Codeine, Heightened Pain Sensitivity and Medication Overuse Headache:

A Neuroimmune Hypothesis and Novel Treatment Strategy

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ABSTRACT

Codeine is the most widely consumed opioid analgesic worldwide. It relies upon partial metabolism to morphine to elicit analgesic effects. Paradoxically, the pain-reliever morphine has previously been linked to states of increased pain sensitivity; such as medication overuse headache and opioid-induced hyperalgesia and allodynia.

Despite the clinical impact of medication overuse headache the pathophysiology behind this disorder remains unclear and mechanism-based treatments are lacking. Although most acute headache treatments are alleged to cause medication overuse headache, within this thesis we conclude from the literature opioids are the drug class most strongly associated with worsening headache. In opioid-induced hyperalgesia and allodynia sensitivity to normally noxious, and non-noxious stimuli respectively, are enhanced due to opioid exposure.

Chronic morphine may exacerbate pain in the long-term by non-specifically activating toll-like receptor-4 (TLR4) on glial cells, resulting in a pro-inflammatory state that manifests clinically as increased pain. Here we hypothesise medication overuse headache is a specific form of opioid-induced hyperalgesia, which derives from a cumulative interaction between central sensitisation and glial priming, due to repeated activation of nociceptive pathways by recurrent headaches, and pain facilitation due to glial activation and subsequent neuroinflammation.

The first part of this thesis examines the efficacy of a glial-attenuating treatment, ibudilast, in the clinical management of medication overuse headache induced by opioid use in a double-blind, randomised, placebo-controlled parallel group study. Patients received ibudilast 40 mg twice daily or placebo for 8 weeks and recorded headache and analgesic intake using a headache diary for 4-weeks prior to randomisation and throughout the treatment phase.

No reduction in headache burden, opioid analgesic intake or headache related quality of life were observed in the ibudilast group compared to placebo, however, valuable safety data were obtained

demonstrating ibudilast 80 mg/day is well tolerated, facilitating the use of similarly high doses in future studies for alternative indications.

Prior to this PhD project the relationship between codeine and increased pain sensitivity had not been investigated. *In silico* docking simulations performed as part of this PhD suggest codeine binds to MD2, an accessory protein for TLR4, signifying it may be able to induce hyperalgesia independent of conversion to morphine. Evidence that codeine can induce hyperalgesia would sit in line with our glial hypothesis for opioid overuse headache. Thus, the second part of this PhD includes a series of preclinical experiments to 1) determine if chronic codeine alters pain sensitivity 2) ascertain if pre-existing glial activation primes for opioid-induced hyperalgesia, 3) investigate signalling pathways involved and 4) assess potential interventions to reverse exacerbated pain sensitivity. Hyperalgesia and allodynia were measured using hot plate and von Frey tests respectively, at baseline, day 3 and day 5 in mice receiving intraperitoneal codeine 21 mg/kg, morphine 20 mg/kg or saline, twice daily.

Our preclinical studies demonstrate that despite providing lesser acute analgesia, equimolar codeine and morphine induced similar hot plate hyperalgesia, suggesting codeine does not rely upon conversion to morphine to increase pain sensitivity, emphasising the non-opioid receptor-dependent nature of this phenomenon. IL-RA reversed codeine-induced hyperalgesia and allodynia, and knock-out of TLR4 protected against codeine-induced pain sensitivity changes. Glial attenuation with ibudilast reversed codeine-induced allodynia and thus could be investigated as potential treatment for conditions involving codeine-induced pain enhancement.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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LIST OF ABBREVIATIONS

ABBREVIATION	DEFINITION
5-HT	5-Hydroxytrptamine, serotonin
CCI	Chronic Constriction Injury
CD11b	Cluster of Differentiation Molecule 11B
CYP	Cytochrome P450
DAMGO	D-Ala2,N-Me-Phe4,Gly-ol5 encephalin
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, Edition IV
GFAP	Glial Fibrillary Acidic Protein
HAD	Hospital Anxiety and Depression
ICHD-I	International Classification of Headache Disorders, First Edition
ICHD-II	International Classification of Headache Disorders, Second Edition
ICV	Intracerebroventricular
ID	Intradermal
IL	Interleukin
IT	Intrathecal
IV	Intravenous
MDQ-H	Medication Dependence Questionnaire in Headache patients
MIDAS	Migraine Disability Assessment
MOH	Medication Overuse Headache
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
PBMC	Peripheral Blood Mononuclear Cell
PREEMPT	Phase III REsearch Evaluating Migraine Prophylaxis Therapy
SC	Subcutaneous
SD	Sprague-Dawley
SF-36	Short Form 36 (36 items)
TLR	Toll-Like Receptor
TLR2	Toll-Like Receptor 2
TLR4	Toll-Like Receptor 4
TNF-α	Tumor Necrosis Factor α
TTH	Tension Type Headache
US	United States

LIST OF PUBLICATIONS DURING CANDIDATURE

Johnson, J, Rolan, P, Johnson, M, Bobrovskaya, L, Johnson, K, Tuke, J, Williams, D & Hutchinson, M 2015 "Codeine-induced hyperalgesia and allodynia: Investigating the role of glial activation." *Trans Psych,* e482–491.

Johnson, J & Rolan, P 2015 "Letter to the Editor: A paradoxical case of topiramate-induced hyperphagia." *J Clin Neurosci, (in press).*

Thomas, J, Mustafa, S, **Johnson, J**, Nicotra, L & Hutchinson, M 2015 " The relationship between opioids and immune signalling in the spinal cord." In Schaible, HG (ed.), *Pain Control. Handbook of Experimental Pharmacology*, vol 227. Springer Berlin Heidelberg. pp 207-238.

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Johnson, J, Rolan P, Williams, D & Hutchinson, M "*Codeine, increasing pain and medication overuse headache: The first evidence of codeine induced hyperalgesia."* Society for Neuroscience Meeting, San Diego, Nov 9-13, 2013.

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Johnson, J & Rolan, P "*Medication overuse headache: A neuroimmune hypothesis and novel approach to treatment.*" Centre for Neuroscience 2012 Collaborators Day, Adelaide, Sep 28, 2012.

Rolan, P & Johnson, J "*Medication overuse headache is a manifestation of opioid induced*

hyperalgesia: A neuroimmune hypothesis and novel approach to treatment." European Headache and Migraine Trust International Congress, London, Sep, 2012.

Johnson, J & Rolan P "*Medication overuse headache is a manifestation of opioid-induced hyperalgesia: A hypothesis and clinical trial design.*" University of Adelaide Faculty of Health Sciences Postgraduate Research Conference, Adelaide, Aug 31, 2012.

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Johnson, J "*Medication overuse headache – the pharmacists role.*" Invited presentation, Flinders Medical Centre Pharmacy Department Continuing Education Seminar Series, Flinders Medical Centre, Adelaide, Feb 23, 2011.

GENERAL INTRODUCTION TO THESIS

Opiates have been vital in pain medicine for millennia, yet there is still much to be discovered in terms of their pharmacology and optimal clinical use. Traditional opioid pharmacology involves agonism of μ-opioid receptors on neurons to reduce nociceptive transmission. However, over the past few decades we have come to understand the importance of the interactions between opioids and glial cells within the central nervous system in the modulation of both opioid analgesia and detrimental opioid effects.

While evidence-based guidelines steer physicians away from using opioids in the routine long-term management of headache, this message is poorly conveyed to the general public. Nine of out ten Australian headache suffers will not seek medical attention for their headaches, opting instead to self medicate as required[1]. In Australia the weak opioid codeine is available in over-the-counter combination analgesic preparations, which are often selected by consumers for the treatment of headaches. In some cases the intake of codeine-containing analgesics for headache becomes frequent and subsequently patients experience an increase in headache frequency, which is now thought to be a consequence of such excessive analgesic intake.

This thesis will discuss the headache disorder known as medication overuse headache, focusing upon that related to opioid intake, and will investigate new mechanisms, treatment options and study methodologies with the aim of improving outcomes for patients suffering from this debilitating condition.

In Chapter 1 an introduction to medication overuse headache will precede a review article, published as a part of this PhD, which ties what is currently known about the

pathophysiology of medication overuse headache and opioid-induced hyperalgesia together to form the basis of our neuroimmune hypothesis for medication overuse headache induced by opioid intake. The review paper then explores novel neuroimmune-based treatment strategies, highlighting the potential utility of ibudilast, a microglial attenuator, to assist opioid withdrawal in the management of medication overuse headache.

Based upon the hypothesis presented in Chapter 1, Chapter 2 details a clinical trial of ibudilast in the treatment of medication overuse headache patients who are overusing opioid analgesics. First the current treatment strategies employed in the management of medication overuse headache, and the shortcomings of such treatments, are discussed. Primary results from the clinical trial are presented in a manuscript, which is followed by an extended, comprehensive critical analysis of the study methodology, with the aim of rationalising study findings and identifying strategies to improve clinical trial design in the future.

To complement this clinical research a series of animal experiments were designed and executed to investigate each aspect of the hypothesis tested in the ibudilast clinical trial. Chapter 3 introduces concepts relating to the assessment of pain in preclinical studies, previously utilised models of opioid-induced hyperalgesia and allodynia and the basics of codeine pharmacology, before presenting a publication documenting the animal studies performed as part of this PhD. This manuscript characterises for the first time, a mouse model of codeine-induced hyperalgesia and allodynia, and subsequently explores the molecular mechanisms behind the development of increased pain sensitivity after codeine exposure.

Finally, Chapters 4 and 5, the discussion and conclusion, summarise all findings, relate the preclinical findings to our clinical study and hypothesis regarding the pathophysiology of medication overuse headache, place the data gained in context with regard to current understandings and clinical practice, and underline the significance of the contribution to knowledge delivered within this PhD thesis.

1. LITERATURE REVIEW AND HYPOTHESIS GENERATION

1.1 THE BURDEN OF HEADACHE

Headache is the most common neurological condition in developed countries[2], causing significant levels of pain and disability among sufferers worldwide[3]. It is the neurological symptom most commonly encountered by both general practitioners and neurologists[4]. Throughout the world almost half (47%) of the adult population suffer from a current headache disorder while the lifetime prevalence of headache has been reported to be greater than an astounding 90%[5].

Migraine was found to be the 19th leading cause of global disability by the World Health Organisation[6] and while no other headache disorders were included in the report, subsequent calculations indicate collectively headache disorders would have almost certainly ranked within the top 10[3]. Headache disorders substantially impair quality of life and impose a significant financial burden upon patients and the community. Notably, unlike other highly prevalent disorder which largely affect the elderly, headache tends to primarily affect individuals during their most productive years when they would otherwise be raising young families and contributing to the work force. The United States economy alone loses US$13 billion annually from the estimated 113 million work days lost due to headache[7]. From a patient's perspective, repeated headaches, coupled with ongoing fear of the next attack during the interictal period, can interfere substantially with employment and impact negatively on family and social relationships[8], highlighting the importance of the endeavour to manage chronic headache more successfully.

The International Headache Society has developed a comprehensive headache classification system, grouping headache disorders based upon their aetiology and phenotype[9]. This

classification separates headache disorders into three overarching groups, 'primary headaches', which are not attributed to an underlying cause, 'secondary headaches', which result from a secondary, treatable cause and finally 'cranial neuralgias central and primary facial pain and other headaches'. Collectively, the two most common primary headache disorders, tension-type headache (TTH) and migraine, and the secondary complication of medication overuse headache, are responsible for the greatest proportion of the public headache burden by far[10].

The acute treatment for both TTH and migraine often involves self-medication with non-prescription analgesics with the aim of attaining symptomatic relief. Such treatments may include simple analgesics such as paracetamol and non-steroidal anti-inflammatory drugs (NSAIDs) including aspirin, or combination preparations containing simple analgesics together with caffeine or low doses of the weak opioid codeine[11, 12]. Migraine specific agents, including the serotonin receptor agonists known as the 'triptans', ergotamine and dihydroergotamine are also used to abort episodes of migraine before they peak. Other prescription agents, including tramadol or products containing higher doses of codeine may also be prescribed for the acute relief of refractory headache. In Australia, the vast majority of patients with headache elect to self-medicate, and formal data regarding local patterns of over-the-counter analgesic use are not available. However, data from general practice indicates paracetamol is the analgesic agent most widely used to treat headache[13]. Codeine is the second most frequently employed analgesic, with greater than 15% of all patients presenting with headache being prescribed a codeine-containing preparation[13].

In some cases patients progress to use such symptomatic treatments on a frequent basis and subsequently suffer a marked worsening of their headache disorder; this condition is

now formally recognised in the International Classification of Headache Disorders II (ICHD-II), as a secondary form of headache called medication overuse headache[9].

1.2 MEDICATION OVERUSE HEADACHE

Medication overuse headache (MOH) is a disorder in which the frequent intake of symptomatic medications plays a role in the transformation of episodic headache into a chronic form[14]. It appears that the overuse of certain headache medications provides only short-term relief, at the expense of increasing headache intractability[15]. While it is difficult to confirm a definitive causal relationship, several lines of evidence, arising from both scientific studies and clinical observation, support the role of medication overuse in the chronification of pre-existing headache. As such, the diagnosis of MOH is now well accepted by clinicians and the scientific headache community.

Although MOH is commonly encountered in clinical practice, many aspects of this condition, including the precise criteria for diagnosis, pathophysiology and optimal management, remain controversial and require further research[16].

The clinical presentation of MOH encompasses huge variability and can depend upon both the patients primary headache disorder as well as the type of medication overused[17]. Patients with migraine and TTH seem to have the greatest potential for MOH[18], however MOH is occasionally reported in patients diagnosed with other primary headache disorders, including cluster headache[19] and hemicrania continua[20]. Medication overuse can also complicate other secondary headache disorders, for example it is reported that up to 42% of post-traumatic headache patients experience co-existing analgesic overuse[21].

Often MOH presents with peculiar pattern-shifting phenotype, with its features changing from those of migraine to those of TTH, even within the same day[9]. In most patients who begin with migraine as their primary headache disorder, overuse of triptans tends to result in an increase in migraine attack frequency or the development of daily migraine-like headaches[17], whereas overuse of analgesics leads to the presentation of a milder, TTH-like daily headache during which superimposed migraine attacks continue[22]. In MOH evolving from TTH the overuse of analgesics generally results in a greater frequency of TTH, and in some cases the TTH becomes constant, lasting 24 h a day every day[17].

1.2.1 Diagnosis and classification of medication overuse headache

Medication overuse headache, specifically ergotamine overuse headache, was first recognised and clearly described in 1951 by Peters and Horton of the Mayo Clinic, Minnesota[23]. Their publication detailed the adverse effects of excessive ergotamine intake, warning clinicians to be "…cautious in the administration of these ergotamine preparations, to instruct the patient to use the drug sparingly and not to abuse its use such as by taking it daily"[23]. In 1963 the pair published a case series describing 52 chronic headache patients who used excessive amounts of ergotamine for long periods and improved clinically following ergotamine withdrawal[24]. Similarly, in 1955 Friedman and colleagues observed the phenomenon of ergotamine overuse headache commenting, "…in some patients the use of ergotamine relieves the headache for which it is administered but at the same time leads to an increased frequency of these headaches"[25].

Analgesic overuse headache was probably first observed by pharmaceutical industry workers in Switzerland, many of whom, following the irresponsible intake of free phenacetin samples, developed chronic headache[26]. In 1982 Kudrow confirmed the paradoxical effects of frequent analgesic use in headache patients in the first placebo-controlled trial which

demonstrated that the mean headache improvement rate of amitriptyline-treated chronic headache patients was greater in those randomised to cease, rather than continue, analgesic use[27]. In the same year Isler highlighted that chronic migraine could be caused by migraine medications[28], and Mathew and colleagues also observed that the excessive use of acute medications could contribute to the transformation of episodic migraine into a chronic headache disorder, coining the term 'transformed migraine' in 1987[29, 30].

Today the classification of chronic daily headaches, particularly when associated with frequent medication intake, remains a source of much controversy. In the past many terms have been used to describe MOH, including rebound headache, drug-induced headache, drug-misuse headache, medication-misuse headache, and medication abuse headache, amongst others [31-34]. The International Headache Society first published the International Classification of Headache Disorders (ICHD-I) in 1988, recognising headache associated with medication overuse as a secondary headache disorder.

The ICHD-I revolutionised the diagnosis of headache, being the first system to provide explicit inclusion and exclusion criteria for a wide range of headache disorders[9]. The classification distinguished between '8.2-Headache induced by chronic substance use or exposure', specifically citing 'Ergotamine overuse headache' and 'Analgesic abuse headache' as sub-forms, and '8.4-Headache from substance withdrawal (chronic use)', with sub-forms 'Ergotamine withdrawal headache', 'Caffeine withdrawal headache' and 'Narcotic abstinence headache'[35]. The ICHD-I criteria for the diagnosis of Headache induced by chronic substance use or exposure are outlined in Appendix 1. The ICHD-I also acknowledged headache induced by chronic intake of ergotamine or analgesics had only been reported in patients using such medications for headache disorders, and not when these substances were used for other conditions.

Whilst development of the ICHD-I signified a major advance in headache classification it struggled to adequately classify those headaches occurring on a daily or near daily basis, known collectively as chronic daily headaches[36]. Following the introduction of the 'triptans' (5-Hydroxytryptamine (5-HT) agonists), in the early 1990's[37], it was soon clear they too were also able to increase the frequency of the headaches for which they are administered[38, 39], necessitating further amendments to the classification of headache induced by chronic use or exposure.

In 2004 the International Headache Society published the second edition of the International Classification of Headache Disorders (ICHD-II), which renamed this condition medication overuse headache, and expanded on the previous diagnostic criteria, with the entity further defined to include 6 sub-types as outlined in Table 1[9]. Although acute headache medication intake frequency may be less than headache frequency (i.e. the patient may not take acute headache medication every time that they have a headache), expert opinion dictates that intake on 10 or 15 days per month (depending on the class of headache medication used) can be sufficient to exacerbate headache, and as such intake equal to or above this frequency has been designated as *overuse.* Shortly after the release of the ICHD-II it became apparent that the criteria for medication overuse headache required re-apprasial. Following much constructive ciriticism at the International Headache Research Seminar in 2004, revised criteria for MOH were published mid 2005[32]. The primary modifications included elimination of specified headache characteristics and the addition of a new sub-form, 'medication overuse headache attributed to a combination of acute medications', to classify those patients who overuse medications from multiple classes without overusing a single class of drug[32].

Despite the initial revision of the ICHD-II controversy regarding the classification and diagnostic criteria for MOH continued. Many clinicians raised concerns about the applicability of the criteria in clinical practice and the research setting, given criteria D, the mandatory requirement of headache improvement following medication withdrawal, included with the aim of establishing causality, meant a definitive diagnosis could only be made in retrospect. For diagnosis to be confirmed headache must resolve following cessation of the overused medication, thus MOH could not be diagnosed during the initial evaluation and the formal diagnosis could only be established when the condition no longer exists. In response to this issue the Headache Classification Subcommittee published amended criteria as an appendix to the ICHD-II, removing the need for improvement following withdrawal. Application of both versions of diagnostic criteria in clinical studies show the appendix criteria are able to diagnose a significantly greater portion of patients than the initially revised ICHD-II criteria (ICHD-IIR), with the ICHD-II identifying a greater portion of patients with probable medication overuse headache.

Table 1. International Headache Society criteria for medication overuse headache published in 2004 (International Classification of Headache Disorders (ICHD)-II). *Removed during revision in 2005 (ICHD-IIR). #Removed in New Appendix Criteria published in 2006.

2004 International Classification of Headache Disorders Criteria for Medication Overuse Headache [9]
A. Headache present of >15 days/month with at least one of the following characteristics and fulfilling criteria C and D 1. Bilateral* 2. Pressing/tightening (non-pulsating) quality* 3. Mild or moderate intensity*
B. Intake of simple analgesics on ≥15 days/month or intake of opioids, ergotamine or triptans on ≥10 days/month, for >3 months
C. Headache has developed or markedly worsened during analgesic overuse
D. Headache resolves or reverts to its previous pattern within 2 months after discontinuation of analgesics#

Today the overuse of analgesics and other headache treatments continues to represent a significant public health issue[40] and the classification of MOH remains a contentious topic. Recently, it was proposed the name for this disorder should again be changed to 'medication adaption headache', to reassign blame from the patient to the underlying mechanism[33]. The Headache Classification Subcommittee is now close to publishing the ICHD-III; a Beta edition, which includes the New Appendix Criteria for MOH published in the main body and an adjustment requiring patients to have a pre-existing headache disorder, has recently been released for field-testing[41].

1.2.2 Epidemiological aspects of medication overuse headache

Epidemiological studies clearly indicate overconsumption of acute headache treatments occurs worldwide, in developing as well as developed countries[42]. Medication overuse headache is reported to be the third most common form of headache observed in clinical practice, following only TTH and migraine[43]. Globally, approximately 1-2% of the general adult population suffer chronic headache associated with medication overuse and the prevalence of MOH appears similar across many countries, although in some regions the prevalence of MOH may be much higher, as summarised in Table 2.

Table 2. Adult prevalence rates and demographic data for medication overuse headache in population based studies. NA = data not available for medication overuse headache patients. *Year of publication.

Location	Year*	Prevalence (%)	Period (months)	Female Sex (%)	Mean Age (years)
Taiwan[44]	2001	1.1	12	62	45 ± 15
Norway[45]	2004	0.9	NA	66	NA
Spain[46]	2004	1.4	NA	92	45
Netherlands[47]	2006	0.7	12	72	43±8
Norway[48]	2008	1.7	12	76	-
Georgia[49]	2009	0.9	12	NA	NA
Brazil[50]	2010	1.4	12	NA	NA
Germany[51]	2010	1.0	6	74	53
Sweden[52]	2011	1.8	NA	76	50.9
Russian Federation[53]	2012	7.2	12	NA	NA
Iran[54]	2012	4.9	12	65	NA
Turkey[55]	2012	2.1	12	NA	NA

Unlike other headache disorders such as migraine and TTH, which decrease in prevalence substantially after peaking during the third and fourth decades of life[56], the prevalence of MOH appears to remain stable with increasing age. Two studies in elderly subjects aged ≥65 years, living in China or Italy, found prevalence rates of 0.98% and 1.7% respectively, comparable to that of the general adult population[57, 58].

Medication overuse headache has also been reported in adolescents and children as young as 5 years old[59, 60]. A study in Norway found the prevalence of daily headache associated with analgesic overuse in adolescents aged 13 to 18 years to be 0.5%[61]; while a large study in Taiwanese adolescents aged 12 to 14 years found a slightly lower prevalence of 0.3%[62].

However, it must be acknowledged that the definite population prevalence of MOH remains unknown as virtually all epidemiological studies in this area are of cross-sectional design and merely assess concurrent chronic daily headache and medication overuse, thus a causal relationship is rarely established. Evaluating the prevalence of MOH is complicated further

by the reluctance of some patients to admit to the true frequency of analgesic consumption. This may stem, at least in part, from the negative connotations of addiction to opioids and other analgesics[63].

While relatively common in the general population, the comparative frequency of MOH is a great deal higher in specialised headache and tertiary care centres. In the United States greater than 60% of all patients attending such centres suffer from MOH[64]. This figure rises to 80% when considering only those patients presenting with chronic daily headache[65]. In Taiwan 48% of patients with chronic daily headache at the outpatient clinic of the Taipei Veterans General Hospital had MOH[66]. In Europe between 25% and 40% of patients presenting to specialist headache centres meet the criteria for MOH[67, 68]. These figures contrast dramatically with those from headache clinics in India where only 3.1% of patients present with MOH[69]. It has been suggested that this difference is due in large part to the preferred practice of applying local pain balms, trialling alternative therapies and delaying the intake of analgesics in India[69].

A preponderance of MOH in women has also been observed in both population and clinic based studies in adults and adolescents (see Table 2)[51, 52, 61, 70]. A meta-analysis of 29 MOH studies found females were more prone to MOH than males, reporting a ratio of 3.5:1, respectively[71], which is slightly higher than that expected based upon the higher incidence of migraine in women[42]. Additionally, medication overuse headache sufferers, like other chronic headache patients, are more likely to have a low education level and low socioeconomic status as compared to the general population[47, 52, 72, 73].

Medication overuse headache impacts considerably on the quality of life of affected individuals, and imposes a large economic burden upon society[74, 75]. As with other forms of

chronic headache MOH places high intangible costs upon sufferers, resulting from disruption of family lives and relationships, impediment of social roles and career opportunities and a reduced sense of individual well-being[76]. Medication overuse headache ensues a large financial burden upon the patient as well as society at large. The economic burden of MOH is a cumulative consequence of the direct expenses associated with utilisation of health care resources and the indirect costs resulting from increased sick leave and reduced performance in the workplace[76].

A number of studies have established the general quality of life of patients with MOH is poorer than that of healthy individuals and patients with episodic headache disorders[46, 47, 77]. MOH patients score significantly lower than healthy individuals in each of the 8 domains of the Medical Outcomes Study Short Form 36 (SF-36), with the most prominent differences observed in the areas of physical role and bodily pain[46]. Chronic daily headache patients overusing analgesics have been found to score lower in all domains of the SF-36 compared with chronic daily headache patients not overusing analgesics[78]. Furthermore, the self-administered Migraine Disability Assessment (MIDAS) questionnaire has demonstrated that MOH elicits a profound impact on daily functioning with the scores for MOH patients established to be greater than three times that of the episodic migraineurs. Additionally, a considerable improvement extending to key life domains (work, home, family, social and recreation) was noted following treatment, indicated by a significantly lower MIDAS score 6 months after inpatient withdrawal and initiation of headache prophylaxis[79]. These results suggest the overuse of acute medications and resultant headache chronification are directly related to disability and reduced daily functioning.

1.2.3 Medication overuse headache as a biobehavioural dependence disorder

For many diseases diagnosis and classification are based upon the pathophysiological changes observed and underlying aetiology, however this is not possible for MOH as the causal pathology has not yet been elucidated. Some aspects reported to contribute to the development of MOH relevant to this thesis are the presence of concurrent psychological conditions and aberrant behaviours, particularly those related to substance dependence.

The complex inter-relationship between emotion and pain is now well recognised, and as in many chronic pain disorders, this dynamic appears to be of importance in both prompting and sustaining MOH[80]. Together behaviour and biology are thought to contribute to the initiation and maintenance of MOH and successful long-term treatment of this disorder depends upon adequate treatment of both elements[80]. Specific psychological states and behaviours found to be important in the development and perpetuation medication overuse include anxiety disorders, depressive disorders and obsessional drug-taking and/or dependence-related behaviours[80, 81].

High rates of anxiety and depression, among other psychiatric disorders, are frequently reported in MOH patients, even when compared with other chronic headache sufferers[82], thus it has been suggested that psychiatric comorbidity may represent an important factor in the transformation of episodic headache disorders to MOH[15]. This preponderance of psychiatric co-morbidity in headache patients with chronic substance use was investigated years before the diagnosis of MOH was proposed[83]. Migraine patients with headache induced by chronic substance use were found not only to be more anxious and more depressed than other migrainous patients but the 'headache induced by chronic substance use' diagnosis was also significantly associated with current major depressive disorder, life time panic disorder, life time social phobia and current social phobia[83]. The authors of this

study highlighted that depression and anxiety could reinforce inappropriate pain coping, which could inturn lead to drug abuse[83].

More recently, the relationship between psychiatric co-morbidity and the progression from episodic migraine to MOH has been investigated[81]. In this study MOH patients were found to suffer more frequently from affective disorders and anxiety than patients with episodic migraine, with the psychiatric disorders largely preceding MOH, supporting their conceptualisation as risk factors for MOH[81]. A small study in Japan found 66.7% of MOH patients suffered from at least one affective disorder compared to only 2.5% of patients in the episodic migraine control group[84]. Although it is conceivable that the increased frequency and duration of headache in MOH, compared to episodic headache disorders, may be responsible for the higher prevalence of depression observed in this patient group, studies utilising chronic headache control groups suggest otherwise[84]. For example, patients with MOH arising from TTH also have a higher frequency of psychiatric co-morbidity than chronic TTH patients, a group that suffer a similar headache burden[85].

According to the operant conditioning theory, there are three types of conditioning factors that can favour dependence-related behaviours in headache sufferers[16]. These factors include positive reinforcement secondary to reward resulting from the psychotropic effects of the medication consumed, such as relaxation after opioid administration; negative reinforcement following removal of the adverse stimulus, i.e. pain relief following medication intake; and finally, punishment due to withdrawal manifestations when the medication is not taken[16]. The relative influence of each of these factors differs between individuals[16], for example some patients may display phobic avoidance associated with fear of headache (termed “cephalalgiaphobia”), thus medication intake is maintained primarily to avoid withdrawal headaches[86], while in patients exhibiting sedation-seeking behaviour

(termed “soporophilia”) the psychotropic effects of headache treatments may be the most important contributor to continued medication intake[80].

In the past MOH patients have also been reported to pre-emptively use headache medications at the first sign of an impending headache or headache related anxiety[15] and to ritualistically take symptomatic headache treatments in the evening before bed and again upon rising in the morning in an attempt to prevent a headache developing[80, 87]. Similarly, many MOH patients take headache treatments prophylactically in an attempt to prevent missing work or social events[88]. Such patterns of behaviour may represent a prelude to psychological dependence[80]. This pre-emptive medicating behaviour may further be reinforced by the patients physician as it is common for doctors to recommend patients take migraine medications early in during the aura phase or at the very beginning of a migraine attack to ensure greatest efficacy – a practice which may lead to unnecessary consumption of medication for headache attacks which could turn out to be minor[88].

Other manifestations of withdrawal such as emotional distress, which is closely linked to the pain experience, can lead to patients presenting with a negative affect in regard to pain. The patient may begin “catastrophising”, which involves a pessimistic outlook when considering pain evolution, feeling that pain is unbearable and surpassing the patients coping abilities[16]. When combined with an external locus of control, catastrophising can result in a situation where the patient relies upon only one coping skill and thus routinely takes medication when faced with pain[16]. As in other chronic pain states catastrophising in MOH patients suffering affective disorders can induce a feeling of helplessness, and patients may restrict social interactions and other pleasure activities, which inturn produces an exclusive focus on pain, forming a vicious cycle that further reduces quality of life[16].

Previously MOH has been described as a clinical condition bordering between drug dependence and a chronic pain disorder[89] and several lines of evidence support this hypothesis, at least in a subset of patients. In the behavioural field the term dependence describes a pattern of behaviours characterised by both recurrent failure to control the behaviour, along with continuation of the behaviour despite the patients awareness of significant negative consequences[90]. A number of clinical features of MOH, such as impaired control over substance use and the propensity to relapse following withdrawal represent behavioural disturbances suggestive of dependence, leading some researchers to propose a pathological relationship between MOH and substance-dependence disorders[91].

At present a number of clinical and biological observations support the theory that at least a proportion of MOH sufferers display behaviours that fall under the spectrum of substance-related disorders. These observations include the presence of dependence related behaviour in MOH patients, co-morbidity between MOH and established substance disorders and biological and genetic studies that identify links between the two disorders[16]. Repeated observations indicate acute headache medications containing psychoactive ingredients are preferred by some MOH patients, and importantly, such medications represent the treatments with the greatest propensity to cause MOH[16, 92].

While acute medication withdrawal is largely an effective treatment for MOH in the short-term[93], a number of long-term follow up studies indicate one third to one half of patients relapse within 1-5 years[94-96]. A review of prospective studies found patients overusing opioids to have the highest relapse rate after withdrawal treatment[9], which may indicate such patients experience psychological dependence that persists after physical drug withdrawal[16]. Alternatively, the higher relapse rates in patients overusing medications with anxiolytic properties including opioids, could also reflect the occurrence of 'rebound' anxiety

upon discontinuation of such agents, or perhaps a state of pre-existing anxiety which both predisposed the patient to originally overuse a sedating medication and may predispose to relapse following withdrawal[80].

The Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) criteria for dependence (see Appendix 2) have been utilised by a number of authors to investigate dependence related behaviour in MOH patients, although it has not been specifically validated for use in the headache population[16]. A large study in Taiwan found 68% of MOH patients met a modified version of the DSM-IV criteria for dependence on acute headache treatments[66]. A study conducted in France found a remarkably similar 67% of MOH sufferers presenting to a specialty headache clinic met the standard DSM-IV criteria for substance dependence[97]. Similarly, another smaller French study involving MOH patients with migraine as their initial primary headache disorder, found 65.9% of those overusing opioids to meet the DSM-IV criteria for substance dependence[81].

A variety of other questionnaires have also been used to examine the dependence characteristics in patients with MOH. Markedly enhanced substance need has been observed in chronic daily headache patients who overuse analgesics, compared with episodic headache suffers, as assessed by the Leeds Dependence Questionnaire[98], a self-administered questionnaire that systematically explores the International Classification of Diseases, 10th edition diagnostic criteria for dependence[99]. In this study the substance need in MOH patients was of similar intensity to that of drug addicts, although some differences in the pattern of responses were noted, with drug addicts scoring higher in items assessing compulsive use and the need to maintain drug effect[98]. Based upon these results the authors conclude that 'dependence' in MOH appears to originate from the necessity for analgesics to cope with everyday life[98].

A second study by the same research group used the Leeds Dependence Questionnaire to compare the relationship between the patient and their analgesics across three distinct groups – episodic migraineurs, chronic migraine patients who overuse medications and individuals suffering rheumatic disease[100]. Patients with chronic migraine overusing medications had the strongest attachment to their analgesics, scoring significantly higher than both other groups[100]. However, the Leeds Dependence Questionnaire was not designed to assess analgesic dependence and further research is required to determine if it is valid for use in the MOH patients[101].

