

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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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 nonhistaminergic 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 of a variety of pruritic conditions in humans, including nonhistaminergic itch.

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 antipruritic efficacy in atopic dermatitis patients (ClinicalTrials.gov ID: NCT03798561).

Introduction

Itch is an unpleasant sensation that provokes the desire to scratch as a primary response 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 after 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 that accompanies several disorders including skin conditions, such as atopic dermatitis, psoriasis, xerosis, and mastocytosis.

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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 (Balaskas et al., 1993; Chia et al., 1998; Yosipovitch et al., 2003) or neurologic 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., 1997, 2003) with free nerve endings located in the epidermis that 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

ABBREVIATIONS: C_{Ave} , average plasma concentration; CNS, central nervous system; CQ, chloroquine; Mrgpr, mas-related G protein-coupled receptor; TRPA1, transient receptor potential cation channel, subfamily A, member 1; TRPV1, transient receptor potential cation channel, subfamily V, member 1.

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 liquid chromatography with tandem mass spectrometry.

Statistical Analysis. The data for each variable was put in tabular form (i.e., Excel worksheet). Summary statistics were computed and include group means, S.D.s, and numbers of animals per group. Statistical analysis was performed using GraphPad Prism 6, version 6.0c (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 and 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 undertaken and

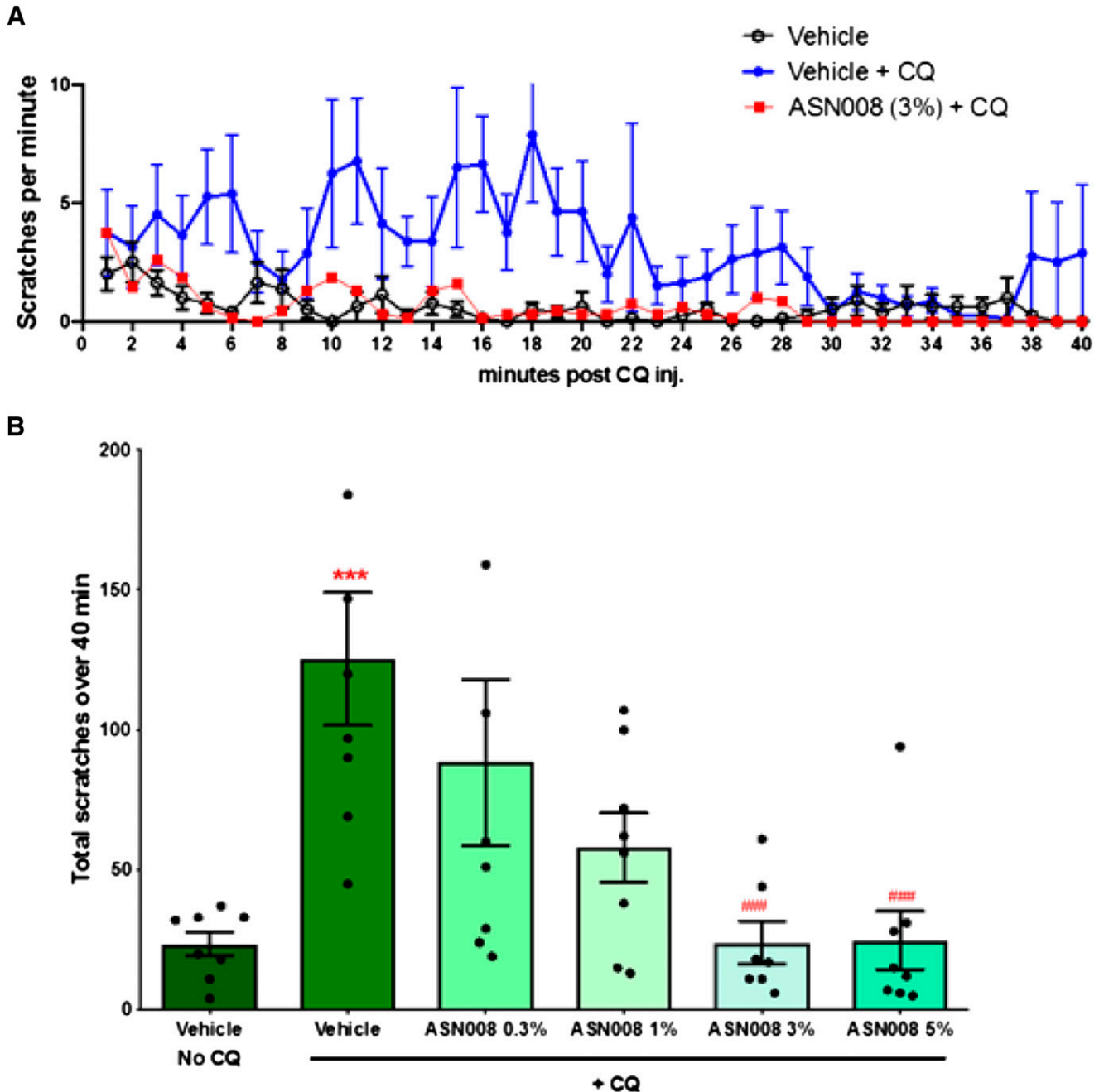


Fig. 2. Concentration-response. (A) Scratches per minute over a period of 40 minutes. (B) Bar graph with scatter dot plot shows the total number of scratches over a 40-minute period with or without intradermal CQ injection 6 hours after the application of gel vehicle and different gel formulations with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

are 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-Induced Scratching. In the absence of the chloroquine stimulus, only occasional brief episodes of grooming involving the hind paw were noted and were counted. After 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 minutes. CQ injected unilaterally induced ipsilateral scratching behavior. The total number of scratches in the 40-minute period showed a significant increase (5-fold) after intradermal injection of CQ (125.4 ± 23.68 scratches) compared with the gel vehicle group (23.50 ± 4.25 scratches), and scratching persisted unabated for intervals in excess of 40–60 minutes (Fig. 2A).

ASN008 Concentration-Response. To determine the minimum effective concentration of a single application of ASN008, a concentration-response study was performed in which the mice were pretreated with different concentrations

