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Effect of a novel, orally active matrix metalloproteinase-2 and-9 inhibitor in spinal and trigeminal rat models of neuropathic pain

Michael A. Henry, DDS, Ph.D.,
Professor, University of Texas Health Science Center at San Antonio

Dara D. Fairchild, B.S.,
Research Assistant, University of Texas Health Science Center at San Antonio

Mayur J. Patil, M.S.,
Graduate Research Assistant, University of Texas Health Science Center at San Antonio

Taleen Hanania, Ph.D.,
Vice President, Behavioral Pharmacology, PsychoGenics Inc., Tarrytown, NY

Heather S. Hain, Ph.D.,
Director, Behavioral Pharmacology, PsychoGenics Inc., Tarrytown, NY

Scott F. Davis, B.S.,
Associate Scientist, Behavioral Pharmacology, PsychoGenics Inc., Tarrytown, NY

Sam A. Malekiani, M.S.,
Scientist, Behavioral Pharmacology, PsychoGenics Inc., Tarrytown, NY

Andrew Hu, M.S.,
Senior Research Associate III, Behavioral Pharmacology, PsychoGenics Inc., Tarrytown, NY

Roy Sucholeiki, M.D.,
Vice President, Clinical Development, Aquilus Pharmaceuticals Inc., Woburn, MA

Darrell Nix, Ph.D.,
Vice President, Research & Development, Aquilus Pharmaceuticals Inc., Woburn, MA

Irving Sucholeiki, Ph.D.
President & CEO, Aquilus Pharmaceuticals Inc., Woburn, MA

Abstract

Aims: To study the effect of a novel matrix metalloproteinase-2 (MMP-2) and MMP–9 inhibitor, AQU-118, on mechanical allodynia in the spinal nerve ligation (SNL) model of neuropathic pain, and the chronic constriction injury of the infraorbital nerve (CCI-IoN) model of neuropathic orofacial pain.

Methods: Five groups of SNL rats were given daily oral doses of either AQU-118 (5, 10, 20mg/Kg), gabapentin (100 mg/Kg) or vehicle (0.5% methylcellulose) and then paw withdrawal threshold was measured with von Frey (VF). Three groups of CCI-IoN rats were given daily oral

Correspondence to: Dr. Irving Sucholeiki, Phone: 617-759-6590, Fax: 781-756-1738, sucholeiki@aquiluspharma.com.

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doses of either AQU-118 (40mg/Kg), gabapentin (100mg/Kg) or vehicle (0.5% methylcellulose) and then mechanical allodynia was measured with facial VF and non-reflex based orofacial stimulation test (OFST) assay. Naïve rats were also tested for effect of AQU-118 (40mg/Kg) on basal sensitivity to mechanical stimulation/locomotive activity.

Results: SNL-mechanical allodynia was attenuated by gabapentin (100mg/Kg) and AQU-118 (in a dose-dependent manner). CCI-IoN-mechanical allodynia was also attenuated by both AQU-118 (40mg/Kg) and gabapentin (100mg/Kg) as measured by both facial VF and OFST assay. Upon cessation of either AQU-118 or gabapentin, VF responses in both models and OFST assay times reverted to levels observed in vehicle-treated lesion control rats.

Conclusion: Results demonstrate that oral AQU-118 attenuates mechanical allodynia in both neuropathic pain models and with efficacies that mirror gabapentin at the 40mg/Kg dose used in the CCI-IoN model but without effect on basal sensitivity to mechanical stimulation/locomotive activity. These findings support a possible role for MMP-2/−9 in the etiology of neuropathic pain and also suggest that inhibition strategies represent a viable treatment option.

Keywords

Matrix metalloproteinase; MMP-2; MMP-9; inhibitor; allodynia; orofacial pain; operant

Introduction

Neuropathic pain conditions affect both the spinal and trigeminal systems and can be resistant to management with currently available medications. This lack of efficacy dictates the need for new treatment approaches based on mechanisms that contribute to the development of neuropathic pain. Use of rodent models has implicated various inflammatory proteins such as cytokines and in particular certain matrix metalloproteinases (MMPs) in the development of neuropathic pain after nerve injury [1–6]. MMPs are a family of structurally related zinc-containing enzymes that have been reported to mediate the breakdown of connective tissue in normal physiological processes such as embryonic development, reproduction, and tissue remodelling. Of particular interest is the observation that the parenteral administration by epineurial (EP), intrathecal (IT) and intraperitoneal (IP) routes of injection of hydroxamate containing MMP inhibitorss can reduce mechanical allodynia in various surgically-induced rodent models of neuropathic pain [7–9]. These include the chronic constriction injury (CCI), spinal nerve ligation (SNL) and the L5-spinal nerve crush (L5-SNC) rodent models [7–9]. The reduction in mechanical allodynia from these MMP inhibitors is currently thought to be due to the inhibition of MMP-9 and/or MMP-2, both of which have been observed to be elevated after surgery [8–10]. MMP-9 knock-out mice show reduced sensitivity to mechanical allodynia after SNL and sciatic nerve crush. Additionally, membrane-type 5 MMP (MT5-MMP/MMP24) (which specifically activates the inactive zymogen form of MMP-2) knock-out mice do not develop mechanical allodynia after sciatic nerve injury [11]. Taken together, these findings support the possible role of MMP-2 and MMP-9 in the generation of neuropathic pain and also suggest that strategies to inhibit these MMPs may be effective in reducing neuropathic pain symptoms.

