The Treatment of Neuropathic Pain with Cannabinoid Based Nanotechnology

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ABSTRACT

The current treatment strategies for neuropathic pain are only partially effective at best. There is a drive to find more effective treatments for chronic forms of pain such as neuropathic pain. Cannabinoids are drugs that are derived from the cannabis plant or mimic the effects or structure of plant-derived cannabinoids. Cannabinoids are moderately effective in the treatment of neuropathic pain. Previous research has investigated several ways of selectively targeting the different cannabinoid receptors to avoid psychoactive side effects. Selective targeting has been unsuccessful as cannabinoids are highly lipophilic and readily enter the brain causing side effects. Current research has shown that different receptor selective cannabinoids typically produce side effects at the therapeutic doses required to treat neuropathic pain.

The research in this thesis investigated the use of nanotechnology for the synthesis of water soluble nanoparticles containing the potent cannabinoid WIN 55,212-2 (WIN). The drug was created to selectively target the site of inflammation in the spinal cord caused by neuropathic pain, while failing to pass through the blood brain barrier. The cannabinoid WIN was successfully encapsulated inside a styrene maleic acid (SMA) based micelle. The now water soluble micelles were assessed for size and release to ensure adequate properties. Of the micelles produced, a loading of 21 % WIN to SMA was decided on for the behavioural studies, due to this loading of micelle having slow release properties and a large diameter. The large size ensured that the micelle would remain in the circulation and not pass fenestrations of the kidneys. The large diameter also ensured they should not pass the blood brain barrier and produce side effects. The release properties of the micelle suggested that it would be relatively stable within the circulation. These properties of enhanced permeability and
retention effect were expected to allow the micelles to bioaccumulate in the area of inflammation in the spinal cord.

Using the chronic constriction injury model of sciatic neuropathy, the SMA-WIN micelles were efficacious in the treatment of neuropathic pain for a prolonged period of time compared to the positive control WIN. Pain relief occurred for up to 8 hours at a dose of 11.5 mg/kg of SMA-WIN micelle. To evaluate cognitive impairment the rotarod assessment was utilised. Results showed initial impairment caused by SMA-WIN micelles to be identical to WIN controls for up to 1.5 hours. These results probably indicate motor impairment from an initial release of WIN from the micelle. However, the SMA-WIN micelle was still able to produce prolonged analgesia with decreased side effects compared to the fat soluble cannabinoids.

The effectiveness of the nanotechnology for the treatment of neuropathic pain is promising. This study has found that the formulation of nanoparticles for the treatment of neuropathic pain may be an exciting new field of research and should be investigated further.
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<tr>
<td>CB1</td>
<td>Cannabinoid receptor type 1</td>
</tr>
<tr>
<td>CB2</td>
<td>Cannabinoid receptor type 2</td>
</tr>
<tr>
<td>CCI</td>
<td>Chronic constriction injury</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>EDAC</td>
<td>N-(3-Dimethylaminopropyl)-N’-ethylcarbiduamide hydrochloride</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>EPR</td>
<td>Enhanced permeability and retention</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GTPγS</td>
<td>Guanosine 5'-O-[gamma-thio]triphosphate</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
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<tr>
<td>IASP</td>
<td>International Association for the Study of Pain</td>
</tr>
<tr>
<td>I.V.</td>
<td>Intravenous</td>
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<td>NaOH</td>
<td>Sodium hydroxide</td>
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CHAPTER ONE

INTRODUCTION

The aim of this thesis is to test the efficacy of micellar delivery of a potent synthetic cannabinoid at reducing neuropathic pain in a rat model. In this introductory chapter the problem of neuropathic pain will be discussed, including its characteristics and prevalence, as well as problems with treatment. Cannabinoids will then be introduced, particularly with respect to their potential as therapeutic agents for neuropathic pain. Finally, the strategy of using nano-micelles to deliver cannabinoids for neuropathic pain will be discussed.

1.1. Neuropathic pain

The current definition of neuropathic pain as provided by a special interest group of the International Association for the Study of Pain (IASP) is ‘pain caused by lesion or disease of the somatosensory nervous system’ (Treede et al., 2008). However, in practice neuropathic pain is hard to classify. This is due to the fact that neuropathic pain is not a single disease, but a syndrome that is caused by a range of different diseases and lesions which manifests in a range of symptoms and signs (Jensen et al., 2011). The current definition by IASP allows a wider range of aetiologies to fall under the umbrella of neuropathic pain. This means neuropathic pain can be viewed as a change in pathological neural plasticity, rather than the underlying cause of the disease leading to neuropathic pain. The causes of peripheral neuropathic pain or central neuropathic pain are now recognised to include; infections, trauma, metabolic abnormalities, chemotherapy, surgery, irradiation, neurotoxins, inherited neuro-degeneration, nerve compression, inflammation, and tumour infiltration (Dworkin et
al., 2003). Some of the most common forms of central neuropathic pain arise from spinal cord injury, stroke, or multiple sclerosis (Ducreux et al., 2006).

Regardless of the cause, neuropathic pain results in maladaptive changes in injured sensory neurons along the entire nociceptive pathway. This can result in spontaneous pain and/or hypersensitivity to painful stimuli. The resulting symptoms vary enormously from individual to individual. Patients can experience symptoms of persistent or paroxysmal pain independent of a stimulus. Alternatively they may experience stimulus evoked pain such as increased sensitivity to pain (hyperalgesia) and/or resultant pain from a stimulus that would not normally cause pain (allodynia) (Woolf et al., 1999).

1.2. Prevalence of neuropathic pain

Neuropathic pain has only recently been shown to be highly prevalent within the general population, with consistent findings in large scale population studies. A UK study surveyed a total of 6000 adults and reported 48 % prevalence for any type of chronic pain, and a prevalence of 8.2 % for pain predominantly of neuropathic origin (Torrance et al., 2006). Similar findings were found by Bouhassira et al., (2008) in a French nationwide postal survey involving 30,155 adult participants. The study reported the prevalence of chronic pain at 31.7 %, and of chronic pain with neuropathic origin at 6.9 %. A more recent study in South America that surveyed 1597 adults found a 42 % prevalence of chronic pain, and a 10 % prevalence of neuropathic origin (Brandao De Moraes Vieira et al., 2012). In contrast an Austrian survey reported a prevalence of neuropathic pain at 3.2 % (Gustorff et al., 2008). The differences in the ratios may be explained by the fact that the Austrian survey was based on an internet questionnaire, and it is likely that this was less accessible to older people. These
widespread multiregional findings suggest that neuropathic pain is highly prevalent across the general population and this is a cause for concern.

Neuropathic pain can have an increased prevalence within subpopulations of patients with underlying medical conditions. In patients with spinal cord injuries the mean prevalence of neuropathic pain is reported to be between 40 % and 46 %, and often becomes chronic over time, with an increased prevalence over time (Budh et al., 2003; Siddall et al., 2003; Werhagen et al., 2004). An even higher prevalence of phantom limb pain exists (a type of neuropathic pain) in amputees, with 72 % to 80 % of patients suffering from the condition (Dijkstra et al., 2002; Ephraim et al., 2005; Richardson et al., 2006).

Neuropathic pain prevalence is not only increased in trauma patients, common diseases such as diabetes also predisposes certain individuals to neuropathic pain. A study by Van Acker et al. (2009) showed diabetic polyneuropathy with neuropathic pain occurred in 14 % of diabetic patients. Neuropathic pain was higher in type 2 diabetic patients (17.9 %) than in type 1 diabetic patients (5.8 %). This prevalence increased with patient age and diabetes duration. The presence of neuropathy in HIV patients ranges in studies between 19 % and 49 %, depending on the study (Cherry et al., 2009; Maritz et al., 2010; Smyth et al., 2007). Another neurological disorder, multiple sclerosis also increases the risk of neuropathic pain, with a meta-analysis showing a risk factor of 28 % (Foley et al., 2012). In stroke victims, 16 % develop central neuropathic pain in the year following a cerebral venous thrombus (Bugnicourt et al., 2011). As these conditions have a high prevalence in the general population, neuropathic pain may be a greater burden on society than was previously realised.

Apart from the human burden of neuropathic pain, the associated costs of neuropathic pain should also be considered. These include not only direct medical expenditures but also loss of productivity. Quantifying the direct burden of neuropathic pain is difficult due to the fact that neuropathic pain patients often generate expenses related to other co-morbidities, which
confound the results (OConnor, 2009). Co-morbidities were also shown to increase in neuropathic pain patients (Berger et al., 2004). Two studies have calculated the direct medical expenditure using age and sex matched controls. The first study used US insurance claims from the year 2000 (Berger et al., 2004). Over one year the medical expenses of the neuropathic pain patients were three fold higher than the medical costs of patients without neuropathic pain ($US 17 355 vs. $US 5715, respectively) (Berger et al., 2004). A Canadian study compared the average annual number of physician visits for patients. It found significantly higher number of visits in the neuropathic pain patients than in the matched controls (14.7 vs. 6.4, respectively). The cost to health care resources was significantly higher in the neuropathic pain patients ($CAD 4,163 versus $CAD 1,846, respectively) (Lachaine et al., 2007). Neuropathic pain impairs people’s ability to work, further adding to the economic burden. A European cross-sectional survey measured that 43 % of neuropathic pain patients reported impairment in their employment status, and 17 % were disabled and unable to work due to neuropathic pain (McDermott et al., 2006). Neuropathic pain is clearly detrimental to our society, both to the individuals that suffer reduced quality of life, and socioeconomically.

1.3. Current treatment strategies

Current first line treatments for neuropathic pain give most patients at best only partial relief. These include tricyclic antidepressants and serotonin-noradrenaline reuptake inhibitors, anticonvulsants, and topical lidocaine (Dworkin et al., 2007). The side effects of these treatments along with only partial efficacy limit their effectiveness as therapeutics (Dworkin et al., 2007). Meta-analysis shows that tricyclic antidepressants only provide a 50 % reduction in pain intensity in 30 % of individuals with neuropathic pain (Finnerup et al., 2007; Sindrup et al., 2000). Second line treatments include tramadol and opiates. These have been shown to
be more effective with acute use but have serious long term safety issues. Opiates lose their efficacy with repetitive dosing and patients run the risk of addiction. Opiates can even enhance hyperalgesia (Fields, 2011). The lack of effective and safe treatments is clearly problematic, and has stimulated an interest in cannabinoid drugs as a novel treatment option.

1.4. Nociception

Nociceptive pain is the ability of the human body to detect the presence of potentially damaging stimuli as an early warning mechanism (von Hehn et al., 2012). The International Association for the Study of Pain (IASP) describes pain as ‘an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage’ (Loeser et al., 2008). Nociception therefore has an important role in human physiology. The sensation of pain is mediated by the nociceptors, the primary sensory neurons which transmit information via nociceptive pathways in the spinal cord to the brain. There are two main types of nociceptive pain sensations, fast and slow pain. Fast pain is mediated primarily by the thinly myelinated Aδ fibres and slow pain transmitted by unmyelinated C fibres (Dubin et al., 2010). These nociceptive neurons express specialised transducer ion channel receptors - mainly transient receptor potential channels - which are tuned to chemical mediators. Nociceptive receptors, termed nociceptors, have high thresholds and normally only respond to stimuli of sufficient energy to potentially or actually damage tissue (Julius et al., 2001). A variety of nociceptors have different forms of receptor expression on the nociceptive neurons, leading to a variety of different pain perceptions. Activation of nociceptors requires that large stimuli depolarise peripheral terminals with a sufficient amplitude and duration, which ensures a prevention of miscoded impulses (Dubin et al., 2010). These high threshold nociceptors are activated by intense mechanical, thermal, or chemical stimuli, and feed this
information to nociceptive neurons in the spinal cord which project via the thalamus to cortical areas in the brain (von Hehn et al., 2012).

1.5. Inflammatory pain

Inflammatory pain can give rise to neuropathic pain, yet the two are distinct pathologies. Injury to tissue induces an inflammatory response causing the release of cytokines, chemokines, and other inflammatory mediators. During inflammation, normally innocuous stimuli can produce pain and responses to noxious stimuli are exaggerated and prolonged. Increased sensitivity occurs at the inflamed area and in surrounding non-inflamed tissue. This is a result of plasticity in peripheral nociceptors and central nociceptive pathway, caused by the inflammatory mediators (Costigan et al., 2009). Hypersensitivity provides a protective physiological response, causing decreased use of the injured area, and so protecting it against subsequent damaging stimuli. Removal of nociceptive neurons, which express a tetrodotoxin-resistant sodium channel, eliminates inflammatory pain but leaves neuropathic pain intact. Inflammatory pain normally disappears after the resolution of the initial injury, marking one major difference between inflammatory pain and neuropathic pain (Huang et al., 2006).

