Effect of styrene maleic acid WIN55,212-2 micelles on neuropathic pain in a rat model

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Abstract
Cannabinoid receptor agonists are moderately effective at reducing neuropathic pain but are limited by psychoactivity. We developed a styrene maleic acid (SMA) based on the cannabinoid WIN 55,212-2 (WIN) and tested in a rat model of neuropathic pain and in the rotarod test. We hypothesized that micelle preparation can ensure prolonged plasma half-life being above the renal threshold of excretion. Furthermore, SMA-WIN could potentially reduce the central nervous system effects of encapsulated WIN by limiting its transport across the blood–brain barrier. 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 compared to control (base WIN). Attenuation of chronic constriction injury-induced mechanical allodynia occurred for up to 8 h at a dose of 11.5 mg/kg of SMA-WIN micelles. To evaluate central effects on motor function, the rotarod assessment was utilized. Results showed initial impairment caused by SMA-WIN micelles to be identical to WIN control for up to 1.5 h. Despite this, the SMA-WIN micelle formulation was able to produce prolonged analgesia over a time when there was decreased impairment in the rotarod test compared with base WIN.

Introduction
Current first-line treatments for neuropathic pain are only partially effective [1]. Tricyclic antidepressants at best provide a 50% reduction in pain intensity in 30% of affected individuals [2,3]. Second-line treatments such as tramadol and opioids have problems with long-term use, and lose efficacy with repeated dosing; opioids may even exacerbate hyperalgesia [4].

Synthetic cannabinoids such as WIN55,212-2 (WIN) [5] and CP55,940 [6] are potent cannabinoid CB1 and CB2 receptor agonists, and attenuate hyperalgesia and allodynia in rat models of neuropathic pain. However, cannabinoids which act on CB1 receptors cause psychotropic effects, limiting their therapeutic use [7]. There is preclinical evidence that peripherally restricted CB1 agonists have analgesic effects [8–10]; however, several peripherally targeting cannabinoids with limited blood–brain barrier penetration have failed to significantly relieve acute pain at tolerable doses in humans [11–14]. Nevertheless, if a cannabinoid drug could be designed to more selectively target nociceptors in pathological peripheral or nervous tissue in persistent pain conditions, it could provide a more effective treatment of chronic pain.

A strategy that might achieve this is selective nanomiceller targeting. Using micelles, it is possible to encapsulate hydrophobic molecules inside a larger hydrophilic structure [15]. In cancer research, it has been shown that tumour targeting may be achieved using such micelles for cytotoxic agents, because nanoparticle-sized drugs in the 10–200 nM range selectively extravasate at areas of disrupted vasculature, such as in tumours or inflamed tissue; part of the so-called enhanced permeability and retention (EPR) effect [16]. The nanomicelles also avoid renal excretion due to their size, and thus remain in circulation for extended periods, prolonging duration of action. We aimed to construct a cannabinoid nanomicelle in this size range using styrene maleic acid (SMA) to encapsulate WIN. SMA micellar systems have been previously used to improve the delivery of various anti-tumour drugs [17–19]. The styrene core encapsulates lipophilic drugs through hydrophobic association, while the maleic acid shell ensures water solubility.

We hypothesized that cannabinoid micelles could selectively target inflamed tissues in neuropathic pain. Some rodent models of peripheral neuropathic pain involve central neuroinflammatory processes, and increased vascular permeability in the spinal cord [20]; this may allow for selective targeting of spinal cannabinoid receptors through the EPR effect, and thus potentially reduce the psychotropic effects of cannabinoids in the brain. In this work, we synthesized and characterized nanomicelles based on SMA and WIN, a potent CB1/CB2 receptor agonist. We then tested the SMA-WIN construct in vivo, using a rodent model of neuropathic pain, chronic constriction injury (CCI), to assess its effects on neuropathic pain behaviours, and a rotarod test to assess its effects on motor impairment.
Methods

Synthesis of WIN nanomicelles using SMA

Cumene-terminated poly(styrene-co-maleic anhydride) (SMA) was obtained from Sigma-Aldrich (St. Louis, MO), supplied with a 1.3:1 mole ratio of styrene:maleic anhydride. N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDAC) was obtained from Sigma-Aldrich (St. Louis, MO). Other reagents were of commercial reagent grade and were used without further purification.