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The aim of the present study was to study the effects of a novel MMP-2 and MMP–9 inhibitor, AQU-118, on mechanical allodynia in the SNL model of neuropathic pain, and the CCI injury of the infraorbital nerve (IoN) model of neuropathic orofacial pain.. AQU-118, (3-(1H-Indol-3-yl)-2-[5-(4-trideuteromethyl-phenylethynyl)-thiophene-2-sulfonylamino]-propionic acid), is a potent small molecule inhibitor of both MMP-2 and MMP-9 with an *in vitro* IC50 of 3 nM and 9 nM respectively and an *in vivo* oral bioavailability in rats of 44% at 20 mg/Kg [12]. This study with AQU-118 encompassed several goals. First, was to evaluate the oral efficacy of AQU-118 in the SNL-rat model because this model is able to differentiate between compounds that are useful in combating neuropathic pain in humans (gabapentin) from those that are not (indomethacin) [13, 14]. Second, was to test AQU-118 in a common rodent model of orofacial neuropathic pain where CCI lesion is performed with chromic suture to the IoN of the trigeminal system [15, 16]. The lesion produces mechanical allodynia as commonly detected by von Frey (VF) filament stimulation of the vibrissal pad and so mimics the tactile allodynia reported by humans in experimental and clinical pain studies [17, 18]. Lastly, it was important to include a non-reflex based pain read-out in the evaluation of orally administered AQU-118. Some recently published reviews have questioned the ability of animal models that rely solely on reflex based read-outs (i.e. paw withdrawal, tail flick and writhing behavior) to accurately predict human efficacy [19, 20]. Thus, an animal model was included that uses an “operant” measure of pain sensitivity [20,21]. This operant model is the orofacial stimulation test (OFST) assay that measures the time spent retrieving a sweetened milk reward while encountering a self-applied mechanical stimulus to the vibrissal pad in animals with a CCI-IoN lesion [22–24]. In addition, mechanical allodynia of the rodent’s vibrissal pad was assessed via VF. This design allowed the use of the same positive control (oral gabapentin, 100 mg/Kg) and rat species in an evaluation of oral AQU-118 efficacy on mechanical allodynia in both the CCI-IoN and SNL-rodent models. Study results show efficacy of an orally administered dual active MMP-2/MMP-9 inhibitor in both the SNL and CCI-IoN rat models for neuropathic pain.

Materials and Methods

Testing of orally administered AQU-118 in the SNL-rat model of neuropathic pain

The use of animals was approved by the Institutional Animal Care and Use Committee at PsychoGenics, Inc. Sprague-Dawley rats (200–225g) from Harlan (Indianapolis, IN) were used in the study. Each treatment group incorporated an equal but mixed distribution of animals to be orally dosed (p.o.) with vehicle, gabapentin and AQU-118 (see protocol Table 1). The doses of AQU-118 (5, 10, 20 mg/Kg, Q.D.) were picked based on initial oral PK studies that showed linear oral bioavailability with doses up to 10 mg/Kg then reaching saturation at the 20 mg/Kg dose.

Spinal Nerve Ligation (SNL) Surgery: Under general anesthesia with continuous inhalation of isoflurane, surgery was performed with aseptic procedures. The skin at the area of the lower lumbar and sacral level of the rat was shaved and disinfected. A longitudinal incision at the lumbar level left of the vertebral column was made and the left paraspinal muscles were separated. The transverse process of L6 was removed and the L5 and L6 spinal nerves exposed. 4–0 silk thread was used to ligate the left L5 spinal nerve. The wound

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was closed by suture and staples. All rats received an analgesic (buprenorphine, 0.05 mg/Kg, s.c.) immediately before and 6 hours after surgery. After recovery from anesthesia, animals were then single-housed for the duration of the study.

Monofilament (VF) Mechanical Stimulation Assay: Withdrawal from a mechanical stimulus was measured by applying VF filaments (Stoelting, Wood Dale, IL) of ascending bending force to the plantar surface of both hind paws, ipsilateral and contralateral to the surgical manipulation. Filaments ranged from 0.69 to 60 g (0.692, 1.202, 1.479, 2.041, 3.63, 6, 8, 10, 15, 26, and 60). Each filament was applied 3 times to determine withdrawal. A positive response was defined as withdrawal from the VF filament. Confirmation of the paw withdrawal threshold (PWT) was tested by assessing the response to the filament above and below the withdrawal response. Rats were brought to the experimental room and allowed to habituate in the room for one hour prior to testing, and acclimated to the observation chambers for at least 15 minutes prior to taking PWT measurements.

Pre-operative Baseline Testing: Prior to surgery, all rats were tested using the VF test. Rats that had an ipsilateral PWT of less than 12 g were excluded from the study.

Post-operative Testing: Three days following surgery, VF responses were obtained and animals were balanced and assigned to treatment groups (n=8 per group) based on their post-operative PWT values. On days 5, 6, 8 and 10 post-surgery, rats were given vehicle, gabapentin, or AQU-118 and tested within 1 hour of administration (see below). PWT values were measured without compound on days 11, 12, and 14. All measurements were taken at approximately the same time every day by observers blinded to the treatment.

Compound Administration: Gabapentin (100 mg/Kg, Q.D.; *Toronto Research Chemicals*) was dissolved in saline and administered p.o. on days 4–10, prior to testing, at a dose volume of 1 mL/Kg. Gabapentin dose was prepared fresh daily. AQU-118 (5, 10, 20 mg/Kg, Q.D.) was dissolved in 0.5% methylcellulose (400 cps) and administered p.o. on days 4–10, prior to testing, at a dose volume of 1 mL/kg. AQU-118 dose was prepared fresh daily. The vehicle was administered at a dose volume equivalent to the test compound administered. Compounds or vehicle were administered in the afternoon at approximately the same time each day.

Statistical Analyses: Data at all time points post-surgery were analyzed by two-way repeated measures analysis of variance (RM ANOVA) with time as the within-subjects factor and treatment as the between-subjects factor. This was followed by Fisher PLSD post-hoc comparisons where appropriate. Pre-surgery baseline paw withdrawal data were analyzed by one-way ANOVA. An effect was considered significant if $p<0.05$. Data are presented as the mean ± standard error of the mean (SEM).

Testing of orally administered AQU-118 in the CCI-IoN model of orofacial neuropathic pain

The use of animals was approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio. Thirty two male Sprague-Dawley

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rats (175–200 grams; Charles River Laboratories, Inc., Wilmington, MA) were used in this study (see protocol Table 2).

