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Topical Application of ASN008, a Permanently charged Sodium Channel Blocker, Shows Robust Efficacy, a Rapid Onset and Long Duration of Action in a Mouse Model of Pruritus

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Running title:
Topical ASN008 and chemically induced itch in a mouse model

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List of nonstandard abbreviations:

C_{Ave}: Average plasma concentration

CQ: Chloroquine

LC-MS/MS: Liquid chromatography with tandem mass spectrometry

Mrgpr: Mas-related G protein-coupled receptor

PMD: Paw motion detection

SEM: Standard error of the mean

TRPA1: Transient receptor potential cation channel, subfamily A, member 1

TRPV1: Transient receptor potential cation channel, subfamily V, member 1

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Abstract

The sensation of pruritus, or itch, is associated with a variety of skin and medical disorders. Itch is transmitted through afferent C-fibers, and sodium channels play a key role in the transmission process. Local anesthetics, which block sodium channels, are used topically to treat itch, but generally have a short duration of action and are not selective for afferent nerves underlying the itch sensation. Accordingly, there is a substantial unmet need for safe, efficacious, long-acting treatments for chronic pruritus, including non-histaminergic itch. We investigated the dose-response, time to onset and duration of action of ASN008 topical gel, which targets small afferent sodium channels, in a murine model of pruritus in which scratching behavior is induced by intradermal injection of chloroquine into the nape of the neck of C57BL/6 mice. Topical application of ASN008 gel resulted in a concentration-dependent reduction of scratching behavior. Onset of action was ≤ 1 hour and duration of scratching inhibition was 15-24 hours. In a further study involving once-daily application for 5 days with chloroquine challenge on day 5, treatment with ASN008 gel again resulted in a concentration-dependent reduction of chloroquine-induced scratching, even when the gel was removed 3 hours after each daily application. In conclusion, topical ASN008 gel produces a dose-dependent reduction of scratching in a mouse model of pruritus, with a rapid onset and long duration of action, and may prove to be an effective, once-daily treatment for a variety of pruritic conditions in humans, including non-histaminergic itch.

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Significance statement

ASN008 gel produces a dose-dependent reduction of scratching in a mouse model of pruritus, with a rapid onset and long duration of action, and may prove to be an effective, once or twice-daily treatment for a variety of pruritic conditions in humans. ASN008 gel has demonstrated good safety and tolerability in healthy volunteers and is currently under investigation in a Phase 1b clinical study to evaluate safety, tolerability, pharmacokinetics and preliminary anti-pruritic efficacy in atopic dermatitis patients (ClinicalTrials.gov ID: NCT03798561).

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Introduction

Itch is an unpleasant sensation that provokes the desire to scratch as a primary response in order to clear the pruritogen and to terminate the itch sensation. Pruriceptive itch is most commonly associated with a primary skin disorder, as seen in atopic dermatitis, and originates following the activation of peripheral sensory nerve terminals associated with allergic reactions either induced by insect bites or when pruritogens come in contact with the skin. Chronic itch is a debilitating and dominating symptom which accompanies several disorders including skin conditions, such as atopic dermatitis (AD), psoriasis, xerosis and mastocytosis. Pruritus in the absence of primary dermatologic etiology may also be a manifestation of underlying systemic diseases, as in cases of renal and liver failure (Patel TS et al, 2007; Alhmada Y, 2017; Yosipovitch et al, 2003) or neurological disorders such as diabetic neuropathy and shingles (Oaklander et al 2003; Ikoma et al, 2006). The transmission of the pruriceptive itch is through the primary afferent nerve fibers, primarily the slow conducting unmyelinated C-fibers and some thinly myelinated A-delta fibers (Schmelz et al, 2003; Schmelz et al, 1997) with free nerve-endings located in the epidermis which act as sensors in detecting and transmitting pruriceptive signals to the higher brain centers.

Histamine is the best studied pruritogen in mediating itch transduction, and pruritus mechanisms are often classified as histamine-dependent or histamine-independent based on the responsiveness of the itch sensitive neurons to histamine. Histamine released from mast cells has been shown to activate a subset of neurons expressing TRPV1 receptors, evidenced by TRPV1 antagonism in histamine evoked activation of dorsal root ganglion (DRG) neurons (Shim et al, 2007) and reduced histamine evoked scratching behavior (Imamachi et al, 2009). However, other pruritogens have emerged in recent years which induce itch via histamine-independent pathways. Chloroquine, an anti-malarial drug, causes intolerable itch sensations via such histamine-independent neural

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pathways (McNaughton et al, 2010) that utilize mas-related G protein coupled receptor (Mrgpr) initiated signaling. Mrgprs have recently emerged as a novel class of receptors in histamine independent itch pathways, and MrprA3 is the receptor for chloroquine. In contrast to histamine, chloroquine utilizes TRPA1 as a key transduction channel downstream to the MrgprA3 receptor (Liu et al, 2009; Wilson, 2011). TRPA1 is highly expressed in TRPV1 positive neurons and is activated downstream to several G protein-coupled receptors that are essential in evoking itch sensations in response to several pruritogens, suggesting TRPA1 as an essential channel in evoking itch (Wilson et al, 2011; Bautista et al, 2014).