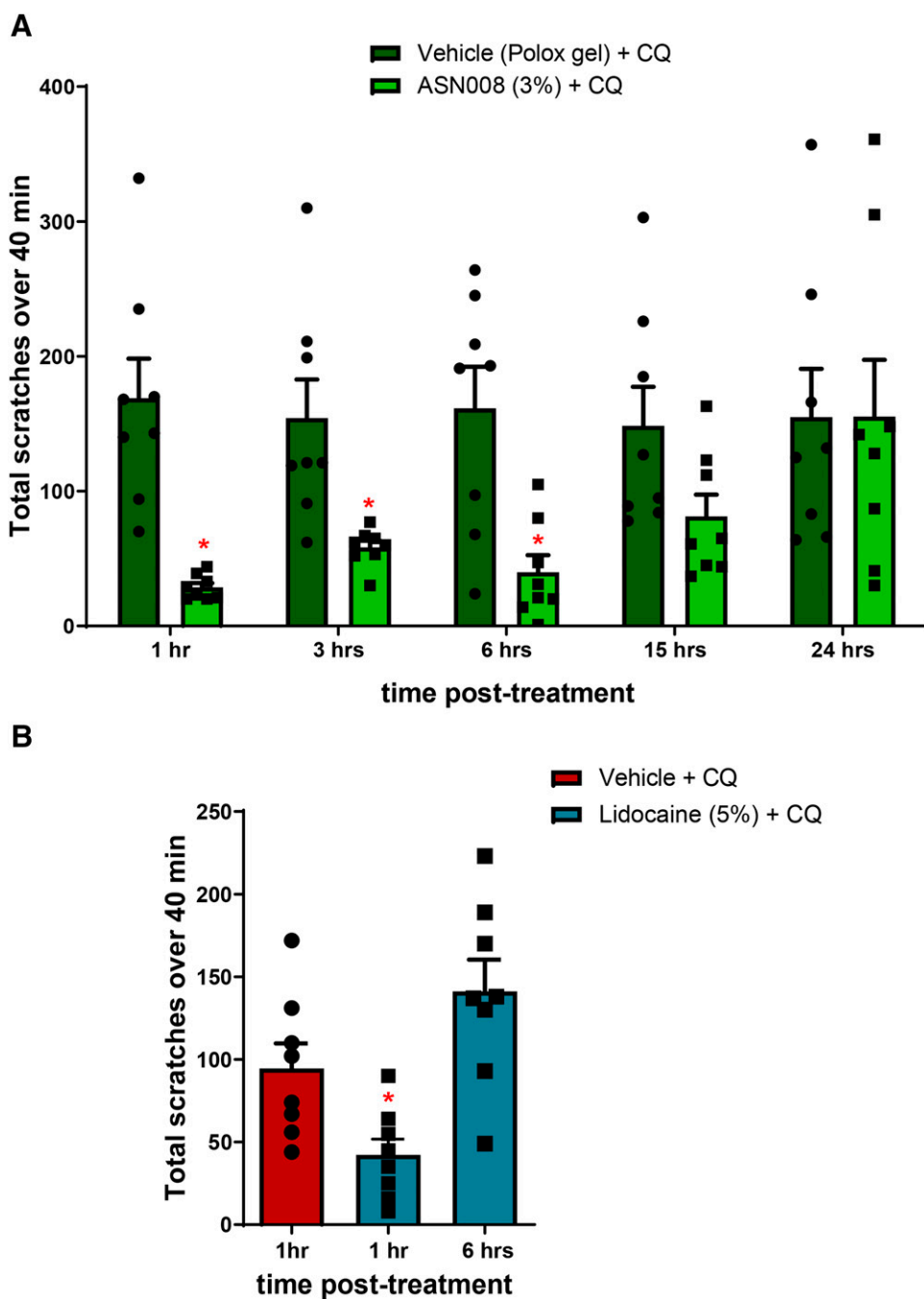


Fig. 3. Bar graph with scatter dot plot depicts total number of scratches over a 40-minute period. (A) Bar graph shows onset and duration of antipruritic action of 3% ASN008 gel on CQ-induced scratching. The onset of antipruritic action of 3% ASN008 gel was ≤ 1 hour (earliest time point measured), and the duration of action was between 15 and 24 hours as compared with the vehicle control at respective time points. Plots indicate means \pm S.E.M. for cumulative scratches. * $P < 0.05$ vs. vehicle at respective time points. (B) Bar graph shows antipruritic action of 5% lidocaine ointment on CQ-induced scratching. Onset of antipruritic action of lidocaine was ≤ 1 hour, and the effect completely reversed by 6 hours. * $P < 0.05$ vs. vehicle at respective time points. $N = 8$ animals per group.

of ASN008 [0% (vehicle), 0.3%, 1%, 3%, and 5%] prior to CQ injection. ASN008 gel formulations (50 μ l) were applied topically to the nape of the neck 6 hours prior to intradermal CQ (4 mg/ml, 50 μ l) injections at the same site. A significant reduction in CQ-induced scratching behavior was observed after topical application of 3% (24.0 \pm 7.74) (Fig. 2) and 5% (24.75 \pm 1.47) (Fig. 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 with treatment with gel vehicle (Fig. 2B). Mice in these groups did not per se display scratching at the site after test article or vehicle application alone. Motor function (measured by placing and stepping) and pinna and corneal reflexes were normal after 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 (Fig. 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 μ l, 4 mg/ml). Another group of mice received the vehicle pretreatment at similar time points. The scratching behavior was recorded over the period of 40 minutes. The vehicle group showed a significant increase in the number of scratches after intradermal CQ injection at all time points (\sim 169 \pm 29.23). The animals pretreated with 3% ASN008 gel showed significant inhibition of CQ-induced scratches, starting at 1 hour (28.62 \pm 3.27) and lasting up to 6 hours (39.87 \pm 12.61), with a partial reversal observed at 15 hours (81.25 \pm 16.20). By 24 hours, complete return of CQ-induced scratching was observed (155.25 \pm 42.07) (Fig. 3A). The effect of 5% lidocaine ointment pretreatment was also studied. The inhibitory effect of 5% lidocaine ointment on CQ-induced scratching was observed as early as 1 hour (42.25 \pm 9.57), as compared with its vehicle control. However, a complete return of CQ-induced scratching was observed by 6 hours after lidocaine application (141.1 \pm 19.26) (Fig. 3B).

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 μ l)-induced scratching were tested in separate groups at three different time points (3, 6, and 15 hours) after 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 (Fig. 4).