Orofacial Stimulation Test Assay: Animals were acclimated for one week after arrival. Animal training with the orofacial stimulation test (OFST) assay was started the second week and included two sessions where animals learned to retrieve sweetened milk (30% sweetened condensed milk; HEB, San Antonio, TX), diluted in tap water, as a reward by placing their head through an opening in the box and licking milk from a bottle spout. Training continued during week three and included three consecutive daily sessions where the animal again retrieved reward but with a mechanical insert placed into the opening. The mechanical insert consists of an array of 16 individual 0.006 inch/diameter nickel titanium wires that protruded 5–7 mm beyond the edge of the metal insert frame (Figure 1) and is different from the one provided by the manufacturer of the OFST assay (Ugo Basile, Comerio, Italy). The mechanical insert provides mechanical stimulation to the center of the vibrissal pad while the animal is retrieving the sweet milk reward. The tip of the milk bottle spout was placed 8 mm from the insert wall to provide maximum contact of wires to vibrissal pads while retrieving milk reward. Each training session consisted of a 10 minute acclimation period where animals were placed in the box in the absence of milk reward followed by a 20 minute period where reward was available. Animals were then food deprived overnight (with access to water) for 18 hours and tested the next day with the OFST assay for a 10 minute period while recording the total time associated with reward retrieval with the mechanical insert in place as a baseline measurement. The cumulative time spent retrieving reward (feeding time) was determined automatically by recording the length of time that a beam of infrared light was interrupted by the snout of the rat that only occurs while retrieving reward. The OFST assay was also performed seven days following placement of the CCI-IoN lesion (see infraorbital nerve lesion), 14 days after the lesion at which time the animals had been treated with eight daily doses of AQU-118, gabapentin or vehicle, and again on day 21 where drug therapy had previously stopped on day 15 (six days earlier). All OFST assay sessions were performed with the use of the same mechanical insert. Each animal undergoing testing with the OFST assay was carefully observed to ensure that reward retrieval events were associated with an interruption of the infrared beam while mechanical stimulation of the vibrissal pad was occurring. Baseline measures were also obtained for VF monofilament stimulation of the vibrissal pad (see below).

Monofilament (VF) Mechanical Stimulation Assay: All animals were also tested for baseline behavioral response following VF monofilament mechanical stimulation to the vibrissal pad (see protocol Table 2). Testing was accomplished by animal placement into a plastic box and a 10 minute acclimation period before testing with Semmes-Weinstein monofilaments (Touch-Test Sensory Evaluator; North Coast Medical Inc., Morgan Hill, CA) was initiated. The middle of the left vibrissal pad was successively tested with the 0.4, 0.6, 1.0, 1.4, 2.0, 4, 6, 8, 10, 15 and 26 gram monofilaments until threshold behavior was recorded. Two minutes were allowed between testing with each filament. Each filament was applied three times and threshold was defined when stimulation with two consecutive filaments of increasing size resulted in two of the three stimulations with each monofilament that produced a behavioral response characterized by any one or combinations of the

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following behaviors; head withdrawal, bite/attack filament, or asymmetrical facial grooming directed to the side of the CCI-IoN lesion. The first (lowest) of these two consecutive monofilaments that showed consecutive threshold responses was defined as the threshold monofilament. Animals were tested prior to CCI-IoN lesion placement (baseline measure), and again 7 (immediately before the initiation of drug/vehicle therapy), 8, 10, 15 (last day drug given), 16, 18 and 21 days after lesion placement.

Infraorbital Nerve Lesion: Three days after baseline measures were obtained with the OFST assay and monofilament stimulation, animals were anesthetized with an intramuscular injection of ketamine (Putney, Portland ME; 75 mg/Kg) and dexmedetomidine (Pfizer, New York, NY; 0.5 mg/Kg) and once pain-free the left infraorbital nerve (IoN) was exposed just distal to the IoN foramen by way of a midline incision over the snout. The IoN was fully exposed as visualized with the use of a surgical microscope (Zeiss). Two 4–0 chromic gut sutures (Ethicon Inc., Somerville, NJ) were placed around the IoN just distal to the foramen and each suture was loosely constricted onto the nerve. The superficial incision was closed with 3–0 black silk suture (Ethicon).

Compound Administration: Three groups of animals were used in the study and included animals with lesions that were treated with vehicle (n=12; lesion with vehicle), animals with lesions treated with gabapentin (Toronto Research Chemicals, North York, ON, Canada; n=10; lesion with gabapentin) and animals with lesions treated with AQU-118 (n = 10; lesion with AQU-118). An AQU-118 dose of 40 mg/Kg was selected based on the results of the SNL-rat study that showed that AQU-118 at the 20 mg/Kg dose exhibited roughly half the efficacy of gabapentin. It was of interest to see if doubling the dose of AQU-118 to 40 mg/Kg would give equipotent reductions in vibrissal pad stimulation as compared to gabapentin. All animals received once daily administration of compound or vehicle on days 7–15 after lesion placement. Post-operative dosing periods after CCI-IoN were different than after SNL since onset of mechanical allodynia is more rapid after SNL [14,15]. All groups received either compound or vehicle by way of gastric lavage feeding tubes (18 gauge/75 mm length, Solomon Scientific, San Antonio, TX). Vehicle was 0.5% methylcellulose (Sigma, St. Louis, MO) in water and this vehicle was used to deliver AQU-118 and used in lesion with vehicle-treated animals. Lesion with vehicle animals received 2 mL of vehicle and lesion with AQU-118-treated animals received a 40 mg/Kg dose in 2 mL of vehicle. Lesion with gabapentin animals received a 100 mg/Kg dose dissolved in saline at a concentration of 100 mg/mL of saline. On the days the animals received compound/vehicle and were tested with either the OFST assay (14 days after lesion placement) or monofilament testing (8, 10, 15 days after lesion placement), testing was initiated one hour after compound/vehicle was administered to each animal.

Statistical Analyses: Statistical analyses were accomplished with the use of GraphPad Prism 5.0 (GraphPad, La Jolla, CA). Differences between groups were evaluated with two-way analysis of variance (ANOVA) with Bonferroni's multiple comparison post-hoc tests and were considered significant when $p<.05$ (*), $p<.01$ (**) and $p<.001$ (***). No animals were excluded from the monofilament analysis, whereas one animal in the AQU-118 group was excluded from the OFST assay analysis since this animal consistently retrieved milk

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reward while avoiding mechanical stimulation of the vibrissal pads by turning of the head. Error bars represent standard error of the means (SEM).

Testing of orally administered AQU-118 on basal sensitivity to mechanical stimulation and locomotive activity in naïve Sprague-Dawley rats.

Sixteen male naïve Sprague-Dawley rats were used in this study (weighing between 200–300 grams and obtained from Charles River Laboratories). Animals were randomly assigned to one of two experimental conditions (n=8 each); either administration of AQU-118 (40mg/Kg) or vehicle (0.5% methylcellulose).

Measure of Tactile Allodynia: To assess effect of AQU-118 on basal mechanical stimulation, a PWT test was conducted 1–2 days before dosing (Day −1, Baseline) and then on Day 1 and Day 3 following consecutive daily dosing with AQU-118 (see below). Animals were placed in a Plexiglas chamber (20×10.5×40.5 cm) and habituated for 10 minutes to derive PWT scores. The chamber was positioned on top of a mesh screen so that mechanical stimuli could be administered to the plantar surface of both hindpaws. Mechanical PWT sensitivity for each hindpaw was measured using the up-and-down method with eight VF monofilaments (0.39, 0.58, 1.00, 1.87, 4.00, 7.88, 13.79, and 25.62 g). Each trial began with a VF force of 1.00 g delivered to each hindpaw for approximately 1 s. If there was no withdrawal response, then the next highest force was delivered, but if there was a response, the next lowest force was delivered. This procedure was repeated until no response was detected at the highest force (25.62 g) or until five total stimuli were administered. This procedure was performed three times and the 50% withdrawal values of each were averaged to determine a mean 50% threshold to tactile stimulation for the right and left paws of each animal.

Measure of Locomotor Activity: Motor activity was measured using a circular open field. The open field consists of a circular base (100-cm diameter) with an aluminum sheet metal wall (height of 45 cm). Each animal was individually placed in the center of the apparatus and the total horizontal distance traveled during a 60-sec test was recorded and calculated using a Med-Associates Ethovision tracking system.

Dosing & Testing: Before dosing, the rats had baseline (pre-drug) measures of PWT and locomotor activity. 24–48 hours later, animals were randomly assigned to receive an oral administration of either vehicle (0.5% methylcellulose, n=8) or AQU-118 (40mg/Kg in 0.5% methylcellulose, n=8) at a volume of 1ml/Kg. Sixty minutes after AQU-118 (or vehicle) administration, animals were tested for PWT and locomotor activity. AQU-118 administration occurred for 3 days, with locomotive and VF testing on Day 1 and Day 3 after drug administration. The compound was made up fresh for each of the 3 drug administration days.

Statistical Analysis: Mixed repeated measures ANOVAs were conducted with condition as the between-subjects factor (drug or vehicle) and time as the within-subjects factor.

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Results

Attenuation of VF mechanical allodynia by oral administration of AQU-118 in the spinal nerve ligation (SNL)-rat model, an ascending dose study.

The study was designed to have in addition to a vehicle (n = 8) and a positive control (n=8, gabapentin, 100 mg/Kg), three doses of AQU-118 (5, 10 and 20 mg/Kg, n=8/dose) were also tested (see protocol Table 1). By the third day post-surgery, rats with L-5 spinal nerve ligation displayed significant mechanical allodynia as compared to pre-operative testing (Figure 2a). Oral dosing of AQU-118 beginning on day 4 caused an increase in the paw withdrawal threshold (PWT) at the 10 mg/Kg ($p < .01$ on day 8) and 20 mg/Kg ($p < .001$ on day 8) dose groups as compared to the vehicle control group. AQU-118 dose dependently increased PWT. The PWT resumed to post-surgical levels upon cessation of AQU-118 after day 10. No statistically significant effect on contralateral PWT was observed with oral dosing of AQU-118 which was comparable to both the vehicle and positive control (gabapentin) arms (Figure 2b).

Attenuation of VF mechanical allodynia by oral administration of AQU-118 in the chronic constriction injury (CCI) of the infraorbital nerve (IoN) rat model.

Before CCI-IoN surgery, mechanical stimulation of the left vibrissal pad with VF monofilaments showed no significant difference between the animals included in the three groups (lesion with vehicle, lesion with 100 mg/Kg gabapentin and lesion with 40 mg/Kg AQU-118) in gram force needed to elicit a behavioral threshold response (vibrissal pad response threshold) (Figure 3; see protocol Table 2). On the seventh day after CCI-IoN surgery, measurement of vibrissal pad response threshold indicated a marked increase in mechanical sensitivity for all groups as compared to pre-operative baseline measures (Figure 3). The lesion with vehicle group showed a gradual and progressive reduction in the gram force needed for a threshold response (increase in mechanical sensitivity) that continued from day 7 after the lesion to day 15 and which was still present on day 21, the last day tested. This decreased gram force needed for threshold behavioral response indicates that mechanical allodynia of the vibrissal pad resulted from the CCI-IoN lesion. Eight consecutive daily oral doses with AQU-118 (40 mg/Kg) or gabapentin (100 mg/Kg) which began 7 days after the CCI-IoN, and after vibrissal pad response threshold measurement was obtained on day 7, continued until day 15. Testing with VF showed a statistically significant reversal of mechanical allodynia in both the AQU-118 and gabapentin groups when compared to the lesion with vehicle group, at both 10 days (four consecutive days of drug) and 15 days (nine consecutive days of drug) after lesion placement. This reversal of mechanical allodynia observed in both drug treatment groups was rapidly lost at the day 16 time point, 24 hours after the last administration of each agent, at which time there was no significant difference between any of the three groups (Figure 3). This lack of significant difference among the three groups remained at the 18 and 21 day time points (after lesion placement).

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Effect of oral administration of AQU-118 on operant behavior after CCI-IoN surgery using orofacial stimulation test assay.

Use of the orofacial stimulation test (OFST) assay to measure the retrieval of a sweetened milk reward while encountering a self-applied mechanical stimulation of the vibrissal pads showed changes in this behavior as a result of the CCI-IoN lesion in the different groups (lesion with vehicle, lesion with 40 mg/Kg AQU-118, and lesion with 100 mg/Kg gabapentin) evaluated (see protocol Table 2). These differences were observed in the cumulative feeding time (Figure 4a) and the cumulative feeding time as a percent difference from baseline for each animal (Figure 4b).

All feeding times were observed to decrease seven days after lesion placement and before initiation of drug/vehicle administration (Figure 4). Cumulative feeding times were significantly increased in both the lesion with gabapentin and lesion with AQU-118 groups when compared to the lesion with vehicle group at the 14 day time period after the lesion following eight daily doses of compound/vehicle administration (Figure 4a). This same difference was apparent when cumulative feeding time was evaluated as a percent difference from each animals baseline measure (Figure 4b). The cumulative feeding times in the drug treated groups was reduced at the day 21 time point which was not different from the vehicle group and where these times approached baseline levels in all three groups (Figure 4).

Effect of oral administration of AQU-118 (40mg/Kg) on basal sensitivity to mechanical stimulation and locomotive activity in naïve Sprague-Dawley rats.

Mechanical sensitivity as measured with PWT showed no significant effect of time, $F(2,28) = 2.84$, $p = .08$, on PWT scores in both AQU-118 and vehicle groups, and with PWT scores that remained stable over time. There was also no significant main effect of treatment condition (AQU-118 as compared to vehicle), $F(1,14) = .003$, $p = .96$, nor a significant interaction of time with treatment condition interaction, $F(2,28) = .62$, $p = .55$ (Figure 5a). Results on locomotive activity showed no significant effect of time, $F(2,28) = 2.45$, $p = .10$, or condition, $F(1,14) = .21$, $p = .66$ and no significant interaction of time with condition, $F(2,28) = 1.37$, $p = .27$ (Figure 5b). These results indicate that oral dosing with AQU-118 at 40 mg/Kg for multiple days in naïve rats does not alter basal sensitivity to mechanical stimulation or induce changes in locomotive activity that could be due to sedative effects.

Discussion

Current treatments for neuropathic pain are often either not effective or only partially effective [25, 26]. Opioids have limited potential for alleviating neuropathic pain and can cause the unwanted side effects of dependency, tolerance, nausea, drowsiness and constipation [27, 28]. Many of the most common treatments, such as the use of gabapentin and more recently pregabalin, generally give less than desired results while still producing significant dose-limiting side effects [29]. In the last few years no oral drug has been approved for pain that is truly novel. In this area where no current single drug treatment is effective in more than 50% of patients, novel therapeutic approaches are urgently needed. Studies showing that MMP-2 and/or MMP-9 become elevated after CCI or SNL-surgeries and that inhibiting these MMPs can reduce mechanical allodynia in rodents presents a novel

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approach by which neuropathic pain may be treated [8–10]. Up until now, however, no translational research has been reported to substantiate this approach. Results show that a dual active MMP-2/MMP-9 inhibitor can attenuate mechanical allodynia when given orally in both the SNL and CCI-IoN rat models of neuropathic pain when compared to vehicle control. A dose response was observed in the SNL study whereby increasing the daily oral dose of AQU-118 increases the paw withdrawal threshold of rodents via VF filaments. The greater reduction in PWT seen with gabapentin as compared to AQU-118 in the SNL rat model could be due to the different effective molar concentrations associated with the doses used for each drug. The molecular weight (Mw) of AQU-118 is almost three times as large as that of gabapentin (Mw of 171 amu for gabapentin versus Mw of 503 amu for AQU-118 as the dihydrate) giving the 20 mg/Kg dose of AQU-118 an effective molarity that is almost 15x less than that of the 100 mg/Kg gabapentin dose (0.584 mmoles/Liter for gabapentin versus 0.0397 mmoles/Liter for AQU-118). However, given that the actual difference in PWT between AQU-118 (20mg/Kg) and gabapentin (100mg/Kg) treated animals was only ~2x, suggests that AQU-118 may have greater efficacy or target tissue penetration than gabapentin. Moreover, these findings also suggest the possibility that a higher dose of AQU-118, such as the 40mg/Kg dose used in the CCI-IoN study, could have produced even greater reductions in PWT scores in SNL animals, although additional studies will be needed to test for this possibility.

The finding that oral dosing with an MMP-2/−9 inhibitor can attenuate mechanical allodynia in the CCI-IoN rat model is significant since it was unknown whether a MMP inhibitor could attenuate mechanical allodynia in this rodent model. This study helps to settle this efficacy question with data showing decreased mechanical sensitivity of the vibrissal pad that was maintained with daily oral dosing with AQU-118 (40 mg/Kg) for eight days, but with a rapid return of mechanical sensitivity seen 24 hours after dosing was terminated (Figure 3). The results obtained with AQU-118 mirror that observed with the positive control (100 mg/Kg, gabapentin) in both the extent of the decrease in the vibrissal pad response threshold as well as the duration of action after its cessation eight days later. A comparison of the effects of AQU-118 to the effects of gabapentin showed greater efficacy of AQU-118 in the CCI-IoN study when compared to the SNL study. Although other explanations are possible, this result could be due to the higher dose of AQU-118 used in the CCI-IoN study (40 mg/Kg) as compared to the 20 mg/Kg dose used in the SNL study. A common finding in both studies was that the positive effects of AQU-118 on mechanical allodynia were maintained throughout the dosing period and apparently without the development of tolerance after a week of treatment. Although the acute effects of AQU-118 on mechanical allodynia were not specifically evaluated, rapid effects were seen after only one day of compound administration (Figures 2 and 3) and these observations suggest that AQU-118 may possess acute analgesic properties.

Because of concerns that reflex based read-outs via VF filaments may not be a predictor of clinical efficacy, evaluation of AQU-118 was augmented with an operant OFST assay. Use of this assay that measured the time spent retrieving a sweetened milk reward while encountering a self-applied mechanical stimulation of the vibrissal pads showed that after eight days of oral dosing with either AQU-118 or gabapentin, the average cumulative feeding time (Figure 4a) and the cumulative feeding time as calculated by the percent

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difference from baseline for each animal (Figure 4b) both showed significant increases in the AQU-118 and gabapentin treated groups as compared to vehicle group. This assay has been used by others [22–24] and also utilized a mechanical insert that varied in design from the one provided by the manufacturer, thus suggesting that design of the insert is an important factor when using this assay. Since the IoN innervates the vibrissal pad and the vibrissal pad is contacted by mechanical stimuli while retrieving reward, the change in feeding times seen in compound treated animals is interpreted as a reduction of mechanical sensitivity due to compound administration. The VF results also help support this possibility.

Although the results obtained in the CCI-IoN subjects with the use of the OFST assay were mostly similar to the results obtained with the VF test, some differences were noted. Similarities included the same positive reduction of both AQU-118/gabapentin on mechanical allodynia in both assays during the active administration phase. In contrast, some differences were noted between the two assays that involved a complete and rapid return of mechanical allodynia one day after drug administration was terminated as shown by VF assay (Figure 3), whereas effects of drug termination in the OFST assay were more modest since cumulative feeding times remained higher than seen 7 days after lesion placement (Figure 4a). One possible explanation for this observation is that the increased cumulative feeding times observed after dosing was discontinued may be an example of conditioned place preference/learned behavior that enhances an animal's pain tolerance [30, 31]. However more studies would be needed to help substantiate this hypothesis. The fact that the cumulative feeding time for the AQU-118 group is significantly greater on day 21 than on day 7 and greater than the vehicle control group on day 21 does suggest that discontinuation of AQU-118 does not cause an increase in pain sensitivity (i.e. rebound effect) as one might expect with morphine [32, 33]. In fact, preliminary experiments involving the testing of AQU-118 in the naloxone precipitated mouse model of morphine withdrawal showed no apparent increase in the level of MMP-2 and MMP-9 after AQU-118 discontinuation [34].

Even though these results are very encouraging in predicting clinical efficacy for AQU-118 in reducing mechanical allodynia, the exact mechanism(s) of action responsible for this reduction is uncertain. The fact that oral treatment with AQU-118 is capable of reducing mechanical allodynia in both the SNL and CCI-IoN models suggests a similar mechanism of action in both animal models. Earlier studies in rodents have found that following injury to dorsal root ganglion primary sensory neurons, MMP-9 induced early neuropathic pain via interleukin-1β cleavage to its active form and microglia activation and MMP-2 induced delayed neuropathic pain via IL-1β cleavage to its active form and astrocyte activation (8). In other rodent studies MMP-9 has been observed to promote Schwann cell-mediated myelin basic protein degradation and macrophage infiltration in the spinal nerve and astrocyte activation in the spinal cord (9). Together, these studies help suggest possible mechanism(s) of action for MMP-2/−9 inhibitors in reducing mechanical allodynia by actions on macrophages and Schwann cells within the injured peripheral nerve and/or actions on glial cells within the spinal cord, yet additional work is needed to define the specific mechanisms involved. In addition, even though investigators were blinded in the SNL study, investigators were not blinded in the CCI-IoN study. Although this represents a possible limitation/bias when interpreting behavioral responses following mechanical stimulation to the vibrissal pad

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in the drug treated animals, the bias from lack of blinding was minimized with the use of the automated OFST assay. Another possible limitation included the lack of sham subjects in both groups of animals (SNL and CCI-IoN), but results showed analgesic-like effects in both groups of drug-treated animals when compared to lesion-vehicle treated animals.

Although there is no definitive clinical study proving that elevated levels of MMP-9 and/or MMP-2 can directly cause neuropathic pain, there have been a few biomarker studies correlating elevated levels of these MMPs in patients suffering from certain types of pain conditions. For example, MMP-9 plasma levels have been found to be elevated in patients suffering from migraines when compared to controls [35]. Elevated levels of MMP-2 and/or MMP-9 have also been observed in chronic inflammatory demyelinating polyneuropathy and nonsystemic vasculitic neuropathy [36,37]. There have also been clinical studies relating elevated levels of MMP-2 and/or MMP-9 with certain diseases known to be responsible for producing specific types of neuropathy. For example, elevated serum levels as well as zymographic activity have been found for both MMP-2 and MMP-9 in type 2 diabetic patients as compared to non-diabetics [38]. Elevated levels and activity of MMP-2 have also been found in the urine and plasma of type 1 diabetic patients as compared to healthy control subjects [39,40]. Together, these findings help support a possible role for MMP-9/MMP-2 in the development of certain pain conditions that include neuropathic pain and provide a basis for the clinical use of inhibitors to these enzymes as a new class of analgesics.

In conclusion, the results of this study demonstrate for the first time attenuation of mechanical allodynia via oral dosing of a dual active MMP-2/MMP-9 inhibitor, AQU-118, in both the SNL rat model of neuropathic pain and the CCI-IoN rat model of neuropathic orofacial pain. In both the SNL and CCI-IoN animal models, the effectiveness seen with AQU-118 paralleled that of the positive control, gabapentin, providing additional evidence that this novel compound may prove to be clinically effective in the treatment of various neuropathic pain conditions.

Acknowledgments

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Fig. 1- Orofacial Stimulation Test Assay Mechanical Insert

The mechanical insert consists of an array of nickel titanium wires that contact the vibrissal pad while the animal is retrieving a sweetened milk reward.

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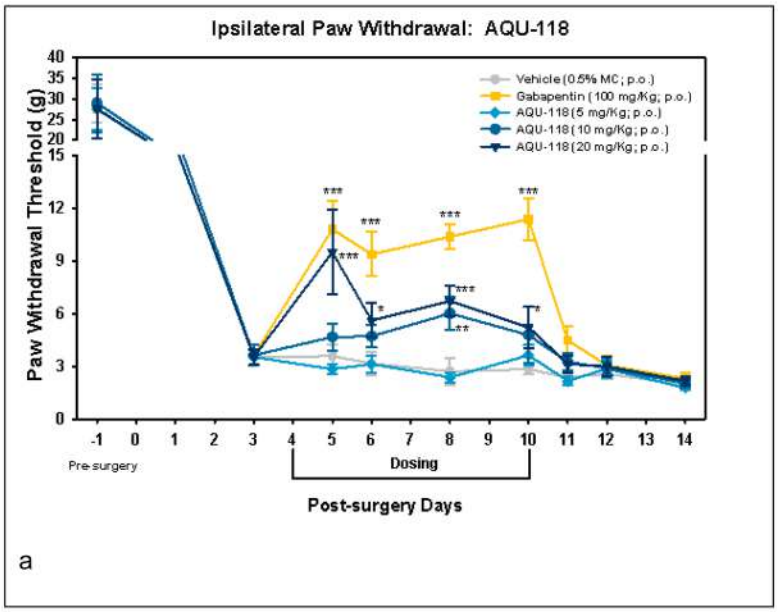

Fig. 2- Mechanical Response Threshold: SNL, Ipsilateral & Contralateral Paw Withdrawal

(a) Paw withdrawal thresholds following SNL-surgery for ipsilateral hind paws (n=8 for all groups). Data are presented as mean, error bars are SEM. *p<.05, **p<.01, ***p<.001 as compared to vehicle ipsilateral threshold. (b) Contralateral paw withdrawal thresholds following surgery and administration of AQU-118, gabapentin or vehicle (n=8). Data are presented as mean, error bars are SEM.

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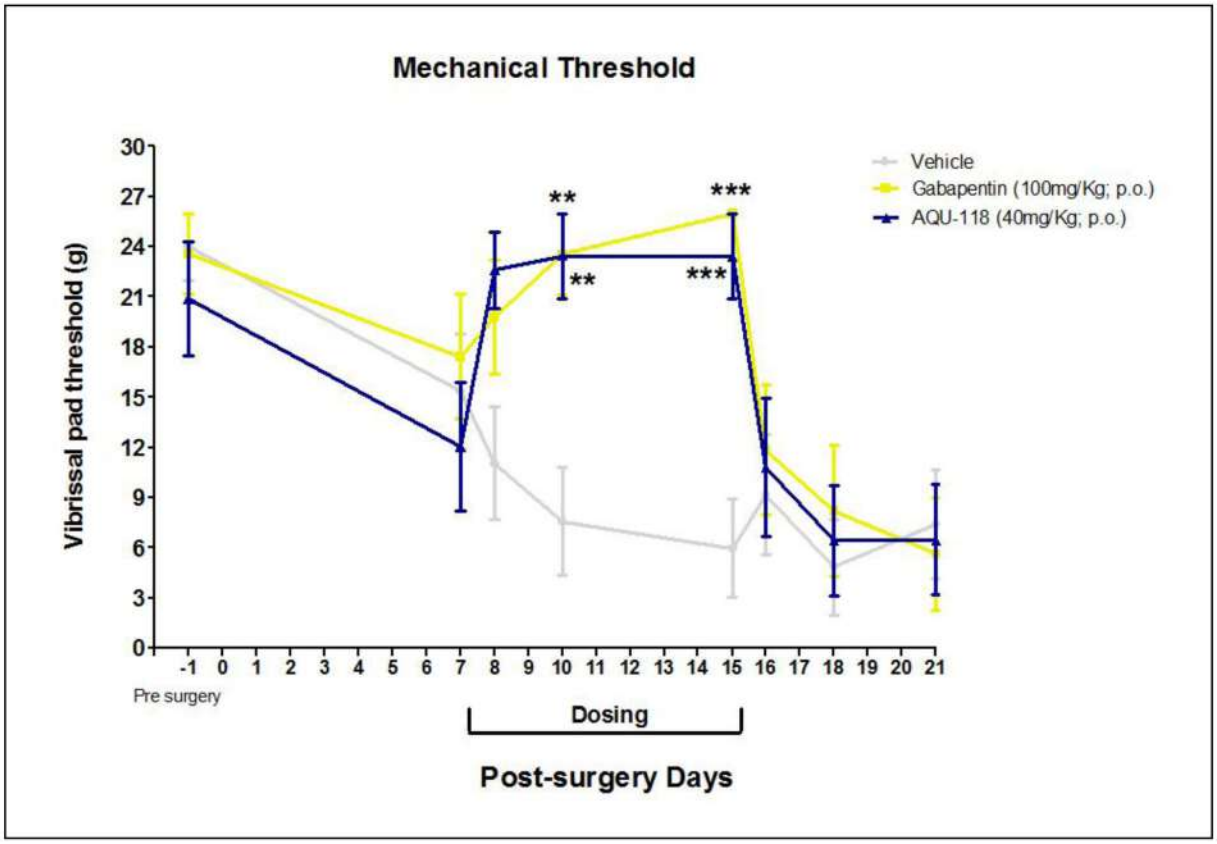

Fig. 3- Mechanical Response Threshold: CCI-IoN, Vibrissal Pad

Vibrissal pad response threshold following CCI-IoN surgery and daily oral administration of AQU-118 (40 mg/Kg), gabapentin (100 mg/Kg) and vehicle (0.5% methylcellulose). Dosing began on Day 7 immediately after Day 7 testing and continued once daily through Day 15. Data are presented as mean, error bars are SEM. **p<.01, ***p<.001 as compared to vehicle.

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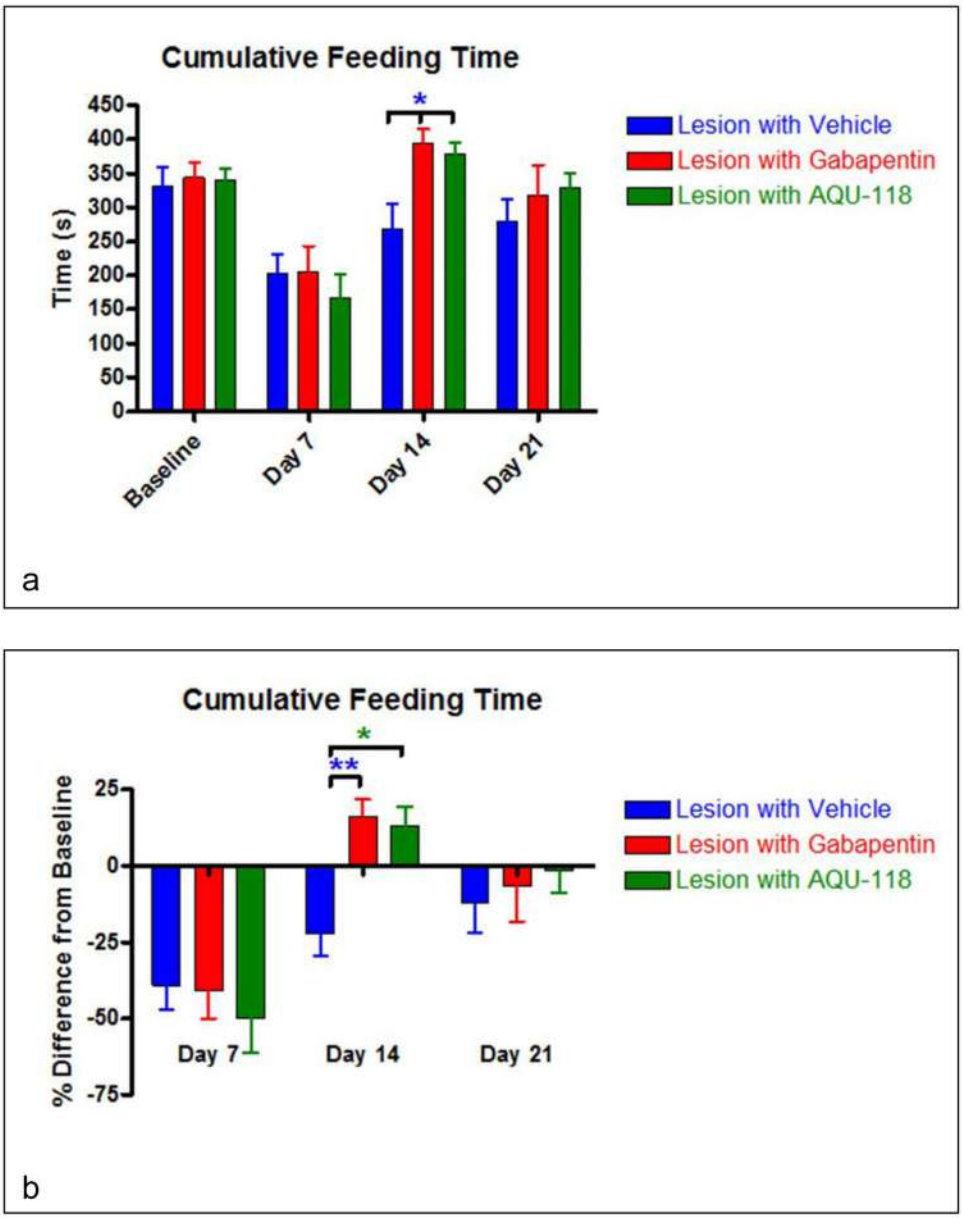

Fig. 4- Feeding Time

(a) Cumulative feeding time before CCI-IoN lesion (Baseline), 7 days after CCI-IoN lesion (Day 7), effect of once daily oral administration of AQU-118 (40 mg/Kg), gabapentin (100 mg/Kg) and vehicle (0.5% methylcellulose) for 8 consecutive days as tested 14 days after lesion (Day 14), and 6 days after cessation of oral dosing (Day 21). Dosing began on Day 7 immediately after Day 7 measurement and continued through Day 15. Data are presented as mean, error bars are SEM. *p<.05 as compared to vehicle. (b) Average percent difference in cumulative feeding time from Baseline (pre-surgical levels) for each animal on Day 7 (seven days after lesion placement), Day 14 (after eight consecutive days of oral dosing) and on Day 21 (six days after cessation of oral dosing). Data are presented as mean, error bars are SEM. *p<.05, **p<0.01 as compared to vehicle.

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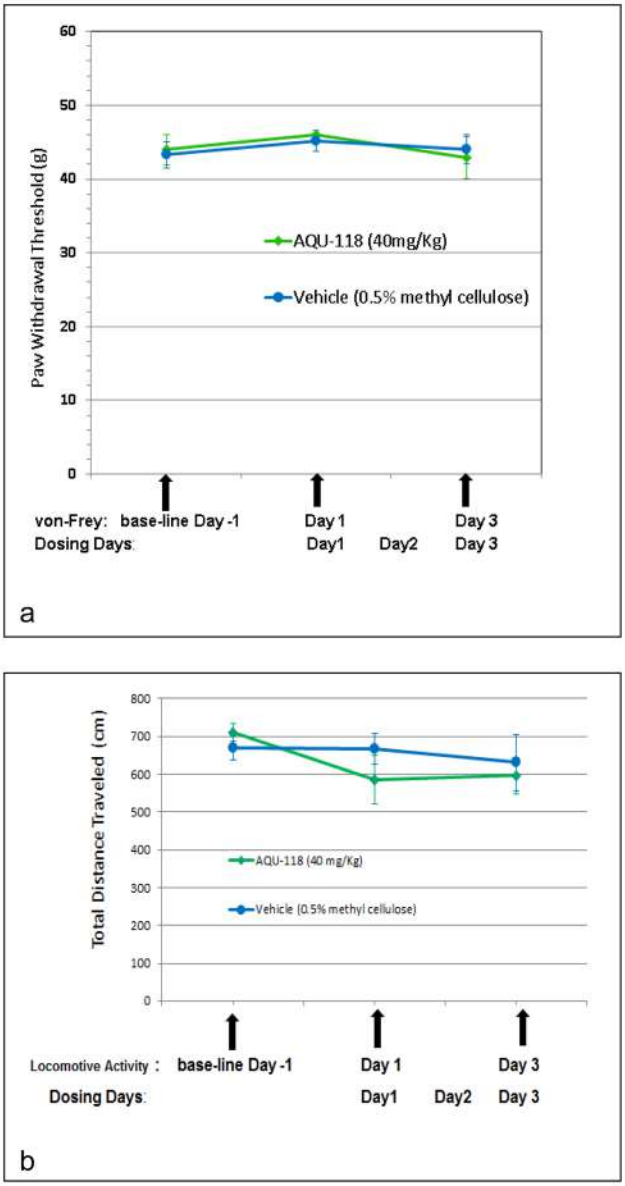

Fig. 5- PWT & Locmotive Activity in naïve rats.

(a) PWT (n=8 for all groups) at Day-1 (baseline, before dosing) and at Day 1 and Day 3 after dosing with either AQU-118 (40 mg/Kg) or vehicle (0.5% methylcellulose). Once per day dosing occurred starting on Day 1 and continued through Day 3. (b) Locomotive activity (n=8 for all groups) at Day-1 (baseline, before dosing) and at Day 1 and Day 3 after dosing with either AQU-118 (40 mg/Kg) or vehicle (0.5% methylcellulose). Once per day dosing occurred starting on Day 1 and continuing until Day 3.

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Table 1.

Protocol for SNL study using male Sprague-Dawley rats.

Group	#Rats	Route	Dose[1] (mg/Kg)	Compound	Dosing Days	VF Testing Days[4]
1	8	p.o.	NA[2]	Vehicle[3]	4–10	3,5,6,8,10–12,14
2	8	p.o.	100	Gabapentin	4–10	3,5,6,8,10–12,14
3	8	p.o.	5	AQU–118	4–10	3,5,6,8,10–12,14
4	8	p.o.	10	AQU–118	4–10	3,5,6,8,10–12,14
5	8	p.o.	20	AQU–118	4–10	3,5,6,8,10–12,14

[1] Once per day dosing via gastric gavage.

[2] NA not applicable

[3] 0.5% methyl cellulose.

[4] A base-line measure was taken via VF before surgery and then on day 3 after surgery. Testing was done on the same time each day and 1 hour after dosing on dosing days.

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Table 2.

Protocol for CCI-IoN study using male Sprague-Dawley rats.

Group	#Rats	Route	Dose[1] (mg/Kg)	Compound	Dosing Days	VF Testing Days[4]	OFST Testing Days[5]
1	12	p.o.	NA[2]	Vehicle[3]	7–15	7,8,10,15,16,18,21	7,14,21
2	10	p.o.	100	Gabapentin	7–15	7,8,10,15,16,18,21	7,14,21
3	10(9)[6]	p.o.	40	AQU–118	7–15	7,8,10,15,16,18,21	7,14,21

[1] Once per day dosing via gastric gavage.

[2] NA not applicable

[3] 0.5% methyl cellulose.

[4] A base-line measure was taken before surgery and then on day 7 after surgery. Testing was done on the same time each day and 1 hour after dosing on dosing days accept on Day 7 where dosing occurred after base-line testing.

[5] A base-line measure was taken before surgery and then on day 7 after surgery. Testing was done on the same time each day and 1 hour after dosing on dosing days accept on Day 7 where dosing occurred after base-line testing. .

[6] One rat was excluded (to make n=9) during the OFST testing due to it's exhibiting certain postures that avoided mechanical stimulation.