Topical local anesthetics are commonly used to treat itch. Conventional local anesthetics are hydrophobic in nature and impair the nociceptive transmission by blocking voltage gated-sodium channels from inside of the cell (Hille, 1977). However, along with short duration of action, these regional anesthetic agents do not specifically target sensory neurons, as they block excitability of all afferent neurons that express sodium channels and hence interferes with the function of autonomic and motor neurons. This highlights a continuing need for new long lasting and targeted local therapeutic modalities. ASN008 is a permanently charged sodium channel blocker that has been formulated in a polymer-based gel. The structure of ASN008 is provided in Figure 1. Due to its positive charge, ASN008 cannot passively gain access to the interior of the cell to block the sodium channel, but it does pass through activated (open) TRPV1 or TRPA1 channels, which are activated during itch and pain conditions. Selective expression of these TRP channels on C- and Aδ-fibers and not on motor neurons allows for nerve-type selective blockade of sodium channels by ASN008. Based on this mechanism, this drug may have a broad application in difficult to treat chronic pruritic diseases such as atopic dermatitis.

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Materials and Methods

Animals

Adult male C57BL/6 mice, 25-30 grams (Harlan Sprague Dawley Inc., Indianapolis, IN, USA), were housed in the vivarium for a minimum of 2 days before use, maintained on a 12/12-hour day-night cycle and given free access to food and water. All studies were conducted in Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved facilities at the University of California, San Diego, and in accordance with protocols and Standard Operating Procedures approved by the Institutional Animal Care and Use Committee of the University of California, San Diego, and in compliance with the USDA Animal Welfare Act (USDA, Title 9, Code of Federal Regulations, Part 3, Federal Register, 15 February 1991). Animals were housed in the UCSD Clinical Teaching Facility Vivarium in a room with temperature maintained in the range of 65-82 °F and relative humidity in the range of 30-70%. The room was illuminated with fluorescent lighting on a daily 12-hour light/dark cycle. Animals were randomly separated into cages containing approved bedding material. Animals were checked a minimum of once daily and cages were cleaned a minimum of once weekly. Animals were randomly assigned to dosing groups based on date of receipt at the testing facility.

Drugs and Test Articles

Drugs employed were Chloroquine (4 mg/mL). Chloroquine solutions were prepared from stock which were then serially diluted to the final concentration in 0.9% saline. All solutions were stored at 4 ºC and brought to room temperature prior to use. ASN008 gel formulations containing 0.3%, 1%, 3% and 5% ASN008, corresponding gel vehicle, lidocaine ointment and corresponding ointment vehicle, manufactured by Amneal Pharmaceuticals, NJ were supplied by Asana

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BioSciences. The 0.1% ASN008 gel formulation was prepared at the University of California San Diego by mixing one part 0.3% ASN008 gel formulation with two parts vehicle gel.

Drug and Test Article Delivery

Mice were anesthetized (2.5% isoflurane, with 80% oxygen and 20% room air) and were shaven on the dorsolateral aspect of the neck and upper shoulder. ASN008 gel (0.1 %, 0.3 %, 3 % and 5%) and gel vehicle were applied topically onto the shaved region. Intradermal injections of a solution of Chloroquine in 0.9% saline (50 μL) were then administered at different timepoints to perform the behavioral studies.

Behavioral Assessment

During testing, animals were placed in plexiglass cylindrical chambers and a detection band was placed around the hind paw ipsilateral to the shaven area. To initiate scratching behavior, intradermal (ID) injection of a solution of Chloroquine in 0.9% saline (50 μL) was administered in the middle of the shaven area of skin using a 29G needle. Scratching behavior was recorded over a period of 40 min using a paw motion detector. The dose of CQ and the period of scratches recorded are based on the validated model of CQ induced scratching over time (Marino et al., 2012, Green AD et al 2006, Ramachandran R et al., 2018).

Paw Motion Detection (PMD) for Measuring Itch Responses

The PMD detects the movement of a 0.1 gram, non-ferrous metal band placed around one hind paw of the mouse (Yaksh et al, 2001). The testing apparatus consists of 8.5 cm diameter, 22.5 cm tall cylindrical chambers. Under each cylinder is a pair of circular concentric electromagnetic coils that serve as antennae for transmission and reception. The outer coil diameter is 12 cm. The transmitter coil assembly is constructed to emit a 5–8 mW, 6–8 kHz, sinusoidal electromagnetic

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field (Blue Max 800 Precision scan search coil, White's Electronics, Inc., 1011 Pleasant Valley Road, Sweet Home, Oregon 97386). The detection principal is that eddy currents created by the movements of the ferrous and nonferrous metals perturb the electromagnetic field. Such perturbations produce an output waveform and are subsequently detected (Yaksh et al, 2001).

Plasma Sample Collection and Analysis

Plasma samples were collected immediately after completion of scratching behavior recording in the two concentration-response studies. For the single-dose concentration-response study, plasma samples were collected from the groups treated with the 3% and 5% ASN008 gels. For the 5-day repeat-dose concentration-response study, plasma samples were collected for all groups treated with ASN008 gels (0.1%, 0.3%, 1% and 3%). Samples were analyzed using LC-MS/MS.

Statistical analysis

The data for each variable was put in tabular form (i.e. Excel worksheet). Summary statistics were computed and include group means, standard deviations and numbers of animals per group. Statistical analysis was performed using GraphPad Prism 6, v6.0c (GraphPad Software, San Diego, CA). Analysis of the automated scratching data sets generated in pooled control groups and in pooled chloroquine treated groups did not reject the hypothesis at the $p<0.05$ level of either normality (D'Agostino & Pearson omnibus normality test) or homogeneous variances (Bartlett's test). Accordingly, for comparison of chloroquine induced scratching, results were compared using a one-way ANOVA across doses or time with Bonferroni post-hoc tests to compare groups at similar doses or times. For all post hoc comparisons, multiplicity adjusted p-values were calculated. In each case, Bonferroni post hoc tests (e.g. t-tests with Bonferroni corrections) were

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undertaken and presented in the graphics and figure legends for values between $p < 0.01$ and $p < 0.0001$.