Effect of the Successive Daily Dosing of Different Concentrations of ASN008 on Chloroquine-Induced Scratching Behavior

This study examined the antipruritic effect of successive once-daily dosing of ASN008 formulations for five 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 after 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 \pm 6.54) or 3% ASN008 gel (35.0 \pm 12.61), reliably resulted in complete blockade of the chloroquine-induced scratching (Fig. 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 (Fig. 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 [average plasma concentration (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

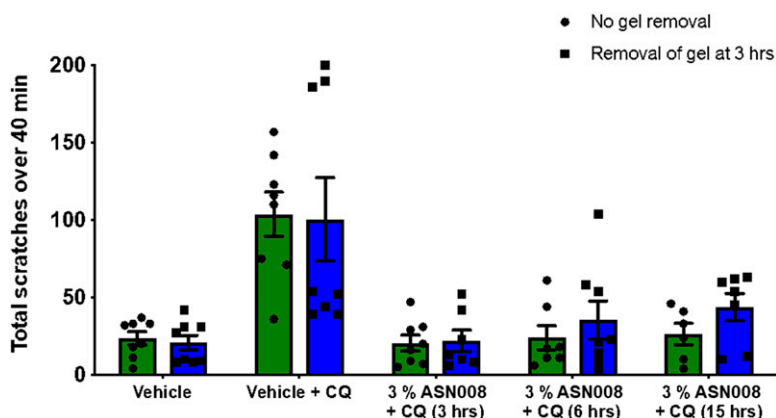


Fig. 4. Bar graph shows total number of scratches in the 40-minute period after CQ injection 3, 6, and 15 hours after the application of ASN008 gel (3%) or gel vehicle alone with or without its removal at 3 hours. Animals pretreated with vehicle gel showed a significant increase in the number of scratches after intradermal chloroquine injection. The animals pretreated 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 with the group that was pretreated only with the vehicle. Plots indicate means \pm S.E.M. for cumulative scratches. $N = 8$ animals per group.

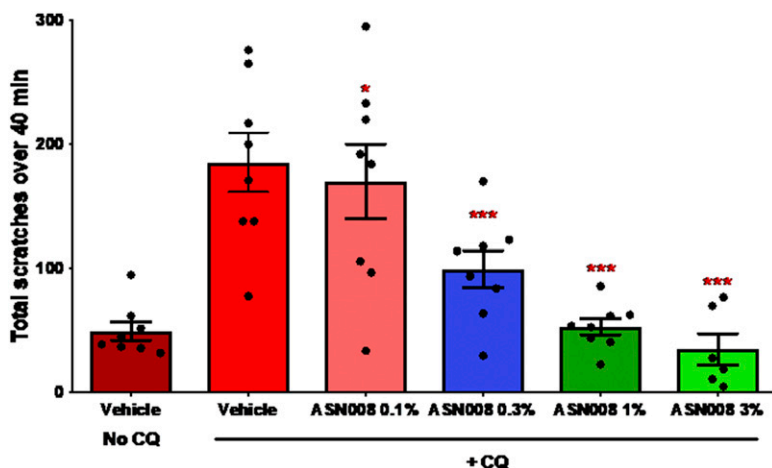


Fig. 5. Bar graph and scatter dot plot represents the total number of scratches after intradermal CQ injection over a 40-minute period on day 5 after once-daily application of ASN008 gel (0.1%, 0.3%, 1%, and 3%) or vehicle gels for 5 days. Successive application of ASN008 for 5 days resulted in significant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

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, whereas 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 and 27 ng/ml, respectively, with CV values of 58% and 34%, respectively (Fig. 6; Table 1).

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; Aghahowa 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; Kopecky et al., 2001; Patel and Yosipovitch, 2010). 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 then has access to the sodium channel. Importantly, it is the charged (quaternary) form of the molecule that 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 that 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 with the conventional local anesthetics such as lidocaine, a tertiary amine that does not bear a permanent positive charge and that has a very

TABLE 1
Plasma levels of ASN008 from concentration-response studies

| | Single application ^a | | | Once-daily application for 5 days ^a | | |
|---------------------------------|---------------------------------|-----|------|--|-----|-----|
| | 3% | 5% | 0.1% | 0.3% | 1% | 3% |
| ASN008 concentration in gel | | | | | | |
| ASN008 C _{Ave} (ng/ml) | 4.4 | 3.2 | <0.5 | 1.0 ^b | 9.6 | 27 |
| S.D. (ng/ml) | 5.2 | 3.8 | N/A | 0.39 | 5.5 | 9.2 |
| Coefficient of variation (%) | 118 | 118 | N/A | 37 | 58 | 34 |

^a $N = 8$ animals/group.

^bPlasma level <0.5 ng/ml in four of eight animals; C_{Ave} calculated from plasma levels in four animals with C_{Ave} values above 0.5 ng/ml.

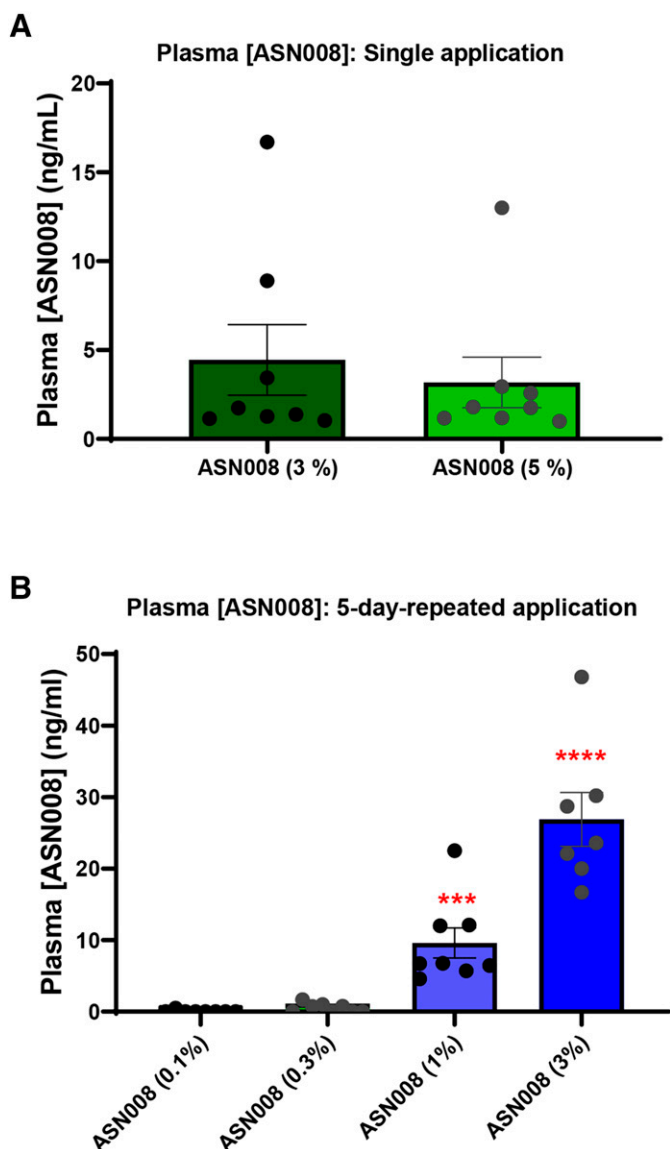


Fig. 6. Bar graph with scatter dot plot represents plasma levels of ASN008 after single application (A) and repeated once-daily application (for 5 days) (B) of ASN008 gel using different concentrations. The plasma levels of ASN008 in the 5-day repeat-dose study were significantly higher in the 1% and 3% groups than the 3% and 5% groups in the single-dose study. Plots indicate means \pm S.E.M. for plasma ASN008 concentration. *** $P < 0.001$; **** $P < 0.0001$ vs. ASN008 (0.1%) and ANS008 (0.3%); $N = 7$ to 8 animals per group.

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 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 $\leq 3\%$ 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 nonadverse, 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 central nervous system (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 per 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, nonadverse increases in respiratory rate and minute volume and lower tidal volume at 30–40 minutes after 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 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.

Conclusions

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 an inviting strategy for developing a selective and well tolerated therapy for pruritus.

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/ chromodacryorrhea, salivation, piloerection, fur appearance, palpebral 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 |
| Physiologic observations | Catalepsy, body temperature, body weight | None |

Authorship Contributions

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

Conducted experiments: Ramachandran, Malkmus.

Performed data analysis: Ramachandran, Malkmus, Yaksh.

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

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