1.6. Endogenous pain inhibition

Endogenous mechanisms exist that diminish pain through inhibition of nociceptive processing. Endogenous pain may be modulated through facilitation or inhibition of nociceptive input by three main mechanisms, which modulate the perceived level of pain. Firstly, modification of synaptic strength by long term potentiation and long term depression
in the spinal dorsal horn may increase or decrease transmission of nociceptive signals to the brain. At the level of the spinal cord, local dorsal horn interneurons are responsible for both feed-forward and feed-back modulation to second order pain neurons. These interneurons involve GABA, glycine or opioids neurotransmitters, with the GABAergic neurons highly expressing cannabinoid receptors (Salio et al., 2002; Seifert et al., 2009). Recruitment of inhibitory interneurons can occur. They can also be suppressed by activation of low threshold mechanoreceptors. Mechanoreceptors activate Aβ afferents nerves, which result in temporarily inhibited nociceptive responses from Aδ and C fibres. This produces a reduction in pain sensation (Galan et al., 2004). Finally, descending inhibition originates in the brainstem, mainly the periaqueductal gray and nucleus raphe magnus. This exerts top-down modulation of nociceptive input at the spinal level. The descending pain modulation system produces hypoalgesia by inhibiting sensory processing within the dorsal horn of the spinal cord. These descending pathways are all regulated by the cannabinoid, serotonergic, noradrenergic and opioid systems (Heinricher et al., 2009). Endocannabinoids can regulate pain sensitivity through endogenous pain inhibition. Exogenous cannabinoids therefore modulate endogenous pain inhibition, therefore producing a variety of results such as nociception (Ossipov et al., 2010).

1.7. Development of peripheral sensitisation in neuropathic pain

Neuropathic pain usually develops from damage to the neuronal system, which results in a cascade of events. These events normally begin locally, but lead to changes in synaptic plasticity and inflammation in the central pain pathways. Most insults initiate peripherally, such as neuronal damage which causes peripheral sensitisation. At the early onset from the peripheral insult, neutrophil granulocyte infiltration occurs next to the immediate vicinity of
the lesion site. This releases chemo-attractants and cytokines, resulting in the recruitment and activation of macrophages (Perkins et al., 2000). These immune cells plus a variety of other monocytes are recruited, and they release cytokines, chemokines, and other inflammatory mediators. The inflammatory mediators produced activate these changes to the nociceptive neurons (Dray, 1995). The threshold for neuronal activation is then reduced and neuronal excitability increases via the insertion of transducer channels and voltage-gated ion channels. This is evident by the spontaneous activity and reduced threshold in both injured and neighbouring uninjured nociceptive afferents (Decosterd et al., 2000; Sorkin et al., 1997).

1.8. Development of central sensitisation in neuropathic pain

Central sensitisation (i.e., pain sensitisation that involves the central nervous system) also often occurs in neuropathic pain, and is a form of use-dependent synaptic plasticity. The rapid firing and highly active nociceptors cause the on-going activity of injured nerves to trigger central sensitisation. This was first shown through high-frequency stimulation of primary afferent fibres. The primary afferent fibres then produced long-term potentiation (LTP) or long-term depression (LTD) of fast excitatory post synaptic potentials, in the spinal cord dorsal horn neurons that are associated with nociception (Randic et al., 1993). This has been further shown with even relatively low-frequency afferent barrage, which is similar to the continuous signals in inflammation, and some types of neuropathic pain (Ikeda et al., 2006). Central sensitisation explains how facilitation develops after the end of conditioning stimuli. Once generated it can remain autonomous, or only require a very low level input to produce perceived nociception. In central sensitisation not only does homosynaptic potentiation occur, but hetrosynaptic potentiation (where subsequent responses are amplified to other non-stimulated non-nociceptor or nociceptor fibres) can also occur (Woolf, 2011). This increased
neuronal activation occurs via four main methods; first, a presynaptic surge in excitatory transmitter release causing increased synaptic strength (Chen et al., 2010); second, an increase in post-synaptic response to the neurotransmitter (Kawasaki et al., 2004); third, a reduction in inhibitory neurons causing less inhibition (Moore et al., 2002); and finally, increased membrane excitability of nociceptive related neurons resulting in subthreshold action potentials turning into suprathreshold action potentials (Balasubramanyan et al., 2006).

However, not only is central sensitisation dependent on synaptic plasticity, but also on changes in microglia, astrocytes, and gene transcription (Colburn et al., 1999; Ji et al., 2003). Astrocytes are a type of glial cell that are responsible for maintenance of endothelial cells in the blood brain barrier, nutrient support of neuronal cells, extracellular ion balance, and repair within the central nervous system (CNS) (Faulkner et al., 2004). Microglia, another type of glial cell functions as a resident immune cell in the brain and spinal cord, and plays an active immune defence roll. Microglia can be activated through changes in the microenvironment, and act the part of a macrophage immune cell (Rabchevsky et al., 1998). Activation of these glial cells has been shown to be essential for the development of neuropathic pain in animal models (Tsuda et al., 2003). Blocking the activation of microglia and astrocytes attenuates the development of neuropathic pain, with the microglia having the more prominent roll (Ledeboer et al., 2005; Mika et al., 2009).

The glial cells have been shown to be essential for the induction and maintenance of pain with a number of mechanisms proposed. Suggested mechanisms involve activation of mitogen-activated kinases, up regulation of P2 purinoceptors, and increased pro-inflammatory cytokines (Gao et al., 2010; Ikeda et al., 2012). Microglial markers have been shown and demonstrated to have significantly increased in neuropathic pain models, whereas astrocyte markers undergo a smaller increase. However an increase in both cell types can result in hyper excitability in the spinal dorsal horn (Ikeda et al., 2012). Central sensitisation also
results in astrocyte-mediated and localised breakdown of the blood brain barrier. This occurs at the level of the spinal cord, where neuroplasticity occurs. Breakdown of the blood brain barrier can result in neurodegeneration (Gordh et al., 2006). These inflammatory measurements are prolonged and can be shown to be increased past 35 days following injury at well above basal levels (DeLeo et al., 1997). All these factors combine to produce neuropathic pain as a chronic lasting disease, suggesting that neuropathic pain is both a neuronal plasticity and inflammatory mediated disease.

1.9. Cannabinoids

Cannabinoids are a class of compounds that activate the cannabinoid receptors. There are three main groups of cannabinoids, the phytocannabinoids; produced by the cannabis plant, endocannabinoids; produced naturally in the body, and synthetic cannabinoids; synthesised chemically by humans (Pertwee, 2009). Medical Cannabis has been used for centuries for the treatment of pain, with the earliest records coming from China and India. There has only been relatively recent interest from Western society (Zuardi, 2006).

There are two main types of cannabinoid receptors: the Cannabinoid type 1 (CB1) receptor and Cannabinoid type 2 (CB2) receptor (Howlett, 2002). The CB1 receptor is highly expressed in the nervous system and has been shown to be in high densities in neurons involved with nociception (Ahluwalia et al., 2000). The CB1 receptor is located preferentially on the presynaptic membrane, and therefore cannabinoids act as retrograde neurotransmitters. Endogenous cannabinoids are released from the post-synaptic membrane and act on the CB1 receptor to decrease neurotransmitter release (Wilson et al., 2002). The CB2 receptor is associated mainly with immune cells. However, this is controversial and the potential role of
CB2 receptors in the brain and nervous system continues to be investigated (Van Sickle et al., 2005).

Cannabinoids act on G-protein coupled receptors, causing activation of Gi/o proteins. Their actions are diverse and regulate many physiological processes. Neuronal CB1 receptors activate inward rectifying $K^+ \text{ channels}$, and the inhibition of voltage sensitive calcium channels. This reduces neuronal excitation and neurotransmitter release (Vásquez et al., 2003). Activation of the receptors also results in inhibition of adenylyl cyclase, causing a cascade of effects, such as reduction of cyclic adenosine monophosphate, which therefore modulates protein kinase A phosphorylation and gene regulation (Kaminski, 1998; Wade et al., 2004). Finally, regulation of the mitogen-activated protein pathway is thought to be effected through coupling with Gi/o proteins. This is the proposed mechanism for CB2 mediated immunosuppression. However, unlike CB1 receptors, CB2 receptors are thought to not activate ion channels (Felder et al., 1995).

1.10. Cannabinoids as a treatment for neuropathic pain

Cannabinoid receptors expressed in the peripheral nervous system and CNS have been shown to have pain relieving effects. Separating out the effects of cannabinoids on the peripheral nervous system from those in the CNS is difficult due to the lipophilic nature of cannabinoids that readily pass the blood brain barrier. This means that cannabinoids acting on the peripheral CB1 receptor can also cause psychotropic symptoms, by acting on the CB1 receptors in the brain (D’Souza et al., 2009). An early paper showed that WIN55, 212-2 (WIN), a potent CB1 and CB2 receptor agonist, attenuated hyperalgesia and allodynia in a rat model of neuropathic pain (Bridges et al., 2001). This result was repeated ten years later using another non-selective
agonist, CP 55,940 (Sain et al., 2009). Both studies showed attenuation of hyperalgesia and allodynia. Where the studies differentiated was Bridges et al. (2001) used a CB1 antagonist SR 141716 to demonstrate pain relief was occurring via the CB1 receptor, while Sain et al. (2009) used CB1 and CB2 knockout mice. The results showed the anti-nociceptive effects occurring via the CB1 receptor, not the CB2 receptor.

Despite these findings the ability of the CB2 receptor to mediate nociception still remains controversial. Many studies have contradicted one another over whether the CB2 receptor can mediate nociceptive pain relief, or if in fact the pain relief occurs via the CB1 receptor through non-specific targeting. Some supporting articles argue for CB1 mediated pain relief (Choong et al., 2007; Kehl et al., 2003; Liu et al., 2006), while other research suggests CB2 selective agonists can mediate the effect (Valenzano et al., 2005; Whiteside et al., 2007). The consequence of this is that if nociception is also CB2 mediated, there could be a potential therapeutic target without the psychotropic effects.

Finally a study carried out in this research lab concluded that the CB1 receptor and not the CB2 receptor is the target in cannabinoid mediated spinal anti-nociception in the chronic concentration injury model (Brownjohn et al., 2012). Using intrathecal administration the experiment administrated selective CB1/CB2 agonists and non-selective agonists. It was argued that the drug concentrations were small enough to avoid acting at the periphery, and due to the lipophilic nature of cannabinoids they should not be acting in the brain. The investigation found that the non-selective CB1/CB2 receptor agonist, WIN reversed mechanical allodynia. In contrast neither of the two CB2 selective agonists, GW405833 and JWH-133, produced a reversal. Brownjohn et al., (2012) also corroborated these results using a [35S] GTPγS binding assay, as both CB2 selective agonists were shown to be insufficiently specific for the detection of functional CB2 in native tissue, while non-selective WIN elicited a result. These results are consistent with other studies, such as (Choong et al., 2007; Kehl et
al., 2003; Liu et al., 2006). The results suggest that anti-nociception is CB1 mediated; therefore psychotropic effects appear to be the leading limitation in cannabinoid treatment for neuropathic pain. If CB2 mediated effects are occurring it is at high doses where the drugs are non-specific and therefore also likely to be acting on the CB1 receptor and also causing side effects. Therefore CB2 agonists could be having an effect, but the controversial and inconsistent results of effective CB2 treatments (discussed above) indicate that the effect size is unlikely to be large enough to be useful clinically. As clearly defined pain relief is needed, and only a small effect size is seen, this leads to the conclusion that CB2 agonists are an unviable treatment for neuropathic pain.

Peripheral cannabinoid induced anti-nociception has been shown to be effective in treating neuropathic pain. A study by Yu et al. (2010) showed that CB1 knockout mice had significantly reduced analgesia produced by local and systemic delivery of cannabinoids using AZ11713908 (a peripherally restricted CB1R agonist) and that CB2 knockout mice were unaffected. Yu et al. (2010) concluded that cannabinoids exerted their pain relief peripherally, and that the CB1 receptors were essential for this effect. Therefore peripherally acting CB1 agonists are a new and promising treatment for chronic pain type diseases. Similar studies have replicated these findings using a variety of treatments and have further implicated the importance of CB1 mediated peripheral nociception (Clapper et al., 2010). Finally, this conclusion has been corroborated using WIN and knockout mice that had the CB1 nociceptive neurons localised in the peripheral nervous system removed, whilst the central CB1 receptors were left intact. The data showed reduced anti-nociception when WIN was administrated systemically, locally, but not intrathecally (Agarwal et al., 2007). This further emphasises the importance of the CB1 receptors in the periphery and also points out that the cannabinoid receptors in the spinal cord are an important target, as they also reduced neuropathic pain.
In clinical trials the effects of smoked marijuana and synthetic cannabinoids have been extensively reviewed for the chronic condition of neuropathic pain. The CB1/CB2 centrally and peripherally acting cannabinoids have been shown to effectively suppress neuropathic pain in a variety of clinical environments (Rahn et al., 2009). Clinical trials using CB2 selective agonists for the treatment of pain have thus far proven ineffective (Ostenfeld et al., 2011). These results demonstrate the need for a selectively targeted drug for the CB1 receptor.