Results

Concentration-dependent effect of single topical application of ASN008 gel on intradermal chloroquine induced scratching

Chloroquine (CQ) induced scratching

In the absence of the chloroquine stimulus, only occasional brief episodes of grooming involving the hind paw were noted and were counted. Following intradermal injection of CQ, the animal displayed a brisk scratching of the injected nape skin with the ipsilateral hind paw. This scratching behavior was characterized by long durations of high frequency bouts of paw movement recorded by the machine detection algorithm as a series of scratch counts (Marino et al, 2012). Scratches were recorded for 40 min. CQ injected unilaterally induced ipsilateral scratching behavior. The total number of scratches in the 40 min period showed a significant increase (five fold) following intradermal injection of CQ (125.4 ± 23.68 scratches) as compared to the gel vehicle group (23.50 ± 4.25 scratches) and scratching persisted unabated for intervals in excess of 40-60 min (Figure 2A).

ASN008 Concentration-response

To determine the minimum effective concentration of a single application of ASN008, a concentration-response study was performed where the mice were pre-treated with different concentrations of ASN008 (0% (vehicle), 0.3%, 1%, 3% and 5 %) prior to CQ injection. ASN008 gel formulations (50 µL) were applied topically to the nape of the neck, 6 hours prior to intradermal CQ (4 mg/mL, 50 µL) injections at the same site. A significant reduction in CQ induced scratching

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behavior was observed following topical application of 3% (24.0 ± 7.74) (Figures 2A and 2B) and 5% (24.75 ± 1.47) (Figure 2B) ASN008 gels. Reductions in scratching behavior were observed after treatment with the 0.3% and 1% ASN008 gels, but they were not statistically significant compared to treatment with gel vehicle (Figure 2B). Mice in these groups did not per se display scratching at the site following test article or vehicle application alone. Motor function (measured by placing and stepping) and pinna and corneal reflexes were normal following the application of the drug. No weaknesses were observed in any of the study animals.

Time of onset and duration of action of 3% ASN008 gel on chloroquine induced scratching

Since, ASN008 (3%) was the most effective concentration that showed a significant inhibition on CQ induced scratching (Figure 2A), we examined the time course effect of the 3% ASN008 gel in the CQ induced pruritus model. Mice were pretreated with 3% ASN008 gel at various time points (1, 3, 6, 15 and 24 hours) prior to the injection of CQ (50 µL, 4 mg/mL). Another group of mice received the vehicle pre-treatment at similar timepoints. The scratching behavior was recorded over the period of 40 min. The vehicle group showed a significant increase in the number of scratches following intradermal CQ injection at all time points (~169 ± 29.23). The animals pre-treated with 3% ASN008 gel showed significant inhibition of CQ-induced scratches, starting at 1 hour (28.62 ± 3.27) and lasting up to 6 hours (39.87 ± 12.61), with a partial reversal observed at 15 hours (81.25 ± 16.20). By 24 hours, complete return of CQ-induced scratching was observed (155.25 ± 42.07) (Figure 3A). The effect of 5% lidocaine ointment pre-treatment was also studied. The inhibitory effect of 5% lidocaine ointment on CQ-induced scratching was observed as early as 1 hour (42.25 ± 9.57), as compared to its vehicle control. However, a complete return of CQ-induced scratching was observed by 6 hours post lidocaine application (141.1 ± 19.26) (Figure 3B).

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In order to evaluate the effect of the absorbed drug and its ongoing efficacy after removal of the gel, 3% ASN008 gel was applied topically to the skin and removed at 3 hours after application by gentle lavage, and the effects on intradermal CQ (4 mg/mL, 50 μL) induced scratching were tested in separate groups at three different time points (3 hours, 6 hours and 15 hours) following test article application. Separate groups without the removal of the drug were also included for reference purposes. The group with the removal of the drug showed similar time-dependent inhibitory effects on CQ induced scratching as observed in the group without the removal of the drug (Figure 4).

Effect of the successive daily dosing of different concentrations of ASN0008 on chloroquine-induced scratching behavior

This study examined the anti-pruritic effect of successive once-daily dosing of ASN0008 formulations for 5 consecutive days at 0.1%, 0.3%, 1% and 3% and vehicle on CQ-induced scratching behavior on day 5. Two other groups were included in this study. One group was examined for repeated application of placebo and the baseline scratching was recorded on day 5 without intradermal CQ administration. The other group received topical application of ASN008 (3%), which was removed with water after 3 hours following the application every day for 5 days. Once-daily application for 5 days with CQ challenge on day 5, treatment with 1% ASN008 (53.25 ± 6.54) or 3% ASN008 gel (35.0 ± 12.61) reliably resulted in complete blockade of the chloroquine-induced scratching (Figure 5), even when the 3% gel was removed 3 hours after each daily application (data not shown), consistent with the results from a single-application study examining gel removal after 3 hours (Figure 4).

