INVESTIGATING THE FDA'S APPRAISAL OF PRECLINICAL STUDIES IN NEW DRUG APPLICATIONS FOR PAIN INDICATIONS AND ITS IMPLICATIONS FOR PEPDUCIN P4PAL-10

by

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Submitted in partial fulfilment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
August 2023

Dalhousie University is located in Mi'kma'ki, the
ancestral and unceded territory of the Mi'kmaq.
We are all Treaty people.

For Micha and Thyme

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Abstract

PURPOSE: Musculoskeletal diseases are responsible for a large proportion of disabling conditions, with chronic pain being the primary concern for patients. Current drug options are limited and often associated with adverse side effects. In the area of arthritis treatment, the proteinase-activated receptor-4 (PAR4) antagonist pepducin P4pal-10 has emerged as a promising target. It has been shown previously that pepducin P4pal-10 can reduce joint pain and inflammation in rodent models of arthritis. The preparatory goal of this project was the confirm the analgesic capacity of pepducin P4pal-10 in the Freund's Complete Adjuvant (FCA) model of inflammatory joint disease.

The United States Food and Drug Administration (FDA) generally require pharmaceutical companies to submit animal studies as part of their New Drug Application (NDA). By understanding the FDA decision-making at the pre-clinical stage, it can help us to identify salient aspects which can then be applied to inform the developmental trajectory of pepducin P4pal-10 and its likelihood as a candidate compound for the treatment of pain and inflammation in osteo- and rheumatoid arthritis. This study investigated: (1) if the FDA performed a robust analysis on the validity and reliability of pre-clinical studies submitted as part of an NDA? (2) if there is a difference in the amount of discussion given to pre-clinical studies during the review process for a drug's first indication compared to subsequent indications? (3) if the preclinical studies for pepducin P4pal-10 have high validity and reliability?

METHODS: The FDA approval package for duloxetine and pregabalin was obtained from the Drugs@FDA webpage. Data related to pre-clinical pharmacology for pain indications were extracted from reading the documents and keyword search. All the animal studies discussed or referenced in the regulatory reports were collected to assemble a package of analyzable material for each drug.

A rubric to evaluate the validity and reliability of preclinical research was developed. Each pre-clinical study received a score for "Study Design-Validity" and "Study Design-Reliability". While the publications were analysed one at a time, the overall idea is that the rubric is applicable to the drug, not the individual publications. An overall quantitative measure of validity and reliability for the drug could then be determined.

RESULTS: The Freund's Complete Adjuvant (FCA) model of rat inflammatory joint pain resulted in demyelination of the saphenous nerve at day 21. Myelin thickness was significantly different in the large diameter axons but not the small diameter axons in the FCA animals compared to the control group (one-way ANOVA, $p<0.001$, $n = 254$-392 fibres from 10-12 animals per group). Treatment with pepducin p4pal-10 caused a reversal of secondary allodynia at day 7 and day 21 post-FCA induction (2way RMANOVA, $p<0.0001$). Deficits in hindlimb weightbearing was not attenuated by pepducin p4pal-10 treatment at day 7 and day 21 (2way RMANOVA, ns).

An analysis of pre-clinical studies for pregabalin and duloxetine revealed that the study design had high validity and moderate reliability. The FDA reviewers considered these studies to be pivotal experiments, however, there was minimal evidence of the FDA evaluating the rigour of those studies. Although the raw data from all the pre-clinical studies were submitted to the FDA, only a summary of the main findings were published in the regulatory reports. The FDA appeared to have taken the conclusions drawn by the study authors at face value, without any regard for the validity or reliability of the study design. These results, together with the high validity and reliability of related studies examining P4pal-10 in preclinical models of joint disease, suggest that pepducin P4pal-10 is a promising compound for the treatment of joint pain.

List of Abbreviations and Symbols Used

5-HT 5-hydroxytryptamine

ALSDAC Arthritis and Life Support Drugs Advisory Committee

ANOVA One-way analysis of variance

ARRIVE Animal Research: Reporting of In Vivo Experiments

CDER Center for Drug Evaluation and Research

CFR Code of Federal Regulations

CGRP Calcitonin gene-related peptide

CHMP Committee for Medicinal Products for Human Use

CIA Collage-induced arthritis

CNS Central nervous system

COS Center for Open Science

DACCADP Division of Anesthetics, Critical Care and Addiction Drug Products

DPN Diabetic peripheral neuropathy

DRG Dorsal root ganglion

E-CAC Executive Committee for Animal Care

ECL Extracellular ligand

EMA European Medicines Agency

EOP2 End of phase 2

FCA Freund's Complete Adjuvant

FDA Food and Drug Administration

GABA gamma-aminobutyric acid

GPCR G-protein coupled receptor

IASP International Association for the Study of Pain

ICL Intracellular ligand

IL-17 Interleukin-17

IL-2 Interleukin-2

IL-6 Interleukin-6

IND Investigational New Drug

LORA Late-onset rheumatoid arthritis

MDD Major Depressive Disorder

MIA Monoiodoacetate

MMT Medial meniscus transection

NCPR Non-clinical pharmacology report

NDA New Drug Application

NE Norepinephrine

NIH National Institute of Health

NMEs New Molecular Entities

NSAIDs Non-steroidal anti-inflammatory drugs

OA Osteoarthritis

OSF Open Science Framework

PAG Periaqueductal gray

PAR4 Proteinase-activated receptor-4

PARs Proteinase-activated receptors

PGE2 Prostaglandin E2

PHN Post-herpetic neuralgia

PPRECISE Preclinical Pain Research Consortium for Investigating Safety and Efficacy

RA Rheumatoid Arthritis

RMANOVA Two-way repeated measures analysis of variance

RSAA Reduction of Spontaneous Activity by Adjuvant

RVM Rostral ventromedial medulla

sNDA Supplemental New Drug Application

TGA Therapeutic Goods Association

TNF-α Tumor necrosis factor alpha

TRP Transient receptor potential channels

VGSCs Voltage-gated sodium channels

YLDs Years of healthy life lost to disability

YLLs Years of life lost to premature mortality

YORA Young-onset rheumatoid arthritis

Acknowledgements

I would like to thank my supervisors Dr. Jason McDougall and Dr. Matthew Herder for their guidance and support throughout my time in graduate school. Your thoughtful suggestions have been instrumental towards the conceptualization and completion of this thesis.

Many thanks to the members of my advisory committee, Dr. Chris Sinal, Dr. Jim Fawcett, Dr. Denis Dupre, for the constructive feedback which have contributed greatly to the actualization of this research project.

I am grateful to the members of the McDougall Lab, Alison Reid, and Melissa O'Brien, for their vital assistance with the technical aspects of this project.

I would like to thank all the members at the Department of Pharmacology for creating a nurturing environment. Special thanks to Luisa Vaughan, Lori Lawson, Sandi Leaf, Sidney Norman, and Tianqi Liu, for taking care of all the administrative tasks essential for the graduate program to operate smoothly.

Finally, I need to thank my family and friends for their unwavering support throughout my studies.

Chapter 1 Introduction

1.1 Overview of Arthritis

Arthritis refers to a group of over 100 diseases characterized by inflammation and pain in the moveable joints or other regions of the body. The most prevalent forms of arthritis are osteoarthritis (OA) and rheumatoid arthritis (RA). OA is caused by biomechanical wear and tear of the joints while RA is an auto-immune disease. The Global Burden of Diseases report published in 2019 estimated a global incidence of 528 million and 36.8 million people living with OA and RA respectively (IHME, 2020; Almutairi et al, 2021). Arthritic diseases have a significant disease burden, as they are a major cause of years lost to premature mortality (YLLs) and years of healthy life lost to disability (YLDs) (World Health Organization, 2019).

Despite an urgent clinical need, few pharmacological therapies exist to treat joint pain and most focus on symptoms such as pain. RA is often treated with NSAIDs, corticosteroids, and most recently biologics such as infliximab and tocilizumab which target TNF-α and IL-6 respectively (Kukar et al, 2009). Antidepressants and anti-epileptics are often used to treat fibromyalgia and other chronic neuropathic pain conditions such as diabetic peripheral neuropathy (Maizels & McCarberg, 2005). These pharmacological options for the management of joint pain have varying effectiveness and many have adverse side-effects when used over an extended time. New pharmacological tools to treat pain effectively are greatly needed, along with a more nuanced understanding of the mechanisms underlying complex pain states.

Rheumatoid arthritis is the most common form of inflammatory joint disease (Romao and Fonseca, 2021). It is a multifactorial disease and much remains unknown about its precise aetiology. Generally, a combination of genetic and environmental risk factors for RA is associated with the development of systemic autoimmunity. The incidence of RA is about three times higher

in women than in men. Women also suffer from a higher rate of premature mortality and disability arising from RA (Almutairi et al, 2021). The sex imbalance is typically attributed to the predisposing effects of pro-inflammatory estrogens, accompanied by a decrease in the levels of progesterone and androgens which can exert anti-inflammatory effects, resulting in an overall net effect of systemic inflammation (Romao and Fonseca, 2021).

The serine proteinases are a group of chemical mediators known to contribute to disease progression in arthritis. Serine proteinases are found at elevated levels within arthritis joints and implicated in the catabolic destruction of joint tissue, resulting in inflammation and pain (Lucena and McDougall, 2021). Blocking of serine proteinases in several preclinical and clinical studies has demonstrated analgesic effects (McDougall et al, 2009; Lucena & McDougall, 2021; McDougall & O'Brien, 2021). An example of a serine proteinase is the proteinase-activated receptor 4 (PAR4). PAR4 signalling can be blocked by a PAR4 antagonist such as pepducin P4pal-10. Pepducins are lipidated peptides that were developed to specifically inhibit many G-protein-coupled receptors (GPCRs) including serine proteinases, for example, proteinase-activated receptor-4 (PAR4) (Zhang et al, 2015). The opening aspect of this study explores the efficacy of pepducin P4pal-10 in modulating RA pain in a preclinical model of RA. and the impact of preclinical studies on regulatory decision-making, to determine the likelihood of pepducin P4pal-10 as a candidate compound for the treatment of pain and inflammation associated with arthritis.

1.2 Animal Models of Inflammatory Joint Disease

Animal models serve a crucial role in furthering our understanding of the pathophysiological pathways underlying inflammatory joint disease and for the preclinical evaluation of therapeutic agents. Rodents such as rats and mice are the most widely utilized animal

species due to practical reasons arising from genetic homogeneity, cost, and reproducibility. The animal models may involve spontaneous emergence of arthritis e.g., TNF-transgenic mice or as inducible models in susceptible strains e.g., CFA.

1.2.1 TNF-transgenic Mouse Model

The TNF-transgenic mouse model expresses a modified human TNF gene lacking post-transcriptional regulatory elements. It provides compelling evidence of TNF involvement in inflammatory arthritis. There are many strain variations available: mouse strains may have a single copy or multiple copies of the TNF transgene. The commonly used 3647-strain TNF-transgenic mouse (single copy of the TNF transgene) has a later onset of joint inflammation in comparison to multiple copy strains (6 to 8 weeks vs. 3 to 4 weeks), slower disease progression, and increased lifespan (Muley et al, 2015). Due to the features of late-onset and slow disease progression, the single-copy TNF-transgenic mouse model is well suited for studies investigating the preclinical stages of disease and progression towards chronicity. While the TNF-transgenic model is not driven by autoimmunity, many of the histopathological findings in human RA and systemic inflammation are present in the model (Muley et al, 2015). Overall, the TNF-transgenic mouse model is a good model for the study of TNF-induced inflammatory pathways implicated in human RA.

1.2.2 Freund's Complete Adjuvant Model

Chronic inflammatory arthritic pain is typically induced in susceptible rat strains e.g., Lewis or Wistar rats by an intra-articular administration of Freund's Complete Adjuvant (FCA) (Muley et al, 2015). FCA is a mixture of heat-killed *Mycobacterium tuberculosis* emulsified in

paraffin oil. Shortly after intra-articular injection of FCA, localized edema, thermal, and mechanical hyperalgesia are evident in the ipsilateral joint. Tissue swelling arising from immunological mechanisms occur seven days after FCA injection. Pain experiments using the FCA paradigm typically allow for treatment to occur during the prophylactic phase (day 0-8) and therapeutic phase (post-day 8). FCA also induces articular hypoxia, which models the low oxygen environment commonly found in joints affected by RA. Previous studies have also revealed that FCA triggers the production and release of several pro-inflammatory mediators including nitric oxide, leukotriene B2, PGE2, TNF-α, IL-2, and IL-17 (Schinnerling et al, 2019). These pro-inflammatory cytokines cause joint inflammation, synovitis, and damage to surrounding bone leading to joint degeneration (Bendele, 2001). These pro-inflammatory mediators also induce joint pain by causing peripheral sensitization.

The extra-articular features of the FCA model closely resemble human RA. However, the severity of cartilage damage arising from FCA is much lower than in human RA, thus not a good representation for the study of cartilage-related aspects of RA. FCA is considered one of the more suitable models for investigating inflammatory joint pain due to the robust nociceptive responses displayed in this model. The joint FCA model has been used widely in industrial and academic research labs to study novel analgesics and promising anti-arthritic molecules.

1.3 Methods of Assessing Joint Pain in Animal Models

Assessment of pain in animals is experimentally and conceptually complex. A significant amount of research has been conducted to standardize protocols, reduce subjectivity, and expand the scope of measurable responses from solely sensory to the inclusion of psycho-affective aspects.

Two of the most frequently used methods to assess joint pain are pain-evoked behaviours and pain-suppressed behaviours.

1.3.1 Pain-evoked Behaviours

Assessment of mechanical pain behaviours in animal models can be measured by paw withdrawal thresholds in response to application of noxious or non-noxious tactile stimuli. The most frequently used mechanical stimulus for this type of assessment in rodents involves the application of Von Frey hairs which are nylon filaments that bend at a predefined force. Von Frey hairs of increasing stiffness are applied onto the plantar surface of the hind paw to quantify the mechanical threshold required to trigger paw withdrawal. As the knee joint was the primary site of tissue injury and inflammation in these studies, it is important to note that von Frey algesiometry measures referred pain or secondary pain. A lower threshold required to trigger evoked responses is indicative of allodynia and hyperalgesia. This approach is limited by the potential for high inter-rater variability due to nuances in the interpretation of paw withdrawal responses.

Spontaneously occurring pain is a common feature across many inflammatory diseases. Assessing non-evoked pain behaviour such as hindlimb weight bearing allows for greater insight into spontaneous pain. Hindlimb incapacitance is an example of a technique used to quantify spontaneous pain, where differences in weight-bearing between a diseased versus “normal” hindlimb is assessed. Rodents typically distribute their body weight equally between their hindlimbs. After the induction of inflammation in one hindlimb, rodents start to put more weight on the non-inflamed limb. The change in weight bearing can be calculated in stationary animals (static weight bearing) and moving animals (dynamic weight bearing). Hindlimb incapacitance assessments are useful for measuring differences in weight-bearing arising from models of

inflammatory arthritis, osteoarthritis, neuropathic pain etc. However, the technique is limited by the fact that repeated testing may affect pain behaviours (habituation).

1.3.2 Pain-suppressed Behaviours

Pain-suppressed behaviours are defined as a decrease in healthy behaviours (e.g., feeding, exploration, grooming) after exposure to a noxious stimulus. Evidence of pain-suppressed behaviours can be measured by a reduction in locomotor activity. Reduced locomotor activity occurs concurrently with pain-like states in rodent models of neuropathic and inflammatory pain (Urban et al, 2011). For example, less exploration was observed in the TNF-transgenic mice model of RA. Measurement of locomotor activity with an assay like the Reduction of Spontaneous Activity by Adjuvant (RSAA) model can be useful to preclinically simulate the reduced physical activity of RA patients (Matson et al, 2010).

1.4 Conundrums in Pharmaceutical Innovation

Despite unprecedented investment in pharmaceutical innovation over the past twenty years, the number of new drugs approved by the Food and Drug Administration (FDA) has been relatively constant since the 1950s (Munos, 2009; Morgan et al, 2011). This is especially true in the context of pain where very few new molecular entities (NMEs) with analgesic properties have entered clinical trials or received FDA approval in recent years. Most newly approved analgesics are an improvement on a previously approved active ingredient, reformulated as a combination drug or involving a novel drug delivery mechanism. Such repackaged drugs typically rely greatly upon the New Drug Application (NDA) for the reference drug and may only need to submit a minimal amount of information demonstrating the safety of the new formulation. In comparison,

it is certainly a more intensive process for an NME to gain regulatory approval as substantial evidence of safety and efficacy is required for its Investigation New Drug (IND) and NDA submissions. This led me to wonder about the drug development process for an NME and the factors that may help or hinder its progression toward regulatory approval.

Research and development for an NME involves a significant amount of preclinical research. Promising results from those preclinical studies may then lead to the next steps of drug development such as an IND submission. However, there is a 90% failure rate for drug candidates entering phase 1 clinical trials (Sun et al, 2022). Tremendous attention has been devoted towards ensuring rigorous design and thorough analysis of clinical trial data, evidenced by clinical trial information being publicly disclosed in regulatory reports published by the FDA. Pharmacological and toxicological preclinical data are essential components of a candidate drug's IND and NDA submission; however, no studies have been conducted to examine their impact on regulatory decision-making. Therefore, this led me to question that rigour in preclinical study design may be lacking and could possibly be a contributor to failure in drug development.

1.5 Pain and Rheumatoid Arthritis

Pain is a primary concern for people living with arthritis. Pain management is frequently a reason for patient visits to their primary care physician. Arthritic pain is often poorly managed due to a limited understanding of the underlying pain mechanisms in RA and the use of analgesics that inadequately address the disease (Walsh & McWilliams, 2014). RA is also known to be a rather heterogeneous disease, which may be an additional factor contributing to poor pain control as each type of pain may require a specific intervention for effective management.

1.5.1 Neurobiology of Pain

Pain is a major problem globally. According to the International Association for the Study of Pain (IASP), pain is defined as "An unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage" (Raja et al, 2020). A person's experience of pain is modulated by biological, psychological, and sociological factors. In most people, pain is experienced as a temporary experience that signals something is amiss (acute pain). Pain persisting for over three months is defined as chronic pain (Treede et al, 2019). A global estimate of one in five people across the lifespan live with chronic pain. In Canada alone, eight million people live with chronic pain. Chronic pain might be the result of an underlying disease e.g., inflammatory arthritis or may have no identifiable cause e.g., fibromyalgia.

Pain is transmitted from the periphery to the central nervous system by two main types of primary afferent fibres: myelinated A-δ fibres (fast-conducting) and unmyelinated C fibres (slow-conducting) The A-δ and C fibres are specialized forms of peripheral nerve fibres known as nociceptors (Basbaum & Jessell, 2000). Upon detection of potentially harmful stimuli, nociceptors initiate the process of transduction, converting the physical stimulus into electrical signals that are then transmitted further along the nervous system (Basbaum et al, 2009). This process involves the depolarization of ion channels such as the transient receptor potential (TRP) channels, acid-sensing ion channels (ASICs), or voltage-gated sodium channels (VGSCs) found on the surface of a nociceptor nerve fibre (Julius & Basbaum, 2001). VGSCs, e.g., $NaV_{1.7}$, $NaV_{1.8}$, and $NaV_{1.9}$, are particularly relevant to pain signalling (Emery et al, 2016). $NaV_{1.7}$ is highly expressed in nociceptors and implicated in inherited pain disorders such as paroxysmal extreme pain disorder (gain-of-function mutation) and congenital insensitivity to pain (loss-of-function mutation). $NaV_{1.8}$ is involved in the initiation and propagation of action potentials in nociceptors and has been

implicated in inflammatory pain. $NaV_{1.9}$ is involved in the initiation and maintenance of action potentials in nociceptors and has been implicated in neuropathic pain. Noxious stimuli that activate specific chemical-, thermal-, or mechanical-sensitive transducer proteins are then converted to a form of membrane depolarization known as generator potential (Bennett et al, 2019). Generator potentials higher than the threshold is amplified by VGSCs to initiate an action potential which is then propagated (Ma et al, 2019).

Once action potentials are generated, they are transmitted to the spinal cord where the nociceptor synapses with second-order nerve cells in the dorsal horn (Basbaum & Jessell, 2000). Neuropeptides are released from the nociceptor spinal terminals, e.g., calcitonin gene-related peptide (CGRP) and substance P, which sensitizes the second-order pain-transmission neurons (Gold & Gebhart, 2010). The second-order neurons cross over to the opposite side of the spinal cord, projecting towards the brainstem and thalamus. The anterolateral quadrant of the spinal cord is the main target for pain transmission. Ascending nociceptive axons may either project directly to the thalamus or the medullary reticular formation of the brain stem (Basbaum et al, 2009). At the thalamus, the pain pathway may terminate at the ventrocaudal or medial thalamus. Spinal neurons project nociceptive input directly to the ventrocaudal thalamus, which then project to the somatosensory cortex. Nociceptive spinoreticular neurons project directly to the medial thalamus, which then projects to many areas in the forebrain including the somatosensory cortex (Bausbaum et al, 2009). Hence, there are two major ascending pain pathways: directly via the lateral spinothalamic pathway and indirectly via the medial spinoreticulothalamic pathway.

Pain signalling at the somatosensory cortex may activate the descending pain modulation pathway (Zhuo, 2017). A dynamic balance between facilitatory and inhibitory mechanisms exists which are affected by pathological, behavioural, emotional, and psychological states (Kwon et al,

2014). Pain modulation begins at the periaqueductal gray (PAG) which processes the nociceptive input, then relays it to the rostral ventromedial medulla (RVM) and finally to the dorsal horn interneurons to transmit a signal for the release of endogenous analgesics, e.g., endorphins, norepinephrine, and serotonin, within the peripheral nervous system (Ren & Dubner, 2009).

1.5.2 Pain Mechanisms in RA

Multiple pain mechanisms contribute to RA pain: joint damage, peripheral sensitization, and central sensitization. Radiographic studies assessing joint degeneration or structural changes in RA patients revealed that joint damage makes a relatively small contribution to RA pain (McWilliams & Walsh, 2017). Joint replacement surgery appears to affect pain by lowering nociceptive drive and reducing synovitis. Improvements in central pain processing may also arise, such as in cases of OA, however, this has not yet been conclusively known to also occur in RA.

Damage to peripheral nerves can directly cause neuropathic pain in the absence of extraneous tissue damage or nociceptive input. Examples of such pain include multiple sclerosis and radicular sciatica. Peripheral neuropathies such as compression (carpal tunnel syndrome), comorbidities (diabetes mellitus), or drug treatments as frequently associated with RA pain. About 56-67% of RA patients describe neuropathic-like symptoms as measured using the painDETECT questionnaire (Walsh & McWilliams, 2014). However, there is an overlap between neuropathic pain and actual neuropathology which cannot be distinguished by the painDETECT scale, so it is unclear which pain mechanisms may be specifically driving neuropathic pain in RA.

Persistent nociceptive input results in changes to central pain processing, such that nociceptive input is heightened after the local sensitization of peripheral afferent fibres at the joint. Synovitis is linked to the accumulation of cytokines and other pro-inflammatory compounds which

lead to the sensitization of peripheral nerves (Schaible et al, 2010). Within the CNS, immune cells like glial cells are directly responsible for the development and maintenance of central sensitization through the production of inflammatory cytokines e.g., IL-1β.

1.6 Proteinase-Activated Receptors

Proteinase-activated receptors (PARs) are a group of G protein-coupled receptors (GPCRs) that are activated by the proteolytic cleavage of the N-terminal sequence, exposing a new amino-terminal sequence which acts as a tethered ligand. The tethered ligand binds then binds to a conserved sequence located at the extracellular loop. This results in the activation of intracellular pathways and conformational changes to the PAR. Four types of PARs have been discovered: PAR1, PAR2, PAR3, and PAR4. Synthetic activating peptides that act as PAR agonists have been developed and used to study the pharmacology of the PAR receptors e.g., PAR4 activating peptide AYPGKF-NH2 (Rudinga et al, 2018). Pepducins are another class of compounds used to study GPCRs including PARs (Zhang et al, 2015). Pepducins are cell-penetrating peptides that can rapidly enter the intracellular space and bind to a sequence within the transmembrane region of the GPCR (O'Callaghan et al, 2012). Pepducin P4pal-10, palmitate-SGRRYGHALR-NH_2, was developed to specifically target the third intracellular loop of PAR4 (Chandrabalan & Ramachandran, 2021). PAR4 antagonist by pepducin P4pal-10 decreased PAR4-mediated calcium signalling, binding to β-arrestin, and Akt phosphorylation but not MAPK signalling (Peach et al, 2023). Over 30% of FDA-approved therapeutics target GPCRs, further highlighting the role of pepducins as mechanistic probes to further our understanding of GPCR signalling (Rask-Andersen et al, 2011).

1.6.1 Role of PARs in Pain, Inflammation, and Arthritis

A substantial body of evidence suggests that the members of the PAR family are involved in the modulation of joint nociception (Lucena & McDougall, 2021). At this time, most studies have focused on investigating the role of PAR1 and PAR2 in the development of inflammation and pain in RA (Xue et al, 2012; McIntosh et al, 2007; Xue et al, 2021). Emerging evidence suggests that PAR3 and PAR4 also contribute to pain and inflammation (Nieuwenhuizen et al, 2015; McDougall et al, 2009; French & Hamilton, 2016). Animal models of RA (antigen- and adjuvant-induced models) revealed elevated expression of PAR1 in the joints (Hirano et al, 2002). PAR1 knockout mice that were induced with arthritis had a less severe disease phenotype, where decreased levels of IL-6, less cartilage damage, and milder synovitis were observed (Song et al, 2005). PAR2 is widely expressed throughout various cell types in arthritis joints (McCullock et al, 2018). Acute and inflammatory joint pain models (kaolin/carrageenan and Freund's Complete Adjuvant respectively) were induced in PAR2 double knockout and wildtype mice; inflammation was much more severe in the wildtype mice and caused cartilage erosion, synovial hyperplasia, and immune cell infiltration (Muley et al, 2016).

1.6.2 Role of PAR4 in Inflammation, Pain, and Arthritis

PAR4 contains seven transmembrane helices, three intracellular ligand (ICL) and three extracellular ligand (ECL) domains, an extracellular NH_2-terminal domain, and an intracellular COOH terminus. Thrombin and trypsin cleave PAR4 at Arg^{47}/Gly^{48} located within the extracellular NH_2-terminal domain, to reveal the tethered ligand GYPGQV (Peach et al, 2023). The tethered ligand binds to Asp residues within ECL2, activating the PAR4 receptor via the canonical mechanism. PAR4 may also be activated by a biased mechanism involving cathepsin G

and cathepsin S cleavage at Ser^{67}/Arg^{68} of the NH_2-terminal domain (Stoller et al, 2022). PAR4 signalling occurs through the G protein signalling pathways involving $G\alpha_{12}$ and $G\alpha_q$ but not $G\alpha_{i/o}$ (Faruqi et al, 2000).

PAR4 is expressed throughout the rat knee joint. Immunofluorescence staining demonstrated the presence of PAR4 in the menisci, synovium, chondrocytes, and subchondral bone (Russell et al, 2010). PAR4 has also been detected throughout the vascular system (Hirano & Kanaide, 2003; Fender et al, 2017). Many early experiments examined the role of PAR4 blockade in anti-platelet therapy as PAR4 mediated signalling is particularly important for platelet activation and thrombus formation (Rudinga et al, 2018). During the development of pharmacological antagonists and synthetic activating peptides to study PAR4, additional observations relating to the potential utility of PAR4 in other tissue types were observed (Mcfarlane et al, 2001). In a study where the PAR4 synthetic activating peptide, AYPGKF-NH2, was injected into the plantar surface of rats, causing prolonged edema (Hoele et al, 2005). The PAR4 active peptide acted as an exogenous ligand by binding to residues within the ECL2 domain of PAR4. Another study pre-treated rats with a bradykinin-2 receptor agonist, resulting in the reversal of AYPGKF-NH2 effects in joints (McDougall et al, 2009). These findings indicate that PAR4 activation is dependent on articular bradykinin-2 receptors.

PAR4 is abundantly expressed in joint tissue and found in over 60% of DRG neurons (Russell et al, 2011). However, not much research has been conducted to investigate the role of PAR4 in arthritis. After the administration of AYPGKF-NH2, mechanical allodynia and increased rate of blood flow was blocked by pre-treatment with either the bradykinin-receptor antagonist (HOE-140) or PAR4 antagonist pepducin P4pal-10 (McDougall et al, 2009). Pre-treatment of kaolin/carrageenan mice with pepducin P4pal-10 greatly reduced synovitis and joint perfusion

(McDougall et al, 2009). Electrophysiological studies were conducted to characterize the effect of PAR4 activation on nociceptor activity (Russell et al, 2010). Rats treated with the PAR4 synthetic activating peptide had a significantly larger number of joint nociceptive afferent fibres firing. Pre-treatment with either HOE-140 or pepducin P4pal-10 blocked the pro-nociceptive effect of AYPGKF-NH2 (Russell et al, 2010). Further studies examining PAR4 involvement in arthritis are essential, considering the promising observations discussed earlier and the limited understanding of PAR4 in chronic inflammatory pain.

1.7 FDA Regulatory Approval Process

Pre-clinical experiments using model animals are conducted during the earliest stages of drug development to characterize a drug's pharmacological and toxicological profile. Regulatory agencies like the FDA generally require pharmaceutical companies to submit animal studies as part of their IND application. The FDA then reviews the submitted data prior to granting permission for the company to begin clinical trials. The company may then submit an NDA after conducting clinical trials. Once a drug is reviewed by the FDA, a regulatory report containing its decision-making process is released as a publicly available document.

Many studies examining the study design of clinical trials have been conducted using the information available through the FDA and Health Canada regulatory reports (Turner et al, 2022; Lythgoe & Middleton, 2021; Lexchin et al, 2021). However, a knowledge gap exists for the validity and reliability of pre-clinical experiments. No studies have been conducted to study the process used by regulatory agencies to evaluate pre-clinical experiments submitted as part of an NDA. The pre-clinical pharmacology studies are an extremely important area to investigate as early decisions about the safety and efficacy of a drug are drawn from this set of studies, which

regulatory agencies use to inform their decision on whether to allow the sponsor to begin first-in-human clinical trials.

Regulatory reports released by the FDA are a relatively under-utilized source of information. They can be mined for greater insight into the drug approval process, which may enable more transparency with respect to regulatory decision-making. To conduct an analysis to analyze the rigour of the pre-clinical pharmacology studies used to support NDAs, this led to several research questions and hypotheses as described in the next section. Salient aspects that contribute strongly to regulatory decision-making were identified, and this was used to inform the drug development for pepducin P4pal-10 and its likelihood as a candidate compound for the treatment of pain and inflammation in RA.

1.8 Study Objectives and Hypotheses

Research question #1: Is the PAR4 antagonist, pepducin P4pal-10, anti-nociceptive in the FCA rat model of chronic inflammatory arthritis?

Hypothesis 1a. The PAR4 antagonist, pepducin P4pal-10, is anti-nociceptive in the FCA rat model of chronic inflammatory arthritis.

Research question #2: Do regulatory agencies perform a robust analysis to validate pre-clinical studies submitted as part of a New Drug Application (NDA)?

Hypothesis 2a. Regulatory agencies do not analyze the validity or reliability of animal studies submitted as part of an NDA.

Hypothesis 2b. The validity and reliability of animal studies submitted in an NDA is low (Less than 4/8 and 6/12 respectively).

Research question #3: Is there a difference in the amount of discussion given to pre-clinical studies during the review process for a drug's first indication compared to subsequent indications?

Hypothesis 3: There is a greater emphasis on pre-clinical studies during the review process for the drug's first indication.

Research question #4: Do the preclinical studies for pepducin P4pal-10 have high validity and reliability?

Hypothesis 4: The preclinical studies in existence for pepducin p4pal-10 have high validity and reliability.

Chapter 2 Methods

This chapter describes the methods used to evaluate the efficacy of pepducin P4pal-10 in an animal model of rheumatoid arthritis and subsequently describes the development of a rubric to analyze the rigour of pre-clinical pain studies. The effects of pepducin P4pal-10 treatment on chronic inflammatory pain was measured through the pain behaviour assessments (von Frey algesiometry and dynamic incapacitance) and saphenous nerve myelination (G-ratio calculations). These techniques described in the first section of this chapter are frequently used in preclinical pain research. To date, no studies have looked at the impact of preclinical pain research on regulatory decision-making. The second part of this chapter details the development of a rubric to analyze the variables that contribute to the validity and reliability of preclinical pain studies submitted to the FDA as part of an NDA. The third part of this chapter describes each variable in detail and includes the statement used to operationalize the variable.

2.1 Animals

The experimental protocol (#21-111) was approved by the Dalhousie University Committee on Laboratory Animals (UCLA) which strictly adheres to the standards set by the Canadian Council on Animal Care (CCAC).

Male Wistar rats (300-330g) were sourced from Charles River Laboratories (Saint Constant, Quebec, Canada). They were acclimated for at least seven days at the Carleton Animal Care Facility located at Dalhousie University (Halifax, Nova Scotia, Canada) before the start of the experiment. The rats were kept in pairs, in ventilated cages maintained at 22°C with lights switched on from 0700 to 1900 daily. The cages contained woodchip bedding, Enviro-dri material, and environmental enrichment. Kibble and water were supplied *ad libitum*.

2.2 Freund's Complete Adjuvant Model of Inflammatory Pain

Male Wistar rats were anaesthetized with 2.5% isoflurane. Once the animal was at the third plane of anesthesia as observed by the absence of the flexor withdrawal reflex, the area surrounding the right knee was shaved. The joint diameter was measured using a vernier caliper. Three measurements were taken and averaged. The knee region was then cleaned thrice with each of the following: chlorohexidine, 70% ethanol, and betadine to disinfect the injection site.

Freund's Complete Adjuvant (FCA: 50 µg in 45.5 µL mineral oil and 4.5 µL mannide monooleate) was injected into the knee joint capsule. Afterwards, the knee was flexed and extended for 30 seconds to distribute the material around the joint capsule.

2.3 Pain Behaviour Assessments

Von Frey hair algesiometry and dynamic incapacitance assessments were conducted on the animals prior to FCA model induction (day 0), at day 7 post-FCA induction, and then at day 21.

2.3.1 Von Frey Hair Algesiometry

Von Frey hair algesiometry was used to assess secondary mechanical allodynia. The rats were placed in a Perspex chamber that had a mesh flooring and allowed to acclimate for about 15 minutes. A set of six von Frey filaments (2, 4, 6, 8, 10, 15g) was used to measure the mechano-sensitivity of the ipsilateral hindpaw. The Dixon's Up-Down method was utilized to calculate the force required to trigger a positive response (Chaplan et al., 1994). A positive response included paw withdrawal, licking, or shaking. The filament was applied to the planar area of the hindpaw till it was slightly bent and held there for 3 seconds. If a positive response was observed, the lower force filament was then applied. If there was no response from the animal, the higher force filament

was applied in the same manner until the 15g filament was reached. The paw withdrawal threshold was calculated as follows: Paw Withdrawal Threshold = $10^{[Xf + k\delta]} / 10000$

2.3.2 Dynamic Incapacitance

Immediately after each von Frey hair measurement, the rats were assessed for dynamic weightbearing of the hindlimb. This is an assessment of spontaneous pain. The rats were placed in a Perspex chamber containing a pressure sensitive floor and a camera to record their movements. Video recordings and pressure readings were taken for three minutes while the animals roamed around the chamber. The videos were analyzed to calculate the percentage weightbearing on the ipsilateral hind paw compared to the weightbearing on the contralateral hind paw.

2.4 Saphenous Nerve Assessment

Electron microscopy was used to image transverse sections of the saphenous nerve. Myelin thickness was assessed by G-ratio analysis to establish if there was neuropathy in the FCA rats.

The animals were deeply anaesthetized with 2.5% isoflurane. Once the absence of sensory reflexes was observed, an intracardiac injection of Euthasol (390 mg/mL sodium pentobarbital, 50 mg/mL sodium phenytoin) was administered to kill the animal. The skin surrounding the ipsilateral hindlimb was cut open to expose the area around the saphenous nerve. The saphenous nerve was carefully separated from the nearby fascia and blood vessels. A segment of the saphenous nerve approximately 5mm in length was harvested. The nerve sample was fixed in a 2.5% glutaraldehyde (dissolved in 0.1M sodium cacodylate), kept at 4°C, and left to stand for seven days. Subsequent steps for sample preparation were done by Mary Ann Trevors at the Electron Microscopy Lab (Dalhousie University) as described below.

The nerve samples were soaked in 1% osmium tetroxide for 2 hours for further fixation, followed by a quick rinse with distilled water. Afterwards, the samples were placed in 0.25% uranyl acetate at 4°C overnight to increase tissue membrane stability and enhance contrast. The samples were dehydrated with a graduated series of acetone (50%, 70%, 95%, 100%). Next, the samples were incubated in varying ratios of 100% Acetone/Epon Araldite Resin solution: 3:1 ratio for 3 hours and 1:3 ratio overnight. The nerve samples were embedded in 100% Epon Araldite Resin and cured in a 60°C oven for 48 hours. Thin sections of about 100 nm thick were cut using a Reichert – Jung Ultracut E Ultramicrotome outfitted with a diamond blade. The finely cut sections were placed a mesh copper grid containing 300 squares/inch. The sections were stained with 2% aqueous uranyl acetate for 10 minutes, rinsed twice with distilled water for 5 minutes, then stained with 3% aqueous lead citrate for four minutes, and rinsed with distilled water. The prepared sections were left to air dry and kept at room temperature.

The saphenous nerve axons were imaged using the JEOL JEM 1230 Transmission Electron Microscope at 80kV. The copper grids were placed in the microscope and a cross-section of the saphenous nerve could be visualized by using a 3X3 grid to separate the nerve into nine quadrants. Representative sampling of the nerves sections was obtained by capturing three micrographs from quadrant one, five and nine. The micrographs were captured at 2500X magnification with a Hamamatsu ORCA-HR digital camera.

Analysis of the micrographs was done using ImageJ software. The G-ratio was used as an assessment of myelin thickness, where G = square root (a/A), where “a” is the internal axon area and “A” is the total axon area. A larger G-ratio indicates lower myelination surrounding the axon. Axon with an internal axon diameter less than 3 μm were classified as small diameter fibres. Axons with an internal axon diameter of larger than 3 μm were classified as large diameter fibres. The 3

μm cut-off for small diameter fibres was obtained from prior studies in the rat model (O'Brien & McDougall, 2020).

2.5 Administration of pepducin P4pal-10

At day 7 and day 21 post-FCA induction, animals were randomly assigned to receive either saline (5 mL/kg, i.p.) or pepducin P4pal-10 (300 μg/kg, i.p.). Pain behaviour measurements were assessed at 30, 60, 120, and 180 minutes after drug administration.

2.6 Materials

Information about all the drugs, reagents, and devices used in this study can be found in Appendix A.

2.7 Statistical Analysis of Preclinical Data

All the preclinical experimental data are presented as mean ± standard error of the mean (SEM). The entire datasets for pain behaviour and G-ratio were normally distributed and were analyzed with parametric statistics. For the G-ratio dataset, one-way analysis of variance (ANOVA) was used. For the pain behaviour dataset, two-way repeated measures (RMANOVA) were used. Variables included in the parametric test include drug treatment and time. The "time" variable was tested as a repeated measure when the same animals were involved. The "treatment" variable was a measure of drug treatment with pepducin P4pal-10. Šídák multiple comparisons tests were performed following ANOVA and RMANOVA. A p value lower than 0.05 was deemed statistically significant.

2.8 Development of a rubric to analyze pre-clinical pain studies

No studies have been conducted to examine the impact of preclinical pain research on regulatory decision-making. This chapter describes the development of a rubric to analyze the variables that contribute to the validity and reliability of preclinical pain studies submitted to the FDA. There are several guidelines and recommendations for rigorous animal research. The PPRECISE considerations, ARRIVE guidelines, and Landis 4 are three notable examples (Andrews et al, 2016; Percie du Sert et al, 2020; Landis et al, 2012). In addition, some primary research has been conducted to identify threats to validity and reliability in pre-clinical studies (Federico et al, 2020; Altmanet al, 1999; Hirst et al, 2014). These literature sources contained recommendations to ensure construct validity, internal validity, external validity, and reliability. They were all used to inform the inclusion of the variables in the rubric. These variables are fully described in the final section of this chapter.

Variables thought to be important aspects of a pre-clinical study were selected. Study design can be thought of as having two main components - validity and reliability - a pre-clinical study would ideally have high validity and high reliability. Thus, two categories were created for the criterion: “study design-validity” and “study design-reliability”. Each variable was then placed into the category that it was related to. As all the selected variables ultimately contribute to a study’s rigour, and it is not quite possible to say with certainty that some variable(s) should/could be given more importance than the others, I chose to use a scoring system of "0” if the variable was absent and “+1” if the variable was present. To operationalize the variables, the description for each variable aimed to make it possible to give a present or absent answer (summarized in Figure 2.1)

A: Study Design – Validity

Variable	Criterion
Multiple animal models	The drug's effect is demonstrated in more than one pain model representative of the condition. (+1)
Multiple pain outputs	At least two pain outputs were measured. e.g., mechanical, thermal, spontaneous. (+1)
Multiple species	The drug is tested in more than one animal species. (+1)
Timing of assessment	The timing of pain behaviour assessments must be within the timeframe considered as the standard for the model. (+1)
Mode of administration	Discussion of the chosen mode of drug administration and its impact on pharmacology and toxicology is included. (+1)
Inclusion of controls	A reasonable explanation for the inclusion of control/comparator group(s) in the study design should be included. (+1)
Dose response curve	Dose response experiments were performed to examine if the drug displays a dose-dependent effect. (+1)
Locomotor assay	A locomotor activity assay was performed to rule out drug-induced motor impairment. (+1)

B: Study Design – Reliability

Variable	Criterion
Number of studies	Multiple studies demonstrating similar findings, can be from within the same laboratory by the original researchers. (+1)
Inter-laboratory reliability	Additional studies from investigators at other research sites, show reproducibility and are in concordance with the original researchers. (+1)
Sample size	The sample size must be of adequate power to draw credible conclusions from the data. (+1)
Sex differences	Researchers examined if there are sex differences in the effect of drug treatment or state a reason for prioritizing one sex over the other or combine both male and female animals into the same model whenever possible. (+1)
Randomization	The animals should be an equal chance of being assigned to any of the study groups at the onset of the experiment. (+1)
Blind outcome assessment	Experimenters are unaware of the given treatment when performing the behavioural assessment(s). (+1)
Publication venue	Article was published in a reputable peer reviewed journal. (+1)
Important experimental parameters described	The instruments used and settings for equipment (e.g., fluorescent microscopy parameters) were included in the article. (+1)
Statistical analysis method	Method used for the statistical analysis was fully described. (+1)
Inclusion/exclusion criteria for data	Description of the reasoning to include and/or exclude data were stated. (+1)
Negative results and/or outliers discussed	Results that did not support the hypothesis were discussed. (+1)
Pre-registration of study prior to experiment initiation	The study was registered prior to the initiation of experiments. (+1)

Figure 2.1 Rubric used to evaluate the validity and reliability of preclinical study design
Each of the variables in this figure were operationalized with a corresponding description that aimed to give an absent or present answer. A score of "0" was given if the variable was absent and a "+1" if the variable was present. The rubric was applied individually to each publication to give a score for validity **(A)** and reliability **(B)**. Once the whole set of selected publications for the drug had been scored, an overall quantitative measure of validity and reliability could then be determined for the drug.

2.9 Validity variables

Two types of validity were explored using the variables in the rubric: internal validity and construct validity. Internal validity refers to the extent to which the experimental study design can identify the pharmacological intervention as the source of the measured outcomes. Construct validity refers to the strength of the association between experimental models and the human disease they were meant to simulate (Federico et al, 2020).

This section will proceed to describe each of the following validity variables in more detail: multiple animal models, multiple pain outputs, multiple species of animal models, timing of assessment, route of administration, inclusion of controls, dose response curve, and locomotive assay.

2.9.1 Multiple animal models

Animals are used in pre-clinical experiments to study pain pathophysiology, develop new treatment strategies, and evaluate the efficacy of novel pharmacological agents. Animal models of human diseases allow for the investigation of variables related to acute pain, along with the variables that contribute to the onset and maintenance of chronic pain. These variables can be systematically analyzed for causality in a manner that is often unfeasible in humans. Many animal models of pain have been developed to mimic human pain conditions. Rodents are most used as they have a high level of genetic and nervous system similarity to humans (Zheng-Bradley et al, 2010). Rodent models are typically inbred strains which means that each generation of animals have genetic uniformity. The use of inbred strains may help to increase reproducibility due to the reduction of variability arising from genetic factors.

Animal models of pain contain two crucial aspects: method of injury and the follow-up endpoint measurement. To ensure construct validity, a suitable animal model produces nociception by reproducing (as closely as possible) the mechanisms responsible for the disease state in humans. In OA research, animal models can be either induced or spontaneous. Induced models are models where OA-like features are produced through surgery or chemical means. Spontaneous models are animals who naturally develop OA while genetically modified animals rapidly develop OA in the absence of any external intervention.

Despite the lack of a gold standard animal model for use in OA research at present, several rat models have been used extensively to study joint pain (Cope et al, 2019). The medial meniscus transection (MMT) and monoiodoacetate (MIA) models are two types of induced models that have been developed to mimic the inflammation, neuropathy, and tissue damage occurring in OA. The MMT model is a surgically-induced model of post-traumatic OA, which displays joint destruction of the cartilage in a manner like the clinical observations of osteoarthritis. The MIA model involves a single intra-articular injection of the reagent, resulting in inflammation and joint destruction. Both the MMT and MIA are reasonably well-characterized models commonly used in the study of pain associated with OA (Gregory et al, 2013). Pre-clinical experiments evaluating the efficacy of a novel analgesic agent for the treatment of OA pain would benefit from testing the compound in more than one animal model. Evidence demonstrating efficacy of the drug treatment in multiple animal models would increase the confidence that the pharmacological intervention is the cause of the changes in pain outcomes (key measure of internal validity).

This variable was assessed with the criterion: "**The drug's effect is demonstrated in more than one pain model representative of the condition.**" For example, in a study examining the

effect of pepducin P4pal-10 on osteoarthritis pain, if two OA animal models like the MMT and MIA models were used, a score of +1 would be given for this variable.

2.9.2 Multiple pain outputs

Pain is a multi-dimensional phenomenon. One of the main drawbacks of animal models is the inability to directly measure pain. Instead, researchers rely on surrogate measures, through the observation of pain behaviours demonstrated on evoked or spontaneous pain assays. The methods selected to quantify pain behaviours are specific to the primary research question and pain model used. In various pain models, investigating both evoked and spontaneous pain are necessary as these behavioural outcomes are affected in the corresponding human pain condition (Tappe-Theodor et al, 2019). When using the Freund's Complete Adjuvant (FCA) rat model of inflammatory joint pain, evoked pain can be measured by von Frey algesiometry while spontaneous pain can be measured by dynamic weight bearing. In general, quantification of evoked pain is useful for investigating the underlying mechanisms responsible for hyperalgesia and allodynia. Quantification of spontaneous pain is often useful for furthering our understanding of modulation in pain processing and the associated cortical mechanisms (Gregory et al, 2013). Measuring multiple pain outputs by using assays to quantify both evoked and spontaneous pain in animal experiments helps to increase construct validity.

This variable was assessed with the criterion: "**At least two pain outputs were measured. e.g., mechanical, thermal, and spontaneous.**" For example, if a study used the tail-flick test and von Frey algesiometry, thermal and mechanical pain outputs were measured respectively. A score of +1 was be awarded to the variable when evidence of two or more pain outputs were measured.

2.9.3 Multiple species

During the pre-clinical stage of drug development, the drug is tested in two or more species of animals. This is essential to characterize the drug's safety and efficacy as a model animal may not metabolize the drug in the same way as humans. Small differences in pain signalling or enzyme pathways may have a large effect in the pharmacology of the drug. To fulfil the IND application animal experimentation requirements, most sponsors would test a drug on a rodent species (rat or mouse) and one non-rodent species such as a dog or monkey. Commonly used model animals include mice, rats, guinea pigs, rabbits, mini-pigs, dogs, and non-human primates. Once evidence of a novel drug's safety and efficacy has been established in multiple animal models, the FDA may approve the IND application, granting the sponsor permission to commence human trials (Center for Drug Evaluation and Research, 2022).

This variable was assessed with the criterion: "**The drug is tested in more than one animal species.**" For example, a study examining the effects of drug treatment in rat and mice models would be given a score of +1.

2.9.4 Timing of assessment

Pain behaviour assessments must be measured during the standard timeframe for the model (Turner et al, 2019). Baseline measurements are taken before the animal is given any treatment. Once pain is present, the frequency of assessment needs to match the anticipated duration of analgesic treatment. This will then allow for a time-course depicting the treatment effect to be plotted. The Freund's Complete Adjuvant (FCA) rat model of inflammatory joint pain, the disease takes about one week to develop after FCA administration, reaches maximum severity within a

few weeks, and remission may occur afterwards. Disease severity and duration may vary across different strains of susceptible rats, though the FCA model is typically used in Lewis or DA rats.

As rodents are nocturnal, pain assessments are likely to be more accurate if the animals were examined during the most active period of their circadian cycle (Turner et al, 2019). Nociception is most pronounced during the dark phase of a rodent's circadian cycle and inflammatory signaling pathways also reach peak activity during this time. However, there are practical difficulties in assessing rodents during the dark phase which would require major changes in animal handling practices. Instead, assessing pain behaviours at a similar time of the day throughout the experiment can be implemented rather easily to rule out differences in pain response from circadian cycle effects.

This variable was assessed with the criterion: "**The timing of pain behaviour assessments must be within the time frame considered as the standard for the model.**" Most of the rodent pain models are well characterized and have standard window of time where the disease model is active. It is during this window that the pain behaviour assessments should be conducted to ensure construct validity. If pain behaviour is assessed at an earlier or later timepoint, confounding variables may arise from physiological changes in the pain model. When the timing of pain behaviour assessments falls within the standard window for the model, a score of +1 was given.

2.9.5 Route of administration

Administering test substances to model animals requires meticulous consideration to optimize the delivery of the substance into the animal while reducing the potential for adverse events (Turner et al, 2011). Substances can be administered via many different routes. Route selection is often determined by whether the substance is being tested or a local, systemic, or

parenteral effect. The parenteral route usually results in the highest bioavailability of the drug because the reagent is not affected by the first-pass effect which occurs during hepatic metabolism of orally administered substances. Parenteral administration also avoids some of the variability resulting from digestive tract absorptive processes. Regulatory requirements may shape the selection of a particular route of administration, for instance, in nonclinical safety testing the method used in animals should closely resemble the projected route in humans (Center for Drug Evaluation and Research, 2022).