The difficulty of using CB1 mediated cannabinoids is in achieving an effective therapeutic index with small adverse side effects and maximum efficacy. One study assessed the treatment of polyneuropathies using a variety of current neuropathic pain treatments (Toth et al., 2008). These included cannabinoids, gabapentanoids, tricyclic antidepressants, and anticonvulsants. The study by Toth et al. (2008) showed that most forms of neuropathic pain had comparable (or slightly lower) efficacy to the other pharmacological treatments, with similar intolerable side effects. Similar results have been reproduced in other studies which have demonstrated comparable efficacies and adverse side effects of cannabinoids to other current treatments (Rahn et al., 2009; Watson et al., 2010). The current consensus statement and guidelines from the Canadian Pain Society state that cannabinoids should be considered when other options have failed or are not possible (Moulin et al., 2007). The adverse side effects of cannabinoids for the treatment of neuropathic pain are most commonly reported as dizziness, drowsiness, intoxication, dry eyes, dry mouth, headache, vertigo, ataxia, and hunger (Karst et al., 2003; Nurmikko et al., 2007; Skrabek et al., 2008). Although adverse events are at similar rates to current interventions, cannabinoids demonstrate low toxicity with no reported respiratory depression or changes in heart rate, with high doses being non-lethal.

Overall the use of clinical CB1 mediated cannabinoids offers little or no added benefit to current neuropathic pain treatments. However, in patients that suffer adverse side effects from current treatments, such as tricyclic antidepressants or gabapentanoids, cannabinoids may
offer an alternative treatment that would potentially have fewer side effects for the individuals.

Periphery targeting CB1 receptor drugs could have the potential to provide anti-nociceptive benefit without producing intoxication or other CNS impairing effects. Several peripherally targeting cannabinoids have been developed such as the orally active AZD 1940, a CB1/CB2 agonist with limited blood brain barrier permeability. Although initially successful in animal studies AZD 1940 has so far failed clinically to reduce acute pain at tolerable doses, and still produced a high level of behavioural side effects (Kalliomäki et al., 2013). AZD 1940 was further reported to be unsuccessful at reducing capsaicin induced pain in healthy volunteers (Kalliomäki et al., 2013). Further research into peripherally restricted cannabinoids is currently occurring with a large range of new animal behavioural studies being carried out (Dziadulewicz et al., 2007). So far clinically no other peripherally acting cannabinoid has been assessed. However, a variety of peripherally restricted cannabinoids have produced successful results in animal studies (Brusberg et al., 2009; Fride et al., 2004). Although promising it would be expected that peripherally acting cannabinoids would exert some side effects such as constipation, hypotension, and possibly weight gain by acting on the peripheral cannabinoid receptors (Kruger et al., 2010). Also the evidence discussed previously suggests the spinal cord to be an important target for cannabinoid mediated pain relief. Ideally, if a cannabinoid drug could be designed to target the peripheral nociceptors and the inflamed spinal cord it could provide a more effective treatment of pain. Such a drug could be safer than non-selective cannabinoids for patients in terms of side effects, and would fill a much needed gap in neuropathic pain treatment.
1.11. Selective nanomiceller targeting

A micelle is an aggregate of surfactant molecules dispersed in a liquid colloid. A typical normal phase micelle forms an aggregate in an aqueous solution, with the hydrophilic head in contact with the surrounding solvent, and the hydrophobic tail regions in the micelle centre. Through the formation of micelles it is possible to encapsulate hydrophobic molecules inside micellular compounds, hence forming nanomiceller drugs (Batrakova et al., 2006).

As it is difficult to selectively target cancer tissues rather than normal tissue nanomicelle drugs have been designed for selectively targeting tumours, which have proven problematic in antitumor therapy. Greish et al., (2005) have shown high tumour targeting and efficacy by using micelles containing pirarubicin, an anthracycline antibiotic, which is effective against a variety of cancers. Pirarubicin micelles were shown to have similar cytotoxic effects to free pirarubicin in vitro preparations on breast cancer and colon cancer cell lines. What make this nanomiceller drug superior to the drugs delivered by conventional means, are the effects in vivo as these large molecules accumulate in the tumour. This is due to the enhanced permeability and retention effect (EPR) of macromolecular drugs in solid tumours. In solid tumour tissue there is enhanced vascular permeability due to a variety of factors. The nanomicelles bioaccumulate in the tumour, and enhance toxicity (Maeda et al., 2000). The pirarubicin micelles also avoid renal excretion due to their large nature so they remain in circulation leading to more selective targeting. In animal testing, this preparation at 20 mg/kg leads to complete tumour eradication (Greish et al., 2005). Nanomicelle formulations have been reproduced with a variety of other anti-tumour drugs, such as doxorubicin and zinc protoporphyrin, providing effective treatments (Greish et al., 2004; Nakamura et al., 2011).

For the reasons listed above, if a micelle preparation could be created with cannabinoids it may be possible to selectively target the periphery and spinal cord. As micelle preparations
remain in circulation and cannot pass the blood brain barrier, peripheral cannabinoid receptors could be targeted (Greish, 2010). Nanomicelles act on an area by being internalised into cells by endocytic pathways. This produces a release of the micellar contents in the presence of membrane components. The micelles also gradually release their contents, as seen in release studies (Nakamura et al., 2011). Neuropathic pain also demonstrates localised vasodilatation and increased permeability at the spinal cord (Gordh et al., 2006), allowing targeting of the cannabinoid receptors through the described EPR effect. This method could potentially avoid the psychotropic effects of cannabinoids by causing micelles to accumulate at areas where the blood brain barrier is disrupted, such as in the spinal cord, but not enter the higher functioning areas in the brain.

Therefore I hypothesise that WIN nanomicelles will produce prolonged analgesia in neuropathic pain, with decreased psychoactive side effects. I aim to address this hypothesis by synthesising SMA-WIN micelles and then testing the efficacy and specificity of effects on neuropathic pain with rat behavioural models.
CHAPTER TWO

THE SYNTHESIS AND CHARACTERISATION OF WIN 55,212-2

NANO- DELIVERY SYSTEM USING STYRENE MALEIC ACID

2.1. Introduction

Research into new treatments for neuropathic pain has mainly focused on designing new drugs to target specific receptors. Little to no work has investigated the possibility of improving the pharmacokinetics profile of existing drugs by using new nano-technology. New research in this field could allow for the development of a new category of analgesic agents. Altered mechanisms of delivery could transform existing drugs to target them to specific parts of the body, improving therapeutic ratios.

The pharmacokinetics of a compound often causes treatments to fail and should be a key part of drug design (Singh, 2006). Nanotechnology can modulate pharmacokinetics by forming a drug into a nanoconstruct with entirely new properties. These nanoparticles typically range between 1-100 nm (The Royal Society, 2004). The larger size of nanoparticles in comparison with conventional drugs enables the drug to exploit the EPR effect. The EPR effect of macromolecular drugs occurs due to increased vasodilation of blood vessels. The large size of macromolecular drugs also stops the excretion via the kidneys. This means that in previously conducted research other constructed nanomicelles for the irradiation of tumours have been able to accumulate in the inflamed tissues (Larsen et al., 2009).

Cannabinoids such as WIN are highly hydrophobic and are thus insoluble in water. This causes the half-life of the drug to be very rapid and produce longer effects in areas of the body such as fat deposits (Valiveti et al., 2004). The blood brain barrier readily allows the
hydrophobic drug across into the brain to exert its psychoactive symptoms (Barna et al., 2009). These properties make it unsuitable for targeting the active site of inflammation in the spinal cord. Micelle formulation would make the cannabinoid water soluble and target the drug to the site of increased vasodilation via the EPR effect. Also due to their large size the micelles should not be able to cross the blood brain barrier therefore reducing the psychoactive symptoms (Greish, 2010).

The purpose of this chapter is to describe the development of a new cannabinoid nanoconstruct for the treatment of neuropathic pain, with the focus being on improving current biodistribution and efficacy profiles. One way of creating nanoparticle drugs is by using styrene maleic acid (SMA) micelle formation methods, to encapsulate the cannabinoids. The SMA micellar systems have been previously used to improve the delivery of various anti-tumour drugs, such as; zinc protoporphyrin IX, doxorubicin, Geldanamycin, and pirarubicin, (Daruwalla et al., 2007; Greish et al., 2004; Nakamura et al., 2011). In this micellar system, the styrene core can encapsulate hydrophobic drugs through hydrophobic association, while the maleic acid shell insures its water solubility. As cannabinoids are lipid soluble, the micelle structure should readily be incorporated into the SMA core, creating a stable construct in the macromolecular size range. Ideally this product, once injected, will be subject to the EPR effect and target inflammation at the level of the neuropathic spinal cord. This should result in higher efficiency in treating neuropathic pain with fewer side effects. In addition, due to the prolonged circulatory half-life known for SMA micelles, it also ensures that the drug remains above the minimum therapeutic level for an extended period. Prolonged circulatory half-life can improve the drug effectiveness with the need for fewer injections.

After successful synthesis of the micelle has been achieved, both release rate and size will be determined. It is important to measure release rate to ensure the stability of the drug in the plasma, and that the treatment will reach its target. A high stability of the micelles at
physiological pH prevents rapid release of the free drug from micelles during circulation. This will cause the free drug to not be able to gain access to critical organs and tissues, which would be protected from the side effects from WIN. Such a stable micelle can allow the EPR affect to occur depending on their size, allowing the drug to reach its target successfully (Nakamura et al., 2011). The importance of size determination is due to the fact that drug uptake into inflammatory sites is inversely correlated with the renal clearance of small molecular compounds (Noguchi et al., 1998). Therefore it is necessary to achieve a size over the renal fenestrations of the kidney so the micelle will remain within the circulation. This allows the micelle to undergo the EPR effect and lead to more selective targeting (Greish et al., 2005).

The central aim of this drug synthesis is therefore to create a new nanomicelle drug with superior qualities to the base drug for the treatment of neuropathic pain.

2.2. Methods

2.2.1 Materials

The high potency CB1/CB2 non selective agonist WIN 55,212-2 (WIN) was obtained from Tocris Bioscience (UK). Cumene terminated poly(styrene-co-maleic anhydride) (SMA) was obtained from Sigma-Alderich (St. Louis, MO, USA) and supplied with a 1.3:1 mole ratio of styrene: maleic anhydride, an average $M_n$ of approximately 1600 as determined by Gel permeation chromatography. $N$-(3-Dimethylaminopropyl)-$N'$-ethylcarbiduumide hydrochloride (EDAC) was obtained from Sigma-Alderich (St. Louis, MO, USA) also. Other reagents were of commercial reagent grade and were used without further purification.
2.2.2 Preparation of SMA-WIN 55,212-2 nanomicelles

Preparation of SMA micelles containing WIN was similar to the method previously reported by Larson et al. (2011) and was achieved in three steps. First, poly(styrene-co-maleic anhydride) was hydrolysed under aqueous alkaline conditions. To achieve this deionised water was adjusted to pH 14, by 1 M NaOH with a temperature of 70 °C. SMA was added to the solution under stirring conditions and was maintained at pH 14 and 70 °C for 4 hours. Under these conditions SMA becomes soluble in weak acid or alkaline condition. The subsequent hydrolysed SMA solution was adjusted to pH 7.0 with 0.1 N HCl, and then diluted to a final concentration of 10 mg/mL and allowed to cool to ambient temperature.

The second stage involved internalisation of WIN into the SMA micelle. Varied ratios of SMA, EDAC, and WIN were used to create different loadings. Set volumes of SMA outlined in table 2.2.1 were pipetted into a small beaker under stirring, and diluted to approximately 50 ml with deionised water. The solution was controlled to pH 5.0. WIN was dissolved in minimal dimethyl sulfoxide (DMSO) and then added to the solution while stirring. The amount of DMSO used was just enough to dissolve WIN, normally around 200 µL with a final concentration well below 0.01%. This was followed rapidly by EDAC dissolved in 8 mL of deionised water. The solution became cloudy and the pH rose. A pH of 5 was maintained using HCl. Once a constant pH 5 was maintained for 30 minutes, addition of NaOH raised the pH to 11. When the pH dropped a half log, the pH was raised to 11. This was repeated until the solution changed from cloudy to clear. Once the pH stabilised it was adjusted back to 7.4.