Plasma Levels of ASN008

Plasma samples were taken from animals approximately 7 hours after application of the 3% or 5% ASN008 gels in the single-dose concentration-response study, and approximately 4 hours after application of the 0.1%, 0.3%, 1% and 3% ASN008 gels on day 5 of the 5-day repeat-dose concentration-response study. Data are summarized in Table 1. Plasma levels of ASN008 in animals receiving 3% or 5% ASN008 gels in the single application study were found to be low and highly variable (C_{Ave} = 4.4 ng/mL, CV = 118% in the 3% group; C_{Ave} = 3.8 ng/mL, CV = 118% in the 5% group). In the 5-day repeat-dose concentration-response study, plasma samples taken after application of the 0.1%, 0.3%, 1% and 3% ASN008 gels were found to be low, although higher than in the single application study, moderately variable and concentration-dependent. In the group treated with the 0.1% ASN008 gel, plasma levels of ASN008 were below the level of quantitation (0.5 ng/mL). In the group treated with the 0.3% gel, four of the animals had plasma levels of ASN008 below the limit of quantitation, while in the remaining four animals, the average level was 1.0 ng/mL with a CV value of 37%. In the groups treated with the 1% and 3% ASN008 gels, the average plasma levels of ASN008 were 9.6 ng/mL and 27 ng/mL, respectively, with CV values of 58% and 34%, respectively (Table 1 and Figure 6).

Table 1. Plasma Levels of ASN008 from Concentration-Response Studies

	Single Application[1]		**Once Daily Application for 5 Days[1]**			
ASN008 Concentration in Gel	**3%**	**5%**	**0.1%**	**0.3%**	**1%**	**3%**
ASN008 C_{Ave} (ng/mL)	4.4	3.2	< 0.5	1.0[2]	9.6	27
Standard Deviation (ng/mL)	5.2	3.8	N/A	0.39	5.5	9.2
Coefficient of Variation (%)	118	118	N/A	37	58	34

[1] N=8 animals/group
[2] Plasma level < 0.5 ng/mL in 4 of 8 animals; C_{Ave} calculated from plasma levels in 4 animals with C_{Ave} values above 0.5 ng/mL.

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Discussion

The present study demonstrates that intradermal injection of chloroquine results in a robust and highly reliable scratching response. Previous work has shown that this chloroquine effect was not blocked by diphenhydramine, consistent with its histamine-independent effect (Marino et al, 2012). This effect of chloroquine is believed to be mediated by activation of Mrg (Liu et al, 2009; McNaughton et al, 2010; Ru et al, 2017), which is expressed in TRPV1- and TRPA1-expressing small primary afferent nerves (Hager et al, 2008; Akiyama and Carstens, 2014).

Mechanisms of therapeutic actions

Targeting the sodium channels with local regional anesthetics such as lidocaine has been shown to have significant effects upon the sensation of pruritus (Layton and Cotterill, 1991; Allenby et al, 1993; Patel and Yosipovitch, 2010; Kopecky et al, 2001). However as local anesthetics typically exert their action from an intracellular site, penetration through the cell membrane requires a lipophilic state to penetrate and then a re-protonation in the more acidic intracellular environment allowing it to it efficaciously block the voltage gated sodium channel (Hille, 1977; Butterworth and Strichartz, 1990). There are two limiting issues related to the use of this therapeutic approach. First, such uptake targets not only the nociceptive or pruriceptive neurons, but also motor and autonomic axons. Second, lipophilicity leads to a more rapid vascular uptake and loss of therapeutic activity. As noted, ASN008 is a permanently charged sodium channel blocker that contains a quaternary amino functionality. Because of its permanent positive charge, ASN008 cannot gain access to the interior of the cell by passively permeating through the cell membrane. However, ASN008 is believed to gain access to the interior of the cell by passage through the large gated pores present in TRPA1 and TRPV1 channels. These channels, when activated, allow for the permissive movement of the quaternary molecule into the intracellular compartment where it

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then has access to the sodium channel. Importantly, it is the charged (quaternary) form of the molecule which is the active entity in such channel blocks. This restricted access of the quaternary form thus endows the approach with an inherent specificity resulting in a block which is limited to membranes with such large access pores and the presence of conditions leading such pores to be activated (opened).

Effect of ASN008, a permanently charged quaternary amine sodium channel blocker

The present study investigated the concentration-response, duration of action and continuous application of the quaternary amine sodium channel blocker ASN008 on Chloroquine induced scratching behavior. Topical application of ASN008 gel resulted in a concentration-dependent reduction of scratching in a mouse model of pruritus. A single application of 3% ASN008 gel significantly reduced chloroquine-induced scratching. A rapid onset and long duration of suppression of this robust scratching behavior was also observed compared to the conventional local anesthetics such as lidocaine, a tertiary amine that does not bear a permanent positive charge and which has a very short duration of action. Repeated application of the ASN008 gel showed no signs of tachyphylaxis, consistent with its anticipated mechanisms of action, blocking of small afferent sodium channels.

Topical application of ASN008 gel resulted in a long duration of action. This duration of effect is considered to reflect the long residence time (e.g., slow clearance) of the quaternary molecule from inside the axon and poor local vascular absorption of the highly polar molecule. Assessment of plasma drug levels revealed a dose-dependent plasma concentration for ASN008, which at the therapeutic concentration of 3% resulted in a peak plasma concentration of < 30 ng/mL. In contrast to these relatively low plasma concentrations after therapeutic dosing of the charged ASN008 in gel formulation (3%), a eutectic mixture of lidocaine 2.5% applied to the normal mouse skin for

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management of soft tissue wound pain revealed considerably greater C_{max} values of 160 ng/mL (Al-Musawi, et al 2016). Also possibly contributing to the long duration of action is the potential for the skin tissue to act as a depot for drug accumulation. This is supported by the observation that plasma levels of ASN008 in the 5-day repeat-dose study were substantially higher in the 1% and 3% groups than the 3% and 5% groups in the single-dose study, indicative of drug accumulation.