Preparation of SMA micelles containing WIN was similar to the method previously reported by Larson et al. [21]. Briefly, hydrolysed SMA solution at pH 5.0 was added to different weight ratios of WIN in the presence of EDAC to create different drug/SMA loadings. Set volumes of SMA were pipetted into a small beaker under continuous stirring, and diluted to 50 mL with deionized water. WIN was dissolved in 200-μL dimethyl sulfoxide (DMSO) and then added to the solution while stirring, to give a final concentration of 0.01%. This was followed by the rapid addition of EDAC dissolved in 8 mL of deionized water. A pH of 5 was maintained for 30 min, followed by addition of NaOH to adjust pH to 11. Once a clear solution was obtained and the pH stabilized it was adjusted back to 7.4.

Purification and concentration of the micelles was carried out by using ultrafiltration using an Amicon ultrafiltration system [YM-10 membrane; molecular weight cutoff (MWCO) 10 kDa]. The SMA-WIN micelles were washed in the ultrafiltration system with deionized water four times to ensure >99.99% purity. Following rotoevaporation, the product was frozen at −80°C and freeze dried, which produced SMA-WIN in powdered form. Loadings of WIN in SMA-WIN micelles were measured by dissolving known weights of SMA-WIN powder in 90% DMSO and measuring absorbance at 328 nm. WIN contents were then calculated based on a standard curve of WIN in 90% DMSO.

Drug release from SMA-WIN micelles

The release of free WIN from the SMA micelles was measured using a dialysis method. SMA-WIN solution (1.5 mL) at 5 mg/mL was pipetted into the inside of each dialysis membrane. Dialysis membranes were submerged in 15 mL of two different solutions inside a falcon tube: either distilled water at pH 7.4 or distilled water at pH 5.5. 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 and 96 h and the amount quantified at 328 nm. Absorbance readings were taken of both the bag solution and surround liquid combined at the end of the experiment. The percentage release was calculated according to Equation (1).

\[
\text{Percentage release} = \frac{\text{Absorbance Sample} \times \text{sample volume}}{\text{Absorbance of SMA} - \text{WIN before dialysis} \times \text{Total volume}} \times 100
\]  

In vivo testing of WIN nanomicelles

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 and 450 g were used for all behavioural testing. Prior to surgical manipulation or behavioural testing, rats were housed for at least 3 days in a 12-h light/dark cycle with ad libitum access to food and water.

Drug administration

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 30–60 min before injections. High and low doses for SMA-WIN were chosen based upon the loading and release of WIN from the micelles in vitro. These criteria are approximations only – stability of the micelles and release rates for WIN in vivo has not yet been determined. The equivalent amount of WIN in SMA-WIN for 1 mg/kg WIN was provided by 4.6 mg/kg SMA-WIN, and the same amount of WIN expected to be released from SMA-WIN by 11.5 mg/kg over the course of 12 h. Injection volume was 1 mL/kg in all cases. Rats were placed inside a restraining device and tails were warmed for 5–10 min to dilate veins, before drugs were administered intravenously (i.v.) with a sterile 28 gauge needle.

Chronic constriction injury

As previously described [22], animals were induced and maintained under 2.5% halothane anaesthesia (Nicholas Piramal Ltd., Maharashtra, India) in medical grade oxygen. 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 of the left hind limb. Initial incision was followed by blunt dissection with glass rods. Once exposed, sciatic nerves were maintained by applying saline (0.9% NaCl in deionized water). Four sutures (4/0 2 metric chromic gut; Ethicon Inc, NJ) were loosely tied (2-mm apart) around the exposed nerve, with no occlusion of the nerve process. The nerves were genitl 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 Inc.) and the skin was stapled. The animals were then allowed to recover and treated for post-operative pain. Buprenorphine (Reckitt Benckiser, Berkshire, UK) was administrated initially after surgery and post-operatively at 12-h intervals for 36 h (0.1 mg/kg short circuit). Animals were regularly monitored...
for 3 days following surgery, and 10 days were allowed for neuropathic pain to develop fully.

