be restored in the human eye, preventing downstream POAG pathologies from being initiated. Here, we have shown that topical ocular administration of 4Br-BnIm significantly reduced myocilin levels in the TM. This suggests that specific Grp94 inhibition is a viable method for restoring normal TM cell function in patients with POAG.

The functional pathologies associated with myocilin-induced POAG include elevated IOP, diminished scotopic/photopic visual responses, and deterioration of RGC health/function. Importantly, topical ocular administration of 4Br-BnIm led to a significant reduction in each of these functional pathologies, demonstrating its broad therapeutic
utility. The effectiveness and potency of specific Grp94 inhibition as a viable treatment for POAG pathologies also highlights the vital role Grp94 plays in POAG disease progression. Prior to our work, Grp94 was mainly studied for its pro-oncogenic properties as it relates to cancer development [78-80]. Specifically, how elevated levels of Grp94 are a molecular hallmark of tumor metastasis [81-83]. With Grp94’s clear involvement in other degenerative diseases, it would be prudent to further explore non-primary open-angle forms of glaucoma, including closed-angle and secondary glaucoma, to determine what role, if any, Grp94 plays in their disease progression.

In conclusion, this study revealed the effectiveness and potency of specific Grp94 inhibition as a viable treatment method for POAG. Autophagic degradation of mutant myocilin following specific Grp94 inhibition leads to restoration of TM cell function, reduced IOP, and preserved retinal cell function, ultimately restoring health and function of the eye (Fig. 3.6). Grp94 inhibition holds considerable promise as a potential disease-modifying treatment of POAG. New compounds that are more potent at reducing intracellular myocilin at lower concentrations, while also readily crossing the corneal epithelial barrier are currently under development. Efforts are also currently underway to determine the most effective means of drug delivery into the eye, to target TM cells while reducing off-target effects. Although topical ocular administration provided significant reductions in pathologies associated with POAG, other means of delivery, such as anterior chamber micro-injections, could prove to be more effective at directly targeting the TM. Studies exploring potency, bioavailability, means of delivery, and disease mechanism will be vital in progressing this research into human clinical trials and developing an effective treatment for patients with POAG.
Figure 3.1 4Br-BnIm reduces intracellular mutant myocilin. (A) Structure of specific Grp94 inhibitor, 4Br-BnIm. (B) Tetracycline-inducible Hek cells (iHEK) overexpressing the disease-associated I477N mutant myocilin were treated with increasing doses of 4Br-BnIm to determine a dose response. Western blot analysis of cell lysates were performed 24 h after drug treatment. (C) Quantification of (B) shows effective reduction of intracellular mutant myocilin when treated with 4Br-BnIm.
Figure 3.2 Topical ocular 4Br-BnIm reduces intraocular pressure in Tg-MYOCY437H mice. (A) Intraocular pressure (IOP) levels as recorded by rebound tonometry for four treatment groups (WT + vehicle (n=6), WT+Grp94 Inhibitor (n=6), Tg + vehicle (n=12), Tg+Grp94 Inhibitor (n=14)). Readings were recorded every two weeks throughout the treatment period. Time=0 represents the IOP of animals prior to treatment initiation. Treatment period began at age=4 months, and occurred 1x/day for the duration of the study. (B) Quantification of (A) comparing average IOP levels of the four treatment groups pre- and post- treatment cycle. Initially, there was no statistical difference between Tg groups prior to treatment (p=.3721) (19.2 to 19.9 mmHg). However, after the treatment period we observed a significant reduction in IOP for the Tg+Grp94 Inhibitor group compared to Tg + vehicle group (p=<.0001) (14.0 to 17.5 mmHg), returning to levels consistent with both WT treatment groups (13mmHg). Statistical analysis carried out using two-tailed t-test. *P<.05, **P<.001, ***P<.0001.
Figure 3.3 Topical ocular 4Br-BnIm reduces intracellular myocilin accumulation in the TM of Tg-MYOCY437H mice. (A) Representative images showing myocilin (Santa Cruz (21243)) accumulation in the TM of WT and Tg-MYOCY437H mice topically treated with vehicle or 4Br-BnIm by fluorescent immunostaining and multiphoton microscopy. Trabecular meshwork (TM) and ciliary body (CB) are marked. (B) Quantification of (A) showing fluorescent intensity translating to myocilin accumulation levels comparing WT + vehicle (n=6) group to WT + 4Br-BnIm (n=4). There is no significant difference between wildtype mice treatment groups (p=.9588). (C) Quantification of (A) showing myocilin accumulation in both Tg mouse treatment groups compared to WT mice. Tg-MYOCY437H + vehicle (n=14) have significantly more myocilin accumulation compared to WT mice (p=.0002). Tg-MYOCY437H + 4Br-BnIm (n=10) are not significantly different in myocilin accumulation compared to WT mice (p=.5933). Scale bar, 40μM. Statistical analysis carried out using two-tailed t-test. *P<.05, **P<.001, ***P<.0001.
Figure 3.4 Topical ocular 4Br-BnIm restores scotopic and photopic vision in Tg-MYOCY437H mice. (A) Representative tracings of average scotopic (N35-P50) wave amplitudes in dark-adapted mice. (B) Quantification of (A) comparing WT treatment groups (WT + vehicle (n=4), WT + 4Br-BnIm (n=6)) N35-P50 wave amplitude. Statistically, there was no difference between WT groups (p=.8255), allowing us to group all WT mice together (n=10) (40.7 ± 2.7uV). (C) Quantification of (A) comparing Tg treatment groups (Tg + vehicle (n=7), Tg+4Br-BnIm (n=8)) to WT group. Tg mice treated with vehicle had significantly diminished (p=.0134) N35-P50 wave amplitude compared to WT mice (28.8 ± 3.0uV to 40.7 ± 2.7uV). Tg mice treated with Grp94 inhibitor were not statistically different (p=.3652) from WT mice (36.5 ± 3.6uV to 40.7 ± 2.7uV). This shows a recovery in N35-P50 wave amplitude in Tg mice following Grp94 Inhibitor treatment. (D) Representative tracings of average photopic P50-N95 wave amplitudes in light-adapted mice. (E) Quantification of (D) comparing WT treatment groups (WT + vehicle (n=4), WT+4Br-BnIm (n=6)) P50-N95 wave amplitude. Statistically, there was no difference (p=.6850) between WT groups, allowing us to group all WT mice together (n=10) (45.0 ± 6.0uV). (F) Quantification of (D) comparing Tg treatment groups (Tg + vehicle (n=10), Tg+4Br-BnIm (n=8)) to WT group. Tg mice treated with vehicle had significantly diminished (p=.0140) P50-N95 wave amplitude compared to WT mice (27.4 ± 2.4uV to 45.0 ± 6.0uV). Tg mice treated with Grp94 inhibitor were not statistically different (p=.5310) from WT mice (39.6 ± 5.6uV to 45.0 ± 6.0uV). This shows a recovery in P50-N95 wave amplitude in Tg mice following Grp94 Inhibitor treatment. Statistical analysis carried out using two-tailed t-test. *P<.05.
Figure 3.5 Topical ocular 4Br-BnIm preserves RGC viability in Tg-MYOCY437H mice. (A) Representative images showing gamma-synuclein (Abcam (55424)) positive cells translating to viable retinal ganglion cells in whole mounted retinas. Positive cell threshold was determined using ImageJ analysis software. (B) Quantification of (A) comparing gamma-synuclein positive retinal ganglion cells in WT (n=3) to Tg mouse treatment groups. Tg + vehicle (n=4) treated mice had significantly less gamma-synuclein positive cells than WT mice (p=.0002). Tg + 4Br-BnIm (n=4) mice did not have a significantly different amount of gamma-synuclein positive cells (p=.7945). (C) Representative images showing gamma-synuclein staining intensity of retinal ganglion cells in whole mounted retinas. (D) Quantification of (C) comparing gamma-synuclein staining intensity in WT (n=3) to Tg mouse treatment groups. Tg + vehicle (n=4) treated mice had significantly lower gamma-synuclein intensity than WT mice (p=.0023). Tg + 4Br-BnIm (n=4) mice did not have a significantly different intensity of gamma-synuclein staining (p=.5846). Scale bar, 40uM. Statistical analysis carried out using two-tailed t-test. *P<.05, **P<.001, ***P<.0001.
Figure 3.6 4Br-BnIm inhibits Grp94 association with mutant myocilin, reducing pathologies associated with POAG. In a healthy eye, myocilin is produced in the ER of TM cells. If there is a mutation in the myocilin genome, a protein product is produced that readily accumulates and aggregates due to recognition by Grp94. If mutant myocilin is allowed to aggregate within the ER of TM cells, cellular dysfunction occurs, leading to POAG associated pathologies. Using the specific Grp94 inhibitor, 4Br-BnIm, Grp94 association with mutant myocilin is inhibited allowing for alternative degradation via autophagy. Myocilin degradation restores TM cell function, leading to reduced IOP and retinal cell preservation.
Chapter Four:

Targeting the ER-Autophagy System in the Trabecular Meshwork to Treat Glaucoma

4.1 Abstract

A major drainage network involved in aqueous humor dynamics is the conventional outflow pathway, which is gated by the trabecular meshwork (TM). The TM acts as a molecular sieve, providing resistance to aqueous outflow, which is responsible for regulating intraocular pressure (IOP). If the TM is damaged, aqueous outflow is impaired, IOP increases and glaucoma can manifest. Mutations in the MYOC gene cause hereditary primary open-angle glaucoma (POAG) by promoting the abnormal amyloidosis of the myocilin protein in the endoplasmic reticulum (ER), leading to ER stress-induced TM cell death. Myocilin accumulation is observed in approximately 70-80% of all glaucoma cases suggesting that environmental or other genetic factors may also promote myocilin toxicity. For example, simply preventing myocilin glycosylation is sufficient to promote its abnormal accretion. These myocilin amyloids are unique as there are no other known pathogenic proteins that accumulate within the ER of TM cells and cause toxicity. Moreover, this pathogenic accumulation only kills TM cells, despite expression of this protein in other cell types, suggesting that another modifier exclusive

1This work was previously published (Stothert AR et al, 2015) and is used with the permission of the publisher
to the TM participates in the proteotoxicity of myocilin. ER autophagy (reticulophagy) is one of the pathways essential for myocilin clearance that can be impacted dramatically by aging and other environmental factors such as nutrition. This review will discuss the link between myocilin and autophagy, evaluating the role of this degradation pathway in glaucoma as well as its potential as a therapeutic target.

4.2 Introduction

Aqueous humor is a watery, ionic fluid, much like plasma in the blood, produced by the ciliary body that fills the anterior chamber of the eye [89]. Unlike the vitreous humor in the vitreous chamber of the eye, aqueous humor is constantly produced; therefore, in order to maintain a homeostatic environment, it must be constantly recycled out of the eye. Regulatory aqueous outflow occurs by two pathways in the anterior chamber: the conventional pathway, comprised mainly of the TM, and the unconventional outflow pathway, comprised of drainage channels located at the angle between the ciliary muscle and the iris. The conventional pathway regulates upwards of 85% of the aqueous outflow that occurs in the anterior chamber [90].

TM is the major component of the conventional outflow pathway, comprised of TM cells, juxtacanalicular connective tissue (JCT), the inner walls of Schlemm’s canal, Schlemm’s canal, the collector channel, and finally the episcleral vein, which carries aqueous humor back to systemic circulation [91, 92]. The TM is located at the iridocorneal junction of the anterior chamber, the area where the iris forms a junction with the cornea. In addition to giving the eye its shape, aqueous humor is responsible for providing nutrients to the avascular structures of the anterior chamber, including the
lens and cornea [89, 90]. However, one of the more important functions aqueous humor provides to the microenvironment of the eye is producing IOP [89], which is largely regulated by resistance of outflow provided by the TM in the conventional outflow pathway. Studies have shown that resistance to outflow is increased with age and in ocular disorders, such as glaucoma, where IOP elevation is a pathological hallmark [90].

Glaucoma is a neurodegenerative protein misfolding disorder, characterized by retinal ganglion cell (RGC) death and optic nerve (ON) axon damage leading to progressive irreversible vision loss [31, 52-55]. Glaucoma is the second leading cause of blindness worldwide, with over 60 million individuals suffering from the disease. In the United States alone, it is estimated that 2-3 million people have glaucoma, with over 120,000 individuals suffering blindness due to the disease [31, 56, 77]. The most common forms of glaucoma include open-angle glaucoma, angle-closure glaucoma, normal-tension glaucoma, and congenital glaucoma. Although all forms of glaucoma are seen throughout the population, over 90% of cases involve a form of POAG [32, 33]. In POAG aqueous drainage channels remain exposed, but the TM network is damaged preventing proper outflow [33]. POAG arising from distinct mechanisms is often associated with accumulation of the glycoprotein myocilin, within the TM and Schlemm’s canal [22, 24, 34, 35, 41].

4.3 Normal Intracellular Myocilin Processing

Understanding the normal features of myocilin protein could help to elucidate why this protein accumulates in the glaucomatous eye. Myocilin is a ubiquitous 504
amino acid (aa) secreted glycoprotein expressed in both ocular and non-ocular tissues. In the eye, myocilin is found in the TM, sclera, ciliary body, choroid, cornea, iris, retina, and ON [93]. Outside the eye, myocilin is expressed in tissues of the peripheral nervous system [94]. Structurally, myocilin consists of an N-terminal region containing a signal peptide sequence (aa 1-32), a helix-turn-helix (aa 18-58) and a leucine zipper domain containing two coiled-coil domains (aa 74-110 and aa 118-186) responsible for coordinating protein-protein interactions [3, 77]. Myocilin is a glycoprotein that normally undergoes asparagine-linked glycosylation at aa residues 57–59 (Asn-Glu-Ser) [22, 95]. The C-terminal globular region contains the olfactomedin (OLF) domain (aa 230-504) [40, 77], an evolutionarily conserved region of amino acids first discovered in the olfactory neuroepithelium. In humans, the OLF domain is found within a group of glycosylated proteins important in the organization of the nervous system during development [34, 96]. Though myocilin is expressed throughout the body, its physiological function is yet unknown. Recent studies have suggested the importance of myocilin in oligodendrocyte differentiation and axonal myelination [97], and myocilin may play a role in retinal cell programmed death during development [98].

The secretory pathway is responsible for synthesis, folding, and delivery of a wide range of cellular proteins, including myocilin [84, 85]. This pathway is particularly important in correctly coordinating the localization of newly formed proteins to specific organelles and to the extracellular space [85]. The pathway is comprised of the rough endoplasmic reticulum (ER), ER-exit sites, ER-to-Golgi intermediate compartment (ERGIC), the Golgi complex, and post-Golgi carriers [84]. During ribosome synthesis of a polypeptide, proteins that are to be secreted are targeted by a signal recognition
particle (SRP), a molecule that targets hydrophobic peptide signal sequences on the N-terminal domain of a protein. SRPs direct the translocation of newly forming polypeptides into the ER membrane via translocons, or channels in the ER membrane, for continued translation and post-translational modifications required for secretion. Once in the ER membrane, post-translational modifications such as signal peptide cleavage, glycosylation, disulfide bond formation, and glycosidase trimming occur to ensure proper protein folding.

The cleavage of the N-terminal signal peptide sequence is a common modification for many proteins that enter the ER, including myocilin. Cleavage of this sequence occurs via signal peptidase complex (SPC), a 4-subunit polypeptide. Most of the SPC subunits are required for cell viability, except the Sec11 subunit, which contains the protease active site [84]. The SPC cleaves off the N-terminal signal peptide sequence, exposing glycosylation sites on the protein [84, 85]. Asparagine–linked (N-linked glycosylation) is a post-translational modification where oligosaccharides are attached to asparagine residues in the N-terminal domain of the protein [99]. Oligosaccharyltransferase enzyme (OST), an 8-subunit polypeptide, carries out the addition of oligosaccharides. Cells require the majority of the OST subunits for viability, though the Stt3 domain is dispensable and only required for catalytic activity. N-linked glycosylation of a protein is thought to be important in thermodynamic stability, solubility, and protein folding [99]. Glycosylated proteins in the ER membrane then form disulfide bonds at free sulfhydryl groups on cysteine residue side chains due to the oxidizing environment within the ER lumen. A specialized family of disulfide isomerases is required for the formation, reduction and isomerization of these disulfide bonds,
ensuring correct protein folding [100]. Once the signal sequence is cleaved, proteins are glycosylated and disulfide bonds form. Following this process, the final step required for proper protein folding is the trimming of glucose residues from the protein oligosaccharide core. Glucosidases (Gls1/Gls2) are enzymes that rapidly trim glucose residues, allowing for proper chaperone folding [101]. Mns1 and Htm1, enzymes that cleave mannose residues, generate signals for protein degradation by recognizing proteins that are terminally misfolded [102].

After post-translational modifications, proteins are exported to ER-exit sites, areas that have a high concentration of coat-protein complex II (COPII) [85, 103]. COPII is a molecule that forms a coat around properly folded proteins, promoting vesicle formation for Golgi transport. COPII vesicle formation is activated by the ER GTPase Sar1, which interacts with Sec proteins responsible for protein recruitment to form Sec23-24 and Sec13-31 complexes. The Sec23-24 complex is vital for protein capture and the Sec13-31 complex is necessary for COPII coat formation [103, 104]. Once COPII vesicles are formed around proteins, they can fuse with each other forming pre-Golgi intermediates [105], or fuse with already made ERGIC [106] and directed to the Golgi complex (Fig. 4.1A), where they are further modified and processed, allowing delivery to their final destination [107, 108]. Each step along this pathway represents a potential point where myocilin processing could go awry. This entire process is particularly relevant for understanding how a group of POAG cases that are caused by mutations in the secreted myocilin protein.
4.4 Myocilin Misfolding Propagates Glaucomatous Pathology

Mutations in the MYOC gene are observed in ~30% of juvenile onset glaucoma cases, and 2-4% of patients with POAG. To date, over 70 glaucoma-causing myocilin mutations have been identified, with >90% found in the C-terminal OLF domain [3]. Less than 10% of disease-causing mutations are located in the N-terminal domain. Mutations in the C-terminus of the myocilin protein result in conformational changes in the tertiary structure of the protein, namely that charged residues important for regulating cleavage and secretion are no longer solvent-exposed [2, 33, 37, 56] (Table 4.1). These mutations thus induce a toxic gain of function for the cell via myocilin protein misfolding and abnormal amyloidosis in the ER of TM cells [43]. Based on the genetic evidence, it is thought that the point mutations in the MYOC gene make the protein prone to misfolding, intracellular retention and subsequent aggregation somewhere along the secretory pathway in TM cells. This leads to ER stress, causing TM cell death, dysfunctional aqueous humor outflow, increased IOP and eventually optic nerve damage [22, 35, 37].

In addition to inherited mutations in myocilin, inhibition of post-translational modifications in WT myocilin can alter secretion leading to intracellular accumulation. Our laboratory examined this phenomenon by utilizing two pharmacological inhibitors, tunicamycin and brefeldin a. Tunicamycin is a well-documented inhibitor of N-linked glycosylation, leading to improper protein folding; Brefeldin A is an inhibitor of ER-Golgi transport. Treatment of cells overexpressing WT myocilin with Tunicamycin or Brefeldin A resulted in increased levels of intracellular myocilin [43]. Moreover, prolonged treatment with glucocorticoids can result in increased myocilin deposition within the ER
of TM cells, which has been documented to cause glaucoma pathology in humans [109-111]. We have shown that WT myocilin, similar to other secreted proteins, is typically degraded through the proteasomal-dependent ER-associated degradation (ERAD) mechanism (Fig. 4.1b) [43, 84, 85]. ERAD is a process that uses the VCP/p97 complex to typically triage misfolded proteins from the ER lumen to the cytosol for ubiquitination and subsequent proteasomal degradation [112]. But in cases where misfolded myocilin is produced, either by mutation or altered post-translational modification, this clearance mechanism fails, leading to myocilin aggregation, ER stress and TM cell toxicity. Therefore, if ERAD could be restored for these misfolded myocilin species, or another clearance mechanism could be engaged, such as autophagy, myocilin toxicity in the TM could be reduced.

4.5 Autophagic Degradation of Misfolded Proteins Promotes Cellular Homeostasis

Even in normally functioning cells, if there is a proteotoxic stress leading to accumulation of misfolded proteins in the ER, the unfolded protein response (UPR) is activated. If the activation is acute, then the UPR is beneficial to the cell, but if the activation is chronic, it can facilitate cell death [86, 113, 114]. In cases of glaucoma caused by mutations in the MYOC gene, the UPR seems to be chronically activated, suggesting that this could be a primary mechanism of TM cell death. While trying to stimulate ERAD of mutant myocilin is one approach for overcoming chronic UPR activation, one caveat of this mechanism is that ubiquitin-proteasome degradation is limited to monomeric proteins. For an amyloidogenic protein like myocilin, this simply
may never work. When the cell needs to degrade damaged organelles or aggregated proteins, a less specific but more robust ER autophagic mechanism of clearance (reticulophagy) is available. Engaging this system may be much more efficient for facilitating removal of myocilin aggregates from the ER [115].

**Autophagy** is a general term used for the process involving degradation by lysosome-containing vesicles. There are at least three types of autophagy, differing by their means of delivering material to the lysosome for degradation: macroautophagy, microautophagy, and chaperone-mediated autophagy [116, 117]. During chaperone-mediated autophagy, substrates are recognized by a particular amino acid peptide sequence and are translocated into the lumen of a lysosome for degradation [28]. Microautophagy is a less specific process where the lysosomal membrane invaginates or sequesters material in the cytosol for degradation within the lysosome [118]. In addition, organelles such as the mitochondria and the ER can degrade their protein content via distinct routes that ultimately utilize macroautophagy [119].

Macroautophagy is a catabolic process necessary for cell homeostasis [53, 117, 120]. It is upregulated when cells require intracellular nutrients and energy such as during starvation, growth factor withdrawal, or in the context of high-energy demand [95]. Additionally, macroautophagy can be upregulated in times of cellular proteotoxic stress, when misfolded proteins accumulate. Macroautophagy is a multi-step process consisting of four stages: initiation, nucleation, maturation, and degradation. Initiation occurs by the down-regulation of the mammalian target of rapamycin (mTOR) pathway [116]. In times of starvation autophagy-related protein 13 (Atg13) and unc51-like autophagy activating kinase (Ulk1/2) are upregulated leading to the inhibition of the
mTOR pathway. Conversely, when growth factors, such as insulin-like growth factor, bind to growth factor receptors, initiation of receptor tyrosine kinase signaling and subsequent activation of protein kinase B (AKT) occurs. AKT activation inhibits the major inhibitory proteins of the mTOR pathway, tuberous sclerosis 1 and 2 (TSC1/2), resulting in the activation of the mTOR pathway and subsequent inhibition of macroautophagy [121]. Additionally, mTOR inhibition leads to ULK1 phosphorylation and activation of autophagic effectors Beclin1 and phosphatidylinositol 3-kinase (Vps34) [95].

Following initiation, nucleation is the process in which the autophagosome, the unique organelle associated with macroautophagy, forms. Phosphorylated Beclin1/Vps34, along with p150, form a stable complex that initiates formation of the, pre-autophagosome, or phagophore [121]. The phagophore matures into the autophagosome and fully surrounds the target cytosolic components. This process involves the elongation of the phagophore membrane via the incorporation of microtubule-associated protein 1A/1B-light chain 3 (LC3) into the membrane. LC3 mediates autophagosome growth and cytoplasmic cargo recruitment, and provides a scaffold to transport fully formed autophagosomes to the lysosomes. Once the autophagosome has fully formed around cytoplasmic constituents, LC3 mediates dynein-dependent microtubule trafficking to lysosomes. Finally, the autophagosome fuses with the lysosome via the SNARE proteins VAMP8, Vti1B, and Lamp2, and results in the formation of the autolysosome. Following autolysosome formation, the autophagic cargo is degraded and then recycled back to the cytosol for cell re-uptake [95, 121].
Until recently macroautophagy was thought of as a non-selective degradation pathway, but current research has proposed the idea of organelle-specific autophagy, including ribophagy (ribosomes), mitophagy (mitochondria) and reticulophagy (ER). While specific details on individual organelle autophagy remain largely uncharacterized, it is postulated that cellular constituents are targeted for these distinct autophagic pathways by Atg upregulation leading to autophagosome formation or possibly by accumulation of intra-ER ubiquitinated proteins [119]. The latter is supported by work from our laboratory showing that only mutant/misfolded myocilin becomes ubiquitinated within the ER following treatment with epoxomicin, a proteasome inhibitor [43]. In this way, the accumulation of misfolded, ubiquitinated myocilin could activate reticulophagic degradation.

4.6 TM Autophagic Dysregulation Promotes Ocular Disorders

Studies have shown that increasing macroautophagy can delay the aging process, and extend tissue longevity. This was first observed in the C.elegans model, where inhibition of the insulin-like growth factor receptor induced autophagy [122]. Conversely, mutation of Atg genes resulted in decreased tissue longevity [122]. Moreover, age-related reductions in Beclin1 [123], Sirtuin1 (A modulator of mTOR inhibition)[124], and Atg5/7 [125] protein levels have been observed in aged tissue. Additionally, decreased levels of ULK1 and LC3 have been observed in aged tissue. As glaucoma is a slowly progressing ocular disorder and pathology increases are associated with aging, it is possible this could largely be an effect of reduced autophagic protein clearance in TM cells.
Studies in primary human TM (hTM) cells have also shown that during glaucoma, the autophagic mechanism in TM cells is dysregulated. Recent research has indicated a dysregulation of the autophagic pathway and reduction of the autophagic response to oxidative stress in TM cells isolated from patients with glaucoma [53]. Interestingly, this research determined an overall reduction of LC3 in TM cells of glaucoma patients compared to controls [53]. Additionally, there was an increase in senescence-associated-β-galactosidase (SA-β-Gal) activity, which is shown to be upregulated when autophagy is inhibited in primary human fibroblasts [126]. Moreover, they observed an increase in lipofuscin, or non-degradable lysosomal content, in TM cells of glaucoma patients compared to control cells [53]. Other proteins associated with autophagy found to be down regulated during glaucoma were sequestosome-1 (p62), a scaffold that targets ubiquitinated proteins for autophagic degradation, scCTSB, a lysosomal protein, and LC3B-II [53]. Taken together, these data show that autophagic clearance mechanisms are dysfunctional in TM tissue of glaucoma patients. Thus, restoring these pathways could be beneficial in disease.

4.7 Grp94 Prevents Misfolded Myocilin Degradation via Autophagy

ER homeostasis, including ERAD and reticulophagy pathways, is mediated by chaperone activity. Chaperones aid in the proper folding of proteins under physiological conditions as well as in times of ER stress [28, 127, 128]. Stress caused by accumulation of terminally misfolded proteins activates the UPR, resulting in cessation of protein translation [113, 127] and increased production of molecular chaperones [114]. We recently performed a small-interfering RNA (siRNA) screen on several ER-
associated chaperones to examine their effects on overexpressed I477N mutant myocilin or WT myocilin in Hek293T cells [43]. Interestingly, only knockdown of glucose-regulated protein 94 (Grp94), the ER-specific isoform of Hsp90, caused intracellular I477N mutant myocilin reduction. Under physiological conditions, Grp94 is inactive [129]. During ER stress, Grp94 is associated with ER quality control and aids in the proper folding of misfolded proteins. Grp94 also associates with the ERAD-associated osteosarcoma amplified 9 (OS-9) protein and acts to shuttle terminally misfolded monomeric protein to the ER membrane for subsequent proteasomal degradation [129, 130]

This intracellular reduction was also observed in cells overexpressing four other common glaucoma-associated myocilin mutations [54]. Interestingly, Grp94 knockdown had no effect on intracellular or secreted levels of WT myocilin. Moreover, following myocilin immunoprecipitation (IP), we found that Grp94 associated with mutant protein but not WT myocilin [43], highlighting the specificity offered by this chaperone. Using a cycloheximide (a potent inhibitor of protein translation) chase experiment, we were able to determine that the half-life of intracellular mutant myocilin was decreased after Grp94 inhibition. Additionally, we observed an increase in high-molecular weight myocilin species with Grp94 overexpression, and conversely, reduction in high-molecular weight myocilin species after Grp94 knockdown [43]. This led us to believe that Grp94 was preserving mutant myocilin, and possibly driving protein aggregation.

Importantly, Grp94 inhibition also reduced intracellular mutant myocilin levels, as well as cellular toxicity associated with mutant myocilin overexpression, in primary hTMs [54]. Interestingly, Grp94 inhibition had no effect on normally folded WT myocilin levels.
However, when N-linked glycosylation of WT myocilin was blocked by tunicamycin, we observed an increased association with WT myocilin and Grp94. This was accompanied by sensitization to Grp94 inhibition [43]. Moreover, Grp94 could stimulate aggregation of WT myocilin OLFs in vitro, a process that could be mitigated by the addition of Grp94 inhibitor [54]. These data led us to the conclusion that Grp94 recognizes misfolded myocilin species within the ER, but iteratively attempts to triage the protein through ERAD unsuccessfully. This leads to myocilin protein aggregation in the ER and subsequent TM cell toxicity (Fig. 4.2).

To explore the feasibility of Grp94 inhibition as a potential therapeutic mechanism, one first must examine the importance of Grp94 in an organism. Grp94, as a molecular chaperone of the ER, has relatively few client proteins [44]. Interestingly, despite this Grp94 is necessary for embryonic development. This has been shown in studies looking at both heterozygous (+/-) and homozygous (-/-) knockdown of Grp94 in murine models and of gp93 in drosophila [131]. While the -/- mice have lethality and a number of other functional deficits, +/- mice survive and seem healthy, suggesting that partial but not complete depletion of Grp94 may be a viable therapeutic strategy [132]. Moreover, recent studies have shown that targeted knockout in discreet tissues does not impact viability [86]. However, Grp94 does play important roles in immune cell and liver function [133, 134], and others, suggesting that caution will be necessary moving forward with therapeutic approaches targeting this protein.

While previous data suggests that the inhibition of Grp94 association with misfolded or mutant myocilin leads to intracellular degradation of the myocilin protein and therefore could be a potential therapeutic target to treat glaucoma, the mechanism
by which degradation of this protein occurs was still not known. To determine if the 
autophagic pathway was involved in misfolded myocilin protein clearance, cells 
overexpressing mutant myocilin (I477N) were co-transfected with Grp94 siRNA and 
either Lamp2 or Beclin1 siRNA, two well-characterized components of the autophagy 
pathway. Indeed the addition of Lamp2 or Beclin1 siRNA abrogated intracellular 
myocilin clearance following Grp94 knockdown [43]. Thus, inhibiting Grp94 shunts 
misfolded myocilin towards the autophagic degradation pathway, a pathway that must 
be functional for mutant myocilin turnover (Fig. 4.3). In fact, since TM cell dysfunction 
prevents normal aqueous humor outflow, nutrient delivery to these cells is impaired, 
which would normally trigger macroautophagy and possibly facilitate misfolded myocilin 
clearance, restoring balance to the outflow pathway. But, because of the interaction of 
Grp94 with myocilin, its autophagic degradation is prevented, allowing glaucomatous 
pathology to perpetuate. Therefore, discovering therapeutic measures to enhance the 
ER autophagic mechanism, allowing for misfolded myocilin to be degraded, could lead 
to restoration of TM cell function and reduction in glaucomatous pathology. In fact, 
perhaps combining Grp94 inhibition with enhancers of reticulophagy could work 
together synergistically to promote myocilin clearance and rescue glaucoma caused by 
myocilin toxicity.
Table 4.1 List of known mutations in the MYOC gene that lead to glaucoma. Bold denotes mutations in the N-terminus; Underline denotes mutations in the C-terminus.

<table>
<thead>
<tr>
<th>Mutation (AA)</th>
<th>Gene Location</th>
<th>Ref</th>
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<tbody>
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Figure 4.1 Normal intracellular processing of myocilin. After signal recognition particle (SRP) targeting and translocation of a newly forming myocilin polypeptide to the endoplasmic reticulum lumen, posttranslational modifications occur to ensure proper folding of the protein. If these modifications are successful (A), the newly formed, properly folded, protein will be sent to the Golgi apparatus via COPII coat formation for packaging and delivery to specific cellular organelles or the extracellular space. However, if these modifications do not properly occur (B), and the protein becomes terminally misfolded, the intra-ER degradation mechanism, known as ERAD, will degrade the protein through a proteasomal dependent manner for peptide recycling. This method requires the ubiquitination of misfolded proteins, and is only substantial if the misfolded protein is monomeric.
Figure 4.2 Grp94 association with mutant myocilin promotes intracellular accumulation. Mutations in the MYOC gene lead to the production of myocilin protein that readily misfolds and is prone to aggregation. Grp94 is an ER-associated co-chaperone that recognizes pre-aggregated misfolded myocilin, preventing ERAD proteasomal degradation, leading to aggregation within the ER. Proteotoxicity caused by aggregated myocilin in the ER leads to TM cell death and aqueous outflow dysfunction, promoting the pathologies associated with glaucoma.
Figure 4.3 Inhibition of Grp94 interaction with mutant myocilin promotes autophagic degradation. Through siRNA knockdown or pharmacological inhibition, Grp94 association with misfolded myocilin can be prevented, inhibiting protein aggregation. After inhibition, misfolded myocilin can be processed into transport vesicles for transport out of the ER lumen to the cell cytosol, where these vesicles fuse with lysosomes promoting autophagic degradation of the misfolded protein. As aggregated misfolded myocilin is cleared from the ER of TM cells, aqueous outflow becomes restored.
Chapter Five: Final Considerations

This study has demonstrated that intra-ER misfolded myocilin mechanics can be altered by the chaperone, Grp94. Specifically, Grp94 recognition of terminally misfolded myocilin prevents ERAD clearance promoting protein accumulation precipitating cellular toxicity. Through pharmacological inhibition, misfolded myocilin clearance and degradation can occur, albeit through the robust autophagic pathway. The major findings of this study demonstrate how the alteration of Grp94 interaction with mutant myocilin can lead to 1) reduced intracellular myocilin accumulation, 2) reduced TM cellular toxicity, 3) promotion of the autophagic clearance mechanism, and 4) pathological rescue of glaucoma phenotypes. We have clearly shown that Grp94 association with misfolded myocilin in the ER of TM cells is a major contributor to myocilin accumulation causing glaucomatous pathologies. This study supports the possibility of developing novel therapeutics inhibiting Grp94’s association with misfolded myocilin as a viable treatment option for POAG.

For quite some time, chaperone modulation has been a therapeutic target for multiple diseases including, Alzheimer’s disease [66, 144], Parkinson’s disease [145, 146], and Cancer [78, 147]. Specifically, Grp94 inhibition has been previously studied as a potential target for cancer treatment [44, 148]. However, until our work outlined in this manuscript, Grp94 was not considered a target for potential glaucoma treatment. In fact, until our work, Grp94 was not known to be associated with myocilin in cases of myocilin-associated glaucoma. With our previously discussed work, we have clearly shown the
importance of Grp94 associating in progressing myocilin-associated glaucoma pathology, as well as discovered a novel target for therapeutic intervention. Moreover, we have shown that topical administration of a Grp94 inhibitor can alleviate pathologies associated with myocilin-induced glaucoma. Development of new Grp94 inhibitors that are more potent at lower concentrations, as well as have greater anterior chamber bioavailability when applied topically, would be vital in progressing this research forward into higher organisms, including humans. These studies support the idea of specific therapeutic intervention targeting the underlying glaucoma disease mechanism as a viable means for not only reducing symptoms, but curing the disease all together.

Clear limitations arise in our approach as currently the underlying disease mechanism of glaucoma is still unknown. While our research has shown a direct link to Grp94 association with misfolded myocilin as a mediating factor of myocilin accumulation and toxicity, the specific disease mechanism must be discovered to fully understand potential therapeutic targets. Additionally, while topical administration of ophthalmic drugs continue to be the gold standard in treating various ailments of the eye, more direct drug delivery may be necessary to reduce unwanted off target effects. Previous clinical trials studying the effects of Hsp90 modulation in the treatment of cancer and other diseases have shown promise, however, off-site toxicities have prevented research from advancing [44]. Additionally, while a majority of glaucoma research centers on myocilin accumulation in the ER of TM cells, the biological relevance of myocilin in the human body still remains unknown. While groups have postulated myocilin’s importance in cell proliferation, peripheral axon myelination, oligodendrocyte differentiation, and optic nerve myelination, its specific function remains
relatively unknown [94, 97, 149]. Therefore, specifically targeting accumulated myocilin in the ER of TM cells would be necessary to prevent off-target toxicities from systemic or broad administration.

Finally, while we have shown promising results in targeting Grp94 as a means for reducing POAG pathology, other forms of glaucoma, including closed-angle and steroid induced glaucoma, remain without a viable curative treatment option. Next steps in our research will center on determining to what extent, if any, myocilin accumulation plays in the development of other subtypes of glaucoma. Determining if myocilin is directly responsible for multiple glaucoma subtypes would allow us to progress our research, determining if Grp94 inhibition is a viable treatment method for all glaucomas. Additionally, determining the specific mechanism of misfolded myocilin degradation following Grp94 inhibition would be imperative in advancing this work into clinical trials. While we have shown evidence linking autophagic degradation to misfolded myocilin clearance, the specific mechanism, and proteins involved are still unknown. Determining the specific mechanism of degradation could also lead to new targets for potential therapeutics. While this research is still in its infancy, promising results could lead to the first curative therapeutic for treating POAG.

Overall our findings suggest there is a direct relationship between Grp94 and misfolded myocilin, which leads to pathologies associated with POAG. Through specific pharmacological inhibition, we were able to alleviate myocilin-associated glaucoma pathology, while promoting misfolded myocilin clearance. This work validates Grp94 as a potential therapeutic target for treatment of myocilin-associated POAG.
References


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11200 Rockville Pike, Rockville, MD
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Appendix A2: Human Molecular Genetics
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Appendix A3: Experimental Eye Research
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Appendix B:

Approved IACUC Protocols

MEMORANDUM

TO: Chad Dickey, Ph.D.

FROM: Forah Mouli, MSPH, IACUC Coordinator
Institutional Animal Care & Use Committee
Research Integrity & Compliance

DATE: 8/7/2015

PROJECT TITLE: Grp94-selective inhibitors to treat hereditary glaucoma

FUNDING SOURCE: USF department, institute, center, etc.

IACUC PROTOCOL #: MI-00000318

PROTOCOL STATUS: APPROVED

Your request for continuation of this study was received and will be reported to the Institutional Animal Care and Use Committee (IACUC). The IACUC acknowledges that this study is currently ongoing as previously approved. Please be advised that continuation of this study is in effect for a one-year period beginning 8/27/2015.

Please take note of the following:

• IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol through the eIACUC system. After these years all continuing studies must be completely re-described in a new electronic application and submitted to IACUC for review.

• All modifications to the IACUC Approved Protocol must be approved by the IACUC prior to initiating the modification. Modifications can be submitted to the IACUC for review and approval as an Amendment or Procedural Change through the eIACUC system. These changes must be within the scope of the original research hypothesis, involve the original species and justify in writing. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application.
MEMORANDUM

TO: Chad Dickey, Ph.D.

FROM: Farah Moulia, MSPH, IACUC Coordinator
Institutional Animal Care & Use Committee
Research Integrity & Compliance

DATE: 3/8/2016

PROJECT TITLE: Gpr14-selective inhibitors to treat hereditary glaucoma

FUNDING SOURCE: Federal government or major agency that awards grants based on peer-reviewed proposals (NIH, NSF, DOD, AHA, ACS, etc.)
National Eye Institute

IACUC PROTOCOL #: R 150000067
PROTOCOL STATUS: APPROVED

Your request for continuation of this study was reviewed and will be reported to the Institutional Animal Care and Use Committee (IACUC). The IACUC acknowledges that this study is currently ongoing as previously approved. Please be advised that continuation of this study is in effect for a one-year period beginning 6/4/2016.

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- All modifications to the [IACUC-Approved Protocol] must be approved by the [IACUC] prior to initiating the modification. Modifications can be submitted to the [IACUC] for review and approval as an Amendment or Procedural Change through the eIACUC system. These changes must be within the scope of the original research hypothesis, involve the original species and method defined in writing. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application.
Appendix C:

**Tg-MYOC\textsuperscript{Y437H} Mouse Breeding Schematic**

- **P1**
  - Tg-MYOC\textsuperscript{Y437H}
  - C57BL/6 x SJL/J
  - Male
  - WT (Jax)
  - C57BL/6
  - Female

- **F1**
  - 50% HET
  - 50% WT (non)

Discard WT littermates, use male HET for breeding.

- **F1 Generation**
  - Male
  - WT (Jax)
  - C57BL/6
  - Female

- **F2**

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<td>HET (Tg)</td>
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<td>WT</td>
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Use F2 generation and beyond for cohorts. HET (Tg) and WT (non) littermates are used for cohorts. Continued breeding is carried out with HET males, and WT females (Jackson Laboratories).