Preclinical Safety

Based on preclinical dermal toxicological evaluation (acute primary skin irritation study in rabbits, skin sensitization in guinea pigs and 28-day repeat dose dermal toxicity study in minipigs), topical administration of ASN008 gel in the same formulation at ≤3% was well tolerated without adverse irritation or sensitization findings on the treated skin. Findings in the dermal toxicity study were limited to the skin (erythema, edema and eschar), were non-adverse and were fully reversible. In addition, based on the preclinical systemic toxicological and safety pharmacology studies (28-day repeat dose intravenous toxicity study in rats and single dose subcutaneous CNS and respiratory studies in rats), there were no adverse findings in any tissues or on CNS or respiratory systems, including motor function up to the maximum doses administered (5 mg/kg/day in the 28-day repeat dose intravenous study, 10 mg/kg in the single dose subcutaneous CNS study and 8 mg/kg in the single dose subcutaneous respiratory study). The battery of assessments conducted in the CNS studies are summarized in Table 2.. The only findings in the respiratory study were transient, non-adverse increases in respiratory rate and minute volume and lower tidal volume at 30-40 minutes post administration of ASN008. Of note, systemic exposure levels of ASN008 measured in these studies (1095 ± 309 ng/mL at 5 mg/kg in the 28-day repeat dose intravenous study, and 1070 ± 188 ng/mL at 10 mg/kg in the single dose subcutaneous study) were over 100-fold higher than

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concentrations measured at the minimum fully efficacious concentration of 1% ASN008 in the 5-day repeat dose concentration-response study in the mouse pruritus model (9.6 ± 5.5 ng/mL, Table 1). Overall, ASN008 gel is well-tolerated in dermal toxicology studies at concentrations that are efficacious in the mouse pruritus model, and in systemic toxicology studies at doses leading to systemic concentrations of ASN008 that are more than 100-fold higher than systemic concentrations measured at the minimum fully efficacious concentration in the mouse pruritus model.

Table 2. Functional Observation Battery Assessments in CNS Safety Pharmacology Studies

Observation Category	Assessment	Findings
Home cage observations	Posture, convulsions/tremors, biting, eyelid closure, feces consistency	None
Handling observations	Ease of removal from cage, ease of handling animal in hand, lacrimation/chromodacryorrea, salivation, piloerection, fur appearance, palbebral closure, respiratory rate/character, eye prominence, mucous membranes/eye/skin color, red/crusty deposits, muscle tone	None
Open field observations	Mobility, rearing, convulsions/tremors, grooming, bizarre/stereotypic behavior, time to first step (seconds), gait, gait score, arousal, backing, urination/defecation	None
Sensory observations	Approach response, touch response, startle response, tail pinch response, pupil response, eyeblink response, forelimb extension, hindlimb extension, air righting reflex, olfactory orientation	None
Neuromuscular observations	Hindlimb extensor strength, hindlimb foot splay, grip strength (hind- and forelimb), rotarod performance	None
Physiological observations	Catalepsy, body temperature, body weight	None

Conclusion

These results with the therapeutic effects of topical ASN008 upon chloroquine induced scratching in the mouse are of particular interest as, unlike other models of pruritus, molecular studies of CQ-induced pruritus suggest a mechanistic phenotype for the pruritus that accompanies other diseases such as chronic kidney disease, chronic liver disease, skin disorders, and burns (McNeil and Dong, 2014; Ajayi, 2019). The rapid onset of action and long-lasting effect of the topical ASN008 application suggest this to be inviting strategy for developing a selective and well tolerated therapy for pruritus.

Authorship contributions:

Contributed to research design: Ramachandran, Thompson, Malkmus, Gupta and Yaksh

Conducted experiments: Ramachandran, Malkmus

Performed data analysis: Ramachandran, Malkmus, Yaksh

Contributed to the writing of the manuscript: Ramachandran, Thompson, Malkmus, Gupta, Lin and Yaksh

References:

Ajayi, AAL (2019) Itching, chloroquine, and malaria: a review of recent molecular and neuroscience advances and their contribution to mechanistic understanding and therapeutics of chronic non-histaminergic pruritus. *Int J Dernatol.* **58**(8): 880-891.

Akiyama T and Carstens E (2014) Spinal Coding of Itch and Pain, in *Itch: Mechanisms and Treatment* (Carstens E, Akiyama T, eds) Chapter 19. Frontiers in Neuroscience, CRC Press/Taylor & Francis, Boca Raton, FL.

Alhmada Y1, Selimovic D1, Murad F1, Hassan SL1, Haikel Y1, Megahed M1, Hannig M1, Hassan M1. Hepatitis C virus-associated pruritus: Etiopathogenesis and therapeutic strategies. World J Gastroenterol. 2017 Feb 7; 23(5): 743–750.

Allenby CF, Johnstone RS, Chatfield S, Pike LC, and Tidy G (1993) PERINAL--a new no-touch spray to relieve the symptoms of pruritus ani. *Int J Colorectal Dis.* **8**: 184-187.