**Von Frey hair analysis**

Behavioural testing for mechanical allodynia was undertaken 1 day prior to surgery (baseline paw withdrawal thresholds), 3 days after surgery (during development of neuropathic pain) and 10 days post-surgery (pre-dose thresholds). Paw withdrawal threshold of the ipsilateral hindpaw was measured immediately prior to (‘‘pre-administration threshold’’) and at multiple time points (0.25, 0.5, 1, 2, 4, 8, 12, 24 and 48 h) after drug administration (‘‘post-administration thresholds’’). The experimenter was blinded to experimental group for all behavioural testing. Prior to behavioural 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 at least 15 min 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 Inc., Morgan Hill, CA) were presented to the left (ipsilateral) hindpaw. The hairs presented had the following log force (10,000 G) intensities: 3.61 (0.407), 3.84 (0.692), 4.08 (1.202), 4.17 (1.479), 4.31 (2.041), 4.56 (3.630), 4.74 (5.495), 4.93 (8.511), 5.07 (11.749) and 5.18 (15.136). A modified form of the up/down procedure was used; monofilaments were applied to the left ventral plantar aspects perpendicular to the hind paw for 3 s. A withdrawal was considered as a paw flinch upon presentation or sustained application of a given monofilament.

Calculation of 50% paw withdrawal thresholds was carried out by application of paw withdrawal response rates to a Gaussian integral psychometric function, using a maximum-likelihood curve fitting method. Conversion of the threshold values creates a continuum of values that are suitable for parametric statistical analysis. The program used to compute the data, PsychoFit, was created by Prof. Lewis O. Harvey at the University of Colorado, and is available as a freeware download (http://psych.colorado.edu/~lharvey/html/software.html). Data was then standardized using Equation (2).

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

(2)

**Rotarod test**

Motor performance was measured using an accelerating rotarod (RotaRod LE8500; Panlab SA, Barcelona, 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 min. Behavioural testing was undertaken 24 h after habituation. The habituation involved two training periods 1–2 h apart. Animals were placed on the accelerating rotarod until they were able to remain there for at least 60 s. 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 s the habituation was restarted from time point zero.

The time taken for the animal to fall off the rotarod was recorded as the latency to fall (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 24 h following i.v. drug treatment, at −0.083, 0.25, 0.5, 1, 1.5, 2, 4, 8, 12 and 24 h. In all experiments a 300-s cutoff was employed. If the animal was unable to stay on the rotarod at 4 rpm, a latency of 0 s was assigned. Data was recorded and expressed as the latency, and also expressed as a percentage disruption of performance [Equation (3)].

\[
\text{Percentage disruption} = 100 - \frac{\text{Post drug latency}}{\text{Pre drug latency}} \times 100
\]  

(3)

**Statistical analysis**

For *in vitro* micelle characterization, results were processed using Prism®, version 6.01 (GraphPad Software Inc., CA). 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. For both neuropathic pain and rotarod experiments, data was analysed with a two-way repeated measures analysis of variance (ANOVA) (time × drug), followed by Bonferroni post hoc testing where appropriate. Assumptions of two-way repeated measures 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, using IBM SPSS Statistics for Windows, version 21.0 (IBM Corp., Armonk, NY).

**Results**

**In vitro characterization of SMA-WIN micelles**

**Preparation and loading efficiency of SMA-WIN**

Our procedure successfully incorporated WIN into SMA micelles. The 5, 10 and 25% aimed loading concentrations produced a 66.8, 71.3 and 73.3% recovery, respectively. The actual loading percentages, calculated from Supplementary Figures S1 and S2 were found to be 5.6%, 14.7 and 27.2%, respectively. The SMA-WIN micelles were soluble in deionized water at all concentrations tested.

**SMA-WIN release profile**

WIN release rates for SMA-WIN micelles were assessed for 5, 15 and 27% loadings, at pH 5.5 and 7.4 (Figure 1). Release rates were increased at the higher pH, and there was a tendency towards slower release rates with increased loading.

