Anti-allodynic Effects of Obtusifolin and Gluco-Obtusifolin against Inflammatory and Neuropathic Pain: Possible Mechanism for Neuroinflammation

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Inflammatory pain and neuropathic pain are major health issues that represent considerable social and economic burden worldwide. In this study we investigated the potential of obtusifolin and gluco-obtusifolin, two anthraquinones found in the seeds of the widely used traditional Chinese medical botanical Cassia obtusifolia, to reduce neuropathic and inflammatory pain. The potential analgesic effects of obtusifolin and gluco-obtusifolin were evaluated by mice formalin test and complete Freund's adjuvant (CFA)-induced nociceptive behaviors in rats. Chronic constriction injury (CCI), L5 spinal nerve ligation (L5 SNL), diabetes, and chemotherapeutics inducing allodynia were used to test whether repeated treatment with obtusifolin and gluco-obtusifolin ameliorated neuropathic pain. Finally, we explored whether obtusifolin and gluco-obtusifolin altered the degree of neuroinflammation in rat spinal cord after CFA administration and CCI induction. Obtusifolin and gluco-obtusifolin (0.25, 0.5, 1, and 2 mg/kg) reversed mechanical allodynia induced by CFA, CCI, L5 SNL, diabetes, and oxaliplatin in a dose-dependent manner in phase 2 of formalin-induced behavior in mice. Furthermore, repeated administration of obtusifolin and gluco-obtusifolin (0.25, 0.5, 1, and 2 mg/kg) reduced licking/biting time in dose-dependent manner in phase 2 of formalin-induced behavior in mice. Unfortunately, repeated administration of obtusifolin and gluco-obtusifolin (0.25, 0.5, 1, and 2 mg/kg) reversed mechanical allodynia induced by CFA, CCI, L5 SNL, diabetes, and oxaliplatin in a dose-dependent manner in rats. Levels of activated nuclear factor-kappa B (NF-κB) and proinflammatory cytokines (interleukin (IL)-1β, IL-6, tumor necrosis factor α (TNF-α)) in lumbar spinal cord were elevated in rats following CFA treatment and CCI induction, and obtusifolin and gluco-obtusifolin significantly inhibited these effects. Our results demonstrate that obtusifolin and gluco-obtusifolin produce significant antinociceptive action in rodent behavioral models of inflammatory/neuropathic pain, and that this activity is associated with modulation of neuroinflammation in spinal cord.

Key words obtusifolin; gluco-obtusifolin; pain; spinal cord; neuroinflammation

Chronic pain is a serious health issue that represents a great social and economic burden in the world. Inflammatory and neuropathic pain is generally viewed to be the most common types of chronic pain. Most current drugs for chronic pain fall into the categories of opioid analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), and antidepressants, etc. Unfortunately, these medications may provide unsatisfactory results, and produce various side effects, which underscore the need for novel analgesics.1,2

Medicinal plants are an important resource for potential novel drugs.3,4 Indeed, many plants exert strong antinociceptive action, and botanical medicines have proven effective for antagonizing different types of chronic pain.1,3

Obtusifolin and gluco-obtusifolin (Fig. 1) are two anthraquinones found in the seeds of Cassia obtusifolia, which are widely used in traditional Chinese medicine.4 Previous reports have shown that gluco-obtusifolin and obtusifolin have inhibitory effects on platelet aggregation and aldose reductase activity, respectively.5 Another report showed that obtusifolin and gluco-obtusifolin ameliorate scopolamine-induced memory impairment in mice.4 However, no reports have been published on the analgesic effects of obtusifolin and gluco-obtusifolin. Thus, the present study was performed to characterize the anti-allodynic effects of obtusifolin and gluco-obtusifolin in diverse models of pain induced by inflammatory (formalin and complete Freund’s adjuvant (CFA) and cancer chemotherapy factors. In this study, we provide evidence that obtusifolin and gluco-obtusifolin afford marked relief of inflammatory and neuropathic pain, and that this analgesic effect is likely associated with inhibition of cytokine (interleukin (IL)-1β, IL-6, and tumor necrosis factor α (TNF-α)) up-regulation in the spinal cord. The antinociceptive effects of obtusifolin and gluco-obtusifolin described in this study provide experimental evidence supporting their use as a novel approach for the treat-

![Chemical Structures of Obtusifolin and Gluco-Obtusifolin](image)

The authors declare no conflict of interest.

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ment of chronic pain.

MATERIALS AND METHODS

Animals, Drugs, and Treatment  ICR mice and Sprague-Dawley rats (Shanghai Laboratory Animal Center, Chinese Academy of Sciences, male, 18–22 g and 180–200 g) were used in this experiment. The animals had free access to food and water except during behavioral test. All rodents were kept in a temperature-controlled room (25 ± 2°C) with a 12-h light/12-h dark cycle. All animals were acclimatized to the animal facility for at least 1 week prior to experiments. All behavioral tests were performed between 09:30 and 18:00.

In each test, the rodents were assigned to obtusifolin and gluco-obtusifolin groups, a positive drug group (except for cytokine assessment), a vehicle group, or a sham group (for CCI-induced and SNL-induced neuropathic pain). They were habituated to the test room for at least 30 min prior to behavioral test. The observer was blinded to drug treatments in all tests. All experiments were conducted in compliance with international laws on animal experimentation and were approved by the Committee of Ethics of Fudan University. All procedures were carried out in accordance with the guidelines for animal care and use at Fudan University.

Obtusifolin and gluco-obtusifolin were purchased from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China) with a purity of 99%. Morphine hydrochloride (Aladdin Industrial Inc., Shanghai, China), aspirin (Sigma), mebhydroxate (Sigma), and gabapentin (Sigma) were used as positive controls. Tween 80 was purchased from Qiaosun chemical (Chengdu, China). CFA and oxaliplatin were obtained from Sigma-Aldrich Corp. (Dongguan, China). Formalin was prepared by mixing 10% formalin and 70% ethanol.

Animals, Drugs, and Treatment  ICR mice and Sprague-Dawley rats (Shanghai Laboratory Animal Center, Chinese Academy of Sciences, male, 18–22 g and 180–200 g) were used in this experiment. The animals had free access to food and water except during behavioral test. All rodents were kept in a temperature-controlled room (25 ± 2°C) with a 12-h light/12-h dark cycle. All animals were acclimatized to the animal facility for at least 1 week prior to experiments. All behavioral tests were performed between 09:30 and 18:00.

In each test, the rodents were assigned to obtusifolin and gluco-obtusifolin groups, a positive drug group (except for cytokine assessment), a vehicle group, or a sham group (for CCI-induced and SNL-induced neuropathic pain). They were habituated to the test room for at least 30 min prior to behavioral test. The observer was blinded to drug treatments in all tests. All experiments were conducted in compliance with international laws on animal experimentation and were approved by the Committee of Ethics of Fudan University. All procedures were carried out in accordance with the guidelines for animal care and use at Fudan University.

Obtusifolin and gluco-obtusifolin were purchased form Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China) with a purity of 99%. Morphine hydrochloride (Aladdin Industrial Inc., Shanghai, China), aspirin (Sigma), mebhydroxate (Sigma), and gabapentin (Sigma) were used as positive controls. Tween 80 was purchased from Qiaosun chemical (Chengdu, China). CFA and oxaliplatin were obtained from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.).

Obtusifolin and gluco-obtusifolin were dissolved in 10% Tween 80 solution, and the dosage ranges of obtusifolin and gluco-obtusifolin treatment were based on a previous study. Gabapentin, morphine, aspirin, and mebhydroxate were dissolved (or diluted) in physiological saline and administered intraperitoneally (i.p.) at a dose volume of 10 mL/kg for mice or 4 mL/kg for rats.

Formalin-Induced Inflammatory Pain in Mouse  The formalin test was adopted by the methods of Dubuisson and Dennis with minor modifications. Mice received 10 μL of 5% formalin into the dorsal surface of right hindpaw. After injection, formalin induced biphasic pain behavior responses that were temporally divided into phase 1 (0–5 min), an interphase period with no pain behaviors, and phase 2 (11–60 min). Time of licking/biting the injected hindpaw was recorded as nociceptive behavior in 5-min bins for 1 h. Obtusifolin and gluco-obtusifolin (0.25, 0.5, 1, and 2 mg/kg), morphine (10 mg/kg, positive control), aspirin (100 mg/kg, positive control), or vehicle was administered i.p. 30 min prior to formalin treatment. The inhibition of nociceptive behavior was calculated using the following formula for each time phase: % inhibition = [(control licking time – test licking time)/control licking time] × 100.

CFA-Induced Persistent Inflammatory Pain Model  To induce persistent inflammatory pain, rats were placed under isoflurane anesthesia and 100 μL of CFA (1 mg/mL Mycobacterium tuberculosis) solution was injected into the plantar side of 1 hind paw, while sham groups were injected with vehicle. Our previous studies showed that significant CFA-induced mechanical pain hypersensitivity developed at 2 h, and reached a peak level between 6 and 24 h that was maintained for at least 7 d. Obtusifolin and gluco-obtusifolin (0.25, 0.5, 1, and 2 mg/kg), mebhydroxate (0.5 mg/kg, positive control), or vehicle was administered i.p. once per day for 5 consecutive days beginning 24 h after CFA injection. The mechanical threshold was measured before CFA injection (baseline), 1 d after CFA injection (predose), and 30 min after drug administration (postdose) on the morning of post-CFA injection days 1 to 5. Mechanical threshold testing was performed using the procedures described below. The inhibitory rate of obtusifolin and gluco-obtusifolin on inflammatory hyperalgesia was expressed as percentage of maximal possible effect (%MPE): MPE% = [(postdose threshold) – (predose threshold)]/[(baseline threshold) – (predose threshold)] × 100. It is possible to obtain a negative MPE if threshold decreases after treatment.

Chronic Constriction Injury Model  The rat CCI neuropathic pain model was implemented according to the method described previously. Rats were anesthetized with chloral hydrate (400 mg/kg i.p., Sigma-Aldrich Co.). The right common sciatic nerve was isolated at the mid-thigh level, and loosely ligated using 4 chormic gut (5-0) ties separated by an interval of 1 mm. All animals were allowed 3 d to recover from the surgery. Obtusifolin and gluco-obtusifolin (0.25, 0.5, 1, and 2 mg/kg), gabapentin (50 mg/kg, positive control), or vehicle was administered i.p. once per day for 7 consecutive days beginning from postoperative day 4. The mechanical threshold was measured before surgery (baseline), after surgery (predose), and 30 min after drug administration (postdose) on the morning of postoperative days 4 to 10. Mechanical threshold testing was performed using the procedures described below. The inhibitory rates of obtusifolin and gluco-obtusifolin for mechanical allodynia were calculated using the same procedures as in the CFA studies.

L5 Spinal Nerve Ligation Model  The rat SNL neuropathic pain model was established according to previously described methods. Rats were placed under anesthesia (chloral hydrate 400 mg/kg i.p.) and a 1.5-cm incision was made dorsal to the lumbosacral plexus. The paraspinal muscles on the right side were separated from the spinal processes, and the L5 spinal nerves were isolated and ligated with 3-0 silk suture distal to the dorsal root ganglion (DRG). Following SNL operation, the rats were given 3-d of recovery. Obtusifolin and gluco-obtusifolin (0.25, 0.5, 1, and 2 mg/kg), gabapentin (50 mg/kg, positive control), or vehicle was administered i.p. once per day for 7 consecutive days beginning from postoperative day 4. Mechanical threshold was measured before surgery (baseline), after surgery (predose), and 30 min after drug administration (postdose) on the morning of postoperative days 4 to 10. Mechanical withdraw threshold testing was performed according to the procedures described below. The inhibitory rates were calculated using the same formula as in the CFA studies.

Diabetic Model of Neuropathic Pain  Rats were given a single i.p. injection of STZ (60 mg/kg, Sigma-Aldrich Co.) or vehicle (0.01 M citrate buffer, pH 4.5) as described previously. Seventy-two hours later, blood (tail vein) glucose level was measured using a commercial glucose meter (OneTouch Ultra, Lifescan [Johnson & Johnson], New Bruns-
The rats with a minimum glucose level of 16.67 mmol/L were used for further experiments. Starting from day 22 after the STZ injection, the rats received daily i.p. injection of obtusifolin and gluco-obtusifolin (0.25, 0.5, 1, and 2 mg/kg), gabapentin (50 mg/kg, positive control), or vehicle for 7 consecutive days. Pain behavior was assessed as described below prior to the STZ injection (baseline), before drug treatment (pre-dosing), and 30 min after each drug injection (post-dosing). The inhibitory rates were calculated using the same formula as in the CFA studies.

Chemotherapy-Induced Neuropathic Pain To study neuropathic pain related to chemotherapy in rats, oxaliplatin (Sigma-Aldrich Co.) was diluted to 2 mg/mL with 5% dextrose in distilled water and injected i.p. at 2 mg/kg on 5 consecutive days. Control animals received vehicle injections. Obtusifolin and gluco-obtusifolin (0.25, 0.5, 1, and 2 mg/kg), gabapentin (50 mg/kg, positive control), or vehicle was administered i.p. once per day for 5 d beginning from 20 d after oxaliplatin injection. Mechanical threshold was measured before oxaliplatin injection (baseline), 20 d after oxaliplatin injection (predose), and 30 min after drug administration (postdose) in the morning. Mechanical withdrawal threshold testing was performed according to the procedures described below. The inhibitory rates were calculated using the same formula as in the CFA studies.

Mechanical Withdrawal Threshold Testing Mechanical withdrawal threshold was measured using an electronic von Frey apparatus (Model 2390, IITC Life Science Inc., Woodland Hills, CA, U.S.A.) as previously described, with minor modifications. Rats were placed into a Plexiglas box on a steel mesh floor and analyses were performed. Stimulation was applied to the center of the hind paw by upward motion of the von Frey filament until the foot was withdrawn, and the threshold was automatically recorded. The maximum strength of the filament used for von Frey testing was 55 g.

Cytokine Determination After CFA and CCI experiments, the animals were sacrificed by decapitation. The L4–5 spinal cords ipsilateral to CFA injection or CCI were obtained. Activation of the transcription factor nuclear factor-kappa B (NF-κB) in the spinal cord was determined using an enzyme-linked immunosorbent assay (ELISA) kit from Active Motif (Cayman Chemical, CA, U.S.A.) based on the principle that only the active form of NF-κB binds to the oligonucleotide containing the DNA consensus site (5'-GGG ACT TTC C-3'). The lumbar section of the spinal cord was homogenized in lysis buffer. Nuclear extract was prepared using a Nuclear Extract Kit (Cayman Chemical) and allowed to bind to a double-stranded oligonucleotide immobilized on the microtiter plate. The primary antibody against the NF-κB p65 subunit was added and a horseradish peroxidase-conjugated secondary antibody was used for detection. TNF-α, IL-6, and IL-1β levels were also quantified using ELISA kits (Abcam, Cam-
Statistical Analysis  Data from behavioral tests and ELISA were analyzed using two-way ANOVA or one-way ANOVA, followed by the Bonferroni or Tukey’s test, respectively, for post hoc analysis. All data are presented as mean±S.E.M. and all statistical analyses were performed using SPSS software version 16.0 (SPSS Inc., Chicago, IL, U.S.A.). A p value of <0.05 was considered statistically significant.

RESULTS

Effects of Obtusifolin and Gluco-Obtusifolin on Formalin-Induced Inflammatory Pain  The effects of obtusifolin and gluco-obtusifolin on formalin-induced nociceptive behaviors are shown in Fig. 2. Vehicle-treated control rats showed overt behavioral signs in response to formalin, showing a peak in activity within 5 min, and a second phase of licking/biting that began at 11 min. Obtusifolin (Fig. 2A), gluco-obtusifolin (Fig. 2B), and aspirin did not reduce phase 1 pain behaviors (p>0.05). In contrast, obtusifolin (Fig. 2C) and gluco-obtusifolin (Fig. 2D) reduced formalin-induced nociceptive behaviors in phase 2 in a dose-dependent manner (F(4, 55)=38.67, p<0.001 for obtusifolin; F(4, 55)=44.19, p<0.001 for gluco-obtusifolin), with obtusifolin (0.25, 0.5, 1, and 2 mg/kg) and gluco-obtusifolin (0.5, 1, and 2 mg/kg) producing a significant reduction in pain behaviors (p<0.001 versus vehicle). The inhibition rates for 0.25, 0.5, 1, and 2 mg/kg obtusifolin were

Fig. 3. Time Course of the Analgesic Effects of Obtusifolin (A) and Gluco-Obtusifolin (B) on CFA Model

Obtusifolin, gluco-obtusifolin (2 mg/kg) or vehicle was administered i.p. 24 h after CFA injection. MWT was expressed as mean±S.E.M. (N=8 per group). ***p<0.001 versus sham group, two-way ANOVA followed by Bonferroni test. ###p<0.001 versus vehicle group, two-way ANOVA followed by Bonferroni test.
Effects of Obtusifolin and Gluco-Obtusifolin on CFA-Induced Chronic Inflammatory Pain

In previous studies, we examined the effects of obtusifolin and gluco-obtusifolin (0.25, 0.5, 1, and 2 mg/kg) in naïve rats and found no significant treatment effects on normal mechanical withdrawal threshold (MWT) (data not shown). Next, the effects of obtusifolin and gluco-obtusifolin on chronic inflammatory pain were examined by CFA model (Figs. 3, 4). The analgesic effects of obtusifolin and gluco-obtusifolin were measured 30 min after administration because the maximal effects were observed 30 min after treatment (Fig. 3). For obtusifolin (Fig. 4A), two-way ANOVA on MWT demonstrated a significant treatment effect between groups ($F(4, 280) = 98.63, p < 0.001$), a significant effect of time ($F(6, 280) = 174.1, p < 0.001$), and an interaction of treatment and time ($F(24, 280) = 7.59, p < 0.001$). Regarding the effect of gluco-obtusifolin on MWT (Fig. 4B), two-way ANOVA also demonstrated a significant treatment effect between groups ($F(4, 280) = 64.65, p < 0.001$), a significant effect of time ($F(6, 280) = 195, p < 0.001$), and an interaction between treatment and time ($F(24, 280) = 4.88, p < 0.001$). As expected, CFA-treated rats that received vehicle developed statistically significant mechanical allodynia ($p < 0.001$ versus sham control). Methotrexate significantly reduced mechanical allodynia ($p < 0.001$ versus vehicle control). Repeated treatment with obtusifolin (Fig. 4A) and gluco-obtusifolin (Fig. 4B) significantly decreased the severity of mechanical allodynia ($p < 0.001$). The analgesic effects of obtusifolin and gluco-obtusifolin were dose-dependent, and there was no indication of the development of tolerance to anti-allodynia effects. The MPE of obtusifolin at doses of 0.25, 0.5, 1, and 2 mg/kg on the 5th day after treatment were 51.13 ± 4.13%, 53.82 ± 5.28%, 86.75 ± 5.63%, and 91.68 ± 8.42%, respectively, compared to 71.01 ± 7.15%, MPE for methotrexate at 0.5 mg/kg (Fig. 4C). The MPE of gluco-obtusifolin at doses of 0.25, 0.5, 1, and 2 mg/kg were 27.83 ± 4.33%, 39.99 ± 6.14%, 51.71 ± 7.18%, and 73.17 ± 4.57%, respectively, while the MPE of methotrexate was 69.45 ± 5.56% (Fig. 4D).

Effects of Obtusifolin and Gluco-Obtusifolin in the CCI Model of Neuropathic Pain

The ability of obtusifolin and gluco-obtusifolin to reduce pain in the formalin and CFA model prompted us to examine the effects of obtusifolin and gluco-obtusifolin on neuropathic pain. Firstly, we measured the effects of obtusifolin and gluco-obtusifolin in the CCI model. For obtusifolin (Fig. 5A), two-way ANOVA on MWT demonstrated a significant treatment effects between groups ($F(4, 315) = 83.08, p < 0.001$), a significant effect of time ($F(8, 315) = 137.9, p < 0.001$), and an interaction between treatment and time ($F(32, 315) = 3.71, p < 0.001$). For gluco-obtusifolin, two-way ANOVA also demonstrated a significant treatment effect between groups ($F(4, 315) = 83.08, p < 0.001$), a significant effect of time ($F(8, 315) = 137.9, p < 0.001$), and an interaction between treatment and time ($F(32, 315) = 3.71, p < 0.001$). For gluco-obtusifolin, the effects of obtusifolin and gluco-obtusifolin on CCI-Induced Mechanical Alldynia in Rats

Obtusifolin, gluco-obtusifolin (0.25, 0.5, 1, and 2 mg/kg), gabapentin (50 mg/kg), or vehicle was administered i.p. once per day for 7 consecutive days, beginning on day 4 after CCI surgery. The time courses of obtusifolin and gluco-obtusifolin treatment effects are shown in (A) and (B). The MPE of obtusifolin and gluco-obtusifolin on the 7th day of treatment is shown in (C) and (D). MWT and MPE data are expressed as mean ± S.E.M. ($N = 8$ per group). ***$p < 0.001$ versus sham group, two-way ANOVA followed by Bonferroni test. **$p < 0.001$ versus vehicle group, one-way or two-way ANOVA followed by Bonferroni or Tukey’s test.
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(Fig. 5B), two-way ANOVA on MWT also demonstrated a significant treatment effects between groups ($F_{(4,315)}=67.78$, $p<0.001$), a significant effect of time ($F_{(8,315)}=144.4$, $p<0.001$), and an interaction between treatment and time ($F_{(32,315)}=4.55$, $p<0.001$). The post hoc Bonferroni’s tests showed that CCI significantly decreased MWT ($p<0.001$ versus sham control), and that gabapentin significantly reduced mechanical allodynia ($p<0.001$ versus vehicle control). Administration of obtusifolin (Fig. 5A) and gluco-obtusifolin (Fig. 5B) (0.25, 0.5, 1, and 2 mg/kg) once per day reversed mechanical allodynia ($p<0.001$). The anti-CCI-induced allodynia effects of obtusifolin and gluco-obtusifolin were dose-dependent, and no development of tolerance to the anti-allodynic effects was seen. The MPE of obtusifolin at doses of 0.25, 0.5, 1, and 2 mg/kg on the 7th day after treatment was $32.24\pm5.78\%$, $37.96\pm5.98\%$, $62.24\pm5.35\%$, and $68.63\pm5.26\%$, respectively, and the MPE of gabapentin was $46.97\pm6.51\%$ (Fig. 5C).

Effects of Obtusifolin and Gluco-Obtusifolin in the SNL Model of Neuropathic Pain  To further evaluate the ability of obtusifolin and gluco-obtusifolin to mitigate neuropathic pain, we measured the effects of obtusifolin and gluco-obtusifolin using the L5 SNL model. For obtusifolin (Fig. 6A), two-way ANOVA on MWT demonstrated a significant treatment effect between groups ($F_{(4,315)}=80.46$, $p<0.001$), a significant effect of time ($F_{(8,315)}=144.4$, $p<0.001$), and an interaction between treatment and time ($F_{(32,315)}=4.55$, $p<0.001$). For gluco-obtusifolin (Fig. 6B), two-way ANOVA on MWT also demonstrated a significant treatment effect between groups ($F_{(4,315)}=88.62$, $p<0.001$), a significant effect of time ($F_{(8,315)}=172$, $p<0.001$), and an interaction between treatment and time ($F_{(32,315)}=5.97$, $p<0.001$). Similar to results for CCI, post hoc Bonferroni’s tests showed SNL significantly decreased MWT ($p<0.001$ versus sham control). Gabapentin significantly reduced mechanical allodynia versus vehicle control ($p<0.001$). Administration of obtusifolin (Fig. 6A) and gluco-obtusifolin (Fig. 6B) (0.25, 0.5, 1, and 2 mg/kg) once per day from days 4 through 10 after SNL also reversed mechanical allodynia ($p<0.001$). The analgesic effects of obtusifolin and gluco-obtusifolin were dose-dependent, and there was no indication of the development of tolerance to anti-allodynia effects. The MPE of obtusifolin at doses of 0.25, 0.5, 1, and 2 mg/kg on the 7th day after treatment was $17.66\pm7.25\%$, $30.98\pm5.32\%$, $62.24\pm5.35\%$, and $68.63\pm5.26\%$, respectively, and the MPE of gabapentin was $46.97\pm6.51\%$ (Fig. 6C).

Effects of Obtusifolin and Gluco-Obtusifolin Neuropathic Pain in a Diabetic Model  Similar to CCI and SNL, diabetes evokes neuropathic pain. To evaluate the effects of obtusifolin and gluco-obtusifolin on the alleviation of diabetic neuropathic pain, we measured the effects of obtusifolin and gluco-obtusifolin using the L5 SNL model. For obtusifolin (Fig. 6A), two-way ANOVA on MWT demonstrated a significant treatment effect between groups ($F_{(4,315)}=80.46$, $p<0.001$), a significant effect of time ($F_{(8,315)}=144.4$, $p<0.001$), and an interaction between treatment and time ($F_{(32,315)}=4.55$, $p<0.001$). For gluco-obtusifolin (Fig. 6B), two-way ANOVA on MWT also demonstrated a significant treatment effect between groups ($F_{(4,315)}=88.62$, $p<0.001$), a significant effect of time ($F_{(8,315)}=172$, $p<0.001$), and an interaction between treatment and time ($F_{(32,315)}=5.97$, $p<0.001$). Similar to results for CCI, post hoc Bonferroni’s tests showed SNL significantly decreased MWT ($p<0.001$ versus sham control). Gabapentin significantly reduced mechanical allodynia versus vehicle control ($p<0.001$). Administration of obtusifolin (Fig. 6A) and gluco-obtusifolin (Fig. 6B) (0.25, 0.5, 1, and 2 mg/kg) once per day from days 4 through 10 after SNL also reversed mechanical allodynia ($p<0.001$). The analgesic effects of obtusifolin and gluco-obtusifolin were dose-dependent, and there was no indication of the development of tolerance to anti-allodynia effects. The MPE of obtusifolin at doses of 0.25, 0.5, 1, and 2 mg/kg on the 7th day after treatment was $17.66\pm7.25\%$, $30.98\pm5.32\%$, $62.24\pm5.35\%$, and $68.63\pm5.26\%$, respectively, and the MPE of gabapentin was $46.97\pm6.51\%$ (Fig. 6C). The MPE for gluco-obtusifolin at doses of 0.25, 0.5, 1, and 2 mg/kg was $32.94\pm4.91\%$, $54.95\pm6.11\%$, $64.07\pm3.42\%$, and $80.55\pm4.17\%$, respectively, while the MPE of gabapentin was $70.6\pm6.39\%$ (Fig. 6D).
neuropathic pain, we examined their effects in STZ-induced diabetes model (Fig. 7). For obtusifolin (Fig. 7A), two-way ANOVA on MWT demonstrated a significant treatment effect between groups ($F_{(4,405)}=113.9$, $p<0.001$), a significant effect of time ($F_{(8,405)}=198.6$, $p<0.001$), and an interaction between treatment and time ($F_{(32,405)}=7.87$, $p<0.001$). For gluco-obtusifolin (Fig. 7B) two-way ANOVA on MWT also demonstrated a significant treatment effect between groups ($F_{(4,405)}=100.3$, $p<0.001$), a significant effect of time ($F_{(8,405)}=189.2$, $p<0.001$), and an interaction between treatment and time ($F_{(32,405)}=7.28$, $p<0.001$). The post hoc tests showed that diabetes significantly decreased MWT to mechanical stimulation ($p<0.001$ versus sham) as expected, and precipitated the development of mechanical allodynia. Gabapentin significantly reduced mechanical allodynia versus vehicle control ($p<0.001$). Obtusifolin (Fig. 7A) and gluco-obtusifolin (Fig. 7B) attenuated diabetes-induced allodynia in a dose-dependent manner ($p<0.001$ versus vehicle) without indication of the development of tolerance. On the 28th day of experimentation (1 week after obtusifolin and gluco-obtusifolin administration), the MPE of obtusifolin at doses of 0.25, 0.5, 1, and 2 mg/kg was 44.59±4.94%, 50.02±4.38%, 59.23±3.27%, and 69.03±5.69%, respectively, and the MPE of gabapentin at 50 mg/kg was 61.97±7.15% (Fig. 7C). The MPE of gluco-obtusifolin at doses of 0.25, 0.5, 1, and 2 mg/kg was 34.19±4.55%, 46.01±3.93%, 49.73±6.45%, and 63.02±3.92%, respectively, while the MPE of gabapentin was 52.39±3.12% (Fig. 7D).

Effects of Obtusifolin and Gluco-Obtusifolin on Chemotherapy-Induced Neuropathic Pain Cancer chemotherapy can produce neuropathic allodynia and hyperalgesia in response to mechanical stimuli in animal models. We used the oxaliplatin neuropathic model to evaluate the effects of obtusifolin and gluco-obtusifolin on neuropathic pain induced by chemotherapy. For obtusifolin (Fig. 8A), two-way ANOVA on MWT in the right hindpaw demonstrated a significant treatment effect between groups ($F_{(4,280)}=48.44$, $p<0.001$), a significant effect of time ($F_{(6,280)}=253.1$, $p<0.001$), and an interaction between treatment and time ($F_{(24,280)}=4.03$, $p<0.001$). For gluco-obtusifolin (Fig. 8B), two-way ANOVA on MWT also demonstrated a significant treatment effect between groups ($F_{(4,280)}=35.34$, $p<0.001$), a significant effect of time ($F_{(6,280)}=211.1$, $p<0.001$), and an interaction between treatment and time ($F_{(24,280)}=3.97$, $p<0.001$). As expected, oxaliplatin-treated rats given vehicle developed statistically significant mechanical allodynia ($p<0.001$, versus sham control). Gabapentin significantly reduced mechanical allodynia versus vehicle control ($p<0.001$). Repeated treatment with obtusifolin (Fig. 8A) and gluco-obtusifolin (Fig. 8B) significantly decreased the severity of mechanical allodynia ($p<0.001$). The analgesic effects of obtusifolin and gluco-obtusifolin were dose-dependent, and there was no indication of the development of tolerance to anti-allodynia effects. The MPE of obtusifolin at doses of 0.25, 0.5, 1, and 2 mg/kg on the 5th day after treatment was 17.34±4.27%, 37.09±4.91%, 38.15±2.75%, and 51.7±4.21%, respectively, and the MPE of gabapentin at 50 mg/kg was 33.7±5.11% (Fig. 8C). The MPE of gluco-obtusifolin at doses of 0.25, 0.5, 1, and 2 mg/kg was 37.63±3.81%, 48.6±3.48%, 46.04±3.94%, and 55.29±3.84%, respectively.

Fig. 7. Effects of Obtusifolin and Gluco-Obtusifolin on Diabetic Mechanical Allodynia in Rats

Obtusifolin, gluco-obtusifolin (0.25, 0.5, 1, and 2 mg/kg), gabapentin (50 mg/kg), or vehicle was administered i.p. once per day for 7 consecutive days, beginning from the 22nd day after STZ injection. The time courses of obtusifolin and gluco-obtusifolin treatment effects are shown in (A) and (B). The MPE of obtusifolin and gluco-obtusifolin on the 7th day of treatment is shown in (C) and (D). MWT and MPE data are expressed as mean±S.E.M. (N=10 per group). *** $p<0.001$ versus sham group, two-way ANOVA followed by Bonferroni test. **** $p<0.001$ versus vehicle group, one-way or two-way ANOVA followed by Bonferroni or Tukey’s test.
Effects of Obtusifolin and Gluco-Obtusifolin on NF-κB Activation and Production of TNF-α, IL-1β, and IL-6 in CFA-Administered Rats and Rats with CCI

CFA is another chronic inflammatory pain model widely used for the evaluation of the pharmacological action of antarthritic agents. In the present study, we found that obtusifolin and gluco-obtusifolin exerted potent analgesic effects in this model that were greater than those produced by methotrexate. Thus, obtusifolin and gluco-obtusifolin likely possess the ability to suppress chronic inflammatory pain.

It has been reported that the second phase of formalin test is comparable to cold allodynia in CCI model of neuropathic pain. Thus, the potent analgesic effects of obtusifolin and gluco-obtusifolin in the second phase of formalin test prompted us to test whether these molecules produce analgesic effects in models of neuropathic pain. A number of animal models, which is considered to be a pain model with very high predictive validity, formalin treatment produces a biphasic behavioral reaction with phases that resemble acute and tonic pain. Central pain modulation occurs in the second phase of the formalin test, through a mechanism thought to involve the release of inflammatory mediators. Our results demonstrated that a single dose of obtusifolin or gluco-obtusifolin produced selective and profound inhibition (greater than aspirin) in the second phase of the formalin test, suggesting that obtusifolin and gluco-obtusifolin produce analgesic action on tonic inflammatory pain by interfering with the release of inflammatory mediators during the central pain modulation response.
models for neuropathic pain have been developed.22,28) And the CCI and SNL models are extensively used rodent models.28,29) These neuropathic pain models successfully produce long-lasting thermal hyperalgesia and mechanical allodynia, which mimic causalgia or complex regional pain syndrome in patients.1,28) Because drug therapies for neuropathic pain are administered over periods of days or weeks,1,22) long-term exposure of obtusifolin and gluco-obtusifolin was used, rather than a single dose.

Repeated administration of obtusifolin and gluco-obtusifolin resulted in dose-dependent reversal of mechanical allodynia (with MPE greater than gabapentin) in CCI and SNL models, suggesting that obtusifolin and gluco-obtusifolin may be efficacious in reducing neuropathic pain. Notably, gabapentin and other agents partially or completely reversed tactile allodynia in animals with SNL, while indomethacin, an NSAID, did not.30) Thus, the analgesic effects of obtusifolin and gluco-obtusifolin in the L5 SNL model suggest that the analgesic actions of obtusifolin and gluco-obtusifolin are different from those of NSAIDs. Moreover, obtusifolin and gluco-obtusifolin did not affect mechanical withdrawal thresholds in unstimulated contralateral hindlimb (data not shown). Thus, the localized action of obtusifolin and gluco-obtusifolin to the area of nerve injury suggests that these molecules function only in afferents stimulated by noxious inputs.

In addition to CCI and SNL, diabetes and chemical insults such as cancer chemotherapeutics also produce neuropathic allodynia in response to mechanical stimuli in animal models. In the clinic, anticonvulsants, anesthetics, antidepressants, and opiates are used to treat these kinds of neuropathic pain, although there is a clear need for new treatments with improved efficacy and safety.31) In this study, we found that obtusifolin and gluco-obtusifolin reversed tactile allodynia in STZ-treated diabetic rats with higher efficacy than gabapentin, without affecting blood glucose levels (data not shown). Furthermore, obtusifolin and gluco-obtusifolin reversed tactile allodynia in oxaliplatin-treated rats in a model of chemotherapy-induced neuropathic pain.

It should be noted that the effects of obtusifolin and gluco-obtusifolin on tactile allodynia were dose-dependent and occurred as early as the first administration in several pain models (CFA, CCI, SNL, diabetes, and oxaliplatin), with higher efficacy than methotrexate and gabapentin. In addition, the trends of mechanical withdrawal threshold curves for groups treated with obtusifolin and gluco-obtusifolin suggest that consecutive administration of these molecules allows different doses of obtusifolin and gluco-obtusifolin to reach higher effective levels. And repeated daily administration of obtusifolin and gluco-obtusifolin reversed the decreased mechanical thresholds, even before the next drug administration, by ameliorating inflammatory and neuropathic pain (data not shown).
neuroimmune and neuroinflammatory activities initiate and maintain inflammatory and neuropathic pain.\textsuperscript{32} Specifically, proinflammatory cytokines such as TNF-\(\alpha\), IL-1\(\beta\), and IL-6 have been strongly implicated in the initiation and development of inflammatory and neuropathic pain,\textsuperscript{32,33} and can activate, or be activated by, NF-xB.\textsuperscript{33} Consistent with previous studies,\textsuperscript{34,35} expression of TNF-\(\alpha\), IL-1\(\beta\), IL-6, and NF-xBp65 were dramatically increased in spinal cord in the CFA and CCI models, and obtusifolin and gluco-obtusifolin treatment significantly inhibited this up-regulation. The finding that obtusifolin and gluco-obtusifolin inhibited over-expression of spinal TNF-\(\alpha\), IL-1\(\beta\), IL-6, and NF-xBp65 associated with inflammatory and neuropathic pain suggests that the action of obtusifolin and gluco-obtusifolin involves regulation of neuro-inflammatory processes and the immune network.

**CONCLUSION**

Our results demonstrate that obtusifolin and gluco-obtusifolin produce significant analgesic action in rodent behavioral models of inflammatory and neuropathic pain, and that this activity is associated with modulation of neuroinflammation in the spinal cord. These results support the further study of obtusifolin and gluco-obtusifolin in preclinical and clinical models of inflammatory and neuropathic pain, because of their potential use in the treatment of a variety of intractable pain conditions in patients.

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