Al-Musawi A, Matar K, Kombian S, and Andersson L (2016) Blood concentration of prilocaine and lidocaine after the use of topical anesthesia (Oraqix®) in lacerated wounds. *Dent Traumatol.* **32**(6): 502-506.

Balaskas EV, Chu M, Uldall RP, Gupta A, and Oreopoulos DG (1993) Pruritus in continuous ambulatory peritoneal dialysis and hemodialysis patients. *Perit Dial Int* **13**, Suppl 2: S527-532.

Bautista DM, Wilson SR, and Hoon MA (2014) Why we scratch an itch: the molecules, cells and circuits of itch. *Nat Neurosci* **17**: 175-182.

Butterworth JF 4th and Strichartz GR (1990) Molecular mechanisms of local anesthesia: a review. *Anesthesiology* **72**(4): 711-734.

Chia S-C, Bergasa NV, Kleiner DE, Goodman Z, Hoofnagle JH, and Di Bisceglie AM (1998) Pruritus as a presenting symptom of chronic hepatitis C. *Dig Dis Sci* **43**: 2177-2183.

Green AD1, Young KK, Lehto SG, Smith SB, Mogil JS. Influence of genotype, dose and sex on pruritogen-induced scratching behavior in the mouse. Pain. 2006 Sep;124(1-2):50-8. Epub 2006 May 11.

Hager UA[1], Hein A, Lennerz JK, Zimmermann K, Neuhuber WL, Reeh PW (2008) Morphological characterization of rat Mas-related G-protein-coupled receptor C and functional analysis of agonists. *Neuroscience* **151**(1): 242-254.

Hille B (1977) Local anesthetics: hydrophilic and hydrophobic pathways for the drug-receptor reaction. *J Gen Physiol* **69**: 497-515.

Ikoma A, Steinhoff M, Ständer S, Yosipovitch G, and Schmelz M (2006) The neurobiology of itch. *Nat Rev Neurosci* **7**: 535-547.

Imamachi N, Park GH, Lee H, Anderson DJ, Simon MI, Basbaum AI, and Han SK (2009) TRPV1-expressing primary afferents generate behavioral responses to pruritogens via multiple mechanisms. *Proc Natl Acad Sci U S A* **106**: 11330-11335.

Kopecky EA, Jacobson S, Bch MB, Hubley P, Palozzi L, Clarke HM, and Koren G (2001) Safety and pharmacokinetics of EMLA in the treatment of postburn pruritus in pediatric patients: a pilot study. *J Burn Care Rehabil* **22**: 235-242.

Layton AM and Cotterill JA (1991) Notalgia paraesthetica--report of three cases and their treatment. *Clin Exp Dermatol.* **16**: 197-198.

Liu Q, Tang Z, Surdenikova L, Kim S, Patel KN, Kim A, Ru F, Guan Y, Weng HJ, Geng Y, Undem BJ, Kollarik M, Chen ZF, Anderson DJ, and Dong X (2009) Sensory neuron-specific GPCR Mrgprs are itch receptors mediating chloroquine-induced pruritus. *Cell* **139**: 1353-1365.

Marino M, Hoang P, Malkmus S, Robertshaw E, Mac EA, Shatterman Y, and Yaksh TL (2012) Development and validation of an automated system for detection and assessment of scratching in the rodent. *J Neurosci Methods* **211**: 1-10.

McNaughton F., Obianwu HO, Isah AO, and Arhewoh IM (2010) Chloroquine-induced Pruritus. *Indian J Pharm Sci* **72**: 283-289.

McNeil B and Dong X (2014) Mrgprs as Itch Receptors, in *Itch: Mechanisms and Treatment* (Carstens E, Akiyama T, eds) Chapter 12. Frontiers in Neuroscience, CRC Press/Taylor & Francis, Boca Raton, FL.

Oaklander AL, Bowsher D, Galer B, Haanpää M, and Jensen MP (2003) Herpes zoster itch: preliminary epidemiologic data. *J Pain* **4**: 338-343.

Patel TS1, Freedman BI, Yosipovitch G. An update on pruritus associated with CKD. Am J Kidney Dis. 2007 Jul;50(1):11-20.

Patel T and Yosipovitch G (2010) Therapy of pruritus. *Expert Opin Pharmacother.* **11**: 1 673-1682.

Ramachandran R1, Marino MJ2, Paul S3, Wang Z4, Mascarenhas NL5, Pellett S6, Johnson EA7, DiNardo A8, Yaksh TL9. A Study and Review of Effects of Botulinum Toxins on Mast Cell Dependent and Independent Pruritus. Toxins (Basel). 2018 Mar 23;10(4). pii: E134. doi: 10.3390/toxins10040134.

Ru F, Sun H, Jurcakova D, Herbstsomer RA, Meixong J, Dong X, and Undem BJ (2017) Mechanisms of pruritogen-induced activation of itch nerves in isolated mouse skin. *J Physiol.* **595**(11): 3651-3666.

Schmelz M, Schmidt R, Bickel A, Handwerker HO, and Torebjörk HE (1997) Specific C-receptors for itch in human skin. *J Neurosci* **17**: 8003-8008.

Schmelz M, Schmidt R, Weidner C, Hilliges M, Torebjörk HE, and Handwerker HO (2003) Chemical response pattern of different classes of C-nociceptors to pruritogens and algogens. *J Neurophysiol* **89**: 2441-2448.