Many of the drug delivery methods used in laboratory animals require sedation, general anesthesia, or restraint. When selecting a route of administration, the impact of such manipulations requires careful consideration so that they are minimally aversive or invasive for the animals. The use of restraints may often be the most aversive effect of an experiment, which is particularly problematic for pain research due to the well-documented phenomenon of stress-induced analgesia. Positive reinforcement conditioning and habituation to restraints may lower the stress response in the animal. Handling of the animals by the same researcher throughout the duration of the experiment may also contribute towards habituation (Mogil et al, 2005).

The advantages and disadvantages of each route need to be carefully thought out in relation to the overall goal of the treatment, as the selected route is likely to have a significant impact on the pharmacokinetics of the drug. Naloxone is an example of a drug that has very different pharmacokinetic effects depending on the route of administration. When administered intravenously, naloxone causes a swift reversal of opioid-induced depression of the central nervous system. Enteral administration of naloxone is used in the treatment of opioid-induced bowel stasis, without the antagonist of systemic opioid receptors (Gibson & Pass, 2014).

This variable was assessed with the criterion: "**Discussion of the chosen mode of drug administration and its impact on pharmacology and toxicology is included.**" A score of +1 was given if the rationale for the route of administration was discussed and/or implications arising from the drug delivery method was included. The route of administration in the animal model should be relevant to the drug delivery method that would be used to treat the human disease. However, more invasive drug delivery methods (that are not typically performed in humans) may be used in animal models to investigate disease etiology.

2.9.6 Inclusion of controls

Details of the groups being compared, including the control groups need to be stated (Percie du Sert et al, 2020). Even if no control group was used, a rationale must be included. Positive controls are used to understand if an expected effect is distinguishable and may also enhance the interpretation of negative results. Negative controls are useful for understanding if a difference between groups arose from treatment intervention (i.e., placebo vs. treatment).

The purpose of the experiment directly influences the study design. Hypothesis-testing experiments investigate clearly defined hypotheses, involving rigorous methods to minimize bias that often includes a statistical analysis plan drawn out prior to the start of the study. In comparison, exploratory research often simultaneously investigates multiple questions which may not involve adhering to rigorous methods. The flexibility of exploratory research allows for the development or testing of novel ideas, for the generation of hypotheses that can then be tested in a more rigorous manner later. Both hypothesis-testing and exploratory studies are important for scientific advances. By clearly reporting the study purpose and inclusion (or lack) of controls, the readers are in a more informed position when deciding how to use the research. These would help the reader to

determine whether the study is adequately rigorous, and findings are robust enough to be use in other research applications or whether the study is ground-breaking but requires further confirmation before it can be applied.

This variable was assessed with the criterion: "**A reasonable explanation for the inclusion of control/comparator group(s) in the study design should be included. (+1)**".

2.9.7 Dose response curve

The concentration of a drug at the target site is responsible for the drug effect. As the relationship between the drug concentration and response may be complex, testing a range of drug concentrations would be useful to reveal the relationship between the drug dose and measurable response. The dose-response data can then be graphed by having the dose on the x-axis and the measured response on the y-axis. A dose response curve allows for several key features of the drug to be calculated: potency (position of curve on the x-axis), maximal efficacy (highest response attainable), and slope (change in response per unit dose). The pharmacological profile of drugs studied under similar conditions can be compared using their respective dose response curves, which may help to ascertain the optimal dose needed to obtain the desired effect. An important aspect of pre-clinical studies is determination of the optimal dose in animals that can then be converted to an equivalent dose during first-in-human clinical trials.

Dose-response data are necessary for the calculation of the therapeutic index for a drug in specific populations. The therapeutic index is a ratio of the minimum toxic drug concentration to the median effective drug concentration. Drugs with a high therapeutic index are generally safer as toxicity is less likely to occur when the dose is increased. Increasing the dosage of a drug with a low therapeutic index has a higher probability of inducing toxicity. The therapeutic index is

affected by population-specific factors such as age, organ function, and pregnancy. Regulatory agencies require non-clinical toxicology information to determine if the drug is safe for humans, and dose-response curves are often used to demonstrate a drug's safety profile (Center for Drug Evaluation and Research, 2022).

This variable was assessed with the criterion: "**Dose response experiments were performed to examine if the drug displays a dose-dependent effect (+1)**".

2.9.8 Locomotor assay

Drug-induced motor impairment is a major confounding variable in pain assessments which can be excluded by performing a locomotor activity assay. Motor impairment arising from drug treatment may result in the animal becoming less responsive to stimuli during pain behaviour assessments. The lower response may then be interpreted as an analgesic effect when the underlying cause is due to motor impairment inhibiting the animal's responsiveness. A straightforward solution to this problem is to conduct a locomotive activity assay comparing the animal's motor behaviour in the presence and absence of the drug. Two groups of animals—one group given the drug treatment and the other group given a sham/control treatment—can be used for a locomotor activity assay. The influence of the drug on the animal's motor behaviour can then be observed directly.

Understanding if/how a drug affects motor behaviour also has clinical implications, as motor impairments are often off-target effects and may result in adverse side-effects in humans. An ideal analgesic drug would not cause changes in motor behaviour; therefore, a finding of motor impairments may result in considerations of whether to continue drug development. However, this is also dependent on an overall risk-benefit analysis for the drug. If the effects on motor behaviour

are relatively mild in comparison to the beneficial impact of the drug, it might be an acceptable trade-off in the eyes of regulatory decision-makers.

All types of pain, and in particular inflammatory pain, can influence an animal's motor behaviour. Both general activity and performance of specific locomotor behaviour may be affected (Whittaker and Howarth, 2014). Assessing locomotor activity can provide an additional measure for characterizing pain. An example of locomotor activity assessment is by measuring the distance travelled by the animal on running wheels or using video tracking software.

This variable was assessed with the criterion: "**A locomotor activity assay was performed to rule out drug-induced motor impairment (+1)**". Motor impairment arising from drug treatment is a major confounding factor in pain behaviour assessments, as motor deficits might cause an animal to become less responsive to stimuli which may then be misinterpreted as reduction in pain behaviours.

2.10 Reliability Variables

Reliability describes the extent to which a causal association can withstand variations in in models, treatments, outcomes, and settings. Two concepts closely connected to reliability are reproducibility and replicability. Reproducibility is defined as acquiring consistent results using the same data or experimental methods as the original researchers. Replicability refers to acquiring consistent results across multiple experiments performed to answer a scientific question, using newly obtained data or variations in experimental design (Miceli, 2019).

This section will focus on explaining each of the following reliability variables in more detail: number of studies, inter-laboratory reliability, sample size, sex differences, randomization, blind outcomes assessment, publication venue, important experimental parameters, statistical

analysis method, inclusion/exclusion criteria for data, negative results or outliers discussed, and pre-registration of study prior to experiment initiation.

2.10.1 Number of studies

A key aspect of reproducibility is the demonstration of similar findings across multiple studies. These studies may be conducted by the original researchers or investigators at other research sites. Advances in science occur through an iterative process, where new ideas are generated by building upon prior work. Reproducibility of results is crucial for the validity and reliability of a publication. Whenever possible, there should be discussion of the results in the context of the current pool of knowledge. This may look like an in-depth discussion of the ways in which the results corroborated and contradicted other publications. The contradictions may lead to an exploration of more specific research questions and may also help to increase reproducibility.

A recent Nature study reported that about 60% of biology researchers were not able to reproduce their own findings (Baker, 2016). A multitude of factors contribute to reproducibility (or the lack thereof), and it is complex problem with no one-size fits all solution. In recent years, several efforts have been put in place to address the lack of reproducibility in science. A set of best practices have emerged that are anticipated to have a positive impact: robust sharing of raw data, use of authenticated reference biomaterials, proper training on study design, pre-registration of studies, publication of negative data, and detailed description of methods (Percie du Sert, 2020; Landis, 2016; Andrews, 2016). These best practices can enhance validity and reliability of a scientific study.

The number of studies was assessed with the criterion: "**Multiple studies demonstrating similar findings, can be from within the same laboratory by the original researchers. (+1)**" Similar results obtained across multiple experiments is an indicator of intra-laboratory reliability.

2.10.2 Inter-laboratory reliability

Additional studies from investigators at other research sites which are in concordance with the original researcher, is an indication of reproducibility. This means the findings are adequately robust and are not significantly affected by variations in methods and equipment. Inter-laboratory reliability helps to establish the validity of data collected and increases confidence in the conclusions drawn from it.

Funding agencies, journal editors, and academic institutions are preoccupied with novelty: novel receptors, novel drugs, novel mechanisms etc. Grant funding is almost always dedicated towards novel research projects (Shin et al, 2022). However, replicability is foundational to good science, but funding sources make such work difficult to achieve. The Nature report also revealed that more than 70% of researchers could not replicate the results of other investigators (Baker, 2016). Freedman et al looked at the costs associated with low rates of reproducibility in preclinical research, they estimated that the USD$28 billion/year was spent on non-reproducible research and as up to 85% of research expenditure was wasted on factors that contribute to non-replicable research (e.g., non-publication of disappointing/ negative results, inadequate description of treatment and methods, flawed study design).

Two organizations have gone to great lengths to increase reproducibility: (1) National Institutes of Health (NIH) – Rigor and Reproducibility guidelines revised grant application instructions with a focus on enhancing experimental design, authenticating biological materials,

and accurate reporting of study findings and (2) Science Exchange & the Center for Open Science – The Reproducibility Project: Cancer Biology. An initiative designed to specifically identify factors affecting reproducibility in cancer research and for the replication of cancer-related experiments by an unbiased third party to determine if results could be consistently reproduced. These efforts encourage greater inter-laboratory reliability and reproducibility in preclinical research.

Inter-laboratory reliability was assessed with the criterion: "**Additional studies from investigators at other research sites, show reproducibility and are in concordance with the original researchers. (+1)**".

2.10.3 Sample size

According to the Landis 4 criteria, ARRIVE guidelines, and PPRECISE considerations, an appropriate sample size is a core component for rigorous study design. The Landis 4 criterion recommends using statistical calculations for sample-size estimation during the time of study design (Landis et al, 2012). An example of a rigorous method used to estimate sample size is the Power analysis: Corrected sample size = Sample size/ (1− [% attrition/100])

The methods used to calculate a sample size of adequate power should be reported. Underpowered experiments are unlikely to uncover meaningful differences between treatment groups, have lower predictive validity, and may often be inconclusive (Landis et al, 2016). Further animal studies may be conducted on erroneous results, leading to the unnecessary use of animal. Minimizing the number of animals used in research is an ethical obligation and often also a funding agency requirement (Percie du Sert et al, 2020).

Sample size estimation according to the Landis 4 guidelines is applicable to most pre-clinical pharmacology studies, except for early-stage exploratory experiments typically conducted using a small sample size for observatory testing. Such exploratory experiments are susceptible to most of the limitations discussed above and should be used only as hypothesis-generating experiments. Potentially novel discoveries emerging from exploratory stage of the research should be backed-up by hypothesis-testing experiments that have a sufficiently large sample size. Thoughtful consideration of an appropriate sample size that can provide adequate statistical power is a crucial contributor to the reliability of the study.

The ARRIVE Essential 10 guidelines (Percie du Sert et al, 2020) recommends reporting the following information: exact number of animals allocated to each group, total number of animals in each experiment, and total number of animals used in the study. Information about the sample size is important for assessing the validity of the statistical analysis and robustness of the study results. As the number of animals allocated to each group at the beginning of the study may differ from the numbers in the analysis, reporting such information allows the reader to understand if there were exclusions or attrition or animals reused in multiple experiments, and the group(s) where they occurred. It is important to explain how the sample size was chosen, details about the sample size calculation should be provided. The sample size needs to be an optimal number in hypothesis-testing experiments, to be able to properly answer the research question. Both small sample sizes (underpowered studies) and overly large sample sizes (overpowered studies) result in problems with validity and reliability of the results. Overpowered studies may incidentally generate statistically significant findings that have no biological relevance. Underpowered studies are likely to miss the detection of real effects, or underestimate true effect size, leading to low internal validity and inconclusive research. If the sample size was determined without power

calculations, this must be stated explicitly along with the reasoning used to select the sample size. When deciding on the sample size, anticipated loss of data or animals due to the exclusion criteria or expected attrition should be taken into consideration.

The Preclinical Pain Research Consortium for Investigating Safety and Efficacy (PPRECISE) Working Group developed a set of guidelines to enhance transparency and minimize methodologically bias in preclinical pain research (Percie du Sert et al., 2020). The PPRECISE working group reported that in preclinical pain research, the median sample size was approximately n=9 mice or rats per group across a large range of assays. There was no indication if the sample size was selected based on formal consideration of power or following convention, because of a lack of reporting about the reasoning underlying sample size determination. The PPRECISE group also recommended a consideration of effect size, transparency on who the sample size was determined, and the use of a power calculation to formally estimate sample size.

Sample size was assessed with the criterion: "**The sample size must be of adequate power to draw credible conclusions from the data. (+1)**". When there was evidence of power calculation or another statistical technique used to determine appropriate sample, a score of +1 was given.

2.10.4 Sex differences

Male and females have different responses to pain. Increased sensitivity to pain and a greater prevalence of pain conditions are observed in women (Bartley and Fillingim, 2013). Sex-based differences in responsivity to pharmacological pain interventions have also been observed (Mogil and Chanda, 2005). These disparities arise are thought to arise from sex hormones, genotype, and endogenous opioid functioning etc. (Prendergast et al, 2014). The underlying

mechanisms responsible for sex differences in pain is complex and not yet fully characterized. It was only in 2014 that the National Institutes of Health required "consideration of sex as a biological variable" in preclinical studies (Arnegard et al, 2020). Historically, preclinical pain studies tend to use only male animals for three main reasons. Firstly, researchers were concerned that using female animals would increase variability due to fluctuating sex hormone levels and result in the need to use a larger sample size. Second, researchers commonly believe the NIH policy requires them to double sample sizes which would increase the cost of experiments significantly. Third, reviewers may request that scientists repeat all experiment during every phase of the oestrous cycle. Despite the 2014 NIH requirement, most articles published in the journal *Pain* in 2015 continued to use only male rodents (Arnegard et al, 2020). Among the preclinical articles analyzed, 56 out of 71 studies tested only males, 6 tested only females, and 6 did not discuss the sex of animals used. Only three articles confirmed the use of both sexes (Arnegard et al, 2020).

Variability in pain data is similar between female and male mice (Mogil & Chanda, 2005). Male animals have a major source of variability arising from cage dominance hierarchies. As male rodents fight with their cage mates for status, pain experiments may be affected by the animal's dominant or submissive position and how recently the aggression occurred. Stress from fights between cage mates would result in higher serum cortisol levels, immune system activation, and systemic inflammation (Mogil & Bailey, 2010). Increased stress levels in rodents may lead to stress-induced analgesia, which would then be a confounding variable in the study. To account for large sex differences, such as pain processing by different types of immune cells, researchers should use a 1:1 ratio of male and female animals. This 50/50 strategy would not detect minor sex

differences, but major sex differences are likely to be uncovered which may then be interesting starting points for future research.

Studies have shown that rodents handled by male experimenters had higher levels of stress hormones, posing a confounding effect on pain behaviour studies (Sorge et al, 2014). Stress-induced analgesia may arise from the heightened corticosterone secretion. Rats and mice given an ankle injection of zymosan showed a 40% decrease in pain response on the grimace scale when a man (compared to a woman) was in the room (Langford et al, 2010). As the sex of the experimental can potentially be a major confounding factor, researchers should report experimenter sex in their publication (Greenspan et al, 2007). To minimize the effect of experimenter sex on pain behaviour studies, whenever possible the same experimenter should carry out all the experiments for the same dataset.

Consideration of sex differences was assessed with the criterion: "**Researchers examined if there are sex differences in the effect of drug treatment or state a reason for prioritizing one sex over the other or combine both male and female animals into the same model whenever possible. (+1)**"

2.10.5 Randomization

The use of appropriate randomization methods is considered an essential aspect of good experimental design across the ARRIVE, PPRECISE, and Landis 4 criteria. A suitable randomization method ensures that each experimental unit has an equal chance of receiving a specific treatment and a balanced number of animals are allocated to each treatment group. An example of an appropriate randomization method is the use of a random number generator (e.g., GraphPad) to assign the treatment administered to each experimental unit. Proper randomization

minimizes selection bias and decreases systematic variability in the allocation of animals to various groups. Randomization is essential for hypothesis-testing experiments, as inferential statistics based on non-randomized group allocation lacks validity and reliability (Altman and Bland, 1999).

It is crucial to note that randomization is distinct from concealed allocation and blinding. Randomization protects against selection bias by ensuring the confounding variables are similar across all the groups, whereas concealed allocation hides the group/treatment of each experimental unit from the experimenter until the time of assignment. Concealing the assignment of the next animal prevents the experimenter from influencing the allocation of given treatment. Blinding is distinct from both randomization and concealed allocation, as it minimizes experimenter bias after allocation. Details about the precise method used to allocate the animals or experimenter group should be provided, so that the reader can ascertain the reliability of the findings and probable limitations.

This variable was assessed with the criterion: "**The animals should be an equal chance of being assigned to any of the study groups at the onset of the experiment. (+1)**"

2.10.6 Blind outcomes assessment

Blinding is part of the ARRIVE essential 10 and PPRECISE considerations. Details about whether researchers were aware of the group allocation at each stage of the experiment (e.g., allocation, conducting the experiment, outcome assessment, data analysis) should be described. Expectation of a specific outcome can unintentionally skew the data collection or data analysis in a manner than supports the expected findings. Blinding is an experimental strategy used to reduce subjective biases. Compelling evidence from systematic reviews showed that non-blinded outcomes assessment in animal research leads to results where the treatment effects are

overestimated, as much as 30-45% increase of effect size (Hirst et al, 2014). All the outcome measures that were assessed (e.g., behavioural changes or molecular markers) should be clearly defined. For hypothesis-testing experiments, the primary outcome measure should be specified

Whenever possible, the researchers should be unaware of the treatment given to each animal through the entire experiment and until data analysis has been performed. If blinding is not possible at every stage of the experiment, blinding should still be utilized at some stages. Assistance from additional persons may be required to facilitate concealed allocation and blinding. Some test conditions might not be possible for true blinding, such as a swollen knee joint, such information should be reported in the manuscript. To minimize the impact of loss of blinding from such conditions, measures such as video recording or automation should be considered whenever possible. The PPRECISE considerations recommended that the use of / lack of blinding in pain experiments should be clearly reported.

Blind outcomes assessment was scored with the criterion: "**Experimenters are unaware of the given treatment when performing the behavioural assessment(s). (+1)**".

2.10.7 Publication venue

There are an estimated 30,000 academic journals publishing approximately two million articles annually. Within a particular field of research, the quality of research can vary greatly across peer-reviewed journals. The top journals typically have the highest standards for quality research and often require researchers to adhere to the ARRIVE guidelines as a prerequisite for the acceptance of their manuscript. Conversely, many lesser journals that publish low or marginal quality research have appeared in recent years. Such journals may have a dubious or non-existent peer-review process and may only require fee payment for publication. Articles published in a

reputable journal are more likely to be reliable as these journals have a team of editors and reviewers who collaboratively ensure that only manuscripts of the highest quality and importance in their discipline are published.

A peer review assesses many aspects of a manuscript, including the key results, validity, significance and originality, methodology, appropriate use of statistical techniques, conclusions, areas for improvement etc. Generally, reviewers are tasked with assessing the validity and reliability of the experimental approach, quality of data, data interpretation to ensure that the entire study is adequately robust. The main purpose of the peer review process is to provide the journal editors with sufficient information to reach a decision on whether (or not) to accept a manuscript for publication. In addition, a peer review often provides suggestions for areas where the paper can be strengthened. The peer review plays a crucial role in ensuring the validity and reliability of published studies.

Several types of peer review processes are used in academic publishing: single-blind peer review, double-blind peer review, and transparent peer review. The single-blind peer review conceals the identity of the reviewer while allowing the author's identity to be known throughout the review process. The double-blind peer review conceals both the identity of the reviewer and author throughout the review process. The transparent peer review conceals the reviewer's identity until the completion of the peer review process. It also allows the author and reviewer to opt-in to the publication of the reviewer reports and/or author rebuttals generated during the peer review process. The double-blind peer review process greatly minimizes the risk of selective publication bias based on the author's reputation and should be used as widely as possible.

Publication venue was assessed with the criterion: "**Article was published in a reputable peer reviewed journal. (+1)**". A journal was deemed to be reputable if one or more of the

following apply: listed in the Directory of Open Access Journals (DOAJ), publisher is a member of the Committee on Publication Ethics (COPE) or indexed in the major bibliographic databases (e.g., Scopus, PubMed, Embase, ScienceDirect, Web of Science etc.).

2.10.8 Important experimental parameters described

Information about important experimental parameters related to the animals and procedures should be provided. Both the ARRIVE and PPRECISE guidelines consider the reporting of important experimental parameters essential for a reliable evaluation of the study in the broader context of other related work (Percie du Sert et al, 2020; Andrews et al, 2016). Clear reporting of this information allows for greater transparency and is more likely to facilitate faithful replication. Critical factors such as species, strain, sex, age, weight of the experimental animals should be provided as these characteristics can greatly influence experimental findings. A comprehensive reporting of the animal characteristics in a manner like the reporting of human patient demographic data supports the validity and reliability of the study results. By providing the reader with all the pertinent animal characteristics, the reader could assess if the animals used for the experiment are appropriate to the research objectives.

The age and weight of animals in each experimental group can be reported as summary statistics (mean and standard deviation). Whenever possible, baseline values for each animal should be provide via a supplementary information section or through a link to a data repository. Other relevant information that should be reported include the origin of the animals, health status, genotype/genetic modification status, and any prior procedures conducted on those animals. These are several factors that can affect the physiology and behaviour of the animals. If genetically modified animals were used, a description of the genetic modification status (e.g., knockout,

overexpression etc.), genotype (e.g., heterozygous, homozygous), modified gene(s), techniques used to generate the modified animal, method used to confirm genetic modification, and information about the control animals should be included. Essentially, every parameter that may influence experimental variability and outcome measures should be reported.

This variable was assessed with the criterion: "**The instruments used and settings for equipment (e.g., fluorescent microscopy parameters) were included in the article. (+1)**".

2.10.9 Statistical analysis method

Details pertaining to the statistical techniques used in each study should be provided. If software was used, the type and version number should also be included. Every statistical model has underlying assumptions, and methods used to determine if the data fit those assumptions must be described. Statistical methods are part of the ARRIVE essential 10 guidelines and the PPRECISE considerations (Percie du Sert et al, 2020; Andrews et al, 2016). An appropriate statistical treatment of data is necessary for confidence in the stated conclusions which are backed up by data collected during the experiment.

Most statistical analysis methods are highly sensitive to missing data points and outliers. While there are some scientifically justifiable reasons to remove data points (e.g., decline in animal health during the study, measurements occurring outside a physiologically plausible range), overzealous "data cleaning" can potentially bias the study. By providing a sound reasoning for the exclusion of certain data points, a differentiation between responsible data presentation and data manipulation can be made. This is especially important since missing data impacts study sensitivity, leading to biased estimates of effect size. In cases where there are missing data points,

the data analysis should also explore the reasons why the data are missing. It is crucial to consider and account for statistical analysis methods that mitigate the missing data.

Issues arising from multiple statistical testing is an area of important consideration in hypothesis-driven preclinical research e.g., 4.6 PPRECISE, Multiplicity (Andrews et al, 2016). Multiple comparisons between groups, outcome variables, time points, statistical methods, secondary analyses, interim analysis are all ways in which multiple statistical testing may occur. The main problem arising from statistical multiplicity is a substantially higher rate of false positives (i.e., type I error) of inconsequential significance. When multiple statistical tests are performed against a significance level of 5%, the probability of finding at least one statistically significant result is greatly increased. However, statistical significance alone does not indicate there is any meaningful connection between the variables tested (inconsequential findings).

For hypothesis-driven preclinical research, the issues related to multiplicity must be acknowledged and ideally addressed at the time of experimental design (Percie du Sert et al, 2020). This can be done through a prioritization of specific comparisons, outcomes, analyses which should be documented prior to beginning the experiment and disclosed in full at the time of publication. Such information would allow for a more accurate interpretation of study findings. Statistical techniques that are designed for multiple testing can also be used to overcome the multiplicity problem. A transparent reporting of all statistical analysis conducted is strongly recommended by all three guidelines as it allows readers to decide if multiplicity has been properly addressed (Percie du Sert et al, 2020; Andrews et al, 2016; Landis et al, 2012).

Statistical analysis method was assessed with the criterion: "**Method used for the statistical analysis was fully described. (+1)**".

2.10.10 Inclusion/exclusion criteria for data

An inclusion and exclusion statement should outline the eligibility or disqualification of animals / data points once the study has begun. The criteria should be defined prior to the commencement of the study and before any data collection. The inclusion/exclusion criteria are often related to animal characteristics or important study parameters. An example of an inclusion is the body weight of experimental animals must be within a certain range. When a dataset is reanalyzed for a different purpose, the original inclusion and exclusion criteria should be provided to give the reader more context for understanding how the data were selected.

The exclusion criteria may arise from animal welfare or technical issues such as surgical complications or compromised model induction. Data exclusion may occur from failure to reach quality control standards, for example, due to unacceptable amounts of contamination, low histology quality, insufficient sample volume etc. (Percie du Sert et al, 2020). The exclusion criteria may also be influenced by the ethical animal use guidelines, particularly humane endpoints. In pain studies, an animal might be removed from the study and euthanized prematurely if the animal is displaying signs of distress like weight loss beyond 20% of its body weight and lack of self-grooming. Data points may be removed from analysis due to the wrong treatment being administered to an animal, equipment malfunction or other forms of human error. If the losses are anticipated, they should be considered when determining the sample size of animals for the study. The exclusion criteria for humane reasons are almost always provided in animal ethics applications and should also be reported in the manuscript as additional context for the reader's interpretation of the data. Other researchers who may be interested in using the model may also benefit from such knowledge.

The ARRIVE, PPRECISE, and Landis 4 guidelines all recommend *a priori* establishment of an inclusion/exclusion criteria and providing an explicit description of the criteria in the manuscript (Percie du Sert et al, 2020; Andrews et al, 2016; Landis et al, 2012). The ARRIVE guidelines additionally recommended the inclusion and exclusion/outlier criteria be submitted as part of a preregistered protocol (Percie du Sert et al, 2020). Most importantly, the exclusion criteria must be independent of treatment assignment to preserve data integrity provided by randomization.

This variable was assessed with the criterion: "**Description of the reasoning to include and/or exclude data were stated. (+1)**."

2.10.11 Negative results or outliers explained

Any animals or data points not included in the statistical analysis should be reported and reasons provided. If there were no excluders or outliers, this should be explicitly stated. Unaccounted for data points or animals can lead to unsubstantiated conclusions. By reporting attritions and exclusions, other researchers are provided with valuable information to evaluate the study or to use the knowledge when replicating the experiment or while testing the treatment effect in other species. Reporting of exclusions arising from adverse effects may also be a source of useful safety information for the planning of human clinical trials (Rice et al, 2008).

The PPRECISE considerations suggested treatment of outliers with the "three-standard deviation rule": exclusion of data that is greater than 3 standard deviations away from the mean (4.5 PPRECISE, Percie du Sert et al, 2020). Bias is introduced when using such a rule, unless there is an understanding of what caused the outlier(s). Conversely, some researchers in the PPRECISE working group believed that all data should be presented, including outliers, to minimize the risk of bias (Andrews et al, 2016). All the researchers agreed that any data excluded must be reported.

This variable was assessed with the criterion: "**Results that did not support the hypothesis were discussed. (+1)**"

2.10.12 Pre-registration of study prior to experiment initiation

An increasing number of journals are requiring study pre-registration prior to experimental initiation to increase reproducibility. Pre-registration of a study involves the submission of the hypotheses, methods, and data analysis plan to a public registry before conducting the study. Study pre-registration has been the norm for clinical trials research since 2000; however, it has not been done as widely in the areas of pre-clinical research. The Open Science Framework (OSF) developed by the Center for Open Science (COS) is an example of a platform used to host registered studies (Foster and Deardorff, 2017). Study registration is a key feature of the OSF as it seeks to preserve, increase access towards, and promote transparency in research (Foster and Deardorff, 2017). Any project can be submitted to the OSF Registries, creating a time-stamped document that cannot be deleted or edited which serves as a preserved copy of the project. The user can choose to withdraw a project, removing the contents of the registered study but a record of it is left behind. Registered studies can be accessible to the public upon submission or embargoed for a maximum of four years. The OSF Registries also has a search feature which allows for searches to be refined by keywords, provider, and type of resource (e.g., data, analytic code, materials, papers, supplements). Some of this information may not be typically available through a peer-reviewed publication and are likely to benefit researchers at all parts of the research lifecycle. Study pre-registration allows research groups to have a better idea of other on-going projects, reducing the likelihood of redundant experiments and possibly even promote more robust research practices when researchers no longer fear being scooped.

Beyond requiring study pre-registration as a prerequisite for manuscript acceptance, some journals have also developed new submission formats for the peer-reviewed publication of Registered Reports (planned research that has not yet been conducted). Currently, just over 300 journals offer a Registered Reports publication format (Chambers and Tzavella, 2022). In March 2020, PLoS ONE introduced *Registered Reports Protocols* and *Registered Reports* (Benetreau, 2021). *Registered Reports Protocol* accepts and publishes planned research that have not been initiated. Studies accepted for *Registered Reports Protocols* are also given provisional acceptance of the completed research for publication in *Registered Reports*. PLoS ONE introduced these new submission formats to encourage a higher uptake of study pre-registration and as a tool to reduce publication bias (Benetreau, 2021).

Registered Reports guarantees acceptance of manuscripts published in *Registered Reports Protocol*, barring major deviation from the published protocol. Manuscripts containing deviations from the published protocol would still be considered when the deviations are acknowledged and justified. Final reports containing unplanned, exploratory, or unregistered analyses are welcomed when they are identified accordingly. This publication format shifts the focus to the significance of the research question and effectiveness of the proposed methodology, rather than the novelty of the results. Peer review of study design allows for more rigorous and creative research (Morton, 2022). External input from reviewers at the study design phase has the power to exert significant downstream changes in a more efficient manner, leading to studies that explore important questions while minimizing confirmation bias and impact bias (Pariente, 2022).

Study pre-registration was assessed with the criterion: "**The study was registered prior to the initiation of experiments. (+1)**". It is worthwhile to note that study pre-registration is a relatively new process for pre-clinical research, implementation of pre-registration is still slow,

and uptake has been low in comparison to clinical research (Heinl et al, 2022; Munoz-Tamayo et al, 2022).

Chapter 3 Efficacy of Pepducin P4pal-10 in Chronic Inflammatory Pain

This chapter examines the efficacy of pepducin P4pal-10 in the Freund's Complete Adjuvant (FCA) rat model of inflammatory arthritis.

3.1 Background and Hypothesis

Serine proteases are a group of enzymes implicated in pain and inflammation (Peach et al., 2023). The protease activated receptors (PARs) are of particular interest as they are expressed in multiple tissue types throughout the joint (Russell et al., 2010). The PARs play an important role in modulating nociceptor sensitivity, vascular reactivity and tissue remodelling. PAR-4 is expressed on joint primary afferents (Russell et al., 2011). Activation of the receptor following a close intra-arterial injection of the PAR-4 active peptide showed an increase in nerve firing, indicating that PAR-4 has a pro-nociceptive role at the joints (McDougall et al., 2009). A PAR-4 antagonist (Pepducin P4pal-10) was able to block the nociceptive and inflammatory effects of PAR-4 activation. Although PAR4 inhibition has been previously evaluated in rodent models of osteoarthritis and acute synovitis, it's efficacy in chronic inflammatory arthritis has not been investigated (O'Brien and McDougall, 2021; McDougall et al., 2009). Peripheral neuropathy is commonly associated with rheumatoid arthritis due to nerve injury arising from joint inflammation and presence of auto-immune antibodies (Scherer et al., 2020). This study sought to assess the effectiveness of PAR4 inhibition, when administered systemically through an intraperitoneal injection, at reducing pain and joint inflammation associated with chronic inflammatory arthritis.

The following hypothesis was evaluated in this study.

1. The PAR4 antagonist, Pepducin P4pal-10, is anti-nociceptive in the FCA rat model of chronic inflammatory arthritis.

3.2 Pain Behaviour in the FCA model of Chronic Inflammatory Pain

The aim of this study was to examine the impact of FCA-induced joint pain and peripheral neuropathy affecting the saphenous nerve.

3.2.1 Methods

On day 0, male Wistar rats were anaesthetized with 2.5% isoflurane. Freund's Complete Adjuvant (FCA: 50 µg in 45.5 µL mineral oil and 7.5 µL mannide monooleate) was injected into the knee joint capsule. Afterwards, the knee was flexed for 30 seconds to distribute the material around the joint capsule. The animals received a treatment of either pepducin P4pal-10 or saline on day 7 and 21. Von Frey hair algesiometry and dynamic incapacitance assessments were conducted on the animals prior to FCA model induction (day 0 baseline), at day 7 post-FCA induction, and at day 21. After all pain behaviour assessments were completed on day 21, the animals were sacrificed. A segment of the saphenous nerve from the region proximal to the ipsilateral knee joint was removed for subsequent analysis of myelin thickness.

3.2.2 Freund's Complete Adjuvant resulted in demyelination of the saphenous nerve

An analysis of saphenous nerve photomicrographs from day 21 showed alterations in the myelin thickness of FCA animals (Figure 3.1). Myelin thickness was measured by a G-ratio analysis, which revealed a significant difference in the large diameter axons but not the small diameter axons in the FCA animals (one-way ANOVA, $p<0.001$, n = 254-392 fibres from 10-12 animals per group).

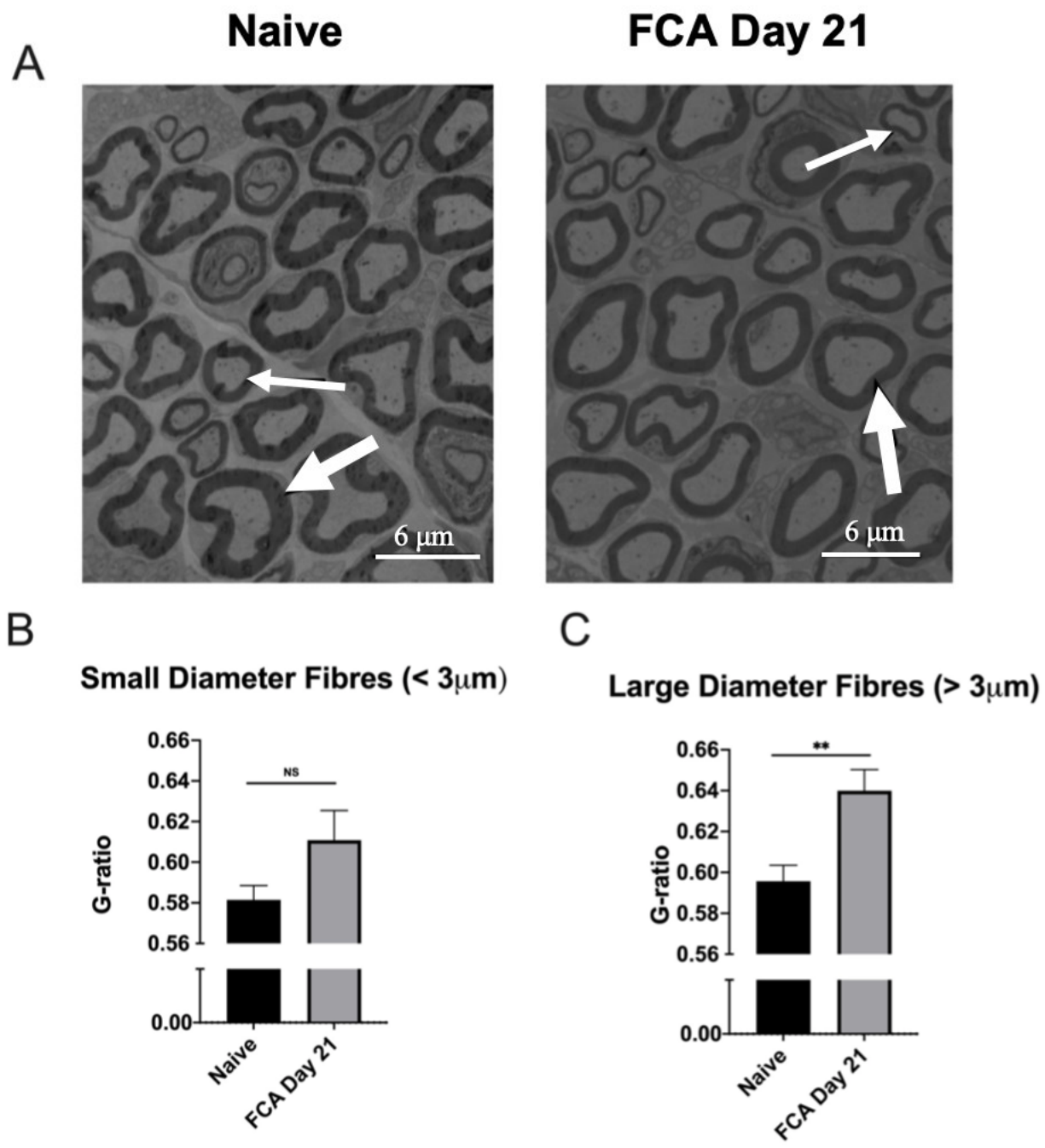

Figure 3.1 FCA-induced nerve damage
(A) Representative electron micrographs of saphenous nerves from naïve and FCA-day 21 animals. Thinner arrow is an example of a small diameter axon. Thicker arrow is an example of a large diameter axon. The scale bar is 6 μm. **(B, C)** G-ratios were calculated for small and large diameter fibers from both naïve and FCA-day 21 animals (n=351-427 axons from 10-12 animals per group). The FCA-treated animals had higher G-ratio values for both small and large diameter fibers. The difference in the large diameter fibers was statistically significant (one-way ANOVA, **$p<0.001$). Data presented as mean ± SEM.

3.2.3 Pepducin P4pal-10 increased ipsilateral paw withdrawal threshold

On day 7 and day 21 post-FCA induction, the animals were treated with an intra-peritoneal injection of either pepducin P4pal-10 or saline. Pain behaviour measurements were taken at 30, 60, 120, and 180 minutes after administration of the treatment. FCA induced inflammatory pain seen in the development of secondary allodynia ($p < 0.001$; $n = 12$; Figure 3.2 A) at the ipsilateral paw. The ipsilateral paw withdrawal threshold decreased from 14.95 ± 0.09 g at baseline (day 0) to 7.43 ± 0.63 g on day 7. On both day 7 and day 21, pepducin P4pal-10 was administered via an intraperitoneal injection at the beginning of the time course. Treatment with pepducin P4pal-10 caused a reversal of secondary allodynia at day 7 and at day 21 post-FCA induction (2way RMANOVA, $p<0.0001$, Figure 3.2A, B).

3.2.4 Pepducin P4pal-10 did not attenuate deficits in hindlimb weightbearing

Hindlimb weightbearing deficits were observed at day 7 post-FCA induction ($p < 0.05$; $n = 12$; Figure 3.2C). Hindlimb weightbearing decreased from 49.82 ± 0.91 g at baseline (day 0) to 38.73 ± 3.65 g at day 7 in the FCA model (Figure 3.2C). Deficits in hindlimb weightbearing were not attenuated by pepducin P4pal-10 treatment at day 7 and day 21 (2way RMANOVA, $p > 0.05$, Figure 3.2C, D).

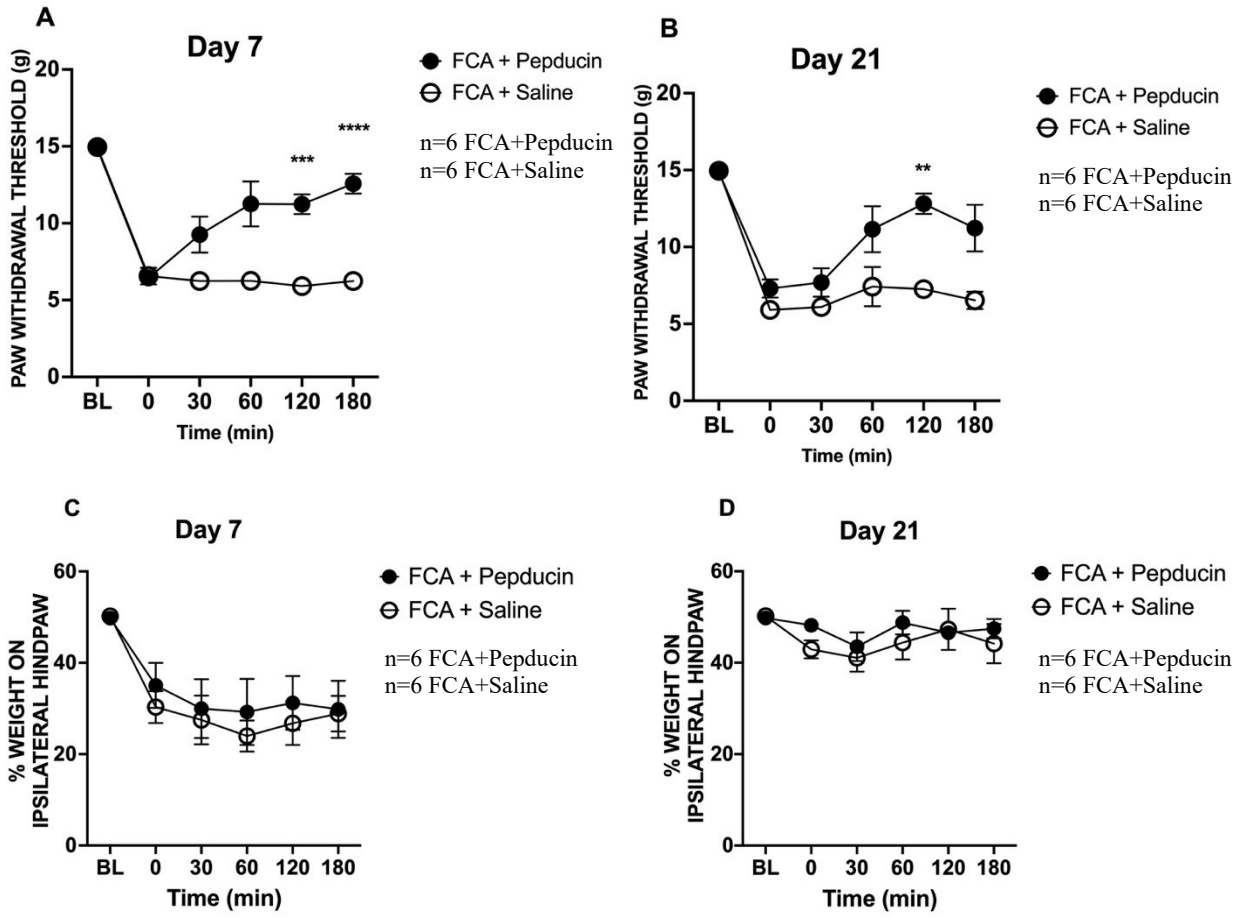

Figure 3.2 Effect of pepducin P4pal-10 on FCA-induced joint pain
(A, B) Animals treated with pepducin P4pal-10 had a greater paw withdrawal threshold compared to the saline-treated animals. A significant difference was detected at both day 7 (2way RMANOVA, $p<0.0001$) and at day 21 (2way RMANOVA, $p<0.0001$). The Sidek multiple comparisons test revealed significant differences at several time points on day 7 t=120 ***$p<0.001$ and t=180 ****$p<0.0001$ and on day 21 t=120 **$p<0.01$. **(C, D)** Hindlimb weight-bearing deficits were observed on day 7 and day 21. There was no difference in the group treated with pepducin P4pal-10 in comparison to the saline group (2way RMANOVA, ns).

3.3 Discussion

The FCA model of chronic inflammatory arthritis study revealed that FCA-induced joint damage resulted in the development of secondary allodynia, hindlimb weightbearing deficits, and demyelination of the saphenous nerve. Treatment with pepducin P4pal-10 caused a reversal of secondary allodynia at day 7 and day 21 post-FCA induction. Deficits in hindlimb weightbearing were not attenuated by pepducin P4pal-10 treatment at day 7 and day 21.

3.3.1 Pepducin P4pal-10 appears to be effective at reducing evoked but not spontaneous pain during the early stage of chronic inflammatory arthritis

The pain behaviour assessments in the FCA model of chronic inflammatory arthritis were conducted at two time points: day 7 and day 21 post-FCA induction. The day 7 time point can be thought of as the early stage while the day 21 time point as the late stage of chronic inflammatory arthritis. Nerve damage is likely to be more extensive at the late stage compared to the early stage. Pepducin P4pal-10 may have a greater effect on evoked pain than on spontaneous pain in the FCA model, as a statistically significant decrease in evoked pain behaviours was observed on day 7 and on day 21. No differences between the groups treated with pepducin P4pal-10 or saline were observed in the pain behaviour assessments of hindlimb weightbearing. Interestingly, a previous study involving MIA and MMT rat models of OA treated with pepducin P4pal-10 also did not show any improvements in hindlimb weightbearing (O'Brien & McDougall, 2021). As bradykinin is known to increase the activity of joint nociceptors and activation of the PAR4-bradykinin pathway leads to an increase in inflammatory joint pain, the anti-nociceptive effects of pepducin P4pal-10 appears to be more effective at ameliorating predominantly inflammatory pain states such as secondary allodynia which was observed in the tests of paw withdrawal thresholds. Since

the deficits in hindlimb weightbearing may be predominantly caused by neuropathic pain and possibly to a lesser extent inflammatory pain, treatment with pepducin P4pal-10 was unlikely to alter spontaneous pain.

Chapter 4 Preclinical Research on Pregabalin (Lyrica)

This chapter explores the role of preclinical pain studies on the FDA approval process for pregabalin. Background related to pregabalin's mechanism of action is described in the first section. The second section explains the key events that occurred during the regulatory approval. In the third section, an analysis of four preclinical publications for pregabalin was presented. The final section is a discussion of main findings from the analyses conducted in this chapter.

4.1 Background and hypotheses

Pregabalin was developed as a successor to gabapentin and is generally thought to be about 2-4 times more potent as an analgesic compound (Lauria-Horner and Pohl, 2003). Pregabalin is structurally related to two amino acids: gamma-aminobutyric acid (GABA) and L-leucine; however, pregabalin does not bind to the GABA receptors or directly modulate GABA signalling. Pregabalin selectively binds to $\alpha_2\delta$ subunit which is an auxiliary protein of the voltage-gated calcium channels (VGCCs). Pregabalin displays similar affinity for the $\alpha_2\delta$-1 and $\alpha_2\delta$-2 subunits. The anti-convulsant, anxiolytic, and analgesic properties of pregabalin appear to arise from the inhibition of $\alpha_2\delta$-1-containing VGCCs. Pregabalin reduces calcium flux in presynaptic terminals, however, it does not fully block calcium channel activity. The pregabalin-induced reduction of calcium flux was more pronounced in inflamed tissue (Fink et al., 2002).

The pre-clinical pharmacology studies are an extremely important area to investigate as early decisions about safety and efficacy of a drug are drawn from this set of studies, which regulatory agencies use to inform their decision on whether to allow the sponsor to begin first-in-human clinical trials. Therefore, knowledge generated from preclinical pain studies may be used

in ways that can significantly impact the safety and disease prognosis for many patients e.g., those enrolled in first-in-human clinical trials.

Regulatory reports released by the FDA are a relatively under-utilized source of information. They can be mined for greater insight into the drug approval process, which may then enable more transparency with respect to regulatory decision-making. Due to the highly influential impact of preclinical pain studies and considering their pivotal role in IND and NDA applications, I chose to take a closer look at how the FDA assessed preclinical studies by using the publications referenced in regulatory reports as a source of information. Pregabalin was specifically selected for analysis because the drug received its first FDA approval for a pain indication, was the first drug to be approved for the treatment of fibromyalgia and went on to receive approval for a total of four pain indications. I decided to conduct an analysis to evaluate the rigour of the pre-clinical pharmacology studies used to support NDAs, and this led to the following hypotheses.

1. Regulatory agencies do not analyse the validity or reliability of animal studies submitted as part of an NDA for pregabalin.
2. There is a greater emphasis on pre-clinical studies during the review process for pregabalin's first indication.

This analysis is focused on NDA #021446 and #021723 as these are the two pain indications for which pregabalin received its initial FDA approval. NDA #021446 was approved for the treatment of pain associated with diabetic peripheral neuropathy (DPN) while NDA #021723 was approved for the treatment of pain associated with post-herpetic neuralgia (PHN).

4.2 Key Events During the Regulatory Approval Process for Pregabalin

4.2.1 Methods

The FDA drug approval packages for pregabalin were downloaded from the Drugs@FDA website (https://www.accessdata.fda.gov/scripts/cder/daf/index.cfm?event=BasicSearch.process). There were four NDA packages for pregabalin (#021446, #021723, #021724, #022488). Each approved indication resulted in the publication of a drug approval package. An FDA drug approval package is a compilation of documents produced by various departments within the FDA during the regulatory approval process. Each drug approval package contained the following documents: approval letter, medical review, chemistry review, pharmacology review, statistical review, clinical pharmacology biopharmaceuticals review, and administrative documents & correspondences. The entire drug approval packages for pregabalin were read thoroughly. All data related to pre-clinical pharmacology and animal experiments were extracted. A key word search was conducted using the terms "pre-clinical", "pharmacology", and "animals" as a secondary measure to ensure all the relevant information had been captured. In certain reports, there were data from the pre-clinical experiments alongside the FDA's comments. In other reports, the FDA referenced published literature as the basis of their decision but did not include any details about the pre-clinical experiments in their review. For such cases, the referenced published studies were obtained from their respective journal websites. As there were insufficient details about experimental design within the pregabalin approval package, I used the corresponding published peer-review article as the source of information for my subsequent scoring of preclinical study design.

Variables thought to be important aspects of a pre-clinical pain behaviour study were selected for inclusion in a rubric. Study design can be thought of as having two main components: validity and reliability. Figure 2.1 shows the rubric used to evaluate the validity (Figure 2.1A) and

reliability (Figure 2.1B) of preclinical study design. The rubric was applied individually to each publication to give a score for validity and reliability. Once the whole set of selected publications for the drug had been scored, an overall quantitative measure of validity and reliability could then be determined for the drug.