In 2006 Radat and colleagues developed the Medication Dependence Questionnaire in Headache patients (MDQ-H), a 21-item, self-rating questionnaire validated for assessing dependence in the headache population[102]. Using this tool Radat and colleagues established patients with MOH have a significantly higher MDQ-H score, indicating a greater degree of dependence, than either episodic migraineurs or TTH patients[102].

Finally, the Severity of Dependence Scale, a simple graded tool designed to measure the psychological aspects of dependence[103], has also been employed to assess dependence on acute headache treatments in MOH patients[104]. A large study found a significantly higher mean Severity of Dependence Scale score in chronic TTH patients who overused medication as compared to chronic TTH patients without medication overuse. The medication overuse group scored 2 points higher on average than the optimal cut-off scores for dependence on more well established addictive substances such as alcohol, amphetamines, cannabis and cocaine[103-108]. Despite the detection of "dependency-like" behaviour in MOH sufferers the authors note it remains uncertain if the high Severity of Dependence Scale scores found in this patient group represent outright addiction or if they simply reflect concerns about losing control over medication intake due to frequent headaches[104]. Taken together these studies

consistently indicate at least a proportion of MOH sufferers possess characteristics of dependence related behaviour[16].

Furthermore, co-morbidity between dependence upon other psychoactive substance and MOH is frequently observed. In the (German) Deutsche Migräne und Kopfschmerz Gesellschaft headache study a higher prevalence of active smoking behaviour was reported in MOH patients when compared to the non-headache controls, however no statistical comparison was presented[51]. Radat and colleagues investigated concurrent dependence on non-migraine abortive substances in MOH patients with a history of migraine and found 13.8% also overused other psychoactive substances such as tobacco, alcohol, caffeine, sedatives/anxiolytics or drugs of abuse, and this overuse was significantly associated with dependence on acute headache treatments[97]. Similarly, dependence on another psychoactive substance, such as alcohol, tobacco or benzodiazepines, was found to be significantly associated with a diagnosis of MOH in a small French study of patients presenting to a specialty headache clinic[81]. In this study 43.9% of the MOH group had a concurrent substance-related disorder as compared to only 14.6% of the migraine group[81]. Also observed was an increased risk of substance abuse disorders among the relatives of MOH patients, compared with relatives of migraine sufferers, even after adjusting for substance-related disorder in the patient, suggesting family cross-transmission for MOH and substance dependence disorders[81].

A familial relationship was again observed by Cervoli and colleagues who reported MOH patients, unlike chronic daily headache patients without medication overuse, were more likely than episodic migraine patients to have a first-degree relative with either MOH or a substance abuse disorder[109]. Ferrari et al. observed a similar trend noting drug and/or alcohol abuse to be significantly higher in first-degree relatives of patients with probable

MOH compared with episodic migraine sufferers[110], yet whether this represents learnt behaviour secondary to environmental influences, stems from a genetic trait or a results from a combination of the two remains to be elucidated[109]. Current research indicates addiction to various substances other than those used for headache relief encompasses genetic components as well as environmental factors[111], and a number of studies have now highlighted a relationship between MOH and some genes associated with substance abuse and dependence disorders[89, 112].

Prognosis following withdrawal has also been linked to dependence related behaviour. Both alcohol consumption and smoking behaviour are predictive of higher relapse rates at 1 year, after adjusting for number of doses per month, outcome at 2 months post withdrawal, and return to abused drug at 2 months[113]. Rate of relapse is likewise dependent on the type of medication overused[95].

1.2.4 Causative medications and pathophysiology of medication overuse headache

Since the recognition of MOH many attempts have been made to determine the relative association between various acute headache treatments and this condition. While it is often stated that all medications used in the acute treatment of headaches, including antipyretic and anti-inflammatory analgesics, opioids, combination analgesics containing simple analgesics with caffeine, barbiturates or opioids, ergotamine and triptans, are capable of inducing chronic daily headache when overused[88], reliable data supporting causal relationships are surprisingly scarce. The type of medication overused varies considerably between studies and is likely to depend on both patient selection and cultural factors[114, 115]. In Australia triptan overuse a less likely than other parts of the world, as here these medications are expensive and only available on prescription, while codeine overuse is more

likely in Australia, given codeine-containing preparations can be readily accessed over the counter.

Studies investigating the propensity of substances to induce MOH are often limited by their cross-sectional design. Many papers have published the prevalence of MOH attributed to various medication classes[52] , however these data have not yet been presented as a percentage of all patients using such medications to allow evaluation of relative risk. Case series similarly suffer shortcomings in this area, as highlighted by Haag[116] in a review that outlined the inappropriateness of clinical case series in determining the differential risks of different medications for provoking MOH, due to inability to control selection bias. This paper suggested instead that Hills criteria for causation could be used to establish the nature of the relationship between a certain drug or drug class and headache progression[116]. Only one class of medication, the opioids, have been shown to play a definitive role in inducing MOH as assessed via Hills criteria of causation, with Bigal and Lipton demonstrating that a causal relationship between excessive opioid use and progression from episodic migraine to chronic daily headache is plausible[117].

In clinic-based studies, case-control studies and longitudinal population-based studies, opioids, unlike other headache treatments, are consistently associated with the development of chronic headache[19, 92, 118-120]. In the Frequent Headache Epidemiology Study, which compared medication use among chronic daily headache patients and episodic headache controls, opioid use was significantly associated with chronic daily headache, where no other medications were [120]. In a German longitudinal study both medication overuse and headache frequency were found to be potent independent risk factors for the development of chronic daily headache, with opioid medications conveying the highest risk[121]. Finally, in a seminal paper published as part of the American Migraine Prevalence and

Prevention study the probability of transformation from episodic migraine in 2005 to chronic migraine in 2006 was modelled in relation to medication use. In unadjusted analyses, intake of preparations containing opioids at any frequency doubled the risk of chronic headache in 2006 as compared to the paracetamol reference group, while triptans and NSAIDs did not significantly increase the likelihood of headache transformation. After adjusting for gender, headache frequency and severity and preventative medication the odds of headache chronification following opioid use remained 1.4 times higher than after using paracetamol[92]. The probability of progression to chronic migraine was also found to correlate with elevated monthly opioid dose[92].

Despite the high prevalence of MOH the mechanisms contributing to the development of this disorder remain unclear[14]. Not all patients who overuse analgesic medications go on to develop MOH; the condition appears to be restricted to patients suffering primary headache disorders[118, 119], indicating the pathophysiology relies upon a specific predisposing factor in this patient group. The reasons why patients with headache who overuse analgesics are more prone to develop chronic headache as compared to other patients who take analgesics frequently remain unknown. While current research suggests several factors could play role in the pathophysiology of MOH, at present it is only possible to summarise mechanisms that appear to be associated with, or may predispose patients to, this condition[74, 115]. From a pharmacological perspective it seems unlikely that the wide spectrum of medications reportedly associated with MOH, which possess varied target sites and pharmacological actions, could result in the same phenomena via the same mechanism.

Drugs with vasodilating properties, such as ergotamine tartrate, caffeine and the triptans, may result in headache simply due to a vasoconstrictive pharmacological rebound. As far back as 1951 Peters and Horton provided a theoretical explanation for this condition,

describing ergotamine overuse headache as a "withdrawal rebound phenomena", which they likened to that "…observed in the nasal mucosa after the effect of vasoconstricting drugs has worn off"[23].

In support of the different pathophysiologies following intake of different drug classes the clinical presentation of triptan overuse headache differs from that of analgesic overuse headache. As discussed previously, triptan overuse can solely increase migraine frequency or cause daily migraine-like headaches while analgesic overuse usually leads to the development of an interictal daily tension-type like headache. Furthermore, triptans can induce MOH faster and at lower doses compared with analgesics, and withdrawal in triptan overuse headache is shorter, easier and associated with lower relapse rates that opioid overuse headache[17].

Opioid overuse headache has been suggested to involve similar pathological processes to opioid-induced hyperalgesia[122], another condition in which opioids are known to paradoxically exacerbate pain[123]. Recently, a role for central immune signalling in the facilitation of pain by morphine has been substantiated[124]. Impelled by this information, we conducted a literature review and subsequently cultivated a neuroimmune hypothesis for the development of opioid overuse headache. The rationale and supporting data for a neuroimmune hypothesis of opioid overuse headache are discussed in detail in the review article published in contribution to this PhD entitled "Medication overuse headache and opioid induced hyperalgesia: A review of mechanisms, a neuroimmune hypothesis and a novel approach to treatment".

In summary, it seems likely that the clinical condition of MOH encompasses a number of different pathological states that present similarly as worsening headache. We hypothesise

true opioid-induced MOH results from activation of glial cells, while if triptan and ergotamine induce headache it may occur due to rebound vasodilation. Headache relating to products containing caffeine may stem from withdrawal. Many patients consume simple analgesics in addition to these other acute headache treatments, thus many MOH suffers also overuse simple analgesics, although clear evidence implicating such painkillers alone in the development MOH is limited.

1.3. MEDICATION OVERUSE HEADACHE AND OPIOID INDUCED HYPERALGESIA: A REVIEW OF MECHANISMS, A NEUROIMMUNE HYPOTHESIS AND A NOVEL APPROACH TO TREATMENT

1.3.1 Statement of authorship

Statement of Authorship

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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Jacinta L Johnson		
Contribution to the Paper	Conceptulised review aims, content and structure, reviewed the literature, drew conclusions from the literature, formed hypothesis presented in the review, wrote the manscript and acted as the corresponding author.		
Signature		Date	14·JUL·2014

Name of Co-Author	Mark R Hutchinson		
Contribution to the Paper	Assisted in forming the hypothesis presented in the review and edited the manuscript.		
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Contribution to the Paper	Assisted in conceptulising review content, forming hypothesis presented in the review reviewed and edited the manuscript.		
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Cephalagia, v. 33 (1), pp. 52-64

2. IBUDILAST IN THE TREATMENT OF MEDICATION OVERUSE HEADACHE

2.1 BACKGROUND AND RATIONALE FOR CLINICAL TRIAL

2.1.1 Current management of medication overuse headache

Medication overuse headache is a particularly difficult condition to successfully treat[125]. Patients often find it extremely difficult and distressing to stop the overuse of medication, and in those who are able to successfully complete detoxification, relapse rates remain high[126]. Current management strategies for MOH are based only upon low-level evidence and expert consensus, as almost all clinical trials in this field are under powered and/or have a high number of dropouts[127]. While it is generally agreed that withdrawal therapy is the treatment of choice, evidence to support this as the optimum recommendation has not been demonstrated unanimously in prospective studies[127], and much controversy still surrounds withdrawal procedures, bridge therapy (treatments of unproven value used during the acute detoxification period) and the initiation of prophylaxis.

Regardless of the setting or withdrawal protocol employed, participant education is paramount, and should be the first step in resolving MOH, as it the patient who decides when to treat and how to treat their headaches[128]. In some patients a brief educational package delivered via correspondence without any additional intervention, is sufficient to improve headache[129]. For example an Italian study demonstrated comparable favourable outcomes both in patients who received advice alone and those who underwent a structured detoxification program, although the study population included patients only with low medical needs[130].

Along with education, withdrawal of the overused medication remains the cornerstone of treatment in MOH, as aside from improving headache withdrawal of overused headache medication is associated with reduced anxiety, depression and disability levels[131]. Withdrawal is also important to prevent somatic complications of medication overuse headache, such as chronic kidney failure, gastrointestinal ulcers, which may arise as a consequence of overusing certain headache drugs[74].

The method of detoxification can differ substantially between treatment sites. The majority of headache specialists opt for abrupt withdrawal of overused medications[132-134], considering this to be more effective, potentially resulting in less protracted suffering and faster resolution of medication-centred pain-coping behaviour. Conversely, others elect to taper medications off slowly in an attempt to minimise withdrawal symptoms, particularly when patients are overusing medication such as opioids or benzodiazepines[135]. Moreover, in some centres withdrawal is conducted in the outpatient setting whereas in others withdrawal is performed as an inpatient procedure. Although conclusive data comparing the two settings are lacking, in uncomplicated cases of MOH it appears outpatient withdrawal can be as successful as inpatient treatment[136]. Expert opinion however recommends inpatient withdrawal for patients who take codeine or other opioids, barbiturates or other tranquilisers, those who present with a high depression score or other psychiatric comorbidities, and patients who have previously failed outpatient detoxification[127]. Inpatient detoxification procedures pose considerable burdens on both patients and the community as they involve greater disruption of employment and other daily activities for patients, increased usage of healthcare resources, and significantly greater costs[135].

Generally during detoxification headache tends to worsen before improving. Withdrawal headaches and additional withdrawal symptoms, such as nausea, vomiting, restlessness,

anxiety, sleep disturbances and tachycardia, usually last between 2 and 10 days (mean 3.5 days)[137] but can persist for up to 4 weeks[135]. The duration and severity of withdrawal appears dependent upon the type of medication overused, with those overusing analgesics, as opposed to triptans, experiencing longer and more severe withdrawal periods[138, 139].

As with the method of detoxification, the type of bridge therapy employed during the withdrawal phase varies considerably between headache centres. Many bridge therapy protocols have been found effective in clinical trials, yet the vast majority of such studies have been uncontrolled, unblinded and have assessed a variety of outcome measures, making them difficult to compare[135]. Corticosteroids are often used during this initial detoxification phase, yet the benefits reported in terms of reduced withdrawal symptoms and improvement in headache are inconsistent and most studies have been small or poorly controlled[140]. Recently a large double-blind, randomised controlled trial found that while prednisolone 100 mg for 5 days did not reduce withdrawal headache it did reduce rescue medication intake vs. placebo, but only during the first 3 days of detoxification[141]. Other protocols employ a vast range of symptomatic treatments from oral medications such as naproxen and metoclopramide[142], to intramuscular preparations of indomethacin or diazepam[143], and intravenously administered dihydroergotamine[144], metoclopramide[143], lignocaine[145] and hydration[143], depending on the type of medication being withdrawn. No evidenced-based recommendation can be made regarding which of these constitutes the most effective therapeutic program for controlling withdrawal symptoms.

The relative importance of medication withdrawal vs. initiation of prophylaxis and the optimal time to commence such prophylactic therapy remain topics of much controversy, as debated recently by experts Diener[146] and Olesen[147]. As studies regarding efficacy of specific headache prophylactic medications in MOH are scarce, if/when prophylaxis is deemed

appropriate selection of an agent is instead based upon the type of primary headache disorder, drug side effect profile, patient comorbidities and previous therapeutic experience[127]. In the past it was generally accepted that MOH patients remain refractory to prophylaxis while medication overuse continues[148]. However, recent studies have cast doubt on these convictions, providing evidence that early induction of prophylactic treatments may in fact be able to reduce headache burden prior to complete medication withdrawal.

Two double-blind studies have investigated the efficacy of topiramate in reducing migraine frequency in patients with chronic migraine while medication overuse continues[149, 150]. In the European topiramate study the number of migraine days per month was significantly reduced by an average of 3.5 days per month in those with medication overuse receiving topiramate compared with placebo, but this reduction was not sufficient to alter headache classification from chronic to episodic[149]. A *post hoc* analysis of the American topiramate study revealed a similar trend, although the difference did not reach statistical significance[151]. The PREEMPT (Phase III REsearch Evaluating Migraine Prophylaxis Therapy) clinical program, which comprised of two large multicentre trials, demonstrated a significant reduction in headache days per month in chronic migraine patients, around 65% of whom were overusing medications, following treatment with onabotulinum toxin A injections compared with placebo injections, yet the absolute difference between the two groups was small (-8.4 vs. -6.6, respectively)[152]. However, results should be interpreted with caution as unblinding of participants due to the visible effects of onabotulinum toxin A may have confounded results. Both groups saw a mean reduction in acute headache medication intake but no between group difference was observed[152]. Finally, a cases series has found a significant decrease in headache days following initiation of sodium valproate in patients with migraine and medication overuse.

As MOH is now recognised a biobehavioural disorder in which pain and emotion are intermingled, it is not surprising that behavioural treatment approaches provide further benefits when added to pharmacological management. Cognitive behavioural stress coping training, biofeedback and relaxation techniques have been used extensively in chronic headache disorders, including MOH. The long-term benefits of such treatments were clearly demonstrated in a study in MOH patients that compared inpatient pharmacological therapy alone or combined with a short course of biofeedback and training in progressive muscle relaxation training[153]. Immediately following treatment and for one year thereafter outcomes were similar, yet at year 3 patients who received the additional behavioural therapy demonstrated greater sustained improvement and lower relapse rates that patients assigned to pharmacological treatment alone[153]. Thus, although independent evidence exists for multiple approaches, further high quality studies are required before a consensus can be reached regarding the optimal approach to the initial management of MOH and its sub-populations.

The long-term management of MOH is presents an even greater challenge than the primary treatment of this condition[134]. In practice, many headache patients find it very difficult to cease opioid medications on a long-term basis, which is reflected in the high relapse rates associated with MOH. Relapse after medication withdrawal in MOH has been investigated in a well-designed prospective study by Katsarava and colleagues, with results finding 31% of patients had relapsed to medication intake on >15 days/month within 6 months[95]. The relapse rate for the unselected MOH cohort increased to approximately 40% at 1 year, and when considering only those who overuse analgesics, this rate rose to greater than 70% at 4 years[96]. Additional studies have consistently reported high relapse rates of between 21-60% following medication withdrawal in mixed MOH cohorts, as outlined in Table 3. It is also

acknowledged in the ICHD-II that relapse rates in MOH patients who overuse opioids are especially high[9].

Table 3. Long-term relapse rates following successful medication withdrawal in heterogeneous medication overuse headache cohorts.

Study	Design	Intervention	Follow-up	Relapse
Grazzi et al. [153]	Prospective, n=38	Inpatient withdrawal + prophylaxis	3 years	42%
Fritsche et al. [154]	Retrospective, n=103	Inpatient withdrawal + prophylaxis	4 years	48.5 %
Hagen and Stovner [155]	Prospective n=50	Outpatient withdrawal	4 years	34%
Katsarava et al. [96]	Prospective, n=96	Inpatient withdrawal	4 years	45%
Pini et al. [156]	Prospective, n=102	No specific intervention	4 years	>60%
Schnider et al. [94]	Prospective, n=38	Inpatient withdrawal	5 years	40%
Suhr et al. [157]	Prospective, n=101	Inpatient and outpatient withdrawal	5.9 years	21%
Tribl et al. [158]	Retrospective, n=55	Inpatient withdrawal + prophylaxis	5 years	33%

All considered, an ideal treatment for MOH would reduce headache pain and subsequent medication intake prior to mandated detoxification to break the cycle of debilitating headache leading to increased medication intake, which further worsens headache. A treatment such as this could significantly reduce discomfort during the withdrawal period, likely increasing adherence to detoxification protocol and offering a significant advantage over current withdrawal procedures. Furthermore, mechanism-based treatments that target specific biological systems involved in dependence may be of benefit in reducing relapse rates.

2.1.2 Evidence for targeting neuroimmune pathways in the treatment of pain and addiction

As introduced in our review paper “Medication overuse headache and opioid induced hyperalgesia: A review of mechanisms, a neuroimmune hypothesis and a novel approach to

treatment", reciprocal signalling between the immunocompetent cells in the central nervous system, including microglial cells and astrocytes, and the neurons has emerged as a key phenomenon involved in pathological and chronic pain mechanisms. Immune mediators released from central nervous system-resident microglia and astrocytes, in addition to classical neuron-derived neurotransmitters, can powerfully enhance neuronal excitability and subsequent nociceptive signal transmission. Furthermore, evidence is mounting that activation of similar cells types and signalling pathways may contribute to dependence and addiction, which is of particular interst given that MOH is proposed to encompass characteristics similar to those of substance dependence disorders[91].

The connection between immune system activation and increased pain has recently been demonstrated in healthy human volunteers[159]. This study established that immune priming, through activation of TLR4 with a low dose endotoxin (lipopolysaccharide, 0.4 ng/kg) intravenous infusion, significantly increases allodynia, hyperalgesia and flare associated with the intradermal capsaicin (50 μg) neuropathic pain model at 3.5 h[159]. Low dose endotoxin administration was also associated with increased levels of interleukin (IL)-6, confirming a previous study which detected endotoxin-induced rises in IL-6 and tumor necrosis factor (TNF)-α that correlated with increased pain sensitivity[160].

Neuroimmune links have also been reported to play a role in headache pain. A number of studies suggest the immune system may be dysfunctional in migraine sufferers, an idea initially proposed due to the reported co-morbidity of migraine and atopic diseases, such as asthma, eczema and hay fever[161-164]. In recent years accumulating evidence has shifted the focus of migraine pathophysiology away from vascular smooth muscle towards cortical and trigeminal sensitivity and neurogenic inflammation, a sterile state of inflammation resulting from the release of neuropeptides and other mediators from peripheral sensory neuron

terminals[165]. This model of sterile neurogenic inflammation highlights a potentially significant role for immune mediators in the precipitation and/or aggravation of migraine[166]. Glial activation specifically, has been proposed by a number of authors to contribute to the neural hypersensitivity present in migraine[167-169]. It is well established that calcitonin gene-related peptide, a potent vasodilator and neuromodulator, is released by neurons during migraine[168]. In response to calcitonin gene-related peptide glial cells release a range of mediators such as IL-1β and fractalkine, which are know to drive pain facilitation and activation of further glial cells. Similarly, the protein S100β, derived from glial cells, has also been found to be elevated in children with migraines[170].

Less is understood regarding the role of the neuroimmune interface in the pathophysiology of tension type headache, in accordance with less being known about the pathogenesis of tension type headache in general. However, levels of TNF-α, a substance released by activated glia recognised to mediate chronic pain states, are raised in chronic headache patients with both tension-type and migrainous headache phenotypes[171]. Furthermore, amitriptyline, a tricyclic antidepressant effective for tension-type headache prophylaxis[172], has recently been found to possess strong TLR4 inhibitory activity[173]. While amitriptyline does not alter baseline pain sensitivity it is able to potentiate morphine analgesia, as are other inhibitors of TLR4 signalling[173], raising the possibility that attenuation of glial activation via TLR4 blockade may contribute to the efficacy of this medication in tension-type headache.

Over reliance on opioids used to relieve pain, as occurs in opioid overuse headache, may also render the brain more excitable and prone to inflammation[15]. Such changes can result in enhanced sensory input, lower pain thresholds and may lead to a prolonged state of hyperalgesia[15, 174]. Similar changes with regard to lowered pain thresholds, presenting as

allodynia, are frequently observed in chronic headache patients including MOH patients, and we hypothesise these adaptations stem, at least in part, from activation of the glial cells, as has been postulated in opioid induced hyperalgesia. Virtually all MOH patients first present with episodic migraine, tension-type headache, or a combination of the two[18] rather than other forms of primary headache such as cluster headache[19]. We hypothesise this selective propensity to develop MOH results from altered pro-inflammatory central immune signalling in patients with migraine and tension-type headache, which renders them particularly susceptible to the effects of opioid-induced glial activation.

As discussed previously, MOH and opioid-overuse headache in particular, shows similarities with drug dependence, and as such MOH has been investigated as a biobehavioural disorder. Like pathological pain, dependence and reward have now been demonstrated to involve activation of glial cells[175].

As our understanding of the importance of the neuroimmune interface has grown a number of trials investigating novel and exciting treatments for chronic pain and substance dependence disorders have commenced, and encouraging results are beginning to emerge.

Both preclinical and clinical evidence exists indicating glial activation and neuroimmune signalling is involved in substance dependence. Ibudilast, given twice daily, has demonstrated efficacy in reducing voluntary alcohol intake using three different rodent models of high alcohol consumption with underlying biological mechanisms thought to reflect human alcohol dependence[176]. Glial cell line-derived neurotrophic factor, which is released by and acts upon glial cells, has been reported to block conditioned place preference, a preclinical behavioural model use to study the rewarding effects of drugs, induced by cocaine[177] and methamphetamine[178]. Ibudilast increases glial production of glial

cell line-derived neurotrophic factor [179] and experimentally is able to attenuate stress-induced and prime-induced reinstatement of extinguished responding previously reinforced with methamphetamine, suggesting efficacy in reducing relapse precipitated by stress and 'slips' during abstinence[180]. Following these positive animal studies a phase Ib clinical trial of ibudilast for methamphetamine dependence has now completed recruitment and partial data analysis, with the small cohort showing ibudilast did offer benefits vs. placebo in a test of sustained of attention[181, 182]. Subsequently, the same research group has now initiated a phase IIb proof-of-concept study to confirm the safety and efficacy of ibudilast in methamphetamine dependence[182].

Of particular relevance, glial cell activation has also been linked with opioid dependence. Ibudilast attenuates morphine-induced elevations in dopamine levels in the nucleus accumbens, indicating reduced reward[183] and ameliorates precipitated of spontaneous opioid withdrawal behaviours[184, 185]. Based upon these successful experimental results a clinical study investigating the efficacy of ibudilast in reducing withdrawal in human heroin addicts has been conducted. During this double-blind, placebo controlled study heroin dependent participants were initially maintained on morphine for two weeks. At the beginning of the second week participants were randomised to receive placebo, low dose ibudilast (20 mg twice daily) or high dose ibudilast (40 mg twice daily), in addition to morphine. At the 3-week time point morphine was ceased, while the study drug (placebo or ibudilast) continued, and subjective opioid withdrawal symptoms and response to opioid analgesia were assessed. The authors preliminary analysis indicates ibudilast is able to dose-dependently reduce both opioid tolerance and dependence[182].

Thus, given the broad package of established preclinical data and the continual emergence of promising clinical results supporting the use of ibudilast in chronic pain and drug

dependence states, ibudilast may be of use in reducing head pain and facilitating decreased opioid intake in patients suffering from opioid-overuse headache.

2.2 CLINICAL TRIAL OF IBUDILAST IN THE TREATMENT OF MEDICATION OVERUSE HEADACHE

2.2.1 Statement of authorship

Statement of Authorship

Title of Paper	Glial attenuation with ibudilast in the treatment of medication overuse headache: A double blind, randomised, placebo controlled pilot trial of efficacy and safety.
Publication Status	○ Published, ○ Accepted for Publication, ○ Submitted for Publication, ◉ Publication style
Publication Details	To be submitted for publication in Brain, Behavior and Immunity.

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Jacinta L Johnson
Contribution to the Paper	Conceptulised study aims and design, obtained ethical approval, recruited participants, co-ordinated the clinical trial, performed quantitative sensory testing, analysed data, interpreted results, wrote the manscript and will act as the corresponding author.
Signature	Date: 14-JUL-2014

Name of Co-Author	Yuen H Kwok
Contribution to the Paper	Conceptulised and performed biomarker sub-study experimental work and analysis and reviewed and edited the manuscript.
Signature	Date: 14/08/14

Name of Co-Author	Nicole M Sumracki
Contribution to the Paper	Performed thermal threshold testing, assisted with quantitative sensory testing and revie
Signature	Date: 14 JUL 2014

Name of Co-Author	James E Swift
Contribution to the Paper	Assisted with participant recruitment, quantitative sensory testing and reviewed and edited the manuscript.
Signature	Date: 14/08/14

Statement of Authorship

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Name of Co-Author	Paul E Rolan
Contribution to the Paper	Contributed to conceptulisation of the study, screened participants for entry into clinical trial, was the medical doctor responsible for participant safety during the trial, reviewed and edited the manuscript.
Signature	Date 9/8/19

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Signature	Date

2.2.2 MANUSCRIPT: Glial attenuation with ibudilast in the treatment of medication overuse headache: A double-blind, randomised, placebo-controlled pilot trial of efficacy and safety.

ORIGINAL MANUSCRIPT

Glial attenuation with ibudilast in the treatment of medication overuse headache: A double blind, randomised, placebo-controlled pilot trial of efficacy and safety.

JL Johnson[1*], YH Kwok[1], NM Sumracki[1], JE Swift[1], MR Hutchinson[2], K Johnson[3], DB Williams[4], J Tuke[5] and PE Rolan[6]

Medication overuse headache (MOH) is a condition that borders between chronic pain and a substance dependence disorder. Activation of immunocompetent glial cells in the central nervous system has been linked to both pathological pain and drug addiction/reward. Pre-clinically, ibudilast attenuates glial activation and is able to reduce neuropathic pain and markers of substance dependence.

To determine the efficacy of ibudilast in the treatment of MOH in patients consuming opioids, a double-blind, randomised, placebo controlled parallel groups study was conducted. Participants with MOH who were overusing opioids were randomised to receive ibudilast 40 mg or placebo twice daily for 8 weeks. Before randomisation participants completed a 4-week baseline headache diary. During treatment, headache diary data collection continued and participants attended 4 study visits during which quantitative sensory testing was performed. Blood samples for immune biomarker analyses were collected before and after treatment in a sub-group of participants.

Thirty four participants were randomised, 15 to ibudilast and 19 to placebo, and 13 and 17 participants respectively, completed treatment. Ibudilast was generally well-tolerated with mild nausea reported as the most common adverse event (66.7% vs. 10.5% in placebo group). No reduction in headache index or frequency, medication intake or headache impact on quality of life was observed in either treatment group. Following stimulation of peripheral blood mononuclear cells with toll-like receptor 2 and 4 agonists, ibudilast significantly reduced release of interleukin-1β, highlighting its potential use as a biomarker for glial-attenuating treatments.

Using the current dosing regime, ibudilast does not improve headache or reduce opioid use in patients with MOH without mandated opioid withdrawal. However, it would be of interest to determine in future trials if ibudilast incorporated into a MOH detoxification program is able to improve ease of withdrawal during a forced opioid down-titration.

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INTRODUCTION

Medication overuse headache

To date, virtually all acute headache treatments have been reported to cause medication overuse headache (MOH), a perplexing condition in which the regular overuse of acute headache treatments paradoxically increases headache frequency and/or severity. While a number of independent trials have attempted to determine the relative tendency of different headache treatments to provoke MOH, most headache patients (approximately 90%) consume multiple headache medications concurrently, making it difficult to confirm distinct causal relationships[1]. It seems likely that distinct mechanisms are responsible for different sub-forms of the disorder, owing to the vastly different pharmacology of implicated drugs.

It is clear from clinical, case-control and longitudinal population-based studies that opioids are consistently associated with the development of chronic daily headache[2-6]. In the Frequent Headache Epidemiology Study, which compared medication use among chronic daily headache patients and episodic headache controls, use of opioids, unlike other medications, was significantly associated with chronic daily headache[5]. Data from the American Migraine Prevalence and Prevention study was used to model the probability of transformation from episodic migraine to chronic migraine over a 1-year period in relation to medication use. In unadjusted analyses, opioid-containing preparations doubled the risk of chronic migraine a year later, while triptans and NSAIDs did not significantly increase the probability of headache transformation compared with paracetamol. The probability of progression to chronic migraine was also found to correlate with elevated monthly opioid dose[6].

In Australia, potent opioids, such as morphine and oxycodone, are rarely prescribed long-term for the management of headache disorders, thus patients are unlikely to have access in adequate quantities for periods sufficient to produce MOH. The weak opioid codeine, however, is readily available over the counter in Australian pharmacies, making it the prime local candidate for the induction of MOH.

Despite the prevalence and clinical impact of MOH its pathophysiology remains unclear. Several features of chronic daily headache imply sensitisation of the trigeminal nociceptive neurons[7] and, facilitation of pain processing due to central sensitisation that normalises following withdrawal, has been established in MOH patients[8, 9]. Thus, we hypothesise that MOH derives from a cumulative interaction between central sensitisation, due to repeated activation of nociceptive pathways by frequent headaches, and activation of glial cells by opioid analgesics, which further facilitates pain, as discussed below.

Involvement of glial activation in pathological pain

A wealth of preclinical evidence indicates neuronal excitability can be powerfully modulated by not only classical neuronally-derived neurotransmitters but also by a range of immune mediators released from activated glial cells, including astrocytes and microglia[10, 11].

Glial activation stimulates a cascade of events leading to the release of a variety of signalling molecules, such as chemokines and pro-inflammatory cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α)[12, 13] that can enhance nociceptive

transmission[12]. Once a stimulus has resolved, microglia can remain “primed”, entering a state in which they over-respond to subsequent stimuli, producing increased pro-inflammatory cytokine release and an exaggerated pain response[14].

Glial cells are sensitive to a range of disturbances in the nervous system[15], and activation can occur in response to trauma, ischaemia, infection, neurodegeneration, persistent pain and opioid exposure[12, 15-17]. Activated spinal glia and their inflammatory mediators have been implicated in the pathophysiology of a diverse range of exaggerated pain states such as neuropathic pain and opioid-induced hyperalgesia. One pathway through which glial cells can become activated is the innate immune toll-like receptor system. In particular, toll-like receptors 2 and 4 (TLR2 and TLR4 respectively) have been implicated in glial activation and the neuroexcitation that follows[15].

Recently, research indicates that morphine, in addition to activating classical neuronal opioid receptors, is able to activate TLR4 on glial cells, triggering the release of mediators which initiate a cascade of events to enhance nociceptive transmission[17]. While at the neurons morphine facilitates pain relief, activated glial cells concurrently produce neuroexcitatory substances that increase pain sensitivity. Initially the net result is analgesia, however, with increasing opioid dose glial activation increases, contributing to opioid tolerance and, following chronic administration, opioid-induced allodynia and hyperalgesia[18]. Furthermore, glial activation is also implicated in the modulation of opioid dependence, withdrawal and reward[19]. The dual activity at both neuronal and glial cells of morphine appears to be shared with other clinically relevant opioids[17].

Activation of glia has also been hypothesised to play a role in the central neural hypersensitivity of migraine[20-22]. The proinflammatory cytokine TNF-α, and S100 calcium binding protein-β, which are both released by activated glia, are elevated in chronic headache patients[23 24]. Taken together, these suggest attenuation of glial activation may represent a valuable, novel approach to managing opioid overuse headache.