Shim W-S, Tak MH, Lee MH, Kim M, Kim M, Koo, JY, Lee, CH, Kim M, and Oh U (2007) TRPV1 mediates histamine-induced itching via the activation of phospholipase A2 and 12-lipoxygenase. *J Neurosci* **27**: 2331-2337.

Wilson SR, Gerhold KA, Bifolck-Fisher A, Liu Q, Patel KN, Dong X, and Bautista DM (2011) TRPA1 is required for histamine-independent, Mas-related G protein-coupled receptor-mediated itch. *Nat Neurosci* **14**: 595-602.

Yaksh TL, Ozaki G, McCumber D, Rathbun M, Svensson C, Malkmus S, and Yaksh MC (2001) An automated flinch detecting system for use in the formalin nociceptive bioassay. *J Appl Physiol* **90**: 2386-2402.

Yosipovitch G, Greaves MW, and Schmelz M (2003) Itch. *Lancet* **361**: 690-694.

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Conflict of interest

These studies were performed at the University of California, Department of Anesthesiology as a Laboratory Service agreement by the University of California, San Diego with Asana Biosciences in the laboratory of Tony L. Yaksh, Ph.D. Scott K. Thompson, Ph.D., Sandeep Gupta, Ph.D. and Jun-Hsiang Lin, Ph.D., DABT are employees of Asana Bioscience.

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Legends for Figures

Figure 1: Chemical structure of ASN008

Figure 2: Concentration-response: (A) Scratches per minute over a period of 40 minutes. (B) Bar graph with scatter dot plot shows the total number of scratches over a 40 min period with or without intradermal chloroquine (CQ) injection 6 hours after the application of gel vehicle and different gel formulations with varying concentrations of ASN008 gel (0.3%, 1%, 3% and 5%). Animals pre-treated with vehicle showed a significant increase in the number of scratches following intradermal chloroquine. The animals pre-treated with 3% and 5% ASN008 gel showed significantly reduced CQ-induced scratching as compared to animals pre-treated with gel vehicle. Plots indicate mean ± SEM for cumulative scratches. ***$P < 0.001$ vs. vehicle with no CQ; $^{###}P<0.001$ as compared to vehicle with CQ, N = 7-8 animals per group.

Figure 3: Bar graph with scatter dot plot depicts total number of scratches over a 40 min period: (A) Bar graph shows onset and duration of anti-pruritic action of 3% ASN008 gel on chloroquine (CQ) induced scratching. The onset of anti-pruritic action of 3% ASN008 gel was ≤ 1 hour (earliest time point measured), and the duration of action was between 15 and 24 hours as compared to the vehicle control at respective timepoints. Plots indicate mean ± SEM for cumulative scratches. *$P < 0.05$ vs. vehicle at respective timepoints. (B) Bar graph shows anti-pruritic action of 5% lidocaine ointment on chloroquine (CQ) induced scratching. Onset of anti-pruritic action of lidocaine was ≤ 1 hour and the effect completely reversed by 6 hours. *$P < 0.05$ vs. vehicle at respective timepoints. N = 8 animals per group.

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Figure 4: Bar graph shows total number of scratches in the 40 min period following CQ injection 3 hours, 6 hours, and 15 hours after the application of ASN008 gel (3 %) or gel vehicle alone with or without its removal at 3 hours. Animals pretreated with vehicle gel showed a significant increase in the number of scratches following intradermal chloroquine injection. The animals pre-treated with ASN008 gel (3%) with or without removal showed similar effects. Chloroquine induced scratching was inhibited in both groups starting at 3 hours and lasted for 15 hours, and this reduction was statistically significant as compared to the group that was pretreated only with the vehicle. Plots indicate mean ± SEM for cumulative scratches. N = 8 animals per group.

Figure 5: Bar graph and scatter dot plot represents the total number of scratches following intradermal chloroquine (CQ) injection over a 40 min period on day 5 following once daily application of ASN008 gel (0.1%, 0.3%, 1% and 3%) or vehicle gels for 5 days. Successive application of ASN008 for 5 days resulted in significant reduction of chloroquine-induced scratching with ASN008 gel (0.3%) and showed a complete blockade with the 1% and 3% ASN008 gels as compared to the vehicle. Plots indicate mean ± SEM for cumulative scratches. *$P < 0.05$ and ***$P < 0.001$ vs. vehicle with CQ; N = 7-8 animals per group.

Figure 6: Bar graph with scatter dot plot represents plasma levels of ASN008 post single application (A) and repeated once-daily application (for 5 days) of ASN008 gel using different concentrations. The plasma levels of ASN008 in the 5-day repeat-dose study were significantly higher in the 1% and 3% groups than the 3% and 5% groups in the single-dose study. Plots indicate mean ± SEM for plasma ASN008 concentration. ***$P < 0.001$, ****$P<0.0001$ vs. ASN008 (0.1%) and ANS008 (0.3%); N = 7-8 animals per group.

