Interleukin-1 receptor modulators: drawing links between structure, biased signaling, and *in vivo* efficacy in models of preterm birth and retinopathy of prematurity

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LIST OF ABBREVIATIONS

Agl	α-amino-β-γ-lactam
ANOVA	Analysis of Variance
AP-1	Activator protein 1
ARVO	Association for Research in Vision and Ophthalmology
BRB	Blood-retinal barrier
BSA	Bovine serum albumin
CD39	Ectonucleoside triphosphate diphosphohydroylase 1
COX-2	Cyclo-oxygenase 2
CXCR4	C-X-C chemokine receptor type 4
DMEM	Dulbecco's modified eagle medium
ECL	Enhanced chemilumiscence
EDTA	Ethylenediaminetetraacetic acid
FcεRI	High affinity IgE receptor
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
GFAP	Glial fibrillary acidic protein
GPCR	G-protein coupled receptor
HEK	Human embryonic kidney
Hgl	α-amino-β-hydroxyl-γ-lactams
HIF-1	Hypoxia-inducible factor 1
HIV-1	Human immunodeficiency virus 1
IAI	Intra-amniotic inflammation
Iba-1	ionized calcium-binding adapter molecule 1
IGF-1	Insulin-like growth factor 1
IL-1	Interleukin 1
IL-18	Interleukin 18
IL-1R	Interleukin 1 receptor
IL-1R1	Interleukin-1 receptor subunit 1
IL-1R2	Interleukin-1 receptor subunit 2
IL-1R3	Interleukin-1 receptor subunit 3
IL-1Ra	Interleukin-1 receptor antagonist
IL-1RacP	Interleukin-1 Receptor Accessory Protein
IL-23	Interleukin 23
IL-33	Interleukin 33
IL-6	Interleukin 6
IL-8	Interleukin 8
IRAK	Interleukin-1 receptor activated kinase
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide

MAPK	Mitogen-activated protein kinase
MMP	Matrix matelloproteinase
MyD88	Myeloid differentiation primary response 88
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3	NACHT, LRR and PYD domains-containing protein 3
OD	Optical density
OIR	Oxygen-induced retinopathy
PAMP	Danger-associated molecular pattern
PAX2	Paired box gene 2
PBS	Phosphate-buffered saline
PDVF	Polyvinylidene difluoride
PMA	Phorbol-12-myristate-13-acetate
PMSF	Phenylmethylsulfonyl fluoride
PRR	Pattern recognition receptor
PTB	Preterm Birth
PTGS2	Prostaglandin endeperoxide synthase 2
PTL	Preterm labour
RGC	Retinal ganglion cell
RNS	Reactive nitrogen species
ROCK	Rho-associated coiled-coil containing protein kinase
ROP	Retinopathy of prematurity
ROS	Reactive oxygen species
SAPK	Stress-activated protein kinase
SDF-1	Stromal derived factor 1
SEAP	Secreted alkaline phosphatase
SEM	Standard error of the mean
Sema3A	Semaphorin 3A
TIR	Toll-IL-1-receptor
TLR9	Toll-like receptor 9
TNBS	2,4,6-trinitrobenzene sulfonic acid
TRAF6	Tumour necrosis factor receptor-associated factor 6
UAP	Uterine-activating protein
V2R	Vasopressin type 2 receptor
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
vMIP-II	Viral macrophage inflammatory protein II

ABSTRACT

Introduction:

Preterm birth (PTB) and subsequently, retinopathy of prematurity (ROP), are two neonatal pathologies associated with a dysregulated inflammatory response. Interleukin (IL)-1 is strongly implicated in both conditions; however, current therapies against IL-1 are large molecules and indiscriminately block all IL-1 signaling pathways, causing undesirable immunosuppressive effects. We previously developed an allosteric modulator of the IL-1 receptor (all-D peptide rytvela, hereafter referred to as 101.10) that was effective in several animal models of inflammatory diseases in an NF-κB-independent manner. To further our understanding of 101.10's structural-activity relationships and to elucidate the roles of the various signaling pathways in PTB and ROP, we synthesised a panel of twelve 101.10 lactam derivatives with varying chiralities in residues 3 and 4 and tested them *in vitro* on cell lines, and *in vivo* in PTB and ROP models.

Methods:

RAW-blue or HEK-blue cells were stimulated with IL-1β after pre-treatment with our derivatives. The QUANTI-blue spectroscopic assay was used to quantify secreted alkaline phosphatase, a reporter gene of NF-κB activity. Western blots were used to quantify phosphorylation of ROCK2, p38 and JNK. Quantiative PCR was also used to determine the expression of pro-inflammatory genes in the cells. The ability of our derivatives to displace 101.10 from the IL-1 receptor was assessed with radioligand binding assays. Derivatives were then tested *in vivo* in a CD-1 mouse model of LPS-induced PTB, and a Sprague Dawley rat model of ROP induced by exposure to 80% O_2.

Results:

All derivatives did not inhibit NF-kB signaling, but most inhibited ROCK2 phosphorylation. Derivatives with an L-valine were stronger inhibitors of p38 phosphorylation and weaker inhibitors of JNK phosphorylation than those with a D-valine. Notably, D-valine derivatives were stronger inhibitors of PTB. The efficacy of D-valine derivatives *in vivo* PTB did not completely translate into efficacy in ROP. Nonetheless, it was observed that both JNK and ROCK2 inhibition were necessary for ROP prevention, while JNK alone prevented PTB.

Conclusions:

Selective modulation of IL-1 signaling, especially JNK and ROCK2 phosphorylation, without affecting NF-κB is a feasible strategy for preventing PTB and ROP. Our small molecules could offer advantages over existing therapies, such as reduced side effects and easier administration.

RÉSUMÉ

Introduction :

La naissance prématurée et, ensuite, la rétinopathie du prématuré sont deux pathologies néonatales associées avec une dérégulation de la réponse inflammatoire. L'interleukine (IL) -1 est fortement impliquée dans les deux états; Cependant, les traitements actuels contre l'IL-1 sont de grosses molécules et bloquent, sans distinction, toutes les voies de signalisation de l'IL-1, engendrant ainsi des effets immunosuppresseurs indésirables. Nous avions précédemment développé un modulateur allostérique du récepteur de l'IL-1 (le peptide tout-D rytvela, ci-après dénommé 101.10), qui était efficace dans plusieurs modèles animaux de maladies inflammatoires, et ce de manière indépendante de la voie ND-κB. Pour approfondir notre compréhension des relations structure-activité de 101.10 et élucider l'implication des différentes voies de signalisation dans la naissance prématurée et la rétinopathie du prématuré, nous avons synthétisé une panoplie de douze dérivés du lactame 101.10 qui varient dans les chiralités dans les résidus 3 et 4 et les avons testés in vitro dans des lignées cellulaires et in vivo dans des modèles de naissance prématurée et de rétinopathie du prématuré.

Méthodes:

Les cellules RAW Bleu ou HEK Belu étaient stimulées par IL-1β après traitement préalable avec nos dérivés. Le test spectroscopique QUANTI-Blue était utilisé pour quantifier la phosphatase alcaline sécrétée, un gène rapporteur de l'activité de NF-κB. L'immunobavardage de type Western était utilisé pour quantifier la phosphorylation de ROCK2, p38 et JNK. La réaction en chaine par polymérase était utilisée pour déterminer l'expression de gènes pro-inflammatoires dans les cellules. La capacité de nos dérivés à déplacer le 101.10 du récepteur de l'IL-1 était évaluée avec des tests de liaison du radioligand. Les dérivés étaient ensuite testés in vivo dans un modèle de naissance prématurée induit par le LPS chez la souris et dans un modèle de rétinopathie du prématuré induit par l'exposition à 80% de O_2 chez le rat Sprague Dawley.

Résultats:

Tous les dérivés n'ont pas inhibé la signalisation NF-κB, mais la plupart ont inhibé la phosphorylation de ROCK2. Les dérivés avec une configuration L-valine étaient des inhibiteurs plus puissants de la phosphorylation de p38 et des inhibiteurs plus faibles de la phosphorylation de JNK par rapport à ceux avec une configuration D-valine. De plus, les dérivés de D-valine démontraient une inhibition du travail préterme induit par le LPS supérieure aux L-valine ce qui permettait de supposer un lien entre l'inhibition de la phosphorylation de JNK et l'inhibition du travail préterme. L'efficacité des dérivés de D-valine in vivo dans la naissance prématurée ne s'est pas complètement traduite par une

efficacité dans la rétinopathie du prématuré. Néanmoins, nous avons constaté que l'inhibition de JNK et de ROCK2 étaient toutes deux nécessaires pour prévenir la rétinopathie du prématuré, alors que l'inhibition de JNK seul empêchait la naissance prématurée.

Conclusions:

La modulation sélective de la signalisation de l'IL-1, en particulier de la phosphorylation de JNK et de ROCK2, sans affecter la voie NF-κB est une stratégie envisageable pour prévenir les naissances prématurées et les rétinopathies du prématuré. Nos petites molécules pourraient offrir des avantages par rapport aux thérapies existantes, tels que la minimisation des effets secondaires et une administration plus facile.

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PREFACE

This thesis is written in the traditional monograph form. The work presented in this thesis has been published in *Frontiers in Chemistry* as a co-first-author publication with Azade Geranurimi (Université de Montréal) (Please refer to Geranurimi **2019**).

This study was designed by Colin WH Cheng, Azade Geranurimi, Dr. William D. Lubell and Dr. Sylvain Chemtob. Colin WH Cheng conducted *in vivo* and *in vitro* experiments, with assistance from Tang Zhu for Western blots and Xin Hou for injections of CD-1 mice for the preterm labour model. The syntheses and purification of the compounds used in this study was performed by Azade Geranurimi and is not covered in detail in this thesis. Most of the data was collected and analysed by Colin WH Cheng. Colin WH Cheng and Azade Geranurimi contributed equally to the writing of the *Frontiers in Chemistry* manuscript, with feedback from Dr. William D. Lubell. All biological sections of the manuscript (i.e. *in vivo* and *in vitro* experiments), and therefore this thesis, were written by Colin WH Cheng.

Chapter 1:
Introduction

Preterm labour (PTL) resulting in preterm birth (PTB), delivery before 37 weeks' gestation, is a serious complication of pregnancy that is associated with considerable morbidity and is the leading cause of death in neonates worldwide (Liu **2012**). It afflicts 5-8 % of pregnancies in Europe, and up to 13% in the United States (Goldenberg **2008**). Inflammation is strongly implicated in PTB (Goldenberg **2008**; Romero **2014**; Menon **2018**) and mounting evidence (Osman **2003**; Romero **1989**; Puchner **2011**), including that of our laboratory (Nadeau-Vallée **2015** and **2017**) shows that interleukin (IL)-1, a pro-inflammatory cytokine, plays a causative role in PTB. Current therapies for PTB, such as indomethacin, mainly focus on inhibiting myometrial contractions (i.e. tocolysis) (Olson **2008**) but fail to address the underlying inflammation and are only modestly effective.

PTB is associated with numerous sequelae but one particularly disabling morbidity is retinopathy of prematurity (ROP). Preterm infants are born with an incomplete retinal vasculature, and the hyperoxia that results from intensive oxygen therapy induces vaso-obliteration in the retina, subsequent retinal hypoxia and lastly neo-vascularisation (Rivera **2011**), potentially leading to blindness. As with PTB, inflammation (Tremblay **2013**; Beaudry-Richard **2018**), and in turn, IL-1 (Zhou **2016**; Rivera **2011**) play a role in ROP, particularly in vaso-obliteration (Rivera **2013**), but none of the current therapies address this inflammation.

Considering the role that IL-1 plays in the pathology of ROP and PTB, antagonism of IL-1 appears to be an attractive therapeutic avenue for these diseases. Current FDA-approved anti-IL-1 therapies consist of competitive antagonists that bind to the orthosteric site on the IL-1 receptor (IL-1R) (Braddock **2004**) and are associated with injection-site reactions and considerable immunosuppression (Dinarello **2012**). These adverse effects

may be due to complete, indiscriminate blockade of IL-1 signaling. For instance, the IL-1-induced nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway is anti-apoptotic (Castro-Alcaraz **2002**) and promotes differentiation of B and T cells (Gerondakis **2010**); its inhibition may be deleterious. To this end, our laboratory has previously developed 101.10 (all-D heptapeptide rytvela), an intramolecular peptide derived from the sequence of the IL-1 receptor accessory protein (IL-1RacP) (Quiniou **2008a**). 101.10 has been shown to be clinically effective in models of PTB (Nadeau-Vallée **2015** and **2017**) and ROP (Rivera **2013**), as well as cutaneous inflammation, colitis (Quiniou **2008a**) and hypoxic-ischemic encephalopathy (Quiniou **2008b**). Moreover, *in vitro* experiments have shown that it does not inhibit NF-kB signaling (Nadeau-Vallée **2015**), a testament to its functional selectivity.

The principal aim of this project is to further understand the structure-activity relationships of 101.10. To accomplish this, we collaborated with the Dr. Lubell's medicinal chemistry laboratory (Université de Montréal), who designed a panel of derivatives that were tested *in vivo* and *in vitro*; the results of this study have been published recently (Geranurimi **2019**). These derivatives are conformationally-constrained by cyclisation of Thr^3 residue into a γ-lactam ring (Geranurimi **2018**) and have varying stereochemistry at the Thr^3 and Val^4 residues. I demonstrate that the *in vivo* efficacy of 101.10 is dependent on its stereochemistry, and that Rho-associated coiled-coil-containing protein kinase 2 (ROCK2) and c-Jun N-terminal kinase (JNK) inhibition are correlated with *in vivo* efficacy. It is hoped that these results will allow for the further optimization of 101.10 for clinical trials, and provide benefits such as improved pharmacokinetics, ease of administration and increased oral bioavailability.

Chapter 2:
Literature Review

2.1 Preterm Birth (PTB)

2.1.1 Epidemiology

PTB and its associated complications are the leading cause of death in the first month of life worldwide, contributing to approximately 1 million deaths in 2010 (Liu **2012**). The incidence of PTB varies significantly among countries but is estimated to be 11.1% of total worldwide births (Blencowe **2012**). Developed countries tend to have lower PTB rates than developing regions; for instance, the East Asian region has a PTB rate of 7.2% while Sub-Saharan Africa's rate is 12.3% (Blencowe **2012**). Surprisingly, the PTB rate is especially high in the United States at 12.7% (Goldenberg **2008**). When classified by gestational age at time of birth, about two-thirds of PTB cases occur at 34-36 weeks' age, while only 5% occur earlier than 28 weeks (Goldenberg **2008**). Despite advances in the medical community's knowledge of PTB mechanisms and risk factors, PTB is becoming increasingly more common in most countries (Goldenberg **2008**; Blencowe **2012**). This highlights the fact that there are still significant unmet therapeutic needs in this field and underscores the need to explore new molecular targets.

2.1.2 The Common Pathway of Parturition

It has been argued that PTB and physiological term labour are similar processes, with the primary difference being the time of occurrence (Romero **2006**). Indeed, there are several biochemical processes that occur in both phenomena, which are collectively referred to as the "common pathway of parturition" (Romero **2014**). The main chain of events in this pathway are 1) transition of myometrium from quiescence to a contractile state, 2) ripening and dilation of the cervix and 3) decidual activation (Romero **2006**),

which are mediated by prostaglandins (Christiaens **2008**). It is striking that both preterm and physiological labour involve an increase in the secretion of pro-inflammatory cytokines such as IL-1β and IL-8 in the fetal membranes and myometrium (Christiaens **2008**), even in the absence of infection at term (Osman **2006**). In other words, these cytokines are likely to play a causal role in labour, both pathologically and physiologically.

2.1.3 Risk Factors

Given the instrumental role that cytokines play in labour, it is logical to propose that infections and the accompanying inflammation are a major risk factor for PTB. Indeed, inflammation is a major risk factor, and the only one in which a causal link has been established (Romero **2014**) and is associated with at least 25% of PTB cases (Goldenberg **2008**). While most of these inflammations are intra-amniotic, extrauterine infections such as periodontitis (infection of the gums) are also known to contribute to PTB (Offenbacher **1996;** Rajakapse **2005**). These infections could affect pregnancy outcomes by the systemic spread of inflammatory mediators from the initial site of infection, bacteremia (systemic migration of bacteria via the bloodstream), or the combined maternal-fetal immune response to the infection (Walia **2015**). The mechanistic details of intra-amniotic inflammation will be discussed in the next section.

Other proposed risk factors for which causality has not been definitively established include:

- Tobacco use, which increases PTB risk by about 50% (Goldenberg **2008**; Cnattigus **2004**). Tobacco smoke is a complex mixture of noxious chemicals, and

promotes systemic vascular inflammation (Bermudez **2002**), which may lead to placental dysfunction (Goldenberg **2008**), contributing to PTB.

- Multiple pregnancies, that is, having more than one developing fetus in the womb. For instance, more than half of all twins will be born preterm, and it is suggested that this is the result of overdistension of the uterus and subsequent premature rupture of fetal membranes leading to labour (Goldenberg **2008**).
- Psychological stress and psychiatric illness, including depression and anxiety (Copper **1996**). There is a growing body of evidence that suggests that psychiatric illnesses are systemic inflammatory diseases (Miller **2016**); it is therefore plausible that the elevated levels of pro-inflammatory cytokines in the circulation could prematurely activate the myometrium leading to PTB.
- Malnutrition (Goldenberg **2008**). Women with a low body mass index (BMI) are at increased risk of PTB (Hendler **2005**). Moreover, deficiencies in specific minerals such as iron and folic acid also increase the risk (Tamura **1992**; Scholl **2005**).
- African ethnicity, which is associated with a 2-3-fold risk of PTB relative to white women; the causes of this disparity are still subject to investigation and are not well-established (Goldenberg **2008**; Collins **1997**).

2.1.4 The placenta and associated membranes

While the structure of the placental membranes is complex, it can generally be summarized as being comprised of three main layers (Bernischke **2012**), described as follows:

- The amnion. This is the innermost layer closest to the developing fetus and is a thin layer of epithelial and connective tissue
- The chorion laeve, which consists of connective tissue and blood vessels of fetal origin and remnants of chorionic villi, the interface for fetal-maternal nutrient exchange.
- The decidua capsularis, which is the only layer of maternal origin and contains immune cells and some vasculature. This layer is contiguous with the decidual basalis of the placenta.

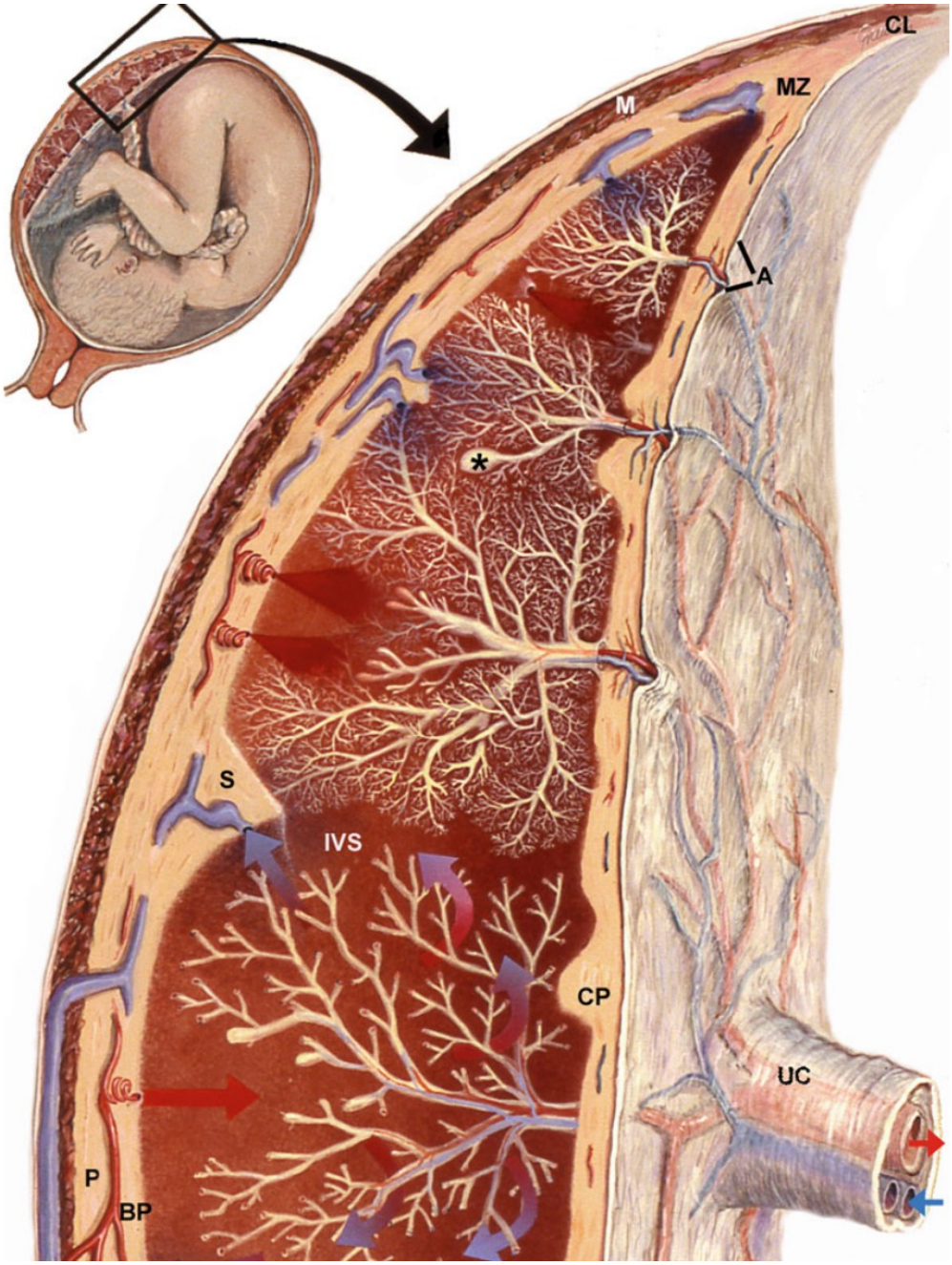


Figure 1. A cross-sectional view of the human placenta and its main anatomical features. Note that this placenta is hemochorial; that is, the intervillous trees, which are of fetal origin, are completely bathed in maternal blood, and there is no maternal epithelium or endothelium between the maternal blood and the fetal blood. UC Umbilical cord; IVS intervillous space; CP chorionic plate; CL Chorion leave; A Amnion; S Septum; M Myometrium; P placental bed; MZ marginal zone; BP basal plate; * cell island connected to a villous tree (From Benirschke **2012**)

These membranes are contiguous with the placenta, which is a transient organ present only during pregnancy, and serves as the main conduit between the developing fetus and the mother for nutrients and waste removal. However, given that the blood type of the mother and fetus are not always immunologically compatible, the placenta has special features to keep the two compartments of blood separate, while maximizing the surface area for nutrient exchange and waste elimination. Between the chorionic plate (facing the fetus) and the basal plate (overlying the maternal endometrium) is the intervillous space, which contains the projections of 30-40 villous trees; each tree branches to form small lobules (Fig. 1), and each lobule represents the smallest unit of fetal-maternal exchange (Burton **2015**). The villous tree is covered in an epithelium known as the syncytiotrophoblast, which contains no intercellular clefts into the intervillous space (to minimize pathogen transmission) but has dense microvilli on its apical surface that maximises the surface area for receptor and transport proteins (Burton **2015**).

It also important to note that the human placenta is hemochorial. This means the syncytiotrophoblastic surface is surrounded in maternal blood without the interference of maternal endothelial tissue and this represents the most invasive form of placentation in mammals (Benirschke **2012**). Notably, rodent placentas are also hemochorial, and this is one of the reasons why mice are the most commonly-used animal model for PTB studies.

Throughout pregnancy, the fetus is surrounded by a layer of amniotic fluid that plays several physiological roles. Firstly, it contains high concentrations of nutrients such as peptides and electrolytes, which promote fetal growth and maturation of the gastro-intestinal tract when it is swallowed (Kwon **2003**). It also contains notable amounts of several growth factors, such as epidermal growth factor, transforming growth factor α and

insulin-like growth factor 1 (IGF-1), which all promote somatic growth (Underwood **2005**). The amniotic fluid protects the fetus physically, but also contains antimicrobial peptides such as α-defensins (Yoshio **2003**). Clinically, amniotic fluid is diagnostically useful as it can be sampled using amniocentesis to assess fetal chromosomes or to evaluate the possibility of microbial invasion (Underwood **2005**). However, the fact that the amniotic fluid is a "closed system" means that the effects of microbial infections can be particularly deleterious to the infant.

2.1.5 Intra-amniotic inflammation (IAI) and preterm labour

IAI is characterized by the presence of elevated levels of pro-inflammatory cytokines and prostaglandins in the amniotic fluid. It should be noted that microbial invasion is not a prerequisite for intra-amniotic inflammation, as it can occur under conditions that are sterile *per se.* In general, sterile inflammation occurs when danger-associated molecular patterns (DAMPs) bind to pattern recognition receptors (PRRs), triggering the inflammatory cascade (Nadeau-Vallée **2016**). PAMPs are typically endogenous molecules that are released extracellularly only after necrosis; for example, cell-free DNA released from dying cells can bind to toll-like-receptor 9 (TLR9), a type of PRR, to induce inflammation (Philippe **2015**).

Microbial invasion of the amniotic cavity also contributes to inflammation and is typically caused by micro-organisms ascending from the cervix, such as group B *Staphylococcus* (Whidbey **2013**; Romero **2014**). This is supported by findings that the micro-organisms detected in amniotic fluid are common constituents of the vaginal microbiota (DiGiulio **2012**) and by experiments in which pregnant mice were infected intravaginally with bioluminescent *E. coli,* resulting in IAI and preterm delivery (Suff **2018**).

However, as mentioned earlier, extrauterine infections can also lead to preterm labour, and to this effect, periodontitis-associated bacteria has been isolated in the amniotic fluid of pregnancies, supporting a possible contributory role of blood-borne dissemination and transplacental transmission (Madianos **2013**). Regardless of the source of the infection, the bacteria, their cell wall components, and their secreted products are detected by PRRs (e.g. TLRs), setting off signaling cascades that upregulate the expression of pro-inflammatory cytokines, matrix matelloproteinases (MMPs), and eventually uterine activating proteins (UAPs) (Elovitz **2003**; Agrawal **2012**). The endpoint of this process is the common pathway of parturition and premature delivery of the neonate.

Interestingly, in the context of evolution, Romero and coworkers propose that preterm labour may have a survival advantage: to eliminate infected fetal tissue, but to the severe detriment of the neonate's health (Romero **2014**).

2.1.6 The role of IL-1

There is a growing body of evidence that points to IL-1 as the primary causative cytokine for preterm labour. Several key studies have contributed to this notion and their implications are as follows:

- Exposure of *ex vivo* human tissues to bacterial products (e.g. lipopolysaccharide) induces IL-1β expression and synthesis (Pavlov **2008**; Himes **2012**)
- IL-1β levels are elevated in the fetal membranes and cervix during PTB (Osman **2003**), with similar trends observed for the amniotic fluid (Puchner **2011**).
- IL-1β administration is sufficient to induce labour; this was first demonstrated in a mouse model (Romero **1991**) and replicated later in a non-human primate model

(Sadowsky **2006**). Therefore, this effect is common to both murine and primate species.

- IL-1β synthesis is mediated by a positive feedback loop; that is, IL-1β upregulates its own synthesis (Weber **2010**) which rapidly magnifies the effects of the ensuing inflammatory cascade.
- The stimulation of human uterine-derived cells with IL-1β results in the in expression of UAPs (Chevillard **2007**). An important example of one such UAP is prostaglandin-enderoperoxide synthase 2 (PTGS2), also known as cyclooxygenase-2 (COX-2) (Zaragoza **2006**; Bartlett **1999**). Prostaglandins have long been known as potent inducers of myometrial contractility and are also clinically used to induce labour (Calder **1973**; Mitchell **1976**).
- IL-1 signaling activates the Rho/ROCK2 pathway (Nadeau-Vallée **2015**), which is implicated in myometrial contractility.
- Antagonism of the IL-1 receptor with IL-1Ra prevents IL-1-induced preterm labour (Romero **1992**) and activation of the Rho/ROCK2 pathway (Nadeau-Vallée **2015**).

Given this strong body of evidence linking IL-1 with PTB, it is disappointing that none of the current PTB therapies directly target IL-1.

2.1.7 Current Therapies and Limitations

To date, no approved therapies for PTB target the underlying inflammatory processes of preterm labour. Instead, current therapies aim to reduce the contractility of the myometrium and are known as tocolytics (Olson **2008**). For instance, indomethacin inhibits PTGS2, and is the most commonly used tocolytic in Canada to date (Hui **2007**).

Unfortunately, the efficacy of such therapies is limited and PTB is only delayed by a maximum of several days, which is insufficient for most cases. Moreover, it is generally more desirable to prevent preterm labour than to treat it when contractions already occurring (Olson **2008**). Therefore, tackling the inflammation that occurs prior to PTB appears to be an attractive and underexploited therapeutic avenue.

2.2 Retinopathy of Prematurity (ROP)

PTB is known to result in numerous sequelae in the neonate, but an especially disabling one is ROP, which can contribute to vision loss in severe cases. To understand the pathological processes underlying ROP, a basic understanding of the structure and function of the retina is required; this is therefore reviewed below.

2.2.1 The Structure of the Retina

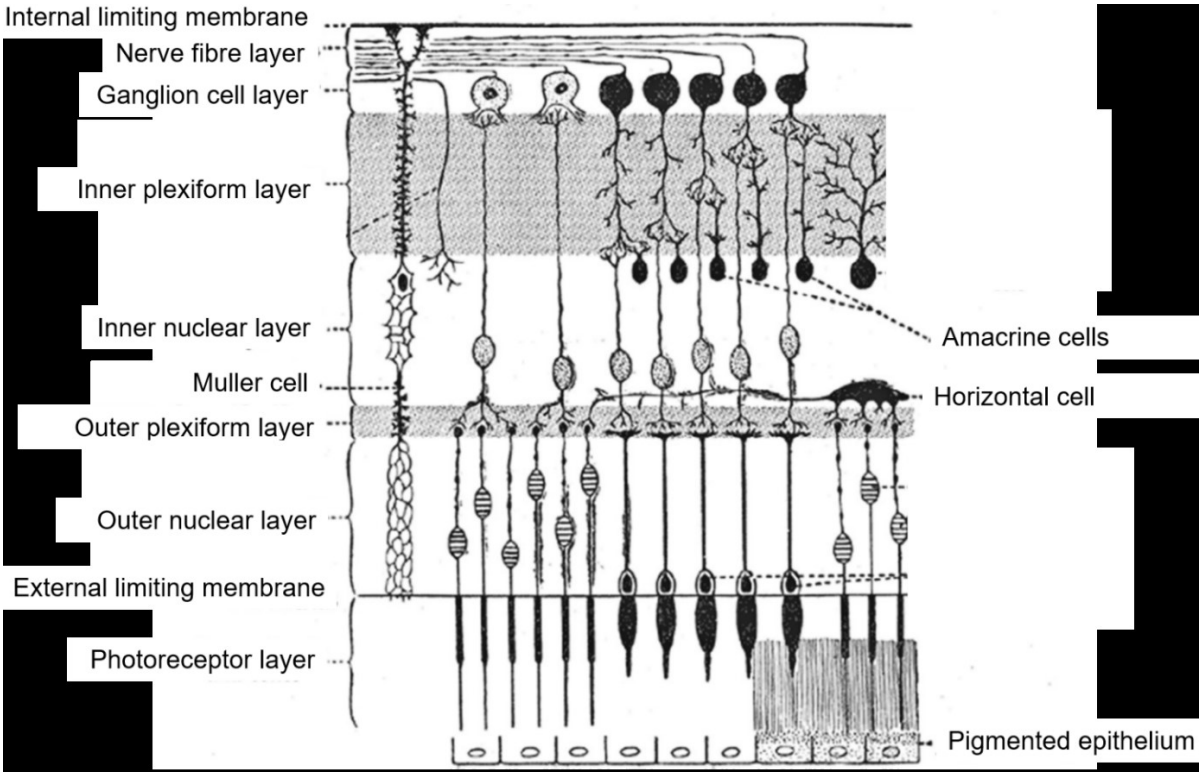


Figure 2. The histological organization of the retina, showing the layers and cell types present. (Adapted from Grossniklaus **2015**)

The retina is one of the most metabolically-active tissues in the body, in terms of oxygen consumption per gram (Wong-Riley **2010**). This is because it is continuously active in receiving light from the surrounding environment and converting it into nervous impulses for transmission to the brain for interpretation. There are several main cell types present in the retina that form the basis of the signal transduction pathway for visual perception, in order from photoreceptor to the optic nerve (Fig. 2, Grossniklaus **2015**):

- Photoreceptors – convert light into neuronal signals. Rods are responsible for vision in low-light conditions and do not provide colour vision, while cones function better in good lighting and mediate colour vision.
- Bipolar cells – bridges photoreceptors to retinal ganglion cells (RGCs), allowing for integration of visual inputs.
- Horizontal and amacrine cells – transmit signals orthogonally or horizontally between bipolar cells and minimizes background noise
- RGCs – integrate impulses from the previous cell types and transmits them to the optic nerve via long axons.

There are also several other types of cells in the retina that do not directly contribute to the above pathway. These include Müller cells which support the retina physically and metabolically, immune cells such as microglia, as well as endothelial cells that form the retinal vasculature.

Understandably, the retina needs to be optimally structured to allow its metabolic needs to be satisfied, while maintaining the transparency needed to receive light. To this

end, the retina is a very intricate tissue with ten layers, described as follows from interior to exterior (Fig. 2, la Cour **2005**):

- Inner limiting membrane – a basement membrane formed from the endfeet of Müller cell processes and demarcates the boundary between the retina and the vitreous cavity of the eye.
- Retinal nerve fibre layer – contains the axons of RGCs which connect to the head of the optic nerve.
- Ganglion cell layer – contains cell bodies of the RGCs.
- Inner plexiform layer – has synapses between amacrine, ganglion, interplexiform and bipolar cells.
- Inner nuclear layer – contains the nuclei of Müller, amacrine, interplexiform and bipolar cells.
- Outer plexiform layer – contains synapses between photoreceptors, horizontal cells, bipolar cells and interplexiform cells.
- Outer nuclear layer – comprises the cell bodies of photoreceptors
- External limiting membrane – not strictly an anatomical structure *per se* but is described as an optical illusion arising from the junction between inner segments of photoreceptors and Müller cells.
- Photoceptor layer – contains the inner and outer segments of photoreceptors.
- Retinal pigment epithelium – a monolayer of pigmented cuboidal epithelial cells that forms the blood-retinal barrier (BRB) together with the Bruch's membrane.

The inner retina (up to the outer nuclear layer) is perfused by the retina's intrinsic vasculature, while the outer retina (beyond the outer nuclear layer) is perfused by the

underlying choroid, a highly vascularized tissue that is not part of the retina *per se* (Grossniklaus **2015**).

2.2.2 Development of the Retinal Vasculature

Compared to the hyaloid and choroidal vasculatures (which develop in the first 5-7 weeks), the retinal vasculature develops relatively late in human gestation, starting only in the 12th week, and reaching maturation near-term at the 37th week (Lutty **2018**).

Vascular progenitors in the developing retina express C-X-C chemokine receptor type 4 (CXCR4), a receptor whose ligand is stromal derived factor-1 (SDF-1); SDF-1 is inducible by retinal hypoxia and is highly expressed by the inner retina, which is not perfused by the relatively mature choroid (Lutty **2018**). Angioblasts, which are vascular progenitors, express high levels of CXCR4, as well as CD39 (ectonucleoside triphosphate diphosphohydroylase-1) (Lutty **2018**). These cells originate and migrate from the optic nerve and aligned parallel to nerve fibres (Hughes **2000**). They later form cords and start to express CD34, an endothelial cell marker, indicating their differentiation into endothelial cells (Chan-Ling **2004**). The growth of CD34^{+} endothelial cells occurs in tandem with GFAP^{+}/PAX2^{+} (Glial fibrillary acidic protein and paired box gene 2 double-positive) astrocytes (Chan-Ling **2004**) and the deep retinal vasculature is usually formed by the 25th week of gestation.

There are several guidance cues involved in vascularization of the retina, which are proposed to be mediated by Müller cells and axons (Lutty **2018**). Müller cells are known to produce Notch1, which is linked to vascular development in many organs (Roca **2007**), and the Müller cells are observed to create cavities in the inner retina where

primitive blood vessels can develop (Lutty **2018**). Vascular endothelial growth factor (VEGF) is also a strong mediator of vasculogenesis and is expressed by Müller cells; VEGF in turn acts on endothelial cells and angioblasts, which express VEGF receptor 2 (VEGFR2) (Chan-Ling **2004**).

2.2.3 Epidemiology

In general, ROP has a higher incidence in severely premature neonates, compared to neonates who are born moderately preterm or near-term; as many as three-quarters of neonates born at 27 weeks' gestation or earlier suffer from ROP (Hellström **2013**). Unfortunately, due to homogeneity in study designs, it is difficult to accurately compare the incidences of ROP among different countries; Nonetheless, it has been thought that the incidence of ROP in developed countries is increasing due to the improved survival of severely premature neonates (Hellström **2013**).

2.2.4 Risk Factors

Most obviously, the largest risk factor for ROP is severe prematurity, although there are several other contributory risk factors:

- Intensive oxygen therapy. Among neonates given supplementary oxygen, those with target oxygen saturation levels of 88-98% required treatment for ROP 4 times as frequently as those who had a lower target of 70-90%; survival rates and the incidence of cerebral palsy was similar in both groups (Tin **2005**).
- Low birthweight, as well as being born small for gestational age, increases the risk of ROP (Darlow **2005**).
- Low serum IGF-1 is associated with a higher incidence of ROP (Hellström **2003**)

- Hyperglycemia and the associated use of insulin also raises the risk of developing ROP (Ertl **2006**)
- Neonatal infections increase the risk of ROP (Manzoni **2006**). This is presumably due to the pro-inflammatory state that occurs (Dammann **2010**), which is detrimental to the retinal vasculature (as described in the next few sections) and is also supported by a recent study by our lab (Beaudry-Richard **2018**).

2.2.5 Pathological processes in ROP

ROP can be simply described as a biphasic disease consisting of initial vaso-obliteration during hyperoxia, and then aberrant neovascularization during the ensuing hypoxia.

During the hyperoxic phase, reactive oxygen species (ROS) accumulate due to the lack of antioxidants in the immature retina (Guzy **2006**; Nielsen **1988**). The ROS can further react with nitric oxide to form reactive nitrogen species (RNS), a significant source of nitrative stress (Kroncke **2003**) that promotes degeneration of the retinal vasculature (Beauchamp **2004**). The polyunsaturated fatty acids in membrane phospholipids also become peroxidated, resulting in compromised cell membrane function and integrity (Arstila **1972**); some products of this fatty acid peroxidation are also pro-inflammatory, and only worsen the damage (Sapieha **2010**).

Other than the direct toxicity of oxygen on the retina, the hyperoxic condition also suppresses the expression of growth factors that are regulated by oxygen. Specifically, this is mediated by the oxygen-sensing transcription factor, hypoxia-inducible factor (HIF) 1 (Wang **1995**). HIF contains two subunits, α and β, and both are required for its activity

(Wang **1995**). The alpha subunit is rapidly degraded under normoxic (and hyperoxic) conditions by HIF-1α prolyl hydroxylases. Because the HIF-1 dimer promotes the transcription of several pro-angiogenic genes such as VEGF (Liu **1995**), the degradation of the α subunit ultimately results in suppression of pro-angiogenic signaling. The end-product of these mechanisms is a heavily vaso-obliterated retina, and the removal of the neonate from oxygen therapy subsequently results in a severely underperfused and hypoxic retina.

Subsequently, in the hypoxic phase of ROP, the metabolically-deprived neurons (Sapieha **2008**) and astrocytes (Dorrell **2002**) of the retina express abnormally high levels of VEGF and erythryopoietin, two pro-angiogenic factors, resulting in excessive and aberrant neovascularization (Sapieha **2010**). HIF contributes significantly to this, as the hypoxic conditions mean that the α subunit is no longer degraded and can translocate to the nucleus to mediate transcription of pro-angiogenic factors (Sapieha **2010**). An important pathological feature of ROP is the formation of neovascular tufts, which are growths of immature vessels that extend into the vitreous. As hypoxia becomes more severe, neurons transition from secreting VEGF to secreting semaphorin 3A (Sema3A), a repulsive cue that guides neovessels out of the retina and into the vitreous (Joyal **2012**). When sufficiently severe, this can lead to the growth of fibrous tissue that exerts traction on the retina and eventually results in retinal detachment and blindness (Sapieha **2010**).

While the choroid is not part of the retina *per se*, it is also detrimentally affected in ROP. Our lab has observed that the choroid involutes and becomes thinner, particularly in the central retina, and that this thinning persists into adulthood in a rat model (Shao

2011). This is particularly damaging to the retina as the choroid is the main source of oxygen for the photoreceptors.

It is important to note that the hypoxic neovascular phase of ROP is dependent on the hyperoxic vaso-obliteration phase. Theoretically, neovascularization should not occur if vaso-obliteration is attenuated sufficiently. Therefore, treating ROP in the early vaso-obliteration phase seems to be an attractive, yet unattained, therapeutic goal.

2.2.6 The role of IL-1

Systemic perinatal inflammation contributes to defects in retinal vasculature and function (Tremblay **2003**), and the effects often linger into adulthood, even after the initial inflammatory insult is no longer present (Beaudry-Richard **2018**). The link between placental pathology, prenatal inflammation and ROP has also been noted clinically in an observational study (Lynch **2018**). In ROP, the specific causes of inflammation are oxidative stress during oxygen therapy, as well as any possible pre-natal inflammation that may have resulted in PTB initially.

As with PTB, IL-1 plays an important role in the inflammatory pathology of ROP, especially in the vaso-obliterative hyperoxic stage.

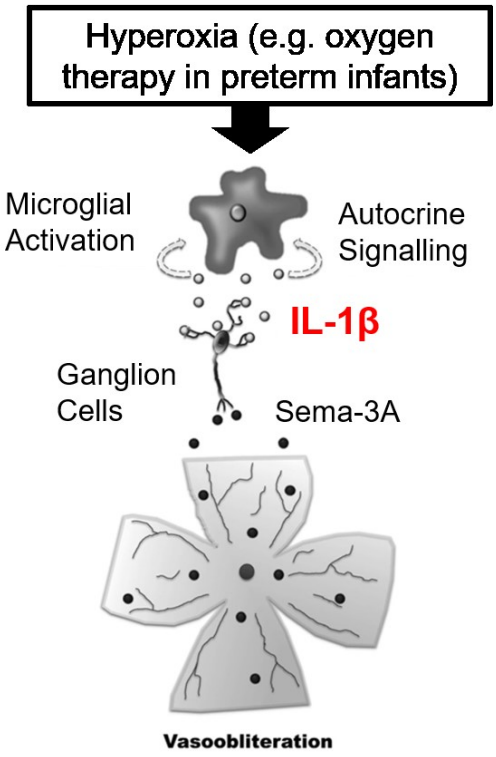


Figure 3. The role of IL-1β in the vaso-obliterative phase of ROP. (Adapted from Rivera **2013**)

Our lab has previously demonstrated that hyperoxia activates retinal microglia, causing them to produce IL-1β (Rivera **2013**). IL-1β is not cytotoxic to endothelial cells *per se*, but instead it causes RGCs to secrete Sema3A, a guidance and repulsive cue for endothelial cells which is pro-apoptotic (Rivera **2013**, Fig. 3). This results in vaso-obliteration in the retina, predisposing it to pathological neovascularization in the later hypoxic stage. Similar observations of vaso-obliteration are also observed in hypoxic-ischemic cerebropathy (Nadeau-Vallée **2017**; Sirinyan **2006**).

Apart from the retinal vasculature, IL-1β is also damaging to the underlying choroid and causes it to involute with long-lasting detrimental effects to photoreceptor function (Zhou **2016**).

2.2.7 Current Therapies and Limitations

The first mainstay of ROP treatment was cryotherapy, which became common in the 1980s, (Cryotherapy for Retinopathy of Prematurity Cooperative Group **2005**) but has since been replaced by laser photocoagulation due to a lower incidence of side effects (Paysse **1998**). Both therapies work on the same principles: ablation of avascular (hypoxic) retina so that it does not produce VEGF and other pro-angiogenic factors, hence preventing neovascularization (Houston **2013**). These therapies have notable side effects such as cataracts, myopia, burns of the cornea and iris, hemorrhages and impaired peripheral vision (Houston **2013**).

Anti-VEGF antibodies, such as bevacizumab, are a newer option for ROP treatment, and work by neutralizing VEGF and preventing its pro-angiogenic effects; they have been widely used in diabetic retinopathy and age-related macular degeneration and are only recently being adopted for ROP (Camphochiaro **2016**). Some of its possible side effects include endophthalmitis (generalized infection of the interior of the eye) and an increased risk of retinal detachment; even when injected intravitreally, bevacizumab enters the systemic circulation, leading to concerns of neurodevelopmental disorders arising from systemic VEGF inhibition (Sisk **2019**).

The most obvious limitation of both treatments is that treatment is only initiated once ROP becomes clinically apparent (that is, in the neovascular phase). As mentioned earlier, if the vaso-obliterative phase can be attenuated, then the risks of neovascularization are largely eliminated, and patient outcomes will be better. To the best of our knowledge, there are no investigational ROP therapies that target

inflammatory processes, such as those mediated by IL-1β. Therefore, it is hoped that 101.10, discussed in the later sections, will form the basis for future anti-ROP therapies.

2.3 Interleukin-1

Interleukin-1 is a pro-inflammatory cytokine that is expressed in two isoforms: IL-1α (31kDa) and IL-1β (17KDa). IL-1α is constitutively expressed and is normally sequestered intracellularly (Rider **2013**). It is released only after cell injury and induces sterile inflammation; it is therefore an alarmin, or a DAMP (Chen **2007**). Conversely, IL-1β expression is inducible and generally only occurs during an infectious or inflammatory insult. While both isoforms are associated with diseases, IL-1β is of greater interest due to the inducible nature of its expression.

2.3.1 Biosynthesis of IL-1β

IL-1β is initially synthesized as an inactive precursor, pro-IL-1β, which must be proteolytically cleaved to produce the biologically-active moiety (Netea **2015**). Caspase-1 is responsible for this enzymatic reaction, and it too is also synthesized as a precursor, pro-caspase-1 (Agostini **2004**). A member of a class of large protein complexes called inflammasomes is required for the conversion of pro-caspase-1 to caspase-1, of which the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome is most well-characterised (Netea **2015**). The NLRP3 inflammasome can be stimulated by a variety of triggers, including ROS (Dostert **2008**), bacterial nucleic acids (Kanneganti **2006**) and cholesterol (Duewell **2010**). Pro-IL-1β can also be cleaved independently of caspase-1, such as in the case of serine proteases from neutrophils (Dinarello **1982**), or proteases secreted by certain pathogenic microbes (Netea **2010**).

2.3.2 The IL-1 receptor complex

IL-1β binds to IL-1 receptor subunit 1 (IL-1R1) on the cell membrane, which then dimerises with the IL-1 receptor accessory protein (IL-1RacP, sometimes referred to as IL-1R3 in the literature) (Wang **2010**). IL-1RacP is necessary for the downstream effects of IL-1 signaling, as it has been shown that in IL-1RacP-deficient mice, IL-1β binds to IL-1R1 without eliciting downstream gene expression (Cullinan **1998**).

IL-1β is also capable of binding to IL-1 receptor subunit 2 (IL-1R2). Unlike IL-1R1, IL-1R2 lacks the intracellular domain required for signaling and therefore regulates IL-1β activity by sequestering excess IL-1β (Colotta **1993**) and IL-1RacP (Lang **1998**). The other mode of IL-1 signaling regulation is performed by the intrinsic IL-1 receptor antagonist (IL-1Ra), which binds to IL-1R1 with high affinity, but prevents IL-1R1 from dimerising with IL-1RacP (Greenfeder **1995**). These two regulatory mechanisms ensure that IL-1 activity is heavily regulated, given that inflammation can be severely detrimental if uncontrolled.

The association of IL-1R1 with IL-1RacP causes their intracellular toll-IL-1-receptor (TIR) domains to overlap, which is a prerequisite for downstream intracellular signaling (Dunne **2003**). This complex recruits myeloid differentiation primary response 88 (MyD88), an adaptor protein (Medzhitov **1998**), and interleukin-1 receptor-activated kinase (IRAK) 4 (Brikos **2007**). IRAK 4 auto-phosphorylates itself, and then phosphorylates IRAK1 and IRAK2, which leads to the recruitment of tumour necrosis factor receptor-associated factor (TRAF) 6 (Cao **1996**). TRAF6 is, in turn, responsible for downstream signaling that results in the upregulation of pro-inflammatory mediators.

2.3.3 IL-1 signal transduction pathways

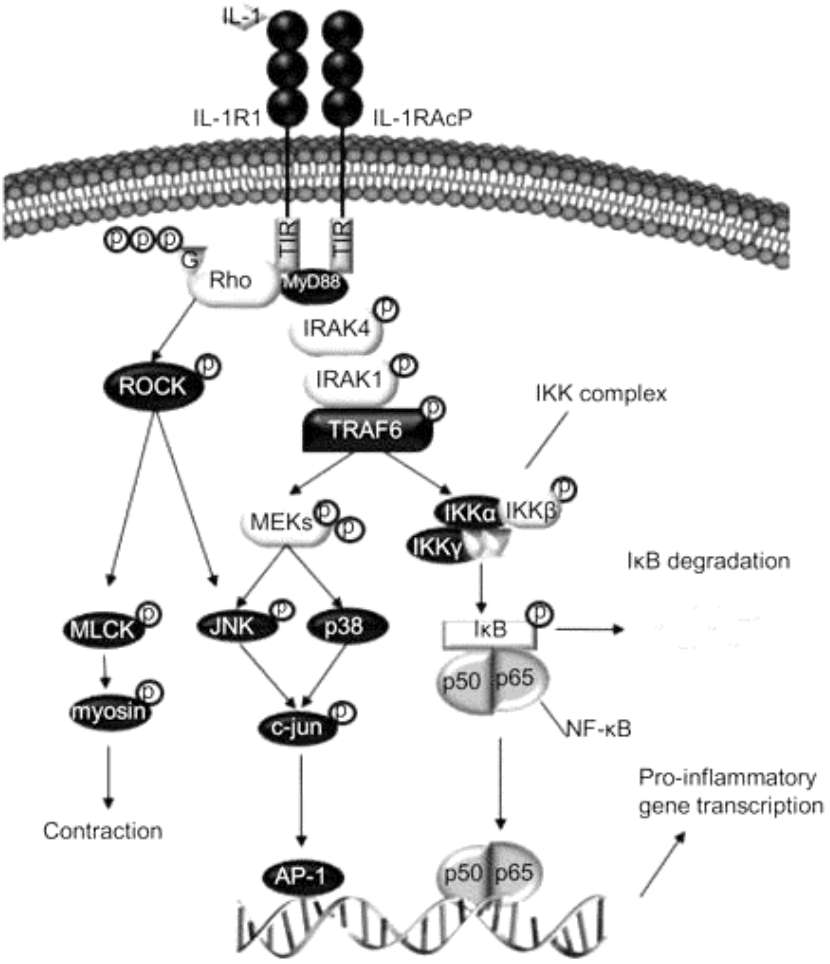


Figure 4. A summary of the principal signaling pathways downstream of IL-1β (Adapted from Nadeau-Vallée **2015**)

There are several signal transduction pathways downstream of IL-1, each with different effects (Fig. 4). They include:

- The Rho/Rho-associated coiled-coil containing protein kinase (ROCK) pathway. ROCKs are serine/threonine kinases mediating actin-myosin contractility and the mobility of the actin cytoskeleton (Loirand **2015**). This pathway is linked to the

increased myometrial contractility that occurs in both term and preterm labour (Goupil **2010**).

- p38 mitogen-activated protein kinase (MAPK) pathway. p38 MAPK mediates the upregulation of pro-inflammatory genes via the transcription factor activator protein (AP)-1 (Nadeau-Vallée **2015**). Other biological consequences of this signaling pathway include apoptosis, cell cycle arrest and cellular senescence mediated by telomere shortening (Zarubin **2005**).
- JNK pathway. As with p38 MAPK, JNK signaling has pro-apoptotic effects and involves AP-1 (Bogyevitch **2010**). JNK is very highly-expressed in the central nervous system and is associated with neurotoxicity, and promotes insulin resistance in diabetes; however, it is beneficial in adaptive immunity in that it promotes polarization of helper T cells (Zeke **2016**).
- Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Unlike the p38 MAPK and JNK pathways, this is cytoprotective and anti-apoptotic (Castro-Alcaraz **2002**) and promotes the maturation of B and T cells for immunovigilance (Pires **2018**). However, this also means that it may promote invasive growth of cells in the context of cancers (Greten **2004**).

2.3.4 Anti-IL-1 therapeutics

Currently, all approved anti-IL-1 therapeutics are large molecules and inhibit IL-1 signaling competitively, albeit with different mechanisms. These therapeutics are as follows (Dinarello **2012**):

- Anakinra (trade name Kineret), a recombinant version of IL-1Ra, manufactured by Swedish Orphan BioVitrum.
- Rilonacept, a soluble decoy receptor that sequesters IL-1β, by Regeneron.
- Canakinumab, an IgG1 monoclonal antibody that neutralizes IL-1β, by Novartis.

The most notable investigational therapy against IL-1 is gevokizumab, which is an allosteric inhibitor, unlike the three aforementioned peptides which are all competitive. Gevokizumab binds to an allosteric site on IL-1β, which then reduces the affinity of IL-1β for IL-1R1, but not the decoy receptor IL-1R2 (Issafras **2013**). An interesting aspect of this inhibitor is that the allosteric site is on the ligand, while the orthosteric site is on the receptor. This in contrast to classical allosteric compounds, for which the allosteric and orthosteric sites are both on the receptor.

It is important to note that all these therapeutics have several characteristics in common that render them undesirable for PTB and ROP treatment. They are:

- Large peptides, which limits their ability to pass through membranes and therefore reduces their oral bioavailability.
- Must be administered systemically by subcutaneous injections, which may reduce patient compliance due to the discomfort of the procedure.
- Associated with injection-site reactions (Satoh **2015**).
- Are immunosuppressive and increase the risk of opportunistic infections (Satoh **2015**). This is especially undesirable in premature infants, who have immature immune systems and insufficient numbers of leukocytes to adequately respond to pathogens (Melville **2013**).

Bearing in mind these limitations, our laboratory had sought to develop novel anti-IL-1 therapeutics using a different biochemical approach, as discussed in the next few sections.

2.4 IL-1 receptor modulators

2.4.1 Receptor antagonists

In the field of pharmacology, receptor antagonists are ligands that bind to receptors, but with the aim of attenuating or blocking the biological response downstream of the receptor. Antagonists can competitive or non-competitive. Competitive inhibitors bind to the orthosteric site of the receptor, but do not activate the receptor; they compete with the native ligand for occupancy of the receptor and this is the mechanism of action of anakinra (kineret), a recombinant version of IL-1Ra. Conversely, non-competitive inhibitors bind to an allosteric site that is separate from the native ligand's binding site, resulting in a conformational change of the receptor that reduces its affinity for the native ligand. This is the mode of pharmacological inhibition utilized by 101.10.

2.4.2 Biased signaling

After a ligand binds to its receptor, the downstream effects are usually mediated by several pathways. In the case of drugs, only a subset of these pathways is responsible for the therapeutic effect, while the remainder may result in undesired side effects. For example, opioids produce analgesia via canonical opioid receptors, but they often impair respiratory function, an effect which appears to be mediated by β-arrestins (Raehal **2005**).

Biased signaling, also known as functional selectivity, is a phenomenon in which a ligand does not uniformly activate (or inhibit) multiple signaling pathways downstream

of its receptor (Kenakin **2013**). In the context of the opioid example, a biased opioid agonist could activate opioid receptor signaling pathways but leave the β-arrestin pathway untouched; such an agonist would be desirable for providing analgesia without the danger of respiratory depression (Bohn **1999**). Taken more broadly, biased ligands may offer the advantage of an improved side-effect profile, while maintaining the same therapeutic effects as traditional unbiased ligands. Biased signaling was originally discovered in G-protein coupled receptors (GPCRs) (eg. The μ-opioid receptor) but is also being studied in other receptor classes such as in cytokine receptors (Nadeau-Vallée **2015**), nuclear hormone receptors and receptor tyrosine kinases (Smith **2018**).

2.4.3 Intramolecular peptides

One of the strategies for designing pharmacological inhibitors is the design of intramolecular peptides. Specifically, the structure of a protein serves as a template for small peptides, which can theoretically compete with the protein for binding to its ligand (McDonnell **1996**). This strategy has resulted in several publications covering different subdisciplines in pharmacology, described as follows:

- McDonnell and coworkers synthesized peptides that inhibit the interaction between IgE and the high-affinity IgE receptor (FcεRI). The peptide sequence was derived from the C-C' loop of the second alpha-chain domain of FcεRI, which is involved in IgE interactions. This is of clinical significance as IgE is a major mediator of allergies (McDonnell **1996**).
- Zhou and coworkers designed peptides with sequences derived from endogenous chemokines, SDF-1 and viral macrophage inflammatory protein II (vMIP-II), which are ligands of the chemokine receptor CXCR4. These peptides were able to

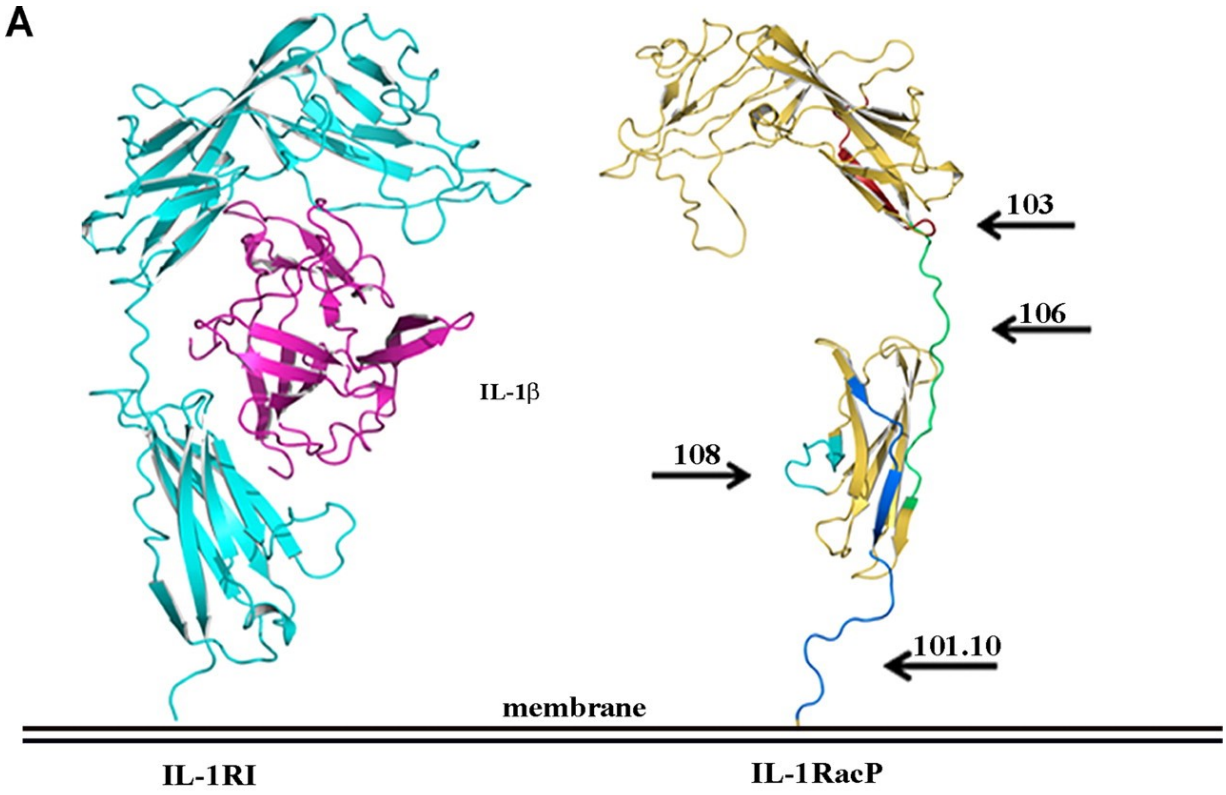
antagonize CXCR4, preventing the entry of human immunodeficiency virus (HIV)-1 into cells and its subsequent replication. (Zhou **2002**)

- Our lab had previously developed an allosteric inhibitor of the vasopressin type 2 receptor (V2R). This peptide (amino acid sequence CRAVKY) was derived from a juxtamembranous region of V2R and exhibits biased signaling in that it inhibits prostaglandin synthesis but did not recruit β-arrestins (Rihakova **2009**).
- Our lab had also designed small peptide non-competitive antagonists of the IL-23 receptor, which were derived from flexible regions of the receptor. As with CRAVKY, these peptides exhibited functional selectivity (Quiniou **2014**).

The development of 101.10 therefore builds on the techniques used in these studies.

2.4.4 The conception and development of 101.10

The intramolecular peptide approach was adopted for the IL-1R1-IL-1RacP signaling complex, in the hope of developing a modulator that exhibited functional selectivity and reduced the side effects associated with currently anti-IL-1 therapies. To this end, the amino acid sequence, and structure, of IL-1RacP was analysed (Fig. 5, Quiniou **2008a**). Loop regions of IL-1RacP were of interest, because they mediate protein-protein interactions and conformational changes (Slingsby **1993**). With the help of computational analysis, fifteen all-D-amino acid peptides were derived from loop sequences. Only four peptides, 101.10, 103, 106 and 108, inhibited IL-1-induced prostaglandin E2 synthesis; moreover, only 101.10 inhibited IL-1-induced p38 phosphorylation and therefore it was selected for further characterization (Quiniou **2008a**). 101.10 is an all-D heptapeptide with the sequence “rytvela”. It should be emphasized that “101.10” is a compound number and is therefore unrelated to its structure.



B **Sequence of the extracellular/transmembrane portion of IL-1RacP: targeted loops/regions are color indicated**

```
  1  MTLLWCVVSL YFYGILQSDA SERCDDWGLD TMRQIQVFED EPARIKCPLF EHFLKFNYST  60
 61  AHSAGLTLIW YWTRQDRDLE EPINFRLPEN RISKEKDVLW FRPTLLNDTG NYTCMLRNTT 120
121  YCSKVAFPLE VVQKDSCFNS PMKLPVHKLY IEYGIQRITC PNVDGYFPSS VKPTITWYMG 180
181  CYKIQNFNNV IPEGMNLSFL IALISNNGNY TCVVTYPENG RTFHLTRTLT VKVVGSPKNA 240
241  VPPVIHSPND HVVYEKEPGE ELLIPCTVYF SFLMDSRNEV WWTIDGKKPD DITIDVTINE 300
301  SISHSRTEDE TRTQILSIKK VTSEDLKRSY VCHARSAKGE VAKAAKVKQK VPAPRYTVEL 360
361  ACGFGATVLL VVILIVVYHV YWL                                        383
```

(transmembrane region)

Figure 5. The conception of 101.10 from a loop sequence in IL-1RacP. Arrows indicate the loop regions from which the four numbered peptides were derived. The coloured amino acids correspond to the loop regions in IL-1RacP (From Quiniou **2008a**).

Subsequent experiments determined that 101.10 (Quiniou **2008a**):

- has an affinity constant of ~5nM for IL1-R1 on thymocytes
- is a non-competitive antagonist of the IL-1 receptor and appears to reduce the affinity of the receptor for IL-1β.

- Exhibits specificity as it does not inhibit the activity of IL-18, another member of the IL-1 cytokine family, and IL-6, an unrelated pro-inflammatory cytokine.

Following up with the *in vitro* efficacy of 101.10, several *in vivo* experiments were performed to demonstrate its efficacy in a variety of inflammatory conditions. In chronological order, 101.10 has been demonstrated to be effective in models of:

- Inflammatory bowel disease, induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS) in rats. 101.10 preserved intestinal integrity and attenuated neutrophil infiltration in the colon (Quiniou **2008a**).
- Cutaneous inflammation induced by phorbol-12-myristate-13-acetate (PMA) ointment application on rat ears. 101.10 notably reduced PMA-induced edema and erythema (Quiniou **2008a**).
- Hypoxic-ischemic encephalopathy of the newborn performed on rat pups. 101.10 reduced inflammation-induced vaso-obliteration and brain infarct size (Quiniou **2008b**).
- ROP, particularly in the vaso-obliterative phase, in rats. 101.10 attenuated oxygen-induced retinal vaso-obliteration and reduced microglial activation (Rivera **2013**).
- PTB in mice, induced experimentally by both IL-1β and lipopolysaccharide (LPS) (Nadeau-Vallée **2015**). 101.10 was also later shown to improve neonatal developmental outcomes, as observed in the lungs, intestines and the brain (Nadeau-Vallée **2017**) as well as the retinal vasculature (Beaudry-Richard **2018**)

Emphasis should also be placed on the functional selectivity of 101.10. Specifically, it was shown to inhibit IL-1β-induced phosphorylation (and hence activity) of p38 MAPK,

JNK and ROCK2, while having no appreciable effect on NF-κB signaling; this contrasts with kineret which indiscriminately blocks all these pathways (Nadeau-Vallée **2015**). A consequence of this biased signaling is that 101.10 maintains the activity of some anti-inflammatory cytokines, such as IL-27, while kineret does not (Beaudry-Richard **2018**). This is consistent with reports that IL-27 signaling is NF-κB-dependent (Guzzo **2010**) and is an important finding since IL-27 can neutralize macrophage activity (Rückerl **2006**).

2.4.5 Conformationally-constrained peptides

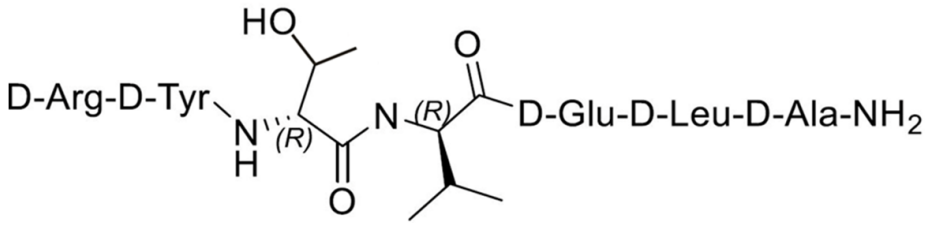
While 101.10 shows promise as a small peptide therapeutic, one of the main challenges of small peptides is that their tertiary structures do not usually resemble the parent protein they are derived from, and a large decrease in entropy is required for them to assume the conformation that gives rise to its biological activity (Robertson **2018**; McDonnell **1996**). To this end, conformational constraints have been employed as a strategy to reduce this entropic cost and improve peptide specificity and efficacy. Examples of chemical modifications that induce conformational constraint in peptides include cyclisation or “stapling” (Walensky **2014**), N-methylation of the backbone (Pelay-Gimeno **2015**) and converting serine/threonine residues into α-amino-β-hydroxyl-γ-lactams (St-Cyr **2010**). Consideration must also be given to the fact that peptides can be subject to proteolytic degradation, and for this reason, protease-resistant D-amino acids are used (Garton **2018**), along with a retro-inverso configuration that reverses the sequence and inverts the chirality of the peptide so that its side chains project with the appropriate orientation (Atzori **2013**).

2.4.6 New derivatives of 101.10

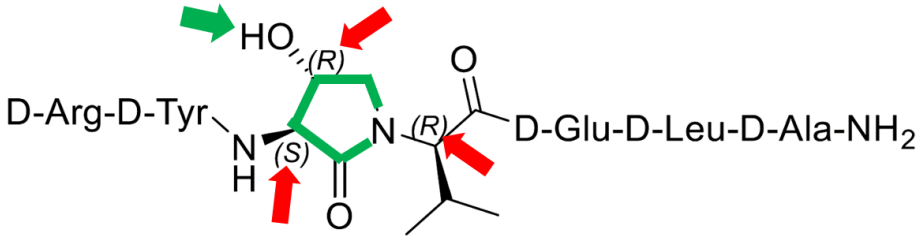
This thesis discusses the biological characterization of a panel of twelve derivatives of 101.10, which are all conformationally-constrained at the D-Thr-D-Val dipeptide residue by conversion to a lactam ring. Eight of these derivatives feature an additional hydroxyl group in the lactam ring and are known as α-amino-β-hydroxyl-γ-lactam (Hgl) derivatives, while the other four lack the hydroxyl group and are referred to as α-amino-β-γ-lactam (Agl) derivatives (Fig. 6). Thr^3 and Val^4 were targeted for modification because we have previously shown that a presence of a fold in the central residues is important to the biological activity of 101.10 (Boutard **2011**). Moreover, the cyclisation of Thr^3 into a lactam constrains the peptide and promotes the formation of β-turns, which can be studied by circular dichroism (St-Cyr **2010**).

The series of derivatives was generated by varying the stereochemistries of the α-carbons of Thr^3 and Val^4 in the Agl and Hgl derivatives, as well as the β-hydroxy carbon in the Hgl derivatives. Combinatorically, this results in a total of eight Agl and four Hgl derivatives, or a total of twelve in the full panel.

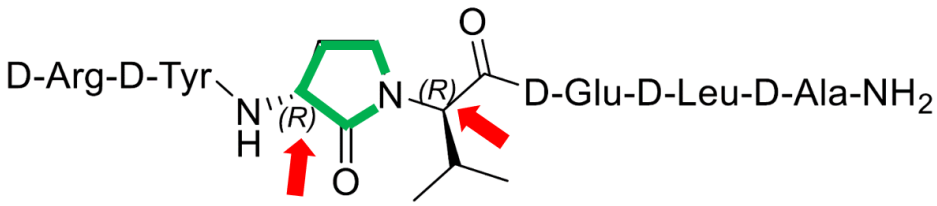
For the purposes of this thesis, the nomenclature used to describe the peptides shall be identical to that presented in Geranurimi **2019**. In other words, 101.10 is designated as peptide **1,** while the Agl derivatives are collectively referred to as peptide **5** and Hgl derivatives are referred to as peptide **6**. This thesis focuses primarily on the biological and pharmacological aspects of our derivatives. For a detailed description of the underlying synthetic chemistry, please consult the main article and supplementary information of Geranurimi **2019**.



101.10 (Peptide **1**)



A Hgl derivative, [(3*S*,4*R*)-**6**], or [(3*S*,4*R*)-Hgl[3]]-**1**



An Agl derivative, [(3*R*,4*R*)-**5**], or [(3*R*,4*R*)-Agl[3]]-**1**

Figure 6. Structures of 101.10 (peptide **1**) and its derivatives. Green lines denote the γ-lactam ring, red arrows denote the chiral centres that were modified, and the green arrow denotes the β-hydroxyl group in the Hgl derivative.

Chapter 3:
Materials & Methods

The Materials and Method section is adapted from my co-first-author publication (Geranurimi **2019**) with minor edits to suit the context of this thesis.

3.1 Chemical Syntheses

All twelve derivatives of 101.10 were synthesized by Azade Geranurimi of Dr William D. Lubell's laboratory, Université de Montréal. The full descriptions of the chemical syntheses can be found in the main text and supplementary material of Geranurimi et al. **2019** and are beyond the scope of this thesis. It should be noted that in these derivatives, only the Thr^3 and Val^4 residues were targeted for modification because we had previously shown that a presence of a fold in the central residues is important to the biological activity of 101.10 (Boutard **2011**).

3.2 Animals

Timed-pregnant CD-1 mice were obtained from Charles River (Saint-Constant, QC, Canada) at gestational day 12 and were acclimatized for 4 days prior to experiments. Animals were used according to a protocol approved by the Animal Care Committee of Hôpital Sainte-Justine in accordance with the principles of the Guide for the Care and Use of Experimental Animals of the Canadian Council on Animal Care. The animals were maintained on standard laboratory chow under a 12h:12h light/dark cycle and allowed free access to chow and water.

Two-day-old (P2) Sprague Dawley rat pups and their mothers were ordered from Charles River (Saint-Constant, QC, Canada) and acclimatized for 3 days. The rats were housed in standard cages with ad libitum access to food and water and kept in a 12h:12h light/dark cycle. To control for the effects of litter size on retinal development, the sizes of

litters were reduced to 12 by sacrificing excess pups by decapitation while under 2% isoflurane anesthesia. All procedures and protocols involving the use of these rats were approved by the Animal Care Committee of the research center of Hôpital Maisonneuve-Rosemont and are in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research approved by the Association for Research in Vision and Ophthalmology (ARVO), and guidelines established by the Canadian Council on Animal Care.

3.3 Reagents

Chemicals were purchased from the following manufacturers: human rIL-1β (200-01B; PeproTech), LPS (L2630; Sigma-Aldrich), 101.10 (peptide 1) (Elim Biopharmaceuticals, Hayward, CA) and Kineret (Anakinra, Sobi, Biovitrum Stockholm, Sweden).

3.4 qPCR experiments

HEK (human embryonic kidney)-Blue IL-33/IL-1β cells were purchased from InvivoGen (San Diego, CA) and used at passages under 15. The cells were cultured in DMEM growth medium supplemented with 10% bovine growth serum, 50 U/mL penicillin, 50 mg/mL streptomycin, 200 mg/mL zeocin, and 100 mg/mL hygromycin. Cells were grown in regular conditions (37 °C, 5% CO_2). Cells were serum starved overnight and treated with 100 ng/mL IL-1β for 4h. Cells were pre-incubated for 30 min with 101.10 (10^{-6}M), its derivatives (10^{-6}M), or Kineret (1.0 mg/mL) prior to the experiments to reach equilibrium (n=4 each treatment). Cells were harvested and incubated for 5 min in RIBOzol (AMRESCO). RNA was extracted according to manufacturer's protocol and

RNA concentration and integrity were measured with a NanoDrop 1000 spectrophotometer. A total of 500 ng RNA was used to synthetize cDNA using iScript Reverse Transcription SuperMix (Bio-Rad, Hercules, CA). Primers were designed using National Center for Biotechnology Information (NCBI) Primer Blast and are shown in Table 1. Quantitative gene expression analysis was performed on Stratagene MXPro3000 (Stratagene) with SYBR Green Master Mix (Bio-Rad). Gene expression levels were normalized to 18S universal primer (Ambion Life Technology, Burlington ON, Canada). Genes analyzed include *IL1β*, *IL6* and *PTGS2* (Prostaglandin H synthetase 2 or cyclooxygenase-2 (COX-2)). Data are representative of 3 experiments (each with n=4 per treatment group).

The purpose of the qPCR experiments is to evaluate the effect of the derivatives on the expression of pro-inflammatory genes in cells stimulated with IL-1β, and therefore determine the ability of our derivatives to inhibit IL-1β-induced gene transcription.

Table 1. List of primers for the human genes assessed by qPCR

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')
IL1β	AGCTGGAGAGTGTAGATCCCAA	ACGGGCATGTTTTCTGCTTG
IL6	TTCAATGAGGAGACTTGCCTGG	CTGGCATTTGTGGTTGGGTC
PTGHS2	ATATTGGTGACCCGTGGAGC	GTTCTCCGTACCTTCACCCC

3.5 NF-kB QUANTI-Blue assay

HEK-Blue IL-33/IL-1β cells (InvivoGen) were pretreated with 101.10 (10^{-6}M), its derivatives (10^{-6}M) or Kineret (1.0 mg/mL) for 30 min, followed by treatment with constant concentration of IL-1β (100 ng/mL), and then incubated at 37 °C for 4 h. Levels of secreted alkaline phosphatase in cell culture supernatant were determined using the QUANTI-Blue assay, according to the manufacturer's instructions (InvivoGen). Alkaline phosphatase activity was assessed by measuring the optical density (OD) at 620–655 nm with a micro plate reader (EnVision Multilabel reader; PerkinElmer, Waltham, MA). Data are representative of 3 experiments (each with n = 4 per treatment group).

This assay was performed to confirm that our derivatives do not inhibit the NF-kB signaling pathway, and hence demonstrate their functional selectivity. The cells used in this assay have been transfected (by the manufacturer) with a gene for secreted alkaline phosphatase (SEAP) that is expressed only when NF-kB is activated. In other words, the SEAP acts as a reporter gene for NF-kB. SEAP is secreted into the conditioned media and can be quantified spectroscopically by a colorimetric assay.

3.6 p38 MAPK, ROCK2 and JNK Phosphorylation Assay

RAW Blue cells (Invivogen) were grown under standard conditions (37 °C, 5% CO_2) and maintained under passage number 15. Cells were equilibrated with 101.10 (10^{-6}M), its derivatives (10^{-6}M), or Kineret (1.0 mg/mL) for 30 min, after which time, they were exposed to IL-1β (100 ng/mL) for 15 min. Cells were harvested and lysed on ice for 30 min using a radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling) supplemented

with 1 mM phenylmethylsulfonyl fluoride (PMSF) and cOmplete™ EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany, prepared according to the manufacturer's instructions). Protein concentrations were determined using a Bradford protein assay (Bio-Rad) on 96-well plates with a microplate reader (EnVision Multilabel reader) measuring OD at 595 mm. Bovine serum albumin serial dilutions were used to generate a standard curve. Lysates were then mixed with 4X reducing sample buffer (Bio-Rad).

Lysates were loaded (30 µg protein per well) in a 5% acrylamide stacking gel, and samples were electrophoresed in a 12% acrylamide resolving gel for 1.5 h at 120 V, followed by a 1 h transfer onto polyvinylidene difluoride (PVDF) membranes at 100 V. Membranes were blocked and incubated with 1:1000 dilution of primary antibody and 1:20,000 dilution of secondary antibodies according to the manufacturer's instructions. Antibodies used were for phospho-p38 MAPK (Cell Signaling, #9211), p38 MAPK (Cell Signaling, #9212), SAPK/JNK (Cell Signaling, #9252), phospho-SAPK/JNK (Cell Signaling, #9251), ROCK2 (Thermo Fisher Scientific PA5-21131), phospho-ROCK2 (Thermo Fisher Scientific PA5-34895), and goat anti-rabbit conjugated to horseradish peroxidase (Abcam, ab6721). Membranes were imaged using an Amersham Imager 600 (GE Healthcare) using Clarity Western ECL Substrate (Bio-Rad). The intensity of protein bands was quantified using ImageJ and standardized using total (phosphorylated + non-phosphorylated) protein content. Data are representative of three independent experiments.

The purpose of the Western Blots is to determine the effects of our derivatives on the three chosen IL-1β-dependent signaling pathways, and to explore the possibility of biased signaling.

3.7 Radioligand Displacement Assay

Displacement of radiolabelled peptide 1 was performed as described previously (Quiniou **2008**). Briefly, RAW-Blue macrophage cells (InVivogen; San Diego, CA) were preincubated for 20 min with 100 µM of non-radiolabelled ("cold") 101.10 or its derivatives, followed by incubation for 2 h at 37 °C with [^{125}I]-101.10 at 600 nM concentration to ensure maximal specific binding (Quiniou **2008**). Cells were washed four times with phosphate-buffered saline (PBS) buffer and lysed with 0.1 M NaOH/0.1% Triton X-100. Bound radioactivity was measured on cell lysates with a Hidex AMG gamma counter (Hidex; Turku Finland). Data are representative of three independent experiments.

This assay was performed to demonstrate the specificity of our derivatives for IL-1RacP (which 101.10 binds to). Displacement of 101.10 implies that the derivative binds, at least partially, to IL-1RacP.

3.8 LPS-induced preterm model in mice (Fig. 7)

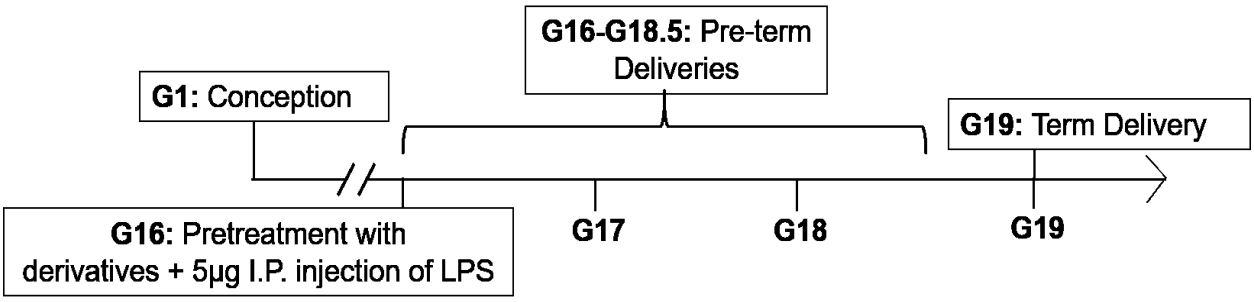


Figure 7. A schematic of the LPS-induced PTB model in CD-1 mice. I.P. intraperitoneal.

Given that 101.10 had previously been effective in this model of PTB (Nadeau-Vallee **2015** and **2017**), we sought to determine if our derivatives had similar efficacies as 101.10.

To this end, timed-pregnant CD-1 mice at 16.5 days of gestation (G16.5) were anesthetized with 2% isoflurane and received an intraperitoneal injection of LPS (n = 4 per group, a single dose of 10 µg) (Nadeau-Vallée **2015**, and Kakinuma **1997**). A dosage of 2 mg/kg/day of 101.10 or its derivatives, or vehicle was injected subcutaneously in the neck, every 12 h until delivery. On G16.5, a dose of 1 mg/kg was injected 30 min before stimulation with LPS (to allow distribution of drugs to target tissues) and 1 mg/kg was injected 12 h after stimulation (n = 4 each treatment). Mice delivery was assessed every hour until term (G19–G19.5). A mouse is considered as delivering prematurely if the first pup is delivered earlier than G18.5.

3.9 Oxygen-induced retinopathy in Sprague Dawley rats (Fig. 8)

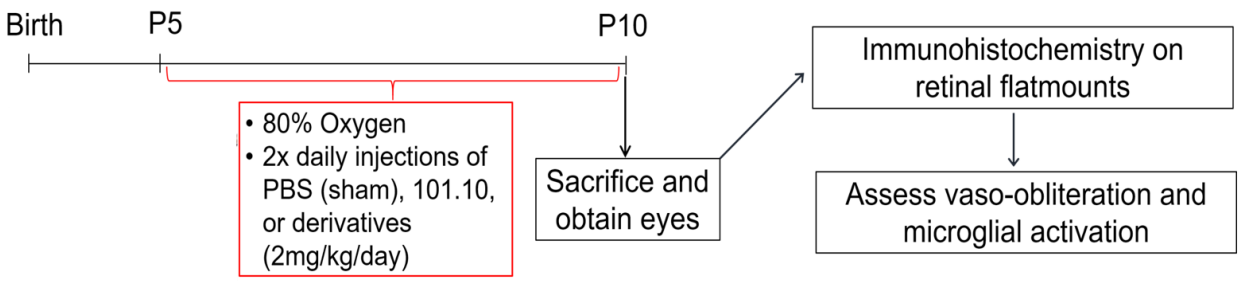


Figure 8. A schematic of the oxygen-induced retinopathy (OIR) 80% oxygen model in Sprague Dawley rats.

The OIR model is (Fig. 8) is another useful experiment which can be used to demonstrate the viability of our derivatives *in* vivo. However, due to constraints in resources at the animal facility and ethical reasons, we opted to only test a subset of our derivatives. Specifically, we selected four derivatives that performed well in the PTB model, one derivative that had no effect on PTB (as a negative control), and a sixth

derivative that had modest efficacy. Retinal flatmounts were performed and immunohistochemistry used to assess vaso-obliteration and macrophage activity in the retina.

As described earlier, Sprague Dawley pups and their mothers arrived at the animal facility on postnatal day 2 (P2) and allowed to acclimatize until P5. On P5, litters were transferred to a controlled hyperoxic environment (Biospherix OxyCycler A84XOV), maintained at 80±1% O_2, which is used to induce vaso-obliteration of the retinal vasculature. At this timepoint, the vasculature is immature and particularly susceptible to hypoxic insults resulting in vaso-obliteration. Control litters were not exposed to hyperoxia and were kept in standard conditions with room air (21% O_2). To control for the effects of hyperoxia on the lactation of the dams, dams of hyperoxic litters were switched with dams of control litters on P8.

In litters exposed to hyperoxia, pups were randomized to receive PBS vehicle, 2 mg/kg/day of 101.10 or derivatives, or 3 mg/kg/day of Kineret from P5 to P10. These doses were determined from our previous study (Rivera **2013**) and administered in twice-daily intra-peritoneal injections titrated to 20 µL per injection with 28-gauge insulin syringes. A total of 6 to 8 pups were used for each treatment group.

On P10, pups were euthanized by decapitation under 2% isoflurane anesthesia. Eyes were enucleated and fixed in 4% paraformaldehyde for 1 h at room temperature before washing twice with PBS and storage at 4 °C in PBS until further processing.

3.10 Retinal Flatmount and Immunohistochemistry

Under a dissecting microscope, the cornea and lens were removed from the eyes gently and remnants of the hyaloid vasculature were removed from the retinas using surgical scissors and tweezers. The retinas were gently removed from the underlying choroid/sclera complex.

Retinas were treated at room temperature for 1 h with a blocking solution consisting of 1% bovine serum albumin (BSA), 1% normal goat serum, 0.1% Triton X-100 and 0.05% Tween-20 in PBS. The retinas were then double-labelled overnight at 4°C with gentle shaking in an antibody solution consisting of 1 mM $CaCl_2$, 1% Triton X-100, 1% FITC-conjugated lectin cell endothelial marker from *Bandeiraea simplicifolia* (Sigma-Aldrich, St Louis, MO) and a 1:500 dilution of rabbit anti-ionized calcium-binding adapter molecule 1 (Iba-1) antibody (Wako Chemicals USA) in PBS. The retinas were then washed thrice with PBS and incubated with a secondary antibody solution consisting of 1% BSA, 0.1% Triton X-100, 0.05% Tween-20 and 1:500 Alexa-594-conjugated donkey anti-rabbit IgG (ThermoFisher Scientific, Rockford, IL) in PBS for 2 h at room temperature. Retinas were washed thrice with PBS and flat-mounted onto glass slides with coverslips and Fluoro-Gel mounting medium (Electron Microscopy Sciences, Hatfield, PA).

3.11 Microscopy

For assessment of vaso-obliteration, retinas were imaged using an epifluorescence microscope at 10X magnification (Zeiss AxioImager Z2) and version 4.8 of the AxioVision software. Images were captured and stitched together using the software's MosaiX feature. Ionized calcium-binding adapter molecule (Iba)-1 staining was

assessed with the same microscope at 20X magnification, and a total of 4 fields per retina were imaged halfway between the optic nerve and the edge of the retina.

Representative images of Iba-1 staining were taken using a laser scanning confocal microscope (Olympus IX81 with Fluoview FV1000 Scanhead) with the Fluoview Software at 30X magnification.

3.12 Quantification and Data Analysis

The FIJI software was used to the quantify the area of vaso-obliteration in each retina, expressed as a percentage of the area of the whole retina. The number of Iba-1-positive cells was counted using the cell counter plug-in in FIJI, and the average of cell counts in 4 fields per retina was calculated.

Data was analyzed using GraphPad Prism 7 with one-way analysis of variance (ANOVA) and the Dunnett's test for multiple comparisons. Outliers were detected using Grubb's test. Results were treated as significant when p was less than 0.05 and expressed as mean ± standard error of the mean (SEM).

Chapter 4:

Results

The Results section is adapted from my co-first-author publication (Geranurimi **2019**) with edits to suit the context of this thesis.

4.1 *In vitro* inhibition of signaling pathways

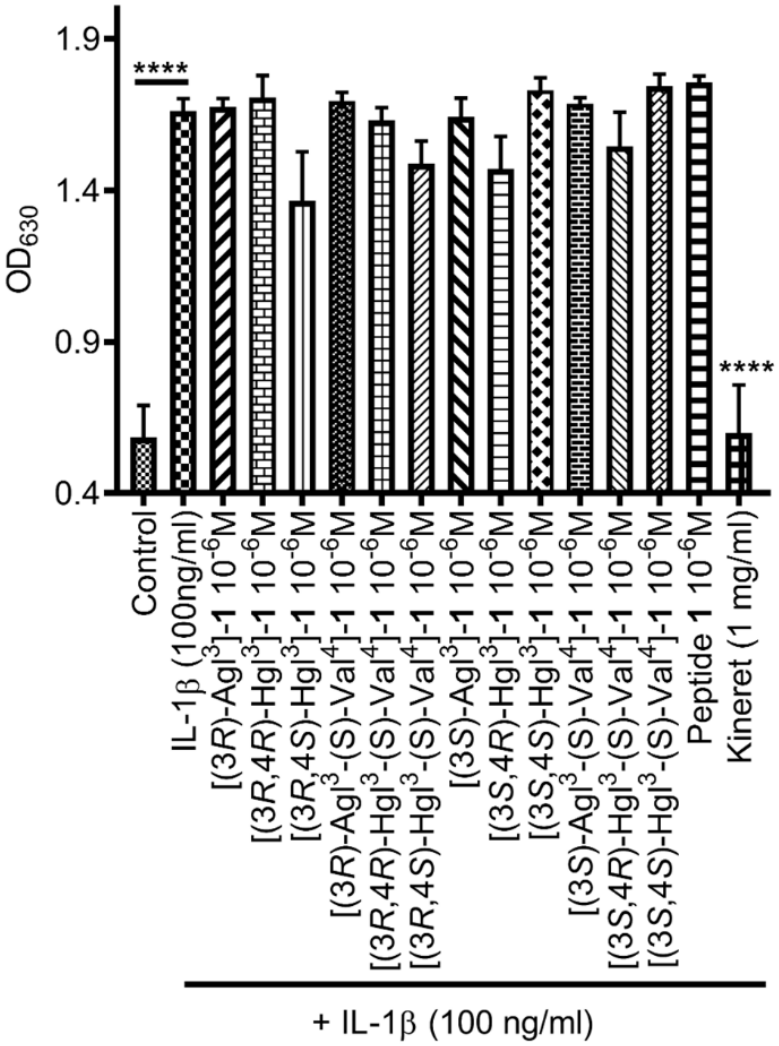


Figure 9. The effects of peptides 1, 5 and 6 on IL-1β-induced NF-κB signaling. NF-κB activation was quantified on HEK-blue cells using the QUANTI-blue assay, which spectroscopically detects secreted alkaline phosphatase, a reporter product from the transcription of the NF-κB gene. HEK-blue cells were pre-incubated with peptides **1**,**5** and **6** or vehicle, then stimulated with IL-1β for 4 h. Data shown represents the average of 3 experiments, n = 6 each. **** p<0.0001 compared to group treated only with IL-1β. Treatment groups that are not labelled with asterisks are statistically non-significant compared to group treated only with IL-1β. (From Geranurimi **2019**).

The biological effects of peptide **1** and derivatives were ascertained *in vitro* in RAW-blue and HEK-blue cells which were stimulated with IL-1β. The QUANTI-blue assay was employed to measure concentrations of secreted alkaline phosphatase, a reporter product from the transcription of the NF-κB gene. All peptides that were tested did not exhibit any noticeable inhibition of NF-κB signaling (Fig. 9). On the other hand, Kineret, which is an unbiased FDA-approved recombinant IL-1 receptor antagonist, inhibited NF-κB as previously reported (Nadeau-Vallée **2015**).

Western Blots were performed to measure phosphorylation of downstream JNK, p38 MAPK and ROCK2 in RAW-blue cells, after pre-incubation with peptides **1**, **5** or **6** and stimulation with IL-1β (Fig. 10 and 11). In contrast to peptide **1** which inhibited the effects of all three kinases, the Agl and Hgl analogs exhibited biased signaling contingent on stereochemistry and structure. For example, JNK phosphorylation was inhibited more profoundly by (*R*)- than (*S*)-Val^4 derivatives **5** and **6**. In the (*R*)-Val series, the Agl and *trans*-Hgl isomers were more effective than the *cis*-Hgl counterparts. Phosphorylation of p-38 was inhibited most effectively by [(3*R*,4*S*)-Hgl^3]- and [(3*R*)-Agl^3-(*S*)-Val^4]-**1**, while [(3*S*)-Agl^3]-, [(3*R*)-Agl^3]-, [(3*R*,4*R*)-Hgl^3]- and [(3*S*,4*S*)-Hgl^3-(*S*)-Val^4]-**1** were inactive. Finally, ROCK2 phosphorylation was inhibited significantly (p<0.05) by most derivatives, except for [(3*S*)-Agl^3-(*R*)-Val^4]- and [(3*R*)-Agl^3-(*R*)-Val^4]-**1**. In contrast to peptide **1** which inhibited effectively all three kinases, the inhibitory activity of the Agl and Hgl derivatives was contingent on configuration and the presence of the hydroxyl group. Conversely, these factors did not play a role in NF-κB signaling, which was universally unaffected by all derivatives **5** and **6**.

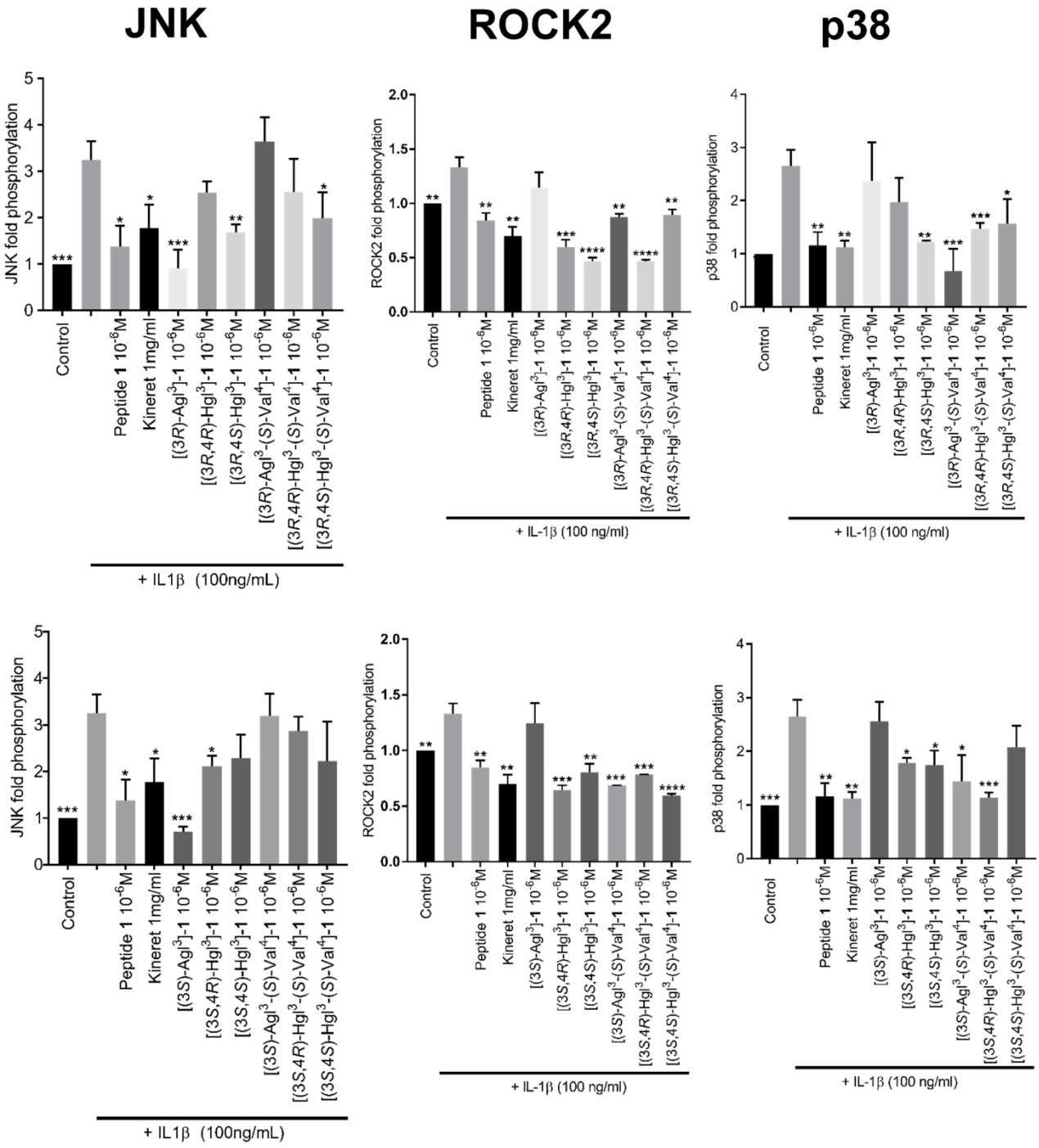


Figure 10. **The effects of peptides 1, 5, and 6 on IL-1β-induced phosphorylation of JNK, ROCK2 and p38 MAPK.** Graphical representations of band density analysis of Western Blots, sorted into columns based on protein of interest (JNK, ROCK2, or p38) and rows by peptide configuration ((3*R*- or (3*S*)). RAW-blue cells were pretreated with peptides **1**, **5**, **6**, Kineret, or vehicle for 30 min and then stimulated with IL-1β for 15 min. Images of representative Western Blots can be found in the Supplementary Figures. Results shown are the average of 3 independent experiments: **p*<0.05, ***p*<0.01, ****p*<0.001, **** *p*<0.0001 compared to group treated only with IL-1β. Treatment groups that are not labelled with asterisks are statistically non-significant compared to group treated only with IL-1β. (From Geranurimi **2019**).

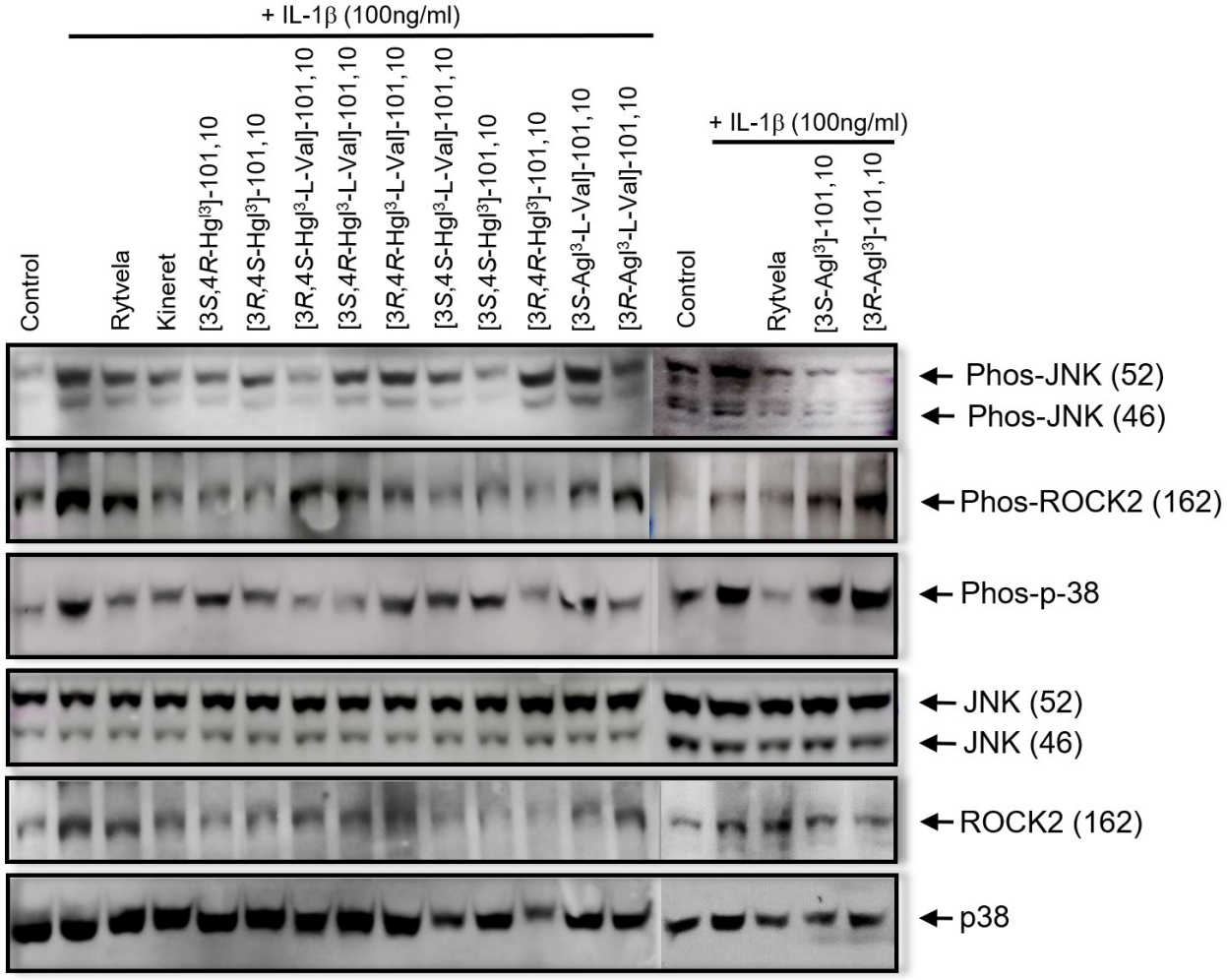


Figure 11. Representative Western Blots of the JNK, ROCK2 and p38 proteins. RAW-blue cells were pre-treated with peptides **1, 5** or **6** for 30 min, then stimulated with IL-1β for 15 min. Cell lysates were prepared and lysates (30µg protein per well) were electrophoresed on 12% resolving gels, transferred to PVDF membranes and incubated with antibodies for these proteins and their phosphorylated forms. Numbers in parentheses indicate the molecular weight of the protein of interest in kilo-daltons (kDa). (From supplementary figure of Geranurimi **2019**)

The effects of peptides **1**, **5** and **6** on the transcription of downstream pro-inflammatory genes that are mediated by IL-1β were measured with focus on IL-6, COX-2 and IL-1β, which upregulates its own expression by a positive feedback loop (Weber **2010**). Quantitative polymerase chain reaction (qPCR) experiments were performed on HEK blue cells to ascertain the expression levels of the mRNA transcripts after pre-treatment with peptides **1**, **5** and **6** and stimulation with IL-1β. Parent peptide **1** exhibited strong suppression of transcription of all three genes. Moreover, Agl and Hgl analogs of **1** maintained similar inhibitory potency as the parent peptide (Fig. 12). On the other hand, ability to suppress the transcription of all three genes was lost in [(3*R*)-Agl^3-(*S*)-Val^4]- and [(3*S*)-Agl^3-(*S*)-Val^4]-**1** and recovered in part in certain Hgl^3-(*S*)-Val^4 analogs with [(3*S*, 4*S*)-Hgl^3-(*S*)-Val^4]-**1** exhibiting ability to inhibit the expression of all three genes.

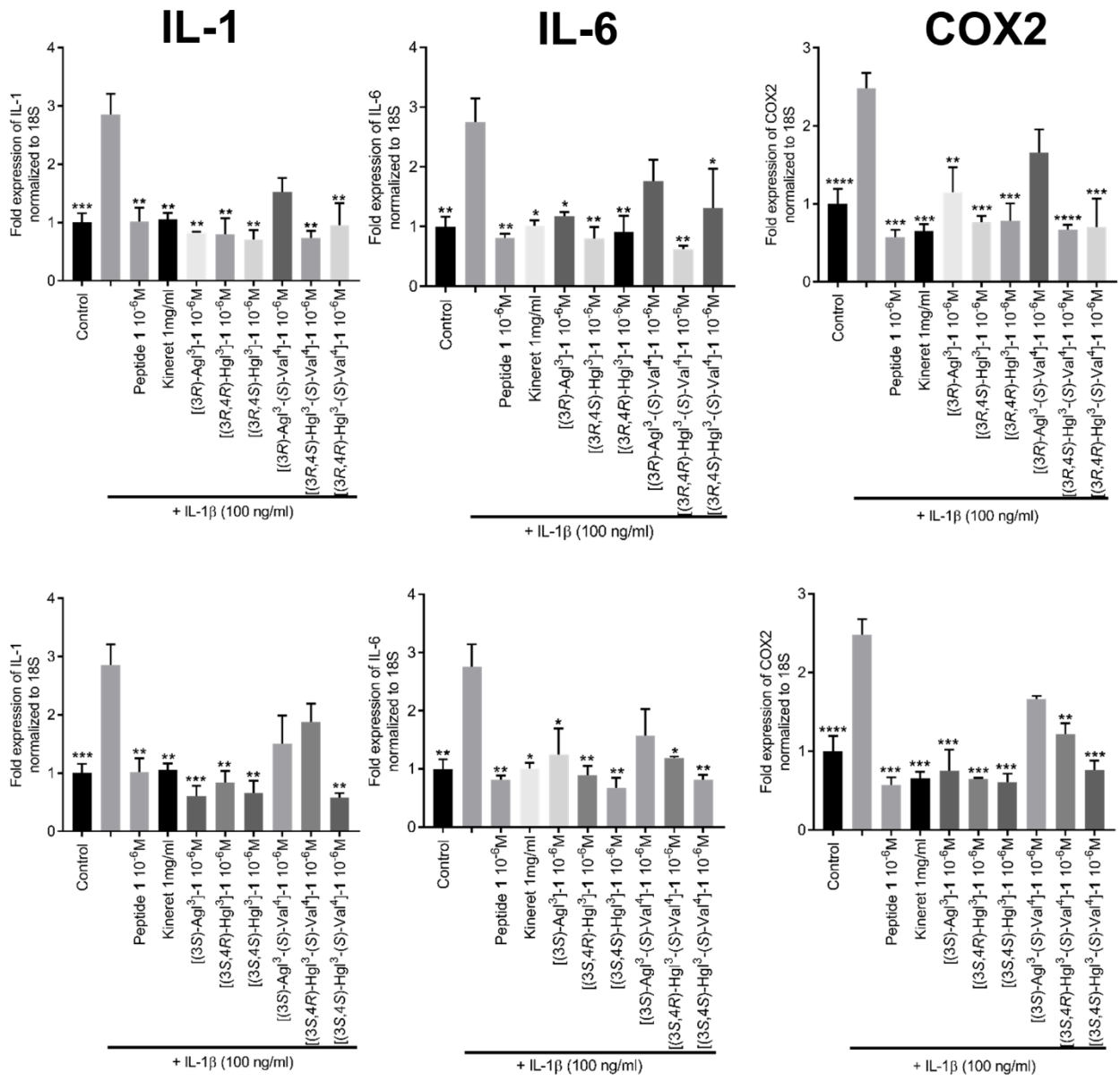


Figure 12. The effects of peptides 1, 5 and 6 on the IL-1β-induced expression of pro-inflammatory genes. HEK-Blue cells were pre-treated with peptides **1**, **5** and **6** as above and stimulated with IL-1β overnight. qPCR was performed on cell lysates using 18S rRNA as internal control. Graphs are sorted in columns by gene of interest (COX-2, IL-1β or IL-6) and rows by peptide configuration ((3*R*) or (3*S*)). Results are representative of an average of 3 independent experiments and are expressed as a fold-change of the non-stimulated control: **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001 compared to group treated only with IL-1β. Treatment groups that are not labelled with asterisks are statistically non-significant compared to group treated only with IL-1β.

4.2 Displacement of radiolabeled peptide 1

Peptide **1** is known to bind to IL-1R (Quiniou **2008**). To determine if the Agl and Hgl derivatives occupied the same binding site as peptide **1**, a radio-ligand displacement assay was used to determine the extent to which peptides **5** and **6** displaced radiolabelled **1**. Compared to cold unlabelled peptide **1**, which was used to set the baseline for specific binding, similar capacity to displace [^{125}I]-**1** was demonstrated by peptides **5** and **6**, except for [(3*R*,4*S*)-Hgl^{3}]-**1**, which exhibited significantly lower (p = 0.0011) ability to compete with radiolabelled peptide **1** (Table 2).

Table 2. The ability of peptides 1, 5 and 6 to displace radio-labelled [^{125}I]-1 in RAW-blue cells. (From Geranurimi **2019)**

Compound	Percentage of displacement of [^{125}I]-**1,** relative to unlabelled peptide **1**, ± SEM	*p*-value
[(3*R*)-Agl^{3}]-**1**	83.55±9.02	0.9908
[(3*R*,4*S*)-Hgl^{3}]-**1***	32.13±16.94	*0.0011*
[(3*R*,4*R*)-Hgl^{3}]-**1**	104.6±6.826	0.9997
[(3*R*)-Agl^{3}-(*S*)-Val^{4}]-**1**	120.7±15.69	0.954
[(3*R*,4*S*)-Hgl^{3}-(*S*)-Val^{4}]-**1**	58.63±13.45	0.0724
[(3*R*,4*R*)-Hgl^{3}-(*S*)-Val^{4}]-**1**	82.12±14.12	0.9308
[(3*S*)-Agl^{3}]-**1**	106.6±12.39	0.9996
[(3*S*,4*R*)-Hgl^{3}]-**1**	78.64±21.22	0.9428
[(3*S*,4*S*)-Hgl^{3}]-**1**	69.73±8.897	0.2864
[(3*S*)-Agl^{3}-(*S*)-Val^{4}]-**1**	102.7±8.637	0.9999
[(3*S*,4*R*)-Hgl^{3}-(*S*)-Val^{4}]-**1**	48.32±21.13	0.0649
[(3*S*,4*S*)-Hgl^{3}-(*S*)-Val^{4}]-**1**	86.06±6.46	0.9806

* p<0.05 relative to cold peptide **1**-treated group

4.3 *In vivo* inhibition of preterm labor

The spectrum of *in vitro* profiles exhibited by peptides **5** and **6** offers a unique means for probing the specific signaling pathways that contribute to the therapeutic potential of IL-1R modulators. Peptides **1**, **5** and **6** were first examined in an established CD-1 mouse model of PTB, induced by intraperitoneal injection of lipopolysaccharide (LPS) into pregnant dams on day 16.5 of gestation. The Agl and *trans*-Hgl analogs of peptide **1** exhibited equivalent potency as the parent peptide **1** in delaying labour (Fig. 13). On the other hand, *cis*-Hgl derivatives of peptide **1** and (*S*)-Val peptides **5** and **6** exhibited little efficacy.

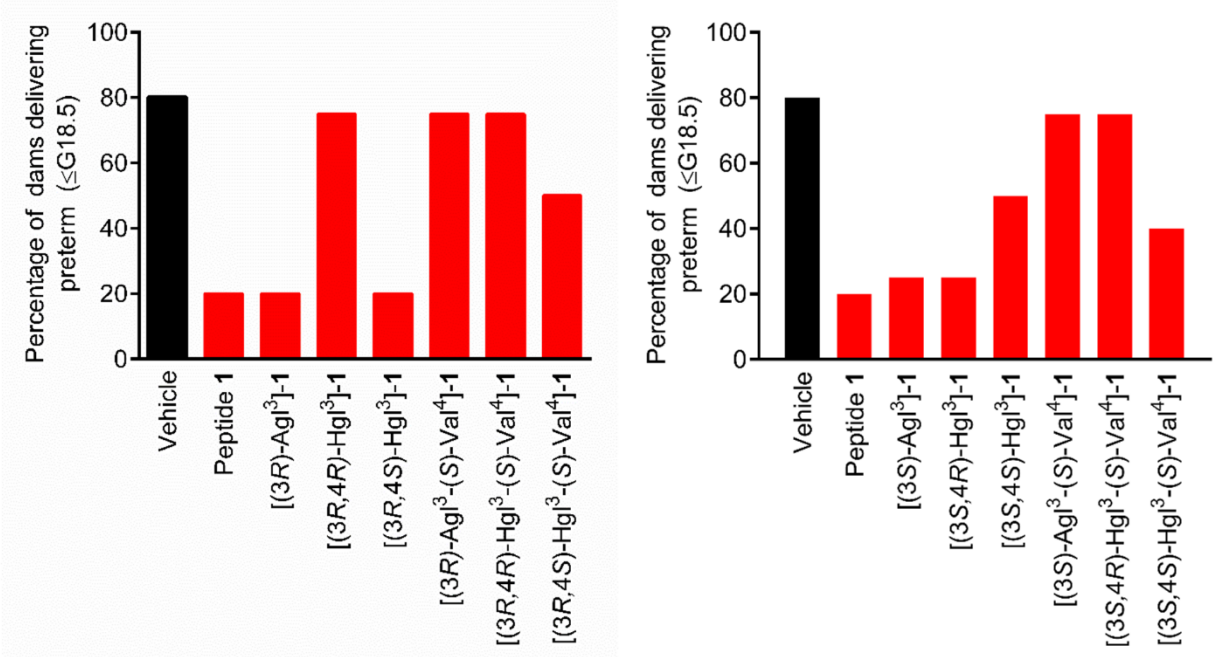


Figure 13. The effects of peptides 1, 5 and 6 on prevention of PTB. (A) Schematic of the CD-1 mouse PTB model. In brief, pregnant dams on day 16.5 of gestation (G16.5) were subcutaneously pretreated with peptides **1**, **5** and **6** or vehicle, followed by LPS, and observed for delivery of pups. A dam was considered as delivering preterm if at least one pup was delivered before G18.5. **(B)** The rates of PTB in dams treated with peptides **1**, **5** and **6** are grouped into (3*R*)- (left) and (3*S*)- (right) derivatives: n = 4-5 dams per treatment group. (Adapted from Geranurimi **2019**).

4.4 *In vivo* inhibition of vaso-obliteration in oxygen-induced retinopathy (OIR)

A model of OIR, analogous to ROP, was next used to examine peptide **1** and a subset of analogs **5** and **6** that were previously tested in the PTB model: e.g., [(3*S*)-Agl^3]-, [(3*R*)-Agl^3]-, [(3*S*,4*R*)-Hgl^3]- and [(3*R*,4*S*)-Hgl^3]-**1** which exhibited the best activity, [(3*S*,4*S*)-Hgl^3]-**1** which had partial efficacy and [3*S*,4*R*-Hgl^3-(*S*)-Val^4]-**1**, which was inactive in delaying labour.

Exposure of rat pups to 80% oxygen from days 5 to 10 of life usually results in vaso-obliteration of ~35% of the retinal capillaries, extending radially from the optic nerve (Fig. 14). Peptide **1** and Kineret both diminished the extent of vaso-obliteration to 15-20% (Fig. 14), consistent with the results of our previous study (Rivera **2013**). Among the four peptides that were strongly effective in the PTB model, only two, [(3*R*,4*S*)-Hgl^3]- and [(3*S*,4*R*)-Hgl^3]-**1** exhibited efficacy in the OIR model and reduced vaso-obliteration to 15-25%. Furthermore, [(3*S*,4*S*)-Hgl^3]- **1**, which was moderately effective (~50% efficacy) in the PTB model, demonstrated efficacy in reducing vaso-obliteration to a somewhat smaller extent than peptide **1**. On the other hand, [(3*S*,4*R*)-Hgl^3-(*S*)-Val^4]-**1**, which had no effect in the PTB model, was similarly ineffective in curbing vaso-obliteration.

Immuno-histochemical staining for Iba-1 was used to assess microglial activation and density, because microglia have been shown to be mediators of vaso-obliteration in the context of OIR (Rivera **2013**). The morphology of microglia has been observed to change at depending on its state of activation: microglia that are inactive and ramified possess numerous processes (branches), which were observed in retina under normoxia and after treatment with peptide **1** (Fig. 15); on the other extreme, activated microglia retract their limbs and become amoeboid (Donat **2017**), as observed in vehicle-treated

retina under hypoxia (Fig 15). Peptides [(3*S*,4*R*)-Hgl[3]]-, [(3*R*,4*S*)-Hgl[3]]- and [(3*S*,4*S*)-Hgl[3]]-**1** partially prevented the activation of microglia; [(3*S*)-Agl[3]]-, [(3*R*)-Agl[3]]- and [(3*S*,4*R*)-Hgl[3]-(*S*)-Val[4]]-**1** had no appreciable effect on microglial morphology (Fig. 15). Quantification of microglial density revealed similar trends (Fig. 15), except for [(3*S*)-Agl[3]]-**1**, which modestly (<20%) reduced microglial density despite not influencing microglial morphology. Peptides **1**, **5** and **6**, which prevented microglia activation, caused a statistically significant reduction in vaso-obliteration area. In summary, certain [Hgl[3]]-**1** analogs behaved like the parent peptide and exhibited protection against vaso-obliteration in the hyperoxic phase of OIR, due at least in part to mitigation of microglial activation.

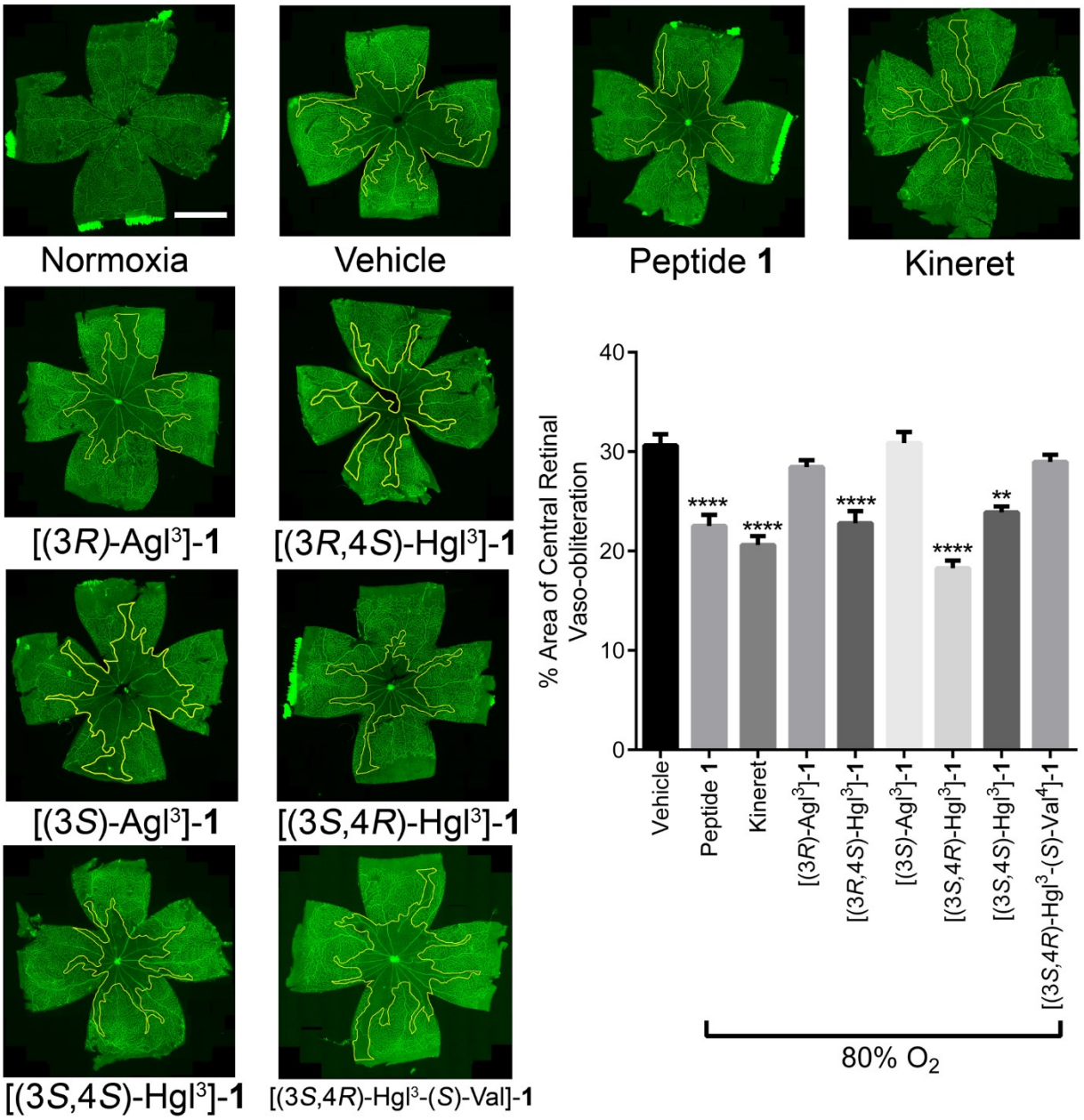


Figure 14. The preventive effects of peptides 1, 5 and 6 against vaso-obliteration in an OIR model. Five-day old Sprague Dawley pups and their mothers were kept in 80% oxygen until the tenth day of life, receiving twice-daily intraperitoneal injections of peptides **1**, **5** and **6** (2mg/kg/day), Kineret (15mg/kg/day) or PBS vehicle (each injection was titrated to a volume of 20 µL). **(A)** Representative retinal flatmounts stained with FITC-conjugated *Bandeiraea simplicifolia* lectin, taken at 10X magnification and stitched with MosaiX in Axiovision 4.8. Yellow lines indicate the central area of vaso-obliteration extending from the optic nerve (centre of each retina), scale bar 2 mm. **(B)** Quantification of area of vaso-obliteration performed using ImageJ, expressed as a percentage of the total retinal area: n = 5-7 of peptides **5** and **6** and Kineret, n = 10-12 for vehicle and peptide **1**; ***p*<0.01, *****p*<0.0001 relative to the vehicle group. Treatment groups that are not labelled with asterisks are statistically non-significant compared to the vehicle group. (From Geranurimi **2019**)

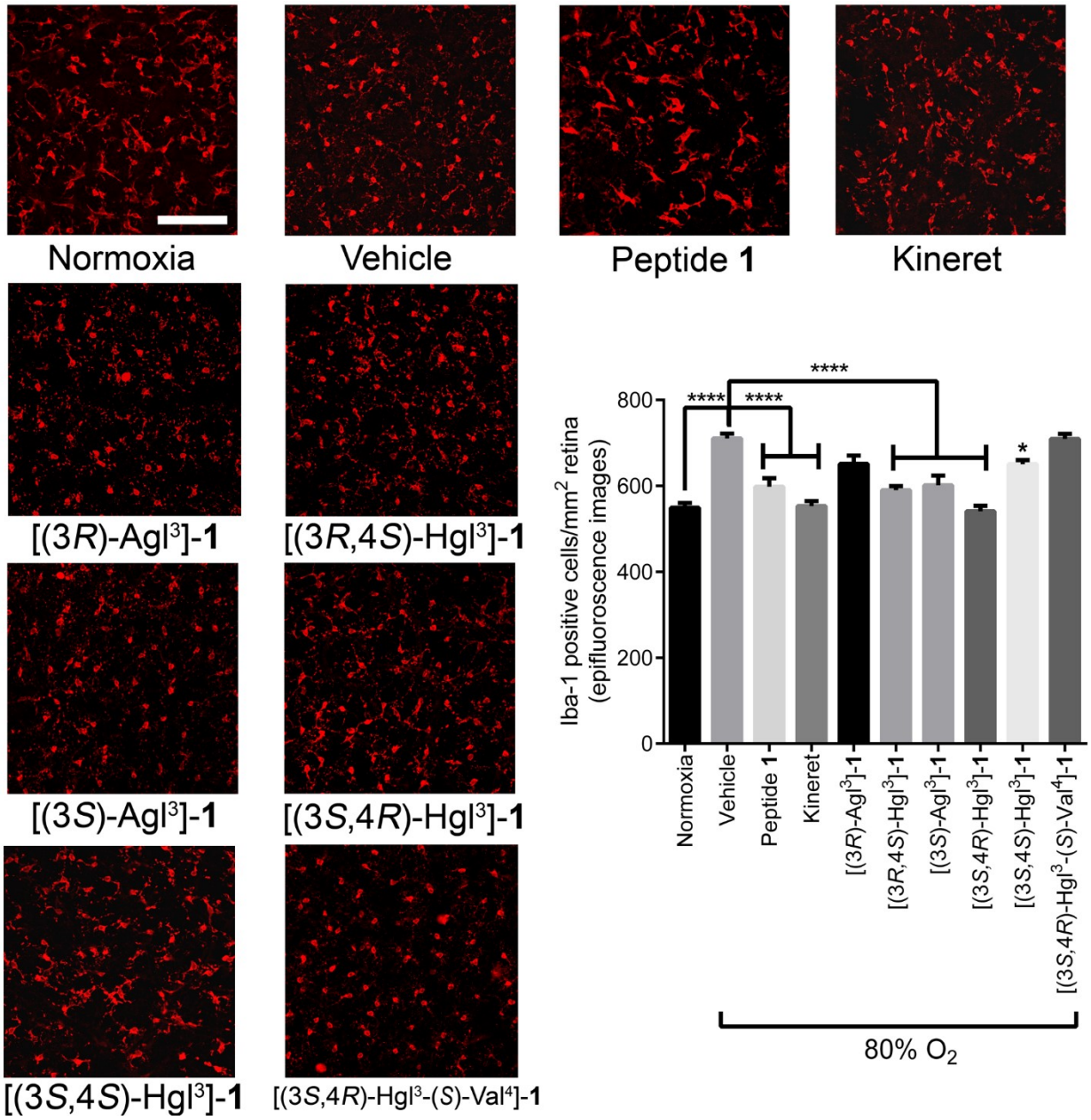


Figure 15. The effects of peptides 1, 5 and 6 on retinal microglial activation and density. Retinas were obtained for immunohistochemistry from rat pups treated with the OIR protocol, and incubated with rabbit anti-iba-1 antibody, followed by donkey anti-rabbit antibody conjugated to Alexa 594. **(A)** Representative confocal images of retinal microglia at 30X magnification: scale bar 100 µm. **(B)** Epifluorescence microscopy images at 20X magnification of retinal microglial density quantified using ImageJ: 4 images per retina were taken at a distance halfway between the optic nerve and the peripheral edge of the retina; n = 5-7 for peptide **1**, **5** and **6**, and Kineret; n = 8-10 for normoxia and vehicle; *p<0.05, ****p<0.0001 relative to the vehicle group. Treatment groups that are not labelled with asterisks are statistically non-significant compared to the vehicle group. (From Geranurimi **2019**)

Chapter 5:
Discussion

Table 3. Heatmap summary of the *in vivo* and *in vitro* effects of peptides 1, 5 and 6.

		Western Blot			qPCR			NF-κB	*In vivo*	
	Structure	JNK	p38	ROCK2	COX2	IL-1β	IL-6		PTB	OIR
	[(3*R*)-Agl^3]-**1**	4	0	0	3	4	3	0	4	0
	[(3*R*,4*R*)-Hgl^3]-**1**	0	0	4	4	4	4	0	0	
	[(3*R*,4*S*)-Hgl^3]-**1**	4	4	4	4	4	4	0	4	4
	[(3*R*)-Agl^3-(*S*)-Val^4]-**1**	0	4	2	1	1	1	0	0	
	[(3*R*,4*R*)-Hgl^3-(*S*)-Val^4]-**1**	1	3	4	4	3	4	0	0	
	[(3*R*,4*S*)-Hgl^3-(*S*)-Val^4]-**1**	2	2	2	4	4	2	0	2	
	[(3*S*)-Agl^3]-**1**	4	0	0	4	4	2	0	4	1
	[(3*S*,4*R*)-Hgl^3]-**1**	3	3	3	4	4	4	0	4	4
	[(3*S*,4*S*)-Hgl^3]-**1**	1	1	3	4	4	4	0	2	3
	[(3*S*)-Agl^3-(*S*)-Val^4]-**1**	0	2	3	1	1	1	0	0	
	[(3*S*,4*R*)-Hgl^3-(*S*)-Val^4]-**1**	0	3	3	3	1	3	0	0	0
	[(3*S*,4*S*)-Hgl^3-(*S*)-Val^4]-**1**	2	0	4	4	4	4	0	2	
Peptide **1**		4	4	4	4	4	4	0	4	4
Kineret		4	4	4	4	4	4	4	0	3

Black = not tested

No effect 0 1 2 3 4 Maximum inhibition/efficacy

PTB and ROP are medical conditions strongly associated with dysregulated inflammation, with IL-1β playing critical roles. Current treatments for PTB, such as oxytocin antagonists and indomethacin, aim to reduce myometrial contractility (tocolysis) but fail to address the underlying inflammatory processes responsible for labor (Olson **2008**). Current ROP treatments employ anti-VEGF antibodies and laser photocoagulation (Hellström **2013**) which treat the proliferative neovascular phase of the disease but fail to address earlier stage vaso-obliteration and its associated inflammation. Modulation of IL-1R signaling offers the potential to mitigate both PTB and ROP as indicated by the *in vivo* results herein and previously reported (Rivera **2013**; Nadeau-Vallée **2015** and **2017**; Beaudry-Richard **2018**).

The effects of our derivatives were assessed in two well-established models of OIR and PTB. While several animal models are available for both pathologies, the CD-1 mouse model was chosen for PTB on the basis that mice are more vulnerable to LPS-induced PTB compared to rats (Elovitz **2004**), and that rodents are similar to humans in that they have a hemochorial placenta (Benirschke **2012**). The Sprague Dawley rat model with exposure to 80% O_2 was utilized as it reliably produces severe vaso-obliteration of the retina; another advantage of using rodents to study ROP is that the retinal vasculature develops after birth in rodents, like that of preterm neonates but not term neonates (Gammons **2016**).

In the *in vitro* assays, peptide **1** exhibited inhibitory activity on the phosphorylation of the three kinases (JNK, p-38 and ROCK2) and on the transcription of downstream pro-inflammatory genes that are mediated by IL-1β (IL-6, COX2 and IL-1β), but did not affect

NF-κB signaling. Moreover, peptide **1** significantly delayed LPS-induced PTB in mice and reduced vaso-obliteration in the OIR model.

Conformational constraint of peptide **1** was performed by replacing (2*R*,3*S*)-Thr^3-(*R*)-Val^4 with all four possible Agl^3-Val^4 (e.g., **5**) and eight possible Hgl^3-Val^4 (e.g., **6**) diastereomers. Among the constrained analogs, [(3*R*,4*S*)-Hgl^3]-**1** exhibited the most similar activity as the parent peptide in the *in vitro* and *in vivo* assays with slightly reduced potency in inhibiting JNK. Moreover, [(3*S*,4*R*)-Hgl^3]-**1** was also typically as potent as **1** but had slightly reduced abilities in inhibiting p38 and ROCK2, both isomers possess *trans*-Hgl residues and (*R*)-Val stereochemistry indicating the importance of the β-hydroxyl group and gauche-(–) χ-dihedral angle side chain geometry for maximal activity.

The contrast of high potency and inactivity exhibited respectively in the PTB and OIR models by both [(3*R*)-Agl^3]- and [(3*S*)-Agl^3]-**1** correlates with their ability to block JNK without inhibitory potency on p-38 and ROCK2. The importance of the hydroxyl group for activity on the latter kinases and for ability to reduce vaso-obliteration in the OIR model is illustrated by the potency of the corresponding Hgl analogs. Notably, [(3*S*,4*S*)-Hgl^3]-**1** which positions the hydroxyl group in a gauche-(+) χ-dihedral angle side chain orientation maintains some potency on all three kinases with best activity on ROCK2 and exhibits respectively moderate and strong activities in the PTB and OIR models.

Notably, the modifications that we have made to the Thr^3 and Val^4 residues in peptide **1** are biologically relevant, because they result in discernable changes to *in vivo* and *in vitro* activity. This observation is in support of previous findings that a fold in the central residues (i.e. Thr^3 and Val^4) of 101.10 is important for its biological activity (St-Cyr **2010**).

When drawing connections between *in vivo* and *in vitro* activity, we note that some inhibitory activity on JNK appears necessary for potency in delaying labor in the PTB model. For example, [(3*R*,4*R*)-Hgl[3]]-**1**, which inhibited strongly ROCK2, but had no effects on JNK and p-38, was inactive in the PTB model. The weaker potency in the PTB assay of the (*S*)-Val analogs correlated with their lack of inhibitory activity on JNK. Although (*R*)- instead of (*S*)-Val may be a prerequisite for binding IL-1R, the latter may also be more susceptible to enzymatic cleavage by proteases (Carmona **2013** and Najjar **2017**).

Correlations between peptide structure, *in vitro* activity and *in vivo* potency highlight the relative importance of blocking specific IL-1 signaling pathways to treat certain pathologies (Table 3). For example, inhibition of JNK phosphorylation was most strongly correlated with effectiveness in PTB inhibition. In agreement with an earlier study in which the use of a specific JNK inhibitor had delayed PTB which was induced by a type of LPS that activated both NF-κB and JNK pathways (Pirianov **2015**), blocking JNK phosphorylation alone was sufficient for PTB prevention. On the other hand, both JNK and ROCK2 phosphorylation were required for attenuation of vaso-obliteration in the OIR model. The latter was in concordance with a study demonstrating the utility of specific ROCK inhibitors an *in vivo* model of OIR (Yamaguchi **2016**). The inactivity of specific compounds may be due to their pharmacokinetic profiles and would require further study to address such issues. For example, delivery of the peptide to its site of action may play a role in efficacy; drug entry into the retina is more challenging than the myometrium, due to the presence of a blood-retina-barrier that limits entrance from systemic circulation (del Amo **2017**) and may account for the enhanced activity of the Hgl relative to the Agl analogs in the OIR model.

Peptide **1** was previously shown to bind to IL-1R (Quiniou **2008**). Most of peptides **5** and **6** displaced radio-labelled **1** to the same extent as cold peptide **1**, suggesting they all compete for the same binding site on IL-1R, though not necessarily with the same binding affinity. The sole exception was [(3*R*,4*S*)-Hgl[3]]-**1**, which displaced radio-labelled **1** to a lesser extent despite being the most efficacious molecule *in vitro* and *in vivo* (Table 2). This contradiction may be due to a different IL-1R binding pattern that retains desirable biased signaling and may require crystallographic analyses to confirm this hypothesis.

Compared to the larger proteins and non-selective competitive inhibitors currently used in anti-IL-1 therapies, peptides **1**, **5** and **6** may offer benefits such as ease of administration, as well as potential to reduce immunosuppression, immunogenicity and related side effects. The small peptides may be further optimized for oral administration, in contrast to antibodies, which must be administered by injection predisposing patients to potentially unpleasant injection-site reactions leading to reduced patient compliance. Notably, anti-VEGF antibodies, a mainstay of ROP treatment, must be injected directly into the vitreous of the eye (i.e., intravitreally), a technique that is invasive, technically demanding and poses the risks of vision-threatening infections such as endophthalmitis (del Amo **2017**).

To date, there are no clinically-approved allosteric inhibitors of IL-1 or its receptor. The anti-IL-1β antibody Gevokizumab, which is currently under investigation, has been reported to bind to an allosteric site on IL-1β (Blech **2013,** Issafras **2014**); however, like other antibodies it may be associated with similar drawbacks including large molecule size and high production costs. Hence, small-molecule allosteric modulation of IL-1R remains an elusive yet important unmet therapeutic need.

Chapter 6:
Conclusions & Future Directions

The current study highlights the utility of targeting IL-1 signaling to treat conditions such as PTB and ROP. It is also, to the best of our knowledge, the first known demonstration of the use of conformational constraint to create folded synthetic peptides in the context of inflammation and cytokine signaling. While all our derivatives and peptide **1** did not inhibit NF-κB, they varied in their ability to suppress the expression of pro-inflammatory factors and the phosphorylation of p38 MAPK, JNK and ROCK2. This means that our peptides could serve as useful probes to identify specific pro-inflammatory signaling pathways in the context of a variety of diseases. They also offer benefits over traditional protein-based anti-IL-1 therapies, such as avoidance of immunosuppression and easier administration, and are promising leads for the field of immunomodulatory therapeutics.

Given that these results have shed light on the importance of the hydroxyl-group in the Hgl^3 residue and (*R*)-Val^4 stereochemistry for *in vivo* and *in vitro* activity, it would be useful to utilize these data for *in silico* analysis of drug-ligand interactions. This is aided by the fact that the crystal structures of the IL-1 receptor complex have previously been elucidated (Thomas **2012**, Wang **2010**, Vigers **1997**). The combination of *in silico* analysis with our biological experiments would allow for the optimized design of the next generation of IL-1 receptor modulators and reduce the amount of labour-intensive trial-and-error in our future drug design regimens.

With regards to the lead molecule 101.10 and its best derivatives, several important hurdles must be overcome before clinical testing. Firstly, it will be important to test several promising candidate molecules in non-human primates, which are more physiologically relevant to humans than the rodents used in this study. Moreover, the

distribution of our peptides in the placenta, fetus and retina should also be assessed using fluorescent-labelled peptides to determine their ability to pass through the blood-retina-barrier and membranes; this has been previously done with the lead compound 101.10 (Rivera **2013**). It also necessary to determine the pharmacokinetic profiles (eg. IC_{50}, K_d, half-life) of our peptides using radioligand assays or surface plasmon resistance experiments. Given the role of IL-1 in numerous other diseases such as type 2 diabetes (Park **2017**) and inflammatory bowel disease (Quiniou **2008a**), our derivatives could be tested in other *in vivo* models to determine the relative contributions of IL-1 signaling pathways to these pathologies.

In summary, while 101.10 continues to be a promising molecule, future studies are still required to consolidate information on its pharmacokinetics and pharmacodynamics before it can enter clinical testing.

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