Ibudilast

Ibudilast is an orally administered anti-inflammatory and neuroprotective agent that has been used in the management of bronchial asthma and post-stroke dizziness in Japan for over 20 years[25]. Ibudilast inhibits phosphodiesterase 4 and 10 (and to a lesser extent 3 and 11) and macrophage migration inhibitory factor, and importantly attenuates activated glial cells. Encouraging preclinical results in a range of central nervous system pathological states have facilitated the clinical development of ibudilast as a potential new treatment for opioid, methamphetamine and alcohol dependence, a variety of chronic pain conditions and progressive multiple sclerosis[26, 27]. As we hypothesise MOH to be a form of opioid-induced hyperalgesia, which may be mediated by glial activation, ibudilast could assist in breaking the headache cycle and reducing headache burden in this condition.

While the gold standard of MOH treatment is to withdraw the overused medication[28], in practice many patients find this to be a very distressing process and fail to complete withdrawal therapy, as headache tends to get worse before it improves[29]. Furthermore, often patients find it extremely difficult to cease opioid medications on a long-term basis, consistent with the theory that MOH is a bio-behavioral disorder that shares neurobiology with

addiction[30, 31]. High relapse rates are common[32], especially in those overusing opioids[29]. Given emerging clinical evidence suggests ibudilast is able to attenuate dependence and withdrawal in opioid addicts, and reduce opioid requirements in chronic pain patients, it may assist MOH patients in ceasing their opioid medications and help to reduce relapse rates.

The objectives of this study were to evaluate the efficacy of ibudilast in reducing indices of headache burden and acute medication intake in patients suffering from MOH who consume opioid analgesics and to determine if ibudilast is able to alter measures of cutaneous sensitivity in the same population. Due to the novel mechanism of action of ibudilast in attenuating glial activation, the results from this study may not only have direct clinical applications in the treatment of MOH, but may also provide valuable insight into the mechanisms behind this challenging condition.

METHODS

Overview

This study was a randomised (1:1), double-blind, placebo controlled, parallel groups trial design, conducted in Australia in accordance with the Declaration of Helsinki ethical principles, Good Clinical Practices, principles of informed consent, and requirements for the public registration of clinical trials (als.gov Identifier NCT01317992). Approval of the study protocol was obtained from the Human Research Ethics Committee and the Investigational Drugs Subcommittee of the Royal Adelaide Hospital. The study data presented were collected between October 2011 and February 2014.

The study consisted of a prospective 4-week baseline phase, followed by an 8-week double-blind treatment phase, during which each participant was randomised to receive either ibudilast or placebo. Throughout the study participants maintained a headache diary, which recorded headache frequency, duration, characteristics and medication intake. At baseline and weeks 2, 4 and 8 of treatment participants visited the Pain and Anaesthesia Research Clinic (PARC), located within the Royal Adelaide Hospital, for additional data collection and safety monitoring. Three months after cessation of treatment a final 4-week headache diary and the two questionnaires were completed via correspondence.

Study participants

Study participants were recruited from the general public in Adelaide, South Australia, via advertisement. All participants received written and verbal participant information and gave their written informed consent prior to study enrolment. The initial recruitment target was 40 participants, 20 per treatment arm. This was revised at the end of 2013 to a target of 30 participants due to prolonged participant recruitment. The initial sample size for this pilot trial was based upon that used in a previous MOH trial[33], which was able to detect significant differences in headache burden between treatment groups.

Screening visit

At screening potential participants underwent a diagnostic interview based on the revised International Classification of Headache Disorders, 2nd Edition (ICHD-IIR) criteria[34] and a

clinical interview to assess for current or past psychiatric and medical conditions and to obtain a complete medication history. The Hospital Anxiety and Depression Scale (HADS)[35] was used to assess concomitant anxiety and depression. A physical examination was conducted, and a urine drug screen was used to confirm the presence of opioids and to screen for illicit drugs. All women of childbearing potential were confirmed as non-pregnant via a urine pregnancy test and agreed to use an approved form of contraception throughout the study. A blood sample from each participant allowed assessment of haematology and blood biochemistry, including renal and hepatic function.

Participant inclusion and exclusion criteria

Eligible participants were predefined as males or females aged 18-65 years with a history of opioid overuse headache meeting the diagnostic criteria listed in the ICHD-IIR[34], i.e. opioid intake on 10 or more calendar days per month for at least 3 months, headache on 15 or more calendar days per month and a history of headache developing or markedly worsening during the period of opioid overuse. All participants were screened by the principal investigator and were excluded if the primary reason for opioid intake was a condition other than headache, if they met the criteria for concurrent triptan overuse headache, had a major psychiatric illness as determined by the principal investigator, history of spinal cord injury, current malignancy, active inflammatory disease, recent significant surgery, recent history of drug or alcohol abuse, physical trauma or infection, significant renal or hepatic impairment or contraindications to ibudilast treatment, including recent history of a cerebrovascular disorder, hypersensitivity to ibudilast or formulation excipients or were currently pregnant or breastfeeding for female participants. Concurrent headache prophylactic medications (for example: anticonvulsants, beta-blockers) were allowed during the study provided they remained unchanged for 12 weeks prior to and throughout the study treatment period. Tramadol use in addition to the overuse of another opioid was allowed but overuse of tramadol alone was not sufficient to meet the inclusion criteria of opioid overuse.

Headache diary

Once confirmed eligible through the initial screening participants completed a headache diary for a baseline period of at least 28 days, immediately preceding the scheduled baseline study visit. In the headache diary participants recorded headache pain characteristics, headache frequency, average headache intensity (11-point numerical rating scale (NRS)), duration of headache (h) and intake of symptomatic headache treatments (time, type, dose of medication consumed). Standardised education and instructions for diary completion were provided to all patients at the completion of the screening visit. At the beginning of the baseline study visit the headache diary was reviewed to confirm the retrospective opioid-overuse headache diagnosis made a screening and to ensure the participant did not meet any medication related exclusion criteria. Participants were formally enrolled in the trial following this final eligibility check.

Study schedule

Following the 28-day baseline headache diary-recording period participants attended the first study visit where they were randomised to treatment for 8 weeks. Participants again returned to the clinic on 3 other occasions at weeks 2, 4 and 8 of treatment (see Figure 1 for a visual representation of the study timeline). At each visit current headache was rated by the

participant on a 100 mm visual analogue scale (VAS) ranging from no pain (0 mm) to worst headache (100 mm) and cutaneous sensitivity to mechanical stimuli was assessed at four sites bilaterally using the brush allodynia test and the von Frey test. Cutaneous sensitivity to thermal stimuli was assessed bilaterally on the palm and cheek using a ramping thermal threshold test. Participants also completed the six-point Headache Impact Test (HIT-6)[36], a reliable and well-validated measure of headache-related impact on quality of life[37, 38], and the 12-point Allodynia Symptom Checklist (ASC-12), a validated tool for detecting allodynia interictally[39]. At each study visit blood samples for safety monitoring were obtained. A further blood sample was collected at baseline and the end of treatment from a sub-group of participants for biomarker sub-study assessment involving stimulation of peripheral blood mononuclear cells (PBMC) with toll-like receptor 2 and 4 agonists.

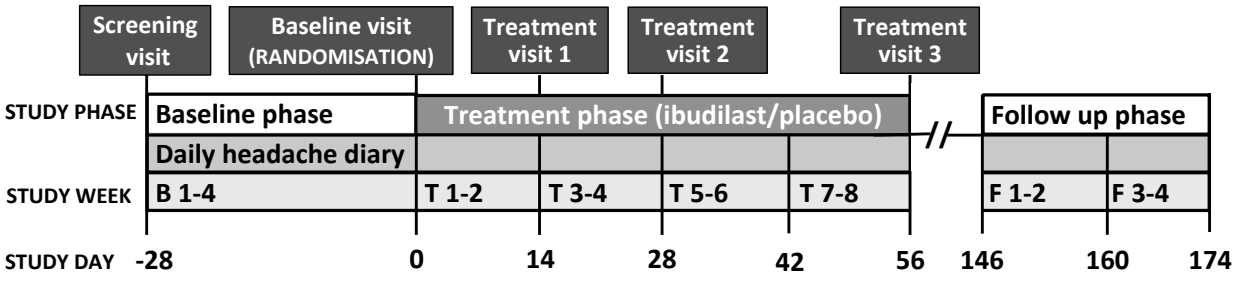

Figure 1. The study design time line. B = baseline weeks. T = treatment weeks. F = follow up weeks.

Randomisation and study medications

Eligible patients were randomised by the Royal Adelaide Hospital Pharmacy Department, using a computer-generated randomisation code, to receive ibudilast 40 mg twice daily or placebo. The initial randomisation schedule provided a 1:1 allocation ratio of ibudilast:placebo. Ibudilast 10 mg capsules (Ketas®, Taisho Pharmaceuticals; via MediciNova) and identical placebo capsules, made by the same manufacturer to contain the same excipients as the active capsules, were packaged in quantities sufficient to last from one study visit to the next. Medication bottles were labeled by the Royal Adelaide Hospital Pharmacy Department as not to reveal treatment allocation to either the investigators or study participants. Participants were required to return any unused capsules or the empty study medication bottles at each study visit to monitor compliance. Additionally participants were instructed to tick a box in the headache diary to confirm that each dose of study medication was taken as directed.

Quantitative sensory testing

At each visit brush allodynia testing was conducted first, followed by von Frey testing and thermal sensitivity testing, which commenced approximately 25 min after completion of the von Frey test. For all quantitative sensory testing procedures test site order was randomised prior to the study visit.

Brush allodynia test

Brush allodynia testing was performed by repetitively stroking a foam brush (2.5 cm wide) across the skin 10 times, at a rate of 1 stroke every 2 s, to a total of eight cutaneous test sites in the trigeminal and cervical distributions. Bilaterally the test sites included the forehead (V_1), maxilla (V_2), mandible (V_3) and distal inner forearm (C_5/T_1). At the completion of the 10^{th} brush stroke at each site the participant quantified the degree of discomfort on a 100 mm VAS scale ranging from no discomfort (0 mm) to very uncomfortable (100 mm), before testing at the next site was performed.

Von Frey test

Von Frey testing was performed at the same sites as the brush allodynia test. Nylon von Frey filaments mounted on plexiglas handles (SENSELab® Anesthesiometer, Somedic, Hörby, Sweden), were applied to produce bowing of 3-5 mm for 1.5 s, in ascending pressure from 0.026 g to a maximum of 110 g force. While keeping their eyes closed throughout the testing procedure, participants were asked to verbally indicate when they were first able to detect the filament on their skin (threshold) and when the sensation first became uncomfortable (tolerance). Each filament was applied 3 times and a verbal indication was required on 2 of 3 occasions for a positive response to be confirmed. Once a positive response was recorded for tolerance, testing was terminated at that test site.

Thermal threshold testing

Participants' individual cold pain thresholds and heat pain thresholds were determined using a PATHWAYS device (model ATS, Medoc, Israel) via the Method of Limits applied bilaterally to the palm and cheek. The thermode (3 cm × 3 cm) was strapped to the palmar surface of the palm, or held against the flesh of the cheek, and the participant was given a hand-held feedback control. The temperature of the thermode was initially set at 32 °C. The temperature of the thermode either heated up (for heat pain) or cooled down (for cold pain) at a constant rate of 1 °C s^{-1}. When the temperature of the thermode was first detected as "just becoming painful," the participant was required to press a button on the hand-held feedback control, which halted the stimulus. The thermode then rapidly returned to 32 °C. The temperature at which the participant halted the stimulus was automatically recorded as the heat or cold pain threshold. After a 20-25 s delay, this procedure was repeated twice more at the same site more to obtain an average; thus, giving a total of 24 test applications (4 sites x 2 temperature protocols x 3 repetitions per protocol).

Biomarker assessment sub-study

Assessment of PBMC reactivity was conducted for a proportion of study participants, forming a sub-study within this clinical trial. Participants were included in the sub-study based upon timing of enrollment; all participants who commenced treatment between the 7^{th} of December 2011 and the 9^{th} of August 2012 were included. Upon arrival at the baseline and week 8 visits, 27 mL of blood was collected into tubes containing EDTA. PBMCs were isolated using Optiprep (Axis-Shield PoC AS, Oslo, Norway) as directed by the manufacturer using the mixer flotation method as previously described[40]. Control wells minus the TLR agonist were also included. Isolated cells were diluted to 1 X 10^6 cells·mL^{-1} in enriched RPMI 1640 (10% foetal

calf serum and 1% penicillin) and plated into 96 well plates (Nunc, Roskilde, Denmark) (100 µL per well). A range of agonist concentrations were added into the wells in triplicate. TLR2 agonist: synthetic triacylated lipoprotein: Pam3CSK4 (Sigma) from 13 pg ml^{-1} to 1 µg ml^{-1} and TLR4 agonist: lipopolysaccharide: LPS (Sigma) from 6 pg·mL^{-1} to 10 µg·mL^{-1}. Plates were incubated for 20 h at 37 °C, 5% CO_2 in a humidified environment (Thermoline Scientific, Australia). IL-1β levels were quantified according to the manufacturers instructions accompanying a commercially available ELISA kit (IL-1β ELISA; BD Bioscience, Australia). Absorbance was quantified on a BMG LABTECHs Polarstar microplate reader (BMG Labtechnologies, Offenburg, Germany) at 450 nm with absorbance at 570 nm subtracted as per manufacturers instructions. The manufacturers limit of quantification of 0.8 pg·mL^{-1} was used.

Outcome measures

Change in average daily headache index from baseline to the final month of treatment was predefined as the primary efficacy outcome. Headache index was calculated daily by multiplying headache duration (h) by average headache intensity (11-point NRS), and was subsequently summated in 4-week (28 day) blocks. This value was then divided by 28 to give an average daily headache index for the baseline period, for weeks 1 to 4 of treatment, weeks 5 to 8 of treatment and for the follow up period, with units h x NRS. Headache index was selected as it captures headache frequency, duration and intensity in one figure, better reflecting the total suffering of headache patients[41]. Secondary efficacy outcomes measures defined *a priori* included headache frequency (days/month), duration (average h on headache days) and intensity (average NRS on headache days) separately, headache impact on quality of life (HIT-6 scores), average daily opioid intake and opioid intake frequency. Tertiary outcomes included changes in cutaneous sensitivity assessed via ASC-12 and quantitative sensory testing.

Statistical analysis

Analysis was conducted on an intention to treat basis for all participants who were deemed eligible for randomisation post baseline headache diary review. Missing values were replaced by the last observed value for that variable. Participant demographics were compared using unpaired t-tests or Fisher's exact tests as appropriate, performed in GraphPad Prism (GraphPad Software; San Diego, CA). Chi-square test with Yates' correction was used to compare doses missed when assessing medication adherence. Fisher's exact tests performed in Graphpad Prism were also used to assess differences between group counts in participant reported adverse events and responder rates. A participant was defined *a priori* to be a 'responder' if percentage change from baseline in the headache outcome of interest was reduced by ≥30%, as a change of this magnitude has previously been reported to be clinically meaningful for chronic headache patients[42].

Participant demographics, headache characteristics and medication intake reported retrospectively during the screening visit and baseline diary headache characteristic and medication intake recordings were analysed to identify predictors of headache index, opioid intake (morphine equivalents (mg)), HIT-6 and ASC-12 scores during the baseline diary data collection period using the generalised linear modeling (glm) function in R statistical language via the graphical user interface RStudio[43]. Subsequently the stepAIC function from the MASS

library{Venables and Ripley, 2002, Fourth Edition. Springer, New York, ISBN 0-387-95457-0}, which performs a stepwise model selection (backward and forward selection) using the Akaike information criterion[44] was performed to identify output variables that contributed to the outcome of interest and eliminate output variables that do not add value to the model thus creating a refined model. The relative contribution of each variable in the refined model was then assessed using the calc.relimp function[45]. Similarly, baseline QST results were analysed to determine which, if any, were able to account for variance in baseline ASC-12 scores using the modeling method described above.

Analysis of variance (ANOVA) tests with Tukey or Sidak adjustments for multiple comparisons were used to compare headache index results, headache duration, frequency and intensity data, average opioid intake and opioid intake frequency data, HIT-6 scores, ASC-12 scores and QST data between groups and over time. Due to prolonged recruitment interim analyses of efficacy and safety were performed at 1 and 2 years after recruitment commenced; no corrections of the reported P values for these interim tests were performed. All results are presented as mean ± SD unless otherwise specified. P values of <0.05 and *F* values of >3 were considered statistically significant.

Analysis of biomarker sub-study data

Graphpad Prism was used for basic statistical analysis of PBMC stimulation data and fitting of concentration-response curves. The concentration-response curve for TLR2 agonist was assessed using a 3 parameter sigmoidal concentration response equation and the bottom responses were fixed at a value of 0, while other parameters were allowed to vary as previously described[40]. For TLR4 a modified biphasic sigmoidal equation was used, as published previously{Kwok et al., 2012, PLoS One, 7, e44232}. The *F*-test was used to ask the question of whether the best-fit curves with the selected parameters (E_{max}, E_{min} and EC_{50}) differ, so that group differences could be identified in the levels of IL-1β released by PBMCs post TLR agonist stimulation. To determine the treatment and visit differences for plasma (IL-1β) a two-way ANOVA with repeated measure was used followed by the Bonferroni *post hoc* test to account for multiple comparisons.

Organisation of and statistical modeling for PBMC stimulation data

The TLR2 and TLR4 agonist concentration-response curves were fitted to a linear regression and the minimum, maximum, slope and intercept were calculated.

All the collected outputs from the participants were imported into RStudio[43]. Generalised linear modelling was used to generate a model to predict which patients received ibudilast; participants on ibudilast were assigned as 1 and participants on placebo was assigned as 0. The stepAIC function, as described above[44], was performed to create a refined model.

A receiver operating characteristic curve (ROC) curve was generated from the refined model and the area under the curve was calculated. To identify non-responders to placebo and ibudilast, the predict function{Venables and Ripley B D, 2002, Fourth Edition. Springer, New York, ISBN 0-387-95457-0} in RStudio was used. A Spearman correlation was used to determine the relationship between the actual treatment assignment and the predicted assignment obtained from the refined model. Participants with the predicted score between 0

and 0.5 were determined to be non-responders to ibudilast and a score above 0.5 determined responder status to ibudilast. The responders of placebo and ibudilast were then selected and a two-way ANOVA with repeated measures was used to identify any treatment differences in headache index scores in the refined population.

RESULTS

Study participants

A total of 34 participants were enrolled in the study and randomised to treatment post baseline headache diary review. At completion 15 participants were allocated to the ibudilast group and 19 to the placebo group. The unbalanced randomisation occurred as a result of revising the original intended enrollment target of 40 participants down to 30 participants due to a prolonged recruitment process. Of the 15 participants randomised to receive ibudilast 13 completed treatment, one withdrew due to treatment emergent adverse effects and one was unable to attend the remaining study visits. Of the 19 participants randomised to placebo 17 completed treatment, one withdrew due to treatment emergent adverse effects and the other was withdrawn due to a change in concurrent medications. All participants who completed treatment went on to complete the 6-month follow up data collection phase. A flow chart describing the participant recruitment process and progression through the trial is presented in Figure 2.

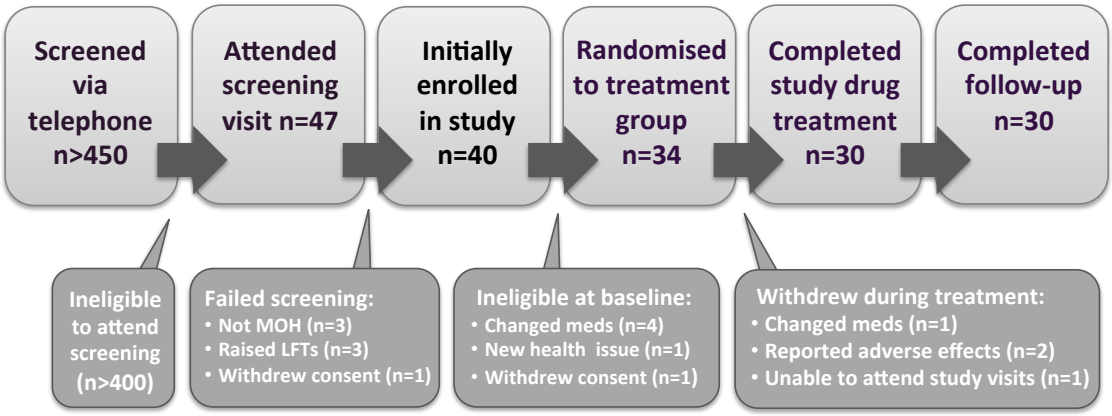

Figure 2. Flow diagram of participant progress through the study phases.

All participants recruited were Caucasian and treatment groups were comparable in age, gender balance, body mass index, and scores for anxiety and depression. While differences were apparent between groups in regard to duration of headache disorder, average daily baseline headache index and initial headache disorder diagnosis, only the difference in duration of chronic headache (≥15 days/month) reached significance, with ibudilast patients suffering from chronic headache for 7 years longer than placebo patients (P=0.03). Baseline headache burden, assessed via the HIT-6 questionnaire, and baseline allodynia during headache, assessed via the ASC-12 questionnaire were not different between the two groups (P=0.41 and P=0.81, respectively). Despite apparent variability in headache, baseline opioid intake was not different between the two groups, in terms of both days of intake per month and average daily morphine equivalents (P=0.70 and P=0.85, respectively). Demographics and baseline medication intake and headache characteristics of the study population are

presented in Table 1. Across groups, opioid intake on 10 or more days per month preceded the onset of chronic headache in 14 patients, began at the same time in 6 patients, and followed the criteria being met for chronic headache in 13 patients.

Table 1. Demographics and baseline headache characteristics for ibudilast and placebo groups. HADS = Hospital anxiety and depression scale. TTH = Tension-type headache. NRS = Numerical rating scale. ME = Morphine equivalents. NSAID = Non-steroidal anti-inflammatory drug. ^ = Data expressed as mean and range, analysed with *t*-test. # = Data expressed as *n* and percentage, analysed with Fisher's exact test. * = Difference is significant.

Study population	Ibudilast (n=15)		Placebo (n=19)		
	^Mean /#*n*	^Range / #%	^Mean / #*n*	^Range / #%	P value
Demographics					
Age (years)^	44	28 - 62	43	23 - 64	0.64
Gender: male#	4	27	5	26	> 0.99
Body mass index^	27.4	17.6 - 40.5	26.7	17.8 - 43.9	0.78
HADS – anxiety score^	10	4-16	7	0 - 17	0.12
HADS – depression score^	7.5	1 - 15	5	0 - 13	0.10
Baseline headache characteristics					
Primary headache diagnosis: TTH#	5	33.3	2	10.5	0.20
Primary headache diagnosis: Migraine#	8	53.3	14	73.7	0.29
Primary headache diagnosis: Mixed#	2	13.3	3	15.8	> 0.99
Age at onset of headache disorder (years)^	19	2 - 41	25	3 - 50	0.17
Headache disorder duration (years)^	25	3 - 52	18	4 - 55	0.16
Duration headache ≥ 15 days/month (years)^	**13**	**1 - 40**	**6**	**1 - 15**	**0.03***
Headache frequency (days per month)^	25	15 - 30	24	15 - 30	0.78
Average daily headache index (h x NRS)^	77	5.5 - 202	57	9 - 109	0.40
Headache impact test score (max 78)^	63.5	46 - 74	65	50 - 72	0.41
Allodynia symptom test score (max 24)^	5	0 - 10	5	0 - 17	0.81
Baseline medication intake					
Opioid intake (days of intake days/month)^	23	11 - 28	22	10 - 28	0.70
Average daily opioid dose (mg ME)^	21	1 - 75.5	19	1.5 - 198.5	0.85
Duration opioid intake ≥10 days/month (years)^	8	1 - 20	7	2 - 19	0.53
Concurrent antidepressant use#	6	40	9	47	0.74
Concurrent antiepileptic use#	2	13	5	26	0.43
Concurrent benzodiazepine use#	4	27	5	26	> 0.99
Concurrent triptan use#	4	27	7	37	0.72
Type of opioid medication used					
Over the counter: codeine + paracetamol use#	9	60	6	32	0.16
Over the counter: codeine + NSAID/aspirin#	8	53	5	26	0.16
Prescription codeine + paracetamol#	4	27	7	37	0.72
Oxycodone#	2	13	3	16	> 0.99
Tramadol#	2	13	1	5.3	0.57
Dextropropoxyphene + paracetamol#	1	7	1	5.3	> 0.99
Morphine#	1	7	1	5.3	> 0.99
Other opioids#	2	13	0	0	0.19

Predictors of baseline headache index, opioid intake and headache impact on quality of life

Using generalised mixed effects modeling a higher headache index over the baseline diary data collection phase was found to correlate with reported throbbing/pulsing headache (P=0.018), characteristic of migraine. However, sensitivity to light during headaches (P=0.003), which is also a characteristic of migraine, was associated with a lower headache index. With regard to demographics, male gender (P=0.027), primary diagnosis of migraine or mixed migraine/TTH (P=0.024), not taking paracetamol (P=0.008), not being prescribed an antiepileptic medication (P<0.001), a higher HIT-6 score (P=0.021) and a higher ASC-12 score (P=0.046) and unsurprisingly, previous headache frequency (P<0.001) were associated with a greater headache index. Higher baseline HIT-6 scores were associated with higher baseline ASC-12 scores (P=0.020) and sensitivity to sound (P=0.030) as recorded in the baseline headache diary. Increased opioid intake in terms of morphine equivalent doses (mg) was associated with female gender (P=0.025), primary diagnosis of migraine (P=0.020), higher headache frequency (P=0.015), not being prescribed a triptan (P=0.002), higher HIT-6 scores (P=0.036) and lower ASC-12 scores (P=0.003). When modeled against the headache diary data higher opioid intake was related to headache that was better (P<0.001) or unchanged (P=0.002) with movement and headache that did not posses tightening/pressing characteristics (P=0.046).

Adherence to study medication

Non-adherence to prescribed clinical trial interventions provides a major obstacle in translating research outcomes to efficacy in clinical practice[46]. In this study adherence to the study medication dosing regime was assessed by participant daily self-report in the headache diary and confirmed by returned capsule count. Participants were considered adherent if >80% of dispensed trial doses were taken over the treatment period, as defined in previous studies[47, 48]. Based upon participant self-report all but one participant (in the placebo group) met the criteria for adequate adherence. Considering the returned capsule count data, again all but one participant (in the ibudilast group) could be considered adherent. Thus, only two discrepancies were noted between subjective self-reported medication intake and objective returned capsule counts. For one participant greater adherence was found via the self-report method, whereas for another participant greater adherence was noted via the returned capsule count. All other participants were found to be adherent using both methods. Doses missed did not differ between treatment groups with an average of 6% of medication doses returned in the ibudilast group compared with 5% (P=0.27) in the placebo group and an average of 8% doses self-reported as missed in the ibudilast group versus 5% in the placebo group (P=0.08). Additional confirmation of adherence was provided by the biomarker sub-study (see section 3.8).

Effect of ibudilast on the primary efficacy outcome - headache index

One participant assigned to the ibudilast treatment group did not complete the headache diary during the baseline period to a standard sufficient to allow inclusion in the efficacy analysis. Thus, the ibudilast group for analysis consisted of 14 participants, while the placebo group for analysis included 19 participants. Although average daily headache index appeared higher in the ibudilast group at baseline (mean 57 h x NRS vs. 74 h x NRS for placebo) and throughout

the study (mean 59 h x NRS vs. 76 h x NRS for placebo over weeks 5-8), it was not significantly different between treatment groups at any of the 4-week time points assessed, as illustrated in Figure 3A. As there was substantial inter-participant variability in headache index, within participant change in headache index from baseline was also calculated, but did not reveal any significant differences between groups over time, see Figure 3B. A responder analysis looking at change in headache index was also conducted as a secondary outcome measure. However, as outlined in Table 2, no significant differences in the proportions of participants who achieved the predefined ≥30% reduction in headache index to be classified as a responder over treatment weeks 1-4 or 5-8 or at follow up vs. baseline were evident between the treatment groups. No association between positive responder status in the ibudilast group at follow-up and likely physical dependence, indicated by higher regular opioid intake, was evident. The three participants who were classified as responders to ibudilast in the headache index analysis recorded average daily baseline opioid intakes of 1, 5.7 and 13.8 mg morphine equivalence, and opioid intake frequencies of 10, 15 and 30 days/month respectively.

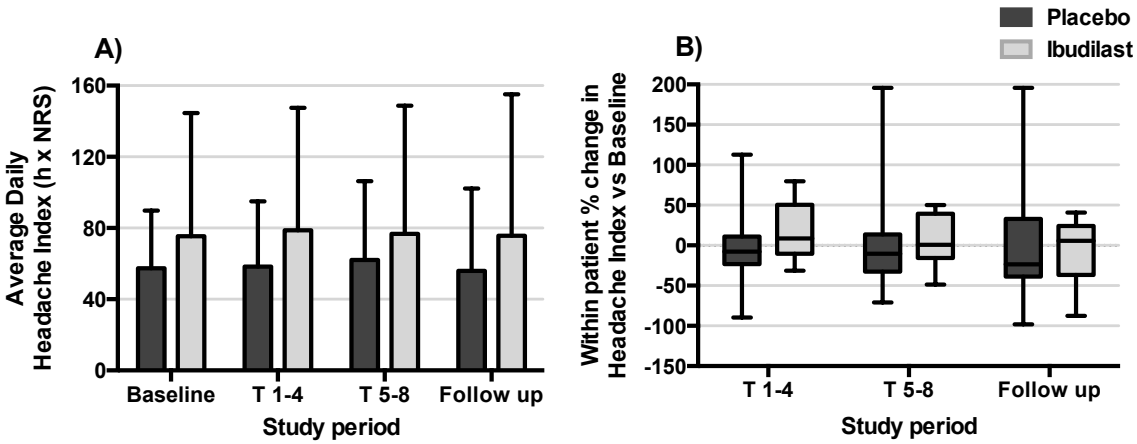

Figure 3. Primary efficacy outcome results. A) Headache index, averaged over 4 week blocks (baseline, treatment weeks 1 to 4 (T 1-4), treatment weeks 5 to 8 (T 5-8) and follow up), did not significantly differ between treatment groups (P=0.35), and did not change over time (P=0.80). Graph presents mean and SD. B) Within participant percentage change in 4-week headache index vs. baseline did not differ between treatment groups (P=0.81) and did not change over time (P=0.31). Graph presents minimum, maximum, interquartile range and median.

Table 2. Responder rates (defined as ≥30% improvement from baseline) for headache indices in ibudilast and placebo groups across the study period. T = treatment weeks, d/m = days per month. NRS = Numerical rating scale.

	Baseline vs. T 1-4			Baseline vs. T 5-8			Baseline vs. Follow up		
Outcome	Ibudilast *n* (%)	Placebo *n* (%)	P value	Ibudilast *n* (%)	Placebo *n* (%)	P value	Ibudilast *n* (%)	Placebo *n* (%)	P value
Headache index	0 (0%)	1 (5%)	>0.99	1 (7%)	5 (26%)	0.21	3 (21%)	7 (37%)	0.46
Headache frequency (d/m)	0 (0%)	2 (11%)	0.50	1 (7%)	2 (11%)	>0.99	1 (7%)	3 (16%)	0.62
Headache duration (h)	1 (7%)	1 (5%)	>0.99	0 (0%)	1 (5%)	>0.99	2 (14%)	3 (16%)	>0.99
Headache intensity (NRS)	0 (0%)	1 (5%)	>0.99	0 (0%)	1 (5%)	>0.99	1 (7%)	3 (16%)	0.62

Effect of ibudilast on secondary outcomes – Additional headache indices and HIT-6 score

No effect of treatment group on headache frequency was detected (P=0.55) as shown in Figure 4A. *Post hoc* Tukey's multiple comparisons tests revealed no difference between ibudilast and placebo groups over weeks 1-4 (P=0.90), weeks 5-8 (P=0.96) or follow up (P=0.93). Furthermore, the only significant within group change over time was between baseline and follow up periods for placebo patients (P=0.01). As shown in Figure 4B and C, headache duration and intensity did not differ by treatment (P=0.68 and P=0.71, respectively) or over time (P=0.44 and P=0.85, respectively). Responder analyses were also conducted, but failed to demonstrate a difference in response rates between ibudilast and placebo groups over treatment weeks 1-4, 5-8 or at follow up vs. baseline with respect to headache frequency, duration or intensity (see Table 2 for response rate details).

Physical, social, and emotional impact of headache on quality of life were assessed using the 6-item short-form Headache Impact Test (HIT-6) questionnaire at baseline, week 4 of treatment, week 8 of treatment and at follow up 6 months post randomisation. Headache was considered to have little to no impact, some impact, substantial impact or severe impact when the HIT-6 score was <49, 50-55, 56-59, or >60, respectively. In all but three participants baseline HIT-6 scores were >60, representing a severe burden of headache across both treatment groups. By week 4 of treatment in the ibudilast group one participant displayed an increase in HIT-6 category, while in the placebo group six participants displayed a decrease in HIT-6 category. By week 8 of treatment in the ibudilast group one participant displayed an increase while one other displayed a decrease in HIT-6 category, whereas in the placebo group one participant displayed an increase while five displayed a decrease in HIT-6 category. Upon statistical analysis HIT-6 score was not found to change significantly with time across the treatment period (P=0.55), and was not influenced by treatment group (P=0.89) as illustrated in Figure 4D. When the HIT-6 score was broken into individual HIT-6 questions, ibudilast did not alter any individual domain over time, and no domain differed between groups at any time point.

Effect of ibudilast on secondary outcome – Acute headache medication intake

A treatment that can reduce the opioid requirements of a headache patient is of clinical interest, even if no change in pain level is recorded. Thus, our secondary efficacy analysis included investigation of acute headache medication intake. Acute headache medication intake was recorded daily in the headache diary and daily opioid intake was converted to an equivalent dose of morphine (mg) based upon the local Royal Adelaide Hospital Pain Management Unit opioid conversion table to allow comparison of patients consuming opioids of differing potency.

Average daily opioid intake was similar between treatment groups at baseline and remained stable throughout the treatment and follow up periods, as shown in Figure 5A. As for headache index, average daily opioid intake presented with great inter-patient variability, thus within participant percentage change in opioid intake from baseline was also calculated for treatment weeks 1-4, 5-8 and the follow up period. Although no overall effects of treatment (P=0.31) and time (P=0.27) were observed on percentage change in opioid intake, the treatment:time interaction emerged as significant (P=0.03). *Post hoc* Tukey's multiple

comparisons tests found no significant differences in percentage change in opioid intake between treatment groups at any time period, and only difference detected a within group difference in ibudilast group between weeks 1-4 vs. follow up (P=0.03), see Figure 5B. In patients receiving ibudilast a greater percentage reduction in opioid intake at weeks 5-8 vs.

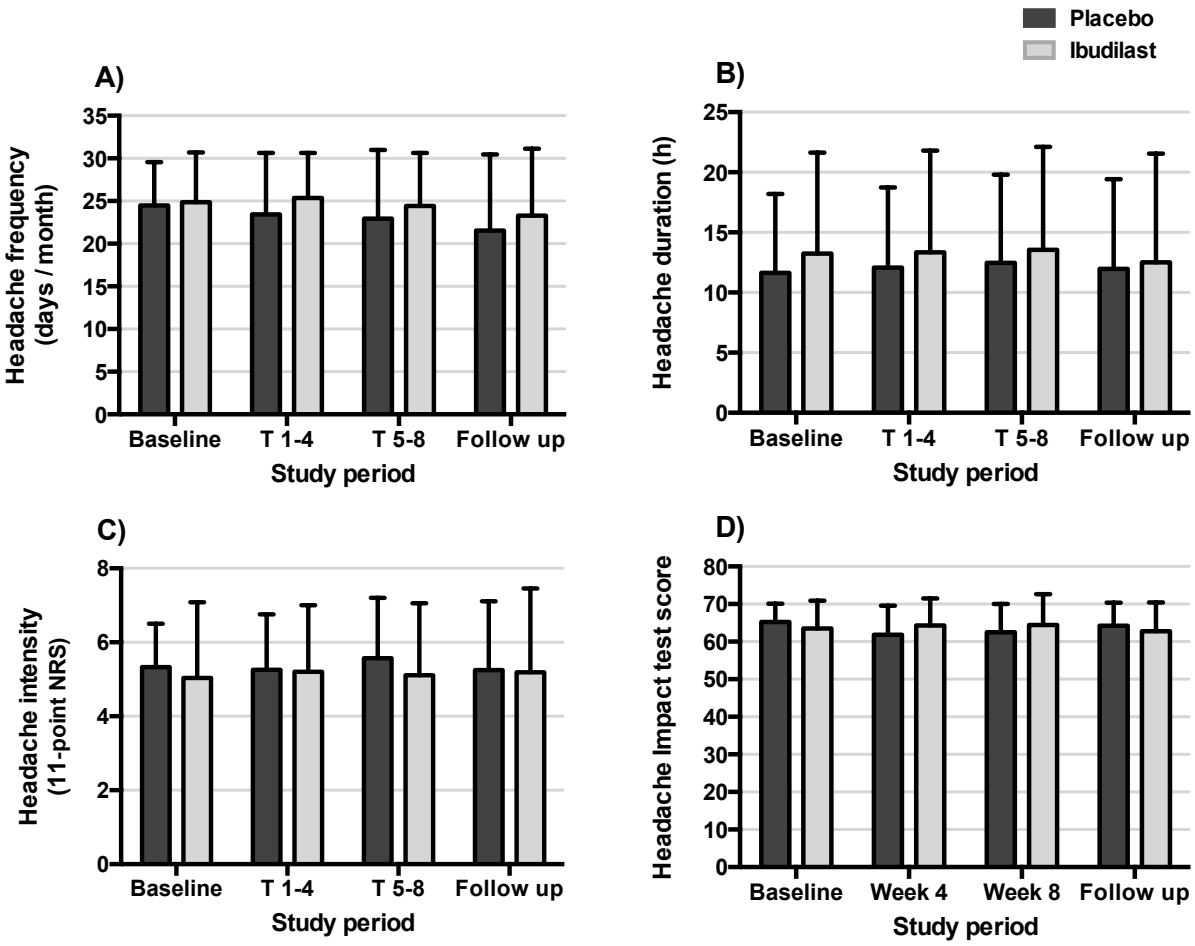

Figure 4. Secondary efficacy outcomes results. A) Headache frequency was not influenced by treatment group (P=0.55). No effect of treatment or time was found for any of remaining the secondary outcomes measures including B) Headache duration (P=0.68 and P=0.44), C) Headache intensity (P=0.71 and P=0.85) and Headache impact test scores (P=0.89 and P=0. 55). NRS = numerical rating score. T = treatment weeks.

baseline was significantly associated with a greater percentage reduction in headache index over the same period (P=0.046), although the direction of causality cannot be confirmed (data not shown). As the diagnostic criteria for medication overuse are based upon frequency of opioid administration, rather than opioid dose, days/month of opioid intake was also investigated as a secondary efficacy outcome. While there was a modest overall reduction in opioid intake frequency (P=0.03) and a greater within patient percentage reduction in opioid intake frequency (P<0.01) over time, *post hoc* Tukey's multiple comparisons tests found within each group for the only significant difference observed across study periods was again in the ibudilast group at T1-4 vs. follow up (P<0.01), see Figures 5C and D. Treatment group in general did not influence opioid intake frequency (P=0.76) or percentage change in opioid intake (P=0.74).

Effect of ibudilast tertiary outcomes – Quantitative sensory testing and ASC-12 score

Four different quantitative sensory tests were employed to evaluate cutaneous sensitivity; the mean result and standard deviation for all sites assessed (left and right sides averaged) in both groups before and after treatment are presented in Table 3. One participant in the

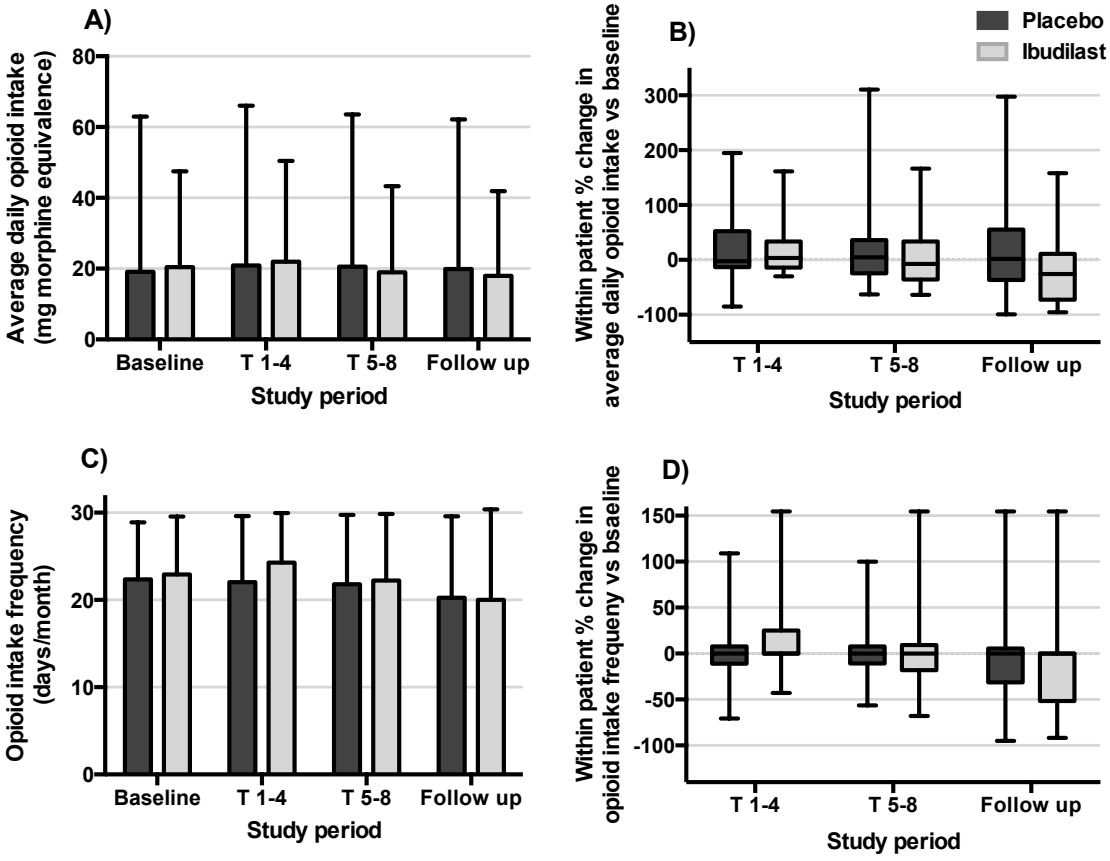

Figure 5. A) Average daily opioid dose did not differ with time (P=0.18) or treatment (P=0.98). B) The treatment:time interaction significantly influenced percentage change in average daily opioid dose (P=0.03), yet treatment (P=0.31) and time (P=0.27) alone did not. C) and D) A modest overall reduction in opioid intake frequency (P=0.03) and a greater within patient percentage reduction in opioid intake frequency (P<0.01) was observed over time, however, treatment group did not influence opioid intake frequency (P=0.76) or percentage change in opioid intake (P=0.74). T = treatment weeks.

placebo group was unable to perform the QST procedures and was therefore excluded from this analysis. At baseline brush allodynia, sensitivity to cold and sensitivity to heat did not vary with site tested (P=0.37, P=0.26 and P=0.08, respectively). A significant site effect was observed when assessing von Frey threshold (P=0.0015) and tolerance (P=0.043), with the jaw displaying a reduced threshold and the cheek displaying both reduced threshold and tolerance, compared with the inner forearm. No between group differences were observed in any of the QST measures at any time point, and no measure changed significantly over the treatment period within each group (week 2 and 4 data not shown).

Self-reported cutaneous allodynia experienced by participants during severe headaches was assessed retrospectively using the 12-point allodynia symptoms checklist at baseline, weeks 2,

4 and 8 of treatment and at follow up 6 months post randomisation. Participants were categorised to experience no, mild, moderate or severe cutaneous allodynia (corresponding to ASC-12 scores of ≤2, 3-5, 6-8, ≥9, respectively), based upon the self-rated frequency of various allodynia symptoms. At baseline overall female participants had more allodynia than males (mean ASC-12 score of 6 vs. 2, respectively P=0.002).

Prior to treatment 71% of participants in the ibudilast group and 67% participants in the placebo group had some degree of allodynia (ASC-12 score ≥3). By week 8 of treatment this had reduced to 43% of participants in ibudilast group and 47% of participants in the placebo group. Statistical analysis demonstrated that ASC-12 score was not influenced by treatment group (P=0.44), and *post hoc* Tukey's multiple comparisons tests found no significant differences between treatment groups at any time point. Overall there was a significant effect of time on ASC-12 scores (P<0.01), and within the ibudilast and placebo groups small but significant reductions in ASC-12 scores were found at week 4 (P=0.003 and P=0.046, respectively) and week 8 (P=0.018 and P=0.008, respectively) vs. baseline as illustrated in Figure 6.

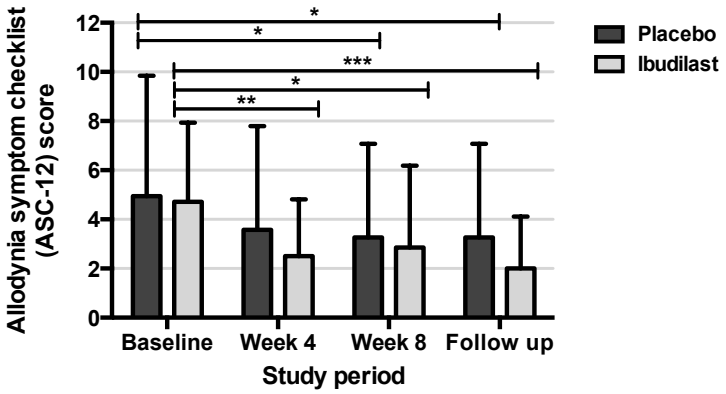

Figure 6. Allodynia symptom checklist scores were modestly reduced in the ibudilast group at week 4 (P=0.003), 8 (P=0.016) and follow up (P<0.001) vs. baseline and in the placebo at week 8 (P=0.01) and follow up (P=0.01) vs. baseline.

Biomarker sub-study

Twenty-three MOH participants were included in the biomarker sub-study, including 9 participants allocated to the ibudilast group and 14 in the placebo group. The characteristics of these participants were similar to the demographics of the total study population, except the difference in duration of headache disorder did not reach significance (P=0.4). Comparisons between the baseline and week 8 visit opioid intake and PBMC output (minimum, maximum, slope and intercept calculated after individual TLR2 and TLR4 agonist concentration-response curves were fitted to a linear regression) are presented for each treatment group in Table 4.

Effect of ibudilast on basal (unstimulated) IL-1β levels in plasma and from PBMCs

No significant treatment (P=0.9) or visit differences (P=0.2) were found between the ibudilast and the placebo group in the plasma IL-1β level. The plasma IL-1β at baseline was 0.5 ± 0.2 $pg \cdot mL^{-1}$ (placebo) vs. 0.6 ± 0.3 $pg \cdot mL^{-1}$ (ibudilast) and after 8 weeks of treatment was 1 ± 0.3 $pg \cdot mL^{-1}$ (placebo) vs. 0.9 ± 0.3 $pg \cdot mL^{-1}$ (ibudilast). Likewise, unstimulated PBMCs did not differ between treatment (P=0.5) or visits (P=0.06). The basal level (unstimulated) IL-1β level

for placebo was 8 ± 3 pg·mL^{-1} and ibudilast was 5 ± 2 pg·mL^{-1}. At the completion of the study, the level of I IL-1β for placebo was 2 ± 0.6 pg·mL^{-1} and for ibudilast was 2 ± 0.4 pg·mL^{-1}.

Table 3. Quantitative sensory testing results for ibudilast and placebo groups at baseline and week 8 of treatment. Sites assessed included regions innervated by each of the trigeminal nerve branches, the ophthalmic nerve (V_1), maxillary nerve (V_2) and the mandibular nerve (V_3). Extracephalic regions assessed included those innervated by the 5th cervical nerve and the 1st thoracic nerve (*C5/T1) and the 7th and 8th cervical nerves (^C7/C8). No significant differences were observed with time or treatment group. Results presented as mean (SD). VAS = visual analogue scale.

	Brush allodynia (VAS score)*				Pressure allodynia Threshold (g)*				Pressure allodynia Tolerance (g)*				Thermal hyperalgesia Heat Tolerance (°C)^				Thermal hyperalgesia Cold Tolerance (°C)^			
	Ibudilast		Placebo		Ibudilast		Placebo		Ibudilast		Placebo		Ibudilast		Placebo		Ibudilast		Placebo	
Study week	**0**	**8**	**0**	**8**	**0**	**8**	**0**	**8**	**0**	**8**	**0**	**8**	**0**	**8**	**0**	**8**	**0**	**8**	**0**	**8**
Site - V_1	7.8 (22.1)	8.4 (21.3)	6.3 (9.9)	6.8 (13.8)	0.12 (0.27)	0.06 (0.09)	0.06 (0.06)	0.06 (0.07)	28.7 (28.9)	22 (15.8)	22.1 (24.8)	28 (29.3)	40.6 (11.6)	41.6 (12)	44 (4.8)	44.3 (4.8)	12.2 (8.2)	13.3 (8.9)	12 (11.3)	12.7 (12)
Site - V_2	3.3 (8.4)	6.3 (15.5)	5.2 (6.7)	8.3 (14)	0.03 (0.02)	0.04 (0.03)	0.03 (0.01)	0.13 (0.39)	18.3 (17)	22.2 (16.7)	15 (10.7)	26.6 (33.6)	-	-	-	-	-	-	-	-
Site - V_3	4 (12.4)	5 (9.9)	4.4 (8.1)	8 (12.2)	0.04 (0.03)	0.06 (0.09)	0.03 (0.02)	1.26 (5.18)	29.4 (23.4)	29.8 (25.3)	18.7 (15.8)	16.5 (20.1)	-	-	-	-	-	-	-	-
Site - Extracephalic	1.6 (3)	3.7 (5.2)	6.3 (9.5)	10.6 (16.8)	0.14 (0.16)	0.16 (0.19)	0.13 (0.18)	1.45 (4.7)	35.1 (36.6)	29.7 (28.4)	24.2 (17)	23 (23.4)	40.9 (11.8)	41.6 (11.9)	42.6 (11.8)	41.8 (11.5)	12.1 (6.5)	12.2 (6.7)	9.3 (9.2)	11.9 (10.4)

Effect of ibudilast on IL-1β levels following stimulation of isolated PBMCs with TLR2 and TLR4 agonists

At baseline there was no treatment difference between the placebo and the ibudilast group following stimulation with a TLR2 agonist (P=0.3, $F_{(3,221)}$=1.2) (see Figure 7A). However after 8 weeks of treatment, a significant decrease in the release of IL-1β was found in the ibudilast group compared with placebo (P<0.0001, $F_{(3,221)}$=11.5) (see Figure 7B). A significant concentration-dependent increase was found at both baseline (P<0.01) and 8 weeks after treatment (P<0.01). Similarly, at baseline, no treatment difference was detected between the placebo and the ibudilast group following stimulation with a TLR4 agonist (P= 0.1, $F_{(1, 159)}$= 2.09) (see Figure 8A). After 8 weeks of treatment significantly less IL-1β (P=0.01, $F_{(4,210)}$= 3.3) (see Figure 8B) was released from the ibudilast group after TLR4 agonist stimulation compared with the placebo group. A significant concentration-dependent increased was also found at both baseline (P< 0.001) and 8 weeks after treatment (P<0.001).

Table 4. Summary of opioid intake and isolated PBMC variables collected from sub-study participants. Data are presented as mean ± SEM. Peripheral blood mononuclear cells (PBMC) were stimulated via with Pam3CSK4 (from 13 pg·mL^{-1} to 1 μg·mL^{-1}) and lipopolysaccharide (LPS) (6 pg·mL^{-1} to 10 μg·mL^{-1}) for 20 h. TLR = toll-like receptor. *Indicates significant result.

	Ibudilast (n=9)	Placebo (n=14)	P value
Opioid intake - baseline (mg morphine eq)			
Daily	17 ± 5.3	9.1 ± 1.9	0.11
Cumulative	478 ± 149	254 ± 54	0.11
Within participant change	4.9 ± 1.7	5.4 ± 0.83	0.7
Opioid intake – treatment weeks 4-8 (mg morphine eq)			
Daily	16 ± 5.5	9.3 ± 2.2	0.18
Cumulative	459 ± 154	261 ± 61	0.18
Within participant change	6.5 ± 2.3	6.2 ± 1.2	0.91
Change in non-stimulated data (baseline vs week 8)			
Plasma IL-β levels (pg·mL^{-1})	0.46 ± 0.31	0.52 ± 0.36	0.91
PBMC count (^6)	1.4 ± 1.6	0.99 ± 2	0.89
PBMC concentration (pg·mL^{-1})	-3.5 ± 1.7	-5.5 ± 3.4	0.67
Change in TLR2 stimulated (Pam3CSK4) IL-1β (pg·mL^{-1}) (baseline vs week 8)			
Minimum	-54 ± 23	-0.0045 ± 14	0.04*
Maximum	-440 ± 247	494 ± 183	0.0055*
Slope	-37 ± 35	93 ± 39	0.03*
Intercept	-224± 154	424 ± 169	0.015*
Change in TLR4 stimulated (LPS) IL-1β (pg·mL^{-1}) (baseline vs week 8)			
Minimum	-124 ± 81	-80 ± 51	0.63
Maximum	-242 ± 184	-30 ± 246	0.54
Slope	69 ± 47	2.4 ± 55	0.40
Intercept	15 ± 155	-108 ± 231	0.70

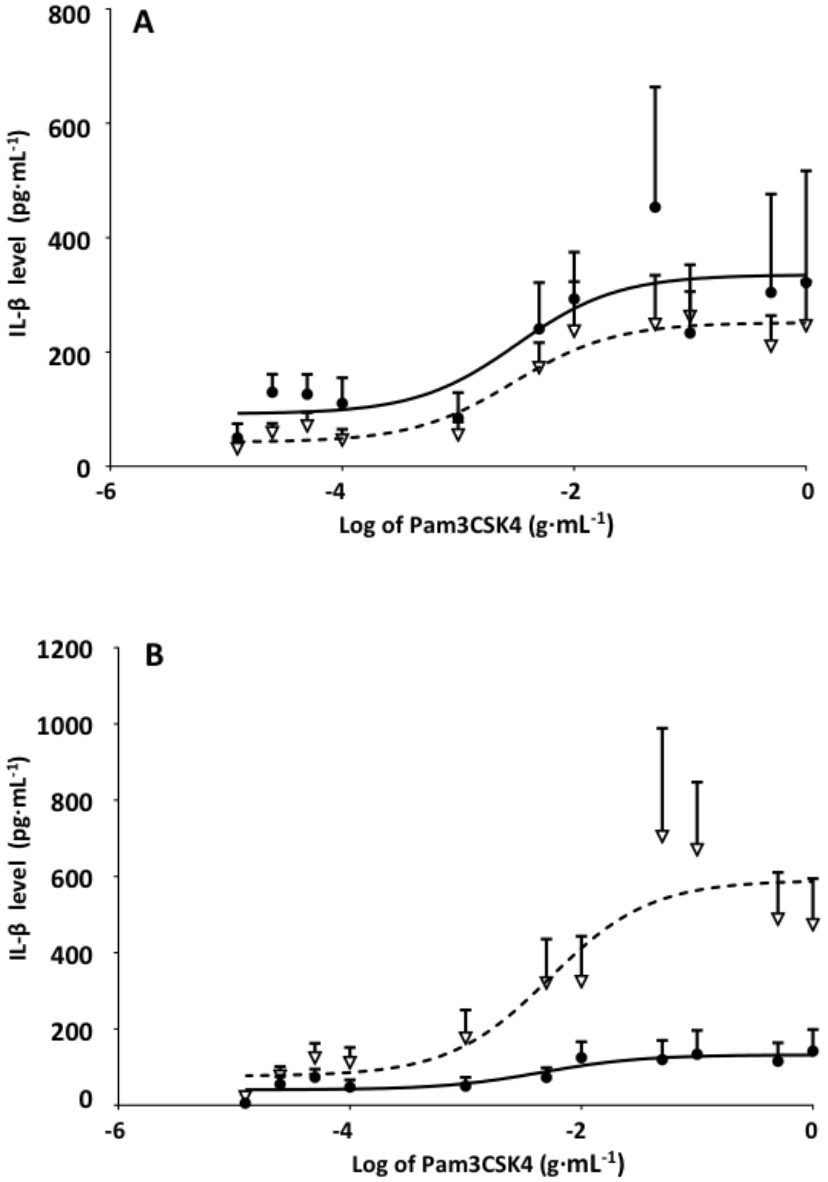

Figure 7. Isolated PBMCs obtained from participants receiving ibudilast treatment (closed circle) and placebo (open triangle) were stimulated with a range of Pam3CSK4 (toll-like receptor 2 agonist) concentrations (13 pg·mL^{-1} to 1 µg·ml^{-1}) to generate the response curves. A) At baseline, there were no group differences. B) After 8 weeks of treatment, ibudilast produced a significant reduction in stimulated interleukin-1β (IL-1β) release ($P<0.0001$). Error bars on graphs represent standard error of the mean.

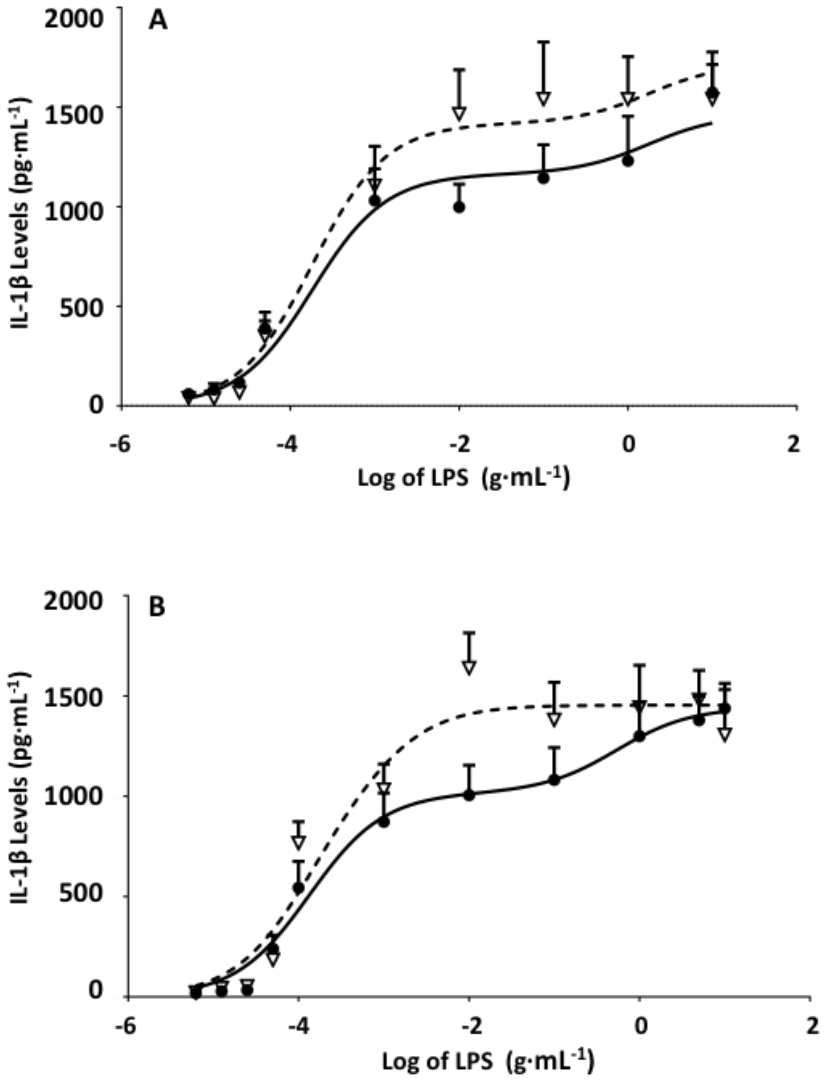

Figure 8. Isolated PBMCs obtained from participants receiving ibudilast treatment (closed circle) and placebo (open triangle) were stimulated with a range of lipopolysaccharide (LPS) (toll-like receptor 4 agonist) concentrations (6 pg·mL^{-1} to 10 µg·mL^{-1}) to generate the response curves. A) At baseline both groups were not different. B) After 8 weeks of treatment, ibudilast produced a significant reduction in stimulated interleukin-1β (IL-1β) release (P=0.01). Error bars on graphs represent standard error of the mean.

Prediction of treatment group using IL-1β output from isolated PBMCs after TLR agonist stimulation

The model constructed with selected output variables (changes from baseline to week 8 of treatment) obtained an area under the receiver operating characteristic (ROC) curve of 0.9 (see Figure 9), and could accurately predict which participants were receiving ibudilast treatment. The panel of selected output variables required to create the model included both TLR2 and TLR4 stimulation data, as shown in Table 5.

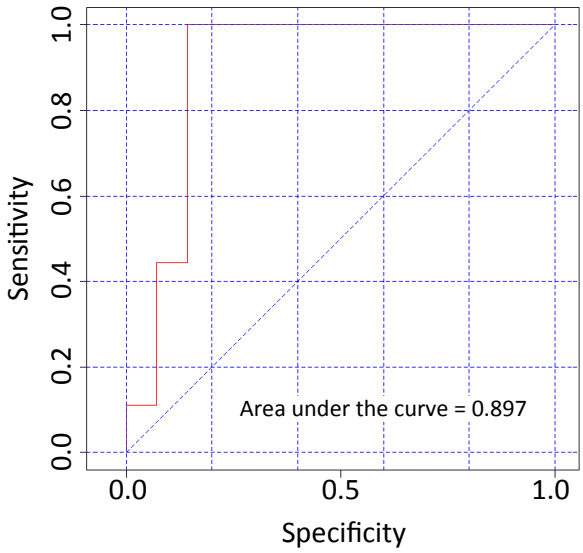

Figure 9. Representation of the receiver operating characteristic (ROC) curve for prediction of treatment. Model generated from data obtained from peripheral collected output variables.

Table 5. Best-fit logistic regression model results for the prediction of treatment, ibudilast or placebo, including discrimination probabilities (D, area under receiver operating characteristic curve (AUC)). The residual deviance for the model includes predictor variables, whereas the null deviance for the model does not. TLR = toll-like receptor.

Output	Estimate	Standard error	P value	Null deviance	df	Residual deviance	df	AUC
Change in TLR4 stimulated min	0.008	0.005	0.11	30.79	22	20.15	18	0.90
Change in TLR4 stimulated max	-0.002	0.0014	0.10					
Change in TLR4 stimulated slope	0.013	0.0079	0.10					
Change in TLR2 stimulated min	-0.03	0.015	0.053					

Exploratory responder analysis based upon PBMC biomarker results

Patients in the ibudilast group who demonstrated reduced stimulated PBMC IL-1β release (6 out of 9 who underwent biomarker testing) were selected, change in average daily headache index score at week 5-8 vs. baseline for this group was recalculated and compared with that

of the entire placebo group. However, even within this refined population no significant treatment effect was evident (P=0.97) (data not shown).

Safety and tolerability of ibudilast

Patients were questioned about adverse events during the study period at each study visit. Moreover, patients were asked contact the study staff and record in the headache diary any adverse events occurring during treatment and the follow-up period. The safety analysis included data from 15 participants receiving ibudilast for a cumulative total of 109 participant weeks, compared with data from 19 participants receiving placebo for a cumulative total of 140 participants weeks.

Generally ibudilast was found to be safe and well tolerated. All participants receiving ibudilast reported at least one adverse event, compared with 68% of patients in the placebo group, a difference that was statistically significant (P=0.02). The most commonly reported adverse event was nausea, which occurred in 66.7% of participants receiving ibudilast, compared to 10.5% of participants receiving placebo (P=0.001). Nausea was generally mild and could be managed by initiating over the counter antiemetics and/or temporarily ceasing the study medication, reinitiating at a lower dose and up-titrating to the original dose. Additional adverse events that were reported more often in the ibudilast group than the placebo group included dysregulation of body temperature, intermittent pruritus and diarrhoea. All but one of the adverse events were classified as mild. One serious adverse event occurred, however this was deemed unrelated to the study medication by the principal investigator as it pertained to a participants preexisting medical condition. No participant was required to withdraw from the study due to safety concerns, although one participant from each treatment group elected to withdraw due to reported worsening of headache.

DISCUSSION

This study involved a group of MOH patients who had suffered from chronic headache for a long time and whom were frequently consuming opioid analgesics without achieving meaningful benefits. On average our patients experienced a severe impact of headache on quality of life, as evidenced by their high baseline HIT-6 scores (63.5 and 65 in ibudilast and placebo groups respectively), which were comparable with previous MOH cohorts[33, 49].

Ibudilast 40 mg twice daily for 8 weeks was not effective in reducing indices of headache burden including headache index, frequency, duration, intensity and headache impact on quality of life (HIT-6 scores). Interestingly, in this study no placebo effect on headache was observed in either treatment group. This is somewhat unusual in clinical trials addressing painful conditions, for example a review of randomised clinical trials of acute migraine medications found an average placebo response rate of 30%. This perhaps could be explained by our patients' extended duration of headache and chronic headache, and subsequent numerous episodes of previous treatment failure. It may be that these patients have trialled so many unsuccessful treatments that they are conditioned to expect little benefit from medication.

Given MOH is thought to involve dependence-like behaviours[49], and that building evidence suggests ibudilast can attenuate opioid withdrawal[50], it was hypothesised that ibudilast may help to ease withdrawal from overused opioid analgesics in MOH, potentially reducing withdrawal-related relapse in such patients. However, in this cohort, no clinically significant reductions in opioid intake were observed following eight weeks of treatment with ibudilast.

In the present study we demonstrated for the first time that ibudilast treatment can reduce the release of IL-1β following the stimulation of isolated PBMCs collected from MOH patients with TLR2 and TLR4 agonists. Importantly, only the evoked response was targeted by ibudilast, suggesting this drug therefore specifically targets only activated cells or pathways, without altering basal function. Using the peripheral PBMC outputs we were able to generate a model that allowed accurate prediction which participants received ibudilast as opposed to placebo treatment. Thus, there is great potential for the use of TLR reactivity assessment in isolated peripheral immune cells as a potential biomarker to evaluate new interventions that target TLR pathways.

Levels of adherence, assessed via participant self-report and returned capsule count, were high within our cohort, suggesting the lack of efficacy observed was not due to insufficient study medication intake. The biomarker responses recorded appear to support these findings, as although no change in the biomarker would not necessarily imply non-adherence, it seems unlikely that a reduction in IL-1β release would occur after the treatment period in the absence of ibudilast ingestion. There is therefore potential for utilisation of PBMC reactivity as a biomarker to confirm adherence to medications such as ibudilast, if dose-response relationships for such treatments can be clearly established.

It is clear that the nociceptive changes following prolonged opioid administration are complex[51] and likely to involve multiple systems. While it is extensive preclinical evidence shows opioids can increase pain through glial-mediated proinflammatory mediator release, long-term opioid use is also associated with a range of other neuroplastic adaptations and alterations in the regulation of various pain transduction pathways[52]. A previous preclinical study performed in our laboratory demonstrated that ibudilast was able to reverse opioid-induced hyperalgesia and allodynia, while opioid delivery continued in an animal model. In this preclinical study experiment opioids were given for 4 days and concurrent ibudilast treatment was commenced from day 2, resulting in a duration of ibudilast treatment equal to that of opioid therapy alone. Thus, although the biomarker study indicates ibudilast was likely to be hitting and inflammatory target, the treatment period may not have been sufficient to reverse potential long-term changes associated with extended opioid exposure. A glial attenuating intervention may therefore be more effective in MOH if implemented earlier in terms of duration of opioid use, in an attempt to prevent pro-nociceptive neuroplastic changes. Alternatively, while it appears ibudilast is effective at a peripheral immune target in our cohort, it is possible that the same effects are not occurring centrally.

It has long been recognised that MOH patients often remain resistant to prophylactic treatments while drug overuse persists[53]. Although ibudilast alone may not be sufficient to reduce headache burden in MOH, it may be able ease a mandated analgesic withdrawal, potentially allowing more patients to successfully undergo detoxification in an outpatient

setting as withdrawal symptoms, and therefore the temptation to reinitiate the overused medication, may be reduced. Regardless, it is clear that multimodal management including patient education, appropriate treatment of concurrent psychological disorders is required to achieve optimal outcomes for patients suffering from MOH.

Although conflicting data exist, some preclinical studies suggest TLR4 and microglia may primarily regulate pain in males, bearing little influence on female pain[54]. If this is the case this could explain the lack of efficacy observed in our largely female cohort. Unfortunately, general the preponderance of MOH in females and subsequent low recruitment of male participants resulted in a male:ibudilast group size that did not allow for a meaningful assessment of sex differences in response to treatment in this clinical trial population.

As discussed, evidence exists to suggest that MOH may be related to the development of central sensitisation[55]. Clinically, central sensitisation presents as allodynia (pain elicited by non-noxious stimuli) and/or hyperalgesia (increased sensitivity to noxious stimuli)[56-58]. Cutaneous allodynia has been studied extensively in migraine and reported in a number of other headaches including TTH, cluster headache[59], and post-traumatic headache[60]. Studies in headache patients utilising multiple experimental pain models have found pain sensitivity is, at least in part, determined by the nature of the nociceptive stimulus and varies with the site of testing[61]. Thus, to gain a complete picture of our patients' pain sensitivity the von Frey filament test and the brush allodynia test were employed to assess static mechanical (pressure) and dynamic mechanical (brush) allodynia, and hot and cold thermal thresholds testing were employed at cephalic and extra-cephalic sites. This study is the first to evaluate cutaneous sensitivity using multimodal stimuli in a well-defined MOH population consuming opioids. Our group has recently collected thermal sensitivity data from a cohort of pain-free volunteers, which can be compared to this data set as the study was designed to ensure the same testing protocol was followed and testing was conducted by the same investigator. In comparison with pain-free volunteers, MOH patients display increased cold pain thresholds, indicating greater sensitivity, at the right cheek, and a non-significant increase in threshold at the left cheek but no difference at the palm on either side. The difference in cold pain threshold between pain-free participants and patients with MOH at the cheek may be due to heightened sensitivity in patients painfully afflicted region, being the trigeminal region (unpublished findings, Sumracki 2014). Further interpretation of the baseline mechanical QST data requires an equivalent pain-free comparator data set.

In other headache patients, primarily suffering from chronic migraine, or episodic migraine with concurrent chronic tension type headache, brush allodynia was found to be more common and severe at the first trigeminal division (V_1)[60]. This was not replicated in our MOH patients, although we did observe reduced sensitivity to pressure in the von Frey test at the inner forearm site. Allodynia was further investigated using the ASC-12 questionnaire. As in other headache cohorts[62], female participants overall reported higher levels of allodynia at baseline, with an average ASC-12 score of 6 (moderate allodynia) vs. 2 (no allodynia) in males. However, a number of ASC-12 items, although not necessarily gender specific, may be more likely to be relevant to female participants (e.g. wearing a necklace, wearing earrings), thus giving females a greater opportunity to contribute to their ASC-12 scores.

Independent of its efficacy in MOH, this study provides important data regarding the safety of ibudilast dosing at 40 mg twice daily for up to 8 weeks. The majority of clinical experience with ibudilast, and resultant safety data, derives from its use in Japan where the standard doses employed are 10 mg twice daily for asthma and 10 mg three times a day for cerebrovascular indications. Recently, clinical trials focusing upon new neurological indications for ibudilast have exposed participants to doses of 30 mg twice daily for extended periods, with some reaching up to 100 mg per day, however chronic dosing data at the larger dose are yet to be published. In this study ibudilast was generally safe and well-tolerated; all adverse events deemed related to the study medication were mild and resolved without sequelae. The most commonly reported adverse event was nausea, which occurred more frequently than in previous studies[63, 64]. The increased incidence of nausea may be related to the higher ibudilast dose, however it should also be noted that the majority of patients in this cohort also experienced nausea, at least episodically, in relation to their headache disorder at baseline. As it is becoming clear that higher doses of ibudilast are associated with greater efficacy in neurological conditions{Johnson et al., 2014, Clin Invest, 4, 1-11}, the safety data generated in our study provide evidence that will be valuable in facilitating approval for higher dose studies required in the future. Given this apparent dose-response relationship for ibudilast in other central nervous system disorders it may be that a higher dose of 50 mg twice daily is required to reach the threshold necessary to elicit a benefit in opioid-overuse headache.

All discussion should be interpreted accordance with the limitations of this trial. The small size of the treatment groups underpins the explanatory nature of this study, however it did preclude a number of desirable subgroup comparisons such as those by gender, primary headache diagnosis or type of opioid overused. A cross-over study design was considered to increase power, but was subsequently ruled out as the intervention was hypothesised to potentially be disease modifying. Heterogeneity in terms of individual patient headache burden and total opioid intake, limited clinical interpretation of mean group changes, thus within patient change and responder rate analyses were also employed. Proper adherence to study protocols, such as daily completion of the headache diary, can never be assumed. Electronic diaries that transmit data in real time would have been useful to identify participants who were back-filling diary pages, and data therefore that may be compromised by greater recall bias. Although in previous pain biomarker studies conducted in our laboratory measurement of IL-1β alone was sufficient to differentiate chronic pain patients from pain-free volunteers[65], quantitating the release of additional proinflammatory cytokines and mediators may have provided further information of interest.

However, despite the small sample size there was no signal to suggest that ibudilast has efficacy at this dose in MOH patients without mandated opioid withdrawal. If ibudilast is confirmed to ease opioid withdrawal in ongoing studies in opioid addicts[66], future trials of ibudilast incorporated into a MOH detoxification program that includes a forced down titration of opioids may show a differential effect.

CONCLUSIONS

Ibudilast dosed at 40 mg twice daily for 8 weeks does not improve headache or reduce opioid use in patients with MOH while frequent opioid intake continues. The ibudilast regimen established in this study proved feasible, safe and generally well-tolerated. Although this study does not establish the efficacy of ibudilast in opioid overuse headache, the encouraging finding of ibudilast-induced reduced reactivity of PBMCs to TLR2/4 agonist stimulation warrants further investigation as clinical biomarker for glial-attenuating treatments in larger trials.

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Conflict of Interest

PR and KJ hold a provisional patent on the use of ibudilast in medication overuse headache. KJ was an employee at Medicinova, a biopharmaceutical company investigating ibudilast, while this study was conducted.

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2.3 CRITICAL ANALYSIS OF CLINICAL TRIAL DESIGN AND EXECUTION

Double-blind, randomised, placebo controlled trials represent the strongest evidence for the effectiveness of a pharmaceutical therapy. There are many possible explanations as to why a drug, which appears promising during laboratory testing, may fail to demonstrate benefit during initial clinical trials. Drugs may fail due to inappropriate selection of the trial population, low statistical power or due to factors pertaining to the sensitivity of outcome measures assessed. A treatment also cannot be effective in a clinical trial if study participants are non-adherent and do not take the study medication. Pharmacokinetic issues need to be taken into consideration; for example a drug not may not reach a concentration at the target site sufficient to elicit the desired activity, due to either inadequate dosing or poor distribution to target tissue. Additionally, treatment duration must be sufficient to induce changes large enough to be detected as clinically meaningful at trial completion. Finally, even with the optimal clinical trial design, a drug may simply fail as it does not possess the desired *in vivo* pharmacodynamic activity in humans, or the selected target may not play the anticipated role in the condition or symptoms being treated. Furthermore, some aspects of clinical trial design may not contribute to the acquisition of useful data and therefore can be discouraged in future studies to improve the logistics and feasibility of clinical trial execution.

2.3.1 Defining and recruiting a suitable medication overuse headache study population

Obtaining a sample size sufficient to provide the power required to detect a meaningful difference is critical when conducting pivotal clinical trials. Recruitment of suitable opioid overuse headache patients for this study proved much more difficult that anticipated given

the estimated population prevalence of MOH of between 1-2% across similar western countries such as the United States and countries in Europe. Although no parallel epidemiological studies have been performed in Australia there is little reason to expect lower rates locally given the prevalence of primary headaches including migraine and TTH headache are similar, and comparable headache treatments are also readily available[186]. Recruitment was conducted via advertisement to the public, through print media, television segments, radio, notice boards situated in public hospitals, universities, and libraries, community pharmacy referral, general practice handouts and social media. When recruitment closed at the end of 2013, a total of 34 participants had been enrolled in our study, and 30 completed all phases of the trial. This was lower than our initial intended enrolment of 40 participants, to be randomised 1:1 to receive ibudilast or placebo. The primary calculation of sample size was based upon a study that utilised the tetracycline antibiotic minocycline, which also has glial attenuating properties, as an add-on therapy for patients which chronic migraine or new daily persistent headache[187]. This study included a total of 40 participants, which was adequate to show a statistically significant improvement headache frequency[187]. However, due to difficulties in recruiting suitable participants, the subsequent costs associated with prolonged study duration, and the exploratory nature of this work, our recruitment target was revised in late 2013 to 30 completers. As we were seeking any signal of efficacy, rather than pivotal evidence, reducing the target number was the most feasible option given the extensive recruitment effort already in place. An *a priori* power calculation was not performed, as estimates for of headache index and likely variability between opioid overuse headache patients were not available in the literature.

A *post hoc* power calculation based upon headache index scores over weeks 5 to 8 was performed using the ClinCalc Post-hoc Power Calculator

(http://clincalc.com/Stats/Power.aspx, accessed 30 May 2014). It was calculated that our study had a 12.6% power to detect the small difference observed between our two treatment groups with an α of 0.05. For future studies, using the mean headache index and standard deviation of our participant cohort at baseline and an α of 0.05 a total of 80 participants (1:1 ratio) would be required to detect a 50% decrease in headache index; this increases to 222 participants (1:1 ratio) if aiming to detect a difference of 30%, as deemed to be clinically meaningful for chronic headache patients. Considering the time required to recruit 34 participants following widespread multimodal advertising campaigns in Adelaide, a city of approximately 1.2 million people, studies aiming to enrol such numbers of opioid overuse headache patients would be better suited to centres with specialty headache clinics and an established MOH population or may require a multicentre design.

Of participants enrolled, 26 were either self-managing their headaches or under the care of their general practitioner, and the remaining 8 participants were seeing a specialist pain management consultant in addition to their general practitioner. This distribution reflects the general MOH population, as the majority of headache sufferers do not receive specialist care[188]. Thus, our results are likely to be generalisable to the MOH population at large. Our trial participants overused a range of opioids, varying from low doses of over-the-counter codeine to high doses of prescription drugs such as oxycodone. A breakdown of the opioids used by the ibudilast and placebo groups is presented in Appendix 6. A higher percentage of patients in the ibudilast group were overusing over-the-counter codeine-containing preparations compared with the placebo group. It is possible the different patterns of opioid overuse between treatment groups could influence results, thus future studies may benefit from stratifying participants according to type of opioid overused.

The ICHD-IIR criteria were used to define MOH, representing the current gold standard in headache research, however this diagnosis still allows for an extremely heterogeneous study population. Participants were eligible if they had a baseline headache burden ranging from moderate to severe headaches present 24 h every day to mild headaches lasting a few hours every second day, thus the average daily baseline headache index was as low as 5.5 for one participant and as high as 202 for another, out of a maximum of 240. In future studies adding inclusion criteria defining a specific range of acceptable baseline headache index scores would provide a more uniform study population, however this would limit generalisability of results to MOH patients presenting with a similar headache burden. Alternatively, a greater sample size and stratification of participants prior to randomisation, followed by sub-group statistical analyses, would allow any differences in treatment response based upon severity of the headache disorder to be detected.

Often discussed in the MOH literature is the need to appropriately address concurrent anxiety and depression to achieve optimal results in the treatment of MOH. The Hospital Anxiety and Depression (HAD) scale was employed at screening during recruitment for our clinical trial to quantitatively and uniformly assess anxiety and depression respectively. The HAD scale is a 14-item self-report scale (7 items for each of the subscales) that asks participants to rate each item on a 4-point Likert scale ranging from 0 to 3 (see Appendix 3). For both the anxiety and depression subscales the overall score is calculated to give a value between 0 and 21[189]. Clinical cut off scores are then imposed to categorise participants as normal (score 0-8), borderline (9-10) or abnormal (≥12) in each domain[189]. Given a large proportion of MOH sufferers experience coexisting anxiety and/or depression including such patients in clinical trials aiming to treat MOH provides greatest generalisability, however patients with such comorbidities are unsurprisingly regarded as more difficult to treat, and

therefore an 'uncomplicated' cohort may have responded differently. Repeating the HAD scale assessment at baseline, weeks 4 and 8 of treatment and at follow up in our ibudilast clinical trial would have provided a more complete picture regarding changes in mood that could have either influenced treatment success, or been altered by treatment success themselves; although determining cause and effect would not have been possible.

In our clinical trial a total participant withdrawal rate of approximately 12% was observed, when divided by treatment group the 13.3% of the ibudilast group withdrew compared with 10.5% of the placebo group. These rates are substantially lower than other headache trials that mandate withdrawal of analgesics, which have reported dropout rates of up to 40%[93, 190, 191]. Patients included in this study had a long history of severe, chronic headaches that had failed to respond to a number of previous treatments. Consequently, participants reported great interest in new headache treatments and subjectively appeared highly motivated to take part in the clinical trial, which may have contributed to the high completion rate.

Approximately 35% of all participants in our trial were taking concurrent prophylactic headache treatments. Generally it is recommended that in trials of new headache prophylactic treatments that existing treatments are withdrawn 3 months prior to participating in a clinical trial[192]. However in this study, rather than being prescribed as a long-term prophylactic medication, ibudilast was trialled as an intermediate add-on treatment to help ease opioid-induced headache long enough for participants to cease overusing their analgesics, which would then in term reduce headache further, at which point ibudilast could be discontinued. Bearing this in mind, along with the additional time that a wash-out period would add to the study duration and the possibility of reduced recruitment or increased withdrawals due to worsening headache during the interim

pre-study period, participants in our study were permitted to continue their prophylactic medications provided they dosing had been stable for 3 months prior and remained unchanged throughout the study.

2.3.2 Suitability of outcome measures selected

The outcomes assessed in the ibudilast clinical trial were separated into primary outcome measures, which were based upon headache index; secondary outcome measures, that could be further divided into headache indices and medication intake outcomes; and tertiary outcomes that included a variety of cutaneous sensitivity measures.

Headache index, also known as 'area under the headache curve' was considered the primary variable of interest as it captures headache frequency, headache duration and headache intensity to give a complete picture of the burden of headache in a single value. It is calculated by summating the product of daily headache duration (h) and headache intensity (11-point numerical rating scale) over a given period, therefore use of such an endpoint is limited as the mathematical transformations required can lead to values which may not be linear. Consequently we included a range of commonly used, well-established, secondary headache outcomes including headache frequency (days/month), average headache duration (h), average headache intensity (11-point numerical rating scale) and headache-related impact on quality of life assessed via the validated 6-item headache impact test (see Appendix 4).

In addition to the role of acute medication use in maintaining MOH, change in medication intake is particularly important as it can reflect changes in headache that may otherwise not be detected[193]. For example if headache index were to remain unchanged yet medication intake was increased, it could be that headache had actually worsened and index has not

changed to reflect this as increasing acute medication intake has reduced headache duration. Surprisingly, a review of long-term outcome studies in patients with MOH conducted after 2006 found only 2 of 9 studies explicitly reported medication use (days/month) at baseline and only 1 study did so at follow-up, although that information is critical in diagnosis and determining relapse rates[194]. In this study we collected details regarding all doses of medication consumed during the baseline, treatment and follow up periods.

Previously, MOH trials have classified a 'responder' as a patient who has <15 days/month of headache and is using acute headache treatments <10 days/month[195]. Although these criteria are consistent with the ICHD-IIR, using this definition, participants who just met the lower end of the inclusion criteria range that experience only a minor reduction in headache or medication intake frequency may be defined as responders, and the subsequent benefit associated with the treatment tested could be overestimated. Thus, in our clinical trial to provide results that were more informative, a responder was defined based upon their individual percentage improvement. Often in pain trials a within patient improvement of ≥50% from baseline is suggested to define a responder, however in chronic headache conditions an improvement of 30% has been found to be clinically meaningful to patients[193]. Regardless of the definition employed, specific responder rates should be defined *a priori*, as was the case in our trial. Consideration could have been given including to patient reported subjective efficacy as an additional endpoint, however as our headache burden efficacy outcomes were based upon the subjective participant-completed headache diary any perceived benefits with regard to headache pain should have been captured.

A pencil-and-paper-based headache diary to be completed daily was used to capture data relating to headache experienced and acute headache medications consumed (see Appendix

5). The diary was based upon that outlined in a review of diaries and calendars for migraines by Nappi and colleagues[196], and was designed to gain only useful information in a simple and logical manner. All participants received standardised information regarding how to complete the diary pages, with directions to fill in the required information at the end of each day. Use of an electronic diary with real-time data transmission would have allowed assessment of daily adherence to diary completion and could have indicated which if any participants were backfilling diary entries. This could then have been taken into account when assessing possible bias brought about by retrospective diary completion. Additionally, the knowledge that investigators would be aware of when diary entries were completed could have provided additional motivation to ensure timely diary data recording.

Given such a wide range of outcomes were assessed, elected in accordance with current guidelines for clinical trials assessing treatments for chronic headache[193, 197], it is improbable the lack of efficacy observed is due to inappropriate outcome selection.

2.3.3 Assessing medication adherence and its impact on ibudilast efficacy

Non-adherence or non-compliance with prescribed clinical trial interventions provides a major obstacle in translating research outcomes to efficacy in clinical practice, yet surprisingly this issue is often overlooked when clinical trial results are published[198]. Previous studies have found 25-50% of headache patients are non-compliant with prescribed prophylactic medication regimes in clinical practice[199, 200]. In recent times a general consensus has been reached regarding a change in preferred terminology; with most clinicians agreeing the term adherence is superior to compliance, as it recognises the autonomy of the patient and puts an emphasis on following the therapeutic agreement between the patient and health care practitioner, rather implying the patient is a passive

responder to the clinicians authoritative directions[201]. The World Health Organisation defines adherence as "the extent to which a persons behaviour corresponds with agreed recommendations from a health care provider"[202], however, this definition does not allow for objective quantitation of adherence. The absence of uniformly accepted criteria to determine the point at which behaviour should be deemed non-adherent is a likely to contributor to the lack of adherence data reported in clinical trials. Different studies have employed different cut-off levels for determining participant to be non-adherent, rarely based upon the clinical significance missing doses. It has been suggested that patients could be considered adherent if data gathered suggest 80% or more of the prescribed doses have been taken over a given time period[202], although some authors use much stricter criteria, classifying a participants as non-adherence when as little as a single dose is missed.

Once the criteria for adherence and non-adherence have been set, another significant issue arises, which method used to measure adherence should be used? A range of both subjective and objective methods have been used previously when investigating adherence to medications, as outlined in Table 4[198].

Table 4. Methods used for assessing medication adherence.[198]

Methods used for assessing medication adherence
Subjective methods • Examining case-note recordings • interviewing patients • Obtaining collateral reports from family or caregivers • Noting the attending physicians' clinical judgment regarding adherence
Objective methods: • Counting the number of tablets left in pill bottles • Accessing data regarding rates of dispensing of repeat prescriptions • Monitoring of serum drug levels • Monitoring the ratio of the plasma drug level and the administered dose (L/D ratio) • Analysis of urine for drugs or their metabolites • Electronic systems that monitoring opening of medication containers

Patient self-report appears to be the most accurate subjective method of assessing adherence[198], yet despite generally possessing high specificity (≈90%), this method is prone to low sensitivity (≈55%)[203]. Although objective methods seem preferable in many respects, a number of issues pertaining to their use must still be taken into account. For example, drug levels in plasma or urine are dependent on a range of pharmacokinetic factors not just drug intake and are only useful in assessing adherence over the a certain period, depending on the half-life of the drug[204]. Recording the opening of a medication bottle may incorrectly assume a dose was taken, while returned pill counts may overestimate compliance if participants discard unused medication[204].

In this study, as briefly discussed in our publication, adherence was assessed via participant daily self-report in the headache diary and via returned capsules count at the end of each treatment period. In line with previous studies, participants were considered adherent if >80% of dispensed trial doses were taken over the treatment period. Despite the relatively laborious study protocol, requiring 4 capsules to be taken twice daily, overall adherence for the cohort assessed via either method was high, with only an average of 6% of medication doses returned in the ibudilast group compared with 5% in the placebo group and an average of 8% doses self-reported as missed in the ibudilast group versus 5% in the placebo group. Despite a greater number of adverse events being reported in the ibudilast group (32 separate events versus 21 in the placebo group), adherence did not differ between the treatment groups, indicating adverse events were not severe enough to deter participants from taking the study drug as prescribed.

When both methods of adherence assessment were compared the adherence classification (adherent versus non-adherent) did not change for the vast majority of participants. Classification differed in only two participants, one was found more adherent via the

returned capsule count, whereas the other was found more adherent when considering self-reported intake. Although classification did not change for most patients, adherence measurements using the different methods were not identical. Within patient, greater adherence was found via self-report in 58.6% of participants, whereas greater adherence was found via returned capsule count in the remaining 42.4%. Such concurrence lends credibility to the combined use of subjective participant daily self-report and objective periodic returned capsule counts in the reliable assessment of adherence in the clinical trial setting.

Furthermore, addition of the peripheral mononuclear blood cell (PBMC) stimulation sub-study, performed by PhD candidate Heilie Kwok, allowed for investigation of a biological marker for compliance in the ibudilast group. While no change in PBMC reactivity would not necessarily imply non-adherence, it would be unlikely that a decrease in PBMC reactivity would occur in the absence of ibudilast ingestion.

In light of the adherence data, the biomarker changes observed and the general attitudes of the study participants towards obtaining relief from their headaches, it seems unlikely that the lack of efficacy observed in our clinical trial results from participants not taking the study medication.

2.3.4 Pharmacokinetic and drug dosing considerations

When ethical approval for this study was sought in 2011, only limited human safety data relating to our 80 mg daily dose were available. In Japan and some other Asian countries where ibudilast has been used to treat asthma for over 20 years, the current recommended dose is 20-30 mg daily[182]. Investigation regarding the use of ibudilast for newer neurological indications has begun with target doses ranging from 60-100 mg per day.

Since commencing our trial, evidence has been mounting to suggest neurological indications require daily doses ≥80 mg[182]. Although dosing at 100 mg per day was considered it was decided that the limited experience with ibudilast dosed at ≥80 mg per day warranted a conservative approach, and thus a daily dose of 80 mg was selected for this trial. If ibudilast is to be further investigated for use in MOH, provided the target is validated, dosing at 50 mg twice daily is likely to provide greater efficacy than 40 mg twice daily as dose response relationship has been demonstrated in both pain and dependence studies[182, 205], and safety data are now robust enough to support such a regimen[182]. For a detailed breakdown of adverse events experienced by participants in our ibudilast clinical trial see Appendix 7.

Data from preclinical rodent studies clearly demonstrate that ibudilast distributes rapidly and extensively into the central nervous system[206]. When a single low (5 mg/kg) dose was given brain and spinal cord concentrations were similar to those achieved in plasma, whereas following repeated high (50 mg/kg) dose oral administration concentrations elicited in the brain and spinal cord were ten times higher than those observed in plasma[206]. While central nervous system levels have not specifically been measured in human studies, evidence of efficacy in endpoints mediated thought central nervous system targets in clinical trials indicate ibudilast does distribute to these regions in humans in doses lower than that used in this clinical trial[205, 207]. Therefore, it is doubtful that poor access to the target site is responsible for our negative trial results.

Often drugs aimed at reducing headache frequency require administration for a number of months before to reach their maximal efficacy[208]. Similarly, when treating MOH through detoxification it has been reported that meaningful post-withdrawal improvement may take 12 weeks or longer. Despite this a treatment period of 8 weeks was selected for our clinical

trial during initial planning. This was determined to be the longest logistically feasible duration, as increasing treatment to 12 weeks would have significantly increased study costs and would have likely increased the drop-out rate, and 8 weeks treatment was considered to be sufficient given the exploratory nature of this study. Future studies investigating ibudilast in headache disorders may benefit from a longer treatment period, as even if no effect were detected it would provide a stronger negative finding.

Sub-optimal dosing and/or duration of treatment may have contributed to the lack of efficacy observed in this trial. However, if ibudilast was likely to provide a clinical meaningful benefit when used in this manner a trend towards reduced headache indices or another signal of efficacy would be still be expected with the current dose and duration.

2.3.5 Potential involvement of additional mechanisms and pathophysiological pathways

Headache patients, similar to patients suffering pain disorders, are well known to respond to placebo treatments[192]. Interestingly, in our study no placebo effect was observed in when considering the mean change in outcomes in either treatment group during our clinical trial. In migraine prophylaxis studies the response rate to placebo (for example patients experiencing a ≥50% reduction in headache or a 50% responder rate) generally ranges between 20% and 40%[192]. In our study assessing response in terms of group responder rates allowed a greater placebo response to be detected. When each participant was considered individually we saw a non-significant between group difference in headache index response rate, with 26% of the placebo group and 7% of the ibudilast group meeting the ≥30% headache reduction criteria. If instead we considered an improvement of ≥50% to constitute a positive response, as per the placebo response rates quoted previously, 0% of our ibudilast

group and 16% of our placebo group would be classified as responders. The greater response across both groups observed when conducting a responder analysis indicates this method may be more sensitive in detecting improvements in trials of headache treatments, but may also be associated with a higher degree of false positive findings.

As the pathophysiology underpinning MOH has not yet been confirmed, although our treatment was based upon the hypothesis of glial cell-derived neuroinflammation potentiated by opioids, the relative contribution of glial mediated pain facilitation may differ as the condition progresses. Perhaps glial cell activation plays a larger role in initiation of headache chronification, with other neuroplastic changes related to opioid exposure or chronic pain driving headache when chronicity has existed for some time. If this were the case a glial attenuating treatment would likely be more beneficial in the early stages of MOH, as opposed to cases where MOH has persisted for many years. Therefore, it would be of interest to investigate differential effects of ibudilast therapy in patients for whom headache chronification is continuing compared with those who have experienced stable chronic headache for a decade or more. Unfortunately our participant numbers in our dataset did not allow such a sub-analysis.

2.3.6 Alternative interventions and other trial design options

Perhaps an alternative glial attenuating agent may have been more successful in treating opioid overuse headache. Minocycline and naltrexone are two other drugs with well-established safety profiles and approval for human use that have been found to attenuate glial activation. Minocycline was decided against as an intervention option in our opioid overuse headache trial as it appears to be more effective in preventing glial activation,

rather than reversing established activation[209, 210], and has not performed particularly well in small, initial clinical trials for other pain states[211, 212].

Naltrexone is both appealing and unappealing for use as a glial attenuating intervention in opioid overuse headache for the same reason; the μ-opioid receptor antagonistic effect of naltrexone blocks acute opioid analgesia. It was decided that a study utilising a glial attenuator without this complication would provide greater information initially, as if naltrexone was used patients would likely cease their opioid analgesics, as they would provide no benefit. Results would then be confounded, as essentially participants would undergo withdrawal treatment, which is the current gold standard for the treatment of medication overuse headache, and benefits due to detoxification and glial attenuation could not be separated. Furthermore, ibudilast was also preferred, as benefits following its use in opioid withdrawal had been demonstrated in humans, not only in animal studies.

As our results suggest ibudilast treatment may not by sufficient to improve headache while opioid overuse continues, it would be of interest to look at glial attenuation as a strategy in combination with, rather than in place of, detoxification. This approach may have a greater chance of success as it has been suggested for some time that medication overuse headache patients remain refractory to drugs that reduce headache frequency while analgesic overuse persists and patients who have previously been resistant to headache prophylactic agents can become responsive to treatment following detoxification[93, 213]. For example, in an early study performed by Kudrow[27] in California, patients suffering the then termed 'chronic scalp muscle contraction headache' were randomised to one of four treatment arms, amitriptyline plus analgesic discontinuation, analgesic discontinuation alone, amitriptyline alone or no change to treatment. This study found the greatest improvement in patients who discontinued analgesics and received amitriptyline followed by those who underwent

detoxification without any additional treatment[27]. Bearing this in mind naltrexone could provide extra benefit in addition to reducing glial activation, in that it could also help to ensure abstinence from opioids during detoxification. Future studies could compare a standard medication withdrawal procedure to medication withdrawal plus naltrexone and medication withdrawal plus placebo to determine if adding naltrexone provides additional benefit.

When designing this study consideration was given to implementing a cross-over study design to provide greater power. This was decided against primarily because it was hypothesised that the ibudilast intervention could be disease modifying, breaking the cycle of headache-medication overuse-further headache providing a carry over effect that may have altered headache in the subsequent treatment period even after cessation of that study drug. Additional drawbacks associated with the cross-over design such as potential unblinding due to differential adverse event profiles and a substantially prolonged trial duration also contributed to the decision to conduct a trial consisting of parallel groups.

After experiencing prolonged recruitment leading to a reduction in sample size and therefore unequal treatment group numbers, it is evident that randomisation using small blocks (for example four patients[197]) could provide a beneficial safeguard for future trials.

In longitudinal clinical trials where participants are assessed repeatedly over time, participant withdrawals and other cases of missing data can be very difficult, if not impossible, to avoid[214]. Methods that can be used to ensure a complete data set for analysis include, but are not limited to, complete case analysis, baseline observation carried forward, last observation carried forward, direct-likelihood and direct-Bayesian analyses. In our clinical trial missing data resulting from participant withdrawals or incomplete headache

diary entries were dealt with by means of last observation carried forward. The last observation carried forward method was selected as it avoids some of the drawbacks associated with complete case analysis and the baseline observation carried forward method. Last observation carried forward circumvents the loss of substantial amounts of information, which can impact dramatically on precision and power, that occurs using the complete case approach[214]. Carrying the last observation forward rather than the baseline observation can remove any advantage that may be obtained by replacing values for a patient who deteriorated during receiving treatment with baseline data for that individual[214]. However, using the last observation carried forward does artificially increase the amount if information in the data, by treating observed data and imputed data equally[214] and can lead to an analysis which is either liberal or conservative. In this study last observation carried forward was deemed likely to provide a conservative estimate as if headache-worsening lead to withdrawal from the study the last observation would capture the increase in headache compared with baseline. In future studies, statistical modeling, for example using mixed model repeated measure techniques, to predict missing values based upon previous data could be employed to ensure the episodic nature of headache and the subsequent day-to-day variability that can occur does not result in misleading data resulting from use of the last observation carried forward.

2.4 CONCLUSIONS AND FUTURE DIRECTIONS FROM CLINICAL TRIAL

While our pilot study results were negative, if ongoing ibudilast studies in opioid-dependent patients show a clear reduction in withdrawal symptoms it would be of interest to trial the addition of ibudilast to an established MOH detoxification protocol to determine if ease and success of mandated withdrawal are improved in those overusing opioids. Such a study would be best suited to a region, or regions if multi-centred, where a specialised headache clinic exists to allow recruitment of an adequately sized cohort and supervision of patients by a physician experienced with opioid overuse headache detoxification procedures.

Finding no evidence of ibudilast efficacy in opioid overuse headache raises the question – was glial cell activation an appropriate target? To investigate if glial activation is likely to be an appropriate target in patients who consuming large amounts of codeine, as the majority of our clinical trial participants did, first it must to demonstrated conclusively that codeine is able to facilitate pain enhancement, as other opioids do. Similarly, the ability of ibudilast or glial-based therapies to reverse increases in nociceptive transmission brought about following codeine exposure must be ascertained. To perform such investigations access to neurological tissue with correlated with behavioural response data is required. Thus, a series of preclinical rodent models of codeine-induced hyperalgesia and allodynia were designed for the second part of this PhD project, to answer the questions left pending following the clinical trial of ibudilast in the treatment of opioid overuse headache.

3. PRE-CLINICAL INVESTIGATIONS OF CODEINE-INDUCED HYPERALGESIA AND ALLODYNIA, FOCUSING ON THE ROLE OF GLIAL ACTIVATION

3.1. INTRODUCTION AND RATIONAL FOR ANIMAL EXPERIMENTS

Preclinical studies in animals allow not only investigations of behaviour that cannot be conducted in a clinical population due difficulties in controlling confounding factors and ethical concerns, but also biochemical analysis of neurological tissue obtained after controlled culling at a selected time point. Consequently, to assist in the interpretation of our clinical trial findings a series of animal experiments were conducted focusing upon codeine-induced changes in pain sensitivity and the potential involvement of activated glial cells in the pathophysiology underlying such changes.

3.1.1. Assessing pain in mice

While much remains unknown in the field of pain research, necessitating ongoing investigations, the study of pain in awake animals raises both technical and also ethical issues[215]. Despite such issues a range of preclinical animal models and tests have been developed utilising thermal, mechanical, electrical and chemical stimuli, paired with assessments of hyperalgesia and allodynia, to facilitate both the primary and translational study of pain[216]. Pain however, is a complex, subjective conscious experience, defined by the International Association for the Study of Pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage"[217]. Thus, it is critical to understand the distinction between pain, as described

above, and nociception, a phenomenon that instead describes only the neuronal encoding and processing of noxious (actually or potentially damaging) stimuli[217].

While pain perception requires cortical involvement and aversive interpretation of the nociceptive signals, nociception encompasses only the mechanisms though which noxious stimuli are detected by peripheral neurons, then transferred and unconsciously treated by the central nervous system[216]. In the clinical setting, evaluation of pain relies almost entirely upon verbal expression by patients, a method of quantification that clearly cannot be used in animal studies[216], necessitating the application of alternative test parameters. The measurement of nociception can be based upon a) the latency to avoidance behaviour, most often the tail or paw withdrawal reflex, b) the stimulus threshold required to elicit avoidance behaviour, or c) observation and subsequent scoring of specific pain-related behaviours[216]. Table 5 provides a summary of the common behavioural tests used to study pain in rodents, the order of mammals most often employed in preclinical pain testing[215].

Table 5. Summary of the characteristics of commonly employed nociceptive tests in mice. Adapted from paper by Barrot[216]. #=Number.

Nociceptive test	Modality	Stimulus	Assessment parameter(s)
Tail flick[218]	Thermal, heat	Fixed T° (beam/water bath)	Withdrawal latency (s)
Hargraeves[219]	Thermal, heat	Fixed T° (beam)	Withdrawal latency (s)
Hot plate[220, 221]	Thermal, heat	Fixed T°	Withdrawal latency (s)
Dynamic hot plate[222]	Thermal, heat	Ramp T°	Scoring (#) & response threshold T°
Cold plate[223]	Thermal, cold	Fixed T°	Scoring (#)
Dynamic cold plate[224]	Thermal, cold	Ramp T°	Scoring (#) & response threshold T°
Von Frey[225]	Mechanical	Multiple fixed pressure	Withdrawal threshold (g)
Randall-Selitto[226]	Mechanical	Ramp pressure	Withdrawal/vocalisation threshold (g)
Strain gauges[227]	Mechanical	Ramp pressure	Withdrawal threshold (g)
Formalin test[228]	Chemical	Paw injection	Scoring (#)

Acute application of high intensity heat stimuli to the skin represents one of the most common methods used to evaluate nociception[229]. Such procedures, including the hotplate test, activate the high threshold sensory fibres that innervate the skin, resulting in a neuronal discharge frequency that is proportional to the intensity of the stimulus[230].

During the hotplate test, the rodent is placed onto a metal surface set at a fixed temperature, and the latency to the appearance of avoidance behaviour is timed with a stopwatch[220]. When the hot plate test was first devised Woolfe and MacDonald[220] evaluated the response to temperatures up to 70 °C. In the mid 70's it was found that a lower hotplate temperature, and therefore lower stimulus intensity, ensures a more sensitive test as demonstrated by Ankier[231] and O'Callaghan and Holtzman[221], who established a 50 °C hotplate surface provides a test that is significantly more sensitive than one in which the surface is set to 55 or 59 °C. Thus, with experience the optimum temperature setting has been revised and today the hotplate is usually set at a temperature between 48 °C and 55 °C[216], depending on the scope of the experiment.

In mice, the response to the hotplate and endpoint for the hotplate assessment can be classified into one of four avoidance behaviour sub-types: 1) withdrawal of the paw away from the hot surface, 2) kicking or shaking of usually the hind limb, 3) licking of the hind or front paw(s), or 4) jumping, ranging from a whole body twitch to a leap[232]. However, many studies will leave the animal on the hotplate until a specific, predetermined response is observed, e.g. paw licking, which needs to be taken into account when comparing data between studies, as this may lead to prolonged response times[232]. While the hotplate test lacks some advantages held by other nociceptive tests, such as the automated and therefore objective response detection and unilateral testing ability of the Hargreaves test[219], it is a

simple test that is able to produce data that are not reproducible only within a laboratory but also across laboratories regardless of variation in climate, location and altitude[233].

The von Frey test is another important tool used in the evaluation of rodent nociceptive sensitivity. Originally derived from a procedure used in the clinical assessment of allodynia[216], this test involves the application of mechanical pressure to the cutaneous receptive field followed by observation of the paw withdrawal response[234].

In the von Frey test, the animal is placed upon wire mesh flooring and pressure is applied to the plantar surface of the hind paw using a range of blunt ended, 5 cm long von Frey filaments of differing diameters. Each filament is pressed against the skin until just bent, at which point it exerts a specific calibrated force. While the flexor reflex can also be elicited by non-nociceptive stimuli, in states of increased pain responses are elicited by pressures that do not lead to responses at baseline or in control animals, indicating the presence of allodynia[216]. Testing can be conducted to determine the lowest filament pressure able to elicit a response or a set number of filament applications can be performed and the parameter recorded is the number of positive withdrawal responses[216]. The von Frey test has advantages over other methods utilising mechanical pressure, such as those which employ Randall-Selitto type devices, as the animal does not require restraint, reducing stress[225].

Initially preclinical pain tests involved only the testing of response to nociceptive stimuli in naïve animals, yet over time models encompassing either a surgical procedure or delivery of exogenous substances to create an altered basal pain state have been characterised, allowing more clinically relevant pain testing to be performed[216]. A vast array of pain state

models have now been developed to facilitate the study of sustained or chronic pain and potential new treatments, as reviewed in detail by Barrot[216] and Gregory et al.[235].

The most studied and therefore most well described general model of neuropathic pain is the chronic constriction injury (CCI) model, in which 4 chromic gut sutures are tied around the primary branch of the sciatic nerve, such that the diameter of the nerve is barely constricted[223]. The CCI procedure results in lasting mechanical allodynia as well as increased sensitivity to thermal heat stimuli, however it is limited in that it produces a severe pain state, rather than replicating the spectrum of pain intensity observed in the clinic. With this in mind a modified version of the CCI model has been devised and tested[236]. In this model the CCI surgical procedure has been altered such that the number of chromic gut sutures tied around the sciatic nerve ranges from 0 to 4. As simply implanting chromic gut produces an inflammatory response that contributes to the nociceptive hypersensitivity associated with the CCI model[237], animals with less than 4 sutures receive additional lengths of chromic gut placed subcutaneously to ensure an equal systemic inflammatory challenge[236]. The degree of resultant allodynia then correlates with the number of nerve ligations, thus when only a single suture is tied around the nerve and 3 pieces of chromic gut are inserted subcutaneously, as employed in our experiment, a mildly allodynic state, which plateaus approximately 14 days post-surgery, is created[236].

As with specific surgical procedures, a number opioid administration paradigms of have been noted to result in increased nociception, and subsequently new models have been described to facilitate the study of this pronociceptive state, known as opioid-induced hyperalgesia.

3.1.2. Pre-clinical evidence and models of opioid-induced hyperalgesia

Since first being investigated in the 1970's, opioid-induced hyperalgesia following the systemic delivery of opioids has been demonstrated repeatedly in a range of *in vivo* preclinical studies, conducted primarily in rats and mice[238]. While such studies initially focused upon hyperalgesia during withdrawal as a measure of opioid dependence, in more recent times investigators have studied the implications of opioid-induced changes in nociceptive sensitivity in relation to clinical pain management[238]. Parameters of laboratory protocols investigating opioid-induced hyperalgesia vary substantially in relation to the opioid analgesic employed, opioid dose, dosing route and time course of administration as well as the timing and type of nociceptive assay utilised[238].

Heightened pain sensitivity induced by exposure to opioids has been demonstrated in many different nociceptive assays measuring response to a variety of stimulation types. To assess sensitivity to hyperalgesia, nociceptive stimuli including thermal tests such as the hot plate test, tail flick test and Hargreaves test, and mechanical tests such as the paw pressure test are used. Allodynia has been investigated using dynamic mechanical stimuli tests, and more commonly, static mechanical stimuli via differing application paradigms utilising von Frey filaments.

In animal studies, morphine, as the prototypical opioid analgesic, is the drug most commonly studied in association with opioid-induced hyperalgesia. Other opioids examined differ from morphine terms of analgesic potency, opioid receptor subtype affinity and intrinsic activity. In addition to morphine, hyperalgesia and/or allodynia have reportedly been induced by the following opioids in rodents: heroin[239, 240], DAMGO ([D-Ala2,N-Me-Phe4,Gly-ol5] encephalin)[241], fentanyl[242-244], alfentanil[245], remifentanil[246], sufentanil [247, 248] and

buprenorphine[249]. Opioid-induced hyperalgesia and allodynia have been established both during continuous drug administration and following acute withdrawal, be it spontaneous or precipitated via an opioid antagonist, indicating that the ability to lower pain thresholds is a property of both opioid administration and withdrawal. As the mechanisms leading to either form of hyperalgesia have not yet been conclusively established it is unclear if the two stem from the same mechanism, share overlapping mechanisms or evolve as a result of separate pathophysiological changes. The parameters of a range of models that have demonstrated opioid-induced hyperalgesia and/or allodynia are summarised in Table 6.

Table 6. Summary of a range of models demonstrating opioid-induced hyperalgesia and allodynia. ICV=Intracerebroventricular, ID=Intradermal, IP=Intraperitoneal, IT=Intrathecal, IV=Intravenous, SC=Subcutaneous, SD=Sprague-Dawley. Continued on page 115.

Drug	Route	Dose	Frequency	Duration	Assessment(s)	Time point	Species
Alfentanil[245]	IV infusion	50 μg/kg bolus, 155 μg/kg/h	Continuous	4 h	Tail compression test	1.5 & 23 h post infusion	Male SD rats
Buprenorphine[249]	IP injection	0.1 μg/kg/ 0.1 μg/kg/	Once/ daily	Single bolus/ 10 days	Tail-flick test	1-4 h post dose	Male SD rats
Buprenorphine[249]	IP injection	100 μg/kg	Twice daily	10 days	Tail-flick test	Days 8-10 post	Male SD rats
DAMGO[241]	IT via osmotic mini-pump	1 nmol/ μl/h	Continuous	7 days	Thermal hyperalgesia Von Frey test	Day 6 of infusion	Male SD rats
DAMGO[250]	ID injection	1 μg	Hourly	3 h	Paw pressure test	Post naloxone at 4 h	Male SD rats
DAMGO[251]	ICV injection	20, 40 & 60 ng/4 μL	Once	Single bolus	Tail-flick test	10 min post dose	Male ICR mice
Fentanyl[244]	IT injection	80 μg/kg x 4 doses	Every 15 min	1 h	Hargreaves test Von Frey test	Days 1, 2 & 4 post dose	Male SD rats
Fentanyl[243]	SC injection	0.3 mg/kg	Twice daily	6 days	Hargreaves test von Frey test	24 h post dose	Male C57BL/6J Mice
Fentanyl[242]	SC injection	40, 60, 80, 100 μg/kg	Every 15 min	1 h	Paw pressure test	5 h to 5 days post final dose	Male SD rats
Heroin[239]	SC injection	1 mg/kg	Once	Single bolus	Tail flick test	Post naloxone	Male SD rats
Heroin[240]	SC injection	0.3 mg/kg	Daily	12 days	Paw pressure test	Days 3 -13	Male SD rats
Methadone[252]	SC via osmotic mini-pump	1 mg/kg /day	Continuous	14 days	Hargreaves test	Days 8, 9, 10, 11, 12, 14, 17	Male SD rats

Drug	Route	Dose	Frequency	Duration	Assessment(s)	Time point	Species
Morphine[253]	SC via osmotic mini-pump	40 mg/kg /day	Continuous	6 days	Hargreaves test Von Frey test	Days 1-3 post pump withdrawal	Male SD rats
Morphine[243]	SC pellet	75 mg /pellet	Continuous	6 days	Hargreaves test von Frey test	24 h	Male C57BL/6J Mice
Morphine[254]	IT catheter	150 μg /3 μL	Once	Single bolus	Spontaneous agitation Touch evoked agitation	30 min post dose	Rats (gender & strain not specified)
Morphine[255]	IV injection	1.25 & 2.5 mg/kg	Once	Single bolus	Tail-flick test Paw pressure test	Post naloxone	Male SD rats
Morphine[256]	Osmotic mini pump	40 mg/kg /24 h	Continuous	10 days	Tail withdrawal test	Days 5-10	Male & female C57BL/6J mice
Morphine[257]	IP injection	10 & 20 mg/kg	Once	Single bolus	Tail-flick test	4 h post dose	Male DBA/2 mice
Morphine[258]	IP injection	10 & 32 mg/kg	Twice daily	3 days	Hargreaves test	Day 3, post naloxone 1 h & 45 min post morphine dose	Male SD rats
Morphine[259]	IP injection	16 mg/kg	Daily	5 days	Hargreaves test Von Frey test	16 & 24 post final dose	Male C57BL/6J mice
Morphine[260]	SC injection	10 mg/kg	Twice daily	6 days	Tail-flick test Paw pressure test Thermal withdrawal test	Day 7	Male mice (strain not specified)
Morphine[261]	SC injection	10 mg/kg (day 1), 20 mg/kg (day 2 & 3), 40 mg/kg (day 4)	Twice daily	4 days	Hargreaves test Von Frey test	Day 5	Male C57BL/6j mice
Remifentanil[246]	SC infusion via pump	40, 80, 100 μg/kg	Continuous	30 or 60 min	Hargreaves test Von Frey test	Days 4-10 (Hargreaves) Days 7-10 (von Frey)	Swiss CD1 mice (gender not specified)
Sufentanil[248]	IP infusion	10 μg/kg x 4 doses	Every 15 min	For 1 h	Hargreaves test Von Frey test Post carrageenan injection	6 h – 14 days post carrageenan injection	Male C57BL/6J mice
Sufentanil[247]	SC	10 μg/kg x 4 doses	Every 15 min	For 1 h	Hot plate fracture of test Post closed tibia surgery		Male C57 BL/6 mice

Currently codeine, despite its long history of use in clinical practice, has not been investigated in a preclinical model of opioid-induced hyperalgesia.

3.1.3. Codeine pharmacology

Codeine is an opiate used extensively for its analgesic, antitussive and, to a lesser extent, its anti-diarrhoeal properties[118, 262]. Codeine has an exceptionally low affinity for μ-opioid receptors, which are responsible for analgesic response, when compared with other commonly used opioids[263] and is thought to rely almost entirely upon metabolic conversion to morphine to exert its analgesic effect[264]. Once absorbed, codeine is metabolised in humans by the cytochrome P450 (CYP) 2D6 isoform to morphine via O-demethylation, as illustrated in Figure 1[265]. However, the CYP2D6 enzyme is polymorphically distributed, with more than 50 different genetic variants known to exist, leading to a broad spectrum of metabolic capabilities within populations. Individuals are usually classed as either poor metabolisers, intermediate metabolisers, extensive metabolisers or ultra-rapid metabolisers depending on the number and functionality genes present and therefore activity the 2D6 enzymes expressed[266]. The majority of the population are classified as extensive metabolisers, converting approximately 10% of the orally delivered codeine dose to morphine[262]. Poor metabolisers make up 5% to 10% of the Caucasian population[267] and are able to form only negligible amounts of morphine following codeine administration[268]. Codeine-induced analgesic effects are far more pronounced in ultra-rapid metabolisers compared with extensive metabolisers as on average they are able to derive approximately 50% higher blood concentrations of morphine and its derivatives following an oral codeine dose[269]. The metabolism of codeine in mice is less well characterised, although it is thought to be similar to that in humans, as discussed in our publication presented on page 124 of this thesis.

Codeine is widely available in Australia as it commonly prescribed by physicians and, unlike other opioid analgesics, can also be purchased over-the-counter in combination

preparations. As codeine-containing products are heavily marketed as ‘stronger’ painkillers such preparations are popular choices when patients are self-medicating in the community. To date no data have been published regarding the ability of codeine to cause opioid-induced hyperalgesia, thus as a much greater proportion of the local population are using this drug, as compared to the tightly regulated, high-potency opioids such are morphine, it is important to determine if chronic codeine administration is also able to induce changes in pain sensitivity.

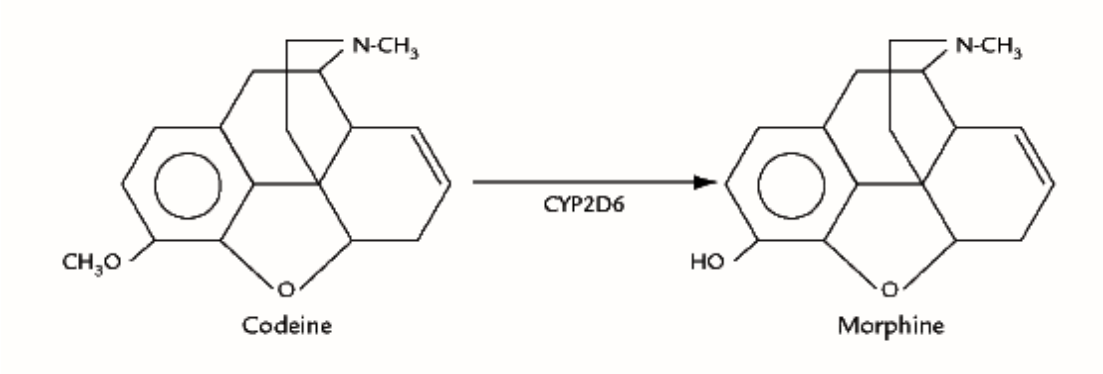
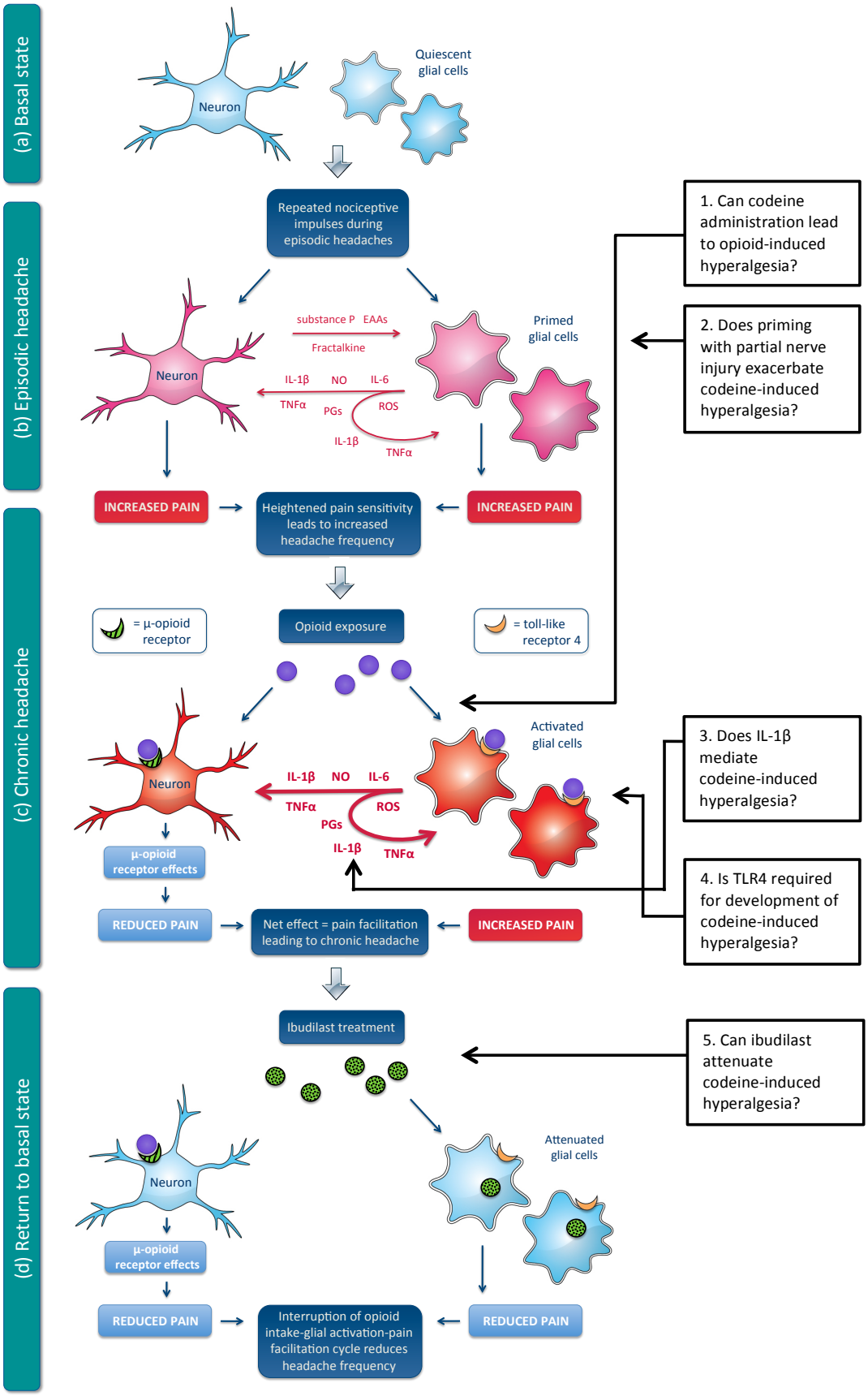


Figure 1. The metabolic activation of codeine to morphine by cytochrome (CYP) 2D6.

Therefore, a series of preclinical studies designed with each aimed to address a specific point in the hypothesised mechanism of pain enhancement by opioids in MOH and reversal by ibudilast, as outlined in Figure 2.

Figure 2. Diagram of questions to be addressed by preclinical studies in relation to the hypothesised mechanism of opioid overuse headache.



3.2. CODEINE-INDUCED HYPERALGESIA AND ALLODYNIA: INVESTIGATING THE ROLE OF GLIAL ACTIVATION

3.2.1. Statement of authorship

Statement of Authorship

Title of Paper	Codeine induced hyperalgesia and allodynia: Investigating the role of glial activation.
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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Jacinta L Johnson
Contribution to the Paper	Conceptulised study aims and design, obtained ethical approval, conducted experimental procedures including all animal handling, behavioral testing, surgical procedures, tissue collection and western blotting, analysed data, interpreted results, wrote the manscript and acted as the corresponding author.
Signature	Date: 14 JUL 2014

Name of Co-Author	Paul E Rolan
Contribution to the Paper	Contributed to conceptulisation of the study and reviewed and edited the manuscript.
Signature	Date: 4.8.14

Name of Co-Author	Michaela E Johnson
Contribution to the Paper	Assisted with tissue collection, blinding for animal drug administration and western blotting and reviewed and edited the manuscript.
Signature	Date: 11.8.2014

Name of Co-Author	Larisa Bobrovskaya
Contribution to the Paper	Developed westen blotting protocol, provided facilities, equipment and consumables for western blotting and reviewed and edited the manuscript.
Signature	Date: 11.8.14

Statement of Authorship

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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Co-Author	Desmond B Williams
Contribution to the Paper	Contributed to conceptulisation of the study, reviewed and edited the manuscript.
Signature	Date: 14/08/14

Name of Co-Author	Kirk Johnson
Contribution to the Paper	Provided input regarding design of ibudilast experiment, provided ibudilast, reviewed and edited the manuscript.
Signature	Date: 8/13/2014

Name of Co-Author	Jonathon Tuke
Contribution to the Paper	Assisted with statistical analysis and reporting of statistical methods, reviewed and edited the manuscript.
Signature	**Date:** 18/7/14.

Name of Co-Author	Mark R Hutchinson
Contribution to the Paper	Contributed to conceptulisation of the study, trained the principal author to perform behavioural assessments and tissue collection, assisted with statistical analysis and interpretation of results, reviewed and edited the manuscript.
Signature	Date: 14/7/14

3.2.2. PUBLICATION: Codeine-induced hyperalgesia and allodynia: Investigating the role of glial activation.

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ORIGINAL ARTICLE

Codeine-induced hyperalgesia and allodynia: investigating the role of glial activation

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Chronic morphine therapy has been associated with paradoxically increased pain. Codeine is a widely used opioid, which is metabolized to morphine to elicit analgesia. Prolonged morphine exposure exacerbates pain by activating the innate immune toll-like receptor-4 (TLR4) in the central nervous system. *In silico* docking simulations indicate codeine also docks to MD2, an accessory protein for TLR4, suggesting potential to induce TLR4-dependent pain facilitation. We hypothesized codeine would cause TLR4-dependent hyperalgesia/allodynia that is disparate from its opioid receptor-dependent analgesic rank potency. Hyperalgesia and allodynia were assessed using hotplate and von Frey tests at days 0, 3 and 5 in mice receiving intraperitoneal equimolar codeine (21 mg kg^{-1}), morphine (20 mg kg^{-1}) or saline, twice daily. This experiment was repeated in animals with prior partial nerve injury and in TLR4 null mutant mice. Interventions with interleukin-1 receptor antagonist (IL-1RA) and glial-attenuating drug ibudilast were assessed. Analyses of glial activation markers (glial fibrillary acid protein and CD11b) in neuronal tissue were conducted at the completion of behavioural testing. Despite providing less acute analgesia ($P = 0.006$), codeine induced similar hotplate hyperalgesia to equimolar morphine vs saline (−9.5 s, $P < 0.01$ and − 7.3 s, $P < 0.01$, respectively), suggesting codeine does not rely upon conversion to morphine to increase pain sensitivity. This highlights the potential non-opioid receptor-dependent nature of codeine-enhanced pain sensitivity—although the involvement of other codeine metabolites cannot be ruled out. IL-1RA reversed codeine-induced hyperalgesia ($P < 0.001$) and allodynia ($P < 0.001$), and TLR4 knock-out protected against codeine-induced changes in pain sensitivity. Glial attenuation with ibudilast reversed codeine-induced allodynia ($P < 0.001$), and thus could be investigated further as potential treatment for codeine-induced pain enhancement.

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INTRODUCTION

Opioid analgesics, used medicinally for millennia, remain vital in pain management. However, convincing preclinical and mounting clinical evidence suggests that long-term opioid use may paradoxically increase pain resulting in opioid-induced hyperalgesia.[1] Opioid-induced hyperalgesia has been reported following the administration of a range of opioids, yet it has not been established if the 'weak' opioid codeine, can induce this phenomenon. Codeine-induced hyperalgesia is of particular interest, as in many regions codeine is available over-the-counter leading to widespread, unregulated consumption.[2,3] Codeine is the most frequently used opioid in several European countries,[4,5] and although most guidelines recommend only short-term codeine treatment, pharmacoepidemiological evidence indicates that >10% of patients prescribed codeine consume more than the 120 defined daily doses per year, indicating chronic use.[6] Codeine has low affinity for μ-opioid receptors, compared with other opioid analgesics,[7] and is considered a prodrug, dependent on transformation to morphine to relieve pain.[8]

Once absorbed, codeine undergoes partial *O*-demethylation to morphine via polymorphic cytochrome P450 isoenzyme 2D6 (CYP2D6).[9,10] Most individuals convert ~10% of an oral codeine dose to morphine.[10] In mice, information regarding the metabolism of codeine is incomplete, yet *O*-demethylation of metoprolol, a typical human CYP2D6 substrate, is similar to that seen in human liver microsomes,[11] providing evidence that BALB/c mice are an acceptable model to assess clinically relevant codeine pharmacodynamics.

Numerous opioid-receptor-dependent neuronal mechanisms of opioid-induced hyperalgesia have been proposed (see Ossipov *et al.*[12] for review), however, substantial preclinical evidence establishes that morphine activates not only classical opioid receptors, but also toll-like receptor-4 (TLR4) on glia, triggering proinflammatory mediator release, initiating a cascade of events that enhance nociception.[13] While neuronal morphine actions are analgesic, concurrent production of neuroexcitatory substances by glial cells (for example, astrocytes, microglia) counteracts this analgesia, to eventually increase pain. Thus, with increasing morphine dose and/or duration, TLR4-dependent glial reactivity increases reducing the analgesic efficacy and ultimately leading to allodynia and hyperalgesia.[14]

This TLR4-glial hypothesis has been established for morphine[15] and oxycodone[16] but remains to be tested for codeine. It is plausible that codeine may induce hyperalgesia indirectly by acting as a prodrug for morphine delivery. Insufficient evidence exists to allow the use of a codeine *O*-demethylation inhibitor to test whether the conversion to morphine is solely responsible for

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any hyperalgesia observed in mice. Instead, here we compared hyperalgesia precipitated by equimolar doses of codeine and morphine. Hypothesizing opioid-induced hyperalgesia to be dose-dependent,[17,18] if codeine were only able to facilitate pain once metabolized to morphine, then significantly less hyperalgesia would be expected in animals receiving codeine vs morphine, as only a minor proportion of the codeine dose is converted to morphine.

In silico docking simulations suggest that codeine docks to TLR4 accessory protein MD2,[19] in a manner similar to morphine,[15,20] indicating codeine has the potential to trigger TLR4-dependent pain enhancement. Owing to codeine's lower μ-opioid receptor affinity, higher doses are required relative to morphine to produce equianalgesia. If codeine activates TLR4, greater glial activation could occur following equianalgesic codeine vs morphine, as a greater number of molecules must be administered to obtain the same therapeutic response. Thus, we hypothesize that the risk (hyperalgesia) to benefit (analgesia) ratio is greater for codeine compared with morphine.

Objectives

The objectives of the experiments presented in this manuscript were as follows: to determine whether chronic codeine administration induces hyperalgesia to the same degree as chronic morphine administration, to ascertain if partial nerve injury primes for codeine-induced hyperalgesia, to investigate the roles of proinflammatory cytokine interleukin-1 and TLR4 in the development of codeine-induced pain enhancement and finally to test the efficacy of a glial-attenuating agent in the reversal of codeine-induced hyperalgesia.

MATERIALS AND METHODS

Animals

Pathogen-free adult male wild-type BALB/c mice were obtained from the University of Adelaide Laboratory Animal Services (Adelaide, SA, Australia). Mice were housed in temperature (18–21 °C) and light-controlled (12 h light/dark cycle; lights on at 0700 h) rooms with standard rodent food and water available *ad libitum*. After arrival, the mice were allowed to acclimate to the facility for at least 5 days and were subsequently handled for a further 5 days before testing. All procedures were approved by the Animal Ethics Committee of the University of Adelaide and were conducted in accordance with the NHMRC Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and the guidelines of the Committee for Research and Ethical Issues of International Association for the Study of Pain.

Drugs

All treatments were administered via intraperitoneal injection at a volume of 10 ml kg^{-1}. Morphine hydrochloride (McFarlan Smith, Sydney, NSW, Australia) in 0.9% saline was administered at 20 mg kg^{-1} (base-corrected). Codeine phosphate (GlaxoSmithKline, Melbourne, VIC, Australia) in 0.9% saline, was administered at 21 mg kg^{-1} (base-corrected equimolar dose to morphine). Anakinra (Kineret, Amgen, Thousand Oaks, CA, USA), a recombinant, nonglycosylated form of the human interleukin-1 receptor antagonist (IL-1RA) in 0.9% saline was administered at 100 mg kg^{-1}. Ibudilast (Medicinova, San Diego, CA, USA) in 35% polyethelene glycol 400 (BDH Laboratory Supplies, Poole, England) in 0.9% saline was administered at 15 mg kg^{-1}. As ibudilast was administered in a single intraperitoneal injection with concomitant codeine or morphine, the total dose volume for both drugs together was adjusted to 10 ml kg^{-1} for consistency between experiments. An equal volume of saline 0.9% was administered to control animals in Experiments 1, 2, 3 and 4, whereas control animals in Experiment 5 received 35% polyethylene glycol in 0.9% saline.

Drug administration

The morphine dose of 20 mg kg^{-1} used throughout the experiments outlined below was based upon that previously used in our laboratory to induce thermal hyperalgesia. The codeine dose was calculated to be equimolar to morphine, not equianalgesic. As briefly discussed in the introduction, only ~10% of the codeine dose is thought to be metabolized to morphine *in vivo*, thus when codeine 21 mg kg^{-1} is administered, the animal will be exposed to substantially less morphine than when dosed with morphine 20 mg kg^{-1}.

Experiments 1a and 1b: Assessment of codeine-induced hyperalgesia and allodynia and impact of prior partial nerve injury. In part 1a, wild-type mice were randomly allocated to receive codeine ($n=8$), morphine ($n=8$) or saline ($n=8$) twice daily for 4 days. In part 1b, all mice underwent a modified version of the chronic constriction injury surgery to induce mild allodynia, as described below. Two weeks post surgery, wild-type mice were randomly allocated to receive codeine ($n=8$), morphine ($n=8$) or saline ($n=8$) twice daily for 4 days. Behavioural assessments were conducted at baseline, before dosing, on day 3 and in the morning of day 5.

Experiment 2: Comparison of acute analgesia between codeine and morphine. Wild-type mice ($n=8$) were randomized to receive a single dose of codeine, morphine and saline on three respective testing days separated by 1-week washout periods. Hotplate testing was conducted three times and averaged to establish baseline sensitivity, and repeated at 15, 30, 40, 50, 60, 75 and 90 min post dose.

Experiment 3: Interleukin-1 receptor antagonist intervention. Wild-type mice were randomly allocated to a 2×2 design of 2 (codeine vs morphine; twice daily for 4 days) $\times$ 2 (IL-1RA vs saline; morning of day 5, 30 min before behavioural testing) $n=8$ per group. Behavioural assessments were conducted at baseline, before dosing on day 3 and in the morning of day 5.

Experiment 4: Assessment of codeine-induced hyperalgesia and allodynia in TLR4 null mutant (TLR4 −/−) mice. TLR4 −/− mice were randomly allocated to receive codeine ($n=8$), morphine ($n=8$) or saline ($n=8$) twice daily for 4 days. Behavioural assessments were conducted at baseline, before dosing on day 3 and in the morning of day 5.

Experiment 5: Glial attenuating intervention. Wild-type mice were randomly allocated to a 2×2 design of 2 (codeine vs morphine; twice daily for 4 days) $\times$ 2 (ibudilast vs PEG400; days 3 and 4 twice daily) $n=8$ per group. Behavioural assessments were conducted at baseline, before dosing, on day 3 and in the morning of day 5.

Partial nerve injury surgery

The Grace model,[21] a modified version of the chronic constriction injury model of sciatic nerve injury[22] was performed at mid-thigh level of the left hind leg under isoflurane anaesthesia (3% in oxygen). Briefly, the sciatic nerve was gently isolated with glass instruments and a single sterile chromic gut suture (cuticular 4-0 chromic gut, FS-2; Ethicon, Somerville, NJ, USA) was loosely tied around the sciatic nerve. The superficial muscle over the nerve was closed and three additional lengths of chromic gut were placed subcutaneously. The Grace model, in which a single chromic gut suture is placed around the sciatic nerve (N) and three pieces of chromic gut are placed subcutaneously (S) is designated N1S3, and creates a mild pain state.[21] All the surgery was performed using aseptic surgical techniques with sterilized instruments. Animals were monitored post-operatively until ambulatory before being returned to their home cage and inspected daily for signs of infection. No such cases occurred in this study. Although rescue morphine analgesia was on hand to administer following the surgery if an adverse event occurred, no such additional analgesia was required.

Behavioural testing

All behavioural testing was conducted during the light phase of the light/dark cycle and followed at least two habituations to the testing environment. The hotplate test and the von Frey test, two robust, well-established methods for assessing opioid analgesia and nociceptive sensitivity in rodents, were selected for use in this study as such tests are simple to conduct and repeated testing can be performed, allowing each animal to function as its own matched control.[23]

Allodynia assessments. The von Frey test was performed within the sciatic innervation region of both hind paws as previously described in detail.[24]

Briefly, mice were placed in individual cylindrical plastic cubicles (10 cm D × 15 cm H) with sufficient room to move freely on a wire mesh (6 mm × 6 mm) platform and allowed to acclimate for ~20 min. A logarithmic series of six calibrated von Frey monofilaments (Touch Test Sensory Evaluator Kit, St Louis, MO, USA), with bending forces that ranged from 0.02–0.4 g, were used to deliver the mechanical stimuli to the left and right hind paws in a random order. Each filament was applied to the left and right hind paw 10 times, and the number of paw-withdrawal responses elicited was recorded. Testing was conducted blind to treatment group allocation.

All animal assessments were conducted at baseline, before drug, dosing on day 3 of the experimental period and in the morning of day 5 of the experimental period. For animals in Experiment 2, additional assessments were conducted on days 3, 7, 11 and 14 post surgery, before randomization to treatment.

Hyperalgesia assessments. In all experiments, hotplate assessments were conducted at baseline, before morning drug dosing on day 3 and the morning of day 5, after von Frey assessments. Mice were placed on a hotplate maintained at 50 ± 0.2 °C,[25] a clean glass cup was placed over the animal and the latency to paw withdrawal (seconds) was recorded. Mice had sufficient room to move freely while under the glass cup. Baseline withdrawal values were calculated from an average of three consecutive latencies, measured at 10 min intervals. A pre-determined cut-off time of 60 s was imposed to prevent tissue damage.[25] Mice were immediately removed from the hotplate surface following the end of a trial due to paw-withdrawal response or elapsed cut-off time.

Western blot analysis of GFAP and CD11b

Four animals from each treatment group were anaesthetized with intraperitoneal sodium pentobarbital (Abbott Laboratories, Chicago, IL, USA) and transcardially perfused with phosphate-buffered saline to flush blood cells from the central nervous system tissue. The lumbar section (L4 to L6) of the spinal column and trigeminal ganglia were dissected out, immediately snap-frozen in liquid nitrogen and stored at −70 °C until processing. In preparation for the analysis, samples were immersed in cell lysis buffer containing 1% protease inhibitor cocktail (Sigma-Aldrich, Sydney, NSW, Australia, catalogue # P8340), sonicated then centrifuged at 14 000 r.p.m. for 5 min. The supernatant was collected and the pellet discarded. Bicinchoninic acid (BCA) assays were performed to determine total protein concentration and subsequently the samples were diluted to allow loading of 30 µg of protein per western blot well. Samples were run on an 8 or 10% sodium dodecyl sulphate-polyacrylamide gel and transferred to nitrocellulose using the wet transfer method. Membranes were immunoblotted with glial fibrillary acid protein (GFAP, 1:3000, Santa Cruz Biotechnology, Dallas, TX, USA, catalogue #sc-6170) or cluster of differentiation molecule 11b (CD11b, 1:2000, Santa Cruz Biotechnology, catalogue #sc-6614) antibodies overnight at 4 °C. Blots were washed and left to incubate with appropriate secondary antibodies for 1 h. Following a 1 min incubation with the enhanced chemiluminescence detection reagent, immunoblots were visualized using a LAS 4000 imaging system (GE Healthcare, Little Chalfont, UK) for the detection of chemiluminescent signals. The density of protein bands of interest were then quantified using ImageQuant TL software (GE Healthcare). Subsequently membranes were washed and then immunoblotted with β-actin antibody (1:10 000, Sigma-Aldrich, catalogue #A3854) as a marker of total protein loaded per each lane. GFAP and CD11b protein levels were normalized relative to β-actin levels.

Statistical analysis

All data are presented as mean ± s.e.m. unless otherwise noted. Statistical analyses were performed using R via RStudio (Version 0.97.312, RStudio, Boston, MA, USA)[26] and GraphPad Prism (Version 6, GraphPad Software, San Diego, CA, USA). Hotplate test data were analysed using linear mixed effects modelling with the R packages lme4,[27] followed by simultaneous tests for general linear hypothesis and adjusted for multiple comparisons using Tukey contrasts, with adjusted *P*-values reported using the single-step method.[28] A one-way repeated measures analysis of variance (ANOVA), corrected with a *post hoc* Tukey's multiple comparisons test,[26] was used to analyse differences in acute analgesia between the treatment groups in Experiment 2. For each von Frey test, von Frey filament number was plotted against percentage response (number of withdrawals per 10 filament applications × 10), giving a slope and intercept for each animal at each test time point using the R package ggplot2.[29] Slope represents percentage change in response as von Frey filament stiffness increases. A positive slope indicates a greater percentage response to high von Frey filament pressures vs low pressures, whereas a negative slope indicates a greater percentage response to low von Frey filament pressures vs high pressures, and as the slope approaches zero the percentage response to low and high von Frey filament pressures become similar. The intercept is an indicator of sensitivity to very low pressures; a greater intercept indicates greater allodynia elicited by low pressures. Slope and intercept were combined to form the allodynia outcome measure and analysed using multivariate ANOVA tests.[26] For simplicity, only von Frey results for the left leg are presented as all the treatments and interventions were delivered systemically or performed on the left side. Western blot results were analysed using two-way ANOVA tests with Bonferroni *post hoc* tests to adjust for multiple comparisons. Correlations between western blot data and behavioural data were investigated using linear mixed effects modelling,[27] followed by AIC stepwise model selection using the stepAIC function from the MASS library.[30] *P*-values < 0.5 and *F*-values > 3 were considered to indicate a significant difference. All R code employed during analysis is available upon request from the authors.

RESULTS

Experiments 1a and 1b: Assessment of codeine-induced hyperalgesia and allodynia and impact of prior partial nerve injury in wild-type mice.

Linear mixed effects modelling including data from both Experiments 1a and 1b highlighted significant effects of drug ($F = 12.6$), day ($F = 36.9$) and surgery ($F = 114.0$) alone, as well as significant drug:day ($F = 8.5$) and day:surgery ($F = 16.0$) interaction effects on hyperalgesia in wild-type mice. Furthermore, as

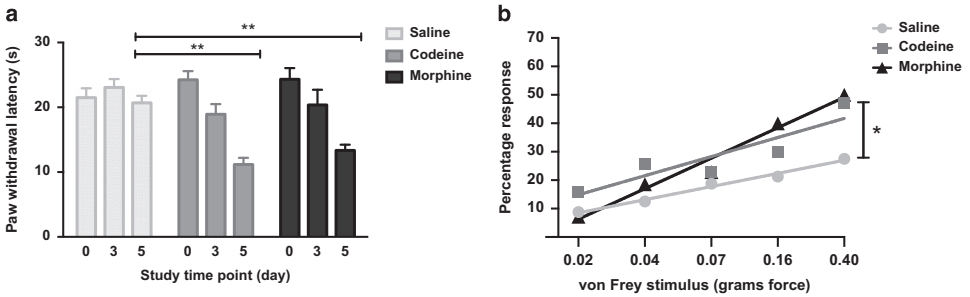


Figure 1. Experiment 1a: mice received intraperitoneal codeine (21 mg kg^{-1}, $n = 8$), morphine (20 mg kg^{-1}, $n = 8$) or saline ($n = 8$) twice daily for 4 days. Hyperalgesia (hotplate) and allodynia (von Frey) were measured on days 0, 3 and 5. (**a**) Codeine and morphine significantly reduced hotplate paw-withdrawal latency at day 5 vs saline. (**b**) There was a significant effect of drug on the development of allodynia in the codeine group (total allodynia was measured by calculating the slope and intercept for the plot of percentage response (number of paw withdrawals per 10 applications x 10) vs von Frey stimulus for each group). *$P < 0.05$, **$P < 0.01$.

illustrated in Figure 1a, Tukey *post hoc* analyses revealed that animals receiving codeine 21 mg kg^{-1} and morphine 20 mg kg^{-1} twice daily for 4 days displayed significantly reduced paw-withdrawal latency, indicative of hyperalgesia, on day 5 compared with saline-treated wild-type mice ($P < 0.01$ and $P < 0.01$, respectively). Paw-withdrawal latency was also reduced on day 5 vs baseline within the codeine ($P < 0.001$) and morphine ($P < 0.001$) groups. In Experiment 1b, partial nerve injury induced hyperalgesia at baseline in all groups, which was equivalent to that present in the codeine and morphine groups who did not undergo surgery on day 5 of the treatment. Basal hyperalgesia was present to such a degree that further decreases in latency in post-surgery animals receiving codeine ($P = 0.5$) or morphine ($P = 0.9$) on day 5 vs baseline, or between post-surgery mice receiving codeine ($P = 0.4$) and morphine ($P = 0.2$) vs saline on day 5 did not reach significance (data not shown). No differences were detected between codeine and morphine groups within Experiments 1a and 1b on days 3 and 5.

Multivariate ANOVA of all von Frey data from Experiments 1a and 1b combined on day 5 highlighted overall significant effects of both drug ($P = 0.007$) and surgery ($P < 0.001$) on allodynia, indicating that both taking codeine or morphine, or undergoing surgery, increases sensitivity to non-noxious stimuli. In the codeine groups, drug ($P = 0.03$, see Figure 1b) and surgery ($P < 0.001$) significantly influenced allodynia, yet there was no significant surgery:drug interaction ($P = 0.91$), demonstrating that codeine-induced allodynia did not differ between surgery and no-surgery animals. In the morphine groups, although surgery alone had a significant effect on allodynia ($P < 0.001$), drug ($P = 0.08$) did not contribute to allodynia until surgery was factored in, as indicated by a significant surgery:drug interaction ($P = 0.04$). This significant surgery:drug interaction demonstrates that the development of allodynia following morphine treatment differs in surgery animals compared with no-surgery animals. Animals who received morphine without surgery displayed lesser sensitivity to low von Frey filament pressures than morphine animals who underwent surgery; however, regardless of surgery status, both groups receiving morphine displayed a high response rate to high von Frey filament pressures.

Behavioural test results for codeine and morphine treatment groups were compared with saline controls on day 5, as well as their respective baseline findings, to control for learned or conditioned behaviour to the testing procedures. However, such factors are unlikely to influence results as no significant change in hotplate latency or response to von Frey filament stimulation was observed in the saline animals across the 5 days of testing.

Experiment 2: Comparison of acute analgesia between codeine 21 mg kg^{-1} and morphine 20 mg kg^{-1}

Acute codeine administration at 21 mg kg^{-1} provided significantly less hotplate analgesia than morphine 20 mg kg^{-1} measured in terms of area under the time-effect (paw-withdrawal latency/analgesia) curve from 0–90 min post dose, as shown in Figure 2.

Experiment 3: Interleukin-1 receptor antagonist intervention

A significant overall effect of intervention (IL-1RA or saline) was detected in both hotplate ($P < 0.001$) and von Frey ($P < 0.001$) tests. As illustrated in Figure 3a1, both codeine and morphine when given with saline only produced significant hyperalgesia ($P < 0.001$ and $P < 0.001$, respectively) at day 5 vs baseline. In codeine and morphine animals receiving IL-1RA before the final hyperalgesia assessment on day 5, paw-withdrawal latency began to return to baseline levels for both opioid groups, with morphine +IL-RA vs morphine+saline ($P < 0.001$) and codeine+IL-RA vs codeine+saline ($P < 0.001$) reaching significance. In the von Frey test, codeine and morphine, given with saline, established allodynia on day 5 vs baseline ($P < 0.001$ and $P < 0.001$, respectively). Partially established allodynia in codeine and morphine groups was abolished by IL-RA on day 5, as depicted in Figure 3a2.

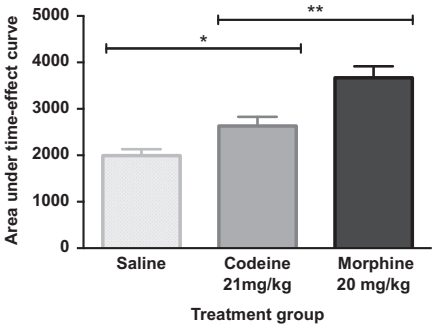


Figure 2. Experiment 2 (acute analgesia): mice ($n = 8$) were randomized to receive a single codeine (21 mg kg^{-1}), morphine (20 mg kg^{-1}) and saline dose on three separate occasions, separated by a washout period of 1 week. Hotplate testing was performed at baseline and 15, 30, 40, 60, 60 and 75 min post dose and total analgesia produced over the 0–90 min testing period for each drug was calculated to give area under the time-effect (analgesia) curve (AUC). Codeine produced a greater AUC from 0–90 min post dose vs saline. Morphine produced a significantly greater AUC from 0–90 min post dose vs an equimolar dose of codeine. *$P < 0.05$, **$P < 0.01$.

Experiment 4: Assessment of codeine-induced hyperalgesia and allodynia in TLR4 −/− mice

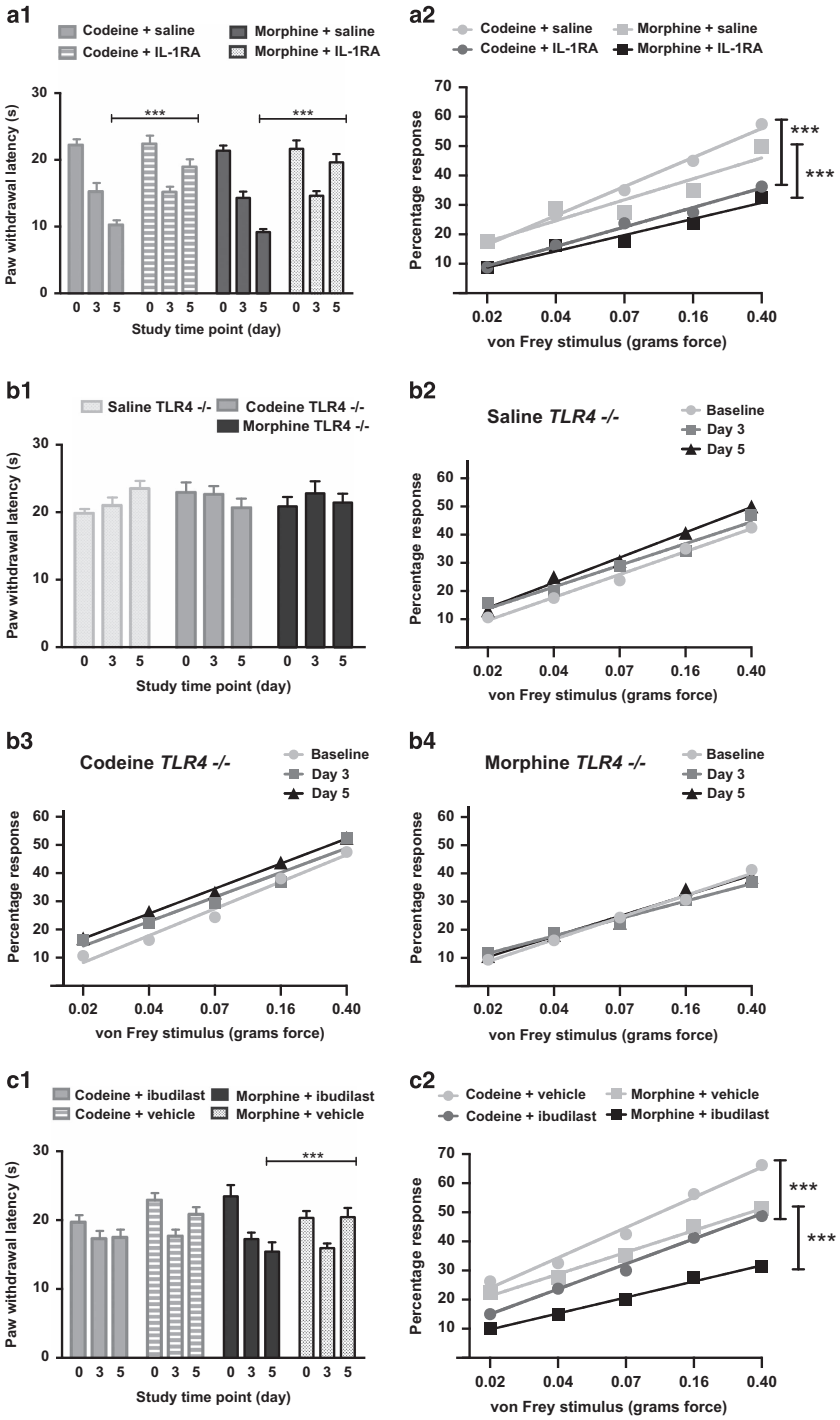
Incorporating data from the no-surgery Experiment 1a mice, linear mixed effects modelling found significant effects of genotype (*TLR4* −/− or wild type) alone ($F = 7.6$), as well as significant drug:genotype ($F = 3.002$) and genotype:day ($F = 21.2$) interactions effects on hyperalgesia. Tukey *post hoc* analyses confirmed no significant differences in paw-withdrawal latency in the hotplate test between treatment groups on day 5 in *TLR4* −/− animals (see Figure 3b1). Similarly, multivariate ANOVA revealed a significant effect of genotype ($P < 0.0001$) on allodynia at day 5 and demonstrated that *TLR4* −/− mice were protected against changes in pain sensitivity in all treatment groups as shown in Figures 3b2, b3 and b4.

Experiment 5: Glial attenuating intervention

A significant overall effect of intervention (ibudilast or vehicle) was detected in both hotplate ($P = 0.002$) and von Frey ($P = 0.003$) tests. As shown in Figure 3c1, ibudilast significantly increased paw-withdrawal latency vs vehicle in the morphine group ($P = 0.035$). The trend of hyperalgesia reversal following ibudilast in the codeine group did not reach significance vs vehicle ($P = 0.246$). Codeine and morphine groups receiving vehicle on days 3 and 4, in addition to their respective opioids, displayed allodynia ($P < 0.001$ and $P = 0.009$, respectively) at day 5 vs baseline. Established allodynia in codeine and morphine groups was abolished by ibudilast on day 5, as illustrated in Figure 3c2.

2.6 western blot analysis of GFAP and CD11b

Following final behavioural testing bilateral trigeminal ganglion tissue and the lumbar section of the spinal cord were dissected and prepared for western blot analysis to investigate levels of markers associated with reactivity of glial cells. Levels of CD11b, an adhesion molecule marker for active macrophages and microglia[31,32] and GFAP, an increase in which accompanies the



reactive response of astrocytes and satellite glial cells after exposure to an insult,[33–35] were quantified.

When all drug treatment groups were taken together, partial nerve injury surgery before opioid administration significantly increased GFAP expression in the trigeminal ganglion ($P < 0.01$) and spinal cord ($P = 0.02$). However, no overall effect of drug was detected ($P = 0.3$). Significant effects of both surgery ($P = 0.02$) and drug ($P < 0.01$), and a surgery:drug ($P < 0.01$) interaction were found on CD11b levels in the spinal cord, although these appear to be driven largely by a difference within the codeine group. Drug also had a significant overall effect on CD11b in the trigeminal ganglion ($P < 0.01$). In Experiments 1a and 1b, linear

Figure 3. (**a**) Experiment 3: mice received intraperitoneal (i.p.) codeine ($n=16$, 21 mg kg^{-1}) or morphine ($n=16$, 20 mg kg^{-1}) twice daily for 4 days. Hyperalgesia (hotplate) and allodynia (von Frey) were measured on days 0, 3 and 5. Thirty minutes before assessments on day 5, half of the mice in each drug group received i.p. interleukin-1 receptor antagonist (IL-1RA, 100 mg kg^{-1}) and the remaining half received saline. (a1) IL-1RA abolished decreases in paw-withdrawal latency in both the codeine and morphine groups at day 5 vs groups receiving saline. (a2) IL-1RA significantly attenuated allodynia induced by codeine and morphine on day 5 vs saline in the left hind paw. (**b**) Experiment 4: toll-like receptor-4 null mutant mice received i.p. codeine ($n=8$, 21 mg kg^{-1}), morphine ($n=8$, 20 mg kg^{-1}) or saline ($n=8$) twice daily for 4 days. Hyperalgesia (hotplate) and allodynia (von Frey) were measured on days 0, 3 and 5. (b1) Codeine ($P=0.996$) and morphine ($P>0.99$) did not alter hotplate paw-withdrawal latency at day 5 vs baseline. Allodynia did not change significantly over time in (b2) saline ($P=0.09$), (b3) codeine ($P=0.051$) or (b4) morphine ($P=0.21$) groups. (**c**) Experiment 5: mice received i.p. codeine ($n=16$, 21 mg kg^{-1}) or morphine ($n=16$, 20 mg kg^{-1}) twice daily for 4 days. On days 3 and 4, half of the mice in each group received i.p. ibudilast (15 mg kg^{-1}) and the remaining half received vehicle twice daily. Hyperalgesia (hotplate) and allodynia (von Frey) were measured on days 0, 3 and 5. (c1) Ibudilast reversed decreases in hotplate paw-withdrawal latency in morphine but not codeine ($P=0.2$) animals at day 5 vs groups receiving saline in addition to their respective opioid. (c2) Ibudilast significantly attenuated allodynia induced by codeine and morphine in the von Frey test on day 5 vs saline in the left hind paw. Total allodynia was measured by calculating the slope and intercept for the plot of number of paw withdrawals per 10 applications vs von Frey stimulus for each group. ***$P<0.001$.

Table 1. Fold change between treatment groups and comparator controls in glial fibrillary acidic protein (GFAP) and cluster of differentiation molecule 11b (CD11b) levels relative to β-actin, in the spinal cord and trigeminal ganglion.

Treatment group	*Comparator group*	*Fold change* (P-*value*)			
		Spinal cord GFAP	*Spinal cord CD11b*	*Trigeminal ganglion GFAP*	*Trigeminal ganglion CD11b*
Codeine only	Saline only	0.93 (>0.99)	1.59 (<0.01)**	0.60 (0.14)	0.25 (<0.01)**
Morphine only	Saline only	0.82 (>0.99)	0.73 (0.23)	0.58 (>0.12)	0.67 (0.35)
Saline *TLR4* −/−	Saline only	1.47 (0.32)	0.46 (<0.01)**	6.33 (0.02)*	Unavailable[a]
Codeine *TLR4* −/−	Saline *TLR4* −/−	0.60 (0.15)	0.97 (>0.99)	0.83 (>0.91)	0.61 (0.03)*
Morphine *TLR4* −/−	Saline *TLR4* −/−	0.43 (0.03)*	0.74 (0.56)	0.25 (>0.07)	1.03 (0.99)
Codeine+ibudilast	Codeine+vehicle	1.32 (0.29)	1.66 (0.14)	0.06 (0.01)*	0.18 (<0.01)**
Morphine+ibudilast	Morphine+vehicle	1.34 (0.48)	1.17 (0.75)	1.78 (>0.99)	1.78 (0.44)

Abbreviation: TLR4, Toll-like receptor-4. Codeine only, codeine *TLR4* −/−, codeine+ibudilast and codeine+vehicle animals received codeine intraperitoneal (i.p.) 21 mg kg^{-1} twice daily for 4 days. Morphine only, morphine *TLR4* −/−, morphine+ibudilast and morphine+vehicle animals received morphine i.p. 20 mg kg^{-1} twice daily for 4 days. Saline only and saline *TLR4* −/− animals received i.p. saline (equal volume to opioids) twice daily for 4 days. Codeine +ibudilast and morphine+ibudilast received i.p. ibudilast 15 mg kg^{-1} (in 35% polyethelene glycol) twice daily on days 3 and 4, in addition to their respective opioids. Codeine+vehicle and morphine+vehicle animals received i.p. 35% polyethelene glycol twice daily on days 3 and 4, in addition to their respective opioids. All tissues samples were obtained on day 5 of the experimental protocols. Values <1 represent a reduction in marker level vs comparator whereas values >1 represent an increase relative to comparator. [a]Unable to quantitate due to poor blot quality.

modelling with stepwise model selection by AIC demonstrates that spinal cord GFAP, CD11b and the interaction between spinal cord GFAP and CD11b are able to predict 82% of the variation in hotplate test scores in the morphine groups, and 35% of hotplate test score variability in the codeine groups, whereas no model based upon the spinal cord markers was able to account for hotplate test variability in the saline-treated mice.

Within *TLR4* −/− animals, codeine and morphine did not increase GFAP or CD11b at either site assessed. The *TLR4* −/− mice displayed reduced CD11b levels in the spinal cord, yet compared with wild-type animals, spinal GFAP was not altered ($P=0.5$), and, in the trigeminal ganglion, CD11b ($P<0.01$) and GFAP ($P<0.01$) appeared elevated. Intervention with ibudilast did not bring about any differences in GFAP or CD11b in the spinal cord; yet in the trigeminal ganglion, codeine produced significant increases in GFAP ($P<0.01$) and CD11b ($P<0.01$) vs morphine, which were abolished following ibudilast administration. A summary of the relevant pair-wise comparisons in glial activation markers throughout all experiments is presented in Table 1 (Specific comparisons between partial nerve injury+drug and drug-only (no surgery) animals are not presented because no differences in hyperalgesia or allodynia were observed after drug treatment). Representative western blot images are presented in Figure 4.

DISCUSSION

In this study, we have demonstrated that codeine is able to induce hyperalgesia and allodynia to the same degree as equimolar morphine. This suggests that codeine may not rely solely upon metabolism to morphine to produce hyperalgesia, as if it was only the morphine metabolite of codeine causing pain sensitivity changes; significantly less hyperalgesia and allodynia would be expected in the codeine group, given that only a small proportion of the codeine dose is converted to morphine. The possibility that codeine is largely being converted to morphine in this mouse strain is unlikely as the hotplate test confirmed that codeine provides significantly less acute analgesia over 90 min post dose than morphine at an equimolar dose in male BALB/c mice, however, we cannot rule out the involvement of other codeine metabolites in the development of increased pain sensitivity. TLR4 and IL-1β appear to have important roles in the development of hyperalgesia as genetic lack of TLR4 and administration of an IL-1RA both abolished codeine-induced increases in pain sensitivity. Furthermore, attenuating glial activation with ibudilast reversed partially established codeine-induced allodynia.

To model the clinical situation in which opioids are administered to patients with an increased basal pain state, we first performed partial nerve injury surgery to prime animals before codeine administration. Relative to the hypothesized involvement of glial cells in morphine-induced hyperalgesia, we expected surgery to first prime the glial cells, triggering them to respond faster with greater magnitude, when subsequently activated following opioid administration.[36] We found that surgery produced hyperalgesia and allodynia at baseline, and although codeine and morphine animals displayed a trend towards a further increase in pain sensitivity, this did not reach significance, likely owing to a floor effect of the behavioural testing employed.

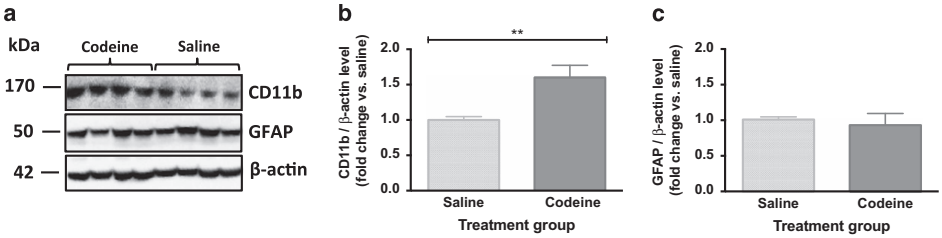


Figure 4. (**a**) Representative western blot images for cluster of differentiation molecule 11b (CD11b), glial fibrillary acid protein (GFAP) and β-actin. Shows samples of the lumbar spinal cord of animals receiving intraperitoneal codeine (21 mg kg^{-1}) or saline, twice daily for 4 days. Tissue samples collected on day 5 of the experimental protocol. (**b**) Fold change in lumbar spinal cord CD11b, normalized for β-actin, in animals receiving codeine (21 mg kg^{-1}) compared with saline controls; corresponds with band at 170 kDa in **a**. (**c**) Fold change in lumbar spinal cord GFAP, normalized for β-actin, in animals receiving codeine (21 mg kg^{-1}) compared with saline controls; corresponds with band at 50 kDa in **a**. $^{**}P < 0.01$.

As in prior experiments,[21,37] partial nerve injury surgery increased the overall levels of a marker of astrocyte activation, GFAP,[38] in the lumbar spinal cord. Administering morphine post surgery further elevated lumbar astrocyte activation, concurring with the results reported by Raghavendra *et al*.,[37] who found a greater magnitude of astrocyte activation following chronic morphine administration in nerve-injured compared with sham-operated rats, yet the same increase was not observed when codeine was given post surgery. Interestingly, unlike previous studies of morphine,[37,39,40] we did not observe an increase in markers of glial activation in no-surgery animals receiving morphine, nor did we find increased glial activation markers in animals receiving chronic codeine, as compared with control animals. The significant hyperalgesia and allodynia present could, however, result from glial activation that was not captured by our tissue collection time point or proinflammation without phenotypic glial cell surface expression changes. While pain behaviour in codeine and morphine animals was comparable, and both groups were sensitive to the same pharmacological interventions (indicating similar mediators are at play), linear modelling suggests that molecular changes, inferred from the phenotypic cellular expression markers, are not identical. Although cellular surface markers represent only surrogates of activity, it is clear the processes underlying opioid-induced changes in pain sensitivity, resulting from the summation of μ-opioid receptor and TLR4 signalling cascades, are indeed complex, particularly following codeine administration. Further studies with greater group numbers for protein analysis and multiple tissue collection time points may assist in clarifying proteomic results.

Toll-like receptor-4, which detects 'danger signals' and microbial associated molecular patterns—such as lipopolysaccharide found in the cell wall of gram-negative bacteria—is a key modulator in innate immune system activation.[41] In our study, mice lacking TLR4 were protected against codeine-induced hyperalgesia and allodynia. Protection against glial activation in *TLR4−/−* mice could not be confirmed as western blot results were inconclusive, however, behavioural findings in *TLR4−/−* mice were robust and agree with previous evidence that pharmacological blockade of TLR4 using (+)-naloxone is able to significantly attenuate paradoxical morphine-induced increases in pain sensitivity.[20]

Once activated, glial cells release a range of proinflammatory mediators such as the inflammatory cytokine IL-1β. Again aligning with a glial activation hypothesis, we have demonstrated that pharmacological antagonism of the IL-1 receptor using anakinra is sufficient to reverse codeine-induced hyperalgesia and allodynia. These results also agree with literature indicating systemically delivered IL-1RA enters the central nervous system[42,43] and is able to reinstate morphine analgesia in animals that display opioid tolerance, a phenomenon which has previously been described as a behavioural manifestation of opioid-induced hyperalgesia.[44]

Treatment with the glial-attenuating agent ibudilast[45] reversed codeine-induced allodynia. Ibudilast's pharmacological actions include inhibition of macrophage migration inhibitory factor[46] and phosphodiesterase 4 and 10 (and to a less extent 3 and 11).[47] Both these inhibitory actions are thought to contribute to its glial-attenuating ability.[48] Previously ibudilast has been shown to potentiate the analgesic potency of morphine in tolerant rodents.[39] Given that blocking IL-1 reversed changes in pain sensitivity, and ibudilast has been shown to prevent the release of IL-1 from microglial cells,[49] it is plausible that the reversal of opioid-induced allodynia by ibudilast is brought about either directly or indirectly through glial modulation. As a phosphodiesterase inhibitor vascular reactivity, and therefore drug distribution, could be altered following ibudilast administration. However, it is unlikely that altered distribution accounts for the reduction in morphine-induced hyperalgesia observed in this study as previous experiments have demonstrated that ibudilast co-administration does not alter morphine pharmacokinetics.[39] Furthermore, multiple studies have established that ibudilast co-administration potentiates morphine analgesia, the opposite of what would be expected if ibudilast resulted in reduced distribution of morphine to the central nervous system.[39,50]

Although ibudilast has previously been reported to produce slight sedation, reduced locomotor activity[50] and reduced sensitivity to touch,[51] it is unlikely that these adverse effects—which occur early (within 30 min of dosing) and are transient[51]—have influenced results, as all testing in our study was performed 16 h post ibudilast dose. While we could not confirm that ibudilast reduced the activation of lumbar spinal glial cells (which would mediate hind paw allodynia), a reduction in glial-mediated inflammation, independent of the cell surface markers assessed, could account for our results. In the trigeminal ganglion, ibudilast significantly reversed substantial codeine-induced satellite glial cell activation, indicating that the trigeminal ganglion glial cells may be particularly sensitive to the glial-activating effects of codeine, and ibudilast may be useful in attenuating activation at this site.

We are confident that our behavioural results are reproducible in male BALB/c mice, yet additional studies are required to determine generalizability to females and other strains of mice, as large variability in opioid response has been reported between female and male rodents[52] and among different mice strains.[53] Regardless, results from previous preclinical studies investigating opioid-induced hyperalgesia following morphine, methadone and buprenorphine dosing have been replicated in human trials, indicating that studies such as this do have positive predictive value in the clinic. In these experiments, pain sensitivity was only assessed the morning after opioid administration (~16h post-dose), thus the hyperalgesia and allodynia displayed may be related to processes involved in opioid withdrawal. In future

studies, additional testing shortly after opioid administration would be of interest to determine if opioid tolerance was also present.

The observation of equal hyperalgesia following equimolar codeine and morphine administration has important clinical implications, as although both drugs appear to increase pain to the same degree, codeine provides only around one-tenth of the analgesia that morphine provides when equal doses are given, thus the risks:benefit ratio may be higher for codeine than for morphine. One example of a clinical pain state that is frequently worsened following codeine use is medication overuse headache, in which patients with an underlying primary headache disorder, progress to experience chronic daily or near-daily headache.[54] We have previously hypothesized that glial priming due to recurrent headaches may be responsible for the specific susceptibility to opioid-induced chronic headache observed in patients with pre-existing headache conditions.[19] Given that the trigeminal ganglion is of particular importance in the pathophysiology of migraine, the exaggerated satellite glial activation observed in the ibudilast intervention experiment suggests that codeine may be particularly detrimental in the management of this condition, and additional studies that include assessment of facial allodynia could provide a useful behavioural correlate to aid in interpreting protein analysis results.

CONCLUSIONS

This study provides the first preclinical evidence that repeated doses of codeine, similar to morphine, are able to induce both hyperalgesia and allodynia. Mechanistically, increased sensitivity to pain following codeine administration may occur as a result of TLR4 activation-dependent proinflammatory cytokine release by glial cells. Glial attenuation with agents such as ibudilast, for which the clinical safety is already established, may prove to be of use in the clinical management of opioid-induced hyperalgesia.

CONFLICT OF INTEREST

KJ was an employee at Medicinova, a biopharmaceutical company investigating ibudilast, while this study was conducted but no longer has material conflicts of interest to declare. The remaining authors declare no conflict of interest.

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4. SUMMARY AND DISCUSSION OF PHD FINDINGS

Codeine is the most commonly consumed opiate worldwide[270], yet surprisingly, given it was first isolated over 180 years ago[271], there is a dearth of clear documentation regarding the safety of long-term codeine use[272]. Over the past few years, in light of numerous reports of serious morbidity and mortality, concern has been mounting that the harms associated with the use of codeine-containing preparations may outweigh the benefits, particularly when contemplating over-the-counter products[273] such as those available in Australia, Canada and the UK. To adequately assess the risk-to-benefit ratio, and therefore determine the appropriateness of codeine therapy for an individual, we must understand all of the potential issues linked with chronic intake including possible of exacerbation of pain, a risk documented previously for codeines' metabolite morphine[274].

One condition for which chronic codeine use appears particular deleterious is headache, as frequent regular intake of codeine, like other opioids, can lead to the development of MOH[117, 275]. Despite the Australian Therapeutic guidelines[276] and National Prescribing Service[277] warning against the use of opioids in migraine management, an analysis of Australian general practitioner consultations conducted between April 2010 and March 2011 indicated over 18% of patients presenting with migraine were prescribed an opioid medication[278]. Currently MOH is cited as the third most common form of headache, representing a huge burden on the community, both financially and in terms of decreased patient quality of life[279]. We hypothesis the condition MOH contains a number of different subtypes with unique pathologies based upon the type of medication overused. While it is often cited that all acute headache treatments can induce MOH, the evidence implicating opioids is clearest, while causal evidence linking the development of MOH following the overuse of simple analgesics alone is scarce. Of all MOH subtypes opioid overuse headache

is particularly difficult to treat. There is a general consensus among experts that patients overusing opioids should undergo gradual detoxification in an inpatient setting[135] and such patients are recognised as having the highest relapse rates, highlighting the complexity of managing this condition in the long term[9]. Therefore, treatments specifically for use in the management of opioid overuse headache that do not require hospitalisation and are able to reduce relapse are of great clinical interest.

While our traditional understanding of pain, focused upon neuronal pathways and mediators, has lead to many theories regarding the genesis of opioid-induced hyperalgesia and allodynia and headache exacerbated by frequent opioid use, clinically successful treatments for these conditions remain elusive. Recent evidence supports an interaction between glial cells and morphine, the active metabolite of codeine, in the pathophysiology of dependence[280], and dependence behaviours have also been linked by multiple groups to MOH[66, 91]. Considering such findings the hypothesis that opioid-overuse headache is a phenomenon similar to opioid-induced hyperalgesia, deriving from a cumulative interaction between central sensitisation, due to repeated activation of nociceptive pathways by recurrent headaches, and pain facilitation due to glial activation, was formulated and created the basis of the studies which constitute this PhD project. Crucially, this hypothesis not only explains only how opioids promote headache chronification over time but also why headache patients, but not other individuals develop MOH when exposed to the exact same drug dosing regimes. Furthermore, this hypothesis brought to light new potential MOH therapies targeting glial activation, such as ibudilast.

In most cases, pharmacological therapies are first evaluated for new indications in relevant preclinical models prior to testing in the clinical trial setting. However, if the safety of a medication has been established and the human condition to be treated is poorly

recapitulated in validated animal models it is reasonable to move directly into the clinical trial phase.

Due to the complex characteristics of MOH, and the lack of understanding regarding not only the pathophysiology of medication-induced headache chronification, but also the predisposing factor(s) that triggers this condition only in patients with a pre-existing headache disorder, no animal model is able to mimic the human presentation of this disorder. Existing models of migraine based upon the familial hemiplegic migraine gene mutations, systemic glyceryl trinitrate administration, and electrical stimulation of trigeminal ganglion or dural vessels or chemical stimulation of the meninges[281] present significant shortcomings[282]. Thus, given the heterogeneity of MOH, particularly that it occurs largely in both migraine and TTH sufferers, as well as the proposed involvement of dependence-like behaviours, the appropriateness of translating of such models to MOH is yet to be determined.

As systemically delivered ibudilast has been available for the clinical management of asthma and post-stroke dizziness in Japan for decades, the favourable safety profile of this medication was clear, and recent trials developing higher dose ibudilast regimes for new central nervous system targeted indications have further extended available promising safety data. Bearing in mind that ibudilast has already been used by millions of patients and that no satisfactory preclinical MOH model exists, it was decided that a clinical trial of ibudilast in MOH patients consuming opioids was warranted, especially given currently available treatments for this disorder are lacking and any positive results could then be directly implemented in clinical practice.

Thus, the first stage of my PhD project involved designing a double-blind, randomised, placebo controlled, parallel groups clinical trial of ibudilast 40 mg twice daily for 8 weeks. This trial was planned in accordance with the International Headache Society guidelines for clinical trials of drugs for the prevention of TTH and chronic migraine, as no trial design guidelines specifically for treatments aimed at reducing the frequency of headache in MOH patients are available. The end points utilised in MOH studies were critically reviewed in 2010, and the results of this review were taken into account when designing the ibudilast clinical trial[194].

This ibudilast clinical trial is the only study to date that has specifically investigated the treatment of MOH in patients who are all overusing opioids. The vast majority of trials include a heterogeneous MOH population including patients who purely overuse simple analgesics, such as paracetamol and non-steroidal anti-inflammatories, patients who overuse triptans or combination analgesics containing caffeine or butalbital and in some cases patients who overuse opioids. However, as these agents possess vastly different pharmacological profiles it is plausible that different mechanisms may be involved in headache chronification brought about by different drug classes. This theory concurs with the differing clinical presentations seen between patients overusing analgesics, who often progress to experience daily or near daily headaches mild headaches, compared with those who overuse triptans, who often experience simply an increase in migraine frequency or more migraine-like headaches[17]. Moreover, in MOH patients following detoxification the characteristics of the withdrawal headache differ according to the type of acute headache treatment that was overused[138]. Thus, if different mechanisms underlie MOH induced by different agents, trials that study such diverse MOH patient populations may fail to detect

treatments which are effective in one sub-type due to masking by a lack of efficacy in other sub-types.

Recruitment for our clinical trial began in September 2010, and by the end of 2013 over 450 patients had undergone initial screening via telephone, leading to a final number of 34. Of these participants, 30 completed the 8-week treatment period and subsequent follow up data collection, 13 in the ibudilast group and 17 in the placebo group. Headache was monitored through use of a headache diary, which participants completed daily.

At study completion it became evident that this ibudilast dosing regimen did not significantly improve any of the headache indices assessed including headache index, headache frequency, average headache duration or intensity, or headache related quality of life, assessed with a self-completed questionnaire, when compared with the placebo group. Medication intake was also closely examined but revealed no significant changes over time between the ibudilast and placebo groups.

Another aspect of this ibudilast clinical trial that sets it apart from the vast majority of MOH studies and headache studies in general, was the intensive longitudinal quantitative sensory testing and biomarker analysis. We measured sensitivity to static and dynamic mechanical stimuli and warm and cool thermal stimuli at cephalic and extra cephalic sites bilaterally at baseline and at weeks 2, 4 and 8 of treatment. Furthermore, as quantitative sensory testing was conducted irrespective of the participants' presenting headache status (experiencing attack or interictal), it was supplemented by a questionnaire evaluating allodynia during the patients' most severe type of headache. However we did not see any alterations in sensitivity measured with either quantitative sensory testing or the questionnaire following ibudilast treatment. Correlation analyses between baseline headache measures, patient

characteristics and medication intake were also performed and gave some interesting findings, including associations between increasing baseline headache index and headache impact on quality of life and greater allodynia symptom checklist scores.

Although no meaningful headache benefits were obtained in the ibudilast group, this study did provide a wealth of safety data, as no previously published trials have used such a high dose for this duration of time. Ibudilast was found to be safe and generally well tolerated, with no serious adverse events occurring that were deemed related to the study medication. The most commonly reported adverse event was nausea, which was found to be mild, transient and responded well to temporary ibudilast cessation and gradual up titration on reinstatement. Thus, these additional data supporting the favourable safety profile of this drug will help to facilitate optimised future investigation of ibudilast for new indications, such as substance dependence disorders, neurodegenerative conditions and varied pathological pain states.

Blood samples were taken from the clinical trial participants to form a PBMC reactivity biomarker study as a part of another students PhD project. The biomarker sub-study found PBMCs isolated from the ibudilast group produced less IL-1β, compared with PBMCs isolated from the placebo group, when stimulated with a TLR2 or TLR4 agonist. The TLR responsiveness PBMCs is thought to mirror TLR responsiveness in the central nervous system[283], thus this finding appears to support our hypothesis in that ibudilast can attenuate TLR responsiveness and therefore glial activation in humans, however such attenuation did not translate to reduce headache pain or medication intake.

The mixed biomarker and headache outcome results in the ibudilast clinical trial left a number of questions relating to my MOH pathophysiology hypothesis unanswered.

Therefore, I designed a series of six preclinical behavioural experiments to address the uncertain aspects of the hypothesis underpinning my clinical trial. The question that needed to be answered was whether chronic codeine administration was in fact able to alter pain sensitivity, as morphine administration can. To confirm this 24 mice were randomised to receive codeine (21 mg/kg), morphine (20 mg/kg, positive control) or saline (equal volume, negative control), twice daily via intraperitoneal injection for 4 days. Prior to dosing on day 1 and day 3 and in the morning of day 5, pain sensitivity was assessed with the hot plate test, employed to detect hyperalgesia, and the von Frey filament test, used to detect allodynia.

This experiment demonstrated clearly for the first time that codeine administration can indeed induce hyperalgesia and allodynia, a novel and important finding given the widespread clinical use of codeine. What was particularly interesting was that equimolar doses of codeine and morphine produced the same degree of hyperalgesia. This could suggest codeine does not rely solely upon *in vivo* metabolism to morphine to alter pain sensitivity, as only approximately 10% of the codeine dose is converted to morphine. Thus, if codeine were producing hyperalgesia by acting purely as a vehicle for morphine delivery, we would expect much less hyperalgesia in the codeine group as they are exposed to much less morphine. Future studies demonstrating a morphine or codeine dose-related effect on hyperalgesia would strengthen this conclusion. It may be that the codeine molecule itself is able to activate glial cells, a hypothesis which fits with docking simulations indicating codeine binds to the myeloid differentiation (MD)2 protein at a location known to be important in activation of the TLR4/MD2 complex. The equal hyperalgesia produced between codeine and the more potent opioid morphine aligns with the clinical observation that regardless of analgesic potency all opioids appear to be associated with the same risk of

MOH in migraine patients, as assessed prospectively in the large population based American Migraine Prevalence and Prevention study (Dr Marcelo Bigal, personal communication, Boston, June 28, 2013).

Furthermore, an additional acute analgesia study was performed using the hot plate test to confirm that the dose of codeine used in the hyperalgesia/allodynia experiments provided less analgesia than the equimolar morphine dose used. Taking into account the discovery that equimolar codeine and morphine produce the same degree of hyperalgesia, yet codeine provides lesser analgesia, it seems that the risk-to-benefit ratio in relation to ongoing pain may be higher for codeine than for morphine.

An important component of my MOH hypothesis was that the priming of glial cells by recurrent headaches leaves headache patients particularly susceptible to chronification following opioid exposure. Thus, although our study involved a peripheral pain state we modelled underlying glial priming prior to opioid exposure by performing a modified version of the chronic constriction injury surgery. This model was developed by our laboratory to provide a spectrum of pathological pain from mild to severe in terms of intensity, as discussed previously. The mildest pain state is induced through a surgical procedure that includes one chromic gut suture tied loosely around the sciatic nerve and 3 equal lengths of chromic gut placed subcutaneously. This procedure was performed two weeks prior to commencing opioid delivery to allow for healing at the incision site and stabilisation of allodynia. In line with my clinical hypothesis I expected that codeine-induced hyperalgesia and allodynia would be exacerbated in these 'primed' animals.

From this experiment it was evident that the modified chronic constriction injury surgery had a profound impact on basal pain sensitivity. Interestingly, the degree of hyperalgesia

present following 4 days of opioid exposure was equivalent to that produced by the modified chronic constriction injury surgery. Basal hyperalgesia was present to such a degree in the primed animals that further decreases in latency, indicating hyperalgesia, following opioid exposure were not detected. Similarly, no consistent increase in allodynia was detected from baseline in the primed animals after opioid administration, however in the morphine group glial priming did increase sensitivity to low-pressure stimuli. Thus, in these animal experiments pre-existing glial priming was not required to facilitate codeine-induced changes in pain sensitivity. If back translated to the clinical scenario this finding could be interpreted to indicate patients without previous glial priming due to recurrent headaches could also develop MOH, which does not agree with the observation that MOH only seems to occur in patients with a primary headache disorder. This deviation from our hypothesis may be related to differences in pain processing and glial cells within the lumbar spinal cord and trigeminal ganglion.

Now that the ability of chronic codeine to exacerbate pain was confirmed, the next aspects of my MOH hypothesis that I wanted to investigate were the involvement of inflammatory cytokines, and the importance of TLR4. To do this, pharmacological blockade of IL-1 using IL-1RA and TLR4 null mutant mice were employed. I established that IL-1RA was able to reverse codeine-induced hyperalgesia and allodynia and that *TLR4-/-* mice were protected against changes in pain sensitivity following codeine administration. Both of these findings agree with my MOH hypothesis, however in our ibudilast clinical trial, unlike in the animal experiment, the reduction of TLR4 agonist stimulated IL-1β in the PBMCs isolated from the active treatment group did not translate to reductions in pain or allodynia.

Finally, the last preclinical study conducted was aimed to determine if ibudilast is able to reverse established codeine-induced hyperalgesia and allodynia. Behaviourally, we found a

significant reduction in pain sensitivity in both animals receiving codeine or morphine when ibudilast was co-administered for the final two days of opioid treatment compared with saline co-administration. This finding was in agreement with out MOH hypothesis, but did not explain the lack of efficacy observed in our clinical trial.

A number of speculative theories could account for this discrepancy. In the animal study ibudilast was administered concurrently with either codeine or morphine for half the duration of opioid administration alone, whereas in the clinical trial participants had been frequently taking opioids for an average of over 7 years, and only received ibudilast for 8 weeks, thus the duration of ibudilast treatment relative to opioid exposure may have been insufficient. Also the treatment initiation point relative to disease/pain onset may have played a role in determining the efficacy of ibudilast as increased pain sensitivity had only just been established in the animal model, whereas the clinical trial participants had suffered from chronic headache for an average of 13 years. It would therefore be interesting to determine if ibudilast retains its efficacy in reversing heightened pain sensitivity in animals that have been receiving opioids for a longer period of time. Similarly, it would be of interest to perform a sub-group analysis of data from the clinical trial in participants who had been overusing opioids for a shorter period of time to determine if their response to ibudilast was any different to those with a long history of frequent opioid use. Unfortunately the small sample size in our clinical trial did not allow for such an analysis.

Additionally, when the human equivalent dose is calculated based upon species weight and body surface area (as recommended by the US Food and Drug Administration), it is clear that the mouse ibudilast dose of 30 mg/kg/day, equivalent to 2.4 mg/kg/day in humans, is lower than the dose used in our clinical trial, which equated to 1.1 mg/kg/day, when considering average participant body weight. The dose used in our clinical trial however was the

maximum dose supported with enough experience to obtain ethics approval, and lower doses have demonstrated clinical efficacy in other central nervous system disorders such as multiple sclerosis[182].

Western blot analysis focusing upon levels of astrocyte and microglial markers, GFAP and CD11b respectively, was preformed for each of the preclinical hyperalgesia and allodynia experiments, however results were largely inconclusive, perhaps as each group only included tissue samples from four animals. Further studies with greater sample numbers for western blotting could provide additional information to verify the role of glial activation in the pain facilitation observed behaviourally. We did see clear results indicating, as expected, greater activation of both astrocytes and microglia in the chronic constriction surgery animals across the board, compared to animals that did not undergo surgery. Although we did not observe differences in glial activation markers at the spinal level, in the trigeminal ganglion tissue we saw a large increase in GFAP and CD11b following codeine exposure that was significantly attenuated by ibudilast. Given the potential differences between glial reactivity in the trigeminal ganglion and lumbar spinal cord, as suggested by some western blotting results, additional studies quantitating cutaneous sensitivity at the trigeminal level would be of interest and relevant to our headache hypothesis.

The results from the preclinical studies within this PhD project further support the co-administration of ibudilast with opioids in the clinical setting, as our finding of reduced hyperalgesia sits alongside others showing potential for reduced hyperalgesia without altering analgesia, perhaps a combination product with codeine/morphine and ibudilast could be trialled in the future with the aim of providing better long-term analgesia.

5. CONCLUSIONS

In summary, the work conducted as part of this PhD has led to the novel finding that chronic codeine administration is able to produce both hyperalgesia and allodynia. The degree of hyperalgesia is no different from that induced by morphine, yet codeine provides significantly less analgesia, indicating the risk:benefit ratio for codeine may be higher than for morphine in terms of pain management. Codeine-induced increases in pain sensitivity appear to be mediated by IL-1 and TLR4. Treatment with ibudilast could represent a new way to manage such pain states, especially given its favourable safety profile, as demonstrated in our clinical trial using 80 mg doses of ibudilast daily on a relatively long-term basis. Although the clinical trial of ibudilast for the treatment of MOH in patients overusing opioids did not provide evidence of efficacy while opioid overuse continued, our hypothesis that glial activation links opioid-induced hyperalgesia and MOH induced by opioids remains as a plausible explanation underpinning the pathophysiology of headache chronification following opioid exposure. Future trials using ibudilast as an add-on therapy to improve ease of opioid withdrawal during standard MOH detoxification procedures would be of great clinical interest.

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APPENDIX

APPENDIX 1. INTERNATIONAL CLASSIFICATION OF HEADACHE DISORDERS CRITERIA FOR HEADACHE INDUCED BY CHRONIC USE OR EXPOSURE.

Table A1. International Headache Society criteria for headache induced by chronic use of exposure published in 1988 (International Classification of Headache Disorders-I)[35].

International Classification of Headache Disorders Criteria for Headache induced by chronic use or exposure
8.2 General Criteria: A. Occurs after daily doses of a substance for ≥3 months B. A certain minimum dose should be indicated C. Headache is chronic (15 days or more a month) D. Headache disappears within 1 month after withdrawal of the substance
8.2.1 Ergotamine Overuse Headache: A. Is preceded by daily ergotamine intake (oral 2 mg, rectal 1 mg) B. Is diffuse, pulsating and distinguished from migraine by absent attack pattern and/or absent associated symptoms
8.2.2 Analgesic Abuse Headache: One or more of the following: 1. ≥50 g of aspirin a month or equivalent of other mild analgesics 2. ≥100 tablets a month of analgesics combined with barbiturates or other non-narcotic compounds 3. One or more narcotic analgesics

APPENDIX 2. DIAGNOSTIC AND STATISTICAL MANUAL OF MENTAL DISORDERS EDITION IV CRITERIA FOR SUBSTANCE DEPENDENCE

Table A2. Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, criteria for substance dependence

Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV) criteria for substance dependence
Tolerance and Physical Dependence 1. Existence of tolerance (either 1a or 1b) a. A need for markedly increased amounts of the substance to achieve intoxication or desired effect b. Markedly diminished effect with continued use of the same amount of the substance 2. Existence of withdrawal symptoms
Lack of control over substance use 3. Intake of larger doses or for longer periods than intended 4. Unsuccessful efforts to reduce or stop substance use 5. Excess time spent to obtain the substance, use the substance or recover from its effects 6. Reduction of normal social, occupational or recreational activities because of substance use
Substance overuse 7. Continued substance use despite knowledge of potential physical problems likely to be caused or exacerbated by the substance

APPENDIX 3. HOSPITAL ANXIETY AND DEPRESSION SCALE

INSTRUCTIONS FOR CALCULATING HADS RESULTS

Participant to tick the most appropriate answer for each questions
Add the scores corresponding to all answers to questions marked 'A' for anxiety total. Add the scores corresponding to all answers to questions marked 'D' for the depression score.
To assign anxiety and depression categories: 0-7=normal, 8-10=Borderline abnormal, 11-21=abnormal.

I feel tense or 'wound up':	**A**	**I feel as if I am slowed down:**	**D**
☐ Most of the time	3	☐ Nearly all the time	3
☐ A lot of the time	2	☐ Very often	2
☐ From time to time, occasionally	1	☐ Sometimes	1
☐ Not at all	0	☐ Not at all	0
I still enjoy the things I used to enjoy:	**D**	**I get sort of frightened feeling like 'butterflies' in the stomach:**	**A**
☐ Definitely as much	0	☐ Not at all	0
☐ Not quite so much	1	☐ Occasionally	1
☐ Only a little	2	☐ Quite often	2
☐ Hardly at all	3	☐ Very often	3
I get a sort of frightened feeling as if something awful is about to happen:	**A**	**I have lost interest in my appearance:**	**D**
☐ Very definitely and quite badly	3	☐ Definitely	3
☐ Yes, but not too badly	2	☐ I don't take as much care as I should	2
☐ A little, but it doesn't worry me	1	☐ I may not take quite as much care	1
☐ Not at all	0	☐ I take just as much care as ever	0
I can laugh and see the funny side of things:	**D**	**I feel restless as I have to be on the move:**	**A**
☐ As much as I always could	0	☐ Very much indeed	3
☐ Not quite so much now	1	☐ Quite a lot	2
☐ Definitely not so much now	2	☐ Not very much	1
☐ Not at all	3	☐ Not at all	0
Worrying thoughts go through my mind:	**A**	**I look forward with enjoyment to things:**	**D**
☐ A great deal of the time	3	☐ As much as I ever did	0
☐ A lot of the time	2	☐ Rather less than I used to	1
☐ From time to time, but not too often	1	☐ Definitely less than I used to	2
☐ Only occasionally	0	☐ Hardly at all	3
I feel cheerful:	**D**	**I get sudden feelings of panic:**	**A**
☐ Not at all	3	☐ Very often indeed	3
☐ Not often	2	☐ Quite often	2
☐ Sometimes	1	☐ Not very often	1
☐ Most of the time	0	☐ Not at all	0
I can sit at ease and feel relaxed:	**A**	**I can enjoy a good book or radio/TV program:**	**D**
☐ Definitely	0	☐ Often	0
☐ Usually	1	☐ Sometimes	1
☐ Not often	2	☐ Not often	2
☐ Not at all	3	☐ Very seldom	3

*Adapted from Zigmond AS, Snaith RP. The hospital anxiety and depression scale. Acta Psychiatr Scand. 1983;67(6):361-370.

APPENDIX 4. HEADACHE IMPACT TEST (HIT-6) QUESTIONNAIRE

HIT-6 HEADACHE IMPACT TEST

This questionnaire was designed to help you describe and communicate the way you feel and what you cannot do because of headaches.

Please tick one answer for each question.

QUESTIONS	Never	Rarely	Sometimes	Very often	Always
When you have headaches, how often is the pain severe?	☐	☐	☐	☐	☐
How often do headaches limit your ability to do usual daily activities including household work, work, school or social activities?	☐	☐	☐	☐	☐
When you have a headache, how often do you wish you could lie down?	☐	☐	☐	☐	☐
In the past 4 weeks how often have you felt too tired to do work or daily activities because of your headaches?	☐	☐	☐	☐	☐
In the past 4 weeks how often have you felt fed up or irritated because of you headaches?	☐	☐	☐	☐	☐
In the past 4 weeks how often did headaches limit your ability to concentrate on work or daily activities?	☐	☐	☐	☐	☐
COLUMN SCORES[#] (study staff only)					
TOTAL HEADACHE IMPACT SCORE (study staff only)					
HEADACHE IMPACT CATEGORY (study staff only)					

* Adapted from http://www.headachetest.com/HIT6/PDFS/English.pdf, The Headache Impact Test™ is a trademark of Quality Metric, Inc. Based upon: Kosinski, M., Bayliss, M., Bjorner, J., Ware Jr, J., Garber, W., Cady, R., Dahlof, C., Dowson, A. & Tepper, S. (2003) "A six-item short-form survey for measuring headache impact: The HIT-6™" *Quality of Life Research*, 12, 963-974.

CALCULATING HIT-6 RESULTS		
First calculate and record the sum of all answers using the allocation of points below:		
PARTICIPANTS ANSWER:	Never	= 6 points
	Rarely	= 8 points
	Sometimes	= 10 points
	Very often	= 11 points
	Always	= 13 points
Next determine and record the participants headache impact category as below:		
PARTICIPANTS SCORE:	<49	= Little to no impact
	50-55	= Some impact
	56-59	= Substantial impact
	>60	= Severe impact

APPENDIX 5. HEADACHE DIARY

HEADACHE DIARY* Please fill in the headache diary **each day**, reflecting on the last 24 hours:

		DATE: ___/____/____ **INITIALS:**	**DATE: ___/____/____** **INITIALS:**	**DATE: ___/____/____** **INITIALS:**
Tick to confirm you have taken the study medication as directed by the study staff:		☐Morning ☐Evening	☐Morning ☐Evening	☐Morning ☐Evening
1. How many hours of headache have you experienced over the last 24 hours?				
2. Rate the average pain intensity of your headache out of 10, where 0=no headache and 10=worst headache imaginable				
3. Was the headache: (tick one answer only)		☐Right-sided ☐Left-sided ☐Both sides	☐Right-sided ☐Left-sided ☐Both sides	☐Right-sided ☐Left-sided ☐Both sides
4. Was the headache:		☐Throbbing/pulsating ☐Pressing/tightening	☐Throbbing/pulsating ☐Pressing/tightening	☐Throbbing/pulsating ☐Pressing/tightening
5. Did the headache change with movement or physical activity?:		☐Unchanged ☐Worse ☐Better	☐Unchanged ☐Worse ☐Better	☐Unchanged ☐Worse ☐Better
6. Did you suffer nausea?		☐Yes ☐No	☐Yes ☐No	☐Yes ☐No
7. Were you bothered by:		☐Lights ☐Sounds ☐Smells	☐Lights ☐Sounds ☐Smells	☐Lights ☐Sounds ☐Smells
8. Did anything trigger this headache? If yes, please specify:				
9. Did you take any medicine to treat your headache? Please record **every different medication** you take for your headache(s) and **every time** you take it. If you require more space to record headache medications taken, please use the additional recording sheet provided at the end of this booklet.	Name:			
	Amount:			
	Time:			
	Name:			
	Amount:			
	Time:			
	Name:			
	Amount:			
	Time:			
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	Amount:			
	Time:			
	Name:			
	Amount:			
	Time:			

* Adapted from Nappi G, Jensen R, Nappi R, Sances G, Torelli P, Olesen J. Diaries and calendars for migraine. A review. Cephalalgia. 2006;26(8):905-916.

HEADACHE DIARY* **ADDITIONAL RECORDING SHEET**

Please use this additional recording sheet if you require more space to record the medications you take to treat your headache(s). Please only use one column per day and make sure to write the date at the top of each column you use.

MEDICATION	DATE: ____/____/____ INITIALS:	DATE: ____/____/____ INITIALS:	DATE: ____/____/____ INITIALS:
Name:			
Amount:			
Time:			
Name:			
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APPENDIX 6. TYPES OF OPIOID ANALGESICS CONSUMED BY PARTICIPANTS IN THE CLINICAL TRIAL OF IBUDILAST IN THE MANAGEMENT OF MEDICATION OVERUSE HEADACHE.

Table A3. Types of analgesics used by participants at baseline and through out the clinical trial of ibudilast in the management of medication overuse headache. Note: percentages total >100% as many participants were consuming more than one type of opioid analgesic.

Type of opioid use	Ibudilast % (n)	Placebo % (n)
Over-the-counter paracetamol + codeine combination products	60% (9)	32% (6)
Over-the-counter NSAID/aspirin + codeine combination products	53% (8)	26% (5)
Prescription paracetamol + codeine combination products	27% (4)	37% (7)
Oxycodone	13% (2)	16% (3)
Tramadol	13% (2)	5.3% (1)
Dextropropoxyphene + paracetamol combination products	7% (1)	5.3% (1)
Morphine	7% (1)	5.3% (1)
Hydromorphone	7% (1)	0% (0)
Pethidine	7% (1)	0% (0)

APPENDIX 7. ADVERSE EVENTS REPORTED DURING THE CLINICAL TRIAL OF IBUDILAST IN THE MANAGEMENT OF MEDICATION OVERUSE HEADACHE.

Table A4. Adverse events reported by treatment group in the clinical trial of ibudilast in the management of medication overuse headache. *Indicates significant difference in incidence between groups assess via two sample t-tests between percentages.

Body system	Adverse event	Ibudilast (%)	Placebo (%)	P value
Neurological	Worsening headache	2 (13.3%)	2 (10.5%)	0.80
	Migraine	2 (13.3%)	0 (0%)	0.11
	Dizziness	1 (6.7%)	1 (5.3%)	0.86
	Feeling dazed	1 (6.7%)	0 (0%)	0.26
	Drowsiness	1 (6.7%)	0 (0%)	0.26
	Irritability	1 (6.7%)	1 (5.3%)	0.86
	Depressed mood	1 (6.7%)	0 (0%)	0.26
	Narcolepsy	1 (6.7%)	0 (0%)	0.26
	Hand tremor	0 (0%)	1 (5.3%)	0.37
Gastrointestinal	Nausea	10 (66.7%)	2 (10.5%)	**<0.01***
	Diarrhoea	2 (13.3%)	0 (0%)	0.11
	Tooth ache	1 (6.7%)	0 (0%)	0.26
	Sour taste in mouth	0 (0%)	1 (5.3%)	0.37
	Stomach cramping	0 (0%)	1 (5.3%)	0.37
Respiratory	Sinus infection	2 (13.3%)	0 (0%)	0.11
	Respiratory infection	1 (6.7%)	2 (10.5%)	0.70
	Coryzal symptoms	1 (6.7%)	0 (0%)	0.26
Ear, eyes, nose and throat	Ear infection	0 (0%)	3 (15.8%)	0.12
	Throat infection	0 (0%)	1 (5.3%)	0.37
	Dry eyes	0 (0%)	1 (5.3%)	0.37
Integumentary	Intermittent pruritus	2 (13.3%)	1 (5.3%)	0.42
	Worsening Eczema	0 (0%)	1 (5.3%)	0.37
Hepatic	Elevated GGT	0 (0%)	1 (5.3%)	0.37
Genitourinary	Poor urine flow	0 (0%)	1 (5.3%)	0.37
Cardiovascular	Chest pain	0 (0%)	1 (5.3%)	0.37
General	Feeling hot	2 (13.3%)	0 (0%)	0.11
	Increased perspiration	1 (6.7%)	0 (0%)	0.26