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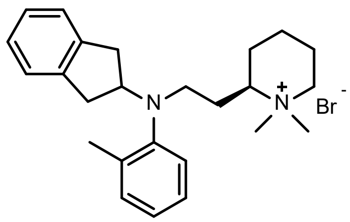
Figures:

Figure 1:



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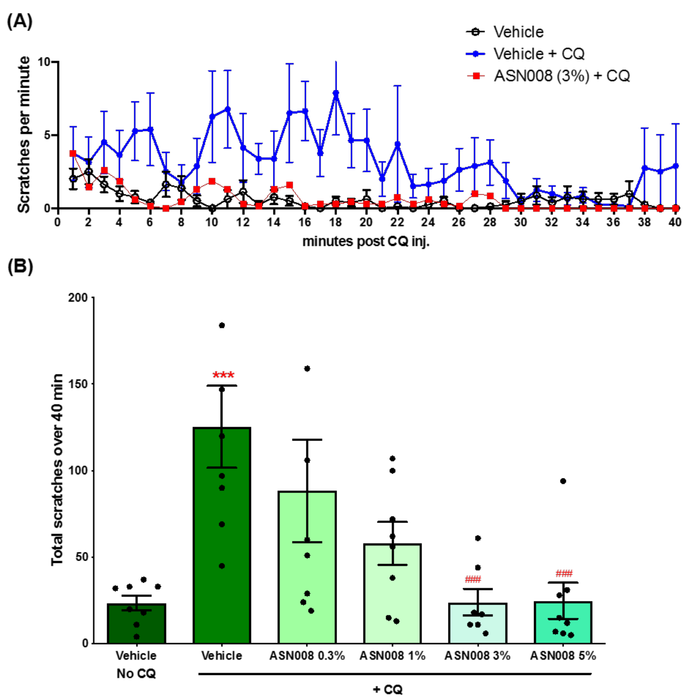

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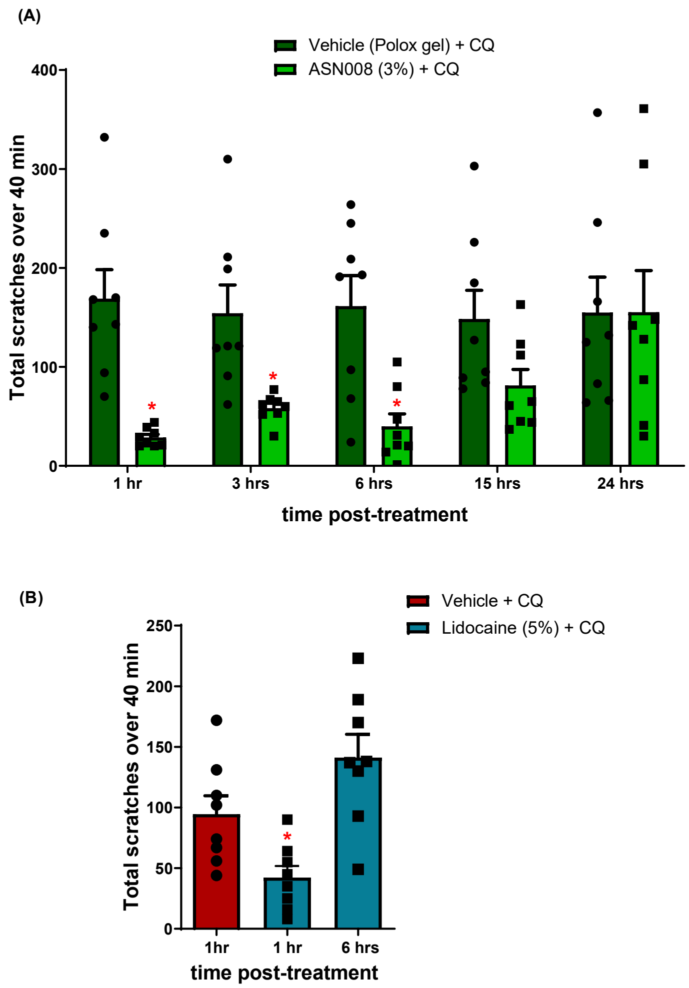

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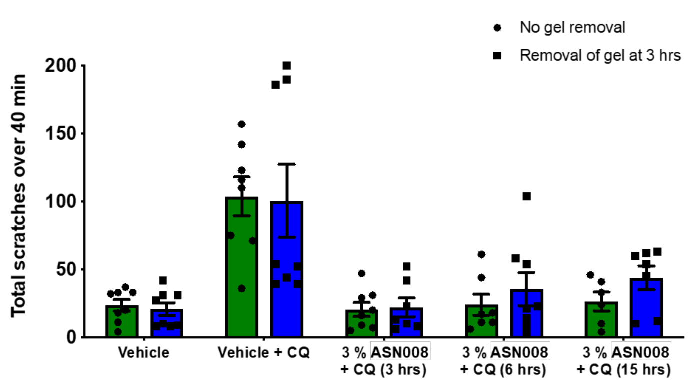

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Figure 5:

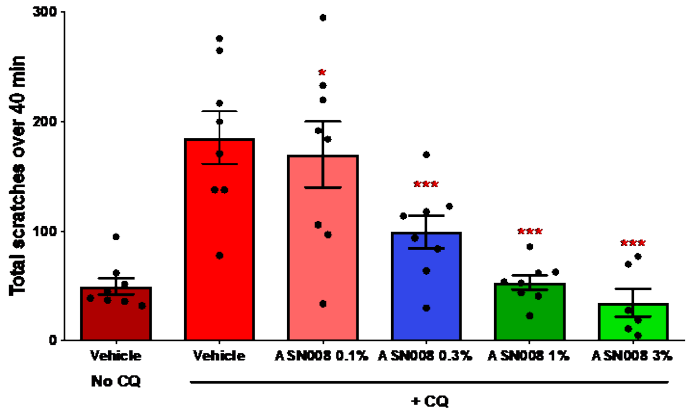

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Figure 6: