INVESTIGATIONS OF SIGMA-1 RECEPTOR AS A NOVEL THERAPEUTIC TARGET FOR GLAUCOMA IN PRECLINICAL MODELS

PhD thesis

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1. INTRODUCTION

Glaucoma is a chronic progressive degenerative optic neuropathy that is the leading causes of irreversible blindness worldwide. Glaucomatous optic neuropathies are associated with characteristic structural deformation at the optic nerve head that may result in progressive retinal ganglion cell (RGC) death and visual field defects. The estimated total population of this sight-threatening eye disease in the world was about 76 million in 2020 and was predicted to increase to 111.8 million in 2040. The worldwide age-standardised prevalence of glaucoma in the population aged 40 years or older is about 3.54%, highest in Africa with 4.79%. Primary open-angle glaucoma (POAG) is the topmost prominent form of glaucoma and its major risk factor is elevated intraocular pressure (IOP), even though other pathogenetic processes may also be involved in the development. In fact, increased IOP is currently the unique modifiable risk factor for glaucoma. At present, the only approach affirmed to be effective to slow the disease progression, sustain visual field and prevent further vision loss is lowering IOP.

IOP homeostasis is maintained by the equilibrium of secretion and drainage of aqueous humor (AH), either through trabecular meshwork (TM) (conventional pathway) or uveoscleral outflow pathway. Most of the AH passes through the TM, traverses Schlemm's canal (SC), collector channels, and finally empties it into episcleral veins. In this passageway, outflow resistance is generated, controlled, and responsible for homeostatic IOP regulation. Impairment of the cellular properties and function in the conventional outflow pathway, and particularly the TM, results in increased outflow resistance that leads to IOP elevation, characteristic of most types of POAG. Currently, the glaucoma therapeutic medications either decrease the inflow of AH (the production of AH at ciliary body) or increase the outflow through the two drainage pathways. The majority of current glaucoma medications that facilitate AH outflow mainly target the uveoscleral drainage which is responsible for only 10 to 15% of total AH egress and even less with age, although POAG prevalence considerably increases with age. Thus,

the other approach, targeting the conventional pathway, seems an attractive therapeutic strategy.

Recent researches report that progressive fibrosis of the tissues on the main outflow, especially the TM, is responsible for most of increased outflow resistance and IOP elevation that results in glaucoma. An increased understanding of the molecular mechanisms for TM malfunction and fibrosis is promising and beneficial for developing therapeutics for this relentlessly progressive disease.

Sigma-1 receptor (S1R), a ligand-operated chaperone protein, is expressed in numerous organs with broad spectrum of biological functions and as a promising target for treating many medical conditions. In addition to the well-documented neuroprotective effects on the central nervous system and the retina, the role of S1R in various other diseases, including cancer, cardiovascular, renal, pulmonary diseases, and COVID-19 have also been reported. S1R has been identified in the TM, however, its subcellular localization in TM cells is still unanswered. There is no evidence about the effect of S1R on TM fibrosis which is associated with elevated IOP, a crucial role in pathogenesis of glaucoma.

Our group's recent patent directed at the use of S1R agonists in the treatment of progressive fibrosis of the kidney and lung has been approved in many countries (the US, EU, and others). Its anti-fibrotic effect in the lung and the heart was also reported. Our group has also published that fluvoxamine (FLU), a specific S1R agonist, triggers the nitric oxide (NO) production in rat kidney, contributing to the vasodilation of peritubular capillary, results in a better blood supply and a milder ischemic injury. In the ocular anterior segment (AS), NO is synthesized endogenously by NO synthases in the ciliary muscle and the outflow tissues, where it takes part in the regulation of IOP. Recently, NO has emerged as a potential novel candidate for IOP lowering in glaucoma treatment. However, there is no evidence of the effect of S1R on NO level in the TM.

Therefore, it is postulated that S1R agonists could have similar effects in the eye. The potential effect of S1R stimulation on TM fibrosis or NO release has not been

elucidated yet. To the best of our knowledge, beside the unique article which revealed that S1R agonist (+)- pentazocine protects TM cells from pressure-induced apoptosis, ours are the first studies investigating the role of S1R on TM cells.

Transforming growth factor-beta2 (TGFβ2) and platelet-derived growth factor (PDGF) are two well-documented fibrotic inducers. TGFβ2, the most abundant TGFβ isoform in the eye, is known to be strongly associated with the development of elevated IOP and POAG. TGFβ2 also plays a key role in glucocorticoids (GCs)-induced ocular hypertension in mice model. Despite GCs-induced glaucoma is a type of secondary iatrogenic open-angle glaucoma, its clinical characteristics are similar to POAG in many ways, including elevated aqueous outflow resistance and morphological and biochemical alterations of the TM. Thus, this glaucomatous model has been used widely in preclinical studies. PDGF has been involved in eye fibrosis, particularly in age-related macular degeneration or proliferative diabetic retinopathy. However, the involvement of PDGF in TM fibrosis is still unclear.

The overall focus of this dissertation is to investigate the novel glaucoma treatments that target the conventional AH outflow route. Thus, this dissertation subjected to the anterior part of the eye, particularly the TM. The posterior part, including the retinal tissues, will not be discussed here.

2. OBJECTIVES

Our research has focused on the investigation of S1R as a novel therapeutic target for glaucoma. To conduct the experiments, I adopted several new methods and experimental models, refining and optimizing them through co-working with my supervisor. The most important established methods were the isolation of primary mouse trabecular meshwork (pMsTM) cells and the dexamethasone-21-acetate (DexAc)-induced ocular hypertensive model in mice.

In our preclinical experiments, we evaluated the *in vitro* effect of FLU using TGFβ2- or PDGF- induced TM models, and explored the IOP lowering effect of FLU eye drop *in vivo* in DexAc-induced mice model.

The following objectives have been set to fulfil the aims:

To assess the effect of S1R agonist FLU on human TM (HTM5) cells

1. Determine cellular localization of S1R in HTM5 cells
2. Study the effect of FLU on fibrotic changes of HTM5 cells
3. Evaluate the stimulating effect of FLU on NO release of HTM5 cells

To assess the effect of S1R activation on pMsTM cells

1. Detect the presence of S1R in pMsTM cells
2. Explore the protective effect of S1R on PDGF-induced pMsTM cells

To assess the IOP lowering effect of FLU on DexAc-induced ocular hypertensive mice model

1. Examine the irritating effect of FLU eye drop on the mice eyes
2. Investigate the protective effect of S1R on this glaucomatous model

3. METHODS

3.1. Assessment the effect of S1R agonist FLU on HTM5 and pMsTM cells

HTM5 cells were treated with pro-fibrotic factors in the presence or absence of S1R agonists FLU. Fibrotic-like changes and NO level were investigated and the effect of FLU was shown (Figure 1, lower panel).

Besides TM cell line, we isolated pMsTM cells from C57BL/6J and S1R$^{-/-}$ mice on a pure C57BL/6J background with positive magnetic bead selection (Figure 1, upper panel). PDGF-induced actin remodelling and alpha smooth muscle actin (αSMA) expression were examined.

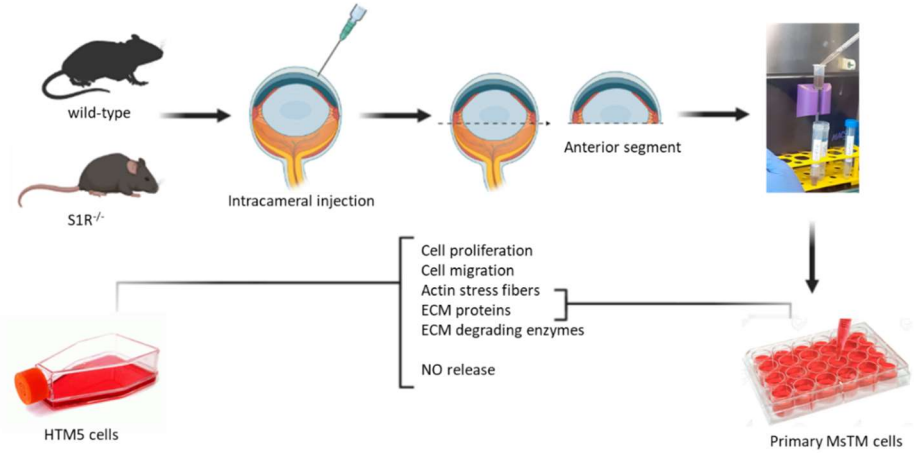

Figure 1. Brief illustration of the experiments with human trabecular meshwork cells (HTM5) and primary mouse trabecular meshwork cells (pMsTM).

3.2. Assessment the effect of S1R on DexAc-induced ocular hypertension in mice model

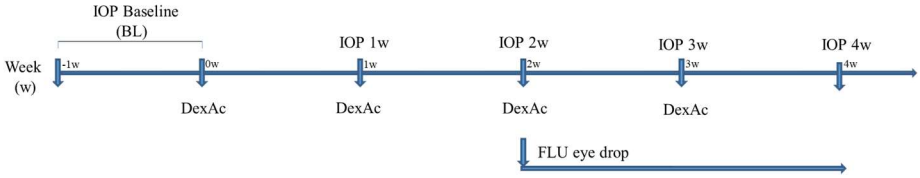

Figure 2. Treatment timeline of the animal experiment. Timeline showing which date intraocular pressure (IOP) measurements were taken and when the first dexamethasone-21-acetate (DexAc) was injected into the eyes (week 0) or the first fluvoxamine (FLU) eye drop was instilled to the eyes (week 2) of wild-type or S1R knockout mice.

Male and female C57BL/6J mice were purchased from Animalab (Budapest, Hungary). The first pair of $S1R^{-/-}$ mice were kindly gifted from Dr. Adrian Y.C.Wong (University of Ottawa, Japan). All animals were bred and housed in the animal facilities of the Pediatric Center at Semmelweis University. The mice were kept in 12-hour light/12-hour dark cycle in constant temperature (22 ± 2°C) with food and water were supplied *ad libitum*. All animal studies were conducted in accordance with the Animal Experiment Regulations of the Animal Welfare Committee of Semmelweis University (PE/EA/916-7/2020), Budapest, Hungary.

The animals were three to four months old at the first injection. Mice were anesthetized intraperitoneally with 90mg/10mg/bwkg of ketamine/xylazine and topical anesthetized with Oxybuprocaine hydrochloride 0.4% w/v (Novesine, OmniVision GmbH). After measuring IOP non-invasively using an Icare Tonolab, 20 µL of dexamethasone-21-acetate (DexAc) or vehicle injection was performed periocularly under the conjunctival fornix by a 32-gauge needle with a Hamilton glass microsyringe (25-µL volume, Hamilton Company, Reno, NV) of both eyes weekly. The duration of the experiments depends on the case (4-5 weeks).

To investigate the IOP lowering effect of FLU eye drop, after two weeks of DexAc injection, eye drop administration began and lasted for 14 days (Figure 2). Micro pipette was used to apply 7 µL of FLU 100 mM or vehicle eye drops topically to both eyes of each animal (one mouse received either FLU or vehicle eye drop to both eyes) twice daily.

3.3. Statistical analysis

Individual sample sizes are specified in each figure legends. Statistical analyses were performed using Prism version 8.0 (GraphPad, USA). Differences among groups were evaluated *via* one-way analysis of variance (ANOVA), and two groups' comparisons were done using Student's *t-test.* Data were presented as mean ± SEM, or mean ± SD, or mean with error, depended on each analysis and described in each figure; $p < 0.05$ was considered as statistical significance.

4. RESULTS

4.1. S1R activation is protective in HTM5 cells

TM cells were characterized by basic, specific morphology and the accelerated expressions of myocilin, αSMA, and fibronectin with dexamethasone induction.

To my knowledge, our group are the first research group who reported that S1R localizes mainly in the endoplasmic reticulum (ER) and is also found in a lesser amount in the cytoplasm of HTM5 cells, due to co-localization of S1R and Grp94.

Two major fibrotic inducers (PDGF and TGFβ2) were used to activate HTM5 cells.

Methyl-Thiazolyldiphenyl-Tetrazolium bromide (MTT) assay and Lactate Dehydrogenase (LDH) assay were applied to test cell viability and toxicity in response to different FLU concentration for 24 hours. The same MTT assay was used to determine the minimum effective concentration of PDGF and TGFβ2 on HTM5 cell growth. All concentrations of FLU tested did not exhibit any toxicity to HTM5 cells. Both PDGF and TGFβ2 modulation resulted in a dose-dependent cell proliferation, with a plateau at 20 ng/mL or a peak at 10 ng/mL, respectively, which are concentrations for further use. 10 μM FLU attenuated HTM5 cell proliferation induced by both cytokine stimuli.

Migration assay or scratch assay was performed in HTM5 cells upon PDGF treatment with and without FLU. By periodic monitoring, every 24 hours, cell-free area was evaluated and compared to this at 0 hour, right after the scratch was made. The results revealed that the effect of PDGF and FLU can be observed obviously after 72 hours. Microscopic images showed that, after 72 hours, accelerated cell movement is observed upon PDGF, while FLU inhibits cell migration.

The next aspect of fibrosis was examined in our project is F-actin reorganization. As actin cytoskeleton is critical not only for maintaining the shape and structure of the cells, but also for a broad variety of functions like cell migration and

division, and so on. Various changes has been described in glaucomatous eyes, actin stress fiber modification is among the most prominent alterations. They lead to the changes in TM stiffness and TM contractility, which in turn result in increased outflow resistance. Both growth factors increased the cell number and cell morphological changes of HTM5 cells as shown in differential interference contrast (DIC) images. All these alterations were prevented by co-treatment with FLU. In parallel, visualization of F-actin with Phalloidin staining and the fluorescence integrated density evaluation revealed a remarked elevated actin stress fiber level with both inducers. Similarly, treatment with FLU demonstrated a prominently less fluorescence and less actin clump and bundle formation. Interestingly, we also provide more evidence that reinforces the protective role of S1R. NE100 (N,N-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)-phenyl]-ethylamine monohydrochloride), a S1R antagonist, reversed the effect of FLU on HTM5 cells as shown on DIC images (cell density and morphology) and fluorescence staining (F-actin).

In the next experiment, we investigated the effect of FLU on extracellular matrix (ECM) deposition that is strongly associated with cytoskeletal remodeling and is a major characteristic in fibrosis. Fibronectin, an important ECM element, considerably increased in response to both growth factors in HTM5 cells. Other crucial ECM proteins, collagen 1a1 and collagen IV also increased in PDGF and TGFβ2-treated HTM5 cells, respectively. In line with the literature, TGFβ2 induced the expression of CTGF, an important modulator of fibrosis, in HTM5 cells. All these cytokines-induced elevated ECM protein levels were suppressed significantly by FLU, except for col1a1 (p value=0.3), which may be due to the small *n* number.

Furthermore, ECM deposition can result from the decreased ECM degrading proteolytic enzymes. Representative western blot showed that TGFβ2 decreases matrix metalloproteinase-2 (MMP2) level. FLU stimulated the expression of two ECM breakdown enzymes. The first one, MMP2 is a member of the MMPs family that is known to be responsible for the degradation of ECM. FLU suspended TGFβ2-induced

suppression of MMP2. The other one, cathepsin K (CTSK) is a lysosomal proteinase, and recently reported to be strongly associated to IOP homeostasis. Immunofluorescence images showed that FLU increases CTSK level in PDGF-treated HTM5 cells.

Collectively, we demonstrated the protective role of FLU against fibrogenic stimuli-induced fibrotic changes in TM cells. To the best of our knowledge, ours is the first study to report the role of FLU in the glaucomatous fibrotic-like changes of TM cells.

Aside from providing evidence of the anti-fibrotic effect of FLU on TM cells, our work presents the novel discovery of the stimulation effect of FLU on NO release in human TM cells. NO is a potent mediator of IOP by facilitating TM outflow and relaxing the TM. In the posterior eye structures, NO also exhibited protective effects in optic nerve and ocular blood vessels. In this project, we explored that FLU increases endogenous NO level in PDGF-treated HTM5 cells.

To provide more evidence about the effect of S1R, on the next step, we conducted the experiments on primary TM cells isolated from WT and $S1R^{-/-}$ mice.

4.2. S1R is protective in pMsTM cells

Based on the specific phagocytosis ability of TM cells, we isolated primary TM cells from C57BL/6J mice following magnetic bead intracameral injection. Immunofluorescence images also revealed the presence of S1R in this cell strain.

Similar to HTM5 cells, pMsTM cells also illustrated elevated actin cytoskeletal remodeling in response to PDGF. This elevation exists in both WT and $S1R^{-/-}$ mice. More importantly, actin clumps and bundles were denser in $S1R^{-/-}$ cells, indicating a protective effect of S1R. As we expected, FLU reduced PDGF-mediated F-actin enhancement on WT pMsTM cells. Interestingly, FLU did not show this protective effect on $S1R^{-/-}$ cells. This again confirms the role of S1R and the effect of FLU due to S1R activation.

In addition to F-actin, αSMA, a key element in fibrosis, was also examined in WT and S1R$^{-/-}$ pMsTM cells. Similarly, PDGF increased the level of αSMA on both kinds of cells. Furthermore, S1R loss led to a higher response compared to WT cells. This, again, emphasizes the involvement of S1R in fibrotic-like changes.

All these findings in pMsTM cells, together with those in HTM5 cells reinforce the protective role of S1R in TM fibrosis, a major causative effect in outflow resistance and IOP elevation.

4.3. S1R effectively lowers IOP in DexAc-induced ocular hypertensive mice model

Prior to investigation the effect of the agonist FLU eye drop and exploration if its molecular mechanism of action due to TM function regulation, we confirmed that S1R expresses in the mouse TM. Confocal immunofluorescence microscope images and western blot illustrated the abundant presence of S1R in TM region of WT C57BL/6J mice. As predicted, S1R has not been detected at all in the AS of S1R$^{-/-}$ mice.

To test the safety of FLU eye drop before using it in our model, the FLU active compound was formulated in various ways and applied 7 µL for each eye, twice daily. The eyes were checked regularly and every other day slit lamp images were taken. Fluorescein tests were performed to detect corneal epithelial cells' damage. We found a well-tolerated and non-irritant FLU eye drop which then used in our study.

We successfully set up DexAc-induced ocular hypertensive mice model. In DexAc-injected groups of WT, IOP increased from baseline 16.83 ± 1.19 to 18.07 ± 1.54 mmHg (+7.37%; $p<0.01$, n=25 mice/group) after 1 week, and then to 18.61 ± 1.91 mmHg (+11.37% *vs* BL; $p<0.01$, n=12 mice) after 4 weeks. Similarly, in S1R$^{-/-}$ mice, IOP of DexAc-injected group increased from baseline 16.51 ± 1.69 to 17.55 ± 1.50 mmHg (+6.30%; $p<0.05$, n=29 mice/group) after 1 week and then to 19.13 ± 1.61 mmHg (+17.72%; $p<0.001$ *vs* BL, n=14 mice, Mann-Whitney test) after 4

weeks. IOP values fluctuated non-significantly in vehicle-injected groups of both WT and $S1R^{-/-}$ mice.

In WT mice, FLU eye drop lowered DexAc-induced increased IOP after 2 weeks of instillation. Obviously, at 4 week, IOP in DexAc-injected group instilled with FLU eye drop was 16.77 ± 1.12 mmHg, significantly lower than DexAc-injected group instilled with vehicle eye drop (18.61 ± 1.91 mmHg) (-9.89%; $p<0.01$, n=12 mice/group)

Comparatively, FLU eye drop did not decrease DexAc-induced elevation of IOP in $S1R^{-/-}$ mice. IOP of FLU-treated group at 4 week (18.88 ± 1.76 mmHg, n=15 mice/group) is even slightly higher than IOP at 2 week and before FLU instillation (17.84 ± 1.25 mmHg) ($p=0.0746$).

Interestingly, in a pilot study with a longer follow-up, in the WT group, IOP returned to baseline by 5 weeks of DexAc treatment while IOP of $S1R^{-/-}$ group did not drop back to the baseline. This phenomenon was also reported previously and suggests that there is a compensatory mechanism which can prevent the secondary GCs response. Of note, in our experiment, lack of S1R made the increased IOP lasted longer than in WT mice, providing more evidence to protective role of S1R.

In summary, we provided evidence and illustrated deeper insights into the protective role of the multifunctional chaperone protein, S1R, in IOP homeostatic regulation. Its IOP lowering effect is likely a consequence of accelerated AH outflow facility through anti-TM fibrosis and released NO stimulation.

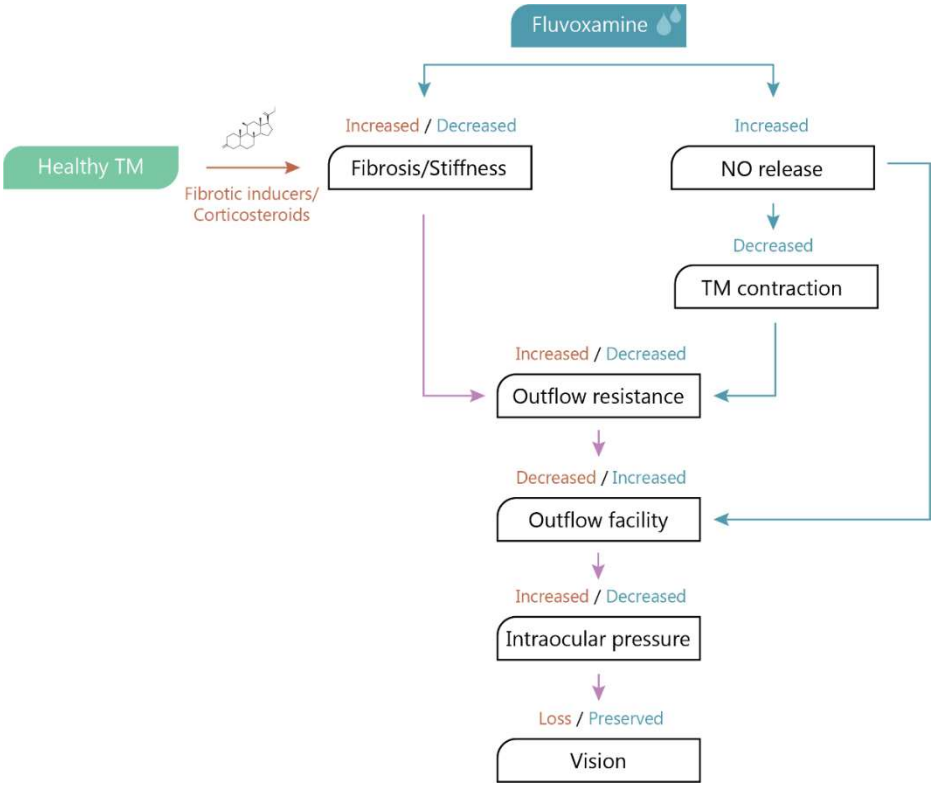

Figure 3. Schematic representation of a model of fibrotic disease in the conventional outflow responsible for ocular hypertension and the potential protective mechanisms of fluvoxamine (FLU). In this study, we triggered this process by fibrotic factors (TGFβ2, PDGF) or corticosteroids administration and restored trabecular meshwork (TM) function by FLU. The flow in red: pathological processes in glaucoma; blue: protective effects of FLU. NO: nitric oxide.

5. CONCLUSIONS

1. In human TM cells, S1R resides mainly in the ER.
2. S1R activation with FLU protects HTM5 cells against TGFβ2- or PDGF-induced fibrotic changes. FLU decreases F-actin, cell proliferation, migration and pro-fibrotic elements such as CTGF, fibronectin, and collagen IV.
3. FLU may facilitate ECM degradation through the elevation of cathepsin K and MMP2.
4. S1R agonist FLU accelerates NO release in HTM5 cells.
5. S1R is protective in PDGF-mediated fibrotic alterations in pMsTM cells.
6. A well-tolerated, FLU- containing, new eye drop has been developed.
7. The FLU eye drop lowers DexAc-induced high IOP in WT mice.
8. S1R is protective in DexAc-induced glaucoma model, since lack of S1R results in a longer lasting and slightly higher IOP increase.

6. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

6.1. Publications related to the PhD thesis

1. **Tran MN**, Medveczki T, Besztercei B, Torok G, Szabo AJ, Gasull X, Kovacs I, Fekete A, Hodrea J. (2023) Sigma-1 Receptor Activation Is Protective against TGFβ2-Induced Extracellular Matrix Changes in Human Trabecular Meshwork Cells. Life, 13: 1581. **IF=3.2**

2. Hodrea J, **Tran MN**, Besztercei B, Medveczki T, Szabo AJ, Őrfi L, Kovacs I, Fekete A. (2023) Sigma-1 Receptor Agonist Fluvoxamine Ameliorates Fibrotic Response of Trabecular Meshwork Cells. Int J Mol Sci, 24: 11646. **IF=5.6**

6.2. Publications not related to the PhD thesis

1. Mai NT, Hoa NT, Nga TV, Linh le D, Chau TT, Sinh DX, Phu NH, Chuong LV, Diep TS, Campbell J, Nghia HD, **Minh TN**, Chau NV, de Jong MD, Chinh NT, Hien TT, Farrar J, and Schultsz C. (2008) *Streptococcus suis* meningitis in adults in Vietnam. Clin Infect Dis, 46: 659-667. **IF=8.266**

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