The next procedure was purification and concentration of the micelles by using ultrafiltration by means of the Amicon ultrafiltration system (YM-10 membrane; cut-off molecular size 10 kDa). The SMA-WIN micelles were washed in the ultrafiltration system with deionised water four times, by reducing concentrations down to one tenth of the original volume (500 ml), and
finally concentrated to about 30 ml. This ensures 99.99% purity of the micellar preparation. Finally the product was frozen at -80 °C and freeze dried, which produced a SMA-WIN in powdered form.

Table 2.1. Loading concentration aims and amounts of measured substrates

<table>
<thead>
<tr>
<th>Loading concentration aim</th>
<th>WIN</th>
<th>SMA</th>
<th>EDAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>5%</td>
<td>5 mg</td>
<td>95 mg</td>
<td>95 mg</td>
</tr>
<tr>
<td>10%</td>
<td>10 mg</td>
<td>90 mg</td>
<td>90 mg</td>
</tr>
<tr>
<td>25%</td>
<td>25 mg</td>
<td>75 mg</td>
<td>75 mg</td>
</tr>
<tr>
<td>20% first batch</td>
<td>40 mg</td>
<td>160 mg</td>
<td>160 mg</td>
</tr>
<tr>
<td>20% second batch</td>
<td>30 mg</td>
<td>120 mg</td>
<td>120 mg</td>
</tr>
</tbody>
</table>

2.2.3 Loading concentration of SMA-WIN 55,212-2 micelles

A standard curve was prepared by serial dilution of WIN in 90% DMSO 10% deionised water. Drug quantification was carried out using UV/Vis spectrometry at 328 nm. Loading concentration was then obtained by dissolving SMA-WIN in 90% DMSO and measuring absorbance at 328 nm. Loading concentration was calculating as percentage of WIN to total weight. WIN content within the micelle was calculated with the standard curve.
2.2.4. Efficiency of SMA-WIN 55,212-2 micelles

Efficiency of the micelle was defined as the total weight of the final SMA-WIN product divided by the initial weight of the drug introduced for micellar preparation. Weights were measured 3 times and averaged.

2.2.5. Drug release from SMA-WIN 55,212-2 micelle

The release of free drug WIN from the SMA micelle preparations was calculated using a dialysis method in vitro. SMA-WIN solutions were prepared in deionised water at a concentration of 5 mg/ml, and a total volume of 6 ml for each loading was created. Measured lengths (20 cm) of dialysis membrane were tied at the ends twice, with a string tied in-between the two knots. Dialysis membranes were activated by partially filling with deionised water. Then they were boiled for 2 minutes. Membranes were washed with deionised water and dried. Inside each dialysis membrane 1.5 ml of the prepared SMA-WIN solution was pipetted in. Dialysis membranes were submerged in 15 ml of two different solutions inside a falcon tube; either distilled water at pH 7.4 (to mimic the plasma pH), or distilled water at pH 5.5 (to mimic the pH in lysosomes upon uptake of the micelle). Two replicates were created for each pH. The falcon tubes were placed inside a shaker at 37 °C at 60 RPM. The WIN released from the dialysis bags was collected at 0.5, 1, 2, 4, 6, 8, 12, 24, 36, 48, 60, 72, 84, 96 hours and the amount quantified at 328 nm. Three samples were taken from each membrane at each time point and measured. Then, samples were returned to the solution. Absorbance readings were taken of both the bag solution and surround liquid combined at the end of the experiment. The readings were used to calculate the total absorbance of solutions. The percentage release was calculated according to equation 1.
CHAPTER TWO: SYNTHESIS & CHARACTERISATION

Equation 1. Percentage release

\[ \text{Percentage release} = \left( \frac{\text{Absorbance sample} \times \text{Sample volume}}{\text{Absorbance applicable water} \times \text{Total volume}} \right) \times 100 \]

2.2.6. Micelle size distribution

SMA-WIN samples were dissolved in deionised water at a concentration of 6.77 mg/ml. All measurements were taken at ambient room temperature. Samples were placed in a 2 ml cuvette and measured using a Malvern Zeta Sizer ZEN3600 (Malvern instruments Inc., Westborough, MA). If quality was low the solution was diluted, or/and a small amount of NaOH was added to force the micelles apart and stop them forming aggregates. All measurements were repeated in triplicate. Three sets of solutions for each SMA-WIN micelle loading concentrations were assessed, indicating the three lines in the recordings, average peak were used to calculated diameter from intensity of the recording using the Malvern Zeta Sizer ZEN3600 software.

2.2.7. Statistical analysis

Computation of results was processed using Prism®, Version 6.01 (GraphPad Software, CA, USA). Linear regression was applied to standard curves to assess the strength of the relationship between independent and dependent variables. A one-phase association non-linear regression was applied to SMA-WIN micelle release, to assess the strength of the relationship between independent and dependent variables.
2.3. Results

2.3.1 WIN 55,212-2 Standard curve in 90 % DMSO

![Graph showing standard curve of WIN 55,212-2 concentrations in 90% DMSO, absorbances read at 328 nm.](image)

*Figure 2.1. Standard curve of WIN 55, 212-2 concentrations in 90 % DMSO, absorbances read at 328 nm. n=3 Data expressed as mean ± SEM.*

A standard curve was produced using two known sample concentrations of WIN, diluted at 7 different concentrations and measured at maximum absorbance. Using linear regression an $R^2$ value of 0.9996 was calculated. This indicated that nearly all change in absorbance within the standard curve was a result of the concentration change. The slope was highly significantly different from zero ($p < 0.0001$). All statistical measures indicated that the standard curve was appropriate for extrapolation of unknown concentrations, shown by the equation below.

*Equation 2. Absorbance*

\[ \text{Absorbance} = 23.145 \times \text{Concentration} - 0.003 \]
2.3.2. Preparation and Loading efficiency of SMA-WIN 55,212-2

The initial 5 %, 10 % and 25 % loading concentration aims were shown to produce a 66.8 %, 71.3 %, and 73.3 % recovery respectively. The actual loading percentages, calculated from figure 2.1, were found to be 5.6 %, 14.7 %, and 27.2 % respectively. These initial batches of SMA-WIN micelles synthesised using protocol 2.2 proved WIN could be successfully incorporated into the SMA micelle. The first batch that aimed to have a loading of 20 % produced a 90.4 % recovery with a loading concentration of 21.7 %. The second batch produced a recovery of 78 % with a loading concentration of 22.4 %. SMA-WIN micelles were soluble in deionised water at all concentrations tested and produced a clear solution when dissolved.

2.3.3. SMA-WIN release profile

![Graph](image)

Figure 2.2. In vitro release rate of the base drug from a range of SMA-WIN 55 212-2 at a range of different loading at pH 7.4. Absorbance is read at 328 nm, n=6. Data expressed as mean ± SEM.
Absorbance of WIN was measured at 328nm and calculated as a percentage of absorbance (Figure 2.2 and Figure 2.3). Using non-linear regression, one-phase association was fitted and values were calculated as; 7.4 pH 5 % loading, 5.5 pH 5 % loading, 7.4 pH 15 % loading, 5.5 pH 15% loading, 7.4 pH 27 % loading, 5.5 pH 27 % loading, gave respective $R^2$ of 0.9548, 0.9825, 0.9659, 0.9274, 0.9199, 0.8787. The high $R^2$ values indicate that nearly all changes in concentration within the fitted curves are a result of the change due to time. Figure 2.2 shows the release profile of SMA-WIN micelles at different loading concentrations. The release of WIN 55, 212-2 micelles appears to have two phases. An initial short term fast release period typically, within the initial 20 hours, this is characterised by the initial steepness of the curve. Followed by a second slow release period, typically after the initial 20 hours, which is shown by the gradual increase of the curve. Differences between the different loading concentrations were evident by the differences in stages of release periods. The lower loading 5 % had a rapid first phase release, followed by a small decreased release over time in comparison to the
over loadings. The 5 % loading also appeared to be less consistent and more variable. The highest 27 % loading had a more gradual phase one and was released more consistently in phase two. The 15 % loading appeared to be a moderation of two, but maintained the consistence of release that the 27 % loading achieved in phase two. Figure 2.3 demonstrates the differences in release profiles during pH changes. The release profile showed an increased release with the higher pH.

2.3. Size distribution of SMA-WIN micelles

![Size Distribution by Volume](image)

*Figure 2.4. Mean size distribution calculated by volume percentage of 5 % loading SMA-WIN nanomicelle.*
Figure 2.5. Mean size distribution calculated by volume percentage of 15 % loading SMA-WIN nanomicelle.

Figure 2.6. Mean size distribution calculated by volume percentage of 27 % loading SMA-WIN nanomicelle.

Micelle size of initial batches was evaluated using protocol 2.4. Solutions were prepared and tested for this experiment. Figure 2.4 shows an inconsistent result quality for the 5 % loading SMA-WIN micelle. Peak 1 recorded a diameter of 686 nm with an intensity of 73.9 %. Peak 2 recorded a diameter of 77 nm with an intensity of 18.2 %. As the quality was poor the
solution may have been forming aggregates, and was therefore not suitable for true interpretation. Due to the limited amount of 5 % WIN-SMA micelle prepared, measurements were not retested. Both 15 % loading and 27 % loading (Figure 2.5 and Figure 2.6 respectively) provided good result quality, indicating results were appropriate for interpretation. 15 % loading SMA-WIN micelle (Figure 2.5) produced an average diameter at peak 1 of 158 nm and a width of 120 nm, with an intensity of 97.7 %. The average diameter size was 116.2 nm for 15 % loading SMA-WIN micelle. The 27 % loading SMA-WIN micelle (Figure 2.6) produced an average diameter at peak 1 of 147 nm, and a width of 97 nm, with an intensity of 87 %. The average diameter size was 101 nm for 27 % loading SMA-WIN micelle.

The spread of the size distribution results appear to be smaller in Figure 2.4 compared to Figure 2.5 and Figure 2.6. However, this is not an indicator of quality. The quality of the results is indicated by the individual readings overall, not the spread of the size distribution. The Malvern Zeta Sizer determines the size by measuring the Brownian motion of the particles in a sample. This is achieved by using dynamic light scattering, and then interpreting the size from established calculations. Therefore the quality refers to the viscosity and aggregation of the sample solutions not the spread of the results. The spread of the results indicate the consistency of the measurements which would be expected to improve with increased sample numbers.
2.4. Conclusion and Discussion

The method described in section 2.2.2 above for synthesising the first batches of micelles was successful. The SMA-WIN micelles were soluble in water and had acceptable loadings ranging from 5.7 % - 27 %. The release profiles showed that lower loading concentrations demonstrated a faster release profile, whereas the higher loading concentration demonstrated a slow maintained release profile. Also, an increased release was observed with higher pH. These properties are consistent with the theory that the reason for these results is due to the interaction between the drug WIN 55, 212-2 and SMA (Greish et al., 2004; Iyer et al., 2007a).

The sizes of the micelles formed were found to be appropriate for drug formulation. Finally, based on the previous results, batches aimed at 20% loading were produced for animal testing.

2.4.1. SMA-WIN recovery and basic properties

All SMA-WIN micelles were readily soluble in water. This property alone is a huge transformation of the properties of WIN. WIN is highly lipid soluble, and like most cannabinoids can be dissolved in water only when using solvents such as DMSO (Pertwee et al., 2000). Dissolving cannabinoids also normally takes a substantial period of time. Water soluble cannabinoids have been achieved through producing cannabinoid esters before, but never through nanomicelle encapsulation (Martin et al., 2006). Although esters initially were reported effective for the treatment of pain, their main advantage was that the esters required no solvents to dissolve in solution. This therefore decreased any potential confounding results produced from ‘active’ solvents required to dissolve regular cannabinoids. Also Pertwee et al. (2000) suggested the esters are actually a pro-drug that elicits responses only after they are hydrolysed to another more lipophilic compound. SMA-WIN micelle formation should prove superior to these constructs as the nanomicelle construct should be able to selectively target the site of inflammation in the spinal cord and treat neuropathic pain.
The recoveries from all batches produced large yields, generally greater than 70%. Variation occurred partly due to filter quality during the washing stage of micelle preparation. Upon purchase of a new filter, the recovery increased to 90.4%. Once the filter had been used several times, batch 2 had a notably decreased yield of 78%. However, the small loss of product indicates that formulation of this drug could be cost effective in production with only a small amount of wastage. High recovery is advantageous for scaling up micelle production. This is highly important for industrial use, where cost to benefit ratios drive will be considered and the synthesis needs to be suitable for up scaling.

2.4.2. SMA-WIN Release rates

The release profile that was measured for the nanomicelles can be explained by the hydrophobic properties of SMA. These properties cause the hydrophobic drug, WIN, to be incorporated into the centre of the hydrophobic moiety of the micelle (Greish et al., 2004). The slow release phase demonstrates the release of WIN that is encapsulated within the micelle. WIN that is not fully encapsulated, but instead associated within the SMA polymer, may explain the fast release phase. Both release profiles appear to be pH and loading dependent (Figure 2.3). The higher WIN loading in the micelle causes the percentage release to be decreased and a greater phase 2 period is therefore seen. In contrast with a lower loading of WIN in the micelle a larger phase one release period is seen (Figure 2.2). The explanation for this is most likely that the micelle with the higher loading may have a larger quantity of WIN encapsulated in the core of the micelle, resulting in a stronger hydrophobic association between the styrene moiety and WIN. The release profile can also be mediated by changes in pH, as stated above (Figure 2.3). As micelles formation is highly pH dependent, an increase in pH results in a decreased stability of the SMA-WIN micelle, therefore increasing release rates. These findings are consistent with other studies that demonstrate that increased pH,
CHAPTER TWO : SYNTHESIS & CHARACTERISATION

decreases stability of the nanoparticle, and that a larger loading increases stability (Greish et al., 2004; Iyer et al., 2007a). Previous research has shown that once at the site of inflammation micelles are internalised into cells by endocytotic pathways. It is then followed by release of the contents in the presence of membrane components (Nakamura et al., 2011). Therefore the release rate may not be the most important factor to determine how a micelle will function, as stability within the plasma may be more critical. The stability of a micelle allows the micelle to remain within the circulation, while the size of the particle controls the localisation of the micelle.

2.4.3. SMA-WIN nanomicelle size

The sizes of the nanoparticles were all over 6nm in diameter. This indicates that they were larger than the fenestrations of the kidney (Bearer et al., 1985). Particles were also larger than the fenestrations found in healthy blood vessels (Braverman, 2000). This should cause the micelles to be retained in the circulation and not pass the blood brain barrier. The size should ensure that the drug has an extended plasma half-life. Ideally, the large size would allow the molecules to bioaccumulate in areas of increased vascular permeability, in a process called EPR effect (Maeda et al., 2000). As neuropathic pain is an inflammatory disease which causes breakdown of the blood brain barrier, it may be possible to target neuropathic pain at the site of action. There is a slight decrease in pH at inflammatory sites (Shah et al., 2008), but this should be negated by the bioaccumulation and potential micelle internalisation, resulting in increased effectiveness.

Unfortunately this selective extravasation of the drug via the EPR effect also occurs in the liver and spleen, which have fenestrations of around 150 nm (Takakura et al., 1998). Therefore nanoparticles also accumulate at these organs (Gaumet et al., 2008). This is a major
route of metabolism for micelles. Previously this has not proven detrimental, partially due to the liver’s large intrinsic antioxidant potential (Iyer et al., 2007b). However, presently the effects of SMA-WIN formulation in the liver and spleen are unknown. This should be investigated, particularly because the high count of CB2 receptors in the spleen (Schatz et al., 1997). Currently there are no nano-constructs related to pain relief. There is however a few Food and Drug Administration (FDA) approved micellar formulations for chemotherapy. These have proved effective, despite the limitations described above (Haley et al., 2008).

2.4.4. Conclusions

The release rates and size of SMA-WIN micelles appear appropriate for the testing of their effect on neuropathic pain. A stable micelle was desired with a small phase one period but slightly increased release during the phase two period. A loading concentration between 15.7 % and 27 % was decided upon and subsequently synthesised, for the testing on neuropathic pain. With a loading concentration aim of 20 %, a final product was successfully created with an actual loading concentration of 21 %. The chemical properties of the micelles appear appropriate for the EPR effect to occur due to their size and release rates. This should cause the micelles to bio-accumulate in the area of inflammation at the spinal cord. The EPR effect of the new micelles should also result in decreased permeability past the blood brain barrier, therefore reducing side effects. As this drug was the first of its kind, very little was known about how it would behave. Potential limitations of the new drug treatment may be revealed upon animal testing. Ideally SMA-WIN micelles offer a new highly viable treatment for neuropathic pain. Significant benefits may also be provided by their ability to manipulate pharmacokinetics causing a subsequent reduction in side effects.
This chapter covers the behavioural testing of the SMA-WIN 55, 212-2 micelle treatment for reducing neuropathic pain. It also investigates into the selectivity of the drug for this effect, i.e., its ability to reduce neuropathic pain whilst avoiding impact on the CNS. Chronic constriction injury in rats was used to induce neuropathic pain and measure the effectiveness of the treatment. The rotarod test was used to measure the permeability of the drug into the CNS and measure behavioural impairment in rats of similar age to chronic constriction injury tested animals. The results obtained from these experiments show the complicated nature of this new formulation.

3.1. Introduction

Neuropathic pain animal models have been designed for the preclinical assessment of potential treatments. The behavioural model used in these experiments, called chronic constriction injury (CCI), is based on the method of Bennett et al. (1988). This research originally described the development of neuropathic pain in rats after tying loose ligations around the sciatic nerve. This contrasts with the older animal models that used transection of the nerve, adapted from Wall et al. (1979). The particular methods in the series of experiments described in the first part of this chapter were based of the protocols developed by Brownjohn et al. (2012), who established the procedures in the Otago Pharmacology and Toxicology department. The established CCI protocol was chosen because it is was cost effective, easy to perform, long lasting and produced less autonomy than the more severe sciatic transections. This results in more consistent basal starting recordings. An additional
benefit was that it is considered more ethical. The use of Von Frey hair filaments allows the assessment of mechanical allodynia and thus measures the effect of the novel drug on the treatment of neuropathic pain.

Motor ataxia was assessed using an accelerated rotarod technique that has been previously used in cannabinoid research as a measure of central cannabinoid activity (Fox et al., 2001; Gutierrez et al., 2011). It is recognised that cannabinoids cause motor impairment by activation of a CB1 receptor in the cerebellum and basal ganglia, which are centrally located (DeSanty et al., 2001; Romero et al., 2002). Therefore, for the cannabinoids to have an ataxic effect they have to pass the blood brain barrier. The rotarod experiment allows cheap and easy testing of this. Therefore, the rotarod test provides a measurement of the ability of SMA-WIN micelle to affect the brain via CB1 receptor activation.

3.2. Methods
3.2.1. Animals

All the experiments conducted were approved by the Animal Ethics Committee at the University of Otago, under guidelines set down for the ethical and humane use of animals in research, following the United Kingdom Act of 1986. Male Wistar rats between 300-450 grams were used for all behavioural testing. Rats were obtained from the Taieri-Hercus Resource Unit (Dunedin, New Zealand). Prior to surgical manipulation or behavioural testing, rats were housed for at least 3 days in a 12 hour light/dark cycle with ad libitum access to food and water. For the duration of the rotarod behavioural experiment, animals were housed under quarantined conditions due to a pinworm outbreak. All animals were assessed for, and shown to be negative for, pinworm.
3.2.2. Intravenous drug administration

Animals were initially weighed and the injection volume of solution was calculated at 1ml/kg. They were placed inside a restraining device and tails were warmed for 5-10 minutes to dilate veins. The tail was held using the same forefingers that supported the sterile 28 gauge needle with 1ml syringe. Starting distally on the tail, blood was taken up into the syringe to confirm successful needle placement into the tail vein. Solutions were then slowly injected.

3.2.3. Drugs

The CB1/CB2 non-selective agonist WIN 55,212-2 (WIN) was obtained from Tocris Bioscience (UK). WIN was dissolved in 2% DMSO, 2% Polysorbate 80 in 0.9 % saline, at the concentration of 1 mg/ml. WIN-SMA was dissolved in saline as previously described (Chapter 2) 30-60 minutes before injections and used as the positive control. A 0.9 % NaCl solution in deionised water was autoclaved and used as saline control. Concentrations of WIN 55,212-2 supplied the equivalent dose of total WIN in the 4.6 mg/kg SMA-WIN micelle and the amount of expected release of WIN from the 11.5 mg/kg. Concentrations of WIN were roughly a third of successful intraperitoneal treatments due to the change in administration (Herzberg et al., 1997).

3.2.4. Chronic constriction injury

Animals were induced under 2.5 % halothane anaesthesia (Nicholas Piramal Ltd., India) in medical grade oxygen. The sufficient depths of anaesthesia were determined by lack of pedal withdrawal reflex and tail withdrawal reflex. Incision sites were determined by superficial
markings between the epiphysis of the femur and the patella. The surgical site on the left hind paw of the animals was shaved, sterilised, and marked.

Initial incisions were made using a scalpel through the superficial fascia exposing the *biceps femoris*. The *pars cranialis* and *pars caudalis* of the *biceps femoris* were separated by blunt dissection to expose the underlying sciatic nerve. Markings for observing the point of tissue separation were achieved by following a white tendinous line and pulling the tissue with forceps. These two muscles twist apart, forming a seam, allowing for identification of the incision point. An initial incision was then made followed by blunt dissection with glass rods.

Once exposed, sciatic nerves were maintained by applying saline (0.9 % NaCl in deionised water). Four sutures (4/0 2 metric chromic gut; Ethicon NJ, USA) were loosely tied 2 mm apart around the exposed nerve, with no occlusion of the nerve process. The nerves were gentled back into position under the *biceps femoris* and allowed to recover. The wounds were closed in layers, initial fascia was stitched (5/0 1 metric silk; Ethicon NJ, USA), and the skin was stapled. The animals were then allowed to recover and treated for post-operative pain. Buprenorphine (Reckitt Benckiser, UK) was administrated initially after surgery and post-operatively at 12 hour intervals for 36 hours (0.1 mg/kg S.C.). Animals were regularly monitored for 3 days following surgery and 10 days were allowed to fully develop neuropathic pain. The short term pain relief that was required for post-operative care does not affect allodynia development (Brownjohn *et al.*, 2012).

3.2.5. *Von Frey hair analysis protocol*

Behavioural testing was undertaken one day prior to surgery (for baseline paw withdrawal thresholds), three days after surgery (for development of neuropathic pain), and 10 days post-surgery (for pre-dose thresholds). Behavioural testing was measured immediately prior to
treatments (I.V.) (“preadministration threshold”) by testing at multiple time points (0.25, 0.5, 1, 2, 4, 8, 12, 24, and 48 hours) (“postadministration thresholds”). All groups were blinded to the experimenter for the testing and released later on for final analysis. On the day of testing, animals were placed on an elevated wire mesh flooring (1.5 mm bars, 14 mm spacing) and contained with a mesh wire cylinder. The animals were habituated for 20-30 minutes in a darkened room, maintained with dull red light.

To calculate 50% paw withdrawal thresholds, a logarithmically graded series of Von Frey hair monofilaments (North Coast medical, Morgan Hill, CA) were presented to the left hindpaw. The hairs presented had the following log force (10,000 X g) intensities: 3.61 (0.407 g), 3.84 (0.692 g), 4.08 (1.202 g), 4.17 (1.479 g), 4.31 (2.041 g), 4.56 (3.630 g), 4.74 (5.495 g), 4.93 (8.511 g), 5.07 (11.749 g), and 5.18 (15.136 g). A modified form of the up/down procedure (Dixon, 1980) was used. Monofilaments were applied to the left ventral plantar aspects of the hind paw at a perpendicular angle for three seconds. A withdrawal was considered as a paw flinch upon presentation or sustained application of a given monofilament.

The up/down procedure testing started with the 2.041 g monofilament, which was presented three times at 30 second intervals. When two or more paw withdrawals were recorded in response to this stimulus, the lowest strength 0.407 g monofilament was presented. In the absence of two or more paw withdrawals with the 2.041 g monofilament, the next strongest 3.630 g monofilament was presented. With the exception of the 2.041 g monofilament, each monofilament was only initially tested once. Monofilaments were tested twice more if a response was elected from the initial stimulus. Once three withdrawals were elected from the same monofilament, testing was stopped, and this was considered the threshold. Presentation of monofilaments continued in ascending order until this threshold was established. An upper cut-off value of 15.136 g monofilament was assigned as the highest stimulus. This was
designated to avoid tissue damage. All withdrawals and non-responses were recorded for analysis, but testing was only completed once each threshold was reached.

Calculation of 50% paw withdrawal was achieved by application of paw withdrawal response rates to a Gaussian integral psychometric function, using a maximum-likelihood fitting method. Conversion of the threshold values creates a continuum of values that are suitable to analyse parametrically. The program used to compute the data, PsychoFit, was created by Professor Lewis O. Harvey Jr. at the University of Colorado, and is available as a freeware download (http://psych.colorado.edu/~lharvey/html/software.html). Data were then standardised using equation 3.