4.2.2 Results

In this section, I will describe the developmental timeline for pregabalin, with a focus on issues related to preclinical studies that arose during its regulatory approval process.

The pregabalin Drug Approval package contained the following documents: approval letter, medical review, chemistry review, pharmacology review, statistical review, clinical pharmacology biopharmaceuticals, and administrative documents & correspondences. All the documents were used as sources of information to compile the timeline describing the regulatory approval process of pregabalin for the management of neuropathic pain associated with diabetic peripheral neuropathy (DPN). The medical review, pharmacology review, and administrative documents & correspondence were documents in which the bulk of the timeline information were found. Most of the information pertaining to preclinical studies conducted on pregabalin were extracted from the medical review and pharmacology review of NDA #021446, and these studies were compiled into a list for subsequent analysis of their study design.

On July 23, 1997, Pfizer (sponsor) initiated the process for an IND application for pregabalin. During a pre-IND meeting between the sponsor and FAAODP on December 18, 1997, the sponsor proposed pregabalin for the broad indication of treating neuropathic pain. The FDA responded by recommending the sponsor perform a set of experiments in at least two neuropathic pain models, demonstrating efficacy in both, while also showing that the apparent benefit for DPN

was not a result of nerve damage. During the pre-NDA meeting on June 7, 2000, the FDA reported to the sponsor that the "data regarding hemangiosarcoma in animal studies could impact approvability of the NDA". A surprisingly high incidence of hemangiosarcoma was identified in two mice strains, and the clinical significance was unknown. Additionally, it was impossible to assess the tumorigenic potential in humans from the pregabalin clinical studies. On July 10, 2000, the IND was transferred to the Division of Anesthetics, Critical Care and Addiction Drug Products (DACCADP). An Executive Committee for Animal Care (E-CAC) meeting was held on December 12, 2000, to discuss the hemangiosarcoma matter. The sponsor proposed that the increased incidence of hemangiosarcoma was unique to the mouse strains tested. The E-CAC disagreed with the sponsor's statement, and stated that the "increased incidence of hemangiosarcoma in mice is indicative of a true tumorigenic response to pregabalin… Another two 2-year bioassay in a different mouse strain, and reanalyse of the rat data, were suggested."

A clinical hold was imposed on January 26, 2001, following the E-CAC conclusions. The clinical hold meant that all the ongoing clinical trials were halted. This was because the small safety margin between mouse exposure and intended human exposure levels led to an unfavourable risk-benefit ratio that did not appear reasonable for further clinical development. The FDA highlighted that "Carcinogenicity of pregabalin is an approvability issue". No further information was available for the time between February 2001 and October 2002. On October 30, 2002, four NDAs for each of following indications were submitted simultaneously: treatment of generalized anxiety disorder, epilepsy, pain associated with diabetic peripheral neuropathy, and pain associated with post-herpetic neuralgia (PHN). The NDA for DPN was the only one accorded priority review status, the other three NDAs went through the standard review pathway. As such, DPN indication

was the first submission to be reviewed by the various FDA departments, and the subsequent NDAs frequently referred to the issues discussed in NDA #21446.

Pregabalin was tested in a wide range of preclinical pain models: a total of eight studies examining drug activity related to analgesia were submitted to the FDA and reviewed by the Division of Anesthetic, Critical Care and Addiction Drug Products as part of the NDAs for pain indications. Those eight studies included animal models of pain arising from acute, inflammatory, neuropathic, and disease states such as cancer. List of preclinical pain behaviour experiments discussed in the pharmacology review package (NDA #21446):

1. Dorsal root reflex response in rat spinal cord (RR 770-00322)
2. Rat model of surgical pain
3. Substance P- or NDMA- induced hyperalgesia
4. Hyperalgesia after thermal injury (RR 770-00304)
5. Thermal pain and hyperalgesia in Rhesus monkeys (RR 740-03528)
6. Streptozocin-treated diabetic rats (RR 770-00295)
7. Rat model of vincristine-induced neuropathy (RR 740-03529)
8. Rat chronic constriction injury and Chung model of neuropathic pain (RR 770-00294)

FDA	Published literature
Dorsal root reflex response in rat spinal cord (RR 770-00322)	Hendrich J, Bauer CS, Dolphin AC. Chronic pregabalin inhibits synaptic transmission between rat dorsal root ganglion and dorsal horn neurons in culture. Channels (Austin). 2012 Mar-Apr;6(2):124-32. doi: 10.4161/chan.19805. Epub 2012 Mar 1. PMID: 22627148; PMCID: PMC3396689.
Rat model of surgical pain	Field MJ, Holloman EF, McCleary S, Hughes J, Singh L. Evaluation of gabapentin and S-(+)-3-isobutylgaba in a rat model of postoperative pain. J Pharmacol Exp Ther. 1997 Sep;282(3):1242-6. PMID: 9316831.
Substance P- or NDMA-induced hyperalgesia	Partridge BJ, Chaplan SR, Sakamoto E, Yaksh TL. Characterization of the effects of gabapentin and 3-isobutyl-gamma-aminobutyric acid on substance P-induced thermal hyperalgesia. Anesthesiology. 1998 Jan;88(1):196-205. doi: 10.1097/00000542-199801000-00028. PMID: 9447873.
Hyperalgesia after thermal injury (RR 770-00304)	Jones DL, Sorkin LS. Systemic gabapentin and S(+)-3-isobutyl-gamma-aminobutyric acid block secondary hyperalgesia. Brain Res. 1998 Nov 9;810(1-2):93-9. doi: 10.1016/s0006-8993(98)00890-7. PMID: 9813259.
Thermal pain and hyperalgesia in Rhesus monkeys (RR 740-03528)	Unpublished.
Streptozocin-treated diabetic rats (RR 770-00295)	Field MJ, McCleary S, Hughes J, Singh L. Gabapentin and pregabalin, but not morphine and amitriptyline, block both static and dynamic components of mechanical allodynia induced by streptozocin in the rat. Pain. 1999; 80 (1): 391-398. doi: 10.1016/S0304-3959(98)00239-5.
Rat model of vincristine-induced neuropathy (RR 740-03529)	Nozaki-Taguchi N, Chaplan SR, Higuera ES, Ajakwe RC, Yaksh TL. Vincristine-induced allodynia in the rat. Pain. 2001 Jul;93(1):69-76. doi: 10.1016/S0304-3959(01)00294-9. PMID: 11406340.
Rat chronic constriction injury and Chung model of neuropathic pain (RR 770-00294)	Chen SR, Xu Z, Pan HL. Stereospecific effect of pregabalin on ectopic afferent discharges and neuropathic pain induced by sciatic nerve ligation in rats. Anesthesiology. 2001 Dec;95(6):1473-9. doi: 10.1097/00000542-200112000-00029. PMID: 11748408.

Figure 4.1 Concordance table of non-clinical pharmacology studies conducted for pregabalin. As there was insufficient information about preclinical study design within the regulatory report, a literature search was conducted to find the corresponding article for each of the preclinical studies discussed in the pharmacology review. Published articles were found for seven out of the eight studies.

On October 30, 2003, Dr. Jerry Cott (primary pharmacology reviewer) and Dr. Daniel Mellon (secondary pharmacology reviewer) recommended against approval of pregabalin for the DNP indication from a toxicology / pharmacology perspective due to unexplored risk related to the diabetic patients being exposed to pregabalin over an extended period. Concerns included elevated incidence of hemangiosarcoma and dermatopathy in animal studies and potential interaction between PPAR-gamma agonist with pregabalin. The pharmacology reviewers recommended that "additional studies should be conducted to investigate the mechanism of dermatopathy in rats and monkeys in order to assist in determining the potential relevance to humans". On June 24, 2004, Dr. Kenneth L. Hastings, Associate Director for Pharmacology and Toxicology at the Office of Drug Evaluation II submits a memorandum recommending approval, along with an explanation for his disagreement with the primary and secondary pharmacology reviewers. Dr. Hastings wrote that (1) no evidence of carcinogenicity was observed in male Wistar rats and (2) skin sores evident in animal studies was not observed in clinical trials. There is no evidence of additional animal studies or analyses submitted prior to Dr. Hastings's evaluation, despite a lapse of 18 months between the clinical hold and subsequent NDA submission. It is possible that additional animal studies and/or re-analyses were indeed submitted by the sponsor during the time prior to the NDA submission, and perhaps filed under supplemental documents to the IND application which may explain why no documentation exists in the NDA package for those 18 months. However, IND packages are not made publicly available by the FDA and so I have no way of further investigating what transpired to influence Dr. Hasting's decision-making to reach a position highly favourable of approving Lyrica.

On December 30, 2004, pregabalin received FDA approval for the management of neuropathic pain associated with DPN under the priority review pathway. Afterwards, several

supplemental NDAs (sNDA) were submitted for additional pain indications: S-010 management of fibromyalgia and S-028 for the management of neuropathic pain associated with spinal cord injury. On June 21, 2007, the FDA approved pregabalin for the management of fibromyalgia. Pregabalin becomes the first drug to gain regulatory approval at the FDA for the fibromyalgia indication. Interestingly, the fibromyalgia sNDA does not contain any additional supporting information submitted for the new indication. There was no drug approval package attached to this sNDA. Only an approval letter and label were available. On June 20, 2012, FDA approves pregabalin for the management of neuropathic pain associated with spinal cord injury (sNDA, S-028).

4.3 Analysis of Pregabalin's preclinical publications

4.3.1 Results

The FDA approval packages for pregabalin discussed a total of eight preclinical pain behaviour studies. Most of the preclinical pain behaviour information and discussion were found in the pharmacology review documents. Out of those eight preclinical pain behaviour studies, four were discussed or referenced on more than one occasion in the pharmacology review (NDA #021446). The multiple references made to those four studies suggested that the pharmacology reviewers deemed them to be of greater relevance. The reviewer's report typically contained a summary of key findings and some general details about the experimental design. Additional documents containing a comprehensive overview of each pain study (documents starting with RR 740- and 770-) were consulted by the reviewers. However, these documents were not made publicly available, and as there were insufficient details within the approval package to analyze the variables of interest, I utilized the corresponding published peer-reviewed articles as a source

of information for my subsequent scoring of preclinical study design. It is unknown if those preclinical pain behaviour study documents starting with RR 740- and 770- were peer-reviewed publications or preclinical study reports prepared in a certain format for the FDA. However, it is worth noting that those four preclinical pain behaviour studies had already been published in a peer-reviewed journal at the time of the FDA's evaluation of the pregabalin NDAs. All the available evidence suggests that the preclinical study reports reviewed by the FDA would be very similar to the peer-reviewed publications, which is why I chose to use the publications as an additional source of information. Prior to coding the four publications, the preclinical study information from the FDA packages were compared to the details reported in the corresponding peer-reviewed publication. No discrepancies were found in any of the four published articles.

Figure 4.2 displays an example of how the rubric was used to analyse the Fields et al., 1999 publication which looked at the effects of pregabalin in a rat model of diabetic pain.

Validity Variable	Scoring	Evidence
Multiple animal models	0	Diabetic rat model of neuropathic pain induced by streptozocin injection.
Multiple pain outputs	1	Static and dynamic allodynia were measured.
Multiple species	0	Male Sprague-Dawley rats.
Timing of assessment	1	Static and dynamic allodynia assessed on several days post-induction of diabetes.
Mode of administration	1	Amitriptyline (p.o), morphie (s.c.), gabapentin (p.o., intraplantar, intrathecal), pregabalin (p.o).
Inclusion of controls	1	Vehicle treated group was included at each time point.
Dose response curve	1	Both gabapentin (10-100 mg/kg) and pregabalin (3-30 mg/kg) dose-dependently blocked static and dynamic allodynia through the p.o. route of administration.
Locomotive assay	0	Not conducted.

Reliability Variable	Scoring	Evidence
Number of studies	1	Anti-allodynic effects of gabapentin and pregabalin are consistent with their prior observations (Field et al., 1997a; Field et al., 1997b).
Inter-laboratory reliability	1	Anti-allodynic effects of gabapentin and pregabalin are consistent with their prior observations (Singh et al., 1996).
Sample size	0	8-10 animals per group.
Sex differences	0	Only male Sprague-Dawley rats were used.
Randomization	0	Not described.
Blind outcome assessment	0	Not described.
Publication venue	1	Pain. March 1, 1999, Volume 80(2).
Important experimental parameters described	1	Drug concentrations, von Frey methods to measure both types of allodynia were described.
Statistical analysis method	1	Fully described.
Inclusion/exclusion criteria for data	0	Not described.
Negative results and/or outliers discussed	0	None described.
Pre-registration of study prior to experiment initiation	0	Protocol was not registered prior to study initiation.

Figure 4.2 Analysis of study design of the Field et al., 1999 publication on pregabalin in a rat model of diabetic pain.
This publication received a validity score of 5/8 and a reliability score of 5/12.

Construct validity is supported by the presence of the following variables: multiple species, age, sex, drug administrative schedule, pain etiology, and pain measures. My analysis showed that the preclinical pain studies for pregabalin had moderate construct validity as the drug had been evaluated in several drug administration schedules, pain aetiologies, and pain measures (Figure 4.3). However, in those four publications, pregabalin was only tested in adult male rats which limited its overall construct validity. It is unclear from the set of analyzed publications if the experimental data gathered may be generalizable across other animal species, juvenile or aged rats, and female rats. Across the four studies, pregabalin was evaluated in animal models of pain arising from spinal neuropathy, diabetes disease model, and inflammation. The efficacy of pregabalin was assayed using multiple pain behaviour measures that encompassed mechanical, thermal, and spontaneous components of pain.

Internal validity is supported by variables such as the denotation of exact sample size, power calculations, random treatment allocation, blinded treatment allocation, blinded outcome assessment, specification of statistical tests, inclusion/exclusion criteria for data, and evaluation of dose-response. The preclinical pain studies for pregabalin had moderate-high internal validity as the following were present: denotation of exact sample size, randomization, blinded outcome assessment, specification of statistical tests, inclusion/exclusion criteria for data, and evaluation of dose-response (Figure 4.3). Reporting of exact sample size and evaluation of dose-response were observed in all the studies. However, the other variables involving random treatment allocation, blinded outcome assessment, inclusion/exclusion criteria for data were each observed in only one study. None of the studies conducted power calculations to determine an appropriate sample size or performed a blinded treatment allocation. Most of the variables aimed at increasing internal validity were utilized, though in a sporadic fashion, across the studies analysed.

Each of the preclinical publications for pregabalin were analyzed in a similar way as the example displayed in Figure 4.2. The full analysis for the remaining three studies can be found in Figure 4.4-4.6, located at the end of the chapter.

Validity Variable	Scoring			
	PMID: 9316831	**PMID: 10534603**	**PMID: 10204753**	**PMID: 9447873**
Multiple animal models	0	1	0	0
Multiple pain outputs	1	1	1	0
Multiple species	0	0	0	0
Timing of assessment	1	1	1	1
Mode of administration	0	0	1	1
Inclusion of controls	1	1	1	1
Dose response curve	1	1	1	1
Locomotive assay	0	0	0	1
Total Score	**4**	**5**	**5**	**5**

Reliability Variable	Scoring			
	PMID: 9316831	**PMID: 10534603**	**PMID: 10204753**	**PMID: 9447873**
Number of studies	1	1	1	1
Inter-laboratory reliability	1	1	1	1
Sample size	0	0	0	0
Sex differences	0	0	0	0
Randomization	0	0	0	1
Blind outcome assessment	0	1	0	0
Publication venue	1	1	1	1
Important experimental parameters described	1	1	1	1
Statistical analysis method	1	1	1	1
Inclusion/exclusion criteria for data	0	0	0	1
Negative results and/or outliers discussed	0	0	0	0
Pre-registration of study	0	0	0	0
Total Score	**5**	**6**	**5**	**7**

Figure 4.3 Overview table to show the scoring for each variable received by each of the four pregabalin preclinical publications.
The PMID number represents the publications analyzed using the rubric. Each of the validity and reliability variables were scored using a rubric with a binary scale. A maximum score of 8 could be obtained for validity and 12 for reliability. Individually, each publication received a low to moderate score for both validity and reliability. When examined as a whole, the set of preclinical studies for pregabalin appear to have moderate validity and moderate reliability.

My analysis revealed that the preclinical pain studies for pregabalin appear to have moderate reliability (Figure 4.3). Two variables essential for establishing reliability were absent from every article. Those variables were discussion of negative results and/or outliers and pre-registration of study prior to experiment initiation. All the other reliability variables appeared in at least one publication. The absence of those two reliability variables suggests the possibility of some publication bias. It is important to note that best practices in preclinical research have evolved greatly over the past twenty years. Study pre-registration for clinical research only became mandatory around the time of pregabalin's FDA approval. Preclinical study pre-registration was almost unheard of at that point in time. Overall, the preclinical publications involving pregabalin appear to have moderate validity and moderate reliability.

4.4 Discussion

The FDA's evaluation of preclinical pain studies for pregabalin was analysed using qualitative and quantitative methods. The qualitative analysis was conducted by a thorough examination of the key events during the regulatory approval process which was described in Section 4.2. Two key findings were obtained: (1) The FDA is not fully transparent in their evaluation of preclinical pain studies; and (2) The FDA does not always analyze the rigor of animal studies. The quantitative analysis was conducted by coding four preclinical pain behaviour publications involving Lyrica that were discussed or referenced in the pharmacology review. This analysis revealed that the greatest emphasis on pre-clinical pain studies occurred during the review process for Lyrica's first indication.

4.4.1 FDA is not fully transparent in their evaluation of preclinical pain studies

Section 4.2 describes the key events during the regulatory process for Lyrica, with a focus on the preclinical aspects. In particular, the actions of the pharmacology reviewers were described and discussed in greater detail to attempt to understand the rationale underlying the regulatory decision-making. A lack of evidence to substantiate the approval recommendation given by the Associate Director for Pharmacology and Toxicology (despite extremely contrasting views put forward by the primary and secondary pharmacology reviewers), along with the 18-month gap where there were no records of additional animal studies or re-analyses, suggests that the FDA has ambiguous standards for the evaluation of preclinical studies or may have withheld the publication of information that were important enough to swing approval decisions.

4.4.2 FDA does not always analyse the rigor of animal studies

The discussion in Section 4.2 suggest that the FDA tends to take the information provided by sponsors at face value in instances where expected results for preclinical experiments are presented. This is apparent through their incorporation of the sponsor's table summarizing a list of preclinical pain behaviour experiments in the pharmacology review, without any discussion or commentary of the results. However, when unexpected results in preclinical experiments are presented e.g., high incidence of hemangiosarcoma in mice strains treated with Lyrica, the FDA would then take a closer look at the study design and initiate an advisory committee meeting.

While the results from animal studies are useful for evaluation of the safety and efficacy of novel drugs, the evidence presented suggests that the FDA tends perform an independent analysis on animal studies only in cases where concerns of safety arise. There was no indication of the FDA performing independent analyses randomly on animal studies with "normal / expected results" to confirm efficacy and safety. This is surprising as the animal studies submitted to the

FDA are often not peer-reviewed, resulting in no other manner of oversight on the overall validity or reliability of those studies.

4.4.3 Greatest emphasis on pre-clinical studies occur during the review process for Lyrica's first indication

The results in Section 4.3 suggest that the greatest emphasis on pre-clinical studies happens during the review process for the drug's initial indication. This is evident as the Lyrica NDA #021446 submitted through the priority review pathway was the first NDA package evaluated by the regulators at the FDA. The preclinical pharmacology review generated for this first indication was then repeatedly referenced in all the subsequent indications that Lyrica was eventually approved for. Hundreds of additional studies investigating Lyrica in animal models of pain have been published since Lyrica's initial FDA approval (Federico et al, 2020). However, in all the approval packages for additional pain indications, no new pre-clinical pain studies were submitted by the sponsor or brought up for discussion by the FDA.

Field MJ, Holloman EF, McCleary S, Hughes J, Singh L. Evaluation of gabapentin and S-(+)-3-isobutylgaba in a rat model of postoperative pain. J Pharmacol Exp Ther. 1997 Sep;282(3):1242-6. PMID: 9316831.

Study Design – Validity

Variable	Scoring	Evidence
Multiple animal models	0	Rat model of post-operative pain: incision of plantaris muscle in the hind paw.
Multiple pain outputs	1	Thermal hyperalgesia and tactile allodynia.
Multiple species	0	Male Sprague-Dawley rats.
Timing of assessment	1	Appropriate timing for measurements.
Mode of administration	0	S.C. dosing of gabapentin and pregabalin.
Inclusion of controls	1	Control group was given isotonic saline.
Dose response curve	1	Gabapentin (3-30 mg/kg s.c.), Pregabalin (3-30 mg/kg s.c.)
Locomotive assay	0	Not described.

Study Design – Reliability

Variable	Scoring	Evidence
Number of studies	1	Field et al, 1997.
Inter-laboratory reliability	1	Lee et al, 1994; Yaksh, 1989.
Sample size	0	8-10 animals per group.
Sex differences	0	Only male rats were used.
Randomization	0	Not described.
Blind outcome assessment	0	Not described.
Publication venue	1	ASPET
Important experimental parameters described	1	Clearly described.
Statistical analysis method	1	Adequately described.
Inclusion/exclusion criteria for data	0	Not described.
Negative results and/or outliers discussed	0	None described.
Pre-registration of study prior to experiment initiation	0	Unknown.

Figure 4.4 Analysis of study design of the Field et al., 1997 publication on pregabalin in a rat model of postoperative pain.
This publication received a validity score of 4/8 and a reliability score of 5/12.

Field MJ, Bramwell S, Hughes J, Singh L. Detection of static and dynamic components of mechanical allodynia in rat models of neuropathic pain: are they signalled by distinct primary sensory neurones? Pain. 1999 Nov;83(2):303-11. doi: 10.1016/s0304-3959(99)00111-6. PMID: 10534603.

Study Design – Validity

Variable	Scoring	Evidence
Multiple animal models	1	Chronic constrictive injury (CCI) and Chung Model
Multiple pain outputs	1	Static and dynamic allodynia. Thermal hyperalgesia
Multiple species	0	Male Sprague-Dawley rats.
Timing of assessment	1	Appropriate timing for measurements.
Mode of administration	0	Pregabalin p.o., morphine s.c., capsaicin (hind paw surface)
Inclusion of controls		"Drug treated groups were compared with the appropriate vehicle treated group."
Dose response curve	1	Pregabalin (3-30 mg/kg, p.o.)
Locomotive assay	0	Not described.

Study Design – Reliability

Variable	Scoring	Evidence
Number of studies	1	Field et al., 1997a,b.
Inter-laboratory reliability	1	Bennett and Xie; Kim and Chung, 1992.
Sample size	0	6-11 animals per group.
Sex differences	0	Only male rats were used.
Randomization	0	Not described.
Blind outcome assessment	1	"All experiments were carried out by an observer blind to drug treatments."
Publication venue	1	Pain
Important experimental parameters described	1	Clearly described.
Statistical analysis method	1	Clearly described.
Inclusion/exclusion criteria for data	0	Not described.
Negative results and/or outliers discussed	0	Not described.
Pre-registration of study prior to experiment initiation	0	Unknown.

Figure 4.5 Analysis of study design of the Field et al., 1999 publication on pregabalin in rat models of neuropathic pain.
This publication received a validity score of 4/8 and a reliability score of 7/12.

Partridge BJ, Chaplan SR, Sakamoto E, Yaksh TL. Characterization of the effects of gabapentin and 3-isobutyl-gamma-aminobutyric acid on substance P-induced thermal hyperalgesia. *Anesthesiology*. 1998 Jan;88(1):196-205. doi: 10.1097/00000542-199801000-00028. PMID: 9447873.

Study Design – Validity

Variable	Scoring	Evidence
Multiple animal models	0	I.t catheters were implanted into the rat.
Multiple pain outputs	0	Thermal escape latency was measured.
Multiple species	0	Only male Holtzmann Sprague-Dawley rats were used.
Timing of assessment	1	Appropriate duration of testing.
Mode of administration	1	Gabapentin and pregabalin: i.p. and i.t.
Inclusion of controls	1	Control groups were injected with comparable volumes of saline.
Dose response curve	1	I.p gabapentin (10-100 mg/kg) and pregabalin (1-30 mg/kg). i.t. gabapentin (30-300 mg) and pregabalin (1-30 mg)
Locomotive assay	1	General behaviours: pinna twitching, blinking, righting reflex were periodically measured to ascertain potential sedative effects.

Study Design – Reliability

Variable	Scoring	Evidence
Number of studies	1	Hwang and Yaksh, 1997. Dirig and Yaksh, 1996.
Inter-laboratory reliability	1	Xiao and Bennett, 1996.
Sample size	0	N=55 at start of study, survived catheter implantation surgery=43, control group=12, treatment: 4-6 /group
Sex differences	0	Only male rats were used.
Randomization	1	"All drug doses were randomized."
Blind outcome assessment	0	Not described.
Publication venue	1	Anesthesiology > Laboratory Investigations.
Important experimental parameters described	1	Clearly described.
Statistical analysis method	1	Clearly described.
Inclusion/exclusion criteria for data	1	Clearly described.
Negative results and/or outliers discussed	0	None described.
Pre-registration of study prior to experiment initiation	0	Unknown.

Figure 4.6 Analysis of study design of the Partridge et al., 1998 publication on pregabalin's effect on substance P-induced thermal hyperalgesia.

This publication received a validity score of 5/8 and a reliability score of 7/12.