**Size distribution of SMA-WIN micelles**

There was a trend towards increasing particle size as micelle loading was increased from 5 to 15% (Figure 2A and B), which reversed upon a further increase in loading to 27% (Figure 2C). SMA-WIN micelles with 15% loading produced a mean diameter at peak 1 of 158 nm and a width of 120 nm, with an intensity of 97.7% and mean diameter size of
116.2 nm; 27% loading produced an average diameter at peak 1 of 147 nm and a width of 97 nm, with an intensity of 87% and diameter size of 101 nm. These particle size distributions are in the desired range for overcoming renal excretion and thereby facilitating a possible EPR effect. On this basis, the decision was made to use an intermediate loading of 20% for in vivo experiments (see below for details).

In vivo characterization of SMA-WIN micelles

Effect of SMA-WIN micelles on CCI-induced mechanical allodynia

Intravenous administration of 1 mg/kg WIN and 11.5 mg/kg SMA-WIN micelles (the dose at which release of WIN is expected to provide a similar dose to 1 mg/kg WIN) significantly attenuated CCI-induced mechanical allodynia in the ipsilateral hindpaw (Figure 3). This was not the case for 4.6 mg/kg SMA-WIN micelles (a dose containing the same amount of WIN as 1 mg/kg WIN). Data are presented to show both the acute (0–2 h, Figure 3A) and sub-acute effects (2–24 h, Figure 3B). Two-way repeated measures ANOVA of paw withdrawal threshold revealed that the interaction between drug and time was significant for the ipsilateral hindpaw \( F_{(32,230)} = 1.755; p = 0.0055 \). Therefore, 11.5 mg/kg SMA-WIN not only reduces allodynia, it produced a prolonged reversal of allodynia compared with the other drug treatments. Specifically, Bonferroni post hoc tests revealed that compared to saline, 11.5 mg/kg SMA-WIN micelles
caused sustained attenuation of mechanical allodynia up to 8 h after administration (p < 0.01 at 8 h).

Post hoc analysis also revealed that the positive control, 1 mg/kg WIN, significantly attenuated CCI-induced mechanical allodynia compared to saline. This peaked at 15 min (p < 0.001), and there were no significant differences by 1-h post-injection (p > 0.05). No significant differences were observed between saline and 9.1 mg/kg SMA-WIN micelles at any time point (p > 0.05 for all comparisons).

**Effect of SMA-WIN micelles on motor performance**

Intravenous administration of 11.5 mg/kg SMA-WIN micelles and 1 mg/kg WIN both produced motor ataxia in rats. Analysis with two-way repeated measures ANOVA of latency to fall showed that the interaction between drug and time was significant [F(18,162) = 9.192, p < 0.001]. This made interpretation of individual time and treatment effects difficult (Figure 4). Bonferroni post hoc testing revealed that compared to saline control, 11.5 mg/kg SMA-WIN micelles caused a significant decrease in latency to fall lasting from 15 min to 1.5 h post-injection (p > 0.05), while WIN caused a significant decrease in latency to fall lasting from 15 min to 1 h post-injection (p < 0.05). There were no significant differences in latency to fall between SMA-WIN micelles and WIN at any time point (p > 0.05 for all comparisons).

**Discussion**

We successfully synthesized SMA-WIN micelles that are soluble in water, with loadings ranging from 5.7 to 27%. Water-soluble cannabinoids such as cannabinoid esters have been produced previously, but not water-soluble cannabinoid nanomicelles [23,24]. The formulations with lower loading concentrations had faster release rates than those with higher loading concentrations. Micelles with higher loadings may have a stronger hydrophobic association between the styrene moiety and WIN, leading to a more stable construct. These findings are consistent with other studies which demonstrate that a larger loading increases stability [18,25].

The micelles produced were all over 6 nm in diameter, i.e. larger than the fenestrations of the kidney [26] and in healthy blood vessels [27]. This should cause the micelles to be retained in the circulation and to not pass through the non-pathological blood–brain barrier. The nanoparticle size, together with extended plasma half-life, should theoretically allow the molecules to accumulate in areas of increased vascular permeability; the so-called EPR effect [16]. As neuropathic pain in animal models has an inflammatory
component, we aimed to use the micelles to target neuropathic pain in pathological tissues; i.e. the inflamed spinal cord and sciatic nerve.

Intravenous delivery of the SMA-WIN micelles attenuated CCI-induced mechanical allodynia in a dose-dependent manner. Allodynia was attenuated by SMA-WIN for 6–8 h longer than by WIN alone. In contrast to this, SMA-WIN micelles impaired performance on the rotarod test with a time course very similar to that of WIN, indicating that significant amounts of WIN penetrated into the CNS very soon after administration of the SMA-WIN.

Our rotarod results using WIN were similar to those reported in other motor impairment studies by Gutierrez et al. [28], in which 0.5 mg/kg of WIN (i.v.) caused motor ataxia for up to 1 h. Nonetheless, as allodynia reversal continued for several hours after performance on the rotarod test had returned to baseline, this indicates that pain relief was at least partially uncoupled from psychoactive effects in our experiments. The prolonged reversal of mechanical allodynia (up to 8 h) with SMA-WIN in our experiments far exceeds that reported in previous studies using WIN in the CCI model. 2.14 mg/kg WIN (i.p.) significantly reduced CCI-induced mechanical allodynia for only 90-min post-administration.

It is not clear why SMA-WIN produced no significant motor impairment after 1.5 h whereas attenuation of mechanical allodynia continued for up to 8 h. This might be due to the accumulation of micelles in inflamed tissue at the injury site in the CCI experiments, or alternatively, the relative insensitivity of the rotarod test compared to Von Frey hair testing. Previous studies using 0.25 mg/kg (i.v.) [29] and 0.5 mg/kg (i.v.) [28] WIN have indicated similar levels of motor impairment in the rotarod test over a comparable timescale to that reported with 1 mg/kg WIN in this study, indicating the poor resolution of this assay for determining the dose dependence of motor impairment. Therefore, although recovery from motor impairment after SMA-WIN micelle treatment and WIN treatment occurred at similar rates, it cannot necessarily be inferred that these two treatments produced similar CNS levels of WIN.

Nevertheless, the rapid onset of ataxia in healthy rats after SMA-WIN administration was unexpected. One possibility is that intact SMA-WIN micelles crossed the blood–brain barrier, and released WIN directly into the brain. This is unlikely, however, due to the large size of the nanoparticles; previous studies have shown a very low distribution of nanoparticles to the non-pathological brain with nanoparticles of this size [18,30]. Another possibility is the inadvertent delivery of an initial bolus of WIN, originating from the micelles prior to administration. However, the micelles were prepared only 1 h prior to testing, and under these conditions it has been calculated that a maximum bolus equivalent to 0.1 mg/kg WIN would have been released from the micelles; a dose below that expected to produce significant motor impairment [28]. A third possibility is that the micelles became less stable once within the plasma. However, previous micelle studies have shown that SMA micelles have a slow release in comparison to other micelle formulations [18]. Nevertheless, some ‘dose dumping’ may have occurred and thus account for the rapid onset of ataxia, with the prolonged analgesic effects caused by the remaining stable micelles.

An important limitation of the present study is that drugs were injected 10 days after CCI, in keeping with established protocols [22]. However, increased vascular permeability may peak at the relevant sites in the spinal cord several days earlier in this model, and largely resolve within 10 days [31]. Therefore, administration of SMA-WIN micelles at an earlier time point, during neuropathic pain induction, may prove more beneficial in future experiments. In addition, the nanomicelles may be more effective in models of inflammatory, rather than neuropathic pain. Although there are behavioural similarities in inflammatory and neuropathic pain conditions, they involve differing pathologies like inflammation-driven hypersensitivity arises in inflamed peripheral tissue, whereas neuropathic hypersensitivity develops in the spinal cord [32].

Cannabinoids, including those with limited blood–brain barrier permeability [10] have been shown to decrease inflammation and inflammatory pain via both CB1 and CB2 activation [32]. Inflammation is characterized by vascular permeability and the extravasation of large structures into the extracellular matrix; ideal conditions for targeted delivery by nanoparticles. This enhance permeability effect of cannabinoid-loaded micelles could directly target of peripheral inflammation, decreasing both the inflammatory response and subsequent pain. Such a strategy has recently been investigated using SMA-encapsulated CO for inflammatory bowel disease [33]. Given the adverse effects that limit the dose and duration of the use of current anti-inflammatory drugs such as corticosteroids, the targeting of peripheral inflammation appears to be the most promising application for SMA-WIN nanomicelles, and requires further experimental exploration.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.
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