Equation 3. Allodynia reversal

\[
\% \text{ Reversal} = \frac{Post \ threshold – predose \ threshold}{Baseline \ threshold – predose \ threshold} \times 100
\]

3.2.6. Rotarod

Motor ataxia was measured using the accelerating rotarod (Panlab RotaRod LE8500, Spain). Animals were placed on the rotating drum and required to walk against the motion. The speed incrementally increased from 4 to 40 rpm over 5 minutes. Behavioural testing was undertaken 24 hours after habituation. The habituation involved two training periods 1 to 2 hours apart. Animals were placed on the accelerating rotarod until they were able to remain there for at least 60 seconds. Animals that climbed off the rotarod during the habituation stage were
placed back on to the rotarod at the same acceleration time point. However, if the animal fell off before the full 60 seconds the habituation was restarted from time point zero.

The time taken for the animal to fall off the rotarod was recorded as the latency (seconds), and was measured by a plastic switch platform to avoid any measurement bias. For testing latencies, animals were measured immediately prior to and up to 12 hours following intravenous drug treatment, at -0.083, 0.083, 0.25, 0.5, 1, 1.5, 2, 4, 8, 12, and 24 hours. In all experiments a 300 second cut off was employed. If the animal was unable to stay on the rotarod at 4 rpm a latency of 0 was assigned. Data was recorded and expressed as the latency, and also expressed as percentage disruption of performance (Equation 4).

\[
\text{Equation 4. Percentage disruption}
\]

\[
\text{Percentage disruption} = 100 - \left( \frac{\text{Post drug latency}}{\text{Pредrug latency}} \right) \times 100
\]

3.2.7. Statistical analysis

Initially the characterisation of the development of neuropathic pain for paw withdrawal thresholds were analysed with a 1-way repeated measure ANOVA, with time as the factor. Newman-Kwels post hoc analysis tests were used to calculate significant differences between time points. Chronic constriction injury drug invention and rotarod analyses were carried out with 2-way repeated measures ANOVA, with time as one factor and the drug as the other. This was followed by Bonferroni post hoc analysis. Assumptions of 2-way repeated measure ANOVA were checked using the Shapiro-Wilk normality test for normality, the Greenhouse-Geisser method for sphericity, and Levene’s test for homogeneity of variance. Initial
computation was calculated using Prism®, Version 6.01 (GraphPad Software, CA, USA) and additional statistics were performed using IBM SPSS Statistics for Windows, Version 21.0 (IBM Corp, NY, USA).

3.3. Results

3.3.1. Mechanical allodynia development in the CCI model

Initial behavioural data from animals within this study were pooled to allow an analysis of the development of mechanical allodynia in the CCI model. Analyses by one way repeated measure ANOVA showed CCI induced mechanical allodynia in the rat. This was evidenced by a significant change in paw withdrawal threshold between groups in the ipsilateral hindpaw (p < 0.0001)(Figure 3.1). Newman-Kwels post hoc analysis revealed that paw withdrawal thresholds were significantly reduced at 3 and 10 days post-surgery compared to pre surgery measurements (p < 0.001 for each comparison). Allodynia was marked 3 days post-surgery, and was fully established by the 10 day time point. At ten days point pharmacological intervention took place.
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Figure 3.1. CCI induces bilateral mechanical allodynia in the left hindpaw of the rat. Paw withdrawal threshold was reduced significantly in the ipsilateral hindpaw following CCI surgery, 3 and 10 days post-surgery. *** = p < 0.001 vs. pre-treatment n = 12 for CCI. Data are expressed as mean ± SEM.

3.3.2. The effect of SMA-WIN intervention on allodynia

Intravenous administration of the higher dose 11.5 mg/kg SMA-WIN micelle, significantly attenuated mechanical allodynia in the ipsilateral hindpaw of the CCI treated rats but not the lower dose 4.6 mg/kg SMA micelle compared to saline. Data is presented to show the acute effects of the treatments (0-2 hours) and the longer term effects (2-24 hours) in separate graphs for clarity (Figure 3.2 and Figure 3.3). A 2-way repeated measure ANOVA of paw withdrawal threshold showed that, ipsilateral to injury, the interaction between treatment and time was significant (F = 1.755 (32, 230); p = 0.0055). The Bonferroni post-hoc test revealed that high dose SMA-WIN micelle caused sustained attenuation of mechanical allodynia compared to saline from 30 minutes to 2 hours. All values were attenuated with a significance of p < 0.01. Significance was lost at 4 hours (p > 0.05), and regained at 8 hours (p < 0.01). Post hoc analysis also revealed the positive control 1mg/kg WIN, significantly attenuated
mechanical allodynia compared to saline. This peaked at 15 minutes ($p < 0.001$) but there were no significant differences by 1 hour post injection ($p > 0.05$). No significant difference was observed between saline and 9.1 mg/kg SMA ($p > 0.05$). Some seizures were observed in the WIN treatment group followed by rapid sedation. No seizures were observed in any other group with only mild sedation seen in high dose SMA-WIN micelle treatment.

Assumptions of the 2-way repeated measure ANOVA were checked. Normality was tested using the Shapiro-Wilk normality test. All groups passed the normality test ($p < 0.05$). Sphericity was calculated using the Greenhouse-Geisser method, giving a value of $p = 0.591$, which showed the required criteria was met. Homogeneity of variance was assessed using Levene’s test across all time points. This was found to be violated at 15 minutes and 30 minutes ($p > 0.05$). All other time points were valid. As measurements at only two time points were violated it is seen as acceptable to still use parametric analysis.

![Figure 3.2. I.V. administration of SMA-WIN and WIN alleviate mechanical allodynia in the ipsilateral hindpaw of the rat during the initial first two hours. Saline n=12, WIN n=7, 4.6 mg/kg SMA-WIN n=6, 11.5mg/kg SMA-WIN n=6, and SMA n=5 (** = $p < 0.01$ vs. vehicle, *** = $p < 0.001$ vs. vehicle). Data expressed as mean ± SEM.](image)
3.3.3. Dose dependant standardised SMA-WIN intervention on allodynia

Raw data for paw withdrawal thresholds were further analysed by converting the data to standardised scores (Equation 1). Both 1mg/kg WIN and 9.1mg SMA were excluded in the graphs as results did not differ from non-standardised data and allowed better interpretation of results with fewer groups. Intravenous administration of the SMA-WIN micelle produced reversal of standardised mechanical allodynia in the ipsilateral hindpaw of the CCI treated rats in a dose dependent manner (Figure 3.4 and Figure 3.5). A 2-way repeated measure ANOVA of paw withdrawal threshold showed that, ipsilateral to injury, the interaction between treatment and time was significant (F = 2.2338 (16, 144) p = 0.004), and therefore interpretation of individual time and treatment effects is difficult. Bonferroni post hoc test revealed that high dose SMA-WIN micelle caused sustained attenuation of mechanical allodynia compared to saline from 30 minutes to 2 hours, with all values at least p < 0.01, and
significance was lost at 4 hours (p > 0.05). Maximum reversal of 11.5 mg/kg WIN-SMA treatment was seen at 30 minutes with 124% reversal in allodynia. This high level of reversal was sustained above 97% for the initial two hours (Figure 3.4.), and slowly decreased over a 24 hour time period to 60% at 4 hours, 47% at 12 hours and finally 24% at 24 hours post injection (Figure 3.5.).

Assumptions of 2-way repeated measure ANOVA were checked. Normality was tested using the Shapiro-Wilk normality test with all groups passing the normality test (p < 0.05). Sphericity was calculated using the Greenhouse-Geisser method giving a value of p = 0.642, which showed the criteria was met. Homogeneity of variance was assessed using Levene’s test across all time points and found to be valid.

![Graph](image.png)

Figure 3.4. I.V. administration of SMA-WIN demonstrates dose dependant standardised percentage reversal of mechanical allodynia in the ipsilateral hindpaw of the rat during the initial first two hours. Saline n=11, 4.6 mg/kg SMA-WIN n=6, and 11.5 mg/kg SMA-WIN n=6 (* = p < 0.05 vs. vehicle, ** = p < 0.01 vs. vehicle, *** = p < 0.001 vs. vehicle). Data expressed as mean ± SEM.
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Figure 3.5. I.V. administration of SMA-WIN demonstrates dose dependant standardised percentage reversal of mechanical allodynia in the ipsilateral hindpaw of the rat from two to 24 hours. Saline n=10, 4.6 mg/kg SMA-WIN n=6, and 11.5 mg/kg SMA-WIN n=6 (*** = p < 0.001 vs. vehicle). Data expressed as mean ± SEM.

3.3.4. SMA-WIN micelle effect on motor ataxia

Intravenous administration of 11.5mg/kg of SMA-WIN micelle and 1mg/kg of WIN produced motor ataxia in rats (Figure 3.6. and Figure 3.7.). A 2-way repeated measure ANOVA of latency to fall, showed the interaction between treatment and time was significant (F = 9.192 (18, 162) p < 0.001). This made interpretation of individual time and treatment effects difficult (Figure 3.6.). Bonferroni post hoc test revealed that the SMA-WIN micelle caused an increase in motor ataxia from 15 minutes to 1.5 hours post injection, compared to saline control (p > 0.05). WIN caused significant motor ataxia from 15 minutes to 1 hour post injection (p > 0.05). There was no significant difference between SMA-WIN micelle and WIN (p > 0.05).
Percentage disruption of motor ataxia (Figure 3.7.) revealed no changes from latency to fall (Figure 3.6.). A 2-way repeated measure ANOVA revealed the interaction between time and treatment was significant (F = 5.565 (18, 153) p > 0.001). The Bonferroni post hoc test revealed the overall column effect demonstrated that both SMA-WIN micelle (p < 0.05) and WIN (p < 0.05) were significant compared to the vehicle control. There was no significant difference between SMA-WIN micelle and WIN (p > 0.05). Increased sedation was observed in both WIN and the SMA-WIN micelle treatment group. Seizures were seen in WIN treatment but not in SMA-WIN micelle.

Assumptions of 2-way repeated measure ANOVA were checked. Normality was tested using the Shapiro-Wilk normality test. All groups passed the normality test (p < 0.05) for both Figures 3.6 and Figure 3.7. Sphericity was calculated using the Greenhouse-Geisser method, and showed the criteria were met. Homogeneity of variance was assessed across all time points using Levene’s test. All time points were found to be valid.

![Figure 3.6. I.V. administration of SMA-WIN and WIN produce motor ataxia in the rat compared to saline control. Time course from baseline pretreatment measurements to 4 hours post injection. Saline n=6, 1 mg/kg WIN, 11.5 mg/kg n=6, and SMA-WIN n=6. (* = p < 0.05 vs. vehicle, ** = p < 0.01 vs. vehicle, *** = p < 0.001 vs. vehicle, all comparisons were made at the same time point). Data are expressed as mean ± SEM.](image)
Figure 3.7. I.V. administration of SMA-WIN and WIN increase motor ataxia in the rat, pretreatment to 12 hours post injection. Saline n=6, 1 mg/kg WIN, 11.5 mg/kg, and SMA-WIN n=6. Both SMA-WIN micelle and WIN are significant different from vehicle (p < 0.05 vs. vehicle). Data expressed as mean ± SEM.
3.4. Conclusions and Discussion

Intravenous delivery of the SMA-WIN micelle significantly attenuated CCI induced mechanical allodynia in a dose dependent manner. Allodynia reversal was for a prolonged period of time compared to WIN. Straight SMA produced no pain relief by itself. This indicates that it was the new SMA-WIN micelle formulation that produced the extended allodynia reversal. As a measure of CNS impairment, motor ataxia was measured. SMA-WIN micelles were shown to impair motor ataxia for a short duration of time, compared to the significantly longer pain reversal. There are several hypotheses of what could be occurring, these are discussed in Chapter 4.

3.4.1. Development of pain behaviour

The validity of this animal model for inducing neuropathic pain was confirmed by the induction of neuropathic pain, with a significant increase in neuropathic pain over the course of 10 days (Figure 3.1). Mechanical allodynia developed in the ipsilateral hindpaw of CCI treated animals, with a stable alldynia level at the 10 day time point. The stable alldynia was evident by the similar decrease in CCI withdrawal responses to past studies that have reassessed the development of alldynia (Brownjohn et al., 2012). This replicated the initial study that described CCI by Bennett et al. (1988). Development of mechanical alldynia indicates that altered morphology has occurred in the spinal cord, inducing a neuropathic pain state. Therefore extensive microgliosis and mild astrogliosis will have occurred in the dorsal horn of the spinal cord, as these are known to be involved in the development of alldynia (Breen et al., 2012). It can be concluded from the findings that this model is valid for the assessment of the efficacy of the SMA-WIN micelle, for the treatment of mechanical alldynia in a stable relevant model of human neuropathic pain. This conclusion is derived
from the evidence of the development of neuropathic pain and previous research that has demonstrated the associated changes in the spinal cord.

3.4.1. SMA-WIN micelle as a treatment for neuropathic pain

Intravenous delivery of the high dose SMA-WIN micelle, significantly attenuated CCI induced mechanical allodynia. This pain relief was mediated for a prolonged period of time of up to 8 hours in the high SMA-WIN dose. This can be compared to the positive control WIN which only produced analgesia for the initial 30 minutes (Figure 3.2 & Figure 3.3). Previous CCI reports of WIN of intraperitoneal administration doses of 2.14 mg/kg have reported significant pain relief of up to 90 minutes (Herzberg et al., 1997). Another example showed 30 μg, intrathecally produced analgesia also for only the initial 30 minutes (Brownjohn et al., 2012). Intravenous administration is known to be significantly faster than intraperitoneal administration, and similar to intrathecal. This is due to the drug being administrated directly to the systemic circulation. It is of note that this new formulation of SMA-WIN micelle has a significantly prolonged period of action (Figure 3.2 & Figure 3.3). This provides an increased bolus treatment time which is essential for effective management of neuropathic pain.

Standard percentage reversal of allodynia was clearly outlined in the dose dependant manner of the SMA-WIN micelle treatment (Figure 3.4 & Figure 3.5). The high dose SMA-WIN micelle treatment group provided prolonged pain relief at a high level of percentage reversal of alldynia. The calculation of standardised percentage reversal of alldynia increased variability by combining time points. This could have clouded the significance of later time points. As the numbers of rats assessed was relatively low, significant differences might be revealed with increasing subject numbers. Overall, evidence for the effectiveness of intravenous SMA-WIN micelles for the treatment of neuropathic pain appears compelling, as
alloydinia was significantly reversed for a prolonged period of time in a dose dependant manner.

Pain reversal was shown to be a result of the SMA-WIN micelle formulation, not due to any effect of the SMA (Figure 3.2 & Figure 3.3). SMA alone produced no noticeable variation compared to vehicle. This indicated that the mechanism of action was not in fact the SMA micelles but the new formulation. Current literature suggests that the SMA molecules act as a carrier molecule rather than an active drug component (Iyer et al., 2007a). Therefore mediated pain relief must be through the actions of WIN released in the systemic circulation, or through SMA-WIN nanomicelle bioaccumulation at the inflammatory sites. These results point to the need for further research in the form of a series of behavioural testing for motor ataxia, as a measure of selective pain targeting.

3.4.2. SMA-WIN micelles effect in the central nervous system

SMA-WIN micelle produced motor ataxia in the rotarod experiment. This indicates that WIN passed the blood brain barrier and was acting in the CNS (Figure 3.5 & Figure 3.6). SMA-WIN micelle treatment was not significantly different from WIN. In both treatments animals recovered by 2 hours post injection and showed no behavioural impairment after 2 hours. The results produced were similar to other motor impairment studies by Gutierrez et al. (2011) where IV 0.5 mg/kg of WIN caused motor ataxia for up to one hour. The recovery of SMA-WIN micelle and WIN treatment for motor impairment occurred at similar rates, SMA-WIN micelle appeared to produce no significant motor impairment after 1.5 hours (Figure 3.6). The duration of analgesia was prolonged after this time, even once motor ataxia abated. At 2 hours post injection, 11.5 mg/kg SMA-WIN micelle produced 97% allodynia reversal (Figure 3.4) but produced no significant motor impairment (Figure 3.6). Further research is needed to fully
understand the reasons for this. Specifically, these findings lead to a series of testable hypotheses (which are expanded upon in Chapter 4). These hypotheses are listed below:

1. The delayed time period of roughly one hour, from preparation of the SMA-WIN micelle to treatment in the rotarod experiment, could have allowed a small percentage of WIN to be released in the solution before injections. This could result in a bolus of WIN also being injected with the micelles, and some micelles may have come unstable once entering the blood. Therefore, WIN could have been released into systemic circulation. This could cause the behavioural effects observed, i.e., large effect caused by free WIN, which is metabolised at the same rate as in free drug control.

2. Micelles release rates are calculated in vitro, not in vivo. The release rate may be faster in vivo. This could result in higher concentration of WIN being released in systemic circulation. The resulting effects could therefore be due to higher concentration of WIN in systemic circulation.

3. Sensitivity of the rotarod test could be a confounding factor. The rotarod may not be able to distinguish between doses, above a certain systemic concentration. This could cause a ceiling effect for higher doses. This could affect the results, due to the fast half-life of WIN and the exponential decay of the drug. As a consequence different doses may be indistinguishable after a short period of time.

4. Finally, the SMA-WIN micelles may pass through the blood brain barrier; however this seems unlikely due to their size (discussed in Chapter 2).

The results showed the 11.5 mg/kg SMA-WIN nanomicelle produced reversal of allodynia in a rat model of neuropathic pain, for a prolonged time. The short term behavioural side effects observed in the rotarod experiment currently remain a point of controversy. Although any of the previous hypotheses are feasible, they can be evaluated for their merits by examining the
literature. Using these evaluative techniques the results from this chapter will now be interpreted in Chapter 4.
4.1. Summary of results

This thesis documents the first study to synthesise SMA-WIN micelles, and to test these micelles for the treatment of neuropathic pain. Micelles were successfully synthesised and were found to be soluble in water. Release rates of the SMA-WIN micelle demonstrated two release phases. An initial fast phase was followed by a slower gradual phase. Both 15.7 % loading and 27 % loading were found to have favourable release profiles. The sizes of the products were found to be appropriate for drug formulation. They were large enough to be contained within the systemic circulation, which is a requirement for enhanced permeability retention in areas of inflammation. Two batches of 20 % loading were produced for animal testing, based on an intermediate with the characteristics of the 15.7 % and 27 % loading.

Rat studies revealed that intravenous delivery of the SMA-WIN micelle, significantly attenuated CCI induced mechanical allodynia, in a dose dependent manner. This allodynia reversal was for a prolonged period of time, compared to WIN in its base form. SMA produced no pain relief by itself. These results show that the new SMA-WIN micelle formulation was responsible for the extended allodynia reversal up to 8 hours post injection. Motor ataxia was calculated, using the rotarod experiment to measure CNS impairment. SMA-WIN micelles were shown to impair motor ataxia, for a short duration of time up to 1.5 hours post injection, but caused no impairment at longer time periods.
To summarise the major findings of this work, synthesis of the new SMA-WIN micelle product was successful and produced favourable physiochemical properties and yields. The behavioural results showed prolonged and significant relief of neuropathic pain even though motor impairment was observed for a short duration. The motor impairment suggests that released WIN was able to pass the blood brain barrier. Therefore, SMA-WIN micelles proved to be a successful novel treatment for neuropathic pain in a rat model, producing prolonged pain relief compared to the base drug. However, the micelles may be limited in their usefulness due to similar side effects of the original cannabinoids, particularly after immediate administration. This problem is discussed further in the section that follows.

4.2. Potential reasons for cognitive impairment the from SMA-WIN micelle

There are several possible reasons for the rotarod results, which show short term motor impairment, which seems to indicate CNS effects from the cannabinoids. Data gathered during the prolonged reversal of alldynia and rotarod experiments in the behavioural experiments, is sufficient to be used to evaluate the cause of the motor impairment. That is to say, motor impairment occurred during the initial 1.5 hours after administration, while neuropathic pain relief was maintained for up to another 8 hours. This data suggests a number of possibilities. In combination with results from current literature on the subject, it is to some degree possible to calculate which explanations are most probable.

One possibility is that SMA-WIN micelles pass the blood brain barrier. This seems unlikely though due to their size (described in Chapter 2). It is also counter indicated because other nanomicelle studies show the biodistributions in the brain to be very low (Greish, 2010; Zhang et al., 2010). If the nanomicelles were indeed nonspecific and entering the brain, it
would be expected that the behavioural effects of motor impairment would have a similar
time frame as the neuropathic pain relief data (Chapter 3).

Another explanation could involve desensitisation of cannabinoid receptors to WIN. If
desensitisation was occurring, it could account for nearly identical recoveries observed at the
higher doses. This could even allow for the possibility of the SMA-WIN micelle releasing
free WIN constantly, but only causing a limited time period of motor ataxia. However, past
research suggests this is not the case. Previous experiments have showed that cannabinoids
that used subcutaneous and intraperitoneal administration for the assessment of motor
impairment caused a more prolonged effect (Hald et al., 2008; Liang et al., 2007; Mitchell et
al., 2005). Compared to intravenous injections these delivery methods caused a more gradual
exposure to the systemic circulation and a slower elimination, as during absorption WIN is
metabolised from the systemic circulation. These studies showed dose dependant impairment
from WIN for a prolonged period of time, even at 3 hours with doses 5 fold higher than the
positive control used within this study. Therefore it can be assumed that desensitisation is not
occurring within the 1.5 hours within this research, as a more prolonged sustained release
caused prolonged impairment.

Another possibility is that the rapid motor impairment seen following injections of the
micelles could be due to an inadvertent delivery of the initial bolus of base WIN 55, 212-2
along with the micelles. This bolus could form in several ways. Firstly, the delayed time
period of roughly one hour from preparation of the SMA-WIN micelle treatment during the
rotarod experiment could have allowed a small percentage of WIN to be released into the
saline solution before injections. During the rotarod experiments injections were delayed by
roughly 1 hour, based on the release studies (Chapter 2, figure 2.2). At 1 hour we would
expect between 2 % and 4 % release of free WIN, from the SMA-WIN micelles. This could
account for a maximum equivalent dose of 0.1 mg/kg, compared to the control of 1 mg/kg.
A third possibility could be that the micelles become less stable once within the plasma. This would result in increased WIN in the systemic circulation. However, previous micelle studies have shown that SMA micelles have a slow release in comparison to other micelle formulations (Greish et al., 2004). These other formulations have release rates that are rapid, which can even be measured on a microseconds time scale (Turro et al., 1980). The gradual release of free drug (such as in SMA-WIN micelles), is due to the solid-like cores of the micelles (La et al., 1996). The majority of micelles have liquid-like cores which quickly release the hydrophobic molecules. This causes ‘dose dumping’ after intravenous injections. This was not expected with the hydrophobic solid-like core of the SMA micelle (Kwon et al., 1997). However the behavioural effects seen indicate that some dose dumping may have occurred, with the prolonged effects caused by the more stable micelles. The lower the surfactant concentration required for micelle formation, the more stable the micelles formed. Therefore, when dilution within a large volume occurs, such as during intravenous administration, only micelles that require small concentrations for formation still exist. Micelles that require higher values become unstable and may dissociate into monomers. This would cause the content to precipitate into the blood, causing dose dumping (Rangel-Yagui et al., 2005). This explanation is plausible because initial volumes of 1.5 ml inside the dialysis bag were introduced to 15 ml of deionised water, while total systemic blood volumes in a rat of the weight used were between 19 ml and 22 ml (Lee et al., 1985). Two other factors remain to be discussed, firstly, while the dialysis membrane is freely permeable to water and small molecules such as WIN, the solution is not in constant circulation in the same manner as systemic circulation. Therefore exposure to the total 16.5 ml (methods 2.2.4) may not occur as rapidly as when the SMA-WIN micelles are injected into the blood. Also, the slight increase of total volume of 15 % - 33 % could increase release of WIN due to the dilution factor stated above (Rangel-Yagui et al., 2005). It is possible that this increase in volume, and total exposure to systemic circulation caused some dose dumping. This may occur at a greater rate
in micelles with an increased phase 1 period. Phase 1 release occurs rapidly, due to less stable micelles and the higher concentrations of micelles available for release. The initial exposure to systemic circulation could increase phase 1 release rate, which produced up to 30% release of WIN. This would account for a dose up to a maximum of 0.43 mg/kg (Chapter 2, figure 2.2). This is still smaller than the WIN control dose. The initial phase 1 period took 12 hours 

in vitro, and should also include the WIN released into the saline injection. This could account for the micelle release rate being faster in vivo than in vitro. This would explain higher concentrations of WIN being initially released into the systemic circulation than was originally hypothesised.

A final possibility is that the relative lack of sensitivity of the rotarod test could be a confounding factor. In this case the test would be insufficient to discriminate between the two different doses used. Once above a certain systemic concentration of free WIN, maximum motor impairment may occur, i.e., a “ceiling effect”. This fits with the data obtained, as only in the WIN group were any seizures observed, and none were seen within micelle treatment (Chapter 3). The seizures may indicate a higher concentration of free WIN in the positive control. The results of motor ataxia in this experiment were similar to the two other intravenous motor impairment studies. Gutierrez et al. (2011) showed that WIN caused motor ataxia for a full 1 hour, even though only half the dose of 0.5 mg/kg was used (compared to the 1 mg/kg used in Chapter 3). In fact, Meng et al. (1998) demonstrated that an intravenous dose of only 0.25 mg/kg can produce similar impairment and recovery to the data presented in this study, at only a quarter of the control dose. Recovery from motor impairment of SMA-WIN micelle treatment and WIN treatment groups occurred at similar rates. This, however, does not mean they had equivalent doses, as half the dose (Gutierrez et al., 2011) or even quarter the dose (Meng et al., 1998) could have caused the same effect. The seizures indicate a higher dose of WIN, even though both WIN and SMA-WIN micelles had the same level of
motor impairment, for the same duration of time. Identical recoveries can also be explained within this time frame, due to fast elimination kinetics and the ceiling effect previously described. First order elimination kinetics state that the plasma concentration of the drug is eliminated at an exponential decay rate. Intravenous WIN has a fast half-life of 7.2 minutes (Valiveti et al., 2004). Therefore the higher concentrations are eliminated at a faster rate, and are indistinguishable from lower doses, due to the initial ceiling effect of the behavioural assessment when using the rotarod.

Of note is the fact that the motor impairment recovery occurred well before the pain relief ceased. This strongly suggests that an initial WIN bolus or dose dumping occurred along with unspecific effects of the rotarod test. The bolus could be as small as 0.25 mg/kg or less (Meng et al., 1998). This is possible with pre-WIN release, along with potential dose dumping. The intravenous method of administration may be flawed, as with any small amount of WIN release, large side effects appear to be produced. The exact systemic concentrations to exert an effect on motor impairment are currently not known. However, it may be possible to minimise the effects (outlined in future directions below).

4.3. SMA-WIN micelles for the treatment of neuropathic pain

The findings of this investigation cast doubt of the initial selectivity for pain of the SMA-WIN nanomicelle over the behavioural side effects for the treatment of neuropathic pain. Nevertheless, the investigation strongly suggests that the new SMA-WIN micelle was effective in the treatment of neuropathic pain and produced prolonged analgesia. Previous research has investigated cannabinoids for the treatment of neuropathic pain. CB1 agonists have been found to be an effective treatment. While CB2 agonists were found to be effective only at doses where the drugs were no longer specific (although this is hotly debated)
Therefore both types of cannabinoids may produce CNS side effects by acting on the CB1 receptor (Fox et al., 2001). In terms of neuropathic pain treatment, cannabinoids can have a more prolonged effect at higher doses subcutaneously and intraperitoneally. However, these higher doses also cause prolonged side effects and impairment by acting on the CB1 receptors in the CNS (Fox et al., 2001). The new SMA-WIN micelle most likely produced impairment initially by a free WIN bolus acting on the CB1 receptors in the CNS. However, once metabolised due to its fast half-life, the treatment produced prolonged pain relief without motor impairment or obvious side effects. Whether or not this prolonged pain relief was caused by enhanced permeability retention of the drug in the site of inflammation, or low level WIN release is unknown. Still release rates should be low after the initial phase 1 period (Chapter 2, figure 2.2). The effects are most likely caused by the enhanced permeability retention effect of the micelles.

**4.4. Evaluation of cognitive impairment from SMA-WIN micelle**

In terms of tolerability, initial impairment from a cannabinoid may be preferable to the intolerable side effects of antidepressants (Dworkin et al., 2007), and also preferable to the additional risk of enhanced hyperalgesia that can be caused by opiates (Fields, 2011). Opiates only have a short term use before their effectiveness reduces (Dellemijn, 1999). There is even evidence that opiate use can prolong recovery (Woller et al., 2013). Administration of opiates is often essential after injuries that lead to neuropathic pain, such as spinal cord injuries. There is mounting evidence that opiate use adversely impacts the recovery and a new treatment may be preferable (Woller et al., 2013). Evidence suggests that repetitive dosing with cannabinoids may be more successful than opiates because efficacy is sustained (Costa et al., 2004; Rashid et al., 2004). While currently the SMA-WIN micelle is intravenously administrated, it has the
potential to provide immediate pain relief in a hospital environment. Many sufferers would not be able to use this route of administration independently. Further development of the principals of the SMA-WIN micelle could lead to improvements within the health system for treatment of neuropathic pain. There is definitely a potential role for nanoparticle based pain relief, but many hurdles remain to be overcome.

4.5. Future directions

In order to determine how CNS side effects might be reduced from the SMA-WIN micelles treatment, more experiments are needed. It may be possible to modulate SMA-WIN micelle side effects by changing the loading concentrations. Accurate behavioural measurements will be needed to be repeated, once this is resolved. However accurate behavioural measurements may represent a major challenge. When administrated systemically WIN appears to exert a high level of impairment even at low doses. This can be deduced from the fact that 0.25 mg/kg causes full motor impairment (Meng et al., 1998). Another study by Li et al. (1999) showed that 10 minutes after intravenous administration, WIN produced no effect at 0.1 mg/kg while 0.2 mg/kg showed impairment. It can be summarised that threshold for impairment to produce maximum effect from intravenous administration should therefore be between 0.1 mg/kg to 0.25 mg/kg. This does provide some promise, as micelles with an initial bolus under the threshold of motor impairment may not be unreasonable, as higher loadings have lower phase 1 release periods.

It could be important to calculate a dose-response for testing motor impairment in the rotarod experiment using I.V. administration. Currently Li et al. (1999) and Meng et al. (1998) are the only studies that give any indication of dosing. The Li et al. (1999) study exerted full motor impairment with a dose of 0.25 mg/kg and the Meng et al. (1998) study exerted only a partial
effect with a similar dosage of 0.2 mg/kg. Either the dose-response curve for motor impairment is very steep, or the studies contradict each other. It is important to resolve this, to calculate whether rotarod is still an appropriate measure for CNS impairment. The rotarod experiment may be limited by the lack of sensitivity to intravenous WIN, and therefore ultimately flawed as a measure of CNS impairment. This may be true with most measures of CNS impairment, when using intravenous injections, due to WINs short half-life (Valiveti et al., 2004).

To determine which was most significant out of pre-administration release of base WIN from the micelles, or dose dumping, a simple release study could use a larger volume of solution based on the protocol from Chapter 2 (methods 2.2.4). Following determination, steps could be taken to reduce the impact of both these effects. A higher loading concentration causes the micelle to form a stronger structure, decreasing phase 1 release, and potentially decreasing the dose dumping effect. Therefore, motor impairment of a new SMA-WIN micelle loading could be retested, with potentially decreased CNS impairment and similar neuropathic pain relief. If that approach fails other nanomicelles with similar properties could be pursued, as the prolonged pain relief appears promising. Chronic constriction injury would also be reassessed, if a product was produced that decreased CNS impairment. Release studies will still be limited as they do not completely represent what actually occur in the body. Finally, a biodistribution study could shed light on what is occurring. This could be adapted from a pharmacokinetics and drug distribution study as outlined by Greish (2010). Using radio-labelled $\text{H}^3$ WIN 55,212-2, micelles could be produced and the distribution throughout the body could be measured using a beta liquid scintillation counter. The biodistribution study could label where the WIN was bioaccumulating in the body and also indicate where it was producing the greatest effect. This could help to determine how the behavioural effects are caused, also indicate areas where metabolism is occurring. The biodistribution study would be
the most informative experiment to use, but it would be costly and time consuming. Every experiment leads to more inquiry and as knowledge increases, potential experiments could be carried on the successful readaptation of an SMA-WIN micelle product.

4.6. Effects of prolonged cannabinoid exposure on the development of neuropathic pain

The effects of prolonged cannabinoid exposure caused by SMA-WIN micelle treatment may have beneficial results for other diseases, not just neuropathic pain. While CB2 receptor activation appears to have little effect on established chronic neuropathy (Brownjohn et al., 2012), when administrated pre-emptively results have been more effective (Leichsenring et al., 2009). Chronic low dose administration of CB2 selective agonists have proven proficient at attenuating the development of mechanical allodynia. This corresponds with a reduction in spinal microglial activation (Hohmann et al., 2004; Leichsenring et al., 2009; Naguib et al., 2008). Neuropathic pain development has also been proven to be successful with treatment of the non-selective cannabinoid WIN (Burgos et al., 2012), or even microinjections that caused no behavioural impairment (Li et al., 1999). Glial cell activation and neuropathic pain development are correlated. Suppression of glial cell activation may result in suppressed attenuation of allodynia during the development phase. The application of SMA-WIN micelles may prove to be beneficial in this area of research to pre-emptively decrease neuropathic pain. This may only require a single injection, instead of multiple injections due to the extended micelle time in plasma. An advantage of this may be that lower doses could be possible. This would potentially decrease side effects and eliminate pain during the initial dosing.
4.7. Potential effects in inflammation

Another possibility for pharmacotherapy is using SMA-WIN micelles for the treatment of inflammatory pain. Although similar, inflammatory and neuropathic pains involve different pathologies. Injury at the tissue site induces inflammation, resulting in increased sensitivity and pain for the duration of the injury (Costigan et al., 2009). It is possible that the enhanced permeability effect could also target this site of inflammation, decreasing the inflammatory response. Cannabinoids have been shown to activate the cannabinoid receptors and decrease signs of inflammation and inflammatory pain, via CB1 and/or CB2 activation (Bolognini et al., 2010). The anti-inflammatory effects have been shown to be mediated peripherally. Cannabinoids that have limited blood brain barrier permeability have been produced and tested for alleviating inflammation (Yu et al., 2010). However, none have been produced with the intention of bioaccumulating and selectively targeting the direct site, which could allow lower dosing. Such a property may prove to make SMA-WIN nanomicelle a more effective treatment.

4.8. Impact on neuropathic pain research

The analgesic properties of cannabinoids appear promising. However, the central nervous system impairment that they cause is undesirable, this research sought to solve this problem. Past research has investigated CB2 receptor agonists as a way to negate this effect. Recent research has suggested that this strategy is unviable (Brownjohn et al., 2012). New research is investigating techniques to bypass the negative effects of cannabinoids, while still maintaining their therapeutic value. Several techniques appear to have promising therapeutic effects and could be useful for cannabinoid based pain relief.
Cannabinoids that do not pass the blood brain barrier have been produced. The idea behind this was to create an orally available drug, with limited blood brain barrier permeability. One example is AZD 1940. AZD portrayed initial promising results as a CB1/CB2 agonist, it had limited blood brain barrier permeability, and animal behavioural results showed a reduction in neuropathic pain. Yet it failed clinically at acute pain reduction within tolerable doses (Kalliomäki et al., 2013). The authors also reported a high level of side effects, around 80%. A less permeable drug, without these side effects is needed for the treatment of neuropathic pain. It may be possible to create a more successful version of this drug. Alternatively another research direction should be pursued.

Another technique that is being explored is targeting enhancement of endocannabinoid levels. Drugs like palmitoylethanolamide compete with endocannabinoids, as a substrate for fatty acid amide hydrolase. It has also been suggested that it activates the peroxisome proliferator-activated receptor α pathway. These mechanisms have been proposed to cause its anti-nociceptive and anti-inflammatory effects (Lambert et al., 2002; LoVerme et al., 2006). Research involving the development of a novel analogue of palmitoylethanolamide has shown successful antinociception properties with no side effects when administered locally (Roa-Coria et al., 2012). However, this still involves a local injection. Other mechanisms of decreasing metabolism, or of reducing uptake of endocannabinoids, may result in increased levels of endocannabinoids in tissues. This can produce behavioural analgesia in models of acute pain. It can also cause cannabinoid receptor activation at sites of high endocannabinoid turnover, as opposed to global CB1 receptor activation that can cause side effects (Naidu et al., 2010). Although this does not remove the possibility of side effects, it certainly appears to decrease them as the side effects are often not detected in animal studies. Unfortunately this efficacy may not be as high for all types of neuropathic pain compared to using cannabinoid ligands (Jayamanne et al., 2006).
Finally, targeting a specific tissue may prove the most effective strategy. When cannabinoids are administrated intrathecally, they treat neuropathic pain effectively (Brownjohn et al., 2012). Cannabinoids are also successful in the treatment of neuropathic pain when administrated locally such as in formalin paw models of inflammatory pain (Fox et al., 2001). Currently these are being administrated locally, or by specific injection sites. The use of SMA-WIN micelles could prove a promising area of research for this field as one intravenous injection can specifically target the site. Nanoparticles for the treatment of neuropathic pain are currently unknown within the literature. The possibility to bioaccumulate nanoparticles in areas of inflammation adds a superior aspect to treatment effectiveness. Although it is still in the development stage, SMA-WIN micelle research may open up a new field of research, due to its promising prolonged analgesia effectiveness.

4.7. Final conclusions

Successful synthesis of the new SMA-WIN micelle product was achieved, and the chemical properties appeared promising for selective targeting of neuropathic pain. The CCI behavioural results proved to be efficacious in the treatment of neuropathic pain. Although side effects were observed initially, they were not prolonged for the entire duration of pain relief. The CNS impairment was probably caused by an initial WIN bolus, associated with the SMA-WIN micelle injection. Even though the SMA-WIN micelle treatment was not entirely successful, with further research, nanomicelle delivery may prove to be a promising new treatment for neuropathic pain.
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