Chapter 5 Preclinical Research on Duloxetine (Cymbalta)

5.1 Background and hypotheses

A growing body of knowledge suggests that imbalance and disinhibition of 5-hydroxytryptamine (5-HT) and norepinephrine (NE) in the endogenous pain inhibitory pathways contribute towards the mechanisms driving persistent pain (Iyengar et al, 2004). Prior studies involving tricyclic antidepressants which are 5-HT and/or NE re-uptake inhibitors were used in the treatment of persistent pain, however, their use was limited due to side effects including cardiovascular arrhythmias, hypotension, anticholinergic effects arising from autonomic receptor binding (Sindrup et al, 2005). Duloxetine is a selective and potent 5-HT and NE reuptake inhibitor which does not have affinity for serotonin, norepinephrine, histamine, adrenergic, dopamine, and opioid receptors, as well as ion channel receptors (Shelton, 2019). Several pre-clinical studies have shown that duloxetine is the first drug that is a balanced inhibitor of both 5-HT and NE (Wong et al, 1993). As 5-HT and NE are thought to be important mediators in descending inhibitory pain pathways, and the effects of simultaneously inhibiting the reuptake of both neurotransmitters on pain is not well understood, researchers at Eli Lilly evaluated duloxetine in rodent models of persistent, neuropathic, and acute nociceptive pain (Jones et al, 2005; Jones et al, 2006; Iyengar et al, 2004).

Regulatory reports released by the FDA are a relatively under-utilized source of information. They can be mined for greater insight into the drug approval process, which may then enable more transparency with respect to regulatory decision-making. Due to the highly influential impact of preclinical pain studies and considering their pivotal role in IND and NDA applications, I chose to take a closer look at how the FDA assessed preclinical studies by using the regulatory reports as a source of information. Like the analysis carried out in Chapter 4, I decided to further

evaluate the rigour of the pre-clinical pharmacology studies used to support NDAs, by testing the same hypotheses in duloxetine.

3. Regulatory agencies do not analyse the validity or reliability of animal studies submitted as part of an NDA for duloxetine.
4. There is a greater emphasis on pre-clinical studies during the review process for duloxetine's first indication.

5.2 Key Events During the Regulatory Approval Process for Duloxetine

5.2.1 Methods

The drug approval package for duloxetine was downloaded from the Drugs@FDA website (https://www.accessdata.fda.gov/drugsatfda_docs/nda/2004/021733s000_CymbaltaTOC.cfm). The entire drug approval packages for duloxetine (NDAs #021427, #021733, #022148, and #022516) were read thoroughly. All data related to pre-clinical pharmacology and animal experiments were extracted. Scanned documents were converted into text files using an optical character recognition (OCR) software. As there were insufficient details about experimental design within the duloxetine approval package, I used the corresponding published peer-review article as the source of information for my subsequent scoring of preclinical study design.

Figure 2.1 shows the rubric used to evaluate the validity (Figure 2.1A) and reliability (Figure 2.1B) of preclinical study design. It is the same rubric that was applied to each publication in Chapter 4. The rubric was applied in a similar manner to score each of the preclinical publications for duloxetine. Once the whole set of selected publications for duloxetine had been scored, an overall quantitative measure of validity and reliability could then be determined for the drug.

5.2.2 Results

In this section, I will describe the developmental timeline for duloxetine, with a focus on issues related to preclinical studies that arose during its regulatory approval process. This analysis involves NDAs #021733, #022148, and #022516) as duloxetine has received FDA approval for the following three pain indications: pain associated with diabetic peripheral neuropathy (2004), fibromyalgia (2008), and management of chronic musculoskeletal pain (2010) respectively.

The duloxetine Drug Approval Package for the DPN indication contained the following documents: approval letter, printed labelling, medical review, chemistry review, environmental assessment, pharmacology review, statistical review, clinical pharmacology biopharmaceuticals review, and administrative documents & correspondence. Apart from the printed labelling and environmental assessment, all the other documents were used as sources of information to compile the timeline describing the regulatory approval process of duloxetine. Most of the information pertaining to preclinical studies conducted on duloxetine were extracted from medical review, pharmacology review, and administrative documents & correspondence of NDA #21733, and these studies were compiled into a list for subsequent analysis of their study design.

On March 14, 2001, Eli Lilly (sponsor) had a pre-IND meeting with the Division of Anaesthetic, Critical Care, and Addiction Drug Products (DACCADP) where the Sponsor was "advised that duloxetine effects on pain must be demonstrated, independent of effects on mood, … for a DPN indication". The Sponsor submitted IND 62,536 on March 19, 2001. On August 8, 2002, an EOP2 meeting was held to "discuss the clinical development plan of duloxetine for pain disorders". As there were presently no drugs approved for the treatment of DPN, the FDA granted a priority review for the duloxetine NDA containing the DPN indication. During a pre-NDA meeting on July 30, 2003, the Sponsor and DACCADP discussed the content and format of an

NDA for duloxetine for the treatment of DPN. On March 3, 2004, Eli Lilly submitted NDA #21733 for the treatment of DPN. The NDA #21733 was reviewed by the following teams: chemistry, manufacturing, and controls (CMC), clinical pharmacology and biopharmaceuticals, pharmacology, medical, and statistics. Duloxetine received unanimous recommendations for approval from all the review teams. On September 3, 2004, duloxetine received FDA approval for the management of neuropathic pain associated with diabetic peripheral neuropathy.

Duloxetine was tested in a wide range of preclinical models. A total of five studies examining drug activity related to analgesia. The results were submitted to the FDA and reviewed by the Division of Anesthetic, Critical Care and Addiction Drug Products (DACCADP) as part of the NDAs for pain indications. Those five studies included animal models of pain arising from acute, inflammatory, and neuropathic aetiologies. The preclinical pain behaviour experiments that were discussed or referenced in the pharmacology review package (NDA #21733) were:

1. Nerve ligation injury models; Seltzer and Chung models (CNS465)
2. Formalin model of persistent pain (CNS466)
3. Acetic-acid writhing test (CNS467)
4. Carrageenan and capsaicin tests (CNS467)
5. Rat model of chronic pain (NCPR48)

Figure 5.1 shows a concordance table of the pre-clinical pharmacology studies discussed or cited in the pharmacology review package of NDA #21733. A literature search for each of the preclinical studies resulted in published articles found for four out of the five studies mentioned in the regulatory report.

FDA	Published literature
Nerve ligation injury models: Seltzer and Chung models (CNS465)	Iyengar S, Webster AA, Hemrick-Luecke SK, Xu JY, Simmons RM. Efficacy of duloxetine, a potent and balanced serotonin-norepinephrine reuptake inhibitor in persistent pain models in rats. J Pharmacol Exp Ther. 2004 Nov;311(2):576-84. doi: 10.1124/jpet.104.070656. Epub 2004 Jul 13. PMID: 15254142.
Formalin model of persistent pain (CNS466)	L. Bardin, S. Gregoire, M. Aliaga, N. Malfetes, O. Vitton, P. Ladure, A. Newman-Tancredi, R. Depoortère. Comparison of milnacipran, duloxetine and pregabalin in the formalin pain test and in a model of stress-induced ultrasonic vocalizations in rats. Neuroscience Research. Volume 66, Issue 2, 2005, 135-140, ISSN 0168-0102, https://doi.org/10.1016/j.neures.2009.10.009.
Acetic-acid writhing test (CNS467)	Jones, C. K., Peters, S. C., & Shannon, H. E. (2005). Efficacy of duloxetine, a potent and balanced serotonergic and noradrenergic reuptake inhibitor, in inflammatory and acute pain models in rodents. *Journal of Pharmacology and Experimental Therapeutics*, *312*(2), 726-732.
Carrageenan and capsaicin tests (CNS467)	Jones CK, Eastwood BJ, Need AB, Shannon HE. Analgesic effects of serotonergic, noradrenergic or dual reuptake inhibitors in the carrageenan test in rats: evidence for synergism between serotonergic and noradrenergic reuptake inhibition. Neuropharmacology. 2006 Dec;51(7-8):1172-80. doi: 10.1016/j.neuropharm.2006.08.005. Epub 2006 Oct 11. PMID: 17045620.
Rat model of chronic pain (NCPR48)	Unpublished.

Figure 5.1 Concordance table of non-clinical pharmacology animal studies conducted for duloxetine. As there was insufficient information about preclinical study design within the regulatory report, a literature search was conducted to find the corresponding article for each of the preclinical studies discussed in the pharmacology review. Published articles were found for four out of the five studies.

On August 17, 2007, Eli Lilly submitted a Type 6 NDA #22148 for duloxetine to be used in the treatment of fibromyalgia. The referenced application was NDA #21427, which was the initial approval of duloxetine for the treatment of major depressive disorder. No new non-clinical pharmacology studies were submitted in NDA #22148. The pharmacology reviewers made extensive referencing to NDA #21427 and #21733, and recommended approval based on the information submitted in those two NDA packages. Despite concerns raised by the statistical reviewer about a lack of efficacy at 6-months and 12-months of treatment, no further investigation was conducted by the regulators and duloxetine received FDA approval for the management of fibromyalgia on June 13, 2008. An example of the statistical reviewers' statement in NDA#22148 regarding approvability of duloxetine for the treatment of fibromyalgia is displayed here:

> "Lastly, there is not enough evidence to demonstrate that duloxetine-treated patients are associated with significant improvements in pain at six months, when an imputation strategy that correctly assigns a bad score to dropouts is applied (in Study HMCJ). Furthermore, there is no evidence that duloxetine continues to demonstrate a clinically meaningful improvement in the BPI average pain score through 12 months of treatment (based on the result from Study HMEH)."

On May 15, 2009, Eli Lilly submitted a Type 6 sNDA #22516 for duloxetine to be used in the management of chronic musculoskeletal pain. There was no new non-clinical pharmacology information submitted with this NDA for the management of chronic musculoskeletal pain. In the pharmacology review, extensive references were made to NDA #21427 (duloxetine for the treatment of major depressive disorder) and #21733 (duloxetine for the treatment of diabetic peripheral neuropathy). The Arthritis and Life Support Drugs Advisory Committee (ALSDAC) was convened to discuss "efficacy and safety data from the NDA application along with extensive

post-marketing safety data for duloxetine, focusing particularly on hepatotoxicity". Most of the advisory committee members supported the use of duloxetine for chronic lower back pain but not for osteoarthritis. All the other reviewers approved the proposed indication of duloxetine for chronic musculoskeletal pain, leading to the FDA approval of this indication on November 4, 2010.

5.3 Analysis of Duloxetine's Preclinical Publications

5.3.1 Results

The FDA approval package for duloxetine discussed a total of five preclinical pain behaviour studies. Most of the preclinical pain behaviour information were found in the pharmacology and medical review documents (NDA #21733). Out of those five preclinical pain behaviour studies, four were published as peer-reviewed articles. The unpublished study was summarized by "duloxetine hydrochloride administration failed to produce analgesia in a rat model of chronic pain" (NCPR48) in the pharmacology review of NDA #21733. The negative outcome of this study might explain why it remained unpublished, as most journals tend to favour the publication of positive results (Duyx et al, 2017; Olson et al, 2002; Jannot et al, 2013). The pharmacology reviewer's report typically contained a summary of key findings and some general details about the experimental design. An example written by Dr. Suzanne R. Thornton-Jones in her pharmacology review of NDA #21733 is shown here:

> "Duloxetine HCL (LY246916) (5, 10, 20, and 30 mg/kg orally) showed dose-dependent reversal of mechanical allodynia behaviour as graded by von Frey filaments in the partial sciatic ligation (Seltzer model) by 4 hours after administration. Reversal of mechanical allodynia was also observed following i.p. (10 and 20 mg/kg) administration in this model."

Additional documents containing a comprehensive overview of each pain study (documents starting with CNS and NCPR) were consulted by the reviewers. However, these documents were not made publicly available, and as there were insufficient details within the approval package to analyze the variables of interest, I utilized the corresponding published peer-reviewed articles as a source of information for my subsequent scoring of preclinical study design. It is unknown if those preclinical pain behaviour study documents starting with CNS and NCPR were peer-reviewed publications or preclinical study reports prepared in a certain format for the FDA. However, it is worth noting that those four preclinical pain behaviour studies had already been published in a peer-reviewed journal at the time of the FDA's evaluation of the duloxetine NDAs. All the available evidence suggests that the preclinical study reports reviewed by the FDA would be very similar to the peer-reviewed publications, which is why I chose to use the publications as an additional source of information. Prior to coding the four publications, the preclinical study information from the FDA packages were compared to the details reported in the corresponding peer-reviewed publication. No discrepancies were found in any of the four published articles.

Figure 5.2 and 5.3 displays an example of how the rubric was used to analyze the Iyengar et al., 2004 publication which looked at the efficacy of duloxetine in rat models of persistent pain.

The Iyengar, et al (2004) article investigated the efficacy of duloxetine in rat models of persistent pain (Chung model and Formalin model). This study was sponsored by Eli Lilly and published in *The Journal of Pharmacology and Experimental Therapeutics*.

Study Design – Validity

Variable	Scoring	Evidence
Multiple animal models	1	L5/L6 nerve ligation (Chung model), Formalin model, tail-flick latency test.
Multiple pain outputs	1	Von Frey algesiometry, paw licking, tail-flick.
Multiple species	0	Adult male Sprague-Dawley rats
Timing of assessment	1	Mechanical allodynia behaviour (in Chung model) measured by Von Frey algesiometry at the same time points (0.5, 1, 2, 3, 4, 6h) after p.o. dosing with the drug or vehicle.
Mode of administration	1	Formalin model: drugs given i.p. 30 mins prior to formalin L5/L6 nerve ligation: drugs given by oral gavage (p.o.) prior to time-course (30 min, 1h, 2h, 3h, 4h, 6h). Tail-flick test: duloxetine given by oral gavage (p.o.) prior to time-course (1h, 2h, 3h, 4h).
Inclusion of controls	1	Vehicle (double-distilled water). Formalin model: Gabapentin as a positive control.
Dose response curve	1	Multiple doses of the drug were included in each assay.
Locomotor assay	1	Rotarod test of sedation/ataxia and neuromuscular function (Fig 6, A-D). Effect of duloxetine, venlafaxine, milnacipran, and amitriptyline on locomotive function was measured.

Figure 5.2 Analysis of validity variables associated with study design for the Iyengar et al., 2004 publication on duloxetine in rat models of persistent pain.
This publication received a validity score of 7/8.

Construct validity variables include species, age, sex, drug administration schedule, pain etiology, and pain measures. My analysis revealed that the preclinical pain studies for duloxetine had high construct validity as the drug had been evaluated in several species, multiple drug administration schedules, pain aetiologies, and pain measures (Figure 5.2). However, in those same studies, duloxetine was only tested in male adult rodents which limited its overall construct validity as questions remain about whether the experimental data gathered may be generalizable across other non-rodent species, juvenile versus aged animals, and female animals. Across the four studies, duloxetine was evaluated in animal models of pain arising from acute, inflammatory, and neuropathic origins. The efficacy of duloxetine was assayed using multiple pain behaviour measures that encompassed mechanical, thermal, and spontaneous aspects of pain.

Study Design – Reliability

Variable	Scoring	Evidence
Number of studies	1	Wong and Bymaster, 2002. Signe F. Bomholt, Jens D. Mikkelsen, Gordon Blackburn-Munro, Antinociceptive effects of the antidepressants amitriptyline, duloxetine, mirtazapine and citalopram in animal models of acute, persistent and neuropathic pain, Neuropharmacology, Volume 48, Issue 2, 2005, Pages 252-263, ISSN 0028-3908, https://doi.org/10.1016/j.neuropharm.2004.09.012.
Inter-laboratory reliability	1	Wong and Bymaster, 2002. Signe et al. 2005
Sample size	0	Formalin model: n= 6-9 Chung model: n= 6=15
Sex differences	0	Only male Sprague-Dawley rats were used. No reason given for prioritization of male rats.
Randomization	0	No description of randomization in methods
Blind outcome assessment	0	No description of blinding in methods.
Publication venue	1	The Journal of pharmacology and experimental therapeutics (JPET)
Important experimental parameters described	1	Instruments used, outcome measures, and interpretation of results were described.
Statistical analysis method	1	ANOVA, Dunnett's t test, and Tukey's test were used. All data presented as mean +/- SEM
Inclusion/exclusion criteria for data	0	No description given
Negative results and/or outliers discussed	0	No description given
Pre-registration of study prior to experiment initiation	0	No prior registration.

Figure 5.3 Analysis of reliability variables associated with study design for the Iyengar et al., 2004 publication on duloxetine in rat models of persistent pain.
This publication received a reliability score of 5/12.

Reliability variables include denoting exact sample size, power calculations, random treatment allocation, blinded treatment allocation, blinded outcome assessment, details of statistical tests, inclusion/exclusion criteria for data, and discussion of negative results / outliers. My analysis revealed that the preclinical pain studies for duloxetine had low reliability as the following design elements were present: exact sample size, specification of statistical tests, and randomization (Figure 5.3). Reporting of exact sample size and specification of statistical tests were observed in each of the four studies. Randomization was used in only one study. None of the studies reported power calculations to determine an appropriate sample size or performed a blinded treatment allocation. The inclusion / exclusion criteria for data were not described in any of the studies. Design elements aimed at increasing reliability were barely utilized across the studies analysed.

Each of the four preclinical pain publications for duloxetine were analyzed using the rubric in a similar way as the example shown in Figure 5.2 and 5.3. Results from the four studies are displayed in the overview table (Figure 5.4). The PMID numbers represent the publications of Iyengar (2004), Jones (2005), Jones (2006), and Bardin (2005) respectively. Figures 5.5-5.7 contain the full analysis for the other three studies, located at the end of the chapter.

Validity Variables	Scoring			
	PMID: 15254142	PMID: 15494550	PMID: 17045620	PMID: 19883699
Multiple animal models	1	1	0	0
Multiple pain outputs	1	1	1	1
Multiple species	0	1	0	0
Timing of assessment	1	1	1	1
Mode of administration	1	1	0	0
Inclusion of controls	1	1	1	1
Dose response curve	1	1	1	1
Locomotive assay	1	1	0	1
Total Score	**7**	**8**	**4**	**5**

Reliability Variables	Scoring			
	PMID: 15254142	PMID: 15494550	PMID: 17045620	PMID: 19883699
Number of studies	1	1	1	0
Inter-laboratory reliability	1	1	1	1
Sample size	0	0	0	0
Sex differences	0	0	0	0
Randomization	0	0	0	1
Blind outcome assessment	0	0	0	0
Publication venue	1	1	1	1
Important experimental parameters described	1	1	1	1
Statistical analysis method	1	1	1	1
Inclusion/exclusion criteria for data	0	0	0	0
Negative results and/or outliers discussed	0	0	0	1
Pre-registration of study	0	0	0	0
Total Score	**5**	**5**	**5**	**6**

Figure 5.4 Overview table to show the scoring for each variable received by each of the four duloxetine preclinical publications.
Each of the validity and reliability variables were scored using a rubric with a binary scale. A maximum score of 8 could be obtained for validity and 12 for reliability. Individually, each publication received a moderate-high score for validity and a low score reliability. When examined as a whole, the set of preclinical studies for duloxetine appear to have high validity and low reliability.

5.4 Discussion

The FDA's evaluation of preclinical pain studies for duloxetine was analysed using qualitative and quantitative methods. The qualitative analysis was conducted by a thorough examination of the key events during the regulatory approval process (described in Section 5.2) while the quantitative analysis was conducted by evaluating the four preclinical pain publications (described in Section 5.3). Two key findings were obtained: (1) The FDA minimally analyzes the validity or reliability of animal studies and (2) The greatest emphasis on pre-clinical pain studies occurred during the review process for duloxetine's first pain indication.

5.4.1 FDA does not analyze the validity or reliability of animal studies

The discussion in Section 5.2 did not reveal any instances where the FDA analyzed the validity or reliability of preclinical pain behaviour studies submitted as part of an NDA. Five preclinical pain studies were presented in the NDA #21733, which was the first pain indication for which duloxetine received approval. The pharmacology reviewers included an overview of the preclinical pain behaviour studies and a summary of the key findings. No additional discussion or commentary of the pain behaviour studies were presented by any of the review teams. Generally, the FDA appeared to be more concerned about the toxicology studies than the pharmacology studies in animals. This was evident through the focus on toxicology issues involving systemic exposure to major metabolites. As no non-clinical studies have investigated the effects of systemic exposure to major human metabolites, the pharmacology/toxicology reviewer (in NDA #21427) recommended that the Sponsor conduct several additional genotoxicity and carcinogenicity studies. However, the supervisory pharmacology reviewer and Associate Director for pharmacology/toxicology overruled those recommendations and deemed them unnecessary.

5.4.2 Greatest emphasis on pre-clinical pain studies occurred during the review process for duloxetine first pain indication

The results in Section 5.3 suggest that the greatest emphasis on pre-clinical pain studies occurs during the review process for the drug's first pain indication. This is evident as NDA #21733 (management of neuropathic pain associated with DPN) was approved for duloxetine's first pain indication and was the only regulatory package where the reviewers presented information pertaining to the five pre-clinical pain behaviour studies submitted by the Sponsor. Interestingly, the pharmacology reviewer wrote that those pre-clinical pain behaviour studies from NDA #21733 had been reviewed in an earlier NDA package #21427 (duloxetine for the Treatment of Major Depressive Disorder). However, there was no mention of preclinical pain behaviour studies having been carried out in any part of the NDA #21427 package.

In the subsequent NDA packages for fibromyalgia (#22148) and chronic musculoskeletal pain (#22516), the pharmacology/toxicology reviewers referred extensively to the data submitted for MDD (#21427) and DPN (#21733). Numerous studies exploring the efficacy of duloxetine in animal models of pain have been published since duloxetine's first pain indication approval. However, no new preclinical pain studies were discussed or cited in the NDA packages for fibromyalgia and chronic musculoskeletal pain. The pharmacology/toxicology reviews were approved entirely on the information submitted in the prior NDAs. This was an interesting finding as the Sponsor and FDA had access to more preclinical information which may support or undermine subsequent NDAs. Instead of utilizing any of the newly available preclinical information, both the Sponsor and the FDA chose to rely solely on the information submitted for the initial NDA.

The Jones et al (2005) article investigated the efficacy of duloxetine in rodent models of acute and inflammatory pain. This study was sponsored by Eli Lilly and published in *Journal of Pharmacology and Experimental Therapeutics*.

A. Study Design – Validity

Variable	Scoring	Evidence
Multiple animal models	1	Acetic acid-induced writhing in mice, carrageenan-induced thermal hyperalgesia and mechanical allodynia, capsaicin-induced allodynia.
Multiple pain outputs	1	Tail-flick test, hot plate test
Multiple species	1	Male CF1 mice, male Sprague-Dawley rats
Timing of assessment	1	Tests were performed between 8am to 6pm. Time-course at 2, 4, 8, 18, 24h post-drug treatment.
Mode of administration	1	Rotorod test: Drug/vehicle given by i.p., p.o., s.c. Carrageenan-induced model: Drug/vehicle given by p.o. or i.p. 90 mins after model injection Tail-flick and hot plate: Drug/vehicle given by s.c. or i.p. Writhing test: Drug/vehicle given by s.c. or p.o. Capsaicin-induced allodynia: Drug given by s.c. or i.p.
Inclusion of controls	1	Vehicle: double deionized water. Active controls were used: duloxetine compared against gabapentin, morphine, and ibuprofen.
Dose response curve	1	A range of drug dosing were tested.
Locomotor assay	1	Rotorod test to assess the effect of duloxetine and morphine on motor performance was conducted.

B. Study Design – Reliability

Variable	Scoring	Evidence
Number of studies	1	Jones, C.K., Peters, S.C. and Shannon, H.E. (2007), Synergistic interactions between the dual serotonergic, noradrenergic reuptake inhibitor duloxetine and the non-steroidal anti-inflammatory drug ibuprofen in inflammatory pain in rodents. European Journal of Pain, 11: 208-215. https://doi.org/10.1016/j.ejpain.2006.02.008
Inter-laboratory reliability	1	Jones et al 2007.
Sample size	1	Mice: n=5-10, rat: n=6-12.
Sex differences	0	Only male rats were used.
Randomization	0	No description given
Blind outcome assessment	0	No description given
Publication venue		The Journal of pharmacology and experimental therapeutics (JPET)
Important experimental parameters described	1	Instruments used, outcome measures, and interpretation of results were described.
Statistical analysis method	1	Determination of ED50 values described, one-way ANOVA, Dunnett's t test.
Inclusion/exclusion criteria for data	0	No description given
Negative results and/or outliers discussed	0	No description given
Pre-registration of study prior to experiment initiation	0	No prior registration

Figure 5.5 Analysis of study design for the Jones et al., 2005 publication on duloxetine in rodent models of acute and inflammatory pain.
This publication received a validity score of 8/8 **(A)** and a reliability score of 5/12 **(B)**.

The Jones et al (2006) article examined the efficacy of duloxetine in a rat model of inflammatory pain. This study was sponsored by Eli Lilly and published in *Neuropharmacology.*

Validity Variable	Scoring	Evidence
Multiple animal models	0	Carrageenan-induced model of inflammatory pain.
Multiple pain outputs	1	Thermal hyperalgesia and mechanical allodynia.
Multiple species	0	Male Sprague-Dawley rats.
Timing of assessment	1	Appropriate timing of measurements.
Mode of administration	0	All drugs were administered by i.p. injections.
Inclusion of controls	1	Vehicle treated control groups.
Dose response curve	1	Venlafaxine (1-100 mg/kg), duloxetine (1-100 mg/kg), desipramine (0.03-30 mg/kg), thionisoxetine (0.03-30 mg/kg).
Locomotor assay	0	Not described.

Reliability Variable	Scoring	Evidence
Number of studies	1	Jones et al., 2005.
Inter-laboratory reliability	1	Lyengar et al., 2004; Bomholt et al., 2005.
Sample size	0	Each group consisted of 6-12 rats.
Sex differences	0	Only male Sprague-Dawley rats were used.
Randomization	0	Not described.
Blind outcome assessment	0	Not described.
Publication venue	1	Neuropharmacology
Important experimental parameters described	1	Clearly described.
Statistical analysis method	1	Clearly described.
Inclusion/exclusion criteria for data	0	Not described.
Negative results and/or outliers discussed	0	Not described.
Pre-registration of study prior to experiment initiation	0	Unknown.

Figure 5.6 Analysis of study design for the Jones et al., 2006 publication on duloxetine in a rat model of inflammatory pain.
This publication received a validity score of 4/8 and a reliability score of 5/12.

The Bardin et al (2005) article investigated the efficacy of duloxetine in a rat model of fibromyalgia. This study was conducted by researchers from the Pierre Fabre Center for Research and published in *Neuroscience Research*.

Validity Variables	Scoring	Evidence
Multiple animal models	0	Two behavioural models - Formalin pain test, stress-induced ultrasonic vocalizations in rats – were used together to screen compounds for anti-fibromyalgia effects.
Multiple pain outputs	1	Acute and late-stage inflammatory pain were measured from the formalin test.
Multiple species	0	Male Sprague-Dawley rats.
Timing of assessment	1	Appropriate timing of measurements.
Mode of administration	0	All the drugs were administered i.p.
Inclusion of controls	1	Control groups injected with vehicle.
Dose response curve	1	Milnacipran (0.16-60 mg/kg), duloxetine (0.16-40 mg/kg), Pregabalin (0.16-160 mg/kg).
Locomotor assay	1	Described at the end in the technical considerations section.

Reliability Variable	Scoring	Evidence
Number of studies	0	Not described.
Inter-laboratory reliability	1	Fields et al, 2006.
Sample size	0	N=7-9 rats per treatment and control group.
Sex differences	0	Only male rats were used.
Randomization	1	"Drug or vehicle was administered randomly during this test period.".
Blind outcome assessment	0	Not described.
Publication venue	1	Neuroscience Research.
Important experimental parameters described	1	Clearly described.
Statistical analysis method	1	Clearly described.
Inclusion/exclusion criteria for data	0	Not described.
Negative results and/or outliers discussed	1	Section on technical considerations concerning the efficacy/potency data.
Pre-registration of study prior to experiment initiation	0	Unknown.

Figure 5.7 Analysis of study design of the Bardin et al., 2005 publication on duloxetine in a rat model of fibromyalgia.
This publication received a validity score of 5/8 and a reliability score of 6/12.

Chapter 6 Preclinical Research on Pepducin P4pal-10

This chapter contains an analysis of several publications that evaluated pepducin P4pal-10 in the context of joint disease and a discussion of the main findings.

6.1 Background and Hypothesis

Preclinical research can generally be broken down into four phases: basic research, drug discovery, lead optimization, and investigational new drug (IND)-optimizing studies. Basic research are studies conducted to understand the pathophysiology of a disease, and discovery of biological pathways that can be modified by drugs to treat the disease. The drug discovery phase involves the testing of drug-like compounds for efficacy and safety, in cellular and animal models of the disease. Compounds which display the most promising results may then enter the lead optimization phase where studies are conducted to determine an effective dosing strategy and chemical modification also be used to improve its potency. Candidate drugs with extremely promising preclinical data may then proceed to IND-enabling studies, which are extensive pharmacology and toxicology studies required by the FDA before the sponsor is granted permission to conduct clinical trials in humans (Center for Drug Evaluation and Research, 2022). Less than 10% of INDs eventually receive FDA approval (Sun et al, 2022). It is not fully understood if poor preclinical study design might be an important factor contributing to the low success rate for promising candidate drugs. This chapter examines the study methodology of several publications involving pepducin P4pal-10, using a rubric to assess validity and reliability.

The following hypothesis was evaluated in this study.

1. The pre-clinical studies for pepducin P4pal-10 have high validity and reliability.

6.2 Analysis of Pepducin P4pal-10 Preclinical Publications

6.2.1 Methods

Variables thought to be important aspects of a pre-clinical pain behaviour study were selected for inclusion in a rubric. Study design can be thought of as having two main components: validity and reliability. Figure 2.1 shows the rubric used to evaluate the validity (Figure 2.1A) and reliability (Figure 2.1B) of preclinical study design. A maximum score of 8 could be obtained for validity and 12 for reliability. The rubric was applied individually to each publication to give a score for validity and reliability. Once the whole set of selected publications for the drug had been scored, an overall quantitative measure of validity and reliability could then be determined for the drug.

6.2.2 Results

Four publications investigating pepducin P4pal-10 in rodent models of arthritis were analyzed. These four articles were selected for analysis as they are currently all the preclinical arthritic pain studies in existence for pepducin P4pal-10. Figure 6.1 displays an example of how the rubric was used to analyze the O'Brien and McDougall, 2021 publication which examined the efficacy of pepducin P4pal-10 treatment in rat models of osteoarthritis pain.

O'Brien MS, McDougall JJ. Targeting Proteinase Activated Receptor-4 Reduces Mechanonociception During the Acute Inflammatory Phase but not the Chronic Neuropathic Phase of Osteoarthritis in Rats. Front Pharmacol. 2021 Dec 22;12:756632. doi: 10.3389/fphar.2021.756632. PMID: 35002698; PMCID: PMC8727523.

A: Study Design – Validity

Variable	Scoring	Evidence
Multiple animal models	1	MMT and MIA models.
Multiple pain outputs	1	von Frey hair algesiometry, dynamic incapacitance, and electrophysiological recordings.
Multiple species	0	Male Wistar rats.
Timing of assessment	1	Early OA assessed at day 3 MIA, day 7 MMT. Established OA assessed at day 14 MIA, day 28 MMT.
Mode of administration	1	Treatment with pepducin P4pal-10 was given intraperitoneally.
Inclusion of controls	1	Drug: Pepducin p4pal-10, vehicle control: saline. The drug and vehicle control were tested in both the MIA and MMT animals.
Dose response curve	0	Unclear how the dose concentration for pepducin p4pal-10 was selected.
Locomotor assay	0	Locomotor activity assay was not conducted.

B: Study Design – Reliability

Variable	Scoring	Evidence
Number of studies	1	Results related to the early inflammatory aspect of the MIA model corroborates with several other published studies.
Inter-laboratory reliability	1	Results were discussed in the context of prior findings and several findings were
Sample size	0	No description of power calculation.
Sex differences	0	Only male Wistar rats were used in this publication.
Randomization	0	No description in the methods.
Blind outcome assessment	0	No description in the methods.
Publication venue	1	Frontiers in Pharmacology.
Important experimental parameters described	1	Instruments used, outcome measures, and interpretation of results were described.
Statistical analysis method	1	Statistical analysis was outlined in the methods section.
Inclusion/exclusion criteria for data	1	Some data not shown but references to similar findings in the published literature were included.
Negative results and/or outliers discussed	1	Results from all the timepoints in both models were reported.
Pre-registration of study prior to experiment initiation	0	No prior registration.

Figure 6.1 Analysis of study design for the O'Brien and McDougall, 2021 publication investigating the efficacy of pepducin P4pal-10 in rat models of osteoarthritis pain.
This publication received a validity score of 5/8 (A) and a reliability score of 7/12 (B).

Each publication received a moderate score for both validity and reliability, as about half of the validity and reliability variables were present in each of the studies analyzed. (Figure 6.2). When examined as a set, most of the validity variables were present at least one study while four of the reliability variables were not present in any of the studies (Figure 6.3). The validity variables not found in any of the studies were “multiple species” and “locomotive assay”. The reliability variables not found in any of the studies were “sample size”, “sex differences”, “blind outcomes assessment”, and “pre-registration of study”. Therefore, this set of studies investigating pepducin P4pal-10’s role in joint disease appear to have high validity and moderate reliability.

Each of the preclinical pain publications for pepducin P4pal-10 were analyzed using the rubric in a similar way as the example shown in Figure 6.1. Results from the four studies are displayed in the overview table (Figure 6.2). The PMID numbers represent the publications of O’Brien (2021), Russell (2009), Russell (2011), and McDougall (2009) respectively. Figures 6.3-6.5 contain the full analysis for the other three studies and are located at the end of the chapter.

Validity Variable	Scoring			
	PMID: 35002698	**PMID: 19889854**	**PMID: 21238854**	**PMID: 19248120**
Multiple animal models	1	0	1	0
Multiple pain outputs	1	1	1	1
Multiple species	0	0	0	0
Timing of assessment	1	1	1	1
Mode of administration	0	1	1	1
Inclusion of controls	1	1	1	1
Dose response curve	1	1	0	0
Locomotor assay	0	0	0	0
Total Score	**5**	**4**	**5**	**4**

Reliability Variable	Scoring			
	PMID: 35002698	**PMID: 19889854**	**PMID: 21238854**	**PMID: 19248120**
Number of studies	1	1	1	0
Inter-laboratory reliability	1	1	1	1
Sample size	0	0	0	0
Sex differences	0	0	0	0
Randomization	0	0	0	1
Blind outcome assessment	0	0	0	0
Publication venue	1	1	1	1
Important experimental parameters described	1	1	1	1
Statistical analysis method	1	1	1	1
Inclusion/exclusion criteria for data	1	0	1	0
Negative results and/or outliers discussed	1	0	1	1
Pre-registration of study	0	0	0	0
Total Score	**7**	**5**	**7**	**6**

Figure 6.2 Overview table to show the scoring for each variable received by each of the four pepducin P4pal-10 preclinical publications.
The PMID numbers represent the four publications analyzed using the rubric. Each of the validity and reliability variables were scored using a rubric with a binary scale. A maximum score of 8 could be obtained for validity and 12 for reliability. Individually, each publication received a moderate score for validity and a moderate reliability. When examined as a whole, almost all the variables were present in at least one study. Therefore, the set of preclinical studies for pepducin P4pal-10 appear to have high validity and moderate reliability.

6.3 Discussion

The analysis of four preclinical publications examining pepducin P4pal-10 in rodent models of joint pain revealed that high validity and moderate reliability.

6.3.1 The preclinical studies for pepducin P4pal-10 have high validity and moderate reliability

Construct validity variables include species, age, sex, drug administrative schedule, pain etiology, and pain measures. My analysis revealed that the preclinical pain studies for pepducin P4pal-10 had high construct validity as the drug had been evaluated in multiple species, several drug administrative schedules, pain aetiologies, and pain measures (Figure 3.5). However, in those same studies, pepducin P4pal-10 was only tested in rodents which limited its overall construct validity as questions remain about whether the experimental data gathered may be generalizable across other non-rodent species, juvenile or aged animals, and female animals. No studies have been done to assess pepducin P4pal-10 in non-rodent pain models, across a range of ages, or in female animals; consideration of these characteristics in future pepducin P4pal-10 studies will certainly be useful to build up the pharmacology / toxicology profile of the compound. Across the four studies, pepducin P4pal-10 was evaluated in animal models of pain arising from acute, inflammatory, and neuropathic aetiologies. The efficacy of pepducin P4pal-10 was assayed using multiple pain behaviour measures that encompassed mechanical, thermal, and spontaneous components of pain.

Reliability variables include denoting exact sample size, power calculations, random treatment allocation, blinded treatment allocation, blinded outcome assessment, specification of statistical tests, inclusion/exclusion criteria for data, and discussion of negative results / outliers. My analysis revealed that the preclinical pain studies for pepducin P4pal-10 had moderate

reliability as the following design elements were present: exact sample size, specification of statistical tests, randomization, and inclusion/exclusion criteria for data (Figure 3.5). Reporting of exact sample size and specification of statistical tests were observed in each of the four studies. Randomization was used in only one study. The inclusion / exclusion criteria for data were described in two of the studies. None of the studies conducted power calculations to determine an appropriate sample size or performed a blinded treatment allocation. Design elements aimed at increasing reliability were infrequently utilized across the studies analysed.

Russell FA, Veldhoen VE, Tchitchkan D, McDougall JJ. Proteinase-activated receptor-4 (PAR4) activation leads to sensitization of rat joint primary afferents via a bradykinin B2 receptor-dependent mechanism. J Neurophysiol. 2010 Jan;103(1):155-63. doi: 10.1152/jn.00486.2009. Epub 2009 Nov 4. PMID: 19889854.

Study Design – Validity

Variable	Scoring	Evidence
Multiple animal models	0	Naïve and pre-treated rats.
Multiple pain outputs	1	Noxious and non-noxious movements.
Multiple species	0	Male Wistar rats.
Timing of assessment	1	Appropriate timing of measurements.
Mode of administration	0	All drugs were administered via i.p. injection.
Inclusion of controls	1	Control peptide was used. Baseline measurements.
Dose response curve	1	PAR4 activating peptide: range of doses 10^{-9} -10^{-5} mol
Locomotor assay	0	Not conducted.

Study Design – Reliability

Variable	Scoring	Evidence
Number of studies	1	Russell and McDougall, 2009. McDougall et al., 2009.
Inter-laboratory reliability	1	Ivanavicious et al., 2004. Salo and Theriault, 1997.
Sample size	0	N=7-12
Sex differences	0	Only male Wistar rats were used.
Randomization	0	Not described.
Blind outcome assessment	0	Not described.
Publication venue	1	Journal of Neurophysiology.
Important experimental parameters described	1	Clearly described.
Statistical analysis method	1	Clearly described.
Inclusion/exclusion criteria for data	0	Not described.
Negative results and/or outliers discussed	0	Not described.
Pre-registration of study prior to experiment initiation	0	Unknown.

Figure 6.3 Analysis of study design for the Russell et al., 2010 publication.
This publication received a validity score of 4/8 (A) and a reliability score of 5/12 (B).

Russell FA, Zhan S, Dumas A, Lagarde S, Pouliot M, McDougall JJ. The pronociceptive effect of proteinase-activated receptor-4 stimulation in rat knee joints is dependent on mast cell activation. Pain. 2011 Feb;152(2):354-360. doi: 10.1016/j.pain.2010.10.038. PMID: 21238854.

Study Design – Validity

Variable	Scoring	Evidence
Multiple animal models	0	Naïve male Wistar rats.
Multiple pain outputs	1	Mechanical allodynia, weight bearing.
Multiple species	0	Male Wistar rats were used.
Timing of assessment	1	Pain behaviour measurements taken prior to drug treatment and 60, 120, 180, 240 mins after i.a. injection of PAR4 activating peptide or inactive peptide.
Mode of administration	1	Saphenous cannulation, s.c. around knee joint.
Inclusion of controls	1	Control peptide.
Dose response curve	1	A range of doses was given for the PAR4 active peptide.
Locomotor assay	0	Not described.

Study Design – Reliability

Variable	Scoring	Evidence
Number of studies	1	McDougall et al., 2009. Russell et al., 2010.
Inter-laboratory reliability	1	Ribeiro et al, 2000. Zuo et al., 2003.
Sample size	0	N=70
Sex differences	0	Only male Wistar rats were used.
Randomization	0	Not described.
Blind outcome assessment	0	Not described.
Publication venue	1	Pain
Important experimental parameters described	1	Clearly described.
Statistical analysis method	1	Clearly described.
Inclusion/exclusion criteria for data	1	Electrophysiology: neurofilaments that elicited a firing response were identified as afferent nerve fibers and included for EP assessment.
Negative results and/or outliers discussed	1	Discrepancy in the role of mast cells discussed.
Pre-registration of study prior to experiment initiation	0	Unknown.

Figure 6.4 Analysis of study design for the Russell et al., 2011 publication.
This publication received a validity score of 5/8 (A) and a reliability score of 7/12 (B).

McDougall, J.J., Zhang, C., Cellars, L., Joubert, E., Dixon, C.M. and Vergnolle, N. "Triggering of Proteinase-Activated Receptor 4 Leads to Joint Pain and Inflammation in Mice." *Arthritis & Rheumatism*, vol. 60, no. 3, Mar. 2009, pp. 728–737., https://doi.org/10.1002/art.24300.

A: Study Design – Validity

Variable	Scoring	Evidence
Multiple animal models	0	C57BL/6 mice were given an intra-articular injection of the PAR-4 activating peptide or an inactive control peptide or vehicle.
Multiple pain outputs	1	Thermal sensitivity (paw withdrawal from radiant heat stimuli) and mechanical nociception (von Frey algesiometry).
Multiple species	0	Only C57BL/6 mice were used in this study.
Timing of assessment	1	The PAR-4 activating peptide/inactive peptide/vehicle control was given 1 hour after the PAR-4 antagonist pepducin p4pal-10 or bradykinin antagonist HOE 140.
Mode of administration	1	Intra-articular injection to induce the model. Intraperitoneal injection to deliver either antagonist.
Inclusion of controls	1	Suitable control groups were used.
Dose response curve	0	Unclear how the dose for pepducin p4pal-10 was selected.
Locomotor assay	0	Locomotor activity assay was not conducted.

B: Study Design – Validity

Variable	Scoring	Evidence
Number of studies	0	First study showing PAR-4 activation increases pain sensitivity and inflammation in joints.
Inter-laboratory reliability	1	PAR-4 activation results in "edema and granulocyte infiltration" – corroborates a finding from another study.
Sample size	0	No description of power calculation.
Sex differences	0	Unknown if male and/or female mice were used.
Randomization	1	Mice were randomly assigned to treatment groups.
Blind outcome assessment	0	No description in the methods.
Publication venue	1	Arthritis & Rheumatism
Important experimental parameters described	1	Instruments used, outcome measures, and interpretation of results were described.
Statistical analysis method	1	Statistical analysis was outlined in the methods section.
Inclusion/exclusion criteria for data	0	Not clear why the results from the joint inflammation and pepducin experiment showed 6-10 mice per group when all the other experiments had 8 mice per group.
Negative results and/or outliers discussed	1	Results from all the timepoints were reported.
Pre-registration of study prior to experiment initiation	0	No prior registration.

Figure 6.5 Analysis of study design for the McDougall et al., 2009 publication.
This publication received a validity score of 4/8 (A) and a reliability score of 6/12 (B).

Chapter 7 Discussion

Pain is a highly debilitating aspect of rheumatoid arthritis which is difficult to manage due to the complicated etiology of the disease and limited efficacy of current analgesics. Inflammatory joint pain affects approximately 15% of the global population (Botz et al, 2017). The prevalence of this condition is expected to increase due to the aging population and the rising incidence of chronic diseases such as arthritis. The management of joint pain typically involves nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, ibuprofen, and naproxen. However, their use is limited by their adverse effects such as gastrointestinal bleeding, renal toxicity, and cardiovascular events (Varrassi et al, 2019). Opiates are also used to manage moderate to severe joint pain, but their use is limited by their potential for addiction, overdose, and liver damage (Yaksh et al, 2015). There is a pressing need for novel analgesics to manage inflammatory joint pain. The limitations of currently available drugs highlight the need for new therapeutic approaches that can provide effective pain relief with fewer adverse effects. The development of novel analgesics is essential to address the unmet needs of patients suffering from joint pain. There are several challenges that pose a barrier to the development and approval of new analgesic drugs. Pain is a highly complex, multifactorial disease. Limitations in our overall understanding of the neurobiology of joint pain, limitations arising from animal models of pain, and limitations of preclinical pain testing are some of the scientific challenges impeding the development of novel analgesics.

FCA injections in the knee joint of rats induced a model of chronic inflammatory arthritis that resembles some of the characteristics of rheumatoid arthritis in humans. Features of rheumatoid arthritis produced in the FCA rat model include tissue inflammation, joint pain, and peripheral nerve damage. Enzymes released into the joint produce inflammation, pain, and

neuropathy. In addition to their catalytic activity, these enzymes also cleave PARs e.g., PAR4. The PAR4 receptor has been identified as a promising target in modulating pain and inflammation in arthritic joints. This two-part study looked at: (1) the efficacy of a PAR4 antagonist, pepducin P4pal-10, at reducing joint pain in a rat model of rheumatoid arthritis; (2) a general assessment of how preclinical pharmacology studies are analyzed by the FDA. The experimental approach used to determine the validity and reliability of study design was then applied to pepducin P4pal-10. Key findings obtained from my assessment of pregabalin and duloxetine revealed details about the FDA's requirements for validity and reliability of preclinical pharmacology studies, which informed my predictions related to the likelihood of pepducin becoming the subject of FDA review for an IND. Transparency issues surrounding preclinical data used to support INDs and NDAs were also discussed. In this chapter, results from this study will be presented to reveal PAR4 involvement in chronic inflammatory arthritis, along with a substantial number of preclinical studies that investigate pepducin P4pal-10 in models of joint pain, indicative of a compelling case for an IND submission.

7.1 Pepducin P4pal-10 Preclinical Studies

Systemic administration of pepducin P4pal-10 improved hind paw withdrawal threshold on day 7 and 21 of the FCA model. The significant improvement in hind paw withdrawal threshold after pepducin P4pal-10 treatment demonstrates a reduction in secondary allodynia (referred pain). McDougall et al (2009) showed that pepducin P4pal-10 pre-treatment of kaolin/carrageenan mice knee joints significantly reduced synovial hyperplasia, cellular infiltration, hyperemia, and joint edema. Other studies investigating the efficacy of pepducin P4pal-10 in osteoarthritis models also displayed an improvement in hind paw withdrawal threshold at certain time points (O'Brien and

McDougall, 2021). Pepducin P4pal-10 did not improve hindlimb weightbearing, indicating it was not efficacious at modulating spontaneous pain. Taken together, these findings reveal that pepducin P4pal-10 has differential effects on distinct aspects of the pain pathway and might be more effective at modulating FCA-induced central sensitization.

This study is the first to investigate the anti-nociceptive effects of a systemically administered PAR4 antagonist in a rat model of chronic inflammatory joint pain. An earlier study in mice showed that PAR4 activation had a pro-nociceptive effect in joints, resulting in pain and inflammation at the joints (McDougall et al, 2009). Pepducin P4pal-10 ameliorated the physiological and clinical aspects of acute joint inflammation in mice (McDougall et al, 2009). In another study involving rat models of osteoarthritis (MMT and MIA), pepducin P4pal-10 reduced both secondary allodynia and joint nociceptor firing during the acute inflammatory phase, but not the chronic neuropathic stage of the disease (O'Brien and McDougall, 2021). My study demonstrated that pepducin P4pal-10 reduced secondary allodynia during the early stage of chronic inflammatory joint pain (day 7 post-FCA) and the late stage (day 21 post-FCA). These findings suggest that pepducin P4pal-10 is effective at reducing mechanonociception arising from acute and chronic forms of inflammatory joint pain.

The mechanisms of action for the PAR4 antagonist, pepducin P4pal-10, has also been evaluated in several other diseases including a visceral model of inflammatory pain, systemic inflammation (sepsis), and airway disease (Annahazi et al, 2012; Slofstra et al, 2007, Carr et al, 2016). Pepducin P4pal-10 exacerbated visceral hypersensitivity in a rat model of ulcerates colitis (Annahazi et al, 2012). Pepducin P4pal-10 dose-dependently decreased the severity of systemic inflammation by preserving kidney, liver, and lung function (Slofstra et al, 2007). Pre-treatment

with pepducin P4pal-10 appeared to inhibit primary human airway smooth muscle growth, which may be a novel approach to treat airway disease (Carr et al, 2007).

None of the preclinical studies involving pepducin P4pal-10 reported adverse side effects arising from the drug treatment. However, most of these studies tested only a single dose and single concentration of pepducin P4pal-10. No dose-response curves were reported. Therefore, information about the therapeutic scope of pepducin P4pal-10 is lacking. The effect of repeated dosing is also unknown. These concepts are typically tested by preclinical research conducted as part of IND-optimization studies. IND-optimization studies may involve testing the safety and efficacy of the drug in multiple species and performing toxicity studies etc. These preclinical pharmacology and toxicology data are an essential component of an IND submission, which is used by the FDA in their decision-making of whether to allow the drug to be used in clinical trials. In recent years, there have been many initiatives to increase transparency in clinical trials, evident in the mandatory registration of clinical trials (clinicaltrials.gov) and meta-analyses conducted on clinical trial data obtained from regulatory reports. In contrast, very little attention has been directed towards improving transparency in preclinical aspects of INDs and NDAs. In the next few sections of this chapter, I will discuss how preclinical pharmacology studies were analyzed by the FDA and potential transparency issues surrounding how preclinical data are used to support INDs and NDAs.

7.2 FDA's Evaluation of Animal Studies

My analysis of the preclinical studies submitted in the NDAs for Lyrica and Cymbalta (Chapter 4 and 5 respectively) revealed that the FDA did not routinely conduct a rigorous analysis of the animal pharmacology and toxicology data. For both drugs, a summary of the main findings

from the pain behaviour studies were included in the pharmacology reviews, along with a few details about the animal models and pain outputs measured. The amount of preclinical information presented in the regulatory packages was much less than the amount of clinical trial information. In addition, those preclinical pain behaviour studies referenced study reports which were not displayed in any other parts of the package. The pain behaviour study reports are distinct from published articles as they are a document containing information about the preclinical study, prepared by the sponsor specifically for an IND or NDA submission. Published articles were occasionally discussed or referenced. It is crucial to note that the FDA is not required by law to include preclinical data in their regulatory approval packages; there are no CDER policies advising how much or what type of preclinical data should be disclosed, and the Code of Federal Regulations Title 21 only governs the release of the NDA reviews as quickly as possible after a drug is approved (Center for Drug Evaluation, 2022; Code of Federal Regulations, 2023). This shows that the FDA is not completely transparent in their disclosure of information from preclinical studies.

A careful analysis of the NDA approval packages for Lyrica and Cymbalta indicated that FDA accepted the preclinical information (typical or expected results) presented by the sponsors at face value, without performing any additional checks for validity or reliability of those animal studies. The FDA only conducted an independent analysis when unexpected toxicology data were uncovered, for instance, the extremely high incidence of hemangiosarcoma in mice treated with Lyrica. From this example, it was evident that the FDA was primarily concerned about safety (toxicology) and not particularly concerned about efficacy during the review of preclinical data submitted as part of an NDA. Perhaps most of the discussions on the demonstration of efficacy through preclinical data were conducted during the IND review, which may explain why this

essential aspect did not appear in the NDA review. However, these regulatory risk-benefit analyses of preclinical data should be published as part of the NDA approval package, as these findings would benefit many parties including prospective clinical trial participants and researchers conducting preclinical studies. Discrepancies between clinical trial results and journal publications of clinical outcomes are a common occurrence, which leads me to postulate that there may also be many discrepancies between preclinical data submitted to regulators and what was published in peer-reviewed journals. Therefore, the FDA can facilitate greater transparency by publishing the IND review as a section in an NDA approval package.

7.3 Animal Studies can be a Deciding Factor in the FDA's Risk-Benefit Analysis

The greatest amount of preclinical pharmacology / toxicology information was presented in an NDA for a drug's initial FDA approval. This was most apparent during the NDA review for the drug's first in human indication, which was observed during the regulatory approval process for Lyrica. It was also evident that in an NDA review, the FDA was more concerned about safety (toxicology) than efficacy (preclinical pharmacology). In the case of Lyrica, animal toxicology studies showed an

> "Increased incidence of hemangiosarcoma in mice is indicative of a true tumorigenic response to pregabalin. Executive Committee for Animal Care (E-CAC) disagrees with Pfizer that hemangiosarcoma are specific to the mouse strain that were studied. Another 2-year bioassay in a different mouse strain, and reanalysis of the rat data, were suggested."

This led to a clinical hold which meant that all ongoing clinical studies involving Lyrica were halted. The FDA informed the sponsor that "based on the E-CAC conclusions and with little safety margin between mouse exposure and intended human exposure levels, the risk-benefit ratio

does not justify continued clinical development. […] Carcinogenicity of pregabalin is an approvability issue." Therefore, animal studies revealing unexpected toxicology findings that had unknown significance in humans became a deciding factor in the FDA's risk-benefit analysis and led to a clinical hold of ongoing trials.

Since the time of the FDA approval of Lyrica and Cymbalta for their initial pain indication, many preclinical studies investigating the safety and efficacy of those drugs have been published (Federico et al, 2020). However, for both drugs, the same set of studies submitted for the initial pain indication was referenced in each of the subsequent NDAs. The newer studies may support or refute the earlier submitted studies. For example, none of the preclinical studies included in Lyrica's NDA approval package examined sex differences in the response to pregabalin. A study published by Ungard et al (2020) showed that efficacy of Lyrica in rat models of cancer and neuropathic pain were significantly affected by sex differences; analgesic effects were much greater among the male animals. Another study identified sex differences in responsivity to Lyrica using a non-human primate model of unilateral spinal nerve ligation, along with differential brain activation, which may contribute to differences in chronic pain perception and responses to analgesics (Murata et al, 2023). It is odd that the regulators do not require the sponsors to submit up-to-date information and there are no requirements outlined in the Code of Federal Regulations (title 21, part 314) stipulating the submission of an updated nonclinical pharmacology & toxicology section.

7.4 Disagreement Between Reviewers are Potential Safety Issues

In the initial NDA review for Lyrica, there was disagreement between the pharmacology reviewers. On Oct 30, 2003, Dr. Jerry Cott (primary pharmacology reviewer) and Dr. Daniel

Mellon (secondary pharmacology reviewer) do not recommend Lyrica for approval for the DNP indication from a toxicology / pharmacology perspective due to unexplored risk related to diabetic patients being exposed to pregabalin over an extended time. Concerns include elevated incidence of hemangiosarcoma and dermatopathy in animal studies and potential interaction between PPAR-gamma agonists and pregabalin. The pharmacology reviewers recommended that "additional studies should be conducted to investigate the mechanism of dermatopathy in rats and monkeys in order to assist in determining the potential relevance to humans". On June 24, 2004, Dr. Kenneth L. Hastings (Associate Director for Pharmacology and Toxicology at the Office of Drug Evaluation II) submits a memorandum recommending approval, along with an explanation for his disagreement with the primary and secondary pharmacology reviewers. Dr. Hastings wrote that (1) no evidence of carcinogenicity was observed in male Wistar rats and (2) skin sores evident in animal studies were not observed in clinical trials. There is no evidence of additional animal studies or analyses submitted prior to Dr. Hastings' evaluation, despite a lapse of 18 months between the clinical hold and subsequent NDA submission. No further pharmacology/toxicology studies were recommended by Dr. Hastings. There is a lack of transparency surrounding Dr. Hasting's decision-making process to reach the incontrovertible stance of approving Lyrica. Perhaps this was a result of the bureaucratic structure of the FDA, where directors have the final say in times of disagreements and may not be required to fully explain their reasoning. Such cases of disagreement between reviewers and directors in which a final decision is made without substantial evidence is suggestive of safety issues that should not be underestimated.

There are some controversies surrounding the FDA approval of Cymbalta and Lyrica for pain indications. Clinical trials demonstrated limited efficacy in the treatment of chronic musculoskeletal pain and fibromyalgia, yet Cymbalta and Lyrica received FDA approval for these

two indications. In stark contrast, Cymbalta was issued a refusal notice for the chronic musculoskeletal pain indication by the European Medicines Agency (EMA) and the Australian. Therapeutic Goods Association (TGA). The conclusions reached by EMA in their review of Cymbalta emphasized that the lack of evidence due to (1) questionable clinical relevance arising from the absence of an active comparator, (2) exacerbation of cardiovascular and GI problems, and (3) limited data on long-term safety and efficacy, especially in the elderly who would consist of a large proportion of the drug's target population. The TGA highlighted their refusal was based on several multiple factors, including the significant risks associated with the use of Cymbalta in patients with reduced hepatic capacity and limitations arising from the narrow scope of the clinical trials which only involved patients with OA of the knee (TGA, 2012). Lyrica received a refusal notice for the fibromyalgia indication from the EMA. As the fibromyalgia clinical trials were conducted in a patient population from the USA, the Committee for Medicinal Products for Human Use (CHMP is a division within the EMA) cited "geographical differences in the way which fibromyalgia is perceived, diagnosed and managed, making studies in the EU population recommendable in view of an approval for the European market" (EMA, 2009). Additional reasons for the refusal included the lack of sufficient evidence supporting clinically relevant benefits in functional improvements and pain over the short-term, insufficient evidence of long-term efficacy, resulting in an overall unfavourable risk/benefit profile. The disagreements between regulators from different countries is further indicative of safety issues.

7.5 Lessons from Pregabalin and Duloxetine: NDA vs. sNDA requirements

A supplementary NDA (sNDA) is closely associated with an existing NDA. An sNDA is submitted to change a label, market new dosage, or change the manufacturing method of a drug.

A sNDA can also be used to add a new indication to an existing NDA in a manner that would require the submission of much less information than an additional NDA. An sNDA was submitted to add fibromyalgia to the list of conditions for which Lyrica was approved. On June 21, 2007, Lyrica became the first drug to received FDA approval for the fibromyalgia pain. Interestingly, no new information, besides the approval letter, related to the fibromyalgia indication could be found on the Drugs@FDA webpage for pregabalin. A proposed new indication is considered a major change to an approved NDA according to Section 506A 314.70(c) of the Federal Food, Drug, and Cosmetic Act (Center for Drug Administration and Research, 2004). The FDA also stated that major changes have a substantial potential to affect the safety or efficacy of the drug product, requiring the submission of an sNDA and approval by the FDA prior to including the proposed changes in the labelling of the drug. Therefore, there is a reasonable expectation that the FDA would publicly disclose their sNDA review for a new drug indication. It is worthwhile to consider that for Lyrica CR (extended-release formulation), the fibromyalgia indication was rejected due to insufficient evidence of efficacy in the two clinical trials. No new preclinical information was submitted in the pregabalin sNDA or for Lyrica CR. Instead, extensive references were made to the initial Lyrica NDA for DPN (#021446).

In contrast, Eli Lilly submitted an NDA (#22148) for duloxetine to be used in the treatment of fibromyalgia. The referenced application was NDA #21-427, which was the initial approval of duloxetine for the treatment of major depressive disorder (MDD). No new non-clinical pharmacology studies were submitted in NDA #22148. The pharmacology reviewers made extensive referencing to NDA #21427 and #21733, and recommended approval based on the information submitted in those two NDA packages. Concerns were raised by the statistical reviewers about the lack of clinical efficacy at 6-months and 12-months of treatment, but no further

investigation was conducted by the regulators and duloxetine received FDA approval for the management of fibromyalgia on June 13, 2008. Overall, the pregabalin sNDA involved much less regulatory scrutiny than the duloxetine NDA for the fibromyalgia indication.

7.6 Implications for Pepducin P4pal-10

My analysis in Chapter 3 revealed that the highlighted set of studies examining pepducin P4pal-10 in joint disease appears to have high validity and moderate reliability. Pepducin P4pal-10 displayed efficacy at reducing acute and chronic inflammatory pain across multiple rodent models, making it an enticing candidate drug for the treatment of inflammatory joint pain. Additional IND-optimization studies are necessary to obtain pharmacokinetic, pharmacodynamic, and toxicology data typically required for an IND application. Other essential experiments for an IND application may include replication experiments of findings obtained by other research labs and in other animal models.

Several similarities and differences were observed for the preclinical evidence underpinning the duloxetine and pregabalin FDA approvals. Both drugs were tested in a multiple pain models and multiple animal species. The drug effects were also measured using multiple pain outputs and when varying the route of drug administration. Dose-response assays and locomotive activity assays were also performed for both drugs. Randomization, blinding, examination of sex differences, and discussion of negative results were not described in any of the pregabalin and duloxetine preclinical studies. Active controls (e.g., duloxetine was compared against gabapentin, morphine, and ibuprofen) were used in the duloxetine study involving rodent models of acute and inflammatory pain, which was an element of experimental design not observed in any of the pregabalin studies. Eight preclinical pain studies were published in pregabalin's initial NDA

approval package while five were published in duloxetine's NDA package for its first pain indication. It is likely that a greater amount of preclinical pain studies was included in Lyrica initial NDA package because the drug was seeking its initial regulatory approval for a pain indication. In contrast, duloxetine was already approved for the treatment of MDD at the time of the NDA review for its first pain indication of DPN. Additionally, there were more discussions related to preclinical data in the initial pregabalin review than in any of the duloxetine reviews for pain indications. This demonstrates that the FDA reviewers pay more attention to the preclinical pain studies during the initial NDA review for a pain indication, and when a drug has already been approved for a non-pain indication, subsequently the regulators are less concerned about the preclinical evidence of efficacy.

A comparison of the preclinical studies for pepducin P4pal-10 showed that most of the variability and reliability variables present in the duloxetine and pregabalin studies were also present in the pepducin P4pal-10 studies. In addition, some of the pepducin P4pal-10 studies described the use of randomization and discussion of negative results, two reliability variables which were not observed in any of the pregabalin or duloxetine studies. However, no dose-response or locomotive activity assays have been performed for pepducin P4pal-10. A minimum of five to eight preclinical pain studies are likely to be required in the IND application for pepducin P4pal-10, the exact number of studies may also greatly depend on the specificity of the proposed indication. A more general indication such as "somatic pain" may require a larger number of preclinical pain studies while a more specific indication such as "musculoskeletal pain" may require a smaller number of supporting preclinical pain studies. It is also possible that the regulators may require a larger number of preclinical pain studies based on the IND being submitted for an entirely new molecular entity in the case of pepducin P4pal-10.

Putting a novel drug through the FDA regulatory approval process is a lengthy and costly endeavour, which is typically only possible when there is major financial backing and strong likelihood of return on investment. While my analysis in Chapter 3 revealed that the set of studies investigating pepducin P4pal-10's role in joint disease appears to have high validity and moderate reliability, a strong base of preclinical studies alone is unlikely to be sufficient for pepducin P4pal-10 to reach the IND stage of drug development. In the case of pregabalin and duloxetine, both compounds had 10+ years of patent exclusivity remaining and were backed by large pharmaceutical companies (Pfizer and Eli Lilly respectively) throughout the IND and NDA process. Largely due to patent exclusivity, pharmaceutical companies were incentivised to support a drug through the costly and multi-year FDA regulatory approval process as they anticipated a massive return on investment (DiMasi et al, 2016). The high costs involved in every stage of drug development means that favourable financial returns are almost always the main driver of pharmaceutical innovation (Morgan et al, 2011). For example, between 2009 and 2018, the median cost of bringing a new drug to market was estimated to be USD$985 million and the average cost was USD$1.3 billion (Wouters et al, 2020).

Pepducin P4pal-10 was patented in the USA by Athan Kuliopulos and Lidija Covic from Tufts Medical Center Inc (Kuliopulos and Covic, 2013). Their utility patent "*G Protein Coupled Receptor Agonists and Antagonists and Methods of Activating and Inhibiting G Protein Coupled Receptors Using the Same*" (US8389480B2) expired on May 26, 2021, due to unpaid maintenance fees. This indicates that the manufacture and use of pepducin P4pal-10 is now in the public domain. All the recent studies conducted on pepducin P4pal-10 were by researchers in academia. While many questions remain about the safety and efficacy of Pepducin P4pal-10, this drug compound

residing in the public domain presents an exciting opportunity for academic researchers to seek out biotech venture capital funding.

7.7 Summary

Pepducin P4pal-10 reduced mechanonociception during the early stage and the late stage of the FCA rat model of chronic inflammatory joint pain. Spontaneous pain was unaltered at both the early and late stages, suggesting that PAR4 is a useful target for addressing inflammatory pain associated with rheumatoid arthritis. Overall, my findings suggest that the set of preclinical studies investigating pepducin P4pal-10 in joint disease has high validity and moderate reliability.

An examination of the NDA approval packages submitted for the pain indications of pregabalin and duloxetine revealed that the regulators do not evaluate validity and reliability of the preclinical data. The set of preclinical studies for pregabalin had moderate validity and moderate-high reliability while those for duloxetine had high validity and low reliability. The NDA submitted for the drugs' first pain indication contained the greatest amount of preclinical information. However, few details related to the impact of preclinical data on regulatory decision-making were included in the NDAs. A lack transparency in the FDA's disclosure of preclinical data was evident.

Based on the quantity and quality of preclinical studies submitted as part of the NDAs for pregabalin and duloxetine, the body of literature available for pepducin P4pal-10 appear to be sufficient for the compound to proceed to the IND stage of drug development. However, putting a candidate drug through the FDA regulatory approval process is time consuming and an extremely costly endeavour. Acquiring sufficient capital to finance the IND submission, IND-optimization

experiments, and clinical trials for a non-patentable drug like pepducin P4pal-10 might be the largest challenge standing in the way of the drug's transfer from the lab to clinic.

7.8 Limitations

7.8.1 Use of animals to study pain and arthritis

No animal model of rheumatoid arthritis fully replicates the disease in humans. Spontaneous forms of rheumatoid arthritis have been observed in non-human primate (NHP) models (e.g., rhesus macaques) which can model the natural disease progression of RA from the acute to late phase of the disease with a similar pathophysiology to human RA (Zhao et al, 2022). However, the use of rodent models is much more widespread in an academic laboratory setting due to practical reasons arising from genetic homogeneity, cost, and reproducibility. Male Wistar rats were used in this study to model chronic inflammatory arthritis which was induced through an intra-articular injection of FCA. A major limitation of the FCA model is the mild cartilage damage which is much lower in severity than in human RA. As a result, the FCA model cannot be used to accurately study histopathological changes that may occur in RA.

Rats used in my study were young adults of approximately 16 weeks old, serving as a model of young-onset rheumatoid arthritis (YORA) which typically occurs in between the ages of 16 to 40 (El-Labban et al, 2010). Human RA, however, occurs more often after the age of 60, in the form of late-onset rheumatoid arthritis (LORA). Differences in disease manifestation and response to disease-modifying drugs exist between YORA and LORA patients (Romao and Fonseca, 2021). It should be noted that RA is 2-4 time more frequent in females, yet my preclinical study was conducted in male rats only. Therefore, assessing the efficacy of pepducin P4pal-10 in

the FCA model involving female rats and older animals is essential to further characterize the effect of sex and age in the RA model.

7.8.2 NDA packages from approved drugs only

Regulatory approval packages were available only for drugs that successfully gained FDA approval. Numerous other INDs and NDAs were certainly submitted for compounds with analgesic properties but were ultimately rejected by the FDA. Reasons for regulatory refusal include clinical trial data showing insufficient evidence of efficacy, unaddressed safety issues in preclinical studies where the clinical significance is unknown, resulting in an unfavourable risk-benefit profile. The FDA does not publish the regulatory review of a rejected drug or details about additional disease indications that were rejected. The regulatory approval packages published by the FDA served as a primary source of information in my study; however, the availability of information meant that I only had access to the packages for successful drugs and pain indications that eventually received FDA approval. Studying successful examples of approved drugs exclusively may result in crucial aspects of the regulatory process being overlooked. By studying both successful and unsuccessful examples, it is more likely that a comprehensive understanding of the salient aspects in preclinical pharmacology which affect regulatory decision-making will be uncovered.

My study also revealed that much of the preclinical pharmacology information and related regulatory discussions occurred during the review of the IND application. As the FDA does not publish the IND review for any drugs, this means that a large amount of preclinical pharmacology information remains inaccessible to the public. The pharmacology review section of the NDA often made extensive references to studies that were previously submitted in the IND; however,

those full preclinical study reports were not included in any other part of the NDA and thus not possible for me to extract for analysis. Instead, I relied on the corresponding peer reviewed publication of the preclinical study that was referenced/discussed in the NDA, under the assumption that the contents of the preclinical study reports and the peer reviewed publications are highly similar. As the IND contains a significant amount of preclinical information used by the FDA in their decision to authorize human clinical trials, publication of IND documents as part of the NDA package would serve to increase transparency in the regulatory process. This is particularly important for rare disease drugs or those that had highly controversial approvals.

7.8.3 Choice of variables in rubric

My decision to include the 20 variables in the rubric was strongly influenced by the ARRIVE, PPRECISE, and Landis 4 guidelines for best practices in animal research. The variables were chosen because they were recommended repeatedly in those three guidelines and a total of 20 variables felt like the upper limit of manageability for my study. While continuing my research, I came across several other important variables that can contribute greatly towards the validity and reliability of study design e.g., blinded treatment allocation, age of animals, and evaluation of anxiolytic-like behaviours. Construct validity may be improved through the consideration of age, as RA is known to have different disease manifestations across the lifespan. For example, this can be achieved by testing the efficacy of a drug in young adult and aged rodents. Internal validity may be improved by blinding the investigator to treatment allocation, and an evaluation of anxiolytic-like behaviour. While my rubric included assessment of locomotor activity to rule out confounding effects arising from motor impairment, anxiolytic activity is likely an additional confounding variable in pain behaviour experiments. Therefore, behavioural methods such as the open field test

and elevated plus-maze should be used to assess anxiety and locomotor activity concurrently in rodent models of pain (Haller et al, 2013; Heredia et al, 2014).

Many reputable journals now require the pre-registration of the study prior to initiation of experimentation, along with declaration of adherence to the ARRIVE 2.0 guidelines. The consideration of sex differences in research is a relatively recent requirement by many funding agencies. These are examples of some of the recent changes that have become commonplace in preclinical animal research. However, almost all the publications for pregabalin, duloxetine, and pepducin P4pal-10 were conducted in the early 2000s, long before those practices were widely adopted.

7.8.4 Binary scoring of variables

As a starting point, a binary scoring system was used to grade each variable. If the variable was present, a score of +1 was given. If the variable was absent, a score of 0 was given. The binary scoring system was selected to give equal weight to each variable as each one was an important contributor to the overall validity and reliability of a study. It was difficult to conclusively ascribe a higher weightage to some of the variables. Instead, I chose to standardize the rubric and scoring by using a binary scale. However, in hindsight some of the variables might not be most appropriately captured by the binary scale. Future refinement of the rubric should include weighting.

7.9 Future Directions

Further experiments investigating the efficacy of pepducin P4pal-10 in the FCA model are necessary to further characterize the role of PAR4 in the rat model of rheumatoid arthritis.

My study can benefit from an analysis of additional drugs that have received FDA approval for musculoskeletal pain indications e.g., milnacipran (Savella) and gabapentin (Neurontin). An analysis of how other regulatory agencies such as Health Canada, Australia's Therapeutic Goods Administration, and the European Medicines Agency analysed the preclinical data for pregabalin and duloxetine may also be an interesting avenue to pursue.

7.9.1 Efficacy of Pepducin P4pal-10 for RA Pain

Pain behaviour measurements should be conducted to examine the efficacy of pepducin P4pal-10 in the FCA model using female rodents and aged animals to further characterize the role of PAR4 in the rat model of chronic inflammatory pain. Performing electron microscopy of the saphenous nerves from day 7 post-FCA animals may also be useful for our understanding of the extend the nerve damage and joint neuropathy as the FCA model develops. It may also be useful to perform electrophysiology experiments to characterize changes in joint nociceptor firing in response to pepducin P4pal-10 treatment. Pepducin P4pal-10 should also be evaluated in additional animal models of RA pain such as the collage-induced arthritis (CIA) model, where an emulsion containing a type II collage and FCA is injected into the base of the tail.

7.9.2 Analysis of additional FDA approved analgesic drugs

At the time of writing, three drugs have received FDA approval for fibromyalgia: Lyrica (2007), Cymbalta (2008), and Savella (2009). Savella (milnacipran) is the only drug that received FDA approval for a single indication – management of fibromyalgia. Interestingly, Savella is approved for fibromyalgia in the USA, Australia, but not in the European Union. As fibromyalgia is often challenging to manage and FDA approvals for fibromyalgia were rather controversial, it

might be worthwhile to examine the preclinical pain studies for milnacipran, to understand the impact of animal studies on regulatory decision-making in this disease.

7.9.3 Analysis of additional regulatory agencies

Regulatory reports are publicly disclosed by Health Canada, Australia's TGA, and the European Union's EMA. While the types of content released by each regulatory agencies may vary, an analysis of the regulatory approval process (focusing on the preclinical data for Lyrica, Cymbalta and Savella) within each of the regulators may reveal some strengths and weaknesses in their respective assessments. The disagreements by reviewers at the same agency may point towards potential safety issues. Furthermore, if/how preclinical studies inform the rationale underlying differences in the final approval decisions for the same drug by each regulatory agency may also be a compelling avenue of further consideration.

6.10 Conclusion

This is the first study to investigate the preclinical decision-making processes necessary for drug approval by the FDA. Duloxetine and pregabalin are two drugs that have received FDA approval for several pain indications; an analysis of their NDA approval packages revealed a lack of transparency in the disclosure of preclinical information. The FDA fell short of scientific rigour by not analyzing the validity or reliability of preclinical studies submitted as part of an NDA. Pepducin P4pal-10 is a promising compound for the treatment of joint pain and inflammation. The results presented in my study demonstrated that pepducin P4pal-10 reduced mechanonociception but not spontaneous pain in the FCA model of RA. The set of publications for pepducin P4pal-10

have high validity and moderate reliability, suggesting that this compound has the potential to move forward to the IND stage of drug development.

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Appendix A: Drugs, Reagents, and Devices

Drug	Manufacturer	Description
Isoflurane	CDMV (Dartmouth, NS, CAD)	Gaseous general anaesthetic
Pepducin P4pal-10	Genescript (Piscataway, NY, USA)	PAR-4 antagonist
Saline (0.9%) NaCl	In-house supplier	Used as a control

Reagent	Manufacturer	Description
Glutaraldehyde	Electron Microscopy Sciences (Hatfield, PA, USA)	Sample fixative for electron microscopy
Sodium cacodylate buffer (1.0M)	Electron Microscopy Sciences (Hatfield, PA, USA)	Buffer for electron microscopy
Freund's Complete Adjuvant	Sigma Aldrich (St. Louis, MO, USA)	To induce FCA model of inflammatory arthritis

Device	Manufacturer	Description
Dynamic Weight Bearing System	Bioseb-DWB-AUTO-R, equipped with DFK22AUC03 camera, Bioseb software 1.4.2.92	Dynamic weight bearing system and software from Bioseb (Boulogne, France) Camera from ImagingSource (Charlotte, NC, USA)
Von Frey Chamber	Custom built	Plexiglass from Concept Plastics Inc. (Dartmouth, NS, CAD)
Von Frey Hairs	Semme Weinstein Microfilaments	North Coast Medical (Gilroy, CA, USA)
Transmission Electron Microscope (TEM)	JEOL JEM-1230, Hamamatsu ORCA-HR camera	TEM from JEOL Corp Ltd (Tokyo, JPN), Camera from Hamamatsu Photonics (Hamamatsu City, JPN)